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DOPAMINERGIC NEURODEGENERATION IN HYPERGLYCAEMIC CONDITIONS

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Per ardua ad astra

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Dear Mom,

I made it. It comes as no surprise, I suspect, as never was there any space to spare for doubt in anyone's mind. Including my own. I propelled myself on this journey, poised and tall and voracious. No space for doubt. Utterly unsuspecting of the obstacle that would lay its livid body on the tracks on my way there. The obstacle was me.

Mom, I met many a great demons within myself: marbles rolling to the back of the mind, dark moths fluttering in the peripheral vision, entire veils obstructing the panorama of a long-time goal. I did not always fight the good fight. At times, I would let them flatten me to sheets and redefine my Self. So I started buttering the plaster and braiding the bricks into a fortress to keep me out of me. A Great Wall if there is one. With every single door leading me back inside.

On this journey inside the outside of myself, I was lent a sea of hands. They reached to me and reeled me onto the continent, out of this illusory pit that we're all so familiar with. And when my identity eluded me – and this, it did many times – they gathered my pieces for when I would return to reclaim them.

Mom, among these hands, there was my research supervisor, the mightiest of lighthouses, who taught me the art of focus, patience, diligence and perseverance against the sternest of storms. You know, Maria has always reminded me of you, in the relentless faith she bore in my aptitude to cut through the crests of waves. Across the ocean that is Research. And during this time in her lab, I owe today a great many other people, in turn, for their patience when I had none, for their incessant support when I was submerged. As you showed me the golden rule of solidarity, my labmates shaped me in its application.

On my journey, I sometimes shared walls with other friends who were enduring the same transformation. I've shared these on more than one continent, countless times rescued by the voices on the other side. In Italy, Annesha, kindred spirit with an insatiable mind and the keenest sense of meta-awareness to whom my heartstrings will always be tied, however far apart our shores grow. Here, in my hometown, these voices came from the loves of my life, Simon and Jo, from the canyon of their hearts wherein whatever I've poured has always echoed back louder. And I know this thing about life now: where hands are outstretched, may they be themselves freezing and reaching to be rescued, they are the most generous source of warmth and solace.

Looking back now at who the people closest to my heart are, I realize that they share this one thing: authenticity. The ineluctable, unsaid necessity to all my relationships, in fact. Because I was raised to be true, to see truth, to earnestly trail the footfall of what is, to me, absolute. Because you raised me to embrace authenticity as the gold standard of virtue. This truth, as found in my friends and harboured in your very DNA, has salvaged my mind and kept me lucid, time and again. This truth, I have found it in one particular person now, Maude, on whose providential shores I washed up. To her, I owe health, I owe resolution, I owe a bewildering encounter with the idea that life may well just be a spectacular collision of souls. And I would be fine with that.

Mom, I made it. I tore down my walls, emptied the water in the belly of my boat, opened my sails like the widest eyes, catching all the colours. I think you know why these words are addressed to you, why there was no doubt that I would succeed to begin with. Because every dimension of my intelligence, of my heart, of my resolve that allowed me to conquer myself, I will forever owe them to you. Even in the remotest moments of this strange, strange drift, Mom, your love, your music, your rightfulness have never eluded me, like an inbuilt compass. To you, I dedicate this thesis, the thickest, starkest endeavour into my mind and soul that I have ever taken on.

To my True North,
from your ever-loving daughter

PREFACE

The work featured in this thesis represents part of the research I performed throughout my doctoral studies. These results were harvested from the Laboratory of Cellular Neurobiology at the University of Québec à Trois-Rivières, Canada, but also from a collaborative effort with the Laboratory of Neuropharmacology at the University of Cagliari, Italy, where I completed six months of research during my doctoral studies. This thesis is presented in the form of scientific articles and manuscripts: as of August 2018, two have been published and one has been submitted. It also refers to two reviews and one book chapter pertinent to the core subject and for which I am lead author. These can be found in the appendices.

As per the authorization granted by the Cellular and Molecular Biology Graduate Program Committee on April 20th, 2017, this thesis is written entirely in English. A French summary of the thesis is provided in the following pages, and French summaries of each of the articles are supplied at the beginning of the corresponding chapters. Please take note that the introduction and discussion sections are written in Canadian English, whereas the articles, manuscripts and reviews are written in American or British English, according to the language requirements specified by the editors of the scientific journals in which they were published or submitted. One of the reviews is also written in French.

All abbreviations and acronyms employed throughout the text or within figures are listed in the table provided for this purpose. Abbreviations in text are defined upon first encounter, except for common biochemical terms or when the fluidity of the text is hindered. Within figures, abbreviations are described in the legend upon first encounter, regardless of whether they were first introduced in the text, but are not defined in subsequent legends. It is important to note that each chapter that features an article stands alone within this thesis and may therefore present terms or abbreviations that are inconsistent with the remainder of the text.

Last, I wish to mention that this thesis explores three distinct subject matters integrated in a single discourse: dopaminergic neurons, hyperglycaemia and the polyphenol resveratrol. Granted the broadness of the topics discussed herein, the introduction is accordingly lengthy, as I was committed to appropriately ventilate all dimensions impinging on the central hypothesis and on future perspectives. Likewise, owing to the multiple techniques and models used to achieve the objectives of this thesis, the methodology is meticulously described in the aim of guiding the reader through the numerous decisions made upstream of the results presented herein.

RÉSUMÉ

Au deuxième rang des maladies neurodégénératives les plus communes après la maladie d'Alzheimer, la maladie de Parkinson atteint quelque 1 % de la population âgée de plus de 60 ans. Cette pathologie est caractérisée par la perte des neurones dopaminergiques logés dans la substance noire pars compacta du mésencéphale, conduisant à une diminution de la dopamine dans le striatum dorsal et à l'apparition de symptômes moteurs. Malgré d'amples efforts dévoués à l'élucidation des ses mécanismes neuropathologiques, la neurodégénérescence sélective des neurones de la voie nigrostriée demeure incomprise. En effet, la voie dopaminergique mésocorticolimbique, provenant du mésencéphale au niveau de l'aire ventrale tegmentale et innervant le striatum ventral et le cortex préfrontal, ne semble pas dégénérer. Une explication voudrait que les neurones de la voie nigrostriée expriment un phénotype distinct les rendant plus vulnérables au stress oxydant. Selon cette conjecture, les neurones dopaminergiques de la voie nigrostriée devraient exhiber une plus grande susceptibilité à toute source de stress oxydant.

Dans cette optique, nous avons émis l'hypothèse que les neurones de la voie nigrostriée sont plus vulnérables au stress oxydant engendré par l'hyperglycémie en comparaison, par exemple, à ceux de la voie mésocorticolimbique. En premier lieu, nous avons vérifié que de fortes concentrations de glucose pouvaient causer un stress oxydant menant à la dégénérescence de neurones dopaminergiques en culture. En effet, chez les cellules PC12 différenciées en neurones dopaminergiques, ces conditions conduisaient à une hausse des niveaux de l'anion superoxyde, une espèce réactive de l'oxygène produite en excès dans les premiers instants suivant une hyperglycémie. En maintenant ces concentrations élevées sur une période de 96 h, les cellules neuronales PC12 entraient en apoptose, tel que le confirmaient la fragmentation de l'ADN et l'altération des profils d'expression de plusieurs marqueurs apoptotiques. De plus, l'usage d'un antioxydant éprouvé, le polyphénol resvératrol, abrogeait la hausse des niveaux d'anion superoxyde et la mort des cellules neuronales PC12.

Suite à la confirmation que de fortes concentrations de glucose pouvaient conduire à la mort de neurones dopaminergiques en culture, nous avons voulu vérifier notre hypothèse centrale dans un modèle rongeur d'hyperglycémie. Nous avons employé un paradigme bien connu qui fait appel à l'administration de streptozotocine, une toxine ciblant les cellules productrices d'insuline, afin de générer un modèle de rat exhibant une hyperglycémie pouvant être maintenue pour une durée de 6 mois. À l'aide de méthodes immunologiques, nous avons démontré qu'une hyperglycémie chronique induisait la dégénérescence des neurones dopaminergiques de la substance noire pars compacta, mais pas de ceux de l'aire ventrale tegmentale. Conséquemment, nous observions une perte des terminaisons dopaminergiques dans le striatum dorsal qui n'était pas perceptible dans le striatum ventral. De plus, par microdialyse intracérébrale, nous avons confirmé une baisse des niveaux de dopamine explicitement dans le striatum dorsal. Cette neurodégénérescence était accompagnée de l'augmentation du nombre

d'astrocytes et de la perte de cellules microgliales au niveau de la substance noire pars compacta et du striatum, mais pas dans l'aire ventrale tegmentale.

En complément, nous avons examiné les aspects comportementaux de notre modèle de rat hyperglycémique à l'aide de tests utilisés chez les rongeurs parkinsoniens. Nos résultats démontrent l'existence de déficits moteurs évoquant ceux retrouvés chez les patients parkinsoniens. Il était intéressant de constater que ces troubles moteurs ainsi que la baisse des niveaux de dopamine se manifestaient avant que ne soit perceptible la neurodégénérescence, indiquant un possible dysfonctionnement du système nigrostrié en amont de la mort neuronale. Puisque la dopamine occupe un rôle prépondérant dans la régulation des comportements sociaux, nous avons également procédé à l'étude des interactions entre paires de rats non-familiers, tout en nous attardant à leurs communications ultrasoniques dont les paramètres sont vraisemblablement modulés par la dopamine. Lors de ces rencontres sociales, nous avons découvert que les rats hyperglycémiques exhibaient une hyper-sociabilité et une agressivité accompagnées de l'accroissement du nombre de vocalisations ultrasoniques émises, suggérant l'existence d'un dysfonctionnement dopaminergique. L'intensité de ces témoignages hyper-réactifs était de plus corrélée au degré de perte d'innervation du striatum dorsal.

La somme de ces résultats nous permet de conclure que les neurones de la voie nigrostriée exhibent une susceptibilité manifeste vis-à-vis de conditions hyperglycémiques soutenues. Ces démonstrations viennent en appui aux études épidémiologiques soulignant un risque accru de développer la maladie de Parkinson chez les patients diabétiques.

Mots-clés : comportements sociaux anormaux, déficits moteurs, diabète, hyperglycémie, maladie de Parkinson, neurones dopaminergiques, stress oxydant, voie mésocorticolimbique, voie nigrostriée.

SUMMARY

Parkinson's disease affects an estimated 1% of the population over the age of 60 years, making it the second most common neurodegenerative disorder after Alzheimer's disease. Fundamentally, this pathology features a progressive loss of dopaminergic neurons harboured in the substantia nigra pars compacta of the midbrain, which leads to decreased dopamine release in the dorsal striatum responsible for the emergence of motor symptoms. Despite arduous efforts deployed to improve our understanding of the neuropathological underpinnings of this disease, researchers are still at loss as to why the nigrostriatal dopaminergic pathway undergoes preferential early degeneration compared, for instance, to the neighbouring mesocorticolimbic pathway originating from the ventral tegmental area in the midbrain and projecting to the ventral striatum and prefrontal cortex. Rapidly gaining momentum is a proposition providing that nigrostriatal dopaminergic neurons possess a distinctive phenotypic liability responsible for their relative susceptibility to oxidative stress. If this holds true, nigrostriatal dopaminergic neurons should be preferentially vulnerable to unspecific oxidative insults.

On these bases, we hypothesized that nigrostriatal dopaminergic neurons are more vulnerable to hyperglycaemia-induced oxidative stress compared to other neuronal populations, expressly ones of the mesocorticolimbic pathway. We began by verifying that high glucose conditions are conducive to the death of dopaminergic neurons in culture and that this degeneration is linked to oxidative stress. When cultured in elevated yet physiological concentrations of glucose, differentiated dopaminergic neuronal PC12 cells promptly exhibited high levels of superoxide anion, a key reactive oxygen species whose overproduction constitutes the earliest event in hyperglycaemia-induced oxidative stress. Sustained for 96 h, high glucose conditions led to the apoptotic death of neuronal PC12 cells substantiated by DNA fragmentation and altered expression profiles of various markers of apoptosis. Treating neuronal dopaminergic PC12 cells with a potent antioxidative polyphenol, resveratrol, attenuated the rise in superoxide anion levels and afforded protection against high glucose-induced apoptosis.

After confirming that high glucose conditions elicit a state of oxidative stress leading to the death of dopaminergic neurons in culture, we set out to verify our central hypothesis in a rat model of long-term hyperglycaemia. We employed a well-known paradigm that utilizes streptozotocin, a toxin that targets insulin-producing pancreatic β cells, to generate a model presenting a hyperglycaemic phenotype that could be maintained for up to 6 months. Employing immunohistochemical and immunoblotting techniques, we demonstrated that long-term hyperglycaemia in rats causes the degeneration of dopaminergic neurons in the substantia nigra pars compacta, but not in the ventral tegmental area. Accordingly, dopaminergic terminal fibres were less dense in the dorsal than in the ventral striatum of hyperglycaemic rats. Utilizing the intracerebral microdialysis technique, we also showed that dopamine release was diminished in the dorsal striatum, but not in the ventral striatum or in the prefrontal cortex. We further discovered a noticeable increase in astrocytes that was neuroanatomically coincidental to

a loss of microglial cells in the substantia nigra pars compacta and striatum, but not in the ventral tegmental area.

Behavioural alterations were assessed in a series of tasks destined to uncover motor deficits in rodent models of Parkinson's disease. Long-term hyperglycaemic rats manifested signs of bradykinesia and gait disturbances reminiscent of parkinsonian motor symptoms. Interestingly, motor deficits and dampened dorsostriatal dopamine release were apparent before neurodegeneration could be discerned, suggesting possible functional impairments of the nigrostriatal pathway upstream of neuronal death. Considering dopamine also exerts an important control on social behaviours, we examined interactions between pairs of unacquainted rats and analyzed dopamine-regulated ultrasonic vocalizations known to reflect the subjects' affective state. In particular, hyperglycaemic rats engaged in markedly hyper-sociable and hyper-aggressive encounters, while emitting a greater number of ultrasonic vocalizations, evocative of a dopaminergic dysfunction. Remarkably, the intensity of these hyper-reactive phenotypes correlated with the degree of dorsostriatal dopaminergic denervation.

Taken together, our data expose the preferential vulnerability of the nigrostriatal pathway to sustained hyperglycaemia, supporting the physiological significance of their phenotypic liability to oxidative stress. Our findings further strengthen the apparent epidemiological link between pre-existing diabetes and an increased risk of developing Parkinson's disease.

Keywords: abnormal social behaviours, diabetes, dopaminergic neurons, hyperglycaemia, mesocorticolimbic pathway, motor deficits, nigrostrial pathway, oxidative stress, Parkinson's disease.

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3-MT	3-Methoxytyramine
3-OMD	3-*O*-Methyldopa
4EBP1	Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
6-OHDA	6-Hydroxydopamine
AADC	Aromatic amino acid decarboxylase
Aβ	Beta-amyloid
Ac	Acetyl group
ac	Anterior commissure
Acetyl-CoA	Acetyl coenzyme A
ACMSD	Aminocarboxymuconate semialdehyde decarboxylase gene
aCSF	Artificial cerebrospinal fluid (CSF)
AD	Aldehyde dehydrogenase or autosomal dominant or Alzheimer's disease
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribosyl
Affin Chrom	Affinity chromatography
AGE	Advanced glycation end-product
AgRP	Agouti-related protein
Akt or AKT-1	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
AN69	Acrylonitrile 69 membrane

ANLS	Astrocyte-neuron lactate shuttle
ANOVA	Analysis of variance
AP-1	Activator protein 1
Apaf-1	Apoptotic protease activating factor 1
APE1/Ref-1	Apurinic/apyramidic endonuclease 1/redox factor-1
Apo2L/TRAIL	Apoptosis antigen 2 ligand/tumour necrosis factor (TNF)-related apoptosis-inducing ligand
aq	Cerebral aqueduct
AR	Autosomal recessive
Arc	Arcuate nucleus
ARE	Antioxidant response element
Arg	Arginine
ARN	*Acide ribonucléique*
Atg13	Autophagy-related protein 13
ATM	Ataxia telangiectasia mutated serine/threonine kinase
ATP	Adenosine triphosphate or *adénosine triphosphate*
AUC	Area under curve
Bad	B cell lymphoma 2-associated death promoter
Bak	B cell lymphoma 2 homologous antagonist/killer
Bax	B cell lymphoma 2-associated X protein
BBB	Blood-brain barrier
BCKDK	Branched chain ketoacid dehydrogenase kinase gene
Bcl-2	B cell lymphoma 2
Bcl-XL	B cell lymphoma-extra large
BDNF	Brain-derived neurotrophic factor
BHE	*Barrière hémato-encéphalique*

BID	B cell lymphoma 2 homology 3 (BH3) interacting-domain death agonist
Bim	B cell lymphoma 2-like protein 11
BSA	Bovine serum albumin
BSLCR	*Barrière sang-liquide céphalo-rachidien*
BST1	Bone marrow stromal cell antigen 1 gene
b.w.	Body weight
¢	Cell
c-FLIP	First apoptosis signal receptor (Fas)-associated protein with death domain (FADD)-like interleukin-1-beta converting enzyme (ICE) (FLICE/caspase 8)-like inhibitory protein
C6orf10	Chromosome 6 open reading frame 10 gene
C	Cytoplasmic fraction
$^{14}CO_2$	Radio-carbon dioxide
CA	*Cornu ammonis*
CamKKβ	Calcium/calmodulin-dependent protein kinase kinase 2 or beta
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated transcript
CB	Cytochalasin B
CBR1	Carbonyl reductase 1
cc	Corpus callosum
CCDC62	Coiled-coil domain containing 62 gene
CCL	Chemokine [C-C motif] ligand
CCR6	Chemokine [C-C motif] receptor type 6
CD	Cluster of differentiation

CD200R	Cluster of differentiation 200 receptor
CD95L	Cluster of differentiation 95 ligand
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
Chem Prot	Chemical proteomics
CMH	*Complex majeur d'histocompatibilité*
CN	Caudate nucleus
CNS	Central nervous system
CNTF	Ciliary neurotropic factor
co	Coeruleus/subcoeruleus complex
COMT	Catechol-*O*-methyltransferase
COX	Cyclooxygenase or *cyclooxygénase*
CR	Complement receptor
CSF	Cerebrospinal fluid
CSF1R	Colony stimulating factor 1 receptor
CTC	Cerebellothalamocortical
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTRL or Ctrl	Control
CX3CL	Chemokine [C-X3-C motif] ligand or fractalkine
CX3CR	Chemokine [C-X3-C motif] receptor
CXCL	Chemokine [C-X-C motif) ligand
Cy3	Cyanine 3
CYP	Cytochrome P450
Cyt *c*	Cytochrome *c*
D1	D1-like dopamine (DA) receptor-expressing medium spiny neurons

D2	D2-like dopamine (DA) receptor-expressing medium spiny neurons
D1/D2 DA-R	D1-like/D2-like dopamine (DA) receptors
DA	Dopamine
DAB	3,3′-Diaminobenzidine
DAergic	Dopaminergic
DAMP	Damage-associated molecular pattern
DAPI	4′,6-Diamidino-2-phenylindole
DAQ	Dopamine quinones
DAT	Dopamine transporter
dc	Dorsal, caudal or dorsocaudal
DDC	3,4-Dihydroxyphenylalanine (DOPA) decarboxylase or diethyldithiocarbamate
DDRGK1	DDRGK domain containing 1 gene
DG	Dentate gyrus
DHAP	Dihydroxyacetone phosphate
DISC	Death-inducing signalling complex
DLS	Dorsolateral striatum
dm	Dorsal IX/X motor nucleus
DMEM	Dulbecco's Modified Eagle medium
DMS	Dorsomedial striatum
DNA	Deoxyribonucleic acid
DOPA or L-DOPA	L-3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic acid
DOPAL	3,4-Dihydroxyphenylacetaldehyde
DR4/DR5	Death receptor 4/5

DS or dStr	Dorsal striatum
dUTP	Deoxyuridine triphosphate
e-	Electron
EGCG	Epigallocatechin-3-gallate
ELK1	E26 transformation-specific (ETS)-like transcription factor
Enz Inhib	Enzyme inhibition assay
Epac1 or EPAC	Exchange factor directly activated by cyclic adenosine monophosphate 1
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-related kinase
Ethidium	3,8-Diamino-5-ethyl-6-phenylphenanthridinium
f	Fornix
F1-ATPase	F_1 portion of adenosine triphosphatase (ATPase or ATP synthase)
F6P	Fructose 6-phosphate
FAD	Flavin adenine dinucleotide (FAD+ oxidized, $FADH_2$ reduced)
FADD	First apoptosis signal receptor (Fas)-associated death domain
FAM47E	Family with sequence similarity 47 member E gene
Fas	First apoptosis signal receptor ligand
FasL	First apoptosis signal receptor (Fas) ligand or *ligand Fas*
FBS	Fetal bovine serum
fc	First order sensory association areas and premotor areas and/or primary sensory and motor fields of the neocortex

FcRβ	Fragment crystallizable region (Fc) receptor beta subunit
Fe^{2+}	Ferrous iron ion (reduced iron 2+)
Fe^{3+}	Ferric iron ion (reduced iron 3+)
FGF	Fibroblast growth factor
FIP200	Retinoblastoma 1 (RB1)-inducible coiled-coil protein 1
FITC	Fluorescein isothiocyanate
Fluor	Fluorescence assay
FM	Frequency modulated
fMRI	Functional magnetic resonance imaging
FOXO	Forkhead box O
FoxO3a	Forkhead box O3
Freq	Frequency
Fru-2,6-P_2	Fructose 2,6-bisphosphate
F.U.	Fluorescence units
G6P	Glucose 6-phosphate
G6PD	Glucose 6-phosphate dehydrogenase
GA3P	Glyceraldehyde 3-phosphate
GABA	Gamma-aminobutyric acid
GAK	Cyclin G associated kinase/auxilin-2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBA	Beta-glucocerebrosidase gene
GCH1	Guanosine triphosphate (GTP) cyclohydrolase 1 gene
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal

Glc	Glucose
Glo	Glyoxalase
Glu	Glutamate
GLUT	Glucose transporter
GMP	Guanosine monophosphate
gp91phox or Nox-2	Cytochrome b-245 heavy chain
GPe	External globus pallidus
GPI	Glucose 6-phosphate isomerase
GPi	Internal globus pallidus
GPNMB	Glycoprotein neuromedin B gene
GRP75 or mtHSP70 or mot-2	Glucose-regulated protein 75 or mortalin or mitochondrial heat shock protein 70
GS•	Glutathione (GSH) radical
GSH	Gamma-L-glutamyl-L-cysteinylglycine or glutathione
GSHR	Growth hormone secretagogue receptor or ghrelin receptor
GSK-3 or GS3K	Glycogen synthase kinase 3
GSSG or GSSH	Glutathione disulphide
GSTP1	Glutathione S-transferase P
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HAD	Human immunodeficiency virus-associated dementia
Hb	Hemoglobin
HbA1c	Glycated hemoglobin subunit alpha 1
HBSS	Hank's balanced salt solution
hc	High order sensory association areas and prefrontal areas of the neocortex

HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
HDACi	Histone deacetylase inhibitor
HF	High fat, high-fat diet or high fat-fed
HG	Hyperglycaemic or high glucose
hg38	Human genome build 38
HIV-1	Human immunodeficiency virus-1
HK	Hexokinase
HLA	Human leucocyte antigen
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1 gene
HMGB1	High mobility group box 1
HMIT	H+/myo-inositol transporter
HO-1	Heme oxygenase-1
HPC	Hippocampus
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
Hsp or HSP	Heat shock protein
HVA	Homovanillic acid
IAP	Inhibitor of apoptosis proteins
Iba1	Ionized calcium-binding adapter molecule 1
IC_{50}	Half maximal inhibitory concentration
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor

IGF1R	Insulin-like growth factor receptor 1
IGF2R	Insulin-like growth factor receptor 2
IGFBP or IGFBF	Insulin-like growth factor binding proteins
IHC	Immunohistochemistry
IL	Infralimbic prefrontal cortex or interleukin or *interleukine*
IL-1R	Interleukin-1 receptor
iNOS	Inducible nitric oxide synthase
INPP5F	Inositol polyphosphate-5-phosphatase F gene
i.p.	Intraperitoneal
IPN	Interpeduncular nucleus
IR or INSR	Insulin receptor
IRS	Insulin receptor substrate
i.v.	Intravenous
JAK	Janus kinase
JAM	Junctional adhesion molecules
JC polyomavirus	John Cunningham polyomavirus
JNK	c-Jun N-terminal kinases
k_{cat}	Maximum turnover rate
K_d	Dissociation constant
Keap1	Kelch-like erythroid cell-derived protein with cap 'n' collar (CNC) homology (ECH)-associated protein 1
K_i	Inhibition constant
KIR	Killer-cell immunoglobulin-like receptor
K_m	Maximum affinity or Michaelis constant
KSRP	K homology (KH)-type splicing regulator protein

LC-MS	Liquid chromatography-mass spectrometry
LCR	*Liquide céphalo-rachidien*
LepR	Leptin receptor
LFA	Lymphocyte function-associated antigen-1
LHA	Lateral hypothalamic area
LKB1	Liver kinase B1
Lmx1a/b	Lin11, Isl-1 and Mec-3 domain (LIM) homeobox transcription factor a/b
LOPD	Late-onset Parkinson's disease
LPS	Lipopolysaccharide
LRP1	Low-density lipoprotein receptor-related protein 1
Lst8	Target of rapamycin complex subunit lethal with SEC13 protein 8
LTA_4H	Leukotriene A4 hydrolase
luc	Luciferase
M	Mitochondrial fraction
MAC-1	Macrophage-1 antigen
MANN	D-Mannitol
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau gene
Mb	Mega base pair
mc	Transentorhinal region and/or ectorhinal region (anteromedial temporal mesocortex)
MC4R	Melanocortin 4 receptor
MCCC1	Methylcrotonoyl-coenzyme A carboxylase 1 gene
Mcl-1	Induced myeloid leukemia cell differentiation protein

MCP-1	Monocyte chemotactic protein-1
MCT	Monocarboxylate transporter
MDM2	Mouse double minute 2 homologue
MG or 3MG	3-*O*-Methylglucose
MGO	Methylglyoxal
MHC	Major histocompatibility complex
MIR4697HG	Microribonucleic acid (miRNA) 4697 host gene
ml	Medial lemniscus
MMP	Matrix metalloproteinases
mn-SOD	Manganese superoxide dismutase
mPGES-1	Inducible microsomal prostaglandin E synthase-1
MPO	Myeloperoxidase
MPP+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
α-MSH	Alpha-melanocyte-stimulating hormone
MT	Mammillothalamic tract
mt	Mitochondria
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVD1	Diphosphomevalonate decarboxylase
N	Nuclear fraction
NA	Nicotinamide

NAcc or NAc	Nucleus accumbens
NAD	Nicotinamide adenine dinucleotide (NAD+ oxidized, NADH reduced)
NADP	Nicotinamide adenine dinucleotide phosphate (NADP+ oxidized, NADPH reduced)
NCAM	Neural cell adhesion molecule
NE	Norepinephrine or noradrenaline
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGA neurons	Neuropeptide Y (NPY)/gamma-aminobutyric acid (GABA)/agouti-related protein neurons (AgRP)
NGF	Nerve growth factor
NIH	National Institute of Health
NK	Natural killer
NO	Nitric oxide
NO_3^-	Peroxynitrite
NOEL	No observed effect level
NOR	Novel object recognition
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NPY	Neuropeptide Y
NQO2	Ribosyldihydronicotinamide dehydrogenase (quinone)
Nr4a2 or Nurr1	Nuclear receptor 4a2
Nrf2	Nuclear factor erythroid-derived 2 like 2
NSERC	Natural Sciences and Engineering Research Council of Canada
NT3	Neurotrophin-3
NTS	Nucleus of the solitary tract

NUCKS1	Nuclear casein kinase and cyclin dependent kinase substrate 1 gene
$\bullet O_2^-$ or $O_2^{\bullet-}$	Superoxide anion
OGTT	Oral glucose tolerance test
OH	Hydroxyl radical
•OH	Hydroxyl radical
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
OS	Oxidative stress
OT	Olfactory tubercle
Ox Phos	Oxidative phosphorylation
P	Phosphate group
P or *p*	Probability
p38MAPK or p38 MAPK	p38 Mitogen-activated protein kinases
p47phox	Neutrophil cytosol factor 1
p66Shc	66 kDa Proto-oncogene Src homologous-collagen homologue (Shc) adaptor protein
PAGvl	Ventrolateral periaqueductal grey
PAMP	Pathogen-associated molecular pattern
PARK-*ATP13A2*	Probable cation-transporting adenosine triphosphatase (ATPase) 13A2 gene
PARK-*DJ1*	Oncogene DJ-1 gene
PARK-*DNAJC6*	Auxilin gene
PARK-*FBXO7*	F-box only protein 7 gene
PARK-*LRRK2*	Leucine-rich repeat kinase 2 gene
PARK-*parkin* or *PARK2*	Parkin gene

PARK-*PINK1*	Phosphatase and tensin homologue (PTEN)-induced putative kinase 1 gene
PARK-*SNCA*	Alpha-synuclein gene
PARK-*SYNJ1*	Synaptojanin 1 gene
PARK-*VPS35*	Vacuolar protein sorting-associated protein 35 gene
PARP or PARP-1	Poly(adenosine diphosphate-ribose) polymerase
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma cell line 12
PD	Parkinson's disease
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PFA	Perifornical area
PFC	Prefrontal cortex
PFK	Phosphofructokinase
Pfkfb3	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGE2	Prostaglandin E2
PGP	Proline glycine proline peptide
P_i	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase or phosphoinositide 3-kinase
PIGD	Postural instability and gait difficulty
PKA	Protein kinase A

PKC	Protein kinase C
PKD	Protein kinase D
PKM	Pyruvate kinase isozymes
PL	Prelimbic prefrontal cortex
PLC	Phospholipase C
PMVK	Phosphomevalonate kinase gene
POHEM	Population health model
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PPP	Pentose phosphate pathway
PRR	Pattern-recognition receptors
PSG	Parkinson's Study Group
PTP	Permeability transition pore
Puma	p53 upregulated modulator of apoptosis
Put	Putamen
PVN	Paraventricular nucleus
Q	Quinone
R•	Free radical
RAS	Rat sarcoma
RBC	Red blood cell
REM	Rapid eye movement
RESV	Resveratrol or *trans*-3,5,4′-trihydroxystilbene
RIPA	Radioimmunoprecipitation assay
RIT2	Ras-like without CAAX 2 gene
RNA	Ribonucleic acid

RNS	Reactive nitrogen species
ROH	Alcohol
ROOH	Hydroperoxide compound
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RRF	Retrorubral field
RT	Room temperature
SCI	Spinal cord injuries
SDF1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Status epilepticus
SEM	Standard error of the mean
SIPA1L2	Signal induced proliferation associated 1 like 2 gene
SIRPα	Signal-regulatory protein alpha
SIRT1	Silent mating type information regulation (Sir) 2 homologue 1
SLC2A	Solute carrier family 2 gene
SLC50A1	Solute carrier family 50 member 1 gene
Smac/DIABLO	Second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP) binding protein with low pI
SN	Substantia nigra
sn	Posterior portion of substantia nigra pars compacta
SNC	*Système nerveux central*
SNc or SNpc	Substantia nigra pars compacta
SNl	Substantia nigra pars lateralis

SNP	Single nucleotide polymorphism
SNr	Substantia nigra pars reticulata
SOCS-1	Suppressor of cytokine signalling-1
SOD	Superoxide dismutase
Spec	Spectroscopic assay
SPECT	Single-photon emission computed tomography
ssDNA	Single-stranded DNA
STAT	Signal transducer and activator of transcription
STK39	Serine/threonine kinase 39 gene
STN	Subthalamic nucleus
STZ	Streptozotocin
T1DM	Type I diabetes mellitus
T2DM	Type II diabetes mellitus
TA	Transaldolase
TBHQ	*Tert*-butylhydroquinone
TBI	Traumatic brain injury
tBID	Truncated B cell lymphoma 2 homology 3 (BH3) interacting-domain death agonist (BID)
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-beta
TH	Tyrosine hydroxylase
Th	T helper cell subtype
TIMP	Tissue inhibitor of metalloproteinase
TK	Transketolase or tyrosine kinase
TLR	Toll-like receptor
TMEM175	Transmembrane protein 175 gene

TNF-α	Tumor necrosis factor-alpha
TPI	Triosephosphate isomerase
Treg	Regulatory T cell
TREM2	Triggering receptor expressed on myeloid cells 2
Trp	Tryptophane
TSB	Thrombospondin
TSC	Tuberous sclerosis complex
TUNEL	Terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling
TyrRS	Tyrosine-transfer RNA ligase
UCP	Uncoupling protein
ULK1	Unc-51 like autophagy activating kinase
UPS	Ubiquitin-proteasome system
USCC	University of California, Santa Cruz
USV	Ultrasonic vocalization
v/v	Volume/volume
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VMAT	Vesicular monoamine transporter
V_{max}	Maximum rate of transport
VPS13C	Vacuolar protein sorting 13 homologue C gene
VS	Ventral striatum
VTA	Ventral tegmental area
WB	Western blotting or immunoblotting
WBSSH	White, Bate-Smith, Swain and Haslam

Xray	X-ray cocrystal structure
Y1R	Neuropeptide Y receptor Y1
YOPD	Young-onset Parkinson's disease
Z-DEVD-FMK	Fluormethylketone-conjugated tetrapeptide (Z-Asp[OMe]-Glu[OMe]-Val-Asp[OMe]-FMK)
ZDF	Zucker diabetic fatty
ZO	*Zona occludens* protein

CHAPTER I

INTRODUCTION

Of all the intricate systems that make up the human organism, no other has elicited more debate or has inspired such a vast array of theories regarding its functions than the central nervous system (CNS). An era's worth of research in neuroscience has amounted to our current appreciation of the various temporal, spatial and thermodynamic circumstances that dictate neural outcomes in countless contexts. Nevertheless, particular modules of the CNS continue to galvanize neuroscientists, in particular the multifarious roles dopaminergic systems fulfil in health and disease. This thesis aims to address one specific aspect of dopaminergic neurons: their selective vulnerability in certain pathological settings, with a keen focus on the nigrostriatal dopaminergic pathway involved in Parkinson's disease.

1.1 Dopaminergic neurons

Possibly the most impressive feature of CNS dopaminergic neurons is their small population size, totalling approximately 200 000 cells per hemisphere in humans (Stark and Pakkenburg, 2004), weighed against the astonishing number of processes they carry out, for instance motor control, cue-related learning, arousal, social play, mood modulation, endocrine regulation and many more. In fact, to sustain these functions, dopaminergic neurons make synapses with no fewer than 200 million other neurons in the striatum, cortex, amygdala and other structures (Stark and Pakkenburg, 2004). Emerging late in prenatal development, it is possible that dopamine holds a particular position in stabilizing and integrating various other brain circuits (Grace, 2016). Conversely, dopaminergic neurons are phylogenetically ancient, occurring in all mammals, birds, reptiles and insects, which allows assumptions on their crucial role in

the adaptation of animal behaviour throughout evolution (Jones and Pilowski, 2002; Smeets and González, 2000; Yamamoto and Vernier, 2011).

Dopaminergic subpopulations were first described in the 1960s upon identification and classification of groups of catecholaminergic neurons (Dahlström and Fuxe, 1964), later updated by others (Hökfelt *et al.*, 1984). Of the designated A1-A17 groups, it is understood today that only groups A8-A11 comprise proper dopaminergic neurons (Björklund and Dunnett, 2007). By definition, dopaminergic neurons utilize the catecholamine neurotransmitter dopamine (3-hydroxytyramine) to communicate and, thus, express the rate-limiting enzyme of catecholamine biosynthesis, tyrosine hydroxylase (TH), as well as aromatic amino acid decarboxylase that ultimately produces dopamine (Björklund and Dunnett, 2007). Conversely, they do not express the enzymes necessary for the conversion of dopamine to the succeeding catecholamines in the biosynthetic pathway, namely noradrenaline and adrenaline (Björklund and Dunnett, 2007). Several other ontological hallmarks are also exclusive to dopaminergic neurons, reviewed in greater detail by Arenas *et al.* (2015)[1].

Of equal importance to their role in the CNS, dopaminergic neurons occupy important functions in the enteric nervous system (Li *et al.*, 2006). They are principally located in the submucosal plexus, only sparingly so in the myenteric plexus, and are distributed more densely in the small intestine in comparison to gastric or colonic tissues (Li *et al.*, 2004). By secreting dopamine onto smooth muscle cells, dopaminergic neurons inhibit gastrointestinal motility (Li *et al.*, 2006). Although the place held by dopaminergic neurons in the proper operation of the enteric nervous system is increasingly acknowledged (see for review Mittal *et al.*, 2017), this thesis will focus on CNS dopaminergic neurons.

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[1] The presence or absence of developmental factors, such as LIM homeobox transcription factors (Lmx1a/b) or the late transcription factor nuclear receptor 4a2 (Nr4a2, better known as Nurr1), determines neuronal identity by suppression of lateral fates or activation of dopaminergic ones. Today, human dopaminergic neurons can be prepared through direct reprogramming of pluripotent stem cells or somatic cells, for the purpose of disease modeling or regenerative therapies (Arenas *et al.*, 2015).

1.1.1 Dopamine metabolism and neurotransmission

Dopamine was first described in the human brain in the 1950s by Kathleen Montagu (Montagu, 1957) and named after its previously achieved chemical synthesis utilizing the precursor L-3,4-dihydroxyphenylalanine (L-DOPA) (Fahn, 2008). Arvid Carlsson and Nils-Åke Hillarp confirmed its identity as a neurotransmitter in the CNS (Carlsson *et al.*, 1958), the former receiving the 2000 Nobel Prize in Physiology and Medicine for this demonstration (Benes, 2001). A host of researchers later confirmed dopamine's implication in Parkinson's disease[2] (Bazelton *et al.*, 1967; Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1966) and behavioural disorders, such as psychoses and addiction (Baldessarini, 1985). Advances in lesion models and dopamine visualization techniques further permitted the discovery of its role in a plethora of brain functions upon which were built entire fields still evolving rapidly today.

Alongside noradrenaline and adrenaline, dopamine belongs to the catecholamine subcategory of monoamine neurotransmitters, all produced from amino acid metabolism and collectively important in various aspects of behaviour. The non-essential amino acid L-tyrosine is the precursor for catecholamines, easily obtained in a balanced diet or derived from L-phenylalanine hydroxylation in the liver, but not in the brain. Tyrosine must gain entry to the CNS via the large neutral amino acid transporter by competing with other amino acids (Duelli *et al.*, 2000). Once inside neurons, the first step consists in the hydroxylation of L-tyrosine to L-DOPA by the rate-limiting enzyme TH. Aromatic amino acid decarboxylase[3] then promptly decarboxylates L-DOPA to dopamine. In adrenergic neurons, dopamine is further hydroxylated into noradrenaline, which is in turn methylated to yield adrenaline (see for review of catecholamine biosynthetic pathways Daubner *et al.*, 2011) (Figure 1.1). Whereas dopamine is produced in the cytosol of neurons, noradrenaline and adrenaline are mainly synthesized in synaptic vesicles. In order to access these vesicles for transformation into other

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[2] Parkinson's disease is later described in section 1.1.3.

[3] This enzyme is also referred to as DOPA decarboxylase despite its lack of substrate specificity.

catecholamines or for storage until its release, dopamine must use vesicular monoamine transporter 2 (VMAT2) (Cartier *et al.*, 2010; Eiden *et al.*, 2004). To reduce dopamine's presence in the cytosol, TH and aromatic amino acid decarboxylase are accordingly complexed to the cytosolic portion of VMAT2, ensuring rapid vesicular uptake and stabilization of dopamine by the acidic environment (Guillot and Miller, 2009). This mechanism is important considering that lingering cytosolic dopamine undergoes deleterious auto-oxidation reactions (see for review Segura-Aguilar *et al.*, 2014), which yield dopamine-derived quinones known to react with nucleophilic components in cells like cysteine residues of proteins (Belluzzi *et al.*, 2012). Quinones also participate in the synthesis of neuromelanin, a pigmented melanin analogue responsible for the dark coloration of certain catecholamine-rich structures[4] (Sulzer *et al.*, 2000).

Dopaminergic neurotransmission occurs by phasic or tonic release (Grace, 2000) (Figure 1.2). Phasic firing involves quick spiking activity produced in dopaminergic neurons in response to a proper stimulus-mediated action potential (Grace and Bunney, 1984*a*) or to presynaptic receptor activation by neighbouring neurons (Rice *et al.*, 2011). Phasic bursts of dopamine carry proper information to targets and provide them with spiking currents necessary for long-term potentiation (Wickens *et al.*, 1996). In opposition, tonic firing represents the slower baseline pacemaker activity of dopaminergic neurons (Grace and Bunney, 1984*b*) and rather fulfil a modulatory role by regulating postsynaptic targets that are innervated by other afferents. Once secreted, dopamine binds G-protein linked metabotropic receptors that exist in five types (Missale *et al.*, 1998) and in heterocomplexes with other dopamine or non-dopamine receptors (Borroto-Escuela *et al.*, 2017), allowing for a great number of outcomes at the postsynaptic membrane. Dopamine receptors can be conveniently separated into the stimulatory, low-affinity D1-like (D1 and D5) and inhibitory, high-affinity D2-like (D2-D4) families. It is commonly accepted that D2-like receptors are more sensitive to tonic neurotransmission than D1-like receptors that rather transduce phasic bursts

[4] Neuromelanin's role in health and disease is unclear. On the one hand, it chelates transition metals like iron ions and may therefore protect catecholaminergic neurons from Fenton reactions that produce reactive oxygen species (ROS). However, under certain circumstances, neuromelanin also departs itself from these metals, acting thereby as an agent of neurotoxicity (Segura-Aguilar *et al.*, 2014).

(Dreyer *et al.*, 2010), but paradigms have been shifting in recent years (Yapo *et al.*, 2017).

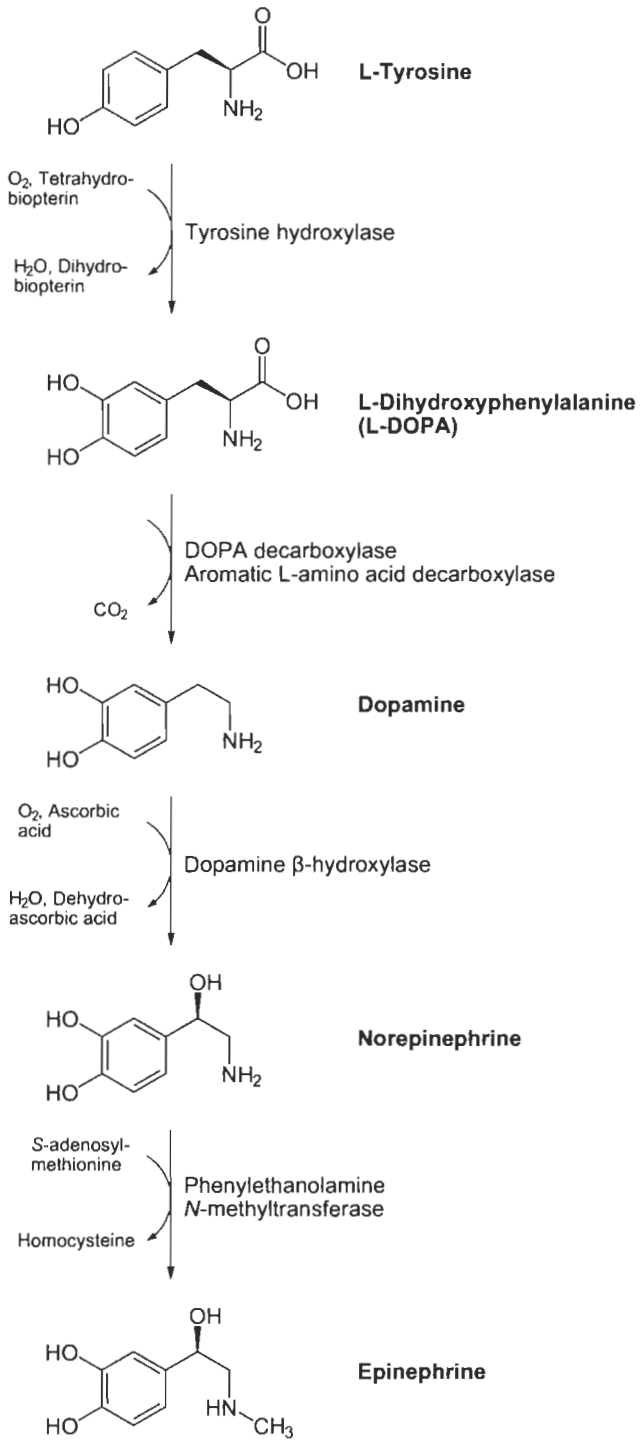

Figure 1.1 **The catecholamine biosynthetic pathway.**
(From Wikimedia Commons: https://commons.wikimedia.org/wiki/File:Biosynthese_Catecholamine.svg.)

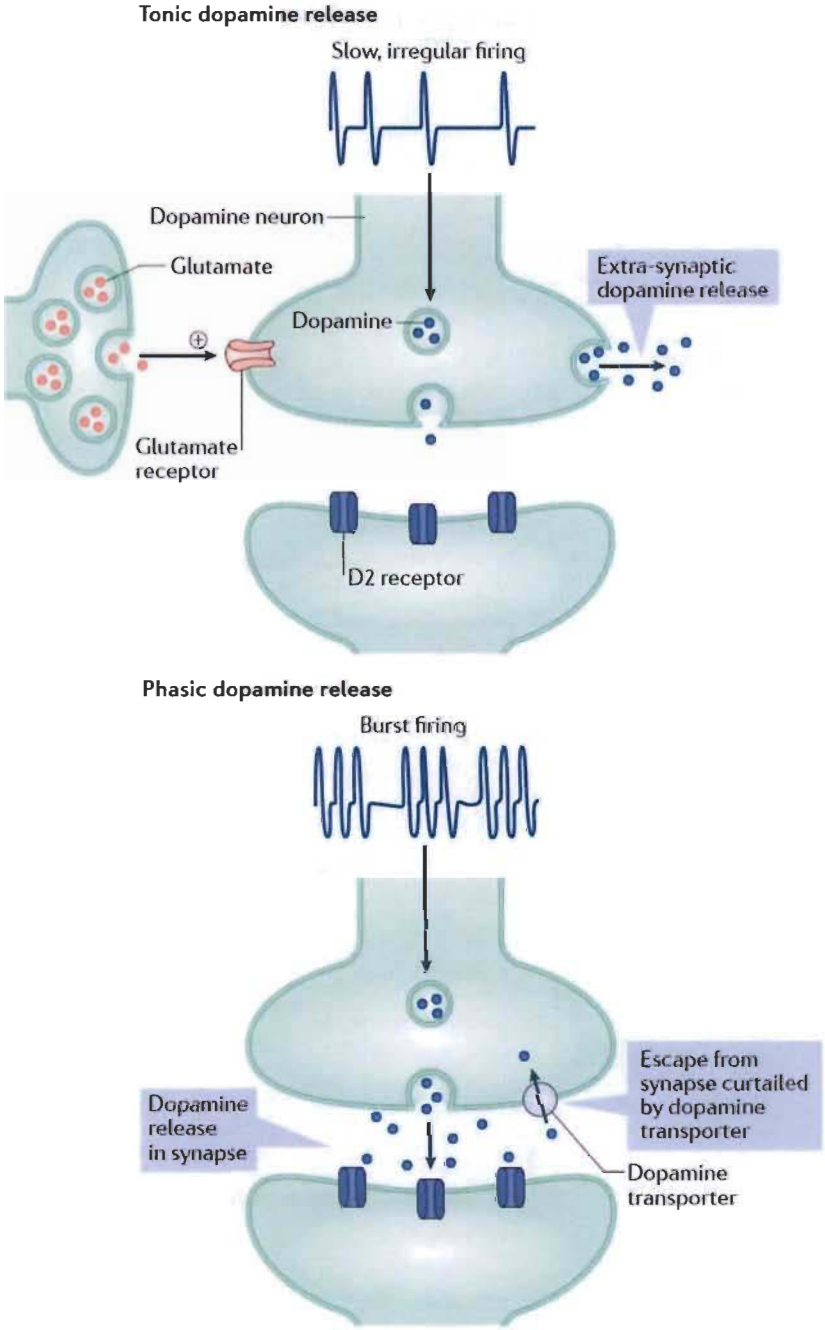

Figure 1.2 **Tonic and phasic dopaminergic neurotransmission.**
Top: Constant low levels of extracellular dopamine are ensured by tonic release, dependent on slow irregular firing and modulated by glutamatergic afferents. Release mainly occurs extrasynaptically where catechol-*O*-methyltransferase ensures dopamine degradation. Bottom: Phasic dopamine neurotransmission is triggered by burst firing of dopaminergic neurons, which release very high levels of dopamine into the synaptic cleft that stimulate postsynaptic dopamine receptors. Phasic dopamine is rapidly inactivated by removal from the synaptic cleft via the dopamine transporter. (Adapted from Grace, 2016.)

Owing to the relatively slow response of metabotropic dopamine receptors, which involves voltage-gated ion channels, dopamine earns the title of neuromodulator, alongside serotonin, noradrenaline and histamine, to name a few. Neuromodulators are characterized by their capacity to regulate diverse populations of neurons, in contrast to other neurotransmitters binding fast-acting ionotropic receptors on more explicitly determined postsynaptic targets. As such, dopamine mainly acts by modulating the excitability of receptive postsynaptic elements to afferent inputs instead of directly affecting their conductance (O'Donnell, 2003). In addition, 60-70% of neurotransmission events occur ectopically, outside the synaptic cleft, implying significant delays in receptor activation, extension of spatial domains to somatodendritic and presynaptic targets, and diluted mediation (Descarries *et al.*, 1996; Sesack *et al.*, 2003; Venton *et al.*, 2003).

Aside from synaptic overflow, negative feedback onto presynaptic D2 autoreceptors and presynaptic influences from other neurotransmitters, dopaminergic neurotransmission is principally regulated by reuptake or catabolism (Figure 1.3). In particular, phasic bursts of dopamine are largely cleared via the Na^+-Cl^- – dependent dopamine transporter (DAT) on presynaptic elements (McElvain and Schenk, 1992; Schroeter *et al.*, 2000). Tonic dopamine, on the other hand, occurs mainly outside of synapses and is therefore predominantly metabolized by catechol-*O*-methyltransferases. Once in the cytosol, dopamine is rapidly catabolized by monoamine oxidase and aldehyde dehydrogenase into 3,4-dihydroxyphenylacetic acid that is further excreted and transformed extracellularly by catechol-*O*-methyltransferase into homovanillic acid (Eisenhofer *et al.*, 2004). Noteworthy, two isoforms of the key degradation enzyme exist: monoamine oxidase A, expressed in CNS neuronal and glial cells but also in peripheral adrenergic neurons, predominantly metabolizes serotonin and catecholamines, whereas monoamine oxidase B, which makes up 80% of this class of enzymes in dopaminergic loci, metabolizes catecholamines with a preference for dopamine[5].

[5] Preferential midbrain and striatal expression renders monoamine oxidase B favoured targets in the elaboration of treatments for Parkinson's disease (Youdim and Weinstock, 2004).

Both of them were found to generate reactive oxygen species (ROS), which contribute to oxidative stress in dopaminergic neurons[6] (Andersen *et al.*, 1994; Graham, 1984).

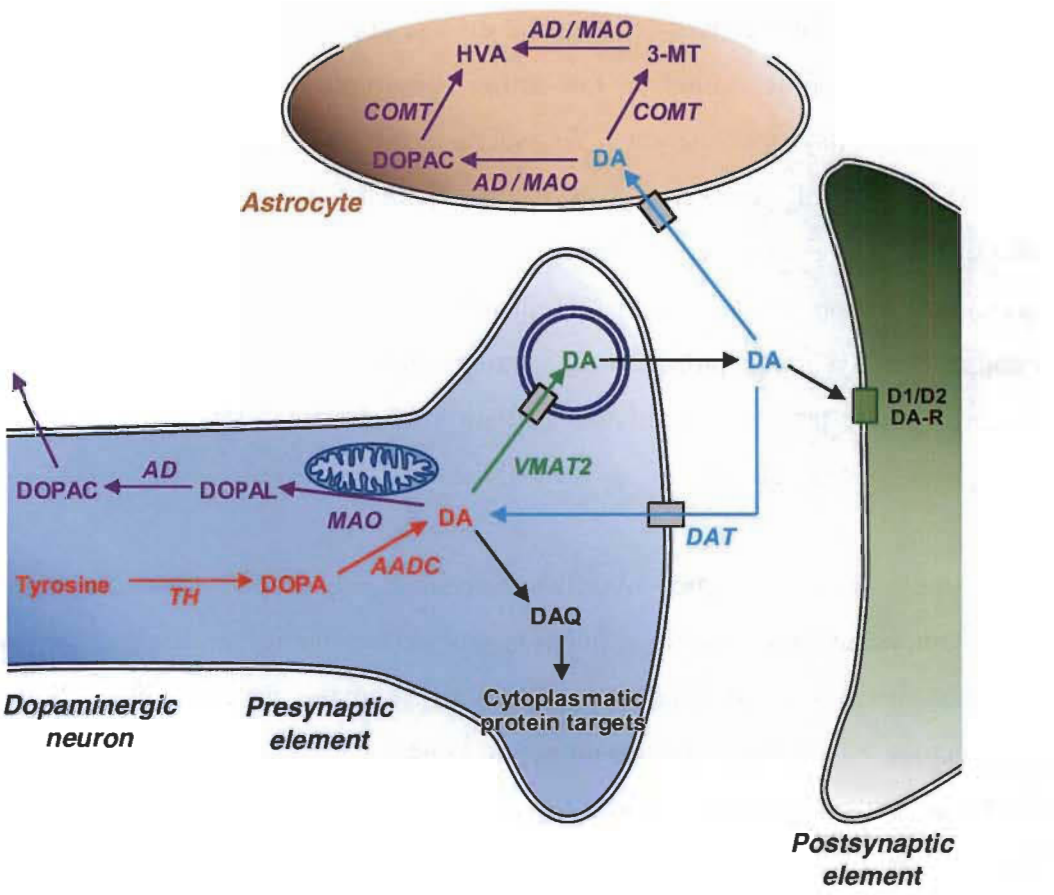

Figure 1.3 **Dopamine reuptake and catabolism.**
Besides presynaptic reuptake and metabolism described in the text, dopamine can also be absorbed by astrocytes via a transporter whose identity remains unknown. There, it undergoes degradation mediated by catechol-*O*-methyltransferase before it is transformed by monoamine oxidase and aldehyde dehydrogenase into homovanillic acid (Dahlin et *al.*, 2007; Hansson and Sellstrom, 1983; Takeda et *al.*, 2002). 3-MT, 3-methoxytyramine; AADC, aromatic amino acid decarboxylase; AD, aldehyde dehydrogenase; COMT, catechol-*O*-methyltransferase; D1/D2 DA-R, D1-like/D2-like dopamine receptors; DA, dopamine; DAQ, dopamine quinones; DAT, dopamine transporter; DOPA, L-3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; HVA, homovanillic acid; MAO, monoamine oxidase; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2.

[6] Oxidative stress is later described in section 1.2.4.

1.1.2 Central dopaminergic systems

As previously mentioned, dopaminergic neurons are divided into groups concurring to cytoarchitectonic and chemoarchitectonic stains, best described in the mouse (Fu *et al.*, 2012) and rat (Ikemoto, 2007). Most of these neuronal clusters are found in midbrain structures. Explicitly, groups A8-A10 correspond neuroanatomically to the retrorubral field, the substantia nigra and the ventral tegmental area, respectively. On the other hand, group A11 is rather located in the hypothalamic arcuate nucleus and projects to the median eminence where it releases dopamine in the circulation via the hypophyseal portal system to influence the secretion of pituitary hormones, notably prolactin (see for review Grattan, 2015). Neurons of the A11 group that constitute this dopaminergic system, termed the tuberoinfundibular pathway, will not be discussed here as its endocrinological role dwells outside of the scope of our present work.

Midbrain dopaminergic neurons receive afferents from the striatum, the brainstem pedunculopontine nucleus, and the lateral habenula via the globus pallidus, the superior colliculus and the rostromedial tegmental nucleus. In turn, they extend their projections to various subcortical and cortical targets, principally the striatum, thalamus, amygdala, globus pallidus and hippocampus (see for reviews Haber, 2014; Lanciego *et al.*, 2012). As such, the midbrain participates in basal ganglia circuitry, principally by providing a dopaminergic input to gamma-aminobutyric acid (GABA)-producing medium spiny neurons of the striatum (Figure 1.4). The latter receive incoming goal-directed messages from various cortical areas, acting as a gateway to basal ganglia that process and manage the information before it is transmitted to the thalamus for action[7] (Schultz, 2002). Basal ganglia allow action selection by exerting a modulatory foothold on cortical information via the stimulatory direct and inhibitory indirect pathways, which oppose each other to activate or silence the thalamus. Midbrain dopamine serves to modulate

[7] There are currently two opposing models to explain action selection by dopaminergic inputs to basal ganglia. One of these is the Go-NoGo model, which stipulates that direct and indirect pathways act in opposition to, respectively, allow actions (Go) or inhibit them (NoGo) (Frank *et al.*, 2004; Shen *et al.*, 2008). Contrarily, the prepare-and-select model grants basal ganglia dopamine more complicated functions, wherein initial direct pathway activation in reaction to stimuli allows the preparation of a set of appropriate responses, and subsequent indirect pathway activity permits the selection of one action among these choices (Keeler *et al.*, 2014).

this tight control and to allow more activation of the thalamus by phasically activating direct pathway medium spiny neurons that harbour stimulatory D1, and by tonically inhibiting indirect pathway medium spiny neurons that harbour inhibitory D2 receptors.

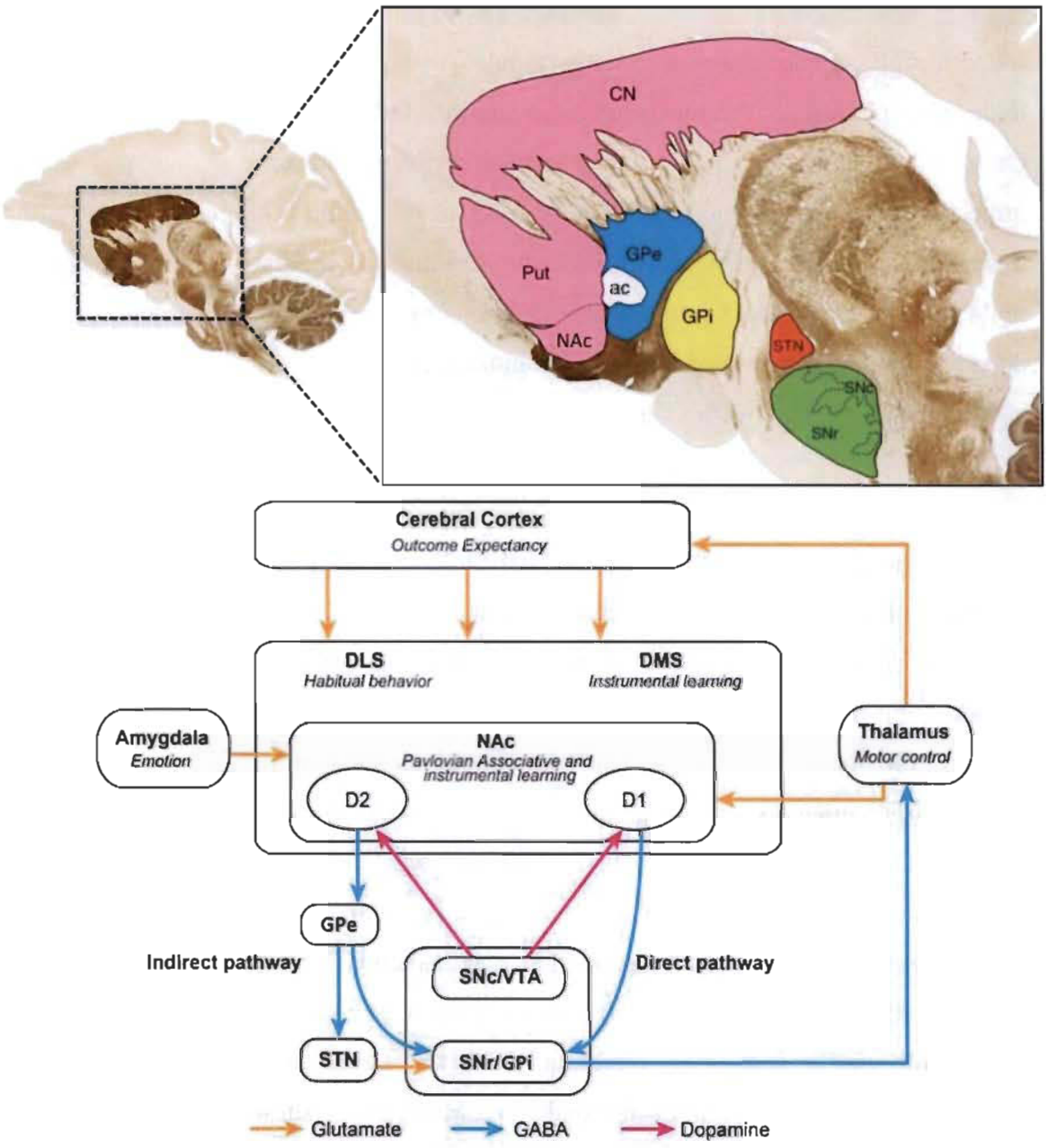

Figure 1.4 **Basal ganglia circuitry in decision-making.**
Top: Basal ganglia comprise the caudate-putamen, and the internal and external globus pallidus. The subthalamic nucleus, substantia nigra pars compacta and ventral tegmental area are sometimes considered as secondary basal ganglia. Bottom: At rest, the thalamus, central activator

of actions, is kept in check by the substantia nigra pars reticulata and internal globus pallidus. This hold can be lifted through the direct pathway when the cerebral cortex activates medium spiny neurons of the striatum, which in turn lifts the inhibition on the thalamus exerted by the internal globus pallidus and substantia nigra pars reticulata, thereby allowing the achievement of the goal. Conversely, a tighter control of the thalamus via the indirect pathway requires the activation of medium spiny neurons that will rather inhibit the external globus pallidus, which ultimately leads to the activation of the internal globus pallidus and substantia nigra pars reticulata responsible for silencing the thalamus and thus thwarting the achievement of the goal. Midbrain dopaminergic neurons fine-tune the sensitive equilibrium of this basal ganglia circuit via aforementioned mechanisms. ac, anterior commissure; CN, caudate nucleus; D1, D1-like dopamine receptor-expressing medium spiny neurons; D2, D2-like dopamine receptor-expressing medium spiny neurons; DLS, dorsolateral striatum; DMS, dorsomedial striatum; GPe, external globus pallidus; GPi, interior globus pallidus; NAc, nucleus accumbens; Put, putamen; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VTA, ventral tegmental area. (Top adapted from Lanciego *et al.*, 2012; bottom adapted from Macpherson *et al.*, 2014.)

1.1.2.1 The nigrostriatal pathway

Restricted to the ventrolateral region of the midbrain, the substantia nigra constitutes the first dopaminergic structure to be identified in 1786 in a study addressing neuromelanin distribution in the human brain (Parent and Parent, 2010; Vicq D'Azyr, 1786). However, it was only much later that its role in motor control was recognized following its association with Parkinson's disease (Bremer, 1920; Brissaud, 1895; Hassler, 1939). The substantia nigra harbours group A9 cells, totalling approximately 16 000 in rats (Oorschot, 1996) and 160 000-232 000 in macaque monkeys (German *et al.*, 1988; Percheron *et al.*, 1989), and is thus recognized as the most dense population of dopaminergic neurons (Figure 1.5). Their localization coincides with the pars compacta region of the substantia nigra, dorsal to the pars reticulata zone that contains more loosely packed GABAergic neurons.

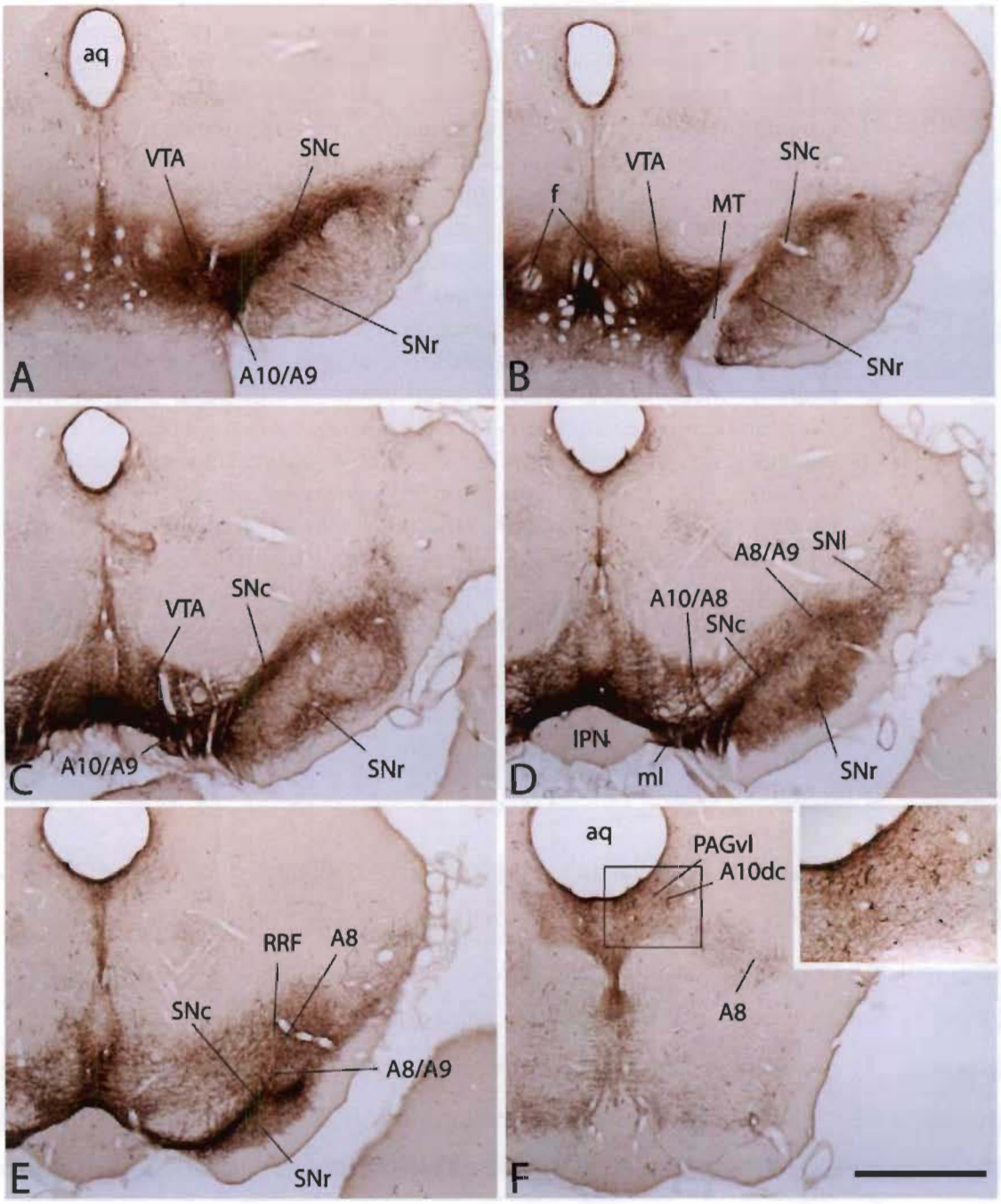

Figure 1.5 **A8-A10 dopaminergic neuronal clusters in the rat brain.** Immunohistochemical staining of dopaminergic neurons in rat brain coronal sections organized rostrocaudally (A-F) depict the neuroanatomical location of each cluster at the level of the midbrain. Particularly important to us, the substantia nigra pars compacta is found laterally to the ventral tegmental area. Scalebars: A-F, 1 mm; inset F, 0.5 mm. aq, cerebral aqueduct; dc, dorsocaudal; f, fornix; IPN, interpeduncular nucleus; ml, medial lemniscus; MT, mammillothalamic tract; PAGvl, ventrolateral periaqueductal grey; RRF, retrorubral field; SNl, substantia nigra pars lateralis. (From Yetnikoff et *al.*, 2014.)

The substantia nigra pars compacta provides the greatest dopaminergic input to the striatum, particularly – but not restricted (Yetnikoff *et al.*, 2014) – to the dorsal striatum better known in primates as the caudate nucleus and putamen or simply the caudate-putamen[8] (Figure 1.6). Nigrostriatal dopaminergic neurons project in a fairly mediolateral topographical manner (Lynd-Balta and Haber, 1994) to an estimated 2.8 million and 31 million striatal neurons in rats and macaques, respectively, each axon ramifying abundantly and harbouring some 500 000 varicosities (Andén *et al.*, 1966). This affords the dorsal striatum with a near-total exposure to dopamine input, implying a significant divergence factor of 300-400 between nigrostriatal neurons and striatal targets. The importance of nigrostriatal arborizations is even better appreciated when taking into account that, at least in rats, one striatal neuron is innervated by an average of 100-200 dopaminergic neurons and that one nigrostriatal axon is estimated to interact with some 75 000 striatal neurons (Matsuda *et al.*, 2009). Such a profuse dopaminergic innervation necessarily infers critical modulatory effects on the dorsal striatum, which receives important glutamatergic inputs from the cerebral cortex, thalamus and amygdala (Donoghue and Herkenham, 1986; Graybiel, 1990; Sadikot *et al.*, 1992).

Striatal modulation by nigrostriatal neurons is best appraised in corticostriatal motor circuits. As it consists in the most important source of tonic dopamine to the striatum, the substantia nigra pars compacta especially inactivates indirect pathway medium spiny neurons expressing D2 receptors, thereby downgrading the inhibition on the thalamus. In Parkinson's disease, degeneration of nigrostriatal dopaminergic neurons disrupts tonic firing in the striatum, which allows for the D2 receptor-modulated indirect pathway to exert its full inhibitory effect on the thalamus. This pathophysiological feature dwells at the core of parkinsonian motor symptoms, chiefly bradkyinesia/akinesia and rigidity (Albin *et al.*, 1989). In fact, several animal models employ nigrostriatal pathway lesions of all sorts to emulate parkinsonian symptoms (Deumens *et al.*, 2002; Duty and Jenner, 2011; Jackson-Lewis *et al.*, 2012; Nagatsu, 1997; Pinna and Morelli, 2014; Ungerstedt, 1968).

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[8] Whether in rodents, primates or humans, the caudate-putamen will hereafter be termed dorsal striatum.

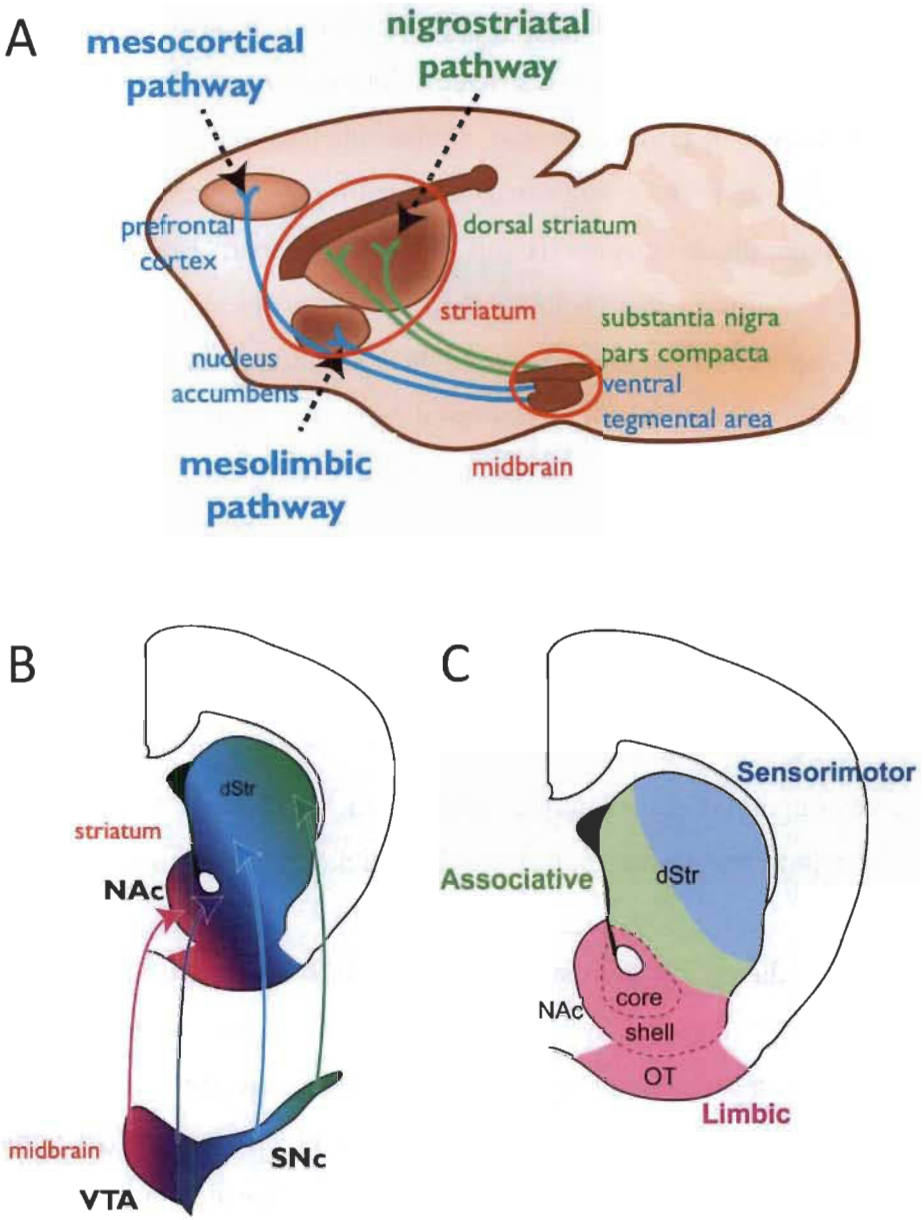

Figure 1.6 **Mesocorticolimbic and nigrostriatal projections.**
Both mesocorticolimbic and nigrostriatal pathways originate in the midbrain, illustrated sagittally (A) and coronally (B). The nigrostriatal pathway projects especially onto dorsal regions of the striatum, while the mesocorticolimbic pathway mainly innervates the ventral striatum (mesolimbic) and the prefrontal cortex (mesocortical). Midbrain projections onto the striatum are fairly topographic, depicted by colour gradients (B), and modulate specific corticostriatal circuits (C). Afferents supplied by sensorimotor, associative and limbic cortical areas are also distributed in a particular neuroanatomical manner, which bears implications on the behavioural programs that different midbrain projections can control (C). dStr, dorsal striatum; OT, olfactory tubercle. (Adapted from Chuhma *et al.*, 2017.)

1.1.2.2 The mesocorticolimbic pathway

In the first half of the 20th century, neuroscientists bore a rather unyielding vision of basal ganglia as movement processing centres, still somewhat enduring today. More complex functions were much later ascribed to these structures upon the identification of corticostriatal circuit loops that did not involve motor areas (Alexander *et al.*, 1990; Haber *et al.*, 1985; Young *et al.*, 1984), and the parallel discovery of a particular striatal subdivision located ventrally and surrounding the anterior commissure termed the nucleus accumbens (Heimer, 1978). In the meantime, a collection of studies were showing rats to electrically self-stimulate certain regions of their brain as positive reinforcement (Olds and Milner, 1954), later found to receive dopaminergic inputs (Crow, 1972) and to be sensitive to dopamine agonists or antagonists (Stein, 1964; Wise and Bozarth, 1987). Pioneering microdialysis work from the laboratory of Prof. Gaetano Di Chiara at the University of Cagliari, Italy, later confirmed the nucleus accumbens identity of this region, shown to be sensitive to dopamine agonizing drugs[9] (Di Chiara and Imperato, 1988).

The nucleus accumbens, found in the ventral striatum, is one of the key dopamine terminals of the mesocorticolimbic pathway. This system is composed of the mesolimbic and mesocortical pathways, originating from groups A8 and A10 neurons (Figure 1.5). Both lodged in the midbrain, group A10 coincides with the ventral tegmental area medial to the substantia nigra pars compacta, whereas group A8 is contained in the retrorubral field[10] located dorsocaudally to the substantia nigra pars compacta (Fu *et al.*, 2012; Halliday *et al.*, 2012*a*). Mesolimbic dopaminergic neurons project to the striatum, preferentially to ventral areas such as the nucleus accumbens and the olfactory tubercle, whereas the mesocortical pathway connects the ventral tegmental area and retrorubral

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[9] Di Chiara's group also made the groundbreaking discovery that the nucleus accumbens is in fact divided into core and shell subregions, which operate very differently in reward valuation (Pontieri *et al.*, 1995).

[10] The retrorubral field does not account for much of the dopaminergic innervation provided by the midbrain and therefore little attention is paid to it in this thesis. Likewise, the olfactory tubercle is a secondary ventral striatum structure whose functions in the mesolimbic pathway will not be granted much focus.

field to the prefrontal cortex, but these connections are much less dense than striatal ones (Figure 1.6).

Akin to nigrostriatal dopaminergic neurons, the ventral tegmental area operates via modulation of corticostriatal circuits at the level of the striatum. However, inasmuch as the nigrostriatal pathway is known for tonically enabling movement, the mesocorticolimbic pathway stands out as a reward processing system endowed with limbic and executive dimensions permitted by phasic bursts of dopamine (Haber *et al.*, 2000). There exists today a wide array of pharmacological paradigms mimicking dysfunctional dopaminergic neurotransmission in the ventral striatum and prefrontal cortex (España and Jones, 2013; Jones *et al.*, 2011). Notably, these models bear a striking likeness to pathologies such as addiction and other neuropsychiatric disorders (Bassareo *et al.*, 2011; Di Chiara *et al.*, 1993; Grace, 2000, 2016; Tanda *et al.*, 1996).

1.1.2.3 Converging and diverging functions

While nigrostriatal and mesocorticolimbic pathways appear to operate very differently considering their fairly different roles in human behaviour and disease, they actually function in similar ways. In fact, aside from their archetypal tonic-motor and phasic-reward instructions, the nigrostriatal and mesocorticolimbic pathways are both endowed with the following characteristics: 1) they comprise dopaminergic neurons that can fire both phasically and tonically depending on the information conveyed by dopamine; 2) they project onto striatal medium spiny neuron D1-like and D2-like receptors thereby modulating corticostriatal circuitry, and; 3) their neuroanatomical origins receive feedback projections from the targets they innervate that refine dopaminergic neurotransmission. Despite the widely acknowledged movement enabling functions of the nigrostriatal pathway, regulation of the striatum by its afferents also holds a role in many other decision-making processes, learned behaviours and habits arising from outcome prediction, especially with regard to sensorimotor functions. These roles may have to do with time scales of dopamine impulses, where sensory-evoked phasic firing encodes teaching signals for the

acquisition of a behaviour, (Chaudhury *et al.*, 2013; Redgrave and Gurney, 2006; Schultz *et al.*, 2006), and where baseline tonic firing enables the proper selection of these pre-learned behaviours by the basal ganglia (Ellwood *et al.*, 2017; Hikosaka *et al.*, 2000; Redgrave *et al.*, 1999). Both firing modes are impaired in Parkinson's disease (Beeler, 2011; Marklund *et al.*, 2009; Redgrave *et al.*, 2010), although the loss of dopamine tone is more typically acknowledged with respect to the motor symptoms it produces. The same holds true for the mesocorticolimbic pathway, which is also devoted to tonic enabling of pre-learned behaviours aimed at seeking pleasure and rewards that had previously required a learning period provided by phasic firing of dopamine (Ellwood *et al.*, 2017).

In a larger sense, nigrostriatal and mesocorticolimbic dopamine operates both as a phasic carrier of predicted outcomes and a tonic enabler of postsynaptic neurons, from which originates its dual informational/modulatory role (Saddoris *et al.*, 2013; Schultz, 1998). Indeed, in both pathways, fast phasic bursts encode reward, slow phasic changes inform on punishing/ambiguous elements, and tonic maintenance enables postsynaptic functions (Schultz, 2007). In this respect, an overarching theory proposes CNS dopamine to mediate the basic reactivity of animals to their environment, ensuring their survival by permitting economic decision-making, reward recognition, monitoring of uncertainty and detection of punishing elements via the prediction of favourable or unfavourable outcomes, wherein dopamine encodes prediction errors (Schultz, 2007) and learning entails progressive reduction of prediction errors. This is applicable to motor, social, cognitive, motivational and reward processes regulated by these pathways.

This begs the following questions: Why are mesocorticolimbic dysfunctions highly associated with addiction, whereas nigrostriatal ones are linked with Parkinson's disease? And why is the mesocorticolimbic pathway largely spared in Parkinson's disease? At first glance, behavioural and pathological disparities in these pathways are the result of rather clear neuroanatomical distinctions between their origins, embedded in different environments, and between their targets, innervated by distinct cortical inputs and expressing differential distribution patterns of D1-like and D2-like

receptors (Bertran-Gonzalez *et al.*, 2008; Centonze *et al.*, 2003; Lanciego *et al.*, 2012). As such, even if both pathways operate with a similar dopamine interface regulating corticostriatal circuitry, they modulate different cortical programs. Other sources of dopaminergic dichotomies in health and disease may rather lie in differential exposures to noise-to-signal (tonic-to-phasic) ratios. Indeed, the dorsal striatum is submitted to a greater noise-to-signal ratio than the ventral striatum, due to the marked tonicity of nigrostriatal neurons responsible for very high basal release of dopamine in the dorsal striatum (Dreyer, 2014; Zhang *et al.*, 2009). This allows two implications: first, phasic firing does not have the same weight in the dorsal striatum than in the ventral striatum, which explains the great differences in the information carried by dopamine in these two areas; second, high basal pacemaking activity may in itself constitute a risk factor in neurodegeneration, later discussed in the context of Parkinson's disease.

In keeping with the discriminating elements that pick these pathways apart, the remainder of this thesis will address the apparent preferential degeneration of nigrostriatal dopaminergic neurons in various conditions, as in Parkinson's disease next addressed, and the behavioural alterations that may arise from this neurodegeneration.

1.1.3 Parkinson's disease

The year 2017 marked the 200th anniversary of Dr. James Parkinson's *An Essay on the Shaking Palsy* wherein were described 6 subjects suffering from a pathological state that he termed at the time *paralysis agitans* (see for historical reviews Obeso *et al.*, 2017; Parent and Parent, 2010). In this essay, Dr. Parkinson illustrated with acute precision several symptomatic aspects exhibited by his subjects that we know today were effectively attributable to Parkinson's disease. Indeed, he accurately identified the gradual nature of both onset and progression, as well as the expression of gait disturbances, forward flexion of the trunk, resting tremors, sleeping troubles and gastrointestinal issues in these individuals (Parkinson, 1817). Charcot later expanded on these observations to include bradykinesia and rigidity to the key features of the disease, and proposed to rename the *paralysis agitans* disorder Parkinson's disease upon

observing that the loss of motor functions was not attributable to weakness (palsy) (Charcot, 1877).

Since the publication of this pioneering report, the field of Parkinson's disease has phenomenally progressed. In this age of effervescent neuroscientific developments, clinical profiles are more accurately drawn, long-term patient management is improving, and the pursuit of etiological and pathophysiological explanations is starting to bear fruit (Chaudhuri and Jenner, 2017). The preferential degeneration of the dopaminergic nigrostriatal pathway is well appreciated today and additional pieces of the puzzle are continuously falling into place, especially with respect to non-dopaminergic affections (Hall *et al.*, 2014; Qamhawi *et al.*, 2015; Remy, 2005). The following section summarizes state-of-the-art knowledge on Parkinson's disease and draws particular attention to the apparent vulnerability of nigrostriatal dopaminergic neurons in this pathology. The current state of the field regarding symptomatology, diagnostics, pathological basis, genetic and idiopathic etiopathogeneses, experimental models, neuroimaging, biomarkers, treatments and unmet needs is extensively reviewed elsewhere by preeminent researchers in a special collaborative publication underlining the second centenary of the shaking palsy (Obeso *et al.*, 2017).

1.1.3.1 Clinical symptomatology and treatments

As highlighted by Dr. Parkinson's and others' early observations, a wide array of motor and non-motor symptoms characterize Parkinson's disease, which both enable and complicate the process for clinicians to arrive to a diagnosis. Indeed, this disorder is pronouncedly heterogeneous in terms of inter-individual symptomatology, but also with respect to disease progression and sensitivity to treatments (Lang and Obeso, 2004) (Figure 1.7). For the sake of example, the age of onset can range from as early as the third decade of life to very old ages (Pagano *et al.*, 2016); initial motor manifestations may affect either upper or lower limbs on either side of the body (Roberts *et al.*, 2017); deterioration rates may present benign or malignant courses (Jankovic *et al.*, 1990), and; distinct subpopulations of patients may respond differently to dopamine treatments

(Miller *et al.*, 2017). The clinical landscape is further convoluted by the existence of idiopathic and genetic forms of the disease. As a result, the dogma according to which Parkinson's disease is a single uniform pathology is slowly ceding to newer concepts handling it as a multisystem syndrome or as Parkinson's *diseases* (Marras and Lang, 2008). Clinicians are only beginning to understand how to harness these inter-individual differences in order to improve treatments and clinical trials (Athauda and Foltynie, 2016*a*; Marras and Chaudhuri, 2016; Nutt, 2016; Payami, 2017).

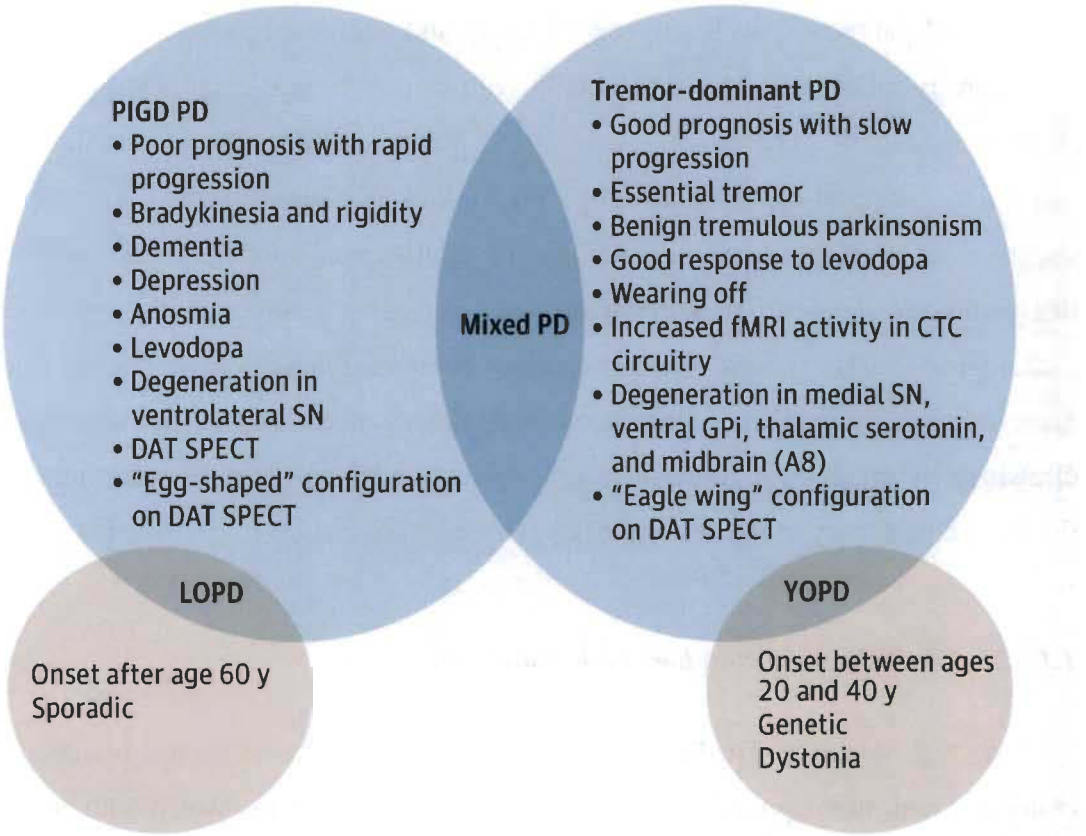

Figure 1.7 **The various forms of Parkinson's disease.**
Parkinson's disease is characterized by a wide spectrum of pathological expressions. Based on symptomatological peculiarities and disease evolution, this pathology can be divided into postural instability and gait difficulty, tremor-dominant, mixed, late-onset and young-onset Parkinson's disease. CTC, cerebellothalamocortical; fMRI, functional magnetic resonance imaging; LOPD, late-onset Parkinson's disease; PD, Parkinson's disease; PIGD, postural instability and gait difficulty; SN, substantia nigra; SPECT, single-photon emission computed tomography; YOPD, young-onset Parkinson's disease. (From Thenganatt and Jankovic, 2014.)

The most prominent feature of Parkinson's disease remains the presence of motor symptoms expressed in a majority of patients as a classical triad of resting tremors, bradykinesia and rigidity; two of them are required for diagnostic purposes (Parkinson Québec, 2018). Later, motor disabilities evolve to implicate gait and postural reflex disturbances, though nowadays they are no longer required to emit a diagnosis. Non-dopaminergic (extranigral) motor features also arise and consist in falls, freezing of gait, speech impairment and difficulty swallowing. Accompanying these motor symptoms are equally disabling non-motor manifestations, such as psychiatric disturbances, dementia and autonomic failure. Granted Parkinson's disease is not a fatal condition, patients are expected to live almost as long as healthy individuals and the primary cause of death appears to be pneumonia (Iwasaki *et al.*, 1990; Pennington *et al.*, 2010), although manifestations of more advanced symptoms, such as falls and swallowing problems, may lead to premature accidental mortalities.

Adequate management of the incapacitating motor symptoms in Parkinson's disease was only made possible 150 years after the issuing of the shaking palsy essay when George C. Cotzias and his colleagues published a trailblazing paper on L-DOPA (Cotzias *et al.*, 1967) that remains, with certain modern adjustments, the benchmark therapy (Freitas *et al.*, 2016, Lotia and Jankovic, 2016). While its function is unmistakably to restore tonic dopaminergic neurotransmission (Dreyer, 2014), exactly how L-DOPA operates remains hazy as of yet. Indeed, L-DOPA may not act as a simple precursor for dopamine allowing for its tonic replenishment in the striatum: some have advanced its possible role as a neurotransmitter itself (LeWitt and Fahn, 2016). Nevertheless, a slew of clinical trials, intervention studies and longitudinal observations ceaselessly validate L-DOPA's efficacy in alleviating motor symptoms (LeWitt and Fahn, 2016; Olanow, 2014; Yahr, 1969), although several collateral effects are manifested by acute and chronic users, the most notable being dyskinesias, impulse control disorders and wearing-off in longstanding administration (Aquino and Fox, 2014; Cilia, 2012; Parkinson Study Group, 2004). In the hopes of delaying the apparition of these side effects, some patients are initially administered dopamine

receptor agonists or monoamine oxidase B inhibitors until these milder medicines can no longer manage the motor symptoms as the disease progresses (Figure 1.8).

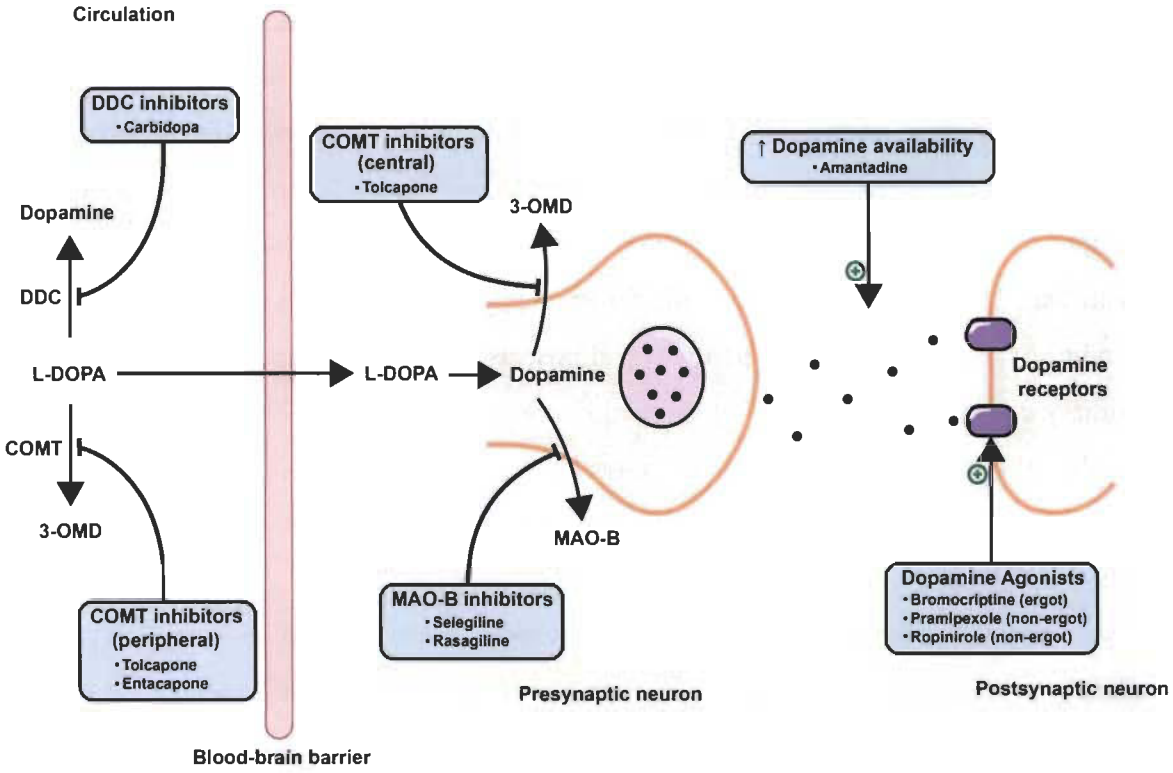

Figure 1.8 **Current parkinsonian drug therapies.**
On the left hand side are depicted adjuvant agents necessary for L-3,4-dihydroxyphenylalanine (L-DOPA) to reach the brain considering its propensity to be metabolized in the periphery, thereby dampening its efficacy. Other drugs are illustrated on the right, including more recent dopamine agonists and inhibitors of the reuptake and metabolism of dopamine. It is important to note that no therapy discovered to date achieves curative endpoints in Parkinson's disease. 3-OMD, 3-*O*-methyldopa; DDC, DOPA decarboxylase. (From http://step2.medbullets.com/step2-3-neurology/121704/parkinsons-disease-drugs.)

Inter-individual irregularities not only complicate diagnosis, but also shape treatment efficacy in Parkinson's disease. Some patients, usually younger diagnostics, will develop a benignly progressive form presenting few non-dopaminergic symptoms and these usually respond well to L-DOPA treatments. Others may suffer from a more malignant, rapidly progressing disease with typically early manifestations of non-dopaminergic motor and non-motor symptoms that resist to L-DOPA interventions

(Lang and Obeso, 2004). Regardless of age at onset, older individuals are more likely to express L-DOPA-resistant motor symptoms in conjunction with cognitive decline and autonomic failure (Kempster *et al.*, 2007). In patients with severe motor fluctuations, other strategies may be employed, for instance deep brain stimulation of the subthalamic nucleus or internal globus pallidus in basal ganglia circuitry, performed in some 150 000 patients (Obeso *et al.*, 2017) since its groundbreaking successful utilization in the 1990s (Pollak *et al.*, 1993). Akin to L-DOPA therapy, the mechanistic underpinnings of deep brain stimulation are ill understood (Lozano and Lipsman, 2013) and adverse effects may supervene in the likes of impaired verbal fluency, depression and even suicidal tendencies (Benabid *et al.*, 2005; Lang *et al.*, 2006). To these motor symptom therapies are sometimes layered supplementary treatments to address psychiatric manifestations arising from the disease or motor symptom treatments themselves, for instance cholinesterase inhibitors for cognitive impairment (Emre *et al.*, 2004), antidepressants and cognitive-behavioural therapy for mood disorders (Dobkin *et al.*, 2011; Menza *et al.*, 2008; Richard *et al.*, 2012) or neuroleptics for psychoses (Cummings *et al.*, 2014).

Today, still, no therapy has been fruitful in modifying the course of Parkinson's disease, even less so in providing hope for curative ends. This may partly be attributable to the failure of clinical trials or therapeutic strategies to take into account the various existing subgroups of Parkinson's disease patients. Sensitive subdivision of clinical subtypes at the early stages of the pathology is among the most pressing research aims to render treatments and their development more discriminative, especially considering the rapid rate at which the population is aging occasioning foreseeable rises in the incidence of age-related neurodegenerative disorders such as Parkinson's disease (Olesen *et al.*, 2012) (Table 1.1). In order to achieve this, reliable identification of premotor phases of the pathology, obviously requiring robust biomarkers, will need to be accomplished first.

Table 1.1

Projected prevalence of select neurological conditions in Canada

	Projection year				
	2011	2016	2021	2026	2031
	Number of prevalent cases * (rate per 100,000 population in projection year) *				
Alzheimer's disease and other dementias	340,200	395,000	461,700	554,200	674,000
	(2,000)	(2,200)	(2,400)	(2,700)	(3,100)
Brain injury (traumatic) †	550,900	595,700	640,100	685,600	730,300
	(1,600)	(1,700)	(1,700)	(1,800)	(1,800)
Cerebral palsy	75,200	79,800	84,300	89,300	94,200
	(200)	(200)	(200)	(200)	(200)
Epilepsy	321,700	345,400	368,100	392,100	415,800
	(1,000)	(1,000)	(1,000)	(1,000)	(1,000)
Multiple sclerosis	98,800	108,600	117,800	126,200	133,600
	(400)	(400)	(400)	(400)	(400)
Parkinson's disease/parkinsonism	84,700	99,000	116,800	138,800	163,700
	(500)	(500)	(600)	(700)	(700)
Spinal cord injury (traumatic) †	35,000	38,400	41,800	45,200	48,100
	(100)	(100)	(100)	(100)	(100)

NOTES:
Alzheimer's disease and other dementias and Parkinson's disease/parkinsonism projections were for a population age 40+ years. Multiple sclerosis projections were for a population age 20+ years. Traumatic spinal cord injury projections were for a population age 5+ years. Traumatic brain injury, cerebral palsy, and epilepsy projections were for a population age 0+ years.

* Data were rounded to the nearest hundred.

† Traumatic brain and spinal cord injuries were based on hospitalized cases, and excluded injuries that did not present to hospital.

SOURCE: POHEM–Neurological (Statistics Canada and Public Health Agency of Canada)[&]

[&] POHEM, population health model.

1.1.3.2 Prodrome and biomarkers

Abnormalities in the health of parkinsonian patients often appear years before a diagnosis is made during a phase termed the prodrome. These premotor or prodromal features are non-motor in nature, the most common manifestations being rapid eye movement sleep behavioural disorder, hyposmia, constipation, and psychiatric disturbances such as depression or anxiety (Pont-Sunyer *et al.*, 2015) (Table 1.2). Seeing as a substantial proportion of patients present one or several signs before onset, efforts have been deployed to elaborate specific and reliable premotor biomarkers to diagnose Parkinson's disease much earlier on (Lang, 2010). A sizeable hurdle to

overcome remains that these symptoms are hardly specific and are often encountered in the general population[11]. For the future development of prodromal biomarkers, the difficulty dwells in selecting individuals who will actually develop Parkinson's disease, which requires setting up arduous longitudinal studies.

Table 1.2

Markers of prodromal Parkinson's disease

Marker	Level of evidence	Approximate relative risk	Lead time	Testing cost/burden
Olfaction	High	5	??	Low / Moderate
REM Sleep Behavior Disorder[&]	High	50	13 years	Low (screens) to High (PSG)[&]
Somnolence	Moderate	1.8	??	Low
Restless legs (late onset)	Low	1.5	Short	Low
Constipation	High	2.5	> 15 years	Low
Orthostatic hypotension	Moderate	? 2-10?	2-5 years?	Low
Urinary dysfunction	Low-Moderate	2.1	?? >5 years	Low
Erectile dysfunction	Low-Moderate	1.2 mild 3.8 severe	5-10 years	Low
Depression/anxiety	High	1.8	Uncertain ?Biphasic	Low, but follow-up higher
Color vision	Low	2.5	>3 years?	Moderate
Subtle parkinsonism	Moderate	10	4-5 years	Moderate - High (Expert)
Quantitative motor testing	Moderate	3-4	5 years	Moderate
SN ultrasound	Moderate	15	Uncertain ? risk marker?	Moderate-High
Dopaminergic PET/SPECT[&]	Low (but high plausibility)	20	5 years	High
PD-related pattern on SPECT/PET	Low	?	?	High
Hippocampal hyperperfusion	Low	?	?	High
GI synuclein pathology[&]	Low	2?	?	High

For this table, only markers with prospective evidence of predictive value are included. For level of evidence, low implies a single study, moderate implies >1 high-quality study, high implies >4 high-quality studies. Lead time refers to the approximate time that the marker deviates from normal values (the time at which testing is reliably abnormal cannot be estimated for most markers). For testing cost, low indicates can be screened by questionnaire (does not require visit), moderate implies in-person assessment required but low cost (eg, research assistant), high implies extensive or expensive evaluation (>$300).

[&] GI, gastrointestinal; PET, positron emission tomography; PSG, Parkinson's Study Group; REM, rapid eye movement. (From Obeso *et al.*, 2017.)

The crying need for prognostic biomarkers in Parkinson's disease arises from its particular pathophysiological course. Indeed, the cardinal motor symptoms permitting diagnosis reflect the loss of nigrostriatal dopaminergic neurons and the ensuing decline in tonic dopamine neurotransmission that can no longer ensure enablement of postsynaptic targets in the dorsal striatum interconnected with corticostriatal circuits of movement production. Owing to compensatory neurocircuit redundancies in the basal

[11] Interestingly, considering the elevated prevalence of hyposmia in premotor patients (~75% when corrected for age) (Haehner *et al.*, 2009), Prof. Johannes Frasnelli's group from the University of Québec recently demonstrated that it was possible to tell apart individuals with Parkinson's disease from individuals with non-parkinsonian olfactory dysfunctions by evaluating olfactory and trigeminal sensitivity. Although both cohorts of subjects displayed impaired olfaction, Parkinsonian patients did not show trigeminal sensitivity deficits, contrarily to hyposmic non-parkinsonian individuals (Tremblay *et al.*, 2017).

ganglia and adjustments of dopamine receptor numbers, anywhere between 30-70% of nigrostriatal dopaminergic neurons and 50-80% of their striatal terminals have perished before the advent of clear motor symptoms (Bernheimer *et al.*, 1973; Cheng *et al.*, 2010; Fearnley and Lees, 1991), dulling any hope for reversal of the pathology. Like many other neurodegenerative disorders, Parkinson's disease begins many years before classical symptoms arise. However, to our advantage, it is a rather special pathology in that neurodegeneration appears to begin in regions outside the substantia nigra pars compacta whose progressive dysfunction may emerge as a phenotypic non-motor signature that could one day be exploited for emitting diagnoses at premotor stages. The following section grants a closer look at the pathophysiological mechanisms subtending the particular course of progression in Parkinson's disease.

1.1.3.3 Pathophysiology

More than a century after its identification by Félix Vicq d'Azyr (Vicq d'Azyr, 1786), the substantia nigra was freshly emerging as the neuroanatomical substrate of the motor syndrome in Parkinson's disease (Blocq and Marinesco, 1892; 1893; Brissaud, 1895). In parallel, Friedrich Heinrich Lewy was conducting histological assessments of parkinsonian brains, discovering the presence of cytoplasmic inclusions of aggregated proteins in the dorsal vagal nucleus and *substantia innominata* (Lewy, 1912), later identified in surviving nigral neurons of other patients and termed Lewy bodies in recognition of the former's seminal work (Trétiakoff, 1919). In the mid 1900s, the role of the substantia nigra in Parkinson's disease was further bolstered by findings enlisting dopamine as a key contributor (Carlsson, 1958; Ehringer and Hornykiewicz, 1960) and demonstrating the projection of nigral neurons to the dorsal striatum (Hornykiewicz, 1966). From these early discoveries were derived the two pathological requirements to emit a definitive post-mortem diagnosis, still standing today: first, the marked loss of pigmented dopaminergic neurons in the substantia nigra pars compacta, and second, the presence of Lewy bodies or their intraneuritic counterparts, Lewy neurites (Dickson *et al.*, 2009). To these findings were added, in later years, the contributions of Langston and colleagues describing parkinsonism in opioid addicts who had

accidentally consumed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston *et al.*, 1983), a by-product of desmethylprodine synthesis that impedes the mitochondrial electron transport chain and triggers the production of ROS[12] (Greenamyre *et al.*, 2001). This discovery also allowed further development of animal models of Parkinson's disease, complementing physical and 6-hydroxydopamine (6-OHDA) lesion paradigms (Ungerstedt, 1968), and coaxed investigations on environmental neurotoxins in the etiopathogenesis of the disease owing to MPTP's resemblance to certain known pesticides.

The latest significant leap forward in the field dates back to the identification of the first gene mutation, explicitly a single point mutation in the *SNCA* gene coding for α-synuclein[13], found to cause a dominant familial form of early onset Parkinson's disease (Polymeropoulos *et al.*, 1997). This event triggered a sequence of discoveries regarding the major constituent of Lewy bodies and neurites, α-synuclein (Spillantini *et al.*, 1997), but also concerning genetic forms of the pathology. Disease-causing duplications (Chartier-Harlin *et al.*, 2004) and triplications (Singleton *et al.*, 2003) of the *SNCA* gene were quickly identified, to which were added in subsequent years several other genes (Table 1.3). It is important to note that idiopathic Parkinson's disease still composes approximately 90-95% of diagnoses and are not always faithfully represented by genetic forms in terms of symptomatology and pathophysiology (see for review Houlden and Singleton, 2012).

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[12] The generation of ROS by the mitochondrial electron transport chain is later described in section 1.2.4.1.

[13] α-Synuclein is an intrinsically unstructured protein, especially localized to presynaptic terminals wherein it cycles between a cytosolic natively unfolded state and a membrane-bound state. Its function in normal physiological conditions remains unclear to date, but it is thought to regulate synaptic activity, vesicular trafficking and several metabolic and transport enzymes specific to dopaminergic neurons (see for review Burré, 2015).

Table 1.3

List of monogenic Parkinson's disease and parkinsonism

Designation and reference[&]	GeneReviews and OMIM[&] Reference	Clinical clues	Inheritance[&]	Previous locus symbol
1. Classical PD				
PARK-*SNCA*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1223/ OMIM 168601	Missense mutations cause classical parkinsonism. Duplication or triplication mutations in this gene cause early onset parkinsonism with prominent dementia.	AD	PARK1
PARK-*LRRK2*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1208/ OMIM 607060	Clinically typical PD	AD	PARK8
PARK-*VPS35*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1223/ OMIM 614203	Clinically typical PD	AD	PARK17
2. Early-onset PD				
PARK-*Parkin*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1155/ OMIM 600116	Often presents with dystonia, typically in a leg	AR	PARK2
PARK-*PINK1*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1223/ OMIM 605909	Often presents with psychiatric features	AR	PARK6
PARK-*DJ1*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1223/ OMIM 606324		AR	PARK7
3. Parkinsonism				
PARK-*ATP13A2*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1223/ OMIM 606693	Kufor-Rakeb syndrome with parkinsonism and dystonia; Additional features: Supranuclear gaze palsy, spasticity/pyramidal signs, dementia, facial-faucial-finger mini-myoclonus, dysphagia, dysarthria, olfactory dysfunction	AR	PARK9
PARK-*FBXO7*	GeneReviews http://www.ncbi.nlm.nih.gov/books/NBK1223/ OMIM: 260300	Early onset parkinsonism with pyramidal signs	AR	PARK15
PARK-*DNAJC6*	GeneReviews: n/a OMIM 615528	May present with mental retardation and seizures	AR	PARK19
PARK-*SYNJ1*	GeneReviews: n/a OMIM 615530	May have seizures, cognitive decline, abnormal eye movements, and dystonia	AR	PARK20

[&] AD, autosomal dominant; AR, autosomal recessive; OMIM, Online Mendelian Inheritance in Man; PARK-*ATP13A2*, probable cation-transporting adenosine triphosphatase 13A2 gene; PARK-*DJ1*, oncogene DJ-1 gene; PARK-*DNAJC6*, auxilin gene; PARK-*FBXO7*, F-box only protein 7 gene; PARK-*LRRK2*, leucine-rich repeat kinase 2 gene; PARK-*parkin*, parkin gene; PARK-*PINK1*, phosphatase and tensin homologue-induced putative kinase 1 gene; PARK-*SNCA*, alpha-synuclein gene; PARK-*SYNJ1*, synaptojanin 1 gene; PARK-*VPS35*, vacuolar protein sorting-associated protein 35 gene.
(From Obeso *et al.*, 2017, adapted from Bar-Gad *et al.*, 2003.)

Another hint pointing to a distinguishing pathological sequence initially surfaced when Kosaka and colleagues described patterns of Lewy body distribution in patients over the course of the disease (Kosaka *et al.*, 1988). This was later confirmed and transmuted into a highly controversial staging framework by a German group

spearheaded by Heiko Braak and Kelly Del Tredici (Braak *et al.*, 2003). They reported that in many idiopathic Parkinson's disease patients, though not all[14] (Kalaitzakis *et al.*, 2008*b*; Milber *et al.*, 2012; Parkkinen *et al.*, 2005; Zaccai *et al.*, 2008), Lewy bodies and neurites appear to colonize the olfactory bulb or the brainstem early in the pathology (stages 1 and 2), spreading sequentially through the midbrain (stages 3 and 4), and ultimately reaching neocortical and limbic areas (stages 5 and 6) (Braak *et al.*, 2003) (Figure 1.9). Interestingly, Lewy bodies had been identified in the brainstem almost one century prior (Lewy, 1912). More recent hypotheses conforming with Braak staging (Braak *et al.*, 2006) go as far as to suggest that the pathology may begin in the periphery downstream from the vagal nerve, for instance in the enteric nervous system, and spread to the CNS as substantiated by evidence in rodents (Holmqvist *et al.*, 2014; Pan-Montojo *et al.*, 2010, 2012; Phillips *et al.*, 2008; Ulusoy *et al.*, 2013), and as alluded to in human studies (Stokholm *et al.*, 2016). Of particular interest, apparition of Lewy bodies in extranigral regions may lead to the dysfunction – but not necessarily to the demise – of these neurons, partly explaining the prodromal phase and later dopamine-unresponsive symptoms. Whether this theory holds true or not, the conjecture that Lewy bodies spread across interconnected neuroanatomical regions has provided a cornerstone for more advanced theories of trans-synaptic neuron-to-neuron propagation of misfolded α-synuclein, for example the discordant prion hypothesis[15], supported by experimental demonstrations (Desplats *et al.*, 2009; Luk *et al.*, 2012; Recasens *et al.*, 2014; Volpicelli-Daley *et al.*, 2011) and observations of host-to-graft spreading of Lewy bodies in humans 10-14 years after transplants (Kordower *et al.*, 2008; Li *et al.*, 2008).

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[14] Many parkinsonian post-mortem samples present Lewy body patterns that deviate from Braak's staging framework (Halliday *et al.*, 2012*b*; Kalaitzakis *et al.*, 2008*a*). In addition, neuroanatomical connectomics do not aid in predicting the apparition of Lewy bodies. Exemplarily, striatal medium spiny neurons, highly interconnected with Lewy body-laden nigrostriatal dopaminergic neurons, hardly ever display Lewy pathology (Halliday *et al.*, 2011; Martin *et al.*, 2008).

[15] Today, no doubt remains that fibrillar α-synuclein can spread from one neuron to another upon experimental inoculation. However, the time course (only 50% of patients show Lewy pathology by 13 years of onset) and infectiousness of α-synuclein in humans are significantly different than those observed in preclinical prion models. In fact, no direct evidence of spreading in humans is available. For many, the question remains whether prion-like spreading and toxicity is the primary governing factor in the selective vulnerability of nigrostriatal dopaminergic neurons in Parkinson's disease (McCann *et al.*, 2015; Surmeier *et al.*, 2017).

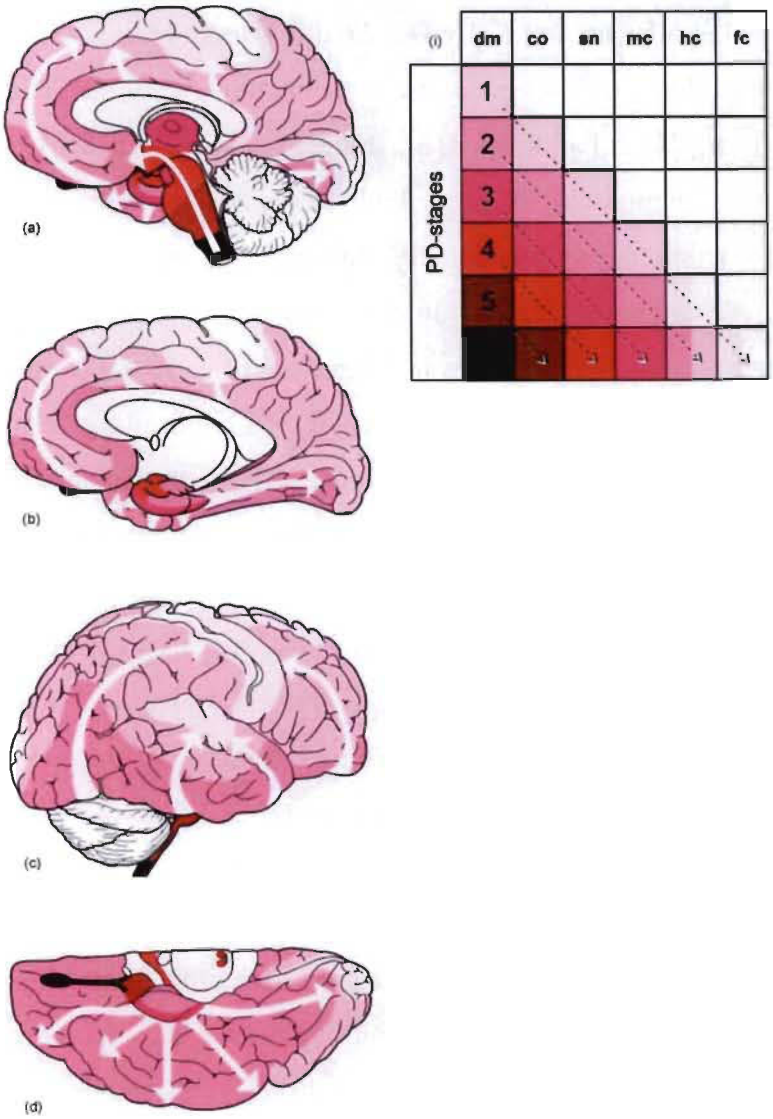

Figure 1.9 **Braak staging of the progression of Parkinson's disease-related Lewy body pathology.**
According to this staging framework, spreading of Lewy body pathology (dm to fc at the top of the graph) begins in the dorsal IX/X motor nucleus (stage 1) but also in the anterior olfactory nucleus, sequentially spreading toward coeruleus regions (stage 2), the midbrain (stage 3), mesocortical entorhinal regions (stage 4), high order cortical areas (stage 5) and finally to first order cortical areas (stage 6). Colour intensity indicates the degree of Lewy body pathology: in the schematic representations of the brain, stage 6 is depicted. co, coeruleus/subcoeruleus complex; dm, dorsal IX/X motor nucleus; fc, first order sensory association areas and premotor areas and/or primary sensory and motor fields of the neocortex; hc, high order sensory association areas and prefrontal areas of the neocortex; mc, transentorhinal region and/or ectorhinal region (anteromedial temporal mesocortex); sn, posterior portion of substantia nigra pars compacta. (Adapted from Braak *et al.*, 2003.)

Connecting this constellation of breakthroughs has led to our current appreciation of the pathophysiology that characterizes Parkinson's disease, without however fully unscrambling its etiopathogenesis. We suspected and have confirmed that the degeneration of nigrostriatal dopaminergic neurons leads to the depletion of dopamine at the striatal interface of basal ganglia circuits, accounting for motor symptoms (Calabresi *et al.*, 2014; Kravitz *et al.*, 2010; Tritsch and Sabatini, 2012). However, formal recognition that Parkinson's disease does not necessarily start in the substantia nigra pars compacta leaves us at loss as to why nigrostriatal dopaminergic neurons degenerate long before other Lewy body-laden neurons do. It is likely that various cellular and molecular mechanisms account for the select vulnerability of nigrostriatal dopaminergic neurons in Parkinson's disease, which only accentuates our awareness of the multiple dimensions that demarcate this pathology.

1.1.3.4 Vulnerability of the nigrostriatal pathway

Identifying exogenous and endogenous risk factors is a sensible place to start on the quest for clues to unravel one of the most important questions left unsettled in Parkinson's disease, that is, the selective early death of nigrostriatal dopaminergic neurons. The incidence of idiopathic Parkinson's disease is clearly linked with environmental factors. The discovery of MPTP as a mitochondria-targeting agent of parkinsonism broke new ground for the exploration of other chemicals, such as pesticides (Furlong *et al.*, 2015; Priyadarshi *et al.*, 2000), solvents (Goldman *et al.*, 2012; Pezzoli and Cereda, 2013), metals (Finkelstein and Jerrett, 2007; Gorell *et al.*, 1997) and organochlorines (Steenland *et al.*, 2006; Weisskopf *et al.*, 2012), subsequently shown to constitute risk factors acting via bioenergetic disruption and oxidative mechanisms. Elements of infection were also suggested long ago to promote Parkinson's disease (Charcot, 1877; Gowers, 1886), substantiated by the occurrence of postencephalitic parkinsonism that succeeded the great pandemic of encephalitis lethargica in the early 1900s (Schwab *et al.*, 1956; Vilensky *et al.*, 2010). Later, studies found certain of these exogenous elements to represent a greater hazard in individuals carrying a specific genetic profile. Indeed, a host of genetic variants are known today

to synergistically increase the odds of developing Parkinson's disease, in gene-environment interactions but also as standalone risk factors (Polito *et al.*, 2016; Warner and Schapira, 2003) (Table 1.4). Monogenic forms of Parkinson's disease also provide supplementary clues pointing to intrinsic issues that bode unfavourable odds. Noteworthy, the abnormal proteic products of these genes interfere with healthy cellular trafficking, protein degradation or mitochondrial function (Figure 1.10).

Aside from these genetic and environmental factors specific to certain individuals, one element of risk stands out due to its universality and that is aging (Reeve *et al.*, 2014). Indeed, of all the risk factors, aging persists as the best-established player in idiopathic Parkinson's disease, as is the case for several other chronic and neurodegenerative illnesses. However, the tightness of this relationship is such that Parkinson's disease is viewed today as the epitome of the synergistic potentiation that occurs between aging and neurodegeneration (Obeso *et al.*, 2017) (Figure 1.11). For example, the substantia nigra pars compacta appears to be relatively susceptible to aging outside of a pathological context (Buchman *et al.*, 2012; Rudow *et al.*, 2008), as cell loss occurs at an estimated rate of 4.7-9.8% per decade in humans (Fearnley and Lees, 1991; Ma *et al.*, 1999). Moreover, iron contents (Bilgic *et al.*, 2012), levels of iron-binding neuromelanin (Double *et al.*, 2003), neuroinflammation (Calabrese *et al.*, 2018), oxidative stress-induced mitochondrial DNA deletions (Reeve *et al.*, 2014), and malfunction of the ubiquitin proteasome system and autophagy clearance pathways (Jana, 2012; Li and Li, 2011; Rubinsztein *et al.*, 2011) were all shown to increase drastically with age, especially so in the substantia nigra pars compacta (Corral-Debrinski *et al.*, 1992; Daugherty and Raz, 2013; Dexter *et al.*, 1989; Fedorow *et al.*, 2006; Soong *et al.*, 1992). Coherently, in the setting of Parkinson's disease, nigrostriatal neurons that contain more neuromelanin are at higher risk of degenerating (Gibb and Lees, 1991; Hirsch *et al.*, 1988; Kastner *et al.*, 1992; Marsden, 1983; Zecca *et al.*, 2001), microgliosis is relatively more pronounced in the substantia nigra pars compacta (Kim *et al.*, 2000; Lawson *et al.*, 1990; McGeer *et al.*, 1988), nigral mitochondrial DNA deletions accumulate (Bender *et al.*, 2006, 2008) and clearance pathways are impaired (Cuervo *et al.*, 2010; Dehay *et al.*, 2010).

Table 1.4

Overview of the 26 genetic risk variants showing consistent association with Parkinson's disease in genome-wide association studies

SNP	Location (hg38)	Nearest gene[&]	Alleles	Risk allele freq	OR	*P* value
rs35749011	1:155,162,810	*SLC50A1 (GBA)*	A/G	0.017	1.824	1.37×10^{-29}
rs114138760*	1:154,925,709	*PMVK (GBA)*	C/G	0.012	1.574	3.80×10^{-7}
rs823118	1:205,754,444	*NUCKS1*	T/C	0.559	1.122	1.66×10^{-16}
rs10797576	1:232,528,865	*SIPA1L2*	T/C	0.140	1.131	4.87×10^{-10}
rs6430538	2:134,782,397	*ACMSD*	C/T	0.570	1.143	9.13×10^{-20}
rs1474055	2:168,253,884	*STK39*	T/C	0.128	1.214	1.15×10^{-20}
rs12637471	3:183,044,649	*MCCC1*	G/A	0.807	1.188	2.14×10^{-21}
rs34311866	4:958,159	*TMEM175 (GAK)*	G/A	0.191	1.272	1.02×10^{-43}
rs34884217*	4:950,422	*TMEM175 (GAK)*	A/C	0.913	1.247	1.10×10^{-6}
rs11724635	4:15,735,728	*BST1*	A/C	0.553	1.126	9.44×10^{-18}
rs6812193	4:76,277,833	*FAM47E*	C/T	0.636	1.103	2.95×10^{-11}
rs356182	4:89,704,960	*SNCA*	C/T	0.367	1.316	4.16×10^{-73}
rs7681154*	4: 89,842,802	*SNCA*	C/A	0.498	1.189	7.09×10^{-19}
rs9275326	6:32,698,883	*HLA-DQB1*	C/T	0.906	1.211	1.19×10^{-12}
rs13201101*	6:32,375,827	*C6orf10*	T/C	0.053	1.192	3.84×10^{-6}
rs199347	7:23,254,127	*GPNMB*	A/G	0.590	1.110	1.18×10^{-12}
rs117896735	7:119,777,065	*INPP5F*	A/G	0.014	1.624	4.34×10^{-13}
rs329648	11:133,895,472	*MIR4697HG*	T/C	0.354	1.105	9.83×10^{-12}
rs76904798	12: 40,220,882	*LRRK2*	T/C	0.143	1.155	5.24×10^{-14}
rs11060180	12:122,819,039	*CCDC62*	A/G	0.558	1.105	6.02×10^{-12}
rs11158026	14:54,882,151	*GCH1*	C/T	0.665	1.106	5.85×10^{-11}
rs2414739	15:61,701,935	*VPS13C*	A/G	0.734	1.113	1.23×10^{-11}
rs14235	16:31,110,472	*BCKDK*	A/G	0.381	1.103	2.43×10^{-12}
rs17649553	17:45,917,282	*MAPT*	G/A	0.774	1.300	2.37×10^{-48}
rs12456492	18:43,093,415	*RIT2*	G/A	0.307	1.106	7.74×10^{-12}
rs8118008	20:3,187,770	*DDRGK1*	A/G	0.657	1.111	3.04×10^{-11}

This table was adapted from Table 1 and Supplementary Table 3 of the original study.[13] It lists the 22 most significant SNPs per locus (defined in 1 Mb boundaries) that showed genome-wide significant ($p < 5 \times 10^{-8}$) association with Parkinson's disease (PD) status.[7] Furthermore, it displays 4 SNPs (labeled with a star [*]) that showed significant association (ie, $p < 1 \times 10^{-5}$ following Bonferroni correction) with PD risk upon conditioning on the most significant SNP in the same genetic region (ie, corresponding to the SNP listed in this table in the preceding line). Note that the nearest gene assigned to each SNP here (as determined according to RefGene as available on the UCSC genome browser (https://genome.ucsc.edu/)) does not necessarily represent the functional element underlying the genetic association. The genes in parentheses refer to the more commonly used gene names for the respective locus. Full names of all official gene names listed here can be found in the EntrezGene database (http://www.ncbi.nlm.nih.gov/gene/). Alleles = the first allele represents the risk allele. hg38, human genome build 38; Freq, frequency; *MAPT*, microtubule-associated protein tau; OR, odds ratio; SNP, single nucleotide polymorphism.

[&] *ACMSD*, aminocarboxymuconate semialdehyde decarboxylase gene; *BCKDK*, branched chain ketoacid dehydrogenase kinase gene; *BST1*, bone marrow stromal cell antigen 1 gene; *C6orf10*, chromosome 6 open reading frame 10 gene; *CCDC62*, coiled-coil domain containing 62 gene; *DDRGK1*, DDRGK domain containing 1 gene; *FAM47E*, family with sequence similarity 47 member E gene; *GAK*, cyclin G associated kinase/auxilin-2; *GBA*, beta-glucocerebrosidase gene; *GCH1*, guanosine triphosphate cyclohydrolase 1 gene; *GPNMB*, glycoprotein neuromedin B gene; *HLA-DQB1*, major histocompatibility complex, class II, DQ beta 1 gene; *INPP5F*, inositol polyphosphate-5-phosphatase F gene; *MCCC1*, methylcrotonoyl-coenzyme A carboxylase 1 gene; *MIR4697HG*, microribonucleic acid 4697 host gene; *NUCKS1*, nuclear casein kinase and cyclin dependent kinase substrate 1 gene; *PMVK*, phosphomevalonate kinase gene; *RIT2*, Ras-like without CAAX 2 gene; *SIPA1L2*, signal induced proliferation associated 1 like 2 gene; *SLC50A1*, solute carrier family 50 member 1 gene; *STK39*, serine/threonine kinase 39 gene; *TMEM175*, transmembrane protein 175 gene; *VPS13C*, vacuolar protein sorting 13 homologue C gene.
(From Obeso *et al.*, 2017, adapted from Fahn and Goetz, 1987.)

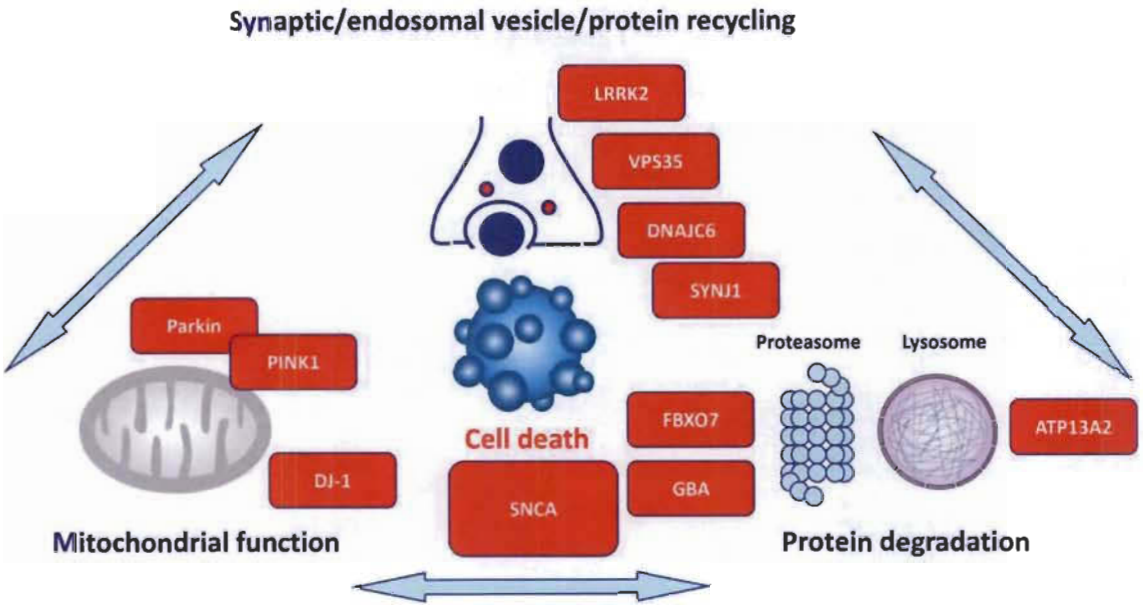

Figure 1.10 Disease mechanisms implicated in Parkinson's disease. This diagram, termed the Bermuda triangle by the authors, highlights three important cellular pathological axes affected in monogenic forms of Parkinson's disease, which may also be implicated in idiopathic forms. Importantly, cellular trafficking, mitochondrial homeostasis and protein degradation are tightly interwoven in the pathophysiology of Parkinson's disease. (From Obeso *et al.*, 2017.)

In considering the sum of exogenous and endogenous risk factors that come into play in Parkinson's disease, several mechanisms proposed to dwell at the core of this illness emerge, notably mitochondrial dysfunction, protein aggregation, iron toxicity, neuroinflammation and impaired cellular trafficking, among others. Despite their apparent disparity, they converge to cause the preferential early degeneration of nigrostriatal neurons in Parkinson's disease and possibly contribute to oxidative stress to which these neurons may be more vulnerable (Brichta and Greengard, 2014; Haddad and Nakamura, 2015; Oliveira *et al.*, 2017; Surmeier, 2007; Surmeier *et al.*, 2017). This concept dates back to the 1980s (Götz *et al.*, 1990; Jenner, 1991; Lenzi *et al.*, 1979; Youdim *et al.*, 1989), but its popularity has waxed and waned at the rhythm of discoveries such as the sequential spreading of Lewy bodies (Braak *et al.*, 2003). Nevertheless, the idea that metabolic oxidative insults may underlie the specific vulnerability of nigrostriatal neurons has lately gained momentum as the prominent philosophies of the 1900s and beginning of the 2000s are failing to satisfy questions

regarding the course of progression of Parkinson's disease (Obeso *et al.*, 2017). Oxidative stress and the propagation of Lewy bodies are not mutually exclusive theories either: oxidative stress is a known facilitator of α-synuclein aggregation and Lewy bodies contribute to oxidative stress (Gupta *et al.*, 2009). As such, they likely engage in an intimate relationship in the etiopathogenesis of Parkinson's disease.

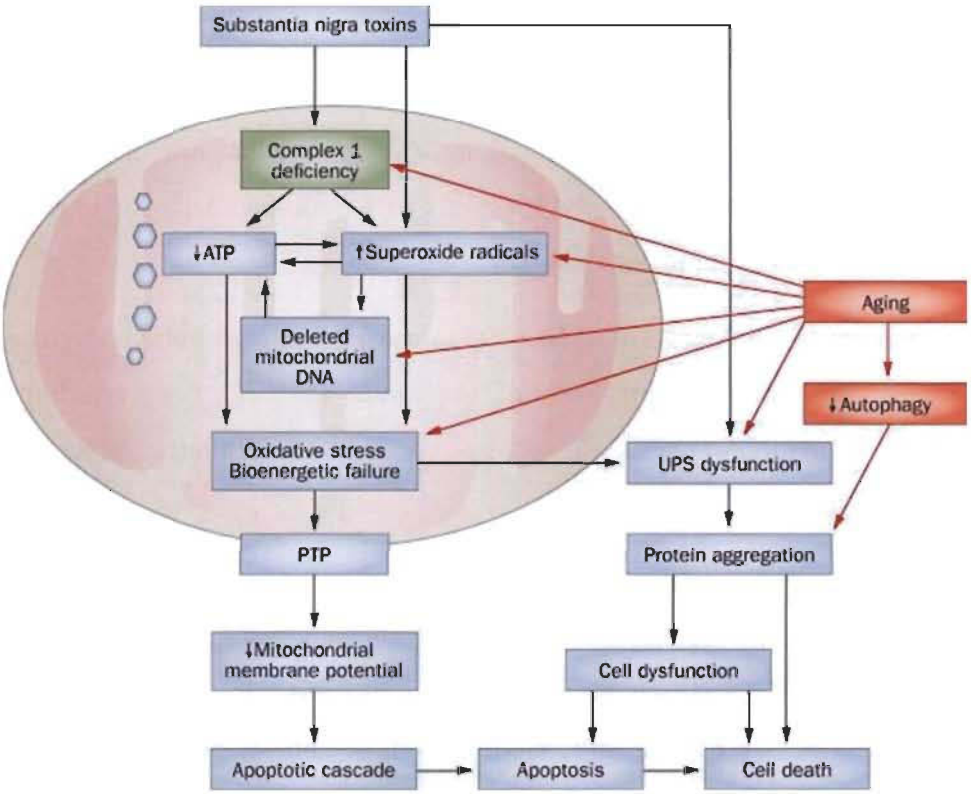

Figure 1.11 Crosstalk between aging and Parkinson's disease.
Aging leads to several pathological events in neurons that are conducive to the development of Parkinson's disease. In particular, oxidative stress emerges as a pivotal factor. ATP, adenosine triphosphate; PTP, permeability transition pore; UPS, ubiquitin-proteasome system. (From Schapira, 2008.)

In order for oxidative stress to incarnate the noxious interface through which all of these risk factors converge onto nigrostriatal dopaminergic neurons, the latter must exhibit exclusive properties discerning them from other neuronal populations and rendering them sensitive to each of these contributing elements. It is critical that nigrostriatal neurons distinguish themselves from other dopaminergic neurons, especially their mesocorticolimbic analogues, as they all share the liability of employing

dopamine as a neurotransmitter, recognized to generate ROS upon auto-oxidation (Segura-Aguilar *et al.*, 2014) and to require ROS-producing monoamine oxidases for their degradation (Andersen *et al.*, 1994; Graham, 1984). In this respect, we argue that nigrostriatal dopaminergic neurons display the following phenotypic idiosyncrasies that may afford their vulnerability to oxidative stress instigated by genetic mutations, environmental toxins and aging:

1) They are embedded in a particular environment, the substantia nigra pars compacta, which stores considerable amounts of iron ions (Chinta and Andersen, 2008; Hirsch and Faucheux, 1998) accumulating with age (Daugherty and Raz, 2013) and known to participate in deleterious Fenton reactions with hydrogen peroxide to produce the very reactive hydroxyl radical (Youdim *et al.*, 1989);

2) They express lower levels of VMAT2 than most other catecholaminergic neurons, implying the protracted presence of dopamine in the cytosol leading to the inevitable formation of toxic oxidative by-products and, ultimately, potentially toxic neuromelanin (Liang *et al.*, 2004);

3) Constituting a very small cluster of cells (German *et al.*, 1988; Percheron *et al.*, 1989), they are nonetheless required to fully innervate a very large surface, the dorsal striatum, which entails tremendous branching of axons harbouring numerous vesicular release sites ridden with potentially pathogenic α-synuclein (Matsuda *et al.*, 2009; Parent and Parent, 2006);

4) To enable corticostriatal circuits, they constitute the greatest striatal input of uninterrupted tonic dopamine relying on autonomous pacemaking activity at the source of high baseline levels of calcium ions, which enhance oxidative phosphorylation, mitochondrial membrane hyperpolarization and superoxide anion production (Guzman *et al.*, 2010; Pacelli *et al.*, 2015);

5) To maintain their neurotransmission activities and drawn-out axonal arbours, these neurons keep an army of mitochondria to fulfil their exorbitant energetic needs, also implying elevated basal rates of oxidative phosphorylation and greater production of electron transport chain superoxide anion by-products (Pacelli *et al.*, 2015);

6) Last, they are endowed with a limited calcium-buffering capacity (Foehring *et al.*, 2009) and scanty endogenous antioxidative defences (Bharath *et al.*, 2002), consequent of their low expression of calbindin (Dopeso-Reyes *et al.*, 2014; Iacopino and Christakos, 1990; Liang *et al.*, 1996; Yamada *et al.*, 1990) and glutathione (Kang *et al.*, 1999), respectively.

In all evidence, these features unite to augment the basal oxidative burden to which are subjected nigrostriatal neurons. Upon further exposure to environmental or genetic insults, endogenous antioxidative defences are eventually overwhelmed: this turning point typified by the failure of neurons to cope with the added oxidative load constitutes the origin of oxidative stress. Sustained in time and enhanced by aging processes, oxidative stress may eventually lead to the death of nigrostriatal neurons in Parkinson's disease. Following this premise, studies have employed primary neuron cultures (Pacelli *et al.*, 2015), acute rat brain slices (Carbone *et al.*, 2017), rodents (Vidyadhara *et al.*, 2016) and primate (Dopeso-Reyes *et al.*, 2014) models to demonstrate that nigrostriatal dopaminergic neurons are more vulnerable to parkinsonian neurotoxins in comparison to other neuronal populations, exemplarily dopaminergic neurons lodged in the neighbouring ventral tegmental area, and that this vulnerability is abated by pharmacologically targeting these specific phenotypic risk features (Carbone *et al.*, 2017; Pacelli *et al.*, 2015).

If indeed nigrostriatal neurons are relatively more susceptible to insults that overwhelm their endogenous coping mechanisms, other sources of oxidative stress that are not specific to Parkinson's disease may yield similar results and shore up the idea of their selective vulnerability. Indications of this are provided by epidemiological evidence showing a higher occurrence of Parkinson's disease in patients suffering from other pathologies with a significant component of oxidative stress, such as diabetes (Cereda *et al.*, 2012; Santiago and Potashkin, 2013; Sun *et al.*, 2012), ischemic stroke (Huang *et al.*, 2013) and sleep apnea (Snyder and Cunningham, 2017). Interestingly, Parkinson's disease is significantly less frequent in patients with the hyperuricemic illness gout wherein the antioxidative effects of uric acid may in fact protect nigrostriatal neurons (Alonso and Sovell, 2010). Although evidence weighs in

favour of the selective vulnerability of the nigrostriatal pathway in pathological states that induce upwelling of systemic oxidation, for instance in a hyperglycaemic setting, studies are required to validate this. The present thesis aims to expound on this question.

1.2 Hyperglycaemia in the central nervous system

Before addressing the mechanisms by which hyperglycaemia[16] may instigate oxidative stress in the CNS, and before laying out the epidemiological evidence in favour of the particular vulnerability of nigrostriatal dopaminergic neurons in these conditions, it is critical that we fully grasp glucose dynamics in the brain. The transport kinetics and metabolism of glucose are highly complex, especially in the CNS, and bear significant implications in the initiation of oxidative stress in neurons. Progressing in this direction, a fundamental appraisal of the brain's energy sources is first required.

1.2.1 Glucose as a preferential fuel for the brain

Long has functional neuroimagery exposed the importance of glucose in the fulfilment of the adult brain's energetic needs (Landau *et al.*, 1955; Schmidt and Kety, 1947; Siesjö, 1978; Sokoloff *et al.*, 1977). In fact, the brain utilizes 25% of total body glucose, mostly to power oxidative metabolism (Heiss, 2016; Kety and Schmidt, 1948; Laughlin and Attwell, 2001; Shulman *et al.*, 2001; Sokoloff *et al.*, 1999). Glucose is indispensable to neurons and thus plays a significant role in CNS health and disease. In settings of extreme dietary and pathological depletion of glucose or its transport, the brain is forced to employ other fuels, although a minimal amount of glucose is always required to maintain normal cerebral functions (Casazza *et al.*, 1984; Owen *et al.*, 1967; VanItallie and Nufert, 2003). In these specific contexts, blood-borne ketone bodies can account for up to 70% of brain energy requirements through its conversion to acetyl coenzyme A, in contrast to fatty acids that inefficiently cross the blood-brain

[16] Normoglycaemia is defined in humans as fasting blood glucose < 5.5 mM (< 100 mg/dL), postprandial blood glucose < 7.8 mM (< 140 mg/dL) or glycated haemoglobin < 5.7%; hyperglycaemia is defined as fasting blood glucose ≥ 7.0 mM (≥ 126 mg/dL), postprandial blood glucose ≥ 11.1 mM (≥ 200 mg/dL) or glycated haemoglobin ≥ 6.5% (American Diabetes Association, 2016).

barrier and are oxidized too slowly for rapid energy provision (Dhopeshwarkar and Mead, 1973; Hamilton and Brunaldi, 2007; Hasselbalch *et al.*, 1994; Morris, 2005; Schönfeld and Reiser, 2013). Likewise, circulating lactate may substitute for glucose and afford basal sustenance, but it does not allow neurons to carry out efficient neurotransmission (Bak *et al.*, 2006, 2012; Ivannikov *et al.*, 2010). In the context of hyperglycaemia, the peripheral production of ketones may arise if insulin levels are too low to supply cells with glucose despite its overabundance (Fukao *et al.*, 2004; Fulop *et al.*, 1999). However, since the brain does not rely on insulin for intracellular import of glucose, as we will see next, the latter remains the preferred substrate, especially considering its elevated circulating concentrations.

1.2.2 Neuronal glucose transport

Neurons have particular needs in terms of glucose transport and availability, as they are required to react extremely rapidly to a perpetually changing intra- and extracellular environment directly ensuing from neurotransmission[17]. Unlike other energy-greedy cells endowed with glycogen stores, like striated muscle cells, neurons cannot count on glycogenolysis for sustenance, as this macromolecule is almost exclusively confined to astrocytes in the brain (Cataldo and Broadwell, 1986; Dienel and Cruz, 2006; Ibrahim, 1975; Koizumi, 1974; Sagar *et al.*, 1987; Watanabe and Passonneau, 1973). Intraneuronal glucose is thus mainly provided from the extracellular environment, which itself is supplied by the circulation. This implies the need for glucose to cross several barriers whose transport kinetics assuredly influence its metabolism in neurons.

1.2.2.1 Transporters and kinetics

Hydrophilic glucose molecules must be transported by facilitated diffusion to reach the intracellular space. Glucose transporters (GLUTs) ubiquitously present at the

[17] In neurons, constant restoration of membrane potentials, clearance of intracellular calcium, synthesis of neurotransmitters, restructuration of synapses and maintenance of highly solicited organelles oblige an uninterrupted energy flux.

surface of cells occupy the function of taking up glucose from the extracellular environment (Table 1.5). Excluding the myo-inositol transporter, all GLUTs identified to date mediate energy-independent facilitative transport (see for review Mueckler and Thorens, 2013; Wood and Trayhurn, 2003).

Table 1.5

Glucose transporter expression sites and substrates

Type	Protein (*gene*)[&]	Sites expressed	Substrate/transports
Facilitative/Sodium-independent	**GLUT1** (*SLC2A1*)	Brain endothelial and epithelial-like brain barriers, glial cells, blood-tissue barriers, eye, pheripheral nerves, placenta, lactating mammary gland (Ubiquitous distribution in most mammalian cells)	>>Glucose, galactose, mannose, glucosamine, ascorbic acid
	GLUT2 (*SLC2A2*)	Kidney, small intestine (epithelial cells), liver, pancreas (islets), brain (astrocytes)	Mannose, galactose, fructose, glucose, glucosamine
	GLUT3 (*SLC2A3*)	Neurons, testis, placenta, brain endothelial cells?	Glucose, galactose, mannose, xylose, dehydroascorbic acid
	GLUT4 (*SLC2A4*) (Insulin-sensitive)	Brown and white adipose tissue, muscle (skeletal), fat, heart (myocardium), hippocampal neurons, cerebellar neurons	Glucose, dehydroascorbic acid, glucosamine
	GLUT5 (*SLC2A5*)	Intestine (jejunum), kidney, testis, brain microglia	Fructose
	GLUT6 (*SLC2A6*)	Brain, peripheral and spleen (leukocytes)	Glucose
	GLUT7 (*SLC2A7*)	Small intestine (mainly in brush border membrane-enterocytes), colon, testis, prostate, liver (associated with endoplasmic reticulum)	>Fructose, glucose
	GLUT8 (*SLC2A8*) (Insulin-responsive?)	Blastocytes, testis, brain (neurons), muscle, adipocytes, mammary gland?	Glucose
	GLUT9 (*SLC2A9*)	Liver, kidney (proximal tubule of epithelial cells), placenta?	Glucose, urate
	GLUT10 (*SLC2A10*)	Liver, pancreas, heart, lung, brain, skeletal muscle, placenta	Glucose, galactose
	GLUT11 (*SLC2A11*)	Iso-form A: Heart, skeletal muscle, kidney Iso-form B: Placenta, adipose tissue, kidney Iso-form C: Adipose tissue, heart, skeletal muscle, pancreas	Fructose, glucose
	GLUT12 (*SLC2A12*) (Insulin-sensitive?)	Heart, skeletal muscle, fat, prostrate, lactating mammary gland ?, spleen ?, breast cancer (Ductal cell carcinoma) tissue	Glucose
	HMIT (*SLC2A13*) (co-transporter)	Brain (neurons intracellular vesicles)	H^+/myo-inositol

[&] GLUT, glucose transporter; HMIT, H+/myo-inositol transporter; *SLC2A*, solute carrier family 2 gene. (Adapted from Simpson *et al.*, 2007.)

Contrary to most peripheral cells that express GLUT4 requiring insulin for membrane translocation, the vast majority of CNS components employ insulin-independent GLUTs (Huang and Czech, 2007; James *et al.*, 1988) (Figure 1.12). GLUT1 is the most widespread isoform in the body and, aside from red blood cells, it is most abundant in the brain microvasculature, evenly distributed among the luminal

and abluminal surfaces of the endothelium (Maher *et al.*, 1994; Simpson *et al.*, 2001). It is also the main transporter employed by glial cells, especially astrocytes (Dick *et al.*, 1984; Gerhart *et al.*, 1989; Maher *et al.*, 1994; Simpson *et al.*, 2001). Neurons, on the other hand, overwhelmingly employ the GLUT3 isoform densely located across the cell surface but more intensely so at the neuropil (Leino *et al.*, 1997; Mantych *et al.*, 1992; Nagamatsu *et al.*, 1993; see for review Simpson *et al.*, 2008). Consequently, to access the brain parenchyma, circulating glucose must penetrate the endothelium and astrocytic endfeet of the blood-brain barrier via GLUT1. In turn, glucose diffuses through the extracellular space where it can be absorbed by neuronal GLUT3.

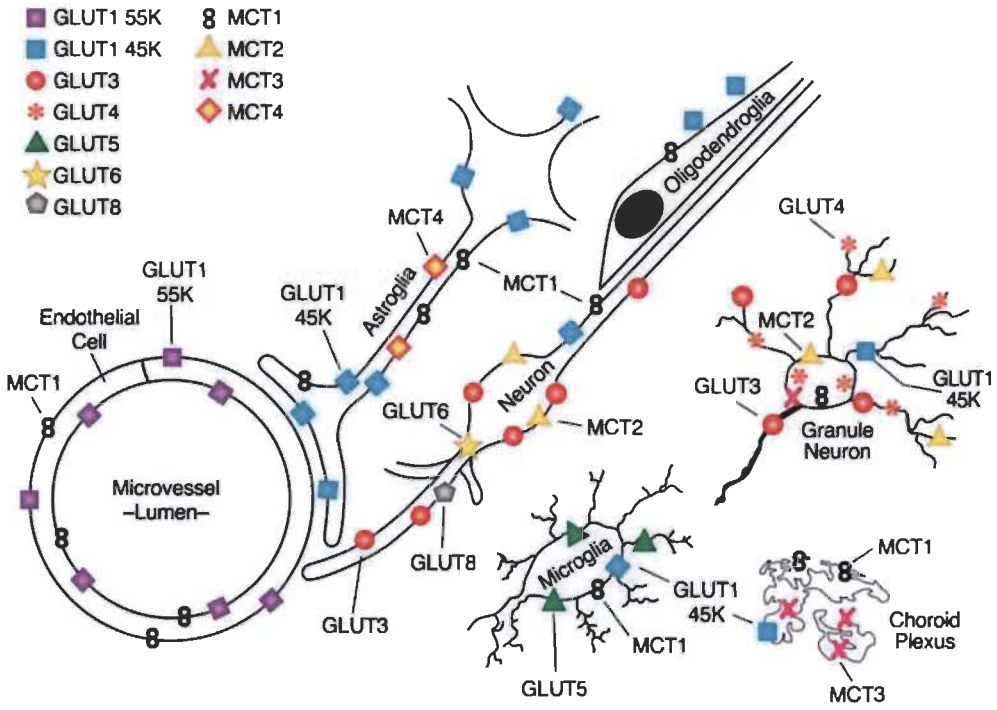

Figure 1.12 Glucose and monocarboxylate transporters in the mammalian brain. Endothelial cells solely express GLUT1, whereas other brain cells usually express multitudinous isoforms. Noteworthy, endothelial cells express the highly glycosylated 55-kDa isoform of GLUT1,while astrocytes and oligodendrocytes express the 45-kDa variant. Neurons mainly express GLUT3, but can also express GLUTS 1, 2, 4, 6, 8 and the myo-inositol transporter, depending on brain regions and isoforms. The fructose transporter GLUT5 is almost only found at the surface of microglial cells, but its function remains unclear in the brain due to the virtual absence of its known substrate in the parenchyma (Jurcovicova, 2014; Maher *et al.*, 1994). MCT, monocarboxylate transporter. (From Simpson *et al.*, 2007, adapted from McKenna *et al.*, 2005.)

Although cellular constituents of the brain do not rely on insulin for glucose uptake, several kinetic factors come into play in limiting glucose permeation between compartments. Seeing as GLUTs are facilitative, bidirectional, energy-independent transporters, they allow not for glucose accumulation but for its equilibration between compartments in a manner that smoothens concentration gradients. Brain GLUTs possess elevated affinities for glucose, highest for neuronal GLUT3 ($K_m \sim 1.5$ mM) (Colville *et al.*, 1993; Maher *et al.*, 1996) (Table 1.6). In fact, GLUT3 also presents the greatest turnover for glucose, defined as the number of transport cycles per transporter per second (Manolescu *et al.*, 2007; Rumsey *et al.*, 1997).

Table 1.6

Glucose transport capacities of brain cells

[a]Cell	TRANSPORTER SPECIES	[Transporter]	[Transporter]	[Transporter]	[b]V_{max} uptake	[b]K_m uptake	[c]substrate/ligand	[d]k_{cat}	[e]predicted V_{max} in model
		pmol/mg membrane protein	carriers/μm^2	pmol/mg total protein	nmol/10^6 cells/min	mM		per sec	mmol/sec
RBC[&]	GLUT1	2000	2083	92	80.4	8	Glc/CB	1166	
Endothelium	GLUT1	400	[f]1000	[g]*18*	*0.21*	*8*	CB	1166	7.8×10^{-15}
Astrocyte	GLUT1	7.3	[f]18	[g]*0.34*	*4.8*	*8*	CB	1166	[j]1.4×10^{-16} [k]1.9×10^{-15}
Neuron	GLUT3	9.5	[f]24	[g]*0.44*	[h]5.0 [i]34.6	2.8	3MG/CB	[h]868 [i]6512	2.3×10^{-14}

[a]Cell in which transport is measured.
[b]Measured or computed maximum rate (V_{max}) or affinity (K_m) for transport.
[c]The substrate employed in transport (Glucose (Glc), 3-O-methylglucose (3MG) or lactate) or ligand GLUT quantitation experiments (Cytochalasin B (CB) binding).
[d]Computed k_{cat} ($[V_{max}/[GLUT]$) for transport.
[e]Predicted V_{max} for transport of glucose and lactate for use in the model.
[f]Assumes 1 mg membrane protein is equivalent to a surface area of 2.4×10^{11} μm^2.
[g]Assumes that the ratio of membrane:total cellular protein is 0.0462 : 1.
[h]Measured at 25°C.
[i]Computed for transport at 37°C assuming that GLUT3 and GLUT1 share the same temperature dependence.
[j]Direction basal lamina to astrocyte.
[k]Direction interstitium to astrocyte. Glucose transport parameters in endothelial cells, astrocytes and neurons were computed using estimated cell membrane GLUT contents and measured k_{cat} parameters. MCT concentrations are *estimated* assuming MCT k_{cat} = GLUT1 k_{cat} and using measured V_{max} values.
[&]RBC, red blood cell.
(From Simpson *et al.*, 2007.)

Since normoglycaemic settings afford much more glucose than transport kinetics require, one would expect brain cells like neurons to contain approximately as much glucose as their immediate extracellular environment. Likewise, brain glucose concentrations should fluctuate as a function of glycaemia, regardless of insulin levels. According to computational studies performed by Simpson and colleagues, neurons and astrocytes only contain ~0.9 mM of glucose in normoglycaemic steady-state conditions (Simpson *et al.*, 2007) (Table 1.7). Similarly, extracellular concentrations of glucose merely reach ~1.4 mM. Even endothelial cells that express GLUT1 and are in direct contact with blood-borne solutes display concentrations of ~3.8 mM. This discrepancy between expected compartment equilibration and markedly lower levels of glucose in parenchymal and endothelial cells may be explained by transport inhibition at the level of the blood-brain barrier. Indeed, endothelial GLUT1 undergoes allosteric inactivation by adenosine triphosphate (ATP) in settings of metabolic abundance (Carruthers and Helgerson, 1989; Levine *et al.*, 1998; Lowe and Walmsley, 1986). Consequently, endothelial cells uptake and provide less glucose to the brain parenchyma in replete, normoglycaemic settings. Conversely, when glycolytic demands increase and ATP runs low, GLUT1 transport is uninhibited and glucose may efficiently be transferred to surrounding tissues (Carruthers, 1986; Cloherty *et al.*, 1996). Globally, the parenchyma's apparent K_m for glucose remains 1-3 mM, signifying that glycaemia must significantly drop before brain uptake deficits are perceived (Rao *et al.*, 2006). This can be interpreted as an evolutionary adaptation in favour of organisms whose neurons were uninterruptedly supplied with fuel.

Table 1.7

Computed compartment glucose levels for the core (primary) model or the astrocyte-neuron lactate shuttle (ANLS)

	[a]Primary Model		[b]ANLS	
Serum [Glc]	6	30	6	30
Endothelial [Glc]	3.8	17.4	3.7	17.0
Basal lamina [Glc]	2.1	10.8	2.0	10.4
Astrocytic [Glc]	0.9	7.9	1.3	9.4
Interstitial [Glc]	1.4	9.9	1.3	9.5
Neuronal [Glc]	1.2	8.8	1.1	8.4
[c]Brain [Glc]	1.2	8.9	1.2	9.1

(From Simpson *et al.*, 2007.)

1.2.2.2 Physiological considerations

Notwithstanding the progress made in elucidating how glucose gains entry to the brain and permeates neurons, the current state of the field leaves many questions unanswered in physiological settings. Converging data on differential expression of GLUTs across brain regions, between species, in health and in disease are lacking. We know that GLUT3 appears to occur in high levels in grey matter, especially in densely populous zones known to be energetically needy; fittingly, neurons from the hippocampal region, cerebral cortex, cerebellum, striatum and midbrain express the greatest amounts in the rat brain (Bondy *et al.*, 1992; Nagamatsu *et al.*, 1993). However, we know little about the transcriptional regulation of brain GLUTs and the stabilization of mRNA transcripts[18]. Best studied is the upregulation of GLUTs in the CNS during hypoglycaemic events (Anitha *et al.*, 2012; Nagamatsu *et al.*, 1994; Santiago *et al.*, 2006; Simpson *et al.*, 1999). Moreover, aside from evidence of cerebral GLUT mobilization in specific regions of the brain during active firing (Ashrafi *et al.*, 2017; Ferreira *et al.*, 2011; Weisova *et al.*, 2009), information is scarce on the recycling and trafficking of these transporters (McClory *et al.*, 2014). Unlike GLUT4, intracellular pools of GLUT1 and 3 are seemingly small and do not require insulin to be upregulated at the cell surface[19].

At any rate, hyperglycaemia, especially sustained for long periods of time, remains a blind spot in the study of cerebral GLUTs and glucose uptake in the brain. In opposition to the hypoglycaemia-induced compensatory upregulation of brain GLUTs, hyperglycaemia has not been demonstrated to afford a clear regulatory effect in rodents, at least on the short term (Anitha *et al.*, 2012; Nagamatsu *et al.*, 1994; Santiago *et al.*, 2006; Simpson *et al.*, 1999). Under normal physiological circumstances, literature further provides that microvasculature density is mostly uniform across brain regions,

[18] There is some evidence in the periphery to support a role for cellular stress in the transcriptional upregulation of GLUT1, explicitly in contexts of oxidative stress, mitochondria inhibition and hypoxia (Anitha *et al.*, 2012; Bashan *et al.*, 1992, 1993; Baumann *et al.*, 2007; Kozlovsky *et al.*, 1997; McMahon and Frost, 1995; Mobasheri *et al.*, 2005; Nagamatsu *et al.*, 1994; Santiago *et al.*, 2006).

[19] Some have raised the possibility that membrane-bound brain GLUTs may be rapidly trafficked in and out of lipid rafts to respond to metabolic signals (Barnes *et al.*, 2004; Rauch *et al.*, 2006).

including the midbrain and the striatum (Ielacqua *et al.*, 2016; Kolinko *et al.*, 2016). However, the possible effects of hyperglycaemia on cerebral blood flow are contradictory. On the one hand, both acute and chronic hyperglycaemic states are known to reduce cerebral blood flow (Duckrow *et al.*, 1987; McCall, 1992; Nishimura *et al.*, 2007). On the other hand, they cause the occurrence of pathological neovascularization, that is, a rise in the volume of capillaries with deficient pericyte coverage (Ergul *et al.*, 2015; Li *et al.*, 2010; Prakash *et al.*, 2013). The ensuing gain of blood-brain barrier permeability might grant glucose the ability to sidestep endothelial ATP-inhibited GLUT1 and to infuse the entire brain more profusely (Carruthers and Helgerson, 1989; Levine *et al.*, 1998; Lowe and Walmsley, 1986).

Despite these remaining ambiguities, various demonstrations strengthen the idea that hyperglycaemia causes a substantial rise in parenchymal glucose concentrations. Early studies demonstrated that brain glucose levels were indeed sensitive to glycaemic circumstances. Indeed, they fall or rise during transient hypoglycaemic or hyperglycaemic challenges, respectively (Abi-Saab *et al.*, 2002; Béland-Millar *et al.*, 2017; Macauley *et al.*, 2015; McCall *et al.*, 1986; Osborne *et al.*, 1997). In rodent models of sustained diabetes-induced[20] hyperglycaemia, intracerebral glucose concentrations are also augmented (de Vries *et al.*, 2003; Gomez and Barros, 2003; Jacob *et al.*, 2002; McCrimmons *et al.*, 2003). These experiments were performed on whole brain tissues and reliable intraneuronal measurements are still lacking[21]. Nevertheless, computational renderings taking into account GLUT kinetics and their distribution predict greater glucose permeation in all brain constituents under hyperglycaemia (Simpson *et al.*, 2007) (Table 1.7). We can therefore cautiously submit to the idea that hyperglycaemia causes an upwelling of intraneuronal glucose likely to hold implications for cellular fitness.

[20] Diabetes mellitus is described in section 1.2.5.1.

[21] Previous studies reporting these concentrations were recently demonstrated to be flawed, owing to differing transport kinetics between glucose and the tagged analogues employed for quantifications (Dienel *et al.*, 2017).

1.2.3 Neuronal glucose metabolism

Before addressing the metabolic singularities that characterize neurons, a brief outline of glucose metabolism is warranted (see for review Stryer *et al.*, 2007) (Figure 1.13). In the cytosol, glucose is first engaged along a glycolytic fate upon irreversible phosphorylation by an important rate-limiting, ATP-consuming enzyme, hexokinase. Resulting glucose 6-phosphate (G6P) is a gateway metabolite, not only to the glycolytic pathway, but also to glycogenesis, relevant to astrocytes, or to the pentose phosphate pathway (PPP)[22]. In glycolysis, G6P undergoes isomerization into fructose 6-phosphate (F6P), which is in turn transformed by phosphofructokinase-1 (PFK) into fructose 1,6-bisphosphate. Importantly, PFK is another rate-limiting, energy-consuming enzyme that drives glycolysis and is allosterically inhibited by high ATP:adenosine monophosphate (AMP) ratios, which equally ensures activation of glycolysis in times of energy depletion. Continuing along the glycolytic pathway, fructose 1,6-bisphosphate is converted into a pair of triose intermediates, glyceraldehyde 3-phosphate (GA3P), further metabolized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) into 1,3-bisphosphoglycerate. From 1,3-bisphosphoglycerate, a series of enzymatic transformations yields the final glycolysis metabolite, pyruvate, through irreversible cleavage of the phosphate group by pyruvate kinase. At this level, pyruvate can enter mitochondria and undergo catalysis mediated by pyruvate dehydrogenase, yielding the gateway intermediate to the tricarboxylic acid cycle, acetyl coenzyme A[23]. In certain circumstances, it can also be metabolized in the cytosol by the bidirectional lactate dehydrogenase enzyme to give lactate, a process termed the Warburg effect[24] (Warburg, 1956). Exogenous lactate may also be provided as a fuel at this level through its transformation to pyruvate, once again via lactate dehydrogenase. Generated along the course of glycolysis and tricarboxylic acid cycling are matrix-dwelling electron donors,

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[22] The PPP is later discussed in section 1.2.3.1. Of note, the PPP yields GA3P and F6P, an additional connection between glycolysis and the PPP.

[23] This is also the access point for alternate fuels, like ketone bodies, provided during glucose depletion, which is beyond the scope of this thesis.

[24] In mammalian cells, the Warburg effect occurs under aerobic conditions, contrarily to the lactate-producing anaerobic glycolytic process. It should not be confused with the Warburg effect that occurs in plants, describing decreased photosynthesis in hyperoxic conditions.

namely reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), whose electrons are funnelled through the electron transport chain of the inner mitochondrial membrane and are relinquished to the terminal electron acceptor, oxygen, to yield water. Concurrently, this electron traffic establishes a transmembrane proton gradient serving to power the synthase that ultimately produces ATP (30-36 per molecule of glucose).

Although this energy production pathway is present in some form or other in all cells, it is highly ramified and shares intermediates with a plethora of other parallel metabolic routes. A few of these are indeed relevant in the brain and in a hyperglycaemic setting, but, for the sake of brevity, only the most salient of these will be discussed in the next subsections.

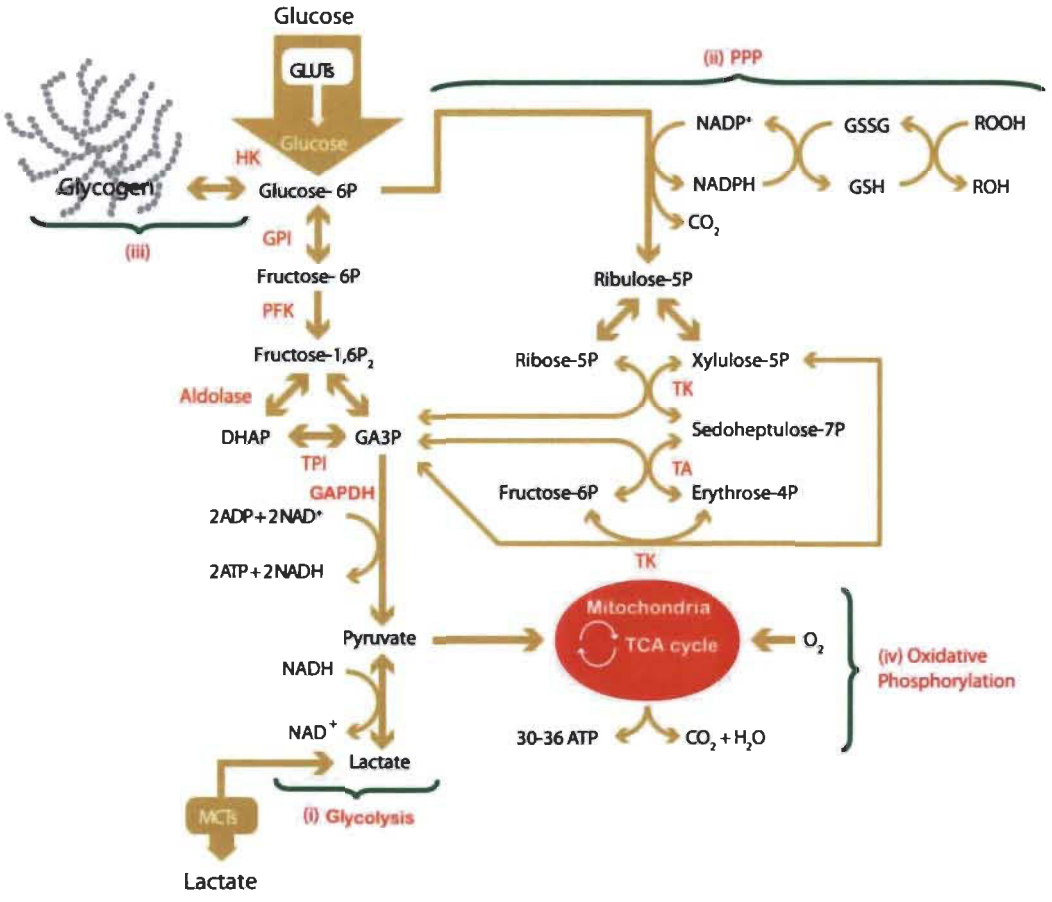

Figure 1.13 Schematic representation of glucose metabolism and pertinent connecting pathways in neurons and astrocytes.
Continued on next page.

(Continued.) Glucose is processed principally via glycolysis (i) leading up to oxidative phosphorylation (iv) with the intent of producing ATP for energy. However, glucose metabolites can also be diverted into tangential pathways. In astrocytes, glucose 6-phosphate (G6P) can be transformed into glycogen by glucokinase (iii). In neurons, the pentose phosphate pathway (PPP) is crucially provided by G6P for the maintenance of a proper redox status via the regeneration of the reduced form of glutathione (ii). ADP, adenosine diphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPI, glucose 6-phosphate isomerase; GSH, glutathione; GSSG, glutathione disulphide; HK, hexokinase; NAD, nicotinamide adenine dinucleotide (NAD+ oxidized, NADH reduced); NADP, nicotinamide adenine dinucleotide phosphate (NADP+ oxidized, NADPH reduced); PFK, phosphofructokinase; ROH, alcohol; ROOH, hydroperoxide compound; TPI, triosephosphate isomerase; TA, transaldolase; TCA, tricarboxylic acid; TK, transketolase. (From Magistretti and Allaman, 2015.)

1.2.3.1 Specific metabolic fates

Extracellular glucose levels in the CNS are always in excess of demand due to favourable GLUT1 kinetics. Yet, to ensure uninterrupted intracellular glucose availability, it is also crucial that the velocities of uptake and metabolism be tightly coupled. In neurons, the first step of glycolysis holds a particular importance in this regard. Indeed, hexokinase phosphorylates glucose more slowly than it is taken up by GLUT3 (Lowry and Passonneau, 1964; Whitesell *et al.*, 1995; Wilson, 2003). In other words, GLUT3 uptake is never limiting and free cytosolic glucose is constantly available (Dienel, 2012). Similarly, to secure glycolysis with a sustained flow of metabolites, especially during neuronal activity, engagement of glucose in the pathway must not be rate-limiting. In this respect, processing of glucose by hexokinase allows neurons to "stock" fuel in the form of G6P. However, since hexokinase is inhibited by G6P, its accumulation is somewhat limited. Nevertheless, ATP levels dwindle during neuronal activation, thereby relieving the inhibition on downstream rate-limiting PFK and favouring the expenditure of accrued metabolites between hexokinase and PFK. In turn, hexokinase is disinhibited due to the consequent reduction of G6P pools and can once again phosphorylate glucose to provide the glycolytic pathway with substrates. These gating processes allowing the accumulation of glycolytic intermediates act as a

buffering system to account for the short-lived gap between activity-induced ATP utilization and its metabolic renewal (Mangia *et al.*, 2011).

As previously stated, the brain almost fully oxidizes the glucose it takes up. Precisely, the oxygen-glucose index ratio (equation 1) in humans is estimated at 5.5 in a resting state and 5.0 during neuronal activation, where 6 represents full stoichiometric oxidation (equation 2) (Shulman *et al.*, 2001; Siesjö, 1978; Sokoloff, 1999).

$$\text{oxygen-glucose index} = \frac{\text{cerebral metabolic rate of } O_2}{\text{cerebral metabolic rate of glucose}} \quad (1)$$

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (2)$$

These observations have been interpreted as the utilization of glucose for non-oxidative processes, which infers bypassing oxidative phosphorylation (Shulman *et al.*, 2001). The engagement of glucose in the Warburg effect[25], the synthesis of amino acids, the glycation of macromolecules and the PPP are all non-oxidative mechanisms suggested to explain the oxygen-glucose index gap. However, within the context of this thesis, we will only discuss the fate of glucose in the PPP (Clarke and Sokoloff, 1999; Dienel, 2009; Shulman *et al.*, 2001).

The PPP is best known for its pivotal role in supplying ribose 5-phosphate for the synthesis of nucleic acids in mitotic cells (see for review Lehninger *et al.*, 1995). However, its importance in neurons rather dwells in the recycling of nicotinamide adenine dinucleotide phosphate (NADPH), a vital redox cofactor (Kletzien *et al.*, 1994). The glycolysis metabolite G6P is the entrance point to the PPP via the rate-limiting enzyme glucose 6-phosphate dehydrogenase (Figure 1.13). A sequence of non-oxidative

[25] The Warburg effect consists in the non-oxidative transformation of 1 molecule of glucose into 2 molecules of pyruvate through glycolysis, which yields 2 ATPs and uses up 2 NAD+ cofactors. Subsequent reduction of 2 pyruvates into 2 lactates via the Warburg effect restores 2 NAD+ cofactors. During sustained neuronal activity, ATP can be produced very rapidly from glucose without the need for oxidative phosphorylation via a self-renewing cycle. The lactate produced can be additionally converted into pyruvate to drive the tricarboxylic acid cycle and, therefore, oxidative phosphorylation. However, its concentrations harvested through the Warburg effect are predicted to outstrip energetic needs and may therefore be released unprocessed into the circulation via monocarboxylate transporters, accounting for part of the 0.5 oxygen-glucose index gap between resting and active states (Shulman *et al.*, 2001).

reactions regenerates 2 molecules of NADPH from G6P and returns the intermediate back on the glycolytic track through the production of GA3P or F6P. Importantly, NADPH produced by the PPP is employed by glutathione reductase to restore oxidized glutathione to its reduced and useful form[26] (Hothersall *et al.*, 1979; Kletzien *et al.*, 1994). Indeed, by virtue of glutathione's detoxifying action on ROS, studies support a potent antioxidative role for the PPP in neurons and astrocytes (Ben-Yoseph *et al.*, 1996*a*, 1996*b*; Bolaños *et al.*, 2008; Herrero-Mendez *et al.*, 2009; Kussmaul *et al.*, 1999; Vaughn and Deshmukh, 2008). Pertaining to the oxygen-glucose index gap, attention must rather be drawn to the shared metabolic intermediate between glycolysis and PPP, F6P, since it stands upstream from any irreversible reactions in the glycolytic pathway, as opposed to GA3P found downstream from the unidirectional PFK enzyme and destined to oxidative phosphorylation. Combined with evidence for a bottleneck effect at the level of PFK in neurons[27] (Bolaños *et al.*, 2010; Herrero-Mendez *et al.*, 2009), some have suggested that F6P is recycled through the PPP by catalytic reversion to G6P (Bouzier-Sore and Bolaños, 2015). Therefore, any fraction of these metabolites that keep cycling through the non-oxidative PPP may well account for a portion of the 0.5 stoichiometric gap between glucose and oxygen use (Bouzier-Sore and Bolaños, 2015).

1.2.3.2 Current hypotheses in neuroenergetics

For the most part, the metabolic processes described above have not been clearly ascribed to neurons or other brain cells on account of the lack of consensus per the proportion of glucose absorbed and metabolized by each compartment. For the past 20 years, the field of neuroenergetics has been a battleground of ideas around this

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[26] Glutathione, or γ-L-glutamyl-L-cysteinylglycine, is a pseudo-tripeptide whose thiol moiety acts as a reducing agent. Glutathione peroxidase catalyzes the transfer of electrons between two glutathiones and one hydrogen peroxide molecule, yielding water and one glutathione disulphide, a condensation of two oxidized glutathiones. Glutathione disulphide is reverted to its useful form, reduced glutathione, by glutathione reductase. Glutathione transferase can also catalyze the addition of glutathione onto potentially toxic xenobiotics.

[27] The bottleneck effect is described in section 1.2.3.2.

enigma, arising mainly from two groups that allot differential importance to these processes in neurons and astrocytes[28].

The first group, spearheaded by Pellerin and Magistretti, defends the position that astrocytes, and not neurons, are the principal site of glucose uptake, especially during neuronal activation (Pellerin and Magistretti, 1994). In turn, astrocytes provide energy to neurons in the form of lactate, a hypothetic process hence termed the astrocyte-neuron lactate shuttle[29] (Figure 1.14). According to this theory, astrocytes are predominantly glycolytic, meaning that they utilize glucose to produce lactate through glycolysis and the Warburg effect. On the other hand, neurons are rather oxidative and transform astrocyte-derived lactate into pyruvate, which is metabolized through the tricarboxylic acid cycle to power oxidative phosphorylation. The authors put forward two central arguments: 1) astrocytes import more glucose than neurons; 2) neurons cannot upregulate their rate of glycolysis. The first contention hinges on evidence demonstrating astrocytes as importing more than 50-90% of available brain glucose (Barros *et al.*, 2009; Nehlig *et al.*, 2004; Vega *et al.*, 2003). The second is based on studies revealing that the levels of a specific enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (Pfkfb3), are markedly downregulated in neurons compared to astrocytes due to constant proteasomal degradation (Bolaños *et al.*, 2010; Herrero-Mendez *et al.*, 2009). Pfkfb3 phosphorylates F6P to produce fructose 2,6-bisphosphate (Fru-2,6-P_2) known to activate one of the key rate-limiting glycolytic enzymes, PFK. The authors therefore contend that sparse expression of Pfkfb3 in neurons explains the bottleneck effect at the level of PFK, liable for its limited glycolytic capacities.

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[28] A heated debate took place a few years ago between these two groups in the *Journal of Cerebral Blood Flow and Metabolism*. A succession of commentaries offers very interesting insight into the arguments hired by both parties to support their theories (Jolivet *et al.*, 2010; Mangia *et al.*, 2011).

[29] Later biophysical computations and physiological applications came in support of this model (Aubert and Costalat, 2005; Aubert *et al.*, 2007; Béland-Millard *et al.*, 2017; Mächler *et al.*, 2016).

The second theory, termed the core model[30], is advocated by Mangia, Simpson and Vannucci, and rather posits that the lactate shuttle effect is negligible (Mangia *et al.*, 2009; Simpson *et al.*, 2007). Their computational model relies on the same variables used by the former group, with the exception that it considers experimentally measured transporter kinetics and distribution patterns throughout the brain. Taking into account these additional parameters, this group arrives at the conclusion that neurons constitute the main site of glucose uptake. To bolster their position, they recapitulated and invalidated the two main arguments in support of the former model. First, regarding the preferred astrocytic uptake of glucose, some of the studies demonstrating a greater glucose influx in favour of astrocytes (80-90%) were performed in cerebellar slices (Barros *et al.*, 2009) and *ex vivo* rat vagus nerves (Vega *et al.*, 2003), which may poorly represent physiological situations. Other reports in freely moving animals indeed moderate these numbers and rather support equal partitioning or even preferential neuronal uptake (50-80%) (Nehlig *et al.*, 2004; Zielke *et al.*, 2007). Second, while the former group argues that glycolytic activity is capped in neurons due to the bottleneck effect at PFK (Bolaños *et al.*, 2010; Herrero-Mendez *et al.*, 2009), Mangia and colleagues contend instead that PFK may be activated by metabolites other than Fru-2,6-P_2, thus, that glycolysis can be enhanced[31] (Ogushi *et al.*, 1990). In support of this, other neuronal monosaccharidic metabolites were found to be more potent activators of PFK, and Fru-2,6-P_2 appears to be expendable in the amplification of glycolysis during neurotransmission (Ogushi *et al.*, 1990; Pauwels and Trouet, 1984). Based on these arguments, the core model stipulates that neurons, and not astrocytes, take up more glucose and account for lactate transients during brain activity.

In a final attempt to reach a consensus, one group gathered both models into a unified computational framework, which ultimately favoured the theory that astrocyte-neuron lactate shuttling is negligible, that neurons are the principal glucose consuming

[30] This position is bolstered by physiological evidence that contradicts the studies in support of the astrocyte-neuron lactate shuttle (Díaz-García *et al.*, 2017; Drulis-Faidasz *et al.*, 2019; Hall *et al.*, 2012).

[31] Simpson and colleagues further provide reports that indirectly contradict the alleged lack of glycolysis upregulation, showing rises in surface GLUT3 in response to neuronal activity and, by the same token, proving that neurons dispose of purposeful pathways for controlling internal energy supplies, likely via glycolytic processes (Ferreira *et al.*, 2011; Weisova *et al.*, 2009).

cells of the CNS, and that they can carry out both glycolysis and oxidative phosphorylation (DiNuzzo *et al.*, 2010). Regardless of whether neurons constitute the preferential consumers of glucose, we can cautiously surmise that intraneuronal glucose levels rise during hyperglycaemia and that they fuel ATP production at least partially through oxidative phosphorylation. However, these models have not been specifically applied to different neuronal subtypes such as dopaminergic neurons or to various brain regions, such as the midbrain and striatum, which all are likely to exhibit distinct metabolic behaviours.

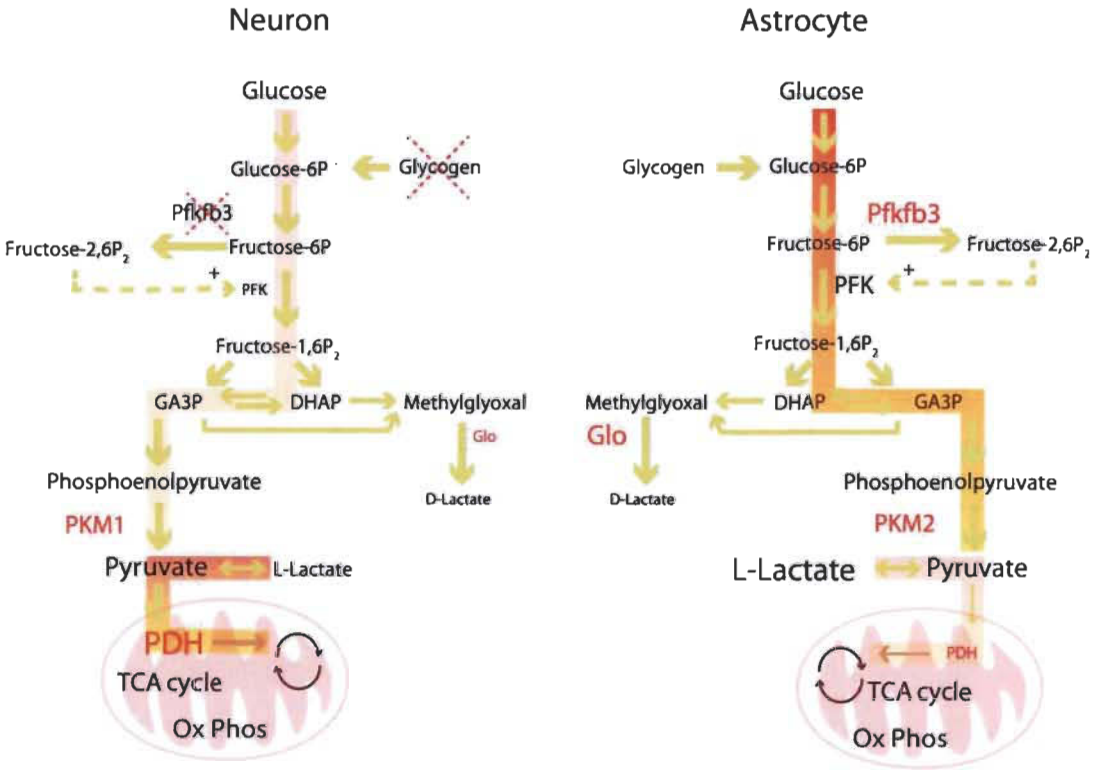

Figure 1.14 A schematic representation of the astrocyte-neuron lactate shuttle theory.
The premise of this school of thought provides that astrocytes are more glycolytic and neurons are more oxidative. It is proposed that the bottleneck effect at the level of PFK is seemingly more pronounced in neurons and accounts for their low glycolytic capacity. A direct corollary of this is that lactate must be shuttled to neurons for it to be able to produce ATP. Glo, glyoxalase; Ox Phos, oxidative phosphorylation; PDH, pyruvate dehydrogenase; Pfkfb3, 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 3; PKM, pyruvate kinase isozymes. (From Magistretti and Allaman, 2015.)

1.2.4 Neuronal oxidative stress in hyperglycaemia

The clinical significance of hyperglycaemia emerged upon its formal identification as the prime culprit in comorbid diabetic complications (Diabetes Control and Complications Trial Research Group, 1993; Gaede *et al.*, 2008; Giacco and Brownlee, 2010; UK Prospective Diabetes Study Group, 1998). The most affected collateral targets in diabetes mellitus are the kidneys, the cardiovascular system and the nervous system (Nathan, 1993). Within each of these tissues dwell cells that are preferentially vulnerable to hyperglycaemia, namely mesangial cells of renal glomeruli, endothelial capillary cells, and brain cells. Despite their many functional and phenotypic differences, these cells share the distinct liability of taking up glucose in a manner that is reliant on extracellular concentrations (Heilig *et al.*, 1995; Kaiser *et al.*, 1993). Indeed, mesangial and endothelial cells predominantly express insulin-independent GLUT1 (Brosius and Heilig, 2005; Mueckler and Thorens, 2013; Sone *et al.*, 2000); likewise, brain cells express several insulin-independent GLUTs, chiefly GLUT1 and GLUT3 (Mantych *et al.*, 1992; Nagamatsu *et al.*, 1993; Simpson *et al.*, 2008). Although it is clear that glucose concentrations rise in these cells under conditions of hyperglycaemia, how exactly cellular damage occurs is not as forthright. The current concensus is that oxidative stress explains hyperglycaemia-induced diabetic complications[32] (Araki and Nishikawa, 2010; Brownlee, 2005; Ceriello, 2003; Giugliano *et al.*, 1996).

Oxidation is a ubiquitous phenomenon common to all organisms that engage in aerobic respiration for energy. Indeed, ROS are mainly produced from the leakage of the mitochondrial electron transport chain during oxidative phosphorylation, but can also arise from other endogenous (e.g., monoamine oxidase activity, dopamine auto-oxidation, Fenton reactions in the presence of transition metals, etc.) or exogenous sources (e.g., exposure to pesticides, smoking, ultraviolet and ionizing radiations, pollution, etc.). Although required for a number of desirable physiological processes, the excessive generation of ROS can affect almost all cellular components, namely

[32] Understanding the link between hyperglycaemia and diabetic complications required the discovery of particular markers in the blood and tissues of diabetic patients indicating a state of oxidative stress (Cheng and González, 1986; Jones *et al.*, 1988; Matkovics *et al.*, 1982).

DNA, lipids and proteins (Barzilai and Yamamoto, 2004; Niki, 2008). To cope with the unavoidable production of ROS, antioxidative defences are provided endogenously, by superoxide dismutase (SOD), catalase, urate, ascorbate and glutathione, or exogenously, by vitamins, polyphenols and other nutrients found in the diet. *Per se*, oxidative stress arises from an imbalance between the generation and clearance of ROS, in favour of the former, and may ultimately provoke cell death. This inability to cope with an oxidative overload constitutes a key pathological element, not only in hyperglycaemia (Brownlee, 2005; Wolff *et al.*, 1991), but also in Parkinson's disease (Hwang, 2013; Schapira and Jenner, 2011; Tsang and Chung, 2009) and aging (Finkel and Holbrook, 2000; Pérez *et al.*, 2009).

In consuming approximately 20% of total inhaled oxygen (Jain *et al.*, 2010; Quastel and Wheatley, 1932), the brain is doubtlessly susceptible to undergo oxidative stress. Indeed, processing of this oxygen implies engagement of oxidative phosphorylation, which inexorably entails the generation of mitochondrial ROS (Kudin *et al.*, 2004; Liu *et al.*, 2002). Owing to their steep demands in ATP, neurons possess a high basal oxidative metabolism compared to other types of cells in the brain or elsewhere, and are thus plausibly more vulnerable to additional sources of stress. Although the literature does not provide an adequate account of the mechanisms underlying hyperglycaemia-induced oxidative stress in the CNS, let alone in nigrostriatal dopaminergic neurons, we can speculate on the events that contribute to this critical coping threshold based on experimental data and our knowledge of neuronal glucose metabolism.

1.2.4.1 Mitochondrial mechanisms

Following an upsurge in intraneuronal glucose, a plausible event that may occur is an amplified flux of electron donors toward oxidative phosphorylation. In a first scenario, high intraneuronal glucose concentrations may provide the glycolysis pathway and tricarboxylic acid cycle with more fuel, culminating in the increased production of electron donors and the improvement of oxidative phosphorylation. In a second scenario,

taking into account the possibility of an astrocyte-neuron lactate shuttle in hyperglycaemia, lactate may be converted to pyruvate to power the tricarboxylic acid cycle in neurons. The end result remains the enhanced solicitation of the electron transport chain.

Many have indeed advocated the theory elaborated by Brownlee and colleagues, postulating that the production of superoxide anion by the overworked electron transport chain constitutes the initial oxidative insult that takes place in cells vulnerable to hyperglycaemia, including neurons (Brownlee, 2005; Du *et al.*, 2001; Giacco and Brownlee, 2010; Nishikawa *et al.*, 2000; Tomlinson and Gardiner, 2008). The premise of this theory hinges on electron transport chain overload, resulting from increased delivery of electrons by donors formed via the glycolytic pathway or the tricarboxylic acid cycle (Figure 1.15). Normally, electron transport chain leaks are estimated to occur at a rate of 1-3% (Boveris, 1977; Kudin *et al.*, 2004; Liu *et al.*, 2002), but other studies demonstrate that hyperglycaemia elicits an early production of superoxide anion beyond basal levels (Du *et al.*, 2001; Nishikawa *et al.*, 2000). Hence, superoxide anion exceeds antioxidative defences and reacts with mitochondrial molecules to form secondary ROS that are apolar, thus, free to diffuse across membranes and to damage the various constituents of the cell, including nuclear DNA. The resulting strand breaks activate the NAD+-dependent DNA repair enzyme, poly(adenosine diphosphate-ribose) polymerase (PARP), whose catalytic activity inhibits a key glycolytic enzyme, GAPDH[33] (Du *et al.*, 2003) (Figure 1.16). Obstruction of glycolysis at GA3P causes upstream intermediates to be rerouted toward deleterious tangential pathways. It is proposed that generation of ROS by these pathways contributes to a self-perpetuating cycle of DNA damage, PARP activation and GAPDH inhibition, leading to a permanent glycolytic impasse and sustained oxidative stress (Brownlee, 2005).

[33] Alongside adenosine diphosphate (ADP)-ribosylation by PARP, oxidative stress also directly inhibits GAPDH. Together, these mechanisms allow for the dynamic rerouting of glucose through the PPP with the aim of producing NADPH cofactors for the renewal of the cofactor glutathione by glutathione reductase (Ralser *et al.*, 2007). The sum result remains the impediment of glycolysis.

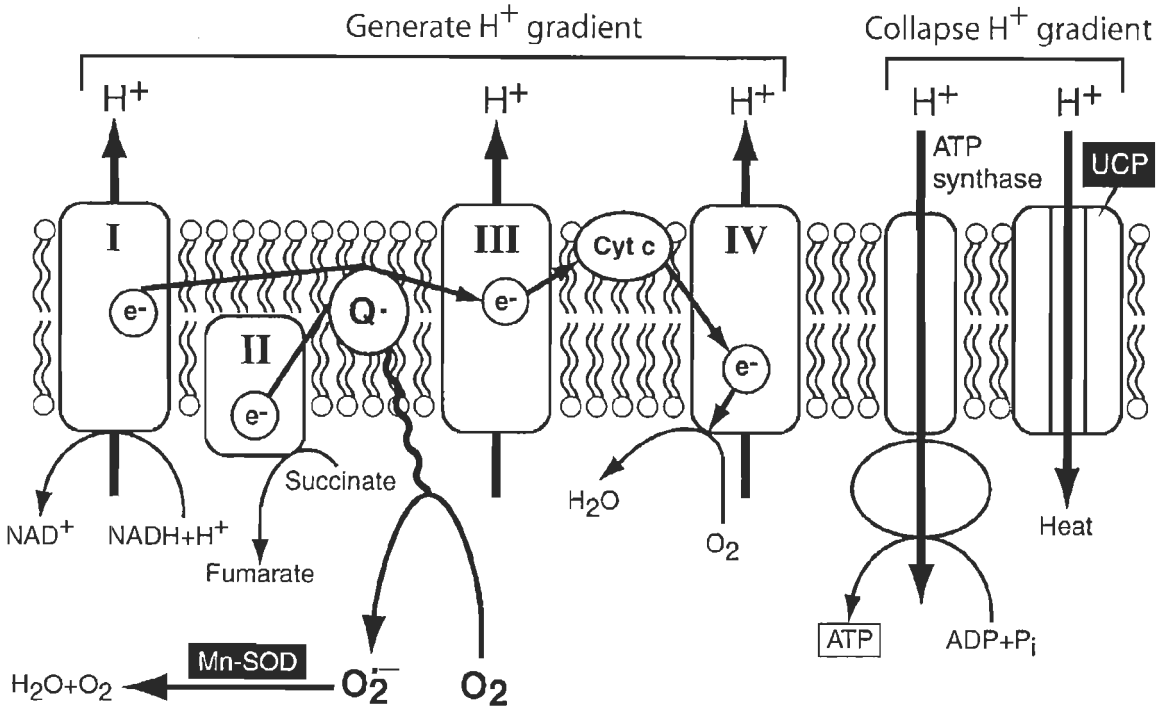

Figure 1.15 Hyperglycaemia-induced generation of superoxide anion at the level of the mitochondrial electron transport chain.
Electron traffic through the complexes (I-IV) of the chain allows the pumping of protons into the mitochondrial intermembrane space, which affords an electrochemical potential difference that powers ATP synthase (Trumpower, 1990). Protons are usually trafficked back into the matrix by ATP synthase, paired to its catalytic activity, and by uncoupling protein-1, which generates heat to alleviate overly steep gradients. However, when electron transport is profuse, the transmembrane potential increases faster than it can be dissipated by ATP synthase and uncoupling protein-1. Above a certain potential threshold, certain complexes, especially complex III, of the electron transport chain undergo inhibition and begin to leak their electrons, which are in turn captured by oxygen to produce superoxide anion (Adam-Vizi, 2005; Korshunov *et al.*, 1997; Kwong and Sohal, 1998; Starkov *et al.*, 2004). Owing to its negative charge that prevents it from crossing phospholipid bilayers, superoxide anion remains trapped within mitochondria. There, it is usually deactivated by the mitochondrial superoxide dismutase (SOD) into peroxide, another reactive oxygen species (ROS) that can cross phospholipid bilayers and damage other components of the cell. An upsurge in oxidative phosphorylation as it occurs in states of high intracellular glucose availability will necessarily increase superoxide anion generation. Cyt c, cytochrome *c*; e-, electron; $O_2^{\bullet-}$, superoxide anion; P_i, inorganic phosphate; Q, quinone; UCP, uncoupling protein. (From Brownlee, 2005.)

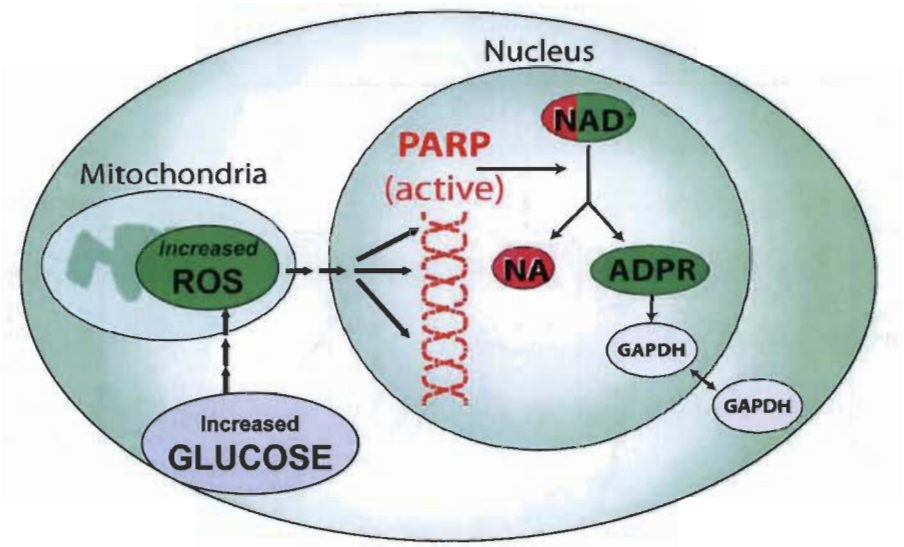

Figure 1.16 Detrimental activation of PARP in response to DNA damage. Oxidative stress leads to ROS-induced nuclear DNA damage responsible for activating poly(adenosine diphosphate-ribose) polymerase (PARP). Besides repairing single-stranded DNA nicks, it also operates as an adenosine diphosphate-ribosyl transferase (Sawa *et al.*, 1997; Schmidtz, 2001). One of its targets is GAPDH, known to shuttle between the cytosol and the nucleus. Upon ADP-ribosylation, GAPDH is inactivated, thereby contributing to the accrual of upstream glycolysis metabolites. Moreover, if PARP is not inactivated, it can lead to the depletion of the NAD+ cofactor. ADPR, adenosine diphosphate ribosyl; NA, nicotinamide. (From Brownlee, 2005.)

It is important to note that the key results in support of Brownlee's theory were obtained in bovine aortic endothelial cells treated with 30 mM of glucose for 7 days (Du *et al.*, 2001; Nishikawa *et al.*, 2000). Consequently, these conditions may not reflect what occurs in the brains of animals exposed to high levels of glucose. A small body of literature investigating these mechanisms in the CNS and the periphery of rodents does not clearly validate this model in neurons. In fact, whether at the level of glycolysis or the tricarboxylic acid cycle, the data inconsistently reports up- or downregulation of these pathways, both in peripheral nerves or brains of hyperglycaemic rodents (Akude *et al.*, 2011; Chowdhury *et al.*, 2010; Hinder *et al.*, 2013; Kaur and Bhardwaj, 1998; Price *et al.*, 2006; Thakran *et al.*, 2003; Thurston *et al.*, 1995; Zheng *et al.*, 2017). However, with regard to oxidative phosphorylation, most studies revealed a decreased activity or expression of electron transport chain constituents in the brain and in peripheral sensory neurons (Aghanoori *et al.*, 2017; Akude *et al.*, 2011; Chowdhury

et al., 2010; Kaur and Bhardwaj, 1998; Stančić *et al.*, 2013). A closer look at the duration of hyperglycaemia affords a clearer understanding of the process. Early events in hyperglycaemia cause the upregulation of glycolysis and tricarboxylic acid cycling (Kaur and Bhardwaj, 1998; Price *et al.*, 2006; Thakran *et al.*, 2003; Thurston *et al.*, 1995). Later, however, these pathways are impeded (Aghanoori *et al.*, 2017; Akude *et al.*, 2011; Chowdhury *et al.*, 2010; Hinder *et al.*, 2013; Zheng *et al.*, 2017). At all time points, oxidative phosphorylation is impaired (Aghanoori *et al.*, 2017; Akude *et al.*, 2011; Chowdhury *et al.*, 2010; Kaur and Bhardwaj, 1998; Stančić *et al.*, 2013). In other words, dysfunction of mitochondrial respiration likely occurs before glycolysis and tricarboxylic acid cycle hindrance. This supports the theory that hyperglycaemia induces an early upsurge in glucose metabolism leading to the initial insult arising from mitochondrial respiration (Brownlee, 2005; Du *et al.*, 2001; Giacco and Brownlee, 2010; Nishikawa *et al.*, 2000; Tomlinson and Gardiner, 2008). However, since the model proposed by Brownlee does not account for precise cellular effects of long-term hyperglycaemia, it cannot explain the global impairment of glucose metabolism observed at later time points.

Among these studies, very few groups measured superoxide anion levels and proper mitochondrial respiration. In these reports, solely performed in peripheral sensory neurons of long-term hyperglycaemic rats, superoxide anion production and respiration rates were downscaled even when corrected for mitochondrial numbers, despite overt manifestations of oxidative stress (Aghanoori *et al.*, 2017; Akude *et al.*, 2011; Chowdhury *et al.*, 2010). This may indicate that sustained hyperglycaemia causes the waning of mitochondrial respiratory functions over time, sparing superoxide anion production while ROS generation is maintained by non-mitochondrial sources. One explanation provides that the initial superoxide anion overload proposed by Brownlee's model may cause deleterious mutations in mitochondrial DNA, leading to defects in electron transport chain components that in turn generate more ROS[34].

[34] Unlike nuclear DNA, mitochondrial DNA lacks protection afforded by histones and repair enzymes. For this reason, the rates of mutagenesis in mitochondria are relatively high. Some subunits of complexes I and IV of the electron transport chain are encoded in mitochondrial DNA and, thus, are highly susceptible to mutation-induced impairments (Wei, 1998).

In the long term, excessive oxidative damage to mitochondrial DNA may impair respiratory chain expression altogether, causing the observed shortfall in superoxide anion[35] (see for reviews Guo *et al.*, 2013; Wei, 1998). Although studies employing proper animal models of hyperglycaemia have not investigated very early mitochondrial events in neurons, a prompt increase in superoxide anion is observed following ischaemia-reperfusion, a paradigm similar in some respects to a transient rise in glycaemia (Iwata *et al.*, 2014; Muranyi and Li, 2006; Won *et al.*, 2015).

1.2.4.2 Rerouting mechanisms: polyol pathway and macromolecule glycation

Aside from ROS generated through mitochondrial failure, hyperglycaemia may trigger the onset of oxidative stress via pathways that dwell upstream in glycolysis. As previously demonstrated, free glucose is present in the cytosol of neurons and its concentrations rise during hyperglycaemia. Under normal circumstances, hexokinase is mandated to transform available glucose into G6P. However, due to substrate-mediated inhibition, it can only engage a fraction of cytosolic glucose along the glycolytic pathway before it is saturated. Excess glucose is therefore consumed by other pathways and is constantly replenished by high extracellular levels. The principal compensatory outlets are the polyol pathway and macromolecule glycation.

The polyol pathway is driven by aldose reductase whose affinity for glucose is relatively low (Gabbay *et al.*, 1966). Under normal glycaemia, aldose reductase does not appoint glucose to the polyol pathway, but high intraneuronal concentrations drive its catalytic activity. Aldose reductase preferentially employs the NADPH cofactor, produced by the PPP, to reduce glucose into the polyalcohol sorbitol (Lee and Chung,

[35] Another explanation is that the increased glucose availability may provide ample supply of ATP via the non-respiratory Warburg effect, thereby circumventing the need for oxidative phosphorylation and culminating in a downward adjustment of mitochondrial functions, akin to the Crabtree effect (Hamberger and Hyden, 1963; Ibsen, 1961). Accounts of this are, however, inconsistent in hyperglycaemic models (Herse and Petchell, 1998; Koziel *et al.*, 2012; Padnick-Silver and Linsenmeier, 2003).

1999)[36]. Sorbitol then undergoes enzymatic conversion to fructose by sorbitol dehydrogenase, which exploits the important cofactor NAD+ (Verdin, 2015; Williamson *et al.*, 1993; Wu *et al.*, 2016) (Figure 1.17). Increased processing of glucose through the polyol pathway thus drains pools of cofactors required for the activity of a plethora of enzymes, including glutathione reductase, GAPDH and others in the glycolytic pathway and tricarboxylic acid cycle (Vander Jagt *et al.*, 1995).

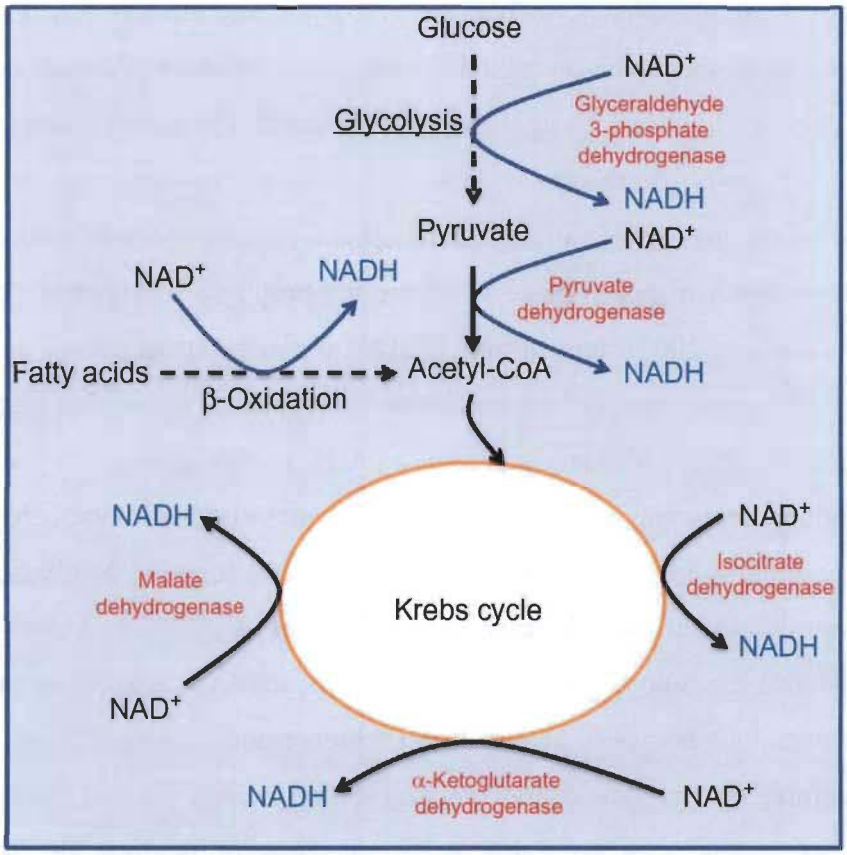

Figure 1.17 NAD in glucose metabolism.
This schematic represents the numerous enzymes that require the NAD+ cofactor to function and highlights the hazard that its depletion represents for the maintenance of normal metabolism. Acetyl-CoA, acetyl coenzyme A. (From Wu *et al.*, 2016.)

[36] A possible noxious effect of driving the polyol pathway was first proposed to be osmotic damage, seeing as cells do not export sorbitol efficiently. However, sorbitol concentrations measured in diabetic nerves and capillaries were too low to endorse this effect (Brownlee, 2001).

The second outlet of excess glucose is via macromolecule glycation, which may indirectly contribute to oxidative stress (Vicente Miranda *et al.*, 2016). Glycation consists of the slow non-enzymatic, energy-independent, covalent addition of a monosaccharide to macromolecules, usually lipids or proteins termed advanced glycation end-products (AGEs)[37] (Figure 1.18). Although glucose is a poor glycating agent, one of its downstream metabolites, methylglyoxal, exceeds its reactivity 20 000 times (Thornalley, 2005). Methylglyoxal is formed from the non-enzymatic degradation of triose phosphates, such as the glycolysis intermediate GA3P, and readily glycates proteins at the level of arginine and lysine residues (Ahmed *et al.*, 1997; Oya *et al.*, 1999; Phillips and Thornalley, 1993; Richard, 1984; Shipanova *et al.*, 1997; Thornalley *et al.*, 1999). Intracellular AGEs produced via this pathway are harmful to cells because of the loss or alteration of their normal protein or lipid functions, but also by virtue of the mechanisms in place to ensure the prophylactic clearance of glycating agents (Ahmed *et al.*, 2003). Indeed, methylglyoxal and other agents are enzymatically degraded by glyoxalase and aldose reductase (Izaguirre *et al.*, 1998; Monder, 1967; Sousa Silva *et al.*, 2013; Vander Jagt *et al.*, 1992). In the setting of hyperglycaemia, excess glycating agents may overwhelm these pathways and lead nonetheless to AGEs, evidenced by the proportional rise in CNS glycation as a function of glycaemia, which can reach an exorbitant 34-fold increase (Uchiki *et al.*, 2012). Most importantly, however, glyoxalase employs gluthathione to deactivate molecules, while we know that aldose reductase depletes pools of NADPH (Schieber and Chandel, 2014; Thornalley, 1988). Therefore, methylglyoxal and other glycating agents derived from glucose or its metabolites indirectly worsen the oxidative status of neurons by monopolizing endogenous antioxidative defences or the cofactors necessary for their restoration.

Since glycation is a non-specific and spontaneous reaction, AGEs may also occur extracellularly. These activate deleterious inflammatory pathways by binding their designated membrane receptor, RAGE (Jakus and Rietbrock, 2004; Singh *et al.*, 2001).

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[37] In diabetic patients, the gold standard in the assessment of systemic glycation, thus of the severity of hyperglycaemia, consists in quantification of circulating glycated haemoglobin (Kovatchev, 2017). In fact, on account of the gradual nature of these reactions, this measure is insensitive to daily glycaemic fluctuations and rather indicates glycaemic control spanning over the previous few weeks.

Binding of RAGEs by AGEs or other endogenous ligands induced by hyperglycaemia is a known mechanism of systemic inflammation in diabetes mellitus and can trigger intracellular signalling cascades that lead to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Bierhaus *et al.*, 2005; Haslbeck *et al.*, 2007; Rong *et al.*, 2005). In the brain parenchyma, neuroinflammation primarily mediated by glial cells can exert additional extraneuronal oxidative injuries[38].

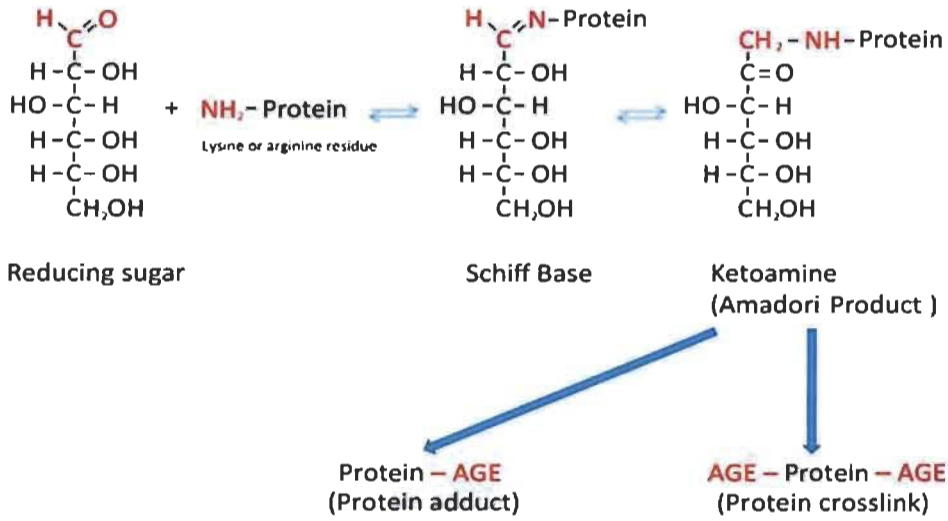

Figure 1.18 The chemistry of protein glycation.
Glycation begins by the formation of a Schiff base wherein the aldehyde group of a glycating agent will bind the amino group of a lysine or arginine molecule in a protein. This is followed by the formation of an Amadori product, consisting in a rearrangement of the hydrogen from the hydroxyl group adjacent to the imine of the Schiff base onto the nitrogen, which yields a ketone function. The ultimate formation of an advanced glycation end-product (AGE) requires the oxidation of the Amadori product, usually catalyzed by transition metals (see for review Jakus and Rietbrock, 2004; Singh *et al.*, 2001). (From Gkogkolou and Böhm, 2012.)

Gathering all the evidence, hyperglycaemia wields its detrimental effects on neurons by an assortment of conceivable mechanisms leading to oxidative stress:

1) The initial rise in glucose metabolism enhances oxidative phosphorylation, which intensifies the rate of superoxide anion formation at the electron transport

[38] We have reviewed the cellular and molecular mechanisms underlying neuroinflammation in Appendix A. The article was published in the French language.

chain. On the one hand, superoxide anion damages mitochondrial DNA, causing the expression of respiratory components prone to generate more ROS, which may lead to global mitochondrial failure in the long term. On the other hand, superoxide anion creates more ROS that cause nuclear DNA strand breaks, thereby activating PARP.

2) Increased expenditure of NAD+ by PARP slows the rates of glycolysis, tricarboxylic acid cycling and oxidative phosphorylation operating via enzymes that require this cofactor. PARP may also ADP-ribosylate and inhibit the glycolytic enzyme GAPDH, which provokes upstream accumulation of metabolites.

3) Accrued levels of GA3P upstream of GAPDH are non-enzymatically converted into the glycating agent methylglyoxal whose decomposition by glyoxalase and aldose reductase expends antioxidative cofactors, such as glutathione and NADPH.

4) Excess glucose can be diverted into the deleterious polyol pathway, powered once again by aldose reductase that further depletes stocks of NADPH required for the renewal of the antioxidative cofactor glutathione.

What emerges here is a number of different pathways that collectively cause a shortage of antioxidant defences layered with mitochondrial dysfunction, two pivotal factors likely responsible for pushing vulnerable cells beyond the critical coping threshold. The sum of these undesirable processes appears to be particularly relevant in neurons, in light of their relatively low glutathione levels and their reliance on the PPP for its rapid regeneration (Bolaños *et al.*, 1995; Kang *et al.*, 1999). In fact, insufficient provision of the PPP leads to poor regeneration of glutathione and neuronal apoptosis (Herrero-Mendez *et al.*, 2009). Neurons may reach the coping threshold quicker than other brain cells, for instance astrocytes that display comparatively greater PPP activity (García-Nogales *et al.*, 2003; Herrero-Mendez *et al.*, 2009).

Most importantly, the proposed molecular events leading to neuronal oxidative stress in hyperglycaemia, explicitly via increased superoxide anion generation (Ziegler *et al.*, 2015), mitochondrial dysfunction (see for review Patti and Corvera, 2010), PARP activation (Obrosova *et al.*, 2005), impairment of the PPP (Ziegler *et al.*, 2017),

glycation (Aubert *et al.*, 2014) and glutathione system deficits (Kasznicki *et al.*, 2012; Mendez *et al.*, 2015), are all cogently established in diabetic patients with neuronal pathology. Remarkably, clinical trials employing pharmacological agents to target some of these pathways, notably aldose reductase inhibitors to diminish polyol pathway influx (Greene *et al.*, 1999; Kawai *et al.*, 2010; Obrosova *et al.*, 2002) or exogenous SOD to quench superoxide anion (Bertolotto and Massone, 2012), are demonstrated to improve diabetic peripheral nerve damage and oxidative status, further solidifying the importance of oxidative stress in neuronal affections arising from hyperglycaemia in humans. Unquestionably, though, glycaemic control remains the most accessible means to preclude oxidative stress-induced diabetic complications in the brain and in peripheral nerves (Van den Berghe *et al.*, 2005).

1.2.5 Vulnerability of the nigrostriatal pathway

In light of their comparatively heightened basal oxidative burden[39], it may be surmised that sustained hyperglycaemia may precipitate the death of nigrostriatal dopaminergic neurons. The validity of this hypothesis transpires from epidemiological evidence suggesting a link between the prime pathologies involving the nigrostriatal pathway and hyperglycaemia, explicitly Parkinson's disease and diabetes mellitus. Before describing how they are pathologically interlaced, the latter disease will be defined in more detail for contextual purposes. Bearing in mind that a handful of other medical conditions also cause hyperglycaemia and may be associated with Parkinson's disease, as is the case for metabolic syndrome (Sääksjärvi *et al.*, 2015), we deliberately hold our focus on diabetes mellitus that offers a more tangible collection of experimental and clinical evidences to work with.

1.2.5.1 Epidemiological basis: Parkinson's disease in diabetic patients

Diabetes mellitus is the most widespread chronic metabolic disorder estimated to affect over 400 million adults worldwide, its prevalence having doubled since 1980 on

[39] Refer to the list of phenotypic liabilities in section 1.1.3.4.

the likely account of the marked rise in overweight or obese phenotypes in the population (World Health Organization, 2016). Today, diabetes has joined the infamous list of leading causes of death, responsible for 8.4% of global mortality in adulthood (International Diabetes Federation, 2013). Both types I and II diabetes occur from the progressive failure of the body to manage circulating blood glucose. However, the etiopathogenesis of type I diabetes (5-10% of diabetic patients) is specifically rooted in the autoimmune destruction of insulin-producing pancreatic β cells and usually, but not always, develops before adulthood (Yoon and Jun, 2001). On the other hand, type II diabetes ensues from peripheral resistance to and deficient production of insulin, alongside other secondary metabolic elements. It is more frequently diagnosed in adults although youth-onset forms are dramatically on the rise (Correia *et al.*, 2008; Nadeau *et al.*, 2016). The perceived relative or absolute lack of insulin control, which especially wields its effects on cells harbouring GLUT4 like striated muscle cells and adipocytes, culminates in prolonged hyperglycaemia and impaired ability to control glycaemic fluctuations, known as glucose intolerance (see for detailed review of their pathophysiology Cnop *et al.*, 2005). Other symptoms also include polyuria, polydipsia and hyperphagia (increased urination, thirst and hunger, respectively).

Granted the multiple aspects of health it impinges on, diabetes nurtures the development of a myriad of diverse comorbid conditions. As previously stated, a number of central (encephalopathies) and peripheral (neuropathies) complications are associated with diabetes, regardless of the type (Biessels *et al.*, 2002). Neuropathies remain the most prevalent neuronal affections in diabetes, as peripheral nerves are devoid of blood-brain barrier protection, which, as we know, serves to limit exposure to circulating glucose (Bansal *et al.*, 2006; Rajabally *et al.*, 2017). Nonetheless, encephalopathies are a widely acknowledged feature of diabetes (DeJong, 1950) and manifest themselves in the form of cognitive decline (Brands *et al.*, 2007; Brayne *et al.*, 2005; Hofman *et al.*, 1997), white and grey matter atrophy (Moran *et al.*, 2013), altered cortical connectivity (Lyoo *et al.*, 2013; van Duinkerken *et al.*, 2012), and neurodegenerative diseases, predominantly Alzheimer's and Parkinson's diseases (Armstrong *et al.*, 2005; Correia *et al.*, 2011; Verdile *et al.*, 2015; Vicente Miranda *et al.*, 2016).

The first accounts of a possible association between diabetes and Parkinson's disease date back to almost 60 years ago. Studies conducted in parallel showed that diabetes exacerbates the progression of motor and cognitive deficits in Parkinson's disease (Schwab, 1960), and that non-diabetic parkinsonian patients present impaired glucose tolerance (Barbeau *et al.*, 1961) and hyperglycaemia (Boyd *et al.*, 1971). Since then, these findings have been reiterated numerous times in treated individuals[40] (Cereda *et al.*, 2012; Lipman *et al.*, 1974; Sandyk, 1993), but also in drug-naïve patients who still displayed higher-than-normal levels of fasting blood glucose, within the prediabetic diagnostic range[41] (Santiago and Potashkin, 2015). In addition, dietary habits featuring an elevated intake of high glycaemic indexed carbohydrates[42] have more recently been associated with greater odds of developing Parkinson's disease (Cheng *et al.*, 2009; Murakami *et al.*, 2010; Okubo *et al.*, 2012; Stafstrom and Rho, 2012; Yang and Cheng, 2010).

In light of accumulating reports showing a possible association between the two pathologies, numerous prospective cohort studies were elaborated to unravel their temporal relationship (see for reviews Santiago and Potashkin, 2013; Wirdefeldt *et al.*, 2011) (Table 1.8). Most, though not all (Becker *et al.*, 2008; Cereda *et al.*, 2011; Driver *et al.*, 2008) showed that pre-existing diabetes is a risk factor for the development of Parkinson's disease (Arvanitakis *et al.*, 2007; Cereda *et al.*, 2012; Hu *et al.*, 2007; Xu *et al.*, 2011). To dispel any doubt, one group addressed the question in a cohort 10 times larger (1.8 million patients) than previously, and robustly confirmed the risk that diabetes represents for the future development of Parkinson's disease (Klimek *et al.*, 2015). Today, little doubt remains as to the hazard that a diabetic prelude represents in the development of Parkinson's disease.

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[40] If anything, many dopaminergic drugs employed in the treatment of Parkinson's disease actually exert a blood glucose lowering effect (Cincotta *et al.*, 1999; Lopez Vicchi *et al.*, 2016).

[41] Prediabetes is defined in humans as fasting blood glucose 5.5-7 mM (100-126 mg/dL), postprandial blood glucose 7.8-11.1 mM (140-200 mg/dL) or glycated haemoglobin 5.7-6.5% (American Diabetes Association, 2016).

[42] The glycaemic index of a food is defined as the rise in glycaemia that it occasions 2 hours after consumption in humans. Low glycaemic indexed nutrients release glucose slowly and steadily, in contrast to high glycaemic indexed foods that cause a rapid hyperglycaemia.

Table 1.8

Recent studies investigating the association between diabetes and Parkinson's disease

Study	Study design	Sample size	Main results[&]
Hu *et al.*, 2007 [5]	Cohort study, Finnish population	PD: 633 Controls: 51 552	T2DM is associated with an increased risk of PD.
Moran *et al.*, 2008 [14]	Meta-analysis	N/A	Shared biological pathways between PD, T2DM, cancer, and inflammation.
D'Amelio *et al.*, 2009 [122]	Case–control	PD: 318 Controls: 318	Inverse association between PD and diabetes preceding PD onset.
Palacios *et al.*, 2011 [101]	Cohort study	PD: 656	No association.
Schernhammer *et al.*, 2011 [123]	Case–control, Danish population	PD: 1931 Controls: 9651	T2DM is associated with an increased risk of PD, especially younger onset PD.
Xu *et al.*, 2011 [4]	Cohort study	Diabetes: 21 611 Controls: 267 051	T2DM is associated with an increased risk of PD.
Bosco *et al.*, 2012 [18]	Case–control, Italian population	PD–dementia: 53 PD: 57	Insulin resistance is associated with an increased risk of dementia in PD.
Menon *et al.*, 2011 [49]	Genome-wide association study	N/A	Shared pathways between PD and T1DM.
Cereda *et al.*, 2011 [121]	Meta-analysis	N/A	Diabetes appears to be a risk factor for PD.
Cereda *et al.*, 2012 [19]	Case–control	Diabetes prior to PD onset: 89 Controls: 89	Onset of T2DM before onset of PD is associated with an increased severity of PD symptoms.
Sun *et al.*, 2012 [124]	Case–control, Chinese population	Diabetes: 603 413 Controls: 472 718	Diabetes is associated with an increased risk of PD onset.
Wahlqvist *et al.*, 2012 [61]	Case–control, Taiwanese population	Diabetes: 64 166 Controls: 698 587	T2DM is associated with an increased risk in PD. Metformin–sulfonylurea therapy reduces the risk of PD.

[&] T1DM, type I diabetes mellitus; T2DM, type II diabetes mellitus.
(From Santiago and Potashkin, 2013.)

From this point onward, the particular bond between diabetes and Parkinson's disease loses focus. What exactly triggers nigrostriatal degeneration in Parkinson's disease among the multifarious metabolic changes that occur in diabetes remains unclear. The two predominant lines of thought grant importance to the dimensions of dysfunctional insulin signalling and oxidative stress. Although insulin occupies an increasingly acknowledged role in the human CNS, by activating neuron-bound insulin receptors responsible for controlling appetite, reward, cognition and memory (Anthony *et al.*, 2006; Benedict *et al.*, 2004, 2007; Craft *et al.*, 1996; Hallschmid *et al.*, 2008; Khanh *et al.*, 2014; Reger *et al.*, 2008), we will address these dimensions in the discussion and maintain our focus on oxidative stress for now.

1.2.5.2 Molecular and cellular bases

Aside from the epidemiological relationship between Parkinson's disease and diabetes, further clues support the possible susceptibility of nigrostriatal dopaminergic neurons in hyperglycaemia. As previously discussed, oxidative stress mediated by sustained hyperglycaemic conditions arises from 1) mitochondrial dysfunction,

2) activation of the polyol pathway, and 3) macromolecule glycation, which together accentuate ROS production and expenditure of antioxidative resources. We will briefly illustrate how these features manifest themselves in Parkinson's disease and how they can be traced back to the phenotypic risk factors identified in nigrostriatal dopaminergic neurons.

One of the most prominent liabilities specific to nigrostriatal dopaminergic neurons consists in their vulnerability to mitochondrial dysfunction. Indeed, their characteristic pacemaking activity leads to steep intracellular calcium fluctuations, known to augment oxidative phosphorylation, mitochondrial membrane hyperpolarization and superoxide anion production (Guzman *et al.*, 2010; Pacelli *et al.*, 2015). On top of this, nigrostriatal neurons have a limited ability to bind excess intracellular calcium (Dopeso-Reyes *et al.*, 2014; Iacopino and Christakos, 1990; Liang *et al.*, 1996; Yamada *et al.*, 1990) and comprise a relatively high number of mitochondria to sustain their dense arbours (Pacelli *et al.*, 2015). It is, therefore, not surprising that mitochondrial dysfunction has long been associated with Parkinson's disease (Perier and Vila, 2012). This pathological node first emerged upon identifying potent parkinsonian toxins (e.g., MPTP and rotenone) whose detrimental effects are principally conveyed by means of complex I inhibition in the electron transport chain (Beal, 2001, 2003; Betarbet *et al.*, 2000; Greenamyre *et al.*, 2001; Langston *et al.*, 1983). The relevance of these findings was then fully recognized upon discerning complex I deficiencies and accumulation of mitochondrial DNA deletions in the substantia nigra pars compacta of parkinsonian patients (Bender *et al.*, 2006, 2008; Schapira *et al.*, 1989). Later, robust support for impaired respiration was provided by the identification of genes that cause familial forms of Parkinson's disease and that are involved in mitochondrial homeostasis, for instance leucine-rich repeat kinase 2 (LRRK2) (Mortiboys *et al.*, 2010; Wang *et al.*, 2012), DJ-1 (Irrcher *et al.*, 2010; Krebiehl *et al.*, 2010), and the mitochondrial duo, PTEN-induced putative kinase 1 (PINK1) and parkin (Ashrafi *et al.*, 2014; Deng *et al.*, 2008; Exner *et al.*, 2007).

Parkinson's disease does not directly point to a hyperactive polyol pathway, although one isolated study showed increased plasma levels of sorbitol in drug-naïve

parkinsonian patients, suggested to arise from glucose metabolism via non-oxidative pathways ensuing from mitochondrial dysfunction (Ahmed *et al.*, 2009). There is some indirect evidence for a link between polyol pathway activation and neurodegeneration, via downregulation of nerve growth factor (NGF) as demonstrated in hyperglycaemia-induced neuropathies (Hounsom *et al.*, 2001, 1998; Obrosova *et al.*, 2001). Levels of brain growth factors, like NGF, that promote neuronal survival and differentiation indeed decline in the substantia nigra pars compacta and dorsal striatum of parkinsonian patients (Mogi *et al.*, 1999). More substantially, though, endogenous antioxidant depletion, provoked by polyol pathway activation, has been repeatedly suggested to partake in the pathogenesis of Parkinson's disease. Indeed, exhaustion of the reduced glutathione cofactor is one of the earliest events to occur in the brains of parkinsonian patients (Jenner, 1993; Pearce *et al.*, 1997; Sian *et al.*, 1994), markedly so in nigrostriatal dopaminergic neurons, which are inherently endowed with limited stocks of glutathione (Bharath *et al.*, 2002; Kang *et al.*, 1999).

Spontaneous methylglyoxal formation and macromolecule glycation are particularly salient elements linking Parkinson's disease to hyperglycaemia. The most obvious connection dwells in the inhibition of the ubiquitin proteasome system caused both by protein and ubiquitin glycation, as it occurs in the CNS (Munch *et al.*, 2012; Rabbini and Thornalley, 2012; Uchiki *et al.*, 2012; Vicente Miranda *et al.*, 2016). In fact, the pathologically relevant α-synuclein protein is glycated in Lewy bodies (Auburger and Kurz, 2011; Cereda *et al.*, 2012; Kurz *et al.*, 2011; Vincent *et al.*, 2012). Interference with the ubiquitin proteasome system may therefore trigger a self-perpetuating cycle, wherein increased levels of glycated α-synuclein further obstruct proteasome function, consequently contributing to accumulation of the former, but also to its oligomerization, aggregation and purported toxicity (Chen *et al.*, 2006; Tanaka *et al.*, 2001). Additionally, RAGE polymorphisms have been shown to both offer protection against and favour the development of Parkinson's disease (Gao *et al.*, 2014). Insightful *in vitro* studies further demonstrate the shielding effect of ablating the receptor in a dopaminergic neuronal model of the disease (Sathe *et al.*, 2012; Teismann *et al.*, 2012). Interestingly, not only can methylglyoxal glycate proteins and erode stocks

of NADPH via glyoxalase and aldose reductase activation, it participates like other aldehydes in deleterious reactions with dopamine to form salsolinol-like compounds (Deng *et al.*, 2012; Song *et al.*, 2014; Szent-Gyorgi and McLaughlin, 1975), which resemble 6-OHDA, MPTP and endogenous dopamine derivatives already recognized for their toxicity (Kurnik-Łucka *et al.*, 2018; Maruyama *et al.*, 1999; Naoi *et al.*, 2002; Su *et al.*, 2013). Of prime significance, parkinsonian patients display particularly elevated levels of salsolinol-like compounds in the substantia nigra pars compacta and dorsal striatum (Deng *et al.*, 2012). Since nigrostriatal neurons express relatively low levels of VMAT2 responsible for the vesicular uptake of lingering cytosolic dopamine, they may also offer ideal temporal conditions for the transformation of the latter into salsolinol-like compounds in a hyperglycaemic setting (Liang *et al.*, 2004).

To summarize, hyperglycaemia represents an additional cause of superoxide anion production, mitochondrial dysfunction, antioxidative bankruptcy, ubiquitin proteasome system failure and dopamine toxicity that may well favour the early demise of nigrostriatal dopaminergic neurons. Although buttressed by a wealth of epidemiological, molecular and cellular evidence, this hypothesis has not been directly tested. In the aim of establishing the selective vulnerability of nigrostriatal neurons to a supplementary oxidative burden, proper demonstrations would require[43] a) the testing of a single exogenous insult, for instance sustained hyperglycaemia; b) the comparison of neurodegeneration in each loci involved in the nigrostriatal and mesocorticolimbic pathways, preferably with concomitant verifications in other brain regions; c) the assessment of neurodegeneration at more than one time point; d) the evaluation of glial populations to gain insight into non-neuronal outcomes, and; e) the full appraisal of behavioural alterations that may arise from nigrostriatal neurodegeneration. It is precisely in this perspective that were elaborated a series of experiments presented

[43] The most pertinent studies in this sense have only demonstrated neurodegeneration in the substantia nigra pars compacta of rodent models of diabetes, without investigating the repercussions on the totality of central dopaminergic pathways, on other populations of brain cells, or on the range of behavioural alterations that may arise from neuronal death (Brambilla Bagatini *et al.*, 2014; do Nascimento *et al.*, 2011). Other studies have also combined various parkinsonian insults with a hyperglycaemic challenge, which does not address the intrinsic hazard that hyperglycaemia represents to nigrostriatal neurons in otherwise healthy subjects (Choi *et al.*, 2005; Morris *et al.*, 2010; Rotermund *et al.*, 2014).

herein to fulfil these interrogations that warrant answers, in light of the epidemiological risk incurred by diabetic patients with regard to the potential development of Parkinson's disease.

1.3 The antioxidative polyphenol resveratrol

The proposal that a heightened vulnerability to oxidative stress is at the origin of the preferential degeneration of nigrostriatal dopaminergic neurons can be tested by verifying the ability of an exogenous antioxidant to prevent this death. In the present thesis, we hire this strategy by employing a well-known antioxidative polyphenol, resveratrol, in dopaminergic neurons exposed to high glucose conditions. Although resveratrol and other dietary polyphenols can offer protection in various disease settings, the scope of our current work does not encompass providing evidence of its already widely acknowledged neuroprotective potential[44]. Used here as an antioxidative tool, the following section thus focuses on resveratrol's ability to preclude oxidative stress. Before detailing its protective mechanisms in dopaminergic neurons, we will nonetheless provide some background information on resveratrol pertaining to its origins, its dietary sources and the chemico-structural peculiarities that shape its antioxidative properties.

1.3.1 Background

1.3.1.1 Historical context

Centuries of traditional medicine practiced in native cultures worldwide have revolved around the potency of edible medicinal herbs and plants rich in polyphenols to ameliorate human health (Manyam *et al.*, 1999; Song *et al.*, 2012). When dietary habits and health status were initially associated, the role of polyphenols in human health

[44] We have already reviewed the therapeutic potential of polyphenols in Parkinson's disease elsewhere (Reglödi, Renaud *et al.*, 2017; Renaud *et al.*, 2015). A critical appraisal of the employment of polyphenols against neurodegenerative diseases is also presented in Appendix B. Specifically regarding resveratrol, our group has extensively discussed its anti-inflammatory mechanisms in the CNS (Appendix C) and has detailed the various demonstrations of its protective properties in preclinical models (Renaud and Martinoli, 2014).

immediately sparked vivid scientific interest. At the root of polyphenol studies, a mixture of flavonoids extracted from citrus fruit was initially coined "vitamin P" because of its anti-scurvy, vitamin C-sparing effects (Bentsath *et al.*, 1936; Rusznyak and Szent-Györgi, 1936). The term was later abandoned upon discerning that "vitamin P" was not truly essential, although the observed health benefits still held true. A host of controlled epidemiological reports have since then supported the protective role of polyphenols in abating numerous health issues, such as cardiovascular diseases (Estruch *et al.*, 2013; Hertog *et al.*, 1993; Joshipura *et al.*, 2001; von Ruesten *et al.*, 2013), diabetes (Carter *et al.*, 2010; Sargeant *et al.*, 2001) and cancer (Lunet *et al.*, 2005; Masala *et al.*, 2012; Steinmetz and Potter, 1996). As evidence amassed, intervention studies were warranted to bolster the significance of these findings, and most, though not all (Anderson *et al.*, 2002; Conquer *et al.*, 1998; Simons *et al.*, 2000), confirmed a positive function for specific dietary polyphenols in human health (see for review Williamson and Manach, 2005). As research progressed, certain biofunctional polyphenols gained more attention than others by virtue of their promising protective competences.

In the field of polyphenol research, resveratrol holds a special place. Indeed, its popularity shot through the roof in the 90s following the airing of an episode of "60 minutes" on the CBS network wherein Dr. Serge Renaud was interviewed on the topic of the "French Paradox", a term henceforth eternalized (Renaud and de Lorgeril, 1992). The expression was employed to describe the apparently low incidence of coronary heart diseases in Southern France populations despite their relatively elevated consumption of saturated fats in comparison to other industrialized countries (Wu *et al.*, 2001). A vital food staple in Southern France, red wine was thought to afford cardioprotection (Renaud and de Lorgeril, 1992) and resveratrol was later proposed to be one of the key bioactive constituents responsible for these observed health benefits (Fauconneau *et al.*, 1997; Frankel *et al.*, 1993; Pace-Asciak *et al.*, 1995). Robust demonstrations that resveratrol exerts antioxidative effects (Frankel *et al.*, 1993; Kerry and Abbey, 1997; Jang *et al.*, 1999) and mitigates carcinogenesis (Della Ragione *et al.*, 1998; Gehm *et al.*, 1997; Hsieh *et al.*, 1999; Jang *et al.*, 1997; Lu and Serrero, 1999; Mgbonyebi *et al.*, 1998) truly launched the field from thereon (Figure 1.19).

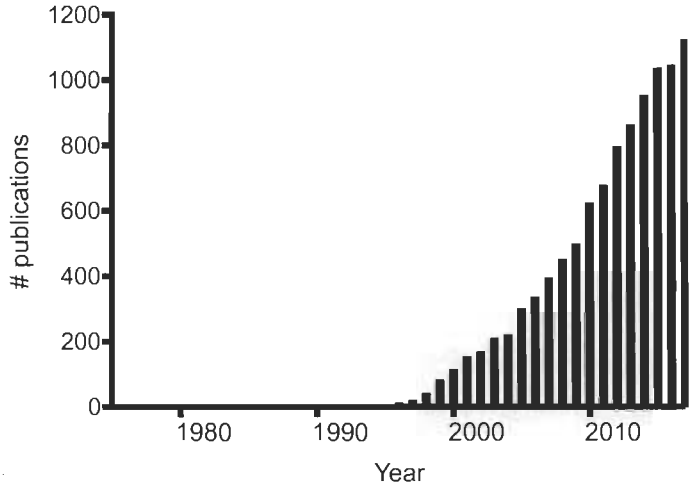

Figure 1.19 Resveratrol in the scientific literature.
A 2018 PubMed search employing the term "resveratrol" yields an approximate total of 10 000 hits since 1987. A trending rise can be observed as early as 1997.

1.3.1.2 Dietary origins

Polyphenols are usually derived from phenylpropanoid metabolism in plants (Parr and Bolwell, 2000). As such, polyphenols usually comprise an assembly of phenyl rings with variable carbon connectors and hydroxylation patterns. Several subclasses arise from the variable complexity of these molecules, for instance phenolic acids (C6-C3 and C6-C1), flavonoids (C6-C3-C6), lignans (C6-C3-C3-C6) and stilbenes (C6-C2-C6) to which belongs *trans*-3,5,4′-trihydroxystilbene, better known as resveratrol (Figure 1.20).

Polyphenols confer plants with evolutively gainful signalling and protective systems. Regarding resveratrol, its exact functions remain uncertain but they are generally recognized to act as phytoalexins (plant-synthetized antimicrobial substances) (Chang *et al.*, 2011). Resveratrol is produced in high concentrations in response to environmental stressors such as pathogenic insults, nutrient deficiency, temperature fluctuations and ultraviolet radiation (Orallo, 2008; Soleas *et al.*, 2001). As such, it is found in substantial quantities in grapevines (*Vitis vinifera*), possibly as an evolutionary adaptation in response to fungal infections (Chong *et al.*, 2009; Dercks and Creasy,

1989). Remarkably, stilbene synthase involved in the biosynthesis of resveratrol promotes disease resistance when artificially expressed in plants (Hain *et al.*, 1993).

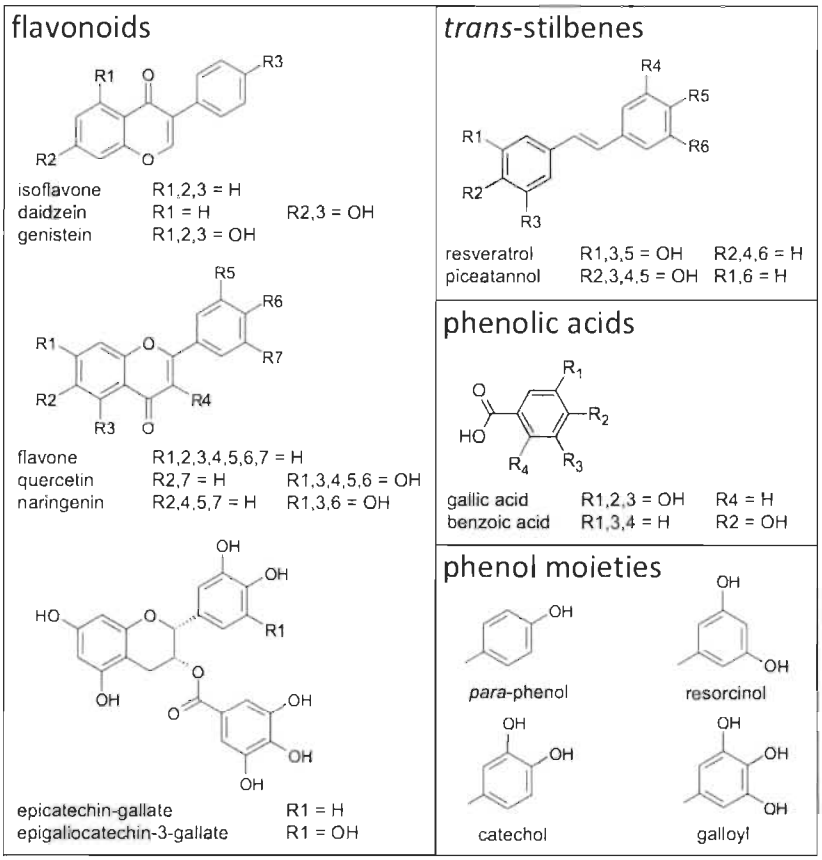

Figure 1.20 Frequently encountered polyphenols.

Found ubiquitously in fresh herbs, fruits, vegetables, nuts, grains and legumes, polyphenols represent on average 1 g/day of a balanced diet (Scalbert and Williamson, 2000). However, resveratrol is one of the least encountered polyphenols in the human diet. Akin to most other polyphenols, stilbenes predominantly accumulate in the outer component of foodstuffs. In the skins of grapes, its levels can reach 50-100 mg/g of fresh weight. Due to the maceration process, red wines generally present greater concentrations of stilbenes than whites or rosés, with resveratrol reaching levels of approximately 0.3-7 mg/L (Bertelli *et al.*, 1998; Lamuela-Raventós and de la Torre-Boronat, 1999; Siemann and Creasy, 1992; Vitrac *et al.*, 2002). Besides grapes, numerous other plants were shown to synthesize stilbenes, though, among the 72 plant

species identified, mulberries and peanuts are the only other typical sources rich in resveratrol in the human diet (Langcake and Pryce, 1977).

1.3.1.3 Structure, chemistry and antioxidative functions

Insofar as the > 8000 polyphenols known to date are structurally diverse, they share several hallmarks that undoubtedly underlie their therapeutic potential in living organisms, namely phenyl rings, hydroxyl moieties and conjugated double bonds. These chemico-structural features grant polyphenols like resveratrol the potential to tackle a keystone of dopaminergic neurodegeneration, explicitly oxidative stress.

Resveratrol wields its antioxidative effects via both direct and indirect mechanisms (Foresti *et al.*, 2013; Leonard *et al.*, 2003). Its hydroxyl groups are thought to account for the near totality of its direct antioxidative actions (Fauconneau *et al.*, 1997; Frémont, 2000; Kawada *et al.*, 1998; Stivala *et al.*, 2001). Indeed, resveratrol scavenges free radical species principally by lending its most acidic hydrogen from the *para*-phenol group or other hydrogen atoms from the *meta*-hydroxyl groups of the resorcinol moiety (Stivala *et al.*, 2001) (Figure 1.20). It may also cede a π bond from the resorcinol ring to accommodate radicals (Khanduja and Bhardwaj, 2003). The radicalized form of resveratrol is stabilized by its multiple conjugated double bonds that allow the delocalization of the unpaired electron (Figure 1.21).

While resveratrol is appreciated for its ability to directly scavenge free radical species, it is not among the most efficient metal chelators. Since divalent transition metals like iron are toxic, cells are endowed with tight control systems to regulate their availability in a free form. When in excess, however, they are known to participate in deleterious reactions that generate ROS[45]. Chelation by bidentate molecules possessing phenol rings is possible, but is best achieved when there are two or three hydroxyl

[45] For example, Fenton reactions occur between ferric ions and superoxide anion to yield ferrous ions and oxygen (Jomova *et al.*, 2010). In turn, Haber-Weiss reactions between ferrous ions and hydrogen peroxide further give ferric ions and hydroxyl radicals, the most reactive kind of ROS (Valko *et al.*, 2004). In the substantia nigra pars compacta endowed with appreciable levels of iron ions, these deleterious reactions are extremely pertinent.

groups next to each other (hydroxyl groups in the *ortho*-position), such as in galloyl or catechol groups (Petry *et al.*, 2010) (Figure 1.20). Resveratrol possesses a resorcinol group wherein two hydroxyl groups are far apart, and, by the same token, it is a much less potent monodentate chelator (Chan *et al.*, 2016; Hider *et al.*, 2001; Purawatt *et al.*, 2007).

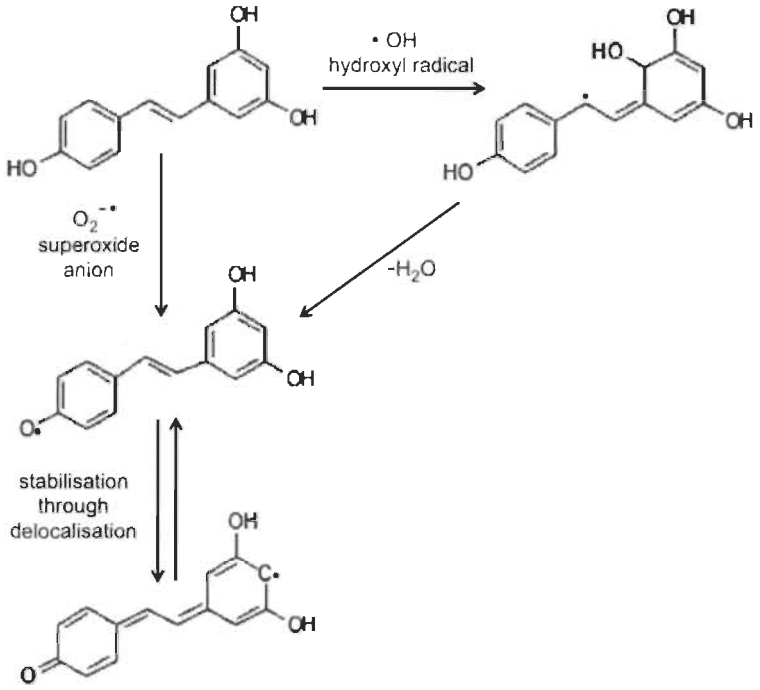

Figure 1.21 Resveratrol's putative direct scavenging mechanisms.
Two examples of reaction partners were provided: superoxide anion and the hydroxyl radical. Superoxide anion is ubiquitous in cells, especially at the level of the electron transport chain. As for the hydroxyl radical, it is the most reactive physiological ROS.

In contrast to direct scavenging mechanisms, the chemico-structural features responsible for polyphenols' indirect antioxidative actions are more difficult to pinpoint. These indirect effects are likely conveyed via the activation of endogenous antioxidative mechanisms and oblige knowledge of polyphenolic interactions. Resveratrol's stilbene carbon scaffold likely contributes to its capacity to interact with molecular effectors of endogenous antioxidative pathways, discussed later. Indeed, resveratrol is planar and relatively hydrophobic, which theoretically should increase its affinity and specificity for hydrophobic pockets found in proteic binding sites (Figure 1.20). In addition, it harbours polar hydroxyl moieties in a conformation that resembles certain endogenous

ligands, such as estrogen. These hydroxyl groups participate in hydrogen bonds with multiple amino acid side chains within hydrophobic pockets. A few studies have also demonstrated the importance of resveratrol's phenol rings, especially the one bearing the *para*-hydroxyl moiety, and its *trans* stereoisomery in binding certain proteins (Nwachukwu *et al.*, 2014; Stivala *et al.*, 2001). In other instances, resveratrol may not require its hydroxyl functions to mediate its neuroprotective effects. Studies tested this hypothesis by preparing a plethora of resveratrol analogs devoid of hydroxyl functions (Heynekamp *et al.*, 2006; Solberg *et al.*, 2014). Despite their inability to scavenge ROS, these stilbene compounds were nonetheless capable of dampening neuroinflammation in a NF-κB-luc reporter cell line (Heynekamp *et al.*, 2006) and reducing β-amyloid plaque density in the CNS of a mouse model of Alzheimer's disease (Solberg *et al.*, 2014). Such studies highlight the importance of resveratrol's multiple chemico-structural singularities in shaping both the nature and the variety of molecular interactions it can engage in, which undoubtedly account for its multifarious modes of action.

1.3.2 Protection of dopaminergic neurons against oxidative stress

Resveratrol's ability to improve various clinical endpoints has been demonstrated in myriads of disease settings, including neurodegenerative diseases (Moussa *et al.*, 2017), diabetes (Goh *et al.*, 2014; Imamura *et al.*, 2017), obesity (Xue *et al.*, 2016), polycystic ovary syndrome (Banaszewska *et al.*, 2016) and ulcerative colitis (Samsamikor *et al.*, 2016). Its antioxidative potential has been shown to contribute to its protective effects in preclinical models of diabetes (Ježek *et al.*, 2014; Jiang *et al.*, 2013; Sadi *et al.*, 2014) and neurological disorders, such as ischemic stroke (Rodrigo *et al.*, 2013; Saleh *et al.*, 2013; Sun *et al.*, 2010), neurodegenerative diseases (Karuppagounder *et al.*, 2009; Maher *et al.*, 2011; Varamini *et al.*, 2014) and traumatic brain injuries (Gatson *et al.*, 2013; Hall *et al.*, 2010; Liu *et al.*, 2011). Specifically related to our work, resveratrol improves the oxidative status of the CNS in rodent models of diabetes (Bagatini *et al.*, 2017; Sadi and Konat, 2016), but this parameter has not been addressed in dopaminergic neurons challenged with high glucose concentrations. Nevertheless, its antioxidative properties have been verified numerous times in preclinical models of Parkinson's disease. As a basis for the employment of resveratrol in dopaminergic

neurons to antioxidative ends, this section will therefore briefly review the findings provided by these studies.

To study Parkinson's disease, several models were developed *in vitro* as well as *in vivo* that depend on pro-oxidative mechanisms to induce dopaminergic neuronal death. Typical parkinsonian toxins include the aforementioned 6-OHDA and 1-methyl-4-phenylpyridinium (MPP+, the active metabolite of MPTP), as well as pesticides like rotenone and paraquat, which instigate ROS overproduction by various means (Deumens *et al.*, 2002; Duty and Jenner, 2011; Jackson-Lewis *et al.*, 2012; Nagatsu, 1997). Accordingly, studies consistently show resveratrol to rescue dopaminergic neurons from the various oxidative assaults that typify these models (Blanchet *et al.*, 2008; Gélinas and Martinoli, 2002; Wu *et al.*, 2011). Neuroprotection is complemented with reduced levels of ROS, like the hydroxyl radical (Lu *et al.*, 2008), which prevents neuronal oxidative damages from arising, as assessed by the measurement of lipid peroxidation and protein carbonyl by-products (Anandhan *et al.*, 2010; Khan *et al.*, 2010; Okawara *et al.*, 2007; Palle and Neerati, 2018). Most remarkable is the recovery of motor behaviour in animal models, which reflects the concrete protection afforded by resveratrol on dopaminergic neurons in models of Parkinson's disease (Anandhan *et al.*, 2010; Jin *et al.*, 2008; Lu *et al.*, 2008; Makhija and Jagtap, 2014; Palle and Neerati *et al.*, 2018; Zhao *et al.*, 2017).

A recurring theme in parkinsonian models is the apoptotic death[46] of dopaminergic neurons. Indeed, this mode of programmed cell death is particularly relevant in Parkinson's disease (Anglade *et al.*, 1997; Mochizuki *et al.*, 1996; Toulorge *et al.*, 2016), but also in diabetes (Maiese *et al.*, 2007; Russell *et al.*, 1999). In this respect, resveratrol is well appreciated for its ability to abrogate oxidation-induced apoptosis. Congruently, resveratrol favourably modulates various pro- or anti-apoptotic proteins, such as p53, protein kinase B (Akt) and glycogen synthase kinase 3 (GSK-3) (Bournival *et al.*, 2009; Feng *et al.*, 2015; Lin *et al.*, 2014; Okawara *et al.*, 2007; Wu *et al.*, 2011; Zeng *et al.*, 2017). As mitochondria are a nexus of apoptotic processes, studies

[46] We address apoptotic mechanisms in greater detail in section 1.5.1.4.

specifically uncovered resveratrol's aptitude to repeal the leakage of mitochondrial pro-apoptotic factors, such as apoptosis inducing factor and cytochrome *c*, likely through modulation of proteins involved in the formation of the mitochondrial permeability transition pore, exemplarily B cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) (Bournival *et al.*, 2009; Mudò *et al.*, 2012; Zeng *et al.*, 2017).

Among these studies, many have reported certain upstream events that may account for the antioxidative and anti-apoptotic effects afforded by resveratrol. On the one hand, resveratrol remarkably restores endogenous antioxidative defences, for instance glutathione, SOD, thioredoxin, heme oxygenase and catalase (Anandhan *et al.*, 2010; Khan *et al.*, 2010; Lin *et al.*, 2014; Mudò *et al.*, 2012; Okawara *et al.*, 2007; Palle and Neerati, 2018; Srivastava *et al.*, 2012). On the other hand, resveratrol displays a striking ability to foster mitochondrial homeostasis, by ameliorating respiration (Palle and Neerati, 2018; Mudò *et al.*, 2012) and providing protection against ultrastructural changes (Jin *et al.*, 2008; Peng *et al.*, 2016). Interestingly, in primary skin fibroblast cultures from patients with early-onset Parkinson's disease caused by different *PARK2* mutations, encoding parkin, resveratrol markedly improved mitochondrial functions, substantiated by increased ATP production, complex I activity, oxygen consumption, and decreased generation of lactate and ROS (Ferretta *et al.*, 2014).

The sum of these results obtained in various cell lines, primary cultures, organotypic midbrain slice models and rodent paradigms leaves no doubt as to the capacity of resveratrol to protect dopaminergic neurons by virtue of its ability to counteract oxidative stress, a key contributor of apoptosis. These neuroprotective benefits seem to stem from the improvement of endogenous antioxidative defences and mitochondrial functions. Taking into consideration the mechanisms by which hyperglycaemia induces oxidative stress, that is, by expending endogenous antioxidants and causing mitochondrial dysfunction, it is reasonable to expect resveratrol to confer protection against high glucose conditions in dopaminergic neurons.

1.3.3 Direct putative targets

Although polyphenols like resveratrol are theoretical ROS scavengers, their ability to do so in a physiological environment remains controversial. First, polyphenol scavenging dictated by the transfer of a proton or the breaking of a double bond must occur faster than any reaction between the free radical species and susceptible constituents of the cell, for instance lipids (Di Meo *et al.*, 2013). Second, in order to offer significant antioxidative support, polyphenols are theoretically required to accrue in similar concentrations to that of endogenous antioxidants such as ascorbate (30-100 μM) and urate (140-200 μM) (Hollman, 2014). However, tissue or plasma concentrations of polyphenols rarely exceed micromolar magnitudes at any given time (Del Rio *et al.*, 2013). Consequently, the direct scavenging properties of polyphenols like resveratrol may be trivial in a physiological context (Benzie *et al.*, 1999; Hollman *et al.*, 2011). In this regard, it is argued that cellular events occurring at nanomolar concentrations might better uphold polyphenols' antioxidative actions. These indirect effects are likely conveyed via the activation of endogenous antioxidative mechanisms and require only very low concentrations owing to the diverging and amplifying nature of many cell signalling cascades.

In an attempt to reconcile resveratrol's bioefficacy with the improbability of firsthand scavenging, efforts have been deployed to identify these putative direct targets (see for review Britton *et al.*, 2015) (Table 1.9). Among the multiple molecules identified to date, a few are directly or indirectly linked to oxidative stress, namely ribosyldihydronicotinamide dehydrogenase (quinone) (NQO2) (Buryanovsky *et al.*, 2004; Wang *et al.*, 2004), phosphodiesterases (PDEs) (Park *et al.*, 2012), and the mammalian target of rapamycin (mTOR) (Park *et al.*, 2016). In neurons, these effectors are notorious for executing noxious processes and their inhibition by resveratrol may explain the latter's beneficial properties in the CNS (Cantó and Auwerx, 2009; Chaturvedi and Beal, 2013; Lee *et al.*, 2012; Li *et al.*, 2010; Sharma *et al.*, 2012; Wang *et al.*, 2008).

Table 1.9

Direct putative targets of resveratrol

Molecular target[&]	Method[&]	Observation[&]
Cyclooxygenase-1	Enz Inhib, Xray	IC_{50} = 0.535 μM
Cyclooxygenase-2	Chem Prot, Enz Inhib	IC_{50} = 0.996 μM
Fatty acid synthase	Enz Inhib	IC_{50} = 8.5 μM
NQO2	Chem Prot, Xray, Enz Inhib	Binding, K_d = 35 nM
GSTP1	Chem Prot	Binding
AKT-1	Chem Prot	Binding
HDACs	Enz Inhib	pan-HDACi
PDE1, 3, and 4	Fluor	IC_{50} = 6, 10, and 14 μM
ATM	Kinase assay	Activation[a]
SIRT1	Fluor	Activation[b]
PKCα, βI, and PKD1	Kinase assay	IC_{50} = 2, 100, and 800 μM
Aromatase	Enz Inhib, *in silico*	IC_{50} = 12.8 μM
DNA/RNA	Spec	Destabilizing
Lipoproteins	HPLC	Binding
DNA polymerase α and δ	Enz Inhib	K_i = 3.3 and 5.0 μM
F1-ATPase	Xray	Binding
CBR1	Chem Prot, Enz Inhib	Binding, K_i = 55.8 μM
LTA_4H	Chem Prot, *in silico*	Binding
PPARγ and δ	Xray, Affin Chrom	K_d = 1.4 and 2.7 μM
Various kinases	Activity assay	Moderate to no effect
TyrRS	Enz Inhib, Xray[c]	K_i = 22 μM

[a]Under oxidizing conditions (H_2O_2).

[b]Direct binding disproved.

[c]Cocrystal with *cis*-resveratrol.

[&]Affin Chrom, affinity chromatography; AKT-1, protein kinase B; ATM, ataxia telangiectasia mutated serine/threonine kinase; Chem Prot, chemical proteomics; CBR1, carbonyl reductase 1; Enz Inhib, enzyme inhibition assay; F1-ATPase, F_1 portion of adenosine triphosphatase; Fluor, fluorescence assay; GSTP1, glutathione S-transferase P; HDAC, histone deacetylase; HPLC, high-performance liquid chromatography; IC_{50}, half maximal inhibitory concentration; K_d, dissociation constant; K_i, inhibition constant, LTA_4H, leukotriene A4 hydrolase; NQO2, ribosyldihydronicotinamide dehydrogenase (quinone); PDE, phosphodiesterases; PKC, protein kinase C; PKD, protein kinase D; PPAR, peroxisome proliferator-activated receptor; SIRT1, silent mating type information regulation 2 homologue 1; Spec, spectroscopic assay; TyrRS, tyrosine-transfer RNA ligase; Xray, X-ray cocrystal structure.

(Adapted from Britton *et al.*, 2015.)

1.3.3.1 Ribosyldihydronicotinamide dehydrogenase (quinone) and oxidative stress

NQO2 is a detoxifying enzyme mandated to catalyze the reduction of quinones to hydroquinones (Monks *et al.*, 1992; Vella *et al.*, 2005). At first sight, it appears to operate as an endogenous antioxidative enzyme, due to its role in reducing quinone

ROS. However, it was recently found to produce ROS by-products during quinone reduction in some settings (Gong *et al.*, 2008; Reybier *et al.*, 2011). In fact, NQO2 appears to impart the noxious effects of certain parkinsonian toxins and of exogenous dopamine on dopaminergic neurons (Janda *et al.*, 2013, 2015; Wang *et al.*, 2008). Interestingly, NQO2 has emerged as a novel risk factor for Parkinson's disease. Indeed, genetic polymorphisms associated with the pathology were initially speculated to result in decreased NQO2 expression (Harada *et al.*, 2001). However, later findings showed these polymorphisms to occur in the promoter and to enhance NQO2 expression, which was further sustained in human fibroblasts of individuals with or without the genetic modification (Wang *et al.*, 2004). In this light, it was concluded that amplified expression of the reductase constitutes a risk factor for the development of Parkinson's disease.

Likely the most relevant target in oxidative stress, NQO2 displays the greatest affinity for resveratrol to date (nanomolar range). Its interaction with the polyphenol has also been ascertained by no less than three methods carried out by two independent groups (Buryanovsky *et al.*, 2004; Wang *et al.*, 2004). It follows that resveratrol's inhibitory action on NQO2 is presumed to confer protection in this setting, but this remains to be confirmed in neurons.

1.3.3.2 Phosphodiesterases and the energy sensing axis

Many of the abovementioned studies demonstrating resveratrol's antioxidative and neuroprotective properties in dopaminergic neurons also expose its modulatory effect on a selection of interrelated bioenergetic power players, namely 5′ adenosine monophosphate-activated protein kinase (AMPK), silent mating type information regulation 2 homologue 1 (SIRT1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Albani *et al.*, 2009; Feng *et al.*, 2015; Ferretta *et al.*, 2014; Mudò *et al.*, 2012; Wu *et al.*, 2011). The signalling axis to which they belong is thought to be responsible for lifespan amelioration provided by exercise and calorie restriction (Tennen *et al.*, 2012).

In this growingly popular axis, PGC-1α is key effector of mitochondrial biogenesis and efficient respiration (Cantó and Auwerx, 2009) (Figure 1.22). As upstream energy sensors, AMPK is activated when cellular ATP:AMP ratios are low and SIRT1 is stimulated by elevated NAD+:NADH ratios (Cantó *et al.*, 2009; Spasić *et al.*, 2009). SIRT1 and AMPK also maintain an intimate bidirectional relationship, since SIRT1 deacetylates liver kinase B1 (LKB1) that in turn phosphorylates and activates AMPK (Hou *et al.*, 2008; Lan *et al.*, 2008), whereas AMPK increases NAD+ levels responsible for stimulating SIRT1 (Cantó *et al.*, 2009). In concert, SIRT1 and AMPK enhance mitochondrial function by deacetylating and phosphorylating PGC-1α, which is required for its activation (Cantó and Auwerx, 2009). Although allosteric activation of SIRT1 was at first greatly advocated to explain resveratrol's effects on this bioenergetic crossroads (Dai *et al.*, 2010; Howitz *et al.*, 2003), methodological oversights came to light and direct binding was refuted (Beher *et al.*, 2009; Borra *et al.*, 2005; Kaeberlein *et al.*, 2004; Pacholec *et al.*, 2010). Later identification of PDEs as direct targets of resveratrol finally shed some light on this cell signalling puzzle (Park *et al.*, 2012).

PDEs play a key modulatory role on intracellular levels of nucleotidic secondary messengers by hydrolysing cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to their respective AMP and GMP forms. By competing with cyclic nucleotides and inhibiting PDEs, resveratrol fosters an environment rich in cAMP, which triggers a series of events leading to the activation of AMPK responsible for the ensuing cascade of events (Park *et al.*, 2012) (Figure 1, Appendix C). The PDE-AMPK-SIRT1-PGC-1α axis is indirectly yet critically involved in ameliorating the oxidative status of neurons. By improving mitochondrial biogenesis and respiration, energy can be afforded to the clearance of ROS and to damaged organelles through activation of autophagy (Cantó and Auwerx, 2009). This axis has congruently triggered discussions on possible therapeutic avenues to explore in Parkinson's disease, in view of the vital role occupied by mitochondrial homeostasis in this disease (Chaturvedi and Beal, 2013; Maiese, 2016).

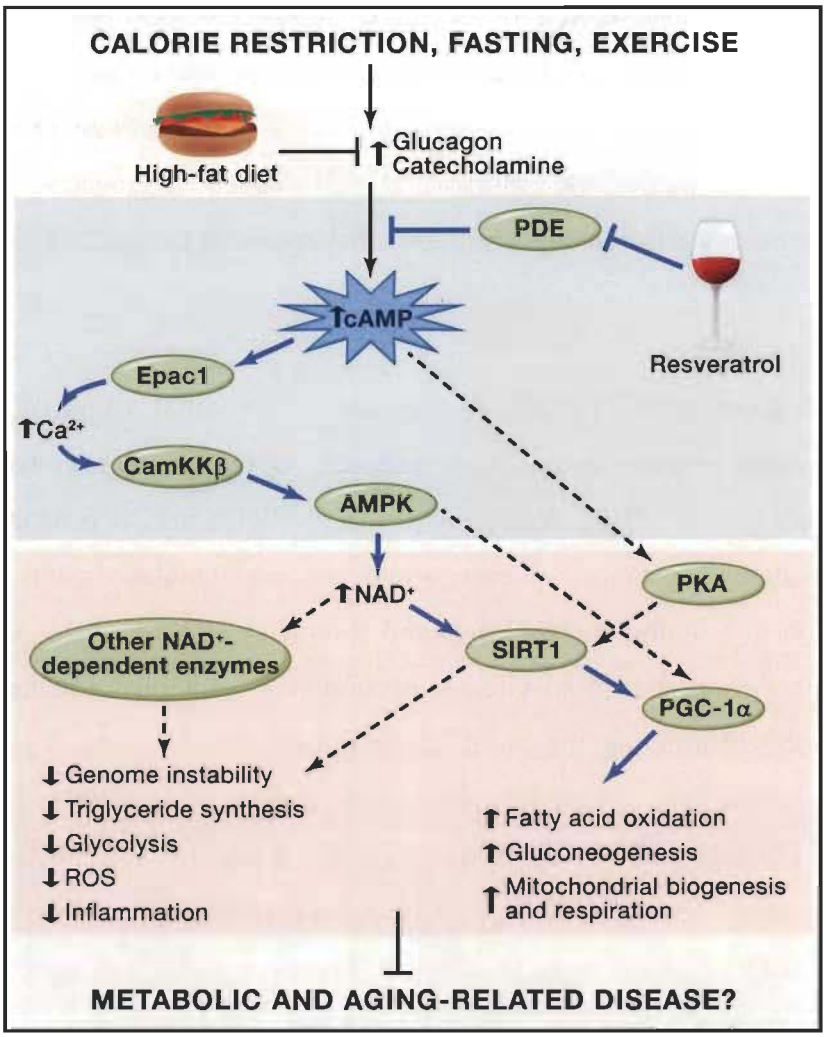

Figure 1.22 **Molecular mainstays of metabolic homeostasis.** By sensing levels of cyclic adenosine monophosphate (cAMP) and NAD+, and by acting on each other, this highly ramified pathway promotes equilibrium in instances of metabolic deficits. Moreover, they protect cells against a slew of noxious molecular events and are thought to mediate the beneficial effects of calorie restriction. AMPK, adenosine monophosphate kinase; CamKKβ, calcium/calmodulin-dependent protein kinase kinase 2 or beta; Epac1, exchange factor directly activated by cyclic adenosine monophosphate 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PKA, protein kinase A. (From Tennen *et al.*, 2012.)

1.3.3.3 Mammalian target of rapamycin and autophagy

Autophagy is an intricately structured cascade of events that promotes the equilibrium between clearance and production of organelles and proteins. *Per se*,

autophagy degrades non-essential or damaged cellular components and is therefore activated in settings of energy depletion and oxidative stress. Moreover, its role in Parkinson's disease has been evoked time and again, as it is a process required to clear misfolded proteins, pathogenic fibrils and full-fledged aggregates expressed in α-synuclein pathology (Hashimoto *et al.*, 2003; Schapira and Gegg, 2011; Schneider and Zhang, 2010).

Focal to autophagy is mTOR, an inhibitor of the initial stages of this process, which is of capital importance in tissues disposing of meagre regenerative capacities, like the CNS (Lee *et al.*, 2012). Akin to AMPK and SIRT1, mTOR is an energy sensor, but it is activated in instances of energy and nutrient abundance, thus occupying a central position in bridging energy levels and autophagy (Kundu, 2011) (Figure 1.23). mTOR phosphorylates the initial effector of autophagy, autophagy-related protein 13 (Atg13), thereby obstructing the onset of autophagy (Chan *et al.*, 2009). However, mTOR is repressed both by AMPK activation (Bolster *et al.*, 2002) and oxidative modifications (Dames *et al.*, 2005), in which context autophagy is accordingly activated to restore cellular homeostasis. mTOR therefore collaborates narrowly with the PDE-AMPK-SIRT1-PGC-1α axis to alleviate oxidative stress and restore favourable bioenergetic dynamics.

Resveratrol was recently shown to bind and inhibit mTOR by competing with ATP, thereby promoting autophagy in proper cellular contexts (Park *et al.*, 2016). Correspondingly, previous reports demonstrating resveratrol's neuroprotective capacities in dopaminergic neurons challenged with rotenone concurrently found it to stimulate the autophagic flux (Lin *et al.*, 2014; Wu *et al.*, 2011). Moreover, suppression of the master metabolic regulator AMPK dampened these beneficial effects, which bolsters the pertinence of bioenergetics-autophagy crosstalk in the neuroprotection afforded by resveratrol against oxidative stress (Wu *et al.*, 2011).

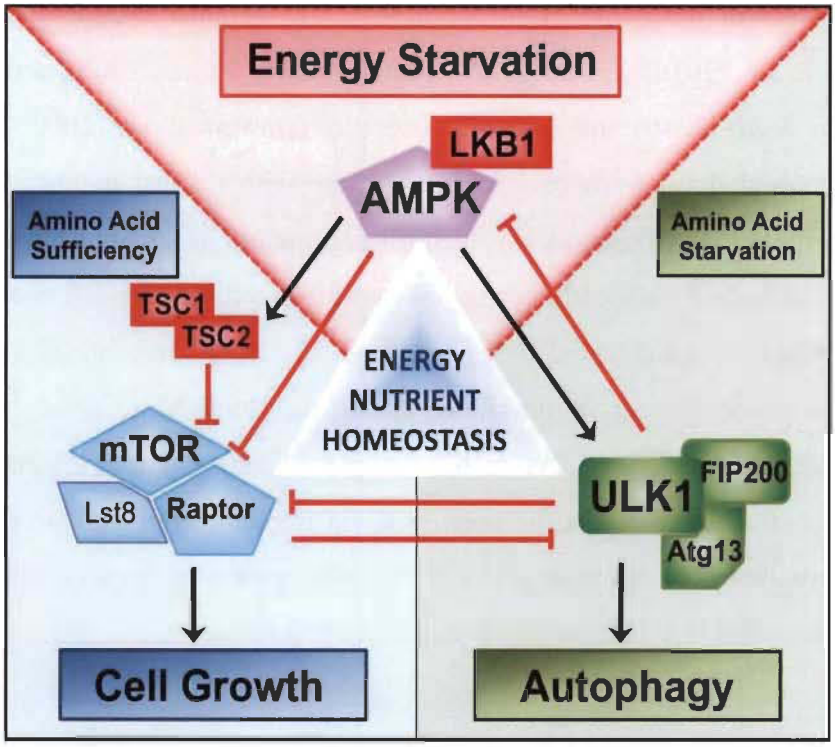

Figure 1.23 Metabolic regulation of cell growth and autophagy dynamics. A triad of kinases senses nutrient and energy availability, acting in concert to promote cellular homeostasis by modulating cell growth and autophagy. The central bioenergetic power player, AMPK, can have opposite effects in the cell depending on the ATP:adenosine monophosphate (AMP) ratio. In a replete, high ATP setting, AMPK will activate the tuberous sclerosis complex (TSC) duo that inhibits mammalian target of rapamycin complex 1 (mTORC1), a complex constituted of mammalian target of rapamycin (mTOR) and its regulators, thereby promoting cell growth and silencing autophagic pathways. In circumstances of energy depletion, AMPK will rather activate Unc-51 like autophagy activating kinase (ULK1), which will stimulate autophagy and inhibit cell growth. This toggle is highly sensitive and meticulously regulated by feedback loops between the different components of this triad. Atg13, autophagy-related protein 13; FIP200, retinoblastoma 1-inducible coiled-coil protein 1; LKB1, liver kinase B1; Lst8, mTORC subunit lethal with SEC13 protein 8. (From Dunlop and Tee, 2013.)

1.3.3.4 Other targets

While focus has only been granted to three of numerous putative direct targets of resveratrol, other binding partners may likely play an indirect though significant role in conveying this polyphenol's antioxidative effects. Worthy of mention is a host of

important players in the arena of inflammation, for instance cyclooxygenases-1 and -2 (Calamini *et al.*, 2010; Murias *et al.*, 2004; Zykova *et al.*, 2008), leukotriene 4A hydrolase (Oi *et al.*, 2010) and estrogen receptors (Bowers *et al.*, 2000; Nwachukwu *et al.*, 2014), which have been reviewed elsewhere with respect to resveratrol's anti-inflammatory potential (Appendix C). Neuroinflammation is greatly acknowledged to actively participate in the etiopathogenesis of Parkinson's disease and is interwoven with oxidative processes, as suggested by microglia-derived oxidative bursts and oxidative stress-induced microglial activation (Langston *et al.*, 1999; More *et al.*, 2013; Russo *et al.*, 2014; Zecca *et al.*, 2008). As such, resveratrol's antioxidative effects in the CNS of rodents and humans may also be conveyed via impeding pro-oxidative enzymes in microglial cells that may injure neurons in the substantia nigra pars compacta, such as myeloperoxidase and NADPH oxidase (Chang *et al.*, 2013).

All evidence considered, its capacity to modulate a wide array of proteic activities affords resveratrol tangible pathway-modifying properties that converge toward the prevention of oxidative stress in dopaminergic neurons. Although mostly tried in parkinsonian paradigms or in non-neuronal diabetic models, the literature grants a credible role for resveratrol in protecting cultured dopaminergic neurons against high glucose conditions.

1.4 Research aims and hypotheses

Provided the premise that dopaminergic neurons of the nigrostriatal pathway are relatively more susceptible to insults that overwhelm their endogenous coping mechanisms in Parkinson's disease, it is reasonable to surmise that other sources of stress, not specific to this pathology, may lead to their death, thereby shoring up this concept. As already mentioned, Parkinson's disease occurs more frequently in patients suffering from pathologies featuring a generalized state of oxidative stress, such as diabetes (Cereda *et al.*, 2012; Santiago and Potashkin, 2013; Sun *et al.*, 2012). Although awareness of these pathological associations has been growing, the selective

vulnerability of nigrostriatal dopaminergic neurons in these settings has never been the object of investigations.

In this respect, the core work of my thesis addresses the following central hypothesis: nigrostriatal dopaminergic neurons are more vulnerable to hyperglycaemic conditions compared to other neuronal populations, expressly the mesocorticolimbic pathway.

Accordingly, we elaborated a series of experiments employing dopaminergic neuronal cultures in high glucose conditions as well as a rat model of hyperglycaemia. We assessed the degeneration of dopaminergic neurons *in vitro* and *in vivo*, and described the ensuing behavioural alterations in rats. By offsetting the production of ROS using a well-known antioxidant, resveratrol, we also verified the role of high glucose-induced oxidative stress in the death of dopaminergic neuronal cultures.

1.4.1 Objective 1: Evaluate the degeneration of cultured dopaminergic neuronal cells in high glucose conditions

We first tested whether dopaminergic neurons in culture undergo degeneration in sustained high glucose conditions. We employed an *in vitro* model of dopaminergic neurons cultured in elevated though physiologically plausible concentrations of glucose. The production of ROS was measured and dopaminergic neuronal cell death was assessed. We specifically quantified the superoxide anion radical, as it is thought to constitute the initial toxic species overproduced in hyperglycaemia and liable for the cascade of oxidative events leading up to cellular injuries (Brownlee, 2005). Next, we investigated the mechanisms by which dopaminergic neuronal cells degenerate in high glucose conditions, focusing on the apoptotic cascade. Particular indices of apoptosis were examined, expressly the terminal events occurring at the level of the DNA supported by changes in the expression of various proteins involved in the process.

1.4.2 Objective 2: Determine the potential of the antioxidative polyphenol resveratrol to hamper the high glucose-induced degeneration of cultured dopaminergic neuronal cells

Alongside objective 1, we employed a strategy aimed at moderating oxidative stress to demonstrate its role in dopaminergic neurodegeneration. In this regard, we selected the stilbene resveratrol whose protective competences were extensively studied by virtue of its antioxidative potential. In line with objective 1, we verified the ability of resveratrol to prevent dopaminergic neuronal cell death, diminish superoxide anion radical production, and impede the apoptotic cascade.

Results for objectives 1 and 2 are reported in the article entitled "Resveratrol protects dopaminergic PC12 cells against high glucose-induced oxidative stress and apoptosis: effect on p53 and glucose-regulated protein 75 colocalization" published in November 2013 in the peer-reviewed journal *Neurotoxicity Research* (see Chapter II).

1.4.3 Objective 3: Characterize dopaminergic neurodegeneration in a rat model of long-term hyperglycaemia

On the grounds of results acquired *in vitro*, the preferential degeneration of the nigrostriatal pathway compared to the mesocorticolimbic pathway was next assessed *in vivo* in a well-established rat model of hyperglycaemia induced by a toxin that targets insulin-producing pancreatic β cells. Neurodegeneration assessments were performed following long-term hyperglycaemia, allowing the gradual instatement and maintenance of oxidative stress required to overwhelm susceptible targets. Accordingly, we measured glucose concentrations at source and terminal regions of the nigrostriatal and mesocorticolimbic pathways to inquire whether all areas were equally exposed to circulating glucose. By immunohistochemical and immunoblotting methods, we appraised neurodegeneration in both pathways as well as in the hippocampus for comparative means. Dopamine was measured at the terminals of these neurocircuits to corroborate observations of neurodegeneration in the midbrain. We also assessed the fate of glial cells in these regions to gain a better understanding of the effects of long-term hyperglycaemia in the substantia nigra pars compacta of rats.

1.4.4 Objective 4: Assess the behavioural alterations resulting from nigrostriatal neurodegeneration in a rat model of long-term hyperglycaemia

Degeneration of neurocircuits involved in the regulation of behaviours, as is the case for the nigrostriatal and mesocorticolimbic pathways, often leads to measurable behavioural alterations. Although both the nigrostriatal and mesocorticolimbic pathways fulfil highly complex mandates that can in some ways overlap, it remains that the former specializes in regulating motor behaviours. In keeping with our hypothesis, motor behavioural assessments were performed in the same subjects as in objective 3 to determine whether observed nigrostriatal neurodegeneration may lead to discernable motor deficits. Rats executed motor tasks typically employed in models of Parkinson's disease. To comparative ends, a cognitive novel object recognition test was performed to evaluate non-spatial working memory regulated by the hippocampus (Bast, 2007; Bast *et al.*, 2009), but also involving the mesocorticolimbic components nucleus accumbens (Annett *et al.*, 1989) and prefrontal cortex (Akirav and Maroun, 2006).

Insights of these behavioural tests in conjunction with the results pertaining to objective 3 are reported in the article entitled "Dopaminergic neurodegeneration in a rat model of long-term hyperglycemia: preferential degeneration of the nigrostriatal motor pathway" published online in May 2018 and issued in September 2018 in the peer-reviewed journal *Neurobiology of Aging* (see Chapter III).

Given the role of dopamine in social reward, social cognition and rough-and-tumble play processes (Narvaes and Martins de Almeida, 2014; Plavén-Sigray *et al.*, 2014; Trezza *et al.*, 2010), we next investigated whether nigrostriatal degeneration disrupts these behaviours in our rat model of long-term hyperglycaemia. In order to evaluate social behaviour, interactions between unacquainted conspecifics and emissions of ultrasonic vocalizations (USVs) were recorded simultaneously. USVs inform on the emotional state of rats during social contexts such as mating, play or aggression (Burgdorf *et al.*, 2008) and are largely regulated by dopamine (Brudzynski, 2009).

Complementary to the evaluation of motor deficits, these inquiries into the social behaviours of our model further extend our comprehension of the various ways that dopaminergic neurodegeneration may manifest itself. These specific results are reported in the article entitled "Long-term hyperglycaemia modifies social behaviour and emission of ultrasonic vocalisations in rats: a possible experimental model of altered sociability in diabetes" (see Chapter IV). The manuscript was submitted to the peer-reviewed journal *Scientific Reports* on July 9th, 2018.

1.5 Methodology

1.5.1 Objective 1: Evaluate the degeneration of cultured dopaminergic neuronal cells in high glucose conditions

1.5.1.1 Cell culture

To elucidate the possible neurodegenerative effects of a hyperglycaemic state on dopaminergic neurons *in vitro*, we employed the well-established model of dopaminergic neurons, the NGF-differentiated pheochromocytoma (PC12) cell line. These cells were initially derived from a neuroendocrine tumour in the adrenal medulla of a rat (Greene and Tischler, 1976), and as such they can be subcultured almost indeterminately. PC12 cells both synthesize and store catecholamines, principally dopamine, in large dense-core vesicles that are released upon depolarization (Greene and Rein, 1977; Greene and Tischler, 1976). PC12 cells share embryonic origins with neuroblastic cells. Thus, they can be differentiated into neuron-like cells with NGF treatments that phosphorylate and activate the tropomyosin receptor kinase A (Huang and Reichardt, 2003). Differentiation to a neuronal phenotype is enhanced by low serum concentrations (Yung *et al.*, 2010). Ensuing signal transduction abrogates cell division and enhances the activity of the rate-limiting enzyme in dopamine synthesis, TH (Schubert *et al.*, 1980), as well as the outgrowth of neurites and the expression of neurofilaments (Greene and Tischler, 1976; Lee *et al.*, 1982). The PC12 cell line also expresses dopamine transport and metabolic machinery, such as DAT, VMAT1

(preferentially expressed in adrenal glands, as opposed to neuronal VMAT2) and monoamine oxidase (Kadota *et al.*, 1996; Liu *et al.*, 1994; Youdim *et al.*, 1986).

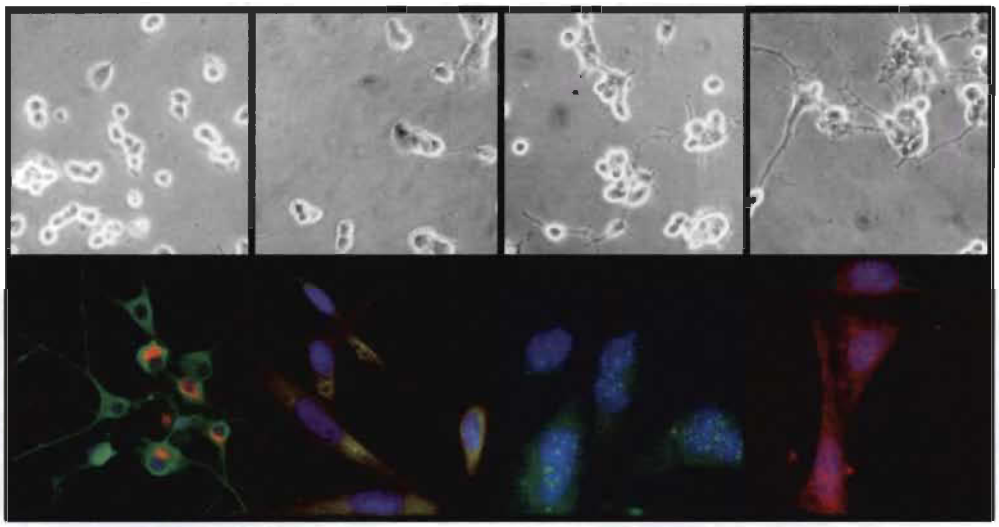

Figure 1.24 Characterization of dopaminergic neuronal cell cultures.
Top: From left to right are shown naïve pheochromocytoma (PC12) cells and 3-, 5-, 7-day nerve growth factor (NGF)-differentiated neuronal PC12 cells. Bottom: Immunofluorescent assessment of the expression of neuronal and dopaminergic markers. From left to right: 7-day differentiated neuronal PC12 cells marked for TH (green), neurofilaments (red) and nuclei (blue); dopaminergic N27 cells with Hoechst-stained nuclei marked for neurofilaments (yellow), DAT (green), and TH (red).

In this respect, our group employs 5-10-day NGF-differentiated neuronal PC12 cells (50 ng/mL) cultured in low concentrations of fetal bovine serum (1%) (Achour *et al.*, 2016; Bournival *et al.*, 2012*a*, 2012*b*; Renaud *et al.*, 2014). Figure 1.24 displays representative microphotographs of naïve (undifferentiated) PC12 cells and neuronal PC12 cells cultured in our laboratory. Pertinent to the present project, they also express markers necessary for a high glucose context, expressly insulin-independent GLUT3[47] (Maher *et al.*, 1991).

[47] PC12 cells also express insulin-dependent GLUT4 (Hudson *et al.*, 1993), as well as insulin-independent GLUT1 and GLUT8 (Maher *et al.*, 1991), although the latter dwells in intracellular compartments and has not yet been found to translocate to the cell surface (Widmer *et al.*, 2005). Given the high affinity and capacity of GLUT3 compared to all other GLUTs (Maher *et al.*, 1996), it is the most relevant glucose transporter in our model in the absence of insulin. Accordingly, midbrain dopaminergic neurons express GLUT3 and only very little GLUT4, at least in the rat brain (El Messari *et al.*, 1998; Maher *et al.*, 1991).

1.5.1.2 High glucose conditions

High glucose conditions *in vitro* aim to emulate the increase in extracellular glucose concentrations that takes place in hyperglycaemia. Depending on the culture medium, typical glucose concentrations range between 5.5 mM (1 g/L) to 17.5 mM (3.5 g/L). Taking into consideration the normal concentrations at which neurons are cultured, careful optimization of D-glucose concentrations and treatment times are required to achieve a faithful model of a physiologically hyperglycaemic state in cell cultures. In this respect, undiagnosed, untreated or uncontrolled diabetes can lead to a rise in glycaemia reaching 25 mM (450 mg/dL), qualified as severe hyperglycaemia (Amblee *et al.*, 2016; Marchese *et al.*, 2017; Saul *et al.*, 2016). In rats exhibiting a glycaemia of 30 mM[48], neurons are predicted to be exposed to ~9 mM of glucose, while at normoglycaemic values of 6 mM, they are exposed to 1.2 mM (Simpson *et al.*, 2007) (Table 1.7). This represents a 7.5-fold increase between normal and severe hyperglycaemic conditions. Since PC12 cells are normally cultivated in 11 mM of glucose, we tested the following concentrations within this physiological 7.5-fold increase in dose-response and time course studies: 25, 50 and 75 mM (Figure 1.25).

Seeing as we were aiming for a moderate cytotoxic effect averaging 30-50% (or 50-70% viability) with the lowest dose of D-glucose, results of these kinetic dose-response studies allowed for the selection of a concentration of 25 mM (4.5 g/L) administered for a duration of 96 h in neuronal PC12 cells. We also tested the effect of D-glucose on naïve PC12 cells and immortalized fetal mesencephalic dopaminergic N27 cells. Akin to PC12 cells, the latter secrete dopamine in addition to expressing the principal dopaminergic neuronal proteins, namely TH, DAT, nestin, neurofilaments and a neuron-specific enolate (Adams *et al.*, 1996; Prasad *et al.*, 1994), also characterized in our laboratory (Figure 1.24). These additional kinetic dose-response investigations ruled out the use of naïve PC12 cells, in light of their proliferative response, at least at 25 mM. These data also confirm that dopaminergic neuronal cells are vulnerable to high glucose

[48] We will later see that this glycaemia was indeed sustainable for 6 months in our rat model.

conditions, even when these are rather moderate (25 mM), strengthened by experiment repetitions in dopaminergic N27 cells.

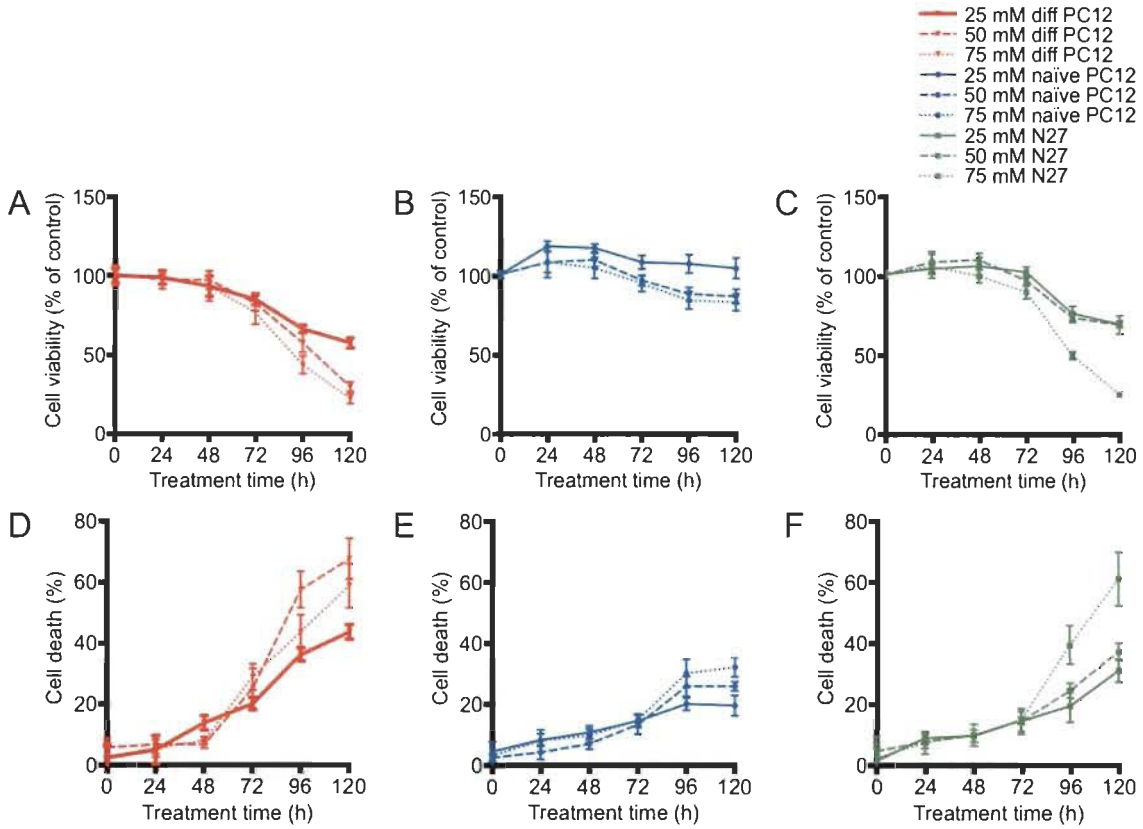

Figure 1.25 Time-course and dose-response study of the toxicity of glucose on dopaminergic cells in culture.
Cells were cultured in high glucose conditions ranging between 25-75 mM for 0-120 h and cell viability (top A-C) or cell death (bottom D-F) were measured by MTT assay and lactate dehydrogenase quantification, respectively. Differentiated neuronal PC12 cells (red lines) demonstrated a greater vulnerability than naïve cells (blue lines). Dopaminergic N27 cells exhibited intermediate susceptibility (green line). There seems to be a slight proliferative effect for non-differentiated cells (B, C), likely owing to the fact that these populations were not in a post-mitotic state: glucose may have stimulated cell growth at low concentrations. This effect was lost at the highest dose. The chosen concentration, 25 mM, is portrayed as a bold line.

As with any cell line, the PC12 model presents limitations. In this regard, we performed tests in a rat model of hyperglycaemia, detailed later. Nonetheless, among

the various utilizations of the PC12 cell line, our paradigm is advantaged by the differentiation of the cells to a clear neuron-like phenotype with pronounced expression of dopaminergic markers and cessation of division. In contrast, most other publications utilizing PC12 cells do not differentiate them, implying a tumoural phenotype and tapered dopaminergic properties, or use much higher concentrations of D-glucose that are not physiologically explainable (75-300 mM)[49] (Aminzadeh, 2017; Fouda and Abdel-Rahman, 2017; Rayegan *et al.*, 2017; Song *et al.*, 2017; Wang *et al.*, 2017; Zhao *et al.*, 2015). Additionally, naïve and NGF-treated PC12 cells are differentially susceptible to oxidative stress particularly caused by methylglyoxal, a by-product of sustained exposure to high glucose concentrations (Okouchi *et al.*, 2005). Differences in cellular redox states, specifically regarding the reduced glutathione-to-oxidized glutathione disulphide ratio, are likely accountable for these distinct responses.

1.5.1.3 Superoxide anion quantification

The primary goal of this part of the thesis was to verify that oxidative stress likely underpins the death of dopaminergic neuronal cells cultured in high glucose conditions. We measured the initial oxidative event that is believed to occur early in response to a glucose overload, that is, the overproduction of superoxide anion (Brownlee, 2005). Quantification of superoxide anion at the level of mitochondria is possible using a modified hydroethidine superoxide anion fluorogenic probe marketed under the name MitoSOX™ (Robinson *et al.*, 2006) (Figure 1.26). Hydroethidine is a reduced form of ethidium (3,8-diamino-5-ethyl-6-phenylphenanthridinium), shown to undergo oxidation by potassium superoxide into a red fluorescent molecule (Rothe and Valet, 1990). Since its discovery, hydroethidine has been widely used as an intracellular superoxide anion detector (Perticarari *et al.*, 1994; Rothe and Valet, 1990; Tarpey *et al.*, 2004). The modified hydroethidine molecule is conjugated with a triphenylphosphonium moiety that steers molecules toward mitochondria. The three lipophilic phenyl groups

[49] Such high concentrations of any solute, in fact, constitute a risk for noxious hypertonic effects that can be mistaken for the toxicity of the molecule *per se*. Although we remained within a reasonable concentration range, our work also provides D-mannitol controls, scarcely metabolized by mammalian cells, to rule out the possibility of a toxic osmotic effect in our model.

facilitate movement across membranes of live cells and the positively charged phosphonium group further favours accumulation in the mitochondrial matrix endowed with a negative membrane potential. As such, MitoSOX™ almost exclusively reacts with mitochondrial superoxide anion (Robinson *et al.*, 2006) (Figure 1.27).

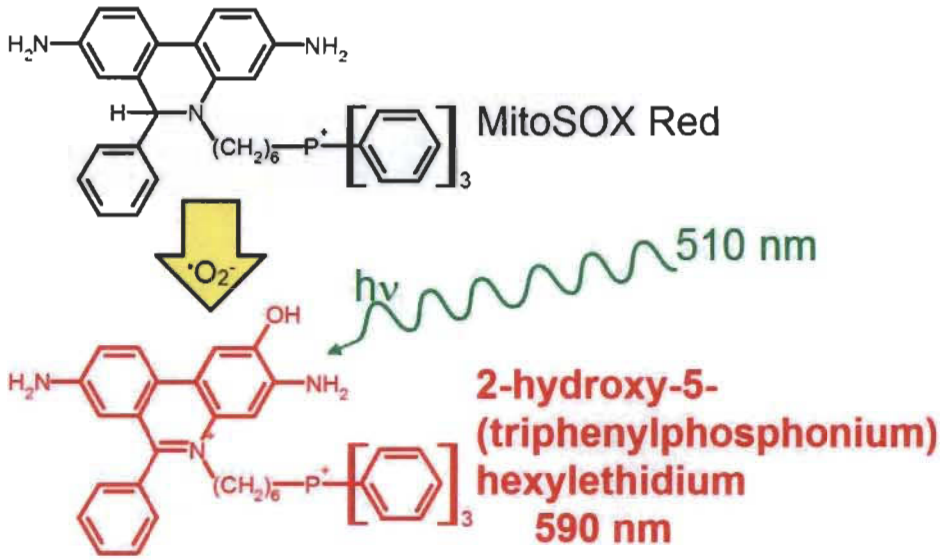

Figure 1.26 Structure and fluorescent mechanism of MitoSOX™ Red.
Upon reacting with superoxide anion, MitoSOX™ adopts a more highly conjugated structure enabling it to produce red fluorescence when it is excited at a wavelength of 510 nm. We can also appreciate the hydroethidine structure to the left and the positively charged triphenylphosphonium responsible for driving MitoSOX™ to mitochondria.

Granted superoxide anion overproduction in response to stress is a very early event in cell cultures (Carange *et al.*, 2011; Ronson *et al.*, 1999), we performed time course studies and identified 3 h as the optimal incubation time when to perform measurements in high glucose conditions. We have also previously shown that levels of nitrates and nitrites, reactive nitrogen species arising from secondary reactions between superoxide anion and nitric oxide (Pacher *et al.*, 2007), are increased at a later time point in our model (Bournival *et al.*, 2012*a*). In the article presented in Chapter II, the use of diethyldithiocarbamate, a selective inhibitor of SOD causing the accumulation of superoxide anion (Khazaei *et al.*, 2009; Puglia and Loeb, 1984), substantiated the specificity of MitoSOX™ in our paradigm.

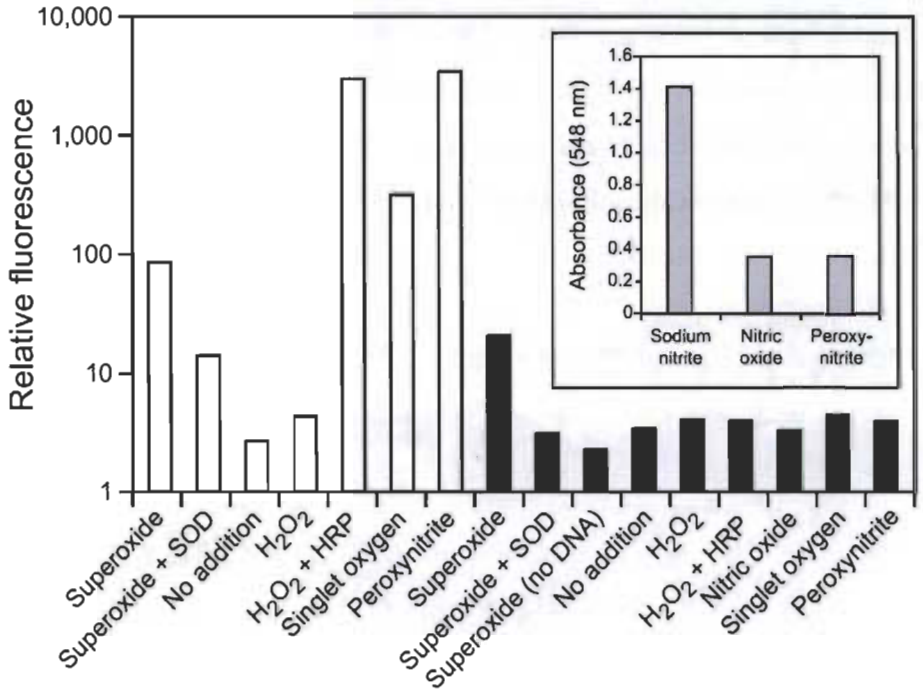

Figure 1.27 Selectivity of MitoSOX™ Red.
The specificity of two ROS probes, dihydrorhodamine 123 (white bars) and MitoSOX™ Red (black bars), were tested on a variety of species. While dihydrorhodamine 123 was not specific for any particular ROS, MitoSOX™ Red more readily scavenged superoxide anion, as evidenced by measures of fluorescence. It remains that this probe emits a weaker signal than dihydrorhodamine 123. The inset graph with grey bars displays Griess nitrite determination results, as a control for the detection of reactive nitrogen species. H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase. (From the product information sheet provided by Molecular Probes, 2015.)

1.5.1.4 Evaluation of apoptotic death

Apoptosis is a key contributor to the degeneration of neurons in Parkinson's disease[50] (Anglade *et al.*, 1997; Mochizuki *et al.*, 1996; Toulorge *et al.*, 2016) as well as in diabetes (Maiese *et al.*, 2007; Russell *et al.*, 1999). Alongside autophagic death, necroptosis, ferroptosis, anoikis and many others, apoptosis belongs to a family of programmed cell death processes that occur in multicellular organisms (Ke *et al.*, 2016)

[50] Several apoptotic hallmarks were revealed in post-mortem substantia nigra pars compacta samples of parkinsonian patients, for instance chromatin condensation and DNA fragmentation (Tatton, 2000), p53 and Bax overexpression (Mogi *et al.*, 2007; Tatton, 2000), and elevated caspase-3 activity (Mogi *et al.*, 2000; Tatton, 2000).

(Figure 1.28). Cellular apoptosis is a profitable course of death for an organism owing to its tight regulation and to the cleanliness of the process that does not induce overt inflammation, as opposed to necrosis (Alberts *et al.*, 2008). The importance of apoptosis becomes all the more apparent in cancer, wherein its pathological impairment is at the basis of several forms of the disease (Evan and Vousden, 2001; Green and Kroemer, 2009). However, excessive apoptosis, due to lax cellular control or elevated death signals in the immediate environment, is also cause for pathology especially in tissues with scant regenerative potential such as the CNS (Bredesen *et al.*, 2006).

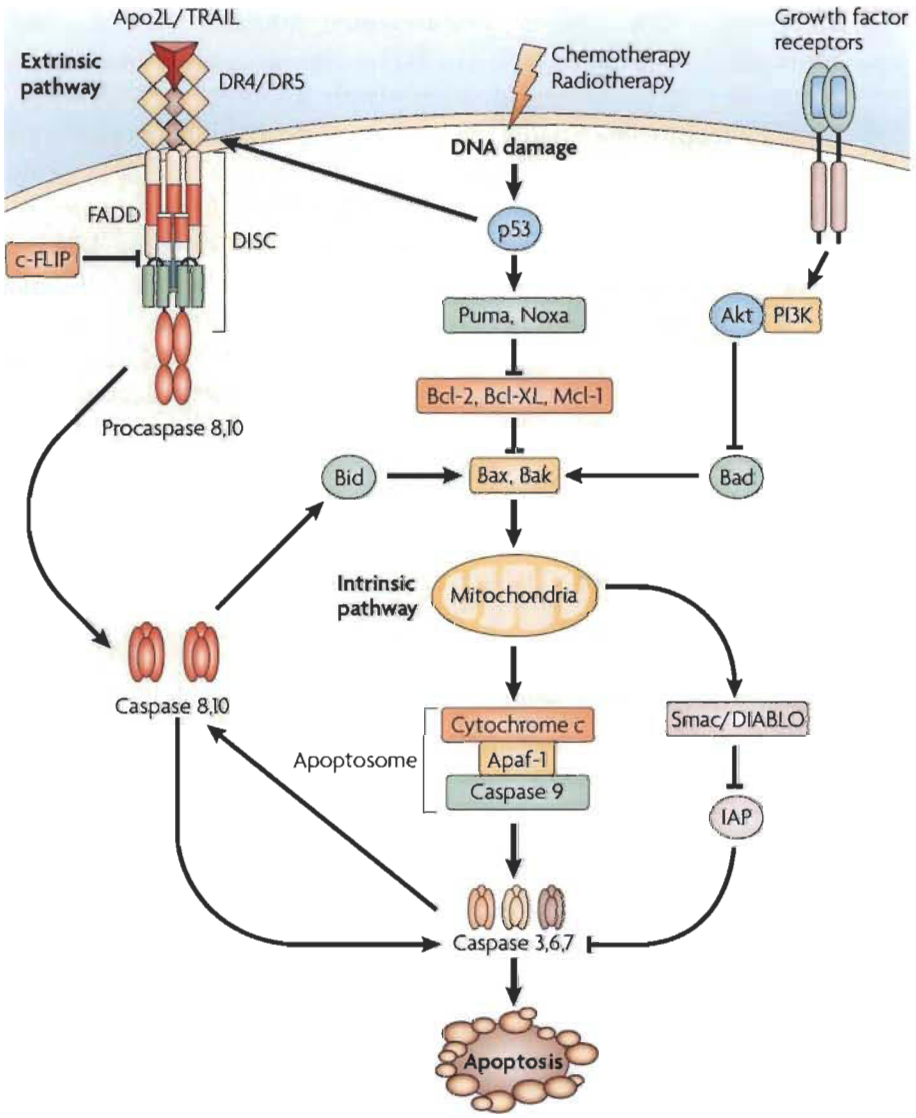

Figure 1.28 **The classic intrinsic and extrinsic pathways in apoptosis.**
Continued on next page.

(Continued.) The intrinsic pathway is usually triggered by an endogenous stress, which activates p53 responsible for the initiation of the apoptotic cascade via transcriptional upregulation of pro-apoptotic factors. These will disrupt mitochondrial homeostasis at the level of the permeability transition pore, allowing for pro-apoptotic factors to escape into the cytoplasm where they will participate in the formation of the apoptosome. This multiproteic structure activates effector caspases, which will then execute the remainder of the apoptotic program. The extrinsic pathway is rather instigated by external death or stress, which will lead to the activation of terminal effector caspases via the stimulation of intermediary caspases. Both pathways are intimately linked. Akt, protein kinase B; Apaf-1, apoptotic protease activating factor 1; Apo2L/TRAIL, apoptosis antigen 2 ligand/tumour necrosis factor-related apoptosis-inducing ligand; Bad, B cell lymphoma 2 (Bcl-2)-associated death promoter; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-XL, B cell lymphoma-extra large; Bid, Bcl-2 homology 3 interacting-domain death agonist; c-FLIP, first apoptosis signal receptor-associated protein with death domain-like interleukin-1-beta converting enzyme (FLICE/caspase 8)-like inhibitory protein; DISC, death-inducing signalling complex; DR4/DR5, death receptor 4/5; FADD, first apoptosis signal receptor-associated death domain; IAP, inhibitor of apoptosis proteins; Mcl-1, induced myeloid leukemia cell differentiation protein; Noxa, phorbol-12-myristate-13-acetate-induced protein 1; PI3K, phosphatidylinositol 3-kinase; Puma, p53 upregulated modulator of apoptosis; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI. (From Ashkenazi, 2008.)

Numerous archetypal events occur over the course of apoptosis, for instance cell shrinkage, chromatin condensation, DNA fragmentation and blebbing, to name a few. The point of no return in apoptosis is often regarded as the modification of chromatin and DNA. We verified this in our model by employing a DNA denaturation method that specifically detects cells whose chromatin is condensed (Frankfurt and Krishan, 2001) (Figure 1.29). This technique harnesses the amplified sensitivity of condensed apoptotic chromatin to thermal denaturation (Allera *et al.*, 1997). Therefore, following high glucose treatments, we heated neuronal PC12 cells and applied formamide to induce denaturation of sensitive apoptotic chromatin. Using a HRP-conjugated monoclonal antibody that specifically targets single-stranded DNA, we were able to measure the proportion of terminally apoptotic neuronal cells by colorimetric detection.

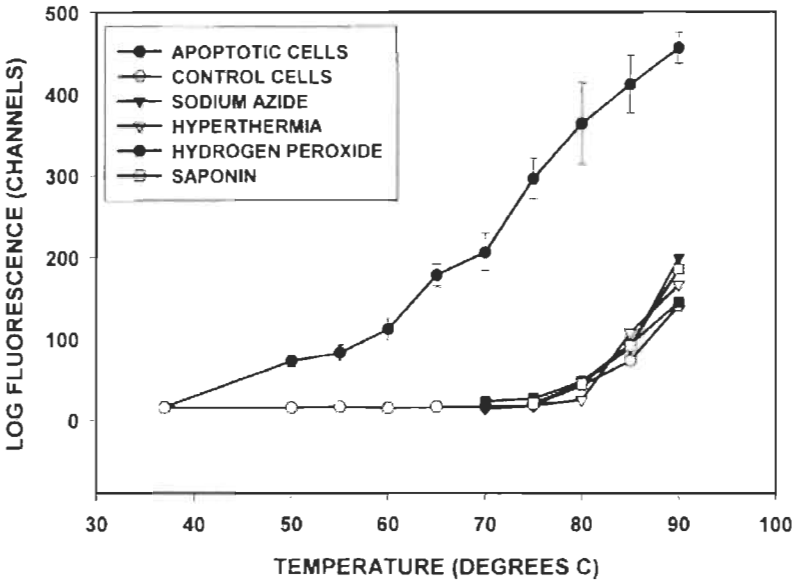

Figure 1.29 Specificity with respect to temperature kinetics of formamide-induced DNA denaturation.
Formamide was applied in cells submitted to several kinds of treatments described in the box. DNA denaturation was quantified over the course of rising temperatures. Apoptotic cells begin to exhibit DNA denaturation at manifestly cooler temperatures compared to other non-apoptotic cells, which corroborates the specificity of this test, performed at 70°C. (From Frankfurt and Krishan, 2001.)

To bolster our findings, we also verified other events that accompany the major phenotypic changes that occur during apoptosis, mainly modifications in the expression, subcellular localization or post-translational alterations of proteins. Activation of the caspase cascade, a sequence of proteic cleavage events controlled by caspase proteases, is a hallmark of apoptosis (Sakahira *et al.*, 1998). Caspase-3 is the terminal effector of this cascade that, upon cleavage, is responsible for the activation of a DNA fragmentation enzyme, caspase-activated deoxyribonuclease. Therefore, we verified terminal caspase activation and DNA fragmentation in our model, by employing immunofluorescence for cleaved caspase-3 labeling combined with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method to isolate cells displaying DNA fragmentation. To ensure the exclusive counting of apoptotic cells, we only identified ones that exhibited both fluorescent signals. The specificity of our results was also substantiated by the use of an irreversible inhibitor of cleaved caspase-3,

the fluormethylketone-conjugated tetrapeptide Z-DEVD-FMK, expected to yield cells with very little or no TUNEL signal.

Next, we evaluated the expression of proteins upstream or downstream from caspase-3 by immunoblotting. Upstream at the level of the mitochondria, one of the pro-apoptotic member of the Bcl-2 family, Bax, spearheads the formation of the mitochondrial permeability transition pore responsible for the cytosolic release of apoptogenic molecules, such as cytochrome *c* (Gollapudi *et al.*, 2003). Bcl-2, an anti-apoptotic member of the Bcl-2 family, interacts with Bax to prevent permeabilization (Renault *et al.*, 2013) (Figure 1.30). It follows that an elevated Bax to Bcl-2 expression ratio is a faithful indicator that apoptosis is on-going (Cory and Adams, 2002). At the level of the nucleus, the DNA repair enzyme PARP constitutes a downstream target of caspase-3 and its cleavage in 24 and 89 kDa fragments is a recognized feature of apoptosis (Kaufmann *et al.*, 1993). Thus, a low ratio of full-length-to-cleaved PARP constitutes a marker of apoptosis. Farther upstream of caspase activation, the tumour suppressor p53 was also assessed in light of its role in evoking apoptosis and sensing oxidative stress. Indeed, p53 is stabilized by oxidative stress and localizes to the nucleus where it induces the transcription of pro-apoptotic factors (Lee *et al.*, 2008; Macip *et al.*, 2003; Nair, 2006). Consequently, we studied the cytoplasmic and nuclear localization of p53 in neuronal PC12 cells following high glucose treatments, a high nuclear-to-cytoplasmic ratio likely indicating its active promotion of apoptosis in our model.

Directly linked to this, we also verified by immunoblotting and immunofluorescence the subcellular localization of a constitutively expressed chaperone of the heat shock protein family, glucose-regulated protein 75 (GRP75), known to sequester p53 in the cytoplasm, thereby preventing apoptosis in multiple models (Kaul *et al.*, 2005). Fluorescence colocalization analyses were performed to evaluate its aptitude to bind p53 in the cytoplasm in conditions of oxidative stress. Normally, GRP75 acts as a mitochondrial chaperone and guardian against oxidative stress among a plethora of other functions. However, its role under conditions of stress is highly variable (Londono *et al.*, 2012) (Figure 1.31). One of our aims was to define its role in

high glucose-induced oxidative stress due to its purported implication in neurodegeneration. Indeed, GRP75 is depleted in the affected brain regions of Parkinson's disease patients (Burbulla *et al.*, 2010; Jin *et al.*, 2006) and knocking out its expression leads to neurodegeneration that can be rescued by the parkinsonian gene product parkin (Yang *et al.*, 2011). In addition, it is a well-recognized binding partner of other genes mutated in Parkinson's disease, namely DJ-1 and α-synuclein (Jin *et al.*, 2006; Li *et al.*, 2005).

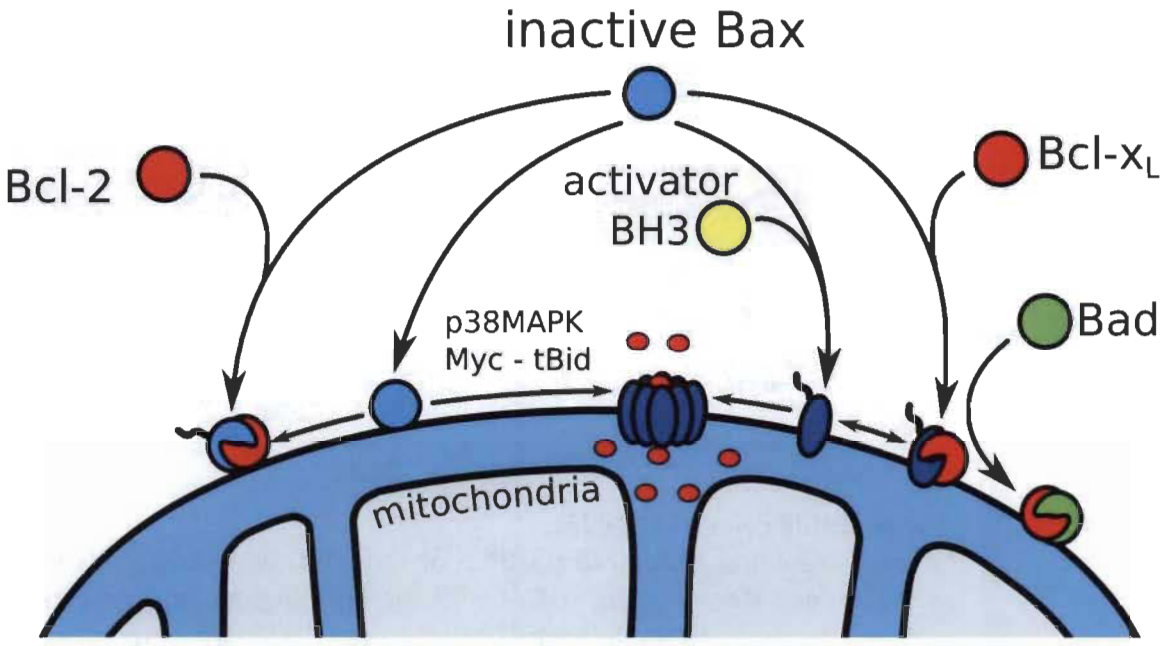

Figure 1.30 Mitochondrial translocation of Bax followed by the formation of the mitochondrial permeability transition pore.
Bax is usually found in an inactive state (light blue) in the cytoplasm. When Bcl-2 homology 3 (BH3)-only proteins such as tBid, Bcl-2-like protein 11 (Bim) or Puma are produced in response to an apoptosis-inducing stress, these can activate Bax, rendering it more liable to insert itself in the mitochondrial membrane leading to the generation of the permeability transition pore. Anti-apoptotic members of the Bcl-2 family, on the other hand, prevent Bax from inserting itself in the membrane. Considering the ratio of these proteins can offer insight into events occurring at the level of the mitochondrial membrane during apoptosis. p38MAPK, p38 mitogen-activated protein kinases. (From Renault *et al.*, 2013.)

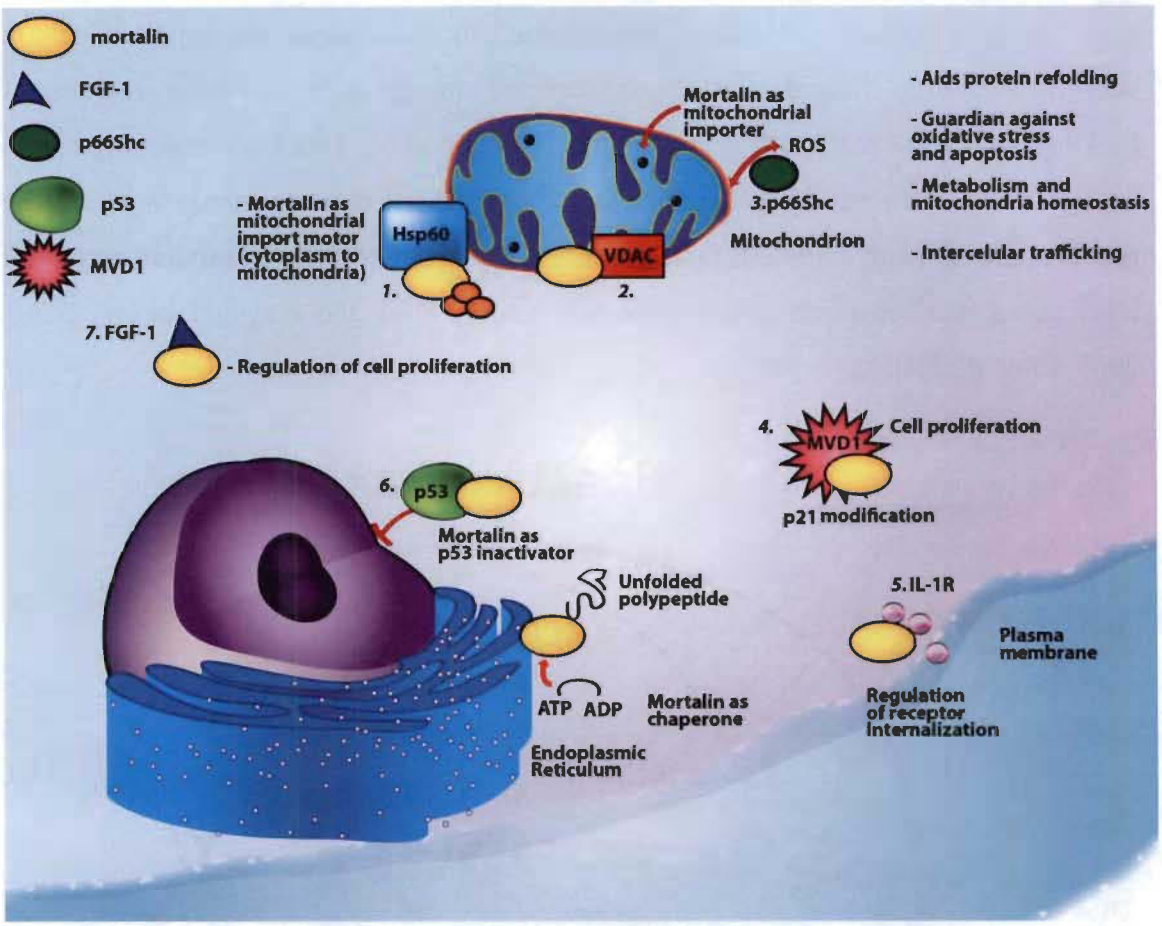

Figure 1.31 The multiple roles of GRP75.
Glucose-regulated protein 75 (GRP75 or mortalin) is primarily involved in the mitochondria, where it fulfils chaperone, import, and oxidation-sensing functions. In the advent of cellular stress, GRP75 is recruited to the cytoplasm where it can regulate several processes, including apoptosis. By binding p53, it abrogates this transcription factor's translocation to the nucleus where it usually performs pro-apoptotic operations. Nevertheless, much remains to be elucidated pertaining to its functions. FGF-1, fibroblast growth factor 1; Hsp, heat shock protein; IL-1R, interleukin-1 receptor; MVD1, diphosphomevalonate decarboxylase; p66Shc, 66 kDa proto-oncogene Src homologous-collagen homologue adaptor protein; VDAC, voltage-dependent anion channel. (From Londono *et al.*, 2012.)

Further methodological details concerning the utilization of neuronal PC12 cells cultured in 25 mM of D-glucose for 96 h, the measurement of superoxide anion at 3 h, or the assessment of apoptosis in our model are provided in Chapter II.

1.5.2 Objective 2: Determine the potential of the antioxidative polyphenol resveratrol to hamper the high glucose-induced degeneration of cultured dopaminergic neuronal cells

1.5.2.1 Resveratrol treatments

In addition to measuring superoxide anion production in neuronal PC12 cells treated with high glucose conditions, we employed an antioxidative strategy to verify the importance of oxidative mechanisms in dopaminergic neurodegeneration *in vitro*. The polyphenol resveratrol was chosen for its potent antioxidative capacities demonstrated in various cellular (Karlsson *et al.*, 2000; Savaskan *et al.*, 2003; Wu *et al.*, 2017; Zhuang *et al.*, 2003) and animal models (Kiziltepe *et al.*, 2004; Palle and Neerati, 2018; Sinha *et al.*, 2002). In neuronal paradigms, resveratrol is best known for its ability to enhance the activity of SOD, an enzyme that catalyzes superoxide anion inactivation[51], both *in vitro* (Bai *et al.*, 2013; Lee *et al.*, 2012; Yuan *et al.*, 2013) and *in vivo* (Cheng *et al.*, 2014; Kesherwani *et al.*, 2013; Nalagoni and Karnati, 2016; Ren *et al.*, 2011).

Very few studies have used resveratrol on neuronal PC12 cells, solely focusing on demonstrating its pro-differentiation competences (Lecomte *et al.*, 2017; Ma *et al.*, 2014; Sugino *et al.*, 2010). These projects established resveratrol's effects at concentrations ranging from 0.5 μM to 20 μM. Most other applications of resveratrol on cultured neurons typically provide its use at 1-120 μM (Calabrese *et al.*, 2010; Jardim *et al.*, 2017). In our paradigm, resveratrol was administered at an optimized concentration of 0.1 μM, constituting the lowest dose that reliably protects our model against various oxidative insults (Bournival *et al.*, 2009; Bureau *et al.*, 2008; Gélinas and Martinoli, 2002). To verify the ability of resveratrol to modulate superoxide anion concentrations in mitochondria, neuronal PC12 cells were co-treated with the polyphenol for the duration of high glucose treatments.

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[51] In purified neuronal mitochondria, resveratrol also demonstrates direct scavenging properties for the superoxide anion (Zini *et al.*, 1999, 2002). One group employed PC12 cells challenged with high glucose concentrations to demonstrate its protective effects via the activation of the phosphatidylinositol 3-kinase/Akt/forkhead box O3 (PI3K/Akt/FoxO3a) pathway (Liu *et al.*, 2015). However, the authors used undifferentiated cells, which, as previously discussed, respond differently to oxidative insults (Okouchi *et al.*, 2005).

The differentiation of PC12 cells with NGF is important, not only to yield a clear neuronal phenotype, but also to prevent adverse effects from resveratrol treatments. Indeed, resveratrol exhibits both protective (Agrawal *et al.*, 2011; Dasgupta and Milbrandt, 2007; Della-Morte *et al.*, 2009) and toxic (Muqbil *et al.*, 2012; Trincheri *et al.*, 2007; Wenner, 2012) properties depending on cellular contexts. In naïve PC12 cells, resveratrol is cytotoxic, whereas it promotes neurite outgrowth, mitochondria renewal and energy balance in NGF-differentiated ones (Hayakawa *et al.*, 2013). For this reason, PC12 cells were differentiated for at least 5 days with NGF before high glucose or resveratrol treatments were employed.

The application of polyphenols in cell cultures also requires certain methodological adjustments. One of these is to ensure the absence of other phenolic substances in the cell culture medium to rule out any effect not attributable to the polyphenol treatment. To that end, all sera used during experiments were charcoal-stripped to remove steroids, the principal phenolic compounds found in these cell culture additives (Bournival *et al.*, 2009; Bureau *et al.*, 2008; Cao *et al.*, 2009; Gélinas and Martinoli, 2002).

Further methodological information concerning the use of resveratrol in our dopaminergic model is provided in Chapter II.

1.5.3 Objective 3: Characterize dopaminergic neurodegeneration in a rat model of long-term hyperglycaemia

1.5.3.1 Rat model of long-term hyperglycaemia

Selection of an appropriate paradigm to simulate long-term hyperglycaemia in rodents likely constituted the most critical aspect of the *in vivo* portion of this thesis. Attempts to model diabetes and other metabolic diseases as working platforms to develop treatments and elucidate pathological mechanisms have yielded today a slew of well-described paradigms (see for reviews Baxter and Duckworth, 2004; Islam and Loots, 2009; Kim *et al.*, 1998; Srinivasan and Ramarao, 2007; Van Belle *et al.*, 2009).

Our choice was founded on accessibility, operational simplicity and hyperglycaemia sustainability. While the aim of this thesis is not to provide an extensive description of the multiple paradigms of diabetes, an overview of the most salient rodent models is offered next with respect to our selection criteria.

Diabetic rodent models are conveniently separated into two categories: genetic and experimentally-induced (Table 1.10). Various genetic and transgenic models of diabetes mellitus have been developed to date, some of the most common being Long Evans Tokushima Fatty rats (cholecystokinin receptor deficiency), Zucker fatty rats (leptin receptor deficiency), and *db/db* (leptin receptor deficiency) or *ob/ob* (leptin deficiency) strains of mice, reflective of a type II diabetes-like state, as well as the non-obese diabetic mouse strain (polygenic cause) and BioBreeding diabetes-prone rats (polygenic cause), rather mimicking a type I-like pathology (Bell and Hye, 1983; Kawano *et al.*, 1992; Shafrir, 2003; Verdaguer *et al.*, 1996). These models bear the advantage of developing a spontaneous pathology. However, they are costly and require meticulous maintenance, which diminishes their accessibility. In addition, frank hyperglycaemia is not observed in some instances, as is the case for Zucker fatty rats or *ob/ob* mice. Conversely, when hyperglycaemia is readily present, for example in *db/db* or non-obese diabetic mice, insulin treatment may be required to maintain the animals.

Table 1.10

Animal models of diabetes

Model Category	Advantages[&]	Disadvantages[&]
I. Spontaneous diabetic animals	Development of type 2 diabetes is of spontaneous origin involving genetic factors and the animals develop characteristic features resembling human type 2 diabetes	Highly inbred, homogenous and mostly monogenic inheritance and development of diabetes is highly genetically determined unlike heterogeneity seen in humans
	Mostly of inbred animal models in which the genetic background is homogeneous and environmental factors can be controlled, allow genetic dissection of this multifactorial disease easy	Limited availability and expensive for the diabetes study
		Mortality due to ketosis problem is high in case of animals with brittle pancreas (db/db, ZDF rat *P. obesus*, *etc.*) and require insulin treatment in later stage for survival
	Variability of results perhaps minimum and require small sample size	Require sophisticated maintenance
II. Diet/Nutrition induced diabetic animals	Develop diabetes associated with obesity as a result of overnutrition as in diabesity syndrome of human population	Mostly require long period of dietary treatment
		No frank hyperglycaemia develops upon simple dietary treatment in genetically normal animals and hence become not suitable for screening antidiabetic agents on circulating glucose parameter
	Toxicity of chemicals on other body vital organs can be avoided	
III. Chemical induced diabetic animals	Selective loss of pancreatic beta cells (alloxan/STZ) leaving other pancreatic alpha and delta cells intact	Hyperglycaemia develops primarily by direct cytotoxic action on the beta cells and insulin deficiency rather than consequence of insulin resistance
	Residual insulin secretion makes the animals live long without insulin treatment	Diabetes induced by chemicals is mostly less stable and at times reversible because of the spontaneous regeneration of beta cells. Hence, care must be taken to assess the pancreatic beta cell function during long-term experiments
	Ketosis and resulting mortality is relatively less	
	Comparatively cheaper, easier to develop and maintain	Chemical produce toxic actions on other body organs as well besides its cytotoxic action on beta cells
		Variability of results on development of hyperglycaemia is perhaps high
IV. Surgical diabetic animals	Avoids cytotoxic effects of chemical diabetogens on other body organs	Involvement of cumbersome technical and post operative procedures
	Resembles human type 2 diabetes due	Occurrence of some other digestive
	to reduced islet beta cell mass	problems (as a result of part of excision of exocrine portion (deficiency of amylase enzyme)
		Dissection of alpha islets (glucagon secreting cells) too along with beta cells leading to problems in counter regulatory response to hypoglycaemia
		Mortality is comparatively higher
V. Transgenic/knock out diabetic animals	Effect of single gene or mutation on diabetes can be investigated *in vivo*	Highly sophisticated and costly procedure for the production and maintenance
	Dissection of complex genetics of type 2 diabetes become easier	Expensive for regular screening experiments

[&] STZ, streptozotocin; ZDF, Zucker diabetic fatty.
(From Srinivasan and Ramarao, 2007.)

Experimentally-induced models are, on the other hand, generally inexpensive, flexible and require little to no special maintenance (Islam and Loots, 2009). This category can further be subdivided into chemical-, diet- and surgically-induced models. Partial or total pancreatectomies were formerly common procedures to generate, respectively, type II or type I diabetes-like phenotypes presenting prominent hyperglycaemia (Foglia, 1950; Pauls and Bancroft, 1950). However, their popularity dwindled with the recognition that much simpler chemical protocols could efficiently deplete insulin-producing pancreatic β cells. By a large amount, the two most prominent toxins are alloxan (Goldner and Gomori, 1943) and streptozotocin (Rakieten *et al.*, 1963). Both operate via oxidative mechanisms to cause the necrosis of pancreatic β cells (Rerup, 1970; Yamamoto *et al.*, 1981) (Figure 1.32). Owing to its greater stability and longer half-life, streptozotocin is sometimes preferred over alloxan in rats and mice, but not in rabbits who are insensitive to the former (Rerup, 1970; Srinivasan and Ramarao, 2007). Depending on the intravenous (i.v.) dose[52], these molecules can yield either type I- (higher dose) or type II-like (lower dose) diabetic features (Rakieten *et al.*, 1963). However, most agree that, regardless of the dose, i.v. alloxan and streptozocin induce a diabetic phenotype that closely resembles type I diabetes with manifestations of acute hypoinsulinaemia instead of insulin resistance. Intraperitoneal (i.p.) injections in adult or neonatal rodents were later adopted to moderate the outcomes and to enhance their maintainability (Ito *et al.*, 1999, 2001; Kodama *et al.*, 1993; Portha *et al.*, 1974, 2007).

Other methods used to generate more sustainable experimentally-induced models consist in layering different treatments to modulate extreme phenotypes. One of the most widely hired strategies in this sense employs single i.p. injections of nicotinamide prior to streptozotocin administration (Masiello *et al.*, 1998; Nakamura *et al.*, 2006). Nicotinamide moderates the necrosis of pancreatic β cells induced by streptozotocin, thereby generating a model that is hypoinsulinaemic and hyperglycaemic, but viable for

[52] When administered i.v., alloxan is usually given in doses ranging from 40-200 mg/kg body weight [b.w.] in rats or 50-200 mg/kg b.w. in mice. Streptozotocin, on the other hand, is administered i.v. in doses ranging from 35-65 mg/kg b.w. in rats or 40-200 mg/kg b.w. in mice.

several months without the need for insulin treatment[53]. In recent years, mixed chemical-diet paradigms were implemented that more faithfully emulate type II diabetes (Reed *et al.*, 2000; Srinivasan *et al.*, 2005). Indeed, standalone specialized diets (high-fat, high-carbohydrate, high-fat low-carbohydrate, etc.) present the disadvantage of causing a very progressive form of metabolic syndrome, characterized mostly by obesity and insulin resistance, but rarely associated with frank hyperglycaemia or glucose intolerance (Houssay and Martinez, 1947; Surwit *et al.*, 1988; Winzell and Ahren, 2004). Combined with a low i.p. dose of streptozotocin, high-fat diets induce increased body weight, hyperglycaemia, hyperinsulinaemia and dyslipidaemia in rodents (Reed *et al.*, 2000; Srinivasan *et al.*, 2005). Uniting insults in the form of diets and toxins is today considered a core framework for the development of long-term type II diabetic-like models (Zhang *et al.*, 2003). Nevertheless, despite attempts to refine existing models, none described to date are fully representative of either type I or II diabetes in humans (Baxter and Duckworth, 2004; Islam and Loots, 2009; Van Belle *et al.*, 2009).

Granted the purpose of our work dwells in evaluating the effects of a sustained hyperglycaemic phenotype and not in faithfully simulating a specific disease, we were principally interested in models displaying this key feature for a long period of time without the need for ectopic insulin interventions. In this spirit, moderate chemical-induced and diet-chemical mixed paradigms constituted the simplest and most accessible protocols. Thus, we tested two well-documented paradigms in parallel: moderate dose nicotinamide-streptozotocin-treated rats and low dose streptozotocin-treated rats fed a high-fat diet (Reed *et al.*, 2000; Srinivasan *et al.*, 2005). In the first protocol, overnight fasted rats were simply injected with nicotinamide (i.p. 100 mg/kg b.w.) 20 min prior to administration of streptozotocin (i.p. 55 mg/kg b.w.), as previously described (Badole *et al.*, 2015; Masiello *et al.*, 1998). In the second protocol, rats were maintained on a 60% high-fat diet adjusted for calories that began two weeks prior to a single i.p. injection of streptozotocin (35 mg/kg b.w., overnight fasted) (Srinivasan *et al.*, 2005). Hyperglycaemia was first assessed 72 h following injections and thereafter measured on

[53] Precisely, nicotinamide inhibits PARP, whose exaggerated activation by streptozotocin-induced DNA alkylation leads to the depletion of NAD+ and ATP, liable for pancreatic β cell necrosis. Nicotinamide also offers protection by serving as a precursor of NAD+ (Szkudelski, 2012).

a regular basis, alongside body weight and food intake. Figure 1.33 summarizes these monitored metabolic parameters in our models.

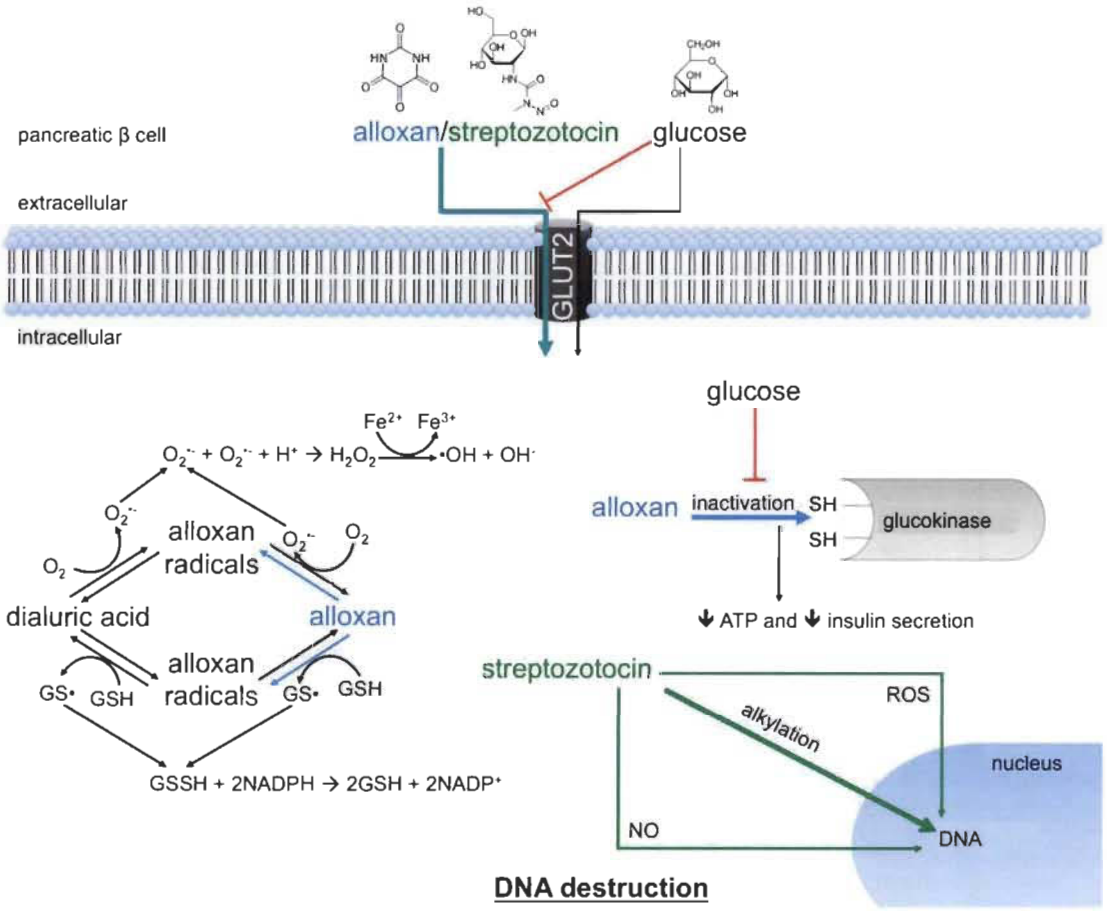

Figure 1.32 Mechanisms of pancreatic β cell death in chemical-induced diabetes. Alloxan and streptozotocin both enter pancreatic β cells via GLUT2, competing with glucose on the way. Alloxan engages in different mechanisms to participate in the death of pancreatic β cells. For instance, alloxan fuels a redox cycle with its reduction product, dialuric acid, thereby producing highly reactive ROS, such as the hydroxyl radical (•OH) via Fenton and Haber-Weiss reactions, and depleting antioxidative cofactors, like GSH and NADPH. Alloxan may also inactivate glucokinase, leading to hampered ATP production and insulin secretion, a mechanism inhibited by the presence of glucose. Contrary to alloxan, streptozotocin's primary mechanism is quite straightforward and implicates firsthand alkylation of DNA. Exposure to either toxin leads to damaged DNA and the activation of the reparation enzyme PARP whose overstimulation will deplete stocks of the NAD+ cofactor: pancreatic β cells thereby undergo necrosis. Fe^{2+}, ferrous iron ions; Fe^{3+}, ferric iron ions; GS•, glutathione radical; GSSH, glutathione disulphide; NO, nitric oxide. (Adapted from Radenković *et al.*, 2016.)

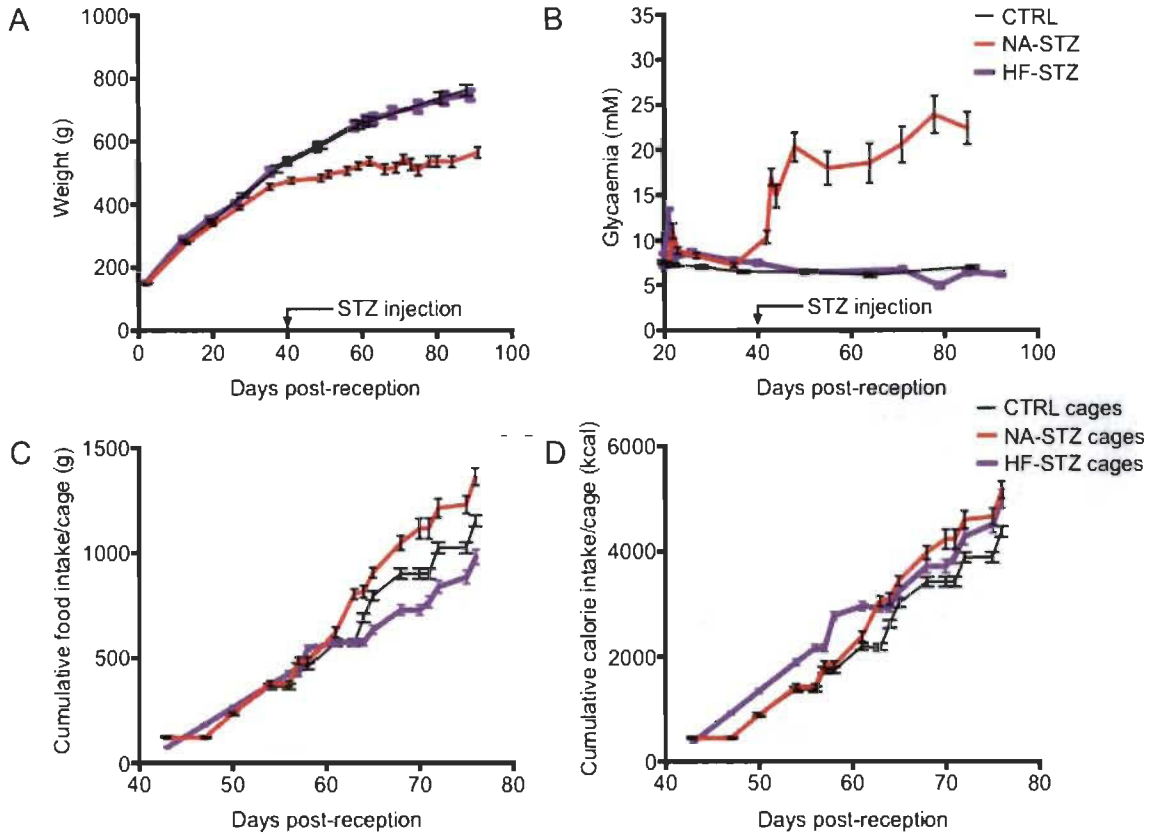

Figure 1.33 Metabolic follow-up of nicotinamide-streptozotocin or high fat-streptozotocin treated rats.
Weight (A), glycaemia (B), cumulative food intake (C) and caloric intake (D) per cage were measured for over two months in two different models of diabetes. High fat-fed rats injected with streptozotocin (purple lines) clearly did not develop hyperglycaemia or hyperphagia, while nicotinamide-streptozotocin rats (red lines) were manifestly hyperglycaemic and hyperphagic. CTRL, control; HF, high-fat diet.

Protocol 1 yielded the best results regarding the maintenance of a moderate to elevated hyperglycaemia. Despite manifestations of a progressive hyperphagia, rats from protocol 1 stopped gaining weight. Conversely, protocol 2 did not render rats either hyperglycaemic or hyperphagic[54]. Indeed, rats administered the high-fat diet did not seem to find the food palatable. Accordingly, they did not gain weight faster than control rats. As the diet contained a great amount of fat (Table 1.11), it is possible that the food pellets became rancid quicker at room temperature upon contact with air. In fact,

[54] We suspected the amount of calories to contribute to the diminished intake of weighted food, but correcting for the energetic value of the diets did not provide further insight.

the high-fat diet did not contain antioxidants to prevent rancidification known to give foods an off flavour. In addition, high-fat food pellets were much softer, which may have contributed to the rats' indifference or dislike toward this diet. The manufacturers of this diet could not provide any explanations, either.

Table 1.11

Ingredient lists of the rat diets provided by Harlan

Ingredients	AIN-93G purified diet (TD.94045) (g/kg)	Adjusted calories diet (60/fat) (TD.06414) (g/kg)
Casein	200	265
L-Cysteine	3	4
Maltodextrin	132	160
Sucrose	100	90
Soybean oil	70	30
Cellulose	50	65.5
Mineral mix	35	48
Vitamin mix	10	21
Choline bitartrate	2.5	3
TBHQ antioxidant[&]	0.014	**0**
Corn starch	397	0
Lard	**0**	**310**
Calcium phosphate dibasic	0	3.4
Blue food colour	0	0.1

[&] TBHQ, *tert*-butylhydroquinone.

The sum of these observations led us to adopt protocol 1 for the project at hand, while rats from protocol 2 were redirected toward another study. Nicotinamide-streptozotocin[55] injected rats, hereafter designated hyperglycaemic, and vehicle-injected control rats fed a normal diet were employed to produce the results presented in Chapter III. Rats were maintained for up to 6 months without the need for insulin interventions. In the brains of our model, we studied the various regions of the nigrostriatal and mesocorticolimbic pathways, explicitly the substantia nigra pars compacta, ventral tegmental area, dorsal striatum, nucleus accumbens and prefrontal cortex, but also the hippocampus for comparative means. In addition, experiments were conducted at two time points, either at 3 or 6 months following induction of

[55] Noteworthy, peripherally administered streptozotocin does not enter the brain (Bhuyan *et al.*, 1974). Other models do employ intracerebroventricular injections of streptozotocin to cause insulin resistance in the CNS, a model of Alzheimer's disease (Corrêia *et al.*, 2011).

hyperglycaemia, in order to gain a clearer insight regarding the time necessary for neurodegeneration to manifest itself in our model. Moreover, many experiments were repeated in different cohorts and in different institutions (Université du Québec à Trois-Rivières, and University of Cagliari, Italy), thereby solidifying our findings. Chapter III offers further details pertaining to the experimental design and the various metabolic parameters monitored along the course of this project, including haemoglobin glycation measurements and a terminal oral glucose tolerance test commonly used in humans and in animal models to assess the severity of hyperglycaemia and glucose intolerance, respectively.

1.5.3.2 Intracerebral glucose measurements

The central hypothesis of this thesis requires firsthand observations of a rise in glucose concentrations in the brain regions of interest, namely the substantia nigra pars compacta and dorsal striatum of the nigrostriatal pathway, and the ventral tegmental area, nucleus accumbens and prefrontal cortex of the mesocorticolimbic pathway. As previously stated, precise quantifications of intraneuronal glucose are lacking in the literature. Extracellular levels of glucose have, however, been appraised in rodents, but reports usually cover one single brain region at a time during specific tasks[56]. More pertinent to our project, several studies demonstrated that hyperglycaemic or hypoglycaemic challenges in otherwise healthy rodents provoke same-direction changes in extracellular glucose levels, as previously discussed (Abi-Saab *et al.*, 2002; Béland-Millar *et al.*, 2017; Macauley *et al.*, 2015; Osborne *et al.*, 1997). In diabetic paradigms, rises in extracellular glucose concentrations were only measured in the inferior colliculus of the brainstem (Jacob *et al.*, 2002; McCrimmons *et al.*, 2003), the striatum (Gomez and Barros, 2003) and the ventromedial hypothalamus (de Vries *et al.*, 2003). Several reports in humans account for cerebral glucose levels but were mostly conducted in pathological states, for instance in partial seizures (Abi-Saab *et al.*, 2002), following

[56] Many experiments have been conducted during various tasks or in response to several stimuli, such as anaesthetics, tail pinching or hypo/hyperoxia. The brain regions targeted during these studies are the striatum (Osborne *et al.*, 1997; Fellows and Boutelle, 1993; Lowry *et al.*, 1998*a*, 1998*b*, 1998*c*), hippocampus (Macauley *et al.*, 2015; McNay *et al.*, 2000, 2001*a*, 2001*b*, 2004; Rex *et al.*, 2009; Su *et al.*, 2015) and motor cortex (Béland-Millar *et al.*, 2017).

a cardiac arrest (Hifumi *et al.*, 2017) and especially in traumatic brain injuries (Jalloh *et al.*, 2013; Rostami, 2014).

Despite these reports indicating a rise in extracellular glucose levels in hyperglycaemic or diabetic states, no adequate account exists to support the supposition that concentrations increase evenly throughout several neuroanatomical locations. We therefore performed glucose measurements in the multiple regions of interest in our hyperglycaemic model by two different methods allowing us to estimate both intracellular and extracellular concentrations. For intracellular glucose assessments, brain homogenates were prepared from the striatal, midbrain, prefrontal cortex and hippocampal regions, the latter serving for comparative means. We used a standard glucose measurement kit that employs a mutarotase enzyme to transform α-D-glucose to β-D-glucose, further oxidized by glucose oxidase. This generates hydrogen peroxide that can be colorimetrically quantified upon its reaction with a chromogenic agent (Crystal Chem, Downers Grove, IL, USA). Since rats were perfused upon sacrifice with ice-cold phosphate-buffered saline, it is possible that the results obtained may provide an underestimation of intracellular glucose concentrations.

For extracellular assessments, the intracerebral microdialysis technique in freely moving and awake rats was employed, later described in greater detail. Seeing as separation of the different subregions of the striatum and midbrain was not possible in the previous method, intracerebral microdialysis advantageously allowed us to specifically target the ventral tegmental area, substantia nigra pars compacta, nucleus accumbens and dorsal striatum by stereotactic vertical insertion of dialysis probes that collect interstitial fluid solutes in real time. Given the various glycaemic and feeding profiles of our rats, especially between control and hyperglycaemic individuals, our protocol required to standardize these parameters. We therefore fasted all rats overnight and administered a specific amount of food diluted in water by intragastric gavage precisely 30 min before the beginning of microdialysis experiments. To obtain a reliable baseline measurement, samples were collected over a 1-hour lapse of time and glucose was quantified using the same kit employed for brain homogenates.

Combining these methods ensured a robust confirmation of the increase in intracerebral glucose levels in our model. It also allowed us to verify that glucose concentrations rise evenly across the brain regions of interest.

1.5.3.3 Assessment of neurodegeneration

Objective 3 aimed to characterize the effects of long-term hyperglycaemia on the nigrostriatal and mesocorticolimbic pathways with a keen focus on neurodegeneration. We combined immunoblotting and immunohistochemical techniques to strengthen any findings of neurodegeneration upon which hinges this part of the project. TH and DAT were employed as dopaminergic markers expressed in the whole length of dopaminergic neurons of either the nigrostriatal or mesocorticolimbic pathways. NeuN served as a general neuronal marker, as it is expressed in all neuron cell bodies, but not in processes, regardless of their type. Harvesting one brain hemisphere for immunoblotting and the other for immunohistochemistry maximized the use of animals.

Immunoblotting assays allowed for a semi-quantitative measurement of the expression levels of dopaminergic and neuronal markers in the midbrain, striatum, prefrontal cortex and hippocampus of our model, 3 and 6 months following induction of hyperglycaemia. Altered TH and DAT expression levels in our model provided hints of dopaminergic modifications, perhaps indicative of neurodegeneration, whereas NeuN measurements served to support these results. We exercised caution when analysing NeuN expression in the striatum, since dopaminergic neuronal cell bodies are not found in this region but rather in the midbrain.

The immunohistochemical method served to confirm whether changes in expression were attributable to a proper loss of dopaminergic neurons. It also permitted the precise neuroanatomical identification of these changes. Since expression levels of TH, DAT and NeuN were altered only at 6 months in the striatum and midbrain of our hyperglycaemic model, we performed immunohistochemical analyses at this time point. Post-fixed frozen hemispheres were sliced rostrocaudally in coronal sections and

neuronal markers were revealed immunohistochemically. In the substantia nigra pars compacta and ventral tegmental area, TH-positive dopaminergic neurons or NeuN-positive overall neurons were counted. In the various regions of the striatum, the density of TH-positive dopaminergic fibres originating from the midbrain was measured by optical densitometry. As such, we were able to verify proper neurodegeneration of either neuronal cell bodies or terminal arbours in both pathways. NeuN-positive neurons were also counted in the striatum, prefrontal cortex and hippocampus to make sure that other non-dopaminergic populations remained unchanged. TH-positive dopaminergic fibre staining was not seen in the prefrontal cortex, as it is only lightly innervated by the ventral tegmental area. Likewise, the hippocampus, which receives minor dopaminergic input from the ventral tegmental area[57] (Lisman and Grace 2005), did not display any staining.

1.5.3.4 Assessment of glial profiles

In an attempt to gain better insight into the changes that take place in the CNS of our hyperglycaemic rat model, the fate of glial populations was studied alongside that of neurons. Astrocyte proliferation, or astrogliosis, is a well-appreciated feature of the diabetic nervous system, both in humans (Araszkiewicz and Zozulinska-Ziolkiewicz, 2016; DeJong, 1977; Lu *et al.*, 2014) and in models (Alomar *et al.*, 2016; Baydas *et al.*, 2003; Duarte *et al.*, 2009; Nagayach *et al.*, 2014; Rostami *et al.*, 2017; Saravia *et al.*, 2002). However, studies have not addressed astrogliosis in multiple regions at a time nor have they tackled this feature in long-term hyperglycaemia. Likewise, microgliosis is an appreciated feature of retinal and peripheral nerve pathologies in models of diabetes (Gaucher *et al.*, 2007; Krady *et al.*, 2005; Mazzeo *et al.*, 2017; Zeng *et al.*, 2000) as well as in patients (Altmann and Schmidt, 2018; Zeng *et al.*, 2008), but many gaps remain pertaining to the CNS (Oliveira *et al.*, 2016; Nagayach *et al.*, 2014). Conversely, the loss of microglial cells or astrocytes may also be indicative of the general distress expressed

[57] The hippocampus and ventral tegmental area form a functional loop. When the hippocampus detects new information that is not stored in long-term memory, it sends a novelty signal through the nucleus accumbens and ventral pallidum to the ventral tegmental area. Then, the ventral tegmental area releases dopamine in the hippocampus, thereby enhancing long-term potentiation and learning. (Lisman and Grace, 2005).

by resident cells of the CNS. Although under-acknowledged, degeneration of glial cells may in fact occur in the context of sustained oxidative stress (Streit *et al.*, 2008).

The importance of addressing glial populations in our models lies in their purported role with regard to neurodegeneration, especially in the context of Parkinson's disease (McGeer *et al.*, 1988; More *et al.*, 2013; Russo *et al.*, 2014). Indeed, glial alterations may imply a neuroinflammatory state likely connected to the death of dopaminergic neurons (Cabezas *et al.*, 2014; Hirsch *et al.*, 2003), as microglial cells were shown to be more abundant in the substantia nigra pars compacta of post-mortem brains of parkinsonian patients (Kim *et al.*, 2000; Lawson *et al.*, 1990).

The knowledge gaps highlighted above alongside the relevance of glial populations in neurodegeneration stress the pertinence of addressing the fate of glial populations as an indicator of the severity of the hyperglycaemic insult in our model. To that end, microglial cells and astrocytes were immunohistochemically detected using antibodies raised against ionized calcium-binding adapter molecule 1 (Iba1) or glial fibrillary acidic protein (GFAP), respectively. Cells were counted in all of the aforementioned brain regions using the method previously described.

1.5.3.5 Intracerebral dopamine measurements

Ideally, observed losses of neurons or their terminals should be supported by further neurochemical investigations. To that end, we measured extracellular dopamine in the terminal regions of the nigrostriatal and mesocorticolimbic pathways, that is, the dorsal striatum, the nucleus accumbens and the prefrontal cortex. The microdialysis technique, also used to measure extracellular brain glucose, allows for the accurate assessment of these regions. However, it entails complicated surgical procedures that could not be performed in our model at the 6-month time point. Instead, we conducted experiments at 3 months to evaluate the possibility that a functional loss of dopaminergic neurotransmission could emerge before the neurodegeneration observed at 6 months. Information provided at this time point was nonetheless relevant to our

interpretation of several behavioural manifestations observed at 3 months in our model, presented next in objective 4.

Ever since its design was optimized (Ungerstedt and Pycock, 1974; Ungerstedt *et al.*, 1982), intracerebral microdialysis has been extensively used to measure small solutes in the interstitial fluid of the brain (see for review Chefer *et al.*, 2009) (Figure 1.34). This technique employs a minute probe composed of a metal cannula that contains in its centre a tubular dialysis membrane permitting free transport of solutes smaller than the molecular weight cut-off, typically of 20 000 Da. The probe is equipped with an inlet, to transport the perfusate, and an outlet, to collect the microdialysate. Referring to an atlas of neuroanatomical coordinates, the probe is vertically inserted in the brain region of choice with the assistance of a stereotactic apparatus. A defined portion of the semipermeable dialysis membrane at the very tip of the probe is directly exposed to the parenchyma; only this surface participates in molecular exchanges and can be modified to accommodate the dorsoventral thickness of the region of interest. Perfusion begins as soon as the probe is inserted and the automatic syringe dispenser is activated. Typical perfusion rates range between 0.3-3 µL/min and have a direct incidence on the recovery rate, that is, the fraction of solute collected by the probe found to be inversely proportional to the speed of its production, diffusion and replacement in the interstitial fluid (Wages *et al.*, 1986; Zetterstrom *et al.*, 1988). Also depending on the perfusion rate as well as on the sensitivity of the analytical apparatus, sample collection times range from 1-20 min. The perfusate classically consists of a solution that most faithfully represents the extracellular environment in which the probe is inserted, except that it is completely depleted in the solute meant to be sampled. Indeed, it is usually accepted that uptake occurs by diffusion along a concentration gradient[58]. In our experiments, we prepared the probes according to the widely employed protocol established in Prof. Gaetano Di Chiara's laboratory (Bassareo *et al.*, 2011; Di Chiara *et al.*, 1993; Tanda *et al.*, 1996). This protocol presents the advantage of employing a dialysis membrane mounted on a tungsten fibre and covered in an impermeable silicone

[58] This also means that retrodialysis is possible if a certain solute is included in the perfusate, but found in lower concentrations in the brain parenchyma (Khan-Dawood *et al.*, 1994; Wei *et al.*, 1997). Such strategies allow for the delivery of drugs to very precise neuroanatomical loci.

sheath. As they are rigid and do not require a cannula to guide them, the brain parenchyma is not in direct contact with metal.

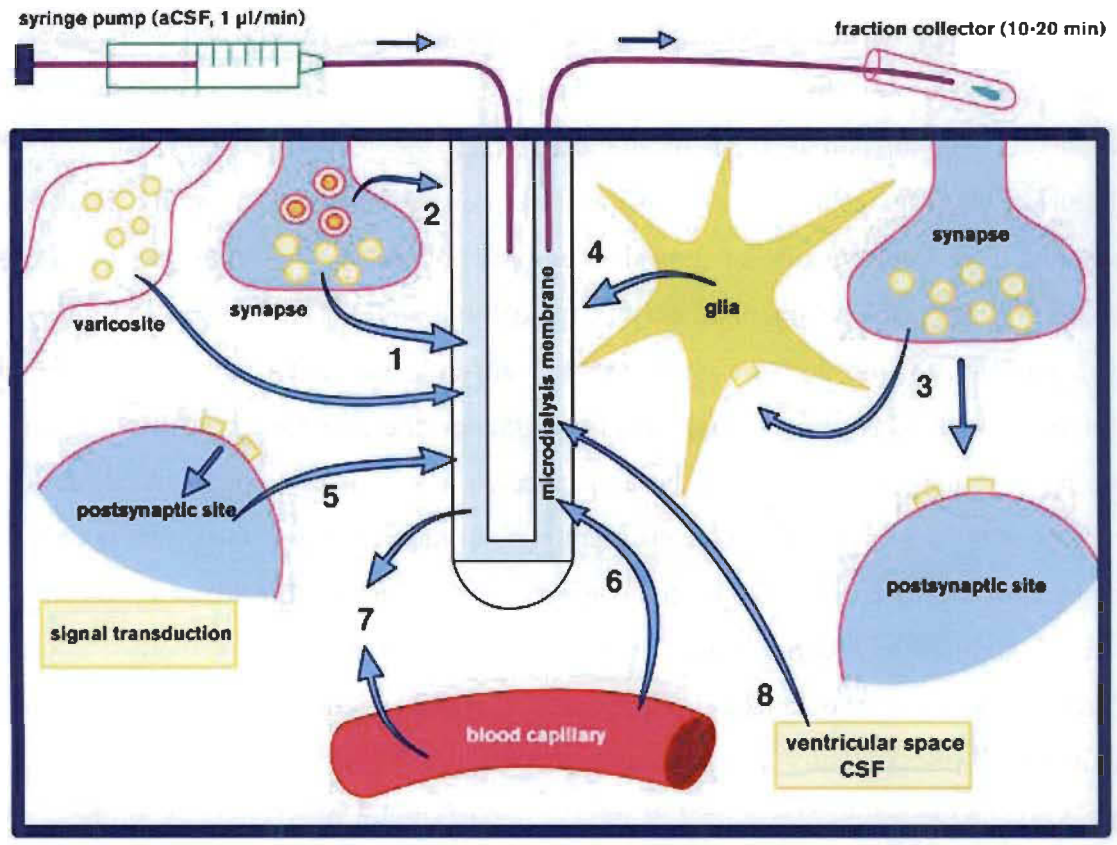

Figure 1.34 Schematic representation of solute exchanges during microdialysis experiments.
Not only do microdialysis probes uptake neurotransmitters, they can also sample most any molecule present in the environment that is smaller than the molecular weight cut-off. These can come from glial cells and blood capillaries, in addition to neurons. Likewise, solutes can be infused in discrete brain regions employing a retrodialysis paradigm, wherein a greater concentration of the substance is supplied in the perfusate (Höcht et al., 2007). aCSF, artificial cerebrospinal fluid; CSF cerebrospinal fluid. (From Bossers *et al.*, 2013.)

Our protocol did not involve behavioural assessments or pharmacological interventions sensitive to the state of the animal that, in these circumstances, must recover for several days. As such, baseline dopamine was measured on the day following acute cannulations in well-rested, awake and healthy rats. An intermediate perfusate rate of 1 µL/min was employed and 9 samples were collected over 20-min

periods for a total of 3 h. Microdialysates were collected and analyzed by high-performance liquid chromatography. This technique separates the various molecules present in a liquid according to their affinity for a stationary (solid column) phase that is usually polar, for instance silica. However, microdialysates usually contain rather hydrophilic solutes whose retention times on the column are too long, hence the use of a reverse-phase high-performance liquid chromatography wherein the stationary phase is non-polar to allow the movement of these molecules (Imperato and Di Chiara, 1984). Solutes are electrochemically detected and their identity is confirmed by appraisal of their signature elution time.

Although microdialysis offers great neuroanatomical precision, it does not offer very good temporal resolution. The sensitivity of the analytical apparatus usually requires samples to be at least a few microlitres, which, at best, implies collection times of 1 min. Consequently, phasic bursts are difficult to quantify by microdialysis as they occur below the 1-second threshold and require voltammetric or electrophysiological methods that are sensitive to infrasecond fluctuations caused by behaviour or stimuli (Hauber, 2010; Schultz, 2010; Segovia *et al.*, 2011; Wightman and Robinson, 2002). Tonic release, however, is feasibly quantified by intracerebral microdialysis experiments that measure events dependent on time constants greater than 1 min (Di Chiara, 1990). Extracellular concentrations of dopamine measured in our experiments therefore represent dopaminergic tonic firing[59] at baseline and hold important implications for behavioural interpretations that we will address in the discussion.

To fulfil objective 3, the joint evaluation of neurodegeneration, glial disturbances and dopamine release in the various brain regions of the nigrostriatal and mesocorticolimbic pathways offered a broad appreciation of the dopaminergic neuronal alterations present in our hyperglycaemic model. Supplementary information, especially pertaining to probe insertion locations in microdialysis experiments, can be found in the article presented in Chapter III.

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[59] We must bear in mind that a loss of tonic firing in the dorsal striatum is most conspicuously observed in Parkinson's disease and leads to bradykinesia and rigidity.

1.5.4 Objective 4: Assess the behavioural alterations resulting from nigrostriatal neurodegeneration in a rat model of long-term hyperglycaemia

Video samples of the behavioural experiments described below can be viewed at the following address: goo.gl/9HF6ux. Copy and paste this shortened URL in your browser to access the Dropbox file.

1.5.4.1 Assessment of motor deficits

In consonance with the three previous objectives, we wanted to achieve a broad perception of the effects of long-term exposure to high concentrations of glucose on dopaminergic neurons. Working with a rodent model allowed us to appreciate behavioural consequences arising from dopaminergic neurodegeneration. Indeed, several well-established tests exist to evaluate nigrostriatal or mesocorticolimbic functions.

As previously mentioned, the nigrostriatal pathway is prominently involved in the production of movement owing to its important pacemaker activity that provides the striatum, especially the dorsal region, with tonic dopamine (Lanciego *et al.*, 2012). Rat models of Parkinson's disease are commonly subjected to specifically designed tests to uncover motor symptoms that may arise from the degeneration of the nigrostriatal pathway (see for reviews Dunnett and Lelos, 2010; Meredith and Kang, 2006; Pinna and Morelli, 2014; Plowman and Kleim, 2011). They are also used to develop treatments to alleviate motor symptoms that are thought to arise from loss of dopamine tonicity (Bergstrom and Garris, 2003; Bergstrom *et al.*, 2011).

Among the great many experimental tools developed to assess motor deficits in rodents, only a few were applicable to our model. Indeed, many tests are designed for asymmetrically lesioned animals induced, for instance, by a unilateral injection of 6-OHDA into the ascending nigrostriatal bundle of rats (Schwarting and Huston, 1996; Ungerstedt and Arbuthnott, 1970; Vellucci *et al.*, 1993). These tests are usually based on the evaluation of turning behaviour in response to stimuli that is due to the enhanced sensitivity of postsynaptic receptors following striatal denervation rather than the proper

loss of dopamine tonicity (Creese *et al.*, 1977). Unilateral utilization of limbs in tasks is another behaviour that can be scored in hemiparkinsonian rodents. However, the CNS of our model is evidently subjected to a wide bilateral exposure to high glucose concentrations and therefore asymmetric neurodegeneration is unlikely. In light of this, we chose tests typically employed in bilateral models of Parkinson's disease or ones that could be adapted to our needs. We did not expect our rats to express overt motor symptoms and, as such, we performed the most sensitive tests possible to uncover bradykinesia or akinesia, as well as gait disturbances and sensorimotor deficits (Pinna and Morelli, 2014). We also selected tests that could be performed at multiple time points, expressly before the induction of hyperglycaemia, then at 3 and 6 months following that mark so as to link results with our neurobiological evaluations.

The stepping test, a form of beam traversal task, is used in rats to assess three different parameters: time of initiation, time to cross and numbers of steps made (Meredith and Kang, 2006; Olsson *et al.*, 1995; Pinna *et al.*, 2007, 2010). The test employs a 20 cm-wide beam inclined at 15 degrees leading to the home cage. Briefly, rats are trained to depart from the hands of the experimenter with one forelimb placed at the beginning of the beam. Protracted initiation of movement toward the home cage and time to cross the beam both uncover bradykinesia or akinesia. The numbers of steps made, usually greater in parkinsonian rodents, inform on the gait. The stepping test therefore reliably picks up subtle basal ganglia alterations and has been used in genetic models and aged animals that present little nigrostriatal damage (Drucker-Colin and Garcia-Hernandez, 1991). In our model based on a bilateral hyperglycaemic insult to the CNS, differences between starting forelimbs were not found and results were pooled.

We also employed the horizontal bar test typically used to measure pharmacologically-induced catalepsy in rodents (Alvarez-Cervera *et al.*, 2005; Ciucci and Connor, 2009; Kuschinsky and Hornykiewicz, 1972; Sanberg *et al.*, 1981, 1988). In our model, however, this test rather served to identify bradykinetic or akinetic individuals. In this task, the experimenter places untrained rats habituated to the test cage in an unusual posture by placing both its front limbs on a cylindrical bar placed at a

convenient height. Normal rodents naturally seek to correct imposed postures and will quickly remove both limbs. Latency to disengage from the bar is thought to expose basal ganglia dysfunction (Duvoisin, 1976; Sanberg, 1980), although it is less sensitive than the previous method, at least in certain genetic models (Kelm-Nelson *et al.*, 2015). This test is however criticized for its sensitivity to recurring trials (Costall and Olley, 1971; Stanley and Click, 1976) likely ensuing from repeated handling that may cause tonic immobility (Sanberg *et al.*, 1980). Since we performed tests at wide intervals, at 0, 3 and 6 months, these confounding events were unlikely. Nevertheless, overt manifestations of proper catalepsy in otherwise active control or hyperglycaemic rats were extremely rare and were omitted from data, as they could have been the result of tonic immobility.

We conducted a third test to evaluate limb gait and bradykinesia, termed the forelimb adjusting step test (Chang *et al.*, 1999; Meredith and Kang, 2006; Pinna *et al.*, 2007, 2010). Briefly, trained rats are held by the torso with only one forelimb placed on a surface and bearing weight. During trials, rats are laterally moved back and forth across the surface at a fixed speed, and forehand (forelimb adjusts toward the torso) or backhand (forelimb adjusts away from the torso) steps are counted (Chang *et al.*, 2003; Kirik *et al.*, 2000, 2002; Olsson *et al.*, 1995; Schallert *et al.*, 2003; Tillerson and Miller, 2002). Akin to the stepping test, the forelimb adjusting step test is performed unilaterally but results were combined upon confirmation that both limbs responded similarly in all animals.

Many other tests were considered for their application in our model but either failed to expose any deficits or were impractical. This was the case for the vibrissae-elicited forelimb placement test wherein a rat's whiskers are brushed upon a surface, which normally evokes a paw placement response (Meredith and Kang, 2006; Pinna *et al.*, 2007, 2010). Sensorimotor integrative deficits are identified when rats fail to respond to brushing of their vibrissae. Our hyperglycaemic model did not perform differently compared to control rats at any time point. We also attempted to perform an adapted pole test for rats, which evaluates coordination and motor skills (Chompoopong

et al., 2016; Zaitone *et al.*, 2012), as well as an adhesive removal task, highly sensitive for uncovering sensorimotor deficits and fine motor impairment in minimally dopamine-deficient animals (Schallert *et al.*, 1982), but rats failed to perform the tasks correctly for various reasons.

Nonetheless, results obtained with the three abovementioned tests were sufficient to draw up a picture of the key motor deficits displayed by our long-term hyperglycaemic model, especially with respect to bradykinesia/akinesia and gait disturbances. Detailed experimental designs followed to fulfil this part of the objective are provided in Chapter III.

1.5.4.2 Evaluation of social behaviour

Although best appreciated in the regulation of motor processes, nigrostriatal dopamine also partakes in the modulation of many other behaviours. Owing to its role in goal-directed conducts and habit learning (Bromberg-Martin *et al.*, 2011; Faure *et al.*, 2005; Gunaydin *et al.*, 2014; Haber *et al.*, 2000; Redgrave *et al.*, 2010; Saddoris *et al.*, 2013; Schultz, 2002; Seger and Spiering, 2011; Wang *et al.*, 2011), nigrostriatal dopamine has recently emerged as a regulator of social interactions and affective-based decisions, which were hitherto exclusively lent to the mesocorticolimbic pathway (Burke *et al.*, 2010; King-Casas *et al.*, 2005; Lamichhane *et al.*, 2014; Narvaes and Martins de Almeida, 2014; Ong *et al.*, 2011; Palmeri *et al.*, 2017; Plavén-Sigray *et al.*, 2014; Stoeckel *et al.*, 2014; Trezza *et al.*, 2010). Although a proper correlation with nigrostriatal degeneration has not been drawn, Parkinson's disease patients do experience an altered quality of social life very early in the pathology. In particular, social interactions, communication and emotion recognition are impaired to variable extents in these individuals (Pell *et al.*, 2006; Schrag *et al.*, 2000; Yoshimura *et al.*, 2005).

For this reason, we designed experiments wherein social behaviours were scored concomitantly to the measurement of an outwardly accessible indicator of dopaminergic

functions modulated in social encounters, namely USV calls (Figure 1.35). In social behaviour research, USVs constitute a growingly valuable instrument in the assessment of the valence of encounters, as they convey information on the affective state of rodents during courtship, rough-and-tumble play, cooperation and aggression (Brudzynski, 2013; Burgdorf *et al.*, 2008; Knutson *et al.*, 1998; Łopuch and Popik, 2011; Wöhr and Schwarting, 2013). Most importantly, however, they provide information on the activation of midbrain dopaminergic pathways involved in their production. The two principal neurotransmitters involved in the emission of ultrasonic calls are dopamine and acetylcholine, which function together as a switch that can quickly activate positive or negative affective states, respectively (Brudzynski, 2007; Cragg, 2006; Rakovska *et al.*, 2003). Dopamine and acetylcholine are sensitively released in response to salient and novel environmental stimuli, including social encounters, and cooperate at the striatal interface to select the appropriate affective state. This is accompanied by the emission of USVs at different ranges of frequency[60]. Dopamine is principally associated with the emission of 50-kHz calls, which are positively valenced and produced during social interactions, such as tickling (Panksepp and Burgdorf, 2000), playing (Brunelli *et al.*, 2006) or mating (Bialy *et al.*, 2000). Acetylcholine is rather linked to 22-kHz USVs reflecting negative affective states in various contexts, for instance social isolation (Francis, 1977) and aggression (Kaltwasser, 1990). Some suggest that 22-kHz calls are also emitted in paradigms of chronic pain (Calvino *et al.*, 1996; Jourdan *et al.*, 1995), but this association was disproven in the streptozotocin-treated rat model of neuropathic pain (Jourdan *et al.*, 2002).

Affiliative/exploratory and aggression-related behaviours were scored in our rats and associated with the emissions of 50- and 22-kHz USVs. Rats interacted in a novel and neutral arena, and not in resident-intruder paradigms (Hilakivi-Clarke *et al.*, 1990; Meehan *et al.*, 1986), to obtain insight into the reactions driven by socialization, which more faithfully represent encounters between humans in day-to-day life. Behaviours and USVs were related to the degree of striatal dopaminergic denervation and to insulin levels and function. This allowed us to draw up the socioaffective profiles of our rat

[60] Examples of USV audiograms are presented in Figure 4.7 of Chapter IV.

model of hyperglycaemia and to identify neurobiological or hormonal influences on social behaviours and associated USV calls. These results also constitute the first report of USV emissions in a rodent paradigm of hyperglycaemia and are found in Chapter IV alongside precise details on the experimental design.

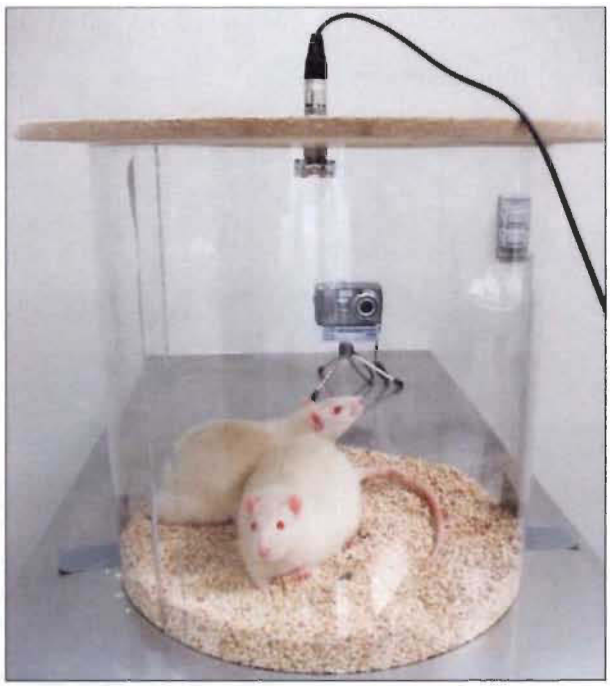

Figure 1.35 Experimental setup of social behaviour assessments.
Pairs of unacquainted rats were placed in a neutral and novel environment for the duration of the tests. A lid with a microphone was placed on top of the cylindrical arena and two cameras were positioned on opposite sides to record ultrasonic vocalizations (USVs) emitted during the encounters.

To briefly summarize the introduction of this thesis, we have laid the foundations for the purpose of our investigations, by discussing the selective vulnerability of the nigrostriatal pathway in Parkinson's disease and by offering robust arguments in support of the hazard that hyperglycaemia may represent to this population of neurons. Precisely, we have committed to demonstrating how nigrostriatal dopaminergic neurons are more vulnerable to oxidative stress and how hyperglycaemia may contribute to oxidative injury in neurons. Both these suppositions are supported by epidemiological evidence. Based on current knowledge, we designed a series of *in vitro* and *in vivo* experiments exploring multiple dimensions of dopaminergic neurodegeneration and

have also used a strategy employing an antioxidant, resveratrol, to offer further validation of the pertinence of oxidative stress in high glucose-induced neuronal death. The results of these experiments are presented in Chapters II, III and IV, and offer molecular, cellular, neurochemical, neuroanatomical and behavioural evidence in support of our central hypothesis that nigrostriatal dopaminergic neurons are more vulnerable to hyperglycaemic conditions compared to other neuronal populations, expressly the mesocorticolimbic pathway.

CHAPTER II

RESVERATROL PROTECTS DOPAMINERGIC PC12 CELLS FROM HIGH GLUCOSE-INDUCED OXIDATIVE STRESS AND APOPTOSIS: EFFECT ON P53 AND GLUCOSE-REGULATED PROTEIN 75 LOCALIZATION

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2.1 Author contributions

Justine Renaud contributed to the design of the study and performed 80% of the manipulations. She wrote 80% of the manuscript, and prepared and edited all the figures. Julie Bournival, a master's student in Maria-Grazia Martinoli's laboratory at the time, contributed to the design of the study and optimized experiments. Ximena Zottig, an undergraduate student in Maria-Grazia Martinoli's laboratory at the time, provided assistance for the immunoblotting experiments. Maria-Grazia Martinoli, Justine Renaud's research supervisor at the Université du Québec à Trois-Rivières, was the guarantor of the work and provided supervision, preparation and editing of the manuscript.

2.2 Résumé

Le resvératrol est un composé polyphénolique dont les propriétés cardioprotectrices et anti-inflammatoires sont déjà bien connues. Il détient également des capacités antioxydantes permettant de protéger des neurones dopaminergiques contre l'apoptose engendrée par le stress oxydant dans des paradigmes de la maladie de Parkinson. De récentes études ont démontré qu'une hyperglycémie soutenue peut causer un stress oxydant pouvant être nocif pour le système nerveux central. À la lumière de ce qui précède, notre étude avait pour but a) d'évaluer le potentiel antioxydant du resvératrol contre des concentrations physiologiquement élevées de glucose dans des neurones dopaminergiques en culture, b) d'étudier les capacités anti-apoptotiques du resvératrol dans ce contexte, et c) d'analyser la relation particulière entre le facteur de transcription pro-apoptotique p53 et son séquestreur cytoplasmique GRP75 dont les niveaux sont faibles dans le cerveau de patients parkinsoniens. Nos résultats démontrent que le resvératrol protège les neurones dopaminergiques en culture contre un stress oxydant induit par de fortes concentrations de glucose, précisément en diminuant les niveaux d'anion superoxyde. De plus, les neurones dopaminergiques en culture traités avec le resvératrol ne succombaient pas à l'apoptose qu'engendrait le glucose à des doses élevées. En particulier, le resvératrol prévenait la fragmentation de l'ADN et l'altération des profils d'expression de plusieurs marqueurs de l'apoptose. De fortes concentrations de glucose causaient une translocation de p53 vers le noyau, et une forte augmentation des niveaux de GRP75 dans le cytoplasme qui répondait à un besoin d'y séquestrer le facteur de transcription pro-apoptotique. En effet, une augmentation du taux de colocalisation entre GRP75 et p53 était observée. À cet égard, le resvératrol était en mesure de prévenir la translocation nucléaire de p53. Ces résultats appuient les études épidémiologiques soulignant que les patients diabétiques courent un plus grand risque de développer la maladie de Parkinson que la population générale. Notre étude suggère également que le resvératrol détient un rôle neuroprotecteur pouvant se prêter au développement de thérapies préventives ou complémentaires contre le développement de complications neuronales dans le diabète.

2.3 Full article in English: Resveratrol protects dopaminergic PC12 cells from high glucose-induced oxidative stress and apoptosis: effect on p53 and glucose-regulated protein 75 localization

Abstract

Resveratrol (RESV), a polyphenolic natural compound, has long been acknowledged to have cardioprotective and anti-inflammatory actions. Evidence suggests that RESV has antioxidant properties that reduce the formation of reactive oxygen species leading to oxidative stress and apoptotic death of dopaminergic (DAergic) neurons in Parkinson's disease (PD). Recent literature has recognized hyperglycemia as a cause of oxidative stress reported to be harmful for the nervous system. In this context, our study aimed a) to evaluate the effect of RESV against high glucose (HG)-induced oxidative stress in DAergic neurons, b) to study the anti-apoptotic properties of RESV in HG condition, and c) to analyze RESV's ability to modulate p53 and GRP75, a p53 inactivator found to be under-expressed in post-mortem PD brains. Our results suggest that RESV protects DAergic neurons against HG-induced oxidative stress by diminishing cellular levels of superoxide anion. Moreover, RESV significantly reduces HG-induced apoptosis in DAergic cells by modulating DNA fragmentation and the expression of several genes implicated in the apoptotic cascade, such as Bax, Bcl-2, cleaved caspase-3, and cleaved PARP-1. RESV also prevents the pro-apoptotic increase of p53 in the nucleus induced by HG. Such data strengthens the correlation between hyperglycemia and neurodegeneration while providing new insight into the high occurrence of PD in patients with diabetes. This study enlightens potent neuroprotective roles for RESV that should be considered as a nutritional recommendation for preventive and/or complementary therapies in controlling neurodegenerative complications in diabetes.

Introduction

Glucose is the essential energy substrate of the central nervous system and large amounts of it are required to fill the high energetic needs of neurons. Unlike muscle cells

or adipocytes that depend on insulin, glucose uptake in neurons depends mainly on its extracellular concentration (Tomlinson and Gardiner, 2008). Persistent episodes of long-term glucose exposure may induce oxidative stress that results in cellular damage (Giaccari *et al.*, 2009), such as neuropathic complications resulting from hyperglycemia in uncontrolled diabetes (Rajabally, 2011). Accumulating evidence has enlightened the relationship between diabetes and neurodegenerative disorders, including Alzheimer's disease (AD) (Vignini *et al.*, 2013) and Parkinson's disease (PD) (Jagota *et al.*, 2012). Recent literature has reported an increased risk of developing PD in patients with type 2 diabetes mellitus (Hu *et al.*, 2007; Sun *et al.*, 2012).

PD is a neurodegenerative disorder characterized by the progressive loss of nigrostriatal dopaminergic (DAergic) neurons in the *substantia nigra pars compacta* (SNpc). DAergic neurons in this region are selectively lost due to the high activity of monoamine oxidase and elevated levels of iron which both lead to increased generation of reactive oxygen species (ROS) (Cui *et al.*, 2012; Pearce *et al.*, 1997). At the cellular level, mechanisms of high glucose (HG)-induced toxicity are similarly sustained by oxidative stress *in vitro* (Bournival *et al.*, 2012; Cao *et al.*, 2012) as well as *in vivo* (Styskal *et al.*, 2012). By increasing aerobic respiration, raised sugar metabolism promotes excessive formation of ROS that, jointly with insufficient antioxidant defences, may damage cells (Apel and Hirt, 2004). Indeed, generation of mitochondrial superoxide is increased and is thought to be at the origin of several HG-induced complications (Brownlee, 2001). Currently, it is well known that oxidative stress may lead to apoptosis (Circu and Aw, 2010) and increased production of ROS in HG conditions may account for glucose neurotoxicity duly observed.

In addition, several genes are known to be implicated in the pathogenesis of PD, such as PINK1 and DJ-1. Glucose-regulated protein 75 (GRP75, also called mortalin/mtHSP70/mot-2), a member of the cytoprotective Hsp70 family of chaperones, interacts with both PINK1 (Jin *et al.*, 2006, 2007; Li *et al.*, 2005; Rakovic *et al.*, 2011) and DJ-1 (Jin *et al.*, 2005; Li *et al.*, 2005). GRP75 is mainly localized within the mitochondria matrix of neurons where it accomplishes several functions such as

mitochondrial import and oxidative stress management (Yaguchi *et al.*, 2007). Overexpression of GRP75 leads to the extension of life span in nematodes and human cells. On the other hand, it serves as a major target for oxidation and it was shown to be involved in aging of nerve cells and in particular in the degeneration of DAergic neurons (Burbulla *et al.*, 2010). In mitotic cells, GRP75 localized in the cytoplasm sequestrates and inactivates p53 preventing its nuclear translocation and apoptosis (Kaul *et al.*, 2001, 2005; Wadhwa *et al.*, 2002). Indeed, p53 is a tumor suppressor protein known to play an important role in evoking apoptosis when located in the nucleus by encouraging the transcription of several pro-apoptotic genes such as Bax (Macip *et al.*, 2003). p53 activity is stabilized in response to oxidative stress through posttranslational modifications disrupting interactions with negative regulators (Neilson *et al.*, 2008). It is also a recurrent target in PD given the involvement of oxidative stress in p53 activation (Nair, 2006) and the evidence of DNA fragmentation and chromatin condensation in DAergic neurons of the SNpc in PD patients (Hartmann and Hirsch, 2001; Tatton, 2000).

Prevention of neuronal loss in PD has not yet been addressed by existing symptomatic treatments. Neuroprotection by dietary polyphenols may be an interesting avenue in current attempts to overcome oxidative stress induced by hyperglycemia. We have recently shown that quercetin and sesamin, antioxidant polyphenols, exert neuroprotective effects in neurons exposed to HG (Bournival *et al.*, 2012). The stilbene resveratrol (RESV) is another polyphenol, primarily found in red wine, known for its potent cardioprotective, anti-inflammatory and anticarcinogenic actions (Aluyen *et al.*, 2012; Rosa *et al.*, 2012). Our group, as well as others, have highlighted its potential in defending neurons against oxidative assaults induced by a spectrum of treatments, including neurotoxins (Blanchet *et al.*, 2008; Bournival *et al.*, 2009; Gélinas and Martinoli, 2002; Peritore *et al.*, 2013) or cerebral ischemic injury (Morris *et al.*, 2011; Simão *et al.*, 2012). Abundant literature suggests that RESV plays a protective role in several neurodegenerative diseases including PD, AD and Huntington's disease (Albani *et al.*, 2010; Hung *et al.*, 2010) as well as against neuroinflammation (Foti Cuzzola *et al.*, 2011).

Although the beneficial properties of RESV in neurodegenerative diseases are extensively depicted in the literature, its role in defending neurons against HG-induced damage has yet to be elucidated. The present study was designed to examine the neuroprotective effects of the polyphenol RESV in differentiated DAergic PC12 cells maintained in HG condition. NGF-differentiated PC12 cells are a reliable model for the investigation of oxidative stress and neuroprotection of DAergic neurons. They express tyrosine hydroxylase (TH), high-affinity dopamine transporter, estrogen receptor-α and -β, neurofilaments and secrete high levels of dopamine (Gélinas and Martinoli, 2002; Kadota *et al.*, 1996; Nilsen *et al.*, 1998). In this comprehensive investigation, we outline the roles of RESV in preventing neural parameters of cellular oxidative stress and apoptosis induced by HG exposure in a cellular DAergic system. Our results demonstrate that RESV can modulate the expression and localization of GPR75 and thus might mediate mitochondria pathways of cell stress.

Materials and methods

Drugs and chemicals

All reagents and chemicals were purchased from Sigma (St. Louis, MO) unless noted otherwise. Mouse anti-GRP75 (raised against amino acids 525-679 of GRP75 of human origin), rabbit anti-p53 (raised against full length p53 of human origin, for immunofluorescence), rabbit anti-Bcl-2 (raised against a peptide mapping at the N-terminus of Bcl-2 of human origin), rabbit anti-Bax (raised against a peptide mapping near the N-terminus of Bax of mouse origin), mouse anti-cleaved PARP-1 (poly(ADP-ribose) polymerase, raised against C-terminal purified thymus PARP-1 of calf origin), goat anti-HDAC1 (histone deacetylase 1, raised against amino acids 450 to C-terminus of human HDAC1), and mouse anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase, raised against recombinant GAPDH of human origin) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-p53 (raised against amino acids surrounding Ser20 of human p53, for Western blotting) and rabbit anti-cleaved caspase-3 (raised against amino-terminal residues surrounding Asp175 in

human caspase-3) antibodies were purchased from Cell Signaling (Boston, MA). Rabbit anti-VDAC (voltage-dependent anion channel, raised against amino acids 152-169 of VDAC of human origin), mouse anti-TH (raised against rat TH) primary antibodies, and anti-mouse and -rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma. Anti-mouse Cy3 (cyanine 3)-conjugated secondary antibody was purchased from Medicorp (Montreal, QC, Canada). Goat anti-rabbit FITC (fluorescein isothiocyanate)-conjugated secondary antibody was purchased from Millipore (Temecula, CA).

Cell culture and treatments

PC12 cells, obtained from American Type Culture Collection (ATCC, Rockville, MD), were maintained in a humidified environment at 37°C and 5% CO_2 atmosphere. Cells were grown in Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated fetal bovine serum (FBS) and gentamicin (50 µg/mL). PC12 cell neuronal differentiation was evoked by administration of nerve growth factor-7S (NGF, 50 ng/mL) in Dulbecco's Modified Eagle medium (DMEM) supplemented with 1% FBS for 7 days, as already described (Bournival *et al.*, 2009, 2012). The DMEM containing 1.0 g/L of D-glucose (Sigma D5523) is further called control (CTRL) medium, whereas HG DMEM containing 4.5 g/L of D-glucose (Sigma D7777) is named HG medium. DAergic PC12 cells were incubated with CTRL or HG medium for 96 h, unless stated otherwise. We previously performed lactate dehydrogenase-based cytotoxicity assays to determine the appropriate time of treatment in order to study the apoptotic process in the remaining live cells (Bournival *et al.*, 2012). For the last 24 h of treatment, DAergic PC12 cells were incubated with or without RESV (0.1 µM). RESV concentration was selected after dose-response and kinetic studies (Bournival *et al.*, 2009; Bureau *et al.*, 2008). An osmotic control consisting of CTRL medium supplemented with 3.5 g/L of D-mannitol (MANN) was used to rule out a hypertonic effect of HG medium on PC12 cells. Charcoal-stripped serum was used in all experiments to ensure that media were free from steroids. For each experiment, initial seeding density was 30 000 cells/cm^2.

Detection of mitochondrial superoxide radical

DAergic PC12 cells were grown and treated on collagen-coated circular glass coverslips (Fisher Scientific, Ottawa, ON, Canada). Intracellular superoxide anion ($^{\bullet}O_2^-$) production was measured with MitoSOX™ Red (Invitrogen, Burlington, ON, Canada), a fluorogenic dye used for the selective detection of superoxide in the mitochondria of live cells. After treating cells with CTRL or HG medium for 3 h with or without RESV, the cells were incubated with MitoSOX™ Red (5 mM) for 10 min at 37°C. MitoSOX™ Red is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, it is oxidized by superoxide and exhibits red fluorescence. Cells were washed with Hank's balanced salt solution (HBSS, Invitrogen), and Hoechst 33342 counterstained all nuclei. Cells were fixed in 4% paraformaldehyde for 6 min at 37°C. Coverslips were mounted with Molecular Probes' ProLong® Antifade Kit (Invitrogen). Images were acquired by a Leica SD AF confocal microscope, and analyzed with Leica Application Suite 3.1.3 software (Leica Microsystems, Concord, ON, Canada). To demonstrate MitoSOX™ Red selectivity, a positive control was performed using sodium diethyldithiocarbamate (DDC), a superoxide dismutase (SOD) inhibitor, in CTRL medium.

Immunofluorescence and terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Apoptotic cells were also detected by both terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Laval, QC, Canada) and activated caspase-3 immunofluorescence. DAergic PC12 cells were grown and treated on collagen-coated circular glass coverslips. Cells were then fixed in 4% paraformaldehyde for 15 min at 37°C, washed with phosphate-buffered saline (PBS) and further incubated in a blocking and permeabilizing solution (1% bovine serum albumin [BSA], 0.18% fish skin gelatin, 0.1% Triton-X and 0.02% sodium azide) for 30 min at RT. Fixed cells were incubated with polyclonal anti-cleaved caspase-3 antibody 1:500 in PBS overnight. The slides were washed and treated with Cy3-conjugated secondary antibody diluted 1:500 in PBS for 4 h and then incubated with the TUNEL enzyme and

fluorescent dUTP mixture for 1 h at 37°C. Nuclei were counterstained 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted with ProLong® Antifade Kit. Images were acquired by a Leica SD AF confocal microscope. DAergic PC12 cells were considered to be apoptotic when they were positive for cleaved caspase-3 and their nuclei were stained with TUNEL. The number of apoptotic DAergic PC12 cells among 300 randomly chosen neuronal was counted on 10 different optical fields from three slides per group, as already reported (Bournival *et al.*, 2009, 2012), with Leica Application Suite 3.1.3 software. In each experiment 50 μM of fluormethylketone-conjugated tetrapeptide Z-DEVD-FMK (Bachem, Torrance, CA), a cell-permeable caspase-3 inhibitor, was used on DAergic PC12 cells in HG and HG RESV conditions as internal control for caspase-3 activation (Bournival *et al.*, 2009, 2012).

Specific apoptotic DNA denaturation analysis

Specific DNA denaturation in apoptotic cells was assessed with a single-stranded DNA (ssDNA) apoptosis ELISA kit (Chemicon International, Temecula, CA). This procedure is based on the selective denaturation of DNA by formamide in apoptotic cells but not in necrotic cells (Frankfurt and Krishan, 2001). After treatment with CTRL or HG medium with or without RESV, denatured DNA was detected with a monoclonal antibody highly specific to ssDNA and a peroxidase-labeled secondary antibody. The reaction was then stopped with a hydrochloric acid solution and ssDNA fragmentation was quantified by measuring absorbance at 405 nm with a Multiscan Ascent microplate reader (Thermolab System, Franklin, MA). ssDNA was quantified with reference to CTRL conditions. Absorbance of positive (wells coated with ssDNA) and negative controls (wells treated with S1 nuclease) served as quality control for the ELISA.

Protein extraction

DAergic PC12 cells were grown and treated in collagen-coated 6-well plates. Total proteins were extracted using a nuclear extraction kit (Active Motif, Carlsbad,

CA). Briefly, cells were washed with a mixture of ice-cold PBS and phosphatase inhibitors, and then harvested in centrifuge tubes. Cell lysis was performed using the supplied buffer and samples were centrifuged to obtain membrane-free supernatants containing total proteins.

Cytoplasmic-nuclear fractionation was achieved using the nuclear extraction kit. Briefly, cells were washed with a mixture of ice-cold PBS and phosphatase inhibitors, and then harvested in centrifuge tubes. Cytoplasmic membranes were ruptured by treatment with a hypotonic buffer and detergent. Samples were centrifuged to pellet the intact nuclei, and soluble material was preserved as the cytoplasmic fraction. Nuclei were then lysed and conserved in the provided lysis buffer.

Mitochondrial-cytoplasmic fractionation was achieved using a mitochondrial extraction kit (Active Motif, Carlsbad, CA). Cells were washed with a mixture of ice-cold PBS and phosphatase inhibitors, and then harvested in centrifuge tubes. Cells were incubated on ice with isotonic cytosol buffer for 15 min. Cell membranes were ruptured with a pestle homogenizer. Intact cells and nuclei were pelleted after two centrifugations and discarded. Supernatants containing cytoplasm and mitochondria were centrifuged twice to obtain a pellet of mitochondria. The resulting supernatant was preserved as the cytoplasmic fraction. Mitochondria were washed with cytosol buffer and lysed with detergent.

Electrophoresis and Western blotting analysis

Protein dosage was performed with a bicinchoninic acid-based sodium dodecyl sulfate (SDS)-compatible Protein Assay Kit (Pierce, Rockfort, IL) for each fraction of every sample. Equal amounts of protein were loaded onto 12% SDS polyacrylamide gels. After electrophoretic separation, the gels were transferred to polyvinylidene difluoride membranes (0.22 μm pore size, BioRad, Hercules, CA). The blots were blocked for 1 h at RT in Blotto B (1% non-fat powdered milk, 1% BSA, 0.05% Tween 20, 0.5 mg/mL sodium azide, in Tris buffered saline). Dilution of primary anti-GRP75,

anti-p53, anti-Bax, anti-Bcl-2, anti-cleaved PARP-1, anti-GAPDH, anti-HDAC1, anti-VDAC and anti-TH (1:200, 1:200, 1:50, 1:50, 1:1000, 1:50, 1:50, 1:500 and 1:10 000 respectively) antibodies was prepared in Blotto B. The blots were then incubated with peroxidase-conjugated secondary antibody (1:10 000) in Blotto B for 2 h at RT and finally developed with an enhanced chemiluminescence substrate solution (Haan and Behrmann, 2007).

Glucose-regulated protein 75-p53 colocalization

DAergic PC12 cells were grown and treated on collagen-coated circular glass coverslips. Then, they were fixed in 4% paraformaldehyde for 15 min at 37°C, washed with PBS and further incubated in a blocking and permeabilizing solution for 30 min at RT. Fixed cells were incubated with both rabbit anti-p53 antibody 1:100 and mouse anti-GRP75 1:100 in PBS overnight. The slides were washed with and subsequently treated with anti-rabbit Cy3-conjugated and anti-rabbit FITC-conjugated secondary antibodies both diluted 1:500 in PBS for 4 h. Nuclei were counterstained with DAPI. Coverslips were mounted with Molecular Probes' ProLong® Antifade Kit. Images were acquired by a Leica SD AF confocal microscope. Colocalization was assessed for 100 randomly chosen PC12 cells on 6 different optical fields from three slides per group with Leica Application Suite 3.1.3 software.

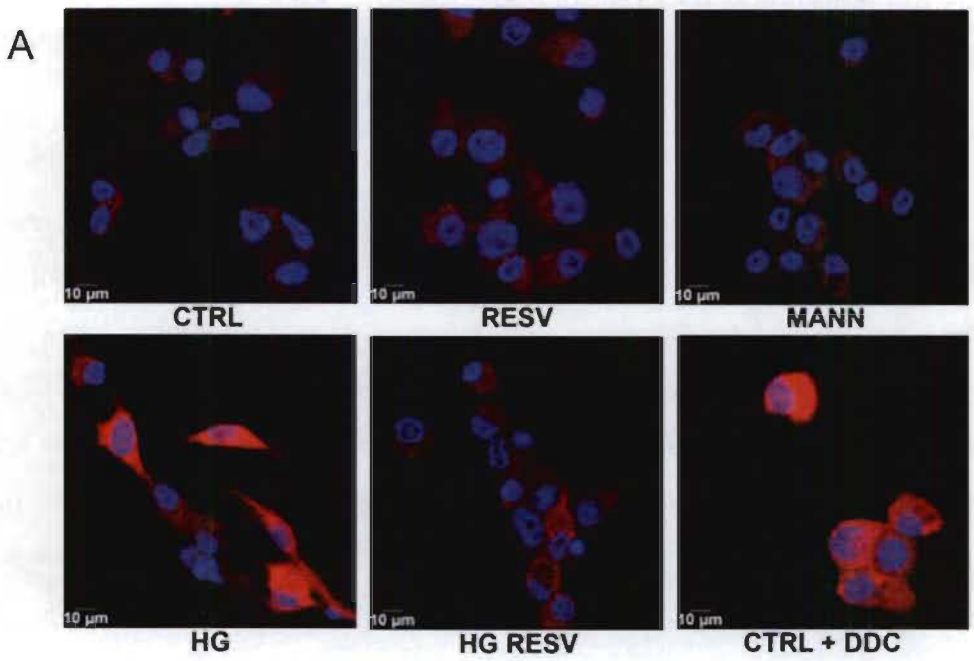
Statistical analysis

Significant differences between groups were ascertained by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis with the GraphPad Instat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval, are expressed as means ± standard error of the mean (SEM) from at least 3 independent experiments. Asterisks indicate statistical differences between the treatment and CTRL condition (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$); plus signs show statistical differences between the treatment and HG condition (+++$p < 0.001$, ++$p < 0.01$ and +$p < 0.05$).

Results

Resveratrol rescues high glucose-induced production of superoxide

To study the mechanisms underlying the neuroprotective effects of RESV against HG, we measured the production of superoxide with a derivative of ethidium bromide, MitoSOX™ Red, after administration of HG with or without RESV for 3 h. This time period was considered since free radical generation and eventually oxidative stress are early events in the causative process of cellular death (Carange *et al.*, 2011; Pérez-De La Cruz *et al.*, 2010; Zhou *et al.*, 2008). Figure 2.1A discloses low fluorescence levels in CTRL and MANN conditions as well as in cells treated with RESV in CTRL medium after 24 h, whereas a marked signal was detected in HG- and DDC-treated cells. When RESV was added to HG medium, fluorescence was strongly reduced. Figure 2.1B also reports the semi-quantitative analysis of mitochondrial superoxide anion presented in Figure 2.1A, revealing high fluorescence levels with HG and positive control DDC as well as a very significant reduction ($p < 0.001$) when DAergic PC12 cells in HG medium were treated with RESV. In the DDC condition, inhibition of SOD supports the specific detection of superoxide anion. All nuclei are stained blue by Hoechst 33342 (Figure 2.1A).



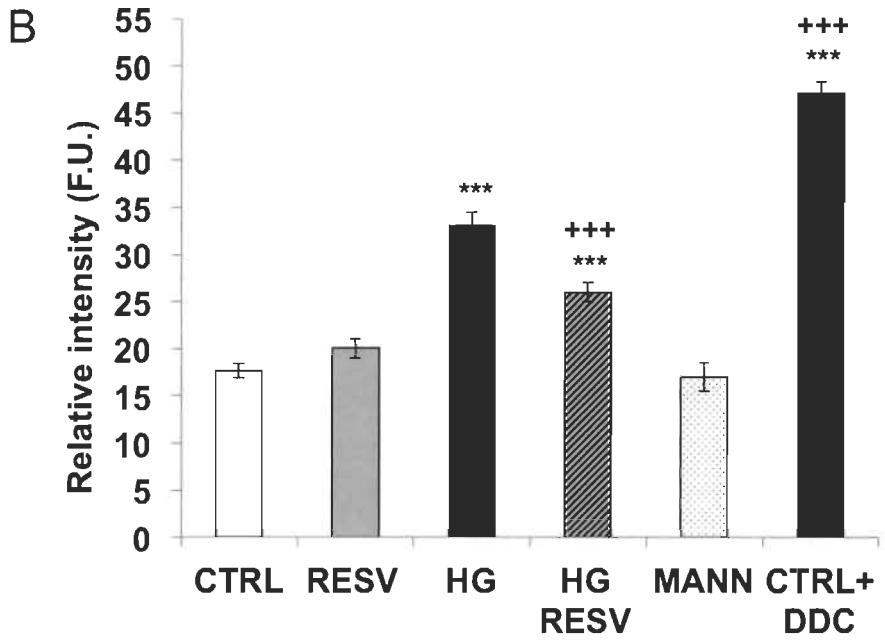
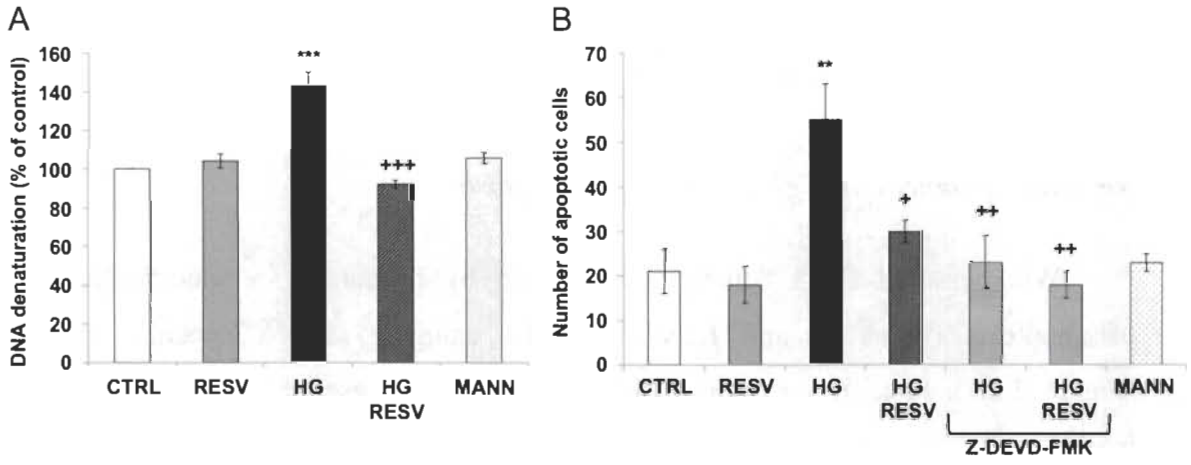


Figure 2.1 RESV reduces HG-induced superoxide anion production in DAergic PC12 cells.
Resveratrol (RESV) reduces high glucose (HG)-induced superoxide anion production in dopaminergic (DAergic) PC12 cells. (A) Fluorescence microphotographs. Blue: DAergic PC12 nuclei counterstained with Hoechst 33342. Red: MitoSOX™ Red superoxide indicator signal. A marked red signal is evident in DAergic PC12 cells treated with HG or diethyldithiocarbamate (DDC) (control [CTRL] + DDC). Red fluorescence was less intense in cells treated with CTRL medium, RESV alone or when RESV was added in HG medium (HG RESV). (B) Semi-quantitative image analysis. $***p < 0.001$ compared with CTRL, $+++p < 0.001$ compared with HG, as determined by one-way one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. Fluorescence units (F.U.).

Resveratrol reduces high glucose-induced apoptosis

We measured DNA denaturation induced by formamide, a specific hallmark of apoptosis (Frankfurt and Krishan, 2001), using a ssDNA specific antibody (Figure 2.2A). Specific apoptotic DNA denaturation is observed in early as well as in late apoptotic cells. HG condition showed a 43% increase in apoptotic cells in comparison to CTRL wells. This increment was fully reversed by RESV treatment in HG medium (Figure 2.2A). MANN medium did not yield significant apoptosis.

We then examined the effect of HG and RESV on later events of the apoptotic cascade leading to DNA fragmentation. Detection of cleaved caspase-3, the terminal effector caspase responsible for late apoptosis-mediated DNA fragmentation (Fan *et al.*, 2005), was conducted by immunofluorescence alongside a TUNEL assay measuring DNA degradation (Figure 2.2B and C). In the HG condition, immunofluorescence revealed the presence of cleaved caspase-3 positive cells (Figure 2.2C, red signal), while TUNEL assay stained numerous nuclei undergoing DNA fragmentation (Figure 2.2C, green signal). Total nuclei were stained with DAPI (Figure 2.2C, blue signal). DAergic PC12 cells were considered to be in late apoptosis when they hosted both caspase-3 activation and DNA fragmentation events (Figure 2.2C, cells pointed by white arrows). Treatment with RESV for 24 h clearly reduced the presence of apoptotic nuclei as implied by the lower number of DAergic PC12 cells exhibiting both green and red fluorescence. The number of apoptotic cells was also counted (Figure 2.2B), as described in the Materials and Methods section. Administration of RESV decreased the number of apoptotic cells compared to the HG condition. MANN medium did not yield a significant rise in apoptotic cells compared to CTRL. To show that caspase-3 activation is a key step in the HG-induced apoptotic pathway, DAergic PC12 cells were pretreated with 50 μM Z-DEVD-FMK, a cell-permeable selective caspase-3 inhibitor, followed by treatment with HG with or without RESV (Figure 2.2B and C).



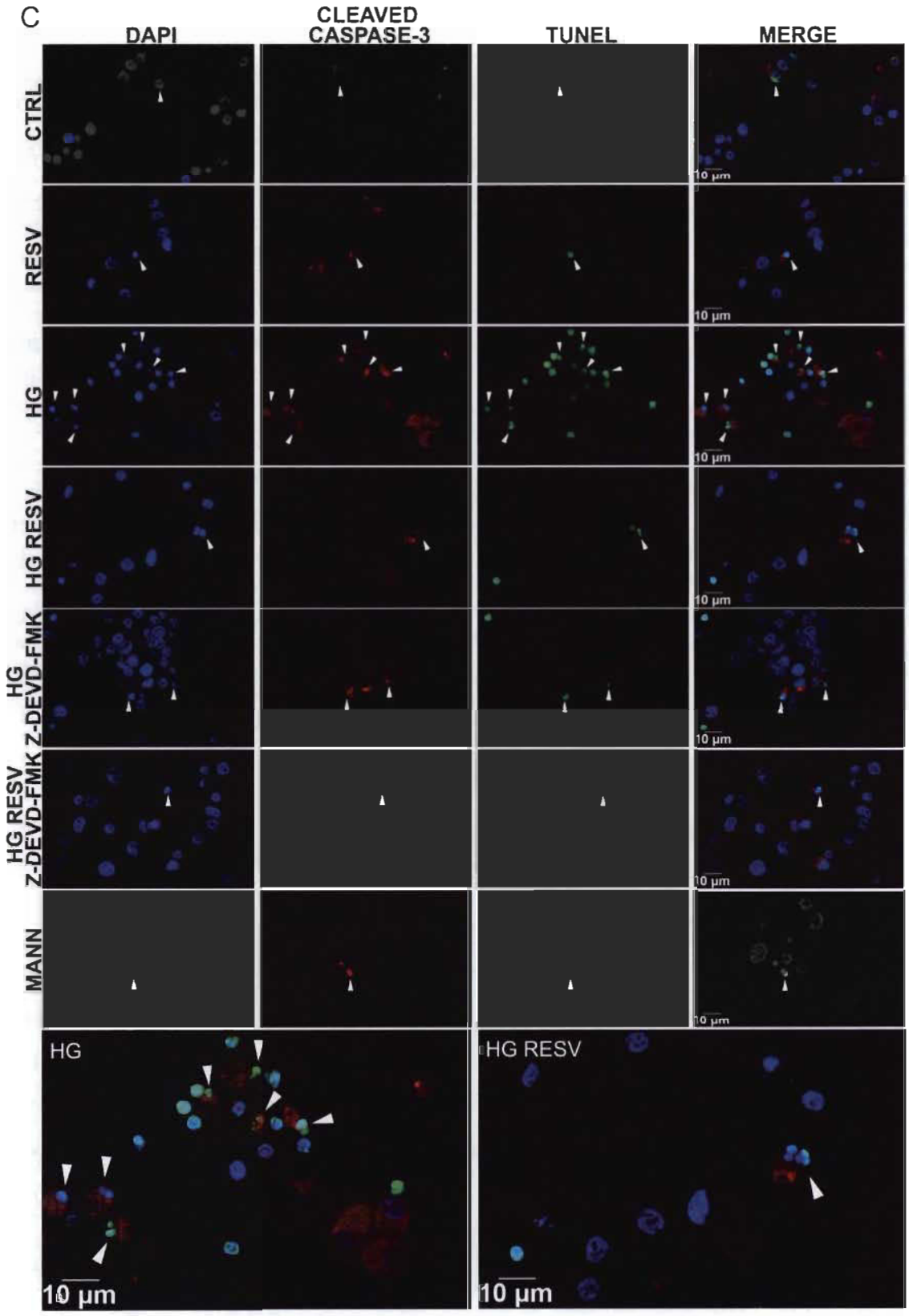


Figure 2.2 **RESV reduces HG-induced apoptosis in DAergic PC12 cells.**
Continued on next page.

(Continued.) (A) Histogram of specific apoptotic DNA denaturation by formamide in DAergic PC12 cells as detected with a monoclonal antibody against single-stranded DNA (ssDNA). CTRL, MANN and RESV alone do not affect specific apoptotic DNA denaturation. HG increases apoptotic DNA denaturation. Treatment of HG-exposed cells with RESV elicits a significant decrease in specific apoptotic DNA denaturation (HG RESV). (B) The number of apoptotic DAergic cells among 300 randomly chosen DAergic cells was counted on 10 different optical fields from 3 slides per group, as illustrated in panel C on a subsequent page. (C) Microphotographs of immunofluorescence detection of apoptotic DAergic PC12 cells. Blue: DAergic PC12 nuclei counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Red: anti-cleaved caspase-3 signal. Green: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of nuclei exhibiting DNA fragmentation. Triple-staining (cells points by white arrows) clearly reveals several apoptotic cells on slides treated with HG and fewer apoptotic cells when DAergic PC12 cells are treated with CTRL medium, RESV alone or when RESV is administered in HG conditions (HG RESV). To show that caspase-3 activation is a key step in the HG-induced apoptotic pathway, DAergic PC12 cells were pretreated with 50 μM of fluormethylketone-conjugated tetrapeptide Z-DEVD-FMK, a cell-permeable caspase-3 inhibitor, followed by treatment with HG, with or without RESV (HG- Z-DEVD-FMK and HG RESV- Z-DEVD-FMK, respectively). MANN condition is similar to CTRL cells. Enlarged microphotographs: HG and HG RESV merge microphotographs, to show apoptotic nuclei in these key conditions. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with CTRL, $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared with HG, as determined by one-way ANOVA, followed by Tukey's multiple-comparison test.

In order to further support these findings, we analyzed the expression of several proteins acting in the apoptotic cascade. Western blotting was performed on total proteins extracted from DAergic PC12 cells treated with HG or CTRL medium, with or without RESV (Figure 2.3). We analyzed the pro-apoptotic Bax and anti-apoptotic Bcl-2 protein ratio (Figure 2.3A) reported to be correlated with apoptosis (Cory and Adams, 2002). A high Bax/Bcl-2 ratio favors the release of mitochondrial factors leading to the activation of effector caspases in the apoptotic cascade (Kang and Reynolds, 2009). Our results demonstrate that the administration of HG medium for 96 h increases the Bax/Bcl-2 ratio two-fold compared to CTRL medium, supporting that HG-induced apoptosis in DAergic PC12 cells is mediated, at least in part, by the mitochondrial pathway (Figure 2.3A, histogram full grey line). The HG-induced raise of the Bax/Bcl-2

ratio was fully reversed in DAergic PC12 cells treated with RESV. Explicitly, HG medium increases Bax expression (Figure 2.3A, histogram white bars, Bax Western bands) but does not modulate Bcl-2 (Figure 2.3A, histogram black bars, Bcl-2 Western bands). RESV reverses the HG-induced increase in Bax expression and increases Bcl-2 expression. We also examined the ratio of full-length PARP-1 on inactivated cleaved PARP-1 (Figure 2.3B). As Chaitanya *et al.* (2010) have demonstrated, PARP-1 is a major player in the prevention of programmed cell death and its cleavage by activated caspase-3 is a hallmark of apoptosis. HG treatment markedly reduced full-length/cleaved ratio, which was fully reversed by RESV administered in HG medium (Figure 2.3B, histogram full grey line). MANN medium did not have a substantial effect on either the Bax/Bcl-2 ratio or the PARP-1 full-length/cleaved ratio. HG increased PARP-1 cleavage while RESV prevented this rise (Figure 2.3B, histogram black bars, cleaved PARP-1 Western bands). Full-length PARP-1expression was not affected in any condition (Figure 2.3B, histogram white bars, full-length PARP-1 Western bands).

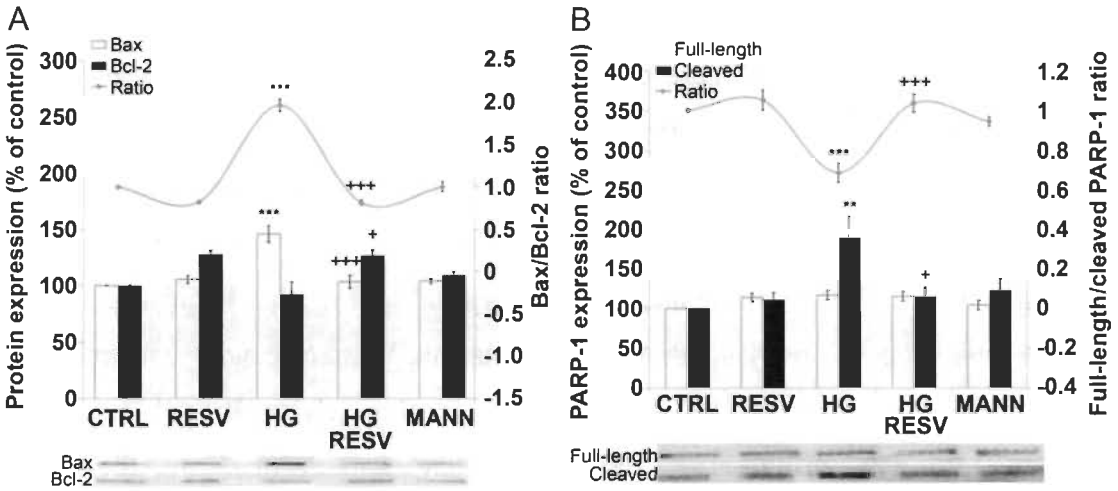


Figure 2.3 RESV modulates the expression of apoptotic protein markers. (A) Effect of RESV on the B cell lymphoma 2 (Bcl-2)-associated X protein (Bax)/Bcl-2 ratio in DAergic PC12 cells (full grey line). CTRL, MANN and RESV alone do not modulate the Bax/Bcl-2 ratio. HG increases the Bax/Bcl-2 ratio significantly and the addition of RESV to HG medium strongly prevents this increment (HG RESV). Bottom: Bax and Bcl-2 bands, as revealed by Western blotting. (B) Analysis of poly(ADP-ribose) polymerase (PARP-1) protein expression. These results are presented as the ratio of full-length (white bars)/cleaved (black bars) PARP-1. CTRL, MANN and RESV alone do not modulate the PARP-1

ratio in DAergic PC12 cells (full grey line). A decrease of PARP-1 ratio is apparent in HG condition. When RESV is delivered in HG condition, a significant increase of full-length/cleaved PARP-1 was evident (HG RESV). Bottom: Western blot bands of full-length and cleaved PARP-1. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with CTRL and $^{+}p < 0.05$, $^{+++}p < 0.001$ compared with HG, as determined by one-way ANOVA, followed by Tukey's multiple-comparison test.

Resveratrol modulates p53 and glucose-regulated protein 75 subcellular localization and colocalization

We studied the expression levels of p53, a tumor suppressor, and GRP75, a stress response protein (Figure 2.4). In several models, GRP75 binds and inactivates pro-apoptotic p53 in the cytosol, therefore helping to prevent apoptosis. In order to elucidate this alleged relationship between both markers, protein levels were measured in the cytoplasm and the nucleus (p53) or in the cytoplasm and the mitochondria (GRP75) (Figure 2.4A and B). Treatment of DAergic PC12 cells with HG medium for 96 h noticeably decreased p53 cytoplasmic/nuclear ratio (Figure 2.4A, histogram full grey line). This was prevented by administration with RESV. Expressly, HG increased p53 expression in the nucleus (Figure 2.4A, histogram black bars, p53 nuclear Western bands) while it did not seem to affect cytoplasmic levels (Figure 2.4A, histogram white bars, p53 cytoplasmic Western bands). RESV in HG medium preserved p53 levels at CTRL range in both compartments. HG administration for 96 h increased GRP75 expression both in the cytoplasm and in the mitochondria (Figure 2.4B, histogram white and black bars, GRP75 mitochondrial and cytoplasmic Western bands). Treatment with RESV in HG medium prevented GRP75 levels from rising in the cytoplasmic fraction only. The result is a small but significant decrease in the GRP75 cytoplasmic/mitochondrial ratio (Figure 2.4B, histogram full grey line). MANN medium did not affect the expression of either GRP75 or p53.

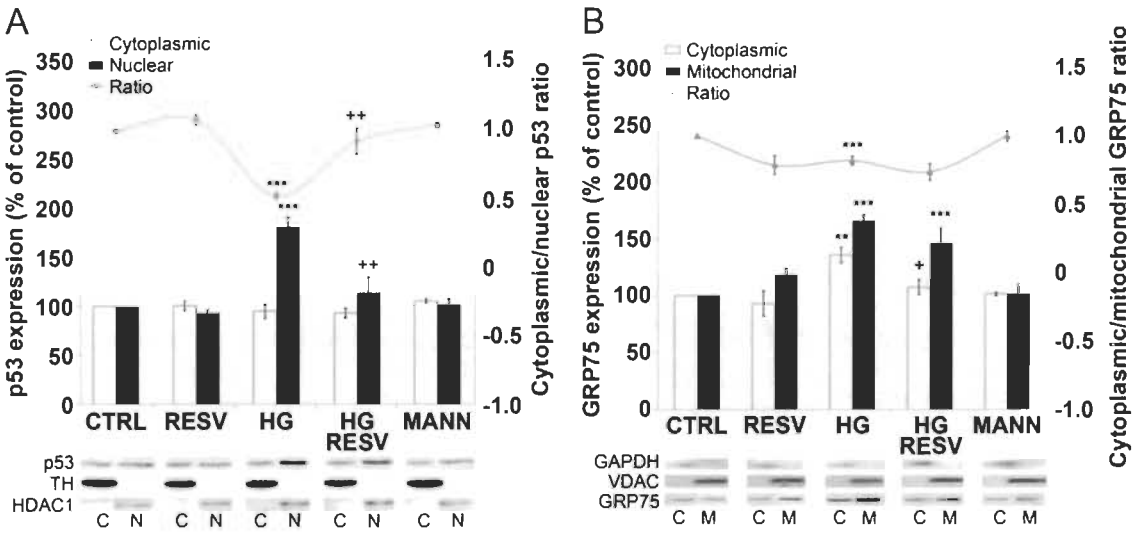


Figure 2.4 **Effect of RESV on the subcellular localization of p53 and GRP75.** (A) HG medium significantly increases nuclear localization of p53 (black bars) and administration of RESV in HG medium prevents this increase (HG RESV). RESV and MANN alone do not modulate p53 cellular localization. Cytoplasmic p53 (white bars) is not affected in any condition. Ratio of cytoplasmic/nuclear p53 is decreased in HG condition, which is prevented by RESV administration (HG RESV, full grey line). Bottom: p53, cytoplasmic fraction (C) purity marker tyrosine hydroxylase (TH) and nuclear fraction (N) purity marker histone deacetylase 1 (HDAC1) bands as revealed by Western blotting. (B) Effect of RESV on the cellular localization of glucose-regulated protein 75 (GRP75) in DAergic PC12 cells. HG treatment increases both cytoplasmic and mitochondrial content of GRP75. Administration of RESV in HG medium (HG RESV) significantly reduces cytoplasmic levels of GRP75 (white bars) while it does not amend mitochondrial levels (black bars). Ratio of cytoplasmic/mitochondria GRP75 is decreased in HG condition, which is prevented by RESV administration (HG RESV, full grey line). Bottom: GRP75, cytoplasmic fraction purity marker glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and mitochondrial fraction (M) purity marker voltage-dependent anion channel (VDAC) bands, as revealed by Western blotting. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with CTRL and $^{+}p < 0.05$, $^{++}p < 0.01$ compared with HG, as determined by one-way ANOVA, followed by Tukey's multiple-comparison test.

Finally, to evaluate the potential for GRP75 and p53 to interact in the cytoplasm, immunofluorescence colocalization measurements were performed following treatment of DAergic PC12 cells with HG medium with or without RESV. Scatter plots show that p53 (Figure 2.5A, green signal distribution) and GRP75 (Figure 2.5A, red signal distribution) signals are mainly independent from one another except for slight

colocalization (Figure 2.5A, plots and micrographs, Figure 2.5B). However, treatment with HG medium still appears to yield more colocalization on the scatter plot (Figure 2.5A, plots), which is also supported by the colocalization rate histogram (Figure 2.5B). Overlaid pictures of p53 and GRP75 staining (Figure 2.5A, micrographs) in CTRL, MANN, and RESV condition show dispersed punctual staining (white signal) in the cytoplasm, while in the HG condition a perinuclear dense staining is clearly visible. Administration of RESV in HG medium reveals a more scattered staining than in HG condition alone.

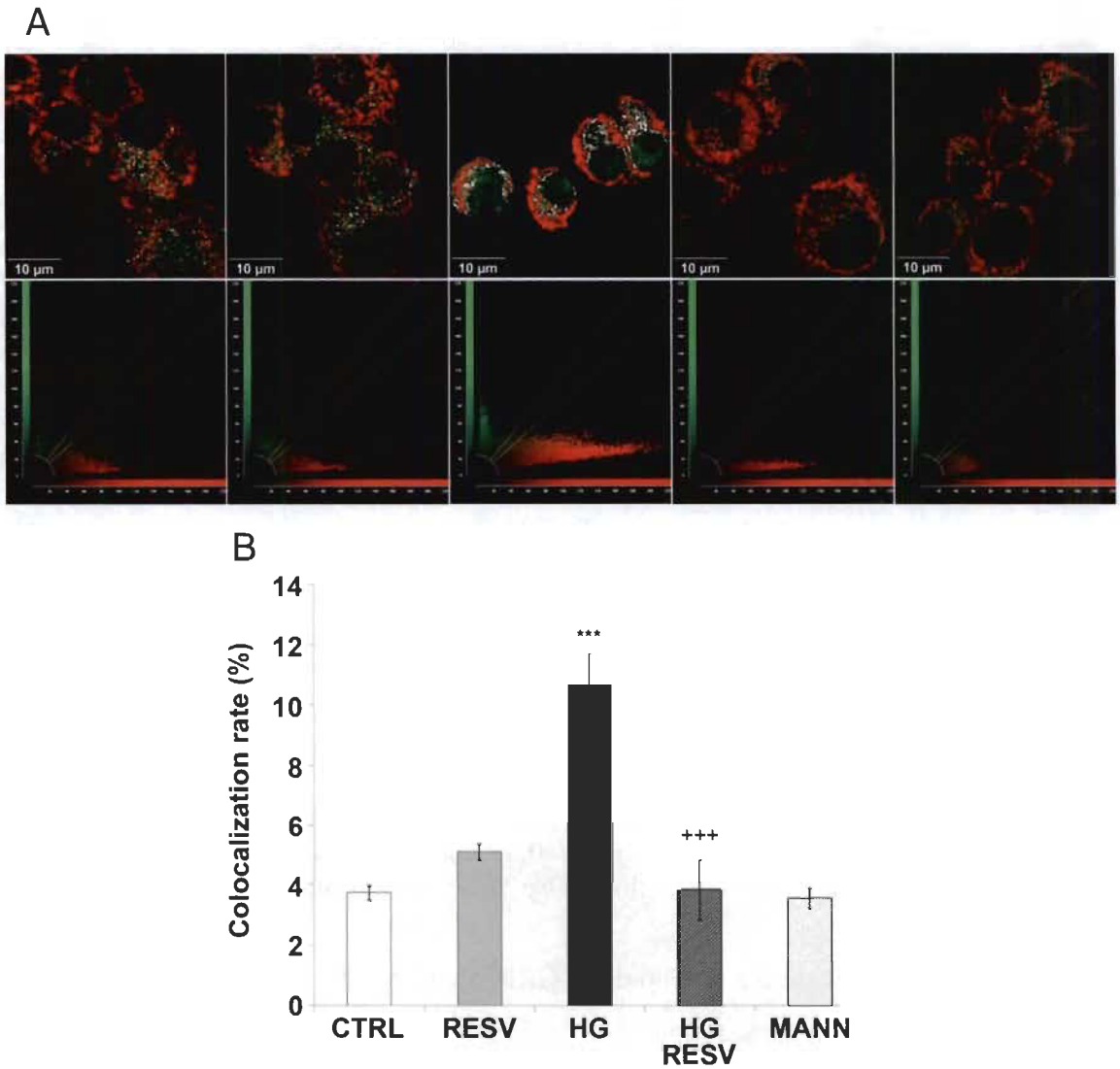


Figure 2.5 **RESV modulates p53 and GRP75 colocalization.**
Continued on next page.

(Continued.) (A) Scatter plots and corresponding overlaid micrographs of p53 and GRP75. Scatter plots show signal intensity for p53 (green signal) on the y axis and GRP75 (red signal) on the x axis. Each dot represents one event of fluorescent signal. Thresholds for green and red signal are optimized at 85% (two white lines) and mean background at 10% (arched delimitation), in each condition. Signal is colocalized when signals in scatter plots are located between the threshold lines on the outside of the background delimitation. White signal in microphotographs indicates high probability of colocalization. CTRL, RESV and MANN conditions are similar in that p53 and GRP75 colocalization is scarce and scattered in the cell cytoplasm. HG increases the white signal around the perinuclear area as well as the number of dots in the region of interest on the plot. Treatment with RESV yields a scatter plot and overlaid white signal (HG RESV) similar to the CTRL condition, suggesting its potential to diminish colocalization between GRP75 and p53. (B) Histogram depicting the colocalization rate of GRP75 and p53 observed in panel A. HG increases the colocalization rate significantly. RESV administration in HG medium reverses this increase in colocalization rate (HG RESV). All other conditions are similar to CTRL. $***p < 0.001$ compared with CTRL and $++p < 0.001$ compared with HG, as determined by one-way ANOVA, followed by Tukey's multiple-comparison test.

Discussion

We previously reported that several natural polyphenols, including the stilbene RESV, exert powerful neuroprotective activity in DAergic PC12 cells against the oxidative burden triggered by the administration of the potent parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *in vivo* (Blanchet *et al.*, 2008) or its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) *in vitro* (Bournival *et al.*, 2009; Gagné *et al.*, 2003; Lahaie-Collins *et al.*, 2008). Since hyperglycemia has also been listed as a growing risk factor for PD (Hu *et al.*, 2007; Jagota *et al.*, 2012; Sun *et al.*, 2012), we focused our study on the neuroprotective effect of RESV on HG-induced oxidative stress and apoptosis in DAergic PC12 cells with regard to GRP75 and p53 localization.

The Diabetes Control and Complications Trial (1993) together with the U.K. Prospective Diabetes Study (1998) have determined that hyperglycemia is the culprit to blame for tissue damage in type I and type II diabetes. Currently, we know that

overproduction of superoxide is the single upstream event leading to the following pathways involved in glucose toxicity (Giacco and Brownlee, 2010): 1) increased flux of glucose and other sugars through the polyol pathway; 2) increased intracellular formation of advanced glycation end-products (AGEs); 3) increased expression of the receptor for AGEs and its activating ligands; 4) activation of protein kinase C (PKC) isoforms; 5) overreactivity of the hexosamine pathway. The formation of AGEs and activation of AGE receptors (Shaikh and Nicholson, 2008), the activation of PKC (Aoki and Li, 2011) and the dysfunction of the polyol pathway (Ahmed *et al.*, 2009) have been identified as contributors in the development of PD. These mechanisms suggest a strong link between neuronal apoptosis observed in PD and hyperglycemic damage in diabetes (Klein *et al.*, 2004; Li *et al.*, 2002; Li *et al.*, 2008).

In this study, we demonstrated the defensive role of RESV in counteracting cellular distress parameters evoked by HG in DAergic PC12 cells. We tested NGF-differentiated PC12 cells, a known, reliable and efficient model for the investigation of oxidative stress, apoptosis and neuroprotection of DAergic neurons (Bournival *et al.*, 2012; Gélinas and Martinoli, 2002; Lahaie-Collins *et al.*, 2008). Since oxidative stress is an essential factor in glucose toxicity and in the pathogenesis of PD, we investigated whether RESV protects DAergic PC12 cells by reducing levels of mitochondrial superoxide in HG condition. Our results show that RESV effectively diminishes superoxide production after as early as 3 h. In PD, superoxide reacts with iron cations to form hydroxyl radical (Ramasarma, 2012), known to exert very deleterious effects on DNA, lipids and proteins. This ROS can also react with nitric oxide, an important signaling molecule in the brain, to form peroxynitrite, a powerful oxidant shown to play a significant role in protein aggregation pertinent to PD (Danielson and Andersen, 2008).

It is currently well known that oxidative stress may cause apoptosis through several pathways: 1) ROS-induced expression or activation of nuclear factor-kappa B (NF-κB) (Gloire *et al.*, 2006); 2) mitochondria-mediated cell apoptosis (Circu and Aw, 2010); 3) ROS-mediated DNA damage and p53 activation (Liu and Xu, 2011);

4) stress-activated protein kinases pathway to apoptosis (Johnson and Nakamura, 2007). We performed a set of experiments to investigate the apoptotic cascade in DAergic PC12 cells ensuing oxidative stress to further demonstrate the preventive role of RESV. A specific apoptotic DNA denaturation assay demonstrated that RESV significantly prevents apoptosis in cells exposed to HG. We further examined markers of late apoptosis to determine whether the protein cascade leads to terminal events such as the irreversible fragmentation of DNA. RESV in HG clearly reduced the number of apoptotic PC12 cells in comparison to the HG condition alone as shown by the decline in TUNEL and cleaved caspase-3 double-positive cells. Another target of activated caspase-3 is PARP-1, a protein known to participate in the repair of damaged DNA (Wang *et al.*, 2012). Our findings reveal that the PARP-1 protein ratio, full-length versus cleaved, was decreased after HG treatment and was then improved by RESV administration, hence supporting once more the neuroprotective anti-apoptotic role of RESV in a HG paradigm. In addition, the Bax and Bcl-2 expression was studied to determine the apoptotic events surrounding the mitochondria. Bax contributes to the leakiness of the outer mitochondrial membrane, while Bcl-2 blocks the permeability transition pore, thus inhibiting mitochondria-mediated programmed cell death (Smith *et al.*, 2008). The rise in the Bax to Bcl-2 ratio is a characteristic feature in apoptosis (Cory and Adams, 2002) equally observed in glucose toxicity (Allen *et al.*, 2005) and in several models of PD including human post-mortem brains (Vila and Perier, 2008). Our data reveal that the Bax/Bcl-2 protein ratio is increased after HG administration, and is decreased by RESV treatment in the HG condition, strongly suggestive of a role for mitochondrial dysfunction in the mechanisms underlying the apoptosis of DAergic neurons in our cellular paradigm of hyperglycemia.

GRP75 has often been linked to PD pathogenesis as reported in studies showing binding properties to PD-associated proteins in the mitochondria (Jin *et al.*, 2005, 2006, 2007; Li *et al.*, 2005; Rakovic *et al.*, 2011) and reduced levels of the protein in post-mortem PD brain samples (Burbulla *et al.*, 2010; Jin *et al.*, 2005; Shi *et al.*, 2008). While GRP75 is mainly confined to the outer membrane of mitochondria, several studies have shown that it may bind and sequestrate pro-apoptotic p53 in the cytosol thereby

preventing its entry in the nucleus, impeding apoptosis and ultimately promoting p53 degradation by the MDM2 proteasome degradation pathway (Kaul *et al.*, 2001, 2005; Wadhwa *et al.*, 2002). Such studies were mainly conducted in cancer cells (Kaul *et al.*, 2001, 2005; Wadhwa *et al.*, 2002) or in naïve, mitotic PC12 cells (Guo *et al.*, 2009; Li *et al.*, 2011). Our results obtained in post-mitotic PC12 cells, show that HG treatment increases GRP75 expression in the cytoplasm as well as in mitochondria thus suggesting that GRP75 is induced by HG cellular stress. While RESV reduced GRP75 levels in the cytoplasm, it did not ensure a significant effect in diminishing mitochondria GRP75 localization. Apparently, in our cellular paradigm, RESV modulates the subcellular distribution of GRP75 by preventing cytoplasmic levels from rising. RESV may be responsible for quenching HG-induced stress signals that promote the induction of GRP75 in the cytoplasm. Moreover, p53 localization is increased in the nucleus, which points toward a pro-apoptotic effect of HG on DAergic PC12 cells. RESV in HG medium maintains the cellular distribution of p53, which partially accounts for its anti-apoptotic properties. Altogether, these results show an increase of GRP75 in the cytoplasm while p53 levels rise significantly in the nucleus in HG condition, suggesting relatively weak interaction between both markers in post-mitotic cells. Colocalization studies deepened our understanding of the relationship between GRP75 and p53 in our cellular model. We show that GRP75 and p53 have a potential to bind in the cytoplasm but to a limited extent. Binding in HG condition is significantly enhanced, perhaps due to increased expression of both proteins in the cytoplasm, but still remains limited. We show for the first time that post-mitotic DAergic PC12 cells exert weak binding of GRP75 and p53, which contrasts with findings in non-differentiated mitotic PC12 cells (Guo *et al.*, 2009; Li *et al.*, 2011). Moreover, GRP75 levels are decreased in post-mortem PD brains (Burbulla *et al.*, 2010; Jin *et al.*, 2005; Shi *et al.*, 2008) while we see an increase of expression in HG condition. Perhaps long-term mitochondrial dysfunction is responsible for the depletion of stress markers such as GRP75 in PD.

Altogether, our results demonstrate that HG-induced oxidative stress and apoptosis of DAergic PC12 cells can be improved by RESV, sustaining an important role for this naturally occurring polyphenol in diabetes treatment. RESV has been the object of

several diabetes studies because of its ability to improve insulin sensitivity, protect pancreatic β cells and control glycaemia (Lee *et al.*, 2012; Milne *et al.*, 2007; Szkudelski and Szkudelska, 2011). Indeed, RESV protects against retinopathy in rats with diabetes (Soufi *et al.*, 2012) and prevents nephropathy in *db/db* mice by inhibiting lipotoxicity-related apoptosis and oxidative stress in the kidney (Kim *et al.*, 2013). Additional beneficial effects of the stilbene RESV may contribute to alleviate obesity-induced metabolic complications (Rosenow *et al.*, 2012) often related with diabetes. A recent clinical study has found oral administration of RESV to be effective in improving glycaemia in type 2 diabetes mellitus (Bhatt *et al.*, 2012).

Even though RESV is principally metabolized into its glucoronide and sulfate conjugates, recent data show that these metabolites may possess beneficial properties (Delmas *et al.*, 2011). Increased bioavailability due to a synergistic effect with other polyphenols or compounds, such as curcumin or the glycemic control drug metformin, must also be taken into account (Bruckbauer and Zernel, 2013; Du *et al.*, 2013). Besides, recent pharmacological advances have improved bioavailability of RESV (for details see Amiot *et al.*, 2013; Neves *et al.*, 2013). Finally, the potential beneficial properties of RESV on human health are broadly displayed in the literature and justify the need to further unravel the powerful cellular role of this dietary polyphenol.

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CHAPTER III

DOPAMINERGIC NEURODEGENERATION IN A RAT MODEL OF LONG-TERM HYPERGLYCEMIA: PREFERENTIAL DEGENERATION OF THE NIGROSTRIATAL MOTOR PATHWAY

Renaud, J., Bassareo, V., Beaulieu, J., Pinna, A., Schlich, M., Lavoie, C., Murtas, D., Simola, N., Martinoli, M. G. *Neurobiol. Aging, 69*:117-128.

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3.1 Author contributions

Justine Renaud designed most of the study and performed 90% of the manipulations at the Université du Québec à Trois-Rivières and at the University of Cagliari, Italy, during a 6-month stay. She wrote 95% of the manuscript, and prepared and edited all the figures. Valentina Bassareo, a researcher affiliated to the National Institute of Neuroscience at the University of Cagliari, performed the microdialysis experiments with Justine Renaud's assistance in preparing the probes, conducting surgeries and collecting the samples. Jimmy Beaulieu, master's student in Maria-Grazia Martinoli's laboratory, provided assistance for the immunoblotting experiments. Annalisa Pinna helped with the motor behaviour tests performed at the University of Cagliari and reviewed the manuscript. Michele Schlich and Daniela Murtas performed glucose measurements in microdialysis samples at the University of Cagliari. Carole Lavoie, a professor from the Department of Medical Biology at the Université du Québec à Trois-Rivières, offered expertise and assistance with the hyperglycaemic model. Nicola Simola supervised the project at the University of Cagliari and reviewed the manuscript. Maria-Grazia Martinoli, Justine Renaud's research supervisor, was the guarantor of the work and provided supervision, preparation and editing of the manuscript.

3.2 Résumé

De nombreuses études épidémiologiques soutiennent une corrélation entre le diabète et les maladies neurodégénératives liées à l'âge, telles les maladies d'Alzheimer et de Parkinson. En effet, l'hyperglycémie engendre un stress oxydant auquel est vulnérable le système nerveux central. Nous avons récemment démontré que des concentrations physiologiquement élevées de glucose causent un stress oxydant menant à la mort de neurones dopaminergiques en culture. Les altérations dopaminergiques survenant avec l'âge dans le diabète demeurent méconnues. Dans cette optique, le but de notre étude était de caractériser l'état des deux voies dopaminergiques principales du système nerveux central dans l'hyperglycémie chronique. Nous avons donc employé un modèle modéré de rat diabétique, soit le paradigme nicotinamide-streptozotocine permettant le maintien d'une hyperglycémie à long terme. Spécifiquement, nous nous sommes attardés à la voie nigrostriée, bien connue pour son rôle dans le contrôle de la motricité et dans la maladie de Parkinson, ainsi qu'à la voie mésocorticolimbique, un circuit voisin davantage associé au système de la récompense. Les neurones et les cellules gliales ont fait l'objet d'analyses 3 et 6 mois suivant l'induction de l'hyperglycémie. Nos résultats démontrent une neurodégénérescence préférentielle de la voie nigrostriée, celle-ci étant accompagnée d'une prolifération marquée des astrocytes et de la mort des cellules microgliales à 6 mois. Des tests comportementaux ont confirmé l'existence de déficits moteurs évoquant les symptômes de la maladie de Parkinson, survenant de cette neurodégénérescence. La somme de ces résultats démontre une relation entre l'hyperglycémie et la mort préférentielle des neurones dopaminergiques de la voie nigrostriée, appuyant de surcroît les études épidémiologiques soulignant le risque que comporte un diabète préexistant pour le développement de la maladie de Parkinson.

3.3 Full article in English: Dopaminergic neurodegeneration in a rat model of long-term hyperglycemia: preferential degeneration of the nigrostriatal motor pathway

Abstract

Epidemiological evidence suggests a correlation between diabetes and age-related neurodegenerative disorders, including Alzheimer's and Parkinson's diseases. Hyperglycemia causes oxidative stress in vulnerable tissues such as the brain. We recently demonstrated that elevated levels of glucose lead to the death of dopaminergic neurons in culture through oxidative mechanisms. Considering the lack of literature addressing dopaminergic alterations in diabetes with age, the goal of this study was to characterize the state of two critical dopaminergic pathways in the nicotinamide-streptozotocin rat model of long-term hyperglycemia, specifically the nigrostriatal motor pathway and the reward-associated mesocorticolimbic pathway. Neuronal and glial alterations were evaluated 3 and 6 months after hyperglycemia induction, demonstrating preferential degeneration of the nigrostriatal pathway complemented by a noticeable astrogliosis and loss of microglial cells throughout aging. Behavioral tests confirmed the existence of motor impairments in hyperglycemic rats that resemble early parkinsonian symptomatology in rats, ensuing from nigrostriatal alterations. These results solidify the relation between hyperglycemia and nigrostriatal dopaminergic neurodegeneration, providing new insight on the higher occurrence of Parkinson's disease in diabetic patients.

Introduction

Glucose is the obligate energy substrate of adult neurons. Owing to the preponderant expression of glucose transporter 1 (GLUT1) at the blood-brain barrier (Anraku *et al.,* 2017) and GLUT3 at the neuron plasma membrane (Patching *et al.,* 2017, Simpson *et al.,* 2007), uptake overwhelmingly occurs in an insulin-independent fashion. Thus, intraneuronal glucose concentrations directly depend on extracellular concentrations, i.e., on plasma glucose concentrations (Jacob *et al.,* 2002).

Indeed, neurons belong to the most severely hit targets in hyperglycemia, along with mesangial and capillary endothelial cells (for review see Brownlee, 2005). Well appreciated is the fact that persistent hyperglycemia induces oxidative stress occasioning damage in the peripheral nervous system, as is the case in comorbid neuropathies of uncontrolled diabetes (Rajabally, 2017). Fewer studies have focused their attention on hyperglycemia in the human central nervous system (Moulton *et al.*, 2015) but go as far as to draw relationships between diabetes and age-related neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (Jagota *et al.*, 2012, Vicente Miranda *et al.*, 2016, Vieira *et al.*, 2017, Vignini *et al.*, 2013).

Notwithstanding the wealth of evidence supporting a toxic role for glucose in neurons of the central nervous system (see for review Tomlinson and Gardiner 2008, Vicente Miranda *et al.*, 2016), details are lacking in the bigger picture. Most studies have addressed neurodegeneration in discrete brain regions on a short-term scale (Do Nascimento *et al.*, 2011, Kamboj and Sandhir, 2011), which do not represent temporal or spatial breadths of hyperglycemic distress in aging diabetic patients. Moreover, literature describing dopaminergic neurodegeneration in hyperglycemic models remains modest. Our group demonstrated a role for chronic glucose exposure in apoptotic death of dopaminergic neurons through oxidative mechanisms (Bournival *et al.*, 2012, Renaud *et al.*, 2014) and several studies report dopaminergic alterations in diabetes or acute hyperglycemia (Lozovsky *et al.*, 1981, Murzi *et al.*, 1996, Sevak *et al.*, 2007). Nevertheless, the state of entire dopaminergic pathways in the long-term has not been inquired.

In order to improve our understanding of central dopaminergic neurodegeneration in hyperglycemia, we focused on two critical central dopaminergic systems, namely the nigrostriatal and mesocorticolimbic pathways (for review see Haber, 2014). The nigrostriatal pathway is primarily involved in the generation of movement. Cell bodies of nigrostriatal neurons are found in the *substantia nigra pars compacta* (SNc), lodged in the ventral midbrain, and extend their dopaminergic fiber terminals to the dorsal striatum (DS). The mesocorticolimbic pathway is often termed the reward system. Cell bodies of mesocorticolimbic neurons are also found in the ventral midbrain,

though in a neighboring subregion named the ventral tegmental area (VTA). This pathway innervates both the ventral striatum (VS, mesolimbic pathway), which contains the nucleus accumbens (NAcc) and the olfactory tubercle (OT), as well as the prefrontal cortex (PFC, mesocortical pathway) (Squire *et al.,* 2008). We thus evaluated neurodegeneration and analyzed glial populations in these regions to obtain a better appreciation of the global state of the dopaminergic pathways in long-term hyperglycemia. Finally, behavioral assessments were carried out to uncover motor and cognitive alterations possibly expressed as a consequence of neurodegeneration.

Research design and methods

Subjects

A total of 91 rats were used in this study. For immunoblotting, immunohistochemical and behavioral studies, two different cohorts totaling 61 male Sprague-Dawley rats (Charles River, St-Constant, Canada) weighing 175-200 g and aged 5-6 weeks were housed under standard laboratory conditions (12 h light/dark cycle). In Italy, motor and cognitive behavior analyses were repeated and complementary microdialysis studies were carried out in 30 male Sprague-Dawley rats (Harlan Italy, Udine, Italy) of the same weight and age, also housed under standard laboratory conditions. In all experiments, standard food and water were available *ad libitum*. Rats were acclimated for 2 weeks and were therefore 7-8 weeks of age upon induction of hyperglycemia described below.

All experiments were conducted in accordance with the guidelines for animal experimentation of the EU directives (2010/63/EU; L.276; 22/09/2010), of the Ethical Committee of the University of Cagliari, of the Animals for Research Act and following the legislation and policies of the Canadian Council on Animal Care, as well as with the guidelines established by the Animal Care Committee of the Université du Québec (Trois-Rivières) (2014-M.G.M.5). Maximal efforts were made to minimize discomfort and numbers of animals used.

Induction of long-term hyperglycemia

Rats were randomly divided into two groups; control (CTRL) and hyperglycemic (HG). Long-term hyperglycemia was induced in fasted rats (overnight, 12-16 h) by a single i.p. injection of freshly dissolved streptozotocin (STZ, 0.1 M cold citrate buffer, pH 4.5, 55 mg/kg b.w.). Nicotinamide (NA, 100 mg/kg b.w.) dissolved in physiological saline was administered i.p. exactly 20 min prior to the STZ injection to minimize the destruction of insulin-producing pancreatic beta cells, yielding HG rats that would survive for 6 months without the need for glycemia-lowering treatments (Badole *et al.,* 2015, Masiello *et al.,* 1998). CTRL rats received i.p. injections of vehicles. All rats were injected on mornings within a time frame of 3 h. Hyperglycemia was confirmed 72 h after NA-STZ injections using a digital glucose meter (UltraMini with One Touch Ultra strips) to analyze blood collected from the tail vein. Only rats with a glycemia steadily above 10 mM were used in this study. Exhaustive metabolic follow-ups were conducted regularly throughout experiments (Supplementary Figure S1).

Motor behavior assessments

We investigated the possibility that the degeneration of neurons and neuronal fibers may be accompanied by alterations in motor behavior. Rats were submitted to motor behavior assessments, beginning with baseline evaluations prior to hyperglycemia induction, followed by trials 3 and 6 months later.

Stepping test and forepaw adjusting step test: Both tests were adapted for non-parkinsonian models, implying bilateral rendering of results, and conducted as previously described (Pinna *et al.,* 2007). The stepping test measures the rapidity of rats to initiate movement and cross a beam. Briefly, rats were held with one forelimb placed at the beginning of a beam leading to the home cage. The initiation time was measured from the moment the free forelimb was placed on the beam until the rat started to step using the same forelimb. Time to reach the home cage and numbers of strides were also recorded. The adjusting step test measures akinesia/bradykinesia (Chang *et al.,* 1999) in rats by dragging their forepaw sideways on a surface at a constant speed.

Briefly, rats were moved slowly sideways along the table surface by an experimenter, first in a forehand and then in a backhand direction. Adjusting steps made in forward and backward directions were counted.

Horizontal bar test: Healthy rats typically correct their position quickly when placed in an awkward posture. To evaluate the ability of rats to correct an externally imposed posture, we performed a horizontal bar test (Sanberg *et al.,* 1981). Following a 10-min habituation phase to the test cage, rats were tested by gently placing both forepaws on a horizontal bar (0.8 cm diameter, 3 cm from floor). Descent latency was measured as the time span from placing the animal on the bar until both paws were removed. The test cage was washed after each animal.

Cognitive behavior

Novel object recognition task: Evaluation of novel object recognition (NOR) performance is widely used for assessing non-spatial working memory in rodents (Ennaceur, 2010), a function regulated by limbic and cortical areas such as the hippocampus (HPC), NAcc and PFC. NOR experiments were performed as previously described (Simola *et al.,* 2008). The experimental procedure consisted of habituation, acquisition and testing phases. Habituation to the test cage took place on the day before tests during a single 5-min trial. The following day, the acquisition phase was performed by placing the rat in the test cage together with two identical copies of an object (familiar objects). Rats were left to freely explore the objects for 3 min. The testing phase took place 60 min after acquisition and consisted in exposing the rat to one copy of the objects presented previously and a novel object. Results were obtained 3 months after injections but not after 6 months since HG rats develop cataracts and their sight is likely to be impaired.

Sacrifices and tissue harvest

At 3 or 6 months, rats were intracardially perfused with ice-cold PBS containing protease and phosphatase inhibitors. The brain was sectioned into hemispheres. One was

immediately used to sequester the striatum, the midbrain and the PFC, which were frozen in dry ice-cooled isopentane and stored at -80°C for later immunoblotting analyses or intracellular glucose quantification in tissues. The HPC was also harvested for comparison purposes, as it constitutes a secondary DA hub receiving dopaminergic input from the VTA through the hippocampal-VTA loop (Lisman and Grace, 2005). The other hemisphere was post-fixed in 4% paraformaldehyde, cryoprotected through gradients of sucrose, and conserved at -80°C for immunohistochemical analyses. The Paxinos and Watson rat brain atlas (1998) served as a reference for all tissue harvests.

Immunohistochemistry

Frozen post-fixed brain hemispheres were cut into 20 µm-thick coronal free-floating serial sections. By systematic random sampling (West, 2012), one out of every six sections was immunoreacted with antibodies raised against either the specific dopaminergic neuron marker anti-tyrosine hydroxylase (anti-TH), the general neuronal marker anti-NeuN, the astrocyte marker anti-glial fibrillary acidic protein (anti-GFAP), or the microglial cell marker anti-ionized calcium-binding adapter molecule 1 (anti-Iba1) (for list of antibodies, concentrations and manufacturers see Supplementary Table S1). Then, sections were incubated with a HRP-conjugated secondary antibody, revealed with 3,3'-diaminobenzidine, mounted on microscope slides, dehydrated, and analyzed under a microscope in brightfield mode (MBF Bioscience, Williston, VT, USA). Neuroanatomical loci were determined according to the atlas of Paxinos and Watson (1998) and were identically delimited in all sections of the same anteriority between animals. Because of the systematic random sampling method, the same numbers of sections at the same anteriorities per region were consistently analyzed in each animal, following guidelines already established for stereology (West, 2012).

Total counts of overall neurons (NeuN+), dopaminergic neurons (TH+), astrocytes (GFAP+) and microglial cells (Iba1+) were performed in the SNc and in the VTA (bregma -4.80 to -6.30 mm) using the manual cell counting marker function provided by the NIH ImageJ software version 1.49. In the DS (lateral and medial, bregma 2.2 to

-1.0 mm), the VS (NAcc and OT, bregma 2.7 to 0.5 mm), the PFC (bregma 4.0 to 2.0 mm) and the HPC (bregma -2.3 to -6.0 mm), we counted total numbers of NeuN+, GFAP+ and Iba+ cells. Density of TH+ dopaminergic terminals in the striatum (lateral, medial, NAcc and OT, refer to aforementioned bregmas) was evaluated by densitometric assays using ImageJ. These were systematically performed on non-overlapping spherical regions of interest (3 x 1 mm^2 for lateral or medial DS; 5 x 0.5 mm^2 for NAcc or OT).

Immunoblotting

Frozen tissues were homogenized by steel ball milling in RIPA buffer and proteins were quantified by the bicinchoninic acid method before running them in a SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes, which in turn were incubated with primary antibodies raised against the dopaminergic markers TH or dopamine transporter (DAT), or against the general neuronal marker NeuN. Following incubation with HRP-conjugated secondary antibodies, blots were finally developed with an enhanced chemiluminescence substrate solution and immunopositive chemiluminescent signals were visualized using the AlphaEase FC imaging system and software (San Leandro, CA, USA). Densitometric blot analyses were performed using ImageJ. β-tubulin blots served as a loading comparative standard and all immunoblotting results have been accordingly normalized to β-tubulin levels.

Intracerebral microdialysis in freely moving rats

Two microdialysis trials in separate experiment-naïve groups of rats were conducted to verify extracellular glucose or DA concentrations in discrete brain regions in order to support and complete data obtained by the other techniques in this study.

Probe preparation: Vertical microdialysis probes were prepared with AN69 membranes (Hospal Dasco, Bologna, Italy) according to the method described by Di Chiara *et al.* (1993) further modified by Tanda *et al.* (1996). Probes had a dialyzing portion of 1.5 mm for the NAcc, the SNc and the VTA, and of 3 mm for the DS and the PFC.

Surgery: Three months after hyperglycemia induction, rats underwent stereotaxic acute probe-insertion surgery as previously described by Bassareo *et al.* (2011). Briefly, the probes were inserted vertically under the following coordinates: NAcc (A: 1.9, L: 1.1 from bregma; V: -7.6 from dura), DS (A: 0.7, L: 2.8 from bregma; V: -6.5 from dura), PFC (A: 3.8, L: 0.9 from bregma; V: -5.0 from dura), SNc (A: -5.8, L: 1.7 from bregma; V: -8.3 from dura) and VTA (A: -6.0, L: 0.5 from bregma; V: -8.2 from dura) according to the atlas of Paxinos and Watson (1998). A maximum of two regions on opposite hemispheres were targeted in each rat (for glucose: VTA and NAcc, VTA and DS, SNc and NAcc, or SNc and DS; DA: DS and NAcc, NAcc and PFC, or PFC and DS), and each subject was tested only once during the experimental session. We collected data in 3-month HG rats for glucose and DA microdialysis experiments. Rats did not undergo surgery at 6 months due to their delicate health.

Methods specific to microdialysis experiments dedicated to glucose measurements: Prior to analytical procedures, rats were fasted overnight to normalize the contribution of feeding on glycemia. Thirty minutes before the start of experiments, each rat was fed with normal chow diluted in water (1.5 g/kg b.w. by intragastric gavage). Glycemia was monitored before, during and after microdialysis experiments to ensure its stability.

Microdiaysis experiments: On the day following surgery, microdialysis experiments were conducted in freely moving and awake rats, as previously described (Bassareo *et al.,* 2011). Briefly, dialysate samples (20 µL) were taken every 20 min, for a total of 9 samples (180 min) for DA quantification and 3 samples (60 min) for glucose measurements. For DA quantification, 20 µL samples were immediately injected into a high-performance liquid chromatography, whereas, for glucose measurements, samples were pooled and immediately frozen for later quantification. Basal dialysate DA was calculated as the area under the curve of the 9 consecutive samples collected during the 180-min session, varying by no more than 10% (for time course see Supplementary Figure S2).

Histology: At the end of experiments, rats were sacrificed and transcardially perfused with 50 mL of saline and 50 mL of a 4% formaldehyde/1% calcium acetate/100 mM NaCl solution. Sections were cut with a vibratome and probe location was ensured as reported by Bassareo *et al.* (2011) (Supplementary Figure S3).

Brain tissue and microdialysate glucose concentrations

In order to investigate whether glucose concentrations may be altered differentially in the various brain regions of interest following long-term hyperglycemia, glucose concentrations in homogenized brain tissue and intracerebral microdialysis samples were measured according to the manufacturer's protocol using a Rat Glucose Assay kit (Crystal Chem, Downers Grove, IL, USA). For homogenized brain tissue, frozen striatum, midbrain, PFC or HPC tissues were homogenized by steel ball milling in volume/weight equivalents of RIPA buffer and immediately deproteinized by trichloroacetic acid precipitation before glucose quantification.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism 7 software (San Diego, CA, USA; http://www.graphpad.com). Significant differences between groups were ascertained by Student's unpaired two-tailed *t*-test, or by two-way ANOVA followed by Sidak's post-hoc analysis. All data, analyzed at the 95% confidence interval, are expressed as means ± SEM. *T* and *F* statistics for each figure are described in Supplementary Table S2.

Results

Glucose concentrations increase in all brain regions of interest

At 3 (Figure 3.1A) and 6 months (Figure 3.1B), glucose concentrations in HG rats (black bars) were increased compared to CTRL rats (white bars) in homogenized tissues

of midbrain, striatum, PFC and HPC. Microdialysis experiments confirmed these results at 3 months in discrete brain regions, namely the SNc and VTA of the midbrain and the DS and NAcc of the striatum (Figure 3.1C).

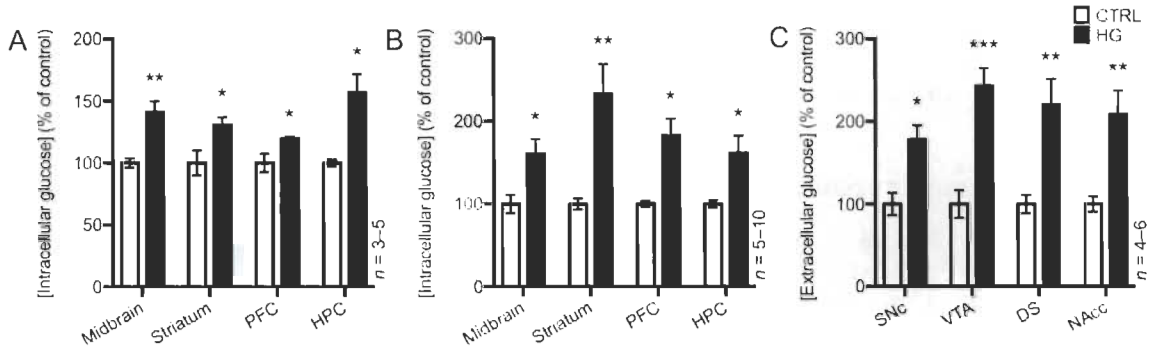

Figure 3.1 Brain glucose concentrations in HG rats compared to CTRL rats. (A, B) Frozen striatum, midbrain, prefrontal cortex (PFC) and hippocampus (HPC) brain tissues were homogenized by steel ball milling and deproteinized before glucose quantification. Three (A) and six (B) months after hyperglycemia-inducing nicotinamide-streptozotocin (NA-STZ) injections, hyperglycemic (HG) rats displayed increased intracellular glucose concentrations in all brain regions analyzed compared to the control (CTRL) group. (C) Three months after injections, microdialysis samples were obtained from acute probes inserted in the nucleus accumbens (NAcc), the dorsal striatum (DS), the substantia nigra pars compacta (SNc), and the ventral tegmental area (VTA). Thirty minutes before the start of experiments, overnight fasted rats were fed with normal chow diluted in water (1.5 g/kg body weight [b.w.] by intragastric gavage). Glucose was measured in the samples, confirming higher extracellular concentrations in all the brain regions of HG rats compared to CTRL rats. Data presented as means ± SEM. Asterisks indicate statistical differences between the HG group and CTRL group (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$).

Long-term hyperglycemia causes preferential degeneration of dopaminergic neurons in the substantia nigra pars compacta

By immunoblotting (Figure 3.2A and B), we observed no difference in the expression levels of TH (Figure 3.2A and C), DAT (Figure 3.2A and D) or NeuN (Figure 3.2A and E) in the midbrain of 3-month HG rats compared to CTRL rats. At 6 months, all three markers were decreased (Figure 3.2B, C, D and E), suggesting dopaminergic degeneration. We confirmed these results by immunohistochemical

analyses, focusing on discrete dopaminergic subregions within the ventral midbrain, namely the SNc and the VTA (Figure 3.2F). Six months after hyperglycemia induction, HG rats displayed a significant loss of dopaminergic neurons in the SNc but not in the VTA, as ascertained by reduced TH+ (Figure 3.2G) and NeuN+ (Figure 3.2H) neuron counts.

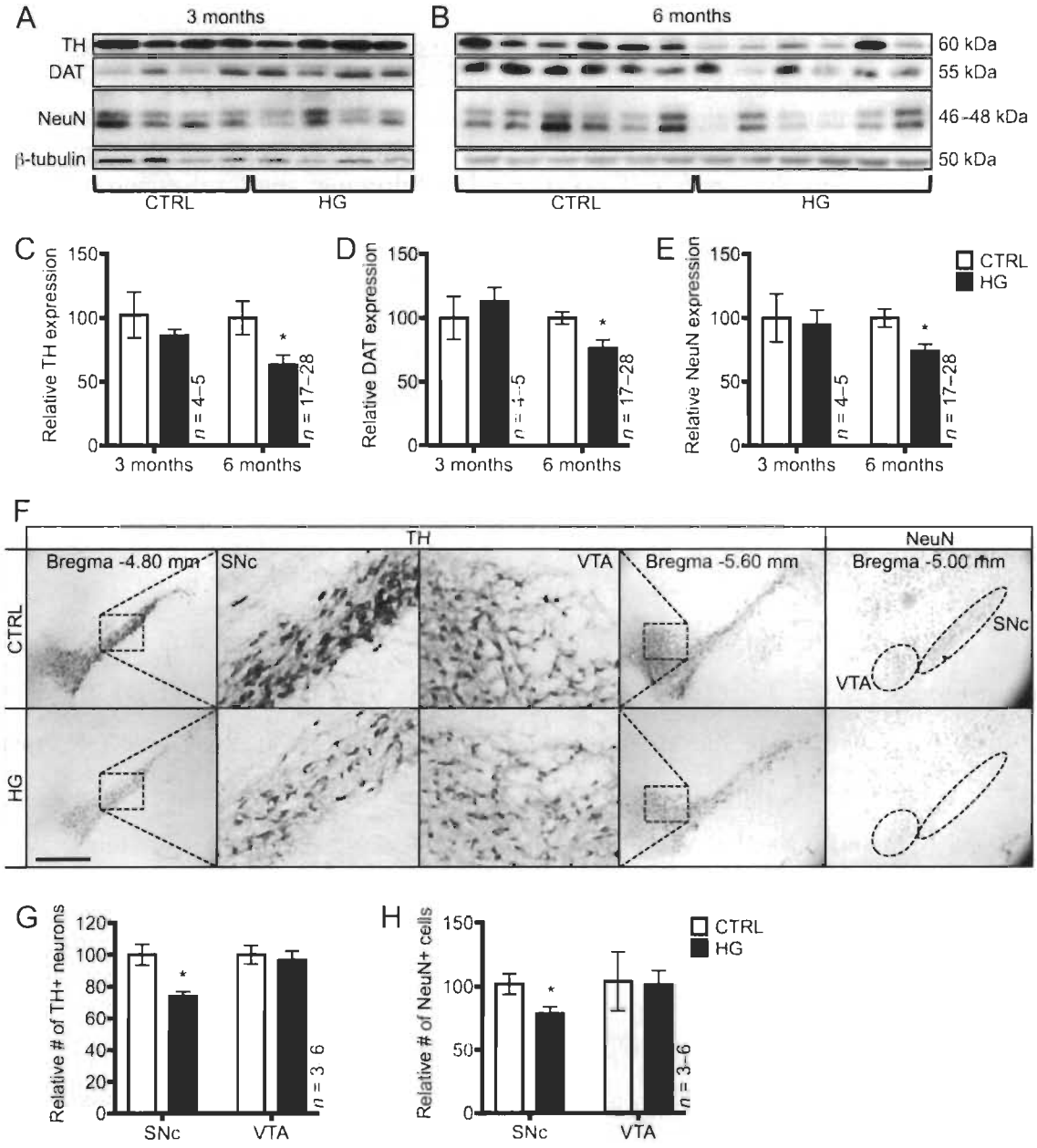

Figure 3.2 **Analyses of midbrain markers of neurodegeneration in HG rats compared to CTRL rats.**

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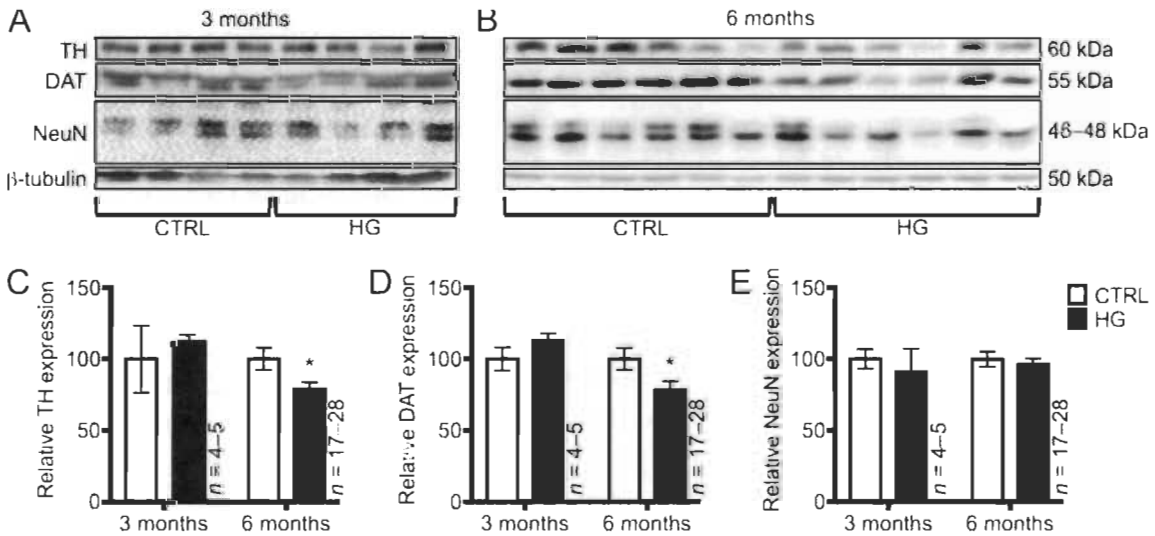
(Continued.) (A-E) Immunoblotting analyses of midbrain homogenates. A SDS-PAGE was performed on steel ball milled frozen midbrain homogenates. The midbrain contains the SNc and the VTA. (A, B) Representative immunoblots of dopaminergic markers TH and dopamine transporter (DAT), and the general neuronal marker NeuN 3 (A) and 6 (B) months following hyperglycemia-inducing NA-STZ injections. (C-E) TH (C), DAT (D) and NeuN (E) expression levels are lower in HG rats 6 months, but not 3 months, after injections compared to CTRL rats. Expression levels are normalized to β-tubulin. Blots were independently repeated at least 3 times each. (F-H) Immunohistochemical analyses of the midbrain region. Six months after hyperglycemia-inducing NA-STZ injections, frozen post-fixed brain hemispheres were cut into 20 µm-thick coronal serial sections and stained for the dopaminergic neuronal marker TH or the general neuronal marker NeuN. (F) Representative midbrain microphotographs of TH staining at 2 different anteriorities and of NeuN staining. The midbrain subregions include the SNc and the VTA, isolated by dotted lines in the NeuN microphotograph. Scalebar 1 mm. (G, H) Cell counts in the SNc and VTA demonstrated lower numbers of both TH+ (G) and NeuN+ (H) cells in the SNc of HG rats compared to CTRL rats. No differences were found in the VTA. All data presented as means ± SEM. Asterisk indicates statistical differences between the HG group and CTRL group ($*p < 0.05$).

Long-term hyperglycemia causes preferential degeneration of dopaminergic fiber terminals in the dorsal striatum

By immunoblotting (Figure 3.3A and B), we observed no difference in the expression levels of TH (Figure 3.3A and C), DAT (Figure 3.3A and D) or NeuN (Figure 3.3A and E) in the striatum of 3-month HG rats compared to CTRL rats. At 6 months, HG rats expressed lower levels of the dopaminergic markers TH (Figure 3.3B and C) and DAT (Figure 3.3A and D), indicative of dopaminergic fiber terminal degeneration in the striatum. NeuN, mainly expressed in the cell bodies of neurons, remained unchanged in HG rats at 6 months (Figure 3.3B and E), suggesting that no neurons were lost in the striatum. Immunohistochemical assays in the various subregions of the striatum (Figure 3.3F) confirmed a significant loss of dopaminergic fiber terminals in 6-month HG rats, especially in the DS, both in the DMS and DLS subregions, as ascertained by quantification of the density of TH+ fibers (Figure 3.3G). No distinctions between 6-month HG and CTRL rats were detected in the ventral regions of the striatum, namely the NAcc and the OT (Figure 3.3G). NeuN+ cell counts

did not differ between CTRL and HG groups in any of the regions investigated (Figure 3.3H), corroborating previous immunoblotting observations of unchanged striatal NeuN expression levels.

Degeneration of dopaminergic fiber terminals may be accompanied by alterations in regional DA release (Górska *et al.,* 2017). To address this possibility, microdialysis samples were collected at 3 months in the main central DA terminals, namely the DS, the NAcc and the PFC. We noticed that, even in the absence of TH loss made apparent at 3 months by immunoblotting (Figure 3.3A and C), HG rats displayed impaired basal DA release in the DS, but not in the other brain regions (Figure 3.3I). Together, these data suggest early (3 months) dopaminergic fiber terminal dysfunction, and later (6 months) degeneration in the DS coinciding with loss of neurons in the SNc where the fibers originate. It is also possible that a moderate amount of neurodegeneration was present at 3 months that we failed to detect by immunoblotting, due to the fewer numbers of rats employed at this time point. If this were the case, dampened dopamine release at 3 months would coincide with this loss of dopaminergic neurons or fibers. Immunohistochemical analyses of brains at 3 months are required to confirm either of these hypotheses. The main finding remains that neurodegeneration was observed in 6-month HG rats, as corroborated by 2 different techniques.



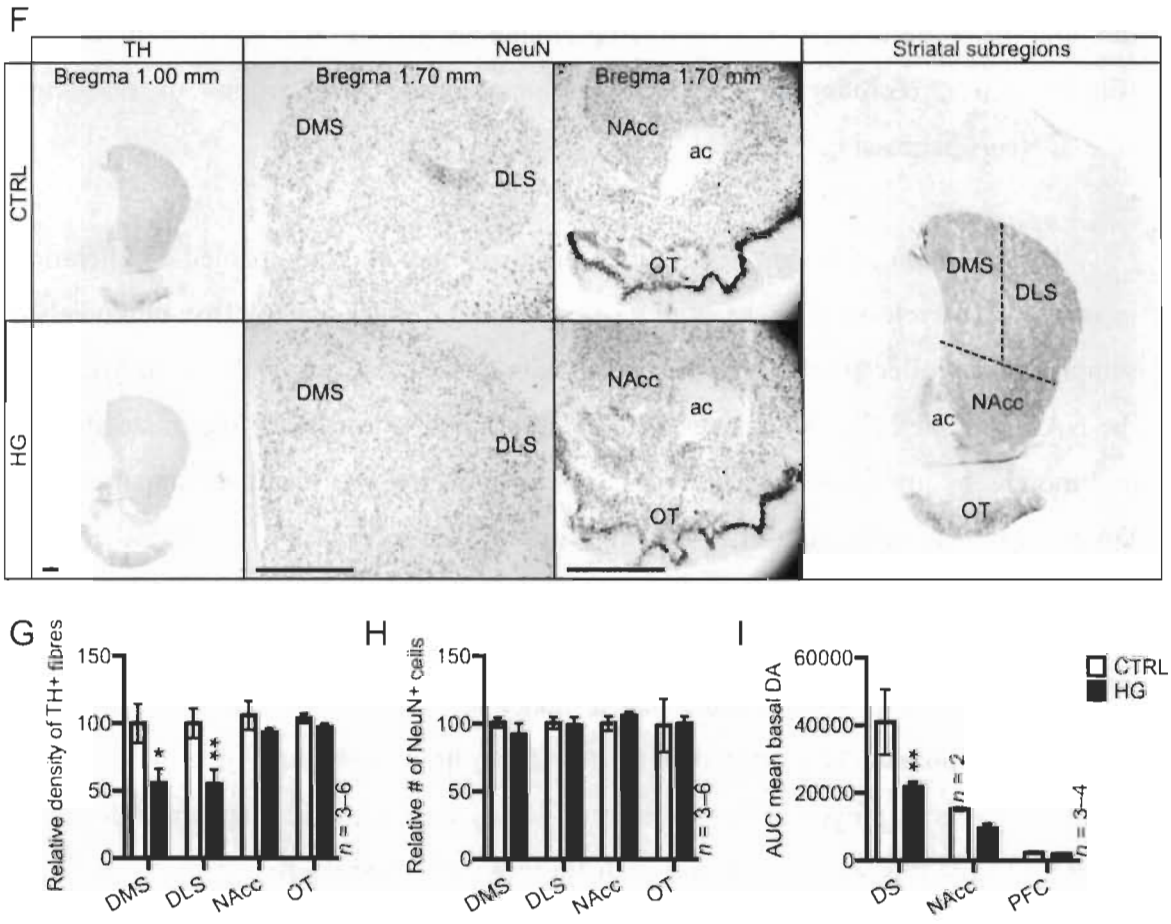


Figure 3.3 **Analyses of striatal markers of neurodegeneration in HG rats compared to CTRL rats.**

(A-E) Immunoblotting analyses of striatum homogenates. A SDS-PAGE was performed on steel ball milled frozen striatum homogenates. The striatum contains the DS and the NAcc. (A, B) Representative immunoblots of dopaminergic markers TH and DAT, and the general neuronal marker NeuN 3 (A) and 6 (B) months following hyperglycemia-inducing NA-STZ injections. (C-E) TH (C) and DAT (D) expression levels are lower in HG rats 6 months, but not 3 months, after injections compared to CTRL rats. No differences in NeuN (E) expression levels were observed at either 3 or 6 months. Expression levels are normalized to β-tubulin. Blots were independently repeated at least 3 times each. (F-H) Immunohistochemical analyses of the striatal region. Six months after hyperglycemia-inducing NA-STZ injections, frozen post-fixed brain hemispheres were cut into 20 μm-thick coronal serial sections and stained for the dopaminergic terminal fiber marker TH or the general neuronal marker NeuN. (F) Representative microphotographs of TH and NeuN staining in the striatum. The striatal subregions include the DS (including the dorsomedial striatum [DMS] and dorsolateral striatum [DLS]), the NAcc and the olfactory tubercle (OT), isolated by dotted lines in the far right panel. Scalebars 1 mm. (G) Density of TH+ fibers was assessed

by densitometric assays in the DMS, DLS, NAcc and the OT, showing less dense dopaminergic terminal fibers in the DMS and DLS, but not in the NAcc or the OT, of HG rats compared to CTRL rats. (H) Cell counts in the DMS, DLS, NAcc and OT demonstrated no differences between HG and CTRL rats. (I) Three months after injections, microdialysis samples were obtained from the NAcc, the DS and the PFC. Measurements of DA in the samples were performed by high-performance liquid chromatography and revealed lower extracellular concentrations in the DS, but not in the NAcc or the PFC, in HG rats compared to CTRL rats. All data presented as means ± SEM. Asterisk indicates statistical differences between the HG group and CTRL group (*$p < 0.05$, **$p < 0.01$). ac, anterior commissure; AUC, area under curve; DA, dopamine.

Long-term hyperglycemia does not cause substantial neurodegeneration in the prefrontal cortex or in the hippocampus

In the PFC and the HPC, we report no sensible changes of TH or DAT expression at either 3 (Figure 3.4A, C and D; Figure 3.5A, C and D) or 6 months (Figure 3.4B, C and D; Figure 3.5B, C and D) as revealed by immunoblotting assays (Figure 3.4A and B; Figure 3.5A and B). In addition, hyperglycemia sustained for 6 months did not modulate NeuN expression (Figure 3.4A, B and E; Figure 3.5A, B and E), suggesting no visible degeneration in neuronal cell bodies housed in the PFC or the HPC. Immunohistochemical analyses confirmed these findings since no changes in NeuN+ cell counts were observed in HG rats at 6 months compared to the CTRL group (Figure 3.4F and G; Figure 3.5F and G). Lack of neuronal degeneration in the HPC is corroborated by another study in long-term diabetic mice (Duarte *et al.,* 2012).

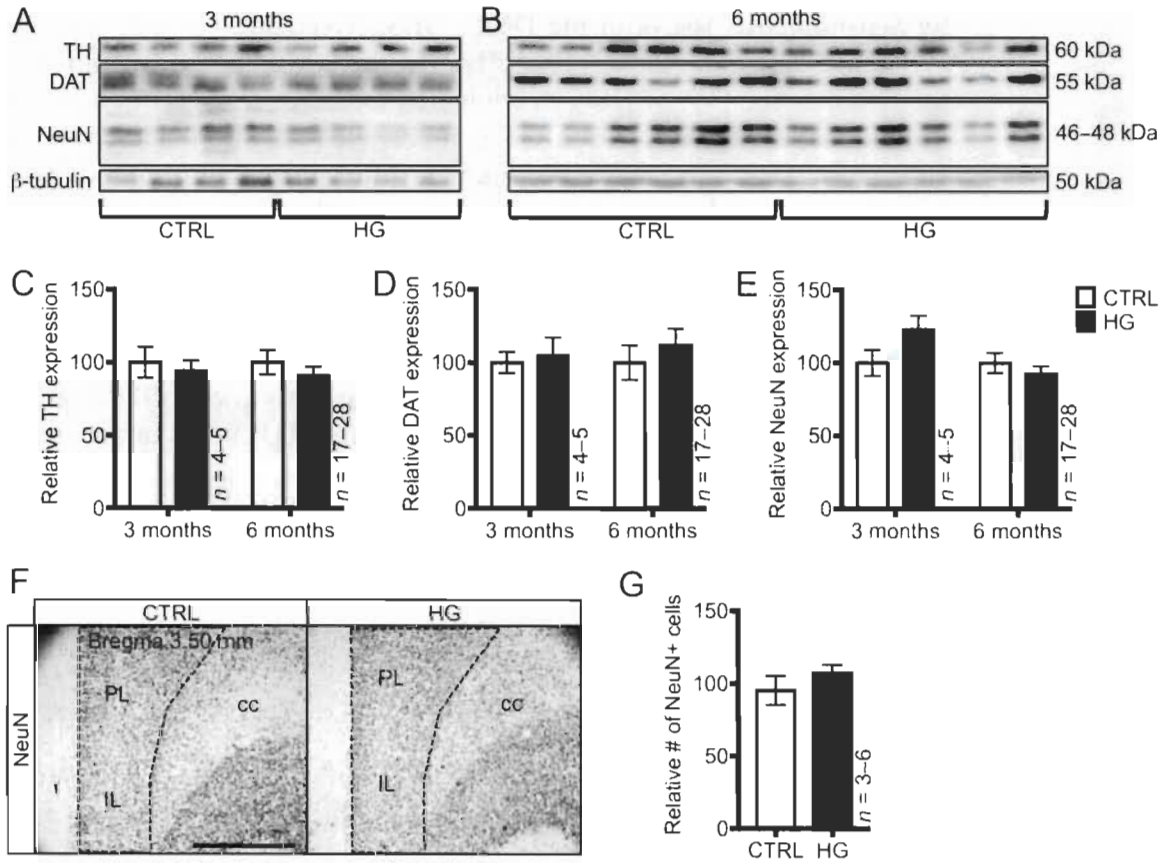


Figure 3.4 **Analyses of prefrontal markers of neurodegeneration in HG rats compared to CTRL rats.**

(A-E) Immunoblotting analyses of PFC homogenates. A SDS-PAGE was performed on steel ball milled frozen PFC homogenates. (A, B) Representative immunoblots of dopaminergic markers TH and DAT, and the general neuronal marker NeuN 3 (A) and 6 (B) months following hyperglycemia-inducing NA-STZ injections. β-tubulin blots served as a loading standard. (C-E) No differences in TH (C), DAT (D) or NeuN (E) expression levels were observed at either 3 or 6 months. Expression levels are normalized to β-tubulin. Blots were independently repeated at least 3 times each. (F, G) Immunohistochemical analyses of the PFC region. Six months after hyperglycemia-inducing NA-STZ injections, frozen post-fixed brain hemispheres were cut into 20 µm-thick coronal serial sections and stained for the general neuronal marker NeuN. (F) Representative microphotographs of NeuN staining. Prelimbic (PL) and infralimbic (IL) subregions of the medial PFC are isolated by dotted lines. Scalebar 1 mm. (G) No differences in NeuN+ cell counts in the PFC of HG rats compared to CTRL rats. All data presented as means ± SEM. cc, corpus callosum.

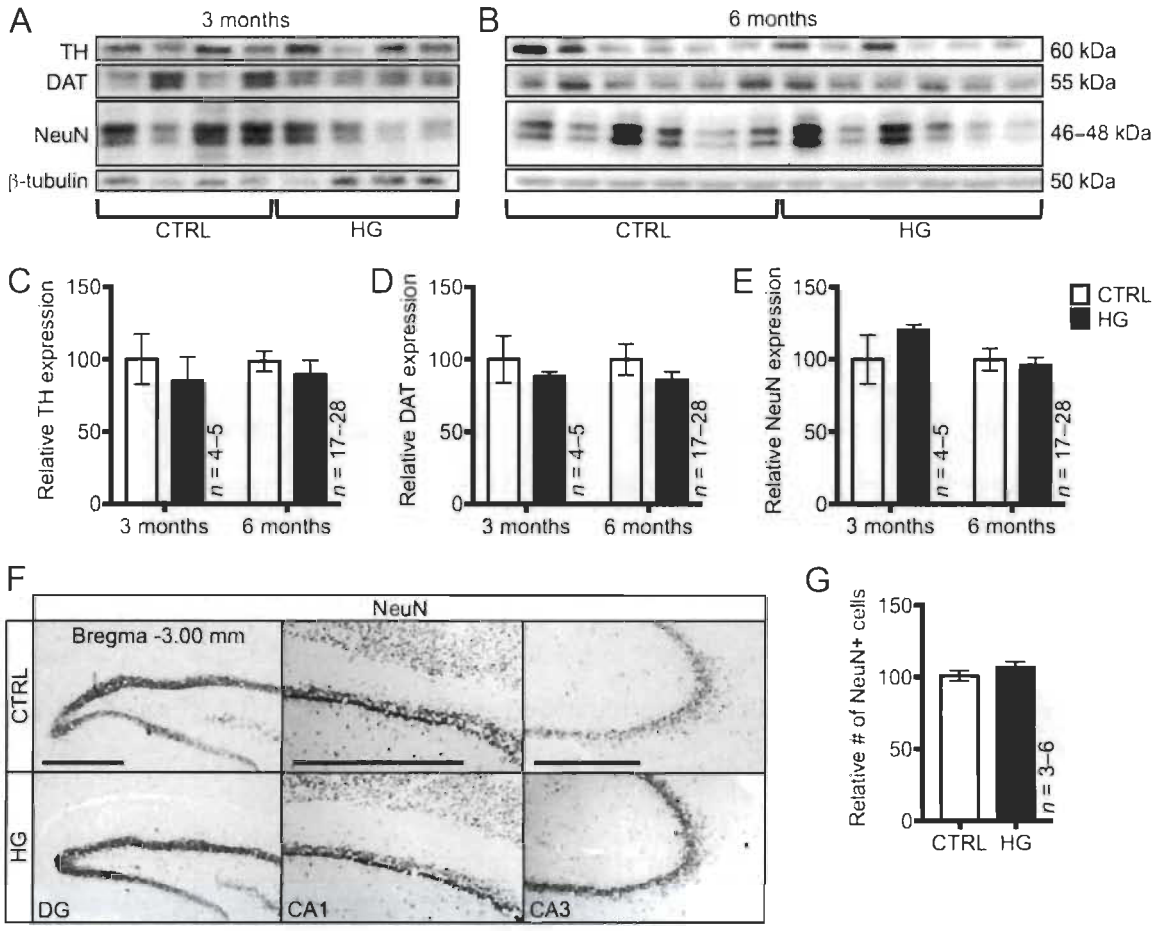
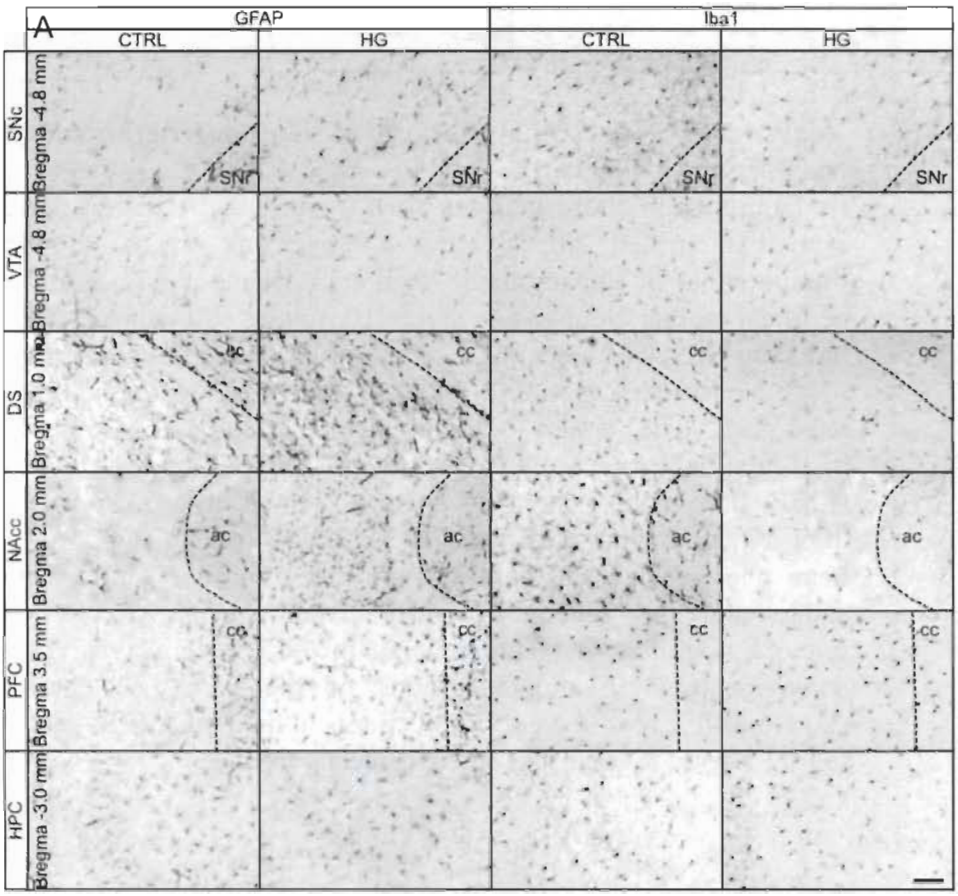


Figure 3.5 **Analyses of hippocampal markers of neurodegeneration in HG rats compared to CTRL rats.**

(A-E) Immunoblotting analyses of HPC. A SDS-PAGE was performed on steel ball milled frozen HPC homogenates. (A, B) Representative immunoblots of dopaminergic markers TH and DAT, and the general neuronal marker NeuN 3 (A) and 6 (B) months following hyperglycemia-inducing NA-STZ injections. β-tubulin blots served as a loading standard. (C-E) No differences in TH (C), DAT (D) or NeuN (E) expression levels were observed at either 3 or 6 months. Expression levels are normalized to β-tubulin. Blots were independently repeated at least 3 times each. (F, G) Immunohistochemical analyses of the HPC region. Six months after hyperglycemia-inducing NA-STZ injections, frozen post-fixed brain hemispheres were cut into 20 µm-thick coronal serial sections and stained for the general neuronal marker NeuN. (F) Representative microphotographs of NeuN staining in the dentage gyrus (DG), *Cornu ammonis* (CA) 1 and 3 regions of the HPC. Scalebars 0.5 mm. (G) No differences in NeuN+ cell counts in the HPC of HG rats compared to CTRL rats. All data presented as means ± SEM.

Long-term hyperglycemic rats display astrogliosis and loss of microglial cells in degenerated dopaminergic regions

Six months following hyperglycemia induction, HG rats exhibited greater GFAP+ staining revealed by immunohistochemistry in the SNc and DS (Figure 3.6A), both regions displaying dopaminergic degeneration. Interestingly, these same regions also showed a significant decrease of Iba+ staining (Figure 3.6A). In 6-month HG rats, this apparent astrogliosis and loss of microglial cells was confirmed by GFAP+ (Figure 3.6B) and Iba1+ (Figure 3.6C) cell counts. Although we could not demonstrate neurodegeneration in the NAcc (Figure 3.3F, G and H), we detected an increase in numbers of astroglial cells (Figure 3.6A and B) together with a loss of microglial cells (Figure 3.6A and C) in HG rats compared to CTRL rats. The PFC, on the other hand, displayed signs of astrogliosis (Figure 3.6A and B) without a decrease in Iba1+ cells (Figure 3.6A and C). No effects were identified in the HPC, the VTA (Figure 3.6A, B and C) or the OT (Figure 3.6B and C).



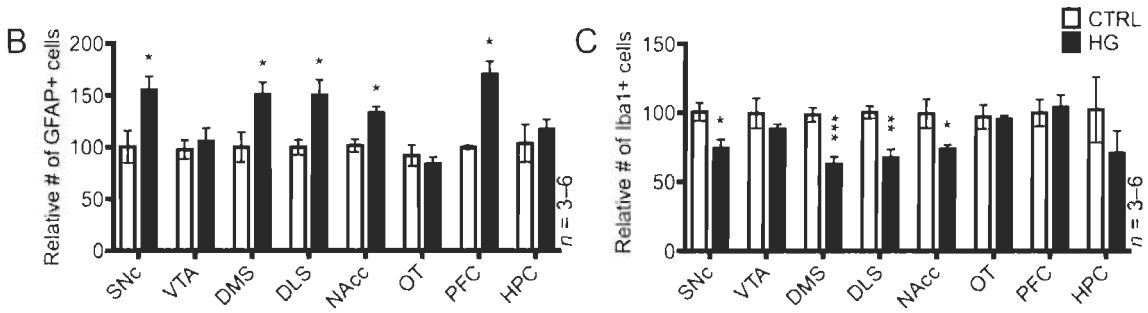


Figure 3.6 Immunohistochemical analyses of astrocytes and microglial cells in the brains of HG rats compared to CTRL rats.
Six months after hyperglycemia-inducing NA-STZ injections, frozen post-fixed brain hemispheres were cut into 20 µm-thick coronal serial sections and stained for the astrocytic marker glial fibrillary acidic protein (GFAP) or the microglial marker ionized calcium-binding adapter molecule 1 (Iba1). (A) Representative microphotographs of GFAP or Iba1 staining in the different brain regions analyzed in this article with neuroanatomical landmarks isolated by dotted lines. The HPC microphotographs are taken from the CA1 region and the PFC microphotographs are taken from the PL region. Not shown: OT and subregions of the DS (DMS and DLS). Scalebar 100 µm. (B) Cell counts in the various regions demonstrated higher numbers of GFAP+ cells in the SNc, DMS, DLS, NAcc and PFC of HG rats compared to CTRL rats. No differences were observed in other regions. (C) Cell counts in the various regions demonstrated lower numbers of Iba1+ cells in the SNc, DMS, DLS and NAcc of HG rats compared to CTRL rats. No differences were observed in other regions. All data presented as means ± SEM. Asterisk indicates statistical differences between the HG group and CTRL group (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$). SNr, substantia nigra pars reticulata.

Long-term hyperglycemic rats show altered motor behaviour

In the stepping test, HG rats took longer to cross the beam (Figure 3.7B) and made more strides (Figure 3.7C) at 3 and 6 months compared to the CTRL group. Time to initiate movement was not different between CTRL and HG groups at any time point (Figure 3.7A). While no changes were detected at the 3-month mark in the horizontal bar test, 6-month HG rats exhibited an increase in the latency to remove paws compared to their baseline results and in relation to the CTRL group (Figure 3.7D). In the adjusting step test, HG rats performed lower numbers of adjustments with either forepaw, in both forehand (white bars for CTRL or black bars for HG) and backhand (horizontally lined

white bars for CTRL or checkered bars for HG) directions, compared to CTRL rats at both time points (Figure 3.7E). In the NOR test, we did not find any difference in the amount of time HG rats spent exploring novel objects compared to the CTRL group (Figure 3.7F). This was confirmed by expressing novel object exploration times in relation to old object exploration times, yielding a discrimination index that did not significantly contrast between CTRL and HG rats (Figure 3.7G).

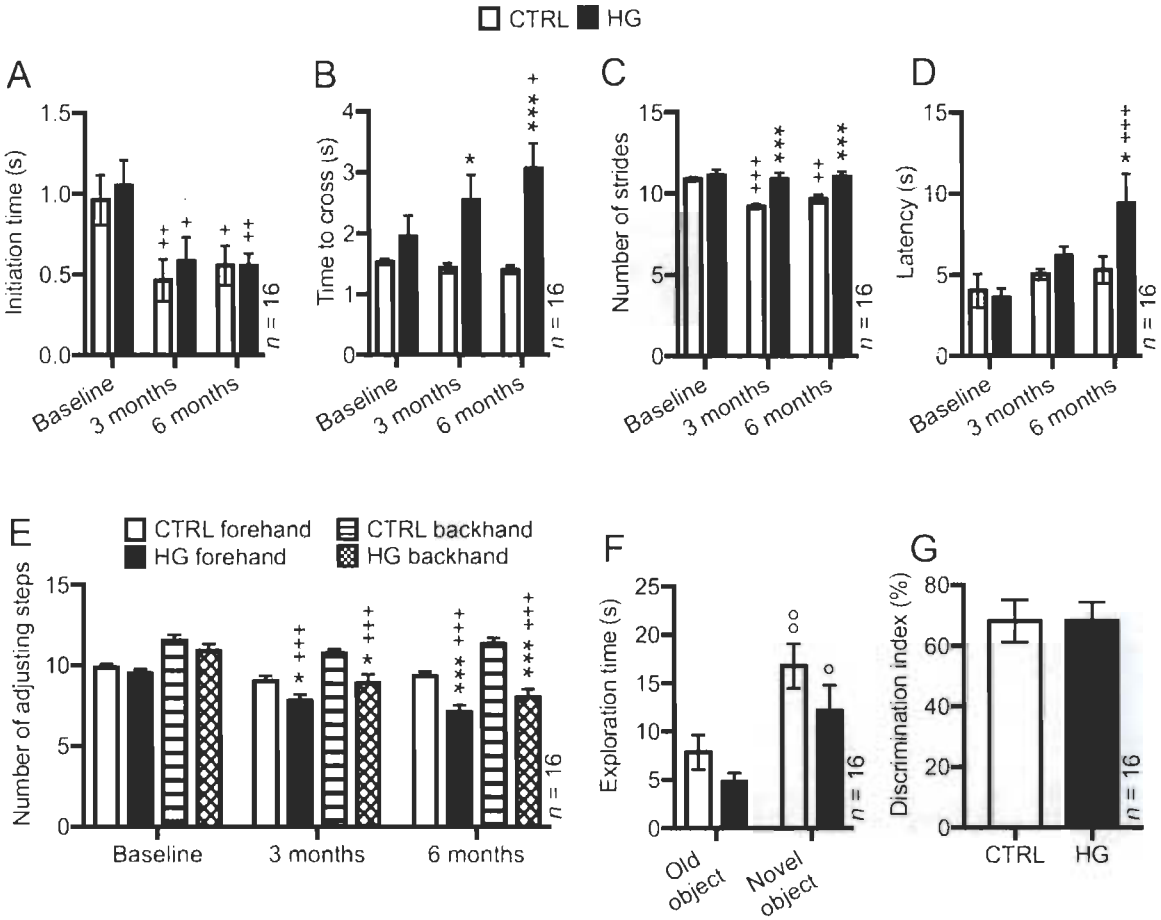


Figure 3.7 **Behavioral assessments of HG rats compared to CTRL rats.**
Three or six months after hyperglycemia-inducing NA-STZ injections, rats were subjected to behavioral tests to evaluate the presence of motor (A-E) or memory impairments (F, G). (A-C) A stepping test was conducted, consisting in a beam-crossing task. Both groups improved their time to initiate movement at 3 and 6 months compared to baseline, although no differences were observed between CTRL and HG rats (A). At 3 and 6 months, however, HG rats took longer than CTRL rats to cross the beam, and their results at 6 months grew significantly different from baseline (B). HG rats also made more strides to cross the beam than

CTRL rats at 3 and 6 months, and only the latter performed better than baseline tests at both 3 and 6 months (C). (D) For the horizontal bar test, descent latency was measured as the time taken for rats to remove both forepaws from a horizontal bar. At 6 months, but not 3 months, HG rats took longer to remove both paws compared to CTRL rats and their own baseline. (E) During the forepaw adjusting step test, the rat was moved slowly sideways along the table surface by an experimenter, first in a forehand and then in a backhand direction. Numbers of adjusting steps made were fewer in HG rats at 3 and 6 months compared to CTRL rats and their own baseline. (F, G) A novel object recognition task, based on exploratory behavior of newly introduced elements in an environment, was used to assess non-spatial working memory in rats at 3 months. Rats were not tested at 6 months due to loss of eyesight (cataracts) of HG rats. Both CTRL and HG groups explored the novel object for a longer period of time than the old object (F). No differences were seen between groups in their capacity to discriminate old and novel objects (G). All data presented as means ± SEM. Asterisk indicates statistical differences between the HG group and CTRL group (***$p < 0.001$ and *$p < 0.05$). Plus signs indicate statistical differences between results at the 3- or 6-month time point and the baseline evaluation (+++$p < 0.001$, ++$p < 0.01$ and +$p < 0.05$). For the novel object recognition (NOR) task, circles indicate statistical differences between exploration time of the novel object and the old object (OO$p < 0.01$ and O$p < 0.05$).

Discussion

The current study sheds a new light on neuronal degeneration in long-term hyperglycemia, innovative in that the condition was extended to a penultimate length of 6 months in the NA-STZ rat model without the need for glycemia-lowering treatments. The noxious effects of long-term hyperglycemia were evaluated in the nigrostriatal and mesocorticolimbic pathways. Our findings are relevant to elucidate the interplay between hyperglycemia and age-related neurodegenerative conditions, such as Parkinson's disease.

To the best of our knowledge, this is the first time that hyperglycemia-induced neuropathological manifestations have been related to glucose measurements in brain tissue homogenates and extracellular intracerebral microdialysates. Since glucose overload is one of the major contributors to diabetic complications (Brownlee, 2005, Tomlinson and Gardiner, 2008), the sole observation that glucose levels are significantly

increased indiscriminately in all brain regions studied herein raises the question as to why some structures degenerate preferentially over time. It is now well appreciated that oxidative stress is a key player in the nigrostriatal degeneration observed in Parkinson's disease (Schapira and Jenner, 2011), a relative susceptibility attributable to various factors: 1) All dopaminergic neurons are submitted to oxidative insults from dopamine auto-oxidation and the elevated activity of monoamine oxidase (Goldstein *et al.,* 2014); 2) Specifically, the SNc holds high levels of iron ions that generate highly reactive hydroxyl radicals, but also possesses low levels of the ubiquitous antioxidant molecule glutathione (Chinta and Andersen, 2008); 3) Dopaminergic nigrostriatal neurons are endowed with an exceptionally dense dendritic arborization, rich in mitochondria and constantly requiring costly metabolic sustenance (Surmeier *et al.,* 2017). On the basis of these physiological peculiarities, we argue that the relative vulnerability of the nigrostriatal pathway, as compared here to the mesocorticolimbic pathway, stems from its elevated basal oxidative burden paired with its scanty survival mechanisms faced to sustained hyperglycemia over the course of aging.

In our long-term model of hyperglycemia, other pathological indices manifested themselves concurrently to dopaminergic neurodegeneration, expressly a prominent astrogliosis and loss of microglial cells both in the SNc and the DS. Astrogliosis has been observed in several animal models of diabetes (Nagayach *et al.,* 2014, Rostami *et al.,* 2017), with implications for dopaminergic neuron death (Cabezas *et al.,* 2014). However, our present study constitutes an original report of the fate of glial cells following long-term hyperglycemia, moreover demonstrating astrogliosis in the SNc of STZ-treated rats for the first time. Microgliosis is well appreciated in retinas and peripheral nerves of diabetic models (Mazzeo *et al.,* 2017) though less well known in the central nervous system (Nagayach *et al.,* 2014, Oliveira *et al.,* 2016). Yet, microglial cell degeneration is an under-recognized feature in diabetes as well as in neuropathologies. In our model, it is likely that microglial cells are lost due to the sustained oxidative burden, supported by observations that this population is susceptible to oxidative stress in health and in disease (Streit *et al.,* 2008). In fact, iron accumulation in the aging human brain promotes oxidative stress and may lead to microglial degeneration

(Streit *et al.,* 2008). Interestingly, in patients deceased from septic shock, microglial apoptosis was correlated to hyperglycemia, possibly due to lack of GLUT5 downregulation (Polito *et al.,* 2011). Other studies have found that advanced-glycation end-products (AGEs), abundant in diabetic and aging brains, cause microglial apoptosis in a dose-dependent manner (Khazaei *et al.,* 2008, Sabokdast *et al.,* 2015). Microglial-derived AGEs were further shown to promote dopaminergic neuron death (Bayarsaikhan *et al.,* 2016). Inasmuch as previous reports reveal microgliosis in rodent models of diabetes within 2 months (Nagayach *et al.,* 2014, Oliveira *et al.,* 2016), we suggest that microglial cells are activated early in hyperglycemia, in turn causing the activation and proliferation of astrocytes (Liddelow *et al.,* 2017). The latter persist in high numbers at 6 months while microglial cells degenerate over time due to the overwhelming oxidative load of sustained hyperglycemia. Although confirmation of this tentative timeline is required, it remains interesting that this astrogliosis paired to microglial cell loss preferentially targets dopaminergic regions.

Motor deficits possibly arising from this nigrostriatal neurodegeneration expressed themselves in tests employed to characterize motor impairment in rat models of Parkinson's disease (Pinna and Morelli, 2014). In particular, HG rats showed bradykinesia and made greater numbers of small steps, bearing likeness to motor symptoms found in parkinsonian patients (Pinna and Morelli, 2014). Together with immunohistochemical, immunoblotting and microdialysis studies, these data provide new insight on the higher occurrence of Parkinson's disease in aging diabetic patients (Cereda *et al.,* 2011, Santiago and Potashkin, 2013, Sun *et al.,* 2012). One study investigating blood biomarkers in 99 *de novo* patients of Parkinson's disease even found their average fasting glycemia (5.64 mM) comparable to that of pre-diabetic individuals (5.55 mM) (Santiago and Potashkin, 2015). Of high interest, recent clinical trials (Barker *et al.,* 2013) are employing drug-repurposing strategies in Parkinson's disease patients using anti-diabetic treatments such as exenatide (Aviles-Olmos *et al.,* 2013) and thiazolidinediones (NET-PD FS-ZONE, 2015). Whereas the link between Parkinson's disease and diabetes remains foggy, some have suggested shared dysfunctional cellular pathways (Santiago and Potashkin, 2013) or neurochemical hypotheses (Deng *et al.,*

2012). Indeed, high reactivity between the glycation metabolite methylglyoxal and DA was recently shown to yield a salsolinol-like toxin specific to dopaminergic neurons (Song *et al.,* 2014). Interestingly, this toxin can be found in brains of parkinsonian patients (Deng *et al.,* 2012).

In summary, our results mend our appreciation of the relationship that may exist between diabetes and Parkinson's disease, by demonstrating the preferential nigrostriatal dopaminergic neurodegeneration that occurs in long-term hyperglycemia. As it is expressed in uncontrolled diabetes, hyperglycemia may cause premature aging of the central nervous system, fostering the development of age-related neurodegenerative disease.

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Supplementary data

Metabolic follow-up and disease progression

Each individual rat's weight (Supplementary Figure S1A), food and water intake (Supplementary Figure S1B), glycemia (Supplementary Figure S1C), and general health were monitored on a regular basis. Glycemia was estimated twice a week and weight was measured weekly. Food and water intake was assessed for group-paired cages, since rats were not housed individually.

Oral glucose tolerance test: On the morning of their sacrifice, 6 months after NA-STZ injections, we performed an oral glucose tolerance test (OGTT; Supplementary Figure S1E and F) on 6 CTRL rats and 16 HG rats. Fasted rats (12-16 h) were administered 2 g/kg of a D-glucose solution by intragastric gavage. Approximately 0.5 mL of blood was collected by the jugular vein right before the OGTT (0 min) and at 30, 60 and 120 min after administration of the bolus. Samples were kept on ice and plasma glucose and insulin were quantified immediately after. Rats were lightly anesthetized by isoflurane inhalation prior to blood sampling. Plasma glucose concentrations following the terminal OGTT were measured using the Rat Glucose Assay kit provided by Crystal Chem (Downers Grove, IL, USA), whereas plasma insulin was quantified using a Milliplex Adipokine Magnetic Bead Multiplex Assay (Millipore; Etobicoke, ON, Canada).

Glycated hemoglobin: In a different group of rats, plasma hemoglobin A1c (HbA1c) levels (Supplementary Figure S1D) were assessed terminally at 3 or 6 months following NA-STZ injections. On the morning of their sacrifice, intracardiac blood samples from CTRL (3 months $n = 4$; 6 months $n = 10$) and HG (3 months $n = 5$; 6 months $n = 16$) rats were drawn when under deep anesthesia by isoflurane inhalation. Glycated Hb was measured immediately using a rat HbA1c assay kit (Crystal Chem; Downers Grove, IL, USA).

Table S1

Antibodies used for immunohistochemical (IHC) or immunoblotting (WB) experiments

Antibody	Antigen	Host/Isotype/ Class	Manufacturer	Catalog number	Dilution
Primary	Rat β-tubulin	mouse/IgG2a κ light chain/monoclonal	Santa Cruz Biotechnology	sc-80005	1:2000
	Dopamine transporter	rabbit/IgG/ polyclonal	Santa Cruz Biotechnology	sc-14002	1:100
	Glial fibrillary acidic protein	rabbit/IgG/ polyclonal	Abcam	ab7260	1:5000 (IHC)
	Ionized calcium-binding adapter molecule 1	rabbit/ polyclonal	Wako	019-19741	1:2500 (IHC)
	NeuN	mouse/IgG1/ monoclonal	Millipore Sigma	MAB377	1:1000 (IHC)
	NeuN	rabbit/IgG/ polyclonal	Cell Signaling Technology	24307	1:1000 (WB)
	Tyrosine hydroxylase	mouse/IgG1/ monoclonal	Millipore Sigma	T1299	1:4000 (IHC) 1:4000 (WB)
Secondary	Rabbit	mouse/IgG/ monoclonal (HRP conjugated)	Santa Cruz Biotechnology	sc-2357	1:5000 (IHC) 1:10 000 (WB)
	Mouse	recombinant IgGκ light chain (HRP conjugated)	Santa Cruz Biotechnology	sc-516102	1:5000 (IHC) 1:10 000 (WB)

Table S2

Detailed statistics

Figure	Statistical test	Factor	*T- or F*-value	*P*-value
Figure 3.1A	Unpaired, two-tailed *t*-test	Midbrain glycemia	$T_{(7)} = 3.823$	$p = 0.0065$
		Striatum glycemia	$T_{(7)} = 2.627$	$p = 0.0341$
		PFC glycemia	$T_{(5)} = 2.982$	$p = 0.0307$
		HPC glycemia	$T_{(4)} = 3.755$	$p = 0.0199$
Figure 3.1B	Unpaired, two-tailed *t*-test	Midbrain glycemia	$T_{(12)} = 2.36$	$p = 0.0360$
		Striatum glycemia	$T_{(15)} = 3.401$	$p = 0.0040$
		PFC glycemia	$T_{(22)} = 2.785$	$p = 0.0108$
		HPC glycemia	$T_{(14)} = 2.181$	$p = 0.0467$
Figure 3.1C	Unpaired, two-tailed *t*-test	SNc glycemia	$T_{(6)} = 3.488$	$p = 0.0130$
		VTA glycemia	$T_{(7)} = 5.515$	$p = 0.0009$
		DS glycemia	$T_{(6)} = 4.03$	$p = 0.0069$
		NAcc glycemia	$T_{(8)} = 3.461$	$p = 0.0086$

Figure	Statistical test	Factor	*T- or F*-value	*P*-value
Figure 3.2C	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.9727$	$p = 0.3631$
		6 months glycemia	$T_{(43)} = 2.589$	$p = 0.013$
Figure 3.2D	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(6)} = 0.6376$	$p = 0.5473$
		6 months glycemia	$T_{(17)} = 2.274$	$p = 0.036$
Figure 3.2E	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.2629$	$p = 0.8002$
		6 months glycemia	$T_{(43)} = 2.462$	$p = 0.018$
Figure 3.2G	Unpaired, two-tailed *t*-test	SNc glycemia	$T_{(7)} = 3.471$	$p = 0.0104$
		VTA glycemia	$T_{(7)} = 0.4764$	$p = 0.6483$
Figure 3.2H	Unpaired, two-tailed *t*-test	SNc glycemia	$T_{(7)} = 2.439$	$p = 0.0448$
		VTA glycemia	$T_{(7)} = 0.1207$	$p = 0.9073$
Figure 3.3C	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.5223$	$p = 0.6176$
		6 months glycemia	$T_{(43)} = 2.432$	$p = 0.019$
Figure 3.3D	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.9898$	$p = 0.3552$
		6 months glycemia	$T_{(10)} = 2.256$	$p = 0.048$
Figure 3.3E	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.4726$	$p = 0.6509$
		6 months glycemia	$T_{(42)} = 0.5358$	$p = 0.595$
Figure 3.3G	Unpaired, two-tailed *t*-test	DMS glycemia	$T_{(7)} = 2.443$	$p = 0.0446$
		DLS glycemia	$T_{(7)} = 2.628$	$p = 0.0340$
		NAcc glycemia	$T_{(7)} = 1.495$	$p = 0.1785$
		OT glycemia	$T_{(7)} = 1.462$	$p = 0.1871$
Figure 3.3H	Unpaired, two-tailed *t*-test	DMS glycemia	$T_{(7)} = 0.7135$	$p = 0.4986$
		DLS glycemia	$T_{(7)} = 0.1507$	$p = 0.8845$
		NAcc glycemia	$T_{(7)} = 1.134$	$p = 0.2942$
		OT glycemia	$T_{(7)} = 0.0886$	$p = 0.9319$
Figure 3.3I	Two-way ANOVA	Region × glycemia	$F_{(2,14)} = 3.60$	$p = 0.0546$
		Region	$F_{(2,14)} = 34.3$	$p < 0.0001$
		Glycemia	$F_{(1,14)} = 7.59$	$p = 0.0155$
Figure 3.4C	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.4794$	$p = 0.6463$
		6 months glycemia	$T_{(44)} = 0.9042$	$p = 0.3708$
Figure 3.4D	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 0.3013$	$p = 0.7719$
		6 months glycemia	$T_{(15)} = 0.7387$	$p = 0.472$
Figure 3.4E	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(7)} = 1.705$	$p = 0.1319$
		6 months glycemia	$T_{(44)} = 0.8821$	$p = 0.3825$
Figure 3.4G	Unpaired, two-tailed *t*-test	PFC glycemia	$T_{(7)} = 1.168$	$p = 0.2810$
Figure 3.5C	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(5)} = 0.6179$	$p = 0.5637$
		6 months glycemia	$T_{(45)} = 0.6687$	$p = 0.5071$
Figure 3.5D	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(5)} = 0.8448$	$p = 0.4368$
		6 months glycemia	$T_{(25)} = 1.272$	$p = 0.215$
Figure 3.5E	Unpaired, two-tailed *t*-test	3 months glycemia	$T_{(5)} = 1.365$	$p = 0.2303$
		6 months glycemia	$T_{(47)} = 0.4349$	$p = 0.6656$
Figure 3.5G	Unpaired, two-tailed *t*-test	HPC glycemia	$T_{(7)} = 0.9185$	$p = 0.3889$

Figure	Statistical test	Factor	*T*- *or F*-value	*P*-value
Figure 3.6B	Unpaired, two-tailed *t*-test	SNc glycemia	$T_{(7)} = 2.546$	$p = 0.0383$
		VTA glycemia	$T_{(7)} = 0.4241$	$p = 0.6842$
		DMS glycemia	$T_{(7)} = 2.637$	$p = 0.0336$
		DLS glycemia	$T_{(7)} = 3.100$	$p = 0.0173$
		NAcc glycemia	$T_{(7)} = 3.432$	$p = 0.0110$
		OT glycemia	$T_{(7)} = 1.038$	$p = 0.3340$
		PFC glycemia	$T_{(7)} = 3.292$	$p = 0.0133$
		HPC glycemia	$T_{(7)} = 0.7282$	$p = 0.4901$
Figure 3.6C	Unpaired, two-tailed *t*-test	SNc glycemia	$T_{(7)} = 2.564$	$p = 0.0373$
		VTA glycemia	$T_{(7)} = 1.295$	$p = 0.2363$
		DMS glycemia	$T_{(7)} = 5.882$	$p = 0.0006$
		DLS glycemia	$T_{(7)} = 3.885$	$p = 0.0060$
		NAcc glycemia	$T_{(7)} = 3.077$	$p = 0.0179$
		OT glycemia	$T_{(7)} = 0.2407$	$p = 0.8167$
		PFC glycemia	$T_{(7)} = 0.2923$	$p = 0.7785$
		HPC glycemia	$T_{(7)} = 1.282$	$p = 0.2405$
Figure 3.7A	Two-way ANOVA	Time × glycemia	$F_{(2,60)} = 0.28$	$p = 0.7554$
		Time	$F_{(2,60)} = 13.7$	$p < 0.0001$
		Glycemia	$F_{(1,30)} = 0.18$	$p = 0.6742$
Figure 3.7B	Two-way ANOVA	Time × glycemia	$F_{(2,60)} = 2.61$	$p = 0.0816$
		Time	$F_{(2,60)} = 1.66$	$p = 0.1972$
		Glycemia	$F_{(1,30)} = 24.2$	$p < 0.0001$
Figure 3.7C	Two-way ANOVA	Time × glycemia	$F_{(2,60)} = 7.20$	$p = 0.0016$
		Time	$F_{(2,60)} = 11.1$	$p < 0.0001$
		Glycemia	$F_{(1,30)} = 10.5$	$p = 0.0029$
Figure 3.7D	Two-way ANOVA	Time × glycemia	$F_{(2,60)} = 3.23$	$p = 0.0463$
		Time	$F_{(2,60)} = 7.51$	$p = 0.0012$
		Glycemia	$F_{(1,30)} = 2.96$	$p = 0.0952$
Figure 3.7E forehand	Two-way ANOVA	Time × glycemia	$F_{(2,60)} = 8.95$	$p = 0.0004$
		Time	$F_{(2,60)} = 23.6$	$p < 0.0001$
		Glycemia	$F_{(1,30)} = 13.3$	$p = 0.0010$
Figure 3.7E backhand	Two-way ANOVA	Time × glycemia	$F_{(2,60)} = 9.99$	$p = 0.0002$
		Time	$F_{(2,60)} = 18.3$	$p < 0.0001$
		Glycemia	$F_{(1,30)} = 10.5$	$p = 0.0028$
Figure 3.7F	Two-way ANOVA	Object × glycemia	$F_{(1,60)} = 0.13$	$p = 0.7102$
		Object	$F_{(1,60)} = 18.4$	$p < 0.0001$
		Glycemia	$F_{(1,60)} = 4.17$	$p = 0.0455$
Figure 3.7G	Unpaired, two-tailed *t*-test	Glycemia	$T_{(30)} = 0.0628$	$p = 0.9503$

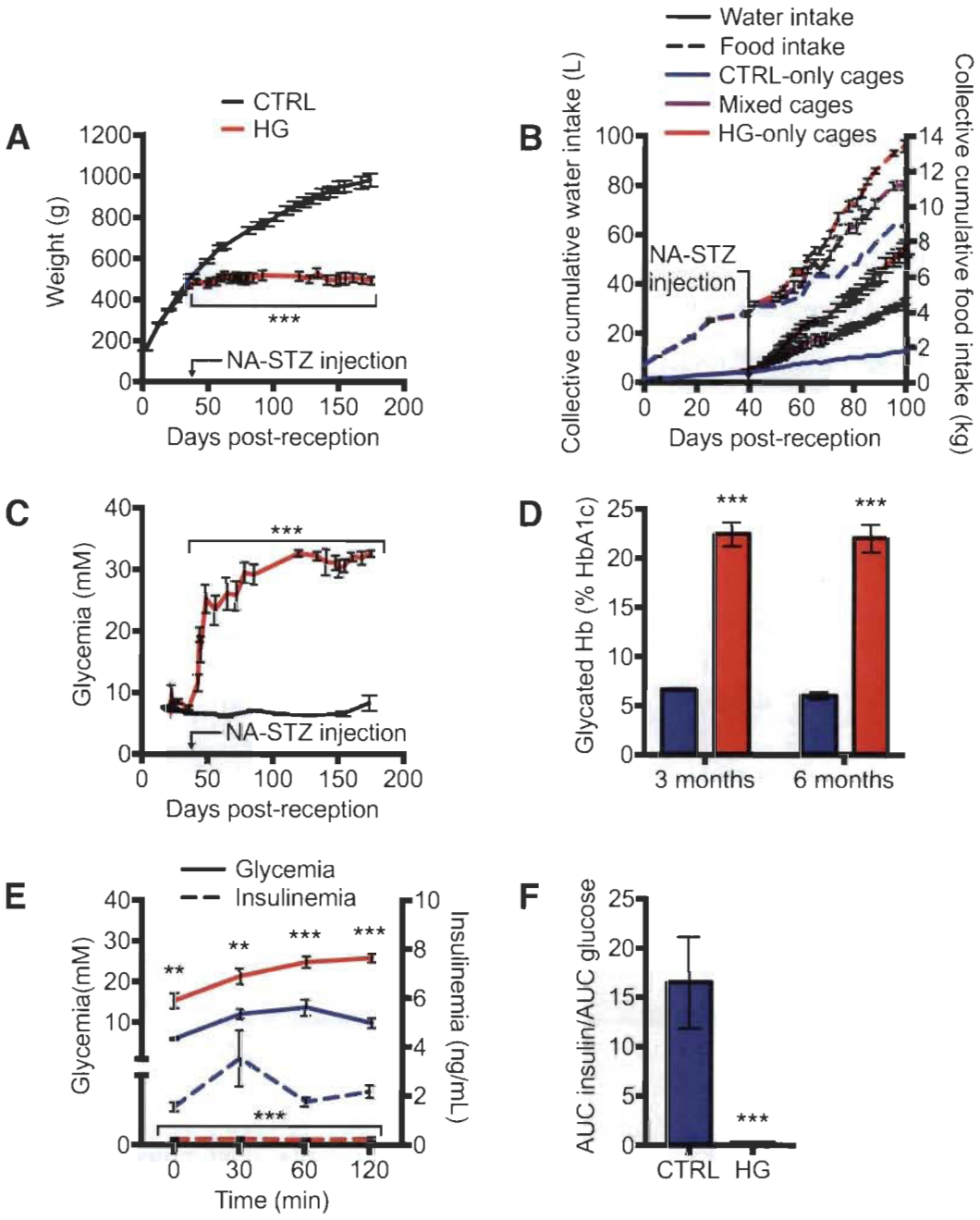

Figure S1 **Metabolic follow-up.**

(A) Mean weight of CTRL and HG rats from the beginning to the end of experiments. (B) Mean total cumulative intake of water (left y-axis) and food (right y-axis) over time. In blue, mean cumulative intake of cages containing solely CTRL animals. In red, mean cumulative intake of cages containing solely HG animals. In purple, mean cumulative intake of cages containing both CTRL and HG animals. (C) Mean glycemia of CTRL and HG rats from the beginning to the end of experiments. (D) Percentage of glycated hemoglobin (Hb) of CTRL and HG animals 3 and 6 months after NA-STZ injections. CTRL 3 months: 6.6%

(49 mmol/mol). CTRL 6 months: 6.0% or (42 mmol/mol). HG 3 months: 22.3% or (220 mmol/mol). HG 6 months: 21.9% or (215 mmol/mol). (E) Oral glucose tolerance test (OGTT) of fasted CTRL and HG rats 6 months after injections. (F) Ratio of AUC of insulin on glucose from the OGTT. Asterisks indicate statistical differences between the HG and the CTRL groups (***$p < 0.001$ and **$p < 0.01$).

Glycated Hb: In a different group of rats, plasma HbA1c levels (Supplementary Figure S1D) were assessed terminally at 3 or 6 months following NA-STZ injections. On the morning of their sacrifice, intracardiac blood samples from CTRL (3 months n = 4; 6 months n = 10) and HG (3 months n = 5; 6 months n = 16) rats were drawn when under deep anesthesia by isoflurane inhalation. Glycated Hb was measured immediately using a rat HbA1c assay kit (Crystal Chem; Downers Grove, IL, USA).

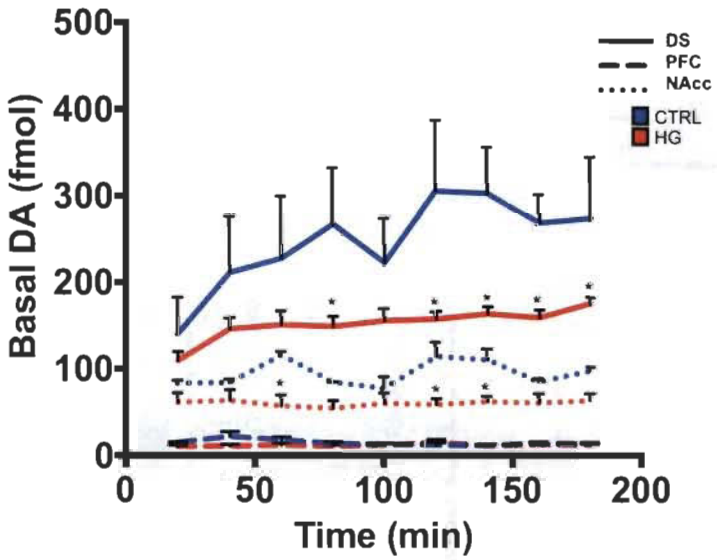

Figure S2 **Time course of microdialysis experiments performed to measure basal DA.**
Three months after injections, microdialysis samples were obtained from the NAcc, the DS and the PFC. Measurements of DA in the samples were performed by high-performance liquid chromatography and revealed lower extracellular concentrations in the DS, but not in the NAcc or the PFC, in HG rats compared to CTRL rats. Data presented as means ± SEM. Statistical analysis was carried out by Statistica for Windows. Basal dialysate DA values, expressed as femtomoles per 20-µL dialysate, were compared between groups by two-way ANOVA. Results showing significant differences between CTRL and HG groups were subjected to Tukey's post-hoc test with $p < 0.05$ (*) as statistically significant.

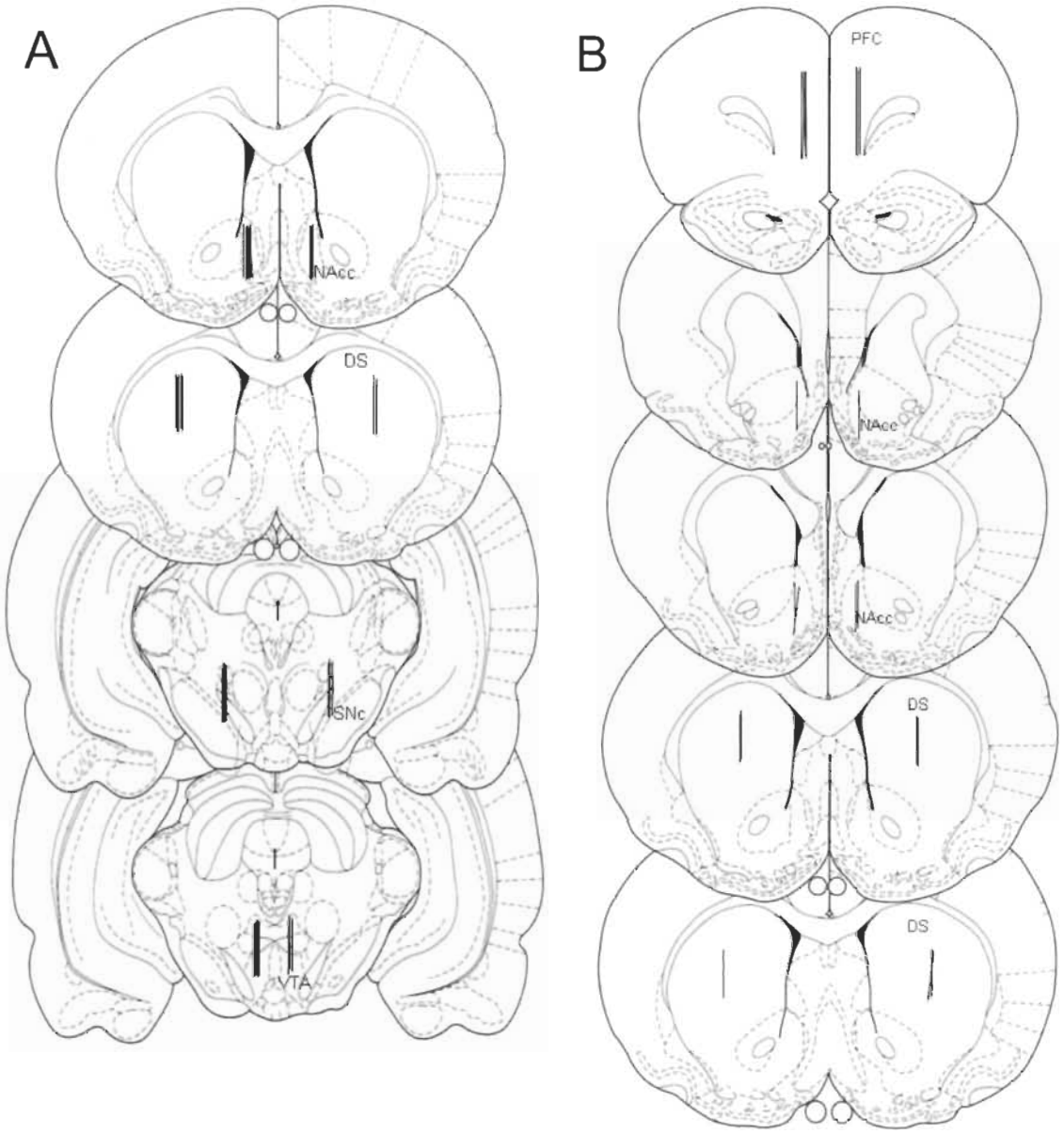

Figure S3 **Localization of dialysis probes (dialyzing portion) in microdialysis experiments performed to measure basal DA or extracellular glucose.** Three months after NA-STZ injections, microdialysis samples were obtained from acute probes inserted in (A) the NAcc, the DS, the VTA and the SNc, for glucose measurements or in the NAcc, the DS, and the PFC for DA measurements (B). At the end of experimental procedures, rats were deeply anesthetized and transcardially perfused with 50 mL of saline and 50 mL of a 4% formaldehyde/1% calcium acetate/100 mM NaCl solution. Sections were cut with a vibratome and probe location was reconstructed by referring to the Paxinos and Watson atlas (1998).

CHAPTER IV

LONG-TERM HYPERGLYCAEMIA MODIFIES SOCIAL BEHAVIOUR AND EMISSION OF ULTRASONIC VOCALISATIONS IN RATS: A POSSIBLE EXPERIMENTAL MODEL OF ALTERED SOCIABILITY IN DIABETES

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4.1 Author contributions

Justine Renaud designed most of the study and performed 90% of the manipulations. She wrote 95% of the manuscript, and prepared and edited all the figures. Jimmy Beaulieu, master's student in Maria-Grazia Martinoli's laboratory, provided assistance for immunohistochemistry experiments. Nicola Simola mentored Justine Renaud at the University of Cagliari during a short 1-month training program to perform USV recordings. He offered expertise and assistance with the design of USV experiments, and analyzed the audiograms. He also provided Figure 4.7. Maria-Grazia Martinoli, Justine Renaud's research supervisor, was the guarantor of the work and provided supervision, preparation and editing of the manuscript.

4.2 Résumé

Il ne fait plus de doute que le diabète soit un facteur de risque contribuant au déclin cognitif et aux troubles de l'humeur se manifestant avec l'âge. Néanmoins, des études adressant les effets de l'hyperglycémie chronique sur les comportements sociaux, tels que le jeu et l'agressivité, se font toujours attendre. Dans cette étude, nous avions pour but d'évaluer les comportements sociaux manifesté par des rats

chroniquement hyperglycémiques. Dans cette optique, nous avons examiné les interactions de type affiliation/exploration et agression entre des paires de rats dans une enceinte neutre, et ce, 5 mois suivant l'induction de l'hyperglycémie dans le paradigme nicotinamide-streptozotocine. Simultanément, les vocalisations ultrasoniques, porteuses de l'état affectif des sujets, étaient enregistrées. Nos résultats démontrent que les rats chroniquement hyperglycémiques sont manifestement agressifs et hyper-sociables dans des contextes de nouveauté sociale. Ceux-ci émettent également beaucoup plus de vocalisations ultrasoniques régulées en partie par le neurotransmetteur dopamine. En fait, ces comportements corrélaient avec le degré de perte d'innervation dopaminergique du striatum, mais pas avec des paramètres liés aux concentrations ou aux fonctions de l'insuline. En somme, nos résultats suggèrent un lien entre l'hyperréactivité sociale causée par l'hyperglycémie chronique et la neurotransmission dopaminergique dans le striatum, déjà reconnu pour son rôle modulateur sur les comportements sociaux. Cette étude jette une nouvelle lumière sur de possibles substrats neurobiologiques, soit la dopamine, pouvant sous-tendre les difficultés psychiatriques et socioaffectives auxquelles font face les patients diabétiques.

4.3 Full article in English: Altered social behaviour in long-term hyperglycemic rats displaying dopaminergic striatal denervation: an ultrasonic vocalisation study

Abstract

Diabetes is a well-known risk factor of cognitive decline and mood disorders. However, there is no adequate account of the effects of long-term hyperglycaemia on the various dimensions of social behaviours, such as play and aggression. In this study, we evaluated the social behaviour presented by the nicotinamide-streptozotocin rat model of long-term hyperglycaemia. Five months following induction of hyperglycaemia, we scored affiliative/exploratory or aggressive social interactions between pairs of unacquainted rats in a neutral arena. Concurrently, we recorded ultrasonic vocalisations (USVs), envisaged as behavioural markers of emotional states in rats. Our results demonstrate alterations in the behaviour of long-term hyperglycaemic rats faced with social novelty. Specifically, hyperglycaemic rats engaged in hyper-sociable and hyper-aggressive encounters, while emitting greater numbers of USVs. The magnitude of social interactivity and vocalisations was associated with the degree of striatal denervation observed in the brains of long-term hyperglycaemic rats, but not with insulin levels or functions. Altogether, our data suggest a lack of social appropriateness in long-term hyperglycaemic rats, which may be partly linked to changes in striatal dopamine known to regulate social behaviour. This study exposes additional dopaminergic neural substrates that may underlie the social dimensions of the psychiatric challenges faced by diabetic patients.

Introduction

The ability to suitably engage in social encounters occupies a significant role in the lives of individuals, epitomized by the debilitating nature of the behavioural inappropriateness manifested in neuropsychiatric disorders like autism, schizophrenia and attention deficit hyperactivity disorder [1-4]. Although less acknowledged, altered social behaviours are also a feature of diabetes. Indeed, recent studies link diabetes with

increased aggression and agitation in patients [5-7]. In addition, both hypoglycaemic (< 70 mg/dL or 3.88 mM) and hyperglycaemic (> 120 mg/dL or 6.66 mM) states are known to foster hostility and aggression [8-11]. To date, very few studies have sought to investigate social interactions in diabetic-like rodents and these have yielded highly inconsistent results [12-14]. Moreover, none have employed long-term paradigms of hyperglycaemia that could better represent the chronicity of diabetes in humans. Perhaps for this reason, the possible neural substrates of inappropriate behaviours in diabetes remain ill defined.

Ultrasonic vocalisations (USVs) are a valuable tool in social behavioural research, carrying information on the affective state of rats during aggressive and playful interactions [15-18]. USVs can be categorized based on average frequency and frequency modulation in the two large families of 22-kHz calls and 50-kHz calls, which are thought to have different behavioural significance [19]. USVs of 22-kHz are considered markers of negative affective states, since rats emit these calls in aversive situations, for example, during aggressive social interactions with conspecifics or upon exposure to predators [20]. On the other hand, rats mainly emit 50-kHz USVs in pleasurable situations such as mating and playful interactions with conspecifics or familiar humans [15]. Moreover, 50-kHz USVs can be further classified in the two subcategories of frequency modulated (FM) and flat calls based on the modulation of sound frequency [16]. FM calls have a sound frequency that varies substantially, whereas flat calls present a sound frequency that remains nearly unchanged within individual calls [16]. Moreover, FM 50-kHz USVs are thought to be tightly linked to positive emotional states, and to function as affiliative calls to elicit approach behaviour and to maintain playful interactions [21, 22]. Flat 50-kHz calls, on the other hand, are unclear in the exact functions they serve [23, 24], but appear to bear a role in pacing social behaviour, supposedly acting as ambivalent contact calls [25, 26].

In addition to conveying information on the affective state of rats, USVs are intimately linked with the activation of specific neuronal circuits [15]. In particular, activation of the ascending mesolimbic dopaminergic system produces 50-kHz USVs

[27, 28], whereas activation of the ascending mesolimbic cholinergic system produces 22-kHz calls [15]. Notably, dopamine and acetylcholine extensively interact at the striatal interface to dynamically regulate the affective state of rodents [15]. As such, dopamine and acetylcholine antagonize each other in a cooperative system wherein one or the other prevails in a context-dependent manner, allowing for the rapid and appropriate activation of one of two states: positive or negative [29]. These states are thus mutually exclusive and are rendered specifically in rats by the categories of USVs emitted [15, 30]. From this perspective, it is reasonable to believe that alterations in mesostriatal dopaminergic neurotransmission may be reflected at the behavioural level by modifications in social interactions and USV emission.

A recent study by our group demonstrated the occurrence of mesostriatal dopaminergic neurodegeneration in a rat model of long-term hyperglycaemia [31]. In light of these results, we evaluated herein the presence of social behavioural abnormalities that may arise from hyperglycaemia-induced dopaminergic denervation in the striatum. In particular, we measured the occurrences of specific affiliative/exploratory as well as aggressive behaviours in pairs of unacquainted rats in a neutral, novel environment. Since no other studies have investigated USVs in hyperglycaemic rats in a social context, we quantified the numbers of 22-kHz, FM 50-kHz and flat 50-kHz calls emitted during these encounters. Lastly, we evaluated the impact of the degree of striatal denervation on the magnitude of social interactivity and USV emissions.

Results

Occurrences of social behaviours and ultrasonic vocalisations

The occurrences of affiliative/exploratory (dorsal contacts, allogrooming, anogenital sniffing and crawl-overs) and aggression-related (freezing, nose-offs, boxing, pins) behaviours were scored during 10-min encounters between pairs of unacquainted rats of the same group, namely control (CTRL) or hyperglycaemic (HG). Noticeably, many behaviours occurred more often between pairs of HG rats compared to duos of

the CTRL group, whether affiliative/exploratory (Figure 4.1A) or aggression-related (Figure 4.1B). In particular, among the affiliative/exploratory behaviours scored, anogenital sniffing and crawl-overs were markedly recurrent. Moreover, aggression events were more numerous between HG rats, as evidenced by significantly greater occurrences of freezing (***$p < 0.001$), boxing (**$p < 0.01$) and pinning events (*$p < 0.05$). USVs were also recorded and analysed for the entire duration of the encounters. Figures 4.1C, D and E show that subjects of the HG group emitted greater numbers of distinct USVs than CTRL rats during these encounters. In particular, HG rats emitted a greater number of negatively valenced 22-kHz (*$p < 0.05$, Figure 4.1C). In addition, calls of the 50-kHz group were significantly more numerous in pairs of HG rats (**$p < 0.01$ for FM 50-kHz calls and ***$p < 0.001$ for flat 50-kHz calls, Figure 4.1D and E). Altogether, these data demonstrate the hyper-sociable and hyper-aggressive nature of social encounters between pairs of HG rats in relation to the CTRL group.

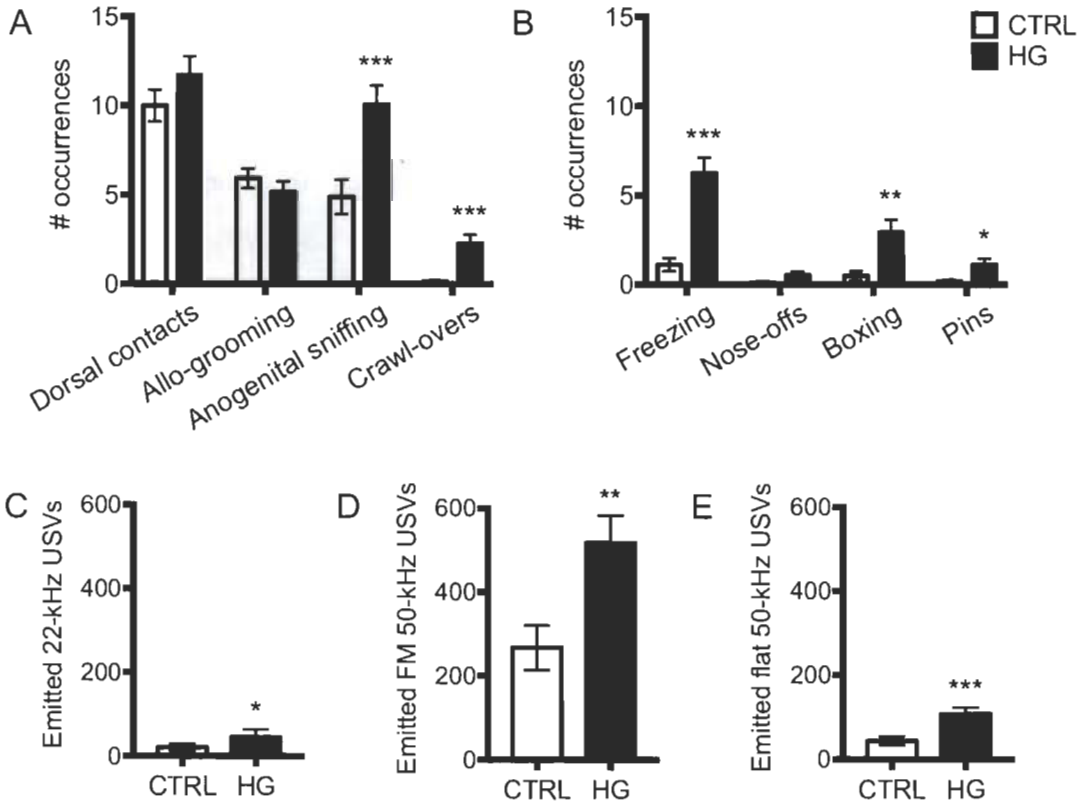

Figure 4.1 Occurrences of social behaviours and USVs.
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(Continued.) The number of occurrences of affiliative/exploratory (A) and aggression-related (B) behaviours were scored during 10-min encounters between pairs of rats of the same group (control [CTRL] or hyperglycaemic [HG]). Negatively valenced (22-kHz) (C), positively valenced (frequency modulated [FM] 50-kHz) (D) and supposed ambivalent (flat 50-kHz) (E) Ultrasonic vocalisations (USVs) emitted by pairs of rats during these confrontations were also scored. All data presented as means ± SEM. Asterisks indicate statistical differences between the HG group and CTRL group, ascertained by a multiple t-test for A and B, or by Mann-Whitney's *U* test for C, D and E ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Behavioural covariance profile

Pearson correlation coefficients were calculated for each pair of behavioural variables measured during encounters between two rats of the same group, and rendered in a covariance matrix wherein values of +1, 0 and -1 signify the tendency for two variables to covary similarly (positive covariance), differently (no covariance) or oppositely (negative covariance), respectively. The CTRL group matrix (Figure 4.2A) revealed tendencies for most behaviours of the affiliative/exploratory type to positively covary together within the same 10-min encounter (upper-left quadrant). In particular, dorsal contacts most positively covaried with crawl-overs. More significantly, aggression-related behaviours positively covaried together within the same encounter between pairs of CTRL rats (lower-right quadrants, $*p < 0.05$ or $***p < 0.001$). Boxing and pins were the strongest associated behaviours of the aggression type ($***p < 0.001$). This is indeed expected in an escalating sequence of rough-and-tumble play where upright boxing between rats usually leads to pinning of one of the animals in a supine position. In addition, bottom-left and upper-right quadrants illustrate that affiliative/exploratory behaviours and aggression behaviours negatively covaried within the same 10-min encounter. This was most significant for allogrooming behaviours ($*p < 0.05$). These data reflect coherence in the interactions between healthy CTRL rats.

Compared to the CTRL group, HG rats displayed a visibly altered behavioural profile (Figure 4.2B). Although there were strong associations between most behaviours of the same nature, whether affiliative/exploratory (upper-left quadrant) or aggression-

related (lower-right quadrant), other behaviours not shown to similarly covary in CTRL rats within the same encounter were markedly positively associated in HG rats. Specifically, the affiliative/exploratory anogenital sniffing behaviour tended to positively covary with aggressive behaviours such as boxing and nose-offs, but was most tightly associated to pins (***$p < 0.001$). Relatedly, we found a strong positive covariance between pins and dorsal contacts (**$p < 0.01$). Despite their higher rate of occurrence (Figure 4.2B), freezing events did not consistently positively covary with any other behaviour during encounters between pairs of HG rats. With respect to the pattern of association displayed by the CTRL group, rats of the HG group seem to engage in incoherent social interactions characterized by episodes of play and fight intertwining within the same encounter.

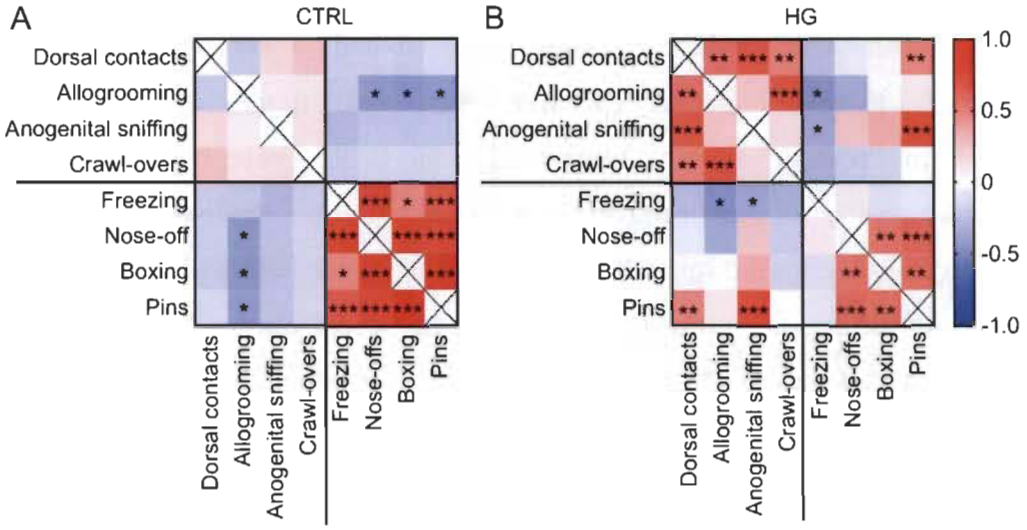

Figure 4.2 Affiliative/exploratory and aggression behavioural covariance matrices.

Matrices show strength of association, expressed as Pearson correlation coefficients, for each combination of behaviour laid out on the x- and y-axes as measured during 10-min encounters between two rats of the same group. Values of 1 (deep red), 0 (white) and -1 (deep blue) signify the tendency for two variables to covary similarly (positive covariance), differently (no covariance) or oppositely (negative covariance), respectively. Affiliative/exploratory behaviours are dorsal contacts, allogrooming, anogenital sniffing and crawl-overs. Aggression behaviours are freezing, nose-offs, boxing and pins. Behavioural profiles are provided for the CTRL (A) and HG (B) groups. Asterisks indicate statistical significance of the positive or negative covariance of two variables (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Behaviour-vocalisation covariance profile

A similar strategy was employed to evaluate whether the emission of certain categories of USVs covaried with distinct types of behaviours within the same 10-min encounter between pairs of same-group rats (Figure 4.3). Between pairs of CTRL rats (left quadrants), the emission of negatively valenced 22-kHz calls and aggression behaviours of all types positively covaried (***$p < 0.001$). In addition, FM 50-kHz USVs positively covaried with one of the most positively valenced behaviours, that is, allogrooming [32] (*$p < 0.05$). Accordingly, 22-kHz USVs negatively covaried with this behaviour (*$p < 0.05$). Flat 50-kHz calls, on the other hand, were more often emitted during encounters wherein anogenital sniffing activities were numerous (**$p < 0.01$), but also slightly positively covaried with allogrooming and pins. This is in line with the proposed ambivalent nature of flat calls, which may have served here to pace intrusive exploratory behaviours, such as anogenital sniffing and allogrooming, or to signal discomfort or uncertainty during pinning events.

With respect to the CTRL group, HG rats (Figure 4.3, right quadrants) presented pronounced distortions in the covariance relationships between the USVs they emitted and the behaviours they exhibited during the same 10-min encounter. Most striking is the lack of a clear positive covariance between 22-kHz calls and behaviours likely to induce these USVs, such as nose-offs, boxing and pins. In addition, FM 50-kHz and affiliative/exploratory behaviours did not positively covary. The most coherent pattern of positive covariance remained the significantly numerous emissions of 22-kHz calls during encounters that also featured numerous freezing events between HG rats (***$p < 0.001$).

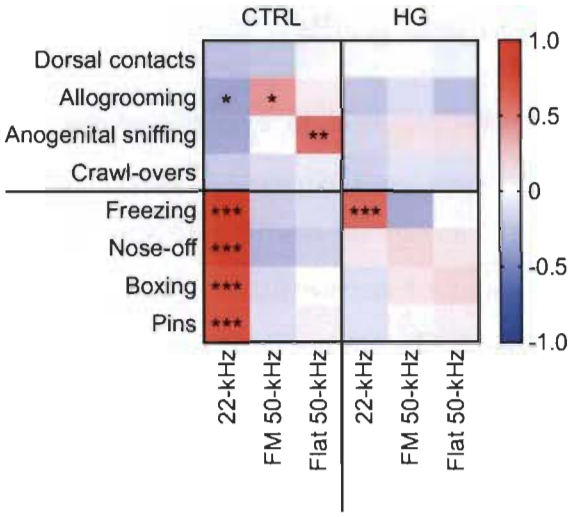

Figure 4.3 Behaviour-vocalisation covariance matrix.
Matrices show strength of association, expressed as Pearson correlation coefficients, for each combination of USV category (x-axis) and type of behaviour (y-axis) as measured during 10-min encounters between two rats of the same group. Values of 1 (deep red), 0 (white) and -1 (deep blue) signify the tendency for two variables to covary similarly (positive covariance), differently (no covariance) or oppositely (negative covariance), respectively. Affiliative/exploratory behaviours are dorsal contacts, allogrooming, anogenital sniffing and crawl-overs. Aggression behaviours consist in freezing, nose-offs, boxing and pins. Twenty-two-kHz USVs are negatively valenced, FM 50-kHz USVs are positively valenced, and flat 50-kHz USVs are supposed ambivalent calls. Behaviour-vocalisation profiles are provided for the CTRL (left quadrants) and HG (right quadrants) groups. Asterisks indicate statistical significance of the positive or negative covariance of two variables (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Magnitude of social interactivity and ultrasonic vocalisations in relation to the degree of striatal denervation, hypoinsulinaemia and glucose intolerance

We performed densitometric immunohistochemical analyses of striatal tyrosine hydroxylase (TH)-positive terminal fibres in the brains of certain CTRL (n = 3) and HG rats (n = 6) utilized in the experiments described above. This allowed for the division of these HG subjects into two distinct subgroups: low denervation and high denervation (Figure 4.4A). Figure 4.4B displays representative microphotographs of the striatal denervation observed in HG brains compared to healthy CTRL rats. The baseline insulinaemia and glucose tolerance of certain CTRL (n = 5-6) and HG (n = 17-20) rats

utilized in the experiments described abovewere also obtained on the day of their sacrifice following an oral glucose tolerance test. This allowed for the division of HG subjects based on the severity of their hypoinsulinaemia (Figure 4.4C, low and high hypoinsulinaemia) or glucose intolerance (Figure 4.4D, low and high glucose intolerance).

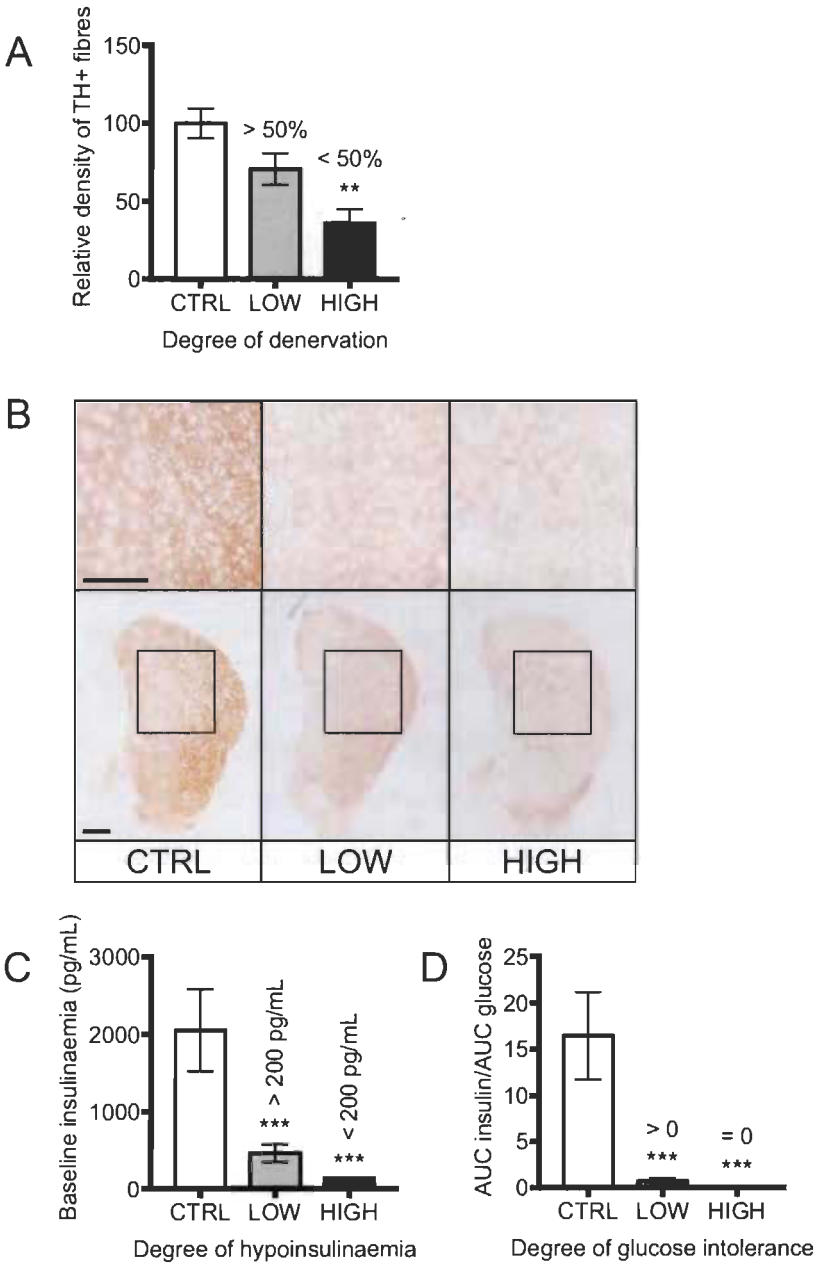

Figure 4.4 **Classification of long-term HG rats based on degree of striatal denervation, hypoinsulinaemia and glucose intolerance.**
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(Continued.) (A, B) Immunohistochemical analyses of the striatal region of CTRL and HG rat brains. At the end of behavioral experiments, frozen post-fixed brain hemispheres were cut into 20 µm-thick coronal serial sections and stained for the dopaminergic terminal fiber marker, tyrosine hydroxylase. A. Densitometric analyses allowed for the distinction of two subgroups, LOW (> 50% of CTRL) or HIGH (< 50% of CTRL), within the HG group concurring to the degree of striatal dopaminergic denervation. B. Representative microphotographs of tyrosine hydroxylase staining in the striatum (Bregma ~1 mm, scalebar 1 mm). C: Baseline insulinemia measurements upon sacrifices allowed for the separation of HG rats between LOW hypoinsulinemia (> 200 pg/mL) and HIGH hypoinsulinemia (< 200 pg/mL). D: Following an oral glucose tolerance test performed on the morning of their sacrifice, areas under the curve of glycemias on areas under the curve of insulinemias yielded indices of glucose intolerance. Rats were separated on this basis between LOW (> 0) and HIGH (0) glucose intolerance. All data presented as means ± SEM. Asterisks indicate statistical differences between the HG group and CTRL group (**$p < 0.01$).

The mean rate of occurrence of specific affiliative/exploratory and aggression-related behaviours was obtained for each abovementioned subgroup ranked by degree of striatal denervation, hypoinsulinaemia and glucose intolerance (Figure 4.5). In particular, HG rats exhibiting the highest levels of striatal denervation engaged in more numerous affiliative/exploratory (Figure 4.5A) and aggression-like (Figure 4.5B) behaviours than rats from the other two subgroups, significantly for dorsal contacts (***$p < 0.001$ compared to CTRL and +$p < 0.05$ compared to the low denervation subgroup), anogenital sniffing (***$p < 0.001$ compared to CTRL and ++$p < 0.01$ compared to the low denervation subgroup), boxing (***$p < 0.001$ compared to CTRL and +++$p < 0.001$ compared to the low denervation subgroup) and pinning (***$p < 0.001$ compared to CTRL and ++$p < 0.01$ compared to the low denervation subgroup). No clear trend for increased affiliative/exploratory or aggression behaviour in relation to the degree of hypoinsulinaemia (Figure 4.5C and D) or glucose intolerance (Figure 4.5E and F) was revealed. In fact, dorsal contacts were less frequent in the high hypoinsulinaemia subgroup than in the low insulinaemia subgroup (+$p < 0.05$).

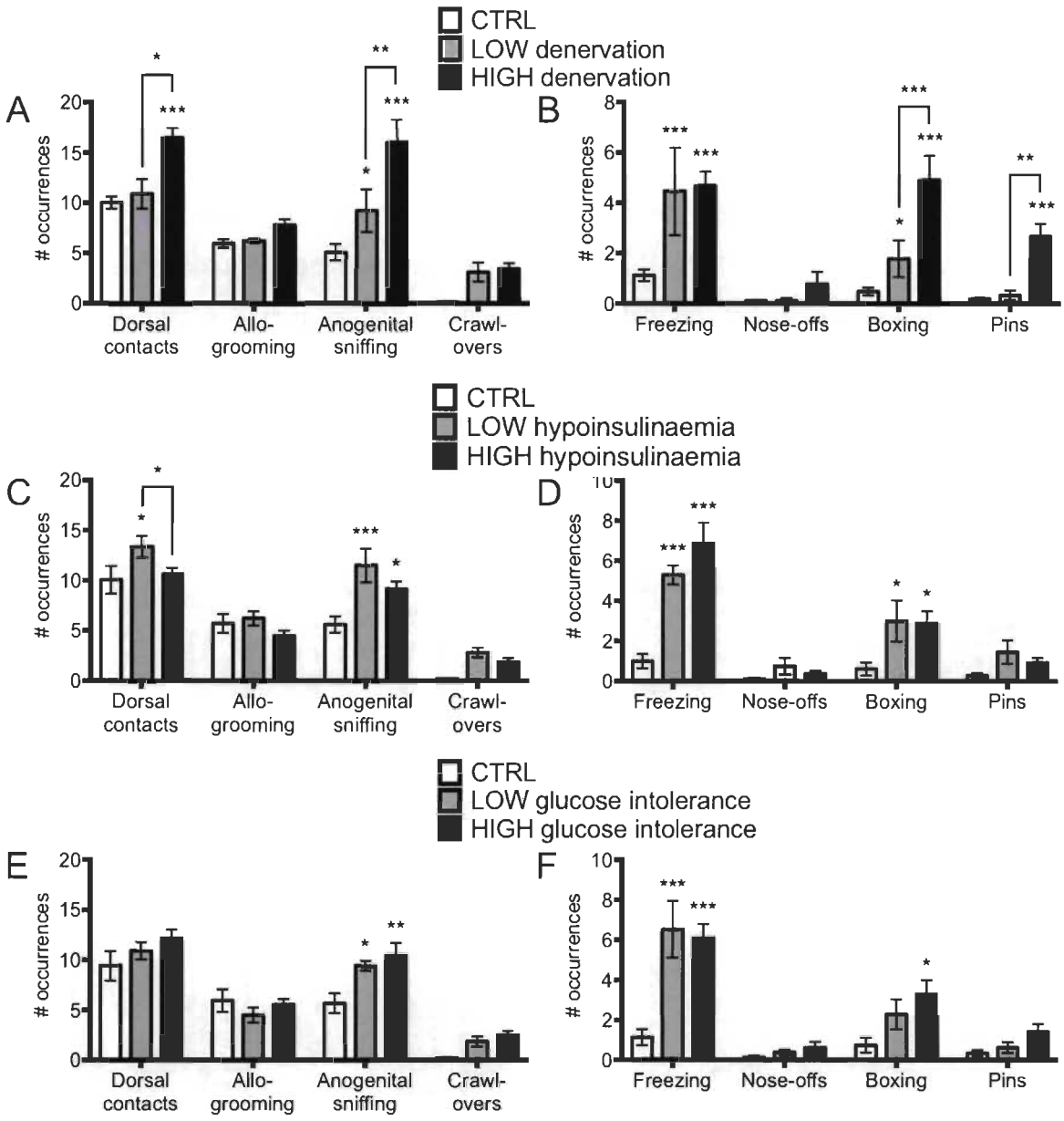

Figure 4.5 Occurrences of social behaviours in relation to the degree of striatal denervation, hypoinsulinaemia or glucose intolerance.
Occurrences of affiliative/exploratory (A, C, E) or aggression-related (B, D, F) behaviours performed by pairs of CTRL rats or pairs of HG rats belonging to the low or high striatal denervation (A, B), hypoinsulinaemia (C, D) or glucose intolerance (E, F) groups were plotted. All data presented as means ± SEM. Asterisks indicate statistical differences between groups and the CTRL group, ascertained by two-way ANOVA followed by Tukey's post-hoc analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Plus signs indicate statistical differences between high and low groups, ascertained by two-way ANOVA followed by Tukey's post-hoc analysis (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$).

Next, the mean rate of occurrence of USVs emitted was obtained for the same defined subgroups (Figure 4.6). In particular, HG rats exhibiting the highest levels of striatal denervation emitted more numerous FM 50-kHz USVs compared to the CTRL subgroup ($*p < 0.05$), which was not the case for the low denervation subgroup (Figure 4.6A). In addition, nonoticeable link was found between the degree of hypoinsulinaemia (Figure 4.6D, E and F) or glucose intolerance (Figure 4.6G, H and I) and emissions of USVs of any category. Together with the previous data, Figure 4.6 supports previous observations of a hyper-sociable and hyper-aggressive behavioural phenotype in long-term HG rats that appears to be partly dictated by the degree of striatal denervation exhibited by individual subjects.

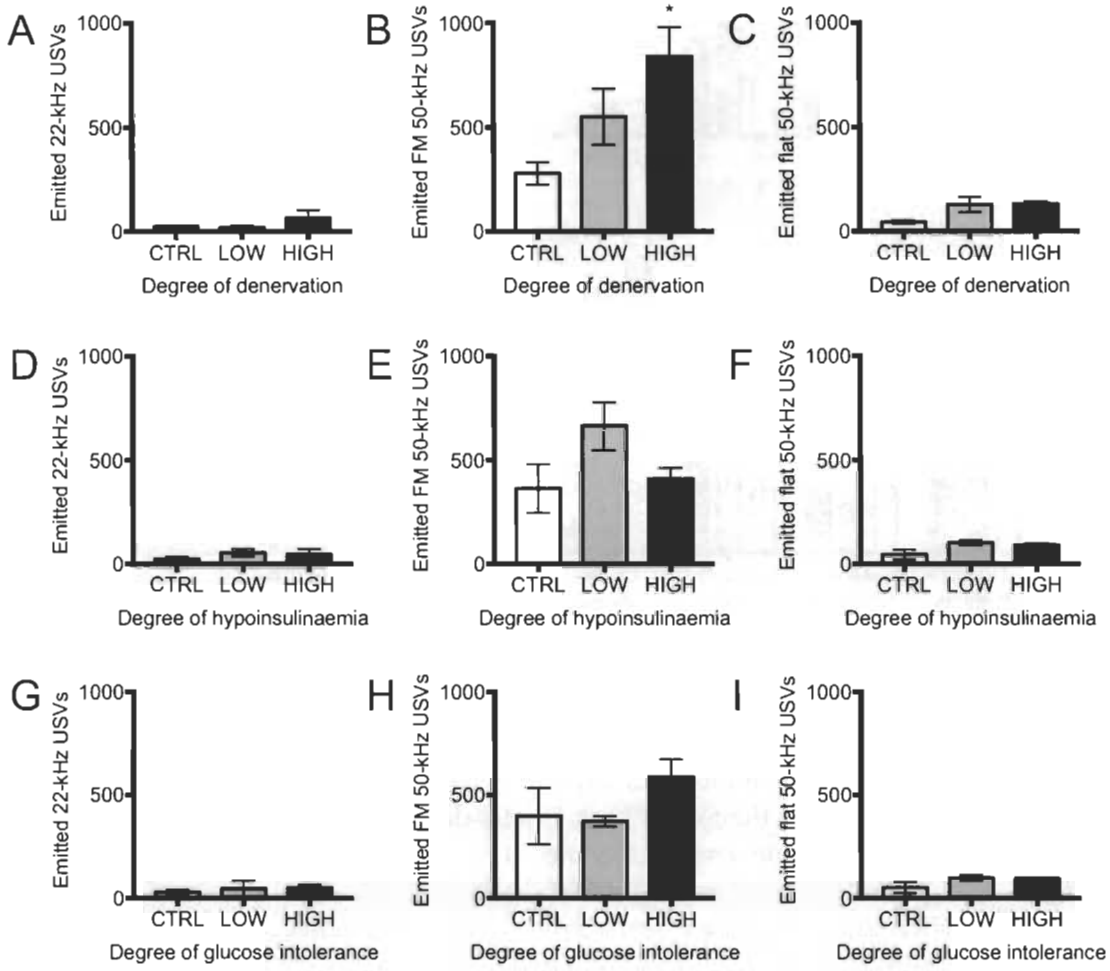

Figure 4.6 Numbers of social behaviours in relation to the degree of striatal denervation, hypoinsulinaemia or glucose intolerance.
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(Continued.) Numbers of USVs emitted by CTRL rats or HG rats belonging to the low or high striatal denervation (A-C), insulinaemia (D-F) or glucose intolerance (G-I) groups were plotted. (A, D, G) Negatively valenced 22-kHz USVs. (B, E, H) Positively valenced FM 50-kHz USVs. (C, F, I) Supposed ambivalent flat 50-kHz USVs. All data presented as means ± SEM. Asterisks indicate statistical differences between groups and the CTRL condition, ascertained by Kruskal-Wallis ANOVA followed by Dunn's post-hoc analysis (*$p < 0.05$).

Discussion

Although awareness of behavioural alterations in diabetic patients has been on the rise lately, research offers limited insight on the physiological factors that may impinge on these issues. While acute hypo or hyperglycaemic states are acknowledged to foster more aggressive states in humans [8-11], little is known about the effects of long-term dysglycaemia. Prior studies have employed acutely hyperglycaemic mice (duration of hyperglycaemia < 1 month) in resident-intruder paradigms [13, 14] or hyperglycaemic rats in mating paradigms [12], but have yielded inconsistent findings on the social effects of hyperglycaemia in paradigms that, further, poorly reflect the chronicity of the diabetic condition.

To the best of our knowledge, the present report constitutes the first detailed account of alterations in social behaviour and USV communications in a rat model of long-term hyperglycaemia. In all evidence, rats of the HG group were hyper-sociable and hyper-aggressive, as supported by marked increases in the occurrences of both affiliative/exploratory and aggression-related behaviours. Accordingly, compared to CTRL rats, they emitted many more USVs thought to operate both as social contact calls [21, 33] and expressions of affective states [15]. In addition to the rise in occurrences of social behavioural or vocalisation events, the covariance profiles of these variables were radically different between CTRL and HG groups. Coherent associations between behaviours of the same kind or between behaviours and USVs that supposedly have the same valence were almost completely lost in HG rats.

In an attempt to better understand the neurobiological underpinnings of impaired social behaviour and disjointed USV communications in our model, results were related to the degree of TH-positive fibres in the striatum. Indeed, focal to social behaviour are a variety of neurotransmitters acting via circuits that can be modulated by pharmacological interventions [34, 35]. One such neurotransmitter is dopamine, exemplarily targeted by dopaminergic antagonists to treat abnormal aggressive behaviours in autism, schizophrenia and excessive stress [36-40]. By releasing dopamine in the striatum, midbrain neurons partake in the regulation of social behaviours but also of other goal-directed operations ranging from movement production to reward-seeking processes [41-44]. In agreement with the purported role of mesostriatal dopamine in social conduct, we found the altered social behaviours between pairs of unacquainted rats to be predicted by the degree of striatal TH-positive fibre loss. This is supported by the differences in 50-kHz USV emissions between CTRL and HG rats, also importantly modulated by mesostriatal dopamine [15]. Nevertheless, we cannot exclude that our results may have been partly influenced by toxic and/or adaptive modifications induced by long-term hyperglycaemia in non-dopaminergic neurotransmitter systems, which also critically regulate social behaviour and emission of USVs [35, 45-50]. For example, based on the loss of striatal TH-positive fibres, it could be hypothesized that hyperglycaemic damage of the noradrenergic system may have participated in the behavioural abnormalities observed here. Further studies will be necessary to precisely determine the interplay between social and vocal behaviour and neurotransmitter systems in HG rats.

The role for increased mesostriatal dopamine neurotransmission in the promotion of impulsive, social and reward-related behaviours as well as in the emission of 50-kHz USVs seems to challenge our results showing an apparent correlation between striatal denervation and increased sociability, aggression and vocalisation. Nevertheless, our findings on the emission of 50-kHz USVs by HG rats are consistent with results from a previous study in male rats bearing a unilateral terminal denervation of the dopaminergic nigrostriatal system [51]. Indeed, the latter study showed that dopamine-denervated rats showed a trend towards an increase in the numbersof 50-kHz USVs emitted during

mating, compared with neurologically intact rats. It is possible that the occurrence of dopamine receptor supersensitivity due to deafferentiation and/or an imbalance in tonic and phasic firing may account for the increased reactivity to social novelty in our model [52]. Indeed, we have previously shown a decrease in tonic dopaminergic transmission in the striatum of long-term HG rats [31]. Tonic firing consists in the slow and irregular single-spike release of dopamine produced by the baseline pacemaker activity of mesostriatal dopaminergic neurons, which maintains a certain level of striatal dopamine "noise" [53]. Tonic dopamine is required to enable striatal postsynaptic targets embedded in corticostriatal circuits involved in the selection of pre-learned programs [54-56]. Phasic firing, on the other hand, involves a stimulus-mediated discharge of spikes in a short amount of time [57]. These bursts raise the levels of striatal dopamine above the noisy tonicity, acting as a "signal" that directs attention to a salient stimulus. When striatal tonicity is low, phasic dopamine becomes much more relevant over the reduced noisy background; thus, when produced in response to salient stimuli like exposure to a novel social context or emission of 50-kHz USVs [58], phasic firing might cause inappropriately intense reactions as observed in our model. Notably, transcending neurodegeneration, receptor supersensitivity, altered dopamine concentrations or imbalances in tonic-to-phasic (noise-to-signal)firing have all been proposed to explain various psychiatric disorders such as schizophrenia [59, 60] and attention deficit hyperactivity disorder [61, 62]. Importantly, hyper-sociable and hyper-aggressive traits are typical of the latter and very similar to those present in our model [63-65].

The overt hyper-sociable and hyper-aggressive behaviours exhibited by HG rats are also reminiscent of impulsive traits found in the streptozotocin model, albeit in other contexts. Indeed, drugs that modulate mesostriatal dopamine are more rewarding in streptozotocin-treated rats than in untreated conspecifics [66-68]. In addition, hyperphagia, a hallmark of the model, is at least partly regulated by mesostriatal dopamine [69, 70]. To all appearances, the streptozotocin model increases the rewarding properties of several environmental stimuli. We show here that this could include social interactions as supported by the concomitant increase in the emission of 50-kHz USVs, which are a marker of positive affective states [15]. While metabolic hormonal

imbalances may be implicated to some extent in the hyper-reactive phenotype of streptozotocin-treated rats, as suggested by some [66, 69], it remains that the extent of behavioural alterations was tightly associated with striatal denervation and not with insulin concentrations or functions in long-term hyperglycaemia. Given its implication in modulating behaviour in short- and medium-term [71-73], a role for the hypothalamic-pituitary-adrenal axis cannot be ruled out in the behavioural abnormalities observed here in streptozotocin-treated rats and should be addressed in future works [14, 74].

In summary, the present results demonstrate explicit manifestations of hyper-sociable and hyper-aggressive traits in our model of long-term hyperglycaemia. The striking overall increases in positively and negatively valenced events, either behavioural or vocal, combined with the loss of coherence between the affective states represented by USVs and the social behaviour displayed may arise from neurobiological alterations. This is supported by the tight association between striatal denervation as opposed to the lack of a clear link between insulin and hyper-reactivity. These findings may ultimately lead to the formal identification of dopaminergic neural substrates that may be targetable by pharmacological treatments, both to prevent striatal denervation and to palliate neurochemical imbalances in diabetic patients faced with psychiatric challenges.

Methods

Subjects

Forty male Sprague-Dawley rats (Charles River, St-Constant, Canada) weighing 175-200 g were housed under standard laboratory conditions (12 h light/dark cycle). In all experiments, standard food and water were available ad libitum. Rats were housed two per cage to rule out any effect of social isolation on their subsequently measured behaviours [75]. Experiments were conducted in accordance with legislation and policies of the Canadian Council on Animal Care, as well as with the guidelines

established by the Animal Care Committee of the Université du Québec (Trois-Rivières) (2014-M.G.M.5).

Induction of hyperglycaemia

Rats were randomly assigned to the control or hyperglycaemic group (CTRL n = 18; HG n = 22). Hyperglycaemia was induced as previously described [31]. Briefly, fasted rats of the HG group were intraperitoneally injected with nicotinamide (100 mg/kg b.w.) followed by the beta cell toxin streptozotocin (55 mg/kg b.w., Enzo Life Sciences, Farmingdale, NY, USA). Pre-injections with nicotinamide restrain the death of insulin-producing pancreatic beta cells and yield long-term HG rats that do not require glycaemia-lowering treatments [31, 76]. CTRL rats received vehicle injections. Throughout experiments, glycaemia was estimated using an UltraMini digital glucose meter and matching strips (One Touch Ultra) both purchased at a local Brunet pharmacy (Trois-Rivières, QC, Canada). Hyperglycaemia was confirmed 3 days after nicotinamide-streptozotocin injections and rats from the HG group that did not display a glycaemia steadily above 10 mM were discarded from this study. Importantly, rats from both groups were matched for age and experience, meaning that they were handled in the same manner and at the same frequency, in light of studies showing the role of age and experience on the emission of USVs [26].

Oral glucose intolerance test and baseline insulinemia

On the morning of their sacrifice, we performed an oral glucose tolerance test (OGTT) on fasted rats, as previously described [31]. Briefly, rats were intragastrically administered 2 g/kg of a D-glucose solution. Glycaemia and insulinaemia were measured right before the OGTT (0 min, baseline) and at 30, 60 and 120 min after administration of D-glucose using the Rat Glucose Assay kit provided by Crystal Chem (Downers Grove, IL, USA) and the Milliplex Adipokine Magnetic Bead Multiplex Assay (Millipore; Etobicoke, ON, Canada), respectively. The ratio of the area under the curve

of plasma insulin concentrations on the area under the curve of plasma glucose concentrations yielded indices of glucose intolerance.

Ultrasonic vocalisations and social behaviour

We began experiments 5 months following the induction of hyperglycaemia, at the time when rats were fit to engage in social interactions. Experiments consisted in 3 trials separated by 7 days each in order to prevent the behavioural habituation of rats to multiple encounters in a short amount of time [77]. Experiments were performed in 3 different quiet rooms to rule out any environmental effects on the behaviour of the rats. USV recordings were performed as previously described [78]. Two group-paired unacquainted rats (CTRL 9 pairs times 3 trials; HG 11 pairs times 3 trials) were placed in a Plexiglas cylinder (diameter, 25 cm; height, 30 cm) with fresh bedding, surrounded by walls to enhance the feeling of security, and topped with a sound-insulating lid. An ultrasonic microphone (CM16/CMPA, Avisoft) was placed through a tight-fitting hole in the lid and was connected to an ultrasound-recording device (UltraSoundGate 116 Hb, Avisoft). During USV recordings, constant gain was maintained. Figure 4.7 shows examples of 22- and 50-kHz USVs recorded during this study. Two video cameras were positioned on opposite sides of the cylinder to record the encounters. USVs and images were recorded for 10 min, starting immediately after both rats were simultaneously placed in the cylinder. Hence, neither rat was an intruder in this context. Recording time was selected based on previous studies that evaluated the emission of USVs by rats that engaged in social contacts [49, 71]. A blinded experimenter scored eight types of interactions provided by the video recordings. Affiliative/exploratory behaviours consisted in dorsal contacts (with both paws), allogrooming, anogenital sniffing, and crawl-overs (one rat crawls over the other rat) [79-81]. Aggression-related behaviours consisted in freezing (a submissive rat remains 5 consecutive seconds or more without movement except sniffing), nose-offs (both rats are immobile, facing each other, four paws on the ground or front paws raised, but do not box), boxing (one or both rats stand on their hind legs and push or paw their opponent), pins (one rat tops another one that is in supine position) [16, 82]. Occurrences of any of these behaviours were

counted for both rats, regardless of the amount of time they lasted. Rats were never opposed to the same partner twice, thus social novelty was preserved throughout trials.

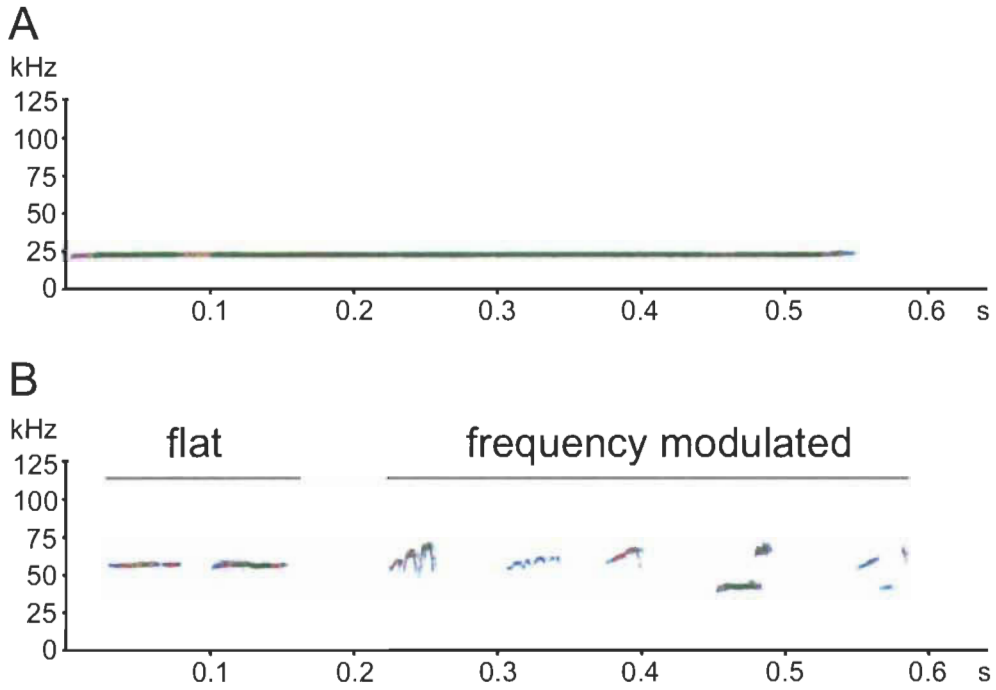

Figure 4.7 **Examples of sonograms of ultrasonic vocalisations.** Representative sonograms of 22-kHz (A) and 50-kHz (B) USVs. FM 50-kHz USVs (B, right) differ from flat ones (A and B left) in that there is a change in frequency within the call. Ultrasonic vocalisations reported are examples of independent calls emitted by different rats.

Immunohistochemistry

At the end of the 3 trials, isoflurane anesthetized rats were intracardially perfused with cold PBS supplemented with protease and phosphatase inhibitors. Brains were harvested and post-fixed in 4% paraformaldehyde, cryoprotected through gradients of sucrose and conserved at -80°C for immunohistochemical analyses [15]. Frozen post-fixed tissues were cut into 20 µm-thick coronal free-floating serial sections and immunoreactions were performed with a primary antibody raised against the rate-limiting enzyme of dopamine synthesis, TH. Sections were then incubated with a horseradish peroxidase-conjugated secondary antibody, exposed with 3,3'-diaminobenzidine, mounted on microscope slides, dehydrated and coverslipped.

Using a microscope in brightfield mode (MBF Bioscience, Williston, VT, USA), the density of striatal terminals positive for TH was quantified densitometrically using the NIH ImageJ software version 1.49, and averaged in 4 slices per animal. A total of 3 brains from the CTRL group and 6 brains from the HG group were randomly selected and analysed. Due to normal interindividual variability, degrees of denervation differed across the 6 HG rats, allowing for the separation of these individuals into high (n = 3) or low (n = 3) denervation subgroups.

Statistical analyses

USV recordings were converted into spectrograms using the software SASLab Pro 4.52 (Avisoft) with the following settings: 512 FFT-length, Hamming window and 75% overlap frame set-up [83]. A skilled experimenter visually inspected and manually cleaned all signals that could not be univocally classified as vocalisations. Numbers of 22-kHz and 50-kHz USVs were then counted using the SASLab Pro 4.52 software. Moreover, an experienced experimenter unaware of treatments manually counted numbers of FM and flat 50-kHz USVs. USVs of 22-kHz were defined as calls with sound frequency maintained between 20- and 35-kHz and long duration, higher than 300 ms for a single call [19]. In the present study, no 22-kHz USVs of duration shorter than 300 ms were observed [84]. USVs of 50-kHz were defined as calls with sound frequency maintained between 35- and 80-kHz and short duration, usually 10-15 ms for a single call [19]. USVs of 50-kHz were further divided in flat and FM USVs, based on the modulation of their sound frequency. Thus, flat calls have the sound frequency within 35-50-kHz that remains nearly unchanged over the duration of the individual USVs [26]. Conversely, FM 50-kHz calls have the sound frequency within 40-80-kHz that shows substantial changes within individual USVs [16].

All data were submitted to statistical analyses performed using GraphPad Prism 7 software (San Diego, CA, USA; http://www.graphpad.com). Data were tested for normality and homoscedasticity, and were analysed accordingly. Behaviour-behaviour and behaviour-USV covariation matrices (Figure 4.2 and Figure 4.3) were derived from

Pearson correlation coefficients calculated for each pair of variables, where +1, 0, and -1 respectively indicate the tendency for two variables to covary similarly (positive covariance), differently (no covariance) or oppositely (negative covariance) [85]. Occurrences of social behaviours, relative density of TH fibres, and measures of insulinaemia and glucose intolerance were parametrically distributed. Thus, significant differences between groups shown in Figure 4.1A and B were ascertained by multiple *t*-tests, ones in Figure 4.4A, C and D were ascertained by one-way ANOVA followed by Tukey's post-hoc analysis, and those shown in Figure 4.5 were determined by two-way ANOVA followed by Tukey's post-hoc analysis. On the other hand, emissions of USVs were non-parametrically distributed. Therefore, significant differences between groups shown in Figure 4.1C, D and E were ascertained by Mann-Whitney's *U* test, while significant differences between groups shown in Figure 4.6 were ascertained by the Kruskal-Wallis ANOVA, followed by Dunn's post-hoc analysis. All data were analysed at the 95% confidence interval and are expressed as means ± SEM.

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CHAPTER V

DISCUSSION

Based on evidence of a unique phenotypic liability, and supported by the greater prevalence of Parkinson's disease in diabetic patients, the work presented in this thesis aimed to verify the hypothesis that nigrostriatal dopaminergic neurons are more vulnerable to hyperglycaemic conditions compared to other neuronal populations, expressly the mesocorticolimbic pathway. In the process, we attempted to cover as many dimensions of dopaminergic functioning as possible, harvesting evidence on a molecular, cellular, neurochemical, neuroanatomical and behavioural level to render a comprehensive picture of hyperglycaemia-induced neurodegeneration. To that end, four objectives were fulfilled and will be examined in light of elements brought to the table in the introduction. I will conclude these discussions by translating the significance of our results to the realities of diabetic and parkinsonian patients.

5.1 Objectives 1 and 2: *In vitro*, high glucose-induced oxidative stress leads to the death of dopaminergic neurons avertible by resveratrol treatments

In Chapter II, we successfully demonstrated that dopaminergic neurons degenerate when cultured in high glucose conditions. Using neuronal PC12 cells treated with the antioxidant resveratrol, we confirmed the implication of oxidative stress in high glucose-induced apoptosis. This first step of the project provided a crucial cornerstone upon which we built subsequent *in vivo* work.

5.1.1 Drawing parallels with Brownlee's theory

Brownlee's theory proposes that superoxide anion production is the earliest event leading up to oxidative stress in a hyperglycaemic setting (Brownlee, 2005; Du *et al.*, 2001; Giacco and Brownlee, 2010; Nishikawa *et al.*, 2000). As previously stated,

early superoxide anion generation has not been investigated in animal models of hyperglycaemia. Our group was the first to verify the production of this specific ROS in cultured neurons exposed to high concentrations of glucose (Bournival *et al.*, 2012*a*). In the present work, we further confirmed that this event occurs as early as 3 h following high glucose treatments, thereby supporting Brownlee's hypothesis.

5.1.1.1 From oxidative stress to apoptosis

Trailing this theory's mechanistic progression, superoxide anion should lead to the formation of other ROS that can damage nuclear DNA. In high glucose conditions, p53 was indeed markedly upregulated[61] (Figure 5.1A and B) and it strongly localized to the nucleus at 96 h, indicating its probable transcriptional activation, stabilization and recruitment to damaged DNA, all ensuing from oxidative insults (Lee *et al.*, 2008; Liu and Xu, 2011; Macip *et al.*, 2003; Norbury and Zhivotovsky, 2004; Sun *et al.*, 1995). Following its nuclear localization, p53 can either induce DNA reparation or prompt the activation of apoptosis when injuries are too substantial (Liu and Kulesz-Martin, 2000). Accordingly, our results show that neuronal PC12 cells undergo apoptosis in high glucose conditions, as evidenced by chromatin modifications, caspase-3-induced DNA fragmentation, PARP cleavage and a rise in Bax-to-Bcl-2 expression ratios.

Supplementary evidence that oxidative stress is ongoing in our model is the rise in GRP75 expression under high glucose conditions (Figure 5.1C and D), which confirms other reports of its induction during cellular stress (Londono *et al.*, 2012). Furthermore, we observe an increase in GRP75 and p53 colocalization in the cytoplasm[62], which likely occurs as a result of oxidative stress (Lee *et al.*, 2007). Indeed, in response to oxidative insults, p53 is phosphorylated, which enables its interaction with GRP75 in the cytoplasm (Figure 5.2). Bound by GRP75, p53 cannot enter the nucleus and induce

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[61] Other groups also found the induction of p53 in response to DNA damage to constitute a key event in the provocation of apoptosis. Indeed, p53 upregulation in dopaminergic neurons challenged with parkinsonian toxins seems to occur before any other apoptotic event (Li *et al.*, 2016; Nair, 2006).

[62] During cytoplasmic sequestration, GRP75-p53 colocalization is noticeably perinuclear in our model, which corroborates the findings of others (Taurin *et al.*, 2002).

apoptosis in otherwise lethal conditions. However, when p53 is steeply expressed, as in our model, it may partially evade GRP75 sequestration in the cytoplasm and enter the nucleus to induce apoptosis.

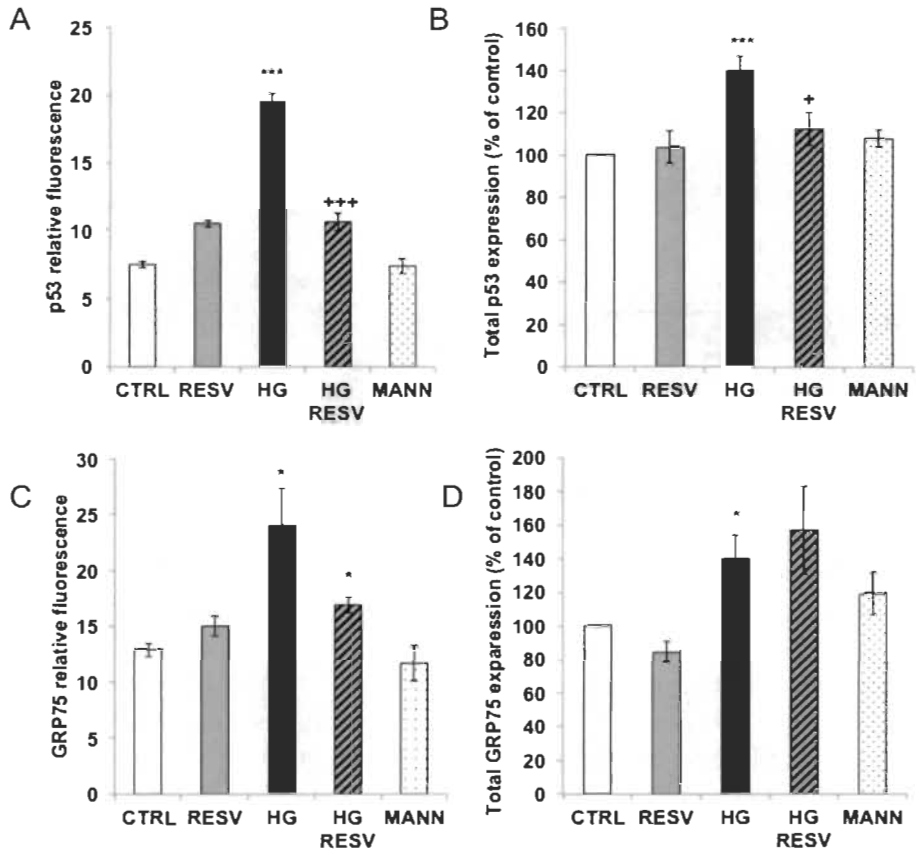

Figure 5.1 Total levels of p53 and GRP75 in neuronal PC12 cells treated with high glucose concentrations.

Previously, we showed subcellular modulation of p53 and GRP75 levels. Here, total cellular levels are addressed. Neuronal PC12 cells were treated with 25 mM of glucose for 96 h, with or without 0.1 μM of resveratrol. Isotonic mannitol served as an osmotic control. (A, C) Relative total p53 or GRP75 immunofluorescence levels. (B, D) Relative total p53 or GRP75 expression measured by immunoblotting. High glucose concentrations increased total p53 expression levels, which was rescued by resveratrol treatments. In response to the glucose-induced rise in p53 levels, GRP75 expression is accordingly enhanced, with or without resveratrol treatments. $*p < 0.05$, $***p < 0.001$ compared with CTRL and $+p < 0.05$, $+++p < 0.001$ compared with HG, as determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. Refer to Chapter II for methodological details.

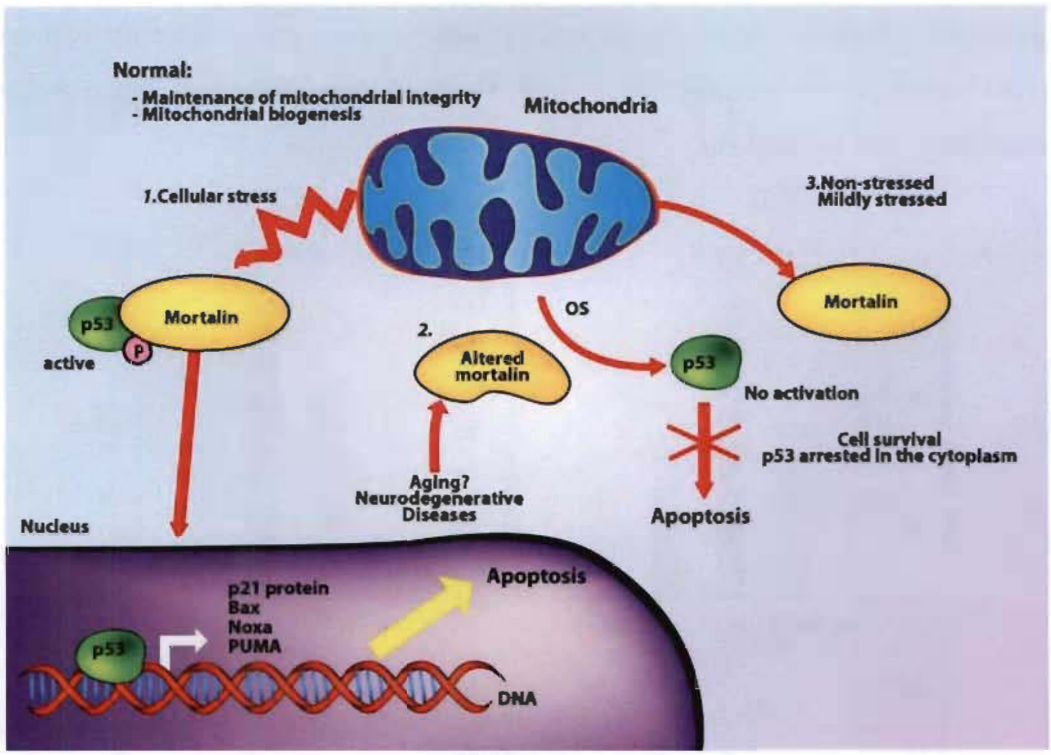

Figure 5.2 **Role of GRP75 in oxidative stress-induced apoptosis.**
As a sensor of oxidative stress and protein chaperone, GRP75 (mortalin) fulfils numerous functions in cells that are dependent on context. Under normal circumstances, GRP75 is thought to promote homeostasis in the subcellular localizations it occupies, expressly mitochondria. In conditions of stress, GRP75 is upregulated and translocates to the cytoplasm where it can also convey protection by preventing apoptosis (1). In particular, GRP75 can bind the phosphorylated form of the pro-apoptotic effector p53. Under sustained amounts of stress, GRP75 can undergo deleterious alterations, which impede its protective functions (2). When the cell is only mildly stressed, GRP75 is poorly recruited to the cytoplasm, likely owing to a lack of significant p53 activation (3). OS, oxidative stress; P, phosphate group. (From Londono *et al.*, 2012.)

5.1.1.2 Paradoxical poly(adenosine diphosphate-ribose) polymerase inactivation

Brownlee's model provides that the activity of the DNA repair enzyme PARP is augmented early in hyperglycaemic conditions, in response to ROS-induced DNA damage. This leads to the ADP-ribosylation of GAPDH and its consequent inhibition, inducing an accumulation of upstream glycolysis metabolites responsible for the rerouting of glucose into deleterious pathways. However, we observe that activated caspase-3 cleaves PARP in its 24 and 89 kDa fragments, thereby preventing its

ADP-ribosylation function (Kaufmann *et al.*, 1993). In fact, PARP cleavage is required in order to carry out apoptosis (Boulares, 1999). Irreversible binding of the 24 kDa portion to damaged DNA inhibits DNA repair and conserves energy pools in anticipation of apoptosis (D'Amours, 2001). Indeed, in its activated form, PARP is a heavy user of NAD+ whose exhaustion can lead to energy depletion-induced necrosis (Boulares, 1999). Since the full-length-to-cleaved ratio was measured in our model after 96 h of exposure to high glucose conditions, it is probable that PARP inactivation occurs late in the apoptotic cascade, which does not contradict a possible transient early increase in its activity.

5.1.1.3 Validating Brownlee's model

Although our results corroborate the implication of oxidative stress in the high glucose-induced apoptosis of dopaminergic neurons in culture, a deeper mechanistic insight is warranted to verify the neuronal suitability of Brownlee's theory, originally elaborated in bovine aortic endothelial cells. In this view, a step-by-step analysis of the events leading up to the death of dopaminergic neurons in culture is presently under way.

The strategy we envision calls upon the inhibition of the most salient checkpoints in glucose metabolism dysfunction in the neuronal PC12 and N27 dopaminergic models. First, we will characterize by immunofluorescence the regulation of GLUT localization at the surface of neuronal cultures in response to glucose in a series of kinetic dose-response studies. In parallel, we will appraise the effect of varying glucose concentrations on its uptake at various time points, keeping in mind that accurate quantifications are not possible with current fluorescent glucose analogues (Dienel *et al.*, 2017). Next, we will verify if GLUT inhibitors, such as cytochalasin B[63], can fully abrogate high glucose-induced neuronal death. Early events of mitochondrial

[63] Cytochalasin B is a competitive inhibitor of all GLUT isoforms. In fact, this inhibitor is used to estimate absolute concentrations of GLUT proteins at the surface of cells, by measuring its equilibrium binding (Kalaria *et al.*, 1988; Maher and Simpson, 1994; Maher *et al.*, 1994). Such measurements were utilized in computing glucose and lactate transport capacities of brain cells in the core model (Simpson *et al.*, 2007) (Table 1.6).

dysfunction will be investigated as per the strategy hired by Brownlee and colleagues, which employed inhibitors of the electron transport chain, like rotenone (complex I inhibitor) and antimycin A (complex III inhibitor), to confirm the site of superoxide anion generation (Du *et al.*, 2001; Nishikawa *et al.*, 2000). Ideally, application of a mitochondrial uncoupler or upregulation of mitochondrial SOD should also be performed to verify, respectively, that dissipating the elevated transmembrane potential induced by the overworked electron transport chain and that quenching superoxide anion at the site of production are individually sufficient to prevent the death of neurons (Du *et al.*, 2001; Nishikawa *et al.*, 2000). We also plan to employ PJ34 and INO-1001, specific PARP inhibitors, to uncover the importance of enhanced PARP activity in the death of neurons, especially in view of our current results demonstrating its cleavage in late apoptosis. Correspondingly, we will assess the level of GAPDH ADP-ribosylation and measure this enzyme's activity in conditions of high glucose. It would also be interesting to verify whether the inhibition of GAPDH by koningic acid is sufficient to elicit the death of neurons in a way that emulates high glucose conditions. Last, the polyol pathway will be investigated, on the one hand, by weighing the benefits of inhibiting aldose reductase with sorbinil in high glucose conditions, and on the other, by measuring the state of reduced glutathione-to-oxidized glutathione in our model.

Considering the lack of evidence pertaining to the chain of early events that lead to neuronal degeneration in high glucose or hyperglycaemic conditions, it would be highly pertinent to determine whether neuronal apoptosis occurs before a tangible loss of mitochondrial respiration. For the moment, the literature only reports a drop in the respiratory rate of cultured peripheral nerves from diabetic rodents at later time points (Aghanoori *et al.*, 2017; Akude *et al.*, 2011; Chowdhury *et al.*, 2010). This question should be investigated both *in vitro* and *in vivo* in order to determine whether cultured neurons are faithful mechanistic models of what occurs in the CNS. The elaboration of proper *in vitro* frameworks provided with a valid precinct of applicability would therefore allow for more cautious and robust conclusions to be drawn from cultured neurons. In our case, we simply aimed to verify the death of cultured dopaminergic

neurons in high glucose conditions, permitting a careful inference of their probable demise in a rodent model of hyperglycaemia.

5.1.2 Resveratrol: partial antioxidative effects, but full neuroprotection

In addition to demonstrating that high glucose concentrations trigger the oxidative stress-induced apoptosis of dopaminergic neurons, Chapter II also provides evidence for the protection conferred by the antioxidant resveratrol in these conditions. These results constitute the first report of the protective effect of resveratrol on neurons challenged with high glucose concentrations or hyperglycaemia. Studies performed in the CNS of diabetic rodents were issued later and support our results (Bagatini *et al.*, 2017; Sadi and Konat, 2016).

Although we did not aim to unravel the mechanistic underpinnings of resveratrol's effects, it is possible to surmise that direct scavenging of ROS occurred in our model, as it was shown to be possible *in vitro* (Fauconneau *et al.*, 1997; Frémont, 2000; Kawada *et al.*, 1998; Stivala *et al.*, 2001). Additional antioxidative mechanisms may have been mediated indirectly through inhibition of PDE, mTOR or NQO2, respectively leading to activation of the AMPK-SIRT1-PGC-1α axis, stimulation of autophagy, and reduced production of cytoplasmic ROS. All of these events may have contributed to mitigate oxidative stress and to prevent the apoptotic death of neuronal PC12 cells in high glucose conditions.

It remains that some of the protective effects exerted by resveratrol treatments were admittedly limited. This was the case for the inhibition of superoxide anion generation and the reversal of GRP75's compensatory overexpression. Our results show that resveratrol did not completely prevent the formation of superoxide anion at 3 h. However, we did not verify this effect at later time points, and the antioxidant may well have exerted its full effect by 96 h. More plausible is that resveratrol's antioxidative effect was indeed partial, but sufficient to confer total neuroprotection, as evidenced by the complete abrogation of apoptosis. The lack of mitochondrial GRP75 downregulation

in neuronal PC12 cells treated with high glucose concentrations and resveratrol indicates a likely need for the chaperone to wield a protective response in this organelle still displaying higher-than-normal levels of superoxide anion. However, in the cytoplasm, resveratrol treatments successfully prevented the overexpression of GRP75. This could be attributable to the fact that DNA damage was no longer critical, that p53 was not upregulated, and that GRP75 was not required to impound this apoptotic factor in the cytoplasm. It follows that resveratrol may have exerted just enough protection to preclude apoptosis by diminishing superoxide anion production to sublethal levels.

Polyphenols often exert partial effects in biological systems that can only partially be optimized by increasing doses. One theory provides that a biphasic dose-response relationship, for instance hormesis, may explain the limited though significant bioefficacy of this class of molecules (Figure 5.3). We have discussed this topic in greater detail elsewhere (Appendix B).

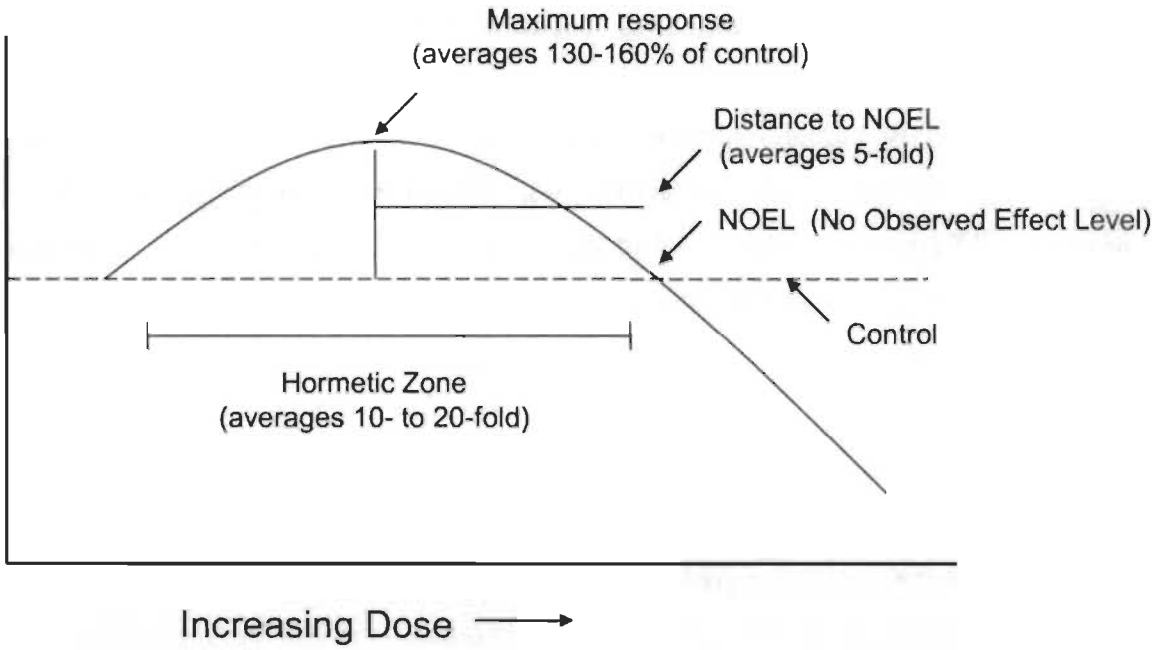

Figure 5.3 **Dose-response curve depicting the quantitative features of hormesis.** Hormesis is most commonly expressed as a J or inverted U dose-response curve, which either dips below or rises above a threshold of efficacy, depending on the observed endpoint. In this hormetic zone, effects are consistently predicted to be moderate, ranging between 30-60%. (From Calabrese *et al.*, 2013.)

5.1.3 The relevance of glucose-regulated protein 75 in Parkinson's disease

Reports showed the depletion of GRP75 in post-mortem substantia nigra pars compacta samples of patients before our paper was published (Burbulla *et al.*, 2010; Jin *et al.*, 2006). Gaining momentum as a possible novel genetic risk factor, reports today are rather equivocal regarding its association with the disease. Indeed, one group found two point mutations, albeit rare, in the GRP75 gene of parkinsonian patients (Wadhwa *et al.*, 2015), whereas another study concluded that this chaperone was not genetically associated to Parkinson's disease (Chung *et al.*, 2017). Nevertheless, evidence converges toward the reduced expression of GRP75 in specific affected brain regions, expressly the substantia nigra pars compacta, both in patients and in rodent models of Parkinson's disease (Cook *et al.*, 2016; Koo *et al.*, 2017; Liu *et al.*, 2015).

In this view, our results rather show a rise in GRP75 expression levels in response to a cellular stress. What occurs in our *in vitro* model challenged with high concentrations of glucose is likely not representative of these physiological observations harvested in brain samples having sustained months, in rodents, or years, in humans, of Parkinson's disease-related stress. It is nevertheless interesting to see how GRP75 is systematically downregulated in the substantia nigra pars compacta and invites the question of whether this protective chaperone may be a supplementary phenotypic liability specific to the neurons harboured in this structure.

5.2 Objectives 3 and 4: *In vivo*, long-term hyperglycaemia causes preferential nigrostriatal dopaminergique neurodegeneration and consequential behavioural alterations

Upon validating that dopaminergic neurons cultured in high glucose conditions undergo cell death, we elaborated a rodent paradigm wherein neurodegeneration could be assessed following long-term hyperglycaemia. Through immunoblotting, immunohistochemical, neurochemical and behavioural analyses, we obtained data presented in Chapter III demonstrating a decrease in dopamine neurotransmission in the dorsal striatum, the degeneration of nigrostriatal neurons, motor impairments and glial

alterations in long-term hyperglycaemic rodents. Chapter IV provided further information on the behavioural manifestations displayed by hyperglycaemic rats in a social context, correlated to the level of striatal denervation.

5.2.1 Intracerebral glucose concentrations

The first element discussed in Chapter III was the rise in CNS glucose concentrations in hyperglycaemic rats. This firsthand demonstration was required to solidify the argument that sustainably elevated intracerebral glucose concentrations may induce neurodegeneration. Precisely, providing proof that glucose concentrations were not altered differentially in the various brain regions of interest was crucial. As previously stressed, all studies reporting intracerebral glucose measurements to date have provided quantifications in, at best, two brain regions at a time (Abi-Saab *et al.*, 2002; Béland-Millar *et al.*, 2017; de Vries *et al.*, 2003; Gomez and Barros, 2003; Jacob *et al.*, 2002; Macauley *et al.*, 2015; McCrimmons *et al.*, 2003; Osborne *et al.*, 1997). Granted inter- and intra-individual variability among hyperglycaemic models, we thus sought to rely on our own measurements to confirm – instead of presuming, like others have – a uniform rise in glucose concentrations throughout various brain regions at a time[64].

In this view, we aimed to verify the rise in brain glucose by more than one single method. Using the microdialysis technique, we were able to obtain estimations of extracellular glucose concentrations in uniformly-fed rats. This technique also allowed us to distinguish midbrain and striatal subareas, owing to the spatial precision afforded by the probes. By homogenizing brain tissues, we also appraised intracellular glucose concentrations, although we were fully aware that terminal perfusion of the animals constituted a cause for underestimations due to efflux through bidirectional GLUTs driven by concentration gradients. Nevertheless, the purpose of these procedures was to assess if there was a significant rise in glucose concentrations throughout the brain,

[64] Speculative computations performed by Simpson and colleagues (2007) still provided a theoretical basis for an initial assumption that glucose concentrations may rise in the brain in hyperglycaemic conditions and for justifying the purpose of our *in vivo* investigations (Table 1.7).

and if there were substantial differences between neuroanatomical loci. We also aimed to verify that intra- and extracellular observations coincided. For these reasons, all results were reported in relative and not absolute values.

Our results provided a firm validation that intracerebral glucose concentrations increase significantly and uniformly throughout the midbrain, striatum, prefrontal cortex and hippocampus of hyperglycaemic rats. Elevated concentrations were also sustained over the course of 6 months. These results were important to unravel several remaining interrogations with respect to a possible downregulation of glucose uptake in long-term hyperglycaemia. Indeed, there is no adequate account of the regulation of GLUTs over long periods of hyperglycaemia (Anitha *et al.*, 2012; Nagamatsu *et al.*, 1994; Santiago *et al.*, 2006; Simpson *et al.*, 1999), nor is there evidence to sustain reduced glucose uptake due to reductions in cerebral blood flow arising from abnormal vascular features in this chronic setting (Carruthers and Helgerson, 1989; Duckrow *et al.*, 1987; Levine *et al.*, 1998; Lowe and Walmsley, 1986; McCall, 1992; Nishimura *et al.*, 2007). We are currently quantifying GLUT expression in brain slices from our model in the aim of filling these knowledge gaps. There is, nevertheless, reason to believe that intracellular glucose concentrations remain significantly elevated after 6 months of sustained hyperglycaemia in our rat model.

5.2.2 Altered glial profiles as an indicator of oxidative stress

On the quest to cover as many aspects of CNS dysfunction in our paradigm, we reported the effects of long-term hyperglycaemia not only in neurons, but also in glial populations. These glial alterations are discussed first on account of the information they can convey with respect to the oxidative status of the CNS, directly linked to our *in vitro* work.

By immunohistochemical staining, we discovered a marked proliferation of astrocytes in the substantia nigra pars compacta, the dorsal striatum, the nucleus accumbens and the prefrontal cortex. Conversely, there was a noticeable loss of

microglial cells in the substantia nigra pars compacta, the dorsal striatum, and the nucleus accumbens. A first appraisal of these results allowed us to conclude that both pathological features are present in the substantia nigra pars compacta, but not in the ventral tegmental area, further underlining a regional vulnerability to pathological alterations ensuing from hyperglycaemia. They also manifested themselves throughout the striatum.

In Chapter III, we discussed the well-appreciated association between astrogliosis and diabetes. We also proposed a role for oxidative stress in causing the death of microglial cells, an under-recognized feature in CNS affections. We can further elaborate on the tentative timeline proposed in this article, which stipulated that an initial microglial cell activation in turn conduces to astroglial activation, followed by the death of the former, which are not equipped to cope with sustained oxidative stress. Indeed, CNS microgliosis occurs early in hyperglycaemic rodent models (Nagayach *et al.*, 2014; Oliveira *et al.*, 2016). Importantly, microgliosis can be triggered by oxidative stress, as demonstrated in various paradigms (Bordt and Polster, 2014; Kang *et al.*, 2001; Mander *et al.*, 2006; Rojo *et al.*, 2010). This event then directly contributes to the activation of astrocytes (Liddelow *et al.*, 2017) whose elevated levels can be appraised in our model 6 months following hyperglycaemia induction. One question that dwells here is the physiological pertinence of upregulating astrocyte proliferation by oxidative stress-activated microglial cells. Recalling the importance of the PPP in protecting against oxidative stress offers one clue. With neurons, astrocytes constitute the main cell type to process important amounts of G6P through the PPP for the recovery of NADPH serving to regenerate the antioxidative cofactor glutathione. In fact, the PPP is 4-5 times more active in astrocytes than in neurons and is upregulated in high glucose conditions[65] (Ben-Yoseph *et al.*, 1996*a*, 1996*b*; García-Nogales *et al.*, 2003; Takahashi *et al.*, 2012) (Figure 5.4). Remarkably, microglial cells can also mediate the increase in astroglial PPP activity, independently of its influence on the proliferation of these cells (Iizumi *et al.*, 2016). It is therefore reasonable to surmise that microglial activation serves to

[65] Similarly, the PPP is downregulated when glucose levels are reduced, thereby dulling the antioxidative potential of neural cells (Takahashi *et al.*, 2012). Some have suggested this phenomenon to explain the risks that sharp glycaemic variations represent in diabetic patients.

promote the proliferation of astrocytes, alongside their PPP flux, in order to respond to oxidative insults. Interestingly, the PPP also serves to generate ribose 5-phosphate for the synthesis of nucleic acids required for mitosis, such as in proliferating astrocytes, constituting yet another way by which this compensatory effect is coupled with purposeful cellular functions (Lehninger *et al.*, 1995).

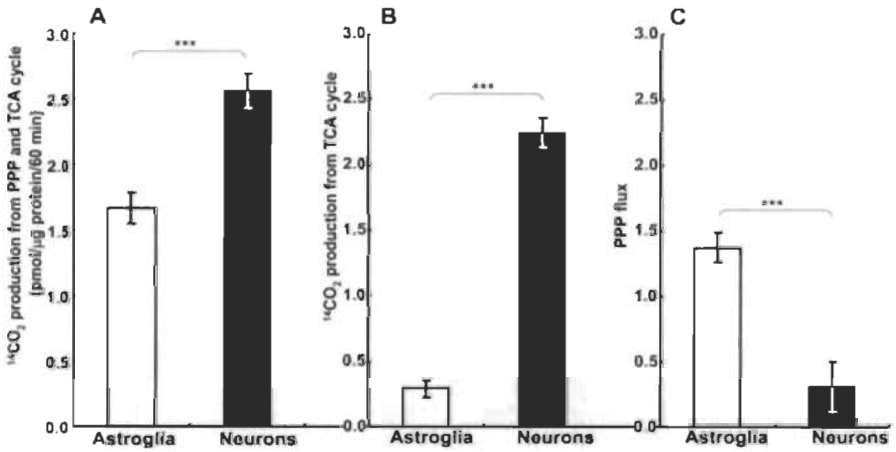

Figure 5.4 Metabolic activities of astrocytes and neurons.
In primary neuronal (black) and astroglial (white) cultures, the rate of PPP activity (C) was obtained by measuring the difference between radio-carbon dioxide ($^{14}CO_2$) production ensuing from the PPP and the tricarboxylic acid cycle (A) or the tricarboxylic acid cycle alone (B) by employing glucose isotopes labelled at the C1 or C6 positions. Focusing on the graph on the right, astroglial cultures display PPP activities 4-5-fold times greater than neuronal cultures. Means ± standard deviation. $*p < 0.05$; $***p < 0.001$ between groups, according to *t*-test. (From Takahashi *et al.*, 2012.)

The functional relationship between oxidative stress-induced microglial activation and astrogliosis clearly emerges upon discerning the near-perfect correspondence between loci displaying high levels of astrocytes and those harbouring significantly fewer microglial cells. If indeed microglial cells perish due to sustained oxidative stress, as granted by several lines of evidence (Khazaei *et al.*, 2008; Sabokdast *et al.*, 2015; Streit *et al.*, 2008), it follows that these identified loci were prone to oxidative events in our model. It is therefore likely that, before their demise, microglial cells were indeed activated by this oxidative stress, as supported by previous demonstrations (Bordt and Polster, 2014; Kang *et al.*, 2001; Mander *et al.*, 2006; Rojo *et al.*, 2010). In these

regions, we can infer that activated microglial cells may have initiated a reactive astrogliosis, still perceptible 6 months following hyperglycaemia. In other words, regions displaying high levels of oxidative stress led to consequent changes in glial profiles to promote ROS clearance via the purposeful activation of the PPP and the generation of antioxidative cofactors. Several supplementary firsthand verifications would be required to support this proposed timeline of events occurring in glial populations. Nonetheless, we can say without a doubt that this possible coping mechanism provided by a reactive astrogliosis did not afford sufficient protection to dopaminergic neurons of the nigrostriatal pathway nor to microglial cells in oxidation-prone areas, which all visibly degenerated in our model 6 months following hyperglycaemia induction.

What remains unclear is why the substantia nigra pars compacta and the striatum were auspicious to oxidative stress-related events, precisely astrogliosis and microglial death, whereas the hippocampus was relatively spared. One explanation might be that these regions share the liability of processing large amounts of dopamine[66], known to react with hyperglycaemia-induced methylglyoxal to form oxidative salsolinol-like compounds (Deng *et al.*, 2012; Song *et al.*, 2014; Szent-Gyorgi and McLaughlin, 1975). Another option is that the substantia nigra pars compacta is rich in iron (Chinta and Andersen, 2008; Hirsch and Faucheux, 1998), whereas the entire striatum is rich in midbrain dopaminergic fibres that harbour a wealth of mitochondria (Pacelli *et al.*, 2015), two independent elements conducive to ROS generation. These lines of thought in fact boil down to the risk phenotypes characteristic of nigrostriatal neurons. We are currently in the process of measuring advanced glycation end-products in these various regions employing antibodies raised against glycated arginine and lysine residues. By these means, we aim to identify differential levels of glycation throughout the CNS, an indirect marker of oxidative stress, and to determine vulnerable cell types between neurons, astrocytes or microglial cells.

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[66] In fact, the prefrontal cortex contains smaller amounts of dopamine than these two regions, as evidenced by our own microdialysis studies (Figure 3.3I), and was also partially spared as it did not display signs of microglial cell death (Figure 3.6A and C); perhaps the proliferation of PPP-utilizing astrocytes in this region was sufficient to partially protect microglial cells against oxidative stress.

5.2.3 Nigrostriatal dopaminergic neuronal death: beyond the validation of our hypothesis

The work presented in Chapter III was pivotal to this project, as it provided a direct confirmation of the core hypothesis. Indeed, our results show that the nigrostriatal pathway is preferentially targeted in a hyperglycaemic setting, compared to mesocorticolimbic neurons. Bridging these findings with observed motor deficits and tracing the sequence of events offer further insight into how nigrostriatal dopaminergic neurodegeneration may have operated in our rat model of long-term hyperglycaemia.

5.2.3.1 Subtle neurodegeneration and motor deficits

Upon inspecting the results presented in Chapter III, it is possible to observe that most changes occurring because of long-term hyperglycaemia are subtle. Indeed, the reduction in the expression of dopaminergic markers in the midbrain and striatum, the relative loss of dopaminergic nigrostriatal neurons, and the dampened tonic dopamine release in the dorsal striatum do not exceed 50% of age-matched controls.

Other groups also observed these modest effects in a similar rodent paradigm (Brambilla Bagatini *et al.*, 2014; do Nascimento *et al.*, 2011). Both groups found small amounts of neurodegeneration in the substantia nigra pars compacta in streptozotocin-treated animals. However, these results were obtained 60 (Brambilla Bagatini *et al.*, 2014) or 90 days (do Nascimento *et al.*, 2011) following streptozotocin injections, while we did not observe neurodegeneration at the 3-month mark, at least when considering expression levels of dopaminergic and neuronal markers[67]. This could be explained by the fact that these studies employed considerably harsher chemical-induced rodent paradigms of diabetes, thereby accelerating the neurodegenerative process. It is unclear whether these models would exhibit more dopaminergic neuronal death at 6 months or

[67] Brambilla Bagatini and colleagues employed i.p. injections of 65 mg/kg b.w. of streptozotocin without nicotinamide, a fairly high concentration in rats affording complete insulin depletion (Brambilla Bagatini *et al.*, 2014). In an even harsher paradigm, the other group administered rats with streptozotocin at a dose of 50 mg/kg b.w. i.v., once again without nicotinamide (do Nascimento *et al.*, 2011).

whether neurodegeneration would slow down to reach a stable degree of modest denervation that could resemble the levels found in our paradigm. However, granted the severity of the hyperglycaemic phenotype, it is unlikely that such models could survive to the 6-month time point.

Other findings corroborating our work contrast with studies performed in paradigms combining parkinsonian and diabetic insults in which overt neurodegeneration can be observed (Choi *et al.*, 2005; Morris *et al.*, 2010; Rotermund *et al.*, 2014). For example, after one week on a high-fat diet following MPTP treatments, mice display almost complete nigrostriatal neurodegeneration (Choi *et al.*, 2005). Although such studies provide information suggesting that metabolic alterations exacerbate the deleterious effects of parkinsonian toxins, they do not provide insight into the natural processes that may underlie neurodegeneration in a longstanding metabolic syndrome. Our model thus presents the advantage of mimicking the dopaminergic neurodegeneration that occurs in a hyperglycaemic setting over the course of aging without other exogenous effects: this neuronal loss is realistically modest.

Most importantly, our findings support the moderate motor deficits observed in our rat model of hyperglycaemia, recalling bradykinesia and gait disturbances in parkinsonian patients. In fact, as already mentioned, some tests did not reveal any differences between control and hyperglycaemic rats, such as in the vibrissae-elicited forelimb placement test used to assess sensorimotor integrative deficits. These observations evoke the compensatory neurocircuit redundancies in the basal ganglia of parkinsonian patients that account for the fact that 30-70% of nigrostriatal dopaminergic neurons and 50-80% of their striatal projections have usually perished before the initiation of gross motor symptoms (Bernheimer *et al.*, 1973; Cheng *et al.*, 2010; Fearnley and Lees, 1991). Accordingly, we found approximately 30% of neuronal loss in the substantia nigra pars compacta and 50% of denervation in the striatum of long-term hyperglycaemic rats. This level of neurodegeneration stands at the threshold of symptomatic detection and likely explains why we observe modest, yet significant, indices of bradykinesia and gait disturbances.

5.2.3.2 Time course of neurodegeneration

In Chapter III, we investigated dopaminergic and motor alterations at 3 and 6 months in our model of hyperglycaemic rats. At 6 months, hyperglycaemic rats displayed clear neurodegeneration of the nigrostriatal pathway, as substantiated by immunoblotting and immunohistochemical assays. However, some indications of dopaminergic dysfunction were already apparent at the 3-month mark before immunoblotting measurements could attest of any neurodegeneration.

Specifically, tonic dopamine release in the dorsal striatum was already dampened at 3 months. We cannot exclude the possibility that the immunoblotting method may not have allowed for the detection of inconspicuous neurodegeneration in striatal dopaminergic fibres, which could have been picked up by more sensitive immunohistochemical analyses at 3 months. Nevertheless, the literature consistently shows a dulling of dopamine neurotransmission occurring without visible neurodegeneration. Indeed, our observations bolster other reports showing that neither TH activity or expression are reduced in the striatum of streptozotocin rats, despite visible downregulation of dopamine neurotransmission as early as 2 weeks following the induction of hyperglycaemia (Bitar *et al.*, 1986; Lim *et al.*, 1994). Depreciation of striatal dopamine neurotransmission despite undetectable neurodegeneration is also typical of some transgenic models of Parkinson's disease (Abeliovich *et al.*, 2000; Li *et al.*, 2009).

Although neurodegeneration was unnoticeable at 3 months, several motor deficits were already apparent, as highlighted by the stepping and forepaw adjusting step tests. In support of our observations, others have reported modest impairments of motor functions in rodent models of Parkinson's disease despite the absence of nigrostriatal neurodegeneration (Abeliovich *et al.*, 2000; Li *et al.*, 2009). Yet, the horizontal bar test did not uncover any motor abnormalities in our model at 3 months. As previously mentioned, it is the least sensitive of the 3 tests, which may explain why modest dopaminergic dysfunctions at 3 months may not have manifested themselves in this specific task (Kelm-Nelson *et al.*, 2015). Another group reported horizontal bar deficits

in a similar model 60 days following the induction of hyperglycaemia. However, these rats were submitted to a harsher streptozotocin regimen and also displayed substantial immobility in the open field test (Brambilla Bagatini *et al.*, 2014). In contrast, although we did not perform this specific test, our rats did not manifest signs of hypolocomotion, on the contrary[68].

All evidence considered, it appears that dopaminergic neurotransmission dysfunctions occur before a detectable loss of nigrostriatal neuron bodies or their projections in our hyperglycaemic rat model. Dampened tonic dopamine release and manifestations of motor deficits preceding neurodegeneration sustain this proposed time course. Nevertheless, a proper immunohistochemical appraisal of the density of striatal dopaminergic fibres at 3 months is warranted to confirm this. It would also be interesting to measure dopamine release at 6 months to assess the extent of neurotransmission deterioration once neurodegeneration is perceptible. However, the surgical procedures inherent to the microdialysis method are risky in our model at that time point, explaining why we didn't perform these analyses.

5.2.4 Hyper-aggressive and hyper-sociable manifestations

In addition to motor deficits highlighted in Chapter III, we discovered hyper-aggressive and hyper-sociable traits in our model of long-term hyperglycaemia. In fact, these results presented in Chapter IV constitute the first adequate account of social behaviour in hyperglycaemic rats obtained in neutral circumstances[69]. In addition, we report the first USV recordings ever performed in a diabetic model, whether chemical-induced, diet-induced or genetic.

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[68] Observations of hyperactivity in our hyperglycaemic model were partly responsible for sparking our interest in their atypical social behaviour, related in Chapter IV.

[69] All other behavioural investigations in similar models relied on resident-intruder paradigms that may uncover submissive or dominant traits, but they do not report normal interspecies interactions (File and Seth, 2003; Hilakivi-Clarke *et al.*, 1990; Meehan *et al.*, 1986).

5.2.4.1 A possible relationship with nigrostriatal dopaminergic neurodegeneration

A rapid analysis of our results reveals much more frequent emissions of all types of USVs and manifestations of affiliation/exploration or aggression-related behaviours made by long-term hyperglycaemic rats. Covariance profiles between kinds of behaviours or between behaviours and USVs are also noticeably altered. Our results converge toward the conclusion that long-term hyperglycaemic rats are hyper-aggressive and hyper-sociable, displaying behaviours that do not coincide with the affective valence of their communications. Importantly, we found a correlation between the degree of striatal denervation and the severity of these phenotypes. Because the article presented in Chapter IV did not precisely address nigrostriatal neurodegeneration, we separated our rats into groups according to overall striatal denervation. However, as we know from Chapter III, this denervation is greatest in the dorsal area; separating the animals on the basis of dorsostriatal denervation still yields the same 3 groups (Figure 5.5). Therefore, although not within the scope of the article presented in Chapter IV, we can still say in the context of this thesis that hyper-aggressive and hyper-sociable manifestations alongside USV emissions were specifically associated with the degree of nigrostriatal dopaminergic neurodegeneration.

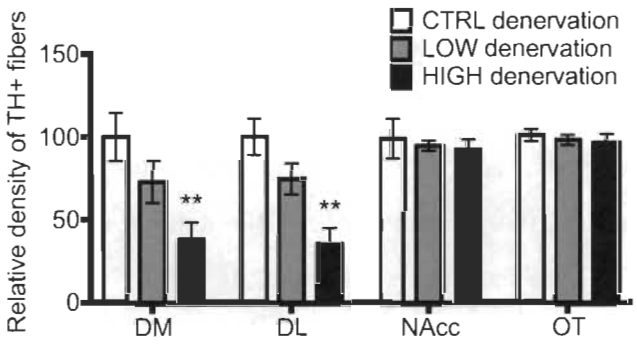

Figure 5.5 Classification of long-term hyperglycaemic rats based on the degree of striatal denervation in multiple regions.
In Figure 4.4, hyperglycaemic rats were already divided in groups according to total striatal denervation. Using the same animals in identical groups, we show here that dorsomedial and dorsolateral denervation are responsible for this total observed striatal denervation reported in Chapter IV. All data presented as means ± SEM. Asterisks indicate statistical differences between the HG group and CTRL group (**$p < 0.01$). Refer to Chapters III and IV for methodological details.

5.2.4.2 A possible relationship with phasic and tonic dopaminergic neurotransmission

Our findings raise the legitimate question of how dampened dorsostriatal dopaminergic neurotransmission can account both for a rise in motor deficits and for abnormal social reward-related behaviours. The link between insufficient tonic firing in the dorsal striatum and manifestations of motor deficiencies reported in our model and manifested in parkinsonian patients in the form of bradykinesia and rigidity is well appreciated (Dreyer, 2014). However, the association between a loss of dopamine tone and increased reactivity to novel or rewarding elements in the environment is much less obvious. In fact, parkinsonian patients usually exhibit apathy or motivational deficits, which starkly contrast with the hyper-sociable and hyper-aggressive traits found in our model (Drui *et al.*, 2014; Magnard *et al.*, 2016). A challenge therefore emerges upon attempting to reconcile these two seemingly divergent behavioural manifestations in light of evidence of depleted dorsostriatal dopamine in our model of long-term hyperglycaemia.

Identifying neuropathologies bearing similar social abnormalities is a sensible place to start in the aim of unravelling this daunting question. Our model quite noticeably exhibits archetypal behavioural features of attention-deficit hyperactivity disorder[70]. This neuropathology is strongly associated with hyper-reactivity to novel elements in the environment, whether social or not, which can manifest itself in the form of inappropriate impulsivity, curiosity and aggression, both in animal models and in humans (Hopkins *et al.*, 2009; Jhang *et al.*, 2017; King *et al.*, 2003; Ko *et al.*, 2013; Rosa-Neto *et al.*, 2005). Like Parkinson's disease, its pathophysiological underpinnings are tightly linked to dopaminergic dysfunction[71] (Levy, 1991; 2004; Sikström and Söderlund, 2007). Recent studies have slowly begun to extricate the neuroanatomical loci implicated in this disease, and the dorsal striatum and substantia nigra pars compacta have emerged among the most important regions. Indeed, patients with

[70] The highly complex epidemiological relationship between Parkinson's disease and attention-deficit hyperactivity disorder is discussed in a short commentary provided in Appendix D.

[71] In fact, the dopamine reuptake inhibitor methylphenidate remains the benchmark treatment.

attention-deficit hyperactivity disorder present an altered nigral morphology (del Campo *et al.*, 2013; Romanos *et al.*, 2010). In addition, others have reported diminished tonic and enhanced phasic dopaminergic neurotransmission in the dorsal striatum of these subjects (Badgaiyan *et al.*, 2015; Sikström and Söderlund, 2007).

This last piece of evidence provides important insight into what may occur in our model of long-term hyperglycaemia. There is cause to believe that low dopamine tonicity, or background noise, favours the saliency of phasic signals. As previously stated, the dorsal striatum is normally submitted to higher noise-to-signal ratios than the ventral striatum[72], owing to the marked pacemaking activity of nigrostriatal neurons (Dreyer, 2014; Zhang *et al.*, 2009). In a context of partial tonic depletion, signals provided by phasic dopamine are overstated when apposed to a diminished background of tonic noise, and also by means of a compensatory upregulation of postsynaptic dopamine receptors[73]. However, when dopaminergic denervation is substantial, as in Parkinson's disease, neither phasic nor tonic dopaminergic neurotransmission is properly fulfilled, giving rise to motor symptoms and loss of hyper-reactivity, or even to the apparition of apathy (Dreyer, 2014). On this basis, we can carefully submit to the idea that our hyperglycaemic paradigm yields a model that is sufficiently denervated to allow the timely overlap between a moderately hypotonic phenotype responsible for subtle motor deficits and a relative hyperphasicity accounting for social hyper-reactivity. We could suppose that if the nigrostriatal pathway had undergone greater levels of degeneration, our model would have more closely resembled the apathetic, motor-deficient parkinsonian phenotype. Noteworthy, proper demonstrations of enhanced dorsostriatal phasic signalling are warranted to confirm this, and would require the employment of methods allowing the infrasecond detection of dopamine transients,

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[72] In fact, the low noise-to-signal ratio in the ventral striatum is conducive to proper phasic signalling by ventral tegmental area neurons, which underlies the mesocorticolimbic pathway's prominent role in encoding reward saliency and value.

[73] Recently emerged complementary evidence in humans and rodents supports a role for nigrostriatal hyperphasicity in behavioural alterations. In particular, self-driven curiosity, motivation, and aggression-related behaviours, all applicable to social settings, are enhanced by phasic nigrostriatal dopaminergic neurotransmission (Di Domenico and Ryan, 2017; Rossi *et al.*, 2013; Skibsted *et al.*, 2017).

for instance fast-scan cyclic voltammetry or electrophysiological techniques (Hauber, 2010; Schultz, 2010; Segovia *et al.*, 2011; Wightman and Robinson, 2002).

5.2.5 Effects attributable to hypoinsulinaemia

To obtain a long-term hyperglycaemic phenotype, rats were rendered permanently hypoinsulinaemic. Given insulin's highly complex roles in the healthy and diseased CNS, extensively reviewed elsewhere (Blázquez *et al.*, 2014; Ghasemi *et al.*, 2013; Gray *et al.*, 2014; Porte *et al.*, 2005), our challenge arises upon attempting to pick apart the effects attributable to hypoinsulinaemia or hyperglycaemia in the multiple neuronal and behavioural alterations observed in our model.

5.2.5.1 Insulin in neurodegeneration

As described in section 1.2.2, insulin is not required for glucose transport in the brain parenchyma or in most resident cells. Nevertheless, this hormone is ubiquitous in the CNS where it mediates developmental, differentiation, plasticity and survival signals. In a normal setting, insulin's concentrations are highest in the pons, medulla and hypothalamus, and lowest in the occipital cortex and thalamus (Banks *et al.*, 1998), our brain regions of interest falling within the average. As well, the insulin receptor is also widely expressed throughout the CNS, especially so at the neuronal surface (Havrankova *et al.*, 1978*a*; Hill *et al.*, 1986; Schulingkamp *et al.*, 2000; Unger *et al.*, 1991), and possesses similar kinetic properties to its peripheral counterpart (LeRoith *et al.*, 1988; Zahniser *et al.*, 1984). Insulin signalling in neurons is quite similar to that in peripheral cells and leads to the activation of PI3K/Akt and RAS-extracellular signal-related kinase (ERK) pathways responsible for the stimulation of synaptic plasticity and gene transcription, among many other effects (Figure 5.6). By modulating these pathways, it is generally accepted that insulin acts as a neurotrophic factor, promoting the survival of neurons. Accordingly, several demonstrations of insulin's neuroprotective competences have been carried out *in vitro*, for instance in settings of excitotoxicity or oxidative stress (Duarte *et al.*, 2006; Kim and Han, 2005; Ribeiro *et al.*,

2014; Sun *et al.*, 2010). *In vivo*, near-direct application of insulin on the CNS, via intranasal or interacerebroventricular treatments, are highly effective in promoting various aspects of neuronal health, as demonstrated in a rat model of Parkinson's disease (Haas *et al.*, 2016; Pang *et al.*, 2016).

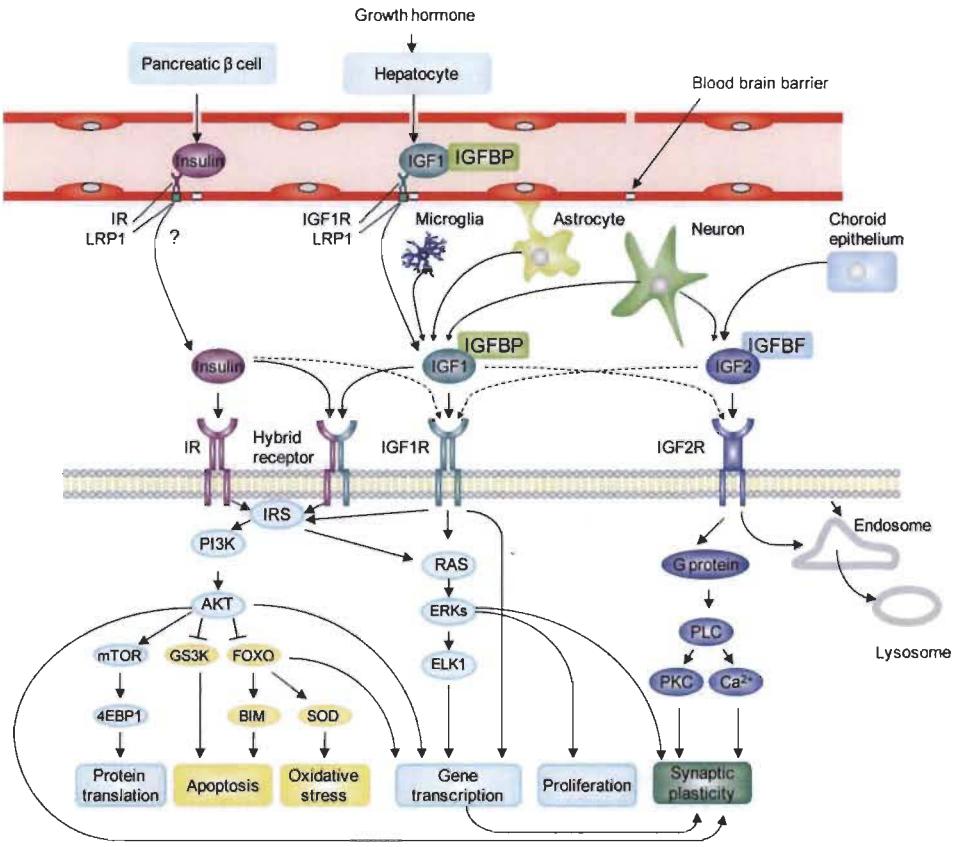

Figure 5.6 **Overview of insulin and insulin-like peptide signalling in the brain.** In the periphery as in the brain, insulin and insulin-like peptides bind 3 kinds of receptors: the insulin receptor (IR), insulin-like growth factor receptors (IGF1R and IGF2R), and hybrid receptors. Insulin preferentially binds the IR but can also bind IGF1R or hybrids. In the central nervous system (CNS), insulin and insulin-like peptides are produced locally or provided by the circulation. Insulin transport across the blood-brain barrier is not well understood. Focusing on insulin, its action on receptors triggers canonical pathways via the insulin receptor substrate that lead to a plethora of cellular changes favouring plasticity and differentiation. 4EBP1, eIF4E-binding protein 1; ELK1, ETS-like transcription factor; ERKs, extracellular signal-related kinases; FOXO, forkhead box O; GS3K, glycogen synthase kinase 3; IGF, insulin-like growth factor; IGFBF and IGFBP, insulin-like growth factor binding proteins; IRS, insulin receptor substrate; LRP1, low-density lipoprotein receptor-related protein 1; PLC, phospholipase C. (From Benarroch, 2012.)

Although the neuroprotective competences of *in situ* insulin treatment are undeniable, the abovementioned reports do not directly address the possible harmful effects of hypoinsulinaemia pertinent to our project. Other studies attempting to demonstrate that neuronal death directly ensues from hypoinsulinaemia employ peripheral insulin administration to reverse cellular or structural damage induced by streptozotocin treatments in the CNS of rodents[74] (Hung *et al.*, 2014; Moreira *et al.*, 2005, 2006; Ramanathan *et al.*, 1999). However, they do not take into account the various peripheral benefits exerted by this hormone replacement therapy, for instance on glycaemic control. Such strategies exploiting organism-wide paradigms to establish causality between hypoinsulinaemia and CNS neuronal death are based on the misconception that intracerebral insulin is solely provided from the circulation, itself supplied by the pancreas. As a matter of fact, insulin concentrations are considerably greater in the brain parenchyma than in the plasma, and are unexpectedly upregulated in hypoinsulinaemic, hyperglycaemic streptozotocin-treated rats (Banks *et al.*, 1997*b*; Gupta *et al.*, 1992; Havrankova *et al.*, 1978*a*, 1978*b*, 1979) (Figure 5.7). In support of this, insulin receptors are modulated in the periphery but not in the brain of streptozotocin models (Pacold and Blackard, 1979; Pezzino *et al.*, 1996). These preliminary findings led to the discovery that, besides being taken up through the blood-brain barrier (Banks *et al.*, 1997*a*; Margolis and Altszuler, 1967; Woods and Porte, 1977), insulin is synthesized by neurons within the mammalian brain (Birch *et al.*, 1984; Deltour *et al.*, 1993; Devaskar *et al.*, 1994; Dorn *et al.*, 1983; Frölich *et al.*, 1998; Mehran *et al.*, 2012; Molnár *et al.*, 2014; Schechter *et al.*, 1994; Young, 1986). Importantly, one group demonstrated that high glucose conditions enhance insulin production by cortical neurons (Molnár *et al.*, 2014), which may be a mechanism to maintain appropriate intracerebral concentrations of insulin when pancreatic supplies plunge (Banks *et al.*, 1997*b*; Gupta *et al.*, 1992; Havrankova *et al.*, 1979). As the aforementioned studies have not quantified insulin in the injured regions of the

[74] One group did claim to show that streptozotocin-induced hypoinsulinaemia to cause CNS injuries, albeit without cell loss, and to further uncover the protective effects of intranasal insulin treatments (Francis *et al.*, 2008). However, this article, along with 7 others from the same group on the same subject, was retracted in 2014.

CNS following its peripheral depletion, there is no way to assertively avow a direct negative influence of hypoinsulinaemia on neurons.

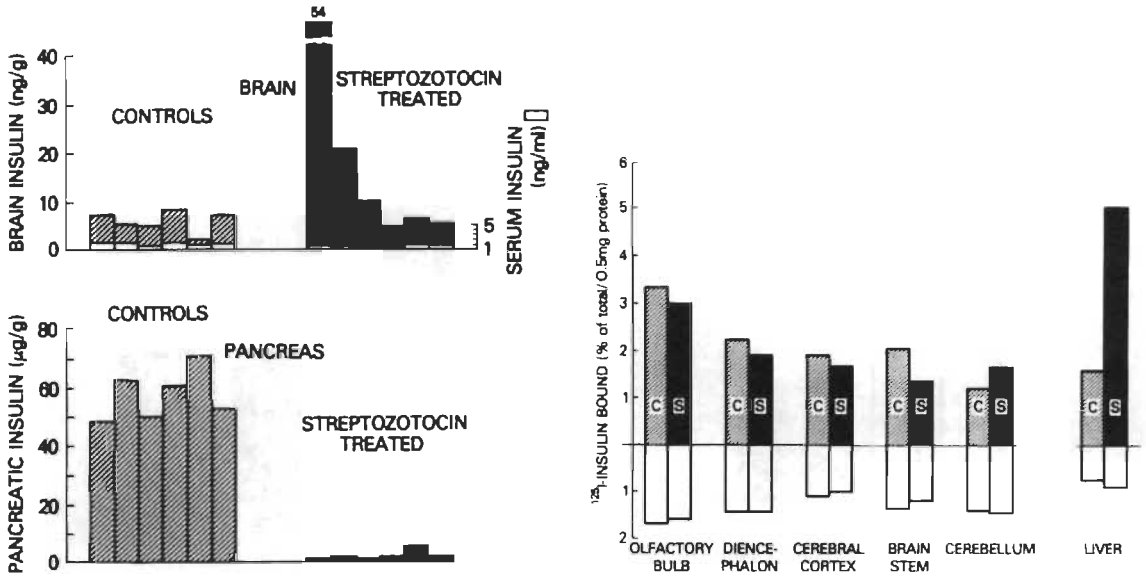

Figure 5.7 Insulin content and binding in the brain of streptozotocin-treated rats.
Left, top: Brain insulin levels (black bars) are not diminished one month after the induction of hyperglycaemia by streptozotocin compared to control conditions (hatched bars), although serum insulin is clearly reduced (white bars). Left, bottom: Pancreatic insulin levels are depleted in the pancreas of streptozocin-treated rats. Right: Insulin binding does not differ between control (C, hatched bars) and streptozotocin-treated (S, black bars) rats across various brain regions. Hepatic insulin binding, on the other hand, is greatly increased in hyperglycaemic rats, owing to an enhanced sensitivity of receptors. White bars indicate non-specific binding. (From Havrankova *et al.*, 1979.)

Regarding our studies, we did not measure insulin concentrations in the brain regions of interest in our long-term hyperglycaemic model. However, faced with the unverified possibility that hypoinsulinaemia can trigger neuronal death, we re-examined our data in a similar manner to Chapter IV by factoring in our rats' insulin profiles. In our model, hypoinsulinaemia and glucose intolerance clearly do not correlate with the loss of nigrostriatal neurons (Figure 5.8). Yet, we strongly agree that future investigations would require quantifications of insulin and its receptor in targeted brain regions to corroborate hypoinsulinaemia's apparent lack of influence on neurodegeneration in our model.

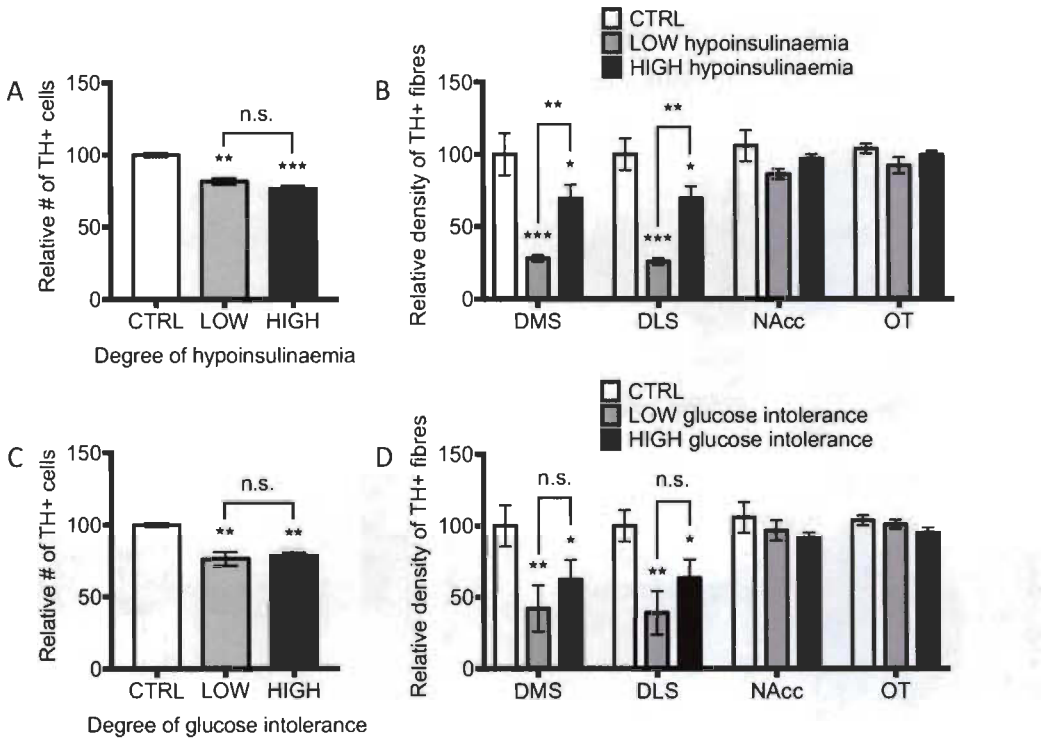

Figure 5.8 **Nigrostriatal dopaminergic neurodegeneration in relation to the degree of hypoinsulinaemia or glucose intolerance.**
Streptozotocin-treated 6-month hyperglycaemic rats were classified according to the degree of hypinsulinaemia (A, B) or glucose intolerance (C, D). Numbers of TH-positive neurons in the substantia nigra pars compacta were related to the degree of hypoinsulinaemia (A) or glucose intolerance (C): no correlation is observed between both variables in these circumstances. Similarly, the density of TH-positive fibres in the various regions of the striatum were related to the degree of hypoinsulinaemia (B) or glucose intolerance (D), and no association is found. All data presented as means ± SEM. Asterisks indicate statistical differences between the LOW or HIGH subgroups within the HG group and the CTRL group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Refer to Chapters III and IV for methodological details.

5.2.5.2 Insulin in behaviour

Not only is insulin endowed with neurotrophic competences, it is a potent regulator of behaviours such as feeding, reward valuation, cognition and memory (Anthony *et al.*, 2006; Benedict *et al.*, 2004, 2007; Craft *et al.*, 1996; Hallschmid *et al.*, 2008; Khanh *et al.*, 2014; Reger *et al.*, 2008). Indeed, intracerebroventricular or intranasal administration of insulin reduces food intake, improves memory,

and ameliorates cognitive performances in humans and rodents (Babri *et al.*, 2007; Benedict *et al.*, 2004; Haj-ali *et al.*, 2009; Park *et al.*, 2000; Stockhorst *et al.*, 2004). Under physiological circumstances, postprandial peripheral insulin most significantly regulates the hypothalamus, directly at the level of the median eminence devoid of blood-brain barrier protection, where it normally conveys satiety signals. All of these regulatory effects, whether physiological or exogenous, are mediated by insulin's ability to promote neuronal plasticity, but also by its modulatory role on neurotransmission itself (Gupta *et al.*, 1992; Ohtani *et al.*, 1997). An emerging and important neurotransmitter on which insulin exercises its control is striatal dopamine. Granted the central position it holds in our project, we will focus here on the relationship between insulin[75] and mesostriatal (nigrostriatal and mesolimbic) dopaminergic signalling, especially pertaining to ensuing effects on food intake behaviours, which are overtly altered in streptozotocin-treated models. This will allow us to draw parallels with the abnormal behaviours we uncovered in our hypoinsulinaemic, hyperglycaemic rat model.

Insulin's effect on the midbrain is primarily indirect. By inhibiting neuropeptide Y /GABA/agouti-related protein (NPY/GABA/AgRP or NGA) neurons and by activating pro-opiomelanocortin/cocaine and amphetamine regulated transcript (POMC/CART) neurons, both located in the arcuate hypothalamus, insulin allows for the stimulation of anorexigenic neurons and the silencing of orexigenic neurons (Abizaid *et al.*, 2006; Cone *et al.*, 2005; Morton *et al.*, 2006) (Figure 5.9). The sum of these effects converges toward decreased food intake and increased catabolism. Pertinent to our project, orexigenic neurons in the lateral hypothalamus innervate many targets including midbrain dopaminergic neurons in the ventral tegmental area and the substantia nigra pars compacta (Sakurai *et al.*, 1998). Impeding orexigenic signals onto midbrain neurons leads to decreased dopaminergic neurotransmission in the striatum and diminished food-seeking behaviours. Although insulin's effects are broadly conveyed to mesostriatal circuits, dopaminergic neurotransmission in the dorsal striatum appears to be essential for feeding, whereas signalling to the nucleus accumbens plays only a secondary role in

[75] In an attempt to simplify this part of the discussion, we will disregard other hormonal changes that occur in our model, namely the depletion of leptin and the rise in ghrelin, which more or less mimic the effects of insulin on behaviour (see for review Palmiter, 2007).

modulating food anticipation behaviours (Baldo and Kelley, 2007; Cannon *et al.*, 2004; Drago *et al.*, 1994; Salamone and Correa, 2002; Ungerstedt, 1971).

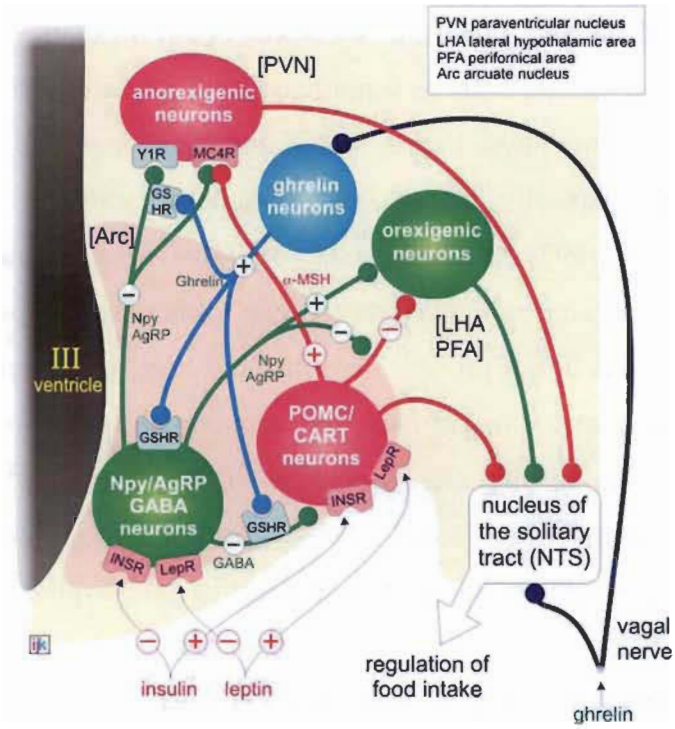

Figure 5.9 **Insulin's action on the hypothalamus.**
The arcuate nucleus is directly exposed to blood-borne hormones, as it is located at the level of the median eminence devoid of the blood-brain barrier. As such, insulin has a direct effect on the two principal populations that dwell therein. Neuropeptide Y (NPY)/gamma-aminobutyric acid (GABA)/agouti-related protein (AgRP) neurons (NGA) neurons innervate the paraventricular nucleus (PVN) and inhibit anorexigenic neurons. Its projections onto orexigenic neurons of the lateral hypothalamic area (LHA) rather activates them. Pro-opiomelanocortin/cocaine and amphetamine regulated transcript (POMC/CART) neurons innervate the same targets but have opposite effects than NGA neurons. Orexigenic and anorexigenic neurons project to the nucleus of the solitary tract (NTS), thereby regulating food intake. Since insulin inhibits NGA neurons and stimulates POMC/CART ones, its sum effect is to inhibit food intake and promote energy expenditure. Not shown here are the LHA's projections to the midbrain. GSHR, growth hormone secretagogue receptor or ghrelin receptor; INSR, insulin receptor; LepR, leptin receptor; MC4R, melanocortin 4 receptor; α-MSH, alpha-melanocyte-stimulating hormone; Y1R, neuropeptide Y receptor Y1. (From http://www.cellbiol.net/ste/alpobesity2.php.)

In this view, one would expect a hypoinsulinaemic setting, resulting from fasting or streptozotocin treatments for instance, to lead to the disinhibition of orexigenic neurons and the enhancement of midbrain dopaminergic neurotransmission; the consequential rise in striatal dopamine, encoding the subjective reward value of food, would compel the organism to feed itself, as it is a goal-directed behaviour. In fact, hyperphagia is archetypal in the spectrum of abnormal behaviours displayed by streptozotocin-treated rodents, confirmed by our own findings (Figure 1.33). However, what actually occurs at the level of striatal dopamine is highly inconsistent with these theoretical extrapolations. Indeed, almost all studies[76] performed in genetic or chemical-induced hypoinsulinaemic models, including ours (Figure 3.3I), report low basal levels of striatal dopamine (Bradberry *et al.*, 1989; Chen and Yang, 1991; Crandall and Fernstrom, 1983; Kono and Takada, 1994; Kowk and Jurio, 1986; Kwok *et al.*, 1985; Lim *et al.*, 1994; O'Dell *et al.*, 2014; Saller, 1984; Samandari *et al.*, 2013; Trulson and Himmel, 1983). Once again, we are faced with ostensibly irreconcilable and opposite outcomes evoked by – what appears to be – one factor.

To date, no adequate explanation has accounted for this dualism. However, we and others propose that, since striatal dopamine transients have never been measured in hypoinsulinaemic models by techniques sensitive enough to detect infrasecond events, it is probable that we are missing very important pieces of the puzzle pertaining to phasic neurotransmission[77] (Palmiter, 2007, 2008). Some indications of a role for phasic firing that could explain overt feeding behaviours in hypoinsulinaemic subjects despite low levels of dopamine are provided by experiments employing psychostimulants. Indeed, streptozotocin-treated rodents manifest a greater sensitivity to addictive substances like nicotine[78] (O'Dell *et al.*, 2014; Samandari *et al.*, 2013). In addition,

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[76] Some reports have shown enhanced dopamine levels in the brains of streptozotocin-treated rodents, but today the consensus remains that concentrations are in fact lower in the striatum (Bitar *et al.*, 1986; Gupta *et al.*, 1992; Lacković *et al.*, 1990).

[77] One study did measure phasic dopamine in striatal neurons by fast-scan cyclic voltammetry, but data were obtained in organotypic slices to which insulin was applied (Stouffer *et al.*, 2015). As we know, it is not clearly known whether peripheral insulin may have a direct effect on regions protected by the blood-brain barrier.

[78] In fact, smoking rates in diabetic patients are higher than in the general population (Bishop *et al.*, 2009).

amphetamine and apomorphine, which flood the striatum with dopamine thereby raising the noise-to-signal ratio, exert a hypophagic effect on fasted, hypoinsulinaemic rats that would normally engage in feeding (Cannon *et al.*, 2004; Sotak *et al.*, 2005). Moreover, knocking out DAT, especially important in clearing phasic dopamine transients from the extracellular space, enhances motivation for food rewards and yields a hyperphagic phenotype (Cagniard *et al.*, 2006; Peciña *et al.*, 2003). Without providing a clear answer, these data do converge toward a heightened production of or sensitivity to phasic dopamine bursts in streptozotocin-treated models like ours.

This proposal bears significant implications for other behaviours mediated by mesostriatal dopaminergic neurotransmission. We observed marked hyper-sociable and hyper-aggressive behaviours in our long-term hypoinsulinaemic, hyperglycaemic rats, which we explained by a possible drop in the noise-to-signal ratio. Admittedly, it is unlikely that the sole decrease in tonic firing in the dorsal striatum arising from hyperglycaemia-induced nigrostriatal neurodegeneration could have accounted for a low-enough noise-to-signal ratio conducive to these hyper-reactive behaviours. Chronic hypoinsulinaemia favouring enhanced mesostriatal dopamine phasicity may well have participated in reducing this ratio. However, our data presented in Chapter IV evokes a lack of correlation between the degree of hypoinsulinaemia or glucose intolerance and the intensity of behavioural abnormalities (Figure 4.5 and Figure 4.6). This can either signify that long-term hypoinsulinaemia does not hold a role in heightening phasicity and hyper-reactivity, or that the low and high subgroups were not different enough to yield a perceptible gradation of behaviour. We reiterate here the need for experiments addressing phasic dopamine transients in hypoinsulinaemic rats.

At any rate, substantial gaps remain in our understanding of the role held by insulin, or lack thereof, on the CNS. Investigations are warranted to explain low dopamine levels in hypoinsulinaemic rats, apparent even after a few days of treatment and, in our model, observed before nigrostriatal neurons had perished at 3 months. It would be imprudent not to contemplate the prospect of a plethora of peripheral and

central substances modulating the effects of insulin on the brain, likely exerting a compensatory effect in times of induced depletion.

5.2.6 Improving the model

The nicotinamide-streptozotocin rat model of long-term hyperglycaemia has allowed us to verify our main hypothesis, but still leaves certain questions pending. Uncertainties remain over the influence that a systemic defect can have on the CNS, a caveat shared by all experimental strategies employing complex peripheral disease models and attempting to ascribe noxious effects on the brain to a single pathological component. In our model, much more than a single variable is tampered with; thus, while it is tempting to attribute selective nigrostriatal dopaminergic neurodegeneration solely to a hyperglycaemic state, we can truly only interpret that the sum of physiological disruptions arising from nicotinamide-streptozotocin injections in our rats maintained over 6 months leads to the preferential death of this subpopulation of neurons. It remains that this multilayered physiological condition constitutes a stress conducive to CNS oxidative stress to which nigrostriatal neurons were manifestly more susceptible. Demonstrations achieved in the introduction section firmly support the legitimacy of this basis upon which hinges our conclusion.

The literature offers a collection of astutely constructed experiments aiming to circumvent this problem. Ideally, the need for tampering with peripheral hormones should be avoided in creating a high glucose environment. In addition, concentrations of glucose should be controlled between individuals. In this view, it is reasonable to propose verifying the selective degeneration of certain populations of neurons throughout the brain by chronic infusions of glucose via the intracerebroventricular route (Park *et al.*, 2009). Worthy of mention, glucose infusion in the brain alone can have peripheral effects by modulating central homeostasis hubs, thus pilot studies would be required to identify these outcomes (Carey *et al.*, 2013; Park *et al.*, 2009). In the same vein, retrodialysis, described earlier, could address certain issues with widespread effects of high or low circulating levels of glucose or insulin on behavioural endpoints

(see for review Höcht *et al.*, 2007). Although this has never been attempted before, glucose could easily be administered directly in the brain regions of interest following a chronic daily regimen. Behaviour or neurotransmission responses could then be measured in acute or chronic paradigms. This would dissipate any doubt regarding the neuroanatomical loci responsible for the behavioural effects observed. Advantageously, *in situ* measurement of oxidative stress could also be conducted in parallel using the salicylate trapping method, which implies administering salicylic acid through the microdialysis cannula and measuring stable oxidized adducts by high-performance liquid chromatography (Mertens *et al.*, 2011).

These kinds of experiments are appealing on the account that they specifically target the CNS or precise regions therein, allowing for the identification of clear-cut causal relationships between high glucose levels, oxidative stress, neuronal death and behavioural outcomes. However, since such experiments imply maintaining a chronic cannula in the CNS of rodents, they cannot be performed for more than a couple of months on end. A methodological compromise would dwell in the employment of a more moderate peripheral phenotype via the maintenance of appreciable levels of circulating insulin without sacrificing the hyperglycaemic condition. To that end, Fernyhough's group designed a strategy utilizing streptozotocin-treated rodents injected with subcutaneous insulin implants into the nape of the neck that can be renewed monthly (Aghanoori *et al.*, 2017; Akude *et al.*, 2011). Using a small implant limits the amount of insulin dispensed and yields a low-maintenance model of long-term hyperglycaemia that does not display overt hypoinsulinaemia. Peripheral alterations are accordingly less pronounced than in the classic streptozotocin model, reducing the risk of inferring inaccurate associations between hyperglycaemia and CNS affections.

5.3 Therapeutic perspectives

This thesis addressed the selective vulnerability of dopaminergic neurons to hyperglycaemia-induced oxidative stress. In the process, we touched upon Parkinson's disease and diabetes whose pathophysiological facets and epidemiological relationship

are directly linked to the core question. We can extend the significance of our findings to these pathologies.

5.3.1 Implications for diabetic patients

Diabetic patients present highly heterogeneous clinical profiles, on account of high inter-individual variations in lifestyle, in disease management and in the presence of additional pathological conditions. However, whether they suffer from type I or type II diabetes mellitus, medical authorities have reached the consensus that glycaemic control constitutes the topmost priority for the prevention of severe comorbidities. Our data provide further support for this approach by demonstrating that uncontrolled hyperglycaemia can, in the long term, lead to dopaminergic neurodegeneration. Importantly, hyperglycaemia on its own was sufficient to cause this modest neuronal death.

The importance of glycaemic control emerged from large trials that marked a milestone in diabetes research by demonstrating that tight blood glucose management could delay the onset of complications (Diabetes Control and Complications Trial Research Group, 1993; Holman *et al.*, 2008; Nathan *et al.*, 2005; UK Prospective Diabetes Study Group, 1998). Most striking was the discovery that these benefits persisted long after efforts of glycaemic control had been abandoned with respect to a cohort of patients who had never undergone tight blood glucose management. This phenomenon is termed "glycaemic memory" and is thought to at least partially arise from oxidative damage to mitochondrial DNA that is vulnerable to permanent mutations leading to irreversible respiratory defects (Giacco and Brownlee, 2010). Seeing as certain neuronal subtypes are intrinsically susceptible to oxidative stress, as we have demonstrated here, there is an imperative need to stress the importance of early blood glucose control in diabetic patients: the mindfulness of practitioners has accordingly evolved in this direction. Because the existence of a glycaemic memory implies that damage appearing at any moment of the disease is irretrievable and accumulative, the current state of the field is more actively striving for the development

of therapies that could reverse cellular defects thought to underlie this phenomenon (Giacco and Brownlee, 2010; Lovre and Fonseca, 2015; Misra and Bloombarden, 2018).

One legitimate question remains concerning the relative risk that constitutes pre-existing diabetes for the development of Parkinson's disease. As previously stated, certain epidemiological studies did not find diabetic patients to present an increased chance of developing this neurodegenerative disease (Becker *et al.*, 2008; Cereda *et al.*, 2011; Driver *et al.*, 2008). However, the sum of the other positive reports converges toward a modest two-fold increase in this risk (Arvanitakis *et al.*, 2007; Cereda *et al.*, 2012; Hu *et al.*, 2007; Klimek *et al.*, 2015; Xu *et al.*, 2011). It is possible to surmise that the life expectancies of patients with type I or II diabetes, respectively shortened by as much as 15 or 5-10 years (Canadian Diabetes Association, 2009), may account for these moderate findings[79]. If diabetic patients lived longer, one could expect the incidence of age-related neurodegenerative disorders like Parkinson's disease to be substantially superior than in the general population. Bearing in mind the idiopathic and multifaceted nature of Parkinson's disease, uncontrolled glycaemia may interact with other risk factors besides aging to additively or synergistically hasten the onset of oxidative stress-induced neurodegeneration; this is plausibly what is picked up by epidemiological studies.

5.3.2 Implications for parkinsonian patients

The keynote finding of our studies provides that hyperglycaemia can lead to the selective degeneration of the nigrostriatal pathway. On the one hand, these results support the hypothesis that nigrostriatal dopaminergic neurons present a characteristic phenotype that renders them susceptible to what we suspect is oxidative stress. On the other, they warn of a possible additional risk factor, especially present in diabetic patients, which could contribute to the development of Parkinson's disease. These results are important in light of persisting doubts regarding the significance of

[79] Other explanations for the heterogeneity of these data could be variations in the type of studies employed (case control vs. cohort), the diagnosis of Parkinson's disease and diabetes (medical examination vs. self-reported) and, as previously stated, the sizes of the populations evaluated.

reports stating that parkinsonian patients display elevated glycaemias (Boyd *et al.*, 1971; Cereda *et al.*, 2012; Lipman *et al.*, 1974; Sandyk, 1993; Santiago and Potashkin, 2015). It is unclear whether hyperglycaemia appears after the onset of the disease or if it manifests itself upstream; a direct corollary of the latter would be that hyperglycaemia may contribute to the development of Parkinson's disease. Our work offers strong support in favour of this second option, without however refuting a possible bidirectional relationship between parkinsonian neurodegeneration and blood glucose control (Brunerova *et al.*, 2013).

For parkinsonian patients who already suffer from this disease, war must be waged on further nigrostriatal dopaminergic neurodegeneration; some rather prioritize tackling the deterioration of their symptoms, which is not always directly linked to further neurodegeneration. Our findings, though not obtained in a parkinsonian model (Choi *et al.*, 2005; Morris *et al.*, 2010; Rotermund *et al.*, 2014), clearly expose the hazard that uncontrolled hyperglycaemia represents for the survival of nigrostriatal dopaminergic neurons. Moreover, other studies provide evidence supporting a possible link between diabetes and the severity of the symptoms in Parkinson's disease (Cereda *et al.*, 2012; Schwab, 1960). Likewise, in older persons without Parkinson's disease, parkinsonian symptoms like gait disturbances are more intimately correlated to existing diabetes (Arvanitakis *et al.*, 2007). Considering the elevated rates of hyperglycaemia in parkinsonian patients (Barbeau *et al.*, 1961; Boyd *et al.*, 1971; Cereda *et al.*, 2012; Lipman *et al.*, 1974; Sandyk, 1993), exercising a tighter glycaemic control constitutes an achievable target to prevent the exacerbation of both neurodegeneration and worsening of symptoms.

Among the most recent clinical advances, anti-diabetic drugs have emerged as strong candidates for the development of disease-modifying therapies. A sharp interest in these substances surfaced following reports evoking a decreased risk of developing Parkinson's disease in diabetic patients employing such drugs. Specifically, the incidence of Parkinson's disease was found to be lower in diabetic patients treated with metformin or thiozolidinediones, two glucose-lowering medications (Brauer *et al.*,

2015; Wahlqvist *et al.*, 2012). For example, current clinical trials are investigating the anti-diabetic and glucose-lowering drug exenatide, a glucagon-like peptide 1 (GLP-1) receptor agonist that largely mimics insulin's action (see for review Athauda and Foltynie, 2017). An initial open label trial carried out in 44 medicated parkinsonian patients showed that twice-daily injections of exenatide for 12 months improved overnight off-medication motor symptoms and dementia, rated according to the unified Parkinson's disease rating scale and the Mattis dementia rating scale, respectively (Aviles-Olmos *et al.*, 2013). This trial employed a washout design wherein exenatide treatments were ceased at 12 months and patients were re-evaluated at later time points. Remarkably, the benefits observed at 12 months still held at 14 (Aviles-Olmos *et al.*, 2013) and 24 months (Aviles-Olmos *et al.*, 2014). This encouraged the elaboration of subsequent double-blinded, placebo-controlled trials once again designed to include a washout period and to measure symptoms in off-medication patients. The benefits of exenatide were reiterated and supplementary DaTscan neuroimaging results, which conveyed information on the amount of presynaptic DAT left in the striatum of patients, confirmed a significant positive difference between treated and control parkinsonian patients (Athauda *et al.*, 2017).

These results are very encouraging and are paving the way for further investigations that will perhaps address the mechanistic underpinnings of exenatide's efficacy. In this regard, it is surprising that glycaemic profiles were not drawn throughout these clinical trials. We can nonetheless suppose that glycaemic control was improved in parkinsonian patients based on robust evidence provided by trials in diabetic patients (Bergenstal *et al.*, 2010; Buse *et al.*, 2009). However, a link between improved glycaemia and symptomatic amelioration in these investigations cannot be presumed. Indeed, GLP-1 and exenatide are neurotrophic factors, akin to insulin (Athauda and Foltynie, 2016*b*; Perry *et al.*, 2002). Exenatide may therefore wield its disease-modifying effects independently of glycaemic ameliorations. It remains that our data grant indirect evidence of the benefits of reducing hyperglycaemia, which we believe is a contributing factor in the efficacy of exenatide treatments in Parkinson's disease.

5.3.3 Employing resveratrol to therapeutic ends

There is compelling evidence to suggest that oxidative stress occupies a central position in both diabetic complications and nigrostriatal dopaminergic neurodegeneration in Parkinson's disease. Accordingly, antioxidants like the polyphenol resveratrol have been the object of much scientific and public interest for their therapeutic potential, either as complementary or preventive treatments. Although not the aim of this thesis, we previously introduced the idea of utilizing resveratrol as a neuroprotective strategy. The many sensitive elements that impinge on the employment of polyphenols in the CNS are reviewed in Appendix B.

5.4 Concluding remarks

The etiopathogenesis of Parkinson's disease remains largely enigmatic, in part due to our lack of comprehension regarding the seemingly selective vulnerability of the nigrostriatal pathway to undergo degeneration, despite the neuroanatomical breadth of neuropathological manifestations in this illness. Ascending hypotheses grant a preeminent role to the existence of a particular phenotypic liability that characterizes neurons of the substantia nigra pars compacta and that may render them more sensitive to oxidative stress. In the aim of verifying the preferred susceptibility of nigrostriatal dopaminergic neurons to oxidative stress, we hired a strategy that employed high glucose conditions to induce the gradual death of neurons *in vitro* and *in vivo*. For the first time, we provided robust evidence for the selective demise of the nigrostriatal pathway compared to mesocorticolimbic neurons over the course of 6 months of sustained hyperglycaemia, attended by alterations in glial profiles that indirectly supported a role for oxidative stress as a key conspirator in this neuronal death, further evinced by *in vitro* groundwork. Despite modest neurodegeneration, hyperglycaemia evoked motor deficits strikingly reminiscent of parkinsonian motor symptoms. We also reported noticeably abnormal social behaviours in a hyperglycaemic model, which further informed us on a possible imbalance between phasic and tonic dopaminergic signalling arising from nigrostriatal neurodegeneration and, perhaps, hypoinsulinaemia.

These findings also direct our attention toward a rising need to better understand how diabetes may alter the quality of patients' social encounters.

Across these experiments, we demonstrated the various advantages that a long-term hyperglycaemic model presents, especially regarding the physiological significance of inducing a gradual and moderate neuronal death without layering additional parkinsonian insults. Indeed, by revealing that hyperglycaemia is sufficient to induce nigrostriatal dopaminergic neurodegeneration, we lend vigorous support to rather modest epidemiological accounts of the relationship between pre-existing diabetes and the development of Parkinson's disease in later years. According to our findings, it is critical that the efforts recently deployed to tighten glycaemic control in diabetic patients be firmly maintained. We further wish to express the tangible risk that an inappropriate glycaemic health may represent for the survival of remaining nigrostriatal dopaminergic neurons in the CNS of parkinsonian patients.

From this wealth of information, we can also fully appreciate the complexity of several dimensions specific to the CNS that, in remaining unsettled, dwell at the core of numerous longstanding debates. There is a profound lack of clarity pertaining to the bond shared between the brain and the periphery, especially in pathological settings. In our project, this was apparent upon attempting to identify the elements responsible for neurodegeneration in a model presenting multifaceted peripheral alterations. Dopaminergic systems also exposed their intricate nature that emerged on our daunting venture to draw connections between neurodegeneration, dopamine functions and behaviour. Most evidently, our understanding of these pending issues is at its infancy, but formal identification of these chasms in our knowledge draws us closer to their unravelling.

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APPENDIX A

LA NEURO-INFLAMMATION : DR JEKYLL OU MR HYDE?

Renaud, J., Thérien, H. M., Plouffe, M., Martinoli, M. G. (2015).
M/S, 31, 979-988.

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Author contributions

Justine Renaud wrote 45% of the manuscript, and prepared and edited all figures and tables. Hélène-Marie Thérien, from the Department of Medical Biology at the Université du Québec à Trois-Rivières, wrote 45% of the manuscript. Marilyn Plouffe, student in the laboratory of Maria-Grazia Martinoli, participated in the writing. Maria-Grazia Martinoli, Justine Renaud's research supervisor, was the guarantor of the work and provided supervision, preparation and editing of the manuscript.

Full review article in French: La neuro-inflammation : Dr Jekyll ou Mr Hyde?

Abstract

Sheltered in a bony cage, populated by cells with little regenerative potential, the central nervous system (CNS) could likely not withstand classic inflammation without risking major sequelae. As a consequence, it had to develop an original way to provide surveillance, defence and reparation, which relies on both, the complex architecture of the periphery-nervous parenchyma exchange zones and the tightly regulated collaboration between all the cell populations that reside in or pass through the CNS. Despite its tight regulation, neuroinflammation is sometimes the cause of irreversible loss but it is also where the solution stands. The specific immune crosstalk that takes place in the CNS needs to be decoded in order to identify the best therapeutic strategies aimed at helping the CNS to restore homeostasis in problematic situations such as is the case in neurodegenerative disorders. This review deals with this double-edged sword nature of neuroinflammation.

Résumé

Enchâssé dans une cage osseuse, peuplé de cellules qui ont peu de pouvoir de régénération, le système nerveux central (SNC) ne peut supporter une réaction inflammatoire telle qu'elle se déroule en périphérie sans en subir de graves conséquences. Il lui a donc fallu développer une façon originale pour assurer surveillance, défense et réparation, qui repose à la fois sur l'architecture complexe des zones d'échange entre la périphérie et le parenchyme nerveux et sur la collaboration hautement contrôlée de toutes les cellules du SNC. Bien que parfois source de problèmes comme c'est le cas dans les maladies neurodégénératives, la neuro-inflammation est aussi porteuse de la solution. C'est de cette double nature dont il est ici question.

Introduction

On a longtemps cru le système nerveux central (SNC) à l'abri de la majorité des effets dévastateurs de la réaction inflammatoire. On le disait immunoprivilégié, protégé qu'il était des agressions étrangères par la barrière hémato-encéphalique (BHE) (Figure 1) et camouflé du système immunitaire par le peu d'expression des produits du complexe majeur d'histocompatibilité (CMH) et l'absence de drainage lymphatique. Mais, au cours de la dernière décennie, l'accumulation des évidences a fait s'écrouler le paradigme. S'il n'y a maintenant plus de doute quant à l'existence d'une réaction inflammatoire dans le SNC et quant à l'utilisation du langage commun de cytokines et de chimiokines pour contrôler ses actions [1], force est aussi de constater que la réaction inflammatoire du SNC se comporte de façon suffisamment différente de son homologue périphérique pour se mériter l'appellation particulière de « neuro-inflammation ». Cette façon unique de gérer l'inflammation et les étapes premières de la réaction immune à travers un drainage soluble plutôt que cellulaire tient aux propriétés particulières des différentes composantes du parenchyme nerveux [pour revue, voir 2]. La notion de privilège, toujours de mise, ne concerne donc plus que le parenchyme, excluant ainsi les zones limitrophes que sont les espaces périvasculaires, les méninges et les ventricules. Privilège bien relatif car, aussi différente soit-elle de l'inflammation, la neuro-inflammation demeure un pari risqué pour un tissu aussi fragile que celui du SNC si on s'en fie aux pertes fonctionnelles auxquelles on l'associe dans les maladies neurodégénératives telles que l'Alzheimer, la maladie de Parkinson ou la sclérose en plaques [3].

La reconnaissance d'une perturbation interne est un prérequis essentiel à l'induction de la réaction inflammatoire. Cette reconnaissance est assurée par un ensemble de récepteurs, baptisés PRR (*pattern-recognition receptors*), dont on connaît pour le moment une vingtaine de membres [4]. Ces PRRs peuvent être solubles, membranaires ou cytosoliques et reconnaissent tout autant les signatures moléculaires de classes de pathogènes (PAMPs pour *pathogen-associated molecular patterns*) que des molécules générées au cours de stress cellulaires autres qu'infectieux (DAMPs pour *damage-associated molecular patterns*). On compte parmi les DAMPs des protéines

agrégées, modifiées ou simplement mal repliées telles l'α-synucléine, la protéine Tau ou la β-amyloïde qui, toutes, ont été associées à des maladies neurodégénératives. On compte aussi parmi les DAMPs de simples molécules comme l'ATP ou le glutamate, abondamment libérées dans le milieu lors de nécroses cellulaires au sein du SNC [5]. L'interaction des PAMPs/DAMPs avec leur récepteur induit une cascade de signalisation qui, en recrutant protéines adaptatrices et kinases, aboutit à l'activation de facteurs de transcription qui modifient de façon drastique le phénotype cellulaire. Cette modification se manifeste tant par l'acquisition de nouveaux récepteurs que par l'expression d'un éventail de cytokines et de chimiokines qui, par des actions à la fois autocrines et paracrines, guident les cellules de défense au site de l'agression, permettent le déploiement focalisé des mécanismes de défense (phagocytose, production de dérivés toxiques de l'oxygène, apoptose) et préparent les étapes de la reconstruction.

Dans le SNC, lorsqu'il est question d'inflammation, c'est la cellule microgliale, la cellule résidente à vocation immune du tissu, qu'on accuse d'emblée de débordements. L'accusation est d'autant plus facile qu'on la retrouve toujours dans les zones inflammées et, qui plus est, avec sa morphologie de cellule « activée » [6]. Cependant, au contraire de ce qu'on observe en périphérie où l'expression constitutive des PRRs est un attribut plutôt spécifique des cellules de la défense innée, on constate que, dans le SNC, les astrocytes, les oligodendrocytes et les neurones expriment leur propre répertoire de PRRs par lequel ils contribuent eux aussi à toutes les étapes de la neuro-inflammation (Tableau 1 et Figure 2) [4].

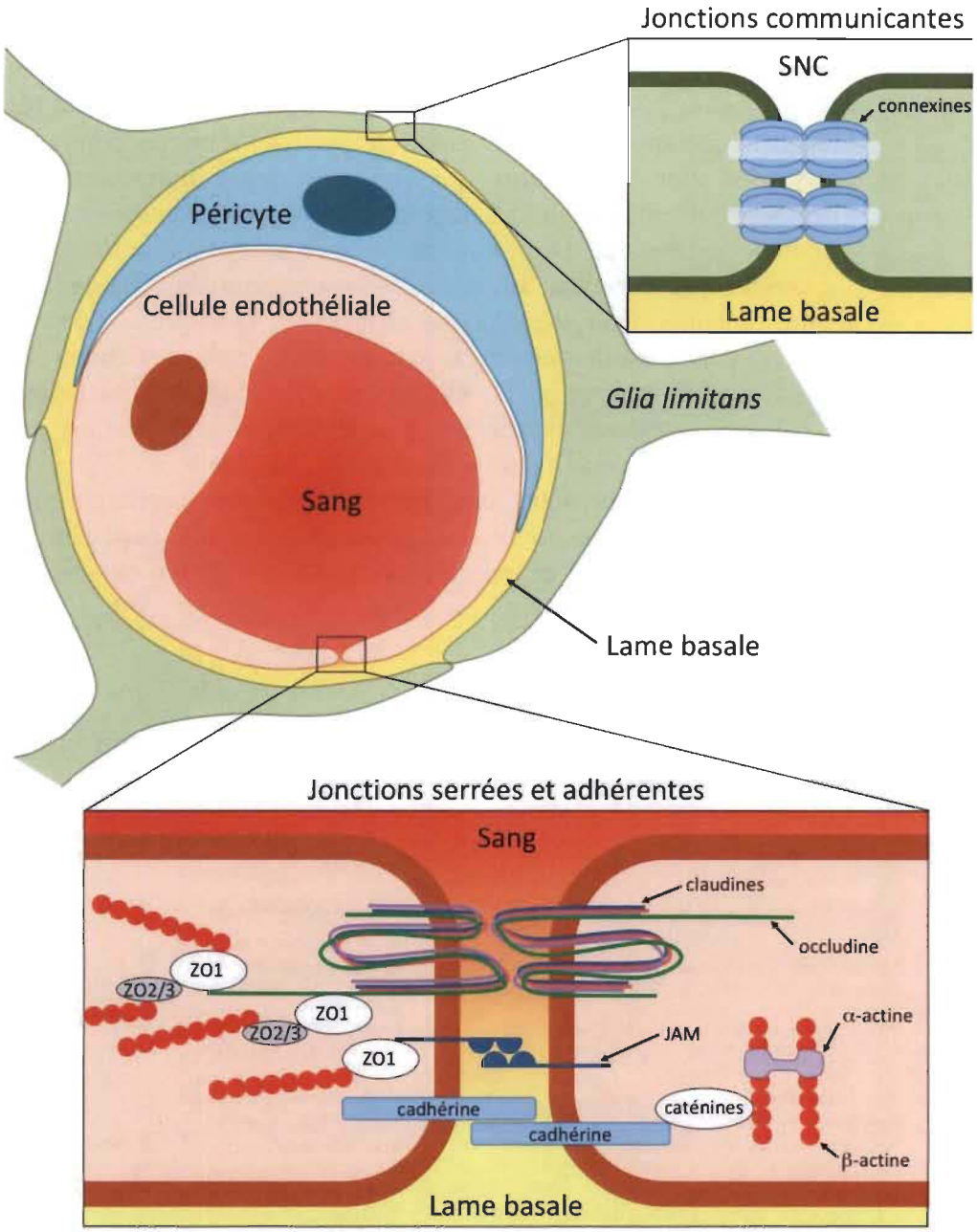

Figure 1 **Schématisation de la barrière hémato-encéphalique.**
La barrière hémato-encéphalique (BHE) empêche la pénétration de la grande majorité des agents et des cellules circulant dans le sang vers le système nerveux central (SNC). Elle est composée de cellules endothéliales, de péricytes, de la lame basale et de la *glia limitans*. Les cellules endothéliales recouvrent complètement la lumière des capillaires du SNC et son non-fenestrées, contrairement à la majorité des cellules endothéliales périphériques. Elles sont reliées entre-elles par des jonctions serrées constituées de protéines transmembranaires – occludines, claudines, et JAM (*junctional adhesion molecules*) – ainsi que par des jonctions adhérentes assurées par l'interaction entre des

cadhérines. Ces protéines transmembranaires sont liées au cytosquelette à l'aide de protéines adaptatrices, tantôt ZO1 et ZO2 (*zonula occludens* protéines 1 et 2), tantôt des caténines. Quant aux péricytes, elles sont distribuées sur 20-30 % de la surface des cellules endothéliales et régulent leur différenciation et prolifération. De par les propriétés contractiles de leurs projections cellulaires qui entourent les cellules endothéliales, elles peuvent modifier le diamètre des vaisseaux sanguins en réponse à l'activité neuronale. De plus, la lame basale tapisse intégralement l'extérieur des péricytes et des cellules endothéliales. La lame basale assure un support physique pour l'arrimage et la migration de certaines cellules par l'expression d'intégrines, mais agit également comme barrière contre le passage de cellules ou de macromolécules indésirables de par sa constitution matricielle. Finalement, la *glia limitans* recouvre > 99 % de la surface des capillaires du SNC. Les pieds astrocytaires qui la forment sont reliés entre eux par des jonctions communicantes et adhérentes. La *glia limitans* régule la morphologie et la perméabilité de la BHE en contrôlant l'expression de certaines protéines des cellules endothéliales.

Tableau 1

Rôles pro- et anti-inflammatoires des divers acteurs cellulaires du SNC dans la neuro-inflammation (suite à la page suivante)

Acteur cellulaire	Promotion de la neuro-inflammation		Inhibition de la neuro-inflammation	
	Actions[&]	Facteurs impliqués[&]	Actions[&]	Facteurs impliqués[&]
Cellules microgliales	Expression de cytokines pro-inflammatoires	TNF-α, IFN-γ, IL-6, IL-1β	Sécrétion de cytokines anti-inflammatoires	IL-4, IL-10, IFN-β, TGF-β
	Expression de protéines membranaires/récepteurs/PRR	TLR, RAGE, LFA-1, MAC-1, CRs, FcRβ	Expression de protéines membranaires/récepteurs*	CD45, CD47, CD56 (NCAM), CD91, CD200R, CD172, CX3CR1, TREM2, TSB, FasL, Fas
	Expression de chimiokines	CXCL1, 2, 12 ; CCL2, 5, 10, 19	Polarisation des cellules Th vers Treg	TGF-β
	Polarisation des Th vers Th1	IL-12, IL-23	Diminution de la production de NO par détournement de l'Arg	Arginase-1
	Présentation d'antigènes soutenant l'activation des cellules Th activées	CMH classe II, facteurs de costimulation	Production de prostaglandines constitutives	COX-1 au détriment de COX-2
	Production de monoxyde d'azote	iNOS	Expression de facteurs neurotrophiques	BDNF, GDNF
	Production de prostaglandines pro-inflammatoires	COX-2	Inhibition de la prolifération des cellules T par détournement du Trp	IDO
	Induction de stress oxydant et nitrosatif	ROS, RNS	Inhibition des MMP	TIMP
	Facilitation de la migration des cellules défense périphériques	MMP	Induction de l'apoptose des cellules défense périphériques et autorégulation	FasL, Fas
	Génération de molécules neurotoxiques	Glu, acide quinolinique (IDO)		
	Soutien de l'immunité innée	molécules du complément		
	Production de DAMP	ATP, HMGB1, HSP		
Neurones	Sécrétion de chimiokines	CXCL10, CCL21	Sécrétion de cytokines	IL-10, TGF-β
	Expression de PRR	TLR	Sécrétion de chimiokines anti-inflammatoires	Fractalkine soluble
	Génération de molécules neurotoxiques	Glu	Expression de protéines membranaires inhibant cellules immunitaires*	CD22, CD47, CD56 (NCAM), CD200, fractalkine, FasL
	Sécrétion de neurotransmetteurs pro-inflammatoires	DA, substance P	Polarisation des Th vers Treg	TGF-β
	Production de monoxyde d'azote	iNOS	Sécrétion de neurotransmetteurs et neuropeptides anti-inflammatoires	GABA, VIP, NE, α-MSH, somatostatine, CGRP
	Facilitation de la migration des cellules de défense périphériques	MMP	Remodelage de la matrice extracellulaire	Protéoglycanes

Acteur cellulaire	Promotion de la neuro-inflammation		Inhibition de la neuro-inflammation	
	Actions[&]	Facteurs impliqués[&]	Actions[&]	Facteurs impliqués[&]
Neurones	Production de DAMP	ATP, HMGB1, HSP, protéines mal repliées (protéinopathies neurodégénératives)	Expression de facteurs neurotrophiques	NGF, BDNF, NT3, GDNF, CNTF
			Induction de l'apoptose des microglies et cellules défense périphériques	FasL
			Absence d'activation des cellules cytotoxiques $CD8^+$	Absence de HLA A, B ou C (CMH classe Ia)
			Inhibition des cellules NK par activation du KIR	HLA G (CMH classe Ib)
Astrocytes	Expression de cytokines pro-inflammatoires	TNF-α, IFN-γ, IL-1β	Sécrétion de cytokines anti-inflammatoires	IL-4, IL-10, IFN-β, TGF-β
	Expression de chimiokines	CXCL1, 2, 12 ; CCL2, 5, 10, 19	Expression de protéines membranaires inhibant cellules immunitaires	Inhibiteurs du complément, CTLA-4
	Expression de protéines membranaires/ récepteurs/PRR	TLR, RAGE, ICAM1, CR	Polarisation des Th vers Treg	TGF-β, CXCL12
	Génération de molécules neurotoxiques	Glu (diminution de son internalisation)	Remodelage de la matrice extracellulaire	Protéoglycanes
	Présentation d'antigènes soutenant l'activation des cellules Th activées ?	CMH classe II, facteurs de costimulation	Expression de facteurs neurotrophiques	BDNF, GDNF, NGF, CTNF, FGF
	Facilitation de la migration des cellules de défense périphériques	MMP	Inhibition des MMP	TIMP
	Production de monoxyde d'azote	iNOS	Induction de l'apoptose des microglies et cellules défense périphériques	FasL, PD-L1 (chez cellules T activées exprimant PD-1)
	Soutien de l'immunité innée	Molécules du complément	Dédifférenciation en *radial-glia-like cells*	FGF2, IGF1, SDF1, VEGF
	Production de DAMP	ATP, HMGB1, HSP		
	Dédifférenciation en *radial-glia-like cells*	CCL2		
Oligodendrocytes	Expression de PRR	TLR	Sécrétion de cytokines	TGF-β
			Expression de protéines membranaires inhibant cellules immunitaires	CD200, fractalkine

[&] Arg, arginine; ATP, adénosine triphosphate; BDNF, *brain-derived neurotrophic factor*; CCL, *chemokine [C-C motif] ligand*; CD, *cluster of differentiation*; CD200R, *cluster of differentiation 200 receptor*; CMH, complex majeur d'histocompatibilité; CNTF; *ciliary neurotrophic factor*; COX-2, cyclooxygénase-2; CR, *complement receptor*; CTLA-4, *cytotoxic T-lymphocyte-associated protein 4*; CX3CR, *chemokine [C-X3-C motif] receptor*; CXCL, *chemokine [C-X-C motif] ligand*; DAMP, *damage-associated molecular pattern*; Fas, *first apoptosis signal receptor*; FasL, *first apoptosis signal receptor ligand* ou ligand Fas; FcRβ, *fragment crystallizable region (Fc) receptor beta subunit*; FGF, *fibroblast growth factor*; GDNF, *glial fibrillary acidic protein*; Glu, glutamate; HLA, *human leucocyte antigen*; HMGB1, *high mobility group box 1*; HSP, *heat shock protein*; ICAM1, *intercellular adhesion molecule*; IDO, *indoleamine 2,3-dioxygenase*; IFN, *interferon*; IGF, *insulin-like growth factor*; IL, interleukine; iNOS, *inducible nitric oxide synthase*; KIR, *killer-cell immunoglobulin-like receptor*; LFA, *lymphocyte function-associated antigen-1*; MAC-1, *macrophage-1 antigen*; MMP, *matrix metalloproteinases*; NCAM, *neural cell adhesion molecule*; NGF, *nerve growth factor*; NK, *natural killer*; NO, oxyde nitrique; NT3, *neurotrophin-3*; PD-1, *programmed cell death protein 1*; PD-L1, *programmed death-ligand 1*; PRR, *pattern-recognition receptors*, RAGE, *receptor for advanced glycation end-products*; RNS, *reactive nitrogen species*; ROS, *reactive oxygen species*; SDF1, *stromal cell-derived factor 1*; TGF-β, *transforming growth factor-beta*; Th, *T helper cell subtype*; TIMP, *tissue inhibitor of metalloproteinase*; TLR, *toll-like receptor*; TNF-α, *tumor necrosis factor-alpha*; Treg, *regulatory T cell*; TREM2, *triggering receptor expressed on myeloid cells 2*; TSB, thrombospondin; VEGF, *vascular endothelial growth factor*.

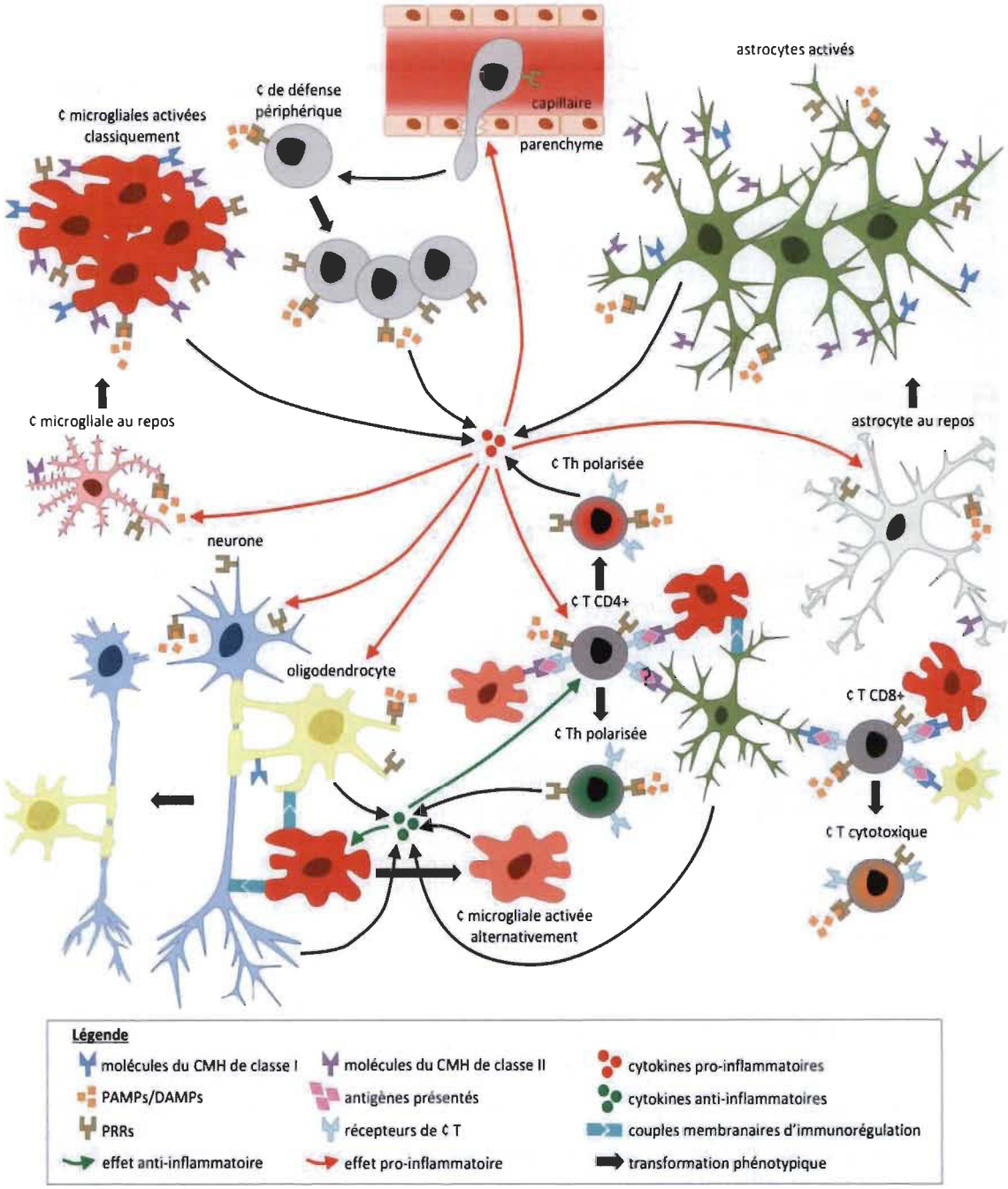

Figure 2 **Dialogues entre les cellules neuronales dans un contexte de neuro-inflammation.**

Dans un contexte de neuro-inflammation, des molécules de danger nommées PAMPs (*pathogen-associated molecular patterns*) ou DAMPs activent les cellules munies de récepteurs PRRs, principalement les cellules microgliales. L'activation de la microglie mène à leur multiplication et à leur transformation vers le phénotype pro-inflammatoire, caractérisé par une forme amiboïde et l'expression accrue de cytokines et de molécules du CMH de classe II. Suite à leur activation par les PAMPs ou DAMPs, les astrocytes subissent également des

changements phénotypiques tendant vers l'expression accrue de cytokines, la présentation d'antigènes et la prolifération. Les cellules du SNC, en particulier les cellules microgliales activées classiquement, produisent des cytokines capables de perméabiliser la BHE et sécrètent des chimiokines, permettant la transvasation de cellules de défense périphériques – lymphocytes, granulocytes, macrophages, etc. – des capillaires vers le parenchyme. Ces cellules peuvent également reconnaître des PAMPs et des DAMPs dans le parenchyme, ce qui soutient leur activation et leur prolifération accompagnées de l'expression de cytokines pro-inflammatoires. Néanmoins, plusieurs acteurs expriment constitutivement des cytokines anti-inflammatoires ou des molécules membranaires donc la fonction immunomodulatrice permet de contenir la réaction inflammatoire, par la polarisation des cellules Th en types anti-inflammatoires et par la transformation de la microglie en un état réparateur. L'exposition à long terme aux cytokines pro-inflammatoires endommage les neurones et les oligodendrocytes, les types cellulaires les plus sensibles à une neuro-inflammation chronique dû à leur faible potentiel régénérateur.

La microglie

La microglie constitue en moyenne 10 % de l'ensemble des cellules gliales. Originant du sac vitellin à partir d'un précurseur érythro-myéloïde CSF1R+ (*colony stimulating factor 1 receptor*) commun aux macrophages résidents [7], les cellules microgliales colonisent très tôt le neuroépithélium où elles poursuivent leur différenciation selon une voie originale dans l'environnement particulier du SNC. Elles conservent tout au long de la vie un certain pouvoir de régénération qui leur permet à la fois de maintenir leur pool et de répondre à des demandes ponctuelles [8]. Après la naissance, une sous-population de cellules microgliales semblerait également provenir de source hématopoïétique par l'infiltration de monocytes dans le parenchyme [9]. Toujours à explorer leur environnement à travers les mouvements rapides de leurs extensions cytoplasmiques qui se chevauchent très peu, dotées d'un très vaste potentiel d'activités, elles se métamorphosent en réponse aux influences qu'elles subissent. Essentielle à la structuration du SNC et à l'exercice des fonctions nerveuses supérieures, un rôle dont on commence à peine à percevoir l'importance [10], c'est cependant pour son rôle en situation traumatique que la microglie est le mieux connue. Exprimant tous les PRRs identifiés à ce jour, les cellules microgliales sont particulièrement bien

équipées pour percevoir les perturbations de leur environnement, fussent-elle causées par la concentration anormale d'une molécule, son format inhabituel ou par l'apparition d'un composé inusité [11]. La reconnaissance induit le passage de l'état de veille à celui d'activation, l'objectif étant, dans un premier temps, d'éliminer la source de la perturbation et, dans un second temps, de favoriser le remodelage et la régénération.

En présence d'un signal de danger, les cellules microgliales adoptent un profil amiboïde qui facilite à la fois leur division et leur déplacement. Elles sont recrutées soit directement par les molécules de danger elles-mêmes, soit par des chimiokines libérées par les autres cellules neuronales alertées du danger. Elles forment autour de la région lésée un bouclier protecteur qui vise à en limiter l'étendue. Les conséquences précises de l'activation dépendent du contexte particulier dans lequel se fait la rencontre du stimulus initiateur, les cellules microgliales oscillant entre deux états extrêmes : un état d'activation classique et un état d'activation alternatif à vocation réparatrice [12].

Dans son état d'activation classique, la microglie exhibe un profil pro-inflammatoire (Tableau 1) caractérisé par 1) la sécrétion d'un large éventail de cytokines pro-inflammatoires et de polarisation Th1 (*T helper cell subtype 1*); 2) l'expression de chimiokines; 3) l'expression d'iNOS (*inducible nitric oxide synthase*) et de COX-2 (cyclooxygénase-2); 4) la production de dérivés toxiques de l'oxygène et de l'azote; 5) l'augmentation de l'expression des molécules du CMH de classe I et II et des facteurs de costimulation; 6) la sécrétion de métalloprotéinases matricielles; et 7) un pouvoir phagocytique accru. Si dans un état pro-inflammatoire la microglie possède l'arsenal nécessaire pour altérer l'étanchéité de la BHE, attirer les leucocytes – principalement les lymphocytes, monocytes et granulocytes –, faciliter leur déplacement dans la matrice extracellulaire, présenter des antigènes, soutenir l'activation de l'immunité spécifique et mettre KO les agents perturbateurs, elle a aussi le pouvoir de causer des dommages collatéraux souvent irréversibles au SNC. Ce potentiel auto-destructeur de la microglie activée classiquement est tempéré par les propriétés immunosuppressives du milieu, en grande partie attribuables aux neurones.

Dans son état réparateur, dicté par le contexte du milieu, la microglie adopte plutôt 1) un métabolisme aérobie; 2) une expression accrue des récepteurs éboueurs; 3) la synthèse de COX-1 au détriment de COX-2; 4) la synthèse d'arginase-1; ainsi que 5) la sécrétion de divers facteurs neurotrophiques et d'éléments matriciels. Ainsi, la microglie détient également le potentiel de participer à la réparation des tissus lésés et le retour à l'homéostasie (Tableau 1), favorisant l'angiogenèse et stimulant la génération de nouveaux oligodendrocytes, astrocytes, voire même neurones, à partir de cellules souches toujours présentes dans le parenchyme nerveux adulte.

Les états d'activation classique et réparateur ne constituent que les deux pôles d'un continuum d'états entre lesquelles les cellules microgliales oscillent en fonction des particularités activatrices du milieu dans lequel elles baignent [13]. Avec l'âge, pour des raisons qu'on s'explique encore mal, l'induction du profil réparateur se fait plus difficile, une situation à mettre en lien avec l'augmentation des maladies neurodégénératives associée au vieillissement [14].

Les neurones

Les neurones jouent un rôle primordial dans l'établissement des privilèges immunitaires du SNC en agissant tant en amont qu'en aval de la réponse [15]. Ils expriment quelques PRRs par lesquels ils perçoivent les signaux de danger présents dans leur environnement et expriment plusieurs récepteurs pour les cytokines qui leur permettent d'ajuster leur contribution à l'immunosuppression en situation d'agression. Les neurones expriment notamment le récepteur TLR3 (*toll-like receptor 3*) dont le PAMP déclencheur est l'ARN double brin. La dimérisation de TLR3 mène à la production d'interférons de type I dont le rôle est crucial dans la défense innée contre les virus, par exemple lors d'une infection par le virus neurotropique de la rage [16].

La microglie avec son imposant arsenal pro-inflammatoire est une des cibles préférentielles de l'attention neuronale qui se veut généralement atténuante. La régulation de la microglie implique à la fois des contacts cellule-cellule et des

facteurs solubles contribuant à freiner l'inflammation (Tableau 1). La majorité de ces protéines sont exprimées de façon constitutive et créent donc d'entrée de jeu un milieu immunosuppresseur capable de contenir les débordements de la microglie. Ces protéines membranaires agissent principalement en interférant avec les voies de signalisation, inhibant des kinases essentielles telle la famille des MAPK (*mitogen-activated protein kinase*) et les Pi3K (phosphoinositide 3-kinases), réduisant l'expression de facteurs de transcription tels c-jun ou c-myc ou inhibant leur translocation nucléaire comme c'est le cas pour Nrf2 (*nuclear factor erythroid 2-related factor 2*) ou NF-κB (*nuclear factor kappa-light-chain-enhancer of activated B cells*). Elles permettent, en conséquence, de diminuer la production de cytokines inflammatoires et de dérivés toxiques de l'oxygène ou de l'azote dès l'engagement des PRRs de la microglie. Les neurones expriment en plus et de façon constitutive le FasL (ligand Fas ou CD95L) par lequel ils contrôlent l'apoptose des cellules microgliales activées. Outre ces facteurs membranaires, les neurones libèrent nombre de facteurs solubles qui contribuent aussi à contraindre l'activation de la microglie. Il y a le TGF-β (*transforming growth factor-beta*) et la fractalkine soluble que les neurones expriment de façon constitutive et dont la production peut être amplifiée en situation traumatique, tandis que l'interleukine-10 (IL-10) est plutôt sécrétée en situation de crise. À ces facteurs immunorégulateurs viennent s'ajouter l'activité neuronale dont l'action anti-inflammatoire est arbitrée par un bon nombre de neurotransmetteurs et plusieurs neurotrophines (Tableau 1). Sous cette influence, les cellules microgliales produisent moins de cytokines inflammatoires, réduisent leur stress oxydant, diminuent leur expression des molécules du CMH et, ainsi, soutiennent moins efficacement l'activation des cellules de défense périphériques ayant gagné accès au parenchyme nerveux. Quant à leur pouvoir phagocytaire, il peut être selon les particularités du contexte soit amplifié, soit diminué.

Le caractère immunosuppresseur des neurones ne contient pas que les ardeurs de la microglie au moment de son activation; il tempère aussi les conséquences de son activation en s'exerçant sur les cellules de la défense périphérique qui, répondant à l'appel, ont gagné ou tentent de s'introduire dans le parenchyme. Ici encore, l'effet s'exerce tantôt par des contacts cellule-cellule impliquant entre autres des cadhérines,

des molécules du CMH, et le FasL, tantôt par l'intermédiaire de facteurs solubles dont le TGF-β et les sémaphorines 3 et 7. Les interactions aboutissent à l'immobilisation des macrophages, la neutralisation des cellules cytotoxiques NK (*natural killer*) et CD8+, la polarisation des Th vers des phénotypes plus immunorégulateurs que pro-inflammatoires tels Th2 (*T helper cell subtype 2*) ou Treg (*regulatory T cell*) et la mort par apoptose des cellules activées.

Les astrocytes

D'origine neuroectodermique, les astrocytes sont les cellules les plus abondantes du SNC, comprenant jusqu'à 70 % de la névroglie. Ce sont des cellules étoilées qui, avec leurs extensions cytoplasmiques, participent à la formation de la glia limitans de la BHE (Figure 1), enrobent les synapses, communiquent entre elles par le biais de jonctions communicantes et contribuent au drainage glymphatique du liquide interstitiel parenchymateux vers le liquide céphalo-rachidien (LCR) [17]. Ils délimitent ainsi un territoire sur lequel ils peuvent exercer leur influence grâce aux récepteurs, canaux ioniques, transporteurs et enzymes de toutes sortes dont ils sont pourvus [18, 19].

En plus de leur rôle clé dans l'homéostasie du SNC, le métabolisme neuronal et la modulation dynamique de la transmission synaptique, les astrocytes sont particulièrement bien outillés pour appuyer la microglie dans sa fonction de sentinelle et de défense [20, 21]. Ils expriment le TLR3 de façon constitutive et peuvent être induits à exprimer plusieurs des PRRs. Tout comme les cellules microgliales, ils sont donc aptes à reconnaître et à réagir à un large éventail de situations dangereuses pour le SNC. En réponse à l'activation, les astrocytes deviennent hypertrophiques, prolifèrent et altèrent leur expression génique. Ils sécrètent nombre de facteurs pro-inflammatoires (Tableau 1) servant à attirer les cellules de défense périphériques et faciliter leur migration dans le parenchyme, expriment des récepteurs éboueurs par lesquels ils contribuent à la phagocytose, et produisent les molécules du CMH de classe II leur permettant de soutenir l'activation des cellules Th activées, bien que leur capacité à présenter des antigènes, comme le fait la microglie, est controversée. L'activation les

amène même à diminuer leur internalisation de glutamate et à perturber le réseau astrocytaire en diminuant la production de connexine 43, ce qui contribue de surcroît au caractère neurotoxique de la réaction inflammatoire.

S'ils participent foncièrement aux processus pro-inflammatoires lorsqu'activés, ls répondent néanmoins à l'IL-1β par la sécrétion de TGF-β et par la libération de différents facteurs neurotrophiques (Tableau 1) qui soutiennent la réparation de la BHE, la remyélinisation, le remodelage de la matrice ainsi que la survie des neurones et des oligodendrocytes. Ils contrôlent l'activation des lymphocytes T par leur expression du récepteur immunorégulateur CTLA-4 (*cytotoxic T-lymphocyte-associated protein 4 ou CD152*) et peuvent déclencher l'apoptose des cellules de défense activées par l'expression constitutive du FasL. En situation d'inflammation chronique, leur capacité d'activer les Th est compensée par une interaction qui biaise la réponse en faveur des Th2, un profil plutôt immunorégulateur que le profil Th1 pour le SNC [22]. De plus, certains signaux d'agression permettent aux astrocytes de se dédifférencier en « *radial-glia-like cells* » capables d'exprimer d'autres facteurs neurotrophiques (Tableau 1) qui ensemble promeuvent la multiplication des cellules souches neuronales et leur migration vers le site de lésion [23]. Enfin, les astrocytes sont particulièrement reconnus pour leur rôle dans la formation de la cicatrice gliale. En effet, lorsqu'activés ils se multiplient abondamment, circonscrivent le site d'inflammation, occupent les espaces créés par la mort ou la phagocytose de cellules neuronales et produisent des éléments de la matrice extracellulaire tels que de l'acide hyaluronique. Cependant, cette réaction de défense qui a pour but d'inhiber la propagation de l'inflammation et d'offrir un effet restructurant a aussi comme conséquence néfaste d'inhiber la migration et la différentiation des cellules souches neuronales [23].

Les oligodendrocytes

Les oligodendrocytes partagent la même origine neuroectodermique que les astrocytes et les neurones. Derniers à entrer en scène, ils établissent avec les neurones une relation symbiotique. Les oligodendrocytes sont tout aussi essentiels au

développement et à la survie des axones que les neurones le sont à la myélinisation [24-26].

Comme les neurones, les oligodendrocytes contribuent au caractère immunosuppresseur du parenchyme nerveux par leur production constitutive de TGF-β et par leur expression du CD200 et de fractalkine. À l'instar des autres cellules du SNC, ils expriment des PRRs, notamment TLR2 et TLR3, qui leur permettent de réagir à certains signaux de danger. Bien que les conséquences de l'engagement de ces récepteurs demeurent pour le moment mal connues, elles devraient se répercuter sur l'ensemble du réseau d'influence des oligodendrocytes. Ce réseau, relativement vaste, implique tout autant les astrocytes avec lesquels les oligodendrocytes sont en lien par le biais de jonctions communicantes que les neurones auxquels ils sont associés par la gaine de myéline, un oligodendrocyte pouvant enrober jusqu'à une cinquantaine d'axones distincts.

Si les oligodendrocytes peuvent participer activement à la mise en place d'une immunité innée par l'intermédiaire de leurs TLRs, ils peuvent aussi être eux-mêmes générateurs de danger. Très sensibles au stress oxydant tout comme ils le sont à la toxicité du glutamate ou à celle de l'ATP, les oligodendrocytes peuvent causer des dommages sérieux à leur environnement et entretenir l'inflammation sans être nécessairement les cellules initialement visées par l'agression.

Les cellules de défense périphériques

Au niveau du SNC sain, rares sont les leucocytes qui arrivent à s'échapper de la vasculature. Leur présence, bien que limitée, permet tout de même d'assurer une immunosurveillance dont l'importance nous apparaît indéniable lorsqu'on mesure les dommages encourus par la suppression de la patrouille des cellules de défense périphériques [pour revue, voir 27]. Dans le cas du JC polyomavirus, un virus ubiquitaire chez l'humain, l'arrêt de l'immunosurveillance secondaire à des thérapies

immunosuppressives entraîne la leucoencéphalopathie multifocale progressive, une maladie du SNC généralement mortelle.

Les leucocytes qui parviennent à pénétrer le SNC, uniquement des mononucléaires, prennent avantage à la fois de leur expression de certaines protéines d'adhésion et de l'existence de zones plus permissives de la BHE, telles que la glande pituitaire, l'éminence médiane, l'aire postrema, l'aire préoptique, la paraphyse, la glande pinéale et l'endothélium des plexus choroïdes. Les monocytes utilisent la voie leptoméningée pour venir peupler les espaces de Virchow-Robin en bordure des artérioles et en continuité avec l'espace sous-arachnoïdien. Quant aux lymphocytes, et uniquement les T activés, ils s'affranchissent de la barrière sang-LCR (BSLCR) en empruntant la voie du plexus choroïdien qui exprime constitutivement des molécules nécessaires à leur admission, telles que le CCL20 (*chemokine [C-C motif] ligand 20*) indispensable au recrutement des cellules CCR6+ (*C-C chemokine receptor type 6*) [28]. Une fois traversés, ils sont entraînés par le LCR, patrouillent les espaces et quittent soit vers le sang par le biais des villosités arachnoïdiennes, soit vers les ganglions cervicaux profonds par le biais de la plaque cribriforme de l'os ethmoïde ou par le biais d'un système lymphatique propre au SNC récemment mis en évidence [29, 30]. On constate donc qu'en situation normale, des cellules de défense d'origine médullaire occupent des niches stratégiques du SNC sans jamais pénétrer le parenchyme, qu'elles exercent patrouille et immunosurveillance comme elles le font en périphérie et qu'elles possèdent tous les attributs qui leur permettent de réagir efficacement à l'agression [27, 31-33].

En réponse à l'agression, les cellules du parenchyme s'activent. Elles libèrent dans le milieu des cytokines pro-inflammatoires qui modifient l'étanchéité de la BHE ainsi que des chimiokines (Tableau 1) qui guident les cellules de défense, y compris les granulocytes, et les autorisent à pénétrer au sein même du parenchyme [34]. Les macrophages périvasculaires participent au recrutement par la production de facteurs de croissance (Tableau 1), la modification des cellules endothéliales et par la sécrétion de métalloprotéinases qui facilitent les déplacements. Bien que nécessaire à la résolution des problèmes, l'afflux de cellules sur le pied de guerre dans le parenchyme

nerveux comporte des risques importants pour un tissu aussi fragile. Pour contrer les risques encourus, les cellules du parenchyme limitent le temps de vie des cellules de défense en provenance de l'extérieur ou les réorientent vers des phénotypes plus immunorégulateurs, notamment par l'expression constitutive de FasL ou de TGF-β de l'environnement nerveux.

Conclusion

La neuro-inflammation est un phénomène hautement complexe par lequel le SNC s'allie au système immunitaire pour assurer sa protection et maintenir son homéostasie [35] (Figure 2). La complexité du phénomène, qui le rend difficilement abordable dans le contexte fragmentaire des études in vitro ou des systèmes modèles, tient tout autant à la multiplicité des intervenants cellulaires qu'à l'enchevêtrement des voies de signalisation et aux effets tantôt synergiques, tantôt antagonistes, tantôt neurotoxiques, tantôt neurotrophiques des signaux émis. Les conséquences en sont difficilement prévisibles car elles dépendent à la fois de la nature, de l'intensité, de la durée que de l'historique de l'agression [36]. Il semble cependant que si elle est source du mal, la neuro-inflammation soit aussi source de la solution [37, 38]. Il nous faut donc apprendre à décoder son langage et à identifier, pour chaque problématique, le maillon faible du réseau afin de mettre au point des stratégies d'intervention ciblées qui permettent de contrer la neurodégénération [39].

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APPENDIX B

OLD MOLECULES, NEW INSIGHTS: THERAPEUTIC CONSIDERATIONS FOR THE USE OF POLYPHENOLS IN NEURODEGENERATIVE DISEASES

Renaud, J., and Martinoli, M. G. (in preparation).

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Author contributions

Justine Renaud wrote 95% of the manuscript. Maria-Grazia Martinoli, Justine Renaud's research supervisor, was the guarantor of the work and provided supervision, preparation and editing of the manuscript.

Full review article in English: Old molecules, new insights: therapeutic considerations for the use of polyphenols in neurodegenerative diseases

Abstract

Over the last two decades, neurodegenerative diseases have received increasing attention due to the rapid rate at which the population is aging and the consequent rise in the incidence of such illnesses, itself entailing a major social and economic burden. Today, an increasingly large body of literature lends functional foods and their biofunctional molecules potential neuroprotective capacities. Among the most prominently studied dietary molecules, polyphenols stand in a class of their own on account of their multiple and often overlapping modes of action. However, ambiguity exists as to the significance of their influence on human health. This review discusses the many characteristics and functions of polyphenols that shape their possible therapeutic applications in neurodegenerative diseases. Knowledge gaps that remain under-explored will be highlighted.

1. Introduction

It has long been acknowledged that dietary habits play a key role in the occurrence and progression of non-communicable diseases. A hoard of epidemiological evidence shows that a diet rich in fruit and vegetables reduces the incidence of cardiovascular diseases [1-4], type 2 diabetes [5, 6], stroke [7, 8] and numerous cancers [9-11]. Others find inverse associations between the consumption of green tea and cognitive decline [12, 13]. These observed health benefits are thought to be at least partly attributable to a class of non-essential nutrients named polyphenols, abundantly found in fruit, vegetables and other edible plants [14, 15].

Alongside cancer and cardiovascular diseases, neurodegenerative disorders constitute a popular field of application for the benefits of polyphenols [see for review refs 16, 17]. This is the case for Parkinson's and Alzheimer's diseases, conditions that are often ill defined and lack a clear etiopathogenetic origin besides that they seem to

arise from the interaction between aging, the environment and genetic risk factors. The etiology of these diseases is further convoluted by a number of proposed causative mechanisms, such as oxidative stress, neuroinflammation, protein aggregation, iron toxicity and mitochondrial dysfunction. As such, the number of proposed actions of polyphenols, both in terms of the numerous disease states they appear to improve and the manifold cellular mechanisms they are reported to modulate, makes their use in complex neurodegenerative disorders compelling. In this review, the factors that impinge on the biofunctionality and bioavailability of dietary polyphenols in the central nervous system (CNS) are discussed with a particular focus on therapeutic applications and limitations.

2. Chemico-structural characteristics

2.1 Classification

Plant polyphenols were originally classified in early literature as "vegetable tannins" owing to their tanning action on animal skins [18]. The first comprehensive description, referred to as the WBSSH definition, recommended that the term polyphenol be exclusively ascribed to water-soluble phenolic compounds having a molecular mass ranging between 500 to 4000 Da, possessing at least 12 phenolic hydroxyl groups, and 5 to 7 aromatic rings per 1000 Da [19]. A less restrictive interpretation was proposed by Quideau, offering a broader view of the WBSSH definition – that was hitherto limited to the structural characteristics common to all phenolics endowed with tanning properties (vegetable tannins) – to include simpler phenolic compounds with potential biological activities others than tanning [20]:

> *The term "polyphenol" should be used to define compounds exclusively derived from the shikimate/phenylpropanoid and/or the polyketide pathway, featuring more than one phenolic unit and deprived of nitrogen-based functions. This definition lets out all monophenolic structures as well as all their naturally occurring derivatives such as phenyl esters, methyl phenyl ethers and O-phenyl glycosides.*

A majority of plant polyphenols originate from phenylalanine that is deaminated to cinnamic acid, which then enters the phenylpropanoid pathway [21]. Plant metabolism utilizes the phenylpropanoid unit C6-C3, a phenol ring with a 3-carbon side chain, as a building block to construct polyphenols. Classification of the resulting molecules is dictated by the number of phenol rings (C6) they contain and the structural elements binding these rings to one another. The main subclasses, varying in complexity, are phenolic acids (C6-C3 and C6-C1), flavonoids (C6-C3-C6), stilbenes (C6-C2-C6) and lignans (C6-C3-C3-C6). Within these subclasses, hydroxylations and O-glycosylations at various positions as well as cis-trans isomerization give rise to the thousands of polyphenols (estimated to > 8000) known to date, offering a tremendously intricate bank of molecules with prospective pharmacological value to explore. These polyphenols alongside their content in various food products are available on databases such as Phenol-Explorer managed by the Institut National de la Recherche Agronomique (www.phenol-explorer.eu).

2.2 Structure versus biofunctionality in neuroprotection

The particular chemico-structural properties shared by polyphenols are pertinent to their therapeutic application, especially in the field of neuroprotection. Indeed, the presence of phenol rings, variable hydroxylation patterns and conjugated double bonds grants polyphenols metal-chelating, fibril-destabilizing, estrogen-like, enzyme-binding and antioxidative properties. These numerous modes of action afford polyphenols the ability to tackle the multifarious pathophysiological aspects of several neurodegenerative diseases, namely oxidative stress, neuroinflammation, protein aggregation, iron toxicity and mitochondrial dysfunction.

The redox properties of divalent metals, such as copper, zinc and iron, are essential for cellular homeostasis. When in excess, however, these metals contribute to generate surplus reactive oxygen species. Polyphenols that comprise at least one galloyl or catechol group (hydroxyl groups in the ortho-position) are powerful bidentate chelators of divalent metals [22], whereas ones having only a phenol substitution (one hydroxyl

function) or possessing a resorcinol group (meta-position hydroxyl pair) are less potent monodentate chelators [23, 24]. For chelation to occur, a deprotonation step of the phenolic group is necessary and has been shown to be possible at physiological pH [23].

Self-assembly of amyloidogenic fibrils involves interactions between aromatic residues [25], as in neuropathologically relevant proteins including tau, beta amyloid (Aβ) and α-synuclein, for example. Using the same kind of aromatic interactions, phenol moieties in polyphenols can interfere with fibril assembly [26], possibly by weakening cross-β structures. This interference seems to arise from hydrophobic and π stacking interactions [27], although the formation of covalent bonds through Schiff base reactions has also been proposed for the green tea polyphenol epigallocatechin-3-gallate (EGCG) [28, 29]. Upon analyzing binding energies between polyphenols and protein fibrils, favorable entropic and enthalpic dynamics were discovered that also suggest the stabilization of H-bonds [30].

Some polyphenols, also referred to as phytoestrogens, have the ability to bind estrogen receptors (ERs), usually with a greater affinity for ERβ [31, 32]. Depending on structure, dose, cell type and estrogen response element (ERE) sequence, some polyphenols have a weak or strong antagonistic or agonistic effect on ERs, affording a very wide spectrum of activities in cells [33-36]. As a requirement to bind ERs, the structure should be composed of a phenolic ring with a configuration resembling that of estradiol, as found in flavonoid isoflavones or the stilbene resveratrol, for instance. Indeed, a specific hydroxylation pattern and an adequate distance between substituted hydroxyl groups are necessary to bind ERs.

Some polyphenols also share structural similarities with endogenous ligands, such as cyclic AMP (cAMP) or nucleoside triphosphates, endowing them with the aptitude to activate or inhibit key enzymes [37, 38]. To date, modulatory effects on enzymes have been confirmed in cellular or animal models for several polyphenols, such as resveratrol on cAMP phosphodiesterases [39], theaflavins on the ATP synthase and respiratory chain [40], and curcumin on glyoxalase 1 [41], to name a few. The presence of appropriately spaced ketone and hydroxyl groups in a planar configuration, bestowing

some polyphenols such as curcumin with the ability to mimic an enediolate intermediate in physiological conditions [42], is a remarkable example of structural elements that make enzyme binding possible.

Notwithstanding the aforementioned functions emerging from their unique chemical structures, the most vastly studied characteristic of polyphenols is their antioxidative action. Polyphenols are thought to exert their antioxidative action directly, by scavenging free radical species firsthand, and/or indirectly, by activating endogenous antioxidative pathways. Direct antioxidative effects usually occur through H-atom transfer from polyphenols' (ArOH) hydroxyl (OH) groups to the free radicals (R•):

$$ArOH + R\bullet \rightarrow ArO\bullet + RH \quad (1)$$

The existence of multiple conjugated double bonds in polyphenols allows for the unpaired electron to be delocalized over the aromatic ring, yielding a much more stable, hence much less reactive, polyphenolic radical (ArO•) (Eq. 1). Some polyphenols also exert indirect antioxidative effects through the Kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2/antioxidant response elements (Keap1/Nrf2/ARE) regulatory pathway made possible by the presence of electrophilic functions (α,β-unsaturated carbonyl group, 1,2- and 1,4-quinones, or other groups) that alkylate thiol sensors in the cysteine pocket of Keap1 [43, 44]. Others, like stilbenes, engage their resorcinol hydroxyl functions in hydrogen bonds with the Kelch pocket of Keap1 [45]. Both these events lead to the disruption of the Keap1/Nrf2 complex, allowing Nrf2 to translocate to the nucleus where it can trigger the expression of antioxidant proteins like heme oxygenase-1 via binding of AREs. This cysteine-modifying function of polyphenols may also bear other implications in various enzymes [44].

3. Factors influencing pharmacokinetics and bioavailability

To be effective in the prevention or amelioration of neurodegenerative diseases, polyphenols must be bioavailable in the CNS. Extensive reports on the bioavailability of

the most common dietary polyphenols can be found elsewhere [46-48]. Within the scope of this review, we will first discuss the principal obstacles that hinder polyphenol bioavailability and later address CNS permeability.

3.1 Food matrix or vehicle

Oral administration is by far the most practical of pharmacological routes, but often raises daunting challenges in terms of bioavailability. Particular factors to consider when developing an oral drug are the molecule's interaction with the vehicle, the transformations it undergoes by digestive and microbial enzymes, and its absorption in the gastrointestinal tract [49].

Acting as the vehicle of administration, food matrices are central to the bioefficacy of polyphenols [50]. Of the few studies conducted, inconsistent results have been obtained, demonstrating either a negligible [51, 52] or a significant [53-56] contribution of the food matrix in polyphenol absorption. For instance, bioavailability differs for quercetin among dietary sources, largely depending on the glycoside form they exist in. Indeed, onions, which contain quercetin conjugated to glucosyl functions, are better sources of bioavailable quercetin than are apples and tea, which contain different quercetin glycosides [57, 58]. It is also unclear whether ethanol bears a role in polyphenol absorption in light of studies showing improved bioavailability of quercetin in rats when administered in a 30% solution of ethanol, an alcohol content that is physiologically improbable in an every day diet [59]. In fact, confounding results were obtained in humans administered red wine or dealcoholized red wine that showed no differences in catechin plasma levels and also demonstrated increased catechin excretion likely due to a diuretic effect [60].

3.2 Gastrointestinal transformations and absorption

Once in transit, some polyphenols remain stable though most are converted with galloyl [61] or methyl [62] groups in the duodenum by digestive enzymes. As a rule,

the best-absorbed polyphenols in humans are isoflavones (in soybean-derived products) and gallic acid (in sumac and green tea), followed by catechins (in green tea), flavanones (in citrus), and quercetin glucosides (in onions), whereas the least absorbed are proanthocyanidins and anthocyanins (in berries), galloylated tea catechins, and stilbenes. Whereas aglycones are normally well absorbed by the small intestine, nutritional polyphenols are more commonly detected in the form of glycosides, esters and polymers, which cannot be efficiently assimilated in the upper portion of the gut.

Molecules not absorbed in the upper gastrointestinal tract continue to the colon to become substrates to the gut microbiota, responsible for a very wide array of reactions, some of which yield monomers from polymers or aglycones from glycosylated polyphenols [see for review ref 63]. Smaller, better-absorbed phenolic acids may also be produced by the gut microbiota. For example, quercetin microbiotic degradation mainly generates 3,4-dihydroxyphenylacetic, 3-methoxy-4-hydroxyphenylacetic (homovanillic acid), and 3-hydroxyphenylacetic acids [64]. Among volunteers challenged with 75 mg of rutin, a quercetin glycoside, the total urinary excretion of microbial metabolites accounted for as much as 50% of the ingested dose [65]. Importantly, the sum of these gastrointestinal transformations and food matrix interactions can either increase or decrease the absorption of the resulting metabolites in the bloodstream.

3.3 Plasma bioavailability, transformations and cellular uptake

Once in the blood, drug-metabolizing enzymes mainly found in the liver and kidneys further modify polyphenols into various conjugated forms, a process that serves to detoxify potentially harmful substances. Molecules are rendered more hydrophilic in order to facilitate their urinary elimination, which usually lowers bioavailability [66, 67]. To date, the green tea polyphenol EGCG remains the only known molecule to abundantly persist in a free form in the human plasma (up to 90%) [68]. While metabolites usually constitute the greatest fraction of circulating polyphenolic species, some forms undergo enterohepatic recirculation via biliary secretion, followed by deconjugation into free polyphenols by the gut microbiota and reabsorption in the

colon [69, 70]. Additional hepatic reactions may also occur thus reverting circulating metabolites back to their free form [71-73], as is the case for the conversion of resveratrol sulphates to bioactive resveratrol by human-expressed sulphatases [73]. Moreover, glucuronide and sulphate metabolites were found to retain some of their beneficial effects in vitro [74, 75]. Chronic administration of polyphenols may be an efficient strategy to increase plasma bioavailability in humans, as reported for EGCG [76].

As the finality of drug administration consists in cellular uptake, the notion of bioavailability encompasses the accessibility of polyphenols to target cells, which depends not only on their aforementioned metabolism but also on their complexation with plasma molecules such as proteins, fatty acids and lipoproteins [77]. In fact, the bioefficacy of therapeutic agents heavily relies on their capacity to bind such serum transporters [78]. Taking resveratrol for example, its lipophilic nature requires it to be conjugated into a more hydrophilic form, by sulphation or glucuronidation, or to be bound to proteins in order to circulate in high concentrations, making its passive unbound transport in the plasma rather arduous [79]. Complexation of resveratrol to transporter proteins, principally albumin [80-82] and lipoproteins [83-86], occurs at the expense of its aptitude to be taken up by cells [79]. Fatty acids are also known to improve the ability of resveratrol to bind transporter proteins [87].

While the binding of polyphenols by transporter proteins may diminish the former's availability in the free form, it is thought to provide a reservoir, playing a focal role in the systemic distribution of bound species [77]. Some studies have even proposed that these complexes could be retained at the cell membrane by albumin and lipoprotein receptors, offering a carrier-mediated mechanism by which polyphenols may gain entry to cells [77] aside from passive diffusion [79]. There remains the possibility that polyphenols need not enter cells to have an effect, as is the case when free resveratrol binds integrin $\alpha V\beta 3$ [88] to fulfill part of its angiosuppressive effects and its ability to trigger p53-dependent apoptosis of breast cancer cells [89].

3.4 Accumulation in the brain parenchyma

Drugs targeted to the brain must ultimately be able to accumulate in the parenchyma, both in a biologically active form and in sufficient concentrations. Three important obstacles stand in the way of brain penetration: the blood-brain barrier (BBB), efflux transporters, and multidrug resistance-associated proteins [90, 91]. Youdim and colleagues were the first to demonstrate polyphenols crossing through the BBB in an in vitro model, describing superior permeation of lipophilic molecules (methylated conjugates) in comparison to hydrophilic ones (sulphated or glucuronidated) [92, 93]. Another team identified a stereoselective process in the passage of flavonoid catechins across the BBB [94]. Yet, elucidation of the exact mechanisms used by polyphenols to penetrate the brain in vivo, either by simple diffusion or by transporters at the BBB, remains to be achieved.

Although information on brain permeation is limited compared to plasma availability, an increasing number of studies have directly detected polyphenols and their metabolites in the brains of rodents and even pigs [95], as reviewed elsewhere [90, 96, 97]. CNS penetration of the most commonly studied polyphenols has been recurrently confirmed in vivo, as is the case for resveratrol [67, 98-101], EGCG [102, 103] and quercetin [93, 104-106].

For example, tritiated resveratrol was administered orally to rats (50 mg/kg b.w.) and levels after 2 hours were found to reach 1.7% of the ingested dose in the plasma and below 0.1% in the brain [67]. Interestingly, 18 hours following administration, the CNS retained 43% of the concentration measured at 2 h, mainly in the form of free resveratrol. Despite its persistence in the brain, resveratrol nonetheless permeates the brain lowest compared to liver, kidney, testicle and lung tissues as confirmed by high-performance liquid chromatography (HPLC) [99]. Despite evidence of entry into the brain, another study was unable to detect the polyphenol or its metabolites in the brains of rats fed a 0.2% resveratrol diet for 45 days using a HPLC assay with a detection limit of 0.5 pmol/ml/mg of tissue [107]. Other teams also used chromatographic quantification in rat brains using different protocols. In one study,

15 mg/kg b.w. of resveratrol were administered intravenously (i.v.) – which by this route constitutes a high dose – and brain tissue concentrations reached ~0.17 nmol/g 90 minutes after injections [99]. Another team administered escalating oral doses of resveratrol (100-400 mg/kg b.w.) for 3 days allowing the detection of ~1.7 nmol/g of brain tissue by liquid chromatography-mass spectrometry [100]. Evidently, the detection of polyphenols in the brain is sensitive to methodological disparities and caution must be exercised when drawing conclusions from these studies.

Some polyphenols are extensively transformed before they reach the brain, which possibly dampens their bioavailability, as discussed above. As an example, curcumin is highly lipophilic and, in theory, should easily gain entry to the brain [108]. However, before reaching the BBB, the free form of curcumin is very rapidly conjugated, rendering it only sparingly bioavailable in the CNS [109]. Conversely, catechins efficiently cross the BBB after oral administration, but are found in glucuronidated and 3'-O-methyl glucuronidated forms in the brain tissue [102, 110]. To date, it remains unclear whether conjugation occurs before or after entry into the brain. Nevertheless, strategies exist to boost CNS concentrations of the aglycone form, for example by continuous administration aimed at promoting tissue accumulation [103]. Following 24 hours of continuous intragastric administration, EGCG levels in the CNS reached 5-10% of concentrations measured in the plasma [103]. These results imply, however, that a very high plasma concentration is needed for EGCG to accumulate in therapeutically reasonable concentrations in the brain. The necessity of maintaining high circulating concentrations may raise questions regarding the safety and tolerability of polyphenols.

3.5 Synergistic effects

Some polyphenols interact beneficially when administered in combination. Synergistic pharmacokinetics are at the basis of emerging multi-drug therapies [111-113] developed to surmount shortcomings such as low efficacy, acquired resistance and undesirable side effects in standalone treatments. Polyphenols exert synergy by

multiple means, extensively reviewed elsewhere [114-116]. Although synergistic chemosensitization properties of polyphenols are well appreciated, for example with respect to EGCG-induced downregulation of the endoplasmic reticulum stress response elements that renders temozolomide treatments synergistically more efficient in a mouse model of glioma [117], what follows will concentrate solely on neuroprotective mechanisms.

Perhaps at the basis of herb and extract efficacies, different polyphenols may concurrently regulate identical or separate targets in cells, resulting in a concerted agonistic effect. For instance, combinations of resveratrol and quercetin [118, 119] or epicatechin and quercetin [120] afford synergistic protection against amyloid-like aggregation, oxidative stress and oxygen-glucose deprivation in vitro. An earlier report of synergy between polyphenols showed that treatment of neuronal PC12 cells with subliminal doses of resveratrol in combination with catechin conferred greater protection against Aβ toxicity than the sum of their individual actions [121]. However, when measuring their direct free radical scavenging capacity, the authors found their combined antioxidative effect to be merely additive, suggesting that their synergistic neuroprotective competences at combined subliminal doses must depend on other cellular mechanisms [121]. Very few studies have addressed neuroprotective synergy in vivo though a combination of polyphenols was found to synergistically rescue photoreceptors in an animal model of retinal degeneration [122].

Synergy can also occur between polyphenols and drugs. Many in vitro reports support this, as is the case for the potentiation of neurite outgrowth by a subeffective dose of brain-derived neurotrophic factor (BDNF) in conjunction with green tea catechins [123, 124], as well as for the protection of primary neurons and astrocytes by a cocktail of subliminal doses of resveratrol and melatonin via upregulation of heme oxygenase-1 [125]. One of the first reports of polyphenol-drug synergy in rodents showed EGCG to favorably interact with rasagiline, an irreversible inhibitor of dopamine-metabolizing monoamine oxidase B (MAO-B) undergoing clinical investigations for the treatment of Parkinson's disease [126, 127]. When administered

alone in subliminal doses, neither EGCG nor rasagiline were capable of rescuing nigrostriatal neurons in a 1,2,3,6-tetrahydropyridine (MPTP)-injured mouse model of Parkinson's disease [128]. However, a combination of these agents at these subeffective doses promoted the survival of the dopaminergic nigrostriatal pathway, demonstrating their synergistic effect. Interestingly, rasagiline's ability to promote the expression of BDNF in concert with EGCG-induced augmentations of protein kinase C at the membrane appeared to produce a sum agonistic effect converging at their downstream effector Akt/protein kinase B, thought to account for their neuroprotective action. Other polyphenol-drug synergies exist for valproate and resveratrol in ischemic stroke [129] as well as for glatiramer acetate and EGCG in experimental autoimmune encephalomyelitis [130], among others.

Many polyphenols readily regulate absorption in the gastrointestinal tract, clearance at the level of the kidneys, and detoxification in the liver by modulating the activity of transport proteins or drug-metabolizing enzymes, which may improve their own oral bioavailability or that of other agents. In this respect, the flavonoid EGCG bears promising potential for use in Parkinson's disease owing to its ability to minimize levodopa methylation in the liver by inhibiting human catechol-O-methyl transferase (COMT), thereby enhancing bioavailability of the drug [131]. Flavonoids in general are also known to be potent inhibitors of cytochrome P450 (CYP) drug-metabolizing enzymes [132, 133] whose aforementioned activity reduces polyphenol bioavailability. This potential to enhance bioavailability of metabolism-sensitive drugs constitutes a clear example of polyphenol synergy that may be possible to harness in humans.

4. Safety and tolerability

Beyond favorable pharmacokinetics, polyphenols must be safe and well-tolerated when employed in humans in the proposed conditions and posology. Several investigations have already addressed safety and tolerability issues in humans [see for review refs 134-137]. What follows will summarize these findings.

4.1 Side effects from dosage and chronicity

Virtually all human investigations performed with a wide array of polyphenol preparations demonstrate that they are safe and tolerable on the short [138, 139], medium [46, 140] and long term [141-143]. Generally, side effects are uncommon, mild and transient, expressing themselves as minor gastrointestinal troubles and, more rarely, in the form of headaches, dizziness and rashes. In a phase II trial, 24 Alzheimer's patients were administered 2 or 4 g of curcuminoids daily for 48 weeks and 3 withdrew due to minor gastrointestinal issues [143]. A study using a single 5 g/70 kg b.w. intake of resveratrol, representing 1/40 of the nephrotoxic dose and 1/4 of the highest dose reported to be safe in rats [144], did not uncover any serious adverse effects [138]. A great number of investigations have also addressed the safety of specific diets enriched in polyphenol-rich functional foods. Of particular interest, black cohosh, soy, and red clover regimens aimed at reducing menopausal symptoms in women have proven to be safe, with occasional manifestations of mild gastrointestinal issues, musculoskeletal and connective tissue troubles, as well as weight gain [see for review ref 134].

4.2 Adverse pharmacological interactions

While a consensus has been reached for the safety and tolerability of polyphenols in most individuals, certain contexts preclude their use. The case of grapefruit juice is a notorious example of the possible noxious effects of polyphenols in specific settings. In 1998, Bailey et al. demonstrated that grapefruit juice potently inhibits drug-detoxifying enzymes, explicitly members of the CYP family responsible for the metabolism of several prescribed drugs [145, 146]. Apigenin, naringenin, nobiletin and hesperetin contained in citrus fruits are thought to be the principal culprits [132, 147, 148]. Interestingly, enzymatic inhibition is apparently irreversible following the ingestion of 200-300 mL of juice, leading to increased drug bioavailability and toxicity for up to 24 hours after intake. With this knowledge, medical professionals are now mindful of the risks of consuming grapefruit juice in individuals already taking antidepressants such as buspirone (Buspar) and sertraline (Zoloft), beta-blockers,

anti-cancer agents, fexofenadine (Allegra), or certain statins (atorvastatin) among many other drugs [149-152]. Several other adverse interactions exist between polyphenols and drugs [153, 154] and have been extensively discussed elsewhere [136].

4.3 Tumorigenicity

As previously discussed, certain polyphenols, termed phytoestrogens, owe part of their biofunctionality to their resemblance to steroid hormones. Members of the flavonoid and stilbene subclasses indeed possess the capacity to bind ERs [155] and testosterone receptors [156], albeit with much lower affinities than endogenous ligands. Many studies find phytoestrogens to be safe with respect to incidences of cancers [157, 158], and support their role in inhibiting aberrant cell proliferation [159-165]. Nevertheless, a few publications draw attention to the possible carcinogenic actions of some phytoestrogens that should not be disregarded [166]. In particular, soy genistein and daidzein (0.001-10 μM) may stimulate the growth of malignant breast tumors, both in vitro and in vivo [166, 167].

In the case of the stilbene resveratrol, studies confirmed its ability to bind both ERs equally [168], however with 7000 times less affinity than estradiol [33]. Interestingly, its effects are ostensible for select EREs regulated by ERα, but not for EREs dependent on ERβ activation. Unlike other ERα agonists, resveratrol does not appear to provoke mammary or uterine tissue proliferation in rats [169] and even promotes neuronal differentiation in vitro [170]. In light of this, resveratrol's favorable effects may in fact partially hinge on tissue-specific expression profiles of ERα and ERβ [171]. More recently, an eloquent study delineated the discriminatory ability of resveratrol to impede inflammation without promoting cell proliferation through pathway-selective ERα activation [172]. Crystallographic renderings of the ligand-binding domain revealed resveratrol to bind in an opposite orientation compared to estradiol, which may be at the core of its pathway selectivity and its proven safety in humans [135], especially concerning risks of carcinogenesis.

5. A portrait of clinical progress

The therapeutic potential of polyphenols is hardly contestable when taking into account the overwhelming body of literature supporting their beneficial effects in countless preclinical disease settings [see for review refs 16, 17]. Notwithstanding the weight of epidemiological, anecdotal and fundamental evidence, translation from bench-to-bedside has proven extremely challenging despite relentless efforts to test polyphenols in clinical trials [see for review ref 90]. As of yet, a single trial addressing polyphenols in neurodegenerative diseases has reached phase III clinical testing [173]. In this prospective, randomized, double-blind, placebo-controlled parallel group study, disease progression will be assessed after 48 weeks of daily oral EGCG treatments in multiple system atrophy patients. Results are still pending as of July 2018.

The case of standardized Ginkgo biloba extracts, rich in flavonoids, yielded particularly disappointing results in light of numerous failed phase I trials with respect to the high hopes held by many [106, 174-176]. These studies addressed dementia prevention in large cohorts of healthy or mildly cognitively impaired elderly individuals administered oral Ginkgo biloba twice daily for several years [177], but no reduction in the incidence of cognitive decline or Alzheimer's disease was found between groups [178-182]. Other phase I and II clinical attempts have also been unsuccessful in confirming the putative positive effects of curcumin in Alzheimer's disease patients [143, 183]. The reasons behind these discrepant results are not understood: have preclinical models failed to fulfill their predictive purpose or are the clinical trials simply incapable of detecting the beneficial effects of polyphenols due to a flawed approach? What is important to keep in mind is that successful clinical trials are not common, on account of the inherent difficulty of translating drug applications between rodent paradigms and humans. Poor choice of participants, administration strategies or clinical endpoints will inexorably undermine the outcomes of these studies. As such, lack of positive results may not in themselves imply invalid preclinical evidence.

In that respect, the required enrolment profile for testing Ginkgo biloba extracts was re-evaluated, yielding positive results in a new round of clinical trials, this time

performed in full-blown Alzheimer's disease and vascular dementia. These trials successfully uncovered the benefits of several months of a daily Ginkgo biloba treatment on cognition and neuropsychiatric symptoms [141, 142]. Changing the endpoints and focusing on prefrontal dopaminergic functions in elderly humans with self-reported mild cognitive decline was another fruitful strategy to expose the beneficial effects of Ginkgo biloba [184]. Nevertheless, the cholinesterase inhibitor rivastigmine, commercially known as Exelon, has been shown to be more efficient than Ginkgo biloba in treating Alzheimer's disease and remains the drug of choice to ameliorate cognitive impairment in mild to moderate forms of the disease [185].

Regarding other phase I trials, several have been successful in confirming discrete positive effects in healthy individuals. A variety of polyphenols, including resveratrol, were found to increase cerebral blood flow without, however, improving cognitive performances in young adults, whether administered in a single dose [186-188] or chronically over 28 days [189]. However, other groups found that longer chronic interventions in elderly humans using either cocoa flavanols or resveratrol enhanced dentate gyrus-related cognitive functions [190] or hippocampal-related memory functions [191], respectively. In Alzheimer's disease patients, resveratrol reached phase II investigations on the basis of its modulatory role on neuroinflammation, cognitive decline, and cerebrospinal fluid (CSF) levels of Aβ40 [192, 193]. Following a twice-daily oral regime for one year, resveratrol and its metabolites were discovered in the CSF, validating its ability to cross the BBB in humans [192]. Despite its relatively low bioavailability, resveratrol maintains the hopes of the scientific community towards its potential use in human neurodegenerative diseases.

6. Future strategies for pharmaceutical development

Polyphenols hold interesting properties that justify the intense scientific efforts devoted to translating their purported neuroprotective effects in humans. However, their questionable bioavailability, their modest effects in humans and the impossibility of applying exclusive patent protection on natural molecules strips dietary polyphenols

of their appeal for pharmaceutical expansion. Nevertheless, several strategies espoused by drug developing teams in recent years have tackled these issues.

6.1 Alternative formulations

Engineering of novel structural analogues inspired by existing polyphenols or formulating specific preparations of polyphenols, such as the well-defined Ginkgo Biloba extract 761, may be patentable options. Among the latest innovations, chemical engineering of pro-drug polyphenolic structures has shown promising results. For instance, acetylation of EGCG or resveratrol via esterification of their hydroxyl moieties yields stable pro-drugs in vivo whose acetyl groups can be hydrolyzed intracellularly by esterases to release the free polyphenol within the cell [194-196]. This strategy minimizes polyphenol auto-oxidation and allows better lipophilicity-dependent cellular uptake [197-199]. Production of conjugates with improved bioefficacy has also been a good experimental approach to promote polyphenol absorption and activity. For example, the glutamoyl diester of curcumin is a more potent neuroprotective agent than is curcumin alone [200]. Similar efforts have been deployed for resveratrol [201-203].

6.2 Alternative drug delivery systems

One of the more hopeful avenues employed by several teams is the development of novel encapsulation technologies. Progress in vehicle formulation made by pharmaceutical companies have allowed the preparation of polyphenols captured in lipid nanocapsules [204-206], nanoparticles [206, 207], exosomes [208], nanocomposites [209], emulsified formulations [206, 210, 211] or in gel form [212]. Promisingly, several reports demonstrate increased bioavailability in rodents administered encapsulated polyphenols [see for review ref 213]. Another unusual packaging approach consists in the administration of biologically compatible carbon nanotubes [214] grafted with polyphenols, such as gallic acid [215]. This method was shown to enhance the antioxidative properties of grafted agents [215] and to improve their ability to traverse

biological barriers [214, 216], although the application of such conjugates is still not common and the outcome of using them has not been sufficiently addressed. Possible health concerns arising from the peculiar physicochemical properties of carbon nanotubes also warrant further investigations [214, 217]. Another simple tactic consists in improving a polyphenol's solubility in circulation, such as for the lipophilic resveratrol [218], via complexation with cyclodextrins whose capacity to form inclusion complexes has already been exploited in other drug delivery strategies [219]. On the whole, each of these methods possesses its own advantages and disadvantages, but brain accessibility is generally augmented owing to improved BBB infiltration by lipophilic vehicles, brain targeting by functionalized capsules, and efficient evasion of metabolism [220].

6.3 Alternative administration routes

In order to target the human brain with more efficiency, the administration route is another variable that can be altered, as long as it remains easy to market. Likely the most interesting of these alternative options is intranasal administration, usually paired with one of the previously mentioned encapsulation technologies, which has produced successful results with brain-bound drugs in humans, at least when considering bioavailability and the capacity to avoid peripheral side effects as clinical endpoints [221, 222]. Notable examples are the administration of insulin for the treatment of Alzheimer's disease [223] and apomorphine for the treatment of Parkinson's disease [224]. The mechanisms by which drugs are delivered to the brain parenchyma have only begun to be elucidated. It would appear that drugs administered nasally either enter the brain through retrograde axonal transport at the level of the olfactory sensory cells, or by their penetration into the CSF across the nasal epithelium [225]. Although studies with polyphenols are scarce in preclinical models [226-228], intranasal curcumin administration has gained much attention [see for review ref 229] due to its very poor oral bioavailability [230] yet promising neuroprotective actions. Curcumin, which is highly lipophilic, may easily cross the BBB [108] if it is efficiently incorporated in the bloodstream and preserved from enzymatic modifications [231]. While it is recognized

as a very safe route, intranasal administration sometimes leads to minor adverse effects, principally nasal irritation, constituting a potential roadblock in the development of intranasal polyphenol administration [224, 232]. Less discussed administration systems verified preclinically with polyphenols include rectal suppositories for efficient systemic distribution, bone-marrow administration to improve immunomodulatory effects in situ and controlled-release implant strategies for direct targeting of tumors, although intrathecal administration for direct distribution in the CSF remains the only other interesting yet invasive option for brain targeting [see for review ref 229].

7. On the topic of dose-response

The previous sections have highlighted studies focused on improving polyphenol bioavailability for their therapeutic use. While bioavailability remains a core issue regarding their therapeutic potential, little is known about the actual dosage required for polyphenols to wield beneficial effects in humans. This constant struggle to prove that polyphenols can accumulate in high-enough concentrations in target tissues is likely deeply rooted in a history of earlier reports that lent these molecules potent antioxidative properties in vitro [233, 234].

More recently, the physiological significance of polyphenols' direct antioxidative actions is met with much skepticism, particularly with regard to the brain, due to their modest gastrointestinal absorption, propensity to undergo heavy biotransformations and rapid excretion by the kidneys [97, 235]. On the one hand, H-atom transfer must always occur faster than at least one of the reactions of free-radical-production cascades (e.g., the limiting propagation step in lipid peroxidation), and this is improbable [236]. On the other, polyphenol concentrations, which rarely exceed micromolar concentrations in plasma or tissues at any given time [237], are substantially inferior to those of endogenous antioxidants such as ascorbate (30-100 μM) and urate (140-200 μM) [238]. Consequently, it is argued that their contribution to plasma's total antioxidative capacity never exceeds 2% and may therefore be irrelevant in a physiological context [235, 239]. In fact, direct antioxidative effects of polyphenols have not been measured in the brain

and some recommend assuming the "absence of evidence as evidence of absence" [97]. Studies demonstrating the anti-inflammatory properties of polyphenol analogues devoid of direct antioxidative capacities challenges the idea by which their health virtues stem from their ability to hamper oxidative stress [201, 202].

Nowadays, it is acknowledged that high circulating concentrations of polyphenols may not be required to achieve certain clinical endpoints. Indeed, by interacting with various enzymatic targets, for instance Keap1, very small doses of polyphenols may benefit from the cascades of events that ensue in cells. Despite this, efforts continue to focus on enhancing bioavailability rather than on identifying an adequate dose-response framework that could predict the behavior of this class of molecules. This oversight may partly account for the apparent difficulty of translating preclinical findings into actual positive outcomes in humans. Where disappointingly modest clinical benefits have been shown, is increasing the dose always a judicious strategy? The answer may not be as obvious as once thought.

Sensible explanations have been proposed to explain the bioefficacy of polyphenols at very low doses. One of these is that polyphenols exert their biological effects in a non-linear fashion, more precisely by abiding to the kinetics of a biphasic dose-response profile. One such model predicts dose-response curves in the shape of a J or an inverted U depending on the endpoint quantified [240, 241]. The biphasic theory stipulates low-dose stimulatory and high-dose inhibitory effects [242, 243]. It describes an agent's direct stimulatory effects at low concentrations followed by the organism's overcompensation riposte at higher doses [244]. In neuroprotection, hormesis predicts very low doses to be beneficial and higher doses to be potentially harmful. The application of this theory is thus intimately linked with whether polyphenols are indeed stressors that induce a defense response in cells. This has yet to be confirmed.

Today, the biphasic hypothesis to explain polyphenols' bioefficacy at very low doses is gaining momentum, resveratrol constituting the best example. A wealth of reports lends resveratrol hormetic properties in various fields of application, ranging

from cancer to neuroscience research, extensively reviewed elsewhere [245]. In some instances, resveratrol stimulates cancer cell proliferation at very low doses, but inhibits carcinogenesis in higher concentrations [246]. Other reports show resveratrol inducing atherosclerotic lesions at high doses, while it remains cardioprotective at lower concentrations [247]. In neurons, resveratrol promotes survival at very low concentrations, but is neurotoxic at higher doses [121, 248]. One study performed in mice and primary cortical neurons proposed a mechanism possibly underlying the biphasic response of energy-depleted neurons to resveratrol, showing protection at low doses and toxicity at higher doses [249]. The authors explained resveratrol's bimodal effects via its stimulatory action on silent mating type information regulation 2 homolog 1 (SIRT1), whose low-grade activity can suppress oxidative stress [250]. However, when stimulated by greater doses of resveratrol, SIRT1 expends too much reduced nicotinamide adenine dinucleotide (NAD+) where neurons are already energetically depleted, causing energy failure. During an ischemic event, resveratrol administration could either be beneficial or detrimental, depending on dosage and timing to which the bioenergetic status of neurons is sensitive.

For now, these studies are more often than not performed in pre-clinical models and do not necessarily reflect what could occur in humans. The best-documented evidence of biphasic dose-responses in humans pertain to radiation events, for instance in cancer treatments or in atomic bomb survivors [251, 252]. However, reservations dwell on the significance of such a dose-response relationship in polyphenols applied to the human brain, seeing as it is highly unlikely that treatments could ever afford to increase their bioavailability in the parenchyma beyond very low concentrations. This could mean that the observed bioefficacy of polyphenols may already be optimal where modest benefits are found in trials. Indeed, one distinct feature of the biphasic hypothesis provides that low dose beneficial effects stem from cellular overcompensation mechanisms in response to the stress charged by polyphenols [253]. Beyond the optimal concentration at which maximal benefits are yielded, this compensation reaction is slowly overwhelmed by the increasing stress polyphenols directly exert on the cell. Even at the optimal concentration, these beneficial effects are

thus thought to be at best partial. If this theory holds true, this could explain the mitigated results clinical trials have harvested to date, even upon increasing dosages.

8. Concluding remarks

In theory, polyphenols hold a very attractive therapeutic potential. Their structure confers them metal-chelating, fibril-destabilizing, estrogen-like, enzyme-binding and indirect antioxidative competences supporting their usefulness in neurodegenerative diseases. Epidemiological evidence shows a strong association between their consumption and a reduced occurrence of various neurodegenerative diseases. Preclinical models lend them neuroprotective properties. Some clinical trials have even been successful in revealing small but noticeable improvements in human health and have confirmed their safety in various settings. Nevertheless, the limited bioavailability of polyphenols faced with their apparent bioefficacy remains an under-explored dimension of their employment in diseases. To achieve clinical dissemination, investigators must demonstrate that polyphenols exert significant health benefits, fulfilling fixed endpoints. However, in neurodegenerative diseases, polyphenol trials consistently fall through in early clinical testing phases. To overcome this, researchers must optimize the design of their trials, with respect to the subjects (disease stage, participant profile, cohort age, medical history), the administration paradigm (polyphenol formulation, route, dosage, frequency, duration) and endpoints (motor symptoms, cognitive decline, neuroinflammation, neuron integrity, CNS vascular health, etc.). This should logically require a clear notion of the relationship between doses and outcomes in very specific human disease settings, as well as the extent to which beneficial effects should be expected to manifest themselves. Indeed, as reviewed here, polyphenols are sensitive to a great number of physiological conditions that impinge on their bioavailability and biofunctionality, which may account for the markedly high interindividual variation observed in clinical investigations. Even biphasic dose-response theories cannot explain this variability.

Despite having collected a large amount of information from many pre-clinical models and applications, the scientific community still cannot agree on a working theoretical framework that could aid in predicting outcomes in humans. Until then, optimization of clinical trials remains an exercise of futile guessing. A priority would consist in determining the maximal health benefits that could be achieved from polyphenol monotherapies as they most usually stand in trials. Can we really expect standalone treatments to fulfill hard-to-reach clinical endpoints? If epidemiological evidence is strong for the protective effects of consuming complex mixtures of polyphenols in food, it may be precarious to expect single molecules to be as effective. Perhaps concentrating on the concerted effects between polyphenols with each other or with other drugs that show partial benefits, such as the MAO-B inhibitor rasagiline [127] or levodopa [131], may overcome the as of yet modest effects in humans. Evaluating polyphenols in preventive clinical paradigms may also constitute a more realistic strategy. Future research should cease to avoid addressing the limitations of polyphenols employed in neurodegenerative diseases; it should rather clearly define and harness these frontiers in emitting educated projections of their true therapeutic potential in human health.

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APPENDIX C

PREVENTION OF NEUROINFLAMMATION BY RESVERATROL: FOCUS ON EXPERIMENTAL MODELS AND MOLECULAR MECHANISMS

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Justine Renaud wrote 90% of the manuscript, and prepared and edited the figure. Maria-Grazia Martinoli, Justine Renaud's research supervisor, was the guarantor of the work and provided supervision, preparation and editing of the manuscript.

Full chapter in English: Prevention of neuroinflammation by resveratrol: focus on experimental models and molecular mechanisms

Abstract

The central nervous system (CNS) is normally protected by the blood brain barrier (BBB) from systemic inflammation, and the role of immunity is fulfilled by its resident cells, mainly microglia. Resveratrol, *trans*-3,5,4′-trihydroxystilbene, has long been acknowledged as a dietary polyphenol bearing potent anti-oxidative and neuroprotective properties in experimental paradigms of neurodegenerative diseases with a component of neuroinflammation, such as Parkinson's disease (PD) and Alzheimer's disease (AD). Nowadays, a growing number of studies indicate that resveratrol is implicated in slowing or altering the pathological progression of neurodegeneration by modulating specific cellular pathways and parameters of neuroinflammation. Here, we present a global portrait of this polyphenol's role in the neural environment with a specific emphasis on the experimental paradigms demonstrating resveratrol's molecular and cellular action on neuroinflammation.

1. Introduction: neuroinflammation

For a long time, the central nervous system (CNS) was considered an immune privileged region of the organism. This apparent state of retreat was attributed to the presence of tight junctions at the boundary between the endothelial membrane and nerve cells named the blood-brain barrier (BBB), and the relatively low expression of major histocompatibility complex (MHC) molecules. Today, we know this paradigm to be inaccurate as major evidence demonstrates support dynamic crosstalk between central and peripheral immunity as established by cytokine-mediated coordination of the inflammatory reaction (1), active immune surveillance by intruding cells of the peripheral immunity (2-4), and even the newfound demonstration of a lymphatic drainage system (5,6).

The CNS remains immunologically specialized inasmuch as resident cells accomplish the bulk of surveillance, defense, and reparation tasks. The principal antigen-presenting immune cells of the CNS are microglia that, in their resting ramified state, monitor their environment for disruptions of the fragile homeostasis required for the proper functioning of nerve cells (7-9). Upon activation, microglia partake in neuroinflammation by secreting inflammatory mediators, producing toxic reactive species, and by executing reparation tasks through phagocytosis of debris (10,11). Astrocytes, which constitute up to 70% of glial cells, also actively participate during the inflammatory reaction by becoming hypertrophic, proliferating (astrogliosis), presenting antigens, expressing both pro- and anti-inflammatory molecules, producing neurotrophic factors, and confining the lesion through the production of a glial scar (12,13). Just as important is the presence of neurons that constitutive express transforming growth factor-beta (TGF-β), soluble fractalkine, and fas ligand (FasL), thus helping to contain inflammation (14,15). As such, all resident cells of the CNS are capable of fulfilling pro- and anti-inflammatory tasks depending on their expression phenotype that greatly depends on surrounding soluble and cellular signals.

Sustained and uncontrolled neuroinflammation can easily take on auto-destructive proportions as observed in several neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease (PD) (16,17). As such, strategies aiming to modulate neuroinflammation are of great interest in the development of neuroprotective therapies for various neurological pathologies.

2. Resveratrol in neuroinflammation

With respect to their proven anti-inflammatory properties, an expanding number of studies have addressed the potential neuroprotective effects of dietary polyphenols in the CNS, especially regarding neurodegenerative diseases (18-20). Among the most promising anti-inflammatory polyphenols, resveratrol (*trans*-3,5,4′-trihydroxystilbene), a stilbenoid found in important quantities in the roots of *Polygonum cuspidatum* (*Fallopia japonica*) and to a lesser extent in red wine, grapes, peanuts, and berries (21),

has attracted much attention over the past twenty years since its anticancer properties were first brought to light (22). Resveratrol has also been shown to possess broad anti-oxidative (23), cardioprotective (24), and anti-inflammatory (25,26) effects. Its neuroprotective actions in a wealth of in vitro and in vivo models (recently reviewed in 27 and 28), together with its sufficient bioavailability, oral safety, and ability to cross the BBB (29,30-36), earn resveratrol a prime role for its potential to counteract neuroinflammation in a pathological context. The following sections review the advance made in the characterization of resveratrol's neuroprotective properties pertaining to its anti-inflammatory actions, with a particular focus on experimental models and the latest proposed molecular mechanisms.

2.1 Resveratrol and neuroinflammation: In vitro models

2.1.1 Microglia monocultures

Resveratrol's anti-inflammatory effects have been tested repeatedly in monocultures of microglial cell lines or primary cultures treated with pro-inflammatory molecules such as the endotoxin lipopolysaccharide (LPS), a component of gram-negative bacteria outer membranes. LPS elicits a potent inflammatory response by binding toll-like receptor 4 (TLR4), which leads to receptor homodimerization and activation that ultimately enhance transcription of pro-inflammatory proteins.

Using BV2 microglial cells treated with LPS, resveratrol attenuated the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (37), as well as it lowered levels (mRNA and protein) of the pro-inflammatory enzymes inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) alongside their products, nitric oxide (NO) and prostaglandin E2 (PGE2), respectively (38). Both mRNA and protein levels of the pro-inflammatory transcription factor κ-light-chain-enhancer of activated B cells subunit RelA (NF-κB/RelA) were reduced as a result of resveratrol's inhibitory effect on upstream regulators, in particular the prevention of mitogen-activated protein kinases (MAPKs) phosphorylation (38). Of importance, these anti-inflammatory effects were fully or partially impaired by a treatment with

rapamycin, meaning that the mechanistic target of rapamycin (mTOR) was required to fulfill resveratrol's beneficial effects (38). When used as a co-treatment in the LPS-activated N9 microglial cells, resveratrol was also shown to be anti-inflammatory by reducing iNOS expression and TNF-α release by acting through inhibition of p38 MAPK phosphorylation (39). More recently, comparable anti-inflammatory effects were observed in the N13 microglial cell line treated with LPS wherein resveratrol upregulated suppressor of cytokine signaling-1 (SOCS-1), a regulator of inflammation that drives a negative feedback loop to temper cytokine production (40). Remarkably, resveratrol was found to inhibit all three pathways downstream of TLR4 activation, namely the MAPK, NF-κB pathway, and janus kinase (JAK)/signal transducer and activator of transcription (STAT) axes, leading to hampered cytokine production. Noteworthy, silencing the expression of SOCS-1 impaired resveratrol's pleiotropic anti-inflammatory actions, suggesting a pivotal role for this regulatory switch in mediating this polyphenol's beneficial effects.

In primary rat microglia treated with LPS, resveratrol reported to prevent microglial activation not only by inhibiting the activity of cyclooxygenases (COXs), but also through the downregulation of the transcription and expression of the inducible microsomal PGE synthase-1 (mPGES-1) known to be importantly involved in LPS-activation (41). This observation was not accompanied by altered COX expression nor modulated by COX-1 inhibitors, suggesting that resveratrol's newfound inhibitory effect on mPGES-1 expression is independent of its action on COX-1.

The use of Aβ in mediating glial activation has become an increasingly popular tool in mimicking neuroinflammation in light of discoveries that demonstrate a clear-cut link between AD and inflammation (42). One study compared the potential of resveratrol to antagonize pro-inflammatory events in BV2 microglial cells activated by either Aβ or LPS (43). Resveratrol inhibited the NF-κB pathway in both models, and apparently interfered with TLR4 oligodimerization upon activation by LPS as shown by co-immunoprecipitation experiments in homogenates from murine bone marrow-derived pro-B Ba/F3 cells coexpressing two differently tagged versions of

TLR4. Although there is no doubt that resveratrol blocked downstream TLR4 pathways, direct interference with TLR4 oligomerization is unlikely given that this polyphenol does not possess the structural motif to perform Michael addition on the receptor (44). Very recently, another group employed oligomeric Aβ instead of Aβ monomers to activate BV2 microglial cells and found that resveratrol successfully mitigated microgliosis and production of cytokines, NO, and reactive oxygen species (ROS) (45). In addition, resveratrol was shown to reduce mRNA and protein levels of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) subunits p47phox and gp91phox, thereby explaining the decrease in ROS formation.

2.1.2 Astrocyte monocultures

As astrocytes are the most abundant type of nerve cell in the CNS, their regulatory role in neuroinflammation cannot be ignored. In the C6 astroglial cell strain derived from a rat glial tumor, resveratrol revoked activation of the NF-κB pathway and impeded the expression of downstream target genes COX-2 and iNOS, following LPS activation (47). Similarly, in primary murine astrocyte cultures activated by LPS, resveratrol was found to reduce levels of several pro-inflammatory cytokines including regulators of T cell polarization, IL-12 subunit β (IL-12p40) and IL-23, and the chemokine monocyte chemotactic protein-1 (MCP-1) (48). Moreover, another study used pharmacological inhibitors of the inducible heme oxygenase-1 (HO-1), ERK1/2, and p38, in primary rat astrocytes, to show that HO-1 was necessary to mediate resveratrol's beneficial effects (49). Of note, HO-1 is a known inhibitor of NF-κB nuclear translocation and of iNOS activity (50). Comparable results were obtained in primary rat astrocyte cultures activated by Aβ, sustaining a role for resveratrol in diminishing COX-2 and iNOS protein levels as well as in impeding astrogliosis (51).

In a comparative study using primary murine microglia and astrocytes as well as the N9 microglial cell, resveratrol precluded the activation of NF-κB in all cell types, but it only successfully inhibited activator protein 1 (AP-1), another key pro-inflammatory transcription factor, in microglia thereby offering a reasonable explanation

for resveratrol's less potent anti-inflammatory response in astrocytes (52). Interestingly, resveratrol had no effect on the phosphorylation levels of all three members of the MAPK family, contrary to other data collected albeit from different experimental models (38-40,53-55). Another comparative study using primary hippocampal astrocyte cultures from newborn, adult, and aged Wistar rats, revealed that pro-inflammatory cytokine production was slightly though significantly more elevated in astrocyte cultures derived from older rats, while expression levels of the anti-oxidant enzyme glutathione synthase and its product, glutathione, were consistently lower (56). Remarkably, resveratrol was able to decrease levels of inflammatory markers and ameliorate the glutathione anti-oxidant response in each one of these unchallenged astrocyte cultures.

2.1.3 Glia-neuron co-cultures

As inflammatory reactions rely heavily on crosstalk between the multiple actors of immunity, co-cultures have emerged as very useful tools to study these interactions. Using LPS-challenged mixed primary cortical cultures, one group showed that resveratrol can reduce microglial activation as demonstrated by a decrease in levels of ionized calcium-binding adapter molecule 1 (Iba1), a marker of activated microglia, and concomitant drops in TNF-α, IL-1β, and NO secretion (57). Similar results were found in mesencephalic mixed primary cultures where resveratrol protected dopaminergic neurons against LPS toxicity (58). The same group further revealed that resveratrol could impair NADPH oxidase activation, an enzyme that like MPO is responsible for respiratory burst, thereby preventing the production of ROS. This was likely due to the inhibition of MAPK phosphorylation, in particular extracellular signal-regulated kinase (ERK1/2), a known activator of NADPH oxidase. Similar NADPH oxidase-inhibition effects for resveratrol had also been previously reported in Aβ-challenged BV2 microglia (45). Moreover, resveratrol could not protect the dopaminergic neurons in mixed cultures from NADPH oxidase-deficient mice, implying that its anti-inflammatory properties may require the inhibition of this ROS-producing enzyme.

It should be noted, however, that mixed cultures cannot distinctively detect interactions that are solely based on paracrine signaling in the absence of cell-cell contact, as is the case for some cellular relationships seen in inflammation. To palliate this problem, the use of conditioned medium was developed as a method for the compartmentalization of cellular populations. Wang and colleagues (59) demonstrated in a conditioned medium experiment that secreted soluble factors from LPS-activated primary microglia reduce the viability of primary hippocampal neurons while resveratrol co-treatment afforded anti-inflammatory effects, leading to decreased secretion of cytokines, lower expression of iNOS, and repression of the NF-κB pathway. Of interest, resveratrol preserved the physiology of the primary neurons treated with conditioned medium on the basis of numbers of growth cones, dendrites, and spines. Ye and colleagues also administered cytokine-rich conditioned medium from LPS-activated BV2 microglia monocultures as a toxic treatment on separate native PC12 cells (60). Resveratrol treatments were performed either on the BV2 microglial or PC12 cell population, resulting in protection against LPS-induced PC12 apoptosis, decreased microglial cytokine release, and all-around increased expression of silent mating type information regulation 2 homologue 1 (SIRT1), a deacetylase with pleiotropic beneficial effects.

While the use of conditioned medium does solve the compartmentalization problems that arise from employing mixed cultures, this method still bears important drawbacks. Indeed, in conditioned medium, the communication occurs unidirectionally instead of bidirectionally, secreted factors become diluted as they diffuse over time, and short-lived molecules, such as ROS that often go hand in hand with inflammatory reactions, decay before the conditioned medium is even transferred to the second population of cells. Currently, insert co-culture systems solve several of these problems. Our group tested the neuroinflammatory paradigm using LPS-activated N9 microglia grown in cell culture inserts overlaid in wells containing nerve growth factor-differentiated dopaminergic PC12 neurons (61). Secreted cytokines diffused through the micropores of the inserts and triggered the apoptotic cascade of the neuronal PC12 neurons underneath, events that were counteracted by a resveratrol pre-treatment.

Similar results were found in the same co-culture neuroinflammation system using the parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPP+) to activate the microglial cell line (62).

One group used an array of co-culture techniques to demonstrate the link between resveratrol neuroprotection and the expression of myeloperoxidase (MPO), a peroxidase enzyme necessary during microglial respiratory burst (46). Resveratrol succeeded to prevent rotenone-induced increases in MPO activity and expression while other anti-inflammatory drugs could not. Remarkably, in a mixed culture of MPO-deficient glial cells, resveratrol greatly ameliorated the impaired inflammatory response to rotenone-induced production of nitrite and transcriptional up-regulation pro-inflammatory markers. In the same study, the authors reported that neurons cultured without microglia, were much more susceptible to rotenone toxicity despite being treated with resveratrol, suggesting that this polyphenol's mode of action may rely at least partly on MPO inhibition. Similar results were also obtained by Zhang et al. showing that solely mixed primary cultures containing microglia were protected against MPP+ by resveratrol administration (58).

One of the earliest studies employing Aβ as a pro-inflammatory insult showed that resveratrol could prevent the activation of the NF-κB axis in a mixed culture of cortical neurons and glia (63). Concomitantly, expression of the deacetylase SIRT1, a known inhibitor of NF-κB activation, was significantly increased. Either overexpressing SIRT1 or treating the mixed culture with resveratrol could suppress increased levels of cathepsin B and iNOS, two effectors of neuronal injury in AD, suggesting a role for this deacetylase in resveratrol's anti-inflammatory actions.

2.1.4 Organotypic slice cultures

Organotypic slice cultures offer interesting advantages over other in vitro platforms such as preservation of tissue architecture, maintenance of intact local synaptic circuitry, and pharmacological accessibility (64). In that respect, resveratrol's

anti-inflammatory potential was investigated in rat hippocampal slice cultures stimulated by Aβ (65). Resveratrol administered both in a free form or loaded in lipid-core nanocapsules was found to diminish cytokine release and ROS formation, thereby protecting the slice cultures against cell death. Remarkably, resveratrol-loaded lipid-core nanocapsules were further able to increase the release of IL-10, an anti-inflammatory cytokine. On the other hand, free form resveratrol was less effective than lipid-core nanocapsules in mediating all-around anti-inflammatory effects and was unable to stimulate IL-10 release, which opens the way for the improvement of administration routes for this polyphenol in pre-clinical experimental models.

Another set of interesting studies employing organotypic rat hippocampal slice cultures have shown the noxious effects of the neurotransmitter glutamate and the neurotoxic viral protein Tat, derived from the human immunodeficiency virus-1 (HIV-1) (54,55). Tat is actively secreted by HIV-infected immune cells and is known to be elevated in the CNS of patients suffering from HIV-associated dementia (HAD) (66), whereas glutamate plays a role in several neurological diseases with a component of excitotoxicity such as AD but also in HAD (66,67). Both toxic treatments increased the expression of pro-inflammatory MCP-1, however they acted through different mechanisms. Whereas glutamate required ERK1/2-induced IL-1β production to mediate its pro-inflammatory action (54), Tat rather necessitated activation of both ERK1/2 and tyrosine kinase (TK) to promote TNF-α release (55). In these paradigms, resveratrol exerted anti-inflammatory protective effects through preventing ERK1/2 phosphorylation and subsequently mitigating cytokine production and ultimately led to decreased expression of MCP-1, as shown previously (48,52). Noteworthy, pharmacological manipulations ruled out the implication of any of the two other MAPKs, which reiterates the pivotal function that ERK1/2 holds in mediating resveratrol's anti-inflammatory effects in these models and others (38-40,53).

2.2 Resveratrol and neuroinflammation: In vivo models

2.2.1 Neurodegenerative disease models

The most common neurodegenerative diseases and brain injuries are attended by inflammatory events that participate either in their onset or progression. In the two most common neurodegenerative disease, AD and PD, neuroinflammation has been broadly characterized ever since seminal work by McGeer and colleagues exposed the existence of unusually elevated levels of MHC class II molecules on the surface of activated microglia in post-mortem brains of PD patients (68).

Experiments performed in rodents challenged with LPS have substantiated resveratrol's general potential to prevent neuroinflammation by improving CNS levels of oxidative markers and pro-inflammatory cytokines, as well as ameliorating locomotor and cognitive behaviors (37,69). Further experimenting using the well-known MPTP mouse model of PD revealed that orally-administered resveratrol could reduce glial activation and dopaminergic neuronal death in the substantia nigra pars compacta, the key region targeted by neurodegeneration in PD. (70). In particular, resveratrol upregulated the transcription and expression of SOCS-1, reduced cytokines release and lowed transcription levels of their respective receptors. In 6-hydroxydopamine (6-OHDA)-treated rats, another rodent model of PD, resveratrol administered per os showed similar anti-inflammatory effects alongside improving dopaminergic neuronal morphology, such as chromatin condensation, mitochondrial tumefaction, and vacuolization (71). Noteworthy, resveratrol was able to attenuate the characteristic apomorphine-induced turning behavior observed in these rats.

Resveratrol's anti-inflammatory properties have further been supported in models of AD. Indeed, resveratrol was shown to diminish plaque area in correlation with impeded microglial activation in the hippocampus of APP/PS1 transgenic mice, a well-documented model of early onset AD (43). Resveratrol mixed in the diet and fed to the mice for a period of one year was found to significantly reduce Aβ plaque density in the cortex, caudate-putamen, and hippocampus (72), and concurrent microglial activation

was hampered in these brain regions. In rats infused with Aβ intracerebroventricularly, resveratrol loaded in lipid-core nanocapsules was shown to improve hippocampal synaptic integrity and gliosis (73) by inhibiting c-Jun N-terminal kinases (JNK) MAPK and the β-catenin pathway through rescuing glycogen synthase kinase 3β (GSK-3β) phosphorylation. In addition, improvement of spatial working memory and long-term recognition memory was observed following treatment with resveratrol-loaded lipid-core nanocapsules (65). Zaky and colleagues reported that resveratrol could reduce NF-κB activation and cytokine production and plaque formation in a rodent model administered with aluminum chloride (74). Besides, resveratrol was able to increase the levels of Aβ40 peptide, a potentially protective form of Aβ40 and rescued the expression of apurinic/apyramidic endonuclease 1/redox factor-1 (APE1/Ref-1), an important player in DNA repair and a redox regulator of transcription factors such as NF-κB and AP-1.

2.2.2 Brain injury models

Traumatic brain injuries (TBI) represent the importance of controlled and well-steered neuroinflammation intended to effectively repair CNS lesions. TBI implies glial activation, cytokine production, debris clearance, and even the recruitment of a substantial cast of blood-derived inflammatory effectors due to loss of BBB integrity (75-77). Thus, it is of prime importance to tackle the inflammatory component of TBI to limit neuronal lesions and dysfunction.

Resveratrol administered post-injury in a murine model of mild TBI, indeed displayed anti-inflammatory actions by attenuating microgliosis in the corpus callosum, cerebral cortex, and dentate gyrus (78), and elevated levels of IL-6 and IL-12 observed in the hippocampus following the injury were rescued by resveratrol administration. As for TBI, patients with spinal cord injuries (SCI) sustain ongoing inflammatory damage long after the traumatic event (75). In a rat model of acute SCI, resveratrol administered post-injury improved the extent of damages in the CNS by reducing necrosis, hemorrhage, oxidative stress, and edema, as well as by rescuing neuronal morphology and apoptosis (79). Alongside these observations, pro-inflammatory

cytokine production and MPO expression were hampered. On the other side, resveratrol also reduced levels of anti-inflammatory IL-10, suggesting an all-around buffering effect of inflammatory signaling. Remarkably, rats treated with resveratrol displayed improved locomotor activity.

Following an ischemic event, reperfusion is a key contributor to the up raise in robust neuroinflammation (76). A pre-treatment of resveratrol in rats challenged with global cerebral ischemia/reperfusion remarkably attenuated glial activation particularly in the CA1 hippocampal region (80). In parallel, NF-κB and JNK activation were hampered, leading to decreased production of COX-2 and iNOS.

Status epilepticus (SE), a state of persistent seizure and a known cause of CNS injury, may implicate neuroinflammation (77). Resveratrol's anti-inflammatory potential has been tested in a rat model of SE, particularly regarding its possible effect on mTOR (81). Indeed, resveratrol could downregulate the activity of mTOR shown to be increased in SE rats, leading to the impediment of the NF-κB pathway and reduction of the classical downstream gene targets. Similar results were obtained with the mTOR inhibitor rapamycin. Remarkably, resveratrol enhanced the levels of mTOR antagonists 5′ adenosine monophosphate-activated protein kinase (AMPK) and SIRT1. However, further experiments showed that pharmacological inhibition of AMPK but not SIRT1 could reverse resveratrol's beneficial effects, implying a role for AMPK activation in this polyphenol's mTOR-inhibitory and anti-inflammatory actions. This is in opposition to results obtained in vitro where resveratrol rather activated mTOR (38).

3. Molecular mechanisms of resveratrol action in neuroinflammation

It is undeniable that resveratrol possesses potent anti-inflammatory properties in light of the compelling in vitro and in vivo evidence that has pointed out its potential to downregulate distinct pathways implicated in inflammation, in particular the NF-κB, AMPK/SIRT1, and MAPKs axes, as well as pivotal actors such as MPO, NADPH oxidase, and HO-1, to name a few. Figure 1 presents an overview of the potential key molecular axes underlying resveratrol's anti-inflammatory benefits.

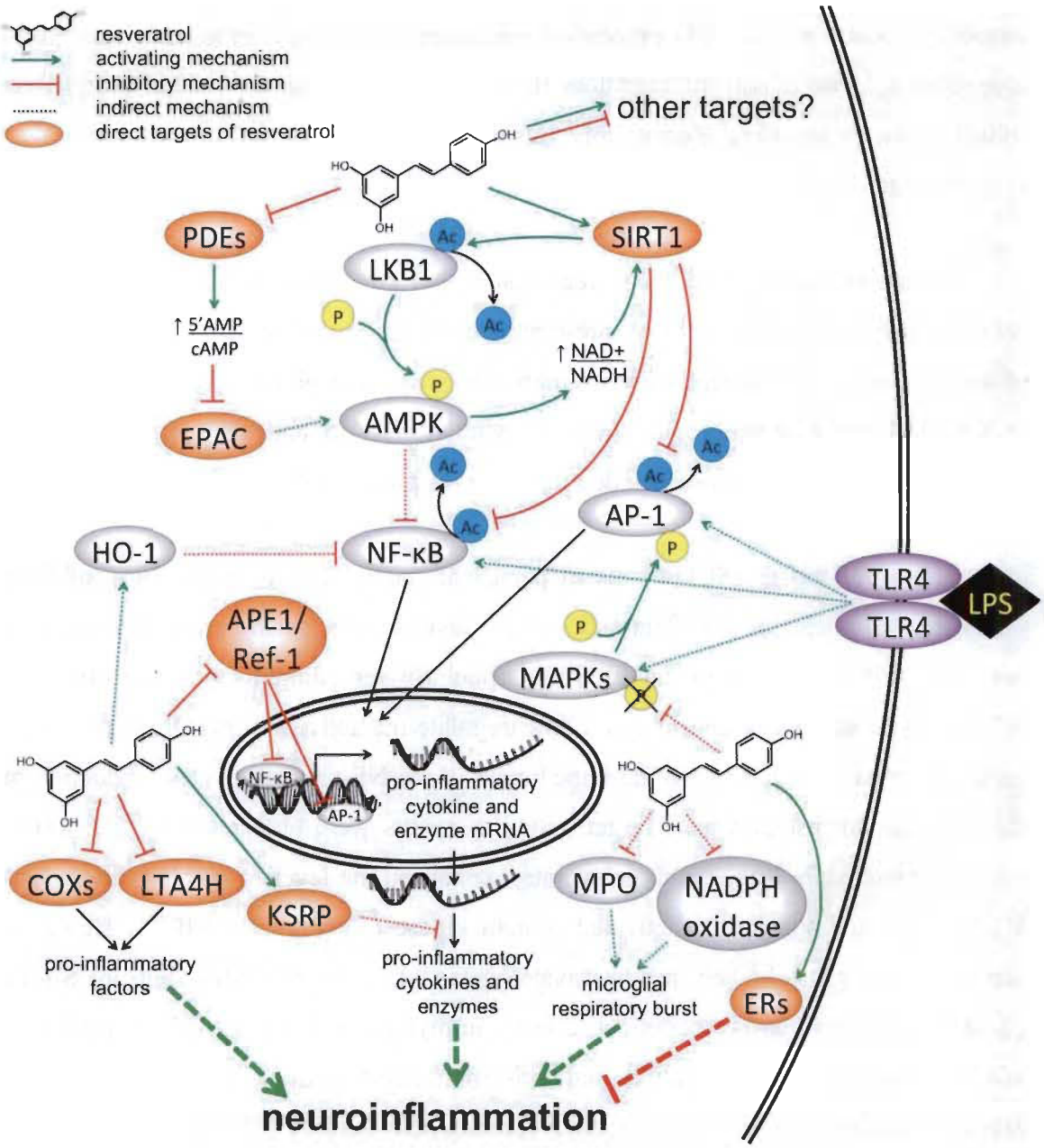

Figure 1 **Possible mechanisms by which resveratrol affords its anti-inflammatory actions, particularly in microglia activated by LPS.** Firstly, resveratrol is suggested to directly inhibit phosphodiesterases (PDEs) or to activate silent mating type information regulation (Sir) 2 homolog 1 (SIRT1). Both targets are responsible for triggering multiple intracellular cascades, some of which depicted herein, leading to the activation of adenosine monophosphate kinase (AMPK). Thereafter, AMPK is capable of inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. Similarly, SIRT1 deacetylates both NF-κB and activator protein1 (AP-1), thereby preventing their activation. Moreover, resveratrol has been demonstrated to directly activate heme oxygenase-1 (HO-1), an inhibitor of NF-κB, and to

stabilize KH-type splicing regulator protein (KSRP), a protein responsible for the degradation of pro-inflammatory mRNAs. As well, resveratrol is a known inhibitor of apurinic/apyramidic endonuclease 1/redox factor-1 (APE1/Ref-1), which potentiates NF-κB- and AP-1-mediated transcription, and of pro-inflammatory enzymes such as cyclooxygenases (COXs) and leukotriene A4 hydrolase (LTA4H), which respectively synthesize the pro-inflammatory mediators prostaglandin E2 (PGE2) and leukotriene B4. Finally, resveratrol's role in directly modulating estrogen receptors (ERs) has also been highlighted as a potential anti-inflammatory pathway. This all-inclusive schematic demonstrates a convergence in the multiple pathways activated or inhibited by resveratrol that ultimately lead to the offset of pro-inflammatory processes in microglia. EPAC, exchange factor directly activated by cyclic adenosine monophosphate 1; LKB1, liver kinase B1; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; TLR4, toll-like receptor 4.

3.1 Pro-inflammatory enzymes

COXs are convincingly some of the most probable direct targets of resveratrol's attention. Both COX-1 and COX-2 catalyze the conversion of arachidonic acid to pro-inflammatory prostaglandins. Pezzuto's group proved resveratrol's inhibitory effect on both COX isoforms, with regard to its anti-cancer potential (22), and these observations have been reinstated in recent days (82). Another inflammation-related protein, leukotriene 4A hydrolase (LTA4H), has recently been shown to be directly inhibited by resveratrol (83). LTA4H catalyzes the final step of the neutrophil chemoattractant leukotriene B4 synthesis pathway. Nonetheless, thwarting results showing LTA4H to degrade another neutrophil chemoattractant, proline glycine proline peptide (PGP), make it difficult to predict the sum anti-inflammatory effect of inhibiting this hydrolase (84,85).

3.2 Pro-inflammatory mRNAs

More recently, resveratrol has also emerged as a potent disruptor of pro-inflammatory mRNAs. Indeed, using a target-fishing method, resveratrol was found to bind KH-type splicing regulator protein (KSRP) whose main activity in the cell consists in binding inherently instable mRNAs, such as the ones encoding pro-inflammatory

proteins IL-8, iNOS, and TNF-α, and recruiting decay machinery (86). Accordingly, resveratrol was able to increase KSRP stability and to concurrently reduce the life of pro-inflammatory mRNAs. In primary cells from mice in which KSRP was inactivated, resveratrol displayed significantly lower anti-inflammatory aptitudes. Interestingly, these effects were observed independently of SIRT1 involvement.

3.3 APE1/Ref1

Resveratrol's anti-inflammatory actions could furthermore operate by inhibiting APE1/Ref-1 (74,87-89), a DNA repair protein that also comprises a redox sensitive catalytic activity, the Ref-1 domain, known to bind to pro-inflammatory factors AP-1 and NF-κB and to activate their transcriptional activity. It would appear that resveratrol not only acts through modulating the cell's redox environment, but also by directly binding pockets located in the redox domain (90). Interestingly, APE1/Ref-1 has also been linked to neurodegenerative diseases and holds a special place among the most recent emerging therapeutic targets (91,92). Conversely, previous work has rather shown that resveratrol may be responsible for increasing APE1/Ref-1 levels, though evidence of direct binding was not provided (74).

3.4 ERs

Another indirect mechanism by which resveratrol could mediate its anti-inflammatory effects in the CNS is through activation of estrogen receptors (ERs), which it is well known to directly bind (93,94). More recently, resveratrol was successfully crystalized in a complex with ERα, and was found to bind the receptor in a unique orientation that would explain why it does not behave like estrogen in several cell types (95). In fact, this "flipped" binding orientation explains why resveratrol acts as a pathway-selective ERα activator, since it perturbs the receptor's coactivator-binding surfaces. In the same published data, resveratrol was proven to require ERα to inhibit IL-6 expression.

3.5 AMPK/SIRT1

The most reputable axis renowned to be activated by resveratrol is the AMPK/SIRT1 pathway. AMPK is a metabolic power player that is thought to afford several of resveratrol's pleiotropic effects, not only pertaining to inflammation. AMPK integrates information on the intracellular energy status, thus acting as an ATP:AMP sensor. Upon ATP depletion, AMPK is activated and increases the NAD+/NADH ratio, itself leading to SIRT1 activation. SIRT1 is also in itself an indirect activator of AMPK as it deacetylates liver kinase B 1 (LKB1) that in turn phosphorylates and activates the kinase. Whether resveratrol binds and activates SIRT1 or whether it inhibits phosphodiesterases (PDEs), which eventually leading to AMPK activation, is still a debated topic (see 27 for a detailed review on this topic), as findings in different models and at different concentrations have yielded confounding results (96-103). The activation of the AMPK/SIRT1 axis may well explain resveratrol's anti-inflammatory effects through 1) deacetylation of AP-1 and NF-κB (104) and 2) NADPH oxidase inactivation by AMPK (105-107).

4. Summary and perspectives

Resveratrol remains today the center of much scientific attention in the global effort to develop therapeutic options to improve or cure neurological diseases, most of which comprise a neuroinflammatory facet. As it has been demonstrated in countless models, resveratrol possesses a very large number of possible modes of action that explain its promiscuity in exerting such a wide range of physiological effects. This chapter has reviewed in details the experimental paradigms that contribute to improve our knowledge of the possible molecular mechanisms of resveratrol action on neuroinflammation. Even if the key direct interactions between resveratrol and its possible molecular target are still under investigation (reviewed recently in 109), resveratrol has been demonstrated as a promising therapeutic strategy in ameliorating neuroinflammation and other pathological expressions with a component of inflammation.

To temper our fervor, it should be reiterated that all the promising anti-inflammatory pathways elicited by resveratrol in nerve cells and reported in this article, derive from cell culture or small animal model systems with no report on resveratrol action on human health or alternate animal models. Indeed, translation of resveratrol body of work from basic sciences to the clinics is still difficult, and the scarce data obtained in controlled studies are disappointing and controversial, especially so with respect to the human response to different doses of resveratrol (reported in 110). It appears capital to us that the next efforts of the resveratrol community should accurately study the molecular target of resveratrol as well as improve the application of pre-clinical advances in large animal models in order to proper understand the unambiguous anti-inflammatory effects, safety and efficacy of resveratrol and any more potential benefits it may have in humans.

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APPENDIX D

THE LINK BETWEEN PARKINSON'S DISEASE AND ATTENTION-DEFICIT HYPERACTIVITY DISORDER: AN ACCOUNT OF THE EVIDENCE

In this thesis, the nicotinamide-streptozotocin model of long-term hyperglycaemia was employed to demonstrate the selective vulnerability of the nigrostriatal pathway to a supplementary source of oxidative stress. In achieving this goal, we were arrested by the remarkably singular behaviours manifested by our model: not only did long-term hyperglycaemic rats display bradykinesia and gait abnormalities, they were also paradoxically hyper-reactive in contexts of social novelty.

Given the paucity of literature addressing behavioural alterations arising from sustained hyperglycaemia, we were solely supplied with our own data and clues from seemingly unrelated neuropsychiatric models on the quest to untangle this enigma. On the one hand, we knew that the dorsal striatum of our model was poorly supplied with dopamine at baseline. On the other hand, the social behaviour of our hyperglycaemic rats bore a striking likeness with that of attention-deficit hyperactivity disorder models. Seeking to sharpen our understanding of this disease, we discovered that attention-deficit hyperactivity disorder and Parkinson's disease share a common dopaminergic neuropathological element. Even more striking is the implication of the nigrostriatal pathway in both disorders. As mentioned in the discussion, patients with attention-deficit hyperactivity disorder present alterations at the level of the substantia nigra pars compacta (del Campo *et al.*, 2013; Romanos *et al.*, 2010) and of the dorsal striatum (Badgaiyan *et al.*, 2015; Sikström and Söderlund, 2007).

While these two neuropathologies appear to be quite distinct, at least by virtue of their symptomatic signatures, it is reasonable to question the possibility that they are more intimately linked than first appearances would allow. In this view, the aim of this appendix is to briefly recount the evidence supporting such an association.

1. Nigral morphology

We mentioned that the substantia nigra pars compacta is altered in patients with attention-deficit hyperactivity disorder (del Campo *et al.*, 2013; Romanos *et al.*, 2010). In fact, these abnormalities were identified with various kinds of imaging techniques more commonly employed in parkinsonian patients, such as transcranial sonography and positron emission tomography.

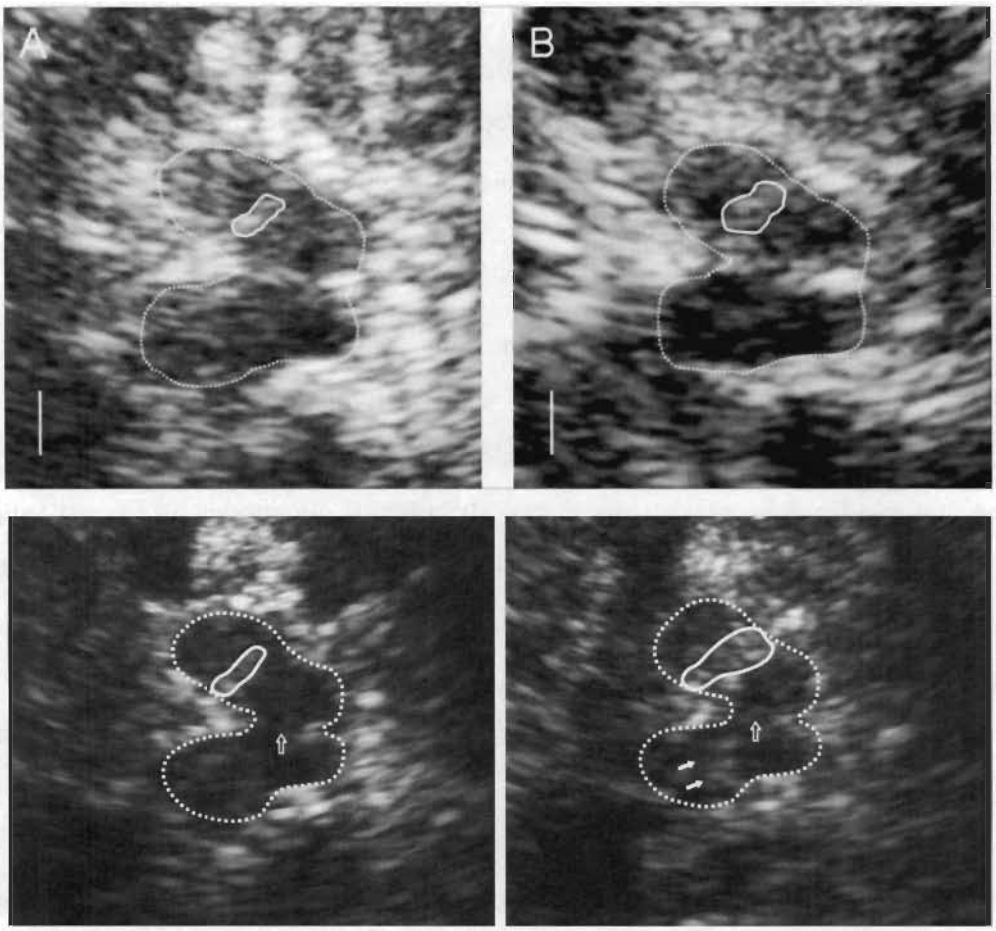

Figure 1 **Transcranial sonographies of the midbrain in patients with attention-deficit hyperactivity disorder and Parkinson's disease.**
In all transcranial sonographies, the midbrain is circled by the dotted line and the echogenic area of the substantia nigra is traced with a full white line. Top: In patients with attention-deficit hyperactivity disorder (B), the echogenic area is enlarged compared with control individuals (A). (From Romanos *et al.*, 2010.) Bottom: Similar findings of an enlarged echogenic substantia nigra pars compacta can be observed in patients with Parkinson's disease (right) in comparison with healthy individuals (left). (From Gaenslen *et al.*, 2008.)

In particular, evidence of an enhanced echogenicity was gathered in children with attention-deficit hyperactivity disorder (Romanos *et al.*, 2010), a pathological sign linked to increased iron content paired with a susceptibility for nigrostriatal dysfunction (Becker *et al.*, 1995; Berg *et al.*, 2002; Gaenslen *et al.*, 2008). Remarkably, the same pattern of echogenicity can be observed in parkinsonian patients (Figure 1). These important findings launched investigations aimed at uncovering the role of oxidative stress, perhaps linked to nigral iron contents, in this neuropsychiatric condition (Hess *et al.*, 2017). It is unclear how iron contents are linked to neuropathogenicity in both these diseases.

2. Animal models

Looking into animal models also provides some indications of a possible link between these pathologies. Quite telling is the existence of a longstanding model of attention-deficit hyperactivity disorder, used for some 40 years now, that employs 6-OHDA. In neonate rats, this parkinsonian toxin destroys the near totality of nigrostriatal neurons, sparing the ventral tegmental area, and causes a compensatory serotoninergic hyperinnervation of the striatum that is thought to underlie behavioural aberrations (Breese *et al.*, 2005; Kostrzewa *et al.*, 2004, 2006). The exact underpinnings of the hyper-reactive phenotype remains ill understood. Noteworthy, total depletion of dorsostriatal dopamine is likely not physiologically relevant in patients with attention-deficit hyperactivity disorder. The hypotonic and hyperphasic aspects thought to underlie this illness are therefore not represented therein (Badgaiyan *et al.*, 2015; Sikström and Söderlund, 2007).

More recently, in an attempt to model Parkinson's disease, one group uncovered a hyper-reactive phenotype resulting from subeffective treatments. Indeed, primates treated with varying chronic doses of the parkinsonian toxin MPTP displayed early premotor social alterations, including enhanced aggressive and affiliative behaviours, highly correlated with dorsostriatal denervation. In the later phase of the pathology wherein all evaluated dopamine-related cortical and subcortical structures were

fully degenerated, these hyper-reactive behaviours were drastically decreased and conspicuous motor symptoms emerged. This study actually sheds light on a possible continuum of nigrostriatal dopaminergic manifestations, ranging from hyper-reactivity at low levels of neurodegeneration to apathy and motor impairments at high degrees of denervation (Durand *et al.*, 2015). In relation to our own findings, we reiterate the possibility that long-term hyperglycaemic rats examined between 3 and 6 months display mixed moderate behaviours representative of both ends of the continuum. It is tempting to propose that some forms of attention-deficit hyperactivity disorder and Parkinson's disease may overlap.

3. Epidemiological evidence

Last, we can address existing epidemiological data suggesting a link between attention-deficit hyperactivity disorder and Parkinson's disease or related pathologies. Indeed, a growing body of literature has addressed the relationship between early-life attention-deficit hyperactivity disorder or its symptoms and late-life parkinsonism (Golimstok *et al.*, 2011; Kim *et al.*, 2013; Walitza *et al.*, 2007). Interestingly, Parkinson's disease and attention-deficit hyperactivity disorder may share certain genetic underpinnings. Specifically, *PARK2*, a causative gene in Parkinson's disease encoding parkin, was found to be associated with attention-deficit hyperactivity disorder (Jarick *et al.*, 2014). For some researchers, this invites the interrogation of whether both diseases are in fact the early- and late-life continuum of a single dopaminergic pathology (Golimstok *et al.*, 2011). We wish to stress, however, that in this setting it is unclear whether treatments addressing attention-deficit hyperactivity disorder, like methylphenidate, may in fact contribute to overt nigrostriatal neurodegeneration found in Parkinson's disease (Sadasivan *et al.*, 2012).

Although any conclusions based on this scanty yet evocative evidence would be deemed premature, the relationship between attention-deficit hyperactivity disorder and Parkinson's disease does merit to be addressed by serious investigations aiming to expose any possible overlapping neurobiological mechanisms. It remains that no study

has appropriately accounted for the history of hyperactivity, aggression or impulsivity in large cohorts of parkinsonian patients.

These findings do, however, provide novel insight into often overlooked non-motor behavioural alterations that may arise from nigrostriatal dopaminergic neurodegeneration. Indeed, the nigrostriatal pathway is just leaving the shadow of the mesocorticolimbic circuit as a regulator of social interactions (Burke *et al.*, 2010; King-Casas *et al.*, 2005; Lamichhane *et al.*, 2014; Ong *et al.*, 2011; Palmeri *et al.*, 2017; Plavén-Sigray *et al.*, 2014; Stoeckel *et al.*, 2014). Impaired social aptitudes recognized to dim the quality of life of parkinsonian patients – even besides the well-appreciated impulse control disorders that ensue from dopaminergic treatments (Cilia, 2012) – are gaining attention in the scientific community (Schrag *et al.*, 2000; Yoshimura *et al.*, 2005; Pell *et al.*, 2006). Granted this era of neuroscientists is shining a powerful limelight on non-motor symptoms in Parkinson's disease, there is hope that these interrogations will soon be addressed.

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