Sara Al-Sweidi

MÉCANISMES D'ACTION IMPLIQUANT LES RÉCEPTEURS OESTROGÉNIQUES DANS LA NEUROPROTECTION CHEZ LA SOURIS MPTP

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Résumé

Des études cliniques concluent que les oestrogènes induisent des effets modulateurs dans le cerveau et des études expérimentales faites par notre laboratoire démontrent que le 17β-oestradiol a des effets bénéfiques dans des modèles animaux du Parkinson. La recherche est en cours pour trouver des nouveaux composés oestrogéniques comme agents neuroprotecteurs au cerveau pour prévenir ou combattre la maladie de Parkinson. Une perte de la dopamine (DA) striatale observée suite a une lésion avec la neurotoxine 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP) chez la souris, le 17β-oestradiol prévient cette perte et cet effet neuroprotecteur peut impliquer les récepteurs oestrogéniques alpha et beta (ERα et ERβ). Les mécanismes impliqués devraient être davantage étudiés et font l'objet de cette thèse.

Les résultats de cette recherche montrent que l'absence des ERs induit des changements dans les niveaux des stéroïdes sanguins, des transporteurs dopaminergiques et par conséquence la susceptibilité des neurones au MPTP. La réponse au traitement avec le 17β-oestradiol, montre une spécificité dans diverses régions du cerveau.

Les souris invalidées pour le ERα (ERKOα) avaient des niveaux élevés d'androgènes et le MPTP cause une diminution de la tyrosine hydroxylase chez les deux groupes ERKOs, les souris ERKOα sont plus sensibles au MPTP que les souris invalidées pour le ERβ (ERKOβ) comparativement aux souris sauvages. Donc, un rôle important des ERα et plus modéré des ERβ est observé au cerveau des souris mâles suite à une lésion au MPTP. Ceci se manifeste sur les concentrations de la DA et aussi sur les transporteurs (DAT et VMAT2) comme marqueurs de l'intégrité des terminaisons dopaminergiques nigro-striatales pré-synaptiques. De plus, les ERs induisent des effets neuroprotecteurs dans le cortex, le striatum et l'hippocampe en modulant les niveaux des récepteurs glutamatergiques NMDA NR1/NR2B et les récepteurs dopaminergiques D1 et D2. Par conséquent, une répercussion se manifeste au niveau post-synaptique dans les voies de signalisation PI3K/Akt et MAPK/ERK où seulement les souris ERKOβ avaient une augmentation de la signalisation Akt/GSK3β, ERK1/ERK2 avec la lésion au MPTP. Ces résultats démontrent que ces deux récepteurs oestrogéniques sont impliqués dans l'effet neuroprotecteur des oestrogènes.

Summary

Clinical studies conclude that estrogens have modulatory effects in the brain and experimental studies done in our laboratory show beneficial actions of 17β-estradiol against MPTP toxicity. We still pursue the concept of using estrogens as neuroprotective agents in order to prevent or fight the neurodegenerative aspects of PD.

Estrogen receptor α (ERα) plays an important neuroprotective role, whereas ERβ produces subtler effects against striatal MPTP toxicity, therefore both ERα and ERβ modulate the degree of vulnerability of dopaminergic neurons to MPTP toxicity through different mechanisms still to be described.

Results show that both ERs are active in the mouse cortex, striatum, and hippocampus; NMDA receptors were affected by the deletion of ERs which in turn affects the response to MPTP and 17β-estradiol treatments with brain region specificity. Other results conclude that ERs affect the response of striatal DA receptors to a MPTP lesion and post-receptor Akt/GSK3β and ERK1/ERK2 signaling.

Foreword

Happiness comes when your work and words are of benefit to yourself and others

- Siddharthe Gautama

I am happy to have finished this thesis and would first like to thank my research director, Dr. Thérèse Di Paolo, for giving me the opportunity to continue my education and providing guidance throughout my research project.

Dr. Marc Morissette and his family deserve my humble gratitude. Technically speaking however, his teachings, patience and great sense of humor and father-like figure proved to be imperative while working in the laboratory.

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List of abbreviations

AARDC: aromatic amino acid decarboxylase

Ach: acetylcholine

Akt: protein kinase B

ALDH: aldehyde-dehydrogenase

AMPA: 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid

AP-1: activator protein-1

BG: basal ganglia

BDNF: brain-derived neurotrophic factor

cDNA: complementary DNA

CNS, central nervous system

COMT: catechol-O-methyltransferase

CREB: cAMP-response element binding protein

DA: dopamine

DARP 32: DA and cAMP-regulated phosphoprotein, 32

DAT: membrane dopaminergic transporter

DHEA: dehydroepiandrosterone

DHT: dihydrotestosterone

3β-diol: 5α-androstan 3-β, 17β-diol

DOPAC: 3, 4-dihydroxyphenylacetic acid

DPN: 2,3-bis(4-hyroxyphenyl) propionitrile

17β-E_2: 17β-estradiol

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

ERE: estrogen-response element

ERK: extracellular-signal-regulated kinase

ERKO: estrogen receptor knock out

E1: estrone

E3: estriol

GABA: γ-aminobutyric acid

GAPDH: glyceraldehyde 3-phospate dehydrogenase

GLU: glutamate

GPe: globus pallidus pars externa

GPi: globus pallidus pars interna

GSK3β: glycogen synthase kinase β

HPLC: high performance liquid chromatography

3β-HSD: 3β-hydroxysteroid dehydrogenase

17β-HSD: 17β-hydroxysteroid dehydrogenase

HVA: homovanilic acid

KA: kainic acid

LB: Lewy body

L-DOPA: L-dihydroxyphenylalanine

LID: L-dopa induced dyskinesia

M1: The primary motor cortex

MAOB: monoamine oxidase B

MAPK: mitogen-activated protein kinase

MPTP: 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine

MPP^+: 1-methyl-4-phenylpyridinium

3-MT: 3-methoxytryptamine

mRNA: messenger ribonucleic acid

NMDA: *N*-methyl-D-aspartate

NF κB: nuclear factor κB

6-OHDA: 6-hydroxydopamine

PD: Parkinson's disease

PKA: protein kinase A

PKC: protein kinase C

P160: 160-kd steroid-receptor coactivation protein

POL II: RNA polymerase II

P450c17: cytochrome P450 17α-hydroxysteroid/C17 20-lyase

PINK1: PTEN-induced kinase 1

PP-1: protein phosphastase-1

PPT: 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole

P450scc: cytochrome P450 side chain cleavage

5α-R: 5α–reductase

ROS: reactive oxygen species

[^{125}I]-RTI-121: 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid

S1: pimary somatosensory area (S1)

SERM: selective estrogen receptor modulator

SN: substantia nigra

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticulata

SP1: specificity protein 1

STN: subthalamic nucleus

TBP: TATA-box-binding protein

[^{3}H]-TBZ-OH: [^{3}H]-dihydrotetrabenazine

TH: tyrosine hydroxylase

UCHL1: ubiquitin carboxyl-terminal hydrolase-1

VMAT2: vesicular monoamine transporter type 2

VTA: ventral tegmental area

WT: wild-type

CHAPTER I

Introduction

I. The Basal Ganglia

In 1664, Thomas Willis anatomically identified distinct subcortical brain structures and the work of Cécile and Oskar Vogt provided a clear description of the brain region grouped and termed as the basal ganglia (BG) in 1941 (Percheron et al., 1994). The BG are located inside the forebrain and are made up of five interconnected brain structures which include the caudate, the putamen, the globus pallidus (GP), the substantia nigra (SN) and the subthalamic nucleus (STN) (Blandini et al., 2000). The first two regions together make up the striatum, the globus pallidus has internal and external parts (GPi and GPe) and the BG includes both the SN *pars reticulata* and *pars compacta* (SNr and SNc). Signaling in the BG involves dopamine (DA) as a main neurotransmitter, it is responsible for the exertion of tonic (homeostatic) and phasic actions (Saint-Cyr, 2005; Schultz, 2002).

The BG and the cerebellum send outputs through the thalamus to cortical motor systems. Cortical motor areas differ in their intrinsic composition as well as their interconnections with the rest of the central nervous system (CNS) (Blandini et al., 2000). The primary motor cortex (M1) receives input from the pimary somatosensory area (S1). The M1 neurons directly control movement kinetics and dynamics (Blandini et al., 2000; Shughrue and Merchenthaler, 2000; Vuckovic et al., 2008). The striatum and STN receive major inputs from outside the BG, which come from the cerebral cortex and the thalamic nuclei (Blandini et al., 2000) (Figure 1).

The striatum, also known as the neostriatum, is a subcortical structure composed of a heterogenous array of neurons, where 96% is occupied by medium spiny projection neurons, which are composed of large dendritic trees spanning 200-500 μm, project to the GP and SN and provoke inhibitory effects by neurotransmitting with γ-aminobutyric acid (GABA) (Blandini et al., 2000; Graveland et al., 1985; Shughrue et al., 1997b). Only 2% of the striatum is arborized by Deiter's neurons, while the rest is home to cholinergic interneurons and GABAergic interneurons

expressing either calretinin, parvalbumin or somatostatin (Iversen et al., 2010). In order to draw a clearer picture, striatal spiny neurons could contain an estimate of 300 synapses (Kawaguchi et al., 1990; Wu et al., 2000), while striatal interneurons could have about 500 synapses (Koos and Tepper, 1999; Tepper and Bolam, 2004).

Moreover, the striatum is spatially divided into dorsal and ventral sectors, where the first contains the caudate-putamen complex and is implicated in the integration of sensorimotor and cognitive information (Iversen et al., 2010). In rodents the caudate and putamen form a single striatal structure, having fibers of the internal capsule passing though, while in primates and humans they are divided by the internal capsule (Blandini et al., 2000; Shughrue et al., 1997b). The ventral striatum contains the nucleus accumbens and the olfactory tubercule and is interconnected to limbic structures like the amygdala, the hippocampus and the thalamus (Parent and Hazrati, 1995). The striatum is highly innervated by the nigrostriatal and mesolimbic systems which are composed of dopaminergic neurons respectively projecting from the SNc and ventral tegmental area (VTA) (Parent and Hazrati, 1995). However, SN neurons contain neuromelanin, which gives them their distinctive pigmentation, these are medium to large sized neurons projecting to the cortex and BG; there is a surprisingly small number of DA neurons in the SNc, which is estimated at 7000-8000 neurons (Nair-Roberts et al., 2008; Oorschot, 1996). A total of 12,000 DA neurons are found in the rat SN, along with another 20,000 in the VTA (Nair-Roberts et al., 2008). In addition, nigrostriatal afferents arising from the SNc synapse on the spine shafts of striatal medium spiny neurons, while other afferents include those of the STN which are glutamatergic and those of the GP which are GABAergic (Rosell and Gimenez-Amaya, 1999). Further, corticostriatal afferent connections are the most numerous, where cortical pyramidal neurons projecting to the striatum arise from cortical layers II-VI, the most dense neuronal fibers

belong to layer V and end on the spines of striatal medium spiny neurons (Rosell and Gimenez-Amaya, 1999).

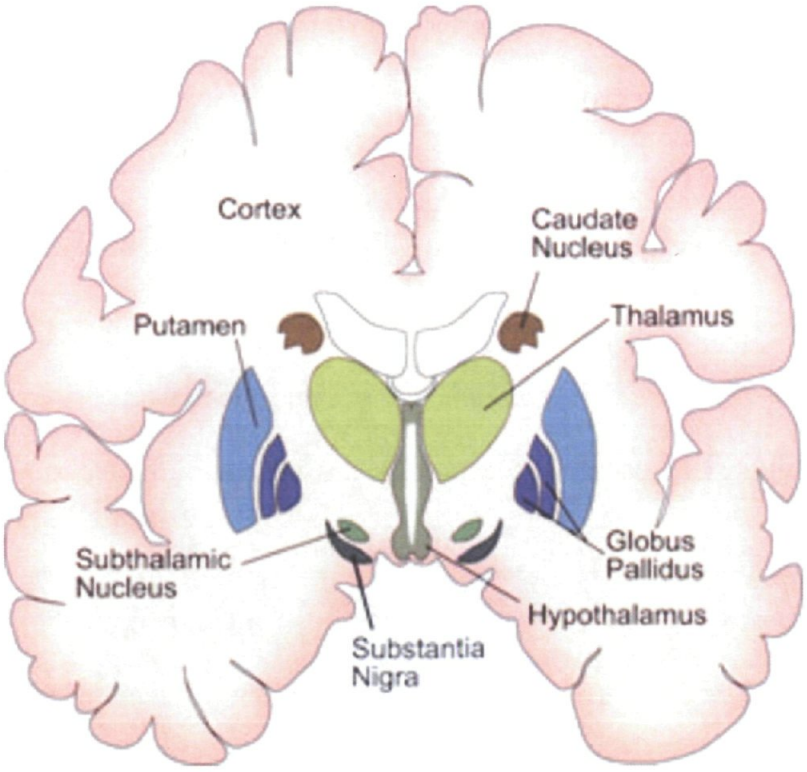

Figure 1. The basal ganglia (BG) are made up of five interconnected brain structures which include the caudate, the putamen, the globus pallidus, the substantia nigra and the subthalamic nucleus. The first two regions together make up the striatum, the globus pallidus has internal and external parts (GPi and GPe) and the BG include both the substantia nigra *pars reticulata* and the substantia nigra *pars compacta* (SNr and SNc), Adapted from (Sukel, 2007).

II. Dopamine

2. 1. *DA synthesis and function*

There are two types of neurotransmitters, the first being slow acting through cascades of biochemical reactions and those which induce their effects rapidly. Fast-acting neurotransmitters are implicated in neuromodulation and long-term regulation, altering target neuron responses to other transmitters (Sidhu et al., 2004).

To merit the 2000 Nobel Prize in physiology of medicine, Arvid Carlsson demonstrated that DA ($C_6H_3(OH)_2$-CH_2-CH_2-NH_2) was a neurotransmitter as well as the precursor of noradrenalin (norepinephrine) and adrenalin (epinephrine) (Carlsson et al., 1957). DA is a naturally produced phenethylamine, a member of the catecholamine family, has emotional cognitive and motor functions (Fauchey et al., 2000; Le Moal and Simon, 1991).

The four major DA pathways in the brain include the nigrostriatal, the mesocortical, the mesolimbic and the tuberoinfundibular pathways (Schultz, 2002). They are respectively responsible for mediating movement, emotions, desire and reward and sensory processing (Schultz, 2002). This neurotransmitter is biosynthesized in the cytosol of nerve terminals. Phenylalanine and tyrosine are DA precursors which can cross the blood-brain barrier (Bohlen et al., 2002) and the first step is the conversion of tyrosine into L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH) which is found in catecholamine-containing neurons (Bohlen et al., 2002). L-DOPA is then decarboxylated to form DA by the aromatic amino acid decarboxylase (AARDC) enzyme (Bohlen et al., 2002).

DA can be degraded into 3,4-dihydroxyphenylacetic acid (DOPAC) by either a monoamine oxidase (MAO) or the aldehyde-dehydrogenase (ALDH), while 3-methoxytyramine (3-MT) results from DA metabolism by catechol-o-methyltransferase (COMT). These two DA

metabolites are then metabolized to form homovanilic acid (HVA) (Bohlen et al., 2002) (Figure 2).

2. 2. *DA receptors*

DA receptors are associated with guanosine triphosphate binding proteins (G-proteins) in order to regulate their actions (Missale et al., 1998). They are divided into two families, the first, termed the D1-like DA receptors includes the D1 and D5 isoforms, while the other siblings (D2, D3 and D4) are called D2-like receptors (Missale et al., 1998).

D1-like and D2-like DA receptors are found all over the brain having an order of abundance as follows: D1>D2>D3>D5>D4 (Missale et al., 1998) and the striatum is known as the brain region with the highest density of D1 and D2 receptors (Zhou et al., 2002). Striatal D1 receptors are localized on post synaptic GABAergic spiny projection neurons, whereas D2 receptors are expressed both presynaptically on nigrostriatal terminals and postsynaptically on GABAergic spiny projection neurons (Roth, 1979) (Figure 2). D2 receptors are also presynaptically expressed on glutamate neurons of corticostriatal neurons (Iversen et al., 2010).

DA receptors were first characterized as modulators of cyclic adenosine monophosphate (cAMP) production (Greengard, 2001). DA receptors use G-protein-mediated signaling to control Ca^{2+} and c-AMP-PKA (protein kinase A) pathways (Mani, 2003). D1 receptors increase cAMP levels and promote the activation of cAMP dependant protein kinases which transfer phosphate molecules from adenosine triphospate to specific substrates (Missale et al., 1998). By contrast, D2 receptors decrease cAMP levels by modulating potassium channels permeability (Missale et al., 1998).

Accordingly, DA receptors regulate fast neurotransmission induced by GABA and glutamate (Greengard, 2001), here the class of D1 receptors are mainly coupled to Gαs, while the

D2 family of dopaminergic receptors are joined to Gαi/o (Missale et al., 1998) and are believed to induce effects using signaling involving protein kinase B (PKB also known as Akt) and glycogen synthase 3β (GSK3β).

Moreover, the extracellular signal-regulated kinase (ERK) is another protein which is greatly implicated in these pathways as a mediator of c-AMP signaling (Beaulieu et al., 2007). Finally, DA increases specific transcription factor phosphorylation leading to an increase in specific gene expression of immediate early genes which in turn activate late genes believed to be very important in modifying synaptic transmission (Girault and Greengard, 2004).

2. 3. *DA transporters*

There are two families of transporters responsible for controlling extracellular DA concentrations; these are the DA transporter (DAT) and the vesicular monoamine transporter 1 and 2 (VMAT) (Lotharius and Brundin, 2002; Wersinger et al., 2003).

The striatum has dense and heterogeneous DAT distribution, the transporter is found on plasma membranes and axon terminals (Sidhu et al., 2004). In fact, immunocytochemistry confirm that DAT is colocalized with tyrosine hydroxylase and the D2 DA receptor (Ciliax et al., 1999).

DAT allows the uptake of DA into the cytoplasm from the extracellular space (Piccini, 2003), while VMAT2 is responsible for storing DA in synaptic vesicles and the reduction of both their levels in the nigrostriatal system is seen in animal models of Parkinson's disease (PD) and in PD patients (Frey et al., 1996) (Figure 2). The actions of these transporters are regulated by presynaptic receptors and protein kinases. Again, DA metabolism includes its extracellular release and once outside DA can interact with target receptors and then be rapidly taken up by DAT and degraded by MAOB (Sidhu et al., 2004). Thus, making the amount of free DA dependant on DAT levels of the plasma membrane and VMAT2's presence on synaptic vesicles.

DA gets across the membrane through DAT using a mechanism where the neurotransmitter's movement is coupled to that of sodium ions moving down their concentration gradient (Sidhu et al., 2004). Here, sodium first binds the transporter's extracellular domain, then DA binds and induces a conformational change which releases both sodium and DA into the cell (Sonders et al., 1997). However, with DAT present on the plasma membrane, DA enters terminals in uncontrolled massive quantities and forms oxidized DA and reactive oxygen species (ROS) which in the end damage the cell (Sidhu et al., 2004). A study reports that DAT could be dephosphorylated by protein phosphastase-1 (PP-1) which is know for its implication in synaptic plasticity, long-term depression and signaling (Cohen, 1989). The resultant effects on DAT phosphorylation still lack a clear understanding but it is theorized that they are implicated in down-regulating the transporter's transportation and internalization (Foster et al., 2003). Moreover, Protein kinase C (PKC) and the mitogen activated kinase pathway (MAPK) have regulatory effects on the transporter's DA movement rate and its internalization (Pristupa et al., 1998).

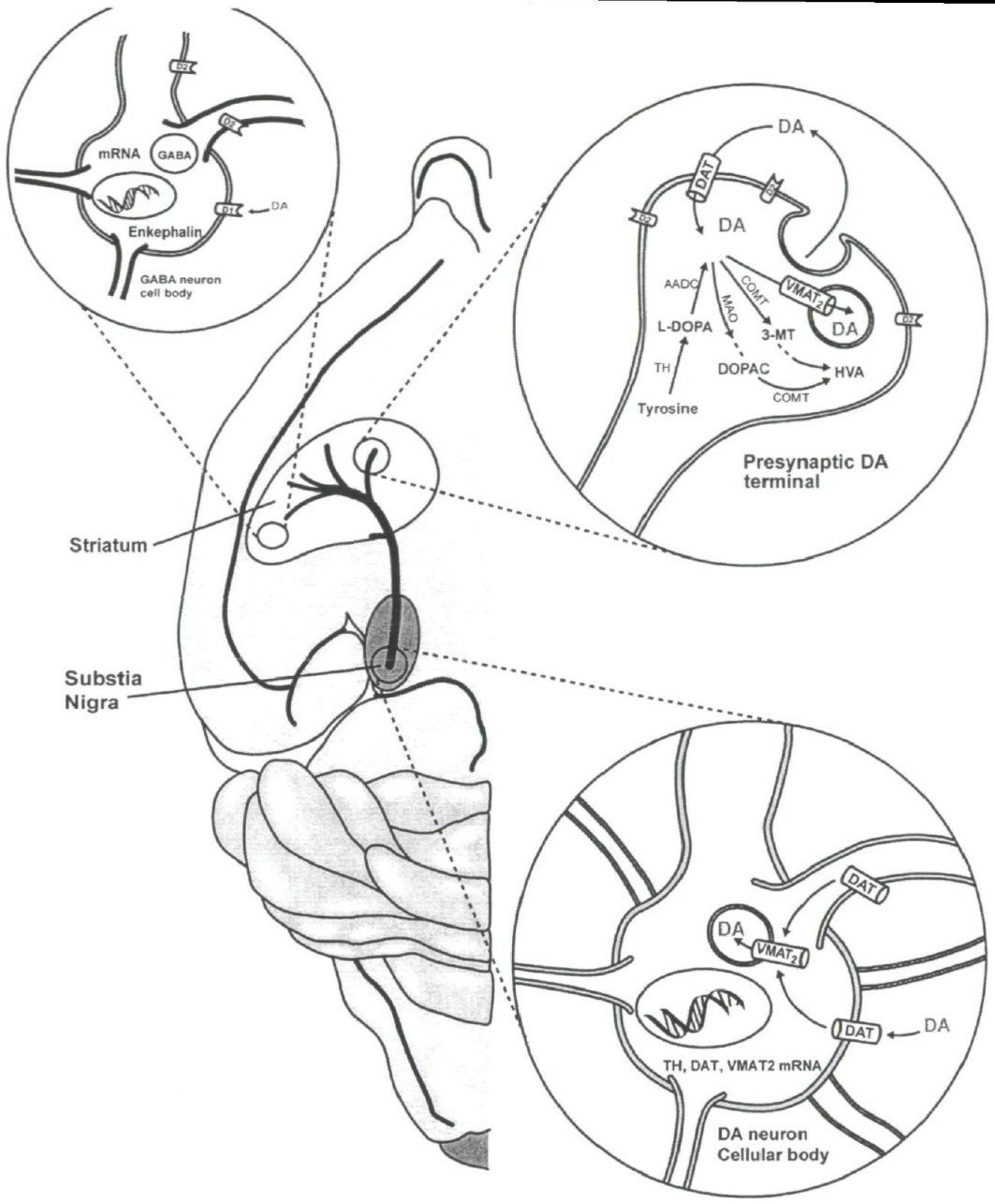

Figure 2. The nigrostriatal DA system. First, tyrosine is converted into L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH) which is found in catecholamine-containing neurons. L-DOPA is then decarboxylated to form DA by the aromatic amino acid decarboxylase (AARDC) enzyme. DA is degraded into 3, 4-dihydroxyphenylacetic acid (DOPAC) by either a monoamine oxidase (MAO) or the aldehyde-dehydrogenase (ALDH), while 3-methoxytyramine (3-MT) results from DA metabolism by catechol-o-methyltransferase (COMT). These two DA metabolites are then metabolized to form homovanilic acid (HVA). The DA transporter (DAT) and the monoamine vesicular DA transporter 2 (VMAT2) are responsible for controlling extracellular DA concentrations. The striatum contains both D1 and D2 DA receptors. Adapted from Bourque et al. (Bourque et al., 2009).

III. Parkinson's Disease

3. 1. *Parkinson's disease, Definition and Symptoms*

Parkinson's disease (PD) is a neurodegenerative disorder which has become more prevalent over the years (Blandini et al., 2000); it is categorized in a group of conditions called movement disorders (Blandini et al., 2000). This chronic disease has no etiology, however the loss of DA-containing neurons in the SN is a main feature of its neurodegenerative process (Blandini et al., 2000). Symptoms include rigidity, bradykinasia, postural instability and tremors at rest. This is a progressive neurological disease having some cases of early onset but for the majority it lies at about the age of 55 along with an exponentially increasing prevalence between 1 and 5% at around 65. The symptoms pf PD were first described in 1817 by a British physician, James Parkinson. In the 1960s, Hornykiewicz deduced that striatal DA depletion defines the disorder (Hornykiewicz, 2002a), leading to the simple interpretation that Parkinson's diseased BG will malfunction by sending wrong signals to recipient lobes and cause locomotor, cognitive and emotional impairment. Thus, what makes PD so infamous is the fact that symptoms appear after a myriad of neurons are lost, with a death toll of dopaminergic neurons going over a critical threshold of 60% in the SNc perikarya and striatal DA levels are depleted by 80% (Bassilana et al., 2005; Bezard et al., 2003; Castaneda et al., 1990) (Figure 3). It is hard to diagnose it before the damage becomes irreversible due to compensatory mechanisms (Saint-Cyr, 2005). Cellular death by apoptosis is thought to take place rather than by necrosis and autophagic cell death due to the characteristic slow and progressive neurodegeneration, appearance of fragmented nuclei and the activation of caspases in PD patients (Tatton, 2000).

The disorder is also referred to as "idiopathic" PD since no known cause is documented, yet some cases may be due to toxicity, drugs, genetic mutation, head trauma, or other medical disorders (Wooten et al., 2004). PD is also characterized as 'sporadic' with no genetic

background in 90-95% of cases and environmental factors or genetic susceptibilities are believed to have a hand in triggering the disease (Braak et al., 2006b).

A hundred years ago Leoux and Gowers each documented that a good percentage of patients affected with PD had relatives suffering from the disease (Rosner et al., 2008). Research on familial forms of PD finds that genetics have an even more prevalent role in the disease. Autosomal dominant or recessive PD are linked to mutations on eight genes and twelve loci have been identified to date. Mutations on nuclear genes encoding α-synuclein, DJ-1, parkin, PTEN-induced kinase 1 (PINK1) and ubiquintin carboxyl-terminal hydrolase-1 (UCHL1) are thought to promote proteasome and mitochondrial malfunction, oxidative stress and synaptic dysfunction (Rochet et al.).

In addition, Lewy bodies (LBs) are seen in many neurodegenerative diseases, being eosinophilic intraneuronal inclusions and a main characteristic of PD, several studies confirmed that antibodies recognize and label their ubiquitinated neurofilaments (Brion and Couck, 1995; Hill et al., 1991) therefore PD is also referred to as a synucleinopathy (Figure 3).

3. 2. *The brain and regions affected by PD*

The neuroanatomical organization of the BG is nomenclaturely divided into three structures: the first is called the neostriatum and comprises the caudate, the putamen and the nucleus accumbens (Saint-Cyr, 2005). Second, the paleostriatum groups the globus pallidus and the substantia nigra, while the amygdala belongs to the third structural compartment called the archistriatum (Saint-Cyr, 2005). The structural domains of the BG have either locomotor, cognitive or emotional (limbic) functions. Domains responsible for locomotor functioning have neuronal circuitry contouring the dorsomedial section of the caudate and neighboring rostral putamen (Parent and Hazrati, 1995). Neurons implicated in emotional functioning lie in the core

of the ventral striatum which groups the nucleus accumbens, the ventral caudate and the amygdala (Saint-Cyr, 2005). Neuronal wiring having cognitive functions mainly runs through parts of the caudate and ventral putamen (de Olmos and Heimer, 1999).

Again, signaling in the BG involves DA as a main neurotransmitter, it is responsible for the exertion of tonic (homeostatic) and phasic actions (Saint-Cyr, 2005; Schultz, 2002) and striatal medium spiny neurons (Graveland et al., 1985) provoke inhibitory effects with GABA (Graveland et al., 1985). Here, glutamate promotes the activation of GABAergic medium spiny neurons and nigrostriatally released DA modulates striatal glutamatergic input (Starr, 1995; Yung et al., 1995). DA neurons project from the SNc and innervate the neostriatum's medium spiny neurons which connect to the SNr and the GPi and this route completes the so called direct pathway (Cardoso et al., 2005). While, the indirect pathway also begins at the SNc but striatal medium spiny neurons project to the GPe before reaching the SNr and GPi (Cardoso et al., 2005). PD spreads by disrupting the harmonic signaling of these pathways (Cardoso et al., 2005) (Figure 3). The medium spiny neurons forming these direct and indirect pathways are approximately equal in number and are separated into two morphologically indistinguishable populations based on the type of neurotransmitter and DA receptor they contain (Smith et al., 1994). The first express D1 DA receptors, use GABA, dynorphin and sustance P (SP), while the second express D2 DA receptors and contain GABA and enkephalin (ENK) (Descarries et al., 1996) and receive input from the cortex (Hersch et al., 1995) (Figure 4).

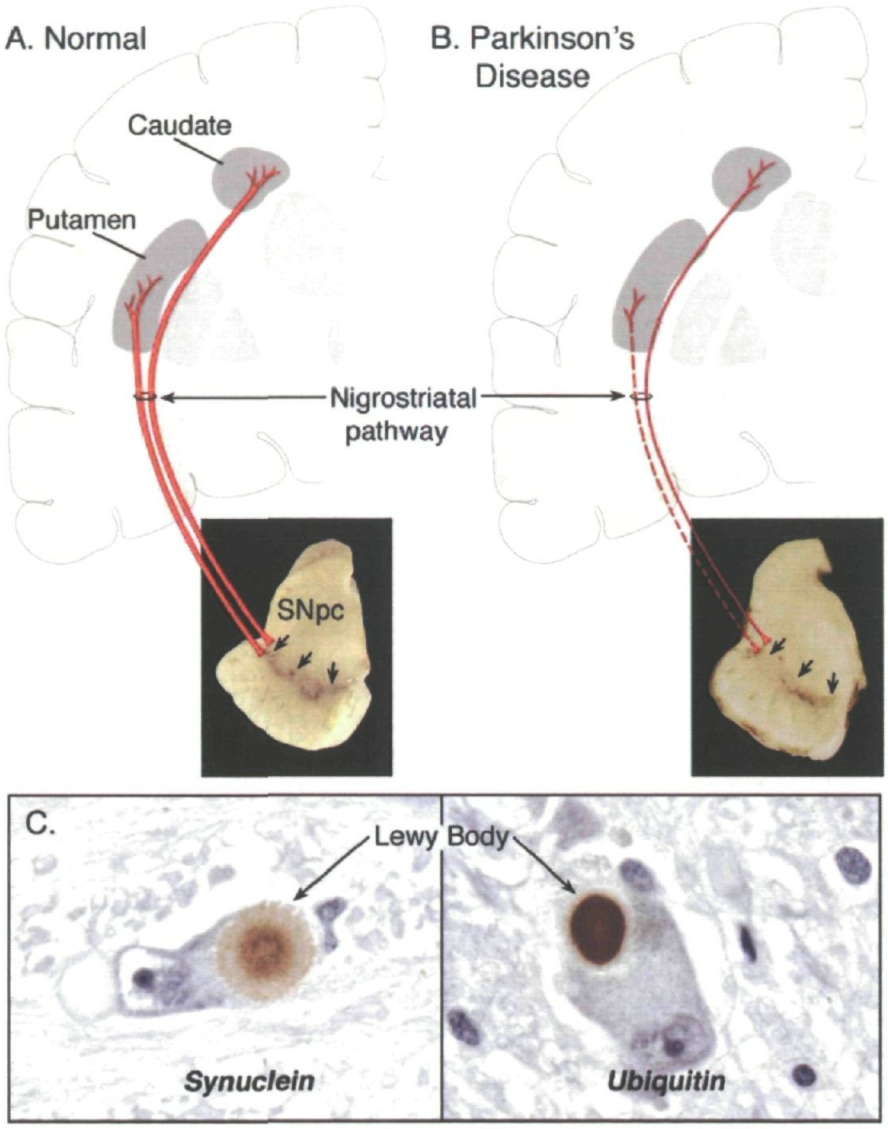

Figure 3. Parkinson's disease (PD) is characterized by the loss of SN neurons which project into the striatum (caudate and putamen). This leads to striatal DA depletion, which results in malfunctioning BG that will send wrong signals to the recipient lobes and cause the locomotor impairement seen as symptoms of the disorder. (A) Normal and (B) diminished nigrostriatal functioning of PD diseased BG. (C) The presence of Lewy bodies in a PD affected brain. Adapted from Dauer et al. 2003 (Dauer and Przedborski, 2003).

3. 3. *The spreading of PD*

Again, sporadic PD is characterized as a multisystem disorder affecting predisposed neuronal cell types of the entire nervous system (Braak and Del Tredici, 2010). Braak et al. conducted detailed analyses of regional patterns of abnormal immunostainning of α-synuclein in the brain (Braak et al., 2006a). Proposed is that the primary stages of PD involve non-dopaminergic structures (Braak and Del Tredici, 2010). The Braak theory depicts a staging procedure for the brain, were the process involves the formation of intraneuronal LBs, beginning at two sites and spreading topographically in six predictable stages involving the olfactory, autonomic, limbic and somatomotor systems (Braak and Del Tredici, 2010).

In the first two stages LBs are observed in anterior olfactory structures and the medulla oblongata/pontine tegmentum. Stages 3-4 are where the SN, the nuclei of the basal mid- and forebrain, as well as the mesocortex are attained, allowing the disorder to clinically manifest (Braak and Del Tredici, 2010). Finally, in the last stages (5-6), lesions are observed in the neocortex along with severe cognitive problems (Emre et al., 2007).

3. 4. *The basal ganglia and PD*

The havok caused by PD eventually leads to changes in acetylcholinergic, GABAergic, glutamatergic, norepinephrinergic and serotoninergic systems, since once SNc dopaminergic neurons are gone the down stream striatum's DA reservoir becomes depleted and result in the hypoactivity of the D1 DA receptors and the hyperactivity of D2 DA receptors expressed on a subset of striatal projecting GABAergic neurons (Cardoso et al., 2005). When the balance is broken the overworking D2 receptors bring on an increased inhibition of the GPe causing an increased activation of the STN. This overly disinhibited STN continues the chain reaction by allowing both the GPi and the SNr to increase inhibition on the cortex through the thalamus

(Cardoso et al., 2005). The end result is the apparition of parkinsonian symptoms (Lang and Obeso, 2004) and it is interesting to note that STN hyperactivity is one of the first regions to malfunction (Cardoso et al., 2005).

STN activity mainly implicates glutamate which is an excitatory neurontransmitter having affinities on ionotropic, like *N*-methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) or kainic acid (KA), and metabotropic receptors, found on nigral dopaminergic neurons (Cardoso et al., 2005). Excitotoxicity could be caused by allowing extracellular calcium influx through over activated NMDA receptors eventually disrupting homeostasis to ultimately lead to cell death (Ozawa et al., 1998). Therefore, a vicious cycle is born because SNc neurons become vulnerable to glutamate induced excitotoxicity brought on through the no longer inhibited STN (Cardoso et al., 2005) (Figure. 4).

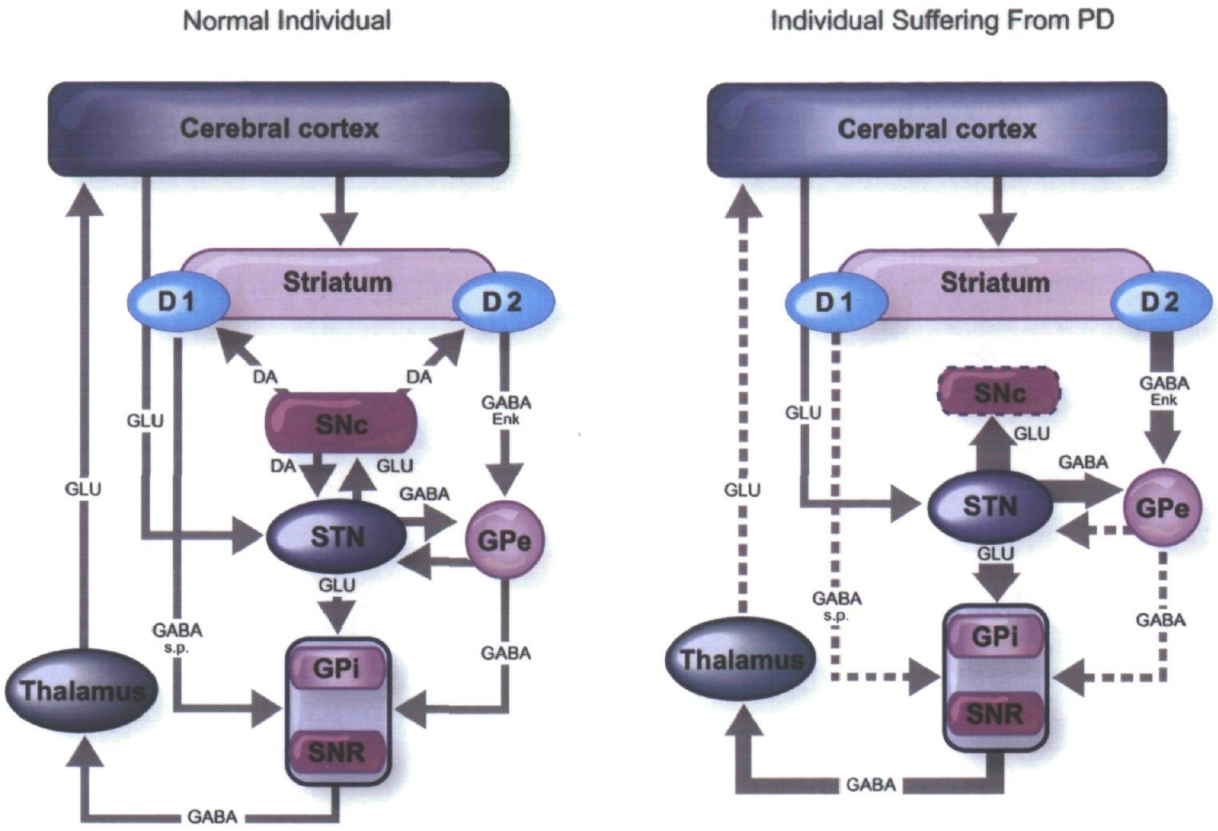

Figure 4. Comparing normal and parkinsonian BG signalization. The loss of SNc dopaminergic neurons promotes the depletion of the downstream striatum's DA reservoir. The consequences include the hypoactivity of D1 DA receptors expressed on a subset of striatal projecting GABAergic neurons. Under parkinsonian conditions, overworked D2 DA receptors increase the inhibition of the GPe, this results in an increased activation of the STN. If uncontrollably disinhibited the STN allows both the GPi and the SNr to increase inhibition on the cortex through the thalamus. The end result is the apparition of parkinsonian symptoms. Adapted from Cardoso et al. 2005 (Cardoso et al., 2005).

3. 4. 1 *NMDA receptors*

Glutamate is the most prominent neurotransmitter in the body, being present in over 50% of nervous tissue (Debanne et al., 2003) and glutamate receptors exist primarily in the CNS and are located on the neuronal membrane. These synaptic receptors can be found on the dendrites of post-synaptic cells. They are expressed in both astrocytes and oligodendrocytes (Steinhauser and

Gallo, 1996). Glutamate receptors are responsible for the glutamate-mediated post-synaptic excitation of neural cells and mediate synaptic plasticity and memory (Debanne et al., 2003). There are two types of glutamate receptors, metabotropic and ionotropic receptors (Ozawa et al., 1998).

NMDA receptors are a type of ionotropic glutamate receptors to which *N*-methyl-D-aspartate, a selective glutamate receptor agonist, binds and induces ion channel opening (Iversen et al., 2010). This receptor in a ligand gated, voltage-dependant and relies on co-activation by both glutamate or aspartate and glycine (Kleckner and Dingledine, 1988). NMDA receptors are composed of a heterotetramer between two obligatory NR1 and two NR2 subunits (Laube et al., 1997).

NMDA receptors isoforms having distinct functional properties in the brain result from NR1 transcript splicing and differential expression of NR2 subunits (Laube et al., 1997).
The NR1 subunits variants include NR1-1a, NR1-1b, which is the most abundantly expressed form, and NR1-2a, NR1-2b, NR1-3a, NR1-3b and NR1-4a, NR1-4b (Laube et al., 1997).
Next, vertebrates express four different isoforms of the NR2 subunit labeled NR2A, NR2B, NR2C and NR2D, which bind glutamate or aspartate and vary in their expression across different cell types (Liu et al., 2004). These subunits specify the elecrophysiological properties of NMDA receptors, like their sensibility to glutamate and permeability to calcium (Cyr et al., 2001) (Figure 5).

The striatum receives two major inputs: a massive excitatory glutamatergic projection from the cerebral cortex and a dopaminergic projection from the SN (Samadi et al., 2007). In PD, the loss of striatal DA is associated with loss of the inhibitory DA control of corticostriatal glutamatergic drive with consequent increased glutamate release (Garcia et al., 2010). In PD, glutamate activity is increased in the BG (Klockgether and Turski, 1993). Excessive levels of

glutamate cause excitotoxicity through the activation of NMDA receptors, promoting neuronal death (Sonsalla et al., 1998).

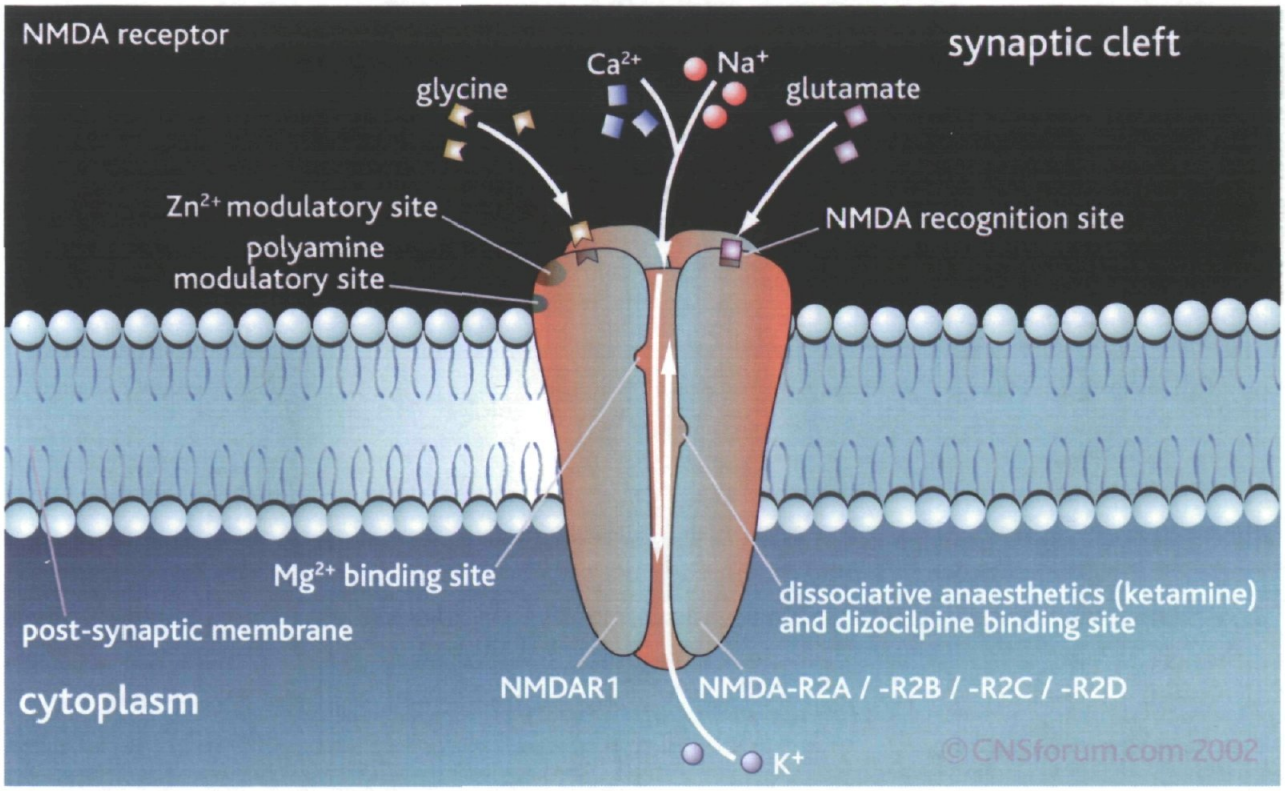

Figure 5. NMDA receptors are a type of ionotropic glutamate receptors to which *N*-methyl-D-aspartate, a selective glutamate receptor agonist, binds and induces ion channel opening. This receptor is a ligand gated, voltage-dependant and relies on co-activation by both glutamate or aspartate and glycine. NMDA receptors are composed of a heterotetramer between two obligatory NR1 and two NR2 subunits (NR2A, NR2B, NR2C or NR2D). This figure was taken from CNSforum.com (CNS Forum, 2002).

3. 5. *Pharmacological and surgical treatments of PD*

Parkinson's disease is multifaceted and its management requires the treatment of the motor and non-motor symptoms for which the options are expanding. The American Academy of Neurology and the Movement Disorder Society published treatment guidelines for both early and late PD (Simuni et al., 2009a).

The Unified Parkinson's Disease Rating Scale (UPDRS) is the most consulted for the assessment of PD symptom severity and is revised to accommodate advances in the treatment of PD (Simuni et al., 2009a). The scores of different subsections are added to provide the total UPDRS score, part I tests mentation, part II looks at daily living activities, part III examines motor function and part IV measures therapy complications (Simuni et al., 2009a).

Again, with no documented cure for PD, the drugs used in treatments focus on symptomatic relief and the most commonly prescribed against PD motor problems is the dopamine precursor L-DOPA (Smith, 2000). L-DOPA was first used in clinical practice in 1967 and was first reported to improve the motor skills of PD patients in 1968 (Cotzias, 1968; Hornykiewicz, 2002b), it is used since DA can not cross the blood-brain barrier. Once in the CNS, L-DOPA is quickly decarboxylated to DA and its peripheral catabolism is prevented by carbidopa a decarboxylase inhibitor drug combined in the treatment (Smith, 2000). This allows smaller doses of L-DOPA to have effect. With a reduction of PD symptoms by 50%, L-DOPA has a 75% satisfactory response in idiopathic PD patients (Smith, 2000). However, harsh side effects are promoted after chronic use and include the development of motor complications such as L-DOPA induced dyskinesias (LIDs) (Smith, 2000). Also, positive feedback of DA replacement therapies are masked by symptoms of the late stages of PD (Lang, 2007).

Next, DAT targeting drugs increase synaptic DA by inhibiting the transporter (Pearce et al., 2002), while selective DA receptor agonists, mimic the effect of DA (Mailman et al., 2001). DA agonist are divided in two subtypes, the classification are ergot derived and non-ergot derived (Simuni et al., 2009a). These are efficacious in the case of early PD, except ergot derived DA agonists like bromocriptine, cabergoline and peroglide were linked to valvular heart disease, therefore approved non-ergot DA agonists include pramipexole, ropinirole and rotigotine (Schade

et al., 2007; Zanettini et al., 2007). For early and advanced PD, pramipexole is administered orally at doses ranging up to 4.5 mg/day (Simuni et al., 2009a). Similarly, ropinirole is given at doses ranging up to 24 mg/day (Simuni et al., 2009a), while rotigotine is the first DA agonist treated through a transdermal system and is used at up to 6 mg/24h for early PD (Simuni et al., 2009a). It has been been shown that the positive outcomes from the use of DA agonists in retarding motor complications depends on the consequent delay of L-DOPA introduction (Holloway et al., 2004; Rascol et al., 2006). Thus, DA agonists are the preferred treatment for early PD and patients needing longterm treatments (Simuni et al., 2009a).

Non-dopaminergic drugs like adenosine A2a receptor antagonists, GABAergic drugs and glutamate antagonists have therapeutic effects inferior to those of L-DOPA, but combining them with it could be promising in delaying the occurrence of drug-induced dyskinesias (Lang and Lozano, 1998). MAOB inhibitors, such as oral selegine, can diminish 'off' time of advanced PD patients and doses range to 10 mg/day and are approved as adjuncts to L-DOPA treatment (Olanow et al., 2001). Finally, when drugs have lost their effectiveness and their side effects combined with the diseases symptoms become overwhelming patients resort to surgical treatments, what with todays more refined sophistication of stereotaxic procedures, brain-imaging technology and computer programming have produced still advancing surgical methods focusing on PD treatment. These include neuronal transplantation, deep brain stimulation and ablative techniques, all of which deal with striatal DA loss and target correcting the consequent functional disturbances in BG circuitry (Bjarkam et al., 2001; Hammerstad and Hogarth, 2001).

3.6. *Pharmacological Treatments and Neuroprotection*

Influencing the etiology or pathogenesis of the neurodegenerative process of PD is the focus of neuroprotective therapies (Shoulson, 1998).

The ELLDOPA, a 2005 study performing statistical analysis comparing the effects of L-DOPA and dominance of handedness over 42 weeks, which randomized 361 early PD subjects to placebo, L-DOPA 150, 300, or 600 mg/day. This trial yielded significantly lower (ameliorated) UPDRS scores in all L-DOPA groups compared to those receiving the placebo treatment (Fahn et al., 2004). Striatal DA terminal DAT and VMAT2 levels are used as markers of neuronal loss because their specific binding correlates with the degree of stiatal DA depletion in the PD patients (Wilson et al., 1996). However, the assessment of PD progression with DAT binding at baseline and 40 weeks using β-CIT single photon emission computed tomography (SPECT) revealed L-DOPA groups to have a graver progression of the disease (Simuni et al., 2009b). A pharmacologic or pharmacodynamic effect of L-DOPA on DAT is hypothesized, since there is so solid proof that L-DOPA either speeds-up or delays PD progression (Fahn et al., 2004). Moreover, mono-therapy using rasagiline or an adjunctive rasagiline therapy to L-DOPA produced symptomatic relief in PD patients (Hoy and Keating, 2012). The ADAGIO study was a placebo-controlled, double-blind, multicentre, delayed-start study, where 1176 patients with untreated early Parkinson's disease were randomly given rasagiline 1 mg or 2 mg per day for 72 weeks (early-start groups) or placebo for 36 weeks followed by rasagiline 1 mg or 2 mg per day for 36 weeks (delayed-start groups) (Hoy and Keating, 2012). Mono-therapy with the recommended dose of 1 mg/day of rasagiline had significant effects on the rate of PD worsening and results from the ADAGIO study concluded delayed clinical progression of PD (Hoy and Keating, 2012).

DA agonists like pramipexole and ropinirole induce neuroprotection in cell cultures, animal models and have also been clinically trialed with SPECT imaging techniques (Simuni et

al., 2009b). Their neuroprotective mechanisms include antioxidant effects and the induction of anti-apoptotic effects (Le and Jankovic, 2001). Also rotigotine has neuroprotective capabilities in both mouse and non-human primate animal models of PD, but has not been tested clinically (Scheller et al., 2007). Next, selegine and rasageline, irreversible MAOB inhibitors, provide mild symptomatic relief in PD patients. Their neuroprotective effects are promted through other mechanisms that include the inhibition of glyceraldehyde 3-phospate dehydrogenase (GAPDH) nuclear translation or the upregulation of Bcl-2 and other anti-apoptotic proteins (Olanow, 2006). However, with all this, no treatment has been able to provide curative neuroprotective effects against PD.

3. 7. *Animal Models of PD*

Again, James Parkinson first described PD almost two hundred years have passed and still no exact cause is documented, only a few pathogenic mechanisms are suggested.

For sporadic PD, the most common form of the disease, neuroinflammation, proteolytic stress, oxidative stress and mitochondrial defects could be involved (Simpkins et al., 2008).

The past two centuries generated deeper understanding and new perspectives on the molecular mechanisms of PD in order to ameliorate or create therapeutic options.

In brief, when selecting an animal model it's limitation and specificities should be taken into consideration. Both toxic and transgenic classes of animal models can provide substantial and reproducible nigrostriatal lesioning.

Toxic animal models are the classic form of experimental PD animal models, which rely on neurotoxins capable of causing most of the pathological and/or phenotypic features of PD in animals (mammals such as *mice or non-human primates).*

3. 7. 1. *MPTP*

In the past forty years, *in vivo* animal models involving neurotoxins administration was either systemic or local depending on the type of the agent and animal species.

PD pathogenesis and mitochondrial dysfunction are entwined, it being a mechanism involved in aging and other neurodegenerative diseases (Abou-Sleiman et al., 2006). A decrease in complex I of the mitochondrial electron transport chain is seen in the SN of PD patients (Schapira et al., 1989), complex I inhibitors include the neurotoxic ion 1-mehyl-4-phenylpyridinium (MPP+, MPTP metabolite), the pesticide rotenone and the herbicide paraquat. Today, MPTP provides an excellent model of PD due to its specific neurotoxicity in animals such as mice, monkeys, cats and pigs, whereas rats are resistant to it (Kopin and Markey, 1988; Przedborski et al., 2001). MPTP was documented as a selective neurotoxin for nigrostriatal tract dopaminergic neurons in the mid 1980s by Dr. Langston. He was the first to describe its effects in young Northern California drug users suffering from parkinsonism attained by intravenous injections of a meperidine analog street preparation containing MPTP (Langston et al., 1983). For the MPTP animal model, some species of mice are used, whereas rats are highly resistant to MPTP for reasons which are not well defined. However, rats are vulnerable to MPP^+ only when it is directly injected into the SNc. It is theorized that the DAT/VMAT2 ratio determines susceptibitlity to MPTP (Joyce et al., 1997).

The major limitations of the MPTP animal model include the lack of a crucial neuropathological hallmark of the disease, because Lewy body cytoplasmic inclusions do not result after exposure to MPTP (Blandini and Armentero, 2012). Controversies regarding the topic arised when attempting to correct this by modifying the MPTP lesioning regimens. First, Fornai and colleagues saw that an osmotic minipump 30 day administration of 30 mg/kg/day of MPTP can result in the formation of nigral inclusions which are immunoreactive for ubiquintin and

alpha-synuclein (Fornai et al., 2005). Dawson et al. did not observe this with an acute (1 day), a semi-chronic (5 days) or a chronic (35 days) MPTP administrations not using osmotic pumps (Shimoji et al., 2005). At the same time, Alvarez-Fischer and colleagues used a chronic MPTP regimen of 40 mg/kg/day, treated using minipumps and resulted in a failed attempt to identify LBs or even significant SNc neuronal loss (Alvarez-Fischer et al., 2008).

Perhaps the development of LBs requires more time than provided in experimental protocols. One could argue that this is due to the toxin's rapid efficiency in replicating PD and the death or scarification of experimental animals before they develop LBs (Przedborski et al., 2001). The other limitation of the MPTP animal model of PD is that with acute (four daily injections) and sub-acute (single daily dose for 5-10 days) administrations, the nigrostriatal DA deficits could be reversible. Accordingly, co-administration with the uricosuric agent *probenecid* can prolongue the effects of MPTP by blocking its clearance from circulation, causing gradual SNc neuron loss (Blandini and Armentero, 2012). This is associated with significant striatal DA depletion lasting for six month after withdrawal from MPTP treatment (Blandini and Armentero, 2012).

Now, MPTP has gained respect as a very potent neurotoxin therefore its safe handling is imperative. The administration of this neurotoxin is done through many routes with different dosing regiments like subcutaneous, intravenous, intraperitoneal and intramuscular. The irreversible effects of MPTP induce tremor, slowness of movement, rigidity, freezing and postural instability, mimicking almost all of the PD characterizing features (Przedborski et al., 2001). As mentioned before this disease takes several years before manifesting, however in the MPTP model the neurodegeneration is at its peak in only a few days after administration (Jackson-Lewis et al., 1995; Langston et al., 1987; Przedborski et al., 2001).

Here, MPTP MPTP crosses the blood-brain barrier is converted into MPP+ by MAOB of glial cells, then MPP^+ enters DA neurons through the presynaptic DA uptake system DAT and

accumulates inside mitochondrion matrixes (Figure 6 A). MPP+ is a very potent neurotoxin which inhibits mitochondrial oxidation of NAD+-linked substrates; it accumulates inside mitochondrion matrixes and inhibits respiration. Next, ATP reservoirs are depleted and a consequent loss of membrane potential follows, resulting in the death of nigrostriatal nerves through mechanisms that would include the loss of calcium homeostasis and the formation of radicals like NO which promote cytotoxic events leading to limited DA release, locomotor defects and finally apoptosis (Przedborski and Vila, 2003) (Figure 6 B). Let us not forget the innate inflammatory mechanisms which could aggravate PD progression (Brooks et al., 2003). Malfunctioning astrogial cell compartments is though to help the progression of selective degradation of the SNc DA neurons in PD and its MPTP model (Przedborski et al., 2001). In accordance, astrocyte MAOB has increased levels in the aging and/or neurodegenerating brain yet it is inhibited by proparylamines (Smith, 2000), therefore selective inhibitors of MAOBs can prevent the harmful effects of MPTP while inhibiting its A isoform does not (Tipton and Singer, 1993). Thus, demonstrating that dopaminergic neurons are susceptible to mitochondrial dysfunction and ROS production (Terzioglu and Galter, 2008).

It is believed that humans intoxicated with this neurotoxin will continue to experience MPTP-induced neurodegeneration (Langston et al., 1999; Vingerhoets et al., 1994) but with the exception of four cases there exists no human pathological material on MPTP intoxication, therefore the comparison of PD and MPTP models is restricted to primates (Forno et al., 1986).

Again, MPTP destroys more dopaminergic neurons of the nigrostriatal pathway in the SNc than in the VTA, making its effects almost identical to those of PD and most publications report that MPTP toxicity touches only the pigmented nuclei of the SNc (Muthane et al., 1994; Seniuk et al., 1990) (Bourque et al., 2009).

Mice are now the preferred species in MPTP models for both technical and financial reasons, despite them being less sensitive to MPTP than monkeys, so much higher doses are administered to induce significant SNc damage (Sonsalla and Heikkila, 1986) therefore MPTP dosing affects the magnitude of nigrostriatal damage. It was demonstrated that neuroprotective effects of tested compounds vary in accordance to MPTP administration which is done using either of these two following protocols.

First, an acute treatment consisting of four 2 hour interval injections in one day, or the second subacute (chronic) protocol dictating one or two MPTP injections per day for a period of five consecutive days (Jackson-Lewis et al., 1995). The former is thought to cause cell death via non-apoptotic mechanisms while the chronic treatment induces cell death through apoptosis (Jackson-Lewis et al., 1995). Hence, cell death mechanisms of idiopathic PD are still under investigation and could involve various apoptotic and/or non-apoptotic pathways and MPTP protocols should prove to be informative for drug screening research for neuroprotection against PD. Moreover, with a MPTP/MPP^+ half/life of approximately two hours in the mouse brain, higher doses of the toxin at shorter intervals result in more damage and therefore prolonging these injection intervals result in diminished MPTP effects (Meredith et al., 2008; Sonsalla and Heikkila, 1986).

In conclusion, PD models using MPTP could enhance our learning on the implication of oxidative stress in the specific destruction of SNc dopaminergic neurons. As mentioned before, MPP^+'s route through DAT gives it access to these neurons. Cellular damage and death arises from this metabolite's ability to deplete ATP from these neurons by blocking complex I of the mitochondrial respiratory chain. Plus, MPP^+ has a high affinity for VMAT2 where it is also given access to dopaminergic vesicles and causing cytosolic DA release and DA-dependant oxidative stress (Cardoso et al., 2005). Again, the demise of more than half of the SNc dopaminergic

neurons will lead to the depletion of about 80% of this neurotransmitter's striatal reservoir, but there are ongoing debates on which mechanisms induce cellular death under these conditions (Figure 6).

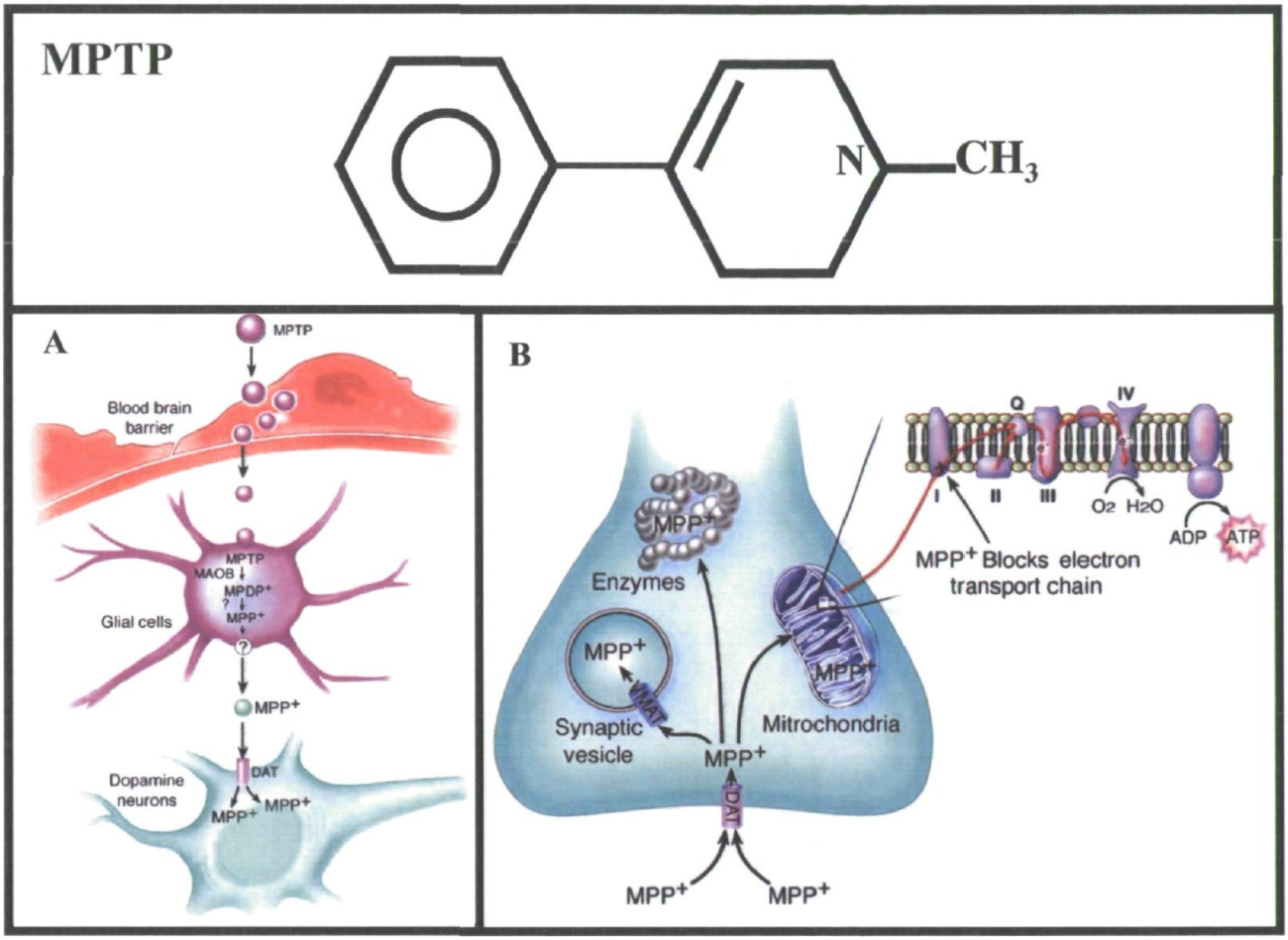

Figure 6. The molecular structure of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). (A) Mechanism of action of MPTP: MPTP crosses the blood-brain barrier is converted into MPP+, a very potent neurotoxin, by MAOB of glial cells, then MPP^+ enters DA neurons through the presynaptic DA uptake system DAT. (B) MPP^+ accumulates inside mitochondrion matrixes and inhibits complex I of the mitochondrial respiratory chain. Next, ATP reservoirs are depleted and nigrostriatal nerves are destroyed through mechanisms that involve the formation of radicals, which promote cytotoxic events leading to apoptosis. This figure was adapted from Przedborski et al. 2003 (Przedborski and Vila, 2003).

3. 7. 2. Pesticides

Neurotoxicity attained with environmental toxins in PD pathogenesis include the use of pesticides. Chronic pesticide exposure has a consequent increased risk of development of PD. Rotenone, another pesticide, is a highly lipophilic flavonoid which can cross the blood-brain barrier, therefore DA neuron entry does not require transportation via DAT (Blandini and Armentero, 2012). Greenamyre et al. described its use in an animal model of chronic intravenous administration to rats (Greenamyre et al., 2010). Like MPTP, rotenone promotes selective SN DA neuron degeneration through massive formation of reactive oxygen species (ROS) and proteasome activity inhibition (Betarbet et al., 2000). This model was the first in the history of PD toxic animal models to yield Lewy body-like cytoplasmic inclusions containing ubiquintin and alpha-synuclein. However, it is no longer used as a valid PD animal model and critical issues include substantial variability in the individual response, high mortality and the specificity of lesioning (Blandini and Armentero, 2012).

3. 7. 3. *Herbicides*

Next, paraquat a nonselective bipyridyl contact herbicide is structurally similar to MPP^+. In fact, MPP^+ was used as a herbicide called cyperquat. However paraquat acts through a different mechanism, entering through a neutral amino acid transporter (L carrier, LAT-1), since it is a charged molecule and cannot cross the blood-brain barrier (Blandini and Armentero, 2012). This toxin causes massive oxidative stress by acting as a redox cycling compound. It generates moderate (20-30%) nigrostriatal DA neuron loss requiring multiple injections (Fei et al., 2008), but the expression and the aggregation of alpha-synuclein in the SNc is detected in paraquat-lesioned mice (Manning-Bog et al., 2002).

3. 7. 4. *Fungicides*

The fungicide manganese ethylene-bis-dithiocarbamate (maneb) can exacerbate paraquat toxicity and co-administration can promote synergic and more significant effects in the SN (Thiruchelvam et al., 2000).

3. 7. 5. *Intracranally injected neurotoxins*

3. 7. 5. 1. *6-Hydroxydopamine (6-OHDA)*

Local intracerebral administration of 6-OHDA was the first PD animal model (Ungerstedt et al., 1974) and provides the highest degree of striatal DA depletion (90-100%), but high mortality rates are associated with bilateral injections. Therefore, unilateral 6-OHDA injection procedures are favored and the contralateral hemisphere is used as a control (Blandini and Armentero, 2012). This hydroxylated DA analogue has high affinity for DAT, does not cross the blood-brain barrier, thus requiring local injection preferably into the medial forebrain bundle or the SNc and causes great anterograde nigrostriatal pathway neurodegeneration. Twelve hours after 6-OHDA administration SNc neurons are lost and this parallels striatal DA loss after 2-3 days (Sauer and Oertel, 1994).

The 6-OHDA neurotoxic mechanism of action relies on its pro-oxidant properties, where cytosolic 6-OHDA accumulates and rapid auto-oxidation induces excessive hydrogen peroxide formation. In addition, 6-OHDA can accumulate in mitochondria and block complex I activity (Blandini et al., 2007).

3. 7. 5. 2. *Lipopolysaccharide*

All currently used neurotoxins in experimental models of PD promote a neuroinflammatory response in the nigrostriatal tract (Blandini and Armentero, 2012). The bacterial endotoxin lipopolysaccharide is used to cause high levels of tissue inflammation when locally injected into the SN or striatum of rats and is accompanied by ipsilateral rotational behavior to the lesioned side in response to amphetamine administration (Blandini and Armentero, 2012).

Here, microglial activation and cytoxic molecule release result in progressive nigrostriatal tract neurodegeneration, where 41% of the SNc is lost after 4 weeks post-injection and the striatal DA levels are reduced by 42%. The presence of alpha-synuclein and ubiquintin in the SNc was observed (Hunter et al., 2009). In addition, it has been shown that injection of lipopolysaccharide into the striatum of rats causes progressive degeneration of the nigrostriatal tract (Hunter et al., 2009).

3. 8. *Transgenic animal models of PD*

A lot of information on PD was achieved by the use of toxic animal models, however the slowly progressive age-dependent lesions accompanied by LBs lack in these models of PD (Blandini and Armentero, 2012). New animal models targeting the disease's pathogenesis by reproducing known PD-related mutations, since genome-wide association studies suggest that familial and sporadic forms of PD could share common genetic backgrounds (Blandini and Armentero, 2012). Transgenic mouse models include mice lacking the following genes: alpha-synuclein, LRRK2, PINK1, Parkin, DJ-1; multiple transgenic mice and the mitoPark mouse (Blandini and Armentero, 2012). The use of non-mammalian organisms, like *Drosophila, C.*

elegans, and *Zebrafish,* is another alternative to study monogenic mutations seen in familial PD patients (Chesselet, 2008). It is believed that sporadic and familial forms of PD could have related genetic background. Therefore, monogenic 'Mendelian' forms of the disorder promoted the development of new animal models, more particularly using mice, having known PD-related mutations (Blandini and Armentero, 2012).

3. 8. 1. *Leucine-rich repeat serine/threonine kinase 2 (LRRK2)*

The most prevalent cause of autosomal dominant PD are caused by mutations in the LRRK2 gene, where six mutations have been documented to date: R1441C, R1441G, N1437H, Y1699C, G2019S and I2020T. It is suggested that LRRK2 modulates DA transmission (Li et al., 2010), since mutated LRRK2 (G2019S) results in age-dependent diminishes striatal levels. Also, mutated LRRK2 promotes α-synuclein aggregation (Kumar and Cookson, 2011). However, the overexpression of the wild-type (WT) or mutant protein does not cause neuronal cell death (Blandini and Armentero, 2012). LRRK2 knock out (KO) mice have no major abnormalities and are not highly sensitive to MPTP (Blandini and Armentero, 2012).

3. 8. 2. *α-synuclein*

Mutations on the α-synuclein gene, the first gene to be linked to familial PD, are a rare autosomal-dominant form of the disorder to which three point mutations are identified: A30P, A53T and E46K. In addition, the duplication or triplication of the gene can induce PD (Blandini and Armentero, 2012). Many α-synuclein transgenic mice were developed, of which some have non-motor dysfunctions seen in early stages of the disease, including olfactory deficits and gastrointestinal alterations (Blandini and Armentero, 2012).

3. 8. 3. *PTEN-induced putative kinase 1 (PINK1)*

The second most common form of autosomal recessive PD is linked to mutations in the PTEN-induced putative kinase 1 (PINK1) gene, which is a cytosolic and mitochondrial serine/theronine kinase (Blandini and Armentero, 2012). PINK1 KO mice have minor mitochondrial and nigrostriatal neurotransmission deficits as well as a high vulnerability to oxidative stress (Gautier et al., 2008), while G309D-PINK1 transgenic mice had age-dependent reduced DA and no LB formation (Gispert et al., 2009).

3. 8. 4. *Parkin*

With over a hundred mutations reported to date, the most common autosomal-recessive PD mutations are on the parkin gene (Blandini and Armentero, 2012).

Parkin, an E3 ubiquintin ligase, is involved in the ubiquintin proteasome system and mutations cause a loss of function, resulting in early-onset PD (Blandini and Armentero, 2012).

Overexpression of the DA neuron selective Q311X parkin mutation causes progressive motor deficits, age-dependent nigrostriatal degeneration and α-synuclein pathology (Lu et al., 2009).

In fact, both PINK1 and parkin regulate mitochondrial control and parkin KO mice are highly vulnerable to neuroinflammation and neurotoxins (Blandini and Armentero, 2012).

3. 8. 5. *DJ-1*

Rare autosomal-recessive PD point mutations are found on the DJ-1 gene, it being a redox-sensitive molecular chaperone protein (Blandini and Armentero, 2012). DJ-1 KO mice are also highly sensitive to toxins and oxidative stress (Paterna et al., 2007).

Finally, multiple transgenic mouse lines have been developed, however they did not yield consistent results (Blandini and Armentero, 2012). They include crossing a-synuclein transgenic mice with parkin or DJ-1 KO mice or simultaneous silencing of PINK1, parkin and DJ-1 (Blandini and Armentero, 2012).

3. 8. 6. *Rat genetic models*

As an alternative to mice, transgenic rat models, which reproduce monogenic PD mutations are now commercially available, the rat circuitry being closer to that of humans (Blandini and Armentero, 2012). Transgenic rat models for PD include the expression of human A53T and A30P α-synuclein mutations under the TH promoter (Blandini and Armentero, 2012). These rats exhibit significant olfactory deficits (Lelan et al., 2011). Whereas, G21019S LRRK2 transgenic rats have impaired DAT function (Zhou et al., 2011).

IV. Estrogens

4. 1. *Gender and PD*

Many studies (Baldereschi et al., 2000; Bower et al., 1999; Kurtzke and Goldberg, 1988; Marder et al., 1996; Mayeux et al., 1992; Mutch et al., 1986), a meta-analysis (Wooten et al., 2004) and reviews (Czlonkowska et al., 2006; Shulman and Bhat, 2006) indicate that PD is more prevalent and has a larger incidence in the male population, while one study documented no such discrepancies (de Rijk et al., 1995). More specifically, it was seen that gender differences exist during the progression of PD and in the responses to L-DOPA treatment (Growdon et al., 1998; Lyons et al., 1998; Zappia et al., 2005). Next, estrogens were observed to have modulatory effects on PD symptoms and LIDs (Bedard et al., 1977; Giladi and Honigman, 1995; Session et al., 1994). A study found that estrogen therapy has positive effects on women in early PD stages,

who have not yet taken L-DOPA (Saunders-Pullman et al., 1999). Also, motor disabilities were remedied by estrogens in postmenopausal women with PD (Tsang et al., 2000). More evidence is seen in a case-study concluding that women who took a postmenopausal estrogenic treatment had a lower risk of developing PD (Currie et al., 2004). Furthermore, one study showed that men suffering from PD will acquire symptomatic medical attention during early stages of the disorder (Saunders-Pullman, 2003), so one could argue that the male sex experiences PD symptoms before the opposite sex or that the disease progresses more rapidly in men, thus supporting that estrogen can provide neuroprotective effects (Saunders-Pullman, 2003). Further, gender differences were also seen in outcome studies of symptoms after stereotactic surgery for PD (Shulman and Bhat, 2006), which brings us to suggest that treating women for PD should be adjusted to hormonal changes.

In the pre-agricultural period not long ago, the average life expectancy of human beings was half what it is today. Back then people had life spans varying between twenty and forty years of age while after just one century it has doubled to an average of 83 years (Gruber et al., 2002). This has drastic effects on women since even though the average onset of menopause is still fifty years of age; it is inevitable that they will have to experience more of the postmenopausal state. In fact, we must not forget that PD is also prevalent in women (Saunders-Pullman, 2003) and some claim that these male/female discrepancies result from hormonal changes before, during and after menopause (Saunders-Pullman, 2003).

4. 2. *Estrogenic compounds*

Steroid hormones are synthesized from cholesterol in the adrenal cortex to produce glucocorticoids, mineralocorticoids and adrenal androgens; in the testes to provide testicular androgens or estrogen; or in the ovaries and placenta to make estrogen, prosgestagens or progestins (Marin et al., 2005). The enzyme aromatase is responsible for their synthesis and Garcia-Segura et al. have observed using rodent reactive astrocytes that the induction of its expression and activation is a potential response to injury (Garcia-Segura et al., 1999). Steroids are lipophilic molecules produced in endocrine glands, they travel through the blood stream with the help the cytoskeleton and carrier proteins, like sterol carrier protein-1, in order to reach their target, once there they can easily pass through the plasma membrane and blood-brain barrier by simple diffusion (Marin et al., 2005). In the end, they are metabolized by sulfation, glucuronidation methylation or hydroxylation mainly in the liver (Gruber et al., 2002).

Estrogens could be either natural steroids, synthetic or non-steroidal, the three natural estrogens include 17β-estadiol (17β-E_2) which is biologically active, estrone (E1) and estriol (E3) which are categorized as C18 steroids (Marin et al., 2005), all of which lack side groups that ameliorate potency and duration of action found in their synthetic siblings (Kompoliti, 2003) (Figure 7).

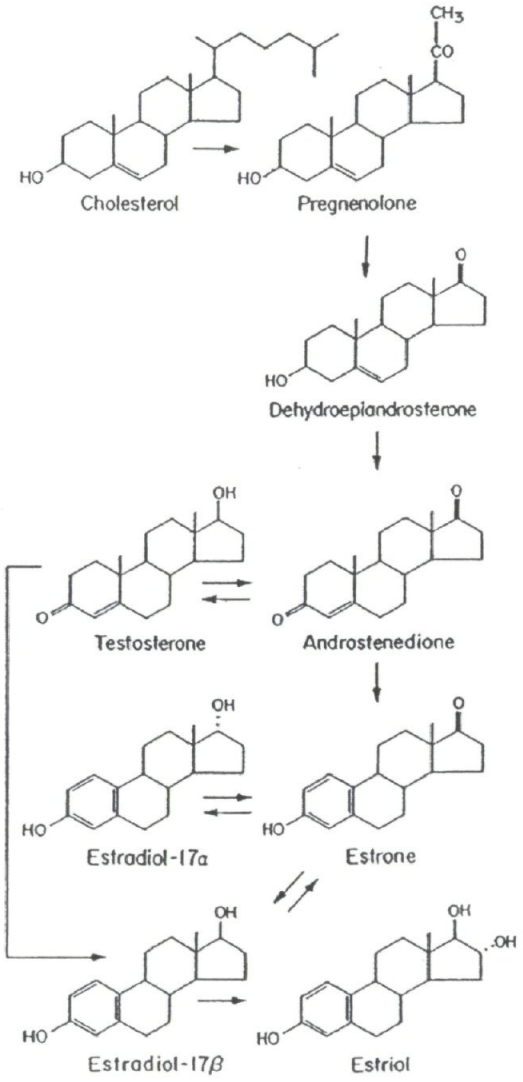

Figure 7. Synthesis of estrogens. Steroid hormones are synthesized from cholesterol by the aromatase enzyme, they include testosterore, 17α-estradiol, 17β-estradiol, estrone and estriol (Garcia-Segura et al., 1999). This demonstrates that testosterone is converted into 17β-estradiol which is the precursor of estrone and estriol. Adapted from Heftmann et al. 1970 (Heftmann, 1970).

4. 3. *Estrogenic functions*

The female reproductive system is orchestrated by the regulatory activities of distinct brain areas including the hypothalamus and anterior pituitary and of course the peripheral organs which are the ovaries and uterus (Marin et al., 2005). From pregnancy to delivery all these are implicated in controlling the feedback actions of the ovarian hormones estradiol and progesterone (Marin et al., 2005). Gonadal hormones have many roles in neuroendocrine system regulation, behavior modulation, promoting plasticity and the differentiation of particular neuronal families (Marin et al., 2005). Estrogens are functionally involved as growth-stimulating agents, they can modulate apoptosis (Arai et al., 1996). They are pleiotropic showing many diverse beneficial and perhaps opposing effects, while studies have shown that they can improve memory and cognitive performance (Sheng et al., 1991) and when used in hormone therapy, show positive effects against cardiovascular disease and fight osteoporosis (Grady et al., 1992).

4. 4. *Estrogen receptor subtypes*

There are two estrogen receptor subtypes documented today, before the second subtype named ERβ (or NR3A2) was discovered and cloned from rat prostate in 1996 the effects of estrogens were thought to be mediated by the original subtype named ERα (or NR3A1) (Kuiper et al., 1996; Walter et al., 1985). The transcriptional information of these two receptors lies on two different genes, ERα is encoded by the *ESR1* gene on chromosome 6, while ERβ is encoded by *ESR2* on chromosome 14 (Nilsson et al., 2011). In fact, twenty splice variants have been documented for ERα and ten for ERβ (Kuiper et al., 1996). When comparing these two, there is little or no conservation in their N-terminal region, but they have a homology of 59% in their ligand-binding domains and are 96% homologous in their DNA-binding domains (Nilsson and Gustafsson, 2011) (Figure 8).

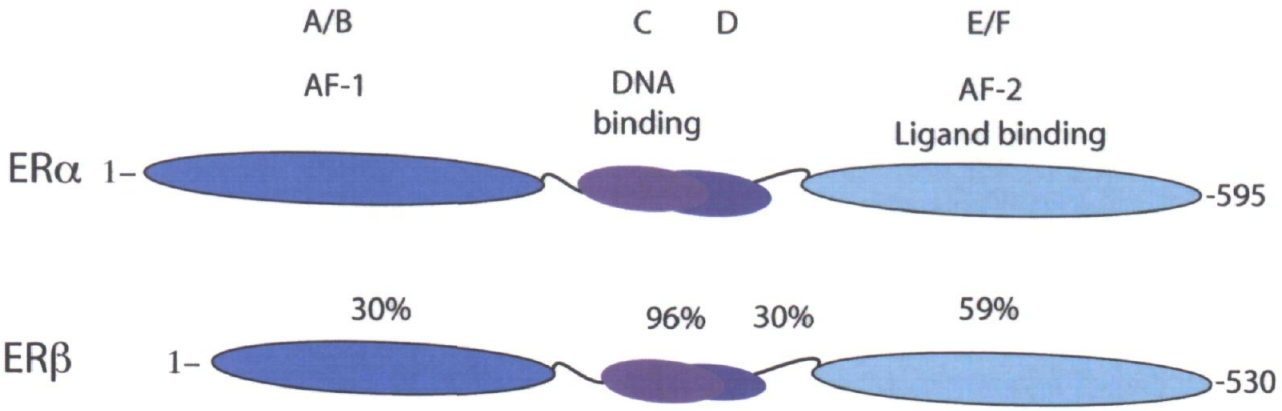

Figure 8. Comparing structural homologies of ER subtypes ERα and ERβ. ERs ERα and ERβs have commun nuclear receptor super family features, these include the amino terminal domain (A/B domain), they have a 59% homology in their ligand-binding domains (E/F domains) and are 96% homologous in their DNA-binding (C/D domains). Adapted from Nilsson and Gustafsson 2011 (Nilsson and Gustafsson, 2011).

Therefore, one could claim that these siblings are engaged in different biological roles (Jin et al., 2008). Supporting this is a study on two different estrogen therapies demonstrating that the expression of ERs can be regulated (Jin et al., 2008). Here, the estrogenic compounds promote different effects through regulating the expression of both ERs and further supporting clinical evidence that various estrogenic treatments are distinct in their effects on target cells, tissues or organs (Jin et al., 2008). Estrogens may differ in their affinity for either ER subtype, for example 17β-estradiol equally binds both ERα and ERβ, while estrone and the selective estrogen receptor modulator (SERM) raloxifene preferentially bind ERα, while estriol has more affinity for ERβ (Kompoliti, 2003). Estrone and estriol are categorized as weak ER agonists (Gruber et al., 2002).

In addition, estrogens can diffuse in and out of cells until their specificities and affinities confine them to target cells though receptors and each ER has its own nonsteroidal agonist synthesized by Katzenellenbogen et al. (Meyers et al., 2001; Stauffer et al., 2000), ERα's is 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazolen (PPT) and 2,3-bis(4-hyroxyphenyl) propionitrile (DPN) is selective for ERβ.

PPT activates gene transcription through its very high affinity and specificity for ERα while showing a 410-fold greater binding selectivity for ERα over ERβ (Stauffer et al., 2000). On the other hand, DPN is a complete ERβ agonist and its binding selectivity is 72-folds greater for it over ERα (Meyers et al., 2001). At low and high concentrations, PPT is known to promote transcription through ERα (Stauffer et al., 2000), while DPN has effects on both ERs with a 78 times greater preference for ERβ at low doses (Meyers et al., 2001) and activates ERα at high doses (Harrington et al., 2003), while 17β-estradiol binds both ERs with equal affinities (Gruber et al., 2002). Hence, binding affinity determines the molecular ability to bind a specific receptor and binding assays determine its capacity to induce genomic transcription once bound to the receptor.

In fact, studies claim that selective ER agonists could regulate cognitive function in postmenopausal women (Shughrue and Merchenthaler, 2000).

Further, it has been observed that growing old has negative effects on ERα mediated events, the aging process is believed to attenuate ER functioning by increased methylation of the ER gene (Post et al., 1999; Smith, 2000).

4. 5. *Estrogen receptor functions and mechanisms*

The plethora of beneficial effects of estrogens in the CNS include inducing an increase in anti-apoptotic proteins, regulating second messenger cascades, modulating glutaminergic activation, maintaining calcium homeostasis, attenuating NMDA receptor activity and providing antioxidant effects (Al Sweidi et al., 2012; Gruber et al., 2002; Sanchez et al., 2010).

Estrogen receptors regulate gene transcription by either having positive or negative effect on the expression of target genes, the two subtypes ERα and ERβ are ligand activated receptors that work with the help of two DNA consensus elements (Sawada and Shimohama, 2003).

In general, the knowledge gathered on ER gene activation mechanisms is mainly due to studying target genes and genetic sequences like the estrogen response element (ERE) (Levy et al., 2008). The genomic mechanism could take two different routes, a direct one and an indirect one. When not bound to a ligand inactive ERs are found as monomers that associate with heat shock protein (Hsp-90) and immunophilins forming a multiprotein complex (Gruber et al., 2002). Once estrogen binds a complementary receptor the phosphorylation of its many different serine/threonine residues is induced, causes them to lose the Hsp-90 and change their conformation to promote their homo or heterodimerization and translocate into the nucleus, a hydrophobic clef is also revealed to bind transcriptional coactivators which will help by initiating a chromatin structure change in target promoters and by recruiting other aiding proteins to the DNA (Gruber et al., 2002; McKenna and O'Malley, 2002; Sukovich et al., 1994). Once inside, ERs interact with estrogen response elements (EREs) on the regulatory sequences of target genes in order to either suppress or activate their transcription while being limited to both promoter and cell-specificities (Kumar and Chambon, 1988). However, they can also make do without EREs with the help of ER-tethering and coactivation of transcription factors bound to the target DNA like the nuclear factor κB (NF κB), specificity protein 1 (SP1), cAMP-response element binding

protein (CREB) and or fos/jun transcription factors, to in turn modulate gene transcription through the activator protein-1 (AP-1) site (Kalaitzidis and Gilmore, 2005; Levin, 2002; McKenna and O'Malley, 2002; Sukovich et al., 1994).

The indirect route is initiated by disinhibitting mitogen-activated protein kinases (MAPK) (Nilsen and Brinton, 2002; Nilsen et al., 2002; Singer et al., 1999) and PKB/Akt signaling cascades (Singh, 2001), these lead to the activation of the transcriptional factor cAMP-response element binding protein (CREB) (Carlstrom et al., 2001; McEwen, 2001). In the end, the modulation of proteins that regulate apoptosis like Bcl-2 and Bad is induced.

In addition, estrogens can induce their effects without ERs interaction with the help of growth factors, which promote protein kinase activity inducing the phosphorylation of different activation sites on the receptors. However, nonnuclear estrogenic signaling is relayed by MAPK mechanisms (Carlstrom et al., 2001; McEwen, 2001) (Figure 9).

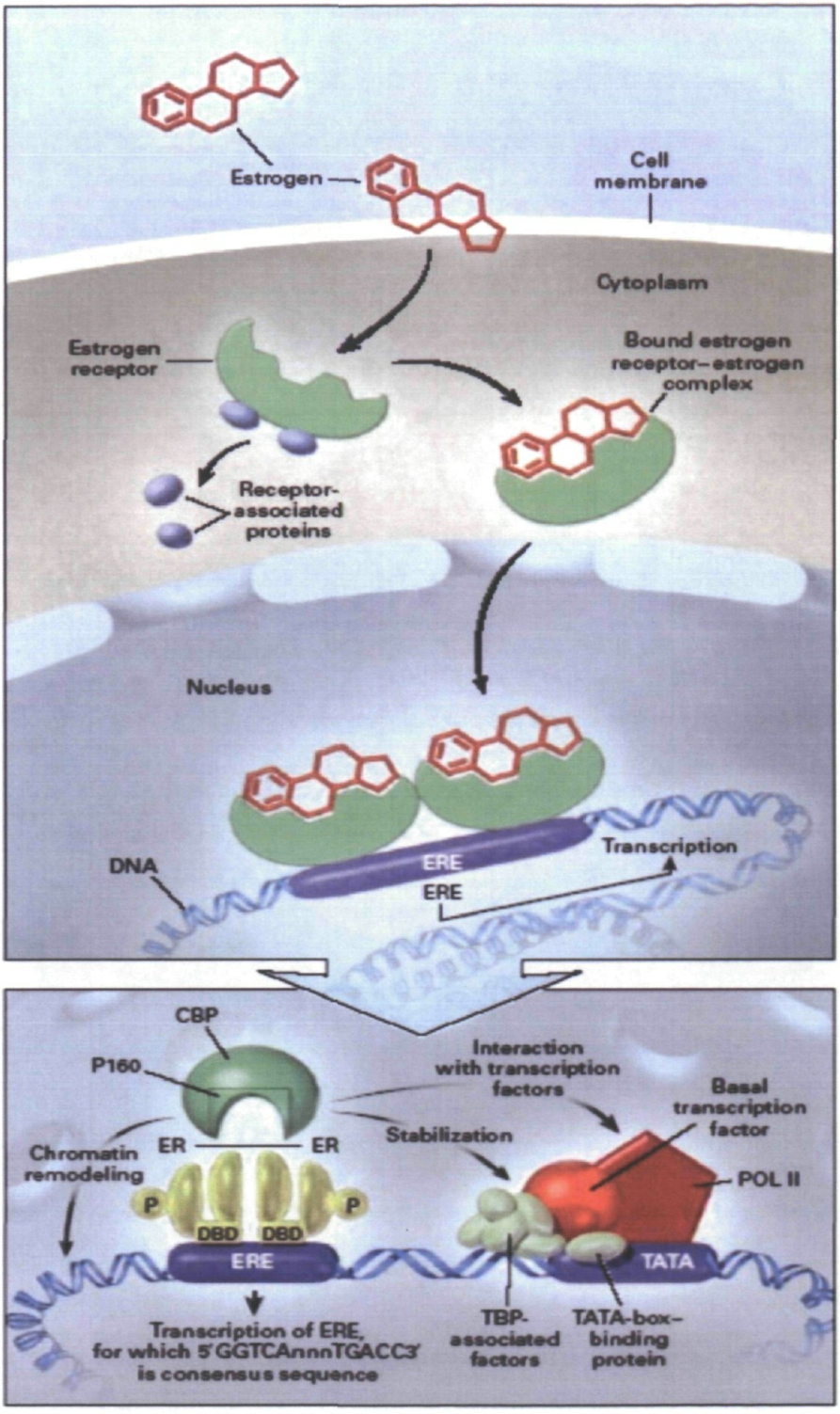

Figure 9. Once estrogens bind their receptors (ERα or ERβ), ERs are released from their bind with cytoplasmic chaperones and travel into the nucleus. Once inside the nucleus, the hormone-receptor complex binds DNA and induces transcription through RNA polymerase II (POL II), TATA-box-binding protein (TBP) and many other transcription factors finalize the preinitiation complex. Once phosphorylated ERs become active, interact with different proteins like 160-kd

steroid-receptor coactivation protein (P160) and p300-cyclic AMP response-element-binding protein (CBP) in order to form a complex that will then bind to the estrogen-response element (ERE) on ER DNA-binding domains (DBD) and initiating transcription. This figure was adapted from Gruber et al. 2002 (Gruber et al., 2002).

4. 6. *Expression of ERα and ERβ*

ERs are expressed all over the human body. ERα is predominantly expressed in bone, the liver, mammary glands, the testes and epididymis and the uterus (Drummond and Fuller, 2010; Taylor and Al-Azzawi, 2000). While, ERβ is found in the bladder, the colon, lungs and the immune system (Drummond and Fuller, 2010; Taylor and Al-Azzawi, 2000). Both ERs are expressed in the heart, vascular system, adipose tissue, prostate and the brain (Drummond and Fuller, 2010; Taylor and Al-Azzawi, 2000).

In the brain, both ERs are are expressed with expression pattern differences (Al Sweidi et al., 2012; Nilsson and Gustafsson, 2011; Weiser et al., 2008).

ERs are minutely or not at all expressed in the SN pars compacta or the striatum and this was assayed *in vivo* using by autoradiography (Shughrue et al., 2002), *in situ* hybridization (Shughrue et al., 1997a; Shughrue et al., 1997b), immunocytochemistry (Shughrue, 2004) and by Western blot (Rodriguez-Navarro et al., 2008; Schultz et al., 2009).

4. 7. *Implication of membrane ERs*

But not all estrogenic effects could be attributed to the nuclear effect of activated ERs, some effects take place within a matter of seconds or minutes, making it obvious that a route other than one implicating classic intranuclear receptor transcription modulation is taken. Membrane-associated ERs could provide reasoning for these observations, this theory gained lots of support over the past 30 years since Pietras and Szego first observed the receptors having rapid

responses to 17β-estradiol and until today evidence of their existence is due to the study of the fast nongenomic effects of 17β-estradiol (Mermelstein et al., 1996). Each ER has many splice variants (Zhao et al., 2005) and research has shown that ERα and ERβ are able to act as plasma membrane receptors (Marin et al., 2006; Mhyre and Dorsa, 2006; Zhao et al., 2005). However, Marin and colleagues conclude that membrane ERα and ERβ are homologs of the nuclear ERs (Marin et al., 2006), so ERKOα and ERKOβ mice should therefore be missing the proper membrane ERα and ERβ. There is also evidence of new plasma ERs categorized as neither ERα or ERβ (Taylor and Al-Azzawi, 2000; Wang et al., 2003) G protein coupled receptors, or having no relation to the known ERs (Zhao et al., 2004) termed membrane ER GPER1 (or GPR30).

4. 7. 1. *Membrane ER GPER1 (GPR30)*

While classic ERs provide links between the effects of steroid hormones and gene transcription, fifteen years ago, a distinct G protein-coupled ER termed GPER1 was discovered (Carmeci et al., 1997; Feng and Gregor, 1997; Kvingedal and Smeland, 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). GPER1 has been shown to be located on the plasma membrane by electron microscopy (Funakoshi et al., 2006). Immunohistological studies determined its expression in the rat CNS, high levels were observed in brain regions including the cortex, the striatum, the hippocampus and the SN (Brailoiu et al., 2007). 17β-Estradiol binds GPER1 with high affinity (Matsuda et al., 2008; Revankar et al., 2005).

V. Estrogen Receptor Knockout (ERKO) mice

Initially, research on the roles of estrogens, SERMs and ER subtype-selective agonists in PD development has relied on the use of animal models (Nilsson and Gustafsson, 2011). Neuroprotection against MPTP-induced loss of striatal DA was observed in both male and female mice (Al Sweidi et al., 2012; Bourque et al., 2009; Sanchez et al., 2012).

Again, genetically modified mouse model use genetic engineering techniques to alter the mouse genome for use in research on human diseases. These include null mutations, an extra gene copy or point mutations (Blandini and Armentero, 2012).

Gene knockin is the genetic engineering method, where a protein coding cDNA sequence is inserted in a chromosomal locus. This is commonly done in mice and what differentiates the knockin method from the transgenic technology, is that the former implicates a "targeted" gene insertion into a specific locus (Crusio et al., 2009).

Next, knockout mice are genetically engineered to have an inactivated existing gene achieved by replacement of the gene or its disruption with an artificial strand of DNA (Crusio et al., 2009).

ERKOα mice are now available since the disruption of the Esr1 gene of ERα produces healthy male and female mice that are sterile. These mice are a good model for studying oestrogen dependent cellular proliferation mechanisms, cell differentiation and metabolism in both reproductive and non-reproductive tissues. Also, the first application listed for the ERKOα mouse is to define the possibly shared or distinct actions of ERα and ERβ. Korach et al. showed that ERKOα male mice have a 10% reduction in vertebral bone density when compared to wildtypes (Korach et al., 1996). We are investigating the brain therefore this likely does not hinder our studies. Next, despite being infertile ERKOα mice have a close to normal hormonal profile and activity of the hypothalamic/pituitary axis (Couse and Korach, 1999). Ogawa et al.

observed that an alteration in neuroendocrine activities and behavioural patterning through reduced aggressive behaviour in male mice (Ogawa et al., 1998).

In the brain, Küppers et al. observed functional alterations of the SN DA system and that TH and the brain-derived neurotrophic factor (BDNF) levels are reduced in both male and female ERKOα mice (Kuppers et al., 2008). Couse et al. conclude that ERβ expression in ERKOα mice is comparable to that of wild-types (Couse et al., 1997).

As for ERKOβ mice, they have been found to be in a state of systemic hypoxia (Morani et al., 2006), since the ERβ is expressed to a high degree in type I and type II pneumocytes. A low number of alveoli (Patrone et al., 2003) and diminished lung volume (Massaro and Massaro, 2006) result from this ER's deletion.

VI. Objectives and Research Hypotheses

6. 1 *Work based hypothesis*

The study of estrogenic compounds is a growing domain since research has labeled them as neuroprotective agents (Morissette et al., 2008). In the case of PD, estrogens have been known to protect the dopaminergic system at the nigrostriatal level (Jourdain et al., 2005). However, the beneficial effects of estrogens remain far from simple; complexity is underlined by their various neuroprotective actions and numerous mechanisms involved. Hence, explaining the relevance of the still ongoing research concerning these mechanisms and new or improved neuroprotective compounds. We propose that neuroprotection arises through ERs and that the extent of the beneficial effects of estrogenic compounds is influenced by the levels and the subtype of ERs.

Susceptibility to "old age" diseases is increasing with today's higher life expectancies. In fact, the prevalence of PD is ever so slightly increasing and there still is no cure. Therapies focusing on symptomatic relief, as well as preventing or slowing PD progression are required.

Research asserts that are estrogens a multifunctional messenger and investigators, such as ourselves, speculate that estrogens could also have positive effects in the prevention or treatment of neurodegenerative diseases (Al Sweidi et al., 2012; Bourque et al., 2009). For example, estrogen have been known to increase DA receptor densities post-synaptically (Sanchez et al., 2010), have controlling effects on the DA system though the inhibition of DAT (Disshon et al., 1998) and decreasing MAO or COMT activity (Kompoliti, 2003).

Even though, gender difference in the case of PD remains a controversial topic since there is a higher rate of occurrence in men based on statistical evidence of death rates (Shulman and Bhat, 2006; Wooten et al., 2004). Analyses confirm that men and women have different symptom profiles and suffer from different severities of depression, cognitive dysfunction, dyskinesia and dysautonomia (Rajput et al., 2002). One study showed that men suffering from PD will acquire symptomatic medical attention during early stages of the disorder (Saunders-Pullman, 2003), so one could claim that the male sex experiences PD symptoms before the opposite sex or that the disease progresses more rapidly in men, thus supporting that estrogen can provide neuroprotective effects (Saunders-Pullman, 2003). In addition, research on genetic linkage suggests the existence of a PD susceptibility gene to the X chromosome (Wooten et al., 2004), which could imply that the disorder could be sex-linked. Further, gender differences were also seen in outcome studies of symptoms after stereotactic surgery for PD (Shulman and Bhat, 2006), bringing us to suggest that treating women for PD should be adjusted to hormonal changes.

Hence, all this put together explains the relevance of the still ongoing research concerning these mechanisms and new or improved neuroprotective compounds.

6. 2. *Research objectives*

The neuroprotective effects of steroidal hormones depend on the type of the steroid, the degree of denervation, the method and timing of administrating the hormonal treatment (Bourque et al., 2009). With this in mind, the hypothesis will be questioned for validity through testing with these objectives:

17β-Estradiol is worthy of research in this field of research since many have documented its significant neuroprotective effects. Thus, this research project revolves around the effects of 17β-estradiol and ERα and ERβ activity.

6. 2 .1. *Objective 1*

A first objective was to see if mice that have been knocked out for either ERα or ERβ react differently to MPTP treatment and attain more information on the effects of MPTP on ERs signaling believed to be neuroprotective. We investigated the neuroprotective contribution of ERα and ERβ against MPTP toxicity by examining DAT, the VMAT2 and TH in ERKOα and ERKOβ C57Bl/6 male mice compared to their plasma steroid levels.

6. 2. 2. *Objective 2*

Our second objective was to observe whether the two ER subtypes are active in cortical, striatal and hippocampal brain regions and then elucidate each receptor's neuroprotective contribution. In order to gain a more detailed comprehension on the neuromodulative effects of ERα and ERβ, the effects of different doses of MPTP and/or 17β-estradiol treatments on ERα and ERβ activity will be compared in this section by analyzing the levels of NMDA receptors containing NR2B subunits in these regions.

6. 2. 3. *Objective 3*

This project's third objective is to compare the neuroprotective effects of 17β-estradiol against MPTP toxicity in order to gain further information on the involvement of ERs for the modulation of DA receptors. Better elucidate if the neuroprotective mechanisms of 17β-estradiol use either ERα and/or ERβ and which pathways are initiated and also gain insight on mechanisms chosen in the sake of neuroprotection by comparing the activity of Akt/GSK3β and ERK1/2, since these proteins are thought to have roles in cell survival signaling.

6. 3. *Research hypotheses*

6. 3. 1 *Hypothesis 1*

The first hypothesis talks of 17β-estradiol induces neuroprotective effects via both ERs.

6. 3. 2 *Hypothesis 2*

Secondly, we hypothesize that both receptors are implicated in neuroprotection and modulate NMDA receptors containing NR2B subunits in different brain regions.

6. 3. 3 *Hypothesis 3*

We predict that mice which do not express the ERα are more vulnerable to the neurodegenerative effects of MPTP, since this receptor is believed to have supremacy for modulating neuroprotection and that 17β-estradiol could combat MPTP toxicity through mechanisms involving the regulation of DA receptors and Akt/GSK3β and ERK1/2 signaling.

CHAPTER II

OESTROGEN RECEPTORS AND SIGNALLING PATHWAYS: IMPLICATIONS FOR NEUROPROTECTIVE EFFECTS OF SEX STEROIDS IN PARKINSON'S DISEASE

Sara Al Sweidi*†, Maria G. Sánchez*†, Mélanie Bourque*†, Marc Morissette†, Dean Dluzen ‡

and Thérèse Di Paolo*†

*Molecular Endocrinology and Genomic Research Center, CHUQ (CHUL), Quebec City, Canada

†Faculty of Pharmacy, Laval University, Quebec City, Canada

‡Department of Anatomy and Neurobiology, Northeastern Ohio Universities College of Medicine and Pharmacy (NEOUCOM), Rootstown, OH, USA

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I. FOREWORD

Sara Al-Sweidi: I treated the mice, participated in the sacrifice and cryostat cutting of mouse brains. I did all the DAT and VMAT2 autoradiography experiments (Data seen in figures 1 and 2) and wrote the review.

Mélanie Bourque: Senior PhD student in our laboratory. She helped cut the mouse brains and participated in the autoradiography and *in situ* hybridizations experiments. She provided the results on GPER1 in the mouse striatum (Figure 3).

Maria G. Sánchez: Senior PhD student in our laboratory. She provided the results concerning GPER1 levels in the hemiparkinsonian monkey striatum (Figure 4).

Marc Morissette: Research assistant in our laboratory who had a lot to do during this project. He extracted mouse brains during the sacrifice, then dissected and prepared the brain structures for HPLC dosing then performed these. Also, he helped with the cutting of the mouse brains and the autoradiography experiments, as well as the writing of the article. He provided the data on protein kinase B (Akt) levels in the hemiparkinsonian monkey striatum (Figure 5).

Thérèse Di Paolo: Research director who undertook main decisions concerning which treatments had to be administered to the knockout mice. She planned and supervised all the protocols from start to finish and made an important contribution in the writing of this review.

II. RÉSUMÉ

Le Parkinson est une maladie neurodégénérative reliée à l'âge qui affecte plus d'hommes que de femmes. Le 17β-oestradiol, et non les androgènes, prévient la perte de la dopamine (DA) striatale observée suite une lésion avec la neurotoxine 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP) et cet effet neuroprotecteur peut impliquer les récepteurs oestrogéniques ERα et ERβ. Ceci a été démonté par une étude chez des souris mâles C57Bl/6 sauvages (WT) ou invalidées (KO) pour les récepteurs ERα et ERβ (ERKOα et ERKOβ) en analysant les concentrations des transporteurs dopaminergiques et vésiculaires, DAT et VMAT2 et la tyrosine hydroxylase (TH). Les souris ERKOβ intacte démontrent une diminution des niveaux striatales de DAT et VMAT2, les souris ERKOα étaient plus sensible au MPTP et avaient des niveaux sanguins élevés d'androgènes. Le traitement au 17β-oestradiol augmente les niveaux sanguins de 17β-oestradiol chez tous les groupes et induit la neuroprotection que chez les WT.

Nous avons également étudié le récepteur oestrogénique couplés aux protéine G (GPER1) chez la souris et chez le singe *Macaca fascicularis* femelle. Les niveaux du GPER1 augmentent dans le noyau caudé et le putamen des singes MPTP et dans le striatum des souris males lésées au MPTP ou à la méthamphétamine.

De plus, les effets neuroprotecteurs des oestrogènes impliques la signalisation anti ou pro-apoptotique, telles la protéine kinase B (Akt) et la kinase GSK3β. Chez les souris et singes intactes ou lésés au MPTP, le traitement avec le 17β-oestradiol augmente les niveaux striatales de pAkt (Ser 473)/βIII-tubulin, pGSK3 (Ser 9)/βIII-tubulin and Akt/βIII-tubulin. L'activation des récepteurs ERα, ERβ et GPER1 par les oestrogènes est importante durant la modulation de la signalisation induite par les ERs, ainsi que le développement de traitements thérapeutiques préventifs.

III. ABSTRACT

Parkinson's disease (PD) is an age-related neurodegenerative disorder with a higher incidence in the male population. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, 17β-oestradiol but not androgens were shown to protect dopamine (DA) neurones. We report that oestrogen receptors alpha and beta (ERα and ERβ) distinctly contribute to neuroprotection against MPTP toxicity as revealed by examining the membrane DA transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) and tyrosine hyroxylase (TH) in ER wild type (WT) and knock out (ERKO) C57Bl/6 male mice. Intact ERKOβ mice had lower levels of striatal DAT and VMAT2, while ERKOα mice were the most sensitive to MPTP toxicity when compared to WT and ERKOβ mice and had the highest levels of plasma androgens. In both ERKO mice groups treatment with 17β-oestradiol did not provide neuroprotection against MPTP in spite of elevated plasma 17β-oestradiol levels. Next, the recently described membrane G protein-coupled oestrogen receptor (GPER1) was examined in female *macaca fascicularis* monkeys and mice. GPER1 levels were increased in the caudate nucleus and the putamen of MPTP-monkeys and in the male mouse striatum lesioned with methamphetamine or MPTP. Moreover, neuroprotective mechanisms in response to oestrogens transmit through Akt/GSK3 signalling. The intact and lesioned striata of 17β-oestradiol treated monkeys, like that of mice, had increased levels of pAkt (Ser 473)/βIII-tubulin, pGSK3 (Ser 9)/βIII-tubulin and Akt/βIII-tubulin. Hence, ERα, ERβ and GPER1 activation by oestrogens is imperative in the modulation of ER signalling and serve as a basis for evaluating nigrostriatal neuroprotection.

IV. INTRODUCTION

Numerous studies (1-7), a meta-analysis (8) and reviews (9, 10) indicate that Parkinson's disease (PD) is more prevalent and has a larger incidence in the male population. A meta-analysis of seven studies that used a stringent inclusion criteria reported that overall PD is 1.5 times greater in men than in women (8). These authors included as possible reasons for this increased risk of PD in men than women toxicant exposure, head trauma, neuroprotection by oestrogens, mitochondrial dysfunction and/or X linkage genetic risk factors (8).

Gender differences were reported during the progression of PD and in the responses to L-dopa treatment (11-13). In the case of PD, oestrogens have been known to protect the nigrostriatal dopaminergic pathway (14, 15). However, the beneficial effects of oestrogens remain far from simple; complexity is underlined by their various neuroprotective actions and numerous mechanisms involved. Hence, research continues concerning these mechanisms and the development of new or improved neuroprotective compounds. We propose that oestrogenic neuroprotection arises through oestrogen receptors (ERs), is influenced by their subtype and review their implication in neuroprotection as derived from results from our laboratory using lesioned primates and mice models of PD and current literature.

V. PD

Characteristics, cause and symptoms of PD

PD is a neurodegenerative disorder that has become more prevalent over the years due to the aging population (16). This disease has no documented etiology, however it is characterized as chronic and progressive with the loss of dopamine (DA) containing neurones in the brain substantia nigra (SN) being a main feature in its neurodegenerative process (16). The disorder is also referred to as "idiopathic" PD since no known cause is documented, yet some cases may be

due to toxicity, drugs, genetic mutation, head trauma, or other medical disorders (16). PD is also characterized as 'sporadic' with no known genetic background in 90-95% of cases and environmental factors or genetic susceptibilities are believed to have a hand in triggering the disease (16).

What makes PD so infamous is the fact that symptoms appear after a myriad of neurones are lost, with a death toll of dopaminergic neurones going over a critical threshold between 70-80% in the SN (16). It is hard to diagnose before the damage becomes irreversible due to compensatory mechanisms (16). Disruption in DA transporters which are responsible for controlling DA concentrations and include the DA transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) are thought to play a role in this pathology (17).

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD

MPTP provides a model of PD due to its specific neurotoxicity in certain species such as mice and monkeys (18, 19). It was discovered as a by-product in the chemical synthesis of a meperidine analog with potent heroin-like effects (19). The irreversible effects of MPTP in man induce tremor, slowness of movement, rigidity, freezing and postural instability, thus mimicking almost all features of PD (19). MPTP enters the brain through the presynaptic DA uptake system where it is converted into the 1-methyl-4-phenylpyridinium (MPP^+) ion by monoamine oxidase B (19). MPP^+ is a very potent neurotoxin which inhibits mitochondrial oxidation of NAD^+-linked substrates. It accumulates inside mitochondrial matrixes and inhibits respiration (19) resulting in death of nigrostriatal nerves through mechanisms that include the loss of calcium homeostasis and formation of radicals which promote cytotoxic events leading to limited DA release, locomotor defects and finally apoptosis (19).

Oestrogens and PD

Several reports indicate that PD has a later age at onset in women and that men are more susceptible (reviewed in (15). Clinical studies demonstrate that there is an increased risk of developing PD under conditions of an early decrease in natural endogenous oestrogens (20, 21). A 2-week double-blind cross-over study, on postmenopausal women suffering from mild to moderate PD, showed that transdermal treatment with a high dose of 17β-oestradiol reduces the antiparkinsonian threshold dose of levodopa (22). Oestrogens were observed to have modulatory effects on PD symptoms and levodopa-induced dyskinesias (23-25) and motor disabilities were remedied by oestrogens in postmenopausal women with PD (26). On the basis of evidence from a case-control design study of 133 female PD cases and 128 female controls, it was concluded that women who took a postmenopausal oestrogen treatment had a lower risk of developing PD (27). Men with PD show symptoms requiring medical attention during earlier stages of the disorder (21) suggesting that the disease progresses more rapidly in men, thus supporting that oestrogen can provide neuroprotective effects (21). Further, gender differences in symptoms were also seen in outcome studies after stereotactic surgery for PD (10).

VI. ERs

Classic ERs

Currently, there are two main documented ER subtypes (28). The transcriptional information for these two receptors lies on two different genes (28). In fact, twenty splice variants have been documented for ERα and ten for ERβ (28). There is little or no conservation in their N-terminal region, but they have a homology of 55% in their ligand-binding domains and are 95% homologous in their DNA-binding domains (28). Oestrogens may differ in their affinity for either ER subtype. For example 17β-oestradiol binds equally with both ERα and ERβ, while oestrone

and the selective oestrogen receptor modulator (SERM) raloxifene preferentially bind ERα and oestriol has more affinity for ERβ (29). Each ER has its own nonsteroidal agonist (30, 31), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) is selective for ERα and 2,3-bis(4-hyroxyphenyl) propionitrile (DPN) for ERβ.

ERα is present in high levels within the cardiovascular system, bone, mammary glands and the uterus, while ERβ is found mainly in the urinary tract, prostate and ovaries (32, 33). At the cellular level, the nucleus is mainly home to ERα whereas ERβ is reported to be localized in the cytoplasm (34). In the CNS, these two receptors have similar patterns of expression in the preoptic area, the cortical amygdaloid nuclei and the bed nucleus of the stria terminalis. In rodents, ERα expression is found exclusively in the ventromedial hypothalamic nucleus and the subfornical organ (35) and it is predominant in mouse hippocampus (36) while ERβ is predominant in rat hippocampus and the cerebral cortex (35). Immunohistochemical and *in situ* hybridization data show that ERα and ERβ are present in rat SN (37). In addition, Kritzer et al. showed the colocalization of ERs and TH in the ventral tegmental area and SN of the rat brain (38) and later specified that ERβ is present on collateral SN pars compacta projections to the ventral striatum (39). While Küppers et al. claim that both ERα and ERβ mRNAs are expressed in GABAergic neurons of the striatum (40).

Nuclear ER activity

ERs regulate gene transcription by exerting positive or negative effects on the expression of target genes. ERα and ERβ are ligand-activated receptors that work with the help of two DNA consensus elements (41). These receptors regulate gene transcription so they are part of a vast family of proteins called ligand-activated transcription factors. Their actions have been

documented to include either genomic or nongenomic mechanisms and the fact that both ERs are found in dopaminergic neurones of the midbrain, although in low abundance, implies that the adult nigrostriatal system is targeted by oestrogens (40, 42). The genomic mechanism could take two different routes, a direct or an indirect one. When not bound to a ligand, ERs are found as monomers that associate with heat shock protein (Hsp-90) and immunophilins forming a multiprotein complex (43). Once oestrogen binds a complementary receptor, the phosphorylation of its many different serine/threonine residues is induced, which causes them to lose the Hsp-90 and change their conformation to promote their homo- or heterodimerization and translocation into the nucleus. A hydrophobic clef is also revealed to bind transcriptional coactivators that help by initiating a chromatin structural change in target promoters (44, 45). Once inside, ERs interact with oestrogen response elements (EREs) on the regulatory sequences of target genes in order to either suppress or activate their transcription while being limited to both promoter and cell-specificities (46). However, they can also function without EREs with the help of ER-tethering and coactivation of transcription factors bound to the target DNA like the transcriptional factor cAMP-response element binding protein (CREB). They can also interact with *fos/jun* transcription factors were they can regulate transcription through activator protein-1 (AP-1) (44, 47-49).

The indirect route is initiated by disinhibiting mitogen-activated protein kinases (MAPK) (50, 51) and protein kinase B (PKB/Akt) signalling cascades (52), thus leading to the activation of CREB (53, 54). Ultimately, the modulation of proteins like Bcl-2 and Bad that regulate apoptosis is induced.

Membrane ERs

Genomic mechanisms require hours to run their course since they involve the transcription and translation of oestrogen regulated genes (55). But not all oestrogenic effects are attributable to nuclear ERs, some effects occur within a matter of seconds or minutes, making it obvious that a route other than one implicating classic intranuclear receptor transcription modulation has transpired. Membrane-associated ERs could provide the basis for these observations. This theory gained support over the past 30 years since Pietras and Szego first observed receptors as having rapid responses to 17β-oestradiol (56) and continue today with evidence of their existence due to the fast nongenomic effects of 17β-oestradiol. While ERα and ERβ are both able to act as plasma membrane receptors (57), there is also evidence of new plasma ERs categorized as G protein-coupled receptors (GPCRs), which have no relation to the known ERs (58). How ERs are brought to the plasma membrane is not well established but research on non-neuronal cells supports the theory that ER palmitoylation could be a mechanism (59, 60). Here, a post-translational addition of a 16-carbon fatty acid (palmitate) to ER ligand-binding domain residues enables targeting of ERs to the plasma membrane (59, 61).

The cloning of a G protein-coupled ER named GPER1 (also known as GPR30) was reported over 15 years ago by several groups (62-67). GPER1 is distinct from the classic ERα and ERβ and has been shown with electron microscopy to be located on the plasma membrane (68). Its expression in the rat CNS, as determined through immunohistological studies, is high in brain regions including the cortex, the striatum, the hippocampus, the SN, the Islands of Calleja, the hypothalamic-pituitary axis and the brainstem autonomic nuclei (69), while its cellular localization also includes the endoplasmic reticulum and the golgi apparatus (70, 71). GPER1 has a high affinity for 17β-oestradiol but 17α-oestradiol failed to significantly displace [^{3}H]oestradiol

binding to GPER1 (70, 71). The competitive binding assay shows that GPER1 does not bind cortisol, progesterone or testosterone while oestrone, oestriol had very low affinities for the receptor (72); tamoxifen, genistein and ICI 182,780 are agonists on GPER1 (73). Hammond and colleagues observed that GPER1 is expressed (0.4-42%) in GABAergic neurons and that it is colocalized in 63-99% of cholinergic neurons in the basal forebrain (74). However, Mufson et al. and Shughrue et al. saw that approximately 30% of the cholinergic neurones also contain ERα (75, 76). In mice, another membrane ER, dubbed ER-X, was found to be expressed in the neocortex, in lung plasma membrane microdomains associated with caveolin, and in the uterus (58, 77, 78).

VII. OESTROGENS, ERs and NEUROPROTECTION

It is well established that aging results in a decline in the production of the female hormone oestrogen, with the most drastic decrease at menopause (79). In addition, it has been observed that with aging negative effects on ERα mediated events are believed to attenuate ER functioning by increased methylation of the ER gene (80, 81). In age-related animal models of neurodegenerative diseases, numerous studies show that oestrogens play an important protective role and accumulating evidence implicates ERs.

It is expected that nuclear ER concentrations in man diminish with aging as reported in rats (82). However in humans, postmortem tests show complex age-related alterations of the canonical ERα and various ERα splice variants in the brain and the expression pattern of certain forms is brain area-specific (83). In the vasopresssinergic supraoptic nucleus and the hippocampus ERα was reported to increase with advancing age in women with higher expression in postmenopausal than in the pre- and perimenopausal women (84). ERα splice variant del.7 (deletion of exon 7)

and del.2 (deletion of exon 2) declined with advancing age (61-84 years old) in the mamillary body but not in the hippocampus (83) whereas no change was observed in an other study for 12 exon-skipping variants with the most common form found being del.5, del.7 and del.2 in people from 29 to 59 years of age (85). The del.7 is a dominant negative variant that can inhibit transcriptional activity of both ERα and ERβ by forming heterodimers (86). Del.4 was observed in the caudate nucleus, putamen and SN of a 71 year old female Alzheimer disease patient but the effect of aging was not reported (83). Del.4 is suggested to be a silent variant without activity of its own but a dominant negative function was ascribed to this slice form through protein-protein interactions with ERα (87). Hence, higher levels of ERs with hindered activity are seen in some brain regions as a function of aging (88).

The effects of MPTP on dopaminergic markers

VMAT2 packages serotonin, histamine, epinephrine, norepinephrine and DA into vesicles and is mainly confined to the CNS (89). The DAT is a specific protein of DA neurons (90). DAT and VMAT2 specific binding under pathological conditions are used as markers to evaluate DA cell body and terminal integrity. Neurotoxicity of SN DAT specific binding and mRNA are less severe than that seen in the striatum of MPTP and methamphetamine lesioned mice (14, 91), suggesting that the presynaptic DA terminals of the striatum are more vulnerable. Accordingly, high doses of neurotoxins are needed to affect SN TH mRNA levels (92). In fact, cell body destruction requires elevated doses of toxins whereas cell terminals are lost with lower doses of the same neurotoxins (92). Hence, different stages exist where neurones are injured but not dying or dead when neuroprotection is feasible by steroids, and in humans oestrogens are only beneficial before starting levodopa therapy in the early stages of PD (93).

Nigrostriatal DA activity regulation depends on DA availability which in turn is controlled by DAT and VMAT2 present in neurons. A role for DAT in astrocytes is also reported as Karakaya et al. observed that DAT is expressed in neonatal astrocytes and 17β-oestradiol dose-dependently down-regulated DAT mRNA by 80% and 60% in the neonatal midbrain and striatal astroglia cultures, respectively (94). It was also noted that 17β-oestradiol inhibits the clearance of extracellular DA by 45% and 35% in the neonatal midbrain and striatal astroglia cultures; this effect was abolished with the use of an ER antagonist ICI 182 780 (94). These authors concluded that the effects of 17β-oestradiol on DAT could be neuroprotective under pathological conditions since the end result is delayed DA uptake by astroglia (94). Thus, 17β-oestradiol induced decrease of astroglial DA uptake may diminish DA metabolisation resulting in increased availability of synaptic DA and subsequently more DA for recycling by neurons. VMAT2 was not detected in astrocytes (94).

Oestradiol neuromodulation and neuroprotection

Oestrogens modulate the nigrostriatal and mesolimbic DA systems' activity at various components of neurotransmission (95-97). We have shown by biochemical and pharmacological studies that 17β-oestradiol can modulate DA receptors (98) and DAT (99-101). Striatal and nucleus accumbens DA D2 receptor and DAT density are increased with chronic 17β-oestradiol treatment without affecting their mRNA levels, implying that 17β-oestradiol activity was non-genomic (102, 103).

Oestrogens produce their modulatory effects through pro- or anti-dopaminergic activity such as on enzymes that synthesize or degrade DA, DAT, VMAT2, DA receptors and DA release (15). In the striatum of ovariectomised rats, DAT density fluctuations are observed during the oestrous

cycle (97) and 17β-oestradiol treatment increased DAT density through acute and chronic treatment (101, 104), while reductions in SN DAT mRNA levels in ovariectomised rats were restored with oestrogens (99).

Postmenopausal women given an oestrogen replacement therapy had increased DAT density in the left anterior putamen (105). There is less data on gonadal hormone modulation of VMAT2, in the rat brain, 17β-oestradiol treatment had no effect on its striatal density (106) and in another study in the SNc chronic 17β-oestradiol treatment did not affect VMAT2 mRNA (107).

Estrogen provide relief from PD symptoms if treatment is given at early stages of the disease (93). Hence, to model this stage of PD we used conditions of moderate nigrostriatal DA loss in MPTP mice when motor behavior is not yet impaired nor is there significant DA cell death. Thus, under conditions of early nigrostriatal DA neuronal degeneration, MPTP mice show a significant reduction of striatal DA concentrations and DA transporter loss. The neuroprotective effects of 17β-oestradiol, when administered prior to the MPTP regimen, are observed upon the prevention of DA and dopaminergic metabolite depletions (108-111), as well as DAT and VMAT2 specific binding loss (112). Also, 17β-oestradiol treatment promotes an increase in TH immunoreactive neurones of the SNc of male mice (113). The neuroprotective effects of 17β-oestradiol upon MPTP are achieved with pretreatment at low doses mimicking physiological levels in male and female mice but treatment with high doses does not prevent MPTP neurotoxicity (114). Oestrogens are thought to convey their neuroprotective effects through genomic mechanisms which signal through ERs or by using non-genomic mechanisms through membrane bound receptors (115-117). Again, we propose that these neuroprotective effects are mediated via an interaction with ERs. This is supported by the observation that 17α-oestradiol, which has a low ER affinity, does not induce neuroprotective effects (91). Moreover, the weak ER agonists

oestrone and oestriol (91) have weak or no neuroprotective potentials against dopaminergic loss caused by MPTP (118).

ERα and ERβ could have distinct roles in neuroprotection against MPTP toxicity (119), with ERα being the dominant receptor involved in neuroprotection (120, 121). In fact, it is speculated that ERβ plays a less dominant role in neuroprotection because its activity is optimal once cellular death is inhibited and regeneration and neurogenesis commence (122). Dubal et al. show that, in a cerebral ischemia model, 17β-oestradiol treatment does not protect the cortex or striatum in ERKOα mice when compared to wild type (WT) and ERKOβ mice (120). Our group has demonstrated that PPT but not DPN provides neuroprotection against MPTP (123, 124). Similarly, PPT but not DPN protects against β-amyloid peptide in cerebrocortical neuronal cultures through a PKC-dependent signalling pathway (125). Others claim that ERβ plays a role in neuroprotection. For example, Carswell et al. provide data suggesting that DPN but not PPT pretreatment reduces ischemic damage in the striatum and CA1 region of the hippocampus (126) and Westberg et al. report that ERβ gene polymorphisms could influence the age of onset of PD (127). We reported that treatment with DPN or 17β-oestradiol but not PPT modulates D2 DA receptors in ovariectomised rats (102), while data from a study with ERKOα and ERKOβ male mice suggest that ERβ affects DA metabolism since ERKOβ mice had a lower DA turnover rate (119). 17β-oestradiol was able to prevent the loss of 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid ([^{125}I]-RTI-121) binding to DAT and [^{3}H]-dihydrotetrabenazine ([^{3}H]-TBZ-OH) binding to VMAT2 in the striatum and SN of MPTP and methamphetamine lesioned mice (114). Moreover, the SN decrease of these transporters' mRNA produced by MPTP was prevented by oestrogen (91). We have also examined the neuroprotective contributions of ERα and ERβ

against MPTP toxicity by examining DAT, VMAT2 and TH in ER knock out (ERKO) C57Bl/6 male mice.

Our results show striatal DAT and VMAT2 levels of intact ERKOβ mice to be lower than WT and ERKOα mice whereas ERKOα had elevated plasma androgens concentrations (two-way ANOVA shows an effect of genotype $p<0.0001$; mean ± SEM, testosterone: WT= 3.5 ± 0.9 ng/ml, ERKOα = 12.5 ± 1.0 ng/ml $p<0.00001$ vs WT and ERKOβ = 6.1 ± 1.2 ng/ml; dihydrotestosterone: WT= 114 ± 32 pg/ml, ERKOα = 997 ± 117 pg/ml $p< 0.0001$ vs WT and ERKOβ =149 ± 59 pg/ml) (128). This is in agreement with a previous report showing a significant increase of testosterone in ERKOα male mice when compared to WT males (129). Despite being infertile, ERKOα mice have a close to normal hormonal profile and activity of the hypothalamic/pituitary axis (130), while ERKOβ mice have been found to be in a state of systemic hypoxia (131). Functional alterations of the SN DA system as well as reduced TH and brain-derived neurotrophic factor (BDNF) levels are observed in both male and female ERKOα mice (132). ERKO could affect the maturation of other components of brain DA transmission such as the DAT and VMAT2, but this possible effect has not been verified.

In WT and ERKO mice, MPTP caused a dose-dependent loss of both striatal transporters (Figure 1) that correlated with their previously reported reductions in striatal DA concentrations (119) (DAT: $R= 0.755$, $p<0.0001$ and VMAT2: $R= 0.787$, $p< 0.0001$). Compared to WT and ERKOβ, DAT, VMAT2 and TH showed a greater sensitivity to MPTP in ERKOα mice (Figure 1 and data not shown). WT mice were compared to ERKO mice pretreated with 17β-oestradiol alone and/or with an effective dose of MPTP. The striatum and SN of ERKOα mice were more vulnerable to MPTP toxicity and 17β-oestradiol protected against this toxicity only in WT mice (Figure 2 and data not shown) in spite of similar plasma 17β-oestradiol concentrations among the three

genotypes (two-way ANOVA shows an effect of 17β-oestradiol treatment $p<0.0001$; mean ± SEM in pg/ml, WT: vehicle: 2.2 ± 1.5 and 17β-oestradiol treated: 10.0 ± 1.4, ERKOα: vehicle: 4.3 ± 1.4 and 17β-oestradiol treated: 11.2 ± 2.0 and ERKOβ: vehicle: 3.3 ± 0.8 and 17β-oestradiol treated: 10.2 ± 1.2). Hence, even though the lack of the ERα caused a more significant susceptibility to MPTP toxicity both ERα and ERβ were shown to be implicated in neuroprotection resulting from 17β-oestradiol.

The absence of ERα and ERβ throughout development in ERKO mice could affect brain organisation and may result in a different adult brain. However, our results on WT mice showing that PPT but not DPN protects striatal DA against MPTP toxicity (123, 124), are in agreement with our results demonstrating a greater susceptibility of the nigrostriatal DA system of ERKOα mice to MPTP (128). Hence, the ERKO mouse model is a valid tool for the study of the role ERs in nigrostriatal DA neuroprotection and supports results using ER specific agonists.

Neuroprotective implication of membrane ERs

Each ER has many splice variants (133) and research has shown that ERα and ERβ are able to act as plasma membrane receptors (133-135). There is also evidence of new plasma ERs categorized as neither ERα nor ERβ (136, 137). These, so called, GPCRs have no relation to the known ERs (138). However, Marin and colleagues conclude that membrane ERα and ERβ are homologs of the nuclear ERs (134). If correct, then membrane ERα and ERβ should be absent in ERKOα and ERKOβ. There is evidence that intracellular ERα and ERβ are transported to the membrane and their interaction with metabotropic glutamate receptors provides many possibilities for membrane associated 17β-oestradiol cell signalling mediation (139). On the cellular membrane, ERα and ERβ activity resembles that of GPCRs and oestradiol modulates membrane associated ERα and

ERβ by inducing their internalization (139). While some controversies underlying its localization and activity still exist (61), it seems clear that GPER1 mediates rapid as well as transcriptional oestrogenic activity in the brain and periphery (73). GPER1 activity is manifested through two plasma membrane associated enzymes, the first is Gs-protein which induces adenylyl cyclase promoting elevated intracellular concentrations of cAMP, the second being Gβγ, results in calcium mobilization and kinase activation (140).

However, the role of GPER1 in the brain DA systems and neurodegenerative disorders has yet to be determined. Potential tools for elaborating the physiological activities of this new ER in the brain include the GPER1 specific agonist, G1 and antagonist, G15 both of which exist without any activity on ERα or ERβ (141, 142). As noted above, a vast amount of literature provides evidence that oestrogens have positive effects on the DA system in the brain and their classical mechanisms on nuclear ERs should be studied along with their potential GPER1 activity. Therefore, we sought to assess the distribution of striatal GPER1 and its response to oestradiol and lesions. High levels of GPER1 were measured in the striatum of male mice and were increased by MPTP lesion (Figure 3). Moreover, we compared methamphetamine-induced neurotoxicity on striatal GPER1 in male and female mice; methamphetamine, which produced significantly decreased striatal DA in males, increased striatal GPER1 levels in male, but not in female mice (143). Ovariectomised female *macaca fascicularis* monkeys with a unilateral MPTP lesion of the nigro-striatal pathway that received a chronic 17β-oestradiol or vehicle treatment for one month were next studied. The lesioned striata of these monkeys were extensively denervated as indicated by reductions in DA concentrations (144). GPER1 levels were abundant in the monkey striatum both in the caudate nucleus and the putamen at the two rostro-caudal levels measured. Similar levels of GPER1 were found in the anterior versus posterior caudate nucleus

and putamen of monkeys (Figure 4). GPER1 levels were higher in the putamen, but not the caudate nucleus, in the MPTP-lesioned side compared to that of intact-side of hemiparkinsonian monkeys. 17β-oestradiol treatment did not significantly change GPER1 levels in the intact or lesioned caudate nucleus and putamen of monkeys (Figure 4). The present results show that GPER1 is abundant in the striatum of monkeys and mice and is increased in response to toxins that target the nigro-striatal pathway.

VIII. Akt and GSK3 SIGNALLING PATHWAYS

Akt signalling in mental and neurodegenerative diseases

In addition to the classical functions of DA receptor-cAMP-dependent mechanisms, striatal DA D2 receptors can also exert physiological effects via PKB (Akt) (145). Akt can be activated following binding of neurotransmitters or growth factors on many specific cell-surface receptors, which in turn initiate a cascade of second messengers related to the phosphatidylinositol 3-kinase (PI3K) pathway (146). Deactivation of Akt after dephosphorylation results in activation of the glycogen synthase kinase-3 (GSK3) mediated signal (147). Dysregulation of Akt/GSK3 signalling is involved in many DA-associated neurological and neuropsychiatric disorders. Reduced Akt activity or expression levels were shown in brains of schizophrenic patients (148, 149) and there are data indicating an involvement of GSK3β in depression and psychosis (150). A report showed an association between Akt1 gene and PD that was a protective haplotype (151). In sections of post-mortem SN, an extensive reduction of pAkt(Thr308) and pAkt(Ser473) in PD patients was observed compared to controls (152).

The implication of PKB/Akt in 17β-oestradiol induced neuroprotection

The PI3-K/Akt and MAPK signalling pathways are associated with 17β-oestradiol activity in the brain (114). ERs relay MAPK signals through sequential activation of Ras, B-raf, MAPK/ERK kinase (MERK1/2), and MAPK (ERK1/2) in order to finally induce various transcriptional factors that promote neuronal survival (115, 135, 153, 154). The effector Akt can be activated via the PI3-K pathway through ERs (123). Akt activity promotes cell survival by modulating the expression of anti-apoptotic proteins like Bcl-2 and apoptotic proteins like Bad and Bax (123) and signalization converges at GSK3β. GSK3β is a highly expressed kinase in the brain and once activated functions in inducing cellular death pathways, therefore its inactivation favours the promotion of cellular survival mechanisms (123). The activity of this kinase is proapoptotic, it is inhibited if phosphorylated on certain serine residues, thus promoting cell survival (155). Moreover, activation of these signalling pathways through either membrane-associated or genomic actions of 17β-oestradiol could be combined and act synergistically in injured neurones to amplify the neuroprotective process (156).

Data from several sources indicate that the MAPK pathway and ERβ contribute to cell survival signalization pathways in various models of neuronal injury (126, 157-159). However, Kahlert and colleagues have observed that ERα is involved in the activation of Akt signalling (160, 161), while ERβ is not (162). Our group in collaboration with L.M. Garcia-Segura documented that treatment with 17β-oestradiol increased phosphorylated PKB/Akt (at serine 473) levels in mice (123). We also observed that GSKβ3 phosphorylation on serine 9 was highly diminished in MPTP mice and that PPT treatment significantly blocked GSK3β activation, but not the treatment with 17β-oestradiol or the ERβ agonist Δ5-diol (123). Only PPT treatment had positive effects by increasing the levels of inhibited GSK3β in MPTP treated mice, and in intact wild-type

mice it was 17β-oestradiol and PPT treatments which increased the levels of activated PKB/Akt and deactivated GSK3β (123). These results support a role for the ERα in the PI3-K pathway as associated with the neuroprotective effects of oestrogenic compounds against MPTP. Moreover, GPER1 activated by 17β-oestradiol was also shown to initiate PI3-K signaling and Akt activity (70, 71).

No data are yet available on the oestrogenic modulation of this signalling pathway in monkeys. We thus measured the effect of a one-month treatment with 17β-oestradiol on the Akt/GSK3 signalling pathway in the brain of ovariectomised monkeys with a unilateral MPTP lesion of the nigro-striatal pathway. 17β-oestradiol treatment induced an increase of pAkt(Ser 473)/βIII-tubulin in the intact and lesioned posterior caudate nucleus and in pGSK3β(Ser 9)/βIII-tubulin in the intact and lesioned anterior putamen compared to vehicle-treated monkeys. In the intact and lesioned putamen, the Akt/βIII-tubulin was also increased in monkeys treated with 17β-oestradiol whereas GSK3β/βIII-tubulin remained unchanged (Figure 5 and 6). These translational results in monkeys, such as our previous findings in mice (123) suggest that activation of the Akt/GSK3 signalling pathway is involved in the 17β-oestradiol effect on the striatal DA system and support a beneficial role of oestrogenic treatment resulting from an increase in the activity of signalling pathways implicated in cell survival.

IX. CONCLUDING REMARKS

The combinations of the effects of nuclear ERα and ERβ and membrane GPER1 signalling pathways are reported to result in cell cycle progression and cell proliferation (73), which is sought as being beneficial in the injured or neurodegenerative brain. ERβ is reported to modulate ERα mediated transcription in mice, therefore in certain tissues and under certain conditions

these ERs are interdependent (163). Moreover, ERα and GPER1 were reported to crosstalk (73). There exists great diversity in possible synergistic or antagonistic interactions between ERs and GPER1 (73) and both membrane and fast transcription activity of GPER1 are reported as leading to the activation of genes like c-*fos* (164).

In summary, a complex cascade of genomic and non-genomic oestrogen induced activity results in neuroprotection, which in turn relies on the neuroanatomical and spatio-temporal organization of ERs and various signalling pathway molecules involved in their crosstalk in different neuronal populations. Oestrogens and oestrogenic drugs modulate and protect nigrostriatal DA activity and our results propose that ERs are implicated in these effects. A role for ERα and its agonists is observed in neuroprotection, while the novel GPER1 could provide a new target to modulate the nigrostriatal DA system. We also demonstrate that the Akt/GSK3 signalling pathway is modulated by oestrogens in intact and MPTP lesioned mice and monkeys. The implication of ERα, ERβ and GPER1 on modulation of nigrostriatal DA activity support the development of a new generation of ER specific drugs for the brain.

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XI. FIGURES

Figure 1.

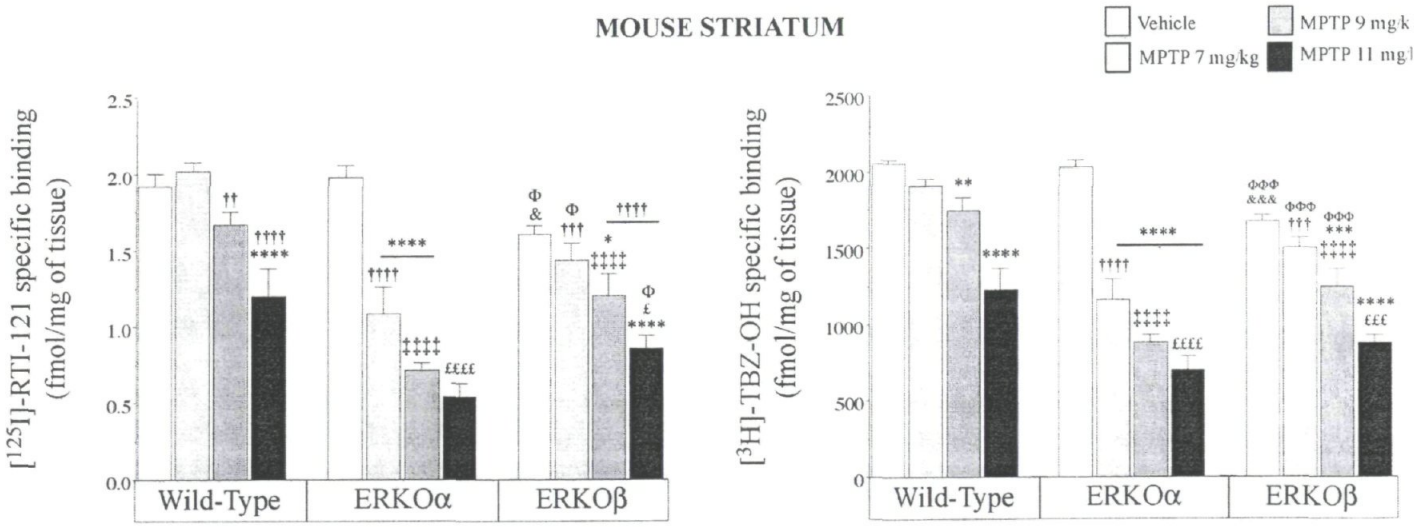


Figure 2.

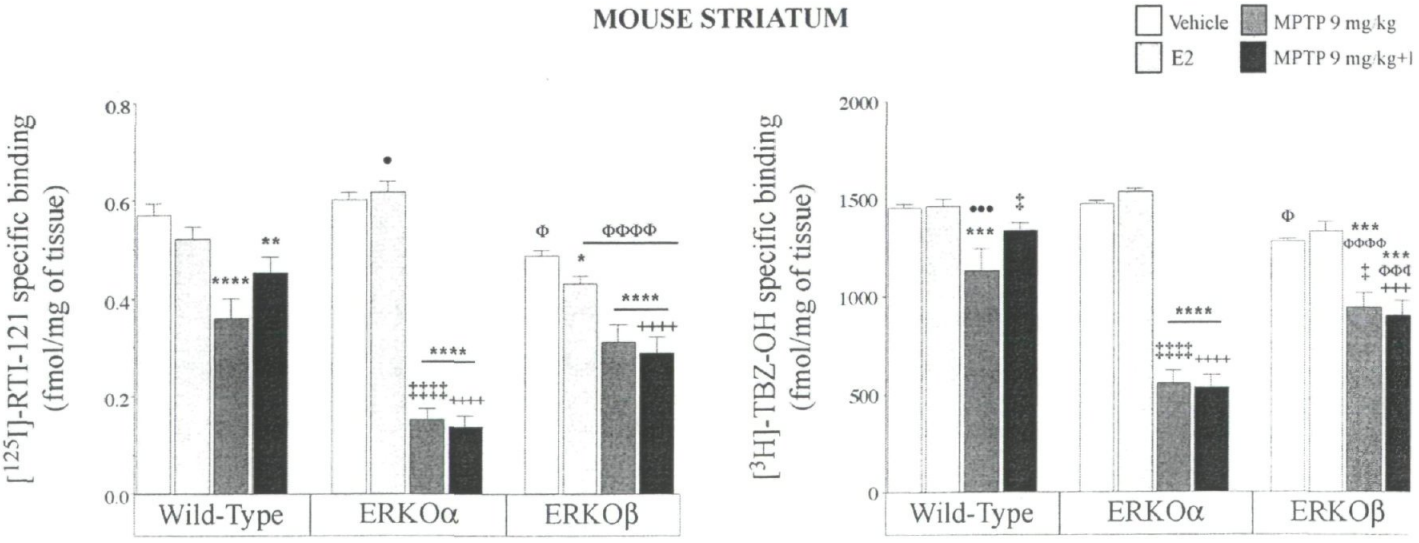


Figure 3.

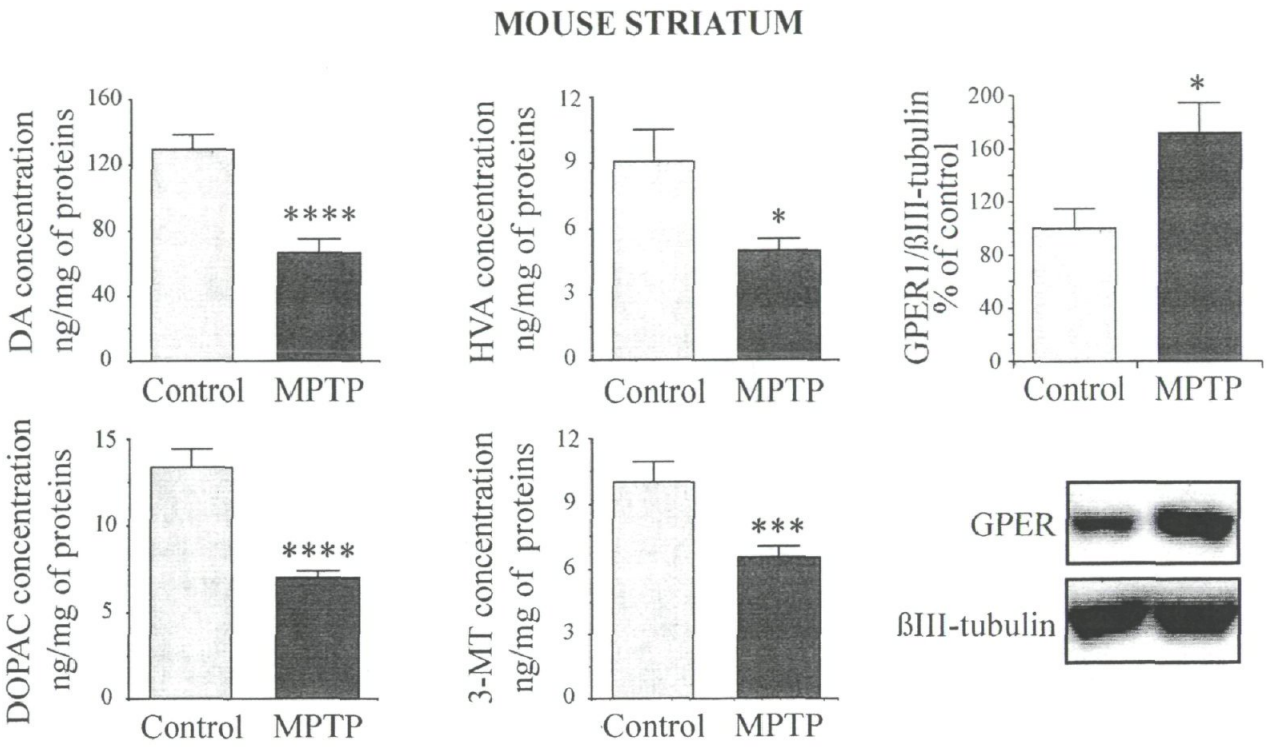

MOUSE STRIATUM
DA concentration ng/mg of proteins
160
120
80
40
0
****
Control
MPTP
HVA concentration ng/mg of proteins
12
9
6
3
0
*
Control
MPTP
GPER1/ßIII-tubulin % of control
200
160
120
80
40
0
*
Control
MPTP
DOPAC concentration ng/mg of proteins
15
10
5
0
****
Control
MPTP
3-MT concentration ng/mg of proteins
12
9
6
3
0
***
Control
MPTP
GPER
ßIII-tubulin

Figure 4.

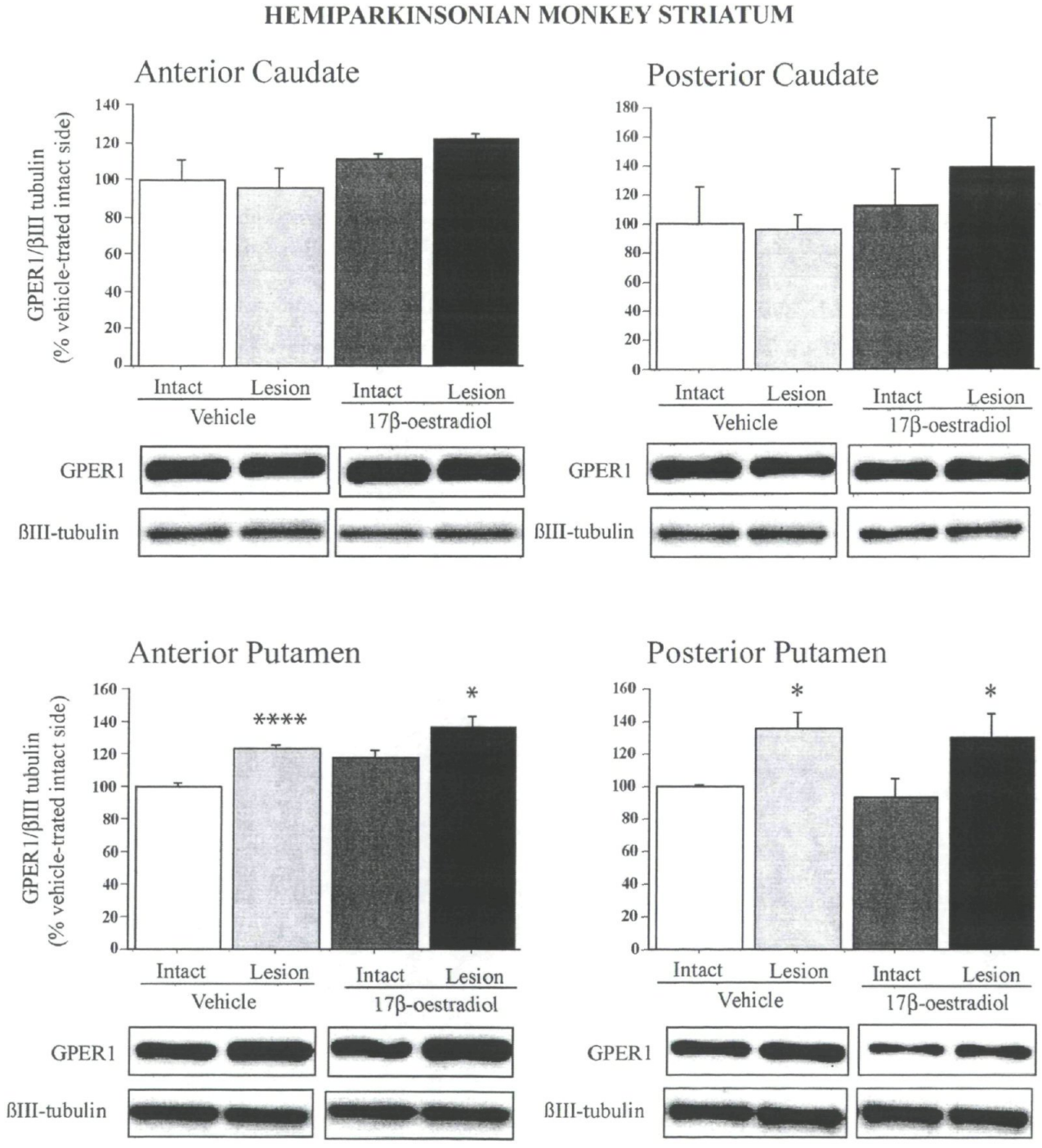

HEMIPARKINSONIAN MONKEY STRIATUM
Anterior Caudate
Posterior Caudate
Anterior Putamen
Posterior Putamen
GPER1/βIII tubulin
(% vehicle-trated intact side)
Intact
Lesion
Vehicle
17β-oestradiol
GPER1
βIII-tubulin
****
*

Figure 5.

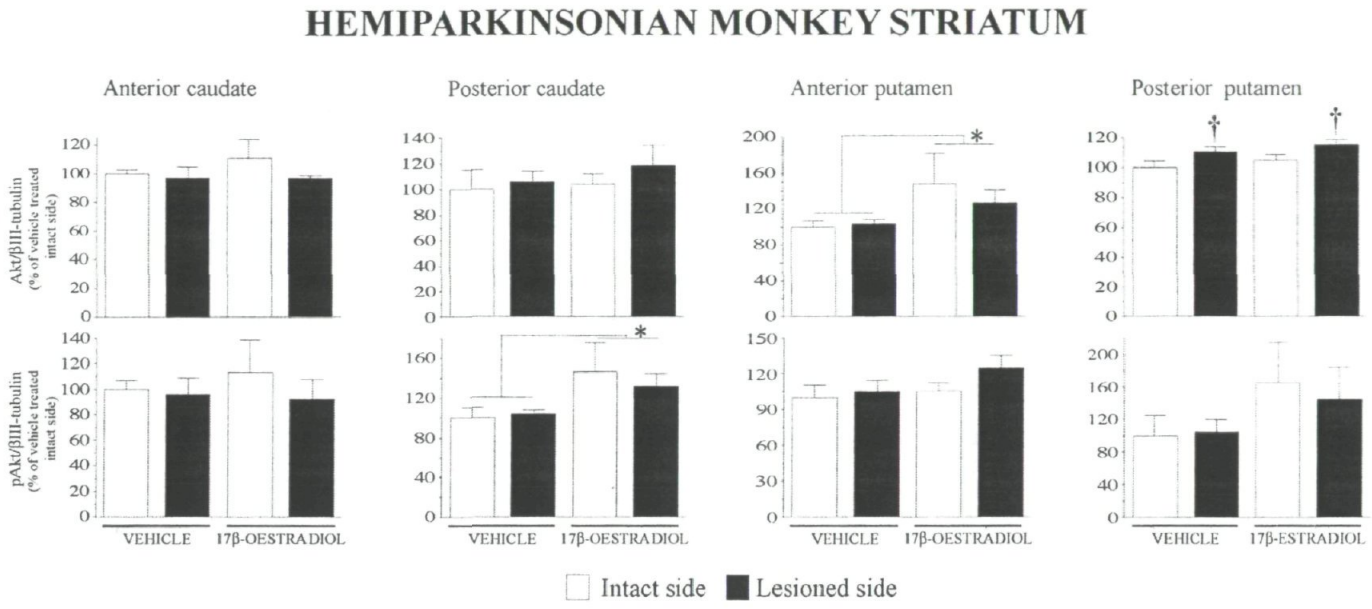
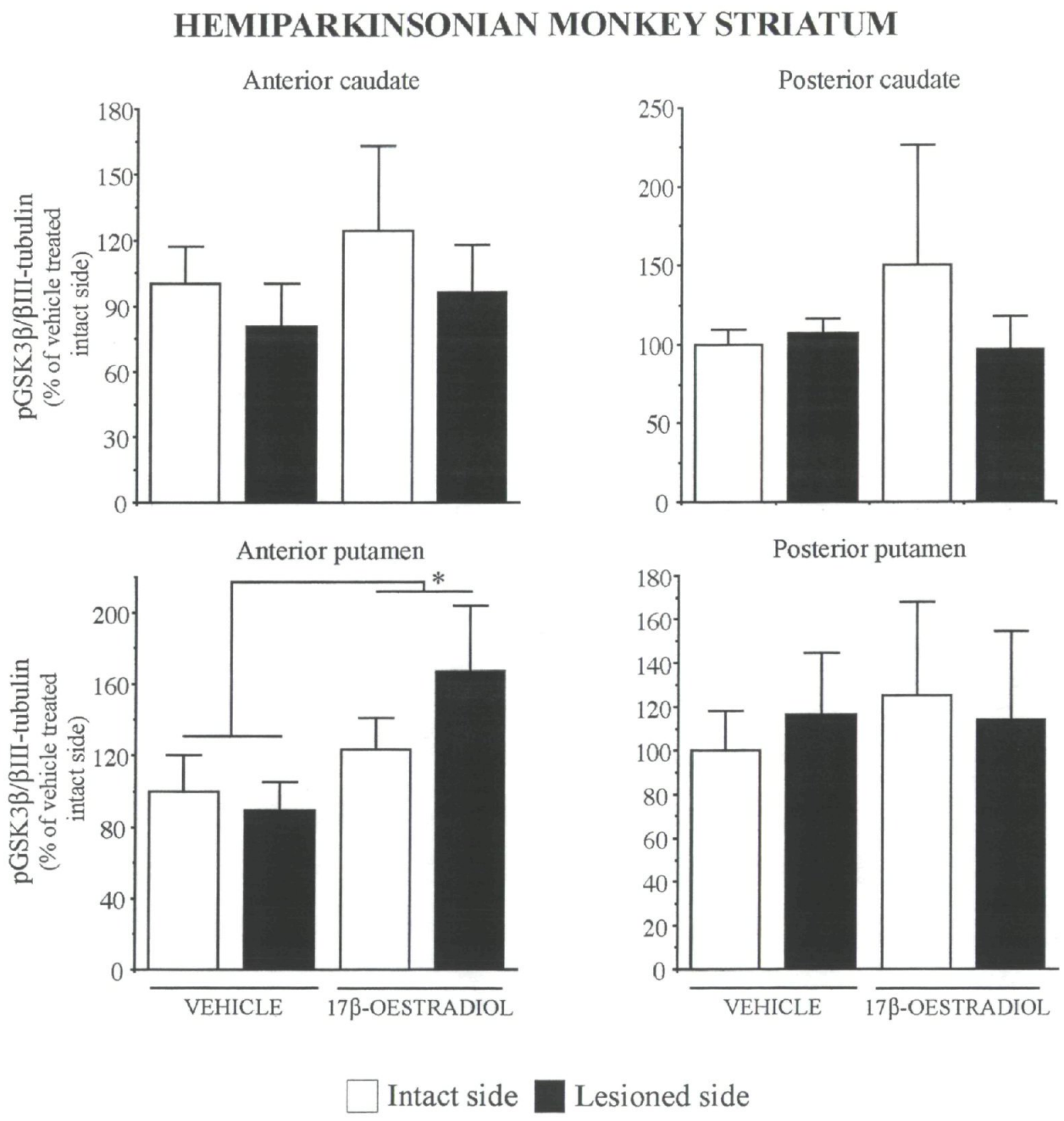

HEMIPARKINSONIAN MONKEY STRIATUM
Anterior caudate
Posterior caudate
Anterior putamen
Posterior putamen
Akt/βIII-tubulin (% of vehicle treated intact side)
pAkt/βIII-tubulin (% of vehicle treated intact side)
VEHICLE
17β-OESTRADIOL
17β-ESTRADIOL
Intact side
Lesioned side

Figure 6.


HEMIPARKINSONIAN MONKEY STRIATUM
Anterior caudate
Posterior caudate
Anterior putamen
Posterior putamen
pGSK3β/βIII-tubulin (% of vehicle treated intact side)
180
150
120
90
60
30
0
250
200
150
100
50
0
200
160
120
80
40
0
*
180
160
140
120
100
80
60
40
20
0
VEHICLE
17β-OESTRADIOL
Intact side
Lesioned side

XII. LEGENDS TO FIGURES

Figure 1. Dose-response effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on striatal dopamine transporter (DAT) and vesicular monoamine 2 (VMAT2) specific binding measured with 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid ([^{125}I]-RTI-121) and [^{3}H]-dihydrotetrabenazine ([3H]-TBZOH) binding respectively in WT compared to oestrogen receptor knockout (ERKO)α and ERKOβ mice using our assay conditions (104, 165). Experimental details of treatments of these mice and their striatal biogenic amine concentrations were previously described (119). DAT: $F_{11,57}$=20.3, p<0.0001 and VMAT2: $F_{11,55}$=30.6, p<0.0001; *p<0.05, **p<0.01, ***p<0.005 and ****p<0.0001 vs respective intact, vehicle (saline/gelatine solution, 0 MPTP); ††p<0.01, †††p<0.005 and ††††p<0.0001 vs WT MPTP 7 mg/kg; ‡‡‡‡p<0.0001 vs WT MPTP 9 mg/kg; £p<0.05, £££p<0.005 and ££££p<0.0001 vs WT MPTP 11 mg/kg; &p<0.05 and &&&p<0.005 vs WT vehicle (0 MPTP); Φp<0.05, and ΦΦΦp<0.005 vs respective experimental ERKOα genotype group.

Figure 2. Effect of treatment with 17β-oestradiol (E_2) (2μg/day) for ten days on anterior striatal DAT and VMAT2 specific binding respectively measured with [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH in intact and MPTP (9 mg/kg) lesioned WT, oestrogen receptor Knockout (ERKO)α and ERKOβ mice. For experimental details see legend to Figure 1. DAT: $F_{11,69}$=32.7, p<0.0001 and VMAT2: $F_{11,68}$=31.2, p<0.0001; *p<0.05, **p<0.01, ***p<0.005, and ****p<0.0001 vs respective intact, vehicle (0 MPTP); ‡p<0.05 and ‡‡‡‡p<0.0001 vs WT MPTP; •p<0.05 and •••p<0.005 vs WT+E_2; ++++p<0.0001 vs WT+MPTP+E_2; Φp<0.05 and ΦΦΦΦp<0.0001 vs respective ERKOα genotype group.

Figure 3. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (11 mg/kg) in C57Bl/6 male mice on striatal DA and its metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT) concentrations as well as G protein-coupled oestrogen receptor (GPER1) levels. Catecholamines concentrations were measured under conditions we previously reported by high performance liquid chromatography with electrochemical detection (119) and GPER1 levels by Western blots (143). *p<0.05, ***p<0.005, and ****p<0.0001 vs respective intact, vehicle (0 MPTP).

Figure 4. G protein-coupled oestrogen receptor (GPER1) levels in the caudate nucleus and putamen of hemiparkinsonian OVX monkeys treated for one month with vehicle (n=4) or with 17β-oestradiol (0.1 mg/kg once daily subcutaneous, n=3). Data is expressed as % intact-side of vehicle treated monkeys. Experimental details of treatments of these monkeys and their striatal biogenic amine concentrations were reported (144). GPER1 and βIII-tubulin were measured by Western blots under conditions we previously described (143). *$p<0.05$ and ****$p<0.0005$ vs intact side of respective treated monkeys.

Figure 5. Relative levels of Akt and its phosphorylated form (pAkt, Ser473) in the caudate and putamen of hemiparkinsonian 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkeys treated for one month with vehicle or 17β-oestradiol. For experimental details see legend to Figure 4. Akt and its phosphorylated form were measured by Western blots under conditions we previously described (166). *$p<0.05$ vs vehicle-treated monkeys; †$p<0.05$ vs respective intact side.

Figure 6. Relative levels of phosphorylated glycogen synthase kinase (pGSK3β, Ser9) in the caudate and putamen of hemiparkinsonian 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkeys treated for one month with vehicle or 17β-oestradiol. For experimental details see legend to Figure 4. GSK3β and its phosphorylated form were measured by Western blots as described (166). GSK3β/βIII-tubulin levels were unchanged by the lesion and 17β-oestradiol treatment (data not shown). *$p<0.05$ vs vehicle-treated monkeys.

CHAPTER III

Estrogen receptors and gonadal steroids in vulnerability and protection of dopamine neurons in a mouse model of Parkinson's disease

Sara Al-Sweidi, Marc Morissette, Mélanie Bourque and Thérèse Di Paolo

Molecular Endocrinology and Genomic Research Center of CHUQ-CHUL

and Faculty of Pharmacy, Laval University, Quebec, Qc, Canada

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The published version is annexed

I. FOREWORD

Sara Al-Sweidi: I treated the mice, participated in the sacrifice, during catecholamine dosing through HPLC and cryostat cutting of mouse brains. I did all the DAT and VMAT2 autoradiography experiments, as well as the DAT, VMAT2 and TH *in situ* hybridizations.
I performed the blood plasma preparations for assaying by gas chromatography and negative chemical ionization mass spectrometry and wrote the article.

Marc Morissette: Research assistant in our laboratory who had a lot to do during this project. He extracted mouse brains during the sacrifice, then dissected and prepared the brain structures for HPLC dosing then performed these. Also, he helped with the cutting of the mouse brains and the autoradiography and *in situ* hybridizations experiments, as well as the writing of the article.

Mélanie Bourque: Senior PhD student in our laboratory. She helped cut the mouse brains and participated in the autoradiography and *in situ* hybridizations experiments.

Thérèse Di Paolo: Research director who undertook main decisions concerning which treatments had to be administered to the knockout mice. She planned and supervised all the protocols from start to finish and made an important contribution in the writing of the article.

II. RÉSUMÉ

Chez la souris, le 17β-oestradiol induit des effets neuroprotecteurs contre la neurotoxine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Cet effet neuroprotecteur peut impliquer les récepteurs oestrogéniques ERα et ERβ et pour évaluer ces mécanismes nous avons fait une étude chez des souris mâles C57Bl/6 invalidées (KO) pour les ERα et ERβ (ERKOα et ERKOβ). Les concentrations des transporteurs dopaminergiques et vésiculaires, DAT, VMAT2 et de la tyrosine hydroxylase (TH) ont été mesurés par autoradiogaphie et par hybridation *in situ,* dans le striatum et la substance noire (SN) et furent comparés aux niveaux sanguins de stéroïdes gonadiques. Des souris sauvages (WT), ERKOα et ERKOβ ont reçue des traitements de MPTP (7, 9, 11 ou 15 mg/kg) et/ou de 17β-oestradiol (2 μg/jour). Les souris ERKOβ intactes avaient des niveaux striatales moins élevés de DAT et de VMAT2 comparativement aux WT et ERKOα. Le MPTP a causé une diminution dose-dependente de ces deux transporteurs et on a observé une corrélation positive entre la liaison spécifique et les niveaux striatals de DA. Les groupes ERKOα sont les plus vulnérables au MPTP et leurs niveaux de DAT, VMAT2 et TH sont affectés par des doses inférieures dans le striatum et la SN. Le traitement au 17β-oestradiol augmente les niveaux sanguins de 17β-oestradiol chez tous les groupes et a induit la neuroprotection que chez les souris WT. Les souris ERKOα avaient des niveaux sanguins élevés de testostérone, dihydrotestostérone et 3β-diol et on observe une corrélation négative des niveaux striatals de DA et TH mRNA dans la SN avec les niveaux sanguins de testostérone et de 3 β-diol. Ces résultats démontrent que l'absence des ERs chez des souris males induit des changements de niveaux des stéroïdes sanguins, des transporteurs DA ainsi que leur susceptibilité au MPTP.

III. ABSTRACT

17β-estradiol is well known to have neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. We investigated the neuroprotective contribution of estrogen receptors (ERα and ERβ) against MPTP toxicity by examining the membrane dopamine (DA) transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) and tyrosine hydroxylase (TH) in ER knock out (ERKO) C57Bl/6 male mice compared to their plasma steroid levels. A dose-response to MPTP comparing wild-type (WT) to ERKO mice was studied. WT mice were also compared to ERKO mice pretreated with 17β-estradiol alone and with MPTP. Specific radioligand binding autoradiography and *in situ* hybridization for DAT, VMAT2 and TH were assayed in the striatum and the substantia nigra (SN). Intact ERKOβ mice had both striatal transporters levels lower than WT and ERKOα mice. MPTP caused a dose-dependant loss of both striatal transporters that correlated with striatal DA concentrations. Compared to WT and ERKOβ mice, ERKOα mice DAT, VMAT2 and TH were affected at lower MPTP doses. In the striatum and SN, ERKOα mice were more vulnerable and 17β-estradiol protected against MPTP toxicity only in WT mice. ERKOα mice blood plasma had higher levels of testosterone, dihydrotestosterone and 3β-diol compared to the plasma of WT and ERKOβ mice. 17β-estradiol treatment increased estradiol plasma levels in all genotypes. Striatal DA concentrations and SN TH mRNA correlated inversely with plasma testosterone and 3β-diol levels. Hence, in male mice the lack of ERα or ERβ altered their basal plasma steroid levels and both striatal DA transporters as well as their susceptibility to MPTP toxicity.

IV. ABBREVIATIONS

CNS, central nervous system; DA, dopamine; DAT, membrane dopamine transporter; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; 3β-diol, 5α-androstan 3-β, 17β-diol; DOPAC, 3,4 dihydroxyphenylacetic acid; 17β-E_2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERKO, estrogen receptor knock out; E1, estrone; ERK, extracellular-signal-regulated kinase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; P450scc, cytochrome P450 side chain cleavage; P450c17, cytochrome P450 17α-hydroxysteroid/C17 20-lyase; PPT, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole; 5α-R, 5α–reductase; [^{125}I]-RTI-121, 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid; SN, substantia nigra; TH: tyrosine hydroxylase; [^{3}H]-TBZ-OH, [^{3}H]-dihydrotetrabenazine; VMAT2, vesicular monoamine transporter type 2; WT, wild-type.

V. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer and is likely to increase due to the aging population (review: Siderowf and Stern, 2003). Motor impairment in PD results from the loss of striatal dopamine (DA), due to the death of DA neurons in the substantia nigra (SN). There is no cure for PD but the motor symptoms are alleviated by replacement of DA by its precursor levodopa (L-DOPA) or by treatment with direct DA receptor agonists (Hornykiewicz, 2002; Olanow et al., 2009). Nevertheless for the majority of PD patients, these therapies eventually loose effectiveness and are associated with side-effects (Katzenschlager and Lees, 2002). Thus, there is a need for therapies to prevent the loss of DA neurons and/or halt disease progression. Estrogenic drugs could bring such disease modifying therapies for PD.

There are two families of transporters responsible for controlling extracellular DA concentrations; these are the DA transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) (Guillot and Miller, 2009). The striatum has dense and heterogeneous DAT distribution, the transporter is found on plasma membranes of axon terminals and immunocytochemistry shows that DAT is colocalized with tyrosine hydroxylase (TH) and the D2 DA receptor (Ciliax et al., 1999). DAT allows the uptake of DA into the cytoplasm from the extracellular space, while VMAT2 is responsible for storing DA in synaptic vesicles and reduction of its levels in the nigrostriatal system is seen in animal models of PD and in PD patients (Guillot and Miller, 2009; Le Saux and Di Paolo, 2006). The actions of these transporters are regulated by presynaptic receptors and protein kinases (Guillot and Miller, 2009). Hence, the amount of free DA depends on DAT levels on the plasma membrane and the presence of VMAT2 on synaptic vesicles.

Epidemiological and clinical studies support a beneficial effect of estrogens against the development and progression of PD. A greater prevalence and incidence of PD is described in men than in women (Shulman and Bhat, 2006; Wooten et al., 2004). Men with PD show symptoms requiring medical attention during earlier stages of the disorder than women suggesting that the disease progresses more rapidly in men, thus supporting that estrogen can provide neuroprotective effects (Saunders-Pullman, 2003). Gender differences in symptoms were also seen in outcome studies after stereotactic surgery for PD (Shulman and Bhat, 2006). Also, an inverse association between factors reducing estrogen stimulation during life and PD is found, supporting the hypothesis that endogenous estrogens play a role in its development (review: Bourque et al., 2009). Therapy with 17β-estradiol was reported to be beneficial at an early stage of PD, before initiation of L-DOPA (review: Bourque et al., 2009).

17β-estradiol is neuroprotective in both male and female mice against a variety of central nervous system (CNS) insults such as protection of DA neurons against the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), where pre-treatment with 17β-estradiol before MPTP prevents the loss of striatal DA and its metabolites (review: Bourque et al., 2009), DAT, and VMAT2 (D'Astous et al., 2003). Dluzen and colleagues suggest that estrogen protects by decreasing DAT's binding affinity, thus not allowing entry of neurotoxic compounds, like the MPTP ion 1-methyl-4-phenylpyridinium (MPP+), into dopaminergic nerve terminals (Dluzen, 2000). Furthermore, the neuroprotective effect of 17β-estradiol appears to be mediated through interaction with estrogen receptors (ERs). In male mice, 17β-estradiol which binds to and activates ERs, is neuroprotective against striatal MPTP toxicity; whereas 17α-estradiol, the isomer with low ER affinity, lacks neuroprotective activity (Callier et al., 2000) and estriol and estrone, weak agonists on ERs, show poor or no activity to protect against MPTP toxicity

(Jourdain et al., 2005). Thus, the potencies of the above compounds to protect against MPTP toxicity parallel their activity on ERs. There are two main ER subtypes, ERα and ERβ (Green et al., 1986; Kuiper and Gustafsson, 1997). ERα is widely expressed throughout the body and mediates most of the feminizing effects of estradiol (Mitra et al., 2003). By contrast, ERβ has a much more restricted distribution, of which expression in the brain is notable (Kuiper and Gustafsson, 1997). Both ERs have been detected in the mouse striatum and SN (Mitra et al., 2003). Moreover, no sex difference was observed for ERα and ERβ levels in mouse striatum during development and in adulthood (Kuppers and Beyer, 1999). Using specific agonists for ERα and ERβ we have previously shown that ERα agonists protect against MPTP toxicity in male mice (D'Astous et al., 2004).

The intact male mouse MPTP animal model of PD is representative of PD pathology and, to unravel the neuroprotective effects of ERs, striatal catecholamine concentrations of ER knock out (ERKOα and ERKOβ) male mice were previously published (Morissette et al., 2007). The degree of MPTP-induced DA and dihydroxyphenylacetic acid (DOPAC) depletions were greater in ERKOα than in wild-type (WT) male mice, whereas ERKOβ mice exhibited no change in MPTP sensitivity but they showed a lower DA turnover than WT and ERKOα mice. 17β-estradiol partially prevented the MPTP-induced decrease in striatal DA and DOPAC levels only in the WT mice (Morissette et al., 2007). Therefore, we hypothesize that sparing of striatal DA concentrations from MPTP toxicity in these WT mice is due to neuroprotection of DA neurons by endogenous steroids and administered 17β-estradiol acting on ERs. Hence, in the present study we investigated the effect of ER genotype on blood steroid levels and various DA markers in these mice. We explored the contribution of the striatal and SN DAT, VMAT2 and TH in MPTP

toxicity and neuroprotection by 17β-estradiol in WT mice compared to ERKOα and ERKOβ male mice.

VI. MATERIALS AND METHODS

6.1. *Animals and treatments*

Adult WT, ERKOα and ERKOβ male C57Bl/6 mice (7-12 weeks, 18-28 g, WT and ERKO mice) were purchased from Taconic Laboratories (Hudson, NY, USA). MPTP and 17β-estradiol were purchased from Sigma Chemical (St-Louis, MO, USA). In order to minimize the possible variability of the response to MPTP treatment, WT and ERKO mice were of C57Bl/6 background and were equally distributed for age and weight in experimental groups of six animals. The Laval University Animal Care Committee approved all the animal studies. All efforts were made to minimize animal suffering and to reduce the number of mice used.

An extended MPTP dose-response up to 20 mg/kg was performed in WT male C57Bl/6 mice and striatal biogenic amine concentrations of these mice was previously reported (Morissette et al., 2007). The MPTP doses (7, 9 and 11 mg/kg) that specifically affected striatal DA while sparing serotonin concentrations in WT mice (Morissette et al., 2007) were used for comparison of MPTP dose-responses of ERKOα, ERKOβ and WT mice. Mice received four 0.1 ml intraperitoneal injections with saline or a saline solution of MPTP at a two-hour interval and were killed 5 days after treatment with MPTP.

The effect of 17β-estradiol and MPTP toxicity in ERKOα and ERKOβ was compared to WT mice. Four groups of both ERKOα and ERKOβ mice were compared to WT mice. An intermediate dose of 9 mg/kg MPTP was selected and we investigated the effect of 17β-estradiol treatment in intact and MPTP mice. Each group received a 5-day pre-treatment of 17β-estradiol

or vehicle prior to MPTP injections. The pre-treatment consisted of two daily subcutaneous injections (in the dorsal part of the neck) of 17β-estradiol, while control mice received injections of vehicle (0.9% saline with 0.3% gelatin). Concentrations used were 2 μg per day for 17β-estradiol such as we used previously (D'Astous et al., 2004; Morissette et al., 2007). On day 5, mice received four injections of MPTP (9 mg/kg, per intraperitoneal injection) at a 2-hour interval, while the control group received saline solution. Treatments with 17β-estradiol or vehicle were continued until day 10. The next day, mice were killed with an air/halothane mixture and decapitated; trunk blood was collected and brains were quickly removed and frozen in a mixture of isopentane/dry ice and then stored at –80°C. Gas chromatography and negative chemical ionization mass spectrometry was used to assay steroid plasma levels as described (Labrie et al., 2007).

6.2. *Preparation of brain tissue*

Frozen brains were cut on a cryostat in 12 μm thick slices at striatal and SNc regions. Coronal sections for the anterior striatum (bregmas between 1.54 and 1.18 mm), middle striatum (bregmas between 0.50 and 0.14 mm), posterior striatum (bregmas between -0.34 and -0.70 mm) and the SN (bregmas -2.70 mm to -3.28 mm) were done according to the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos, 1997).

6.3. *DAT and VMAT2 autoradiography*

Autoradiography using isopropylester 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid ([^{125}I]-RTI-121) to the DAT was done on the striatum and the SN as previously described (Callier et al., 2001). DAT specific binding was measured using 20 pM [^{125} I]-RTI-121(2200 Ci/mmol,

Perkin-Elmer, Woodbridge, ON, Canada) in the presence of 100 nM of mazindol to estimate nonspecific binding. The striatal slices were exposed to Kodak BIOMAX film for two days and the SN slices for four days.

Autoradiography using [^{3}H]-dihydrotetrabenazine ([^{3}H]-TBZ-OH) for VMAT2 binding was done on the striatum and the SN using 20 nM of [^{3}H]-TBZ-OH (20 Ci/mmol, ARC, Saint Louis, MO USA) in the presence of 1 μM of cold TBZ-OH to estimate nonspecific binding as described (Kilbourn and Frey, 1996). Striatal slices were exposed to sensitive Kodak BIOMAX film for four weeks and the SN slices for six weeks.

6.4. *DAT, VMAT2 and TH in situ hybridization*

DAT mRNA levels in the SN were measured by *in situ* hybridization using a sequence encoding the entire rat DAT (Callier et al., 2000). The percentage of homologies between the rat and the mouse cDNA sequences used to generate the radioactive probe for the DAT transporter is 95% (GenBank accession no. NM_012694 and NM_010020.3). The whole rat DAT sequence was subcloned into the *Eco*RI site of pBlueScript and used in the preparation of sense and anti sense strands of cRNA probes. Uridine 5'([α-^{35}S]thio)triphosphate-labeled probes were prepared by *in vitro* transcription of linearized templates (sense, Kpn1; antisense, Xba1) with T3 and T7 RNA polymerases to generate sense and antisense probes respectively. The prehybridization, hybridization and post-hybridization procedures were followed as previously described (Callier et al., 2000; Jourdain et al., 2005).

VMAT2 and TH mRNA levels in the SN were measured by *in situ* hybridization using cDNA oligonucleotide probes as previously described (Calon et al., 2002; D'Astous et al., 2003). *In situ* hybridization was done using an oligonucleotide complementary to bases 1321-1366

(GenBank accession no. NM_172523) of the mouse VMAT2 sequence and an oligonucleotide complementary to bases 1435-1482 (GenBank accession no. M69200) of the mouse TH sequence. These probes were labeled at the 3' end by adenosine 3'([α-^{35}S]thio)triphosphate. The pre-hybridization, hybridization and post-hybridization procedures were followed as previously described (Calon et al., 2002; Jourdain et al., 2005).

Slide-mounted tissue sections were apposed to sensitive Kodak BIOMAX film where DAT *in situ* hybridization samples were exposed for three days, VMAT2 for four weeks and TH for three weeks.

6.5. *Statistical analysis*

Autoradiograms of specific binding and mRNA relative optical densities were analyzed using Scion Image 1.63 software. The striatum was analyzed at three rostro-caudal coordinates which include the anterior, middle and the posterior striatum; each of these regions were subdivided in half into medial and lateral regions. The statistical analyses were done using Statview 4.51 for Macintosh Computer software. A one-way ANOVA was used to compare MPTP and/or 17β-estradiol treatments followed by post-hoc analysis with Fisher probability of least significance difference test. A simple regression model (Pearson) was used to determine the coefficient of correlation and the significance of the degree of the linear relationship between variables. A $p \leq 0.05$ was required for the results to be considered statistically significant.

VII. RESULTS

7.1. *Dose-response of MPTP on DAT, VMAT2 and TH*

The effects of MPTP in ERKO and WT mice is shown in Figure 1. Similar results were observed in the medial and lateral parts of the three rostro-caudal striatal coordinates measured therefore these results were grouped and the anterior striatum is shown as a representative, while detailed results on the middle and posterior striata are represented as supplementary data. In the anterior striatum, DAT specific binding was higher by 27% in vehicle-treated ERKOα mice compared to vehicle-treated WT mice (Figure 1A) while no difference was observed in the middle and posterior striatum (Supplementary data 1, S1). VMAT2 specific binding was similar in vehicle-treated ERKOα mice compared to vehicle-treated WT mice at the three rostro-caudal striatal levels measured (Figure 1B and S1). By contrast, vehicle-treated ERKOβ mice had significantly lower DAT specific binding in the middle (23%) and posterior (21%) striatum, as well as lower VMAT2 specific binding in the anterior (19%) and posterior (25%) striatum compared to vehicle-treated WT mice (Figure 1B and S1). Moreover, DAT specific binding was lower in vehicle-treated ERKOβ mice compared to vehicle-treated ERKOα mice in the anterior and middle striatum as well as lower VMAT2 specific binding at all three rostro-caudal striatal levels measured (Figure 1B and S1).

MPTP dose-dependently decreased striatal DAT and VMAT2 specific binding of ERKO mice and ERKOα mice were more vulnerable to MPTP toxicity than WT and ERKOβ mice. The ERKOα mice showed decreased DAT and VMAT2 specific binding at lower MPTP doses than ERKOβ mice (Figure 1). In addition, the loss of striatal DAT and VMAT2 specific binding positively correlated with striatal DA concentrations in the anterior striatum (DAT: R= 0.766,

$p<0.0001$, Figure 3A and VMAT2: $R= 0.757$, $p<0.0001$, Figure 3B), as well as in the middle and posterior striatum (data not shown).

In the SN, DAT and VMAT2 specific binding and mRNA remained unchanged, in the WT groups as well as in both ERKOα and ERKOβ mice, at the three MPTP doses tested (data not shown). SN TH mRNA levels were similar between genotypes of vehicle-treated mice and decreased with increasing doses of MPTP; this was significant and to a similar extent in both ERKOα and ERKOβ mice, while only a tendency to decrease was seen in the WT group at the MPTP doses tested (Figure 4A).

7.2. *Effect of 17β-estradiol in intact and MPTP WT compared to ERKOα and ERKOβ mice*

The intermediate dose of 9 mg/kg of MPTP was chosen to investigate the neuroprotective effect of 17β-estradiol in the three groups of mice to further explore the differences between genotypes observed in the MPTP dose-response results. Similar findings were observed at the three striatal rostro-caudal coordinates analyzed as well as in their medial and lateral sub regions, therefore the anterior striatum results are shown as representative.

Similar differences in striatal DAT and VMAT2 specific binding were seen between the intact WT versus ERKOα and ERKOβ mice of the controls of the MPTP/17β-estradiol experiment shown in Figure 2 as compared to data of the control mice of these genotypes in the MPTP dose-response study of Figure 1. Vehicle-treated ERKOα mice in Figure 2A had higher DAT levels in the anterior striatum. Moreover, DAT and VMAT2 specific binding was lower in vehicle-treated ERKOβ compared to ERKOα mice (Figure 2).

No significant effect of 17β-estradiol treatment was measured on DAT specific binding in the anterior striata of unlesioned WT or ERKOα mice whereas it decreased in ERKOβ mice

(Figure 2A). No significant effect of 17β-estradiol treatment was measured on VMAT2 specific binding of unlesioned WT, ERKOα or ERKOβ mice (Figure 2 B).

In WT mice, DAT and VMAT2 specific binding were decreased by MPTP. DAT levels decreased by 42%, (Figure 2A) while VMAT2 levels were lowered by 21% in the anterior striatum (Figure 2B). Treatment with 17β-estradiol prevented this in the striata of MPTP WT mice. By contrast, in ERKOα or ERKOβ mice, 17β-estradiol did not protect striatal DAT and VMAT2 specific binding against MPTP toxicity, their specific binding remaining decreased. Moreover, MPTP-lesioned ERKOα mice had significantly lower striatal DAT specific binding compared to ERKOβ mice. In the anterior striata of MPTP mice, DAT levels decreased by 81% in ERKOα mice; whereas in ERKOβ mice DAT levels decreased by 38% compared to unlesioned ERKO mice. The same pattern was observed for VMAT2 specific binding, where its levels decreased by 65% in ERKOα mice and by 23% in ERKOβ mice compared to unlesioned ERKO mice (Figure 2B).

There were positive correlations between [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH specific binding and DA concentrations in the anterior striatum of WT mice, ERKOα and ERKOβ mice of the MPTP dose-response (Figures 3A and 3B) and the MPTP/17β-estradiol experiments (Figures 3C and 3D).

In the SN, treatment with 17β-estradiol of unlesioned mice left unchanged the TH mRNA levels of all WT, ERKOα and ERKOβ mice (Figure 4B). In ERKOα and ERKOβ mice, a 9 mg/kg MPTP treatment caused a significant decrease of TH mRNA levels whereas the decrease was nonsignificant for WT mice. SN TH mRNA levels of 17β-estradiol-treated MPTP ERKOβ mice were higher than for 17β-estradiol-treated MPTP ERKOα mice (Figure 4B).

Plasma estrogen and androgen concentrations were measured in order to investigate the effect of absence of ERα and ERβ on basal plasma steroid levels as well as on the metabolism of administered 17β-estradiol. We assayed and quantified the endogenous androgens testosterone, dihydrotestosterone (DHT) and 5α-androstan 3-β, 17β-diol (3β-diol) (Figure 5). Blood levels were below limit of quantification for dehydroepiandrosterone (DHEA), androst-ene-3β, 17β-diol (5-diol), androstenedione and estrone (Figure 5).

The 17β-estradiol treatment significantly increased this steroid's plasma concentration, compared to intact and MPTP treated mice that had low 17β-estradiol levels (Figure 6B). 17β-estradiol treated MPTP WT mice had similar plasma levels to that of 17β-estradiol treated intact WT mice. By contrast, 17β-estradiol treated MPTP ERKOα mice had higher plasma 17β-estradiol levels than the 17β-estradiol treated ERKOα mice, while the opposite was seen for ERKOβ mice.

Testosterone plasma levels were higher in ERKOα mice than in WT or ERKOβ mice, the latter two groups having similar values (Figure 6A). A similar pattern was observed for DHT and 3β-diol plasma levels that were also higher in ERKOα mice when compared to WT and ERKOβ mice (Figures 6C and 6D). The latter plasma steroid concentrations were also of similar levels in WT and ERKOβ mice. Both striatal DA concentrations (Figure 7A and 7B) and SN TH mRNA levels (Figure 7C and 7D) correlated negatively with either testosterone or 3β-diol plasma concentrations.

VIII. DISCUSSION

In DA neurons, the DAT is the port of entry of several toxins such as MPTP (Dauer and Przedborski, 2003). This transporter is abundant in DA neurons of the nigro-striatal pathway and has been used to target these neurons to model their loss as occurs in PD (Dauer and Przedborski, 2003). In post-mortem brains of PD patients, opposing roles of DAT and VMAT2 are documented where lower DAT levels decrease toxin entry in DA neurons and higher VMAT2 concentrations promote the sequestering of these toxins in cell bodies of remaining DA neurons that resisted to the PD pathology (Joyce et al., 1997). This supports a role for these transporters in the vulnerability of DA neurons that was explored in the present study in relation to estradiol-induced neuroprotection.

In response to a variety of toxins, striatal DA terminals are more sensitive to damage than their cell bodies in the SN (Bywood and Johnson, 2000). Striatal DAT and VMAT2 are located on DA terminals and were used here as markers of terminal loss by MPTP toxicity. In neuroprotection against MPTP in mice treated with 17β-estradiol there is a high correlation between striatal DA loss and/or protection with striatal DAT and VMAT2 specific binding, suggesting that in this paradigm changes in their specific binding are associated with the loss or sparing of striatal DA terminals. In intact female rats, we observe that DAT density varies along with the estrous cycle (Morissette and Di Paolo, 1993), while acute and chronic 17β-estradiol treatment increases striatal DAT specific binding in rats (review: Sanchez et al., 2010). ERs regulate most of the effects of estrogens and ERβ effects are not highly feminizing (Gruber et al., 2002). In a rat animal model, we saw that ERβ regulates a 17β-estradiol-induced increase of striatal DAT expression, therefore it is possible that a lack of ERs would lower DAT levels (Le Saux and Di Paolo, 2006). Thus, it was interesting to investigate the roles of ERs in mediating

the neuroprotective effects of 17β-estradiol against the loss of DA nerve terminals caused by MPTP in male mice. Two complementary paradigms were used. First, we investigated whether genetic deletion of either ERα or ERβ altered the vulnerability of DA markers to MPTP. We then determined whether deletion of ERs altered the sensitivity to the neuroprotective effects of exogenous 17β-estradiol treatment.

In intact male mice, the lack of an ER affected striatal DAT and VMAT2 specific binding and the differences observed in the MPTP dose-response study between the WT and ERKO mice could be influenced by the initial differences of density of these markers. In 2 year-old ERKOβ mice of a different background than ours, Wang et al. found shrinkage of SN neurons as measured with Nissl staining compared to WT mice (Wang et al., 2001). In 22 months old ERKOβ mice we found a small reduction of SN TH mRNA levels compared to young mice (Morissette et al., 2008). No change of SN TH mRNA was observed in our young ERKOβ mice compared to WT or ERKOα mice but they showed lower striatal DAT and VMAT2 specific binding compared to the other genotypes. Compared to WT and ERKOβ mice, the ERKOα mice, having higher striatal DAT but not VMAT2 specific binding, showed a greater susceptibility to MPTP with decreases of striatal DAT and VMAT2 at lower MPTP doses. The results follow the pattern we previously reported in these mice with their striatal DA levels (Morissette et al., 2007) and a significant correlations between striatal DAT and VMAT2 specific binding and DA levels in these mice were noted. Hence, changes of striatal DAT and VMAT2 specific binding is likely reflecting the extent of loss or sparing of DA terminals in the striatum. The present results showed that treatment with 7-11 mg/kg of MPTP spared the SN of WT mice, providing conditions where neurons are injured with loss of terminals but could be healed. It is noteworthy

that the ERα had a protecting role here in male mice suggesting that endogenous estrogens acting on ERα play an important role in neuroprotection.

When mice were administered a small dose of MPTP, differences in response to 17β-estradiol treatment emerged between the genotypes that was less prominent under basal conditions. 17β-estradiol protected against the MPTP-induced loss of DAT and VMAT2 specific binding and TH mRNA in WT but not in ERKOα or ERKOβ mice. Preventing the decrease of striatal DAT and VMAT2 levels as well as DA concentrations is likely due to neuroprotection of DA neurons rather than activity on DA synthesis and/or metabolism enzymes since all these markers were protected in WT mice. Our results show that ERα plays an important role against striatal MPTP toxicity, whereas the ERβ appears to play a subtler role. ERKOα mice were more sensitive than WT and ERKOβ mice to the effects of MPTP on the dopaminergic system. ERKOβ were also vulnerable to the effects of MPTP, but their response to the neurotoxin was more similar to that of WT mice. Hence, endogenous and exogenous estrogens acting on ERs are shown here to play an important protective role against MPTP toxicity and ERα plays the most significant role in mediating this protective effect. ERβ is also implicated in modulating the sensitivity of DA neurons to MPTP toxicity, since the protective effects of 17β-estradiol were also absent in ERKOβ mice. Hence, ERα and ERβ mechanisms of action likely differ. In 17β-estradiol induced neuroprotection against cortical injury, ERα was documented to have an essential role against ischemia induced cell death and ERβ activity was necessary during the recovery phase (Dubal et al., 2006). This could also be the case in MPTP toxicity. The absence of ERβ would leave only the early ERα activity dependent phase of neuroprotection. Our laboratory documented that an ERβ but not an ERα agonist can regulate rat striatal D2 DA receptors and DAT levels (Le Saux et al., 2006). Thus, in 17β-estradiol-induced neuroprotection of DA

neurons, ERα would play the primary role against MPTP toxicity and ERβ supports these neurons' equilibrium, activity and recovery.

Estrogen effects on nigro-striatal DA is well documented (review: Bourque et al., 2009), even though ERα and ERβ are scarce within the striatum and that their expression is not increased with the MPTP lesion or an estradiol treatment (Shughrue, 2004). Neural tissues express multiple ERs including many spliced variants of both ERα and ERβ (Toran-Allerand, 2004). The structure-activity of estrogenic compounds for striatal neuroprotection (Callier et al., 2000; Jourdain et al., 2005) points to an effect via ERs. ERs are present in the nucleus and also at the membrane level (Marin et al., 2006; Mhyre and Dorsa, 2006; Mitra et al., 2003). Membrane ERα and ERβ were shown to be homologous to their intracellular counterparts (Marin et al., 2006) suggesting a common origin. Hence ERKOα and ERKOβ mice likely also lack their respective membrane ERα and ERβ. Accumulating evidence support an important trophic and protective role in the brain of estrogens acting on membrane ERs through various signal transduction pathways (Mhyre and Dorsa, 2006). Perhaps the scarce nigro-striatal ERs, localized on plasma membranes, and their interaction with signaling molecules confers signal amplification but this localization may render them more difficult to detect. Our results on neuroprotection by 17β-estradiol in MPTP mice show an implication of PI3K/Akt pathway shared by the ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) (D'Astous et al., 2006). These data support the implication of a membrane ERα in MPTP neuroprotection. The implication of ERβ in MPTP neuroprotection may involve different signaling pathways in neurons as well as in glia (Mhyre and Dorsa, 2006). Estradiol is demonstrated to activate both extracellular-signal-regulated kinase (ERK) and Akt signaling pathways in the same population of cortical neurons within a time frame consistent with that of membrane ER signaling, this implies that estradiol-

induced neuroprotection is mediated by membrane ER-induced signaling (Mannella and Brinton, 2006).

Our laboratory previously and extensively published on the neuroprotective effects of 17β-estradiol administration in MPTP treated mice (review: Bourque et al., 2009) and the androgens such as testosterone and DHT had no beneficial effects (Ekue et al., 2002). Nevertheless, endogenous levels of these steroids have not been previously investigated in this paradigm. We thus sought if the endocrine status of these animals was altered with the MPTP lesion and/or the ERα and ERβ knockouts.

The overall results of assayed steroid plasma levels reveal that ERKOα mice had elevated testosterone, DHT and 3β-diol concentrations when compared to WT and ERKOβ mice. This is in agreement with a previous report of a significant increase of testosterone in ERKOα male mice when compared to WT males (Rissman et al., 1997). Moreover, we saw that the loss of nigro-striatal DA significantly inversely correlates with testosterone and 3β-diol plasma levels.

We observed an increase of 17β-estradiol plasma levels in mice treated with 17β-estradiol in all genotypes of mice investigated; the lack of response of ERKO mice to the 17β-estradiol treatment was therefore not because of lower circulating concentrations of 17β-estradiol due to higher metabolism of exogenous administered 17β-estradiol in the knockout mice. Nevertheless we observed some differences in the plasma 17β-estradiol concentrations, being higher in ERKOα and lower in ERKOβ MPTP mice compared to the unlesioned 17β-estradiol treated respective ERKO mice. We also observed an inverse correlation between SN TH mRNA and the plasma levels of testosterone and 3β-diol. The observation that striatal DA and SN TH mRNA decrease as testosterone levels increase is consistent with our previous findings showing lower SN DAT mRNA levels in testosterone treated MPTP male mice (Ekue et al., 2002). In addition,

the deleterious effect of testosterone was also observed in methamphetamine toxicity on mice (Lewis and Dluzen, 2008). In humans, this may also be consistent with a higher incidence of PD in men than women (Wooten et al., 2004).

We find an important role of ERα and a lesser role of ERβ in DA sensitivity to MPTP in male mice, while androgens have no affinity for this receptor. This shows the importance of ER in male mice and a possible target for neuroprotection. While 17β-estradiol treatment in males may not be acceptable, a selective estrogen receptor modulator, raloxifene was administered to men without deleterious effects. Raloxifene administered for 3 months in healthy elderly men was reported, using functional magnetic imaging, to enhance brain activation in various cortical areas, suggesting an effect on cortical arousal; no significant side-effects were reported (Goekoop et al., 2005). Hence, raloxifene is showing beneficial effects in aged men. Therefore, drugs with estrogenic activity such as raloxifene, that we reported to protect against MPTP toxicity in intact male mice (Callier et al., 2001), could be useful in men since androgens have no protective activity (Ekue et al., 2002).

In conclusion, our results suggest an important role of ERs on brain nigro-striatal DA markers of male mice: 1- Intact ERKOβ mice had striatal DA transporter levels lower than WT and ERKOα mice. 2- ERα neuroprotective activity is dominant since ERKOα mice are more susceptible to MPTP toxicity, as assessed with loss of DAT and VMAT2 transporters specific binding, than WT or ERKOβ mice, but both ERs are necessary for neuroprotection because exogenous 17β-estradiol fails to protect ERKOα or ERKOβ mice from MPTP toxicity. 3- ERKOα mice had elevated plasma androgen concentration compared to WT and ERKOβ mice and 4- both striatal DA concentrations and SN TH mRNA levels correlated negatively with either testosterone or 3β-diol plasma concentrations of mice of all three genotypes. In brief, the absence

of ERα in male mice resulted in alteration of basal plasma steroid concentrations and levels of striatal DA transporters along with highest susceptibility to MPTP. The absence of ERβ in male mice resulted in lower basal striatal DA transporter levels and higher susceptibility to MPTP than WT mice.

IX. ACKNOWLEDGEMENTS

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Disclosure Statement

There are no conflicts of interest for any of the authors of this manuscript.

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XI. FIGURES

Figure 1.

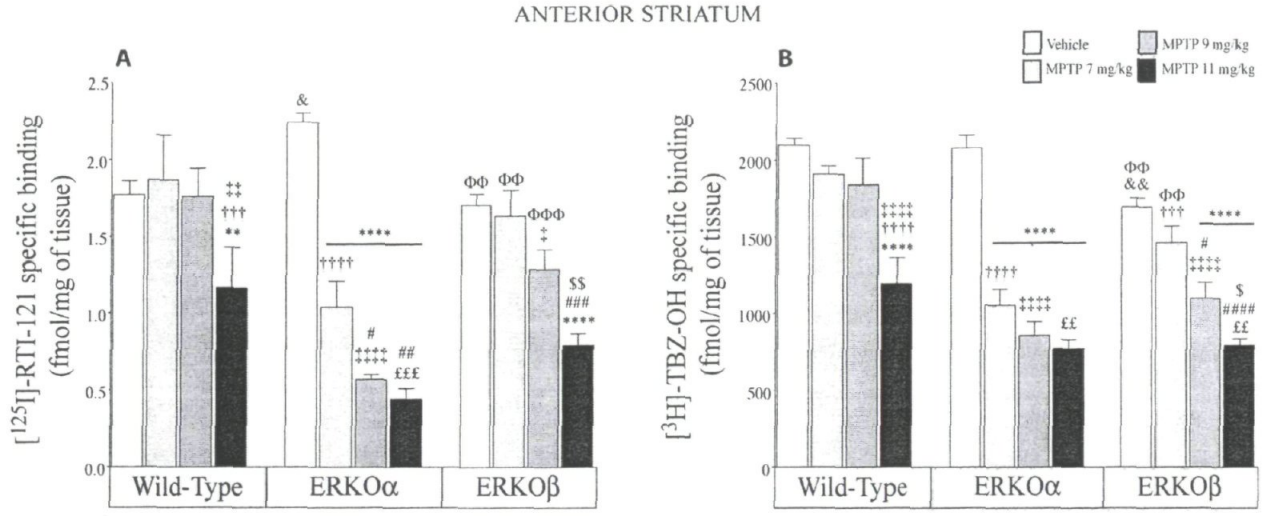
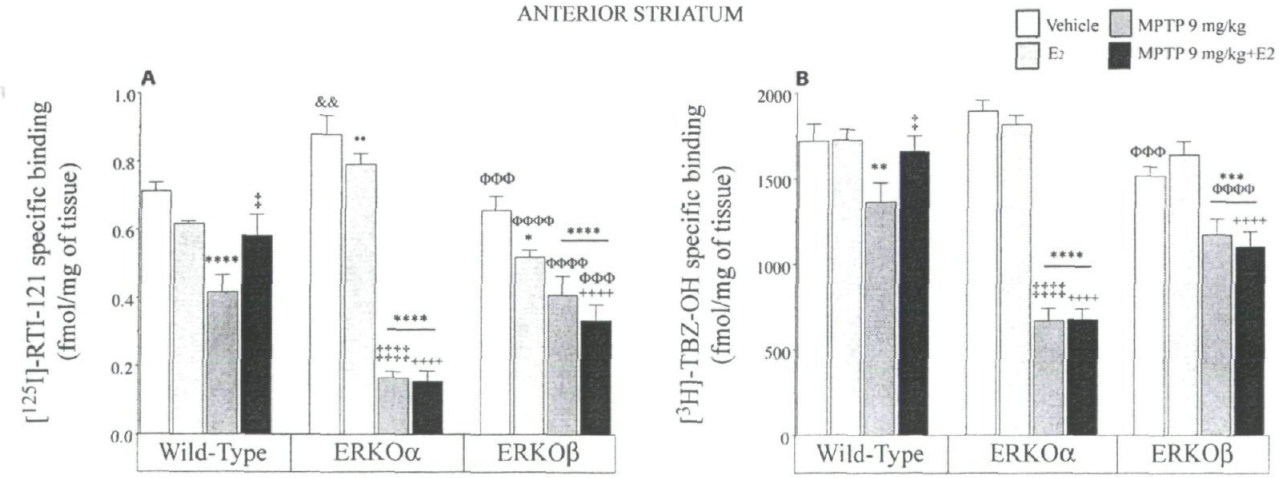


Figure 2.



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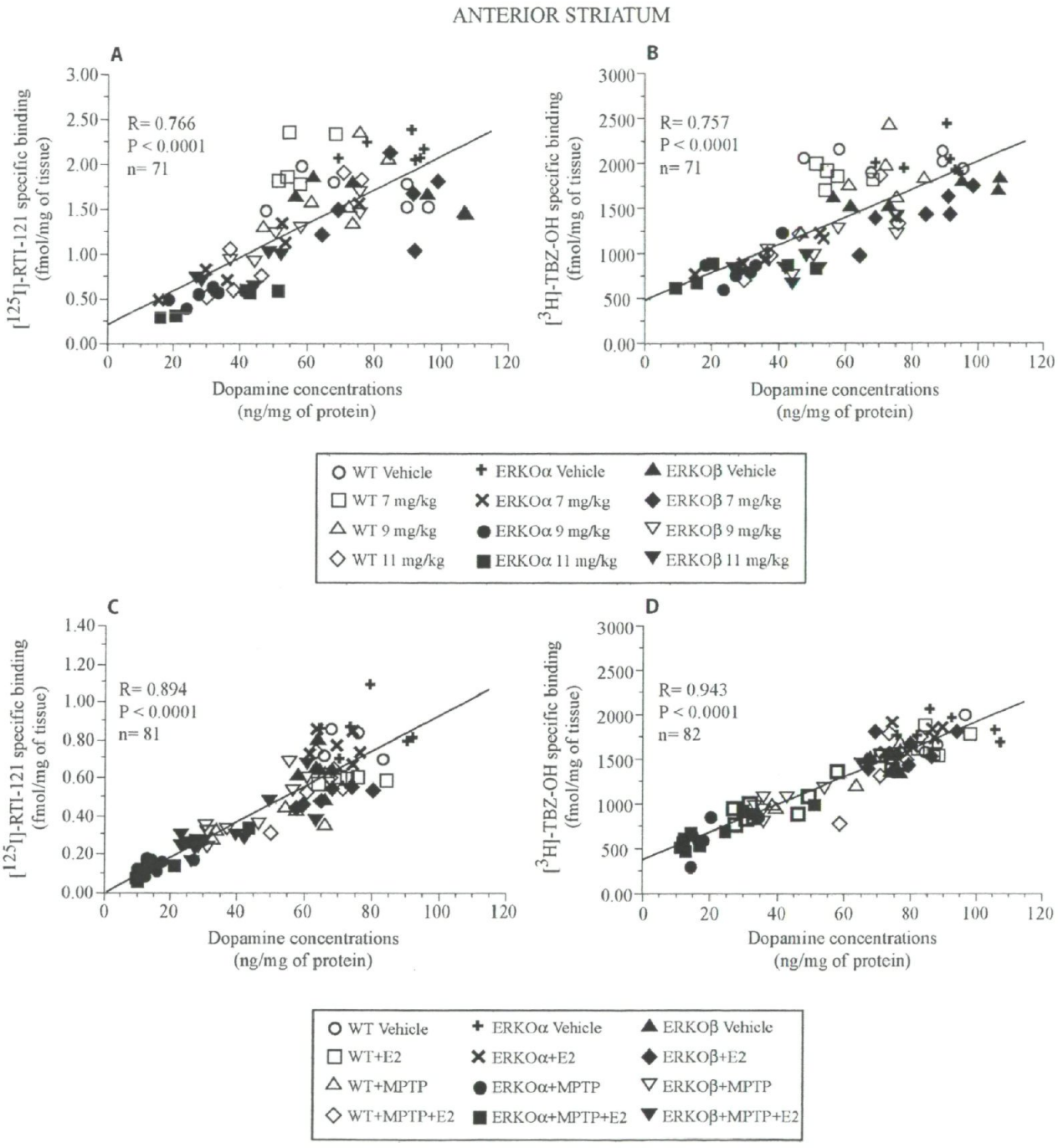

ANTERIOR STRIATUM
A
[125I]-RTI-121 specific binding (fmol/mg of tissue)
R= 0.766
P < 0.0001
n= 71
Dopamine concentrations (ng/mg of protein)
B
[3H]-TBZ-OH specific binding (fmol/mg of tissue)
R= 0.757
P < 0.0001
n= 71
Dopamine concentrations (ng/mg of protein)
WT Vehicle
WT 7 mg/kg
WT 9 mg/kg
WT 11 mg/kg
ERKOα Vehicle
ERKOα 7 mg/kg
ERKOα 9 mg/kg
ERKOα 11 mg/kg
ERKOβ Vehicle
ERKOβ 7 mg/kg
ERKOβ 9 mg/kg
ERKOβ 11 mg/kg
C
[125I]-RTI-121 specific binding (fmol/mg of tissue)
R= 0.894
P < 0.0001
n= 81
Dopamine concentrations (ng/mg of protein)
D
[3H]-TBZ-OH specific binding (fmol/mg of tissue)
R= 0.943
P < 0.0001
n= 82
Dopamine concentrations (ng/mg of protein)
WT Vehicle
WT+E2
WT+MPTP
WT+MPTP+E2
ERKOα Vehicle
ERKOα+E2
ERKOα+MPTP
ERKOα+MPTP+E2
ERKOβ Vehicle
ERKOβ+E2
ERKOβ+MPTP
ERKOβ+MPTP+E2

Figure 4.

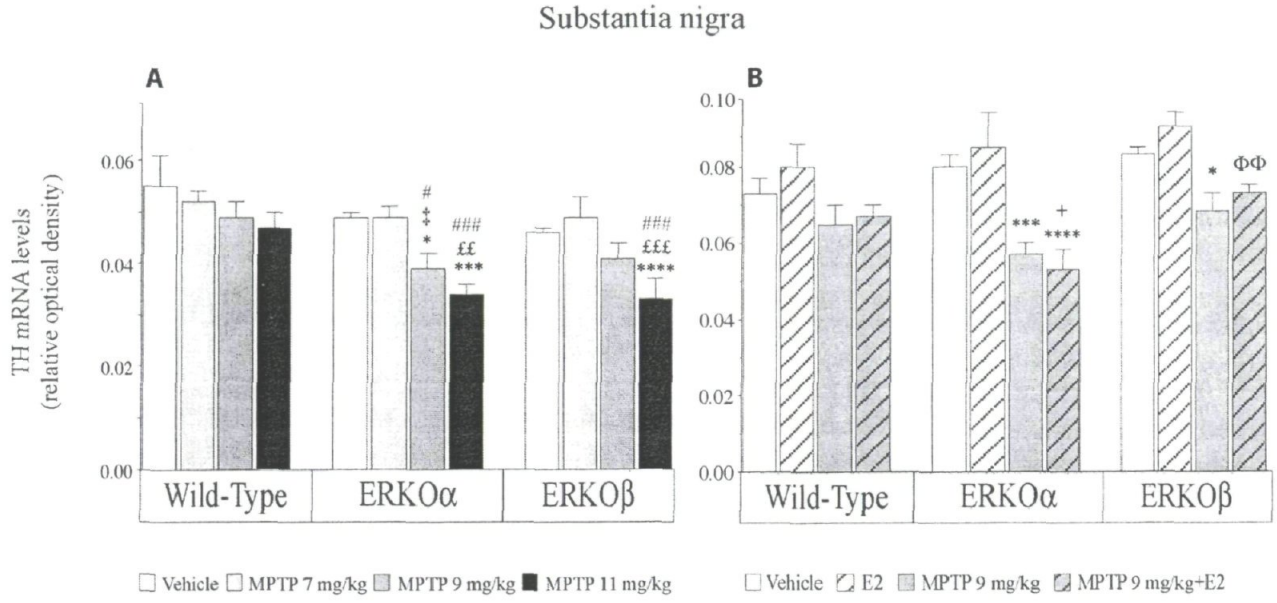

Substantia nigra
A
B
TH mRNA levels
(relative optical density)
0.00
0.02
0.04
0.06
0.08
0.10
Wild-Type
ERKOα
ERKOβ
Vehicle
MPTP 7 mg/kg
MPTP 9 mg/kg
MPTP 11 mg/kg
E2
MPTP 9 mg/kg+E2

Figure 5.

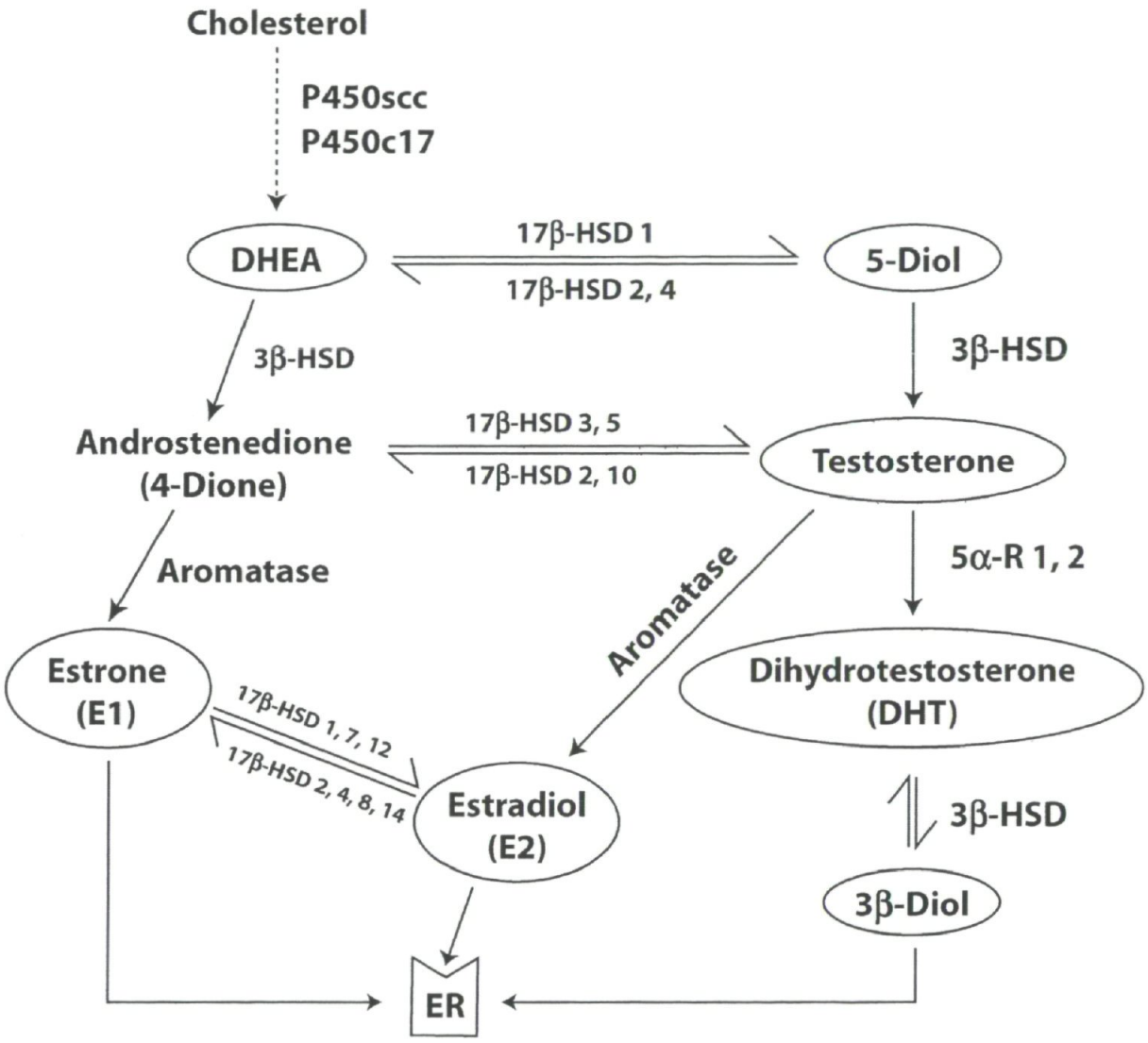

Cholesterol
P450scc
P450c17
DHEA
17β-HSD 1
17β-HSD 2, 4
5-Diol
3β-HSD
3β-HSD
Androstenedione
(4-Dione)
17β-HSD 3, 5
17β-HSD 2, 10
Testosterone
Aromatase
5α-R 1, 2
Aromatase
Estrone
(E1)
17β-HSD 1, 7, 12
17β-HSD 2, 4, 8, 14
Dihydrotestosterone
(DHT)
Estradiol
(E2)
3β-HSD
3β-Diol
ER

Figure 6.

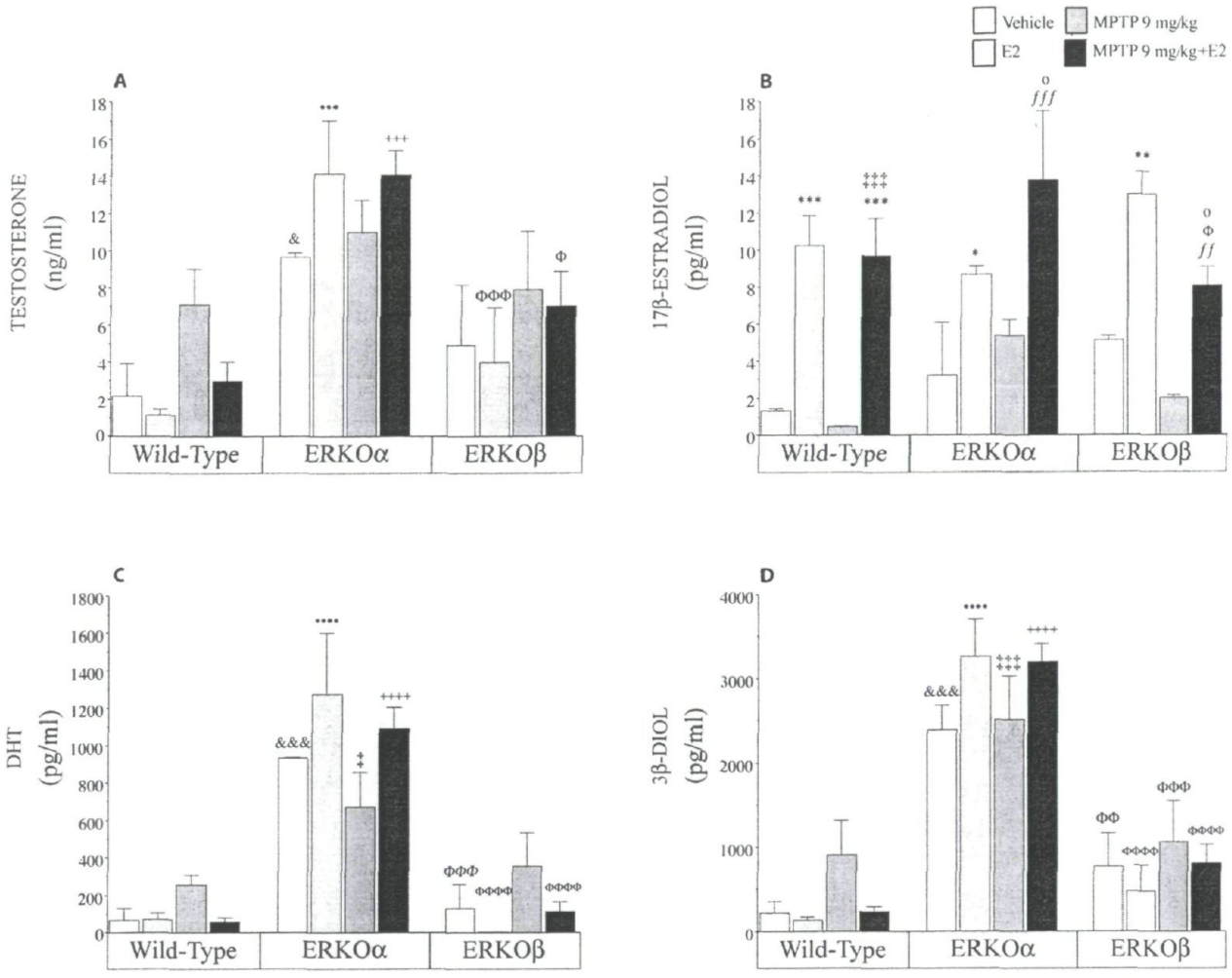
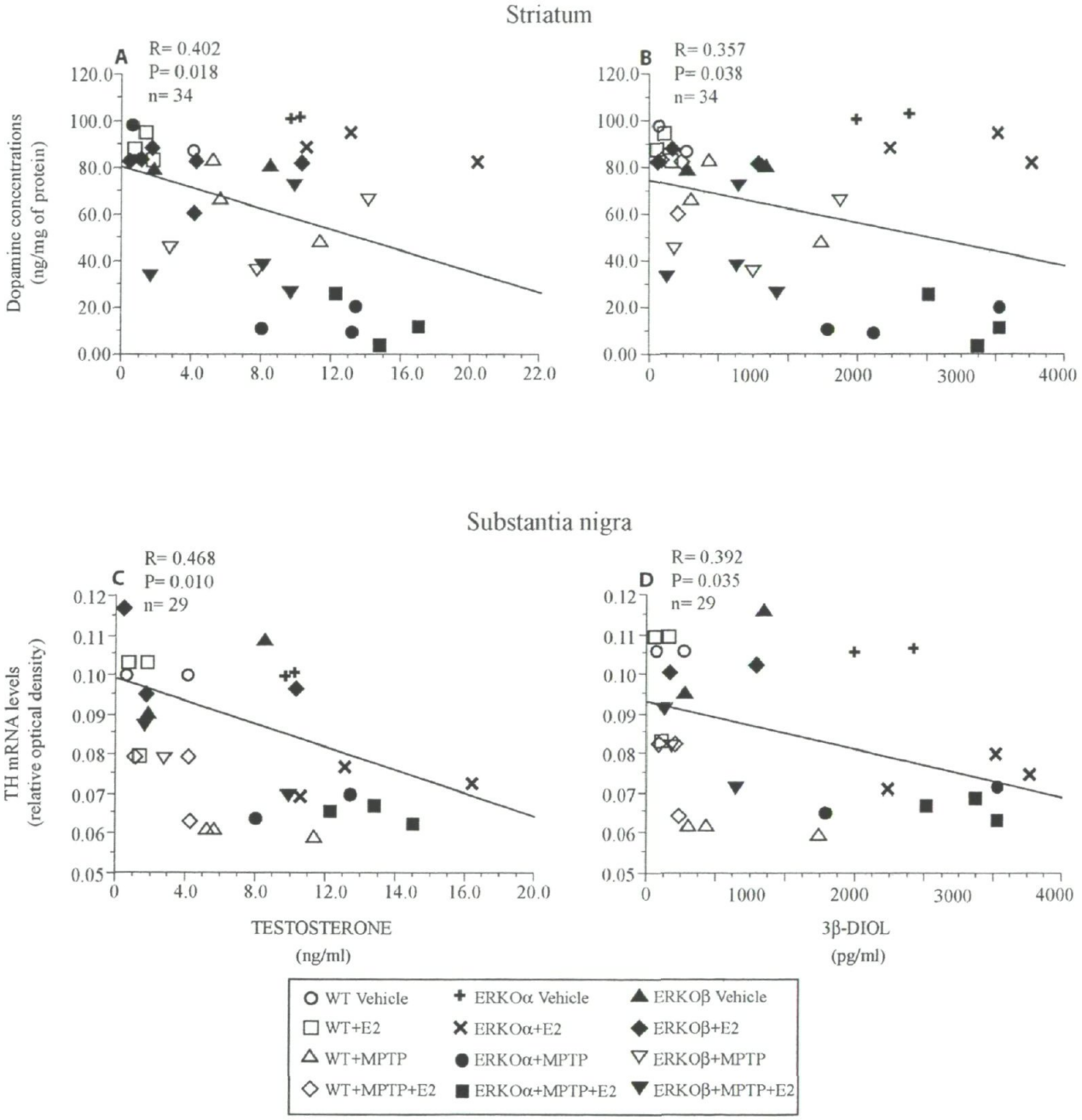

A
TESTOSTERONE (ng/ml)
Wild-Type
ERKOα
ERKOβ
B
17β-ESTRADIOL (pg/ml)
Vehicle
E2
MPTP 9 mg/kg
MPTP 9 mg/kg+E2
C
DHT (pg/ml)
D
3β-DIOL (pg/ml)

Figure 7.


Striatum
A
R= 0.402
P= 0.018
n= 34
B
R= 0.357
P= 0.038
n= 34
Dopamine concentrations (ng/mg of protein)
Substantia nigra
C
R= 0.468
P= 0.010
n= 29
D
R= 0.392
P= 0.035
n= 29
TH mRNA levels (relative optical density)
TESTOSTERONE (ng/ml)
3β-DIOL (pg/ml)
WT Vehicle
ERKOα Vehicle
ERKOβ Vehicle
WT+E2
ERKOα+E2
ERKOβ+E2
WT+MPTP
ERKOα+MPTP
ERKOβ+MPTP
WT+MPTP+E2
ERKOα+MPTP+E2
ERKOβ+MPTP+E2

XI. LEGENDS TO FIGURES

Figure 1. Dose-response effect of MPTP on striatal DAT (A) and VMAT2 (B) specific binding respectively measured with [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH binding in wild-type compared to ERKOα and ERKOβ mice. There was a statistically significant effect of MPTP in the anterior striatum (DAT: $F_{11,59}$=12.50, p<0.0001 and VMAT2: $F_{11,58}$=23.73, p<0.0001). **p<0.01 and ****p<0.0001 vs respective intact, vehicle (0 MPTP); †††p<0.005 and ††††p<0.0001 vs WT MPTP 7 mg/kg; ‡p<0.05, ‡‡p<0.01 and ‡‡‡‡p<0.0001 vs WT MPTP 9 mg/kg; ££ p<0.001 and £££p<0.005 vs WT MPTP 11 mg/kg; #p<0.05, ##p<0.01, ###p<0.005 and ####p<0.0001 vs respective ERKO+MPTP 7 mg/kg; $p<0.05 and $$p<0.01 vs respective ERKO+MPTP 9 mg/kg; &p<0.05 and &&p<0.01 vs WT vehicle (0 MPTP); ΦΦp<0.01 and ΦΦΦp<0.005 vs respective experimental ERKOα genotype group. One of the six mice died in the ERKOα MPTP 11 mg/kg group.

Figure 2. Effect of treatment with 17β-estradiol (E_2) (2μg/day) for ten days on anterior striatal DA transporters DAT (A) and VMAT2 (B) specific binding respectively measured with [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. There was a statistically significant effect of treatments for DAT ($F_{11,71}$=29.13, p<0.0001) and VMAT2 ($F_{11,71}$=26.94, p<0.0001) specific binding. *p<0.05, **p<0.01, ***p<0.005, and ****p<0.0001 vs respective intact, vehicle (0 MPTP); ‡p<0.05 and ‡‡‡‡p<0.0001 vs WT MPTP; ••p<0.01 vs WT+E2; ++++p<0.0001 vs WT+MPTP+E2; &&p<0.01 vs WT vehicle (0 MPTP); ΦΦΦp<0.005 and ΦΦΦΦp<0.0001 vs respective experimental ERKOα genotype group.

Figure 3. Correlations between [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH specific binding and DA concentrations in the anterior striatum of WT, ERKOα and ERKOβ mice of the MPTP dose-response (A and B) and the MPTP/17β-estradiol experiments (C and D). Striatal DA concentrations of these mice are from Morissette et al. 2007.

Figure 4. A) Dose-response effect of MPTP on SN tyrosine hydroxylase (TH) mRNA levels measured by *in situ* hybridization in wild-type compared to ERKOα and ERKOβ mice ($F_{11,52}$=4.75, p<0.0001). B) Effect of MPTP and 17β-estradiol (E_2) (2μg/day) treatments on SN TH mRNA levels in wild-type compared to ERKOα and ERKOβ mice ($F_{11,51}$=5.81, p<0.0001). *p<0.05, ***p<0.005 and ****p<0.0001 vs respective intact, vehicle (0 MPTP); ‡p<0.05 vs WT MPTP 9 mg/kg; ££p<0.01 and £££p<0.005 vs WT MPTP 11 mg/kg; #p<0.05 and ###p<0.005 vs respective ERKO+MPTP 7 mg/kg; +p<0.05 vs WT+MPTP+E_2; and ΦΦp p<0.01 vs respective experimental ERKOα genotype group.

Figure 5. Simplified schematic representation of biosynthesis of the steroids assayed. The dotted arrow implies that several stages and intermediate steroids were implicated in the biosynthesis of the final product. Encircled steroids were assayed. Testosterone is converted into 17β-estradiol and 3β-diol through the actions of 5α-R, aromatase and 3β-HSD (Gruber et al., 2002). Estrone is produced from DHEA by the enzymes 3β-HSD and aromatase (Gruber et al., 2002). P450scc, cytochrome P450 sidechain cleavage; P450c17, cytochrome P450 17α-hydroxysteroid/C17, 20-lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-R, 5α–reductase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; 3β-diol, 5α-androstan 3-β, 17β-diol; E1, estrone, ER, estrogen receptor.

Figure 6. Effect of MPTP (9 mg/kg) and 17β-estradiol treatment on blood plasma levels of (A) testosterone ($F_{11,22}$=4.20, p=0.0021) , (B) 17β-estradiol (E_2) ($F_{11,21}$=7.22, p<0.0001), (C) DHT ($F_{11,22}$=10.57, p<0.0001) and (D) 3β-Diol ($F_{11,22}$=12.04, p<0.0001) of wild-type compared to ERKOα and ERKOβ mice. Plasmas of three mice were pooled (according to similar striatal DA concentrations) in order to produce quantities sufficient for steroid detection and quantification. *p<0.05, **p<0.01 and ***p<0.005 vs respective intact, vehicle (0 MPTP); ‡p<0.05 and ‡‡‡p<0.005 vs WT MPTP; •••p<0.005 and ••••p<0.0001 vs WT+E_2; +++p<0.005 and ++++p<0.0001 vs WT+MPTP+E_2; &p<0.05 and &&&p<0.005 vs WT vehicle (0 MPTP); op<0.05 vs respective ERKO E_2; *fff*p<0.01 and *fff*p<0.005 vs respective ERKO MPTP; Φp<0.05, ΦΦp<0.01, ΦΦΦp<0.005 and ΦΦΦΦp<0.0001 vs respective experimental ERKOα genotype group.

Figure 7. Correlations between striatal DA concentrations (A and B) and SN TH mRNA levels (C and D) and testosterone or 3β-diol plasma levels of WT, ERKOα and ERKOβ mice.

XII. SUPPLEMENTARY MATERIAL

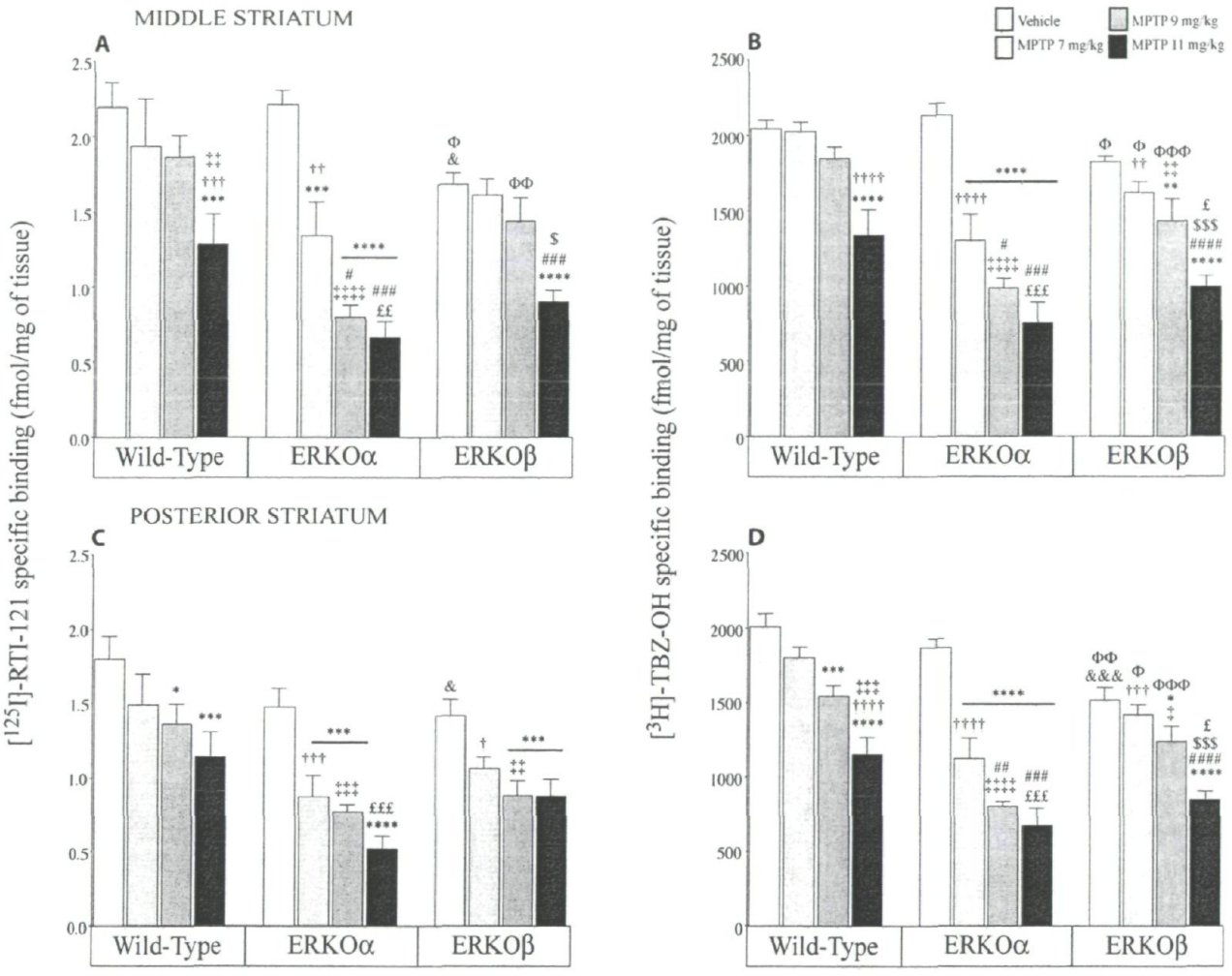


Figure 1 supplementary data. Dose-response effect of MPTP on striatal DAT (A and C) and VMAT2 (B and D) specific binding respectively measured with [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH binding in wild-type compared to ERKOα and ERKOβ mice. There was a statistically significant effect of MPTP in the middle (DAT: $F_{11,58}$=10.16, p<0.0001 and VMAT2: $F_{11,56}$=19.27, p<0.0001) and posterior (DAT: $F_{11,59}$=7.77, p<0.0001 and VMAT2: $F_{11,57}$=23.26, p<0.0001) striatum. *p<0.05, **p<0.01, ***p<0.005 and ****p<0.0001 vs respective intact, vehicle (0 MPTP); †p<0.05, ††p<0.01, †††p<0.005 and ††††p<0.0001 vs WT MPTP 7 mg/kg; ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.005 and ‡‡‡‡p<0.0001 vs WT MPTP 9 mg/kg; £p<0.05, ££ p<0.001 and £££p<0.005 vs WT MPTP 11 mg/kg; #p<0.05, ##p<0.01, ###p<0.005 and ####p<0.0001 vs respective ERKO+MPTP 7 mg/kg; $p<0.05 and $$$p<0.005 respective ERKO+MPTP 9 mg/kg; &p<0.05 and &&&p<0.005 vs WT vehicle (0 MPTP); Φp<0.05, ΦΦp<0.01 and ΦΦΦp<0.005 vs respective experimental ERKOα genotype group. One of the six mice died in the ERKOα MPTP 11 mg/kg group.

CHAPTER IV

Effect of oestrogen receptors on brain NMDA receptors of MPTP mice

Sara Al-Sweidi, Marc Morissette and Thérèse Di Paolo

Molecular Endocrinology and Genomic Research Center of CHUQ-CHUL

and Faculty of Pharmacy, Laval University, Quebec, Qc, Canada

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I. FOREWORD

Sara Al-Sweidi: I treated the mice, participated in the sacrifice, during catecholamine dosing through HPLC and cryostat cutting of mouse brains. I did all the NMDA NR1/NR2B [^{3}H]Ro 25-6981 specific binding autoradiography experiments. Previously performed the blood plasma preparations for assaying by gas chromatography and negative chemical ionization mass spectrometry. Completed all the autoradiogram and statistical analysis and wrote the article.

Marc Morissette: Research assistant in our laboratory who had a lot to do during this project. He extracted mouse brains during the sacrifice, then dissected and prepared the brain structures for HPLC dosing then performed these. Also, he helped with the cutting of the mouse brains and the autoradiography experiments, as well as the writing of the article.

Thérèse Di Paolo: Research director who undertook main decisions concerning which treatments had to be administered to the knockout mice. She planned and supervised all the protocols from start to finish and made an important contribution in the writing of the article.

II. RÉSUMÉ

Le Parkinson est une maladie dégénérative caractérisée par la perte des neurones dopaminergiques de la voie nigrostriatale et une hyperactivité glutamatergique. Plusieurs études suggèrent une implication des oestrogènes dans la neuroprotection contre la maladie de Parkinson. Le 17β-oestradiol prévient la perte de dopamine (DA) striatale causée par la neurotoxine 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP) chez la souris; et module les récepteurs N-méthyl-*D*-aspartate (NMDA). Chez la souris, le 17β-oestradiol protège contre la perte de la dopamine striatale causée par la neurotoxine MPTP et cet effet neuroprotecteur peut impliquer les récepteurs oestrogéniques ERα et ERβ. Afin de évaluer le role des ERs dans la réponse des récepteurs NMDA à une lesion, nous avons fait une étude chez des souris mâles C57Bl/6 invalidées (KO) pour les ERα ou ERβ (ERKOα et ERKOβ). Des souris sauvages (WT), ERKOα et ERKOβ ont reçu un traitement de MPTP (7, 9, 11 mg/kg) et ont été comparées à des contrôles (injectées au véhicule). De plus, des souris WT, ERKOα et ERKOβ traitées au MPTP (9 mg/kg) ont reçu un traitement avec le 17β-oestradiol (2 μg/jour). Le cortex, le striatum et l'hippocampe ont été analysés pour des concentrations des récepteurs ayant les sous unités NR2B (NMDA/NR2B) par autoradiogaphie utilisant le ligand [^{3}H]Ro 25-6981. Dans le cortex frontal, le cortex cingulé et le striatum, les souris WT traitées au véhicule avaient une plus grande liaison spécifique que les souris ERKO. La liaison spécifique corticale a diminué avec l'augmentation de la dose de MPTP chez les souris WT et ERKOα, ainsi que dans le striatum des souris WT. Toutfois, les souris invalidées pour les ERα et ERβ avaient des niveaux striatales de récepteurs NMDA diminués. Le traitement avec le 17β-oestradiol n'avait pas d'effet dans le cortex de souris des trois génotypes (intactes ou lésées) avec le MPTP. Dans le striatum, le 17β-oestradiol augemente la liaison spécifique de [^{3}H]Ro 25-6981 chez les souris intactes ERKOβ et WT lésées au MPTP. De plus, la liaison spécifique striatale corrèle positivement avec les concentrations de dopamine striatale seulement chez les souris WT.

Finalement, les traitements MPTP et 17β-oestradiol avaient des effets limités dans l'hippocampe, dans les sous-régions CA3 et le gyrus denté, les souris ERKOα traitées au véhicule ou avec le 17β-oestradiol avaient des niveaux augmentés de la liaison spécifique. Le MPTP diminue la liaison spécifique chez les souris ERKOα dans le CA1, CA2 et le CA3. Donc, les récepteurs

NMDA sont affectés par le manque de ER et cela peut ensuite affecter la réponse aux traitements de MPTP et 17β-oestradiol avec une spécificité dans diverses régions du cerveau.

III. ABSTRACT

Parkinson's disease (PD) is characterized by the loss of nigrostriatal dopamine (DA) neurons and glutamate overactivity. There is substantial evidence to suggest that oestrogens prevent or delay the disease. 17β-oestradiol, has neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD and modulates brain N-methyl-*D*-aspartate (NMDA) receptors. In MPTP-lesioned mice oestrogen receptors ERα and ERβ are important in 17β-oestradiol induced neuroprotection. In order to evaluate the role of ERs in the response of NMDA receptors to lesion, we compared wild-type (WT) to ER knockout (KO) C57Bl/6 male mice that received 7, 9 or 11 mg/kg of MPTP. These mice were also treated with MPTP (9 mg/kg) and 17β-oestradiol. [^{3}H]Ro 25-6981 specific binding autoradiography was used to label NMDA receptors containing NR2B subunits. In the frontal and cingulate cortex and striatum, vehicle-treated WT mice had higher [^{3}H]Ro 25-6981 specific binding compared to ERKO mice. Cortical [^{3}H]Ro 25-6981 specific binding decreased with increasing doses of MPTP in WT and ERKOα but not ERKOβ mice, while a dose related decrease was only observed in the striatum of WT mice remaining low in ERKOα and ERKOβ mice. No effect of 17β-oestradiol treatment in intact or MPTP-lesioned mice of all three genotypes was observed in cortex, while it increased striatal specific binding of intact ERKOβ and MPTP-lesioned WT mice. Striatal [^{3}H]Ro 25-6981 specific binding positively correlated with striatal DA concentrations only in WT mice. MPTP and 17β-oestradiol treatments had more limited effects in hippocampus. Only in CA3 and dentate gyrus did vehicle and 17β-oestradiol-treated ERKOα mice have higher [^{3}H]Ro 25-6981 specific binding than WT and ERKOβ mice, while MPTP decreased this specific binding only in CA1, CA2 and CA3 of ERKOα mice. Hence, brain NMDA receptors were affected by deletion of ERs which affect the response to MPTP and 17β-oestradiol treatments with brain region specificity.

IV. INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and its incidence is likely to increase due to the aging population (1). PD principally involves the death of dopamine (DA) neurones in the substantia nigra (SN) but other neurotransmitters and neuromodulators are also affected. Gene mutations in familial PD are reported but the cause of the majority of PD cases is currently unknown (2) and there is no cure for PD. Neuroprotection or disease modification defined as an intervention that would protect or rescue vulnerable neurones, thereby slowing, stopping, or reversing disease progression, is not yet available for PD (2). Restoring deficient DA with its precursor levodopa remains the most effective PD treatment, but a majority of patients develop abnormal involuntary movements called dyskinesias after 5-10 years of treatment; dyskinesias are very difficult to manage (3, 4). No drug is yet available for dyskinesias, aside from a modest benefit with amantadine, a drug with antagonistic glutamate activity, in some PD patients (5). Moreover, while levodopa and DA agonists, currently used in the treatment of PD, are effective at reversing the motor symptoms of the disease they do little to combat the underlying degeneration of DA neurones.

Glutamate is the most abundant excitatory neurotransmitter, mediating as much as 70% of brain synaptic transmission (6). Glutamate activity is increased in the basal ganglia in PD (6). The striatum receives two major inputs: a massive excitatory glutamatergic projection from the cerebral cortex and a dopaminergic projection from the SN (7). In PD, loss of striatal DA is associated with the loss of the inhibitory DA control of cortico-striatal glutamatergic drive and with the consequent increased glutamate release (8).

Ionotropic glutamate receptors mediate fast excitatory neurotransmission and include N-Methyl-*D*-aspartic acid or N-Methyl-*D*-aspartate (NMDA), 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-

yl)propanoic acid (AMPA) and kainate receptors (9, 10). Reducing glutamate overactivity can be achieved by blocking post-synaptic glutamate receptors with antagonists. Ionotropic glutamate receptor antagonists, mainly NMDA and AMPA receptor antagonists, have been investigated but cause significant adverse effects in their different clinical applications including PD, limiting their usefulness (11, 12).

Oestrogens induce their transcriptional activity through two distinct classical receptors, oestrogen receptors alpha (ERα) and beta (ERβ). Both ERα and ERβ mRNAs are expressed in the cortex (13) and in GABAergic neurons of the striatum (14). Immunohistochemistry and *in situ* hybridization confirm that both ERs are present in the hippocampus, (15). Also, ERα and ERβ are present in the SN (16) and their colocalization with tyrosine hydroxylase was observed in the ventral tegmental area and the SN of the rat brain (17).

Oestrogens are well documented to be neuroprotective of nigro-striatal DA against various toxins such as MPTP and methamphetamine (18, 19). Oestrogens have been shown to modulate NMDA and AMPA receptors in the prefrontal and cingulate cortex as well as in the striatum and hippocampus of ovariectomised female rats (20, 21) increasing or decreasing these receptors depending on the brain region. The neuromodulatory effects of oestrogenic drugs on glutamate receptors in male mice is well documented (21), but little information is available on the oestrogenic modulation of glutamate receptors in animal models of PD. Oestrogens acting through their respective receptors can modulate glutamate receptors, therefore the hypothesis is that oestrogenic modulation of glutamate receptors plays a role in oestrogenic neuroprotection. Hence, the aim of the present study was to investigate the effect of 17β-oestradiol treatment on NMDA receptors in intact mice and mice lesioned with MPTP as well as the contribution of ERα and ERβ by comparing wild-type (WT) and ER knockout (KO) male mice.

V. MATERIALS and METHODS

Animals and treatments

Adult WT, ERKOα and ERKOβ male C57Bl/6 mice (7-12 weeks, 18-28 g, WT and ERKO mice) were purchased from Taconic Laboratories (Hudson, NY, USA). MPTP and 17β-oestradiol were purchased from Sigma Chemical (St-Louis, MO, USA). WT and ERKO mice were equally distributed for age and weight in experimental groups of six animals. The Laval University Animal Care Committee approved all the animal studies. All efforts were made to minimise animal suffering and to reduce the number of mice used.

An extended MPTP dose-response up to 20 mg/kg was performed in WT male C57Bl/6 mice and striatal biogenic amine concentrations of these mice was previously reported (22). The MPTP doses (7, 9 and 11 mg/kg), that specifically affected striatal DA while sparing serotonin concentrations in WT mice, were used for comparison of MPTP dose-responses of ERKOα, ERKOβ and WT mice (22). Mice received four 0.1 ml intraperitoneal injections with saline or a saline solution of MPTP at a two-hour interval and were killed 5 days after treatment with MPTP.

The effect of 17β-oestradiol and MPTP toxicity in ERKOα and ERKOβ was compared to WT mice. Four groups of both ERKOα and ERKOβ mice were compared to WT mice. An intermediate dose of 9 mg/kg MPTP was selected and the effect of 17β-oestradiol treatment was investigated in intact and MPTP mice. Each group received a 5-day pre-treatment of 17β-oestradiol or vehicle prior to MPTP injections. The pre-treatment consisted of two daily subcutaneous injections (in the dorsal part of the neck) of 17β-oestradiol, while control mice received injections of vehicle (0.9% saline with 0.3% gelatin). Physiological concentrations of 17β-oestradiol were used, 2 μg per day as previously published (22, 23). 17β-oestradiol at 1-2 μg are beneficially effective against neurotoxicity (19).

On day 5, mice received four injections of MPTP (9 mg/kg, per intraperitoneal injection) at a 2-hour interval, while the control group received saline solution. Treatments with 17β-oestradiol or vehicle were continued until day 10. The next day, mice were euthanised with an air/halothane mixture and decapitated; trunk blood was collected and brains were quickly removed and frozen in a mixture of isopentane/dry ice and then stored at –80°C.

Preparation of brain tissue

Frozen brains were cut on a cryostat in 12 μm thick slices at cortical, striatal and hippocampal regions. Coronal sections for the anterior cortex (bregma 2.46 mm), anterior striatum (bregma 1.18 mm) and hippocampus (bregma –1.94 mm) were cut according to the mouse brain atlas by Franklin and Paxinos (24). Histological sections were thaw-mounted on superfrost slides (Trade Mark, Fisher, On, Canada) and stored at -80°C.

[^{3}H]Ro 25-6981 *Autoradiography*

Autoradiography using [^{3}H]Ro 25-6981 (26 Ci/mmol, gift from F. Hoffman-La Roche Ltd., Basel, Switzerland), an NMDA antagonist selective for the NR1/NR2B assembly, was done on the cortex, the striatum and the hippocampus according to our previously published procedure (20). The slides were briefly preincubated twice for ten minutes in a Tris-HCl 50 mM, EDTA 10 mM, pH 7.6 buffer at room temperature. Then sections were incubated with 5 nM of [^{3}H]Ro 25-6981 in the same buffer for 90 minutes at 4°C. Afterwards, the sections were rinsed three times (2 X 5 min and 1 X 15 min) in the Tris-HCl/EDTA buffer at 4°C. Non-specific binding was determined by adding 10 μM Ro 04-5595 hydrochloride (gift from F. Hoffman-La Roche Ltd.) to the incubation buffer. Slides were exposed to Kodak BIOMAX MR film (Kodak, Rochester, NY,

USA) with calibrated [^{3}H] standards (Microscales, Amersham, Arlington Heights, IL, USA). Cortical, striatal and hippocampal slides were exposed for thirty five days and [^{3}H]Ro 25-6981 specific binding was calculated by subtracting the measured non-specific binding from the total binding.

Statistical analysis

Autoradiograms of specific binding relative optical densities were analyzed using Scion Image 1.63 software. The anterior cortex was subdivided into the cingulate and the frontal motor cortex, while the anterior striatum was subdivided in half into medial and lateral regions for analysis. The hippocampus was analysed in subregions including the CA1 strata oriens (or) and radiatum (rad); CA2 (or and rad), CA3 and dentate gyrus. For each group, the mean of 6-9 animals and the mean of six brain sections per animal were measured. The statistical analyses were performed using Statview 4.51 for Macintosh Computer software. A one-way ANOVA was used to compare MPTP and/or 17β-oestradiol treatments followed by post-hoc analysis with Fisher probability of least significance difference test. A $p < 0.05$ was required for the results to be considered statistically significant.

VI. RESULTS

The effect of the MPTP lesion on NMDA receptors in the WT compared to the ERKO mice was first investigated and showed a difference between the genotypes. Control ERKOα had lower [^{3}H]Ro 25-6981 specific binding in the cortex, both the frontal ($F_{11,57}$=8.38, p<0.0001) and cingulate cortex ($F_{11,57}$=6.48, p<0.0001), being at 82% and 88% respectively of control WT mice values (Figure 1A and 1B). Moreover, compared to WT mice the intact ERKOβ mice also had low NMDA receptor specific binding observed at 79% in the frontal cortex and 81% in the cingulate cortex. A dose related decrease of cortical NMDA receptor specific binding caused by MPTP was observed in ERKOα mice but not in ERKOβ mice. In the frontal cortex and cingulate, NMDA receptor specific binding of WT mice decreased only with 9 mg/kg of MPTP. In ERKOβ mice, NMDA receptor specific binding increased with 11 mg/kg of MPTP when compared to respective ERKOα experimental group in both cortical regions (Figure 1).

In the MPTP and 17β-oestradiol treatment experiment, as in the MPTP dose-response experiment, vehicle treated ERKOα mice had lower cortical [^{3}H]Ro 25-6981 specific binding, at 71% in the frontal cortex and 62% in the cingulate when compared to control WT mice values (Figure 2A and 2B). Similarly, lower values were also measured in the cortex of control ERKOβ mice compared to control WT mice values, being at 79% in the frontal cortex ($F_{11,72}$=5.02, p<0.0001) and 74% in the cingulate ($F_{11,75}$=8.77, p<0.0001) (Figure 2A and 2B). 17β-oestradiol treatment did not influence NMDA specific binding in the cortex of all three non-lesioned genotypes (Figure 2). 9 mg/kg of MPTP increased NMDA specific binding only in ERKOβ mice and this effect was significant in the cingulate, however treatment with 17β-oestradiol did not bring back NMDA receptor specific binding of MPTP lesioned ERKOβ mice to that of respective

controls (Figure 2B). No effect of 17β-oestradiol treatment was observed in WT and ERKOα MPTP lesioned mice.

Similar to the results observed in the cortex, lower values were also measured in the striatum of control ERKOα and ERKOβ mice, when compared to control WT mice values (Figure 3A and 3B). In the MPTP dose-response experiment, control ERKOα mice had much lower striatal NMDA NR1/2B specific binding, seen at 57% in the medial striatum ($F_{11,58}$=10.42, $p<0.0001$) and at 59% in the lateral striatum ($F_{11,58}$=12.95, $p<0.0001$) when compared to control WT mice values. Lower values were also measured in the anterior striatum of control ERKOβ mice but to a smaller extent, 72% in the medial striatum and at 75% in the lateral striatum when compared to control WT mice values and significantly higher than that of control ERKOα mice (Figure 3).

In addition, a dose-related decrease of striatal NMDA receptor specific binding with increasing doses of MPTP was observed in the striatum of WT but not in ERKOα and ERKOβ mice. MPTP had no dose-response effect in the striatum of ERKOα or ERKOβ mice. These effects are almost identical in the medial and lateral striatum (Figure 3). Striatal [^{3}H]Ro 25-6981 specific binding positively correlated with striatal DA concentrations of these WT mice (Figure 3C), whereas no correlation was obtained for ERKOα (Figure 3D) or ERKOβ (Figure 3E) mice.

In the MPTP lesion and 17β-oestradiol treatment experiment, like in the MPTP dose-response experiment, vehicle-treated ERKOα mice had lower striatal NMDA NR1/2B specific binding at 82% of vehicle-treated WT mice values in both the medial ($F_{11,78}$=13.62, $p<0.0001$) and lateral ($F_{11,78}$=13.76, $p<0.0001$) striatum (Figure 4A and 4B). Lower values were also measured in the striatum of vehicle-treated ERKOβ mice, being at 91% and 94% of vehicle-treated WT mice values in the medial and lateral striatum, respectively (Figure 4).

The 17β-oestradiol treatment had no effect on non-lesioned WT and ERKOα mice, but increased NMDA specific binding in the striatum (lateral as well as medial parts) of non-lesionded ERKOβ mice (Figure 4A and 4B). MPTP (9 mg/kg) was only effective in WT mice, significantly decreasing striatal NMDA specific binding and 17β-oestradiol co-treatment prevented this decrease. No effect of 17β-oestradiol treatment was observed in ERKOα or ERKOβ MPTP lesioned mice. These effects were closely identical in the medial and lateral parts of the striatum (Figure 4A and 4B). In this experiment also striatal [^{3}H]Ro 25-6981 specific binding positively correlated with striatal DA concentrations of these WT mice (Figure 4C) whereas no correlation was obtained for ERKOα (Figure 4D) or ERKOβ (Figure 4E) mice.

While the cortical and the striatal NMDA receptors responded similarly to absence of ERα or ERβ and were affected by the MPTP lesion, a different pattern was observed in the hippocampus. No effect of the lesion at 7, 9 and 11 mg/kg MPTP doses on NMDA specific binding was measured in the CA1(or), CA2 (or and rad) and dentate gyrus, while a small decrease (17%) was observed in the CA1(rad) and CA3 (data not shown).

In the CA1 subregion of the hippocampus, (oriens: $F_{11,78}$=2.10, p=0.03 and radiatum $F_{11,78}$=3.82, p=0.0002), intact ERKOβ mice had lower (9%) NMDA receptor specific binding when compared to intact ERKOα mice. 17β-oestradiol treatment had no effect on NMDA receptor specific binding of all three genotypes. MPTP alone and combined with the 17β-oestradiol treatment significantly decreased NMDA receptor specific binding of ERKOα mice in both the CA1 or and rad (Figure 5A and B).

Similar results to the CA1 were observed in the radiatum ($F_{11,78}$=2.47, p=0.01) of the CA2 hippocampal subregion (Figure 6B), as well as in the CA2 oriens ($F_{11,78}$=1.76, p=0.07) but did not reach statistical significance in the latter (Figure 6A).

Intact ERKOα mice had higher NMDA receptor specific binding levels when compared to the non-lesioned WT and ERKOβ mice in both the CA3 ($F_{11,78}$=3.08, p= 0.001) and dentate gyrus ($F_{11,78}$=2.80, p=0.004) (Figure 7A and B). Treatment with 17β-oestradiol did not affect NMDA receptor specific binding of all three non-lesioned genotype experimental groups. MPTP significantly decreased NMDA receptor specific binding of only the ERKOα mice in the CA3 hippocampal subregion and its combination with 17β-oestradiol decreased these levels even more in both the CA3 and dentate gyrus (Figure 7).

Summaries of genotype differences in [^{3}H]Ro 25-6981 specific binding to NMDA receptors as well as the differences in response to MPTP and 17β-oestradiol treatments in the brain regions investigated of WT, ERKOα and ERKOβ mice are included in Tables 1 and 2.

VII. DISCUSSION

The present study showed an oestrogen-glutamate interaction involving NMDA/NR2B receptors in the effects of MPTP and/or 17β-oestradiol treatment in male mice.

Effect of lesion on NMDA receptors

Overactive glutamate neurotransmission is well known in PD as a consequence of removing the inhibitory dopaminergic tone on cortico-striatal glutamate (25). NMDA and AMPA receptors specific binding were observed to be lower in striatum of PD patients (26) and of MPTP-lesioned monkeys (27). The reduced striatal NMDA receptors in PD and MPTP-lesioned animals could be a compensatory response to oppose overactive glutamate transmission. As in human and non-human primates, reduced cortical and striatal NMDA receptors were also observed here in WT MPTP-lesioned mice compared to control mice which could also be explained by a compensatory mechanism. By contrast, this response of NMDA receptors to glutamate overactivity in the striatum of WT mice was not observed in mice lacking ERα or ERβ, while in the cortex it is not observed in mice lacking ERβ, suggesting disrupted compensation.

Neurotransmitter and regional specificity of the MPTP lesion

We previously reported striatal concentrations of serotonin and its metabolites 5-hydroxyindoelacetic acid (5-HIAA) in these mice (22); they remained unchanged at doses of MPTP up to 11 mg/kg suggesting that under the present conditions the lesion was specific to nigrostriatal DA, serotonin remaining unchanged. Higher doses of MPTP will affect striatal serotonin and 5-HIAA concentrations as we have previously reported (22). This is our first

detailed investigation of brain regions other than the striatum and the SN using our MPTP mouse model of oestrogenic neuroprotection. A paradigm similar to ours, using acute lesioning with 20 mg/kg of MPTP of 8-10 week old male C57BL/6 mice, provided HPLC analysis reporting that DA concentrations are depleted in striatum and frontal cortex (28). DA concentrations were low in the frontal cortex (4.0 ng/mg of protein) of control mice compared to the striatum (141 ng/mg protein) and at 7 days post-MPTP striatal DA was depleted by 95% in the dorsal striatum, 86% in the ventral striatum and 88% in frontal cortex (28). In their study, serotonin loss following MPTP was modest compared to that of DA with a 48% loss in the dorsal striatum, 39% in the ventral striatum and 48% in the frontal cortex. Based on these results, in our experiment the frontal cortex DA depletion is likely to be similar to that seen in striatum, but both structures would be depleted to a lesser extent since we used lower doses of MPTP to be neurotransmitter specific for DA leaving serotonin levels unchanged. Hence, the effects of MPTP on NMDA receptors in the frontal cortex could be due to decreased cortical DA concentrations, but could also be indirect since striatal DA concentrations are much higher than cortical levels and nigrostriatal DA exerts an inhibitory control on cortico-striatal glutamate (29).

In another paradigm also similar to ours, reduced striatal and hippocampal DA concentrations were measured in 8 weeks old male C57BL/6 mice administered 20 mg/kg of MPTP (four i.p. injections two hours apart) (30). DA concentrations were low in the hippocampus (0.25 μg/g) of control mice compared to the striatum (9.34 μg/g); at 7 days post-MPTP striatal DA was depleted to 34% of controls and hippocampal DA concentrations to 52% of controls (30). Depletion of hippocampal DA levels was less than in the striatum but it attenuated NMDA receptor mediated synaptic transmission, altered the time course of long-term potentiation (LTP) and long-term depression (LTD) by impairing the induction of LTP and prolonging the duration of LTD in these mice (30). Therefore, DA loss alters NMDA receptor-

mediated synaptic transmission and activity-dependant synaptic plasticity (30). Based on Zhu et al. 2011 results, in our experiment DA depletion was also likely to be less in the hippocampus than in the striatum and depleted to a lesser extent in both regions since we used lower doses of MPTP. This would be consistent with our observed general lack of effect of MPTP on NMDA receptors in the hippocampal subregions since DA depletion is likely modest. Similarly, here also the effects of MPTP in the hippocampus could be due to decreased hippocampal DA concentrations, but could also be indirect since striatal DA concentrations are much higher than the hippocampal levels.

Plasma 17β-oestradiol levels

We previously reported the blood plasma steroid concentrations of the mice used in the MPTP lesion and 17β-oestradiol treatments experiment (31). An increase of 17β-oestradiol plasma levels was measured in mice treated with 17β-oestradiol of all the genotypes investigated; the lack of response of ERKO mice to the 17β-oestradiol treatment was therefore not because of lower circulating concentrations of 17β-oestradiol due to higher metabolism of exogenous administered 17β-oestradiol in these knockout mice. Nevertheless, we observed some differences in plasma 17β-oestradiol concentrations, being higher in ERKOα and lower in ERKOβ MPTP mice compared to the unlesioned 17β-oestradiol treated respective ERKO mice (22).

Effect of MPTP, 17β-oestradiol and gonadectomy on NMDA receptors

In 17β-oestradiol treated and MPTP-lesioned WT mice, nigro-striatal DA degeneration is prevented and this is possibly associated with diminished cortico-striatal glutamate overactivity since DA is less depleted. Therefore, higher NMDA receptor specific binding may be due to

lesser decreased levels of this receptor in order to compensate for overactive glutamate activity, since the effects of MPTP are dampened by 17β-oestradiol induced neuroprotection. Higher NMDA receptor specific binding may also be due to the effect of 17β-oestradiol increasing the synthesis of this receptor. Accordingly, using specific agonists for ERα and ERβ we reported that ERα agonists protect against MPTP toxicity in mice (32), that ERKOα mice are more sensitive to the loss of striatal DA concentrations by MPTP and both ERKOα and ERKOβ mice are not protected by 17β-oestradiol against MPTP induced striatal DA loss (22).

We previously reported the effect of ovariectomy and oestrogen treatment in ovariectomised rats on NMDA receptors containing NR2B subunits with [^{3}H]Ro 25-6981 binding autoradiography and *in situ* hybridisation of NMDAR1/2B subunits (20, 33, 34). In the cingulate and the prefrontal cortex, ovariectomy increased [^{3}H]Ro 25-6981 specific binding compared to intact rats and this was corrected with a 17β-oestradiol treatment, while it was not significant with the ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) or the ERβ agonist 2,3-bis(4-hyroxyphenyl) propionitrile (DPN) (21). Both the selective oestrogen receptor modulators tamoxifen and raloxifene, like 17β-oestradiol, decrease [^{3}H]Ro 25-6981 specific binding in the cingulate cortex of ovariectomised rats and increase this binding in hippocampal regions (20). We also investigated NMDA receptors in intact, short-term (2 weeks) and long-term (10 months) ovariectomised rats using NMDA-displaceable [^{3}H]glutamate specific binding. In the hippocampus, we saw a decrease with ovariectomy that was corrected with 17β-oestradiol, tamoxifen and raloxifene treatments, while in the cortex an increase was seen with ovariectomy that was corrected with these oestrogenic drug treatments (20, 21, 35). Striatal NMDA receptors were decreased with 17β-oestradiol, tamoxifen and raloxifene treatments. NMDA-displaceable [^{3}H]glutamate binding was also reported to increase in the hippocampal CA1 of ovariectomised

rats administered 17β-oestradiol benzoate 72 and 48 h before death and was associated with improved arm-choice accuracy in a working memory task (36). By contrast, using [^{3}H]MK-801 specific binding to label NMDA receptors in the hippocampus, a decrease of specific binding was observed following 10 weeks of 17β-oestradiol treatment associated with an improved memory performance (37). In the CA1, CA2/3 and dendate gyrus hippocampal regions, changes in both NMDA receptor [^{3}H]Ro 25-6981 specific binding and NMDA subunits 1 and 2B mRNA levels were positively correlated, suggesting that changes in specific binding was due to changes in synthesis of these receptors, decreasing with gonadal hormone withdrawal by ovariectomy and increasing with chronic treatments of 17β-oestradiol or PPT, while DPN had no significant effect (20, 21).

The functional effects of 17β-oestradiol on hippocampal NMDA receptors is well documented in female animals using various experimental approaches; 17β-oestradiol regulates hippocampal dendritic spine density via an NMDA receptor-dependent mechanism (38). An NMDA receptor increase promoted by a 17β-oestradiol treatment, measured using [^{3}H]-glutamate autoradiography, was shown to be required for 17β-oestradiol induced hippocampal CA1 spinogenesis in ovariectomised female rats (38). However, 17β-oestradiol fails to modulate NMDA receptor binding in gonadectomised males and does not increase spine density; it is argued that the lack of oestrogenic activity is due to a paucity of ERs (38). The absence of oestrogenic effects on hippocampal NMDA receptors in males is also related to 17β-oestradiol's inability to modulate cholinergic activity (important in oestradiol modulation of NMDA receptors) in the forebrain and the hippocampus due to a lack of ERs, since females express ERα in cholinergic cell bodies of the forebrain and in hippocampal cholinergic terminals (39, 40).

Effect of lesion, 17β-oestradiol and ERKO on NMDA receptors

In ERKO animal models, the absence of either ERα or ERβ in female mice was reported to have negative effects on hippocampal-dependant learning tasks and impaired synaptic plasticity in the CA1 was observed in ERKOβ female mice (41). The present results showed lower NMDA receptor specific binding in the frontal and the cingulate cortex as well as in the striatum of ERKOα and ERKOβ compared to WT male mice. This could be explained by a stimulatory effect of the low levels of endogenous oestrogens in males that could maintain higher NMDA receptors levels and in absence of ERs upon which 17β-oestradiol is acting this effect would be lost.

NMDA receptors were not decreased in ERKO compared to WT mice in all the hippocampal subregions measured. By contrast, higher NMDA specific binding in the CA3 and dendate gyrus of ERKOα mice was observed compared to WT and ERKOβ mice as well as in the CA1 and CA2 in ERKOα compared to ERKOβ mice. The androgen 5α-dihydrotestosterone (DHT) is reported to increase CA1 strata oriens and radiatum NMDA receptor binding density in castrated male rats (42). Little data is available for other brain regions, testosterone treatment had no effect on [^{3}H]CGP 39653 binding to NMDA receptors containing NR1/2A in the cerebral cortex (43) and no data is reported for the striatum. Our previous results on these mice showed that ERKOα mice have higher blood concentrations of endogenous androgens including: testosterone, DHT and 5α-androstan 3-β, 17β-diol (3β-diol) compared to WT and ERKOβ mice (31). Hence, higher levels of NMDA receptors in hippocampal regions of ERKOα but not of WT or ERKOβ mice could be due to the effect of androgens on this glutamate receptor. However, in contrast to the well-documented neuroprotective activity of oestrogens, elevated androgen levels

are not neuroprotective of nigro-striatal DA, testosterone and DHT have no neuroprotective effects against MPTP (44) and ERKOα mice are more sensitive to MPTP (22, 31).

The results on intact mice of all three genotypes revealed that 17β-oestradiol treatment generally left NMDA specific binding unchanged in cortical, striatal and hippocampal subregions assayed except for an increase in the striatum of ERKOβ mice, while deletion of ERα or ERβ also had very little effect on male hippocampal NMDA receptors. Using NMDA-displaceable [^{3}H]glutamate specific binding in the striatum, we previously observed no effect of 17β-oestradiol treatment in MPTP lesioned male mice (45), this is at variance with the present results and may be because a more specific ligand for NMDA receptors containing NR2B was used in the present study allowing to measure a change with the lesion and 17β-oestradiol treatment. Here, ERKOα MPTP-lesioned mice treated with 17β-oestradiol showed a generalised decrease of [^{3}H]Ro 25-6981 specific binding in hippocampal subregions while this was observed only in the CA1 of WT mice and no decrease for ERKOβ mice. Moreover, intact ERKOα mice had higher NMDA receptors compared to WT and ERKOβ mice. In CA3 neurones of adult male rat hippocampus, a rapid (within 2 hours) decrease was reported in hippocampal thorns and spines by 17β-oestradiol, PPT but not DPN (46). Hence, overall these results demonstrate that both male mice (in the present study) and rats (46) are responsive to 17β-oestradiol treatment in the hippocampus but the time course, ER specificity and regional effect vary.

Link between striatal NMDA receptors and DA concentrations

A significant (positive) correlation between striatal NMDA receptor specific binding and DA concentrations was observed in WT mice of both experiments (Figures 3 and 4), whereas there was no correlation in ERKOα or ERKOβ mice. This could be explained by the MPTP-

induced decrease of DA removing the DA inhibitory control on glutamate, thus resulting in increased glutamate activity. In turn, glutamate overactivity could be compensated to restore homeostasis by a reduction of NMDA receptors. The lack of correlation in the ERKO mice suggests that removing ERα or ERβ had a disruptive effect on the adaptation of NMDA receptors to the lesion and 17β-oestradiol treatment.

Conclusion

The present *in vivo* experiments in male mice showed that brain NMDA receptors were affected by deletion of ERs that in turn affected the response to MPTP and 17β-oestradiol treatments with brain region specificity. Taken together these studies support an important role of ERs in glutamate neurotransmission; glutamate being the most abundant excitatory brain neurotransmitter this has important functional significance in intact as well in neurodegenerative conditions.

VIII. ACKNOWLEDGEMENTS

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X. FIGURES

Figure 1.

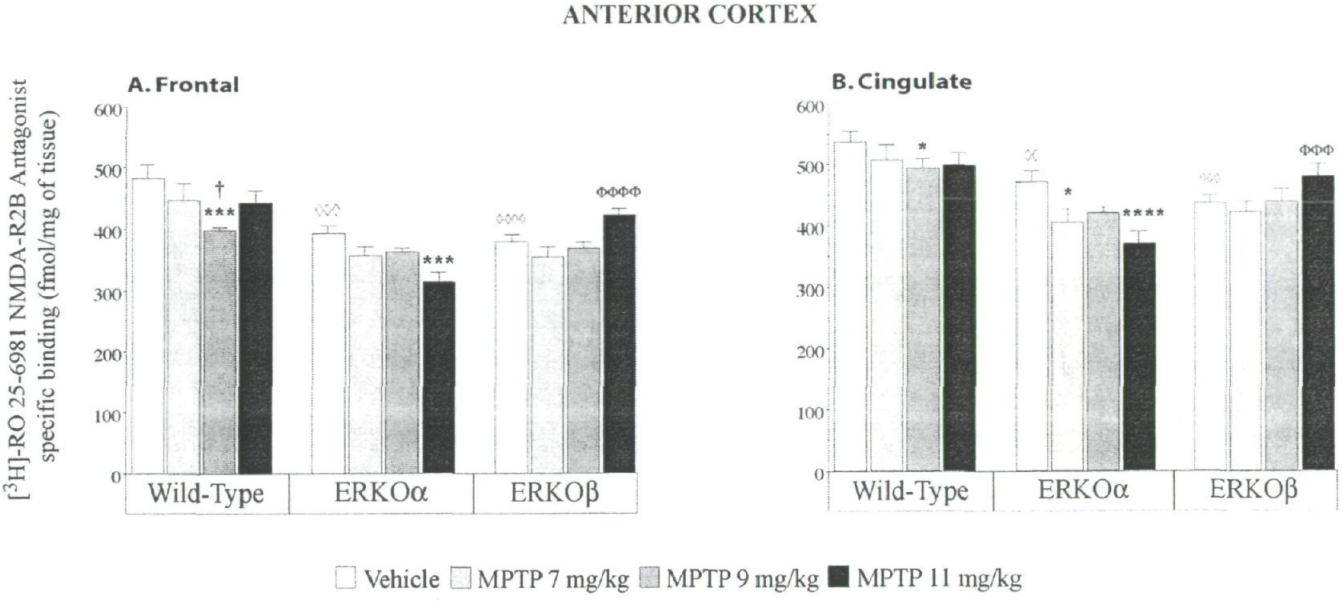


Figure 2.

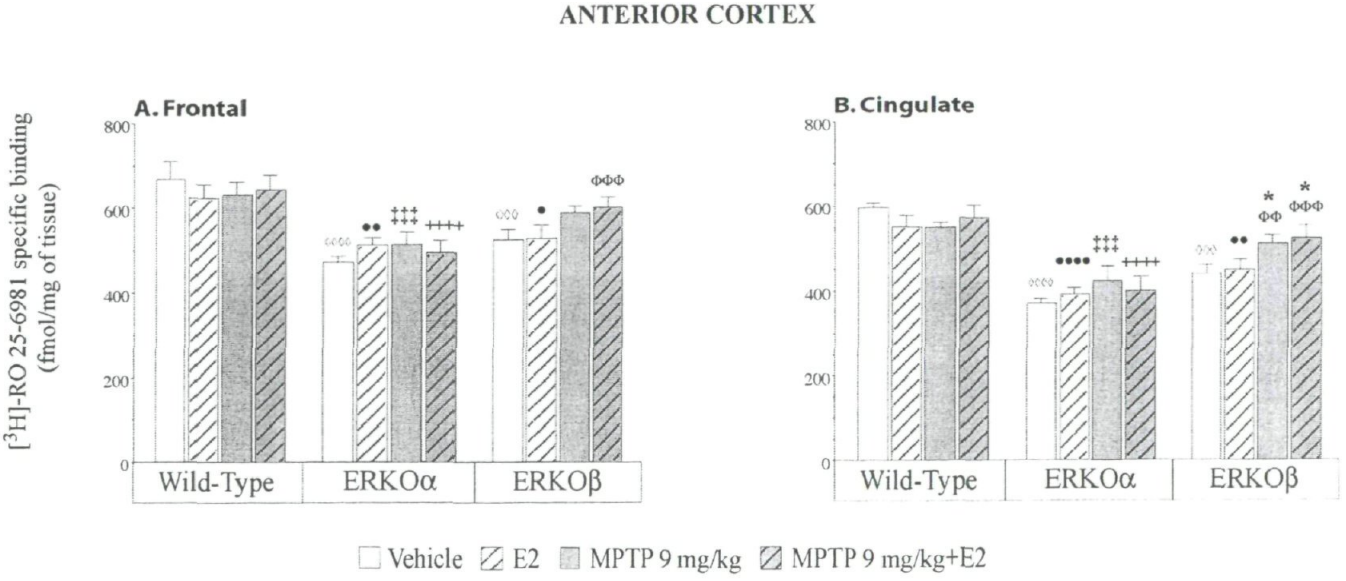


Figure 3.

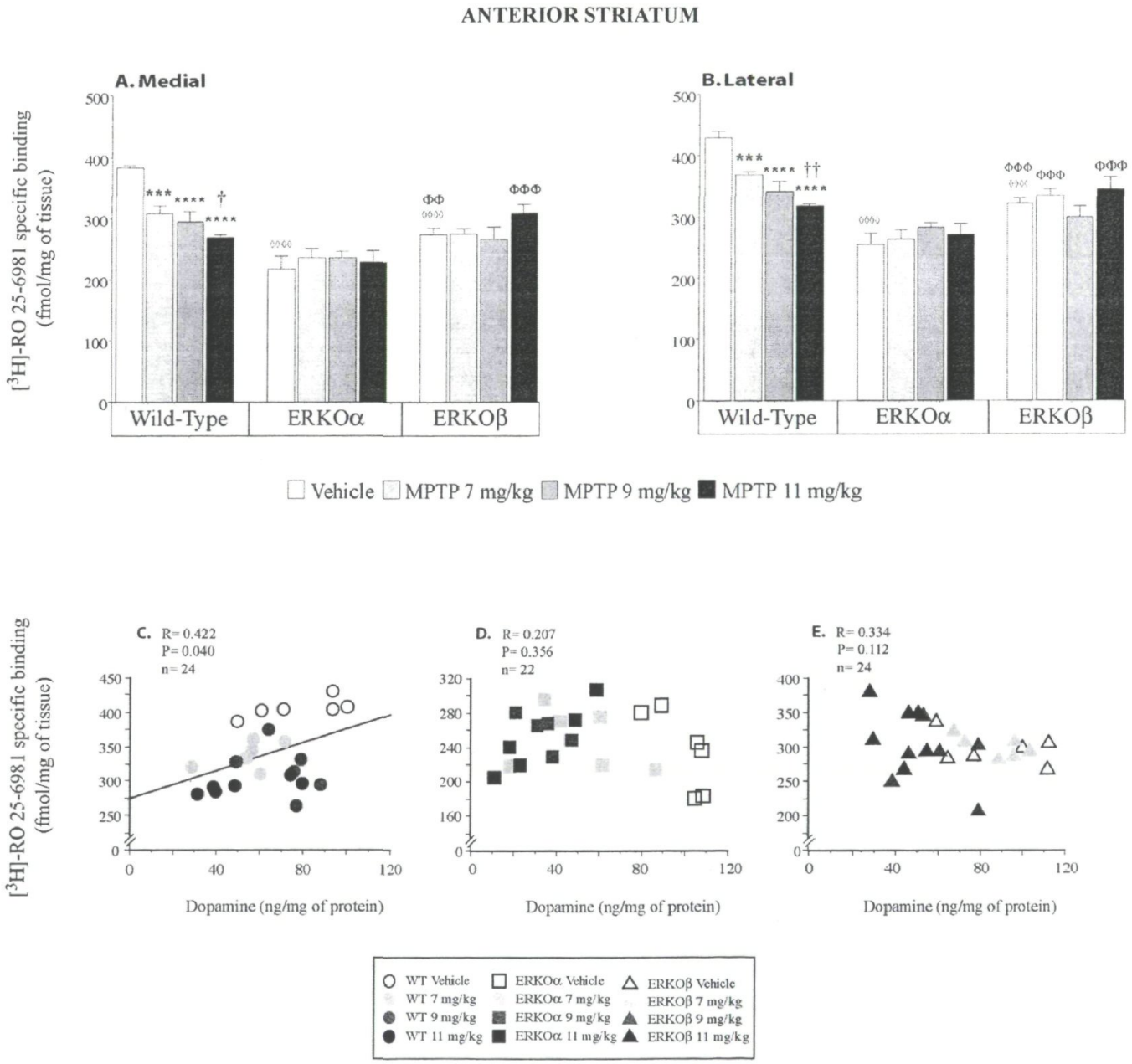

ANTERIOR STRIATUM
A. Medial
B. Lateral
[3H]-RO 25-6981 specific binding (fmol/mg of tissue)
Wild-Type
ERKOα
ERKOβ
Vehicle
MPTP 7 mg/kg
MPTP 9 mg/kg
MPTP 11 mg/kg
C. R= 0.422 P= 0.040 n= 24
D. R= 0.207 P= 0.356 n= 22
E. R= 0.334 P= 0.112 n= 24
Dopamine (ng/mg of protein)
WT Vehicle
WT 7 mg/kg
WT 9 mg/kg
WT 11 mg/kg
ERKOα Vehicle
ERKOα 7 mg/kg
ERKOα 9 mg/kg
ERKOα 11 mg/kg
ERKOβ Vehicle
ERKOβ 7 mg/kg
ERKOβ 9 mg/kg
ERKOβ 11 mg/kg

Figure 4.

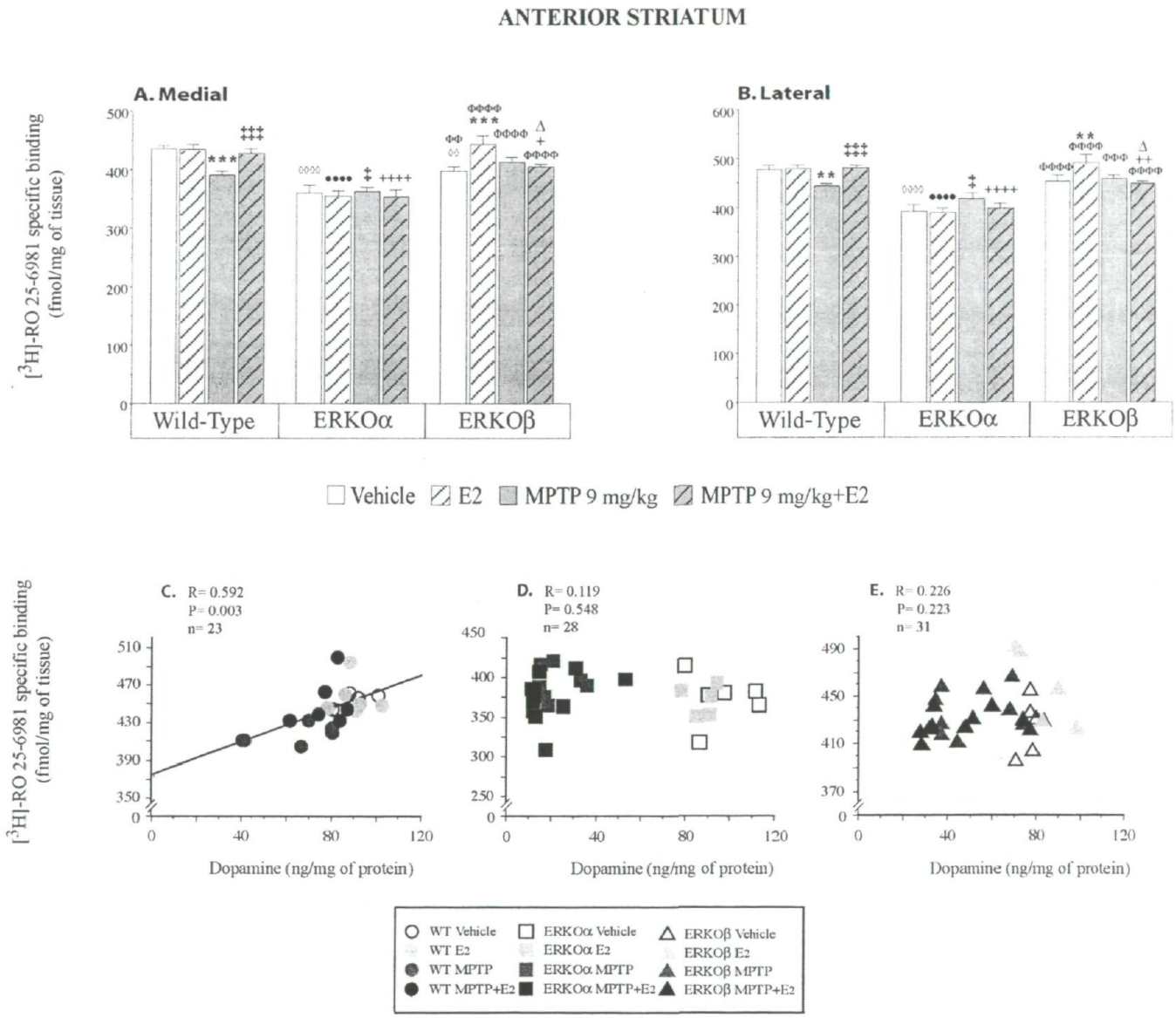
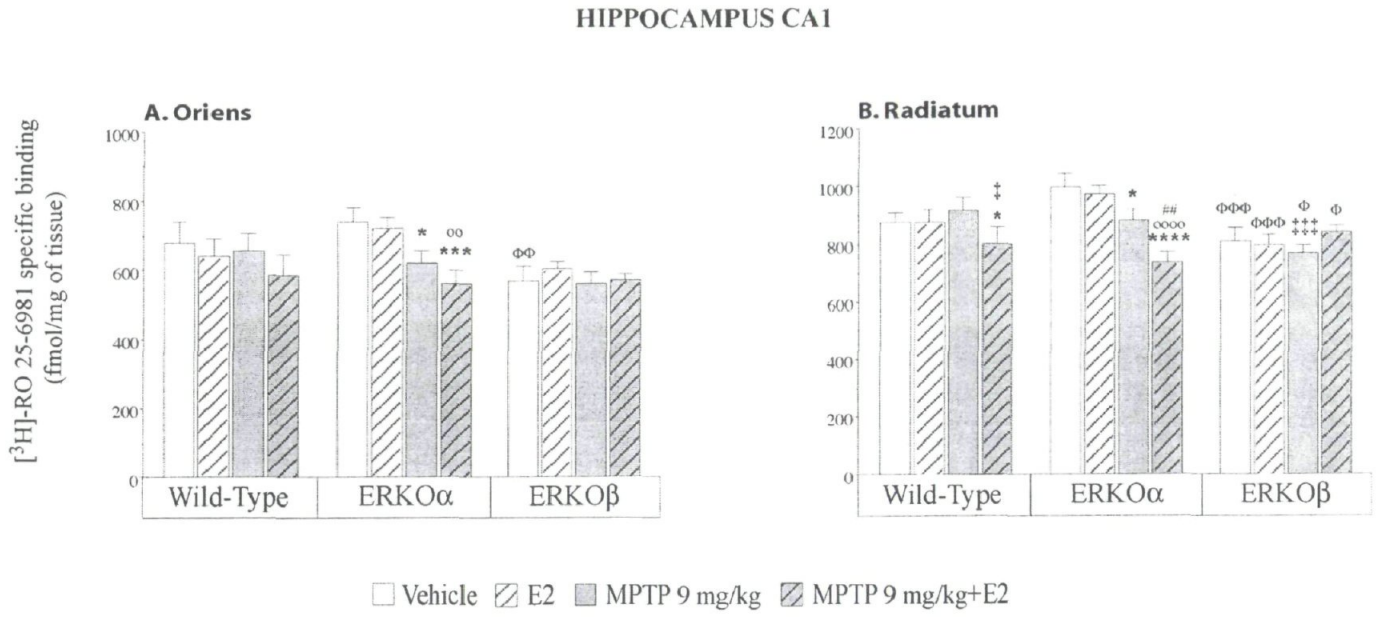

ANTERIOR STRIATUM
A. Medial
B. Lateral
[3H]-RO 25-6981 specific binding (fmol/mg of tissue)
Wild-Type
ERKOα
ERKOβ
Vehicle
E2
MPTP 9 mg/kg
MPTP 9 mg/kg+E2
C. R= 0.592 P= 0.003 n= 23
D. R= 0.119 P= 0.548 n= 28
E. R= 0.226 P= 0.223 n= 31
Dopamine (ng/mg of protein)
WT Vehicle
WT E2
WT MPTP
WT MPTP+E2
ERKOα Vehicle
ERKOα E2
ERKOα MPTP
ERKOα MPTP+E2
ERKOβ Vehicle
ERKOβ E2
ERKOβ MPTP
ERKOβ MPTP+E2

Figure 5.



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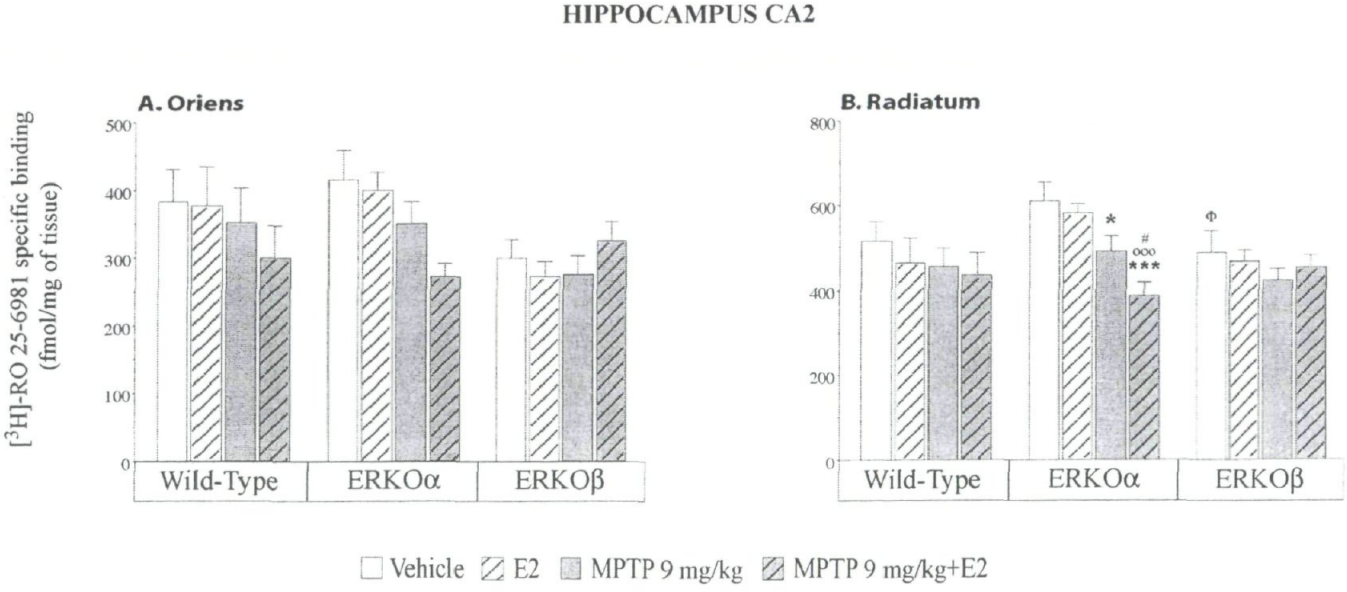

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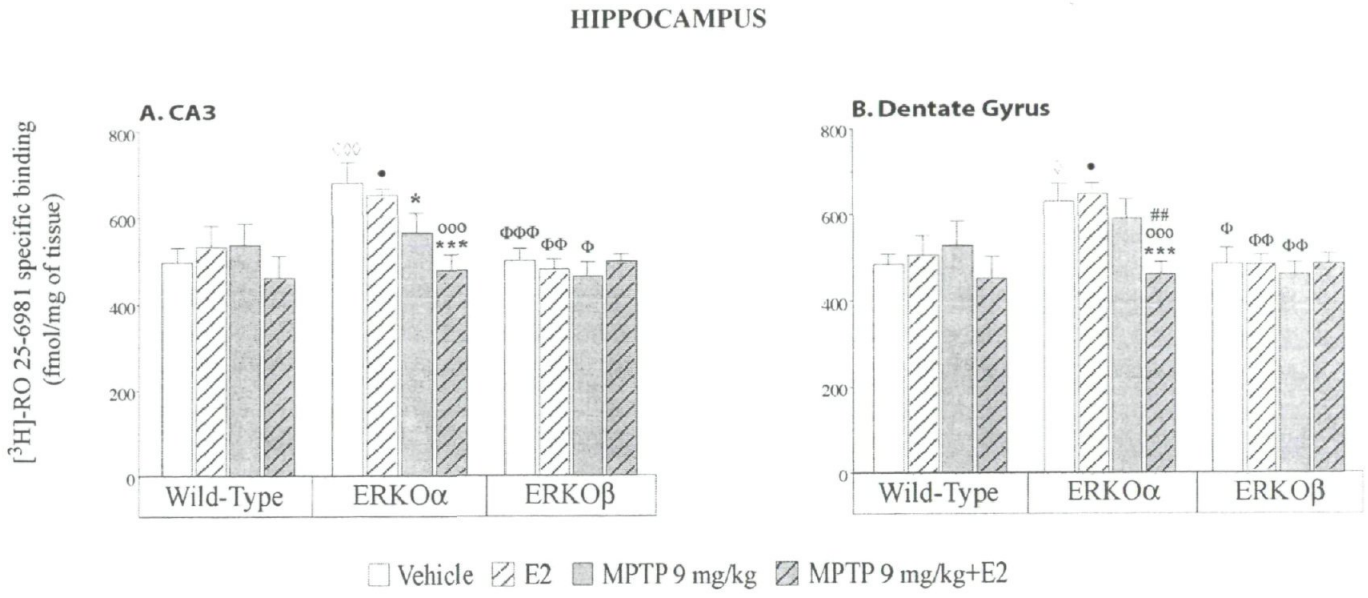
HIPPOCAMPUS
A. CA3
B. Dentate Gyrus
[3H]-RO 25-6981 specific binding (fmol/mg of tissue)
800
600
400
200
0
Wild-Type
ERKOα
ERKOβ
Vehicle
E2
MPTP 9 mg/kg
MPTP 9 mg/kg+E2

XI. TABLES

Table 1. Summary of genotype differences in mouse brain NMDA receptors

Brain Region	ERKOα versus WT mice	ERKOβ versus WT mice	ERKOα versus ERKOβ mice
Cortex			
Frontal	–	–	0
Cingulate	–	–	0
Striatum			
Medial	–	–	–
Lateral	–	–	–
Hippocampus			
CA1 Oriens	0	0	+
CA1 radiatum	0	0	+
CA2 Oriens	0	0	0
CA2 radiatum	0	0	+
CA3	+	0	+
Dentate gyrus	+	0	+

0, –, +: No difference, lower and higher NMDA receptor [^{3}H]Ro 25-6981 specific binding levels

Table 2. Summary of differences in brain NMDA receptor specific binding response to MPTP and 17β-oestradiol treatments in WT, ERKOα and ERKOβ mice.

Brain Region	WT mice			ERKOα mice			ERKOβ mice		
	MPTP	17β-E_2	MPTP +17β-E_2	MPTP	17β-E_2	MPTP +17β-E_2	MPTP	17β-E_2	MPTP +17β-E_2
Cortex									
Frontal	–	0	0	–	0	0	0	0	0
Cingulate	–	0	0	–	0	0	0	0	+
Striatum									
Medial	–	0	0, ↑	0	0	0	0	+	0
Lateral	–	0	0, ↑	0	0	0	0	+	0
Hippocampus									
CA1 Oriens	0	0	0	–	0	–	0	0	0
CA1 radiatum	0	0	–	–	0	–	0	0	0
CA2 Oriens	0	0	0	0	0	0	0	0	0
CA2 radiatum	0	0	0	–	0	–	0	0	0
CA3	0	0	0	–	0	–	0	0	0
Dentate gyrus	0	0	0	0	0	–	0	0	0

0, –, +: No effect, decreased and increased NMDA receptor levels versus respective intact vehicle treated mice

↑: Increased NMDA receptor levels versus respective MPTP treated mice

XII. FIGURE LEDGENDS

Figure 1. Dose-response effect of MPTP on cortical NMDA receptors measured with [^{3}H]RO 25-6981 NMDA-R2B antagonist specific binding in wild-type compared to ERKOα and ERKOβ mice. *p<0.05, ***p<0.005, and ****p<0.0001 vs respective intact, vehicle; †p<0.05 vs WT+MPTP 7 mg/kg; ◊◊ p<0.01, ◊◊◊p<0.005 and ◊◊◊◊p<0.0001 vs WT vehicle; ΦΦΦp<0.005 and ΦΦΦΦp<0.0001 vs respective ERKOα experimental group.

Figure 2. Effect of treatment with 17β-oestradiol (E_2) 2μg/day for ten days on cortical [^{3}H]RO 25-6981 specific binding in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. *p<0.05 vs respective intact, vehicle; ‡‡‡p<0.005 vs WT+MPTP; •p<0.05, ••p<0.01 and ••••p<0.0001 vs WT+E2; ++++p<0.0001 vs WT+MPTP+E2; ◊◊◊p<0.005 and ◊◊◊◊p<0.0001 vs WT vehicle; ΦΦp<0.01 and ΦΦΦp<0.005 vs respective ERKOα experimental group.

Figure 3. Dose-response effect of MPTP on striatal (A. Medial and B. lateral) [^{3}H]RO 25-6981 specific binding in wild-type compared to ERKOα and ERKOβ mice. C, D and E- correlations of [^{3}H]RO 25-6981 specific binding with striatal dopamine concentrations in wild-type, ERKOα and ERKOβ mice. Striatal dopamine concentrations of these mice were previously measured (22). ***p<0.005, and ****p<0.0001 vs respective intact, vehicle; †p<0.05 and ††p<0.01 vs WT+MPTP 7 mg/kg; ◊◊◊◊p<0.0001 vs WT vehicle; ΦΦp<0.01 and ΦΦΦp<0.005 vs respective ERKOα exprimental group.

Figure 4. Effect of treatment with 17β-oestradiol (E_2) 2μg/day for ten days on striatal (A. medial and B. lateral) [^{3}H]RO 25-6981 specific binding in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. C, D and E- correlation of [^{3}H]RO 25-6981 specific binding with striatal dopamine concentrations (22) in wild-type compared to ERKOα and ERKOβ mice. **p<0.01 and ***p<0.005 vs respective intact, vehicle; ‡ p<0.05 and ‡‡‡p<0.005 vs WT+MPTP; ••••p<0.0001 vs WT+E2; Δp<0.005 vs ERKOβ+E2; +p<0.05, ++ p<0.01 and ++++p<0.0001 vs WT+MPTP+E2; ◊◊p<0.01 and ◊◊◊◊p<0.0001 vs WT vehicle; ΦΦp<0.01, ΦΦΦp<0.005 and ΦΦΦΦp<0.0001 vs the respective ERKOα experimental group.

Figure 5. Effect of treatment with 17β-oestradiol (E_2) 2μg/day for ten days on hippocampal CA1 Oriens (A) and radiatum (B) [^{3}H]RO 25-6981 specific binding in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. *p<0.05, ***p<0.005 and **** p<0.0001 vs respective intact, vehicle; ‡p<0.05 and ‡‡‡p<0.005 vs WT+MPTP; Φp<0.05,ΦΦp<0.01 and ΦΦΦp<0.005 vs respective ERKOα experimental group; oop<0.01, oooop<0.0001vs ERKOα+E2 and ##p<0.01 vs ERKOα+MPTP.

Figure 6. Effect of treatment with 17β-oestradiol (E_2) 2μg/day for ten days on hippocampal CA2 Oriens (A) and radiatum (B) [^{3}H]RO 25-6981 specific binding in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. *p<0.05 and ***p<0.005 vs respective intact, vehicle; Φp<0.05 vs respective ERKOα experimental group; ooop<0.005 vs ERKOα+E2 and #p<0.05 vs ERKOα+MPTP.

Figure 7. Effect of treatment with 17β-oestradiol (E_2) 2μg/day for ten days on hippocampal CA3 (A) and dendate gyrus (B) [^{3}H]RO 25-6981 specific binding in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. *p<0.05 and ***p<0.005 vs respective intact, vehicle; ◊p<0.05 and ◊◊◊p<0.005 vs WT vehicle; •p<0.05 vs WT+E2; Φp<0.05, ΦΦp<0.01 and ΦΦΦp<0.005 vs respective ERKOα experimental group; ooop<0.005 vs ERKOα+E2 and ##p<0.01 vs ERKOα+MPTP.

Table 1. Summary of genotype differences in mouse brain NMDA receptors.

0, –, +: No difference, lower and higher NMDA receptor [^{3}H]Ro 25-6981 specific binding levels.

Table 2. Summary of differences in brain NMDA receptor specific binding response to MPTP and 17β-oestradiol treatments in WT, ERKOα and ERKOβ mice.

0, –, +: No effect, decreased and increased NMDA receptor levels versus respective intact vehicle treated mice. ↑: Increased NMDA receptor levels versus respective MPTP treated mice.

CHAPTER V

Estrogen receptors and lesion-induced response of striatal dopamine receptors

Sara Al-Sweidi, Marc Morissette and Thérèse Di Paolo

Molecular Endocrinology and Genomic Research Center of CHUQ-CHUL

and Faculty of Pharmacy, Laval University, Quebec, Qc, Canada

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I. FOREWORD

Sara Al-Sweidi: I treated the mice, participated in the sacrifice, during catecholamine dosing through HPLC and cryostat cutting of mouse brains. I did all the experiments on striatal D1 and D2 dopamine receptors, which were measured by autoradiography with the specific ligands [^{3}H]-SCH 23390 and [^{3}H]-raclopride, respectively. Completed all the autoradiogram and statistical analysis and wrote the article.

Marc Morissette: Research assistant in our laboratory who had a lot to do during this project. He extracted mouse brains during the sacrifice, then dissected and prepared the brain structures for HPLC dosing then performed these. He provided the results on protein kinase B (Akt), Glycogen synthase kinase (GSK3β) and extracellular–regulated kinases (ERK) signaling by Western blot. Also, he helped with the cutting of the mouse brains and the autoradiography experiments, as well as the writing of the article.

Thérèse Di Paolo: Research director who undertook main decisions concerning which treatments had to be administered to the knockout mice. She planned and supervised all the protocols from start to finish and made an important contribution in the writing of the article.

II. RÉSUMÉ

On a précédemment démontré que la neuroprotection induit par le 17β-oestradiol, un agoniste des récepteurs oestrogéniques (ERs), prévient les effets de la neurotoxine 1-méthyl-4-phényl-1,2,3,6-tétrahydropyridine (MPTP) chez la souris et cet effet neuroprotecteur peut impliquer la protéine kinase B (Akt). Afin d'évaluer les mécanismes associés, nous avons réalisé une étude chez des souris mâles C57Bl/6 sauvages (WT) ou invalidées (KO) pour les ERα et ERβ (ERKOα et ERKOβ) qui ont reçu des traitements de MPTP (0, 7, 9, 11 mg/kg) et/ou de 17β-oestradiol. Les niveaux des récepteurs dopaminergiques D1 et D2 ont été mesurés par autoradiographie utilisant les ligands [^{3}H]-SCH 23390 et [^{3}H]-raclopride, respectivement. Ensuite, les concentrations striatales des protéines impliquées dans la signalisation anti ou pro-apoptotique, l'Akt et la kinase GSK3β respectivement, les protéines ERK1 et ERK2 ainsi que leurs formes phosphorylées ont été quantifiées par immunobuvardage de type Western. Les souris intactes ERKOβ avaient des niveaux bas de la liaison spécifique [^{3}H]-SCH 23390 striatale et les souris ERKOα et ERKOβ avaient des niveaux bas de la liaison spécifique [^{3}H]-raclopride. Le MPTP a causé une diminution des niveaux du récepteur D1 chez les souris ERKOα et cela corrèle avec leurs niveaux de DA striatales, ainsi qu'une diminution des niveaux du récepteur D2 qui corrèle avec les niveaux de DA striatales des les souris sauvages et ERKOα. De plus, le MPTP augmente les niveaux du récepteur D2 chez les souris ERKOβ. Le 17β-oestradiol induit une augmenation des niveaux du récepteur D1 chez les souris ERKOα et ERKOβ et du récepteur D2 chez les souris sauvages et ERKOβ lésées au MPTP. Seulement les souris ERKOβ montrent une augmentation de la signalisation Akt/GSK3β, pERK1/ERK1 et pERK2/ERK2 sans ou avec la lésion au MPTP. Donc, les ERs affectent la réponse des récepteurs dopaminergiques striatales à une lésion au MPTP ansi que les voies de signalisation post récepteurs.

III. ABSTRACT

We previously showed that neuroprotection induced by 17β-estradiol, an estrogen receptor (ER) agonist, against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mice implicates protein kinase B (Akt). In order to evaluate the associated mechanisms, this study compared ERα and ERβ intact or knockout (KO) and wild-type (WT) C57Bl/6 male mice following MPTP treatment of 7, 9, 11 mg/kg and/or 17β-estradiol. Striatal D1 and D2 dopamine receptors were measured by autoradiography with the specific ligand [^{3}H]-SCH 23390 and [^{3}H]-raclopride, respectively and signaling by Western blot for Akt, glycogen synthase kinase 3β (GSK3β) and extracellular-regulated signal kinases (ERK1 and ERK2). Control ERKOβ mice had lower striatal [^{3}H]-SCH 23390 specific binding than WT and ERKOα mice; both KO mice had lower [^{3}H]-raclopride specific binding. Striatal D1 receptors decreased with increasing doses of MPTP in correlation with striatal DA concentrations in ERKOα mice and remained unchanged in WT and ERKOβ mice. Striatal D2 receptors decreased with increasing doses of MPTP in correlation with striatal DA concentrations in WT and ERKOα mice and increased in ERKOβ mice. In MPTP lesioned mice, 17β-estradiol treatment increased D1 receptors in both ERKOα and ERKOβ mice and D2 receptors in WT and ERKOβ mice. MPTP left unchanged striatal pAkt/Akt and pGSK3β/GSK3β levels in WT and ERKOα mice while in vehicle-treated ERKOβ mice these levels were higher and increased with MPTP lesioning. Striatal pERK1/ERK1 and pERK2/ERK2 levels showed to lesser extent a similar pattern. In conclusion, ERs affected the response of striatal DA receptors to a MPTP lesion and post receptor signaling.

Running title: Dopamine receptors in estrogen knockout MPTP mice.

List of abbreviations: Akt, protein kinase B; BSA, bovine serum albumin; DA, dopamine; D1, D1 dopamine receptor; D2, D2 dopamine receptor; EEDQ, N-ehtoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; 17β-E_2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERKO, estrogen receptor knock out; ERK, extracellular-signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; MAPK, mitogen-activated protein kinase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate buffered saline; PD, Parkinson's disease; PVDF, polyvinylidine difluoride; SDS, sodium dodecyl sulfate; SN, substantia nigra; TBS, Tris buffered saline; WT wild-type.

IV. INTRODUCTION

Dopamine (DA) is an important brain neurotransmitter playing a major role in locomotion, motivation and cognitive processes (Bjorklund and Dunnett, 2007a, b). Brain DA signaling is mediated through a family of receptors coupled to G proteins. These receptors are grouped into two classes on the basis of the G-proteins to which they couple: D1 and D5 called D1-like DA receptors which stimulate adenylyl cylase, while the D2-like receptors including D2, D3 and D4 receptors are negatively coupled to adenylyl cylase (Missale et al., 1998). The striatum is known as the brain region with the highest density of D1 and D2 receptors (Missale et al., 1998). In rodents, these two receptors are segregated in the striatal output pathways with D1 receptors in the direct and D2 receptors in the indirect pathway (Gerfen and Surmeier, 2011). Striatal D1 receptors are localized on post synaptic GABAergic spiny projection neurons whereas D2 receptors are expressed both pre synaptically on nigrostriatal terminals and postsynaptically on GABAergic spiny projection neurons (Roth, 1979).

The striatum is implicated in a wide variety of psychomotor disorders such as Parkinson's disease (PD), schizophrenia and drug abuse; altered DA neurotransmission is reported to play an important role in these diseases (Gerfen and Surmeier, 2011). Drug treatments of these mental and neurodegenerative diseases involves regulation of dopaminergic neurotransmission and DA receptors are an important target. PD involves principally death of DA neurons in the substantia nigra projecting to the striatum and the gold standard treatment is to replace the lost DA by the precursor L-DOPA (Gerfen and Surmeier, 2011). By contrast, in schizophrenia overactive DA neurotransmission is treated with antipsychotics that block D2 receptors (Shin et al., 2011). The long-term adaptation of DA systems to the lack of DA or alternatively to its overactivity is important to understand since D2 receptors are a target of numerous therapeutic agents.

D1 and D2 receptors are reported to change in parkinsonian rodents and monkeys as well as humans suffering from PD, with an increase for striatal D2 receptors measured in numerous studies whereas for D1 receptors increases, decreases and no change are documented (Laihinen et al., 1994, Morissette et al., 1996, Antonini et al., 1997, Goulet et al., 1997, Surmeier et al., 2010). Estrogen receptors (ER) alpha (ERα) and beta (ERβ) are present in the nigro-striatal pathway (Kuppers and Beyer, 1999, Kuppers et al., 2000, Shughrue et al., 2000, Mitra et al., 2003). It is well documented that 17β-estradiol modulate both D1 and D2 DA receptors in the striatum (Bourque et al., 2009, Sanchez et al., 2010); this was not correlated with changes of mRNA levels of these receptors (Le Saux et al., 2006) but was associated with reduced receptor degradation as evaluated with these DA receptor kinetics after irreversible inhibition with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Morissette et al., 1992).

Both D1 and D2 receptors are reported to stimulate mitogen-activated protein kinase (MAPK)/ extracellular–regulated kinases (ERK) signaling (Cai et al., 2000) whereas the phosphatidylinositol-3 kinase/Akt /GSK3β signaling pathway is shown to be affected by a D2 but not D1 receptor agonist and antagonist (Sutton and Rushlow 2011). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesion and dopaminergic treatments affect these signaling pathways (Bychkov et al., 2007, Morissette et al., 2010). Hence, ERs modulate neuroprotection and 17β-estradiol could combat MPTP toxicity through mechanisms involving the regulation of receptors and Akt/GSK3β and ERK1/2 signaling.

The aims of the present study were to seek the response of striatal D1 and D2 receptors to a MPTP lesion and the implication of ERα and ERβ using knockout (KO) mice for these receptors. MPTP is known as a selective neurotoxin for nigrostriatal tract and used to model PD pathology (Przedborski et al., 2001) and male mice were used in order to reproduce the

epidemiologic results demonstrating that more men than women develop PD (Wooten et al., 2004, Shulman, 2007). Receptor specific binding to striatal D1 and D2 receptors were measured and compared to DA levels and to the ERK1/2 and Akt/GSK3β signaling.

V. EXPERIMENTAL PROCEDURES

Animals and treatments

Adult male C57Bl/6 WT, ERKOα and ERKOβ mice (7-12 weeks, 18-28 g) were purchased from Taconic Laboratories (Hudson, NY, USA). MPTP and 17β-estradiol were purchased from Sigma Chemical (St-Louis, MO, USA). Mice were equally distributed for age and weight in experimental groups of six animals. All efforts were made to minimise animal suffering and to reduce the number of mice used. The Laval University Animal Care Committee approved all the animal studies.

We previously reported striatal biogenic amine concentrations of WT C57Bl/6 male mice following an extended MPTP dose-response study up to 20 mg/kg (Morissette et al., 2007). The MPTP doses (7, 9 and 11 mg/kg), that specifically affected striatal DA while sparing serotonin concentrations in WT mice, were thus used for comparison of MPTP dose-responses of ERKOα, ERKOβ and WT mice (Morissette et al., 2007). Mice received four 0.1 ml intraperitoneal injections with saline or a saline solution of MPTP at a two-hour interval and were killed 5 days after treatment with MPTP.

Then, the effect of 17β-estradiol and MPTP in WT, ERKOα and ERKOβ were compared. Four groups of both ERKOα and ERKOβ mice were compared to WT mice. An intermediate dose of 9 mg/kg MPTP was selected and the effect of 17β-estradiol treatment was investigated in intact and MPTP mice. Each group received a 5-day pre-treatment of 17β-estradiol or vehicle prior to MPTP injections. The pre-treatment consisted of two daily subcutaneous injections (in the dorsal part of the neck) of 17β-estradiol, 2 μg per day as we previously used (Callier et al., 2000, Morissette et al., 2007) while control mice received injections of vehicle (0.9% saline with 0.3% gelatine). On day 5, mice received four separate 0.1 ml intraperitoneal injections of the

vehicle treatment or a saline solution of MPTP (9 mg/kg of MPTP per injection) at two-hour intervals. Treatments with 17β-estradiol or vehicle were continued until day 10. Mice were killed five days after the MPTP lesioning with an air/halothane mixture and decapitated; trunk blood was collected and brains were quickly removed and frozen in a mixture of isopentane/dry ice and then stored at –80°C.

Binding Autoradiography

Frozen brains were cut on a cryostat in 12 μm thick slices. Coronal sections for anterior striatum (bregma 1.18 mm) were cut according to a mouse brain atlas (Franklin and Paxinos, 1997). Histological sections were thaw-mounted on superfrost slides (Trade Mark, Fisher, Nepean, On, Canada) and stored at –80°C. Autoradiography using [^{3}H]-SCH 23390 (86 Ci/mmol) and [^{3}H]raclopride (74.4 Ci/mmol) (PerkinElmer, Life Sciences, Inc. Boston, MA, USA) were used to label D1 and D2 receptors, respectively according to our previously published procedures (Gagnon et al., 1995, Landry et al., 2002). Brain sections for each binding assay were exposed to Kodak BIOMAX MR film (Kodak, Rochester, NY, USA) with calibrated [^{3}H] standards (Microscales, Amersham, Arlington Heights, IL, USA). Slides labelled with [^{3}H]-SCH 23390 were exposed for six weeks and those with [^{3}H]raclopride for two weeks. Autoradiograms relative optical densities were analyzed using Scion Image 1.63 software. For each group, the mean of 6-9 animals and the mean of six brain sections per animal were measured. [^{3}H]-SCH 23390 and [^{3}H]raclopride specific binding were calculated by subtracting the measured non-specific binding from the total binding.

Western blot

The left anterior striata conserved at -80°C were dissected from 50 μm thick brain slices cut on a cryostat for analysis by Western blotting (Bregma 0.68-0.50 mm). Frozen tissue pieces were first homogenized in RIPA 1X lysis buffer (1X Tris buffered saline (TBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), 0.004% sodium azide) (Santa Cruz Biotechnology, Inc.). 1% protease cocktail and 1% phosphatase inhibitor cocktail (Sigma Oakville, ON, Canada) were added to the buffer prior to use. Next, homogenates were left on ice to solubilize for 15 minutes and then centrifuged at 16,000g for 15 minutes. The protein content of the supernatent was measured using a modified Lowry assay (Micro BCA protein assay kit, Pierce, USA). All sample concentrations were adjusted as to load an equal amount of protein (5 μg) along with a prestained protein ladder for the determination of molecular weights.

Protein samples were heated for 5 minutes at 95°C and then 12% SDS-polyacrylamide gels were used for electrophoresis and run through a Triple wide mini-vertical gel system (C.B.S. Scientific Company, Inc). Proteins were then electrophoretically transferred to polyvinylidine difluoride (PVDF) membranes and afterwards membranes were blocked using 5% bovine serum albumin (BSA) diluted in a 0.01% Tween-20/phosphate buffered saline (PBS) 1X solution. Next, membranes were incubated by gentle shaking overnight at 4°C with the primary antibody diluted in 1X PBS solution containing 0.1% Tween-20 and the 5% BSA. Primary specific antibodies consisted of those against Akt (diluted 1:1000), pAkt (Ser 473, diluted 1:1000), GSK3β (diluted 1:1000), pGSK3β (Ser 9, diluted 1:1000), phospho-p44/42 MAP kinase (pERK1/2 diluted 1:1000), p44 MAP kinase (ERK1 diluted 1:1000) and p42 MAP kinase (ERK2 diluted 1:1000) were supplied by Cell Signaling Technology, Inc (Beverly, MA), while the antibody against βIII-tubulin (diluted 1:1000) was purchased from Chemicon International (Temecula, CA) and used as

loading control. Afterwards, membranes were incubated with a horseradish peroxidase-coupled secondary antibody (diluted 1:5000); Cell Signaling Technology, Inc. Beverly, MA). Finally, immunoreactive bands were revealed with an enhanced chemiluminesecence system (KPL, LumiGlo reserve substrate kit, Mandel Scientific Company Inc.). When needed, membranes were stripped using a solution containing 62.5 mM (Tris[Hydroxymethyl]aminomethane) (TRIS), 2% SDS, and 0.5% β-mercaptoethanol.

The exposed membranes were analyzed using an Alpha Innotech FluorChem Q MultiImage III camera. Bands were semi-quantitatively analyzed through scanning with the Alpha Innotech-Alpha View image acquisition and analysis software version 3.1.1.0 (Alpha Innotech Corporation, Copyrights 1993-2009). All individual band densities were normalized with respect to their internal control β-III-tubulin values in order to express arbitrary units of relative kinase abundance. Experiments were repeated 2-3 times and phosphorylated protein levels were normalized to their respective total protein.

Statistical analysis

Autoradiograms of specific binding relative optical densities were analyzed using Scion Image 1.63 software. The striatum was analyzed at an anterior coordinate and was subdivided in half into medial and lateral regions. The statistical analyses were performed using Statview 4.51 for Macintosh Computer software. A one-way ANOVA was used to compare MPTP and/or 17β-estradiol treatments followed by post-hoc analysis with Fisher probability of least significance difference test. A simple regression model (Pearson) was used to determine the coefficient of correlation and the significance of the degree of the linear relationship between variables. A $p \leq 0.05$ was required for the results to be considered statistically significant.

VI. RESULTS

The response of D1 receptors to a MPTP lesion in WT compared to ERKO mice was first investigated and showed a difference between the genotypes. Control ERKOβ had slightly 9% but significantly lower [^{3}H]-SCH 23390 specific binding in the striatum when compared respectively to vehicle treated WT and ERKOα mice, mice of the latter genotypes having similar D1 receptor specific binding (Figure 1A). A dose related decrease of striatal D1 receptor specific binding caused by MPTP was observed in ERKOα mice while it remained unchanged in WT and ERKOβ mice. In ERKOα mice, striatal D1 receptor specific binding decreased with 9 and 11 mg/kg of MPTP (Figure 1A and 1B). As striatal dopamine loss increases with increasing doses of MPTP, this lesion may be compensated by an increase of post-synaptic D1 and D2 receptors to restore homeostasis. Moreover, as the MPTP lesion increases with increasing doses of the toxin, pre-synaptic terminals bearing D2 receptors are progressively lost. Hence, to investigate the contribution of ERs to the MPTP lesion-induced response of DA receptors, we correlated the striatal DA receptor and DA concentration data. Striatal D1 receptor specific binding positively correlated with striatal DA concentrations of these ERKOα mice (Figure 1 C) whereas no correlation was obtained for WT (Figure 1B) or ERKOβ (Figure 1D) mice.

The response of D2 receptors to a MPTP lesion in the WT compared to the ERKO mice also showed a difference between the genotypes. In the WT experimental groups, MPTP caused a decrease in striatal D2 receptor levels when compared to the intact vehicle treated group that was more extensive at the highest dose of 11 mg/kg MPTP. Control ERKOα and ERKOβ mice had 30% and 33% respectively lower striatal [^{3}H]-raclopride specific binding compared to the vehicle treated WT mice (Figure 2A). A dose-related decrease of striatal D2 receptor specific binding caused by MPTP was observed in ERKOα mice groups reaching statistical significance at 11

mg/kg. Moreover, MPTP-treated ERKOα mice had lower striatal [^{3}H]-raclopride specific binding compared to the respective WT mice treated with 7, 9 and 11 mg/kg MPTP. By contrast, in ERKOβ mice, a dose-related increase of D2 receptor specific binding with increasing doses of MPTP was observed, with a significant increase at 11 mg/kg MPTP (Figure 2A). While an increase was observed with the lesion, MPTP-treated ERKOβ mice still had lower striatal [^{3}H]-raclopride specific binding compared to the respective WT mice treated with 7, 9 mg/kg of MPTP, whereas 11 mg/kg MPTP-treated ERKOβ mice had higher striatal [^{3}H]-raclopride specific binding than the respective WT and ERKOα mice. Striatal [^{3}H]-raclopride specific binding positively correlated with striatal DA concentrations of WT and ERKOα mice, while a negative correlation was observed in ERKOβ mice (Figure 2 B, C and D).

The correlations observed in figures 1 and 2 highlight clear differences between WT, ERKOα and ERKOβ mice and contribute to overall understanding of ER-dopamine interactions within a dopamine dense brain region that is central to multiple DA pathways. The present results provide significant implications for DA-related pathologies in males.

Next, signaling proteins in the striatum of these mice showed that ERKOβ mice displayed 50% higher basal levels of striatal pAkt/Akt when compared to that of WT and ERKOα mice. A dose-dependent increase of pAkt/Akt in the striatum of ERKOβ mice was observed with increasing doses of MPTP with an extensive increase of 150 % with MPTP doses of 9 and 11 mg/kg (Figure 3 A). No difference of striatal pAkt/Akt levels was observed between WT and ERKOα mice and after MPTP lesion. Striatal pGSK3β/GSK3β levels were similar between WT and ERKOα mice and remained unchanged with the MPTP lesion. By contrast, striatal pGSK3β/GSK3β levels were 80% higher in ERKOβ mice compared to the other mice genotypes.

Moreover, in ERKOβ mice striatal pGSK3β/GSK3β levels doubled with 9 or 11 mg/kg of MPTP compared to the WT and ERKOα mice (Figure 3 B).

Striatal pERK1/ERK1 levels of WT mice dose-dependently decreased with increasing doses of MPTP with significantly lower values with 9 mg/kg of MPTP compared to vehicle treated mice or mice treated with 7 mg/kg MPTP, no decrease was measured at the highest dose of 11 mg/kg MPTP (Figure 4A). By contrast, striatal pERK1/ERK1 levels did not decrease with MPTP in the ERKOα and ERKOβ mice and were higher in both knockout groups compared to WT mice treated with MPTP 9 mg/kg. Moreover, ERKOβ mice treated with MPTP 11 mg/kg had higher striatal pERK1/ERK1 levels compared to WT and ERKOα mice treated with 11 mg/kg MPTP.

Striatal pERK2/ERK2 levels remained unchanged at all doses of MPTP in WT mice (Figure 4B). MPTP at 9 mg/kg increased striatal pERK2/ERK2 ratios of ERKOα and ERKOβ mice and the increase was greater at 11 mg/kg compared to 9 mg/kg MPTP in ERKOβ mice (Figure 4 B).

In the MPTP and 17β-estradiol treatments experiment, as in the MPTP dose-response experiment, ERKOβ vehicle treated mice had a slightly 8% lower striatal [^{3}H]-SCH 23390 specific binding compared to ERKOα mice (Figure 5A). MPTP at 9 mg/kg had no effect on striatal [^{3}H]-SCH 23390 specific binding in WT mice, while ERKOα mice had decreased levels (Figure 5 A). 17β-estradiol treatment decreased [^{3}H]-SCH 23390 specific binding in ERKOα mice and the combination of MPTP and 17β-estradiol increased this specific binding in both ERKOα and ERKOβ mice (Figure 5 A).

Moreover, in the MPTP and 17β-estradiol treatments experiment as in the MPTP dose-response experiment, ERKOα and ERKOβ vehicle treated mice had respectively 24% and 32%

lower striatal [^{3}H]-raclopride specific binding compared to respective WT mice (Figure 5B). In this last experiment, WT mice had decreased [^{3}H]-raclopride specific binding levels with 9 mg/kg of MPTP and combined treatment with 17β-estradiol prevented this decrease (Figure 5 B). As in the MPTP dose-response experiment, MPTP at 9 mg/kg had no effect on [^{3}H]-raclopride specific binding in ERKOα and ERKOβ mice (Figure 5B). Interestingly in ERKOβ mice, the combined MPTP and 17β-estradiol treatments increased [^{3}H]-raclopride specific binding (Figure 5 B).

VII. **DISCUSSION**

The present results showed that in male mice both ERα and ERβ distinctly affected striatal D1 and D2 DA receptors, their response to a MPTP lesion and 17β-estradiol treatment as well as post receptor signaling.

Numerous studies have shown in rats the effects of estrogens to increase striatal D1 and D2 receptor density in ovariectomized female (Morissette and Di Paolo, 1993b, a) and intact male rats (Hruska and Nowak, 1988, Chavez et al., 2010). Our group has shown that D2 receptors are lower in the striatum of ovariectomized compared to intact female rats (Le Saux et al., 2006). In the present study, the striatum of vehicle treated ERKOα and ERKOβ male mice had lower D2 receptor specific binding that WT mice. Removing the ERs in male mice or removing the endogenous source of estrogens in female rats led to a reduction of striatal D2 receptors. The role of ERα and ERβ in the effect of estradiol on striatal D2 receptors has been also studied using a pharmacological approach with specific estrogen receptor agonists; these receptors increased in ovariectomized female rats following a chronic treatment of two weeks with a ERβ agonist, the increase with an ERα agonist was not significant and the effect of 17β-estradiol was greater then either of the specific receptor agonists (Le Saux et al., 2006). Overall, this suggests that estrogen acting on ERα or ERβ receptors have a tonic stimulatory effect on striatal D2 receptors.

The effect of removing the ovarian estrogens in females or removing ER in males on striatal D1 receptors is not well documented. The present results showed slightly but significantly lower D1 receptors in the striatum of vehicle-treated ERKOβ mice but not ERKOα mice supporting a lesser tonic role of ERs on striatal D1 than D2 receptors and more specifically a role for ERβ. An imbalance between the striatal D1 and D2 receptors in the ERKO mice was

observed compared to the WT (in favor of the direct compared to the indirect striatal output pathways), D2 receptor specific binding being lower than for the D1 subtype in the ERKO mice.

D1 receptors are located mostly post-synaptically in the striatum (Roth, 1979). In Parkinsonian patients and animal models striatal D1 receptors were reported to either increase (Rioux et al., 2001), decrease (Turjanski et al., 1997) or remain unchanged (Laihinen et al., 1994). In the present study MPTP left unchanged striatal D1 receptor specific binding in WT and ERKOβ mice while a decrease was observed in ERKOα mice. The decrease of D1 receptors in ERKOα mice was positively correlated with their striatal DA concentrations. The different response of D1 receptors between the mice genotypes could be because of the lack of ERα. Alternatively, all genotypes could possibly respond to loss of DA and the effect may be significant only with an extensive striatal DA depletion as only occurs with MPTP 9 and 11 mg/kg in ERKOα mice (Morissette et al., 2007).

Striatal D2 receptors are located pre-synaptically on DA terminals and post-synaptically; [^{3}H]raclopride binds to both of these receptor populations (Roth, 1979). Loss of DA neurons in PD and in animal models is associated with loss of pre-synaptic D2 receptors and also as compensatory mechanism a post-synaptic D2 receptors increase (Falardeau et al., 1988, Girault and Greengard, 2004). This is accordance with the observation in PD where the symptoms of the disease only become apparent when striatal DA concentrations are reduced by about 80% (Hornykiewicz, 1998, Hirsch et al., 2000). Before this threshold is reached compensatory mechanisms occur to maintain DA neurotransmission including increased turnover of DA and increased DA receptors. In early stage PD patients or patients not taking DA replacement therapy, an increase in striatal D2 receptors is observed by *in vitro* (Piggott and Marshall, 1996) and *in vivo* imaging (Antonini et al., 1997). 6-OHDA or MPTP unilateral lesions of the SN pars

compacta is reported to increase striatal [^{3}H]-raclopride specific binding and reduce [^{3}H]-SCH 23390 specific binding (Da Cunha et al., 2008). These DA receptors response to lesion develop over time and an acute treatment of MPTP was shown to leave unchanged [^{3}H]-SCH 23390 specific binding in the striatum and the SN, while [^{3}H]-raclopride specific binding was decreased in the SN of mice (Araki et al., 2001). We reported a logarithmic correlation between DA concentrations and density of D2 receptor binding in the caudate nucleus, putamen and nucleus accumbens of MPTP monkeys with lower than intact animal values at moderate DA depletion and large increases as the lesion became extensive (Falardeau et al., 1988). Initial decrease of D2 receptors may be due to loss of these receptors located pre-synaptically whereas the post-synaptic adaptation and increase of these receptors develop over time and with an extensive lesion to compensate for reduced neurotransmission.

In the present experiment, mice were investigated five days after the lesion. This short time interval may not have been enough for D2 receptors to adapt and increase. Hence, we have observed a dose-related decrease in WT mice of D2 receptors with increasing doses of MPTP and this was positively correlated with striatal DA concentrations. Similarly in ERKOα mice striatal D2 receptors decreased with increasing doses of MPTP in correlation with DA concentrations. This could be explained by the effect of MPTP to destroy D2 receptor bearing DA terminals and that in this the early stage after the lesion the post-synaptic D2 receptors have yet to increase. By contrast, D2 specific binding in ERKOβ did not decrease with the lesion but dose-dependently increased with increasing doses of MPTP. Contrary to the WT and ERKOα mice in the ERKOβ mice a negative correlation between D2 receptors binding and DA concentrations was observed. It could be that ERβ opposes the ERα response of D2 receptors to the lesion. In ERKOβ mice, the adaptation of D2 receptors to lost DA may be accelerated.

17β-estradiol treatment did not increase striatal [^{3}H]-SCH 23390 or [^{3}H]-raclopride specific binding of the unlesioned mice of all three genotypes these receptors remaining unchanged or decreasing in ERKOα mice for D1 receptors. As in the dose effect of MPTP experiment MPTP caused a decrease of DA receptors (D1 receptor for ERKOα mice and D2 receptors for WT mice) and 17β-estradiol–treated MPTP mice had higher D1 (ERKOα and ERKOβ mice) and D2 receptors (WT and ERKOβ mice). These results suggest that 17β-estradiol acting on its receptors accelerated the lesion-induced compensatory increase of D1 and D2 receptors.

We have previously shown that 17β-estradiol and specific ER agonists increase striatal Akt phosphorylation and its downstream substrate GSK3β in intact male mice and that these increases are also observed in the 17β-estradiol-treated MPTP mice suggesting implication of these signaling pathways in 17β-estradiol neuroprotection (D'Astous et al., 2006). In fact, in response to several hormones (17β-estradiol) and growth factors, Akt deactivates GSK3β by phosphorylation on its regulatory N-terminal domain serine 9, favoring the activation of anti-apoptotic intracellular signaling (Hetman et al., 2000) like GSK3β which is a constitutively active and highly expressed kinase in the brain, inducing cellular death pathways (D'Astous et al., 2006). We also reported in ovariectomized monkeys an increase with a chronic estradiol treatment of striatal pAkt (Ser473)/βIII-tubulin, pAkt (Thr308)/βIII-tubulin, Akt/βIII-tubulin and pGSK3β (Ser9)/βIII-tubulin whereas pGSK3β (Tyr216)/βIII-tubulin and pGSK3α (Ser21)/βIII-tubulin remained unchanged (Sanchez et al., 2012).

DA receptors are also reported to signal through the Akt/GSK3β pathway (Beaulieu et al., 2005, Beaulieu et al., 2007, Sutton and Rushlow, 2011). Treatment for seven days of male rats with the D2 receptor antagonist raclopride increases phosphorylation of Akt and GSK3β in the

prefrontal cortex whereas the opposite is measured with the D2 receptor agonist quinpirole and no effect with the D1 antagonist SCH23390 (Sutton and Rushlow, 2011).

In our MPTP male mice paradigm receiving four injections of the toxin two hours apart and investigation of the animals 5 days afterwards we observed no change of Akt and pAkt Ser473)/Akt and a decrease of pGSK3β(Ser9)/GSK3β (D'Astous et al., 2006). In ovariectomized MPTP monkeys extensively denervated we measured a decrease of pAkt Ser473)/Akt in the caudate nucleus and putamen and a decrease of pGSK3β(Ser9)/GSK3β in the putamen while the other phosphorylated forms of these proteins were unchanged by the lesion (Morissette et al., 2010). A recent study of Durgadoss et al. investigated the effect of acute and chronic MPTP in male mice on Akt and GSK3β midbrain and striatum and observed time, and brain region and phosphorylation site specific effects (Durgadoss et al., 2011). A single dose of MPTP 30 mg/kg left unchanged striatal phosphorylated sites of Akt and GSK3β, Akt and GSK3β levels whereas a chronic MPTP treatment of 8 days increased Akt, decreased pAkt(Thr308) and left unchanged pAkt(Ser473) (Durgadoss et al., 2011). Hence, overall these various studies in mice and monkeys suggest a long-term adaptation of Akt/GSK3β to a MPTP lesion. In the present study, no change of Akt or GSK3β levels were observed, so the phosphorylated states were expressed over the total protein and also were unchanged by the MPTP lesion in WT and ERKOα mice. By contrast, the intact ERKOβ mice striatal pAkt/Akt and pGSK3β/GSK3β were elevated and had lower D2 receptors. This may be causally related since blocking D2 receptors with an antagonist increases pAkt and pGSK3β but this explanation may be too simplistic, ERKOα mice having also lower striatal D2 receptors compared to WT mice but their pAkt and pGSK3β levels were normal. Not only were the striatal pAkt and pGSK3β levels increased in intact ERKOβ mice compared to the other genotypes, they increased two to four fold with MPTP. This is the first such observation

and is difficult to explain. Perhaps compensatory mechanisms are induced in the ERKOβ mice since their DA depletion (but not the DA metabolites 3-methoxytyramine and homovanillic acid) following MPTP is similar to the WT (Morissette et al., 2007).

The two isoforms ERK1 and ERK2 (Pearson et al., 2001) are documented to be indispensable in neuroprotection against oxidative stress, DNA damage, and trophic factor deprivation, all insults believed to be implicated in neurodegeneration (Xia et al., 1995). Diseased brains show abnormalities in their immunohistochemical staining patterns for phosphorylated ERKs (Perry et al., 1999) and prolonged ERK activation could lead to cell death (Kulich and Chu, 2001). MPTP induced phosphorylation of both ERK1 and ERK2 was seen in MPTP mice (De Girolamo and Billett, 2006) and MPTP monkeys (Bezard et al., 2005). It was observed that inhibiting ERK1/2 promotes activation of GSK3β (Hetman et al., 2002). By contrast in dopaminergic cell lines ERK1/2 were shown to play a neuroprotective role in basal dopaminergic neuron survival and neuroprotection against oxidative stress (Cavanaugh et al., 2006). In the present experiment no effect of genotype was observed on pERK1/ERK1 and pERK2/ERK2 levels. A decrease with the MPTP lesion was only observed for striatal pERK1/ERK1 in WT mice whereas no effect of lesion was observed in the ERKOα mice compared to their respective controls. An increase was only observed with the highest dose of MPTP of both striatal pERK1/ERK1 and pERK2/ERK2 levels in the ERKOβ mice and this was associated with the increase of D2 receptors. As proposed above for Akt/GSK3β signaling in MPTP treated ERKOβ mice may be more responsive to the lesion and an accelerated adaptation.

VIII. CONCLUSION

Both ERα and ERβ affected the response of striatal D1 and D2 receptors to a MPTP lesion, 17β-estradiol treatment as well as Akt/GSK3β and ERK1/2 signaling in male mice. The effect of ER on the adaptation of striatal DA receptors and their signaling are important to understand since D2 receptors are key players in mental and neurodegenenerative diseases and are a target of numerous therapeutic agents.

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XI. FIGURES

Figure 1.

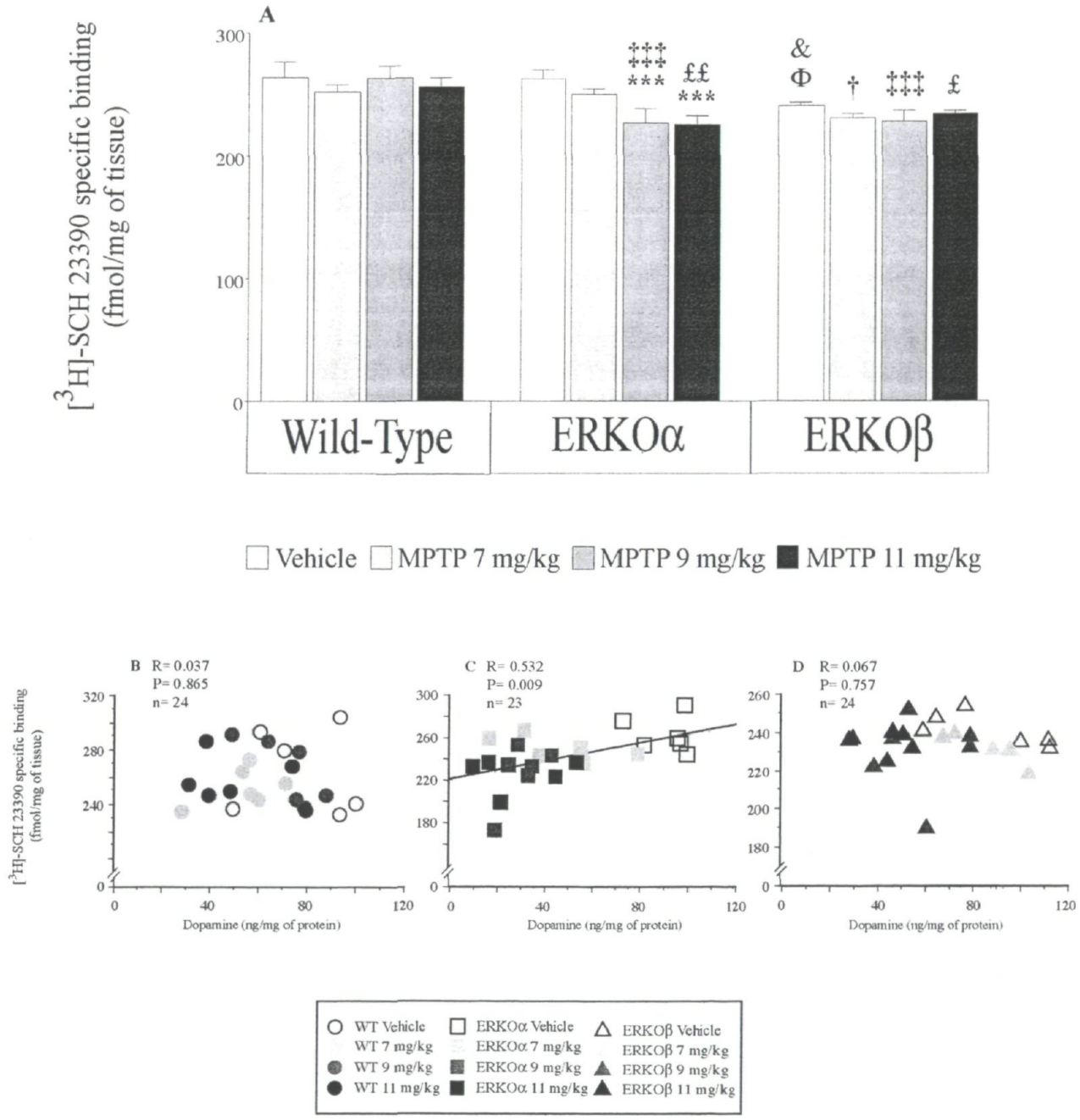


Figure 2.

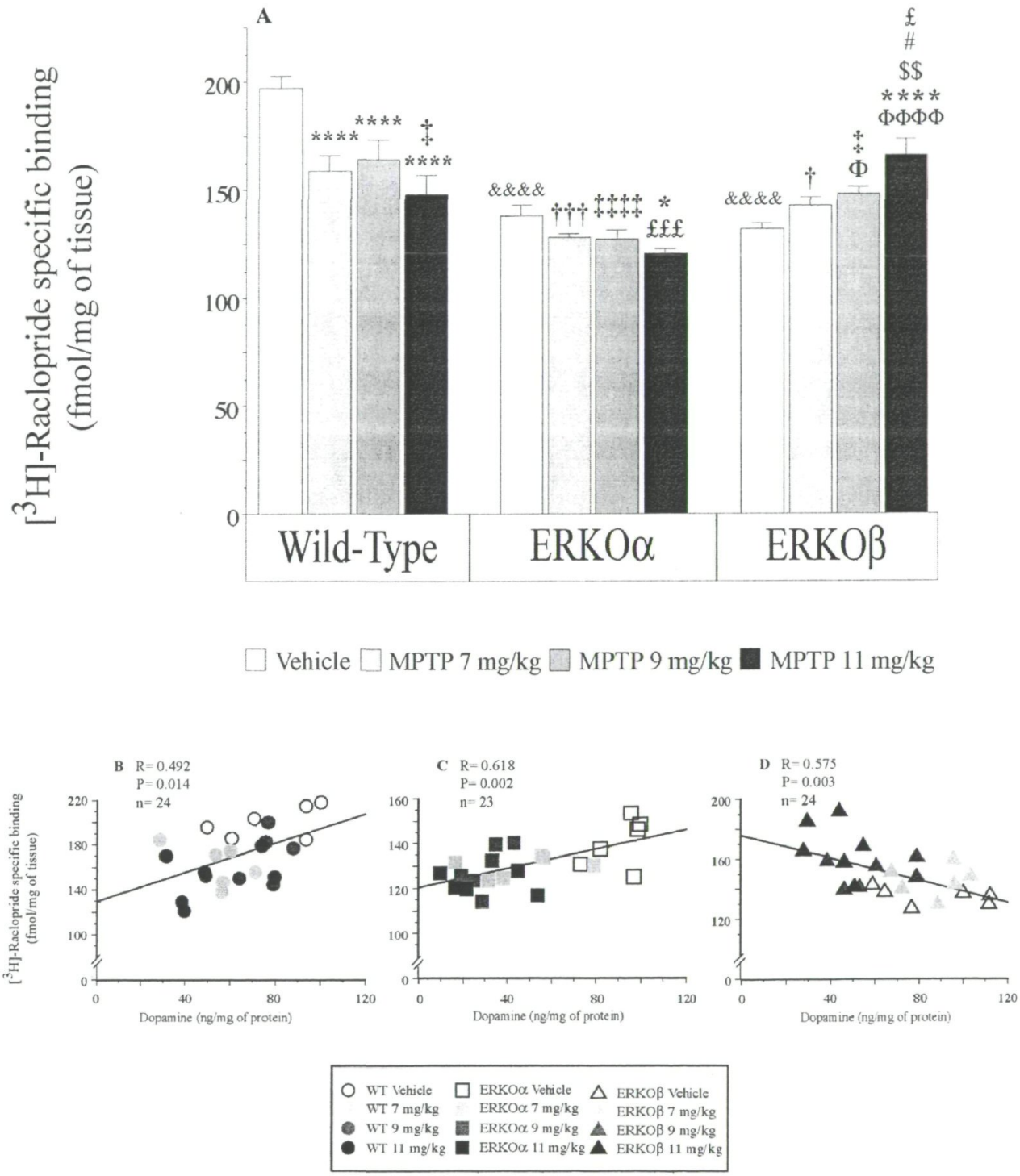

A
[3H]-Raclopride specific binding (fmol/mg of tissue)
Wild-Type
ERKOα
ERKOβ
Vehicle
MPTP 7 mg/kg
MPTP 9 mg/kg
MPTP 11 mg/kg
B R= 0.492 P= 0.014 n= 24
C R= 0.618 P= 0.002 n= 23
D R= 0.575 P= 0.003 n= 24
Dopamine (ng/mg of protein)
WT Vehicle
WT 7 mg/kg
WT 9 mg/kg
WT 11 mg/kg
ERKOα Vehicle
ERKOα 7 mg/kg
ERKOα 9 mg/kg
ERKOα 11 mg/kg
ERKOβ Vehicle
ERKOβ 7 mg/kg
ERKOβ 9 mg/kg
ERKOβ 11 mg/kg

Figure 3.

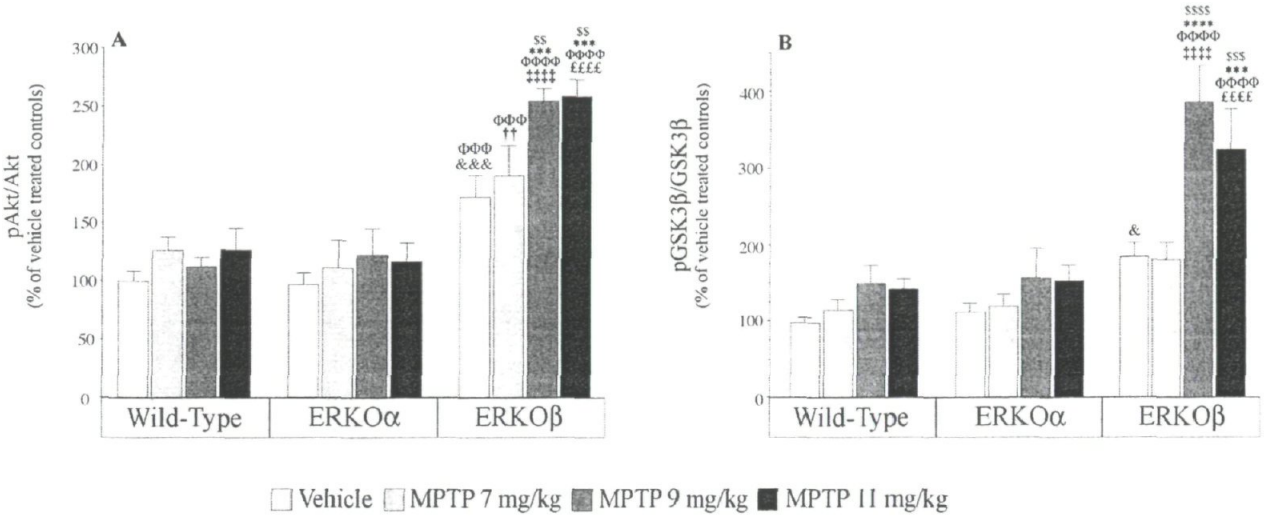


Figure 4.

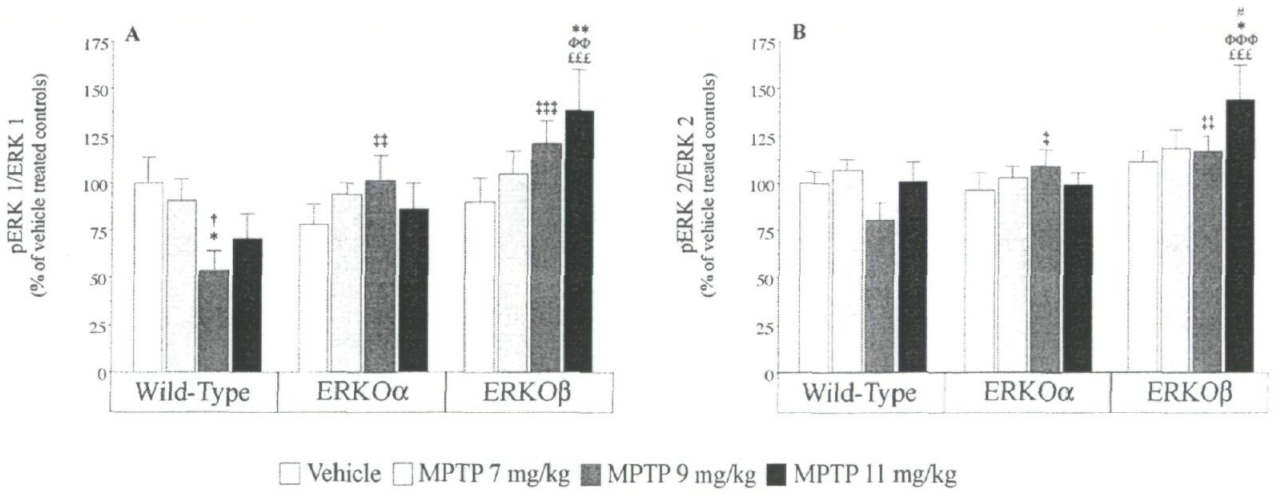
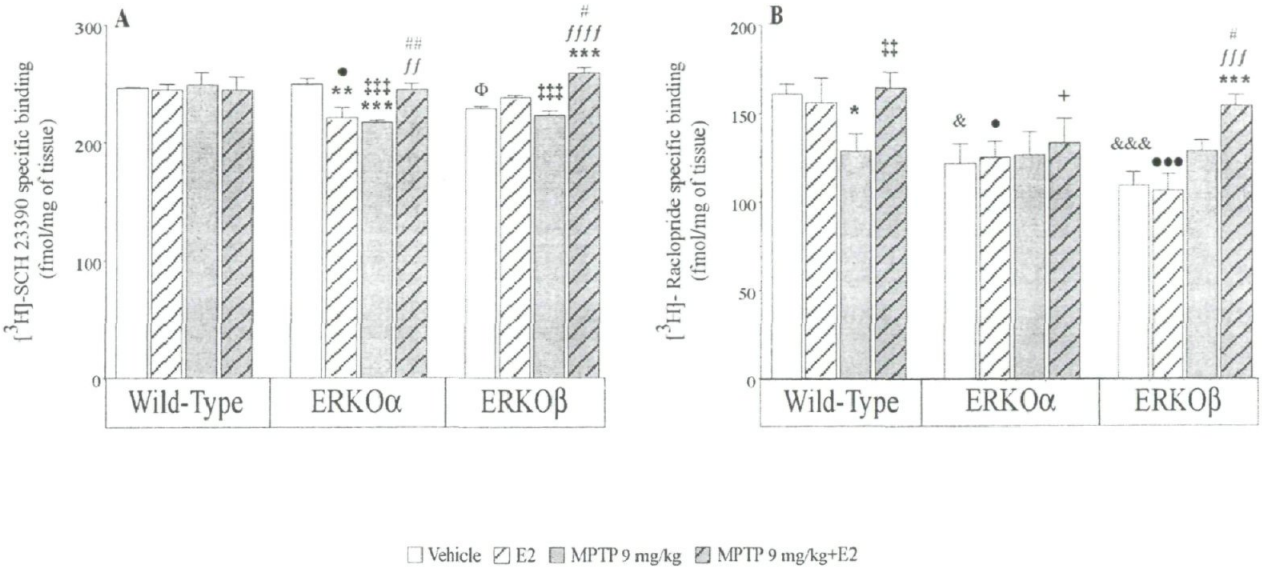


Figure 5.


A
B
[3H]-SCH 23390 specific binding (fmol/mg of tissue)
[3H]- Raclopride specific binding (fmol/mg of tissue)
Wild-Type
ERKOα
ERKOβ
Vehicle
E2
MPTP 9 mg/kg
MPTP 9 mg/kg+E2

XII. LEGENDS to FIGURES

Figure 1 Dose-response effect of MPTP on A- striatal dopamine D1 receptors measured with [^{3}H]-SCH23390 specific binding ($F_{11,59}$=3.94, p=0.0003) and correlations of D1 receptor specific binding with striatal dopamine concentrations in wild-type (B), ERKOα (C) and ERKOβ (D) mice. Striatal dopamine concentrations of these mice were previously measured (Morissette et al., 2007).

***p<0.005 vs the respective genotype, vehicle; &p<0.05 vs WT, vehicle; †p<0.05 vs WT MPTP, 7 mg/kg; ‡‡‡p<0.005 vs WT MPTP, 9 mg/kg; £p<0.05 and ££p<0.01 WT MPTP, 11 mg/kg; Φp<0.05 vs the respective ERKOα genotype experimental group.

Figure 2 Dose-response effect of MPTP on A- striatal dopamine D2 receptors measured with [^{3}H]-Raclopride specific binding ($F_{11,59}$=13.53, p<0.0001) and correlation of D2 receptor specific binding with striatal dopamine concentrations (Morissette et al., 2007) in wild-type (B) compared to ERKOα (C) and ERKOβ (D) mice.

*p<0.05 and ****p<0.0001 vs the respective genotype, vehicle; &&&&p<0.0001 vs WT, vehicle; †p<0.05 and †††p<0.005 vs WT MPTP, 7 mg/kg; ‡p<0.05 and ‡‡‡‡p<0.0001 vs WT MPTP, 9 mg/kg; £ p<0.05 and £££p<0.005 WT MPTP, 11 mg/kg; Φp<0.05 and ΦΦΦΦp<0.0001 vs the respective ERKOα genotype exprimental group; $$p<0.01 vs ERKOβ MPTP, 7 mg/kg; #p<0.05 vs respective ERKO MPTP, 9 mg/kg.

Figure 3 Dose-response effect of MPTP on striatal A- Akt ($F_{11,48}$=11.45, p<0.0001) and B-GSK3β ($F_{11,49}$=10.28, p<0.0001) signaling measured by Western blot in wild-type compared to ERKOα and ERKOβ mice.

***p<0.005 and ****p<0.0001 vs the respective genotype, vehicle; &p<0.05 and &&&p<0.005 vs WT, vehicle; ††p<0.01 vs WT MPTP, 7 mg/kg; ‡‡‡‡p<0.0001 vs WT MPTP, 9 mg/kg; £££p<0.0001 vs WT MPTP, 11 mg/kg; ΦΦΦp<0.005 and ΦΦΦΦp<0.0001 vs the respective ERKOα genotype exprimental group; $$p<0.01, $$$p<0.005 and $$$$p<0.0001 vs ERKOβ MPTP, 7 mg/kg.

Figure 4 Dose-response effect of MPTP on striatal A- ERK1 ($F_{11,58}$=2.97, p=0.0035) and B-ERK2 ($F_{11,59}$=2.71, p=0.0068) signaling measured by Western blot in wild-type compared to ERKOα and ERKOβ mice.

*p<0.05 and **p<0.01 vs the respective genotype, vehicle; †p<0.05 vs WT MPTP, 7 mg/kg; ‡p<0.05, ‡‡p<0.01 and ‡‡‡p<0.005 vs WT MPTP, 9 mg/kg; £££p<0.005 vs WT MPTP, 11 mg/kg; ΦΦp<0.01 and ΦΦΦp<0.005 vs the respective ERKOα genotype exprimental group; #p<0.05 vs ERKOβ MPTP, 9 mg/kg.

Figure 5. Effect of treatment with 17β-estradiol (E_2) 2μg/day for ten days on striatal A- [^{3}H]-SCH23390 specific binding ($F_{11,77}$=4.42, p<0.0001) and B- [^{3}H]-Raclopride specific binding ($F_{11,75}$=3.44, p=0.0007) in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice.

*p<0.05, **p<0.01 and ***p<0.005 vs the respective genotype, vehicle; &p<0.05 and &&&p<0.005 vs WT, vehicle; ‡‡p<0.01 and ‡‡‡p<0.005 vs WT + MPTP; •p<0.05 and •••p<0.005 vs WT+ E2; +p<0.05 vs WT + MPTP + E2; Φp<0.05 vs the respective ERKOα genotype exprimental group; *ff*p<0.01, *fff*p<0.005 and *ffff*p<0.0001 vs respective ERKO + E2; #p<0.05 and ##p<0.01 vs respective ERKO + MPTP.

CHAPTER VI

Discussion

6. 1. *The effects of MPTP on dopaminergic markers*

Evidence gathered from neuropathologic research claims that the SNc is not the starting point of PD (Lang, 2007) as supported by the Braak theory, the SN in affected during stages 3 and 4 as the symptoms start to appear (Braak and Del Tredici, 2010).

It is suggested that when the disease reaches the SNc beginning with pathobiological processes like protein aggregations, which are then followed by dopaminergic neuron specific processes like oxidative stresses and increased DA turnover (Lang, 2007). Other triggered mechanisms include altered calcium homeostasis and excitotoxicity which lead to the rapid loss of SNc neurons (Lang, 2007). Once degeneration in this region reaches a threshold allowing the manifestation of symptoms and progression is accelerated (Lang, 2007). Hence, neuroprotective remedies which concentrate on general biologic processes will not promote beneficial effects against the loss of dopaminergic neurons, accelerated nigral degeneration, or retard the disease's progression. Suggesting that neuroprotection is flawed since it targets relief after dopaminergic cell death and has no interest in stopping the progression of non-dopaminergic symptoms which are responsible for major disability during the disease's final stages (Lang, 2007).

The expression of DAT is higher in females both clinically (Lavalaye et al., 2000; Mozley et al., 2001) and experimental studies (McArthur et al., 2007) and this linked to larger DA terminal density in the striatum. However, the comparison of male and female striatal DAT densities does not support the claim that DAT levels affect intrinsic sex differences of experimental susceptibility (McArthur et al., 2007).

It is suggested that estrogens suppress DAT expression at the time of neuronal insult as a mechanism of neuroprotection (Dluzen and Horstink, 2003; McArthur et al., 2007; Murray et al.,

2003). Our laboratory observed that 17β-estradiol up-regulates striatal DAT in females (Morissette and Di Paolo, 1993a, b), while others saw the opposite effect (Thompson, 1999).

Further supporting the theory that PD treatment should be a combination of therapies formulated to target basic mechanisms of neurodegeneration and those related to the biologic processes of the SNc (Lang, 2007). Histological experiments reveal that ERα was the prevalently detected in the male mouse striatum and SNc, however double-label immunocytochemical results show that it is not colocalized with TH in the SNc and no specific staining of ERβ mRNA or protein was detected in the nigrostriatal system (Shughrue, 2004). Shurghrue and colleagues also demonstrate that MPTP lesioning does not affect the nigrostriatal expression pattern of ERs (Shughrue, 2004), implying that estrogen activity through nuclear receptors does not involve mechanisms modulating ER expression in order to induce the survival of striatal DA neurons. Our laboratory and many others confirmed that MPTP induces a decrease of DAT levels and that it is at the same degree of striatal DA loss (Callier et al., 2000; Callier et al., 2001; Jourdain et al., 2005). We observed that MPTP mice had low DAT concentrations in the SN and that the decrease was not as high as seen in the striatum (Jourdain et al., 2005). We theorized that the cell bodies of DA neurons in the SN are less vulnerable to MPTP than are their dopaminergic terminals in the striatum (Jourdain et al., 2005). Also, moderate MPTP doses cause more severe damage on DA terminals than their cell bodies (Jourdain et al., 2005). Treating MPTP mice with 17β-estradiol blocks the loss of DAT in the striatum and SN (Callier et al., 2000; Callier et al., 2001; Jourdain et al., 2005). The degree of intrinsic neuroprotection is therefore limited by the extent of neuronal damage. As for hormonal influences in the partially injured SNc, significantly more DA neurons survive in the female nigrostriatal pathway than that of males (Murray et al., 2003). It is suggested that hormonal actions target the activity rather than the survival of the

remaining neurons, thus having modulatory effects on their adaptation to mild injury by altering striatal DA content without influencing SN neuronal numbers (Ferraz et al., 2003; McArthur et al., 2007). Results shown in chapter 2 reveal that intact ERKOβ mice had lower striatal DAT and VMAT2 levels than those of WT and ERKOα mice. MPTP caused a dose-dependant loss of both striatal transporters that correlated with striatal DA concentrations. Compared to WT and ERKOβ mice, ERKOα mice DAT, VMAT2 and TH levels were affected at lower MPTP doses. In the striatum and SN, ERKOα mice were more vulnerable and 17β-estradiol protected against MPTP toxicity only in WT mice. Perhaps hormonal actions on ERβ influence the activity of the remaining neurons, while ERα activity modulates the survival of the remaining neurons. Indeed, Motissette et al. suggested a role for ERβ in DA metabolism, since ERKOβ mice show a lower DA turnover (Morissette et al., 2007) (Figure 10 and Table 3 and 4).

In addition, we saw that MPTP had similar effects on $VMAT_2$ in both the striatum and the SN (Ekue et al., 2002; Jourdain et al., 2005). It was also observed that DAT (Callier et al., 2000; D'Astous et al., 2003; Ekue et al., 2002) and TH (D'Astous et al., 2003) mRNA levels were reduced in the SN of mice that were denervated between 70-80%, while if DA depletion was milder (45%) these markers were unchanged (Jourdain et al., 2005). Hence, lesioning must be excessive in order to modulate DAT and TH mRNA and the same was concluded for cell bodies damage. In fact, different toxins are administered at elevated doses to cause cell body destruction when cell terminals are lost with lower doses of the same neurotoxins (Bywood and Johnson, 2000). Therefore, the loss of cell terminals was labeled as a marker of neurodegeneration, as were DAT and $VMAT_2$ specific binding on striatal dopaminergic terminals in the case of PD.

Moreover, in nonlesioned mice DAT and TH mRNA did not fluctuate due to estrogenic treatment (Bosse et al., 1997; Joyce et al., 1997), whereas it was suggested that 17β-estradiol induces DAT transcription in MPTP mice as a mechanism of compensation (Ekue et al., 2002).

6. 2. *17β-Estradiol-induced neuroprotection*

Elwood Jensen was the first to confirm the existence of an intracellular receptor regulating action of 17β-estradiol in the female reproductive tract using ^{3}H-labeled 17β-estradiol (Fan et al., 2010) and research shows that with mild to moderate neurodegeneration, treatment with 17β-estradiol is could restore DA and DAT levels at normal (Callier et al., 2001).

The three subtypes of estrogen-induced neuroprotection mechanisms target antioxidation, defense and viability (Brinton, 2001). It was determined that 17β-estradiol is capable of neuroprotection under moderate neurodegenerative conditions since DAT mRNA levels were not affected by estradiol in the SN (Callier et al., 2001). The conclusion was that the neuroprotective effects of 17β-estradiol do not implicate the synthesis of DAT (Callier et al., 2001), implying that a neuropotective agent can induce different effects under different situations.

As mentioned before, investigators have immensely focused their work on ovarian factors, mainly 17β-estradiol, with the male and the female brain being used for research (Gillies and McArthur, 2010). The brain can produce its own levels of 17β-estradiol *de novo* which is involved in physiological and pathological processes implicating neurotransmission and neuroprotection, having effects on cognition and neurodegenerative disease (Garcia-Ovejero et al., 2005; Garcia-Segura, 2008).

Male sex and age together makeup a strong risk factor, here men are twice as much at risk for developing PD than are women, at all ages and all nationalities studied (Baldereschi et al.,

2000; Cantuti-Castelvetri et al., 2007; Diamond et al., 1990; Haaxma et al., 2007; Schrag et al., 2000; Shulman and Bhat, 2006). In fact, epidemiological and clinical data provide evidence on the neuroprotective effects of estrogens in women (Dluzen and Horstink, 2003; Dluzen, 2000; Shulman and Bhat, 2006) and a large number of animal studies are supportive of the clinical evidence on estrogen's multitude of benefits (Bourque et al., 2009). Research using animal models of PD also demonstrates the male-dominant sex difference in susceptibility, thus providing a justification for the appropriatness of the use of male animal models for studying the roles of steroidal hormones (Miller et al., 1998; Murray et al., 2003). Administration of physiological concentrations of 17β-estradiol reverse the effects of castration in the lesioned nigrostriatal dopaminergic pathway and this could justify the use of physiological levels of 17β-estradiol in male animal models of experimental PD (McArthur et al., 2007; Murray et al., 2003).

Moreover, it was shown that neither castration nor testosterone replacement can change MPTP-induced striatal DA depletion in C57Bl/6 mice (Dluzen et al., 1994). In fact, testosterone of testicular origin could aggravate the susceptibility of the male nigrostriatal neurons to injury even after its aromatization to 17β-estradiol (Dluzen et al., 1994; Lewis and Dluzen, 2008; Murray et al., 2003). Circulating testosterone could be converted into 17β-estradiol by aromatases which are expressed in the striatum (Kuppers et al., 2000) but the question is whether physiological concentration of 17β-estradiol are sufficient to induce DA neuron survival through local steroid synthesis at the site of injury (Gillies and McArthur, 2010).

Our laboratory previously and extensively published on the neuroprotective effects of 17β-estradiol administration in MPTP treated mice (Bourque et al., 2009) and the androgens such as testosterone and DHT had no beneficial effects (Ekue et al., 2002).

The endogenous levels of these steroids have not been previously investigated in this paradigm. We thus sought if the endocrine status of these animals was altered with the MPTP lesion and/or the ERα and ERβ knockouts.

As examined in chapter three, the assayed steroid plasma levels show that ERKOα mice had elevated testosterone, DHT and 3β-diol concentrations when compared to WT and ERKOβ mice (Al-Sweidi et al., 2011), which is in agreement with a previous report noting a significant increase of testosterone in ERKOα male mice when compared to WT males (Rissman et al., 1997). We observed that the loss of nigrostriatal DA significantly inversely correlates with testosterone and 3β-diol plasma levels. Also, there was an increase of 17β-estradiol plasma levels in mice treated with 17β-estradiol in all the investigated genotypes; the lack of response of ERKO mice to the 17β-estradiol treatment was therefore not because of lower circulating concentrations of 17β-estradiol due to higher metabolism of exogenous administered 17β-estradiol in the knockout mice. Gillies and colleagues suggest that the effects of supra-physiological circulating levels of 17β-estradiol are not specific to the nigrostriatal pathway (Gillies and McArthur, 2010). In fact, the presence of steroid synthesizing enzymes implies that striatal 17β-estradiol concentration could be independent of peripheral steroid synthesis and may be six times higher than plasma levels (Mukai et al., 2006), as well as synaptically greater (Balthazart and Ball, 2006).

Nevertheless, we observed some differences in the plasma 17β-estradiol concentrations, being higher in ERKOα and lower in ERKOβ MPTP mice compared to the intact 17β-estradiol treated respective ERKO mice (Al-Sweidi et al., 2011). There is an inverse correlation between SN TH mRNA and the plasma levels of testosterone and 3β-diol (Al-Sweidi et al., 2011). The observation that striatal DA and SN TH mRNA decrease as testosterone levels increase is

consistent with our previous findings showing lower SN DAT mRNA levels in testosterone treated MPTP male mice (Ekue et al., 2002). In addition, the deleterious effect of testosterone was also observed in methamphetamine toxicity on mice (Lewis and Dluzen, 2008). In humans, this may also be consistent with a higher incidence of PD in men than women (Wooten et al., 2004).

However, the aromatization of circulating testosterone into 17β-estradiol could be neuroprotective in the hippocampus (Azcoitia et al., 2001).

DHT, the non-aromatized androgen, did not reverse the effects of gonadectomy in the lesioned nigrostriatal dopaminergic pathway but, on the other hand, physiological levels of 17β-estradiol did (Gillies and McArthur, 2010; Murray et al., 2003). DHT is reported to increase CA1 strata oriens and radiatum NMDA receptor binding density in castrated male rats (Romeo et al., 2005). Little data is available for other brain regions, testosterone treatment had no effect on [^{3}H]CGP 39653 binding to NMDA receptors containing NR1/2A in the cerebral cortex (Brann et al., 1993) and no data is reported for the striatum. Therefore, higher levels of NMDA receptors in hippocampal regions of ERKOα but not of WT or ERKOβ mice could be due to the effect of androgens on this glutamate receptor.

6. 3. *ERs and neuroprotection*

The last three decades have yielded much evidence on estrogen's neuroprotective abilities in PD (Gillies and McArthur, 2010) and proof that 17β-estradiol is capable of inducing neuroprotection in the lesioned male nigrostriatal pathway has great therapeutic potential.

Many have observed the neuroprotective effects of 17β-estradiol, when administered prior to the MPTP regimen, through the prevention or cessation of DA and dopaminergic metabolite loss

(Callier et al., 2000; Miller et al., 1998; Ramirez et al., 2003), or the loss of DAT and VMAT2 (D'Astous et al., 2003). Estrogens are thought to convey their neuroprotective effects through genomic mechanisms which signal through ERs or by using non-genomic mechanisms by inducing antioxidant effects (Behl, 2002; Green and Simpkins, 2000) or through membrane bound receptors (Behl, 2002; Bjornstrom and Sjoberg, 2005). Again, it is important to note that ERα is well-expressed throughout the body while ERβ's distribution is restricted to the brain and therefore is of interest when studying neuroprotection (Kuiper and Gustafsson, 1997; Kuppers and Beyer, 1999; Mitra et al., 2003). The brains of both males and females are exposed to estrogens, obviously circulating concentrations are higher in females (Gruber et al., 2002; Handa et al., 2012) and the probability that ERs are found in dopaminergic neurons of the midbrain implies that the adult nigrostriatal system is targeted by estrogens (Gundlah et al., 2000; Handa et al., 2012; Kuppers and Beyer, 1999). Therefore, the effects of 17β-estradiol are not restricted by genetic sex. Investigators have observed the neuroprotective effects of 17β-estradiol, when administered prior to the MPTP regimen, through the prevention or cessation of DA and metabolite loss (Callier et al., 2000; Dluzen et al., 1996; Miller et al., 1998; Ramirez et al., 2003), or the loss of DAT and VMAT2 (D'Astous et al., 2003). Also, clinically 17β-estradiol provides relief from PD symptoms if treatment is given at early stages of the disease (Saunders-Pullman et al., 1999). In fact, 17β-estradiol's effects are well documented for their involvement in the potentiation of DA release (Becker, 1990) and alteration of DA receptor expression (Bosse et al., 1997). On the other hand, 17α-estradiol does not induce neuroprotective effects being receptor-inactive and having the same antioxidant activity as its receptor-active isomer 17β-estradiol (Jourdain et al., 2005). Other estrogens like estrone and estriol are categorized as weak ER agonists (Gruber et al., 2002). It was seen that neuroprotective potentials of estrogens vary in

accordance with the degree of dopaminergic loss caused by MPTP (Grandbois et al., 2000). More precisely, what was observed was that at high levels of striatal DA loss, 17β-estradiol was capable of inducing neuroprotection, but was not able to rectify the levels of DAT (Callier et al., 2000). In this situation, it was concluded that 17β-estradiol stimulates the remaining neurons and this results in the increased striatal DA concentrations (Callier et al., 2000).

Polymorphisms in ERα (Maraganore et al., 2002) and ERβ (Westberg et al., 2004) are not risk factors for PD, but the latter was observed to be more prevalent in patients having an early age of onset (Hakansson et al., 2005; Westberg et al., 2004). Our laboratory published the neuroprotective effects of 17β-estradiol and PPT in MPTP treated male mice (D'Astous et al., 2004). While, testosterone had no beneficial effects under similar conditions (Ekue et al., 2002) and since DPN did not have significant neuroprotective effects, it was concluded that neuroprotection is a main task of ERα (D'Astous et al., 2004). Baraka et al. compared the effects of 17β-estradiol, tamoxifen, raloxifene, PPT and DPN on behavioral and biochemical in 6-OHDA-lesioned rats and saw that only 17β-estradiol, raloxifene and PPT induced significant ameliorations. Similar to our results published in Morissette et al. (2009) on female hemiparkinsonian monkeys (Morissette and Di Paolo, 2009).

In addition, neuroinflammation is seen in MPTP-lesioned mice (Morale et al., 2006), as well as PD patients (Tansey and Goldberg, 2010) and the overactivation of pro-inflammatory cytokines by glial cells could promote neuronal cell death (Tansey and Goldberg, 2010). In fact, astrocyte activation was observed after MPTP lesioning in the striatum and SN (Morale et al., 2006) and once activated microglia and astrocytes express high levels of inducible-nitric oxide synthase, a cytotoxic mediator of DA neuron injury (Morale et al., 2006). 17β-estradiol pre-treatment lowers the levels of activated astrocytes, blocks the production of inducible-nitric oxide

synthase-derived nitrites and reduces DA neuron toxicity and death in MPTP-lesioned mice (Morale et al., 2006). Therefore, 17β-estradiol-induced neuroprotective effects regulate the neuroinflammatory responses by mediating the actions of glial cells.

It was observed that pretreatment aromatse inhibitors worsen ischemic injury in rodents and aromatase KO mice (Santen et al., 2010) and ICI 182,780 treatment prior to an induced stroke will exacerbate the infraction (Liao et al., 2001). Glial cells express various enzymes involved in neuroprotection, including aromatase, which is overexpressed in astrocytes after brain injury (Garcia-Segura, 2008). In fact, it was observed that aromatase is found near the injury site (Garcia-Segura, 2008).

Moreover, astrocytes express ERs and 17β-estradiol neuroprotective effects could include reducing the production of pro-inflammatory molecules and increase the production of trophic factors and favor neuronal survival (Azcoitia et al., 2010).

Brown and colleagues suggest that ERα and ERβ regulate cytokine and chemokine-mediated neuroinflammation response through ligand-dependent mechanisms. They used ERKOα and ERKOβ mice and saw that both ERs are involved in 17β-estradiol dependent or independent regulation of cytokine and chemokine levels during neuroinflammation (Brown et al., 2010). However, ERα is thought to be required in 17β-estradiol-induced suppression of blood-brain-barrier permeability (Brown et al., 2010).

Here, we demonstrate that ERα plays an important role against striatal MPTP toxicity, whereas the ERβ appears to induce subtler effects. In other words, endogenous estrogens acting on ERs are shown to play an important neuroprotective role against MPTP toxicity and ERα has a more significant role in mediating these protective effects of endogenous estrogens that modulate the sensitivity of dopaminergic neurons to MPTP toxicity. Nonetheless, the results of chapter two

unveil a role for ERβ in modulating the degree of vulnerability of dopaminergic neurons to MPTP toxicity (Figure 10 and Table 3 and 4).

Navarro et al. saw different patterns of ERa localization in the diencephalon and the telencephalon in three experimental groups which include control, ovariectomized and 17β-estradiol treated rats (Navarro et al., 2012). They saw that young rats of all three groups had nuclear immunostaining in the telencephalon, while aging changes ERα localization from the nucleus to extra-nuclear sites (Navarro et al., 2012). Accordingly, it was seen that estrogen promotes ERα trafficking to the plasma membrane and ERβ trafficking away from the plasma membrane (Alyea et al., 2008) and it is suggested that ERβ mediates the induction of apoptosis (Nilsen et al., 2000), while ERα is the dominant receptor modulating estrogen-induced DA efflux, with inhibitory effects by ERβ (Alyea et al., 2008). Thus, making ERα responsible for non-genomic estrogen-induced effects on DAT activity (Alyea et al., 2008). Also, Johnson et al. suggest that ERα has an important role in the maintenance of SN neuron number (Johnson et al., 2010). Hence, neuroprotection relies on mixed genomic and non-genomic mechanisms, which in turn depend on ER distribution and the availability of different DNA response elements and coregulator proteins (Figure 10).

6. 4 *NMDA NR1/2B receptors and 17β-estradiol induced neuroprotection*

The human brain occupies only 5% of the body, however it controls 20% of respiration (Simpkins et al., 2008). Thus, due to its extraordinary aerobic poise, the CNS is highly vulnerable to mitochondrial impairment.

Simpkins and colleagues demonstrate that ERβ is imported into mitochondria and it mediates various estrogen dependent actions (Simpkins et al., 2008). Here ERβ is translocated

through tethering to cytosolic chaperone proteins and/or direct interaction with mitochondrial importation proteins (Simpkins et al., 2008). Once inside, ERβ is implicated in the modulation of the transcription of mitochondrial genes by binding EREs or through protein-protein interactions with mitochondrial transcription factors (Simpkins et al., 2008).

Moreover, accumulating proof demonstrates that ERα and ERβ regulate distinct sets of gene expression, even though they have almost identical DNA binding domains (Katzenellenbogen and Katzenellenbogen, 2000; O'Lone et al., 2007).

It is believed that different coactivation and adaptor proteins having roles in ERs binding and transcriptional activation, as well as different compartmentalization of ERα and ERβ contributes to their distinct activities (Simpkins et al., 2008).

As mentioned when discussing the mechanisms of MPTP neurotoxicity, the neuronal energy depends on mitochondrial ATP production (Simpkins et al., 2008). Again, necrosis and apoptosis result after mitochondrial damage demolishes ATP production and increases ROS (Kroemer and Reed, 2000; Lemasters, 1999). Also, excessive calcium levels resulting from the activation of glutamate receptors can cause mitochondrial calcium loading, ATP production interruption, ROS generation and finally cell death (Lemasters, 1999). In fact, it is suggested that mitochondria modulate viability during excitoxicity (Simpkins et al., 2008) and many of the ERβ regulated genes are mitochondrial structure proteins implicated in oxidative phosphorylation (O'Lone et al., 2007). In the case of transgenic mouse models, phenotypic discrepancies have been noted (Antal et al., 2008; Harris, 2007) these could be due to transcript variants (Simpkins et al., 2008). Prokai and colleagues determined that the estrogenic-induced mitochondrial protective effects are antioxidative (Prokai et al., 2003) but the relative neuroprotective proportion of ERβ modulated mitochondrial effects is not known. With ERα being the dominant neuroprotective ER

in our paradigm, this could be an explanation as to why ERKOβ mice are intermediately susceptible to MPTP toxicity and we previously suggested that the critically potent effects of 17β-estradiol on MPTP-induced mitochondrial death predominantly involve ERα activity (Morissette et al., 2007).

Altered cognitive functioning has been related to menopause (Neugarten and Kraines, 1965) and recent advances reveal that estrogen induces effects in the cortex and the hippocampus (Shughrue and Merchenthaler, 2000). The temporal events mediated by different but potentially interacting neuroprotective pathways of which include estrogen-induced activation of src tyrosine kinase that phosphorylates NMDA receptors involved in the development of hippocampal dendritic spines (Yu et al., 1997). Animal experiments and clinical data have shown that 17β-estradiol activity on ERs has effects on the development and functioning of the hippocampus (Fan et al., 2010). Using ERKOα and ERKOβ mice showed that both receptors affect different types of learning, where ERα is involved in emotional learning and ERβ affects spatial learning (Amin et al., 2005). In fact, the hippocampus is able to synthesize 17β-estradiol (Prange-Kiel and Rune, 2006) and 17β-estradiol-induced synaptogenesis depends on post-synaptic NMDA receptor activity (Jelks et al., 2007; Smith et al., 2009).

Interestingly, increased estrogen-inducible excitatory synapses increases NMDA receptor agonist binding sites and NMDAR1 subunit immunoreactivity (Gazzaley et al., 1996; Weiland, 1992). In addition, both ERs estrogen-dependently bind the tyrosine kinase src, which phosphorylates NMDA receptors (Whitfield et al., 1999), resulting in decreased calcium influx (Yu et al., 1997) and increased downstream phosphorylated CREB (Murphy and Segal, 1997). Further, both PPT and DPN dose-dependently induced neuroprotection in the hippocampus against glutamate-induced cell death (Zhao et al., 2004), while Mazzucco et al. saw that the

activation of both ERα and ERβ increases cell proliferation in the dentate gyrus (Mazzucco et al., 2006). Therefore, synapse formation is modulated by both ERs but through different mechanisms and synaptic proteins. More specifically, 17β-estradiol modulates NMDA NR1/2B receptors, here the gene promotor of NMDA receptors contains an SP1 sequence through which ERα can activate transcription, therefore ERα is involved in the promotion of increased NMDA receptor density (Fan et al., 2010). In fact, ovariectomized rats had decreased NMDA NR1/2B receptor levels in the CA1 oriens and CA1 radiatum hippocampal subregions and 17β-estradiol or PPT, but not DPN treatment reversed this (Jelks et al., 2007).

Dubal and colleagues observed ERKOα and not ERKOβ mice to be resistant to the effects of low concentrations of chronically administered 17β-estradiol (Dubal et al., 2001). Agreeing with our work (Al-Sweidi et al., 2011; D'Astous et al., 2006; Dubal et al., 2001; Morissette et al., 2007), they conclude that ERα activity is mechanistically important in the regulation of 17β-estradiol-induced neuroprotection in an ischemia lesioned ERKO mouse model, since neuroprotection was not present in ERKOα mice (Dubal et al., 2001). However, McCullough et al. observed neuroprotection with pharmacological pre-treatment of estrogen in ERKOα mice (McCullough et al., 2001).

Accordingly, it was demonstrated that estrogens enhance calcium influx during mild NMDA receptor mediated glutamate stimulation (Brinton, 2001; Nilsen et al., 2002; Zhao et al., 2004) and prevent cytosolic as well as mitochondrial calcium influx during high excitotoxic stimulation (Nilsen et al., 2002; Wang et al., 2001; Wang et al., 2006). Hence, it could be suggested that 17β-estradiol-induced neuroprotection involves the controlling of neurotoxic

calcium influx levels and Morissette et al. conclude that ERα could have a role in maintaining mitochondrial activity since ERKOα mice are more susceptible to MPTP toxicity than WT or ERKOβ mice (Morissette et al., 2007) and we observed that NMDA receptors are affected by the deletion of ERs which affects the response to MPTP and 17β-estradiol treatments with brain region specificity (Figure 10 and Table 3 and 4).

In chapter four, we discussed the effects of MPTP and 17β-estradiol in the anterior cortex and the striatum, we report that cortical [^{3}H]Ro 25-6981 specific binding decreased with increasing doses of MPTP in WT and ERKOα but not ERKOβ mice, while a dose related decrease of NMDA NR1/2B receptors was only observed in the striatum of WT mice, having a positive correlation with striatal DA concentrations. Plus, 17β-estradiol treatment increased striatal NMDA receptor levels of MPTP-lesioned WT mice. Hence, what could be asserted again is that both ERs are required to alleviate the neurotoxic effects MPTP. Interestingly, 17β-estradiol increased striatal NMDA NR1/2B receptor levels of intact ERKOβ mice, with levels being the same whether treated with vehicle or lesioned with 9 mg/kg of MPTP, suggests that ERβ attenuate ERα activity (Figure 10 and Table 3 and 4). Further, compared to the MPTP-lesioned striatum, DA turnover significantly increased after 7 and 30 days post-lesioning in the cortex (Vuckovic et al., 2008).

As mentioned before, SNc DA neurons synapse on the spine shafts of striatal medium spiny neurons. While, corticostriatal axons, terminating on striatal neurons, synapse on the spines of striatal medium spiny neurons. Hence, the location of dopaminergic terminals provides the capability of modulating transmissions from the cortex to the striatum, if DA input could change

the sensitivity of striatal neurons to cortical inputs, therefore SNc activity affects the response of striatal neurons to cortical inputs.

Gingerich and colleagues demonstrated that ERα and GPER1 activity is neuroprotective against ischemic neuronal damage in the hippocampus (Gingerich et al., 2010) and it is shown that MPTP effects can reach the hippocampus (Zhu et al.). In mammals and humans only two brain regions undergo neurogenesis they include the dentate gyrus and the lateral walls of the lateral ventricles and a decrease in neuronal generation parallels the apparition of Alzheimer's pathology markers in the brains of transgenic rodents (Demars et al., 2010; Rodriguez et al., 2008; Wang et al., 2010). Hence, it could be suggested that neurodegenerative conditions such as Alzheimer's and PD could be alleviated through neurogenesis. Accordingly, perhaps this is why the results discussed in chapter four show that MPTP and 17β-estradiol treatments had limited effects in hippocampus.

6. 5 *The implication of PKB/Akt in 17β-estradiol induced neuroprotection*

The PI3-K cascade promotes the activation of PKB/Akt a kinase implicated in cell survival mechanisms (D'Astous et al., 2006). Akt positively modulates Bcl-2 levels (Pugazhenthi et al., 2000) and deactivates pro-apoptotic protein GSK3 by inducing its phosphorylation on serine 9 and 21 (Cohen and Frame, 2001) which favors the activation on anti-apoptotic neurotransmission (Hetman et al., 2000). Furthermore, Kahlert and colleagues have observed that ERα is involved in the activation of Akt signaling (Kahlert et al., 2000; Mendez et al., 2003), while ERβ is not (Mendez et al., 2005).

Our team documented that treatment with 17β-estradiol increased pAkt (serine 473) levels (D'Astous et al., 2006). Also seen was that GSKβ3 phosphorylation on serine 9 was highly

diminished in MPTP mice and the PPT treatment significantly blocked GSK activation but not the treatment with 17β-estradiol or the ERβ agonist Δ5-diol (D'Astous et al., 2006). It was found that only the PPT treatment had positive effects by increasing the levels of deactivated GSK3β in MPTP treated mice, but in intact wild-type mice it was the 17β-estradiol and the PPT treatments which increased the levels of activated Akt and deactivated GSK3β (D'Astous et al., 2006). Therefore, ERα and the PI3-K pathway are associated with the neuroprotective effects of estrogenic compounds against MPTP (D'Astous et al., 2006).

How ERs activate Akt and ERK1/2 pathways involves multiple interactions with signaling proteins. When localized in the plasma membrane in calveolae ERs can easily signal through PI3K/Akt and ERK1/2 pathways (Marino et al., 2006). Various scaffold-proteins and signaling molecules are known to promote kinase regulation via 17β-estradiol on membrane ERs these include calveolin proteins, Src, the 85α regulatory subunit of PI3K, receptor tyrosine kinases and G proteins (Marino et al., 2006).

For example, the interaction between ERα and the 85α regulatory subunit of PI3K was enhanced by 17β-estradiol, but this was not observed for ERβ (Mendez et al., 2003). In addition, G protein activation is believed to be implicated in the rapid effects of 17β-estradiol (Mermelstein et al., 1996). Colocalization and immunoprecipitation reveal that ERs directly interact with metabotropic glutamate receptors (mGluR) (Dewing et al., 2007) and only ERα regulates MAPK-dependent CREB phosphorylation via mGluR5 signaling (Grove-Strawser et al., 2010).

6. 6. *ERKs and neuroprotection*

Diseased brains show abnormalities in their immunohistochemical staining patterns for phosphorylated ERKs (Perry et al., 1999), so there must be a better understanding of oxidative mechanisms and the members of the ERK signaling cascade since prolonged ERK activation could lead to cell death (Kulich and Chu, 2001). In fact, it was concluded that MPTP induced the phosphorylation of both ERK1 and ERK2. This was seen in MPTP mice (De Girolamo and Billett, 2006) and MPTP monkeys (Bezard et al., 2005).

Cultured primary ERK1 mutant cells demonstrate ERK2 hyperactivation after being DA and glutamine challenged. Formulated was the opinion claiming that ERK1's *in vivo* functional purpose is to down regulate ERK2's actions (Brambilla, 2003). It is suggested that this is achieved by ERK1 competing with an upstream kinase MEK in order to attenuate the ERK2 pathway (Brambilla, 2003).

Again, our group demonstrated that inhibiting GSK3β is a mechanism by which the PI3K signaling cascade promotes cell survival (D'Astous et al., 2006). In fact, it was observed that inhibiting ERK1 and ERK2 promotes the activation of GSK3β (Hetman et al., 2002).

In the third and forth chapters, the mechanisms of estrogen-induced neuroprotection via ERα and ERβ against neurotoxic insult and glutamate excitotoxicity were investigated, while in chapter five, we examined a 17β-estradiol neuroprotection paradigm comparing D1 and D2 DA receptor activity and the lesioned striatum (Table 4). It is suggested that both glutamatergic and dopaminergic receptor activation induces Ras and ERK2 activity and that ERK1 could negatively modulate ERK2 in the striatum (Brambilla, 2003) and it was previously seen that ERK1 and ERK2 are active only in the DA depleted striatum and this seems to depend on DA and cAMP-regulated phosphoprotein, 32 kDa (DARP 32) (Gerfen et al., 2002; Valjent et al., 2005). By

contrast, D1 DA receptor or DARP 32 KO mice demonstrate psychostimulant activation of ERK1 and ERK2 in the dorsal striatum (Berke et al., 1998). Further, D1 DA receptor KO mice, treated with L-DOPA, do not demonstrate induced activation of ERK1 and ERK2 in the DA depleted striatum (Gerfen et al., 2008). Accordingly, estrogen-induced activation of MAP kinase signalization leads to increased phosphorylation of ERK1 and it was seen that inhibition of this pathway blocks of estrogen-induced neuroprotection against glutamate excitotoxicity (Singer et al., 1999).

In chapter five, we saw that ERs affect the response of striatal DA receptors to a MPTP lesion and post receptor signaling. Here, intact ERKOβ mice had low D1 DA receptor levels, while both ERKO genotypes had lower D2 DA receptor levels in the striatum. Striatal D1 DA receptors decreased with increasing doses of MPTP and correlated with striatal DA concentrations in ERKOα mice and remained unchanged in WT and ERKOβ mice. Striatal D2 DA receptors decreased with increasing doses of MPTP and correlated with striatal DA concentrations in WT and ERKOα mice and increased in ERKOβ mice (Table 3).

Hence, because D1 DA receptor levels of WT mice were not affected by 17β-estradiol, MPTP or 17β-estradiol+MPTP treatments, while under MPTP lesioning 17β-estradiol treatment increased D1 receptors in both ERKOα and ERKOβ mice and D2 receptors in WT and ERKOβ mice. Therefore both ERs modulate striatal D1 DA receptors, while ERα modulates D2 DA receptors (Figure 10 and Table 3 and 4).

Also, MPTP did not affect striatal pAkt/Akt and pGSK3β/GSK3β levels in WT and ERKOα mice while in vehicle-treated ERKOβ mice these levels were higher and increased with MPTP lesioning. The measured striatal pERK1/ERK1 and pERK2/ERK2 levels showed a moderately similar pattern. Increased levels of phosphorylated Akt and GSK3β were highly

significant in ERKOβ mice, therefore with only ERα being active without the possibility of being attenuated by ERβ (Jin et al., 2008), survival mechanisms implicating Akt and ERK signaling are overactive (Figures 3 and 4 of chapter 5).

In vivo studies were also successful, for example rats receiving an intracerebroventrical administration of estrogen had increased ERK activation in the CA1 region and the dentate gyrus of the hippocampus only five minutes after the treatment (Kuroki et al., 2000). Rats receiving a subcutaneous treatment of estrogen also had enhanced ERK phosphorylation in various brain structures within 20 minutes of the administration (Bryant et al., 2005). Accordingly, many have demonstrated, under *in vitro* conditions, that estrogen-induced rapid initiation of ERK signaling results in neuroprotective effects and treatment with a MEK inhibitor disrupts this (Kuroki et al., 2000; Numakawa et al., 2007). Similarly, *in vivo* researching done by Jover-Mengual and colleagues also demonstrated that the actions of estrogens on ERKs are neuroprotective in CA1 hippocampal region subjected to global cerebral ischemia, and these beneficial effects were erased by MEK inhibition (Jover-Mengual et al., 2007). Furthermore, Singh et al. saw that ERKOα mice are capable of rapid estrogen-induced ERK activity (Singh et al., 2000). While, Abraham and colleages claim the contrary since both ERKOα and ERKOβ lost this fast neuroprotective ability and they theorized that both ERs regulate these rapid actions of ERK modulatory signaling (Abraham et al., 2004). Even though our research on neurorprtection targets the genomic effects of estrogens, we observed similar results. Recall in chapter 2, where 17β-estradiol did not protect against MPTP neurotoxicity in both ERKOα and ERKOβ mice, also suggests that both ERs are involved in neuroprotection (Morissette et al., 2007). In addition, since ERα and ERβ have distinct distribution patterns in the male and female nigrostriatal system (Merchenthaler et al., 2004; Mitra et al., 2003; Shughrue, 2004), these studies concluded that

estrogens neuroprotect by inducing nuclear receptor-mediated mechanisms which could be distinct in males and females.

6. 7. *GPER1, ERα and ERβ*

Since 17β-estradiol works on ERs and GPER1, it is feasible that genomice and non-genomic neuroprotective mechanisms could converge (Figure 10).

Not many have examined the effects of MPTP toxicity on ERs levels in the brain. Neither MPTP lesioning nor 17β-estradiol treatment was reported to increase nigrostriatal ERα or ERβ levels (Shughrue, 2004). On the other hand, recently reported increased striatal GPER1 levels of MPTP-lesioned male mice and hemiparkinsonian monkeys (Al Sweidi et al., 2012; Bourque et al., 2011). Liu and colleagues conclude that GPER1 activates ERK signaling which attenuates NMDA excitotoxicity through the depression of NR2B-containing NMDA receptors (Liu et al., 2012).

Again, non-genomic effects could be relayed to the nucleus and promote genomic regulation (Scott et al., 2012), but controversies still exist concerning the topic of ER distributed in the CNS. Shurghrue et al. did not detect ER mRNA in the dorsal striatum of female rats using *in situ* hybridization (Shughrue et al., 1997b), real-time PCR revealed ERα and ERβ mRNA in the dorsal striatum (Kuppers and Beyer, 1999) and light microscopy provided low nuclear immunolabeling of these receptors in female mice (Mitra et al., 2003). ERα IR by electron microscopy showed ERα, ERβ and GPER1 to be present in the dorsal striatal neurons and glia at extranuclear sites of female rats (Almey et al., 2012). Colocalization with TH or vesicular acetylcholine transporter in the striatum yielded that both ERα and GPER1 are expressed in cholinergic interneurons and not on dopaminergic terminals of the striatum (Almey et al., 2012).

Brake and colleagues conclude that because acetylcholine regulates striatal DA transmission, therefore 17β-estradiol may indirectly modulate DA transmission via cholinergic transmission (Almey et al., 2012). ERα is found on axons, axon terminals and glia, while ERβ was seen in axons and glial cells (Almey et al., 2012). Hence, it is suggested that since ERβ has very low IR on axon terminals, it therefore is not involved in the modulation of synaptic transmission. This could be an explanation as to why ERα activity is dominant in 17β-estradiol-induced neuroprotection. On the other hand, GPER1 is located on dedrites and glial cells, where it could modulate neurotransmission through post-synaptic signaling (Almey et al., 2012).

Furthermore, all three receptors are found on the plasma membrane of glial cells. In fact, 17β-estradiol was shown to regulate glial cell-induced neuroprotection via GPER1 (Liu et al., 2011). However, these effects were not significant against MPTP lesioning in ERKO male mice (Al Sweidi et al., 2012; Al-Sweidi et al., 2011; Morissette et al., 2007).

Also, ERα and GPER1 were colocalized in the SN (Brailoiu et al., 2007; Kuppers et al., 2000), therefore estrogens could directly target SN dopaminergic neurons and modulate dorsal striatum DA release and reuptake (Kuppers et al., 2000). Perhaps this is why ERKOβ mice are not as susceptible as ERKOα mice are to MPTP (Al Sweidi et al., 2012; Al-Sweidi et al., 2011; Morissette et al., 2007).

Further, Bourque et al. demonstrated that treatment with the GPER1 agonist reproduces similar neuroprotective effects as seen for 17β-estradiol treatment of MPTP-lesioned male mice (Bourque et al., 2012). These effects include increased striatal DA metabolite levels and DA turnover, protected against the loss of nigrostriatal DA, DAT and VMAT2 (Bourque et al., 2012). Treatment with the GPER1 antagonist G15 completely inhibits G1 activity, while the effects 17β-estradiol were partially blocked by G15 (Bourque et al., 2012). Also, G15 treated mice were

highly susceptible to MPTP toxicity, showing a high reduction of striatal DA levels and DAT specific binding as were MPTP-lesioned ERKOα mice (Al Sweidi et al., 2012; Al-Sweidi et al., 2011; Bourque et al., 2012; Morissette et al., 2007). They saw that G15 completely blocks 17β-estradiol-induced striatal neuroprotection, but these effects were partial in the SN (Bourque et al., 2012). They suggest that striatal neuroprotection mainly involves GPER1, however in chapters 2 and 3 17β-estradiol-induced striatal neuroprotection was not observed in both ERKOα and ERKOβ mice against MPTP toxicity. Hence, striatal neuroprotection requires ERα, ERβ and GPER1 (Figure 10).

6.8. *Conclusion*

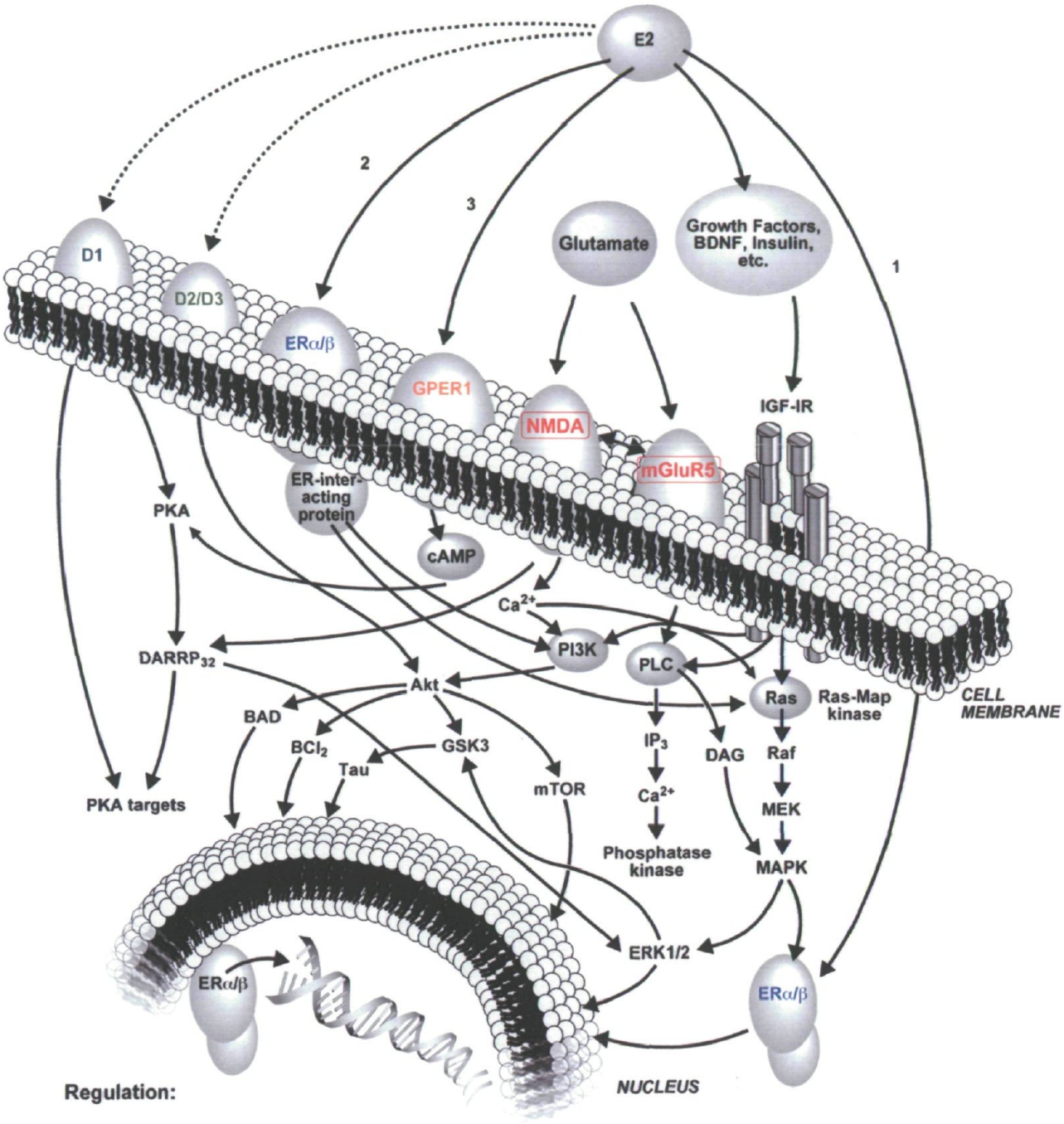
We suggested that these neuroprotective effects are mediated by both ERα and ERβ and since ERs are able of heterodimerization (Pettersson et al., 1997), it is suggested that a spatio-temporal as well as a functional difference in the roles of ERs may exist and would explain the discrepancies seen once challenged with MPTP, some claim that ERβ is involved in neurogenerative events taking place after cellular death. Hence, neuroprotection induced by the two ERs complement one-another. Thus, the actions of ERα and ERβ are strongly inter-related during 17β-estradiol-induced neuroprotection and their roles within different neuronal circuits maybe synergic or antagonistic.

6.9. *Perspectives*

A better understanding of the mechanisms induced by central and systemic estrogens as well as the implicated neuromodulative and neuroprotective roles of ERα and ERβ should be pursued. Differentiation between adaptive or compensatory responses of surviving striatal neurons and actually influencing the survival of the nigrostriatal pathway remains a goal.

The different neuronal populations communicating via ER induced signaling pathways should be determined. In addition, a list of pro or anti-apoptotic proteins, such as DARP 32, Bcl-2, and Bad that could be implicated in these mechanisms are still to be targeted. Next, studying ERs and their signal transductions at a more delayed time frame, that is more than five days after MPTP, would allow DA receptor adaptations to occur and compare the modulative roles of ER subtypes. Further, the use of ER subtype-selective agonists or targeting receptors, like GPER1, with non-feminizing responses has potential. Another option is the modulation of rate-limiting neurosteroidogenesis enzymes like aromatase or 5α-reductase.

Finally, since ERα and ERβ are interdependent the neuroprotective contribution of membrane GPER1 activity in these ERKO mice is also important to examine.



Regulation:

Gene transpription
Neuronal survival
Axonal / dendritic growth
Synaptic potentiation / plasticity

Figure 10. Summary of the effects implicating neuromodulative and neuroprotective estrogenic activity on DA cell survival and plasticity. Pathway 1 demonstrates the "classical" effects of ERs having roles as nuclear transcription factors. First, 17β-estradiol binds ERs, which in turn bind EREs on DNA with the help of coregulator proteins and modulate the transcription of target genes. Pathway 2 requires cytoplasmic or membrane-localized ERs, which initiate crosstalk using membrane-associated receptors like mGlu receptor and cytoplasmic signal transduction pathways. Pathway 3 is induced as17β-estradiol binds to a membrane-associated receptor GPR1/GPER30 and signaling is brought-on by different cytoplasmic kinases. Pathway 1 is a simplified representation of the 17β-estradiol-induced "genomic" response, while pathways 2 and 3 are rapid estrogenic responses which could have both cytoplasmic and nuclear targets (Nilsson and Gustafsson, 2011). Dotted lines represent indirect effects. Adapted from Bourque et al. 2009 (Bourque et al., 2009).

Table 3. Summary of genotype differences in mouse striatal DA receptors, transporters and NMDA receptors.

Striatum	ERKOα versus WT mice	ERKOβ versus WT mice	ERKOα versus ERKOβ mice
DAT	0	–	–
VMAT2	0	–	–
D1 receptor	0	–	–
D2 receptor	–	–	0
NMDA receptor	–	–	–

Table 4. Summary of differences in striatal DA receptors, transporters and NMDA receptor specific binding response to MPTP and 17β-oestradiol treatments in WT, ERKOα and ERKOβ mice.

Striatum	WT mice			ERKOα mice			ERKOβ mice		
	MPTP	17β-E_2	MPTP +17β-E_2	MPTP	17β-E_2	MPTP +17β-E_2	MPTP	17β-E_2	MPTP +17β-E_2
DAT	–	0	+	–	+	–	–	0	–
VMAT2	–	0	+	–	0	–	–	0	–
D1 receptor	0	0	0	–	–	+	0	0	+
D2 receptor	–	0	+	0	0	0	0	0	+
NMDA receptor	–	0	0, ↑	0	0	0	0	+	0

0, –, +: No difference, lower and higher receptor or transporter specific binding levels.
↑: Increased receptor levels versus respective MPTP treated mice.

CHAPTER VII

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Annexe

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REVIEW ARTICLE

Oestrogen Receptors and Signalling Pathways: Implications for Neuroprotective Effects of Sex Steroids in Parkinson's Disease

S. Al Sweidi*†, M. G. Sánchez*†, M. Bourque*†, M. Morissette*, D. Dluzen‡ and T. Di Paolo*†

*Molecular Endocrinology and Genomic Research Center, CHUQ (CHUL), Quebec City, Canada.

†Faculty of Pharmacy, Laval University, Quebec City, Canada.

‡Department of Anatomy and Neurobiology, Northeastern Ohio Universities College of Medicine and Pharmacy (NEOUCOM), Rootstown, OH, USA.

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Parkinson's disease (PD) is an age-related neurodegenerative disorder with a higher incidence in the male population. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, 17β-oestradiol but not androgens were shown to protect dopamine (DA) neurones. We report that oestrogen receptors (ER)α and β distinctly contribute to neuroprotection against MPTP toxicity, as revealed by examining the membrane DA transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) and tyrosine hyroxylase in ER wild-type (WT) and knockout (ERKO) C57Bl/6 male mice. Intact ERKOβ mice had lower levels of striatal DAT and VMAT2, whereas ERKOα mice were the most sensitive to MPTP toxicity compared to WT and ERKOβ mice and had the highest levels of plasma androgens. In both ERKO mice groups, treatment with 17β-oestradiol did not provide neuroprotection against MPTP, despite elevated plasma 17β-oestradiol levels. Next, the recently described membrane G protein-coupled oestrogen receptor (GPER1) was examined in female *Macaca fascicularis* monkeys and mice. GPER1 levels were increased in the caudate nucleus and the putamen of MPTP-monkeys and in the male mouse striatum lesioned with methamphetamine or MPTP. Moreover, neuroprotective mechanisms in response to oestrogens transmit via Akt/glycogen synthase kinase-3 (GSK3) signalling. The intact and lesioned striata of 17β-oestradiol treated monkeys, similar to that of mice, had increased levels of pAkt (Ser 473)/βIII-tubulin, pGSK3 (Ser 9)/βIII-tubulin and Akt/βIII-tubulin. Hence, ERα, ERβ and GPER1 activation by oestrogens is imperative in the modulation of ER signalling and serves as a basis for evaluating nigrostriatal neuroprotection.

Key words: MPTP, 17β-oestradiol, dopamine transporter, Akt/GSK3β, GPER1.

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Correspondence to:
Dr Thérèse Di Paolo, Molecular Endocrinology and Genomic Research Center, CHUQ-CHUL, 2705 Laurier Boulevard, Quebec City, QC, Canada G1V 4G2 (e-mail: therese.dipaolo@crchul.ulaval.ca).

Introduction

Numerous studies (1–7), a meta-analysis (8) and reviews (9, 10) indicate that Parkinson's disease (PD) is more prevalent and has a larger incidence in the male population. A meta-analysis of seven studies that used a stringent inclusion criteria reported that overall PD is 1.5-fold greater in men than in women (8). The possible reasons proposed for this increased risk of PD in men than in women include toxicant exposure, head trauma, neuroprotection by oestrogens, mitochondrial dysfunction and/or X linkage genetic risk factors (8).

Gender differences were reported during the progression of PD and in the responses to L-dopa treatment (11–13). In the case of PD, oestrogens have been known to protect the nigrostriatal dopaminergic pathway (14, 15). However, the beneficial effects of oestrogens remain far from simple; complexity is underlined by their various neuroprotective actions and numerous mechanisms involved. Hence, research continues concerning these mechanisms and the development of new or improved neuroprotective compounds. We propose that oestrogenic neuroprotection arises through oestrogen receptors (ERs), is influenced by their subtype and we review their implication in neuroprotection as derived from

results from our laboratory using lesioned primates and mice models of PD, as well as the current literature.

PD

Characteristics, cause and symptoms of PD

PD is a neurodegenerative disorder that has become more prevalent over the years as a result of an ageing population (16). This disease has no documented aetiology; however, it is characterised as chronic and progressive, with the loss of dopamine (DA)-containing neurones in the brain substantia nigra (SN) being a main feature in its neurodegenerative process (16). The disorder is also referred to as 'idiopathic' PD because no known cause is documented, yet some cases may be a result of toxicity, drugs, genetic mutation, head trauma or other medical disorders (16). PD is also characterised as 'sporadic' with no known genetic background in 90–95% of cases, and environmental factors or genetic susceptibilities are considered to have a hand in triggering the disease (16).

What makes PD so infamous is the fact that symptoms appear after a myriad of neurones are lost, with a death toll of dopaminergic neurones exceeding a critical threshold of between 70% and 80% in the SN (16). It is hard to diagnose before the damage becomes irreversible as a result of compensatory mechanisms (16). Disruption in DA transporters that are responsible for controlling DA concentrations, and include the DA transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2), are considered to play a role in this pathology (17).

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD

MPTP provides a model of PD as a result of its specific neurotoxicity in certain species such as mice and monkeys (18, 19). It was discovered as a by-product in the chemical synthesis of a meperidine analog with potent heroin-like effects (19). The irreversible effects of MPTP in man induce tremor, slowness of movement, rigidity, freezing and postural instability, thus mimicking almost all features of PD (19). MPTP enters the brain through the presynaptic DA uptake system where it is converted into the 1-methyl-4-phenylpyridinium (MPP^+) ion by monoamine oxidase B (19). MPP^+ is a very potent neurotoxin that inhibits the mitochondrial oxidation of NAD^+-linked substrates. It accumulates inside mitochondrial matrixes and inhibits respiration (19), resulting in the death of nigrostriatal nerves through mechanisms that include the loss of calcium homeostasis and the formation of radicals that promote cytotoxic events leading to limited DA release, locomotor defects and finally apoptosis (19).

Oestrogens and PD

Several studies indicate that PD has a later age at onset in women and that men are more susceptible (15). Clinical studies demonstrate that there is an increased risk of developing PD under conditions of an early decrease in natural endogenous oestrogens (20, 21).

A 2-week double-blind cross-over study, on postmenopausal women suffering from mild to moderate PD showed that transdermal treatment with a high dose of 17β-oestradiol reduces the antiparkinsonian threshold dose of levodopa (22). Oestrogens were observed to have modulatory effects on PD symptoms and levodopa-induced dyskinesias (23–25) and motor disabilities were remedied by oestrogens in postmenopausal women with PD (26). On the basis of evidence from a case-control design study of 133 female PD cases and 128 female controls, it was concluded that women who took a postmenopausal oestrogen treatment had a lower risk of developing PD (27). Men with PD show symptoms requiring medical attention during earlier stages of the disorder (21), suggesting that the disease progresses more rapidly in men, thus supporting the idea that oestrogen can provide neuroprotective effects (21). Furthermore, gender differences in symptoms were also seen in outcome studies after stereotactic surgery for PD (10).

ERs

Classic ERs

Currently, there are two main documented ER subtypes (28). The transcriptional information for these two receptors lies on two different genes (28). Twenty splice variants have been documented for ERα and ten for ERβ (28). There is little or no conservation in their N-terminal region, although they have a homology of 55% in their ligand-binding domains and are 95% homologous in their DNA-binding domains (28). Oestrogens may differ in their affinity for either ER subtype. For example, 17β-oestradiol binds equally with both ERα and ERβ, whereas oestrone and the selective oestrogen receptor modulator, raloxifene, preferentially bind ERα and oestriol has more affinity for ERβ (29). Each ER has its own nonsteroidal agonist (30, 31); 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) is selective for ERα and 2,3-bis(4-hyroxyphenyl) propionitrile (DPN) is selective for ERβ.

ERα is present in high levels within the cardiovascular system, bone, mammary glands and the uterus, whereas ERβ is found mainly in the urinary tract, prostate and ovaries (32, 33). At the cellular level, the nucleus is mainly home to ERα, whereas ERβ is reported to be localised in the cytoplasm (34). In the central nervous system (CNS), these two receptors have similar patterns of expression in the preoptic area, the cortical amygdaloid nuclei and the bed nucleus of the stria terminalis. In rodents, ERα expression is found exclusively in the ventromedial hypothalamic nucleus and the subfornical organ (35) and it is predominant in mouse hippocampus (36), whereas ERβ is predominant in rat hippocampus and the cerebral cortex (35). Immunohistochemical and *in situ* hybridisation data show that ERα and ERβ are present in rat SN (37). In addition, Kritzer *et al.* (38) showed the co-localisation of ERs and tyrosine hyroxylase (TH) in the ventral tegmental area and SN of the rat brain and subsequently specified that ERβ is present on collateral SN pars compacta projections to the ventral striatum (39). Küppers *et al.* (40) claim that both ERα and ERβ mRNAs are expressed in GABAergic neurones of the striatum.

Nuclear ER activity

ERs regulate gene transcription by exerting positive or negative effects on the expression of target genes. ERα and ERβ are ligand-activated receptors that work with the help of two DNA consensus elements (41). These receptors regulate gene transcription and so they are part of a vast family of proteins called ligand-activated transcription factors. Their actions have been documented to include either genomic or nongenomic mechanisms and the fact that both ERs are found in dopaminergic neurones of the midbrain, although in low abundance, implies that the adult nigrostriatal system is targeted by oestrogens (40, 42). The genomic mechanism could take two different routes: either direct or indirect. When not bound to a ligand, ERs are found as monomers that associate with heat shock protein (Hsp-90) and immunophilins forming a multiprotein complex (43). Once oestrogen binds a complementary receptor, the phosphorylation of its many different serine/threonine residues is induced, which causes them to lose the Hsp-90 and change their conformation to promote their homo- or heterodimerisation and translocation into the nucleus. A hydrophobic clef is also revealed to bind transcriptional coactivators that help by initiating a chromatin structural change in target promoters (44, 45). Once inside, ERs interact with oestrogen response elements (EREs) on the regulatory sequences of target genes to either suppress or activate their transcription at the same time as being limited to both promoter and cell specificities (46). However, they can also function without EREs with the help of ER-tethering and coactivation of transcription factors bound to the target DNA, such as the transcriptional factor cAMP-response element binding protein. They can also interact with *fos*/jun transcription factors were they can regulate transcription through activator protein-1 (44, 47–49).

The indirect route is initiated by disinhibiting mitogen-activated protein kinases (MAPK) (50, 51) and protein kinase B (PKB/Akt) signalling cascades (52), thus leading to the activation of cAMP-response element binding protein (53, 54). Ultimately, the modulation of proteins such as Bcl-2 and Bad that regulate apoptosis is induced.

Membrane ERs

Genomic mechanisms require hours to run their course because they involve the transcription and translation of oestrogen-regulated genes (55). However, not all oestrogenic effects are attributable to nuclear ERs; some effects occur within a matter of seconds or minutes, making it obvious that a route other than one implicating classic intranuclear receptor transcription modulation has transpired. Membrane-associated ERs could provide the basis for these observations. This theory gained support over the past 30 years ever since Pietras and Szego (56) first observed receptors as having rapid responses to 17β-oestradiol and continues today with evidence of their existence as a result of the fast nongenomic effects of 17β-oestradiol. Although ERα and ERβ are both able to act as plasma membrane receptors (57), there is also evidence for new plasma ERs categorised as G protein-coupled receptors (GPCRs), which have no relation to the known ERs (58). How ERs are brought to the plasma membrane is not well established, although research on non-neuronal cells supports the theory that ER palmitoylation could be a mechanism (59, 60). Here, a post-translational addition of a 16-carbon fatty acid (palmitate) to ER ligand-binding domain residues enables targeting of ERs to the plasma membrane (59, 61).

The cloning of a G protein-coupled ER named GPER1 (also known as GPR30) was reported over 15 years ago in several studies (62–67). GPER1 is distinct from the classic ERα and ERβ and has been shown by electron microscopy to be located on the plasma membrane (68). Its expression in the rat CNS, as determined through immunohistological studies, is high in brain regions, including the cortex, striatum, hippocampus, SN, islands of Calleja, hypothalamic-pituitary axis and the brainstem autonomic nuclei (69), whereas its cellular localisation also includes the endoplasmic reticulum and the Golgi apparatus (70, 71). GPER1 has a high affinity for 17β-oestradiol but 17α-oestradiol failed to significantly displace [^{3}H]oestradiol binding to GPER1 (70, 71). The competitive binding assay shows that GPER1 does not bind cortisol, progesterone or testosterone, whereas oestrone, oestriol had very low affinities for the receptor (72); tamoxifen, genistein and ICI 182,780 are agonists on GPER1 (73). Hammond *et al.* (74) observed that GPER1 is expressed (0.4–42%) in GABAergic neurones and that it is colocalised in 63–99% of cholinergic neurones in the basal forebrain. However, Mufson *et al.* (75) and Shughrue *et al.* (76) observed that approximately 30% of the cholinergic neurones also contain ERα. In mice, another membrane ER, named ER-X, was found to be expressed in the neocortex, in lung plasma membrane microdomains associated with caveolin, and also in the uterus (58, 77, 78).

Oestrogens, ERs and neuroprotection

It is well established that ageing results in a decline in the production of the female hormone oestrogen, with the most drastic decrease at menopause (79). In addition, it has been observed that, with ageing, negative effects on ERα-mediated events are assumed to attenuate ER functioning by increased methylation of the ER gene (80, 81). In age-related animal models of neurodegenerative diseases, numerous studies show that oestrogens play an important protective role and there is accumulating evidence implicating ERs.

It is expected that nuclear ER concentrations in man diminish with ageing, as reported in rats (82). However, in humans, postmortem tests show complex age-related alterations of the canonical ERα and various ERα splice variants in the brain and the expression pattern of certain forms is brain area-specific (83). In the vasopresssinergic supraoptic nucleus and the hippocampus, ERα was reported to increase with advancing age in women with higher expression in postmenopausal than in pre- and perimenopausal women (84). ERα splice variant del.7 (deletion of exon 7) and del.2 (deletion of exon 2) declined with advancing age (61–84 years old) in the mamillary body but not in the hippocampus (83), whereas no change was observed in another study of 12 exon-skipping variants, with the most common form found being del.5, del.7 and del.2 in people aged 29–59 years (85). The del.7 is a dominant negative variant that can inhibit transcriptional activity of both ERα and ERβ

by forming heterodimers (86). del.4 was observed in the caudate nucleus, putamen and SN of a 71-year-old female Alzheimer's disease patient but the effect of ageing was not reported (83). del.4 is suggested to be a silent variant without activity of its own, although a dominant negative function was ascribed to this slice form through protein-protein interactions with ERα (87). Hence, higher levels of ERs with hindered activity are seen in some brain regions as a function of ageing (88).

The effects of MPTP on dopaminergic markers

Vesicular monoamine transporter 2 packages serotonin, histamine, epinephrine, norepinephrine and DA into vesicles and is mainly confined to the CNS (89). The DAT is a specific protein of DA neurones (90). DAT and VMAT2-specific binding under pathological conditions is used as a marker to evaluate DA cell body and terminal integrity. Neurotoxicity of SN DAT-specific binding and mRNA are less severe than that seen in the striatum of MPTP and methamphetamine lesioned mice (14, 91), suggesting that the presynaptic DA terminals of the striatum are more vulnerable. Accordingly, high doses of neurotoxins are needed to affect SN TH mRNA levels (92). Indeed, cell body destruction requires elevated doses of toxins, whereas cell terminals are lost with lower doses of the same neurotoxins (92). Hence, different stages exist where neurones are injured but not dying or dead when neuroprotection is feasible by steroids and, in humans, oestrogens are only beneficial before starting levodopa therapy in the early stages of PD (93).

Nigrostriatal DA activity regulation depends on DA availability, which in turn is controlled by DAT and VMAT2 present in neurones. A role for DAT in astrocytes is also reported because Karakaya *et al.* (94) observed that DAT is expressed in neonatal astrocytes and that 17β-oestradiol dose-dependently down-regulated DAT mRNA by 80% and 60% in the neonatal midbrain and striatal astroglia cultures, respectively. It was also noted that 17β-oestradiol inhibits the clearance of extracellular DA by 45% and 35% in the neonatal midbrain and striatal astroglia cultures; this effect was abolished with the use of an ER antagonist ICI 182, 780 (94). It was concluded that the effects of 17β-oestradiol on DAT could be neuroprotective under pathological conditions because the end result is delayed DA uptake by astroglia (94). Thus, the 17β-oestradiol-induced decrease of astroglial DA uptake may diminish DA metabolisation, resulting in an increased availability of synaptic DA and, subsequently, more DA for recycling by neurones. VMAT2 was not detected in astrocytes (94).

Oestradiol neuromodulation and neuroprotection

Oestrogens modulate the nigrostriatal and mesolimbic DA systems' activity at various components of neurotransmission (95–97). We have shown, by biochemical and pharmacological studies, that 17β-oestradiol can modulate DA receptors (98) and DAT (99–101). Striatal and nucleus accumbens DA D_2 receptor and DAT density are increased with chronic 17β-oestradiol treatment without affecting their mRNA levels, implying that 17β-oestradiol activity was nongenomic (102, 103).

Oestrogens produce their modulatory effects through pro- or anti-dopaminergic activity, such as on enzymes that synthesise or degrade DA, DAT, VMAT2, DA receptors and DA release (15). In the striatum of ovariectomised rats, DAT density fluctuations are observed during the oestrous cycle (97) and 17β-oestradiol treatment increased DAT density through acute and chronic treatment (101, 104), whereas reductions in SN DAT mRNA levels in ovariectomised rats were restored with oestrogens (99).

Postmenopausal women given an oestrogen replacement therapy had increased DAT density in the left anterior putamen (105). There is less data available on the gonadal hormone modulation of VMAT2; in the rat brain, 17β-oestradiol treatment had no effect on its striatal density (106) and, in another study in the SN *pars compacta*, chronic 17β-oestradiol treatment did not affect VMAT2 mRNA (107).

Oestrogens provide relief from PD symptoms if treatment is given at early stages of the disease (93). Hence, to model this stage of PD, we used conditions of moderate nigrostriatal DA loss in MPTP mice when motor behaviour is not yet impaired, nor is there significant DA cell death. Thus, under conditions of early nigrostriatal DA neuronal degeneration, MPTP mice show a significant reduction of striatal DA concentrations and DA transporter loss. The neuroprotective effects of 17β-oestradiol, when administered before the MPTP regimen, are observed upon the prevention of DA and dopaminergic metabolite depletions (108–111), as well as DAT and VMAT2-specific binding loss (112). In addition, 17β-oestradiol treatment promotes an increase in TH immunoreactive neurones of the SN *pars compacta* of male mice (113). The neuroprotective effects of 17β-oestradiol upon MPTP are achieved with pretreatment at low doses mimicking physiological levels in male and female mice, although treatment with high doses does not prevent MPTP neurotoxicity (114). Oestrogens are considered to convey their neuroprotective effects through genomic mechanisms that signal through ERs or by using nongenomic mechanisms through membrane bound receptors (115–117). Again, we propose that these neuroprotective effects are mediated via an interaction with ERs. This is supported by the observation that 17α-oestradiol, which has a low ER affinity, does not induce neuroprotective effects (91). Moreover, the weak ER agonists, oestrone and oestriol (91), have weak or no neuroprotective potentials against dopaminergic loss caused by MPTP (118).

ERα and ERβ could have distinct roles in neuroprotection against MPTP toxicity (119), with ERα being the dominant receptor involved in neuroprotection (120, 121). Indeed, it is speculated that ERβ plays a less dominant role in neuroprotection because its activity is optimal once cellular death is inhibited and regeneration and neurogenesis commence (122). Dubal *et al.* (120) show that, in a cerebral ischaemia model, 17β-oestradiol treatment does not protect the cortex or striatum in ER knockout (ERKO)α mice compared to wild-type (WT) and ERKOβ mice. Our group has demonstrated that PPT but not DPN provides neuroprotection against MPTP (123, 124). Similarly, PPT but not DPN protects against β-amyloid peptide in cerebrocortical neuronal cultures via a protein kinase C-dependent signalling pathway (125). Other studies claim that ERβ plays a role in neuroprotection. For example, Carswell *et al.* (126) provide data

suggesting that DPN but not PPT pretreatment reduces ischaemic damage in the striatum and CA1 region of the hippocampus and Westberg *et al.* (127) report that ERβ gene polymorphisms could influence the age of onset of PD. We reported that treatment with DPN or 17β-oestradiol but not PPT modulates D_2 DA receptors in ovariectomised rats (102), whereas data obtained from a study with ERKOα and ERKOβ male mice suggest that ERβ affects DA metabolism because ERKOβ mice had a lower DA turnover rate (119). 17β-Oestradiol was able to prevent the loss of 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid binding to DAT and [^{3}H]-dihydrotetrabenazine binding to VMAT2 in the striatum and SN of MPTP and methamphetamine lesioned mice (114). Moreover, the SN decrease of these transporters' mRNA produced by MPTP was prevented by oestrogen (91). We have also examined the neuroprotective contributions of ERα and ERβ against MPTP toxicity by examining DAT, VMAT2 and TH in ERKO C57Bl/6 male mice.

Our results show striatal DAT and VMAT2 levels of intact ERKOβ mice to be lower than WT and ERKOα mice, whereas ERKOα had elevated plasma androgen concentrations (two-way ANOVA shows an effect of genotype, $P < 0.0001$; mean $\pm$ SEM, testosterone: WT = 3.5 $\pm$ 0.9 ng/ml, ERKOα = 12.5 $\pm$ 1.0 ng/ml, $P < 0.00001$, versus WT and ERKOβ = 6.1 $\pm$ 1.2 ng/ml; dihydrotestosterone: WT = 114 $\pm$ 32 pg/ml, ERKOα = 997 $\pm$ 117 pg/ml, $P < 0.0001$, versus WT and ERKOβ = 149 $\pm$ 59 pg/ml) (128). This is in agreement with a previous report showing a significant increase of testosterone in ERKOα male mice compared to WT males (129). Despite being infertile, ERKOα mice have a close to normal hormonal profile and activity of the hypothalamic/pituitary axis (130), whereas ERKOβ mice have been found to be in a state of systemic hypoxia (131). Functional alterations of the SN DA system, as well as reduced TH and brain-derived neurotrophic factor levels, are observed in both male and female ERKOα mice (132). ERKO could affect the maturation of other components of brain DA transmission, such as the DAT and VMAT2, although this possible effect has not been verified.

In WT and ERKO mice, MPTP caused a dose-dependent loss of both striatal transporters (Fig. 1) that correlated with their previously reported reductions in striatal DA concentrations (119) (DAT: $R = 0.755$, $P < 0.0001$; VMAT2: $R = 0.787$, $P < 0.0001$). Compared to WT and ERKOβ, DAT, VMAT2 and TH showed a greater sensitivity to MPTP in ERKOα mice (Fig. 1, and data not shown). WT mice were compared with ERKO mice pretreated with 17β-oestradiol alone and/or with an effective dose of MPTP. The striatum and SN of ERKOα mice were more vulnerable to MPTP toxicity and 17β-oestradiol protected against this toxicity only in WT mice (Fig. 2, and data not shown) despite similar plasma 17β-oestradiol concentrations among the three genotypes (two-way ANOVA shows an effect of 17β-oestradiol treatment, $P < 0.0001$; mean $\pm$ SEM in pg/ml, WT: vehicle: 2.2 $\pm$ 1.5 and 17β-oestradiol treated: 10.0 $\pm$ 1.4; ERKOα: vehicle: 4.3 $\pm$ 1.4 and 17β-oestradiol treated: 11.2 $\pm$ 2.0; ERKOβ: vehicle: 3.3 $\pm$ 0.8 and 17β-oestradiol treated: 10.2 $\pm$ 1.2). Hence, even though the lack of the ERα caused a more significant susceptibility to MPTP toxicity, both ERα and ERβ were shown to be implicated in neuroprotection resulting from 17β-oestradiol.

The absence of ERα and ERβ throughout development in ERKO mice could affect brain organisation and may result in a different adult brain. However, our results on WT mice showing that PPT but not DPN protects striatal DA against MPTP toxicity (123, 124) are in agreement with our results demonstrating a greater susceptibility of the nigrostriatal DA system of ERKOα mice to MPTP (128). Hence, the ERKO mouse model is a valid tool for the study of the role of ERs in nigrostriatal DA neuroprotection and supports the results obtained using ER-specific agonists.

Neuroprotective implication of membrane ERs

Each ER has many splice variants (133) and research has shown that ERα and ERβ are able to act as plasma membrane receptors (133–135). There is also evidence of new plasma ERs categorised as

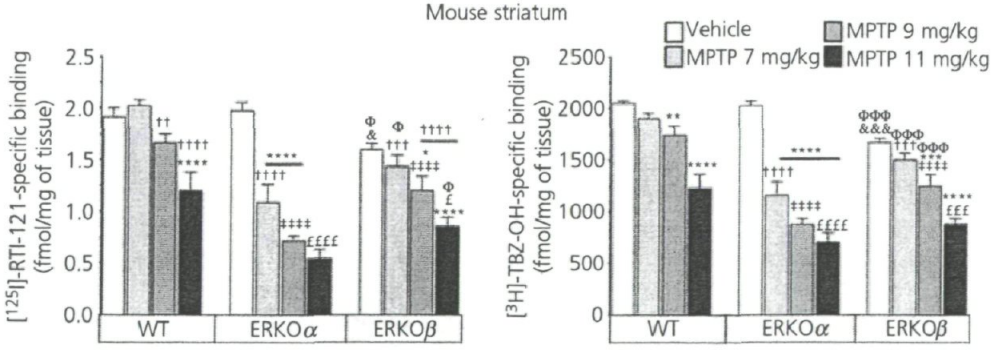

Fig. 1. Dose-response effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on striatal dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2)-specific binding measured with 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid ([^{125}I]-RTI-121) and [^{3}H]-dihydrotetrabenazine ([^{3}H]-TBZ-OH) binding respectively in wild-type (WT) compared to oestrogen receptor knockout (ERKO)α and ERKOβ mice using our assay conditions (104, 165). Experimental details of treatments of these mice and their striatal biogenic amine concentrations were described previously (119). DAT: $F_{11,57} = 20.3$, $P < 0.0001$ and VMAT2: $F_{11,55} = 30.6$, $P < 0.0001$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ and $^{****}P < 0.0001$ versus respective intact, vehicle (saline/gelatine solution, 0 MPTP); $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.005$ and $^{\dagger\dagger\dagger\dagger}P < 0.0001$ versus WT MPTP 7 mg/kg; $^{\ddagger\ddagger\ddagger\ddagger}P < 0.0001$ versus WT MPTP 9 mg/kg; $^{\text{£}}P < 0.05$, $^{\text{£££}}P < 0.005$ and $^{\text{££££}}P < 0.0001$ versus WT MPTP 11 mg/kg; $^{\text{¢}}P < 0.05$ and $^{\text{¢¢¢¢}}P < 0.005$ versus WT vehicle (0 MPTP); $^{\Phi}P < 0.05$, and $^{\Phi\Phi\Phi}P < 0.005$ versus respective experimental ERKOα genotype group.

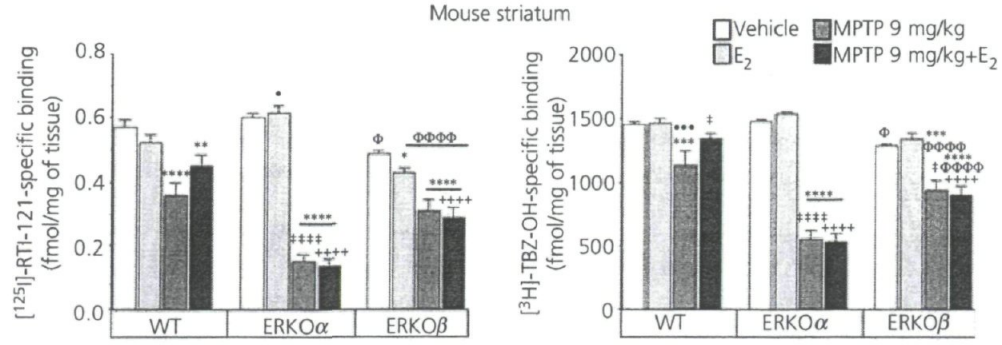

Fig. 2. Effect of treatment with 17β-oestradiol (E_2) (2 μg/day) for 10 days on anterior striatal dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2)-specific binding, measured with 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid ([^{125}I]-RTI-121) and [^{3}H]-dihydrotetrabenazine ([^{3}H]-TBZ-OH), respectively, in intact and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (9 mg/kg) lesioned wild-type (WT), oestrogen receptor knockout (ERKO)α and ERKOβ mice. For experimental details, see Fig. 1. DAT: $F_{11,69}$ = 32.7, $P < 0.0001$ and VMAT2: $F_{11,68}$ = 31.2, $P < 0.0001$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.0001$ versus respective intact, vehicle (0 MPTP); $^{\ddagger}P < 0.05$ and $^{\ddagger\ddagger\ddagger\ddagger}P < 0.0001$ versus WT MPTP; $^{\bullet}P < 0.05$ and $^{\bullet\bullet\bullet}P < 0.005$ versus WT + E_2; $^{++++}P < 0.0001$ versus WT + MPTP + E_2; $^{\Phi}P < 0.05$ and $^{\Phi\Phi\Phi\Phi}P < 0.0001$ versus respective ERKOα genotype group.

neither ERα, nor ERβ (136, 137). These so-called, GPCRs have no relation to the known ERs (138). However, Marin *et al.* (134) conclude that membrane ERα and ERβ are homologues of the nuclear ERs. If correct, then membrane ERα and ERβ should be absent in ERKOα and ERKOβ. There is evidence that intracellular ERα and ERβ are transported to the membrane and their interaction with metabotropic glutamate receptors provides many possibilities for membrane associated 17β-oestradiol cell signalling mediation (139). On the cellular membrane, ERα and ERβ activity resembles that of GPCRs and oestradiol modulates membrane-associated ERα and ERβ by inducing their internalisation (139). Although some controversies underlying its localisation and activity still exist (61), it is clear that GPER1 mediates rapid as well as transcriptional oestrogenic activity in the brain and periphery (73). GPER1 activity is manifested through two plasma membrane-associated enzymes; the first is Gs-protein, which induces adenylyl cyclase promoting elevated intracellular concentrations of cAMP, and the second is G$\beta\gamma$, which results in calcium mobilisation and kinase activation (140).

However, the role of GPER1 in the brain DA systems and neurodegenerative disorders has yet to be determined. Potential tools for elaborating the physiological activities of this new ER in the brain include the GPER1-specific agonist, G1 and antagonist, G15, both of which exist without any activity on ERα or ERβ (141, 142). As noted above, a vast amount of literature provides evidence that oestrogens have positive effects on the DA system in the brain, and their classical mechanisms on nuclear ERs should be studied along

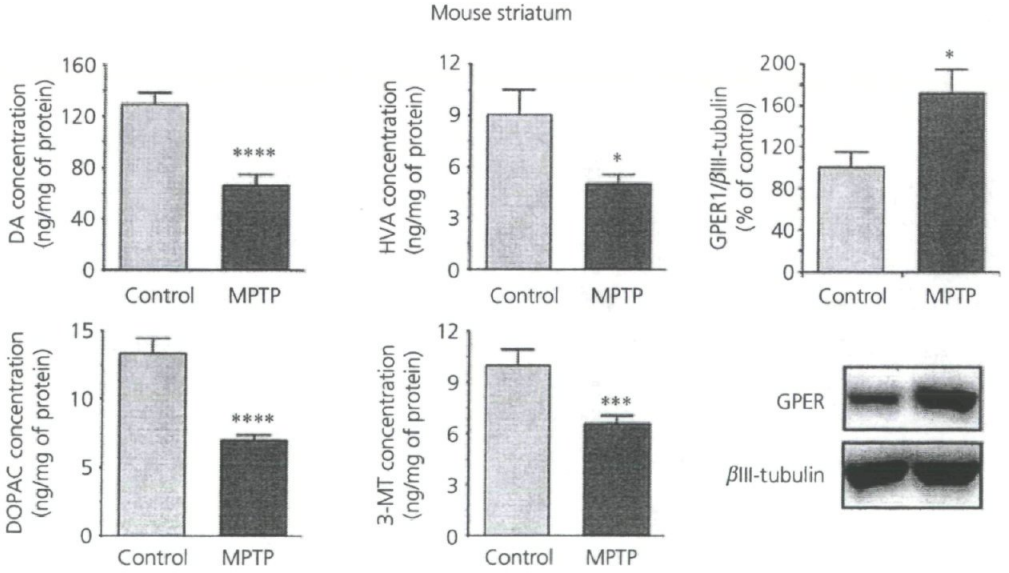

Fig. 3. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (11 mg/kg) in C57Bl/6 male mice on striatal dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) concentrations, as well as G protein-coupled oestrogen receptor (GPER1) levels. Catecholamines concentrations were measured under conditions previously reported by high-performance liquid chromatography with electrochemical detection (119) and GPER1 levels by western blotting (143). $^{*}P < 0.05$, $^{***}P < 0.005$, and $^{****}P < 0.0001$ versus respective intact, vehicle (0 MPTP).

with their potential GPER1 activity. Therefore, we aimed to assess the distribution of striatal GPER1 and its response to oestradiol and lesions. High levels of GPER1 were measured in the striatum of male mice and were increased by MPTP lesion (Fig. 3). Moreover, we compared methamphetamine-induced neurotoxicity on striatal GPER1 in male and female mice; methamphetamine, which produced significantly decreased striatal DA in males, increased striatal GPER1 levels in male, but not in female mice (143). Ovariectomised female *Macaca fascicularis* monkeys with a unilateral MPTP lesion of the nigrostriatal pathway that received a chronic 17β-oestradiol or vehicle treatment for 1 month were studied next. The lesioned striata of these monkeys were extensively denervated, as indicated by reductions in DA concentrations (144). GPER1 levels were abundant in the monkey striatum, both in the caudate nucleus and the putamen, at the two rostro-caudal levels measured. Similar levels of GPER1 were found in the anterior versus posterior caudate nucleus and putamen of monkeys (Fig. 4). GPER1 levels were higher in the putamen, but not the caudate nucleus, in the MPTP-lesioned side compared to that of the intact side of hemiparkinsonian monkeys. 17β-Oestradiol treatment did not significantly change GPER1 levels in the intact or lesioned caudate nucleus and putamen of monkeys (Fig. 4). The present results show that GPER1 is abundant in the striatum of monkeys and mice and is increased in response to toxins that target the nigrostriatal pathway.

Akt and GSK3 signalling pathways

Akt signalling in mental and neurodegenerative diseases

In addition to the classical functions of DA receptor-cAMP-dependent mechanisms, striatal DA D_2 receptors can also exert physiological effects via PKB (Akt) (145). Akt can be activated after the

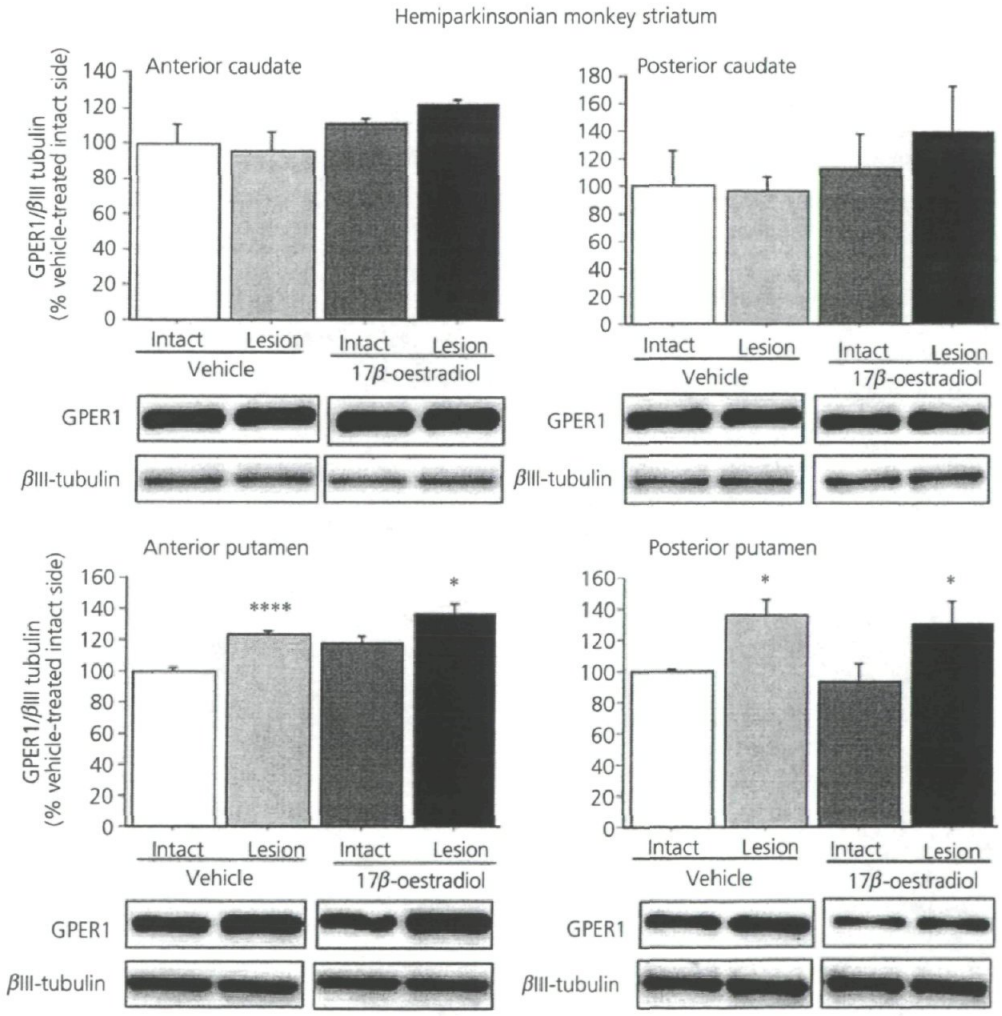

Fig. 4. G protein-coupled oestrogen receptor (GPER1) levels in the caudate nucleus and putamen of hemiparkinsonian ovariectomised monkeys treated for 1 month with vehicle (n = 4) or with 17β-oestradiol (0.1 mg/kg once daily subcutaneous, n = 3). Data are expressed as a percentage of the intact side of vehicle-treated monkeys. Experimental details of treatments of these monkeys and their striatal biogenic amine concentrations were reported previously (144). GPER1 and βIII-tubulin were measured by western blotting under conditions described previously (143). *P < 0.05 and ****P < 0.0005 versus the intact side of respective treated monkeys.

binding of neurotransmitters or growth factors on many specific cell-surface receptors, which in turn initiate a cascade of second messengers related to the phosphatidylinositol 3-kinase (PI3-K) pathway (146). Deactivation of Akt after dephosphorylation results in activation of the glycogen synthase kinase-3 (GSK3)-mediated signal (147). Dysregulation of Akt/GSK3 signalling is involved in many DA-associated neurological and neuropsychiatric disorders. Reduced Akt activity or expression levels were shown in brains of schizophrenic patients (148, 149) and there are data indicating an involvement of GSK3β in depression and psychosis (150). A report showed an association between the Akt1 gene and PD that was a protective haplotype (151). In sections of post-mortem SN, an extensive reduction of pAkt(Thr308) and pAkt(Ser473) in PD patients was observed compared to controls (152).

The implication of PKB/Akt in 17β-oestradiol induced neuroprotection

The PI3-K/Akt and MAPK signalling pathways are associated with 17β-oestradiol activity in the brain (114). ERs relay MAPK signals through sequential activation of Ras, B-raf, MAPK/ERK kinase (MERK1/2) and MAPK (ERK1/2) to finally induce various transcriptional factors that promote neuronal survival (115, 135, 153, 154). The effector Akt can be activated via the PI3-K pathway through ERs (123). Akt activity promotes cell survival by modulating the expression of anti-apoptotic proteins such as Bcl-2 and apoptotic proteins like Bad and Bax (123) and signalling converges at GSK3β. GSK3β is a highly expressed kinase in the brain and, once activated, functions to induce cellular death pathways; therefore, its inactivation favours the promotion of cellular survival mechanisms (123). The activity of this kinase III is proapoptotic; it is inhibited if phosphorylated on certain serine residues, thus promoting cell survival (155) and activated if phosphorylated on tyrosine residues (115). Moreover, activation of these signalling pathways through either membrane-associated or genomic actions of 17β-oestradiol could be combined to act synergistically in injured neurones and amplify the neuroprotective process (156).

Data from several sources indicate that the MAPK pathway and ERβ contribute to cell survival signalling pathways in various models of neuronal injury (126, 157–159). However, Kahlert and colleagues have observed that ERα is involved in the activation of Akt signalling (160, 161), whereas ERβ is not (162). Our group, in collaboration with L. M. Garcia-Segura, reported that treatment with 17β-oestradiol increased phosphorylated PKB/Akt (at serine 473) levels in mice (123). We also observed that GSKβ3 phosphorylation on serine 9 was highly diminished in MPTP mice and that PPT treatment significantly blocked GSK3β activation, but not treatment with 17β-oestradiol or the ERβ agonist Δ5-diol (123). Only PPT treatment had positive effects by increasing the levels of inhibited GSK3β in MPTP treated mice and, in intact wild-type mice, it was the 17β-oestradiol and PPT treatments that increased the levels of activated PKB/Akt and deactivated GSK3β (123). These results support a role for the ERα in the PI3-K pathway being associated with the neuroprotective effects of oestrogenic compounds against MPTP. Moreover, GPER1 activated by 17β-oestradiol was also shown to initiate PI3-K signaling and Akt activity (70, 71).

No data are yet available on the oestrogenic modulation of this signalling pathway in monkeys. We thus measured the effect of 1 month of treatment with 17β-oestradiol on the Akt/GSK3 signalling pathway in the brain of ovariectomised monkeys with a unilateral MPTP lesion of the nigrostriatal pathway. 17β-Oestradiol treatment induced an increase of pAkt(Ser 473)/βIII-tubulin in the

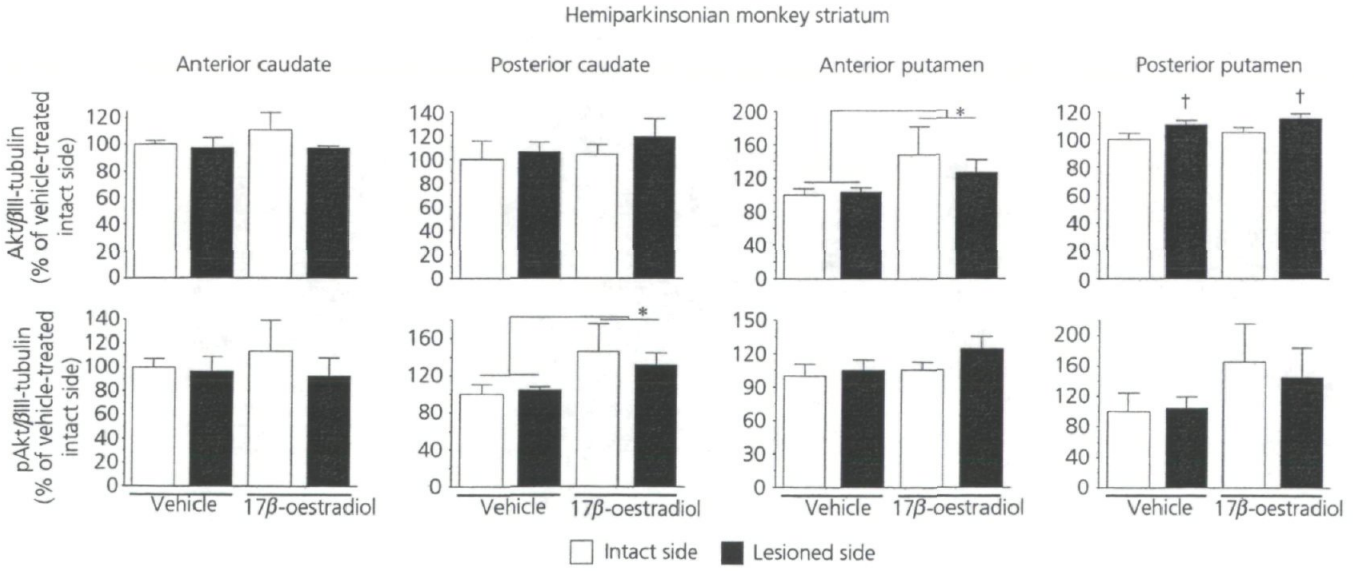

Fig. 5. Relative levels of Akt and its phosphorylated form (pAkt, Ser473) in the caudate and putamen of hemiparkinsonian 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkeys treated for 1 month with vehicle or 17β-oestradiol. For experimental details, see Fig. 4. Akt and its phosphorylated form were measured by western blotting under conditions described previously (166). *$P < 0.05$ versus vehicle-treated monkeys; †$P < 0.05$ versus the respective intact side.

intact and lesioned posterior caudate nucleus and in pGSK3β(Ser 9)/βIII-tubulin in the intact and lesioned anterior putamen compared to vehicle-treated monkeys. In the intact and lesioned putamen, the Akt/βIII-tubulin was also increased in monkeys treated with 17β-oestradiol, whereas GSK3β/βIII-tubulin remained unchanged (Figs 5 and 6). These translational results in monkeys, similar to our previous findings in mice (123) suggest that activation of the Akt/GSK3 signalling pathway is involved in the 17β-oestradiol effect on the striatal DA system and support a beneficial role of oestrogenic treatment resulting from an increase in the activity of signalling pathways implicated in cell survival.

Concluding remarks

The combinations of the effects of nuclear ERα and ERβ and membrane GPER1 signalling pathways are reported to result in cell cycle progression and cell proliferation (73), which is beneficial in the injured or neurodegenerative brain. ERβ is reported to modulate ERα-mediated transcription in mice; therefore, in certain tissues and under certain conditions, these ERs are interdependent (163). Moreover, ERα and GPER1 were reported to cross-talk (73). There is great diversity in the possible synergistic or antagonistic interactions between ERs and GPER1 (73) and both membrane and fast transcription activity of GPER1 are reported to result in the activation of genes such as c-*fos* (164).

In summary, a complex cascade of genomic and nongenomic oestrogen-induced activity results in neuroprotection, which in turn relies on the neuroanatomical and spatio-temporal organisation of ERs and various signalling pathway molecules involved in their cross-talk in different neuronal populations. Oestrogens and oestrogenic drugs modulate and protect nigrostriatal DA activity and our results propose that ERs are implicated in these effects. A role for ERα and its agonists is observed in neuroprotection, whereas the novel GPER1 could provide a new target for modulating the nigro-

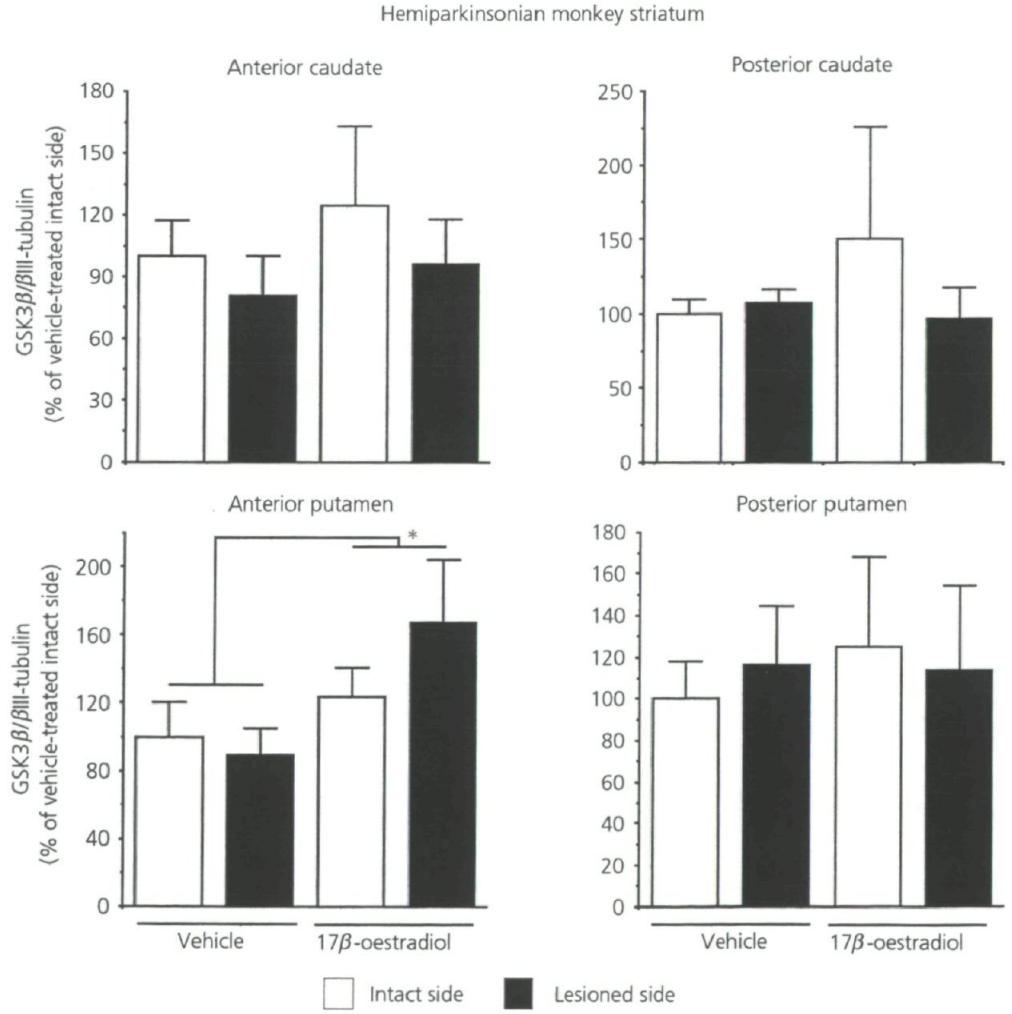

Fig. 6. Relative levels of phosphorylated glycogen synthase kinase-3β (GSK3β) (pGSK3β, Ser9) in the caudate and putamen of hemiparkinsonian 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkeys treated for 1 month with vehicle or 17β-oestradiol. For experimental details, see Fig. 4. GSK3β and its phosphorylated form were measured by western blotting as described previously (166). GSK3β/βIII-tubulin levels were unchanged by the lesion and 17β-oestradiol treatment (data not shown). *$P < 0.05$ versus vehicle-treated monkeys.

striatal DA system. We also demonstrate that the Akt/GSK3 signalling pathway is modulated by oestrogens in intact and MPTP lesioned mice and monkeys. The implication of ERα, ERβ and GPER1 for modulation of nigrostriatal DA activity supports the development of a new generation of ER-specific drugs for the brain.

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Estrogen receptors and gonadal steroids in vulnerability and protection of dopamine neurons in a mouse model of Parkinson's disease

Sara Al-Sweidi, Marc Morissette, Mélanie Bourque, Thérèse Di Paolo*

Molecular Endocrinology and Genomic Research Center of CHUQ-CHUL, and Faculty of Pharmacy, Laval University, 2705 Laurier Boulevard, Quebec City, QC, Canada G1V 4G2

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ABSTRACT

17β-estradiol is well known to have neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. We investigated the neuroprotective contribution of estrogen receptors (ERα and ERβ) against MPTP toxicity by examining the membrane dopamine (DA) transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) and tyrosine hydroxylase (TH) in ER knock out (ERKO) C57Bl/6 male mice compared to their plasma steroid levels. A dose–response to MPTP comparing wild-type (WT) to ERKO mice was studied. WT mice were also compared to ERKO mice pretreated with 17β-estradiol alone and with MPTP. Specific radioligand binding autoradiography and *in situ* hybridization for DAT, VMAT2 and TH were assayed in the striatum and the substantia nigra (SN). Intact ERKOβ mice had both striatal transporters levels lower than WT and ERKOα mice. MPTP caused a dose-dependant loss of both striatal transporters that correlated with striatal DA concentrations. Compared to WT and ERKOβ mice, ERKOα mice DAT, VMAT2 and TH were affected at lower MPTP doses. In the striatum and SN, ERKOα mice were more vulnerable and 17β-estradiol protected against MPTP toxicity only in WT mice. ERKOα mice blood plasma had higher levels of testosterone, dihydrotestosterone and 3β-diol compared to the plasma of WT and ERKOβ mice. 17β-estradiol treatment increased estradiol plasma levels in all genotypes. Striatal DA concentrations and SN TH mRNA correlated inversely with plasma testosterone and 3β-diol levels. Hence, in male mice the lack of ERα or ERβ altered their basal plasma steroid levels and both striatal DA transporters as well as their susceptibility to MPTP toxicity.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer and is likely to increase due to the aging population (review: Siderowf and Stern, 2003). Motor impairment in PD results from the loss of striatal dopamine (DA), due to the death of DA neurons in the substantia nigra (SN). There is no cure for PD but the motor symptoms are alleviated by replacement of DA by its precursor levodopa (L-DOPA) or by treatment with direct DA receptor agonists (Hornykiewicz, 2002; Olanow et al., 2009). Nevertheless for the majority of PD patients, these therapies eventually loose effectiveness and are associated with side-effects (Katzenschlager and Lees, 2002). Thus, there is a need for therapies to prevent the loss of DA neurons and/or halt disease progression. Estrogenic drugs could bring such disease modifying therapies for PD.

There are two families of transporters responsible for controlling extracellular DA concentrations; these are the DA transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) (Guillot and Miller, 2009). The striatum has dense and heterogeneous DAT distribution, the transporter is found on plasma membranes of axon terminals and immunocytochemistry shows that DAT is colocalized with tyrosine hydroxylase (TH) and the D2 DA receptor (Ciliax et al., 1999). DAT allows the uptake of DA into the cytoplasm from the extracellular space, while VMAT2 is responsible for storing DA in synaptic vesicles and reduction of its levels in the nigrostriatal system is seen in animal models of PD and in PD patients

Abbreviations: CNS, central nervous system; DA, dopamine; DAT, membrane dopamine transporter; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; 3β-diol, 5α-androstan 3-β, 17β-diol; DOPAC, 3,4-Dihydroxyphenylacetic acid; 17β-E_2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERKO, estrogen receptor knock out; E1, estrone; ERK, extracellular-signal-regulated kinase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; P450scc, cytochrome P450 sidechain cleavage; P450c17, cytochrome P450 17α-hydroxysteroid/C17 20-lyase; PPT, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; 5α-R, 5α-reductase; [^{125}I]-RTI-121, 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid; SN, substantia nigra; TH, tyrosine hydroxylase; [^{3}H]-TBZ-OH, [^{3}H]-dihydrotetrabenazine; VMAT2, vesicular monoamine transporter type 2; WT, wild-type.

* Corresponding author. Tel.: +1 418 654 2296; fax: +1 418 654 2761.
E-mail address: therese.dipaolo@crchul.ulaval.ca (T. Di Paolo).

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(Guillot and Miller, 2009; Le Saux and Di Paolo, 2006). The actions of these transporters are regulated by presynaptic receptors and protein kinases (Guillot and Miller, 2009). Hence, the amount of free DA depends on DAT levels on the plasma membrane and the presence of VMAT2 on synaptic vesicles.

Epidemiological and clinical studies support a beneficial effect of estrogens against the development and progression of PD. A greater prevalence and incidence of PD is described in men than in women (Shulman and Bhat, 2006; Wooten et al., 2004). Men with PD show symptoms requiring medical attention during earlier stages of the disorder than women suggesting that the disease progresses more rapidly in men, thus supporting that estrogen can provide neuroprotective effects (Saunders-Pullman, 2003). Gender differences in symptoms were also seen in outcome studies after stereotactic surgery for PD (Shulman and Bhat, 2006). Also, an inverse association between factors reducing estrogen stimulation during life and PD is found, supporting the hypothesis that endogenous estrogens play a role in its development (review: Bourque et al., 2009). Therapy with 17β-estradiol was reported to be beneficial at an early stage of PD, before initiation of L-DOPA (review: Bourque et al., 2009).

17β-estradiol is neuroprotective in both male and female mice against a variety of central nervous system (CNS) insults such as protection of DA neurons against the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), where pre-treatment with 17β-estradiol before MPTP prevents the loss of striatal DA and its metabolites (review: Bourque et al., 2009), DAT, and VMAT2 (D'Astous et al., 2003). Dluzen and colleagues suggest that estrogen protects by decreasing DAT's binding affinity, thus not allowing entry of neurotoxic compounds, like the MPTP ion 1-methyl-4-phenylpyridinium (MPP+), into dopaminergic nerve terminals (Dluzen, 2000). Furthermore, the neuroprotective effect of 17β-estradiol appears to be mediated through interaction with estrogen receptors (ERs). In male mice, 17β-estradiol which binds to and activates ERs, is neuroprotective against striatal MPTP toxicity; whereas 17α-estradiol, the isomer with low ER affinity, lacks neuroprotective activity (Callier et al., 2000) and estriol and estrone, weak agonists on ERs, show poor or no activity to protect against MPTP toxicity (Jourdain et al., 2005). Thus, the potencies of the above compounds to protect against MPTP toxicity parallel their activity on ERs. There are two main ER subtypes, ERα and ERβ (Green et al., 1986; Kuiper and Gustafsson, 1997). ERα is widely expressed throughout the body and mediates most of the feminizing effects of estradiol (Mitra et al., 2003). By contrast, ERβ has a much more restricted distribution, of which expression in the brain is notable (Kuiper and Gustafsson, 1997). Both ERs have been detected in the mouse striatum and SN (Mitra et al., 2003). Moreover, no sex difference was observed for ERα and ERβ levels in mouse striatum during development and in adulthood (Kuppers and Beyer, 1999). Using specific agonists for ERα and ERβ we have previously shown that ERα agonists protect against MPTP toxicity in male mice (D'Astous et al., 2004).

The intact male mouse MPTP animal model of PD is representative of PD pathology and, to unravel the neuroprotective effects of ERs, striatal catecholamine concentrations of ER knock out (ERKOα and ERKOβ) male mice were previously published (Morissette et al., 2007). The degree of MPTP-induced DA and DOPAC depletion was greater in ERKOα than in wild-type (WT) male mice, whereas ERKOβ mice exhibited no change in MPTP sensitivity but they showed a lower DA turnover than WT and ERKOα mice. 17β-estradiol partially prevented the MPTP-induced decrease in striatal DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels only in the WT mice (Morissette et al., 2007). Therefore, we hypothesize that sparing of striatal DA concentrations from MPTP toxicity in these WT mice is due to neuroprotection of DA neurons by endogenous steroids and administered 17β-estradiol acting on ERs. Hence, in the present study we investigated the effect of ER genotype on blood steroid levels and various DA markers in these mice. We explored the contribution of the striatal and SN DAT, VMAT2 and TH in MPTP toxicity and neuroprotection by 17β-estradiol in WT mice compared to ERKOα and ERKOβ male mice.

2. Materials and methods

2.1. Animals and treatments

Adult WT, ERKOα and ERKOβ male C57Bl/6 mice (7–12 weeks, 18–28 g, WT and ERKO mice) were purchased from Taconic Laboratories (Hudson, NY, USA). MPTP and 17β-estradiol were purchased from Sigma Chemical (St-Louis, MO, USA). In order to minimize the possible variability of the response to MPTP treatment, WT and ERKO mice were of C57Bl/6 background and were equally distributed for age and weight in experimental groups of six animals. The Laval University Animal Care Committee approved all the animal studies. All efforts were made to minimize animal suffering and to reduce the number of mice used.

An extended MPTP dose–response up to 20 mg/kg was performed in WT male C57Bl/6 mice and striatal biogenic amine concentrations of these mice was previously reported (Morissette et al., 2007). The MPTP doses (7, 9 and 11 mg/kg) that specifically affected striatal DA while sparing serotonin concentrations in WT mice (Morissette et al., 2007) were used for comparison of MPTP dose–responses of ERKOα, ERKOβ and WT mice. Mice received four 0.1 ml intraperitoneal injections with saline or a saline solution of MPTP at a two-hour interval and were killed 5 days after treatment with MPTP.

The effect of 17β-estradiol and MPTP toxicity in ERKOα and ERKOβ was compared to WT mice. Four groups of both ERKOα and ERKOβ mice were compared to WT mice. An intermediate dose of 9 mg/kg MPTP was selected and we investigated the effect of 17β-estradiol treatment in intact and MPTP mice. Each group received a 5-day pre-treatment of 17β-estradiol or vehicle prior to MPTP injections. The pre-treatment consisted of two daily subcutaneous injections (in the dorsal part of the neck) of 17β-estradiol, while control mice received injections of vehicle (0.9% saline with 0.3% gelatin). Concentrations used were 2 μg per day for 17β-estradiol such as we used previously (D'Astous et al., 2004; Morissette et al., 2007). On day 5, mice received four injections of MPTP (9 mg/kg, per intraperitoneal injection) at a 2-h interval, while the control group received saline solution. Treatments with 17β-estradiol or vehicle were continued until day 10. The next day, mice were killed with an air/halothane mixture and decapitated; trunk blood was collected and brains were quickly removed and frozen in a mixture of isopentane/dry ice and then stored at −80 °C. Gas chromatography and negative chemical ionization mass spectrometry was used to assay steroid plasma levels as described (Labrie et al., 2007).

2.2. Preparation of brain tissue

Frozen brains were cut on a cryostat in 12 μm thick slices at striatal and SN *pars compacta* regions. Coronal sections for the anterior striatum (bregmas between 1.54 and 1.18 mm), middle striatum (bregmas between 0.50 and 0.14 mm), posterior striatum (bregmas between −0.34 and −0.70 mm) and the SN (bregmas −2.70 mm to −3.28 mm) were done according to the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos, 1997).

2.3. DAT and VMAT2 autoradiography

Autoradiography using isopropylester 3β-(4-^{125}I-iodophenyl)trophane-2β-carboxylic acid ([^{125}I]-RTI-121) to the DAT was done on the striatum and the SN as previously described (Callier et al., 2001). DAT specific binding was measured using 20 pM [^{125}I]-RTI-121 (2200 Ci/mmol, Perkin–Elmer, Woodbridge, ON, Canada) in the presence of 100 nM of mazindol to estimate nonspecific binding. The striatal slices were exposed to Kodak BIOMAX film for two days and the SN slices for four days.

Autoradiography using [^{3}H]-dihydrotetrabenazine ([^{3}H]-TBZ-OH) for VMAT2 binding was done on the striatum and the SN using 20 nM of [^{3}H]-TBZ-OH (20 Ci/mmol, ARC, Saint Louis, MO USA) in the presence of 1 μM of cold TBZ-OH to estimate nonspecific binding as described (Kilbourn and Frey, 1996). Striatal slices were exposed to sensitive Kodak BIOMAX film for four weeks and the SN slices for six weeks.

2.4. DAT, VMAT2 and TH in situ hybridization

DAT mRNA levels in the SN were measured by *in situ* hybridization using a sequence encoding the entire rat DAT (Callier et al., 2000). The percentage of homologies between the rat and the mouse cDNA sequences used to generate the radioactive probe for the DAT transporter is 95% (GenBank accession no. NM_012694 and NM_010020.3). The whole rat DAT sequence was subcloned into the *Eco*RI site of pBlueScript and used in the preparation of sense and antisense strands of cRNA

probes. Uridine 5′([α-^{35}S]thio)triphosphate-labeled probes were prepared by *in vitro* transcription of linearized templates (sense, Kpn1; antisense, Xba1) with T3 and T7 RNA polymerases to generate sense and antisense probes respectively. The pre-hybridization, hybridization and post-hybridization procedures were followed as previously described (Callier et al., 2000; Jourdain et al., 2005).

VMAT2 and TH mRNA levels in the SN were measured by *in situ* hybridization using cDNA oligonucleotide probes as previously described (Calon et al., 2002; D'Astous et al., 2003). *In situ* hybridization was done using an oligonucleotide complementary to bases 1321–1366 (GenBank accession no. NM_172523) of the mouse VMAT2 sequence and an oligonucleotide complementary to bases 1435–1482 (GenBank accession no. M69200) of the mouse TH sequence. These probes were labeled at the 3′ end by adenosine 3′([α-^{35}S]thio)triphosphate. The pre-hybridization, hybridization and post-hybridization procedures were followed as previously described (Calon et al., 2002; Jourdain et al., 2005).

Slide-mounted tissue sections were apposed to sensitive Kodak BIOMAX film where DAT *in situ* hybridization samples were exposed for three days, VMAT2 for four weeks and TH for three weeks.

2.5. Statistical analysis

Autoradiograms of specific binding and mRNA relative optical densities were analyzed using Scion Image 1.63 software. The striatum was analyzed at three rostro-caudal coordinates which include the anterior, middle and the posterior striatum; each of these regions were subdivided in half into medial and lateral regions. The statistical analyses were done using Statview 4.51 for Macintosh Computer software. A one-way ANOVA was used to compare MPTP and/or 17β-estradiol treatments followed by post-hoc analysis with Fisher probability of least significance difference test. A simple regression model (Pearson) was used to determine the coefficient of correlation and the significance of the degree of the linear relationship between variables. A $p \leq 0.05$ was required for the results to be considered statistically significant.

3. Results

3.1. Dose–response of MPTP on DAT, VMAT2 and TH

The effects of MPTP in ERKO and WT mice is shown in Fig. 1. Similar results were observed in the medial and lateral parts of the three rostro-caudal striatal coordinates measured therefore these results were grouped and the anterior striatum is shown as a representative, while detailed results on the middle and posterior striata are represented as Supplementary data. In the anterior striatum, DAT specific binding was higher by 27% in vehicle-treated ERKOα mice compared to vehicle-treated WT mice (Fig. 1A) while no difference was observed in the middle and posterior striatum (Supplementary data 1, S1). VMAT2 specific binding was similar in vehicle-treated ERKOα mice compared to vehicle-treated WT mice at the three rostro-caudal striatal levels measured (Fig. 1B and Fig. S1). By contrast, vehicle-treated ERKOβ mice had significantly lower DAT specific binding in the middle (23%) and posterior (21%) striatum, as well as lower VMAT2 specific binding in the anterior (19%) and posterior (25%) striatum compared to vehicle-treated WT mice (Fig. 1B and Fig. S1). Moreover, DAT specific binding was lower in vehicle-treated ERKOβ mice compared to vehicle-treated ERKOα mice in the anterior and middle striatum as well as lower VMAT2 specific binding at all three rostro-caudal striatal levels measured (Fig. 1B and Fig. S1).

MPTP dose-dependently decreased striatal DAT and VMAT2 specific binding of ERKO mice and ERKOα mice were more vulnerable to MPTP toxicity than WT and ERKOβ mice. The ERKOα mice showed decreased DAT and VMAT2 specific binding at lower MPTP doses than ERKOβ mice (Fig. 1). In addition, the loss of striatal DAT and VMAT2 specific binding positively correlated with striatal DA concentrations in the anterior striatum (DAT: $R = 0.766$, $p < 0.0001$, Fig. 3A and VMAT2: $R = 0.757$, $p < 0.0001$, Fig. 3B) as well as in the middle and posterior striatum (data not shown).

In the SN, DAT and VMAT2 specific binding and mRNA remained unchanged, in the WT groups as well as in both ERKOα and ERKOβ mice, at the three MPTP doses tested (data not shown). SN TH mRNA levels were similar between genotypes of vehicle-treated mice and decreased with increasing doses of MPTP; this was significant and to a similar extent in both ERKOα and ERKOβ mice, while only a tendency to decrease was seen in the WT group at the MPTP doses tested (Fig. 4A).

3.2. Effect of 17β-estradiol in intact and MPTP WT compared to ERKOα and ERKOβ mice

The intermediate dose of 9 mg/kg of MPTP was chosen to investigate the neuroprotective effect of 17β-estradiol in the three groups of mice to further explore the differences between genotypes observed in the MPTP dose–response results. Similar findings were observed at the three striatal rostro-caudal coordinates analyzed as well as in their medial and lateral sub regions, therefore the anterior striatum results are shown as representative.

Similar differences in striatal DAT and VMAT2 specific binding were seen between the intact WT versus ERKOα and ERKOβ mice of the controls of the MPTP/17β-estradiol experiment shown in Fig. 2 as compared to data of the control mice of these genotypes in the MPTP dose–response study of Fig. 1. Vehicle-treated ERKOα mice in Fig. 2A had higher DAT levels in the anterior striatum. Moreover,

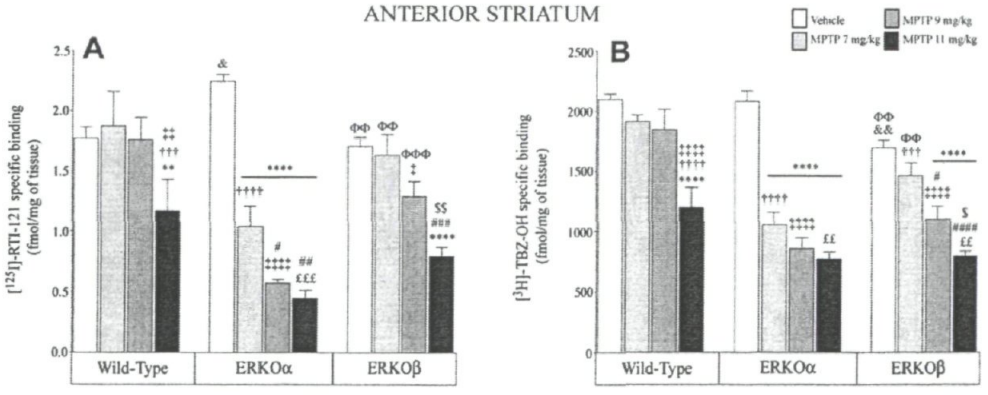

Fig. 1. Dose–response effect of MPTP on striatal DAT (A) and VMAT2 (B) specific binding respectively measured with [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH binding in wild-type compared to ERKOα and ERKOβ mice. There was a statistically significant effect of MPTP in the anterior striatum (DAT: $F_{11,59} = 12.50$, $p < 0.0001$ and VMAT2: $F_{11,58} = 23.73$, $p < 0.0001$). $^{**}p < 0.01$ and $^{****}p < 0.0001$ vs respective intact, vehicle (0 MPTP); †††$p < 0.005$ and ††††$p < 0.0001$ vs WT MPTP 7 mg/kg; ‡$p < 0.05$, ‡‡$p < 0.01$ and ‡‡‡‡$p < 0.0001$ vs WT MPTP 9 mg/kg; £££$p < 0.001$ and ££££$p < 0.005$ vs WT MPTP 11 mg/kg; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.005$ and ####$p < 0.0001$ vs respective ERKO + MPTP 7 mg/kg; $$p < 0.05$ and $$$p < 0.01$ vs respective ERKO + MPTP 9 mg/kg; &$p < 0.05$ and &&$p < 0.01$ vs WT vehicle (0 MPTP); ΦΦ$p < 0.01$ and ΦΦΦ$p < 0.005$ vs respective experimental ERKOα genotype group. One of the six mice died in the ERKOα MPTP 11 mg/kg group.

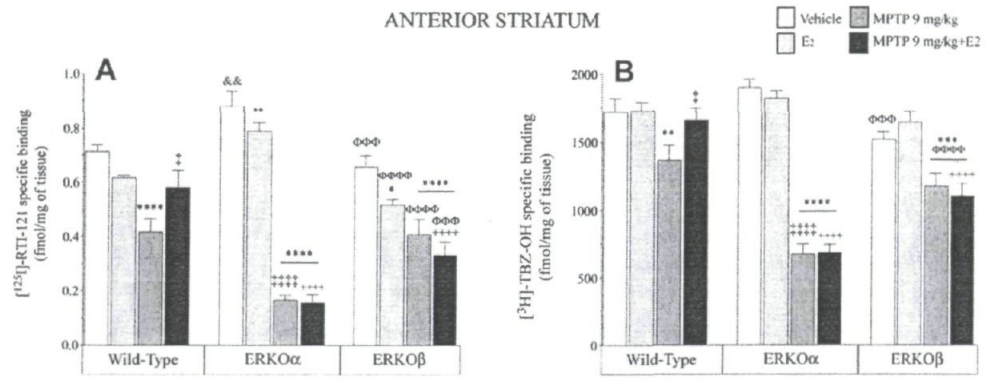

Fig. 2. Effect of treatment with 17β-estradiol (E_2) (2 μg/day) for ten days on anterior striatal DA transporters DAT (A) and VMAT2 (B) specific binding respectively measured with [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH in intact and MPTP (9 mg/kg) lesioned WT, ERKOα and ERKOβ mice. There was a statistically significant effect of treatments for DAT ($F_{11,71} = 29.13$, $p < 0.0001$) and VMAT2 ($F_{11,71} = 26.94$, $p < 0.0001$) specific binding. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.0001$ vs respective intact, vehicle (0 MPTP); ‡$p < 0.05$ and ‡‡‡‡$p < 0.0001$ vs WT MPTP; ••$p < 0.01$ vs WT + E_2; ++++$p < 0.0001$ vs WT + MPTP + E_2; &&$p < 0.01$ vs WT vehicle (0 MPTP); ΦΦΦ$p < 0.005$ and ΦΦΦΦ$p < 0.0001$ vs respective experimental ERKOα genotype group.

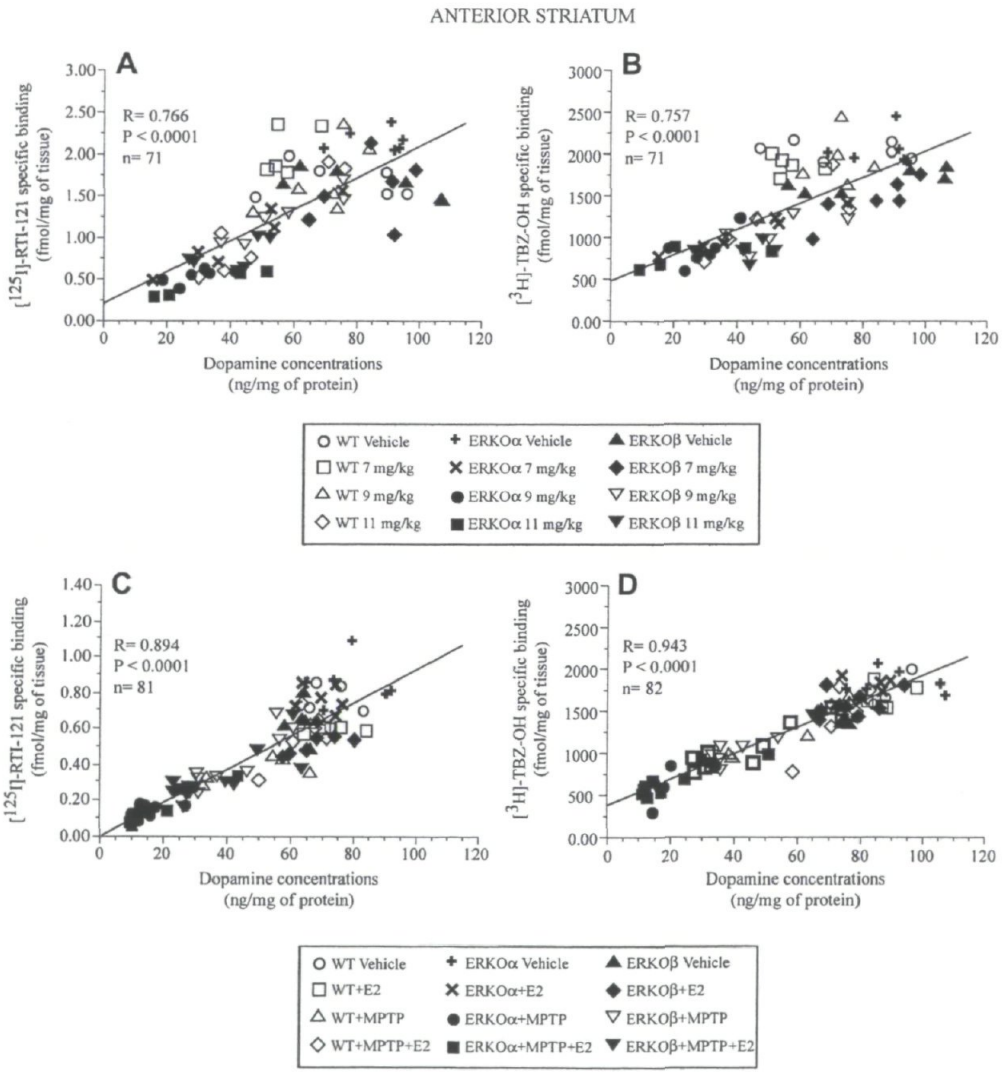

Fig. 3. Correlations between [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH specific binding and DA concentrations in the anterior striatum of WT, ERKOα and ERKOβ mice of the MPTP dose–response (A and B) and the MPTP/17β-estradiol experiments (C and D). Striatal DA concentrations of these mice are from Morissette et al. (2007).

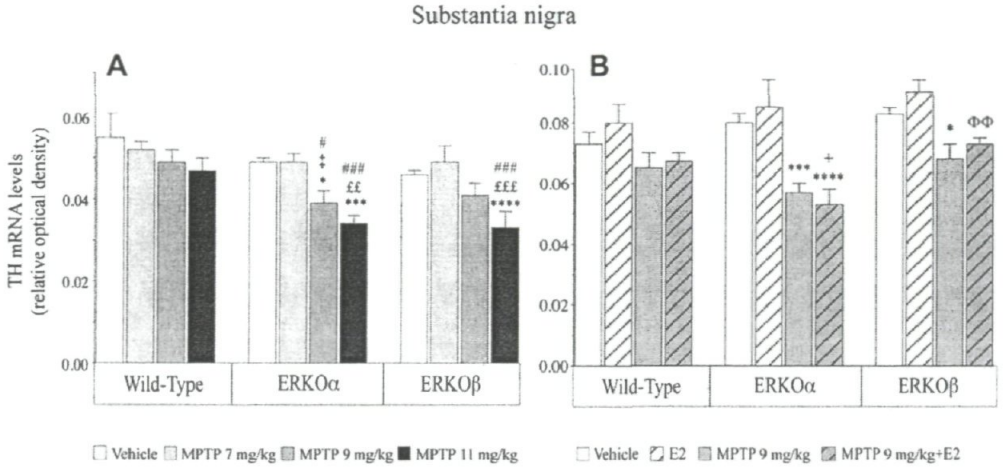

Fig. 4. A) Dose–response effect of MPTP on SN tyrosine hydroxylase (TH) mRNA levels measured by *in situ* hybridization in wild-type compared to ERKOα and ERKOβ mice ($F_{11,52} = 4.75$, $p < 0.0001$). B) Effect of MPTP and 17β-estradiol (E_2) (2 μg/day) treatments on SN TH mRNA levels in wild-type compared to ERKOα and ERKOβ mice ($F_{11,51} = 5.81$, $p < 0.0001$). *$p < 0.05$, ***$p < 0.005$ and ****$p < 0.0001$ vs respective intact, vehicle (0 MPTP); ‡$p < 0.05$ vs WT MPTP 9 mg/kg; ££$p < 0.01$ and £££$p < 0.005$ vs WT MPTP 11 mg/kg; #$p < 0.05$ and ###$p < 0.005$ vs respective ERKO + MPTP 7 mg/kg; +$p < 0.05$ vs WT + MPTP + E_2; and ΦΦ$p < 0.01$ vs respective experimental ERKOα genotype group.

DAT and VMAT2 specific binding was lower in vehicle-treated ERKOβ compared to ERKOα mice (Fig. 2).

No significant effect of 17β-estradiol treatment was measured on DAT specific binding in the anterior striata of unlesioned WT or ERKOα mice whereas it decreased in ERKOβ mice (Fig. 2A). No significant effect of 17β-estradiol treatment was measured on VMAT2 specific binding of unlesioned WT, ERKOα or ERKOβ mice (Fig. 2B).

In WT mice, DAT and VMAT2 specific binding were decreased by MPTP. DAT levels decreased by 42% (Fig. 2A), while VMAT2 levels were lowered by 21% in the anterior striatum (Fig. 2B). Treatment with 17β-estradiol prevented this in the striata of MPTP WT mice. By contrast, in ERKOα or ERKOβ mice, 17β-estradiol did not protect striatal DAT and VMAT2 specific binding against MPTP toxicity, their specific binding remaining decreased. Moreover, MPTP-lesioned ERKOα mice had significantly lower striatal DAT specific binding compared to ERKOβ mice. In the anterior striata of MPTP mice, DAT levels decreased by 81% in ERKOα mice; whereas in ERKOβ mice DAT levels decreased by 38% compared to unlesioned ERKO mice. The same pattern was observed for VMAT2 specific binding, where its levels decreased by 65% in ERKOα mice and by 23% in ERKOβ mice compared to unlesioned ERKO mice (Fig. 2B).

There were positive correlations between [^{125}I]-RTI-121 and [^{3}H]-TBZ-OH specific binding and DA concentrations in the anterior striatum of WT mice, ERKOα and ERKOβ mice of the MPTP dose–response (Fig. 3A and B) and the MPTP/17β-estradiol experiments (Fig. 3C and D).

In the SN, treatment with 17β-estradiol of unlesioned mice left unchanged the TH mRNA levels of all WT, ERKOα and ERKOβ mice (Fig. 4B). In ERKOα and ERKOβ mice, a 9 mg/kg MPTP treatment caused a significant decrease of TH mRNA levels whereas the decrease was nonsignificant for WT mice. SN TH mRNA levels of 17β-estradiol-treated MPTP ERKOβ mice were higher than for 17β-estradiol-treated MPTP ERKOα mice (Fig. 4B).

Plasma estrogen and androgen concentrations were measured in order to investigate the effect of absence of ERα and ERβ on basal plasma steroid levels as well as on the metabolism of administered 17β-estradiol. We assayed and quantified the endogenous androgens testosterone, dihydrotestosterone (DHT) and 5α-androstan 3-β, 17β-diol (3β-diol) (Fig. 5). Blood levels were below limit of quantification for dehydroepiandrosterone (DHEA), androst-ene-3β, 17β-diol (5-diol), androstenedione and estrone (Fig. 5).

The 17β-estradiol treatment significantly increased this steroid's plasma concentration, compared to intact and MPTP treated mice that had low 17β-estradiol levels (Fig. 6B). 17β-estradiol-treated MPTP WT mice had similar plasma levels to that of 17β-estradiol treated intact WT mice. By contrast, 17β-estradiol-treated MPTP ERKOα mice had higher plasma 17β-estradiol levels than the 17β-estradiol treated ERKOα mice, while the opposite was seen for ERKOβ mice.

Testosterone plasma levels were higher in ERKOα mice than in WT or ERKOβ mice, the latter two groups having similar values

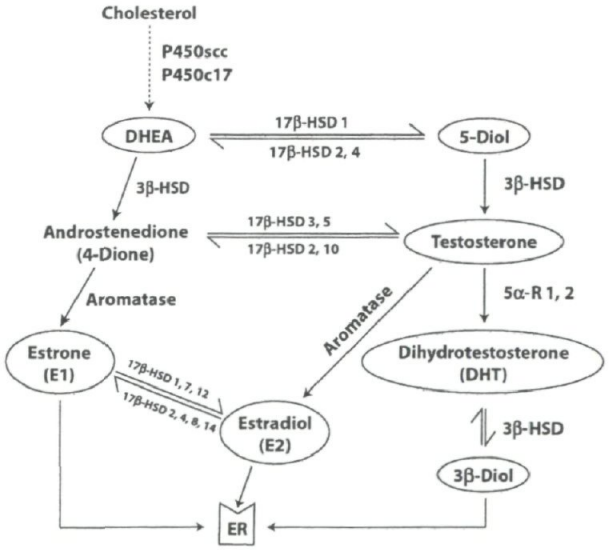

Fig. 5. Simplified schematic representation of biosynthesis of the steroids assayed. The dotted arrow implies that several stages and intermediate steroids were implicated in the biosynthesis of the final product. Encircled steroids were assayed. Testosterone is converted into 17β-estradiol and 3β-diol through the actions of 5α-R, aromatase and 3β-HSD (Gruber et al., 2002). Estrone is produced from DHEA by the enzymes 3β-HSD and aromatase (Gruber et al., 2002). P450scc, cytochrome P450 sidechain cleavage; P450c17, cytochrome P450 17α-hydroxysteroid/C17, 20-lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-R, 5α-reductase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; 3β-diol, 5α-androstan 3-β, 17β-diol; E1, estrone, ER, estrogen receptor.

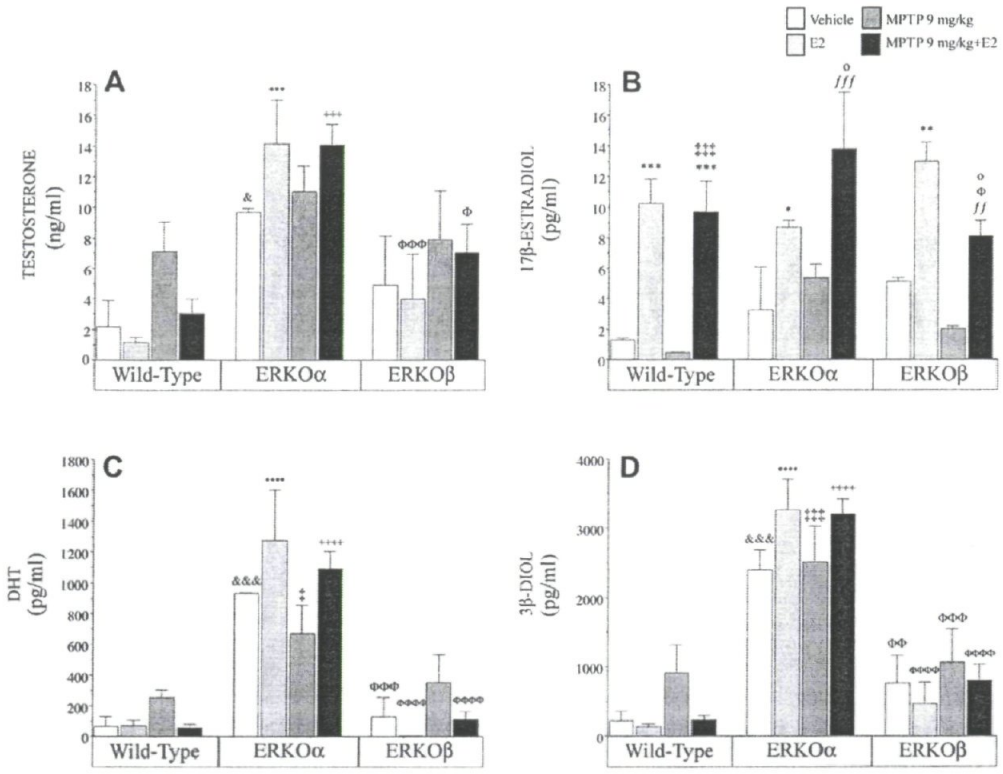

Fig. 6. Effect of MPTP (9 mg/kg) and 17β-estradiol treatment on blood plasma levels of (A) testosterone ($F_{11,22} = 4.20$, $p = 0.0021$), (B) 17β-estradiol (E_2) ($F_{11,21} = 7.22$, $p < 0.0001$), (C) DHT ($F_{11,22} = 10.57$, $p < 0.0001$) and (D) 3β-Diol ($F_{11,22} = 12.04$, $p < 0.0001$) of wild-type compared to ERKOα and ERKOβ mice. Plasmas of three mice were pooled (according to similar striatal DA concentrations) in order to produce quantities sufficient for steroid detection and quantification. $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ vs respective intact, vehicle (0 MPTP); $‡p < 0.05$ and $‡‡‡p < 0.005$ vs WT MPTP; $•••p < 0.005$ and $••••p < 0.0001$ vs WT + E_2; $+++p < 0.005$ and $++++p < 0.0001$ vs WT + MPTP + E_2; $\&p < 0.05$ and $\&\&\&p < 0.005$ vs WT vehicle (0 MPTP); $op < 0.05$ vs respective ERKO E_2; $fffp < 0.01$ and $ffffp < 0.005$ vs respective ERKO MPTP; $\Phi p < 0.05$, $\Phi\Phi p < 0.01$, $\Phi\Phi\Phi p < 0.005$ and $\Phi\Phi\Phi\Phi p < 0.0001$ vs respective experimental ERKOα genotype group.

(Fig. 6A). A similar pattern was observed for DHT and 3β-diol plasma levels that were also higher in ERKOα mice when compared to WT and ERKOβ mice (Fig. 6C and D). The latter plasma steroid concentrations were also of similar levels in WT and ERKOβ mice. Both striatal DA concentrations (Fig. 7A and B) and SN TH mRNA levels (Fig. 7C and D) correlated negatively with either testosterone or 3β-diol plasma concentrations.

4. Discussion

In DA neurons, the DAT is the port of entry of several toxins such as MPTP (Dauer and Przedborski, 2003). This transporter is abundant in DA neurons of the nigro-striatal pathway and has been used to target these neurons to model their loss as occurs in PD (Dauer and Przedborski, 2003). In post-mortem brains of PD patients, opposing roles of DAT and VMAT2 are documented where lower DAT levels decrease toxin entry in DA neurons and higher VMAT2 concentrations promote the sequestering of these toxins in cell bodies of remaining DA neurons that resisted to the PD pathology (Joyce et al., 1997). This supports a role for these transporters in the vulnerability of DA neurons that was explored in the present study in relation to estradiol-induced neuroprotection.

In response to a variety of toxins, striatal DA terminals are more sensitive to damage than their cell bodies in the SN (Bywood and Johnson, 2000). Striatal DAT and VMAT2 are located on DA terminals and were used here as markers of terminal loss by MPTP toxicity. In neuroprotection against MPTP in mice treated with 17β-estradiol there is a high correlation between striatal DA loss and/or protection with striatal DAT and VMAT2 specific binding, suggesting that in this paradigm changes in their specific binding are associated with the loss or sparing of striatal DA terminals. In intact female rats, we observe that DAT density varies along with the estrous cycle (Morissette and Di Paolo, 1993), while acute and chronic 17β-estradiol treatment increases striatal DAT specific binding in rats (review: Sanchez et al., 2010). ERs regulate most of the effects of estrogens and ERβ effects are not highly feminizing (Gruber et al., 2002). In a rat animal model, we saw that ERβ regulates a 17β-estradiol-induced increase of striatal DAT expression, therefore it is possible that a lack of ERs would lower DAT levels (Le Saux and Di Paolo, 2006). Thus, it was interesting to investigate the roles of ERs in mediating the neuroprotective effects of 17β-estradiol against the loss of DA nerve terminals caused by MPTP in male mice. Two complementary paradigms were used. First, we investigated whether genetic deletion of either ERα or ERβ altered the vulnerability of DA markers to MPTP. We then determined whether deletion of ERs altered the sensitivity to the neuroprotective effects of exogenous 17β-estradiol treatment.

In intact male mice, the lack of an ER affected striatal DAT and VMAT2 specific binding and the differences observed in the MPTP dose–response study between the WT and ERKO mice could be influenced by the initial differences of density of these markers. In 2 year-old ERKOβ mice of a different background than ours, Wang et al. found shrinkage of SN neurons as measured with Nissl staining compared to WT mice (Wang et al., 2001). In 22 months old ERKOβ mice we found a small reduction of SN TH mRNA levels

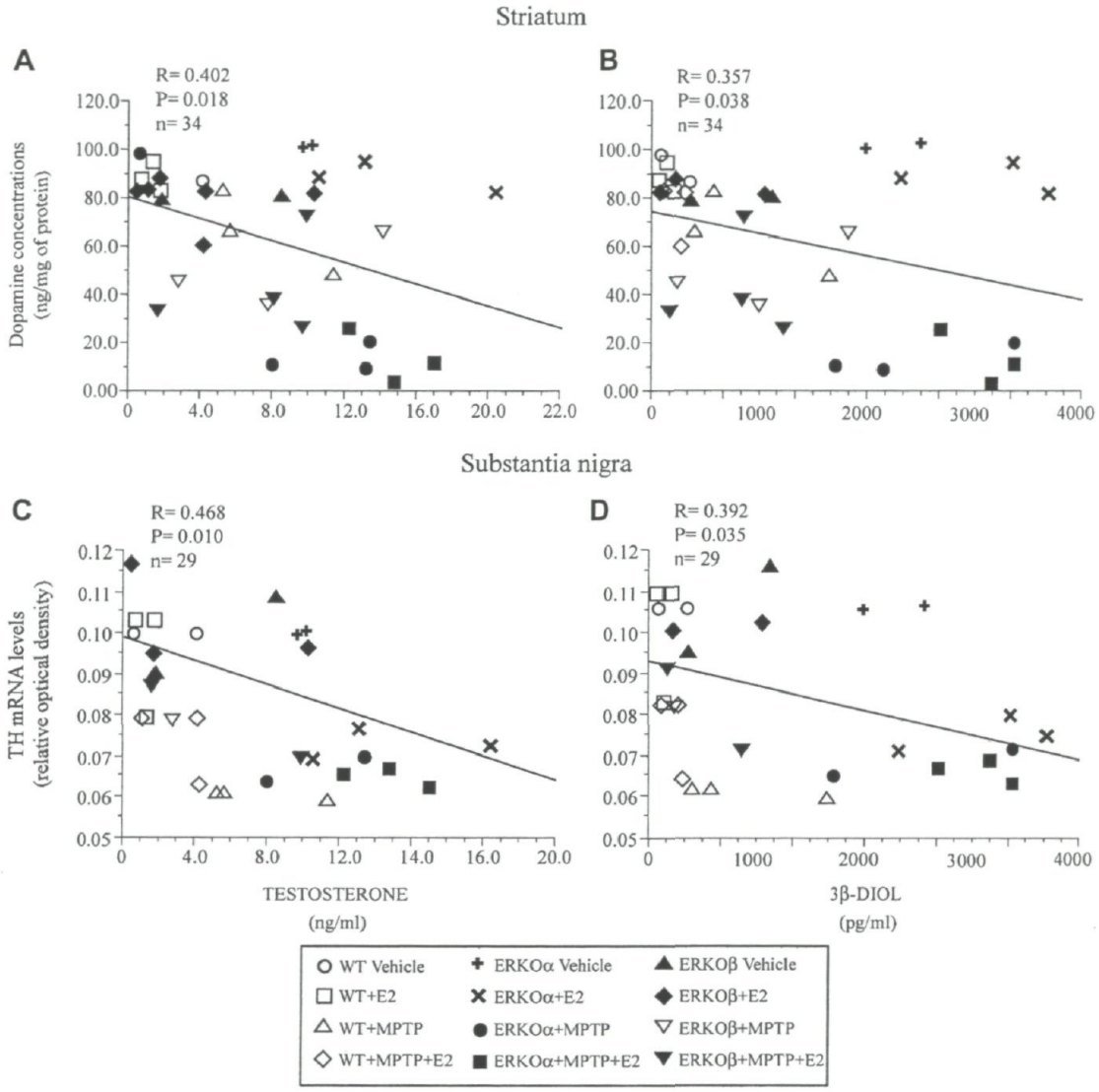

Fig. 7. Correlations between striatal DA concentrations (A and B) and SN TH mRNA levels (C and D) and testosterone or 3β-diol plasma levels of WT, ERKOα and ERKOβ mice.

compared to young mice (Morissette et al., 2008). No change of SN TH mRNA was observed in our young ERKOβ mice compared to WT or ERKOα mice but they showed lower striatal DAT and VMAT2 specific binding compared to the other genotypes. Compared to WT and ERKOβ mice, the ERKOα mice, having higher striatal DAT but not VMAT2 specific binding, showed a greater susceptibility to MPTP with decreases of striatal DAT and VMAT2 at lower MPTP doses. The results follow the pattern we previously reported in these mice with their striatal DA levels (Morissette et al., 2007) and a significant correlations between striatal DAT and VMAT2 specific binding and DA levels in these mice were noted. Hence, changes of striatal DAT and VMAT2 specific binding is likely reflecting the extent of loss or sparing of DA terminals in the striatum. The present results showed that treatment with 7–11 mg/kg of MPTP spared the SN of WT mice, providing conditions where neurons are injured with loss of terminals but could be healed. It is noteworthy that the ERα had a protecting role here in male mice suggesting that endogenous estrogens acting on ERα play an important role in neuroprotection.

When mice were administered a small dose of MPTP, differences in response to 17β-estradiol treatment emerged between the genotypes that was less prominent under basal conditions. 17β-estradiol protected against the MPTP-induced loss of DAT and VMAT2 specific binding and TH mRNA in WT but not in ERKOα or ERKOβ mice. Preventing the decrease of striatal DAT and VMAT2 levels as well as DA concentrations is likely due to neuroprotection of DA neurons rather than activity on DA synthesis and/or metabolism enzymes since all these markers were protected in WT mice. Our results show that ERα plays an important role against striatal MPTP toxicity, whereas the ERβ appears to play a subtler role. ERKOα mice were more sensitive than WT and ERKOβ mice to the effects of MPTP on the dopaminergic system. ERKOβ were also vulnerable to the effects of MPTP, but their response to the neurotoxin was more similar to that of WT mice. Hence, endogenous and exogenous estrogens acting on ERs are shown here to play an important protective role against MPTP toxicity and ERα plays the most significant role in mediating this protective effect. ERβ is also implicated in modulating the sensitivity of DA neurons to MPTP toxicity, since the protective

effects of 17β-estradiol were also absent in ERKOβ mice. Hence, ERα and ERβ mechanisms of action likely differ. In estradiol-induced neuroprotection against cortical injury, ERα was documented to have an essential role against ischemia induced cell death and ERβ activity was necessary during the recovery phase (Dubal et al., 2006). This could also be the case in MPTP toxicity. The absence of ERβ would leave only the early ERα activity dependent phase of neuroprotection. Our laboratory documented that an ERβ but not an ERα agonist can regulate rat striatal D2 DA receptors and DAT levels (Le Saux et al., 2006). Thus, in 17β-estradiol-induced neuroprotection of DA neurons, ERα would play the primary role against MPTP toxicity and ERβ supports these neurons' equilibrium, activity and recovery.

Estrogen effects on nigro-striatal DA is well documented (review: Bourque et al., 2009), even though ERα and ERβ are scarce within the striatum and that their expression is not increased with the MPTP lesion or an estradiol treatment (Shughrue, 2004). Neural tissues express multiple ERs including many spliced variants of both ERα and ERβ (Toran-Allerand, 2004). The structure-activity of estrogenic compounds for striatal neuroprotection (Callier et al., 2000; Jourdain et al., 2005) points to an effect via ERs. ERs are present in the nucleus and also at the membrane level (Marin et al., 2006; Mhyre and Dorsa, 2006; Mitra et al., 2003). Membrane ERα and ERβ were shown to be homologous to their intracellular counterparts (Marin et al., 2006) suggesting a common origin. Hence ERKOα and ERKOβ mice likely also lack their respective membrane ERα and ERβ. Accumulating evidence support an important trophic and protective role in the brain of estrogens acting on membrane ERs through various signal transduction pathways (Mhyre and Dorsa, 2006). Perhaps the scarce nigro-striatal ERs, localized on plasma membranes, and their interaction with signaling molecules confers signal amplification but this localization may render them more difficult to detect. Our results on neuroprotection by 17β-estradiol in MPTP mice show an implication of PI3K/Akt pathway shared by the ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) (D'Astous et al., 2006). These data support the implication of a membrane ERα in MPTP neuroprotection. The implication of ERβ in MPTP neuroprotection may involve different signaling pathways in neurons as well as in glia (Mhyre and Dorsa, 2006). Estradiol is demonstrated to activate both extracellular-signal-regulated kinase (ERK) and Akt signaling pathways in the same population of cortical neurons within a time frame consistent with that of membrane ER signaling, this implies that estradiol-induced neuroprotection is mediated by membrane ER-induced signaling (Mannella and Brinton, 2006).

Our laboratory previously and extensively published on the neuroprotective effects of 17β-estradiol administration in MPTP treated mice (review: Bourque et al., 2009) and the androgens such as testosterone and DHT had no beneficial effects (Ekue et al., 2002). Nevertheless, endogenous levels of these steroids have not been previously investigated in this paradigm. We thus sought if the endocrine status of these animals was altered with the MPTP lesion and/or the ERα and ERβ knockouts.

The overall results of assayed steroid plasma levels reveal that ERKOα mice had elevated testosterone, DHT and 3β-diol concentrations when compared to WT and ERKOβ mice. This is in agreement with a previous report of a significant increase of testosterone in ERKOα male mice when compared to WT males (Rissman et al., 1997). Moreover, we saw that the loss of nigro-striatal DA significantly inversely correlates with testosterone and 3β-diol plasma levels.

We observed an increase of 17β-estradiol plasma levels in mice treated with 17β-estradiol in all genotypes of mice investigated; the lack of response of ERKO mice to the 17β-estradiol treatment was therefore not because of lower circulating concentrations of 17β-estradiol due to higher metabolism of exogenous administered 17β-estradiol in the knock out mice. Nevertheless we observed some differences in the plasma 17β-estradiol concentrations, being higher in ERKOα and lower in ERKOβ MPTP mice compared to the unlesioned 17β-estradiol treated respective ERKO mice. We also observed an inverse correlation between SN TH mRNA and the plasma levels of testosterone and 3β-diol. The observation that striatal DA and SN TH mRNA decrease as testosterone levels increase is consistent with our previous findings showing lower SN DAT mRNA levels in testosterone treated MPTP male mice (Ekue et al., 2002). In addition, the deleterious effect of testosterone was also observed in methamphetamine toxicity on mice (Lewis and Dluzen, 2008). In humans, this may also be consistent with a higher incidence of PD in men than women (Wooten et al., 2004).

We find an important role of ERα and a lesser role of ERβ in DA sensitivity to MPTP in male mice, while androgens have no affinity for this receptor. This shows the importance of ER in male mice and a possible target for neuroprotection. While estradiol treatment in males may not be acceptable, a selective estrogen receptor modulator, raloxifene was administered to men without deleterious effects. Raloxifene administered for 3 months in healthy elderly men was reported, using functional magnetic imaging, to enhance brain activation in various cortical areas, suggesting an effect on cortical arousal; no significant side-effects were reported (Goekoop et al., 2005). Hence, raloxifene is showing beneficial effects in aged men. Therefore, drugs with estrogenic activity such as raloxifene, that we reported to protect against MPTP toxicity in intact male mice (Callier et al., 2001), could be useful in men since androgens have no protective activity (Ekue et al., 2002).

In conclusion, our results suggest an important role of ERs on brain nigro-striatal DA markers of male mice: 1- Intact ERKOβ mice had striatal DA transporter levels lower than WT and ERKOα mice. 2- ERα neuroprotective activity is dominant since ERKOα mice are more susceptible to MPTP toxicity, as assessed with loss of DAT and VMAT2 transporters specific binding, than WT or ERKOβ mice, but both ERs are necessary for neuroprotection because exogenous 17β-estradiol fails to protect ERKOα or ERKOβ mice from MPTP toxicity. 3- ERKOα mice had elevated plasma androgen concentration compared to WT and ERKOβ mice and 4- both striatal DA concentrations and SN TH mRNA levels correlated negatively with either testosterone or 3β-diol plasma concentrations of mice of all three genotypes. In brief, the absence of ERα in male mice resulted in alteration of basal plasma steroid concentrations and levels of striatal DA transporters along with highest susceptibility to MPTP. The absence of ERβ in male mice resulted in lower basal striatal DA transporter levels and higher susceptibility to MPTP than WT mice.

Disclosure statement

There are no conflicts of interest for any of the authors of this manuscript.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.neuropharm.2011.04.031.

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ORIGINAL ARTICLE

Effect of Oestrogen Receptors on Brain NMDA Receptors of 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mice

S. Al-Sweidi*†, M. Morissette* and T. Di Paolo*†

*Endocrinology and Genomics Research Axis of the CHUQ, CHUL, Quebec City, Quebec, Canada.

†Faculty of Pharmacy, Laval University, Quebec City, Quebec, Canada.

Journal of Neuroendocrinology

Parkinson's disease (PD) is characterised by the loss of nigrostriatal dopamine (DA) neurones and glutamate overactivity. There is substantial evidence to suggest that oestrogens prevent or delay the disease. 17β-oestradiol has neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD and modulates brain NMDA receptors. In MPTP-lesioned mice, oestrogen receptor (ER)α and ERβ are important in 17β-oestradiol-induced neuroprotection. To evaluate the role of ERs in the response of NMDA receptors to lesion, we compared wild-type (WT) with ER knockout (KO) C57Bl/6 male mice that received 7, 9 or 11 mg/kg of MPTP. These mice were also treated with MPTP (9 mg/kg) and 17β-oestradiol. [^{3}H]Ro 25-6981 specific binding autoradiography was used to label NMDA receptors containing NR2B subunits. In the frontal and cingulate cortex and striatum, vehicle-treated WT mice had higher [^{3}H]Ro 25-6981 specific binding compared to ERKO mice. Cortical [^{3}H]Ro 25-6981 specific binding decreased with increasing doses of MPTP in WT and ERKOα but not ERKOβ mice, whereas a dose-related decrease was only observed in the striatum of WT mice remaining low in ERKOα and ERKOβ mice. No effect of 17β-oestradiol treatment in intact or MPTP-lesioned mice of all three genotypes was observed in the cortex, whereas it increased striatal specific binding of intact ERKOβ and MPTP-lesioned WT mice. Striatal [^{3}H]Ro 25-6981 specific binding positively correlated with striatal DA concentrations only in WT mice. MPTP and 17β-oestradiol treatments had more limited effects in the hippocampus. Only in the CA3 and dentate gyrus did vehicle and 17β-oestradiol-treated ERKOα mice have higher [^{3}H]Ro 25-6981 specific binding than WT and ERKOβ mice, whereas MPTP decreased this specific binding only in the CA1, CA2 and CA3 of ERKOα mice. Hence, brain NMDA receptors were affected by the deletion of ERs, which affect the response to MPTP and 17β-oestradiol treatments with brain region specificity.

Key words: Glutamate receptor, Parkinson's disease, estrogen receptor knockout mouse, cortex, striatum, hippocampus.

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Correspondence to:
Thérèse Di Paolo, Molecular Endocrinology and Genomic Research Centre, CHUQ-CHUL, 2705 Laurier Boulevard, Quebec City, QC, G1V 4G2, Canada (e-mail: therese.dipaolo@crchul.ulaval.ca).

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and its incidence is likely to increase as a result of the ageing population (1). PD principally involves the death of dopamine (DA) neurones in the substantia nigra (SN), although other neurotransmitters and neuromodulators are also affected. Gene mutations in familial PD are reported, although the cause of the majority of PD cases is currently unknown (2) and there is no cure for PD. Neuroprotection or disease modification defined as an intervention that would protect or rescue vulnerable neurones (thereby slowing, stopping or reversing disease progression) is not yet available for PD (2). Restoring deficient DA with its precursor levodopa remains the most effective PD treatment, although a majority of patients develop abnormal involuntary movements called dyskinesias after 5–10 years of treatment; dyskinesias are very difficult to manage (3, 4). No drug is yet available for dyskinesias, aside from a modest benefit with amantadine, a drug with antagonistic glutamate activity, in some PD patients (5). Moreover, although levodopa and DA agonists, currently used in the treatment of PD, are effective at

reversing the motor symptoms of the disease, they do little to combat the underlying degeneration of DA neurones.

Glutamate is the most abundant excitatory neurotransmitter, mediating as much as 70% of brain synaptic transmission (6). Glutamate activity is increased in the basal ganglia in PD (6). The striatum receives two major inputs: a massive excitatory glutamatergic projection from the cerebral cortex and a dopaminergic projection from the SN (7). In PD, a loss of striatal DA is associated with the loss of the inhibitory DA control of cortico-striatal glutamatergic drive and also with the consequent increased glutamate release (8).

Ionotrophic glutamate receptors mediate fast excitatory neurotransmission and include NMDA, AMPA and kainate receptors (9, 10). Reducing glutamate overactivity can be achieved by blocking post-synaptic glutamate receptors with antagonists. Ionotrophic glutamate receptor antagonists, mainly NMDA and AMPA receptor antagonists, have been investigated, although they cause significant adverse effects in their different clinical applications, including PD, which limits their usefulness (11, 12).

Oestrogens induce their transcriptional activity through two distinct classical receptors: oestrogen receptor (ER)α and ERβ. Both ERα and ERβ mRNAs are expressed in the cortex (13) and in GABAergic neurones of the striatum (14). Immunohistochemistry and *in situ* hybridisation confirm that both ERs are present in the hippocampus (15). Furthermore, ERα and ERβ are present in the SN (16) and their colocalisation with tyrosine hydroxylase was observed in the ventral tegmental area and the SN of the rat brain (17).

Oestrogens are well documented to be neuroprotective of nigrostriatal DA against various toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and methamphetamine (18, 19). Oestrogens have been shown to modulate NMDA and AMPA receptors in the prefrontal and cingulate cortex, as well as in the striatum and hippocampus of ovariectomised female rats (20, 21), increasing or decreasing these receptors depending on the brain region. The neuromodulatory effects of oestrogenic drugs on glutamate receptors in male mice is well documented (21), although little information is available on the oestrogenic modulation of glutamate receptors in animal models of PD. Oestrogens acting through their respective receptors can modulate glutamate receptors; therefore, the hypothesis is that oestrogenic modulation of glutamate receptors plays a role in oestrogenic neuroprotection. The present study aimed to investigate the effect of 17β-oestradiol treatment on NMDA receptors in intact mice and mice lesioned with MPTP, as well as the contribution of ERα and ERβ, by comparing wild-type (WT) and ER knockout (KO) male mice.

Materials and methods

Animals and treatments

Adult WT, ERKOα and ERKOβ male C57Bl/6 mice (7–12 weeks, 18–28 g, WT and ERKO mice) were purchased from Taconic Laboratories (Hudson, NY, USA). MPTP and 17β-oestradiol were purchased from Sigma Chemical (St Louis, MO, USA). WT and ERKO mice were equally distributed for age and weight in experimental groups of six animals. The Laval University Animal Care Committee approved all the animal studies. All efforts were made to minimise animal suffering and to reduce the number of mice used.

An extended MPTP dose-response up to 20 mg/kg was performed in WT male C57Bl/6 mice and striatal biogenic amine concentrations of these mice was previously reported (22). The MPTP doses (7,9 and 11 mg/kg), that specifically affected striatal DA while sparing serotonin concentrations in WT mice, were used for comparison of MPTP dose-responses of ERKOα, ERKOβ and WT mice (22). Mice received four 0.1-ml i.p. injections with saline or a saline solution of MPTP at a 2-h interval and were killed 5 days after treatment with MPTP.

The effect of 17β-oestradiol and MPTP toxicity in ERKOα and ERKOβ was compared with WT mice. Four groups of both ERKOα and ERKOβ mice were compared with WT mice. An intermediate dose of 9 mg/kg MPTP was selected and the effect of 17β-oestradiol treatment was investigated in intact and MPTP mice. Each group received a 5-day pre-treatment of 17β-oestradiol or vehicle before the MPTP injections. The pre-treatment consisted of two daily s.c. injections (in the dorsal part of the neck) of 17β-oestradiol, whereas control mice received injections of vehicle (0.9% saline with 0.3% gelatin). Physiological concentrations of 17β-oestradiol were used (2 μg/day) as reported previously (22, 23). 17β-oestradiol at 1–2 μg are beneficially effective against neurotoxicity (19).

On day 5, mice received four injections of MPTP (9 mg/kg, per i.p. injection) at a 2-h interval, whereas the control group received saline solution. Treatments with 17β-oestradiol or vehicle were continued until day 10. The next day, mice were euthanised with an air/halothane mixture and decapitated; trunk blood was collected and brains were quickly removed and frozen in a mixture of isopentane/dry ice and then stored at −80 °C.

Preparation of brain tissue

Frozen brains were cut on a cryostat in 12-μm thick slices at cortical, striatal and hippocampal regions. Coronal sections for the anterior cortex (bregma 2.46 mm), anterior striatum (bregma 1.18 mm) and hippocampus (bregma −1.94 mm) were cut according to the mouse brain atlas of Franklin and Paxinos (24). Histological sections were thaw-mounted on superfrost slides (Trade Mark, Fisher, ON, Canada) and stored at −80 °C.

[^{3}H]Ro 25-6981 autoradiography

Autoradiography using [^{3}H]Ro 25-6981 (26 Ci/mmol, gift from F. Hoffman-La Roche Ltd, Basel, Switzerland), an NMDA antagonist selective for the NR1/NR2B assembly, was performed on the cortex, striatum and hippocampus in accordance with our previously published procedure (20). The slides were briefly preincubated twice for 10 min in a Tris–HCl 50 mM, ethylenediaminetetraacetic acid (EDTA) 10 mM (pH 7.6) buffer at room temperature. Then sections were incubated with 5 nM of [^{3}H]Ro 25-6981 in the same buffer for 90 min at 4 °C. Afterwards, the sections were rinsed three times (2 × 5 min and 1 × 15 min) in the Tris–HCl/EDTA buffer at 4 °C. Nonspecific binding was determined by adding 10 μM Ro 04-5595 hydrochloride (gift from F. Hoffman-La Roche Ltd) to the incubation buffer. Slides were exposed to Kodak Biomax MR film (Kodak, Rochester, NY, USA) with calibrated [^{3}H] standards (Microscales; Amersham, Arlington Heights, IL, USA). Cortical, striatal and hippocampal slides were exposed for 35 days and [^{3}H]Ro 25-6981 specific binding was calculated by subtracting the measured nonspecific binding from the total binding.

Statistical analysis

Autoradiograms of specific binding relative optical densities were analysed using SCION IMAGE, version 1.63 (Scion Corp., Frederick, MD, USA). The anterior cortex was subdivided into the cingulate and the frontal motor cortex, whereas the anterior striatum was subdivided in half into medial and lateral regions for analysis. The hippocampus was analysed in subregions including the CA1 strata oriens (or) and radiatum (rad), CA2 (or

and rad), CA3 and dentate gyrus. For each group, the mean of six to nine animals and the mean of six brain sections per animal were measured. The statistical analyses were performed using STATVIEW, version 4.51 (SAS Institute Inc., Cary, NC, USA). A one-way ANOVA was used to compare MPTP and/or 17β-oestradiol treatments followed by post-hoc analysis with a Fisher's least significance difference test. $P < 0.05$ was considered statistically significant.

Results

The effect of the MPTP lesion on NMDA receptors in the WT compared to the ERKO mice was first investigated and showed a difference between the genotypes. Control ERKOα mice had lower [^{3}H]Ro 25-6981 specific binding in the cortex for both the frontal ($F_{11,57} = 8.38$, $P < 0.0001$) and cingulate cortex ($F_{11,57} = 6.48$, $P < 0.0001$), at 82% and 88%, respectively, compared to control WT mice (Fig. 1). Moreover, compared to WT mice, the intact ERKOβ mice also had low NMDA receptor specific binding, at 79% in the frontal cortex and 81% in the cingulate cortex. A dose-related decrease of cortical NMDA receptor specific binding caused by MPTP was observed in ERKOα mice but not in ERKOβ mice. In the frontal cortex and cingulate, NMDA receptor specific binding of WT mice decreased only with 9 mg/kg of MPTP. In ERKOβ mice, NMDA receptor specific binding increased with 11 mg/kg of MPTP compared to the respective ERKOα experimental group in both cortical regions (Fig. 1).

In the MPTP and 17β-oestradiol treatment experiment, as in the MPTP dose-response experiment, vehicle-treated ERKOα mice had lower cortical [^{3}H]Ro 25-6981 specific binding, at 71% in the frontal cortex and 62% in the cingulate compared to control WT mice (Fig. 2). Similarly, lower values were also measured in the cortex of control ERKOβ mice compared to control WT mice, at 79% in the frontal cortex ($F_{11,72} = 5.02$, $P < 0.0001$) and 74% in the cingulate ($F_{11,75} = 8.77$, $P < 0.0001$) (Fig. 2). 17β-oestradiol treatment did not influence NMDA specific binding in the cortex of all three nonlesioned genotypes (Fig. 2). Nine milligrams per kilogram of MPTP increased NMDA specific binding only in ERKOβ mice and this effect was significant in the cingulate; however, treatment with 17β-oestradiol did not return NMDA receptor specific binding values of MPTP lesioned ERKOβ mice to those of the respective controls (Fig. 2B). No effect of 17β-oestradiol treatment was observed in WT and ERKOα MPTP lesioned mice.

Similar to the results observed in the cortex, lower values were also measured in the striatum of control ERKOα and ERKOβ mice compared to control WT mice (Fig. 3). In the MPTP dose-response experiment, control ERKOα mice had much lower striatal NMDA NR1/2B specific binding, at 57% in the medial striatum ($F_{11,58} = 10.42$, $P < 0.0001$) and 59% in the lateral striatum ($F_{11,58} = 12.95$, $P < 0.0001$) compared to control WT mice. Lower values were also measured in the anterior striatum of control ERKOβ mice, although to a smaller extent, at 72% in the medial striatum and 75% in the lateral striatum compared to control WT mice and significantly higher than that of control ERKOα mice (Fig. 3).

In addition, a dose-related decrease of striatal NMDA receptor specific binding with increasing doses of MPTP was observed in the striatum of WT but not in ERKOα and ERKOβ mice. MPTP had no dose-response effect in the striatum of ERKOα or ERKOβ mice. These effects are almost identical in the medial and lateral striatum (Fig. 3). Striatal [^{3}H]Ro 25-6981 specific binding positively correlated with striatal DA concentrations of these WT mice (Fig. 3C), whereas no correlation was obtained for ERKOα (Fig. 3D) or ERKOβ (Fig. 3E) mice.

In the MPTP lesion and 17β-oestradiol treatment experiment, similar to the MPTP dose-response experiment, vehicle-treated ERKOα mice had lower striatal NMDA NR1/2B specific binding, at 82% of vehicle-treated WT mice values in both the medial ($F_{11,78} = 13.62$, $P < 0.0001$) and lateral ($F_{11,78} = 13.76$, $P < 0.0001$) striatum (Fig. 4A,B). Lower values were also measured in the striatum of vehicle-treated ERKOβ mice, at 91% and 94% compared to

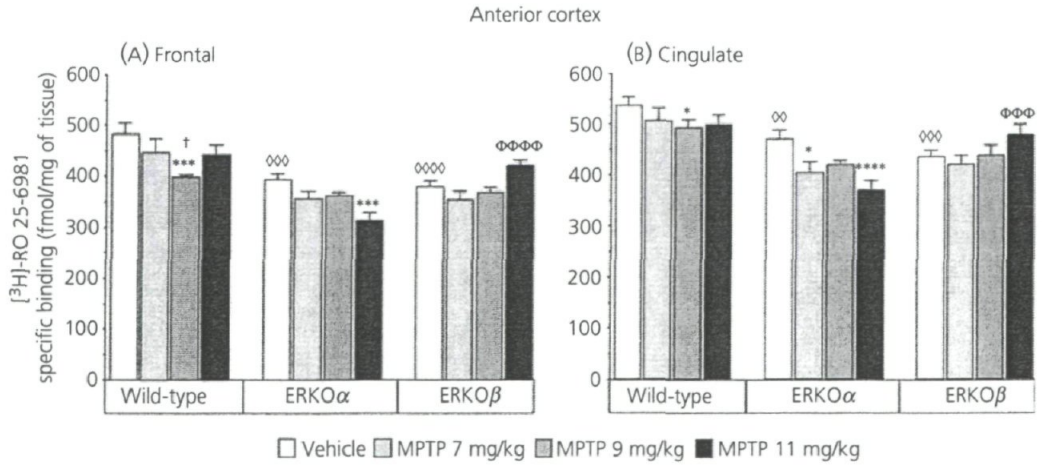

Fig. 1. Dose-response effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on cortical (A) frontal and (B) cingulate NMDA receptors measured with [^{3}H]RO 25-6981 NMDA-R2B antagonist specific binding in wild-type (WT) compared to oestrogen receptor knockout (ERKO)α and ERKOβ mice. *$P < 0.05$, ***$P < 0.005$ and ****$P < 0.0001$ versus respective intact, vehicle; $^{\dagger}P < 0.05$ versus WT + MPTP 7 mg/kg; $^{\diamond\diamond}P < 0.01$, $^{\diamond\diamond\diamond}P < 0.005$ and $^{\diamond\diamond\diamond\diamond}P < 0.0001$ versus WT vehicle; $^{\Phi\Phi\Phi}P < 0.005$ and $^{\Phi\Phi\Phi\Phi}P < 0.0001$ versus respective ERKOα experimental group.

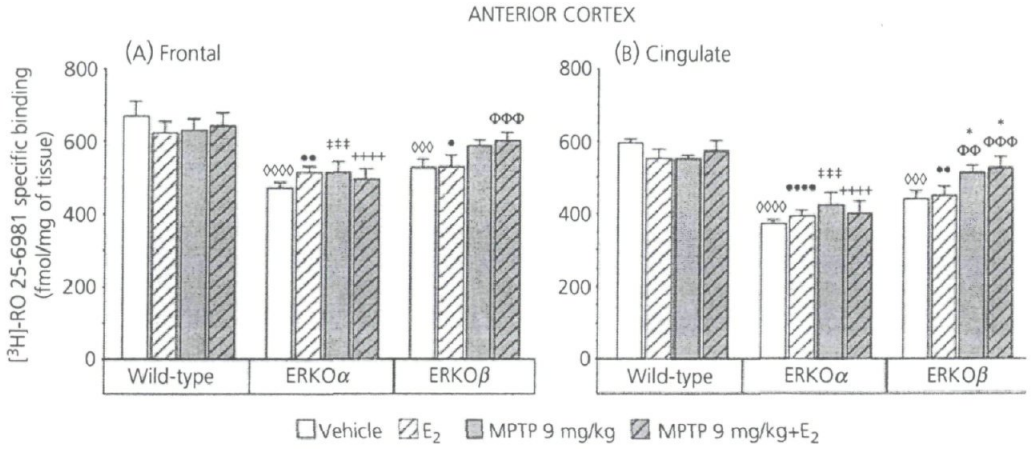

Fig. 2. Effect of treatment with 17β-oestradiol (E_2) 2 μg/day for 10 days on cortical (A) frontal and (B) cingulate [^{3}H]RO 25-6981 specific binding in intact and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (9 mg/kg) lesioned wild-type (WT), oestrogen receptor knockout (ERKO)α and ERKOβ mice. $^{*}P < 0.05$ versus respective intact, vehicle; $^{\ddagger\ddagger\ddagger}P < 0.005$ versus WT + MPTP; $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ and $^{\bullet\bullet\bullet\bullet}P < 0.0001$ versus WT + E_2; $^{++++}P < 0.0001$ versus WT + MPTP + E_2; $^{\diamond\diamond\diamond}P < 0.005$ and $^{\diamond\diamond\diamond\diamond}P < 0.0001$ versus WT vehicle; $^{\Phi\Phi}P < 0.01$ and $^{\Phi\Phi\Phi}P < 0.005$ versus respective ERKOα experimental group.

vehicle-treated WT mice in the medial and lateral striatum, respectively (Fig. 4).

The 17β-oestradiol treatment had no effect on nonlesioned WT and ERKOα mice, although it increased NMDA specific binding in the striatum (lateral as well as medial parts) of nonlesioned ERKOβ mice (Fig. 4A,B). MPTP (9 mg/kg) was only effective in WT mice, significantly decreasing striatal NMDA specific binding, and 17β-oestradiol co-treatment prevented this decrease. No effect of 17β-oestradiol treatment was observed in ERKOα or ERKOβ MPTP lesioned mice. These effects were closely identical in the medial and lateral parts of the striatum (Fig. 4A,B). In this experiment, striatal [^{3}H]Ro 25-6981 specific binding also positively correlated with striatal DA concentrations of these WT mice (Fig. 4C), whereas no correlation was obtained for ERKOα (Fig. 4D) or ERKOβ mice (Fig. 4E).

Although the cortical and the striatal NMDA receptors responded similarly to the absence of ERα or ERβ and were affected by the MPTP lesion, a different pattern was observed in the hippocampus. No effect of the lesion on NMDA specific binding at MPTP doses of 7, 9 and 11 mg/kg was observed in the CA1(or), CA2 (or and rad) and dentate gyrus, whereas a small decrease (17%) was observed in the CA1(rad) and CA3 (data not shown).

In the CA1 subregion of the hippocampus, (or: $F_{11,78} = 2.10$, $P = 0.03$; rad: $F_{11,78} = 3.82$, $P = 0.0002$), intact ERKOβ mice had lower (by 9%) NMDA receptor specific binding compared to intact ERKOα mice. 17β-oestradiol treatment had no effect on NMDA receptor specific binding for all three genotypes. MPTP alone and combined with the 17β-oestradiol treatment significantly decreased NMDA receptor specific binding of ERKOα mice in both the CA1 or and rad (Fig. 5A,B).

Similar results to the CA1 were observed in the radiatum ($F_{11,78} = 2.47$, $P = 0.01$) of the CA2 hippocampal subregion (Fig. 6B), as well as in the CA2 oriens ($F_{11,78} = 1.76$, $P = 0.07$), although this did not reach statistical significance in the latter (Fig. 6A).

Intact ERKOα mice had higher NMDA receptor specific binding levels compared to the nonlesioned WT and ERKOβ mice in both the CA3 ($F_{11,78} = 3.08$, $P = 0.001$) and dentate gyrus ($F_{11,78} = 2.80$, $P = 0.004$) (Fig. 7). Treatment with 17β-oestradiol did not affect NMDA receptor specific binding of all three nonlesioned genotype experimental groups. MPTP significantly decreased NMDA receptor specific binding of only the ERKOα mice in the CA3 hippocampal subregion, and its combination with 17β-oestradiol decreased these levels even more in both the CA3 and dentate gyrus (Fig. 7).

Summaries of the genotype differences in [^{3}H]Ro 25-6981 specific binding to NMDA receptors, as well as the differences in response to MPTP and 17β-oestradiol treatments in the brain regions investigated, of WT, ERKOα and ERKOβ mice are provided in Tables 1 and 2.

Discussion

The present study showed an oestrogen-glutamate interaction involving NMDA/NR2B receptors in the effects of MPTP and/or 17β-oestradiol treatment in male mice.

Effect of lesion on NMDA receptors

Overactive glutamate neurotransmission is well known in PD as a consequence of removing the inhibitory dopaminergic tone on cortico-striatal glutamate (25). NMDA and AMPA receptors specific binding were observed to be lower in the striatum of PD patients (26) and of MPTP-lesioned monkeys (27). The reduced striatal NMDA receptors in PD and MPTP-lesioned animals could be a compensatory response to oppose overactive glutamate transmission. As in human and nonhuman primates, reduced cortical and striatal NMDA receptors were also observed in WT MPTP-lesioned mice compared to control mice, which could be explained by a compensatory mechanism. By contrast, this response of NMDA receptors to

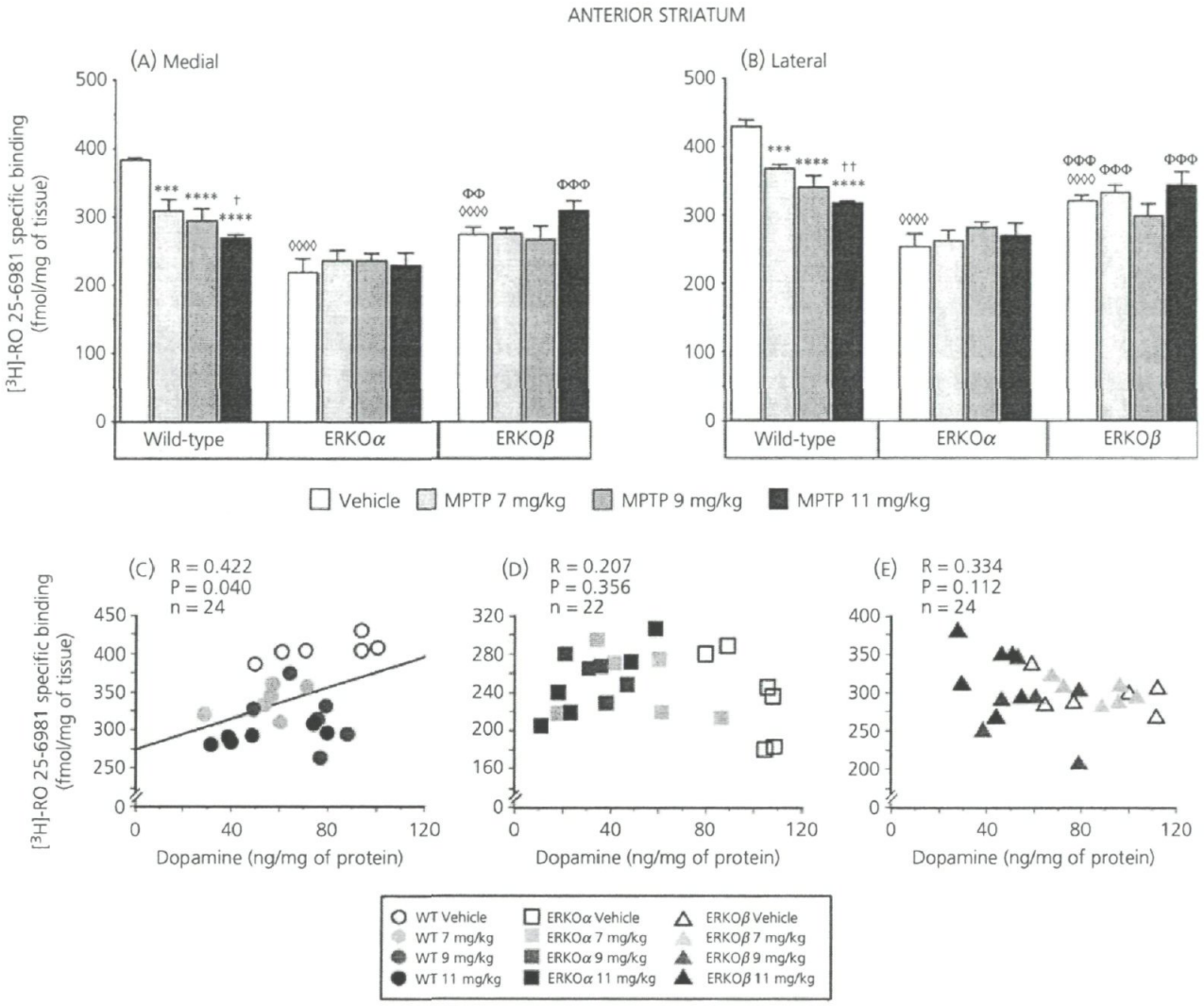

Fig. 3. Dose-response effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on (A) medial and (B) lateral striatal [^{3}H]RO 25-6981 specific binding in wild-type (WT) compared to oestrogen receptor knockout (ERKO)α and ERKOβ mice. (C, D, E) Correlations of striatal [^{3}H]RO 25-6981 specific binding with striatal dopamine concentrations in WT, ERKOα and ERKOβ mice. Striatal dopamine concentrations of these mice were measured previously (22). ***$P < 0.005$ and ****$P < 0.0001$ versus respective intact, vehicle; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ versus WT + MPTP 7 mg/kg; $^{\diamond\diamond\diamond\diamond}P < 0.0001$ versus WT vehicle; $^{\Phi\Phi}P < 0.01$ and $^{\Phi\Phi\Phi}P < 0.005$ versus respective ERKOα exprimental group.

glutamate overactivity in the striatum of WT mice was not observed in mice lacking ERα or ERβ, whereas, in the cortex, it is not observed in mice lacking ERβ, suggesting disrupted compensation.

Neurotransmitter and regional specificity of the MPTP lesion

We previously reported striatal concentrations of serotonin and its metabolites 5-hydroxyindoelacetic acid (5-HIAA) in these mice (22); they remained unchanged at doses of MPTP up to 11 mg/kg, suggesting that, under the present conditions, the lesion was specific to nigrostriatal DA, with serotonin remaining unchanged. Higher doses of MPTP will affect striatal serotonin and 5-HIAA concentrations, as previously reported (22). This is our first detailed investigation of brain regions other than the striatum and the SN using our MPTP mouse model of oestrogenic neuroprotection. A paradigm similar to ours, using acute lesioning with 20 mg/kg of MPTP of 8–10 week-old male C57BL/6 mice, provided high-performance liquid chromatography analysis reporting that DA concentrations are depleted in the striatum and frontal cortex (28). DA concentrations were low in the frontal cortex (4.0 ng/mg of protein) of control mice compared to the striatum (141 ng/mg protein) and, at 7 days post-MPTP, striatal DA was depleted by 95% in the dorsal striatum, by 86% in the ventral striatum and by 88% in frontal cortex (28). In their study, serotonin loss after MPTP was modest compared to that of DA, with a 48% loss in the dorsal striatum, a 39% loss in the ventral striatum and a 48% loss in the frontal cortex. Based on these results, in our experiment, frontal cortex DA

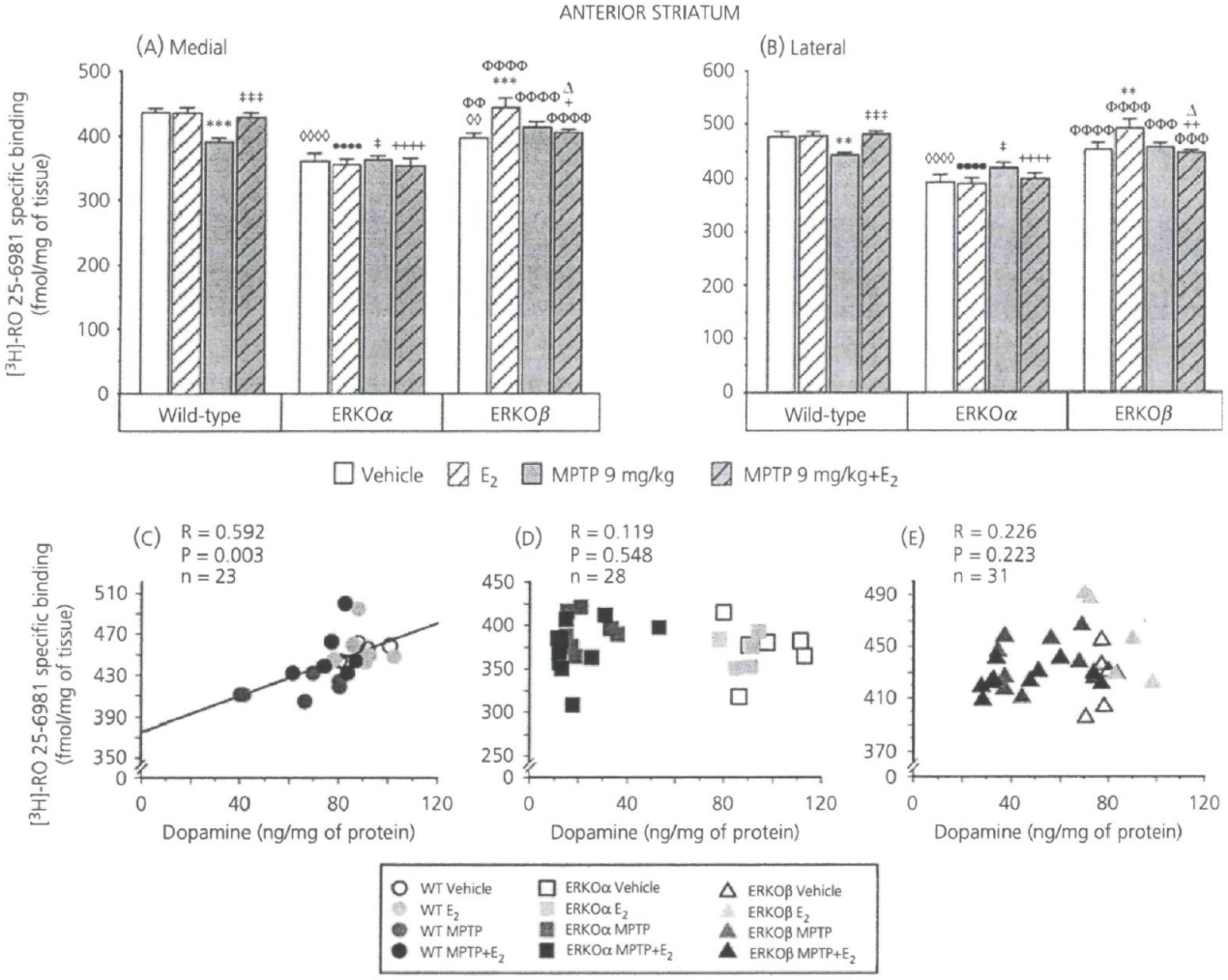

Fig. 4. Effect of treatment with 17β-oestradiol (E_2) 2 μg/day for 10 days on (A) medial and (B) lateral striatal [^{3}H]RO 25-6981 specific binding in intact and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (9 mg/kg) lesioned wild-type (WT), oestrogen receptor knockout (ERKO)α and ERKOβ mice. (C, D, E) Correlations of striatal [^{3}H]RO 25-6981 specific binding with striatal dopamine concentrations (22) in WT compared to ERKOα and ERKOβ mice. $^{**}P < 0.01$ and $^{***}P < 0.005$ versus respective intact, vehicle; $^{\ddagger}P < 0.05$ and $^{\ddagger\ddagger\ddagger}P < 0.005$ versus WT + MPTP; $^{\bullet\bullet\bullet\bullet}P < 0.0001$ versus WT + E_2; $^{\Delta}P < 0.005$ versus ERKOβ + E_2; $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{++++}P < 0.0001$ versus WT + MPTP + E_2; $^{\diamond\diamond}P < 0.01$ and $^{\diamond\diamond\diamond\diamond}P < 0.0001$ versus WT vehicle; $^{\Phi\Phi}P < 0.01$, $^{\Phi\Phi\Phi}P < 0.005$ and $^{\Phi\Phi\Phi\Phi}P < 0.0001$ versus the respective ERKOα experimental group.

depletion is likely to be similar to that seen in striatum, although both structures would be depleted to a lesser extent because we used lower doses of MPTP to be neurotransmitter specific for DA, leaving serotonin levels unchanged. Hence, the effects of MPTP on NMDA receptors in the frontal cortex could not only be a result of decreased cortical DA concentrations, but also be indirect because striatal DA concentrations are much higher than cortical levels and nigrostriatal DA exerts an inhibitory control on cortico-striatal glutamate (29).

In another paradigm that is also similar to ours, reduced striatal and hippocampal DA concentrations were measured in 8-week-old male C57BL/6 mice administered 20 mg/kg of MPTP (four i.p. injections 2 h apart) (30). DA concentrations were low in the hippocampus (0.25 μg/g) of control mice compared to the striatum (9.34 μg/g); at 7 days post-MPTP, striatal DA was depleted to 34% of controls and hippocampal DA concentrations were depleted to 52% of controls (30). Depletion of hippocampal DA levels was less than in the striatum, although it attenuated NMDA receptor mediated synaptic transmission, and altered the time course of long-term potentiation (LTP) and long-term depression (LTD) by impairing the induction of LTP and prolonging the duration of LTD in these mice (30). Therefore, DA loss alters NMDA receptor-mediated synaptic transmission and activity-dependent synaptic plasticity (30). Based on the results of a study by Zhu *et al.* (30), in our experiment, DA depletion was also likely to be less in the hippocampus than in the striatum and to be depleted to a lesser extent in both regions because we used lower doses of MPTP. This would be consistent with our observed general lack of effect of MPTP on NMDA receptors in the hippocampal subregions because DA depletion is likely modest. Similarly, the effects of MPTP in the hippocampus

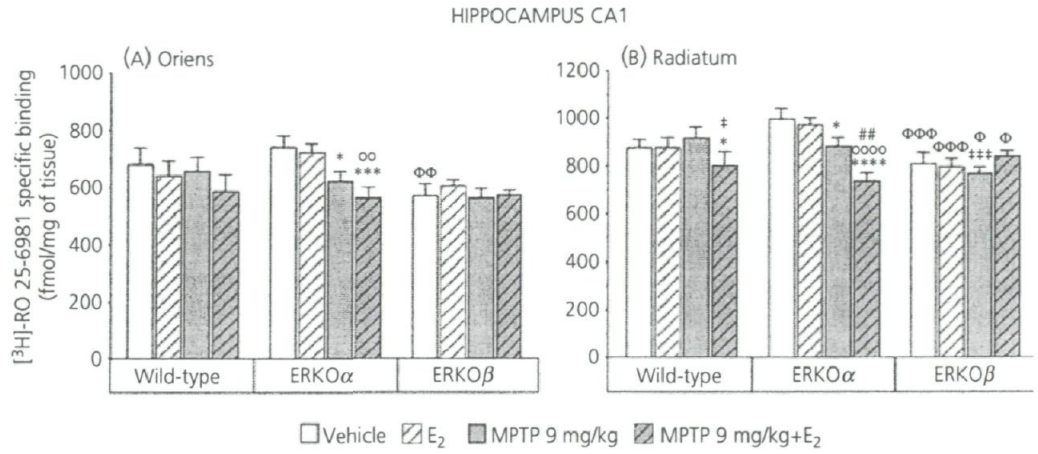

Fig. 5. Effect of treatment with 17β-oestradiol (E_2) 2 μg/day for 10 days on hippocampal CA1 (A) oriens and (B) radiatum [^{3}H]RO 25-6981 specific binding in intact and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (9 mg/kg) lesioned wild-type (WT), oestrogen receptor knockout (ERKO)α and ERKOβ mice. $^{*}P < 0.05$, $^{***}P < 0.005$ and $^{****}P < 0.0001$ versus respective intact, vehicle; $^{\ddagger}P < 0.05$ and $^{\ddagger\ddagger\ddagger}P < 0.005$ versus WT + MPTP; $^{\Phi}P < 0.05$, $^{\Phi\Phi}P < 0.01$ and $^{\Phi\Phi\Phi}P < 0.005$ versus respective ERKOα experimental group; $^{oo}P < 0.01$; $^{oooo}P < 0.0001$ versus ERKOα + E_2 and $^{\#\#}P < 0.01$ versus ERKOα + MPTP.

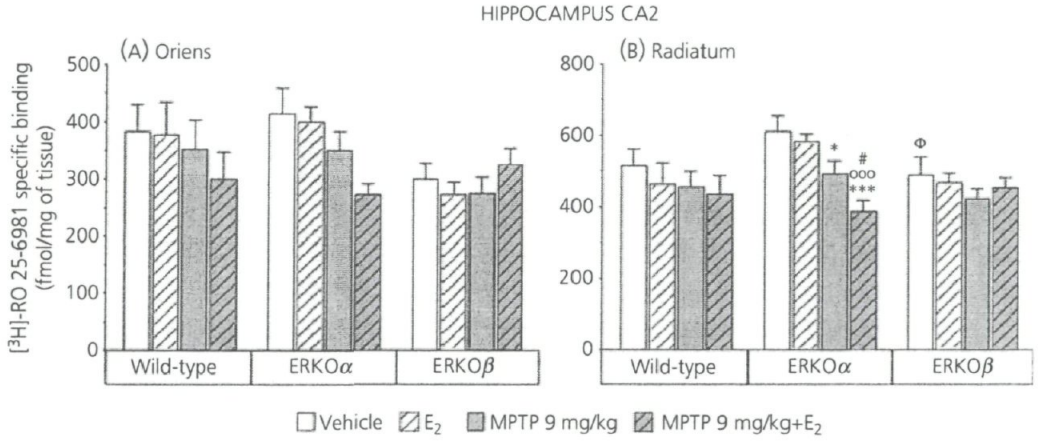

Fig. 6. Effect of treatment with 17β-oestradiol (E_2) 2 μg/day for 10 days on hippocampal CA2 (A) oriens and (B) radiatum [^{3}H]RO 25-6981 specific binding in intact and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (9 mg/kg) lesioned wild-type (WT), oestrogen receptor knockout (ERKO)α and ERKOβ mice. $^{*}P < 0.05$ and $^{***}P < 0.005$ versus respective intact, vehicle; $^{\Phi}P<0.05$ versus respective ERKOα experimental group; $^{ooo}P < 0.005$ versus ERKOα + E_2 and $^{\#}P < 0.05$ versus ERKOα + MPTP.

could not only be a result of decreased hippocampal DA concentrations, but also be indirect because striatal DA concentrations are much higher than the hippocampal levels.

Plasma 17β-oestradiol levels

We previously reported the blood plasma steroid concentrations of the mice used in the MPTP lesion and 17β-oestradiol treatments experiment (31). An increase of 17β-oestradiol plasma levels was measured in mice treated with 17β-oestradiol for all the genotypes investigated; the lack of response of ERKO mice to the 17β-oestradiol treatment was therefore not a result of the lower circulating concentrations of 17β-oestradiol because of the higher metabolism of exogenous administered 17β-oestradiol in these knockout mice. Nevertheless, we observed some differences in plasma 17β-oestradiol concentrations, which were higher in ERKOα and lower in ERKOβ MPTP mice compared to the respective unlesioned 17β-oestradiol-treated ERKO mice (22).

Effect of MPTP, 17β-oestradiol and gonadectomy on NMDA receptors

In 17β-oestradiol-treated and MPTP-lesioned WT mice, nigrostriatal DA degeneration is prevented and this is possibly associated with diminished cortico-striatal glutamate overactivity because DA is less depleted. Therefore, higher NMDA receptor specific binding may be a result of lesser decreased levels of this receptor to compensate for overactive glutamate activity because the effects of MPTP are

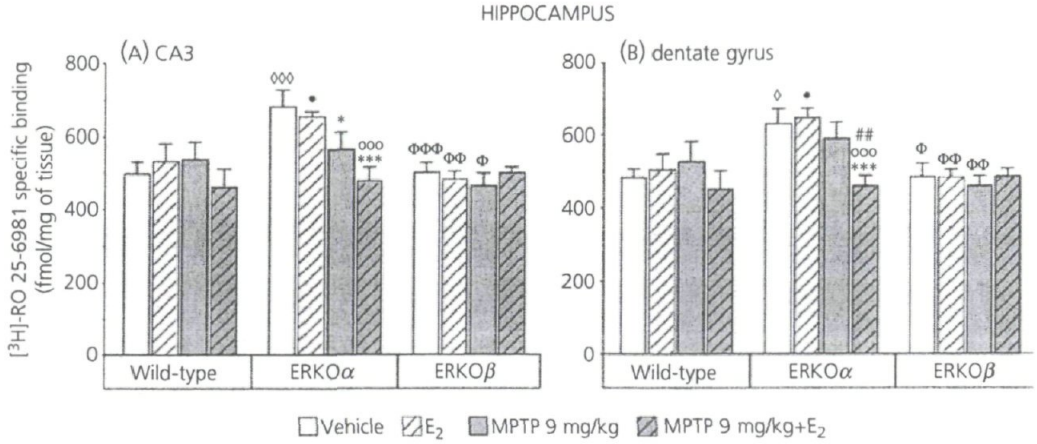

Fig. 7. Effect of treatment with 17β-oestradiol (E_2) 2 μg/day for 10 days on (A) hippocampal CA3 and (B) dendate gyrus [^{3}H]RO 25-6981 specific binding in intact and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (9 mg/kg) lesioned wild-type (WT), oestrogen receptor knockout (ERKO)α and ERKOβ mice. $^{*}P < 0.05$ and $^{***}P < 0.005$ versus respective intact, vehicle; $^{\diamond}P < 0.05$ and $^{\diamond\diamond\diamond}P < 0.005$ versus WT vehicle; $^{\bullet}P < 0.05$ versus WT + E_2; $^{\Phi}P < 0.05$, $^{\Phi\Phi}P < 0.01$ and $^{\Phi\Phi\Phi}P < 0.005$ versus respective ERKOα experimental group; $^{ooo}p<0.005$ versus ERKOα + E_2 and $^{\#\#}P < 0.01$ versus ERKOα + MPTP.

Table 1. Summary of Genotype Differences in Mouse Brain NMDA Receptors.

Brain region	ERKOα versus WT mice	ERKOβ versus WT mice	ERKOα versus ERKOβ mice
Cortex			
Frontal	–	–	0
Cingulate	–	–	0
Striatum			
Medial	–	–	–
Lateral	–	–	–
Hippocampus			
CA1 oriens	0	0	+
CA1 radiatum	0	0	+
CA2 oriens	0	0	0
CA2 radiatum	0	0	+
CA3	+	0	+
Dentate gyrus	+	0	+

0, –, +, No difference, lower and higher NMDA receptor [^{3}H]Ro 25-6981 specific binding levels. ERKO, oestrogen receptor knockout; WT, wild-type.

dampened by 17β-oestradiol-induced neuroprotection. Higher NMDA receptor specific binding may also be a result of the effect of 17β-oestradiol increasing the synthesis of this receptor. Accordingly, using specific agonists for ERα and ERβ, we reported that ERα agonists protect against MPTP toxicity in mice (32), ERKOα mice are more sensitive to the loss of striatal DA concentrations by MPTP, and both ERKOα and ERKOβ mice are not protected by 17β-oestradiol against MPTP-induced striatal DA loss (22).

We previously reported the effect of ovariectomy and oestrogen treatment in ovariectomised rats on NMDA receptors containing NR2B subunits using [^{3}H]Ro 25-6981 binding autoradiography and *in situ* hybridisation of NMDAR1/2B subunits (20, 33, 34). In the cingulate and the prefrontal cortex, ovariectomy increased [^{3}H]Ro 25-6981 specific binding compared to intact rats and this was corrected with a 17β-oestradiol treatment, whereas it was not significant with the ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) or the ERβ agonist 2,3-bis(4-hyroxyphenyl) propionitrile (DPN) (21). Both the selective oestrogen receptor modulators tamoxifen and raloxifene, similar to 17β-oestradiol, decrease [^{3}H]Ro 25-6981 specific binding in the cingulate cortex of ovariectomised rats and increase this binding in hippocampal regions (20). We also investigated NMDA receptors in intact, short-term (2 weeks) and long-term (10 months) ovariectomised rats using NMDA-displaceable [^{3}H]glutamate specific binding. In the hippocampus, we saw a decrease with ovariectomy that was corrected with 17β-oestradiol, tamoxifen and raloxifene treatments, whereas, in the cortex, an increase was seen with ovariectomy that was corrected with these oestrogenic drug treatments (20, 21, 35). Striatal NMDA receptors were decreased with 17β-oestradiol, tamoxifen and raloxifene treatments. NMDA-displaceable [^{3}H]glutamate binding was also reported to increase in the hippocampal CA1 of ovariectomised rats administered 17β-oestradiol benzoate 72 and 48 h before death and was associated with improved arm-choice accuracy in a working memory task (36). By contrast, using [^{3}H]MK-801 specific binding to label NMDA receptors in the hippocampus, a decrease of specific binding was observed after 10 weeks of 17β-oestradiol treatment associated with an improved memory performance (37). In the CA1, CA2/3 and dendate gyrus hippocampal regions, changes in both NMDA receptor [^{3}H]Ro 25-6981 specific binding and NMDA subunits 1 and 2B mRNA levels were positively correlated, suggesting that changes in specific binding were a result of changes in the synthesis of these receptors, decreasing with gonadal hormone withdrawal by ovariectomy and increasing with chronic treatments of 17β-oestradiol or PPT, whereas DPN had no significant effect (20, 21).

The functional effects of 17β-oestradiol on hippocampal NMDA receptors is well documented in female animals using various experimental approaches; 17β-oestradiol regulates hippocampal

Table 2. Summary of Differences in Brain NMDA Receptor Specific Binding Response to 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) and 17β-Oestradiol (E_2) Treatments in Wild-Type (WT), Oestrogen Receptor Knockout (ERKO)α and ERKOβ Mice.

	WT mice			ERKOα mice			ERKOβ mice		
Brain region	MPTP	17β-E_2	MPTP +17β-E_2	MPTP	17β-E_2	MPTP +17β-E_2	MPTP	17β-E_2	MPTP +17β-E_2
Cortex									
Frontal	–	0	0	–	0	0	0	0	0
Cingulate	–	0	0	–	0	0	0	0	+
Striatum									
Medial	–	0	0, ↑	0	0	0	0	+	0
Lateral	–	0	0, ↑	0	0	0	0	+	0
Hippocampus									
CA1 oriens	0	0	0	–	0	–	0	0	0
CA1 radiatum	0	0	–	–	0	–	0	0	0
CA2 oriens	0	0	0	0	0	0	0	0	0
CA2 radiatum	0	0	0	–	0	–	0	0	0
CA3	0	0	0	–	0	–	0	0	0
Dentate gyrus	0	0	0	0	0	–	0	0	0

0, –, +, No effect, decreased and increased NMDA receptor levels versus respective intact vehicle-treated mice.
↑, Increased NMDA receptor levels versus respective MPTP-treated mice

dendritic spine density via an NMDA receptor-dependent mechanism (38). An NMDA receptor increase promoted by a 17β-oestradiol treatment, measured using [^{3}H]-glutamate autoradiography, was shown to be required for 17β-oestradiol induced hippocampal CA1 spinogenesis in ovariectomised female rats (38). However, 17β-oestradiol fails to modulate NMDA receptor binding in gonadectomised males and does not increase spine density; it is argued that the lack of oestrogenic activity is the result of a paucity of ERs (38). The absence of oestrogenic effects on hippocampal NMDA receptors in males is also related to the inability of 17β-oestradiol to modulate cholinergic activity (important in oestradiol modulation of NMDA receptors) in the forebrain and the hippocampus as a result of a lack of ERs because females express ERα in cholinergic cell bodies of the forebrain and in hippocampal cholinergic terminals (39, 40).

Effect of lesion, 17β-oestradiol and ERKO on NMDA receptors

In ERKO animal models, the absence of either ERα or ERβ in female mice was reported to have negative effects on hippocampal-dependent learning tasks and impaired synaptic plasticity in the CA1 was observed in ERKOβ female mice (41). The present results showed lower NMDA receptor specific binding in the frontal and the cingulate cortex, as well as in the striatum of ERKOα and ERKOβ, compared to WT male mice. This could be explained by a stimulatory effect of the low levels of endogenous oestrogens in males that could maintain higher NMDA receptors levels. In the absence of ERs upon which 17β-oestradiol is acting, this effect would be lost.

NMDA receptors were not decreased in ERKO compared to WT mice in all the hippocampal subregions measured. By contrast, higher NMDA specific binding in the CA3 and dendate gyrus of ERKOα mice was observed compared to WT and ERKOβ mice, as well as in the CA1 and CA2 in ERKOα compared to ERKOβ mice. The androgen 5α-dihydrotestosterone (DHT) is reported to increase CA1 strata oriens and radiatum NMDA receptor binding density in castrated male rats (42). Few data are available for other brain regions. Testosterone treatment had no effect on [^{3}H]CGP 39653 binding to NMDA receptors containing NR1/2A in the cerebral cortex (43) and no data are reported for the striatum. Our previous results obtained in these mice showed that ERKOα mice have higher blood concentrations of endogenous androgens, including testosterone, DHT and 5α-androstan 3-β, 17β-diol (3β-diol), compared to WT and ERKOβ mice (31). Hence, higher levels of NMDA receptors in hippocampal regions of ERKOα but not of WT or ERKOβ mice could be a result of the effect of androgens on this glutamate receptor. However, in contrast to the well-documented neuroprotective activity of oestrogens, elevated androgen levels are not neuroprotective of nigrostriatal DA, testosterone and DHT have no neuroprotective effects against MPTP (44) and ERKOα mice are more sensitive to MPTP (22, 31).

The results for intact mice of all three genotypes revealed that 17β-oestradiol treatment generally left NMDA specific binding unchanged in the cortical, striatal and hippocampal subregions assayed, except for an increase in the striatum of ERKOβ mice, whereas deletion of ERα or ERβ also had very little effect on male hippocampal NMDA receptors. Using NMDA-displaceable [^{3}H]glutamate specific binding in the striatum, we previously observed no effect of 17β-oestradiol treatment in MPTP lesioned male mice (45), which is at variance with the present results, possibly because a more specific ligand for NMDA receptors containing NR2B was used in the present study, allowing the measurement of a change with the lesion and 17β-oestradiol treatment. In the present study, ERKOα MPTP-lesioned mice treated with 17β-oestradiol showed a generalised decrease of [^{3}H]Ro 25-6981 specific binding in

hippocampal subregions, whereas this was observed only in the CA1 of WT mice and there was no decrease for ERKOβ mice. Moreover, intact ERKOα mice had higher NMDA receptors compared to WT and ERKOβ mice. In CA3 neurones of adult male rat hippocampus, a rapid (within 2 h) decrease was reported in hippocampal thorns and spines as a result of 17β-oestradiol and PPT but not DPN (46). Hence, overall, these results demonstrate that both male mice (in the present study) and rats (46) are responsive to 17β-oestradiol treatment in the hippocampus, although the time course, ER specificity and regional effect vary.

Link between striatal NMDA receptors and DA concentrations

A significant (positive) correlation between striatal NMDA receptor specific binding and DA concentrations was observed in the WT mice of both experiments (Figs 3 and 4), whereas there was no correlation in ERKOα or ERKOβ mice. This could be explained by the MPTP-induced decrease of DA removing the DA inhibitory control on glutamate, thus resulting in increased glutamate activity. In turn, glutamate overactivity could be compensated to restore homeostasis by a reduction of NMDA receptors. The lack of correlation in the ERKO mice suggests that removing ERα or ERβ had a disruptive effect on the adaptation of NMDA receptors to the lesion and 17β-oestradiol treatment.

Conclusions

The present *in vivo* experiments in male mice showed that brain NMDA receptors were affected by the deletion of ERs, which in turn affected the response to MPTP and 17β-oestradiol treatments with brain region specificity. Taken together, these studies support an important role of ERs in glutamate neurotransmission. Because glutamate is the most abundant excitatory brain neurotransmitter, this has important functional significance with respect to both intact and neurodegenerative conditions.

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