The Role of Autophagy in the Growth and Guidance of Midbrain Dopaminergic Neurons

Thèse

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Résumé

Les neurones dopaminergiques mésodiencephaliques jouent un rôle central dans la régulation d'un large éventail de fonctions cérébrales allant des mouvements volontaires aux comportements associés. Ces fonctions sont régulées par des sous-types distincts de neurones dopaminergiques situés à la base du cerveausoit l'air tegmentaireventrale et la substance noire compacte. Ces neurones innerventdifférentes régions du cerveau en formant les voies nigrostriatales, mésolimbiques et mésocorticales. Les mécanismes moléculaires qui régissent la formation de voies dopaminergiques dans le cerveau sont en grande partie inconnus. L'autophagie est la principale voie de renouvellement cytoplasmatique et s'est révélée importante pour le développement du système nerveux. Nous montrons ici que les protéines nécessaires à l'autophagie sont présentes dans les cônes de croissance des neurones dopaminergiques et qu'elles sontrégulées temporellement pendant leur développement. En outre, le niveau d'autophagie change de façon dynamique dans les neurones dopaminergiques en réponse à des signaux de guidage chimio-répulsifs et chimio-attractifs. Pour caractériser le rôle de l'autophagie dans la croissance / guidage des axones dopaminergiques, nous avons utilisé la méthode d'édition du génome CRISPR-Cas9 ainsi qu'une souris knock-out conditionnelle (cKO) pour les gènes essentiels de l'autophagie (Atg12, Atg5) spécifiquement dans les neurones dopaminergiques. Les axones ATG5 cKO présentent des renflements axonaux et une diminution du nombre de ramifications in vitro et in vivo, probablement en raison de la formation de boucles de microtubules aberrantes. De manière frappante, la suppression de gènes liés à l'autophagie a complètement bloqué la réponse des neurones dopaminergiques aux signaux de guidage chimio-répulsifs et chimio-attractifs. Nos données démontrent que l'autophagie joue un rôle central dans la régulation du développement des neurones dopaminergiques et dans l'amélioration de notre compréhension des processus physiologiques régissant la croissance et le guidage axonal.

Abstract

Mesodiencephalic dopamine neurons play a central role in the regulation of a wide range of brain functions ranging from voluntary movement to reward associated behaviours. These functions are regulated by distinct subtypes of dopamine neurons located in the ventral midbrain substantia nigra pars compacta and ventral tegmental area that project to different brain regions by forming the nigrostriatal, mesolimbic, and mesocortical pathways. The molecular mechanisms that drive the midbrain dopaminergic trajectory formation are largely unknown. Autophagy is the major cytoplasmatic turnover pathway and has been shown to be important to neural system development. Here we show that autophagy machinery is present in the growth cones of dopaminergic neurons and is temporally regulated during their growth and guidance. Furthermore, autophagy level changes dynamically in dopaminergic neurons in response to both chemo-repulsive and chemo-attractive guidance cues. To characterize the role of autophagy in dopaminergic axon growth/guidance, we used CRISPR-Cas9 gene editing as well as a conditional knock-out mice (cKO) for the essential autophagy genes (*Atg12, Atg5)* deleted in dopaminergic neurons. ATG5 cKO axons exhibit axonal swellings and decreased branching *in vitro* and *in vivo*, likely due to aberrant microtubule looping. Strikingly, deletion of autophagy-related genes blunted completely the response of dopaminergic neurons to chemo-repulsive and chemo-attractive guidance cues. Our data demonstrate that autophagy plays a central role to tightly regulate dopaminergic neurons development and improve our understanding about basic physiological processes orchestrating axonal growth and guidance.

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List of abbreviations

3D – three dimension
AADC - aromatic l-amino acid decarboxylase
AD – Alzheimer's disease
ADHD - attention deficit hyperactivity disorder
Akt – protein kinase B
Aldh1a1 - Aldehyde Dehydrogenase 1 Family Member A1
Alfy/WDFY3 - Autophagy linked FYVE protein
Ambra1 - Activating molecule in Beclin 1-regulated autophagy protein 1
Ascl1 - Achaete-scute homolog 1
ATGs - autophagy-related
ATP - Adenosine triphosphate
BDNF - Brain-derived neurotrophic factor
C-X-C - C-X-C motif chemokine
Ca^{2+} - Calcium ions
CA - catecholamine
Calb1 - Calbindin 1
cAMP - Cyclic adenosine monophosphate
cGMP - Cyclic guanosine monophosphate
cKO – conditional knockout
CMA – chaperone-mediated autophagy
CNA - central nucleus of the amygdala
CNG - cyclic nucleotide-gated
Cpc – caudal *caudate putamen*
Cpi – intermediate *caudate putamen*
Cpr – rostral *caudate putamen*
CPt – tail *caudate putamen*
CPu – *caudate putamen*
Cre - Cre Recombinase
CRISPR - clusters of regularly interspaced short palindromic repeats
CXCL12 - C-X-C Motif Chemokine Ligand 12
CXCR4 - C-X-C chemokine receptor type 4
DA - dopaminergic
Dab1 – disabled-1
DAT – dopamine transporter
DNA - Deoxyribonucleic acid
DRG – dorsal root ganglion
En1/2 - Engrailed 1 and Engrailed 2
ER – endoplasmic reticulum
ERC - entorhinal cortex
FGF8 - fibroblast growth factor 8
FoxA1/2 - Forkhead box protein A1 and A2
FTD - frontotemporal dementia
GC – growth cone
GDP - Guanosine diphosphate
GFP – green fluorescent protein
Gli2 - GLI Family Zinc Finger 2
GPCR - G-protein coupled receptors
GPI - glycosylphosphatidylinositol anchor
GTP - Guanosine-5'-triphosphate

HD – Huntington's disease
iPSCs – induced pluripotent stem cells
KO - knockout
L-DOPA - l-3,4-dihydroxyphenylalanine
LC3 - Microtubule-associated protein 1A/1B-light chain 3
lge - lateral ganglionic eminence
LIMK - LIM kinase
Lmx - LIM homeobox transcription factor
Mash1 - mammalian achaete scute homolog-1
mDA – midbrain dopaminergic
MFB – medial forebrain bundle
mge - medial ganglionic eminence
Msx1/2 - Msh homeobox 1 and 2
mTOR – mammalian target of rapamycin
NAc – *nucleus accumbens*
NBR1 - Next to BRCA1 gene 1 protein
Ndnf - Neuron Derived Neurotrophic Factor
Neurog2 - Neurogenin 2
NSC - neural stem cells
Nurr1 - Nur-related factor1
OT – olfactory tubercule
Otx1/2 - orthodenticle homeobox 1 and 2
p1-p3 - prosomeres 1–3
PAK - p21-activated kinase
PD – Parkinson's disease
PFC – prefrontal cortex
PI3K - Phosphoinositide 3-kinases
Pitx3 - paired like homeodomain transcription factor 3
PTEN - Phosphatase and tensin homolog
RFP – red fluorescent protein
Rho - Ras-homologous
RNA - Ribonucleic acid
ROCKs - Rho kinases
RRF - retrerubral field
Sema - semaphorin
Shh - Sonic Hedgehog Signaling Molecule
SNpc – *Substantia nigra pars compacta*
STED - stimulated emission depletion
TH – tyrosine hydroxylase
TRP - transient receptor potential
ULK1/2 - Unc-51 like autophagy activating kinase
VAMP2 - vesicle-associated membrane protein 2
Vglut2 - Vesicular Glutamate Transporter 2
VMAT2 - vesicular monoamine transporter type 2
VTA - VTA
VZ – ventricular zone

Foreword

This thesis is part of the final steps in the PhD program in Neurobiology at Université Laval. I have performed all my research in Dr. Martin Lévesque and Dr. Armen Saghatelyan laboratories in the CERVO Brain Research Centre.

In the first part of the manuscript, I have introduced the background and rationale for my Ph.D. project and article. The second part is the article and core of my thesis that I have worked on during my doctoral studies, which has been submitted to Cell Reports on the 23rd of July of 2019 and is currently under revision. In this study, we show that autophagy is required for proper midbrain dopaminergic axonal morphology and branching during development. Moreover, we demonstrate for the first time that autophagy is pivotal in mediating the responses of dopaminergic axons to guidance cues, and that autophagy ablation by Atg5 knock-out in midbrain dopaminergic neurons completely blunts axon growth/guidance in response to major chemorepellant and chemoattractive signals, such as Sema7a and Netrin-1. Therefore, our data reveal a central role of autophagy in dopaminergic system development by regulating axonal growth/guidance and mediating the responses of these cells to extrinsic guidance cues. In this project, I have contributed to the planning of the research, experimental design, performed and analyzed all experiments and written the manuscript. The reference for the aforementioned manuscript is as follows:

- **Marcos Schaan Profes**, Armen Saghatelyan and Martin Lévesque. Autophagy controls midbrain dopaminergic axon development and their responsiveness to guidance cues. Submitted to Cell Reports on July 23rd, 2019.

The next section discusses the importance of this project as well as its pitfalls and future experiments relevant to the matter. Lastly, is the conclusion to my thesis.

During my doctoral studies, my expertise in autophagy, cell culture, biochemistry and live-imaging made me an ideal candidate for some collaborations. Indeed, I was fortunate to collaborate in a few other projects that are listed bellow:

- Bérard, Morgan and Sheta, Razan and Malvaut, Sarah and Turmel, Roxanne and Alpaugh, Melanie Jeanne and Dubois, Marilyn and Dahmene, Manel and Salesse, Charleen and **Profes, Marcos Schaan** and Lamontagne- Proulx, Jérôme and Qazi, Raza and Tavassoly, Omid and Soulet, Denis and Lévesque, Martin and Jeong, Jae- Woong and Cicchetti, Francesca and Fon, Edward A. and Saghatelyan, Armen and Oueslati, Abid. (2019) Optogenetic-Mediated Spatiotemporal Control of α-Synuclein Aggregation Disrupts Nigrostriatal Transmission and Precipitates Neurodegeneration. Neuron. Under revision. (Posted: 9 July 2019 and available at SSRN: https:// ssrn.com/abstract=3416893 or http://dx.doi.org/10.2139/ssrn.3416893)

In this study, a light-inducible protein aggregation (LIPA) system was developed to model α-synucleinopathies and hence to better understand how α-synuclein (α-syn) aggregation affects neuronal homeostasis leading to neurodegeneration. This system allows for real-time induction of α-syn inclusions formation with remarkable spatial and temporal resolution in both *in vitro* and *in vivo* paradigms. LIPA-induced aggregates auto-perpetuate after transient light induction, leading to Lewy Bodys formation *in vivo* and in cell culture, which compromised the nigrostriatal transmission, induced a significant

dopaminergic neuronal loss and behavioural impairment in mice. Therefore, my expertise in live-imaging made me a valuable candidate for this collaboration in which I performed time-lapse imaging *in vitro* to characterize the LIPA-induced α-synuclein aggragation.

– Audrey Chabrat, Guillaume Brisson, Hélène Doucet-Beaupré, Charleen Salesse, **Marcos Schaan Profes**, Axelle Dovonou, Cléophace Akitegetse, Julien Charest, Suzanne Lemstra, Daniel Côté, R. Jeroen Pasterkamp, Monica I. Abrudan, Emmanouil Metzakopian, Siew-Lan Ang & Martin Lévesque. (2017). Transcriptional repression of Plxnc1 by Lmx1a and Lmx1b directs topographic dopaminergic circuit formation. Nat Commun. 2017 Oct 16;8(1):933. doi: 10.1038/s41467-017-01042-0.

In this paper, we show that the transcription factors Lmx1a, Lmx1b, and Otx2 control subtype-specific midbrain dopamine neurons and their appropriate axon innervation. Additionally, we show that Plxnc1, an axon guidance receptor, is repressed by Lmx1a/b and enhanced by Otx2 and that Sema7a/Plxnc1 interactions are responsible for the segregation of nigrostriatal and mesolimbic dopaminergic pathways. Due to my knowledge in *in vitro* midbrain dopaminergic explant cultures, in axon guidance assays and biochemistry assays, I could collaborate and help to the understanding on how Sema7a/Plxnc1 interaction is important to the midbrain dopaminergic axon segregation in the striatum.

- Hélène Doucet-Beaupré; Catherine Gilbert; **Marcos Schaan Profes**; Audrey Chabrat; Consiglia Pacelli; Nicolas Giguère; Julien Charest; Véronique Rioux; Ariadna Laguna; Qiaolin Deng; Thomas Perlmann; Johan Ericson; Siew- Lan Ang; Francesca Cicchetti; Martin Parent; Louis-Eric Trudeau; Martin Lévesque. (2016). Lmx1a and Lmx1b regulate mitochondrial functions and survival of adult midbrain dopaminergic neurons. Proc Natl Acad Sci U S A. 2016 Jul 26;113(30):E4387-96. doi: 10.1073/pnas.1520387113.

In this manuscript we described that the survival of dopaminergic neurons requires the ongoing action of the transcription factors Lmx1a and Lmx1b. We unveiled an Lmx1a/b-dependent pathway involved in the maintenance of mitochondrial functions in midbrain dopaminergic neurons. Accordingly, ablation of Lmx1a/b results in impaired respiratory chain activity, increased oxidative stress, and mitochondrial DNA damage and causes Lewy neurite-like pathology. For this manuscript, my expertise in autophagy and biochemical assays were important in order to characterize the importance of Lmx1a/b in the maintenance of midbrain dopaminergic neurons.

- Chris Law; **Marcos Schaan Profes**; Martin Lévesque; Julia Kaltschmidt; Matthijs Verhage. (2016). Normal molecular specification and neurodegenerative disease-like death of spinal neurons lacking the SNARE-associated synaptic protein Munc18-1. J Neurosci. 2016 Jan 13;36(2):561-76. doi: 10.1523/JNEUROSCI.1964-15.2016.

In this work, it was demonstrated the lack of requirement for regulated neurotransmitter release in the assembly of early neuronal circuits. This observation was done by assaying transcriptional identity, axon growth and guidance, and mRNA expression in Munc18-1-null mice. Additionally, Munc18-1 mutants neurodegeneration was characterized as cell-autonomous and does not appear to be a result of defects in growth factor signaling or ER stress caused by protein trafficking defects. Despite that, pathological hallmarks of Alzheimer's disease were observed in these mutants, suggesting a parallel between the degeneration in these mutants and neurodegenerative conditions. My knowledge in tauopathies and ubiquitin system made me an ideal candidate to collaborate in this project and help in the characterization of the phenotype.

I. Introduction

The brain is an astonishing structure and the most complex organ in the body. It is responsible for controlling a wide range of functions and behaviours, regulating other organs functions, movement control, emotion and thoughts formation. It is perhaps the biggest puzzle in biology whether we look from molecular, cellular, systemic or even behavioural or cognitive perspectives (Purves, 2004).

Formed by highly organized circuits, the brain is characterized by an ensemble of neurons and their projections and glial cells with of specific anatomical locations that are responsible for processing variety types of information. In general terms, three main functions can be separated: (1) sensory, linking the organism to its environment; (2) motor, responsible for generating actions; and (3) associative, the link between sensory and motor systems, therefore forming the basis for complex functions – e.g. attention, rational thinking, emotions (Purves, 2004).

In order for neuronal circuits to be formed, neurons need to elongate and project their processes (dendrites and axons) toward target areas. Axonal development is a multi step process, starting from growth cone formation, axonal growth and branching, which are coordinated by intrinsic mechanisms and extrinsic cues from the surrounding microenvironment that drive axonal growth and guidance (Tamariz and Varela-Echavarría, 2015). Midbrain dopaminergic (mDA) neurons located in the ventral tegmental area (VTA) and *substantia nigra pars compacta* (SNpc) control a wide range of behavioural responses. The medially located neurons in the VTA project axons to different regions implicated in the reward pathway, including *nucleus accumbens* (NAc) and the prefrontal cortex (PFC), constituting the mesocorticolimbic pathway. Dopamine deregulation in the mesocorticolimbic pathway has been linked to depression, drug addiction, attention deficit hyperactivity disorder and schizophrenia (Van den Heuvel and Pasterkamp, 2008). SNpc neurons, on the other hand, are laterally located in the ventral midbrain and their axons target the dorsolateral striatum (*caudate putamen* - CPu) – forming the nigrostriatal pathway. The loss of nigrostriatal pathway is the main histopathological feature of Parkinson's disease (PD) (Van den Heuvel and Pasterkamp, 2008).

Much remains to be discovered about the detailed development of brain connections. Understanding how neurons develop and precisely form a network of synapses that underlie

our every behaviour is one of the most challenging difficulties in neuroscience. Despite the growing body of evidence that describes axonal guidance and guidance molecules and their receptors, the cellular integration of these different signals is still poorly understood. Autophagy is the main cytoplasmic degradation system that has been shown to play central roles in different cell types (Glick et al., 2010). More specifically, recent studies have highlighted the importance of autophagy in neural system development (Dragich et al., 2016; Fimia et al., 2007; Hara et al., 2006; Hernandez et al., 2012; Yang et al., 2017). However, its contribution on axon guidance had not been addressed until now. Therefore, this thesis is focused on understanding the role of autophagy in axon growth and guidance in midbrain dopaminergic neurons. The advances in understanding mDA axon development and the discovery of neural stem cells (NSC) and induced pluripotent stem cells (iPSC) raised the exciting possibility of developing new cell replacement strategies to cure neurodegenerative diseases, such as PD. Despite that, the success of such strategies will depend upon the full understanding of the mechanisms that regulate survival and axonal pathfinding of grafted cells, such as autophagy.

I.1. Dopamine system

Most cells of the dopaminergic system are part of the midbrain or mesencephalon, and they secrete a specific neurotransmitter: dopamine. This system comprises four different pathways: mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular pathways (Björklund and Dunnett, 2007). Formed by dopaminergic neurons from the VTA that project to the ventral striatum (the *nucleus accumbens* - NAc) the mesolimbic pathway (Figure I-1) plays an important roles in the reward system, feeding and olfaction (Dreyer, 2010). VTA neurons also project to the prefrontal cortex (PFC) forming the mesocortical pathway (Figure I-1) and regulating cognitive, learning, memory and emotional functions (Gorelova et al., 2012). The nigrostriatal pathway (Figure I-1), which is mostly involved in motor behaviour, is constituted by neurons from the SNpc that innervate the dorsal striatum (*caudate nucleus* and *putamen*) (Björklund and Dunnett, 2007). Lastly, the tuberoinfundibular pathway, which is responsible for the secretion of hormones such as prolactin upon dopamine release, is formed by hypothalamic neurons projecting to the pituitary median eminence (Stahl, 2013).

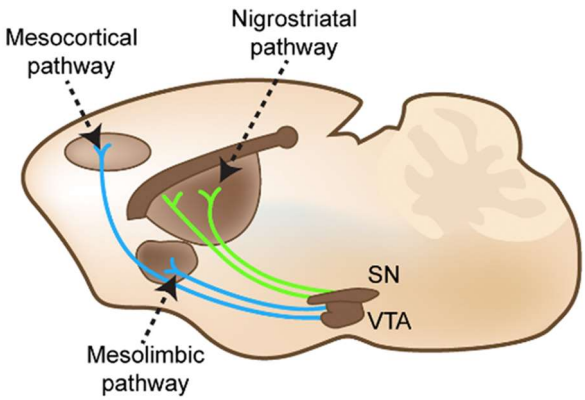

Figure I-1: Nigrostriatal, mesocortical, and mesolimbic pathways represented in an adult mouse brain in the sagittal plane.
SNpc projects to dorsal striatum. The VTA projects to both ventral striatum and PFC. [Modified from (Money and Stanwood, 2013)].

I.1.1. Dopamine

Dopaminergic neurons synthesize dopamine, the neurotransmitter essential for the modulation of their neuronal pathways and ultimately to transfer information through synapses. Dopamine is synthesized from tyrosine with the involvement of two different enzymes: tyrosine hydroxylase (TH) and aromatic l-amino acid decarboxylase (AADC). In order to transform tyrosine into dopamine, TH hydroxylates the aromatic cycle leading to the formation of L-DOPA. Such molecule constitutes the most effective treatment for PD up-to-date. AADC, on the other hand, acts on L-DOPA and the decarboxylation of this molecule leads to dopamine formation (Figure I-2) (Goridis and Rohrer, 2002).

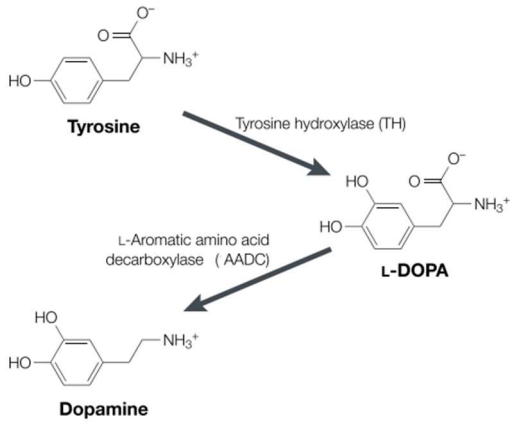

Figure I-2: Dopamine synthesis.
Two enzymatic reactions are responsible for Dopamine synthesis. Enzymes involved are the TH, and the AADC [modified from (Goridis and Rohrer, 2002)]

Synthesized dopamine is concentrated into synaptic vesicles by the action of the vesicular monoamine transporter type 2 (VMAT2) within axon at the presynaptic boutons. An action potential is then required for dopamine release at the synaptic cleft (Giros and Caron, 1993). Once in the synapse, the neurotransmitter binds to dopamine receptors (D1-like and D2-like – see bellow for more details) which can be postsynaptic - located on the dendrites - or presynaptic D2-autoreceptors (Beaulieu and Gainetdinov, 2011). An evoked postsynaptic neuron action potential culminates in dopamine unbinding from its receptors and consequently neurotransmitter reuptake mediated mostly by the dopaminergic transporter (DAT) takes place. Back in the cytosol, dopamine can be concentrated back to the synaptic vesicles for an eventual re-release or broken down by the action of monoamine oxidase enzyme (Figure I-3) (Eiden et al., 2004).

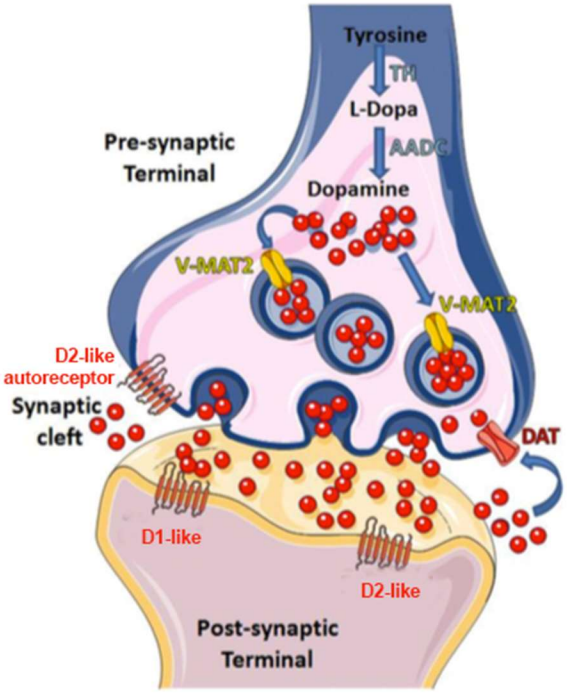

Figure I-3: Schematic representation of dopaminergic synapse.
Dopamine is synthesized in the cytoplasm of mDA neurons and concentrated into synaptic vesicles via VMAT2. When an action potential occurs, fusion of these vesicles with the cytoplasm membrane happens leading to dopamine release in the synaptic cleft. Dopamine released in the cleft can bind in the presynaptic site to D2-autoreceptors or to DAT, or to D1- and D2-class receptors in the postsynaptic site (Giros and Caron, 1993). Modified from (Chabrat, 2016).

I.1.1.1. Dopamine receptors and transporter

Dopamine released by presynaptic terminals activates G-protein coupled receptors (GPCR) (Beaulieu and Gainetdinov, 2011). These receptors have seven transmembrane domains (Beaulieu et al., 2015) and are divided into two main groups: D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors (Andersen et al., 1990; Beaulieu and Gainetdinov, 2011; Missale et al., 1998; Sibley and Monsma, 1992). D1-like receptors activate the $G\alpha_{s/off}$ family of G proteins stimulating the production of the second messenger cyclicadenosine monophosphate (cAMP) by adenylyl cyclase. The activation of D1-like receptors, that are mainly found on postsynaptic sites of dopamine-sensitive cells (e.g. medium spiny neurons), leads to inhibition of the evoked activity at hyperpolarizing membrane potentials (Calabresi et al., 1987) and facilitation of action potential activation when the cell is at a depolarizing potential (Hernández-López et al., 1997). On the other hand, D2-like receptors activate the type $G\alpha_{i/o}$ family of G-protein and consequently inhibits adenylyl cyclase (Beaulieu and Gainetdinov, 2011). Activation of D2-like receptors results in the reduction of action potential firing when cells are depolarized (Hernádez-López et al., 2000). D2 and D3 receptors are expressed both on the postsynaptic cell and on the presynaptic side of the dopaminergic cell (Rondou et al., 2010; Sokoloff et al., 2008). D1-like and D2-like receptors function have been linked to neurodevelopmental disorders and drug seeking behaviours (Akil et al., 1999; D'haenen and Bossuyt, 1994; Kalivas et al., 2005; Okubo et al., 1997; Piazza et al., 1991; Shah et al., 1997).

DAT is a transmembrane protein that mediates a rapid uptake of dopamine from the synaptic cleft back into the cytosol terminating the dopaminergic neurotransmission. To perform the reuptake, DAT requires the biding and co-transport of two Na^+ ions and one Cl^- ion with dopamine. DAT harnesses stored energy in the Na^+ gradient that is generated by the membrane-bound Na^+/K^+ ATPase to drive the uphill transport of dopamine across the cell membrane (Nielsen et al., 2019; Torres et al., 2003). The role of DAT in dopaminergic neurotransmission regulation is underscored by the fact that its malfunctions is associated with neurological disorders including early onset parkinsonism and attention deficit hyperactivity disorder (ADHD). Additionally, inhibition of DAT function is also related to the addictive effects of illicit drugs (e.g. cocaine and amphetamines). Altogether, it makes DAT a possible target for therapeutic drugs for dopaminergic-related disorders (Nielsen et al., 2019).

Therefore, it is important to understand the mechanisms (including downstream effectors) involved in dopamine transduction function in order to identify possible pharmacological targets to develop new strategies to treat diseases involving the dopaminergic system.

I.2. Dopaminergic neurons

Dopaminergic (DA) neurons are a part of catecholamine (CA) neurons that synthesize dopamine. CA neurons are organized according to their localization in the mammalian brain from A1 to A17 neuronal nuclei – ranging from medulla oblongata to the retina. Among these, nine dopamine-containing neuronal cell groups can be found – A8-A17 –, which are located in the ventral midbrain, hypothalamus, olfactory bulb, and retina (Figure I-4) (Björklund and Dunnett, 2007; Dahlstroem, A; Fuxe, 1964).

DA neurons grouped from A8 to A11 are localized in the midbrain; A12 in the hypothalamus; A13 in the diencephalon; A14 to A16 in the olfactory bulb; and A17 (not shown in Figure I-4) in the retina (Björklund and Dunnett, 2007; Dahlstroem, A; Fuxe, 1964).

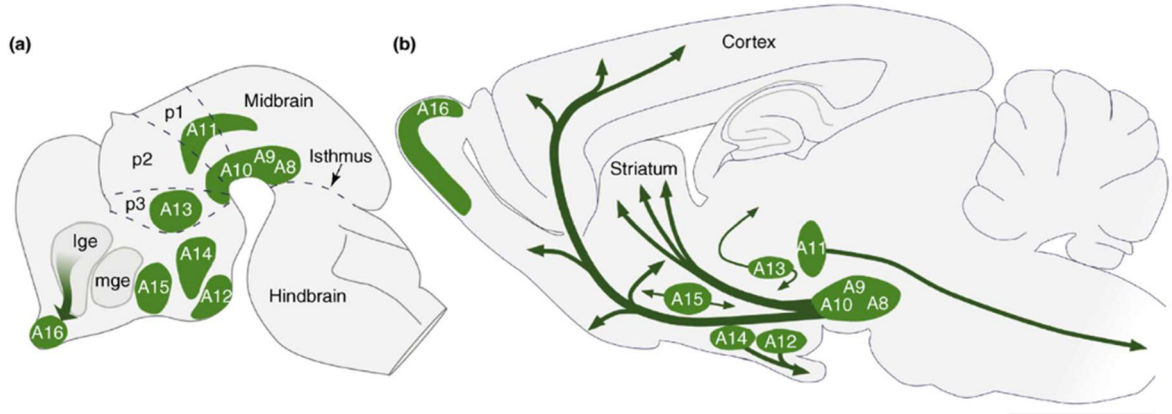

Figure I-4: Distribution of DA neurons nuclei in the rodent brain.
DA neurons are distributed in nine recognized nuclei from the mesencephalon to the olfactory bulb, as illustrated schematically, in a sagittal view, in (a) the developing and (b) the adult rat brain. The numbering separation of the nuclei – from A8 to A16 - was introduced by study of Dahlström and Fuxe in 1964 (Dahlstroem, A; Fuxe, 1964). DA neurons belonging from A8 to A11 are localized in the midbrain, A12 in the hypothalamus, A13 in the diencephalon, A14 to A16 in the olfactory bulb, A17 (not shown) in the retina. (b) The arrows represent the main projections of the DA cell groups. Abbreviations: lge, lateral ganglionic eminence; mge, medial ganglionic eminence; p1–p3, prosomeres 1–3. Adapted from (Björklund and Dunnett, 2007).

I.2.1. Midbrain dopaminergic neurons

mDA neurons are organized into the A8, A9 and A10 neuronal nuclei and constitute about 75% of the total number of DA neurons (Ang, 2006). They are composed of neurons in the medial located VTA (area A10), in the laterally located SNpc (area A9) and in the retrerubral field (RRF, area A8) located dorsally and caudally of A10 at the level of the red nucleus. Efferent projections to specific regions in the forebrain arise from these mDA neuronal nuclei. However, such lateral-to-medial topographic gradient roughly describes such neuronal population and indeed mDA neurons comprise at least 7 neuron subpopulations with distinct patterns of gene expression, electrophysiologic properties, projections to the striatum, cerebral cortex and amygdala, and response to environmental stimuli (Arenas et al., 2015; Björklund and Dunnett, 2007; Bodea and Blaess, 2015; Hassan and Benarroch, 2015; Poulin et al., 2014, 2018; Tiklová et al., 2019).

I.2.1.1. Axonal projections of mDA neurons

Laterally located mDA neurons from SNpc (A9 neurons) establish connections to the dorsal striatum forming the nigrostriatal pathway. Additionally, A9 neurons, together with A8 and A10 neurons innervate the pyriform cortex (Björklund and Dunnett, 2007).

A10 medially located mDA neurons (dorsolateral VTA) project to the lateral regions of the NAc and ventromedial VTA establish connections with the medial regions of the NAc and the PFC constituting the mesocorticolimbic pathway. A10 neurons also project to the olfactory tubercle, the lateral septum, the amygdala, the habenula, the hippocampus and the *locus coeruleus* (Arenas et al., 2015; Van den Heuvel and Pasterkamp, 2008).

Importantly, mesostriatal pathway plays an important role in the control of motor function and its degeneration results in the motor symptoms of PD (e.g. resting tremor, rigidity and bradykinesia). The mesocorticolimbic system regulates working memory, attention, decision-making and reward-associated behaviour. Imbalances of dopamine input in the mesocorticolimbic pathway have been linked to drug abuse disorders, depression, ADHD and schizophrenia (Björklund and Dunnett, 2007; Bodea and Blaess, 2015; Hassan and Benarroch, 2015).

Of note is that RRF neurons (A8) also project to striatum, limbic and cortical areas (Björklund and Dunnett, 2007). Indeed, the NAc is not only innervated by neurons from VTA

but also by neurons from the A8 cluster and RRF neurons also project to the olfactory tubercle (OT), the central nucleus of the amygdala (CNA), and the entorhinal cortex (ERC) - all of which are targeted by VTA axons (Arenas et al., 2015).

The aforementioned characterization of the dopaminergic system was mainly based on traditional methods of cytology and anatomy, however the central nervous system is a very heterozygous organ with a multitude of neuronal types and sub-types. Progress in identifying and mapping such subtypes have been hampered by the lack of genetic tools to study neuron subtypes. Despite that, recently, Poulin et al. developed, based on combinatorial gene expression, intersectional genetic labeling strategies and mapped the projections of mDA neuron subtypes. They demonstrated that at least 7 subpopulations are present in the mDA domain that display distinct but partly overlapping patterns of projections (Poulin et al., 2018). From the SNpc 3 subpopulations were discriminated. The first was the *Aldh1a1*+/Sox6+/Ndnf+ neuronal group. These neurons projected mainly to the locomotor areas of the striatum – the CPu rostral (CPr), intermediate (CPi) and caudal (CPc) – which were shown to be particularly vulnerable in a mouse model of PD (Poulin et al., 2014). The second subpopulation consisted of *Calb1*+/Sox6+/*Aldh1a1*– dorsal SNpc neurons with projections to the medial CPr and the ventromedial regions of the CPi and CPc. Lastly, in the lateral SNpc, the *Vglut2+/Calb1+*/Sox6– mDA neuron subtype projected densely to the tail CPu (CPt). In the VTA, 4 subpopulations projections were characterized. Projecting densely to the medial shell of the NAc, olfactory tubercule and lateral septum are the *Aldh1a1*+ VTA neurons. This pathway might be important in drug addictive behaviours as rats learn to self-administer amphetamine, cocaine or dopamine agonists directly into the NAc medial shell and olfactory tubercule, differently from when lateral shell or core of the NAc is targeted in which they do not learn to self-administer the drugs (Ikemoto, 2007). The other 3 VTA mDA subpopulations pathways characterized were the *Vip*+ (projecting to central amygdala and stria terminalis), the *Calb1*+/Sox6+/*Ndnf*+ (projecting to lateral shell of NAc and medial CPu) and the *Vglut2+/Aldh1a1*– (projecting to the entorhinal cortex and PFC) (Poulin et al., 2018).

The understanding of mDA axonal projections and how they are formed will provide important insights in understanding how this system develops, hence helping to depict the etiology of neurodevelopmental disorders and identifying important players that could be used as therapeutical targets for developmental and degenerative disorders involving the dopaminergic system.

I.2.1.2. Neurodevelopment of Midbrain Dopaminergic Neurons

I.2.1.2.1. Origin, Migration and Differentiation

Dopaminergic neurons development is characterized by a multi-step process that starts with the ventral midbrain induction, followed by specification of distinct mDA progenitor domain in the floor plate that undergoes proliferation, neurogenesis and ultimately differentiation (Figure I-5). mDA neurons neurogenesis occurs at the ventricular zone (VZ) of the floor plate where neural progenitors are located, and VZ expression of developmental factors such as orthodenticle homeobox 1 (Otx1), orthodenticle homeobox 2 (Otx2), sonic hedgehog (Shh) and LIM homeobox transcription factor (Lmx) influences cell fate along a dorsal and ventral axis (Puelles et al., 2004; Smidt and Burbach, 2007; Smits et al., 2006; Vernay et al., 2005). The floor plate located at the ventral midline of the neural tube acts as an organizing centre extending from the spinal cord to the posterior diencephalon (Blaess and Ang, 2015; Placzek and Briscoe, 2005). This “organizing centre” monitors the specification of neuronal and glial identities by secreting the morphogen Shh, however, by non-prolonging Shh signalling the floor plate keeps its ventral identity (Metzakopian et al., 2012; Ribes et al., 2010). The isthmus organizer in the mid/hindbrain boundary expresses fibroblast growth factor 8 (FGF8), and during a specific time window when there is concomitant expression of FGF8 and Shh (by the isthmus organizer and floor plate, respectively), the birth of mDA progenitors occurs (Hynes et al., 1995).

Early expression of transcription factors, including Otx2, Lmx1a/b, Forkhead box protein A1 and A2 (FoxA1/2) and Engrailed 1 and Engrailed 2 (En1/2) homeodomain regulates the differentiation process of mDA neurons. Importantly, each of these transcription factors is incapable of inducing a complete dopaminergic phenotype, which suggests a cooperative work and they remain expressed by postmitotic dopaminergic cells (Hegarty et al., 2013). The first dopaminergic neurons appear at E10.5 in rodents, whereas in humans, neurogenesis of dopaminergic neurons begins between the fifth and sixth week of embryonic development ending before the end of the eleventh week (Almqvist et al., 1996; Freeman et al., 1991; Nelander et al., 2009).

Of notice is that at least three steps have been well characterized on the mDA development based on the expression of molecular markers. The first step is regional specification is the first of them and is characterized by the expression of Otx, Lmx1a and

Lmx1b (Lmx1a/b), En1/2, Msx1 and Msx2 (Msx1/2), Raldh 1, Neurogenin 2 (Neurog2) and Ascl1 (Mash1). In this step neuroepithelial cells and radial glia become proliferative progenitors. Foxa2 then activates Shh during specification, which also regulates Foxa2 via Gli2. To allow floor plate specification, at this step, Foxa2 inhibits NK2 transcription factor-related locus 2 (Nkx2-2). Foxa2 also regulates the expression of neurogenin 2 (Neurog2), Msx1, Wnt1, En1/2 and Nur-related factor1 (Nurr1). Indeed, En1/2 and Nurr1 expression regulation by Foxa2 leads to the differentiation of DA precursors into DA neurons (Arenas et al., 2015; Blaess and Ang, 2015). The second step is the early differentiation or neurogenesis. It can be recognized by the expression of Lmx1a/b, Neurog2, Nurr1, En1/2, Raldh 1 and βIII-tubulin. *Neurog2* gene is an important regulator of neurogenesis and of cellular fate specification in many areas of the nervous system (Ang, 2006). Indeed, specifically for mDA system this gene has important roles in neuronal commitment, cell cycle withdrawal, neuronal differentiation, and survival (Lacomme et al., 2012). Both mDA progenitors and postmitotic immature mDA neurons express Neurog2. Later in this differentiation step, Neurog2 induces the expression of Nurr1+. It is also responsible for inducing the differentiation of Sox2-positive ventricular zone progenitors into dopaminergic postmitotic neuron precursors in the intermediate zone (Andersson et al., 2006; Kele et al., 2006). The third step is the late differentiation and characterizes by the expression of Lmx1a/b, Nurr1, En1/2, paired like homeodomain transcription factor 3 (Pitx3), TH, AADC, Raldh 1 and βIII-tubulin (Ang, 2006). Immature neurons finally emerge in the marginal zone as mDA neurons expressing markers such as TH, AADC, Pitx3, and DAT (Andersson et al., 2006; Kele et al., 2006; Puelles et al., 2003; Yan et al., 2011). Importantly, the dopamine neurotransmitter phenotype is dependent on Nurr1 expression as this transcription factor regulates the expression of proteins crucial for dopamine synthesis such as TH, VMAT2 and DAT (Saucedo-Cardenas et al., 1998; Smits et al., 2003; Wallén et al., 2001; Zetterström et al., 1997). Although the above mentioned literature describe mDA neurons development, VTA and SNpc are populated with distinct neuronal populations as described previously (see section I.2.1 and I.2.1.1. for more details). However, little is known about the generation of specific dopaminergic subtypes. Interestingly, Sox6, Otx2, and Nolz1 have been shown to play a role in subtype specification of VTA and SNpc already at the neural progenitor cell stage (Panman et al., 2014). Indeed, by cell-cycle exit, Otx2 and Nolz1 are expressed in mDA neuron progenitors that will form the VTA, whereas Sox6 is expressed in SNpc progenitor neurons. Additionally, *Otx2* null embryos displayed decreased VTA markers expression with an increase in the expression of SNpc markers, while *Sox6* null

embryos showed lower SNpc markers and increased VTA markers levels. Lastly, Sox6 levels were shown to be reduced in PD patients (Panman et al., 2014). Altogether, these findings link Sox6, Otx2, and Nolz1 to VTA vs. SNpc specification and start to depict how dopaminergic subtypes are formed.

Throughout the differentiation described above, mDA neurons migrate forming the SNpc, VTA and retrorubral area and eventually establish their axonal projections and synapses. mDA progenitors migrate from the floor plate following a radial glia and later laterally ultimately populating the areas that will form the VTA and SNpc (Kawano et al., 1995; Shults et al., 1990). The extracellular matrix Reelin, the chemokine receptor C-X-C chemokine receptor type 4 (CXCR4) and Netrin-1 have been shown to play a role in mDA migration (Bodea et al., 2014; Kang et al., 2010; Li et al., 2014; Lysko et al., 2011; Manitt et al., 2010; Nishikawa et al., 2003; Sharaf et al., 2013; Stumm et al., 2003; Sun et al., 2011; Xu et al., 2010; Yang et al., 2013; Zhao and Frotscher, 2010; Zhu et al., 2009). In mice lacking Reelin, SNpc neurons fail to migrate laterally becoming clustered close to the VTA (Nishikawa et al., 2003) and consistent to Reelin SNpc migration role, Disabled1 (Dab1), a intracellular effector of Reelin, is restricted to laterally migrating mDA neurons (Bodea et al., 2014). Lastly, Reelin was recently shown to be involved in laterally-biased movements in mDA neurons during their slow migration mode and to increase the probability of laterally-directed fast migration required to SNpc neuronal setup (Vaswani et al., 2019). CXCR4 receptor is expressed in mDA neurons undergoing radial migration and appears to be downregulated once the cells switch to tangential migration (Bodea et al., 2014; Yang et al., 2013). CXCR4 is the cognate receptor of chemokine (C-X-C motif) ligand 12 (CXCL12) (Yang et al., 2013) and CXCR4/CXCL12 signaling modulates mDA neurons initial migration (Bodea et al., 2014). Indeed, loss of CXCR4/CXCL12 signaling leads to a transient mDA neurons ectopic accumulation close to the progenitor zone during development (Bodea et al., 2014; Yang et al., 2013). The guidance molecule Netrin-1, is expressed during embryogenesis in the VTA and medial SNpc (Li et al., 2014; Sun et al., 2011) and its receptor deleted in colorectal (DCC), have been shown to be expressed by mDA neurons during migration (Manitt et al., 2010). In agreement with a possible Netrin-1 role in mDA migration, mice deficient for DCC or Netrin-1 show mDA neurons ectopically located in a dorsolateral position between the VTA and SNpc (Li et al., 2014; Xu et al., 2010). Despite that, further studies making use of time-lapse imaging and gene inactivation are required to

better understand the requirement of different molecular targets in regulating mDA neuronal migration.

In practice, by applying the acquired knowledge it is now possible to better identify mDA neuronal progenitors and their further differentiated lineage (immature and mature mDA neurons). Additionally, it makes the transcription factors mentioned above strong candidates to regulate and promote progenitors specification and/or differentiation and most importantly to promote stem cells mDA differentiation (Ang, 2006).

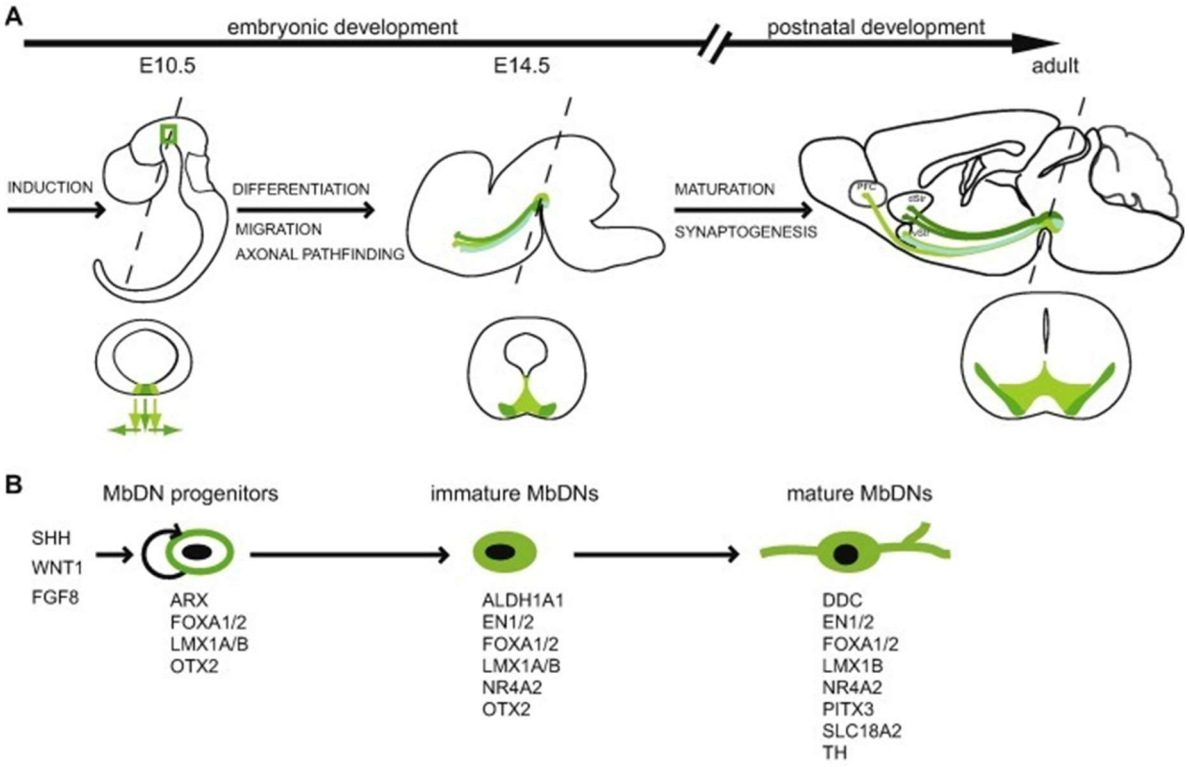

Figure I-5. Schematic representation of mDA neurons development.
(A) mDA neuronal progenitors induced in the ventral midline of the midbrain differentiate, migrate, project their axons towards the forebrain and ultimatelly establish synaptic connections with neurons in their target areas. SNpc neurons are shown in dark green and VTA neurons in light green. Bellow the coronal section of E10.5, the arrows indicate the migratory paths for SNpc and VTA mDA neurons. (B) During mDA neuron development, different signaling molecules and transcription factors are involved in the different steps that lead from progenitors to immature and to mature mDA neurons.

I.2.1.2.2. mDA neuronal axon growth and guidance

Development of neural circuits is a tightly regulated process that relies on a precise series of molecular and cellular events. Once neurons acquire their mature state, they project axons and dendrites forming organized routes to establish highly specific

connections with their targets. The aforementioned mDA pathways formation requires proper axonal and dendritic growth/guidance and also branching and pruning to finally form a functional synapse. To this end, axons are equipped with a highly sensitive and motile structure – the growth cone (GC). To follow highly specific trajectories, the GCs are guided by guidance molecules, topography of the developing brain and interactions with other cells (e.g. neurons and glia). Due to these interactions that are sensed by receptor complexes in the tip of the GCs, intracellular signalling cascades are triggered infringing upon the cytoskeleton and therefore affecting GC motility and steering. Several conserved families of guidance molecules have been identified and they can act as attractants or repellents (e.g. ephrins, Netrins, semaphorins, and slits) (Figure I-6). mDA pathways formations were investigated in rodents and at least three main steps have been identified and are crucial for mDA axon guidance throughout mouse embryogenesis. (1) From E11.5 to E13.5, the mDA neuronal axons of the mesencephalon extend dorsally from the ventrocaudal region of the midbrain, and then deflect rostrally. (2) At E13.5, these axons navigate longitudinally through the midbrain and the diencephalon leading to the formation of the medial forebrain bundle (MFB). (3) During E14.5-E18.5, they reach the region of the forebrain innervating the striatum, limbic system and the neocortex (Van den Heuvel and Pasterkamp, 2008; Prestoz et al., 2012). Several insights into how this tightly regulated circuit formation have been demonstrated based on the knowledge of the presence of guidance cues.

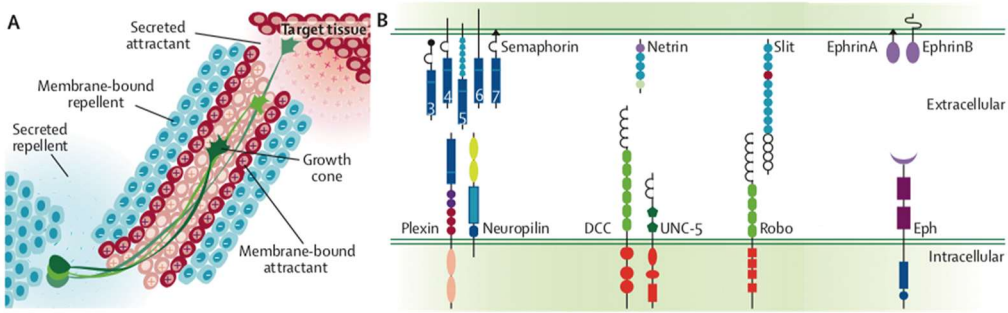

Figure I-6: Axon guidance principle and axon guidance molecules.
(A) Within the membrane surface of the GC, receptor proteins are able to bind to axon guidance molecules inducing axon steering. Guidance molecules have been identified and they can act as attractants, here shown in pink, or repellents, shown in blue. These guidance molecules can either be secreted proteins forming a gradient in the extracellular matrix or be membrane-bound proteins with short-range effects. (B) There are several conserved families of guidance molecules. Shown here are Ephrins, Netrins, Semaphorins, Slits (upper part) and their cognate receptors (lower part). Modified from (van Battum et al., 2014).

I.2.1.2.2.1. Ephs and Ephrins

Ephrins are cell-surface-tethered guidance molecules that bind to Eph tyrosine kinase receptors. Interestingly, Ephs can also bind to Ephrins by a “reverse signalling” mechanism (Van den Heuvel and Pasterkamp, 2008). Eph receptors are the largest tyrosine kinase receptor family and are subdivided EphA and EphB subclasses, based on sequence similarity and their preference for binding to a particular subclass of ephrins. Receptors of the subclasse EphA usually bind to glycosylphosphatidylinositol anchor (GPI)-linked ephrin A proteins whereas EphB receptors bind to transmembrane ephrin B proteins and during development these interactions have been shown to promote attraction, repulsion and to promote growth for neurites (Van den Heuvel and Pasterkamp, 2008; Klein, 2004).

Even though mDA neurons seem to express Ephs and Ephrins during development and adulthood, our knowledge in their spatiotemporal localization during those developmental windows remain elusive (Van den Heuvel and Pasterkamp, 2008; Klein, 2004). Yu et al showed that EphB1 is differentially expressed in VTA vs. SNpc neurons (SNpc with higher expression levels than VTA) and that ephrinB2 had a spatial preference in the striatum, being highly expressed in the NAc and olfactory tubercule but with lower expression in the CPu (Yue et al., 1999). These data together with axon outgrowth *in vitro* assays comparing VTA vs. SNpc axon growth in the presence of ephrinB2 strongly suggested that EphB1/ephrinB2 interaction could be implicated in the organization mDA striatal projection (Yue et al., 1999). However, Richards et al data contrasts this idea where *EphB1* mutant mice showed no anatomical defects in the striatal projections (Richards et al., 2007). Such discrepancy might be due to the fact that mDA neurons could use other Eph receptors other than EphB1 to interact with ephrinB2 in the striatum. Therefore, ephrinB2 *in vivo* role in the mDA projection organization remains to be elucidated. On the other hand, the manipulations in ephrinA/EphA interaction have provided more prominent phenotypes regarding mDA development. Indeed, mice lacking ephrinA5 display reduced striatal innervation (Cooper et al., 2009). Additionally ectopic expression of a soluble antagonist of ephrinA signalling – EphA5-Fc - by astrocites or neurons also rendered reduced TH+ striatal innervation together with behavioural changes characteristic to the phenotype (Halladay et al., 2004; Sieber et al., 2004). Of important notice is that the TH+ neuronal number in the ventral midbrain of these studies were not altered, strongly suggesting that the phenotype is related to growth or misguidance defects which remain to be determined.

I.2.1.2.2.2. Robo and Slits

Slits (Slit1, Slit2 and Slit3) are guidance cues that can repel axon outgrowth (including mDA axons) and branching and bind to the roundabout (Robo) receptors (Robo1, Robo2 and Robo3/Rig1). Both of them have been shown to be present and developmentally regulated in dopaminergic neurons, with Slits being present predominantly in postnatal stages (Van den Heuvel and Pasterkamp, 2008). Despite that, mDA neurons surrounding environment exhibit Slit expression (Marillat et al., 2002) and deficient mice for Slits and Robos display severe axonal defects phenotype including desorganization of the MFB (Bagri et al., 2002; López-Bendito et al., 2007). Altogether, it seems logical to speculate that Slit/Robo interaction could play a role in limiting mDA projections caudally and helping to organize the MFB and the dopaminergic projections towards the forebrain. In fact, *Slit1* and *Slit2* double mutant KO mice show a division in the MFB with many axons defasciculating and projecting towards the hypothalamus, even crossing the midline in the basal telencephalon (Bagri et al., 2002). Moreover, *Robo1* mutant KO mice display similar mDA axonal defective phenotypes as described above for *Slit* mutants. They also display an mDA axon bundle that projected dorsally from the mesodiencephalic region along the dorsal thalamus-pretectum limit. Lastly, *Robo1* and *Robo2* double mutant have even more pronounced mDA axonal phenotypes with mDA axons that failed to reach rostral regions, reached the ventral midline, wandered dorsally, formed a wider tract and projected dorsally in the mesodiencephalon just like *Robo1* mutants (Dugan et al., 2011). The presence of these dorsally projecting fibers in Robo1 mutant and absence in Slits mutants indicates that Robo1 might have Slit-independent functions in guiding mDA axons in a ventral path for the proper formation of the MFB.

I.2.1.2.2.3. DCC and Netrin

Netrins are guidance cues that can act as attractants, repellents and can also induce axon growth. Two receptors have been identified to interact with Netrins: deleted in colorectal cancer (DCC) that induces attractive effects and UNC-5 that induces repellent effects (van Battum et al., 2014). Both DCC and UNC-5 are expressed in mDA neurons during development, however UNC-5 is present at later stages to adulthood (Xu et al., 2010).

Due to the presence of both receptors in mDA development, it was hypothesized that Netrins could play a role in mDA axonal organization. Xu et al, analyzed *DCC* mutants.

Homozygous null for *DCC* displayed prominent developmental defects, including decreased mDA number in the ventral midbrain, mDA neuronal migration defects and axonal issues. Therefore, they further analyzed heterozigous *DCC* mutants in which although they still had decreased mDA neuronal numbers, the ventral striatum innervation was not altered, but contrastly mDA innervation of the medial prefrontal cortex was increased. Consistent to their hypothesis that DCC regulates mDA axonal projections, *in vitro* assays where DCC function was disrupted inhibited Netrin-1 induced mDA axon extension and axon branching. This study therefore highlighted the importance of DCC in regulating mDA development, most specifically precursor cell migration, axon guidance, and arborization by DA neurons (Xu et al., 2010).

In another interesting work, however focusing on Netrin-1 effects on mDA axon guidance, Li et al hypothesized that this guidance molecule could be involved in the dorsal vs. ventral patterning of mDA segregation from the VTA and SNpc. Their *in vitro* co-culture explants experiments showed that mDA axons are attracted to COS cells secreting Netrin-1 and to striatum explants. Additionally, in the co-culture of mDA explants and striatum, this preference was lost when anti-DCC antibody was added to the medium. Moreover, *Netrin* heterozygous embryos showed a tropism of mDA axons towards the striatum which was less observed in the *Netrin* null embryos. Analyzing later developmental stages, *Netrin-1* null mice eventually exhibit mDA striatal innervation, but in a disorganized manner - decreased dorsal striatum innervation and oriented medial-laterally instead of dorso-ventral. Importantly, Netrin-1 expression in the striatum shows a gradient with the highest concentration in the ventral and lateral parts of the striatum, and lowest in the dorsal striatum (Li et al., 2014). Thus, these results revealed novel actions for Netrin-1 providing evidence for a mechanism that is involved in mDA axons selective segregation into their target areas in the striatum.

I.2.1.2.2.4. Plexins, Neuropilins and Semaphorins

Semaphorins comprise a large family (more than twenty have been identified among eight classes) of secreted and transmembrane proteins that have a ~500 amino acid conserved extracellular Sema domain. They are important signalling proteins that have been linked to axon and dendritic growth, branching, guidance and pruning. Sema1-3 comprise secreted semaphorins while Sema4-7 are membrane associated semaphorins. Class 8 are virally encoded semaphorins. Two receptor families have been identified to interact in

response to semaphorins: plexins and neuropilins (Kumanogoh and Kikutani, 2013; Pasterkamp, 2012).

At least three semaphorins have been implicated in guiding embryonic mdDA axons: Sema3A, Sema7F and Sema7A. Sema3F expressed in the ventral midbrain and Sema3A expressed in the midline seem to participate in tightening the MFB and maintaining these fibers in an ipsilateral trajectory due to their interaction with mDA neurons receptors (Kawano et al., 2003; Kolk et al., 2009; Torre et al., 2010). Despite that, Sema3A relevancy to mDA axon guidance remains unclear as some studies show presence of its receptors in the MFB while others failed to show significant levels Sema3A bound to receptor NP-1 (Chung et al., 2005; Grimm et al., 2004; Hermanson et al., 2006; Hernández-Montiel et al., 2008; Kawano et al., 2003). Reports also indicate that Sema3A can act both as repellent and attractant (Castellani et al., 2000) and mDA explants treated with Sema3A showed increase axonal outgrowth (Hernández-Montiel et al., 2008).

Sema7a's relevancy for the topographic segregation of mDA axons in the striatum was demonstrated by Chabrat et al. (Chabrat et al., 2017). In this study, the Sema7A/PlexinC1 interaction and its contribution to the differential innervation of the striatum with axons derived from VTA and SNpc was characterized. They demonstrated that VTA explant axons are repelled by Sema7a while SNpc are not. Additionally, it was demonstrated that PlexinC1 expression is regulated by Lmx1a, Lmx1b and Otx2 transcription factors in mice, with Lmx1a/b expressed in both VTA and SNpc repressing PlexinC1, and with Otx2 expressed in the VTA inducing the receptor expression. Importantly, similar to Netrin-1, Sema7A was shown to be differentially expressed in a gradient manner in the striatum of mice (with higher protein levels in dorsal striatum in comparison to ventral striatum). Confirming the importance of these interactions, conditional knockouts for Lmx1a/b in mDA neurons showed decreased innervation of dorsal striatum whereas Sema7A KO displayed more PlexinC1-positive axons reaching dorsally. Thus, this data clearly shows the importance of Sema7A/PlexinC1 interaction in the segregation of the nigrostriatal and mesolimbic pathways (Chabrat et al., 2017).

I.2.1.2.2.5. Cellular integration of guidance signals

Despite the bulk knowledge acquired in extrinsic cue-derived axonal guidance in the past 20 years, intrinsic cellular mechanisms that could fine-tune the axonal cue response

and lead to differential steering of the axon are still scarce. The ability of the GC to guide the growth of axons is largely dependent on its highly dynamic cytoskeleton and guidance receptors sensitivity. Therefore, much of the data gathered until know focused on those factors and, indeed, important cellular players are emerging, such as second messangers Ca^{2+}, cyclic AMP (cAMP) and cyclic GMP (cGMP), Rho family GTPases and endocytosis (Akiyama and Kamiguchi, 2010; Borisoff et al., 2003; Gomez and Zheng, 2006; Henley and Poo, 2004; Hines et al., 2010; Itofusa and Kamiguchi, 2011; Luo et al., 1997; Tojima et al., 2011).

I.2.1.2.2.5.1. Second messengers and axon guidance signalling

Upon guidance signal from the environment, the primary means by which GC interprets and amplifies signals are second messenger signalling networks that involves Ca^{2+}, cAMP and cGMP. *In vitro* Ca^{2+} studies show that GC exposed to gradients of guidance cues react by inducing a corresponding gradient of Ca^{2+} elevation (Gomez and Zheng, 2006; Henley and Poo, 2004). More specifically, Netrin and BDNF mediated attraction have been shown to use transient receptor potential (TRP) Ca^{2+} channels contributing to membrane depolarization, and Ca^{2+} influx by these channels seemed to be required to chemoattraction as pharmacological blockage of TRP abolished attraction (Li et al., 2005; Wang and Poo, 2005). On the other hand, repellants such as Slits and Semaphorins have been linked to hyperpolarization (Henley et al., 2004; Nishiyama et al., 2008; Togashi et al., 2008). An example is Sema3A signalling that leads to the production of cGMP, which in turn is required for activation of cyclic nucleotide-gated (CNG) Ca^{2+} channels leading to membrane hyperpolarization and GC repulsion (Togashi et al., 2008). As a matter of fact, cyclic nucleotides just as Ca^{2+} impact guidance responses. The ratio of cAMP to cGMP have been proposed to determine if attraction of repulsion would take place with high cAMP/cGMP levels favouring attraction and low favouring repulsion (Nishiyama et al., 2003; Song et al., 1998, 1997). As mentioned before, Sema3A signalling modulates cGMP (Togashi et al., 2008) and genetic studies in *Drosophila* support the requirement of cGMP signalling in Sema/Plexin-mediated repulsion. Indeed, mutation of guanylyl cyclase receptor Gyc76C that has been linked to Sema/Plexin pathway results in motor axon defects comparable to phenotypes observed in Sema and Plexin mutants (Ayoob et al., 2004). There are evidences that Netrin guidance responses also lead to modulation of cAMP levels in *Xenopus* neurons (Corset et al., 2000; Höpker et al., 1999) and in rat DRG neurons (Wu et al., 2006). However,

mutations on soluble adenylyl cyclase failed to result in commissural axon guidance defects in mice (Moore et al., 2008a). Despite that, mounting evidences favour cyclic nucleotides role in axon guidance, but they argue against a generality in Netrin-mediated responses. Therefore, more studies are required to better understand the role of these second messengers in axon pathfinding.

I.2.1.2.2.5.2. Rho family GTPases

Rho-family GTPases are important in rearrangement of actin and microtubules cytoskeleton. They catalyze the hydrolysis of GTP to GDP, switching from active GTP-bound to inactive GDP-bound states. Rac, Cdc42 and RhoA are well studied members of the Rho-family (Hodge and Ridley, 2016) and downstream effectors include Rho kinases (ROCKs), LIM-kinase (LIMK), cofilin and p21-activated kinase (PAK). Importantly, Rho GTPases have been shown to be regulated by Slits, Ephrins, Semaphorins and Netrins. Mediated by Robo, Slits lead to Rac and Rho activation and inhibition of Cdc42 (Fan et al., 2003; Wong et al., 2001). In retinal ganglion cells, ephrins signalling lead to RhoA and Rac activity (the later only transiently) (Jurney et al., 2002; Wahl et al., 2000), and the “reverse signalling” Eph-ephrin results in axon pruning with Rac and Cdc42 activation (Xu and Henkemeyer, 2009). Sema3A activates Rac via PlexinA (Turner et al., 2004), and Semaphorin-mediated PlexinB1 signalling results in RhoA activation (Swiercz et al., 2002) and sequestering of active Rac (Hu et al., 2001). DCC-mediated Netrin responses were shown to increase Rac activity in fibroblasts (Li et al., 2002), to inhibit RhoA activity and to increase Cdc42 and Rac activities in comminssural neurons (Moore et al., 2008b; Shekarabi et al., 2005). Additionally, blocking ROCK with the inhibitor Y27632 led to axon guidance defects (Loudon et al., 2006) and influenced axon growth of chick DRG *in vitro* (Borisoff et al., 2003). Moreover, cofilin that has been linked to regulation of polimerization/depolimerization of F-actin (Lappalainen et al., 1951; Maekawa et al., 1999) was implicated in restoring attractive guidance cue responses in injured axons from hippocampal cultures (Tilve et al., 2015). PAK activation downstream to Rac was also linked to actin dynamics and controlling axon guidance in *Drosophila* (Newsome et al., 2000). Despite that, our knowledge about downstream signalling pathways to axon guidance is still fragmented and more data is required to better understand the contribution of these molecular players in different neuronal systems, including in the dopaminergic system.

I.2.1.2.2.5.3. Endocytosis and Exocytosis

Developing axons undergo dramatic morphological rearrangements that require changes in the surface area. Such conception led to the hypothesis that asymmetric endocytosis and exocytosis could drive these changes, and it is now clear that this mechanism is required in axon guidance (Hines et al., 2010; Kolpak et al., 2009; Tojima et al., 2007, 2010). Indeed, when in presence of guidance cues gradients, the GC will lead to a Ca^{2+} signalling and one of the consequences is modulation of endo/exocytic pathways across the GC (Tojima et al., 2014). Chemoattractants signalling normally promotes vesicle-associated membrane protein 2 (VAMP2) mediated exocytosis on the side encountering the cue gradient whereas chemorepellents result in clathrin-dependent endocytosis on the side facing the gradient (Hines et al., 2010; Kolpak et al., 2009; Tojima et al., 2007, 2010). Endocytosis is also required for efficient cell detachment which happens in cohort with proteolytic cleavage. Ephrin/Eph signalling is one example, and Vav family guanine nucleotide exchange factors were implicated in the endocytic regulation and detachment followed by this interaction. Indeed, *Vav2* and *Vav3* mice mutants display guidance defects consistent with a role as mediators of Eph repellent signalling. Moreover, neurons lacking Vav2 and Vav3 failed to respond to soluble ephrinA-Fc not leading to ephrinA/EphA complex endocytosis (Cowan et al., 2005).

Additionally to regulating the turning polarity of the GCs, these pathways are fundamental in regulating guidance receptors availability on the membrane surface, thus modulating GC responses to guidance cues. Mounting evidences show that receptors such as DCC, UNC-5 and neuropilin 1 have their levels at the GC membrane regulated by the endocytic pathway (Bartoe et al., 2006; Bouchard et al., 2004; Piper et al., 2005). Indeed, GC sensitivity to guidance signals can be adjusted by a process called “adaptation” (Ming et al., 2002). “Adaptation” refers to endocytosis-mediated desensitization when guidance receptors are internalized in the GC transiently desensitizing the GC with posterior protein synthesis-dependant resensitization (Ming et al., 2002; Piper et al., 2005). Examples of this adaptive process was described both upon Sema3A and Netrin-1 signalling using collapse assay of *Xenopus laevis* retinal GCs (Piper et al., 2005). Of note is that the generation of intracellular signals might require receptor processing into early endosomes (Joset et al., 2010; Mann et al., 2003; Zweifel et al., 2005).

I.2.1.2.2.5.4. Could autophagy play a role downstream to guidance signalling?

As mentioned above, during development, axons undergo dramatic morphological rearrangements and for these to take place, protein recycling pathways need to be active. Autophagy is the major cytoplasmic turnover pathway, therefore it is logical to think that this central process could be involved in the protein recycling required in growing axons. Additionally, Rho GTPases (Bento et al., 2013), second messengers (Wauson et al., 2014) and endocytosis (Tooze et al., 2014), all of which are downstream regulators of axon guidance, can modulate autophagy. Moreover, knockout models, such as shown by Hernandez et al., Dragich et al., Ban et al. and Yang et al., also highlight the autophagic importance in the axon. These studies show that axonal structure and presynaptic function are altered in Atg7 conditional KO (cKO) in mDA neurons (Hernandez et al., 2012), that major axonal tracts in the brain are defective in Alfy/WDFY3 - an autophagy adaptor protein – KO (Dragich et al., 2016), and linked autophagy with axon growth and branching (Ban et al., 2013; Yang et al., 2017). These data, however, refer mostly to autophagic adaptor and/or regulator proteins not excluding that the effects seen might be related to their non-autophagical roles. Additionally, most of these studies used non-neuronal specific knockouts and used knockouts that could influence neuronal differentiation raising the possibility that the effects seen could be due to defects in neuronal development rather than axon specific development. Therefore, although evidences point-out to a central role of autophagy in axonal guidance, the limitations on the models used on these studies limit the conclusions that can be taken about the involvement of autophagy in axon guidance. In light of that, more studies are warranted to properly dissect the involvement of this process in axonal development.

I.3. Autophagy

Autophagy, from the Greek “auto” oneself, and “phagos” to eat, is every cellular degradative pathway that involves the delivery of cytoplasmic constituents to the lysosome. There are at least three forms of autophagy: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. CMA has only been described in mammals and involves a selectively deliver of proteins from the cytoplasm to the lysosome in a process that depends of a recognition by receptors from the lysosome of a motif present in cytosolic proteins. Microautophagy refers to a process in which portions of cytoplasm are sequestered by invagination of the lysosomal or vacuole membrane to be degraded. Macroautophagy is

the major regulated catabolic mechanism in eukaryotic cells that is responsible for the removal of long-lived proteins and damage organelles by the lysosome or vacuole (Levine and Kroemer, 2008; Marino and Lopez-Otin, 2004). This dynamic self-digesting process will hereafter be referred as autophagy. In virtually all cells, autophagy is active at a basal level performing homeostatic functions such as protein and organelle turnover, and it is up-regulated in response to both extracellular and intracellular stress conditions such as nutrient starvation, growth factor withdrawal, hypoxia, aggregation of proteins, accumulation of damaged mitochondria, oxidative stress, or high bioenergetics demands. It is also stimulated when cells undergo structural remodeling (Levine and Kroemer, 2008). This pro-survival mechanism is essential for mammalian development. Indeed, it has been shown to be necessary for the Oocyte to embryo transition and important in the embryo to neonate transition (Mizushima and Levine, 2010).

Autophagy is a very conserved process that relies on several protein complexes that are well characterized (Kaur and Debnath, 2015; Lee et al., 2012). These proteins have been identified and named autophagy- related proteins – ATGs – (Figure I-7) (Kaur and Debnath, 2015; Lee et al., 2012). In response to the stimulus, autophagy begins in the cytoplasm with a “C” shaped double membrane structure – phagophore. In yeast, the pre-autophagosomal structure has been assumed as the origin of the phagophore and localizes in the perivacuolar region, where almost all ATG proteins assemble. However such structure has not been observed in mammalian cells and the origin of the membranes that form the autophagic vesicles have not been determined in mammals yet. The initial steps of autophagy include the vesicle nucleation (formation) and vesicle elongation (expansion) of the phagophore. The phagophore edges then fuse themselves engulfing cytoplasmatic cargo and forming the autophagosome (vesicle completion). At this point the autophagosome may fuse with an endosome originating an acidic vesicle – amphisome. Autophagosome or amphisome subsequently fuse with lysosomes enabling the degradation of the cargo by hydrolases. The products of this degradation (e.g. amino acids, lipids) are then exported from the lysosome and recycled for new biosynthetic demands (Levine and Kroemer, 2008). Therefore, autophagy acts by recycling essential “building blocks” to help sustain cell viability.

Until recently autophagy was considered to be a non-selective degradation process in which cytosolic materials were randomly engulfed. However, recent evidences support that, in most cases, cargo is specifically recognized by a growing number of cargo-

recognition molecules or autophagy receptors that physically interact with the material tagged for degradation and with effectors of the autophagy process (Park and Cuervo, 2013). Depending on the cargo to be degraded, new terms to describe this selective nature of autophagy have been proposed, such as reticulophagy for the degradation of endoplasmic reticulum (ER), mitophagy for mitochondrial degradation, ribophagy for ribosomes, xenophagy for pathogens, pexophagy for peroxisomes, lipophagy for lipid droplets, and aggrephagy for aggregates (Park and Cuervo, 2013). This selectivity is ensured by a family of cargo-recognition proteins such as p62 and next to BRCA1 gene 1 protein (NBR1) that are able to interact with microtubule-associated protein light chain 3 (LC3) bound to the double membrane as well as with specific tags in the cargo to be cleared.

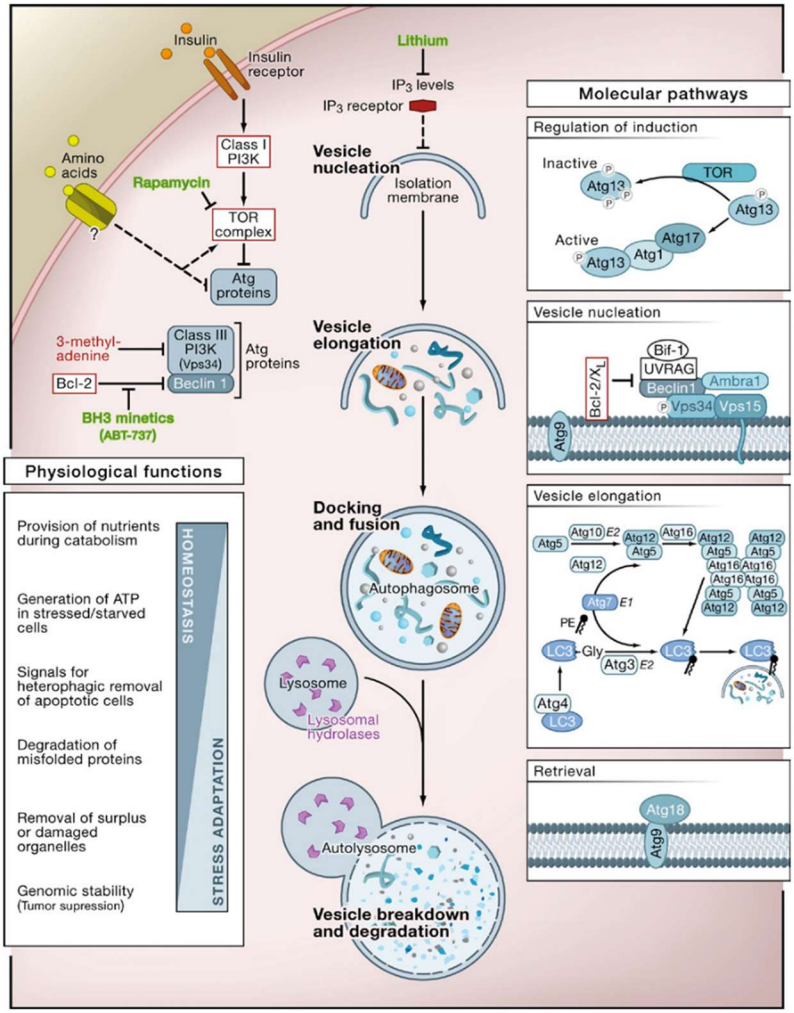

Figure I-7: Overview of the autophagy pathway.
Autophagy is a homeostatic conserved multi-step degradation pathway that leads to cytoplasmic contents degradation within the lysosome. For its proper function, it relies in

several protein complexes from its induction to the final step the degradation, as seen on the right located panels above (Levine and Kroemer, 2008).

I.3.1. Neuronal autophagy

Autophagy is constitutively active in healthy neurons (Lee, 2012; Nixon et al., 2005; Xilouri and Stefanis, 2010). Indeed, neurons are postmitotic and their vast majority are born during embryogenesis having to survive for an entire lifetime. Therefore they are particularly dependent on active degradation pathways such as autophagy to maintain homeostasis and viability as they cannot dilute out proteotoxins simply by cell division (Kulkarni et al., 2018).

Underscoring the importance of autophagy in neurons are several mice models. *Atg5* and *Atg7* deletion from the central nervous system of mice results in accumulation of ubiquitin-positive inclusions, neurodegeneration and motor dysfunction (Hara et al., 2006; Komatsu et al., 2007), indicating the importance of autophagy in the constitutive proteome quality in neurons. Alterations in autophagy have been in fact linked to neurodegenerative disorders, such as Alzheimer's disease (AD), PD, Huntington's disease (HD), and frontotemporal dementia (FTD) (Boland and Nixon, 2006; Lee, 2012; Yue et al., 2009).

Autophagy has also been shown important to neurodevelopment. Examples are Ambra1 KO, Atg7 mDA conditional knockout (cKO), Alfy/WDFY3 KO and Mir505-3p KO (Dragich et al., 2016; Fimia et al., 2007; Hernandez et al., 2012; Yang et al., 2017), all of which displayed structural brain defects. Agreeing with these models is that autophagy has been linked to neurodevelopmental disorders (Lee et al., 2013; Tang et al., 2014).

Despite that, most of the studies regarding neuronal autophagy have been made in mature neurons in an attempt to link autophagy to neurodegeneration. Therefore, the role of autophagy during neuronal development is still fragmented.

I.3.1.1. Axonal autophagy

Neurons are characterized by a highly complex and polarized morphology with extended axonal and dendritic processes. They face the challenging panorama of executing autophagy over the extended distance of the axon. Although axonal autophagy evidences have been present since the 60’s and 70’s (Bunge, 1973; Riley and Chapman, 1967), only recently this process started to be elucidated. With live imaging making use of LC3 (an important autophagic marker) tagged with fluorescent proteins it could be observed that, in

primary neurons, autophagosomes initiate distally and mature into degradative organelles during transport toward the cell soma (Maday et al., 2012). Their transport towards the soma is driven by the microtubule-associated protein dynein (Cheng et al., 2015) and these degradative structures transport engulfed soluble and organelle cargoes such as ubiquitin and mitochondrial fragments (Maday et al., 2012). Despite that, acute and localized axonal stress can lead to execution of autophagy locally. Ashrafi et al., recently demonstrated that in focally-induced depolarization of mitochondria along the axon recruits LC3 and neighbouring lysosomes for local degradation of damaged mitochondria in a PINK1 and Parkin dependent manner (Ashrafi et al., 2014).

Knockout models also highlight the importance of this process in the axon. Axonal structure and presynaptic function are altered in Atg7 conditional KO (cKO) in mDA neurons (Hernandez et al., 2012), and axon major tracts in the brain are defective in Alfy/WDFY3 - an autophagy adaptor protein – KO (Dragich et al., 2016). Interestingly, there are also evidences that suggest the involvement of autophagy in axon growth and branching (Ban et al., 2013; Yang et al., 2017).

Mounting evidences suggest a role of autophagy in axonal circuit development, but despite that, the comprehension of the autophagy involvement in such process is still rudimentary and more data is demanded to fully corroborate the autophagy role in axon growth/guidance.

I.4. Rationale and aim of the project

One of the most challenging difficulties in biology is to understand how billions of nerve cells form their precise connections that underlie complex behaviours. Axon pathfinding has been well described in spinal cord; however, not extensively in the brain. In fact, the molecular mechanisms that drive the midbrain dopaminergic trajectory formation are largely unknown. Although several important proteins have been identified to guide growing axons, the cellular integration of these different signals is still poorly understood. In this context, autophagy, the major cytoplasmic turnover pathway, has been shown to play central roles in different cell types (Levine and Kroemer, 2008). Additionally, known downstream pathways to guidance signals, such as derived from Rho GTPases (Bento et al., 2013), second messengers (Wauson et al., 2014) and endocytosis (Tooze et al., 2014) can regulate autophagy. This information together with our initial data showing that

autophagy machinery is present and timely regulated in mDA neurons during development and enriched in mDA growth cones suggest a prominent role for this this process in mDA development.

Therefore, this PhD project aimed at depicting the role of autophagy in mDA axonal development. To dissect such panorama, we focus on two main objectives:

(1) The role of autophagy in mDA axonal morphology.

Hypothesis: Autophagy is important and it regulates mDA axonal morphology and arborization.

Objective: To characterize the importance of autophagy in the axonal morphology maintenance and arborization complexity throughout mDA development *in vitro*, *in vivo* and *ex-vivo* by making use of Atg5 cKO.

(2) Reveal the role of autophagy in mDA axon growth and guidance.

Hypothesis: Autophagy is required downstream to guidance cues signals in mDA axonal growth and guidance.

Objective: To evaluate the requirement of autophagy in the mDA axonal growth and guidance downstream to guidance cues.

Chapter 1: Autophagy is required for midbrain dopaminergic axon development and their responsiveness to guidance cues

1.1 Résumé

Les neurones dopaminergiques mésodiencephaliques (mDA) jouent un large éventail de fonctions cérébrales. Des sous-types distincts de neurones mDA régulent ces fonctions, mais les mécanismes moléculaires à la base de la formation du circuit mDA sont en grande partie inconnus. Nous montrons ici que l'autophagie, principale voie de recyclage cellulaire, est présente dans les cônes de croissance des neurones mDA en développement et que son niveau change de manière dynamique en réponse aux signaux de guidage. Pour caractériser le rôle de l'autophagie dans la croissance / le guidage des axones mDA, nous avons neutralisé (KO) les gènes essentiels de l'autophagie (Atg12, Atg5) dans des neurones mDA de souris. Les axones mDA déficients en autophagie présentent des gonflements axonaux et une diminution du nombre de ramifications in vitro et in vivo, probablement en raison de la formation de boucles de microtubules aberrantes. De manière frappante, la suppression de gènes liés à l'autophagie a complètement émoussé la réponse des neurones mDA aux signaux de guidage chimio-répulsifs et chimio-attractifs. Nos données démontrent que l'autophagie joue un rôle central dans la régulation du développement des neurones mDA, orchestrant la croissance axonale et l'orientation.

Autophagy is required for midbrain dopaminergic axon development and their responsiveness to guidance cues

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1.2 Abstract

Mesodiencephalic dopamine (mDA) neurons play a wide range of brain functions. Distinct subtypes of mDA neurons regulate these functions but the molecular mechanisms that drive the mDA circuit formation are largely unknown. Here we show that autophagy, the main recycling cellular pathway, is present in the growth cones of developing mDA neurons and its level changes dynamically in response to guidance cues. To characterize the role of autophagy in mDA axon growth/guidance, we knocked-out (KO) essential autophagy genes (*Atg12, Atg5)* in mice mDA neurons. Autophagy deficient mDA axons exhibit axonal swellings and decreased branching both *in vitro* and *in vivo*, likely due to aberrant microtubule looping. Strikingly, deletion of autophagy-related genes, blunted completely the response of mDA neurons to chemo-repulsive and chemo-attractive guidance cues. Our data demonstrate that autophagy plays a central role in regulating mDA neurons development, orchestrating axonal growth and guidance.

1.3. Introduction

During mammalian embryogenesis, neurons form a highly interconnected network by elongating and projecting their processes toward their target areas. Axonal development

is organized into multiple steps starting from growth cone formation, axonal growth and branching, which are coordinated by both intrinsic mechanisms and extrinsic cues from the surrounding microenvironment that shape axonal growth and guidance (Tamariz and Varela-Echavarría, 2015). Midbrain dopaminergic (mDA) neurons located in the ventral tegmental area (VTA) and *substantia nigra pars compacta* (SNpc) project their axons to different brain regions and control a wide range of behavioral responses. VTA neurons are medially located in the ventral midbrain and project to different regions implicated in the reward pathway, including the *nucleus accumbens* and the prefrontal cortex (PFC), constituting the mesocorticolimbic pathway. Imbalances of dopamine input in the mesocorticolimbic pathway have been linked to depression, drug addiction, attention deficit hyperactivity disorder and schizophrenia (Van den Heuvel and Pasterkamp, 2008). Laterally located SNpc neurons target the dorsolateral striatum (caudate putamen), forming the nigrostriatal pathway. Nigrostriatal connectivity loss is the main histopathological feature of Parkinson's disease (PD) (Van den Heuvel and Pasterkamp, 2008). Several axonal guidance molecules have been described and shown to be crucial for the proper growth and targeting of mDA neuron axons. Sema7a and Netrin-1 seem to be of special importance in guiding such axons to their proper targets in the striatum and in the PFC (Chabrat et al., 2017; Flores, 2011).

Despite the growing body of evidence that describes axonal guidance and guidance molecules and their receptors, the downstream effectors of such important proteins remain elusive. Interestingly, mRNA translation, Ca^{2+} signaling and local protein degradation have been implicated as downstream effectors of axonal guidance (Campbell and Holt, 2001; Sutherland et al., 2014). Additionally, endocytosis and exocytosis in the growth cone have been suggested to be involved in the specific turning of the growth cone toward or away from certain environmental cues (Tojima et al., 2011). Interestingly, there is commonly cross-talk between the endocytosis and autophagy pathways, resulting in endocytic cargo being recycled by autophagy (Lamb et al., 2013). Furthermore, macroautophagy is the major catabolic mechanism in eukaryotic cells that is responsible for the removal of long-lived proteins and damaged organelles in the lysosome (Kaur and Debnath, 2015; Lee et al., 2012). This dynamic self-digesting process will hereafter be referred to as autophagy. Autophagy is a very conserved process and relies on several protein complexes that are well characterized and are implicated from the initiation to the degradation step (Kaur and Debnath, 2015; Lee et al., 2012). Autophagy proteins have been identified and named

autophagy-related proteins (ATGs) (Kaur and Debnath, 2015; Lee et al., 2012). In virtually all cells, autophagy is active at a basal level, performing homeostatic functions such as protein and organelle turnover. This housekeeping process is upregulated in response to both extracellular and intracellular stress conditions, such as nutrient starvation, growth factor withdrawal, hypoxia, aggregation of proteins, accumulation of damaged mitochondria, oxidative stress and high bioenergetic demands (Kaur and Debnath, 2015; Lee et al., 2012). Autophagy is also stimulated when cells undergo structural remodeling (Mizushima and Levine, 2010). Notably, interfering with the expression of different autophagy proteins has been reported to cause brain defects. Knockout embryos for Ambra1, an autophagy-related protein, display accentuated mid-hindbrain exencephaly (Fimia et al., 2007), whereas Atg7 conditional knockout (cKO) in mDA neurons leads to aberrant axonal morphology and altered dopamine release in young animals (Hernandez et al., 2012). Moreover, Alfy/WDFY3, an autophagy adaptor protein, has been shown to regulate the formation of major axonal tracts in the brain as well as in the spinal cord (Dragich et al., 2016), and microRNA Mir505-3p, which targets *Atg12*, a core autophagic protein, regulates axonal elongation and branching *in vitro* and *in vivo* (Yang et al., 2017). Much of this evidence, however, comes from experiments with autophagic adaptor and/or regulatory proteins, not core autophagic proteins; therefore, it cannot be excluded that some of the brain defects reported might be related to their non-autophagic roles. Additionally, most of the KO models used were nonneuronal-specific; thus, one cannot exclude the possibility that the observed axon defects could be due to alterations in the cellular organization of nonneuronal cells in the brain. Finally, little is known about the role of autophagy in axonal growth and the guidance of dopaminergic neurons and how this major catabolic pathway is regulated in response to different microenvironmental cues. Therefore, more data are needed to fully corroborate a central role of autophagy in brain development and its implications in the axon growth/guidance of dopaminergic neurons.

In this study, using CRISPR-Cas9 gene editing and cKO of Atg5 specifically in dopaminergic neurons, we show that autophagy is required for proper mDA axonal morphology and branching during development. Moreover, we demonstrate that autophagy plays a central role in mediating the responses of dopaminergic axons to guidance cues, and ablation of Atg5 in mDA neurons completely blunts axon growth/guidance in response to major chemorepellant and chemoattractive signals, such as Sema7a and Netrin-1. Our data reveal a central role of autophagy in dopaminergic system development by regulating

axonal growth/guidance and mediating the responses of these cells to extrinsic guidance cues.

1.4. Results

1.4.1. Autophagy machinery is present in mDA neuronal soma and axons and is enriched in growth cones.

To determine whether autophagy could potentially be involved in mDA development, we first performed immunofluorescent staining on brain sections at different maturational stages. The presence of autophagy was assessed in midbrain sections collected from embryonic day 14 (E14) to postnatal day 7 (P7) through TH (tyrosine hydroxylase) marker and LC3 (an essential autophagy protein) colabeling. This developmental window from E14 to P7 corresponds to the entry of mDA axons in the ganglionic eminence (E14.5) and was chosen because mDA axons are growing toward their forebrain targets in the embryonic stage, having their connections consolidated at approximately P7. LC3 is expressed in TH+ mDA neurons in the VTA and their axon projections in the medial forebrain bundle throughout development (**Fig. 1-1A and B**). To investigate the expression of LC3 in growth cones (GCs), we employed explant cultures. Immunofluorescent signals for LC3 were highly expressed in TH+ GCs from 3 day *in vitro* (3DIV) explant cultures (**Fig. 1-1C**). To further characterize autophagy in mDA development, we performed western blotting from ventral midbrain samples collected from wild-type mice. Samples were collected from embryonic stages to adulthood to comprise the developmental window described above. Our western blot analysis of the autophagic marker LC3-II, which represents the lipidated autophagosomal-associated form of LC3; the Atg5-Atg12 complex, which is required for autophagy initiation; and p62, which is the main autophagic substrate, showed similar temporal profiles. These markers displayed increasing levels from embryonic to postnatal stages, reaching their highest levels at P7 with a subsequent decrease with further postnatal development (**Fig. 1-1D**). Interestingly, P7 corresponds to a developmental stage where axonal pruning occurs, a mechanism that requires dynamic axonal changes (Hu et al., 2004). This structural plasticity is required for mDA connection consolidation and fine-tuning. Hence, the presence and temporal regulation of autophagy markers throughout mDA neuron development and LC3 enrichment in GCs suggest that autophagy may play a major role in dopaminergic circuit formation and axon growth/guidance.

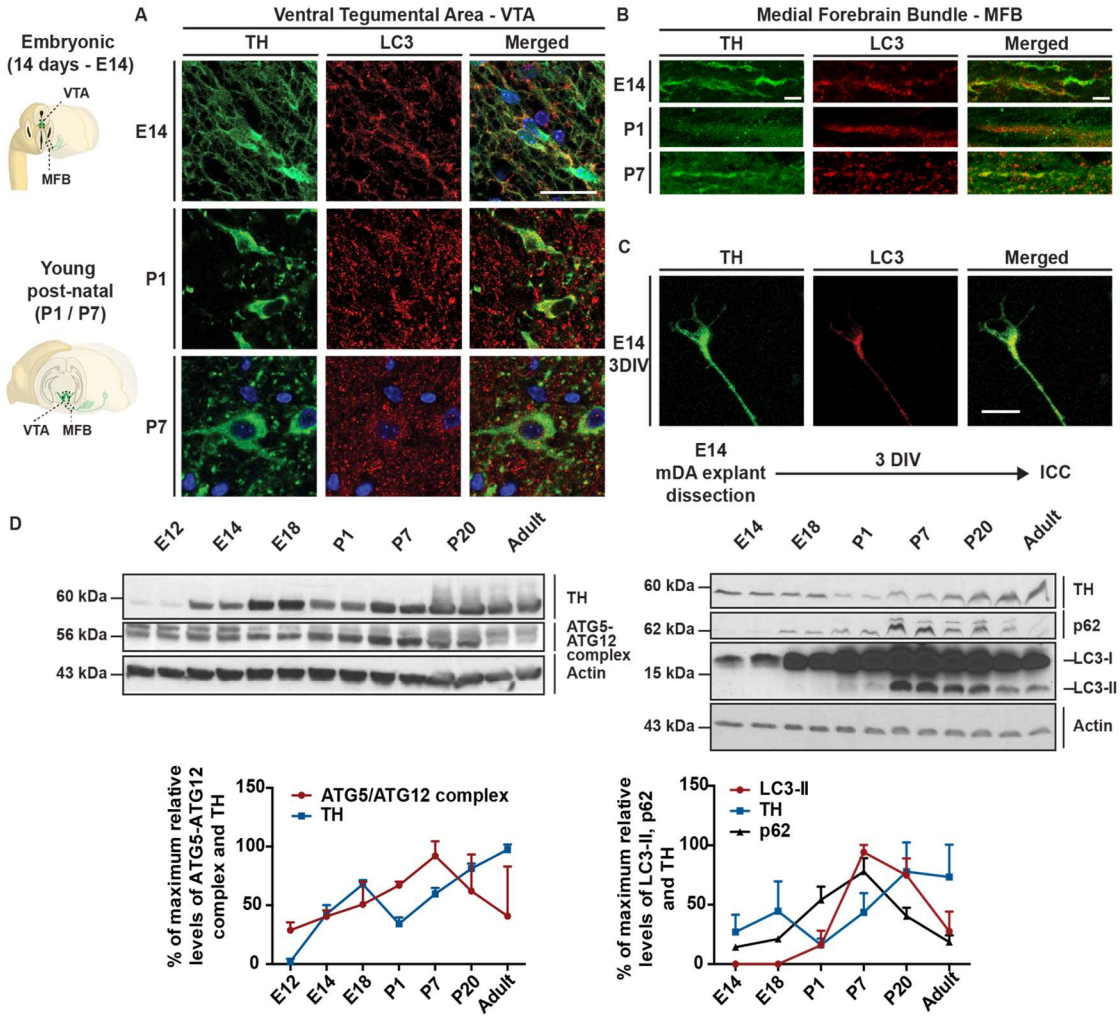

Figure 1-1: Autophagy is present and is timely regulated in midbrain dopaminergic neurons and axons throughout development.
Representative confocal images of immunohistochemical analysis of the autophagic marker LC3 in the VTA (A) and in the medial forebrain bundle (B) from E14 to P7 (n=3 animals with an average of 3 sections per animal). (C) Immunolabeling for LC3 in E14 explant cultures showing enrichment of autophagy markers in the GCs of TH+ axons (n=3 explants from 3 individual embryos). (D) Western blot analysis of autophagic markers LC3-II, Atg55-Atg512 complex and p62 displays time-dependent changes in the protein levels during the developmental window (embryonic to postnatal stages, n=3 animals per age). Scale bars: A, 25 µm, and B and C, 10 µm.

1.4.2. Autophagy regulates mDA axonal morphology and arborization.

To test the role of autophagy during mDA circuit development, we generated autophagy-deficient conditional mutant mice in mDA neurons by crossing *Dat*$^{Cre/+}$ mice with *Atg5*$^{flox/flox}$ mice (hereafter referred to as Atg55 cKO). Dat$^{+/+}$Atg5f$^{/f}$ mice were used as

controls, unless noted otherwise in the text. *Cre* recombinase expressed under the control of the *Dat* promoter was efficient in deleting *ATG5* from mDA neurons as seen by western blotting performed on P7 ventral midbrain samples (**Fig. 1-S1**). Importantly, because of the DAT expression pattern, Atg5 deletion occurs only in postmitotic mDA neurons just prior to their axonal growth toward the forebrain (Prestoz et al., 2012), allowing blockage of autophagy at a developmental time point that does not interfere with mDA neuron differentiation from their progenitors.

Since autophagy is known to be involved in cytoskeletal remodeling, we first examined its requirement for mDA axonal growth and morphology. We used explants of the VTA of E14 Atg5 cKO mice and their control littermates cultured for 3DIV and measured axon diameter and GC area. Axons and GCs from Atg5 cKO explants displayed an abnormal morphology and showed 2.6- and 1.7-fold increases in the axon diameter and GC area, respectively, compared to axons in the control explants (**Fig. 1-2A**). Sholl analysis also showed a reduced number of intersections of axons in Atg5 cKO explants compared to control explants (**Fig. 1-2B**). Importantly, the length of axons and their number that initially grew outward from the explants did not differ significantly between mutants and control explants. To test whether similar morphological defects are observed following manipulation of other autophagy-related genes in mDA neurons, we next performed CRISPR-Cas9 gene editing for *Atg12,* a core autophagy-related protein required for autophagic vesicle formation (Kaur and Debnath, 2015; Lee et al., 2012). Similar to Atg5 cKO, CRISPR-Cas9 gene editing for *Atg12* led to GCs and axonal enlargements with similar fold-change to Atg5 cKO (2.6- and 1.3-fold changes, respectively; Fig. S2).

To determine the requirement of autophagy for mDA axonal morphology *in vivo,* we performed immunohistochemistry for TH on P11 nigrostriatal brain sections derived from Atg5 cKO and control mice. P11 was chosen since the nigrostriatal dopaminergic connections are established around P7, which allows us to assess the impact of autophagy on the establishment and/or maintenance of mDA axons. To ensure that the lack of autophagy ablation did not induce the loss of dopaminergic neurons and that any possible defects in the number or density of axonal profiles in the striatum were not due to lower number of mDA neurons, we performed stereological counting of TH+ neurons in the SNpc and VTA. Neuronal counting did not reveal any difference in the total number of mDA neurons between mutant and control mice (**Fig. 1-3A and B**). To study the axonal projection morphology *in vivo*, we counted the number and size of TH+ profiles (see arrowheads in

Fig. 1-3A) on matched coronal sections from Atg5 cKO mice and control littermates. Our analysis revealed a 1.5-fold enlargement of TH+ axonal profiles and a 0.7-fold reduction in their number when comparing mutants and controls. These data are in line with our *in vitro* analysis showing enlarged GCs and reduced axonal branching and indicate that autophagy is required for proper morphological development and/or maintenance of mDA axons.

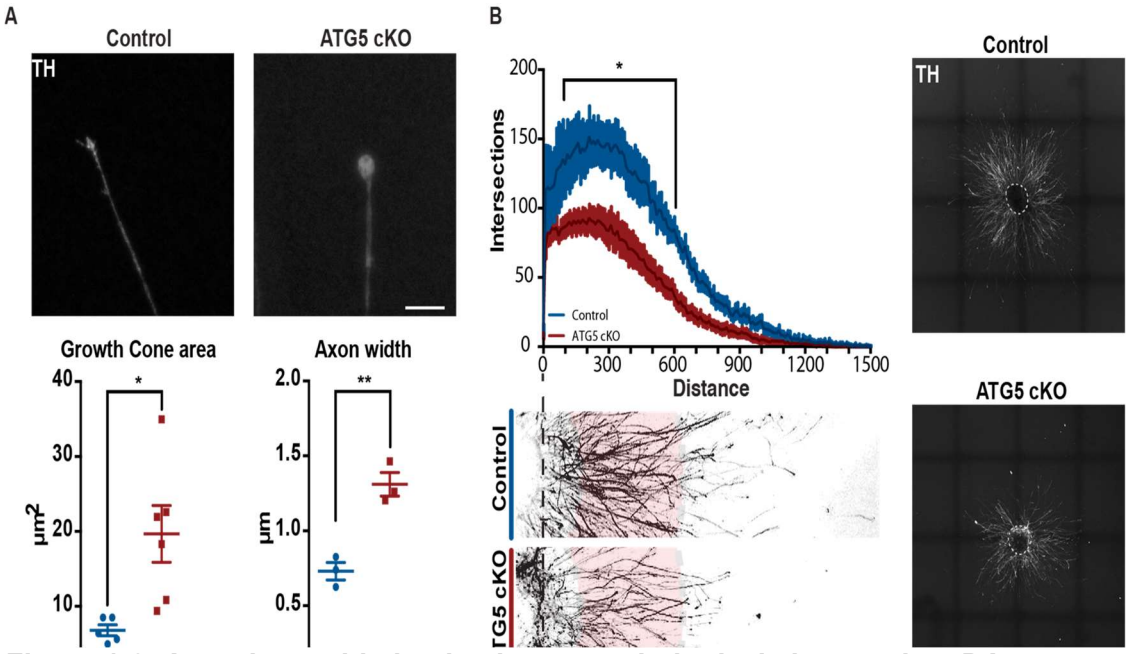

Figure 1-2: Autophagy ablation leads to morphological changes in mDA axons and GCs.
(A) E14 VTA from (a) *Dat*$^{+/+}$*Atg5*$^{flox/flox}$ (control) and *Dat*$^{Cre/+}$*Atg5*$^{flox/flox}$ (Atg5 cKO) mice were dissected into explants and cultured in 3D collagen matrix for 3DIV prior to fixation and ICC. GC area: n=5 explants for control samples and n=6 explants for Atg5 cKO samples dissected from 4 animals for each genotype. For each explant, an average of 20 GCs were analyzed, and the average per explant is plotted (mean values= 6.78 for control and 19.66 for Atg5 cKO; *p*=0.0142; two-tailed t test). Axon width: n=3 explants dissected from 3 animals for each genotype per condition with an average of 20 axons analyzed per explant and averaged (mean values= 0.73 for control and 1.3 for Atg5 cKO; *p*= 0.0041; two-tailed t test). (B) Sholl analysis of E14 explants at 3DIV reveals axonal arborization differences between Atg5 cKO and wild-type samples. n=7 explants for controls and n=10 for Atg5 cKO samples dissected from an average of 6 animals. Two-way ANOVA and the Sidak test were used for post hoc comparisons, *p < 0.05). Scale bar A, 10 μm, and B, 500 μm.

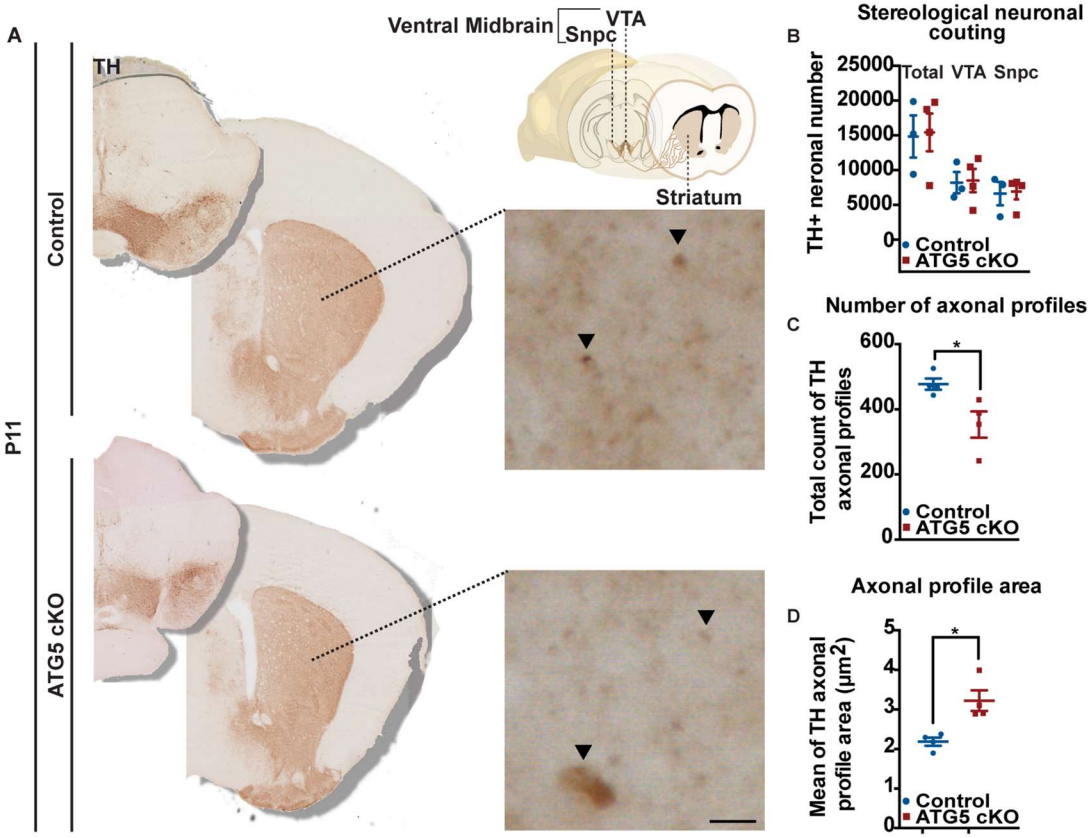

Figure 1-3: Autophagy regulates dopaminergic axonal morphology and arborization *in vivo*.
(A) Immunohistological images reveal different striatal TH staining patterns when comparing P11 controls and Atg5 cKO mice. A higher-magnification image shows the abnormal axon coronal profile enlargements of TH+ axons. (B) mDA neuronal stereological counting shows no difference between groups, n=3 animals per condition (3 sections per animal). Quantification of higher-magnification images showing a reduced number of coronal axon profiles reaching the striatum (C) and the enlargements of the aforementioned profiles (D). N=4 animals per condition (3 sections per animal) and p=0.03 (mean values= 477.23 for control and 353.27 for Atg5 cKO) and p=0.0104 (mean values= 2.18 for control and 3.22 for Atg5 cKO) for C and D, respectively (two-tailed t test). Scale bar, low magnification 50 µm and higher magnification 10 µm.

1.4.3. Autophagy ablation leads to the formation of aberrant microtubule loops within GCs.

It has been previously shown that enlarged GCs can be characterized by a round-shaped tip with looped microtubules (Dent et al., 1999). We thus tested whether autophagy ablation could lead to the aberrant formation of microtubule loops. We performed primary ventral midbrain culture from Atg5 cKO mice and control littermates and performed immunostaining for TH as an mDA neuronal marker in combination with alpha-tubulin immunolabeling to visualize microtubule organization. GCs from midbrain cultures of Atg5

cKO mice displayed an increased prevalence of intra-axonal loops compared to controls (**Fig. 1-4**). Measurement of these loops also revealed that microtubule loops from Atg5 cKO mice were 1.7-fold larger than the loops found in the GCs of control animals. TH+ GCs containing two or more microtubule loops were also observed, and the percentage of GCs with 2 or more loops was much more frequent in Atg5 cKO cultures than in control cultures (2.3-fold change). In sum, autophagy ablation leads to abnormal microtubule phenotypes in dopaminergic GCs and most likely underlies the enlarged GCs observed in Atg5 cKO Gcs.

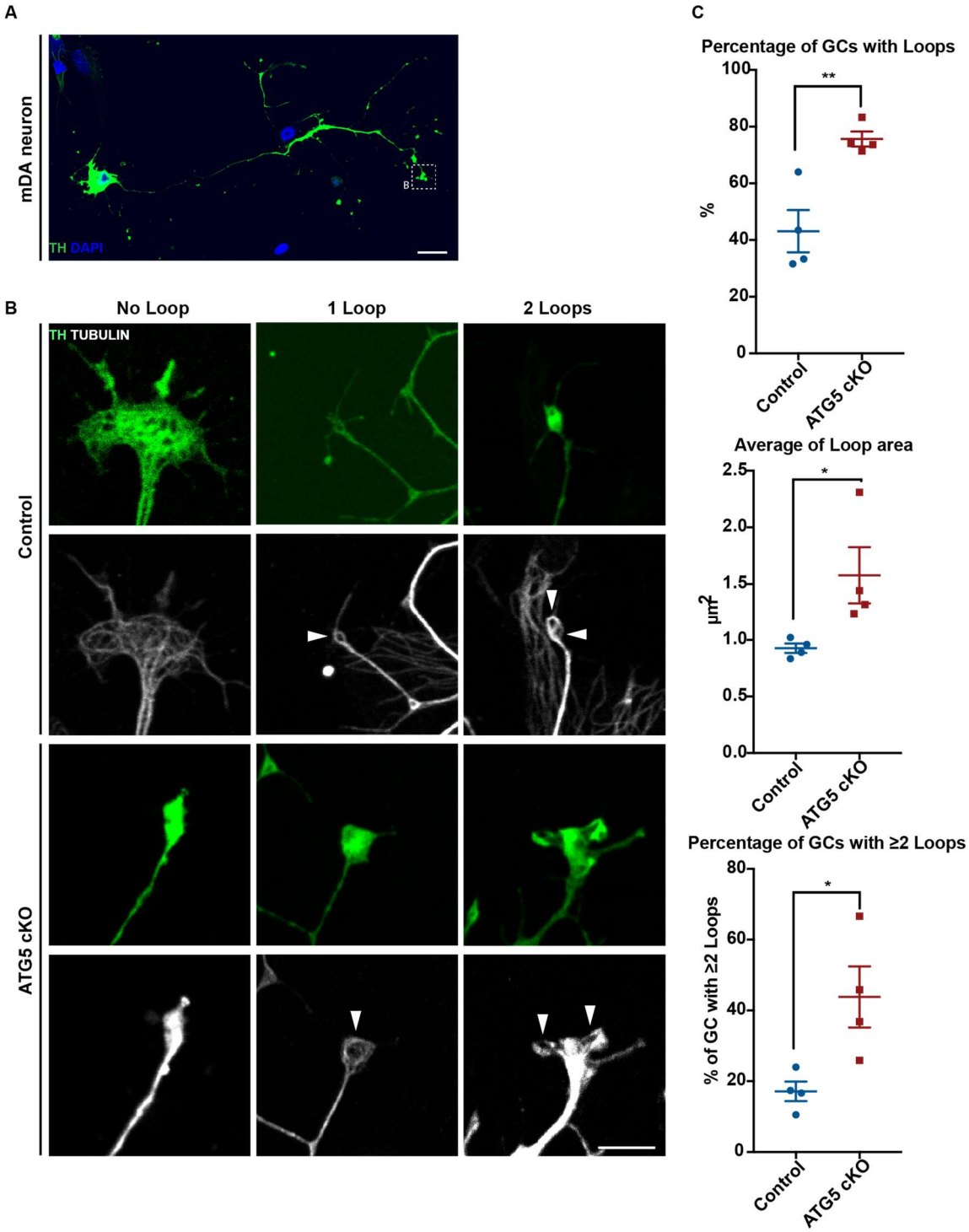

Figure 1-4: Autophagy ablation in mDA neurons leads to the formation of aberrant microtubule loops in GCs.

(A) Representative immunohistological image of an mDA primary culture neuron at 3DIV. Dashed B square depicts an example of GC. (B) Representative high-magnification images of GCs colabeled with TH and tubulin. From left to right, images are separated into GCs without any loop, those with one loop and those with two loops. (C) Quantification of high-magnification images showing from top to bottom that Atg5 cKO cultures display a higher prevalence of loops in GCs than controls (75% in comparison to 43%; *p*=0.0062), a 1.7-fold

larger area (control mean value= 0.92; Atg5 cKO mean value= 1.57; *p*=0.0413) and a higher prevalence of 2 or more loops within GCs (control= 17%; Atg5 cKO= 43.8%; *p*=0.0259). Arrowheads indicate loop presence in the image. The top two rows are images derived from control cultures, and the bottom rows are images from Atg5 cKO cultures. The results shown here are averaged from 4 independent experiments (n=4) with an average of 10 GCs analyzed per experiment. Scale bar, low magnification 50 µm and higher magnification 10 µm.

1.4.4. Autophagy is induced in GCs upon guidance cue exposure in mDA primary cultures.

Autophagy has been shown to regulate the cytoskeleton (He et al., 2016), cellular energy balance (Kaur and Debnath, 2015) and receptor turnover (Khan et al., 2014), all of which have been reported to be involved in axon guidance. Hence, we hypothesized that autophagy could regulate GC responsiveness to guidance cues. To test this hypothesis, wild-type mDA primary cultures were incubated with Sema7a (250 ng/mL), Netrin-1 (200 ng/mL) or control vehicle (1× PBS) for 4 h at 3DIV followed by immunolabeling for TH and LC3. Sema7a and Netrin-1 are important guidance cues for mDA axons, controlling proper innervation of the striatum and the prefrontal cortex. Since autophagosomal LC3 is represented by a dotted pattern (Klionsky et al., 2016), we quantified the number of LC3 puncta in TH+ axons and GCs. Cultures treated with Sema7a and Netrin-1 displayed 1.6- and 1.5-fold increases in the number of LC3 dots, respectively, in the GCs when compared to control conditions (**Fig. 1-5A-D)**. When analyzing the number of LC3 dots found in the axon (40 µm away from the GC), the results show a decrease in the number of LC3+ dots following Sema7a and Netrin-1 treatment (0.6- and 0.44-fold decreases, respectively, **Fig. 1-5C and B**). These data indicate a shift in the localization of the autophagosomes rather than changes in the overall level of autophagy after exposure to guidance cues. In line with this, western blot quantification of LC3-II protein levels from total culture extracts upon guidance cue exposure did not demonstrate any differences between control, Sema7a and Netrin-1 treatments (**Fig. 1-5E**). These data suggest that under our experimental conditions, guidance cue treatment induces little or no autophagosomal biogenesis. Rather, guidance cue stimuli affect the dynamics of already existing autophagosomes and induce their local recruitment to GCs.

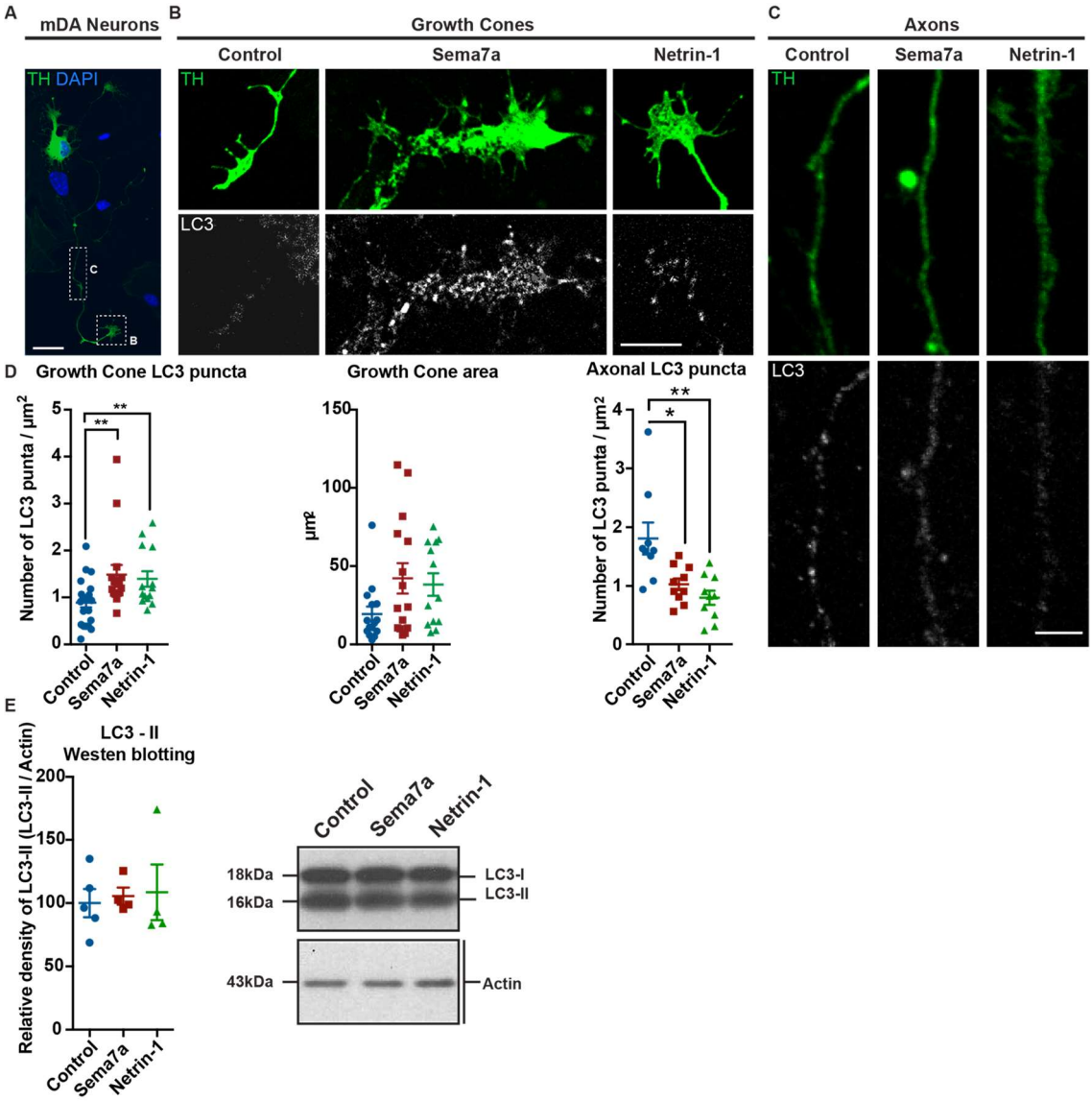

Figure 1-5: Sema7a and Netrin-1 regulate autophagy in mDA axons and GCs.
(A) Representative confocal lower-magnification image of mDA 3DIV primary culture neuron. Dashed B square in the image depicts the identification of axonal GCs and dashed C rectangle depicts the axonal region quantified (40 µm away from the GC). (B) Representative higher-magnification images of TH and LC3 immunolabeling in GCs (B) and axons (C) after 4 h of treatment with PBS (control), Sema7a (250 ng/mL) and Netrin-1 (200 ng/mL). (D) LC3 dots quantification within the GCs [two-tailed t test, p=0.0083 for control (mean value= 0.89; n=22 GCs) vs. Sema7a (mean value= 1.48; n=14 GCs)-treated GCs and p=0.0096 for control vs. Netrin-1 (mean value= 1.39; n=14 GCs)-treated GCs] and axons [two-tailed t test, p=0.0122 for control (mean value= 1.8; n=9 axons) vs. Sema7a (mean value= 1.02; n=10 axons)-treated axons and p=0.0027 for control vs. Netrin-1 (mean value= 0.8; n=10 axons)-treated axons] after guidance cue exposure. GC area quantification revealed a trend to increase after cue exposure. (E) Western blot analysis of 3DIV mDA primary cultures treated for 4 h with PBS (control), Sema7a (250 ng/mL) and Netrin-1 (200 ng/mL). The results shown here are from at least 3 independent experiments.

1.4.5. Autophagy ablation blunts Sema7a and Netrin-1 guidance effects in mDA cultures.

Since autophagy is dynamically regulated in response to guidance cues, we next asked whether autophagy disruption could lead to altered responses of GCs to these guidance cues and affect axonal dynamics and growth. We thus performed time-lapse imaging of GCs *in vitro* and quantified the average displacement rate under baseline, Sema7a and Netrin-1 applications in control and Atg5 cKO cells. Atg5 cKO axons displayed a lower average displacement rate than axons derived from wild-type animals (**Fig. 1-6A and B**), underscoring the importance of autophagy in axonal growth homeostasis. Interestingly, while Sema7a application decreased the average displacement rate of control GCs, no effect of this chemorepulsive molecule on GCs of Atg5 cKO was observed (**Fig. 1-6A, B and C**). Similarly, upon application of the chemoattractant Netrin-1, control axons increased their average displacement rate; however, this effect was blunted in Atg5 cKO axons (**Fig. 1-6A, B and D**). These striking results indicate that autophagy mediates the effects of chemorepulsive and chemoattractive cues on GC growth and dynamics in mDA neurons and that a lack of autophagy completely blunts neuronal responses to guidance cues. Since Sema7a is a membrane-associated glycophosphatidylinositol-anchored (GPI-anchored) protein and not a diffusible cue, we also tested the axon response of Atg5 cKO and control mDA explants grown on alternating stripes of Sema7a. Axons from mDA explants cultured in a Sema7a stripe assay displayed a clear avoidance for Sema7a stripes when provided with a choice between the control and Sema7a substrate (**Fig. 1-6F**). Strikingly, axons from Atg5 cKO explants in the same conditions avoided significantly fewer Sema7a stripes (**Fig. 1-6F**). Altogether, our observations show that autophagy is required downstream of Sema7a and Netrin-1 signals in mDA axon guidance.

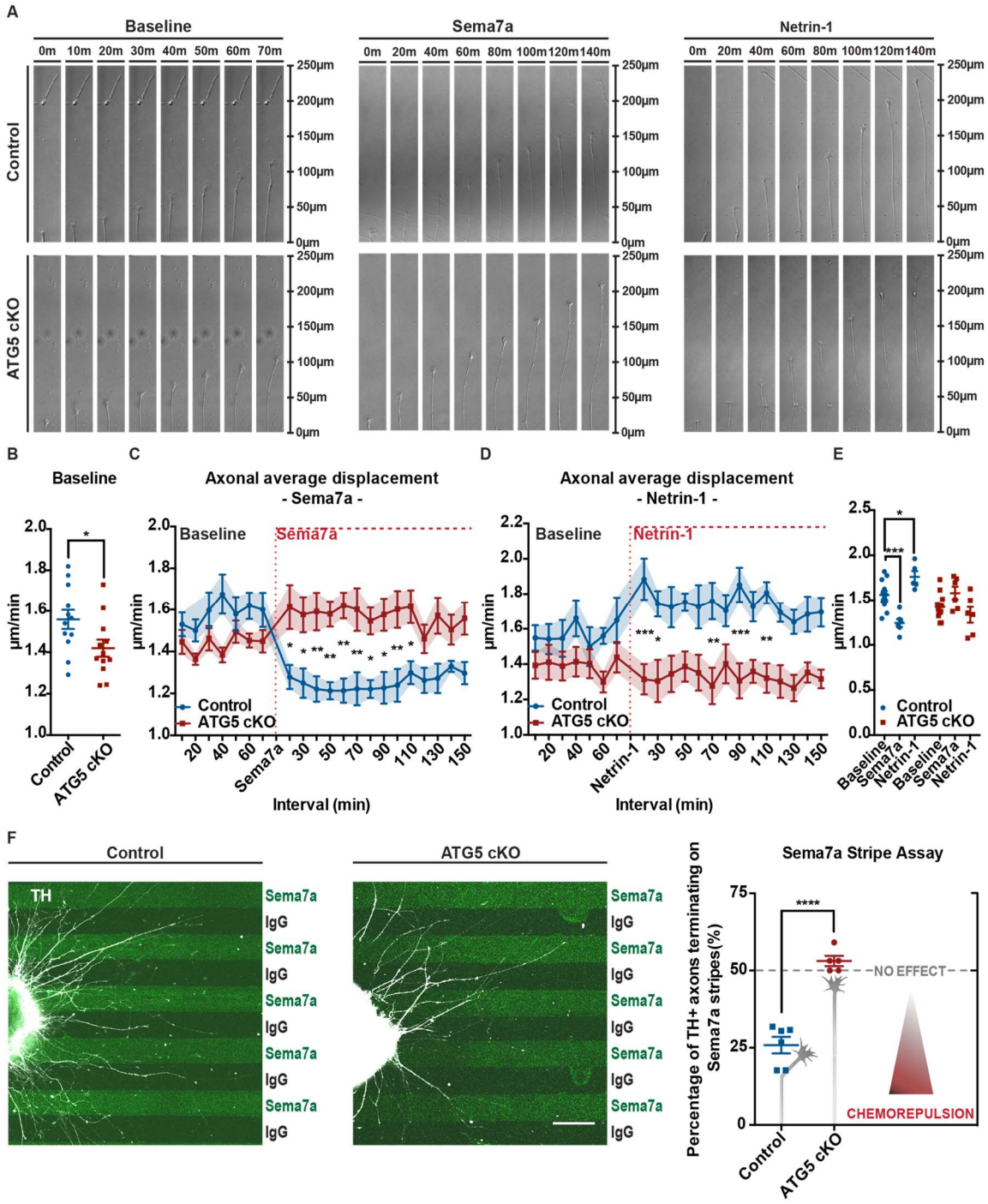

Figure 1-6: Autophagy is required for GC responsiveness to Sema7a and Netrin-1 guidance cues.

(A) Time-lapse images from control and Atg5 cKO axons before and after guidance cue. (B) Baseline average displacement rate (n=12 explants per condition, average of 10 GCs per explant, two-tailed t-test; mean values=1.55 for control and 1.41 for Atg5 cKO; p=0.0357). (C) Displacement curves including baseline and postexposure intervals for Sema7a and (D) Netrin-1 (n=6 explants per condition with an average of 10 GCs analyzed per explant, two-way ANOVA with post hoc Sidak test, *p< 0.05, **p < 0.005 and ***p < 0.0005); and (E) grouped per genotype analysis of average baseline displacement, post Sema7a and Netrin-

1 exposure average displacement rate [baseline (n=12 explants, an average of 10 GCs) and Sema7a and Netrin-1 treatments (n=6 explants, an average of 10 GCs), two-tailed t-test p=0.0005 for control/PBS vs. control/Sema7a and p=0.0285 for control/PBS vs. control/Netrin-1 (mean values=1.55 for control/PBS; 1.21 for control/Sema7a and 1.76 for control/Netrin-1). (F) VTA axons stripe assay images and quantification (n=6 explants for the control and 5 for the Atg5 cKO; data are from 5 independent experiments). Two-tailed unpaired t-test; mean values=0.51 for control and 1.06 for Atg5 cKO; p<0.0001. Scale bar, 500µm.

1.5. Discussion

Here, we show that autophagy is necessary for the morphological maintenance and axon guidance of mDA neurons in mice. More importantly, we reveal for the first time that autophagy is required for GC responsiveness to guidance cues to regulate proper axon guidance.

Axon development is a highly complex process that requires tight regulation and cellular homeostasis. It involves axonal elongation, pathfinding and branching. These processes require dynamic changes in the cytoskeleton (Dent and Kalil, 2001) and cytoplasmic membrane (Pfenninger et al., 2003), mitohondrial energy production homeostasis (Vaarmann et al., 2016) and axonal/GC proteome turnover (Campbell and Holt, 2001), all of which have in common autophagy as a key regulator (Jin et al., 2017; Kast and Dominguez, 2018; Lee et al., 2012). Our observations that autophagy is present in mDA developing neurons and axons and that autophagy markers display a gradual increase during the mDA axonal developmental window, peaking at P7 with a posterior decrease toward adulthood, underscore the relevance of autophagy.

Autophagy dysregulation by *Atg5 or Atg12* knockout in mDA neurons resulted in enlarged axons and GCs, both *in vitro* and *in vivo.* Interestingly, dopaminergic axonal profile enlargements were also observed in $Dat^{Cre/+}Atg7^{flox/flox}$ animals (Hernandez et al., 2012). These observations underscore the notion that autophagy biogenesis in neurons occurs distally in axons with retrograde transport of autophagosomes to the soma where lysis occurs (Maday et al., 2012). This is particularly relevant for mDA neurons that display long and highly metabolic axons (Pacelli et al., 2015). It has been previously shown that mitophagy occurs locally in the distal axons of mDA neurons (Ashrafi et al., 2014), and it is conceivable that macroautophagy also occurs in the distal growing axon and GCs, where there is the necessity of rapid turnover of cytoskeletal proteins. In line with this, our data show that the GCs of autophagy-deficient cells displayed aberrant increased numbers of

microtubule loops, and it has been previously shown that GC area depends on microtubule looping (Purro et al., 2008). We also observed an increased area of these loops. The increased looping prevalence and size can explain the enlargements that we observed, although we cannot exclude the possibility of protein and organelle accumulation due to lack of autophagic degradation.

The lack of autophagy not only affected the GC area but also led to a lower axonal complexity by decreasing the number of branching points. No difference was observed, however, in the initial number of axons that grew out of the explant or in the distance that the axons grew. These data are also in line with our *in vivo* analysis in the developing P11 mice. Atg*5* cKO mice displayed a decreased number of axonal profiles in the striatum, corroborating the *in vitro* Sholl analysis. Additionally, using time-lapse imaging of axons derived from cultured explants of Atg5 cKO mice and their littermates, we observed a decreased displacement rate of GCs following autophagy impairment. Altogether, these data strongly argue that autophagy is required for proper mDA axonal growth. As already mentioned, this process requires very dynamic structural plasticity, and our data suggest that such plasticity would be achieved only with the involvement of autophagy. Our data are in contrast with those of Ban et al. (Ban et al., 2013) and Yang et al. (Yang et al., 2017), where autophagy seemed to negatively regulate axon growth in cortical neurons. However, this difference might be explained by neuronal type-specific differences (dopaminergic neurons vs. cortical neurons) and by the experimental approach. Ban et al. made use of pharmacological (rapamycin) and interference RNA approaches, both of which can act on more than one target. Rapamycin is an mTOR inhibitor, which in turn is a protein hub controlling the phosphorylation of many proteins important in cell homeostasis, including, but not limited to, autophagy (Saxton and Sabatini, 2017). Yang et al. identified a microRNA that targets *Atg12*. Nevertheless, microRNAs are known to be polyvalent and are able to regulate more than one target. To minimize off-target effects from our analysis, we used a genetic Cre model, where we specifically knocked out Atg5, a core protein required for autophagy. Hence, Atg5 cKO results in halting autophagy, different from models of autophagy adaptor and/or regulator protein interference that likely partially disrupt autophagy. Mirroring our data with Atg5 cKO are our results with the CRISPR-Cas9 gene editing of Atg12, another core autophagy protein. It should also be mentioned that changes in axonal morphology and growth do not result from the degeneration of mDA neurons in the midbrain following a lack of autophagy. While it is well established that many

neurodegenerative disorders are linked to metabolic stress (Jha et al., 2017) and autophagy dysfunction (Tan et al., 2014), the numbers of TH+ neurons in the VTA and SNpc were unaltered. Additionally, autophagy conditional knockout mice models specific to dopaminergic neurons (conditional knockouts of *Atg7* under DAT and TH promoters) display degeneration signs only after 1 month in the striatum and after at least 4 months at the midbrain level (Friedman et al., 2012; Hernandez et al., 2012). Although these data are from *Atg7* conditional knockouts, both Atg7 and Atg5 act at the same autophagy conjugation machinery, and mice deficient for these proteins display similar phenotypes (Nishiyama et al., 2007). Therefore, considering our stereological data and the observations from other studies in which degeneration only occurs later than our analysis age (P11), this suggests that the effects we observe are developmental defects rather than degeneration.

Axon growth and guidance, although conceptually distinct processes, occur jointly, making it difficult to completely separate one from the other. Interestingly, rapid local degradation in the axon by the proteasome system has been implicated in chemotropic responses to cues (Campbell and Holt, 2001). Surprisingly, autophagy has not been addressed in such a paradigm, and the specific involvement of this degradation pathway in axon guidance has not been studied. Therefore, we challenged control cultures and Atg5 cKO cultures with Sema7a (chemorepulsive) and Netrin-1 (chemoattractant) guidance cues. Such cues are of great importance for mDA axon guidance and for the consolidation of the dopaminergic system (Chabrat et al., 2017; Flores, 2011; Van den Heuvel and Pasterkamp, 2008). Sema7a is a chemorepulsive cue that has been recently shown to be required for dorso-ventral mDA axonal organization in the striatum (Chabrat et al., 2017). By contrast, Netrin-1 is mostly known to be a chemoattractant cue that is required for proper guidance of mDA axons toward several targets, including the striatum and PFC (Flores, 2011). Our data reveal that autophagy is dynamically modulated by guidance cues. Both Sema7a and Netrin-1 exert similar effects on axonal autophagy by leading to an enrichment in LC3 dots (autophagosome-related phenotype) in the GCs. Interestingly, axons that were not adjacent to GCs (40 µm away) showed a decrease in the number of LC3 dots in the same mDA neurons. Such an observation suggests that upon guidance cues interaction with their receptors, autophagosomes might be recruited and relocated to the GCs. Further corroborating such hypotheses are our western blot data showing similar LC3-II levels under baseline conditions and following Sema7a and Netrin-1 applications. Hence, it is possible that the total level of autophagy is not altered, but it might be redirected to where it is mostly

needed at the moment. In light of these data, we challenged Atg5 cKO cultures with these guidance cues. Strikingly, when explant cultures were incubated with Sema7a and Netrin-1, Atg5 cKO explants failed to react to these cues as revealed by our time-lapse imaging. By contrast, axons from control explants decreased their average displacement when incubated with Sema7a and increased it when incubated with Netrin-1. Altogether, these observations underscore the importance and requirement for autophagy downstream of these guidance cues in mDA axon guidance. In contrast to Netrin-1, which is a diffusible cue, Sema7a is a membrane-associated GPI-anchored protein. Therefore, we also challenged the axonal response of VTA explants growing on alternating stripes of Sema7a. As we previously showed (Chabrat et al., 2017), wild-type cultures grown on control stripes showed no preference, growing randomly, but those cultured on Sema7a-containing carpets displayed clear avoidance of Sema7a stripes, growing preferentially onto control stripes. Remarkably, the effect on Sema7a is completely blunted in explants from Atg5 cKO mice. This finding and our time-lapse imaging indicate that autophagy is required for normal pathfinding behavior in mDA axons in response to guidance cues. Our data become particularly relevant considering that Ambra1 KO (Fimia et al., 2007), mir505-p3 KO (Yang et al., 2017), Alfy/WDFY3 (Dragich et al., 2016) and ULK1/2 whole-brain KO (Wang et al., 2018), all of which are autophagy regulators, result in axonal defects. Importantly, the possibility cannot be excluded that these effects are non-autophagy related, as these are not core autophagy proteins, meaning that autophagy was still occurring in those models and that these proteins are likely related to regulatory functions outside the scope of autophagy. Indeed, Wang et al. claim that the defects seen in their model are noncanonical. Although autophagy levels are not altered in their model, this does not exclude the possibility that autophagy might still be differentially regulated. Localization, for instance, might be altered, or specific autophagy cargo degradation might be deregulated — none of which would likely appear in overall autophagy marker quantifications. By contrast, all of these studies were performed in KO models that are not specific to a distinct cell population. For this reason, it cannot be excluded that these axonal defects could be related to other nonneuronal specific effects. Indeed, if autophagy in glial guideposts is altered, it could also interfere with cellular organization in the brain, which in turn could lead to axonal pathfinding issues. Indeed, one of the phenotypes described by Drajich et al. (Drajich et al., 2016) with the loss of Alfy/WDFY3 is the disruption in the localization of glial guidepost cells.

In sum, increasing evidence around autophagy in brain development shows that autophagy likely has important roles in axon growth and guidance. Our findings reveal autophagy as a key player in the maintenance of mDA axonal morphology and as a prominent mechanism downstream of guidance cue-related effects. Therefore, autophagy appears to be a central mechanism that tightly regulates mDA system development.

1.6. Materials and Methods

1.6.1. Animals

All animal experiments were performed in accordance with the Canadian Guide for the Care and Use of Laboratory Animals and were approved by the Université Laval Animal Protection Committee. *Atg5*$^{f/f}$ (Hara et al., 2006) and *Dat*$^{Cre/+}$*(Zhuang et al., 2005)* mice were genotyped as previously described. *Atg5* cKO mice were generated by intercrossing *Dat*$^{Cre/+}$ males *and Atg5f*^{f} females. *Atg5*$^{f/f}$ mice were used as controls, and *Dat*$^{Cre/+}$*Atg5*$^{f/f}$ – *Atg5* cKO mice were used as the experimental animals. *Atg5* transgenic mice were identified by PCR with forward primers in the *Atg5* sequence, 5-GAATATGAAGGCACACCCCTGAAATG-3, and reverse primers 5- ACAACGTCGAGCACAGCTGCGCAAGG-3 and 5-GTACTGCATAATGGTTTAACTCTTGC-3, identifying heterozygous cassettes and those homozygous for *floxed*, respectively.

1.6.2. Tissue analysis

Mouse brains at P1 were incubated in 4% paraformaldehyde in PBS at 4 °C, followed by cryoprotection in 30% sucrose in PBS, before they were frozen in dry ice. For mice older than P1, perfusion using 4% paraformaldehyde in PBS was instead performed. After cryostat sectioning at 60 µm, sections were washed in PBS and then blocked with 1% normal donkey serum (NDS) and 0.2% Triton X-100 for at least 30 min. When rabbit anti-LC3 was used, sections were instead blocked with 1% normal donkey serum (NDS), 5% milk and 0.2% Triton X-100 for at least 30 min. Primary incubation was performed overnight at 4 °C, except for rabbit anti-LC3, for which a two overnight incubation at 4 °C was performed. The primary antibodies used in this study were rabbit anti-TH (Pel-Freez Biologicals, P40101, 1:1000), sheep anti-TH (Pel-Freez Biologicals, P60101, 1:1000) and rabbit anti-LC3 (Abgent, AP1801a, 1:250). The secondary antibodies used in this study were donkey Alexa-Fluor-488, donkey Alexa-Fluor-555 or donkey Alexa-Fluor-647 (Life Technologies), used at 1:400, and donkey Cy3 and donkey FITC (Jackson Immunoresearch), used at 1:200.

1.6.3. Stereological neuron counting

The number of TH+ and GFP+ neurons within the VTA and SNpc of mutants and controls was quantified using the optical fractionator stereological method (Stereo Investigator; MBF Bioscience). The following analytic parameters were applied: 100×100 µm counting frame size, 15 µm optical dissector height, and 1 in 3 section interval. The coefficients of error (Gundersen m=1) were less than 0.10.

1.6.4. Western blotting

Ventral midbrain from control *Pitx3*$^{GFP/+}$ mice and from *Dat*$^{+/+}$ *Atg5*$^{f/f}$ and *Dat*$^{Cre/+}$*Atg5*$^{f/f}$ brains at E12, E14, E18, P1, and P7 and adult mice were dissected, and samples were then snap frozen. Sample lysis was performed in radioimmunoprecipitation (RIPA) buffer complemented with protease inhibitor and phosphatase inhibitor cocktails (Roche) (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaCl, pH 7.4). Quantification of protein content in the samples was performed using a DC-protein assay (Bio-Rad). Protein extracts (15 µg or 30 µg for LC3-II detection) were separated by SDS-polyacrylamide gel electrophoresis (12% and 15% SDS-PAGE Tris-glycine gels). Nitrocellulose and PVDF (for LC3-II detection) membranes (Bio-Rad) were used for the transfer. Blots were blocked in 7% milk in 1× TBS and 0.01% Tween-20 (Sigma) and immunostained overnight at 4 °C with primary antibodies in blocking solution. For LC3-II detection, 2 overnight incubation was performed. The primary antibodies mouse anti-actin (1:10,000; Millipore, MAB1501), rabbit anti-LC3 (1:500; Abcam, ab AP1801a), sheep anti-TH (1:1000; Pel-Freez Biologicals, P60101), rabbit anti-Atg5 (1:500; Abcam, AP1812b) and rabbit anti-p62 (1:1000; Proteintech, 18420-1-AP) were diluted in blocking solution. Blots were washed 3 × 5 min with 1× TBS and 0.01% Tween-20, and immune complexes were detected with species-appropriate secondary antibodies conjugated to HRP, including goat anti-rabbit HRP (1:3000; CST 7074), goat anti-mouse HRP (1:5000; Life Technologies, G-21040) and donkey anti-sheep HRP (1:5000; Santa Cruz, sc-2473). Membranes were covered with ECL for 1 min (Western Lightning Plus-ECL, PerkinElmer), and chemiluminescence was then documented by exposing the membranes to Pierce CL-Xposure films (Thermo Scientific). Films were scanned and analyzed using the ImageJ64 program.

1.6.5. Ventral midbrain primary cell and explant cultures

P1 mouse ventral mesencephalons were dissected in L-15 medium (Life Technologies) and dissociated in papain solution [12 U/mL papain (Worthington Biochemical), 250 U/mL DNase I type IV (Sigma), 3.5 mM L-cysteine (Sigma-Aldrich), 0.215% $NaHCO_3$ (Sigma-Aldrich), 5 mM EDTA (Life Technologies), 0.2% Phenol Red (Sigma-Aldrich), 1 mM sodium pyruvate (Life Technologies), 1.8 mg/mL D-glucose (Sigma-Aldrich), 50 U/mL penicillin and 50 µg/mL streptomycin (Life Technologies) in HBSS without Ca^{2+} and Mg^{2+} (Life Technologies)]. Mechanical trituration was then performed in trituration solution [0.2% BSA (Sigma-Aldrich), 50 U/mL penicillin, 50 µg/mL streptomycin, 1 mM sodium pyruvate, 1.8 mg/mL glucose, and 250 U/mL DNase I type IV in neurobasal medium (Life Technologies)]. Cells purification was performed using BSA columns [1.8% (wt/vol) BSA, 50 U/mL penicillin, 50 µg/mL streptomycin, 1 mM sodium pyruvate, 1.8 mg/mL glucose, 250 U/mL DNase I type IV, 3 mM NaOH (Fisher Scientific) in neurobasal medium] and centrifugation at 800 × *g* for 5 min. Cells were seeded on top of 12-mm coverslips coated with 0.003% poly-L-ornithine (Sigma) and 10 µg/mL laminin (Life Technologies) (Fisher Scientific) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in complete growth medium [10% (vol/vol) FBS (Life Technologies), 1:50 B27 supplement (Life Technologies), 1:100 GlutaMAX (Life Technologies), and1.2 mg/mL D-glucose in neurobasal medium] at low density (3,000,000 cells/mL). After 3DIV cells were fixed for 30 min at 4 °C in fixative solution (4% PFA, 4% sucrose, in 1× PBS), immunostaining was performed. TH (sheep anti-TH; Pel-Freez Biologicals, P60101; 1:1000) and LC3 (rabbit anti-LC3; Abgent, AP1801a; 1:500) colabeling was performed with two overnight incubations in 5% milk, 1% NDS and 0.2% Triton X-100 in 1× PBS. TH (sheep anti-TH; Pel-Freez Biologicals, P60101; 1:1000) and tubulin (mouse anti-alpha-tubulin, Sigma-Aldrich, T5168; 1:250) colabeling was performed overnight with 1% NDS and 0.2% Triton X-100 in 1× PBS. LC3 dots and tubulin loops were manually counted using confocal images and ImageJ software.

Embryonic ventral midbrain explants were dissected from E14.5 $Pitx3^{GFP/+}$ (electroporation assay), $Dat^{Cre/+}$ $Atg5^{f/f}$ and $Dat^{+/+}$ $Atg5^{f/f}$ embryos in ice-cold L15 with 5% FBS. Explants were grown on 12-mm diameter glass coverslips coated with 20 µg/mL laminin for 2D cultures used for time-lapse imaging or coated with 7 µl of Matrigel™ (BD Biosciences, Mississauga, ON, Canada) for 3D cultures used for morphological analysis. 2D explants were then cultured in 1 ml of neurobasal medium complemented with B27,

PenStrep, GlutaMAX, sodium pyruvate, and FBS (0.4% methyl cellulose 1500 centipoise, 86.8% neurobasal medium, 5% PenStrep, 2% B27, 0.2% GlutaMAX, 1% sodium pyruvate, and 5% FBS) for 1 day at 37 °C, 5% CO_2 prior to time-lapse imaging. 3D explants were each covered by 7 µl of Matrigel and cultured in 1 ml of neurobasal medium complemented with B27, PenStrep, GlutaMAX, sodium pyruvate, and FBS (86.8% neurobasal medium, 5% P/S, 2% B27, 0.2% GlutaMAX, 1% sodium pyruvate, and 5% FBS) for 3 days at 37 °C, 5% CO_2. After 3 DIVs, 3D explants were fixed for 30 min at 4 °C in fixative solution (4% PFA, 4% sucrose, in 1× PBS). TH was immunostained by overnight incubation with sheep anti-TH (Pel-Freez, 1:1000) in 1% NDS and 0.2% Triton X-100 in 1× PBS. Time-lapse analysis was performed on DiC images using MTrackJ plugin. Sholl analysis was performed on confocal images of the TH signal using the Neurite-J plug-in.

1.6.6. Stripe assay

The stripe assay was performed as described by Knoll, B. et al. (Knöll et al., 2007). Glass coverslips were coated with alternating stripes (IgG or Sema7a 100 µg/ml, R&D Systems), which were then covered by laminin (20 µg/ml). Explants from Atg5 cKO mice and control littermates were seeded on top of the coatings, cultured for 3 days, and fixed with 4% PFA, 4% sucrose, in 1× PBS before immunostaining. TH was immunostained by overnight incubation with sheep anti-TH (Pel-Freez, 1:1000) in 1% NDS and 0.2% Triton X-100 in 1× PBS. For quantification, the number of neurites terminating on control vs. Sema7a stripes was manually counted for each explant using confocal images and ImageJ software.

1.6.7. Statistical analyses

GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) software was used for the statistical analyses. The differences between two groups were determined by Student's t test. Sholl analysis and time-lapse data were analyzed by two-way analysis of variance, and the Sidak test was used for post hoc comparisons. All data are represented as mean ± SEM, and significance is defined as *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$.

1.6.8. Microscopes

Immunofluorescence images were acquired using a Zeiss LSM5 Pascal confocal microscope or Zeiss LSM700 confocal microscope and then processed using ImageJ software and Adobe Photoshop CS6. Bright-field pictures were acquired using a Leica

DMRB equipped with a digital camera. Bright-field images were acquired using a TISSUEscope™ 4000 (Huron Technologies).

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1.8. Acknowledgments

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1.9. Author Contributions

Conceptualization, M.S.P., A.S. and M.L.; investigation, M.S.P., A.S. and M.L.; writing of the manuscript, M.S.P., A.S. and M.L.; supervision, A.S. and M.L.

1.10. Supplementary Figures

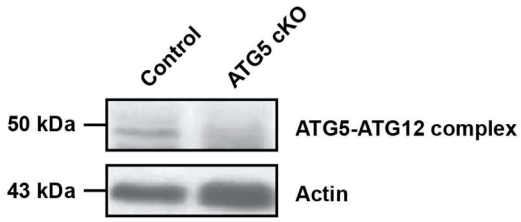

Fig. 1-S1. Cre recombinase under Dat transporter promoter effectively knockouts floxed *Atg5* gene.
Western blot of autophagic marker Atg5-Atg12 complex displays lack of Atg5-Atg12 protein complex in P7 Atg5 cKO animals in comparison to control littermates (n= 3 animals per group).

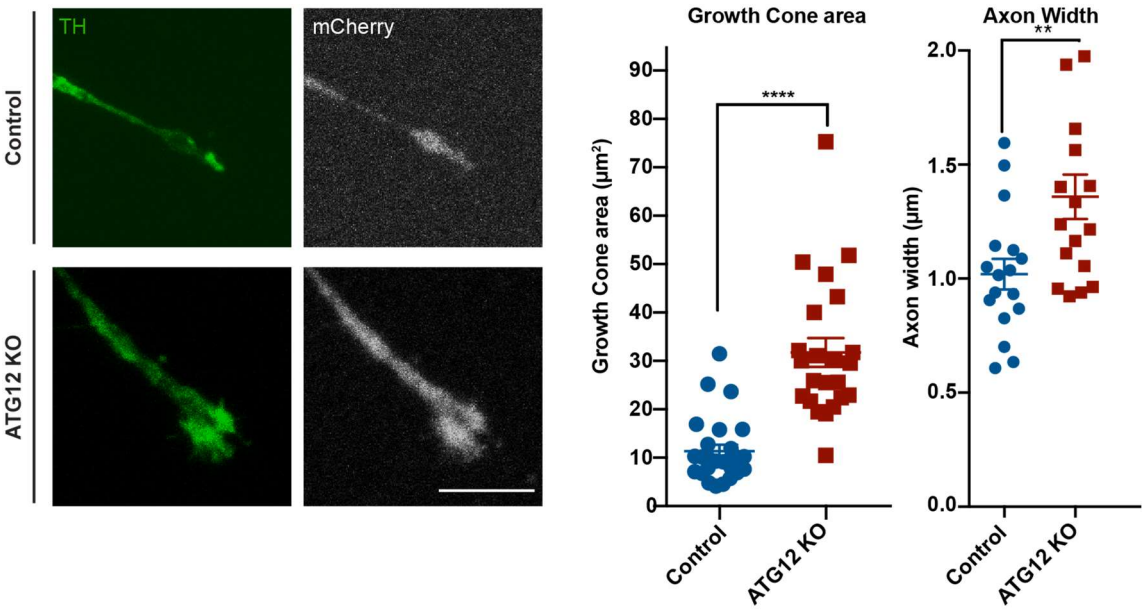

Fig. 1-S2. Autophagy ablation by CRSPR/Cas9 *Atg12* knockout in mDA explants leads to aberrant axon morphology.
E14 VTA from control mice were dissected into explants, electroporated with expression plasmids containing Cas9 and *LacZ* guide as a control or *Atg12* guides for experimental explants and cultured in 3D collagen matrix for 3DIV prior to fixation and ICC for TH labelling. mCherry signal is the result of the direct overexpression of the plasmid marker without ICC. GC area: n=26 GCs for control samples and n=23 GCs for Atg12 KO samples from an average of 4 explants from 3 independent experiments (mean values= 11.37 for control and 31.73 for Atg12 KO; p<0.0001; two-tailed t test). Axon width: n=17 axons for control samples and n=17 axons for Atg12 KO samples from 4 explants from 3 independent experiments (mean values= 1.01 for control and 1.35 for Atg12 KO; p= 0.0071; two-tailed t-test). Scale bar, 10 µm.

Discussion

In this study, the role of autophagy in mDA axon growth and guidance was investigated. Previous studies have focused on the role of autophagy in maintenance and survival of mDA neurons (Hernandez et al., 2012; Levine and Kroemer, 2008). By making use of cKO of *Atg5* and CRISPR-Cas9 gene editing system we provide evidence that autophagy is required for proper mDA axonal morphology and branching *in vitro* and *in vivo,* and it is timely regulated during the development of the dopaminergic system in mice. Moreover, we show for the first time that this process is required for proper response to Sema7a and Netrin-1-mediated axonal guidance in mDA neuronal cultures. This requirement reveals a central role of autophagy in dopaminergic system development by regulating axonal growth/guidance and mediating the responses of these cells to extrinsic guidance cues. Therefore, understanding autophagy in the context of axon growth/guidance is fundamental to improve our knowledge about the developing brain.

Autophagy in the cellular integration of axon development: a rationale

Axon development is a highly complex process that requires a tight regulation and cellular homeostasis. It entails axonal elongation, pathfinding, branching and consolidation; and deregulation of these processes has been linked to neurodevelopmental disorders (Marsh and Dragich, 2018; Zoghbi and Bear, 2012). These processes require dynamic changes in the cytoskeleton (Dent and Kalil, 2001; Kabir et al., 2001; Schaefer et al., 2002) and cytoplasmic membrane (Dai and Sheetz, 1995; Loverde et al., 2015; Pfenninger et al., 2003), mitochondrial energy production homeostasis (Han et al., 2017; Vaarmann et al., 2016; Zhou et al., 2016) and axonal/GC proteome turnover (Campbell and Holt, 2001; Deglincerti et al., 2015), all of which have in common autophagy as a key regulator. Our observations that autophagy is present in mDA developing neurons and axons and that it is timely regulated during the dopaminergic developmental window in mice underscore its relevancy. Such relevancy can be strengthened by the fact that mDA neurons have long and highly branched axons that require high energy for their maintenance (Björklund and Dunnett, 2007; Van den Heuvel and Pasterkamp, 2008; Pacelli et al., 2015; Pissadaki and Bolam, 2013), which further supports the need for autophagy in maintaining mDA neuronal homeostasis. Despite that, autophagy is a general mechanism present and likely required by all neuronal cell types. Indeed, as previously mentioned, autophagy ablation leads to several brain defects related to different brain regions and neuronal types. Therefore,

although the severity of phenotypes differs among the different neuronal types, the presence of brain defects argues in favour of the general requirement of this catabolic process and also highlights that some neurons might be more susceptible than others.

Despite the growing body of evidence that describes axonal guidance and guidance molecules and their receptors, the downstream effectors of such important proteins remain elusive. Interestingly, mRNA translation, Ca^{2+} signalling and local protein degradation have been implicated as downstream effectors to axonal guidance (Campbell and Holt, 2001; Deglincerti et al., 2015; Gomez and Zheng, 2006; Jaffrey et al., 2005; Sutherland et al., 2014). Additionally, endocytosis and exocytosis in the growth cone have been suggested to be implicated in the specific turning of the growth cone towards or away to a certain environment (Kolpak et al., 2009; Tojima et al., 2006, 2010). Importantly, autophagy and endocytosis pathways cross-talk leading to endocytic components, including cell surface receptors, to be recycled by autophagy (Tooze et al., 2014), and in certain cases the autophagy machinery regulates endocytosis (Münz, 2017). The latter happens by Atg8/LC3 lipidation phagosomes facilitating the internalization of cell membrane receptors by a clathrin-mediated endocytosis (Münz, 2017). This is relevant as guidance cues receptor availability is regulated by endocytosis, – as described in section I.2.1.2.2.5.3.. It is, thus, possible that autophagy is acting downstream to this pathway. It would indeed be interesting to test such hypothesis by western blotting in different subcellular sample fractions and/or live imaging by inducing receptor internalization with increasing concentrations of guidance cues and then comparing the data between our Atg5 cKO and control littermates. As a result, one could expect that the incremental presence of guidance cues would lead to receptor/ligand binding-mediated endocytosis in controls with consequent degradation by autophagy. However, as autophagy might act downstream to receptor endocytosis, it could regulate this process. Therefore, one could hypothesize that in Atg5 cKO, or there would be a failure in the internalization of the receptors resulting in accumulated receptors in the plasma membrane or there could be an internalization with failure of receptor turnover which, in turn, would result in increased receptors in the endocytic fraction or co-localizing to endosomes in live-imaging. An additional reason to investigate autophagy involvement downstream to growth and guidance signals is the fact that guidance-implicated molecular pathways such as through Rho GTPases, PI3K/Akt and PTEN have been described in regulating autophagy. Rho GTPases, as described in section I.2.1.2.2.5.2., are implicated in both cue-mediated attraction and repulsion, and can modulate autophagy through

Rac1/Bcl-2 complex (Natsvlishvili et al., 2015). PI3K/Akt pathway, on the other hand, is one of the main autophagy modulatory pathways and autophagy is inhibited when Akt is in active state (Heras-Sandoval et al., 2014). In axon guidance, Akt activation followed chemoattractive signals (Henle et al., 2011) and PTEN down-regulation blocked negative remodelling of β1-integrin adhesions, yet when treated with chemoattractant permitted integrin clustering in *Xenopus laevis* spinal neurons (Henle et al., 2013) thus being implicated in chemorepulsion. Therefore, it would be compelling to test how these pathways, upon guidance signals, modulate autophagy by biochemistry and live-imaging using LC3-GFP-RFP tandem construct to visualize and quantify the autophagic flux. These approaches together with the use of loss of function and gain of function assays could provide interesting data on the mobilization of autophagy through these pathways in axon guidance.

Atg5 cKO pros and cons

Several knockout (KO) models underscore the importance of autophagy in neuronal development and maintenance. Ambra1 (an autophagy-related protein) KO embryos display overgrowth of the nervous system (accentuated mid-hindbrain exencephaly) (Fimia et al., 2007). Atg7 cKO in mDA neurons display aberrant axonal morphology and altered dopamine release in young animals (Hernandez et al., 2012). Moreover, Alfy/WDFY3, an autophagy adaptor protein, has been shown to be implicated in the formation of major axonal tracts in the brain as well as in the spinal cord (Dragich et al., 2016). In similar notes, the microRNA Mir505-3p that targets *Atg12*, a core autophagic protein, might be implicated in axonal elongation and branching *in vitro* and *in vivo* (Yang et al., 2017); and autophagy has been suggested to negatively impact early axon extension in cortical primary cultures manipulated pharmacologically and by interference RNA (Ban et al., 2013). Most of these data, however, refer to autophagic adaptor and/or regulator proteins, and it cannot be excluded that the effects seen might be related to their non-autophagical roles. In fact, adaptor and regulator autophagy proteins usually are not central to autophagy, influencing specific autophagic pathways rather than globally. Atg5, on the other hand, is a core effector for autophagy and its KO virtually eliminates this degradative process within the cells. Consequently, Atg5 KO gives us the ability to identify autophagy-related phenotypes by globally influencing its outcome. However, it also broadens the spectrum of possibilities as it influences many molecular pathways, making it harder to identify deeply influencing specific pathways. Despite that, one could use that to their benefit by using RNAseq or microArray set of data to identify relevant genes to axon guidance that are influenced by Atg5 KO. From there,

hypothesis could be traced around such specific genes to understand specific molecular pathways required for autophagy-dependant guidance effects. Additionally, most of the KO models used were non-neuronal specific, thus one cannot exclude that the axonal phenotypes seen could be due to altered brain topography and also that pathways can be differently regulated in different cellular systems. In light of this, the use of Atg5 cKO brings us several advantages. By knocking-out a gene under DAT promoter, we remove all the noise that would come from a whole organism knockout, making it easier to identify phenotypes and to make direct cause-consequences conclusions. Therefore, this model is more suitable to understand specific functions of the KO gene product. Also, as mentioned above, autophagy modulation can lead to severe developmental phenotypes, including issues with cell differentiation and migration that would likely influence axonal pathfinding as the axons would develop from a neuron not necessarily committed to the dopaminergic phenotype and/or in the wrong environment. Thus, choosing to drive Cre expression under DAT gives us the opportunity to analyze axonal development from already committed postmitotic mDA neurons as DAT is only expressed in postmitotic mDA neurons from E12.5 onwards in mice (Prestoz et al., 2012), hence isolating the specific effect of autophagy on axon growth and guidance.

Finally, little is known about the role of autophagy in axonal growth and the guidance of dopaminergic neurons and how this major catabolic pathway is regulated in response to different microenvironmental cues. So, by choosing the dopaminergic system, aside of studying a relevant system for degenerative and developmental disorders, we have a predetermined system with published literature about guidance that we can apply as tools to better depict the autophagy involvement in axonal development.

Autophagy and axon morphology

Axonal arbor diversity across different neuronal subtypes is pivotal in regulating the functional connectivity of the brain. Therefore, a tight regulation of intrinsic and extrinsic molecular mechanisms driving axonal specification, maintenance and architecture are required for proper neuronal function. Although autophagy has long been associated to neuronal homeostasis and development (Mizushima and Levine, 2010), its developmental role in promoting axonal arbor diversity remains unknown. Thus, we analyzed our Atg5 cKO to characterize whether autophagy plays a role in mDA axonal arborization.

Autophagy depletion by *Atg5* and *Atg12* KOs in mDA neurons resulted in enlarged axons, both *in vitro* and *in vivo,* consistent to what was observed by Hernandez et al. *in* $Dat^{Cre/+}Atg7^{flox/flox}$ animals (Hernandez et al., 2012). Taking together the GC swelling and the fact that we observed an enrichment of the autophagic marker LC3 in wild-type axon tips, these data strongly suggest a functional importance of autophagy in the growth/guidance and maintenance of this motile tip in mDA axons, and highlight the notion that autophagy biogenesis in neurons occurs distally in axons with retrograde transport of autophagossomes to the soma where lysis occur (Maday et al., 2012). Despite that, this tropism to the GC and the fact that mDA neurons display long and highly metabolic axons suggest that it is likely that a certain amount of autophagy should occur locally in the axon. It has been shown that mitophagy occurs locally in distal axons (Ashrafi et al., 2014) and it is reasonable to think that in dynamic growing axons where there is the necessity of rapid turnover of cytoskeletal proteins, plasmatic membrane and receptors, at least in part degradation could occur within the distal axon as it probably would not be sustainable for autophagy only to happen in the soma. Additionally, one could assume that deregulation of cytoskeletal protein turn-over could lead to disorganization of these structures within axons and GC, and indeed our data, as already mentioned, depicted that halting autophagy lead to GC enlargements. The increase of the GC area could be due to microtubule looping (Purro et al., 2008; Yogev et al., 2017) and/or due to accumulation of protein and organelles due to lack of turn-over. Interestingly, our Atg5 cKO cultures displayed aberrant increased numbers of microtubules loops within their GCs in comparison to controls, as well as increased area of these loops. The looping increased prevalence and size can explain the enlargements that we observed, although we cannot exclude the possibility of protein and organelle accumulation due to lack of degradation could also be occurring. It would be interesting to visualize, in our knockout model and control samples, tubulin and actin using superresolution microscopy such as stimulated emission depletion (STED) microscopy and/or dynamics with fluorescent dyes within GCs and axons in live-imaging. With such approach we could measure stabilization and destabilization dynamics of the cytoskeleton and we could use co-localization assays with LysoTracker to confirm the intrinsic relationship between autophagy and cytoskeleton dynamics. Additionally to that, immnohistochemistry to label p62, proteins and organelles relevant to axon growth could also provide insights in whether there is abnormal accumulation of cargo leading to the axonal enlargements. This last experiment could provide insights in whether effector proteins might be physically trapped within the microtubule loops, as it was proposed by

Yogev et al (Yogev et al., 2017), which could help to explain the growth/guidance phenotypes shown in this thesis. Lastly, taxol-based experiments could also prove insightful. Taxol is a known stabilizer of microtubules and also induces microtubules loops (Yogev et al., 2017), and the use of this agent along with gain and loss of autophagy function assays could link autophagy to microtubules stabilization process by possibly showing a rescue effect.

Interestingly, when performing sholl analysis on our explant cultures, ablating autophagy rendered lower axonal arborization complexity in comparison to the controls. However, neither the initial number nor the distance that the axons reached were on average unaltered. Consequently, we could conclude that the branching of these axons was affected. This data was also reflected *in vivo* in developing P1 (data not shown) and P11 mice where Atg5 cKO mice displayed decreased number of axonal profiles reaching the striatum. We could also observe that the axonal baseline average displacement when autophagy is impaired was reduced by making use of time-lapse images on our Atg5 cKO and their control littermates. Although these loops are seen in normal conditions in the transition from quiescent to growing axonal state (Dent et al., 1999), the enrichment of the presence of these structures depicts an aberrant and pathological situation. Of particular note, our data is consistent with the fact that GCs displaying loops are pausing or slower axons (Dent et al., 1999). Altogether, these data strongly argue that autophagy is required for the structural plasticity necessary for proper mDA axonal growth. Despite that, it would be of interest to repeat our live-imaging experiment with a shorter time-lapse interval. By doing so, we would be able to differentiate resting periods from growing periods, and by doing so we would have a better temporal resolution to compare the phenotypes. Interestingly, our observations go in the opposite direction of what has been shown by Ban et al. (Ban et al., 2013) in cortical neurons *in vitro and* Yang et al. (Yang et al., 2017), where autophagy seemed to negatively regulate axon growth. However, this difference might be explained by the distinct neuronal types analyzed - dopaminergic neurons vs. cortical neurons - and also by the experimental approach. Ban et al. made use of pharmacological (rapamycin) and interference RNA approaches, both of which can act on more than one target. As a matter of fact, rapamycin is a phosphorylation inhibitor that has as main target mTOR, which in turn controls the phosphorylation of many proteins important in cell homeostasis – including, but no only, autophagy (Saxton and Sabatini, 2017). Yang et al., on the other hand identified a microRNA that targets *Atg12*. Despite that, microRNAs are known to be polivalent, being able to

regulate more than one target. Additionally, it is unlikely that a single microRNA would, alone, regulate *Atg12* activity – indeed, *Atg12* being a prominent effector of autophagy would likely require a more complex regulation even with redundant effectors. To minimize off-target effects from our analysis we decided to use the conditional KO for Atg5 as already discussed in the section «Atg5 cKO pros and cons». This resulted in a model of halting autophagy which minimizes the analytical "noise", encountered in models where adaptor and/or regulator proteins are used. In the later case, loss of function of these proteins likely influence only partially autophagy resulting in models where autophagy is still occurring, without mentioning the influence of non-autophagy roles of the adaptor protein in the data. Consequently, our approach facilitates the identification and isolation of autophagy-related effects by making use of proper controls. Indeed, mirroring our data by blunting autophagy using the KO of other core autophagic proteins could act as an off-target control. As previously discussed, Atg12 KO displayed similar morphological differences when compared to our Atg5 model, further strengthening our data that depict the impact of autophagy ablation in these models and not off-target effects. On the other hand, mDA axons are highly arborized and such characteristic can impose a metabolic stress that, along with other homeostatic dysfunctions, could lead to neurodegeneration (Pacelli et al., 2015; Pissadaki and Bolam, 2013). Indeed, many neurodegenerative disorders have been linked to metabolic stress (Jha et al., 2017; Liu et al., 2017) and autophagy dysfunction (Nixon, 2013; Son et al., 2012; Tan et al., 2014). However, the number of TH+ neurons in the VTA and SNpc were unaltered at the age analyzed and autophagy KO mice models specific to dopaminergic neurons (conditional knockouts of *Atg7* under DAT and TH promoters) display degeneration signs only later that our window of analysis (Friedman et al., 2012; Hernandez et al., 2012). Of notice is that both Atg7 and Atg5 act at the same autophagy conjugation machinery and mice deficient for these proteins display similar phenotypes (Komatsu et al., 2007; Nishiyama et al., 2007). Altogether, we can conclude that the effects we observed are developmental defect rather than related to neurodegeneration.

Autophagy a new regulator of mDA axon growth and guidance

Axon growth and guidance are two distinct processes that rely on each other and are central in neuronal networking development. As already mentioned, many processes need to be regulated for proper axon growth and guidance to take place, including cytoskeleton remodelling, *de novo* local synthesis of proteins and energy demands - all of which have been linked to autophagy (Koh et al., 2016; Nazio et al., 2016). Of particular notice is that

rapid local degradation in the axon by the proteasome system has been implicated in chemotropic responses to cues (Campbell and Holt, 2001), but surprisingly, autophagy has not been addressed in such paradigm. Therefore we decided to tackle the importance of autophagy in mDA axon guidance as the specific involvement of this degradation pathway in axon guidance has not been properly investigated.

For such purpose, we made use of experiments in which we treated mDA cultures with Sema7a (chemo-repulsive) and Netrin-1 (chemoattractant) guidance cues resulting in autophagy modulation. Both Sema7a and Netrin-1 exerted similar effects on axonal autophagy: an enrichment of LC3 dots (autophagossome-related phenotype) in the GC with a decrease of the same dots in axons not adjacent to GCs (40µm away) as revealed by ICC in wild-type cultures with no global increase of LC3-II as measured by WB. Such observation suggests that upon guidance cues interaction with their receptors, autophagossomes might be recruited and relocated to the GCs. Despite that, we cannot exclude that autophagosomes biogenesis might be happening in the GC concomitantly with recycling of axonal autophagic vacuoles. To test such possibilities, live imaging of axons in microfluidic chambers to visualize LC3 dots upon Sema7a and Netrin-1 challenge would prove insightful. By using this approach one could observe in live motion if there is biogenesis or indeed relocation to the GC of LC3-positive puncta.

We also challenged our Atg5 cKO cultures with these guidance cues and performed time-lapse imaging. Control axons increased or decreased their average displacement accordingly to the presence of Sema7a and Netrin-1, respectively. Atg5 cKO explants, on the other hand, failed to react to these cues. We also performed stripe assay for Sema7a, as it is a membrane-associated GPI-anchored protein unlike Netrin-1, which is a diffusible cue. As such, stripe assay better mimics *in vitro* how these proteins are presented to the receptors on the GC surface for these GPI-anchored cues. Corroborating our live-imaging data, Atg5 cKO axons fail to respond to Sema7a stripes. This is a particularly important data as it is the first direct report showing that autophagy is required for normal pathfinding behaviour in mDA axons upon cue. Strengthening our data, it has been reported that Ambra1 KO (Fimia et al., 2007), mir505-p3 KO (Yang et al., 2017), Alfy/WDFY3 (Dragich et al., 2016) and ULK1/2 whole brain KO (Wang et al., 2018) - all of which are autophagy regulators - result in axonal defects in other neuronal systems. However, as already discussed in the section «Atg5 cKO pros and cons», one cannot exclude off-target effects, as these are not core autophagy proteins nor topographic influences due to the fact that the

KOs were not neuronal specific (e.g. glial guideposts disruption) that could influence axon guidance. Indeed, Wang et al. claim that the defects seen on their model were autophagy-independent as autophagy markers were unaltered and the phenotype was not recapitulated in mice lacking other autophagy genes (Wang et al., 2018). Although in their model autophagy levels are not altered, it does not exclude the possibility that autophagy might still be differentially regulated. Localization, for instance, might be altered, or specific autophagy cargo degradation might be deregulated – none of which would likely show in overall autophagy markers quantifications. Altogether, further analysis of sections and brains of our Atg5 cKO would provide us with interesting data regarding autophagy requirement for mDA guidance *in vivo*. It would be interesting to investigate VTA vs. SNpc innervation of different targets by making use of concomitant stereotaxic injections of AAv-Flex viral system with fluorescent reporters. However, this must be done at early postnatal stages in order to minimize effects from degeneration due to the lack of autophagy in the system. By doing this experiment, we would be able to visualize the innervation patterning from these regions and check for abnormal phenotypes. Indeed, 3D lightsheet microscopy would be useful for this approach as it would improve the spatial resolution and give us an overall view of the dopaminergic system in Atg5 cKO mice. Of notice, is that by inspecting Atg5 cKO sections we could not observe any strong misguidance dopaminergic phenotype, hence further strengthening the need for better spatial resolution *in vivo* analysis methodologies as the described above. The reason that we do not see *in vivo* strong guidance phenotypes could be explained by the far more complex environment that the axons are surrounded when in comparison to *in vitro* approaches. Indeed, *in vitro* there is a lack of the topographic environment, as well as, it poses an oversimplified guidance test approach where usually only one guidance molecule is tested at a time. Therefore, it is very difficult to account for the many variables present *in vivo*, where, for example, several guidance cues are likely working together to create the environment for axons to grow. Additionally, as autophagy is a general mechanism and influences both chemoattractant and chemorepellent signalling *in vitro*, it is possible that in some environmental cases the loss of reactivity for both guidance classes could result in no change whatsoever as the GC is would not favour attraction nor repulsion.

Our data depict that autophagy is required for proper mDA axon pathfinding behaviour, but the mechanisms by which autophagy controls axon guidance remain elusive. As autophagy is a broad influencing pathway involved from bionergetic demands to

cytoskeleton remodelling, it is likely that it influences axon development in diverse ways. Despite that, cytoskeleton is central to this question as it is its plasticity that drives axon guidance and growth. It is possible that microtubules disorganization seen in our model could lead to improper pathfinding behaviour and lack of reactivity to guidance cues. Another possibility is that these aberrant loops could act as traps (Yogev et al., 2017) for important players downstream to guidance cue signalling, leading to a blunted guidance cue effect. Other important factor to be accounted for is that autophagy is also known to regulate receptor recycling downstream to endocytosis. Therefore, the loss of guidance seen in our model might relate to disorganization of receptor turn-over leading to improper signalling. These explanations are plausible and can possibly happen together. It would indeed be interesting to address these questions. Experiments using taxol in loss and gain of function of autophagy (as described in section «Autophagy and axon morphology») together with guidance cue challenges could provide some information in whether microtubules loops could be underneath the loss of reactivity to Sema7a/Netrin-1. Additionally, live-imaging of mDA axons to visualize tubulin dynamics in controls and Atg5 cKO with or without guidance cues treatment could also give insights and shed some light in how microtubules could be involved in the phenotype observed. Also, WB with subcellular fractionation from Atg5 cKO and control littermates to probe for PlexinC1, β1 integrin, DCC and UNC-5 in the presence of increasing concentrations of Sema7a and Netrin-1 could indicate whether autophagy could be acting in the turnover of these receptors. Of notice is that both hypothesis, receptor turn-over issues and microtubules disorganization/desensitization to guidance signalling, could explain the lack of reactivity of Atg5 cKO axons to attractant and repellant signalling. Lastly, RNAseq or microArray analysis of our mutant focused on guidance and cytoskeletal pathways could provide targets to be further explored as downstream effectors of axon guidance that require autophagy as a regulator.

Could autophagy be a new therapeutical avenue for axon regeneration in spinal cord injury or neurodegenerative disorders?

Autophagy is required for cellular homoeostasis due to its bulk or selective degradation of cytoplasmic components. Such components include organelles, long-lived proteins, and protein aggregates. As such, autophagy dysfunction has been implicated in the etiology of neurodegenerative and neurodevelopmental disorders (Boland and Nixon, 2006; Lee, 2012; Yue et al., 2009; Lee et al., 2013; Tang et al., 2014). As autophagy is a central degradation pathway and it has been shown to be downregulated in

neurodegenerative disorders, the hypothesis that manipulating this pathway could provide a therapeutical avenue to clear protein aggregates and halt the progression of proteinopaties was easily made and is a hot topic in the neurobiology field of study. However, its role in axon regeneration remains speculative and poorly studied. Our data strongly shows that autophagy is involved in both axon growth and guidance, thus, it raises the possibility that autophagy manipulation could be used as a strategy for reinnervation. Indeed, in spinal cord injury models, autophagy stimulation has been shown to be beneficial for axonal re-growth (He et al., 2016; Romeo-Guitart et al., 2019). He et al. showed that this effect was dependent on microtubule stabilization. Interestingly, as discussed above, we also observed microtubule phenotypes in our results. Despite that, the mechanistic behind how autophagy could manipulate axonal growth/guidance need to be further explored. Moving forward, it will be important to characterize how autophagy can regulate the cytoskeleton, cellular energy balance and receptor turnover in the context of axon guidance. Additionally, manipulating autophagy in studies with grafted neural stem cells (NSC) and induced pluripotent stem cells (iPSC) will provide useful and could potentially point us toward the possibility of using autophagy manipulation in regenerative strategies in the brain.

Conclusion

For decades now autophagy has been a hot topic within neurodegenerative disorders due to its involvement on proteinopaties pathogenesis (Lee, 2012; Takalo et al., 2013; Tan et al., 2014; Tung et al., 2012). The discovery of neural stem cells (NSC) and induced pluripotent stem cells (iPSC) raised the exciting possibility of developing new cell replacement strategies to cure neurodegenerative diseases. Yet, although promising, the use of NSC and iPSC to heal these devastating disorders is far from reach and, clearly, the success of such treatment will depend upon the full understanding of the mechanisms that regulate survival and axonal pathfinding of grafted cells. Therefore, our data starts to depict a, until now, rather neglected field of study in axon pathfinding – the downstream pathways to guidance cues required for proper axon pathfinding. Despite that, this major degradation pathway has also been shown to be linked to neurodevelopmental disorders as autism spectrum disorders and schizophrenia (Lee et al., 2013; Marsh and Dragich, 2018; Dere et al., 2014),which highlights its importance in brain development. Surprisingly, until now such relevancy was not properly addressed.

In sum, our findings show autophagy as a key player in the maintenance of mDA axonal morphology and as a prominent mechanism downstream to guidance-related effects. Therefore, autophagy appears to be a central mechanism to tightly regulate mDA system development and to improve our understanding about basic physiological processes of axon growth/guidance and maintenance in the developing brain. Consequently, the work presented here revels new roles for autophagy in neurons and urges for more studies to further elucidate the role of autophagy in axon development and to test whether this mechanism can also be applied to the entire CNS.

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