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Role of Lmx1a and Lmx1b Transcription Factors in Post-Mitotic Midbrain Dopaminergic Neurons

Thèse

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Sous la supervision de:

Martin Lévesque, Directeur de recherche

RÉSUMÉ

Lmx1a et Lmx1b sont des facteurs de transcription connus pour leur rôle au cours du développement des neurones dopaminergiques du mésencéphale (mDA). Ils ont été montrés comme essentiels à chacune des étapes de différentiation des progéniteurs en neurones dopaminergiques matures. Des études récentes ont également mis en évidence l'importance de ces deux facteurs de transcription dans les neurones dopaminergiques chez l'adulte. Lmx1a/b sont impliqués dans la régulation de gènes mitochondriaux ainsi que dans l'autophagie. Cependant, jusqu'à présent, rien n'est connu sur le rôle de Lmx1a/b dans les neurones dopaminergiques post-mitotiques. Le but de cette thèse est d'élucider le rôle de Lmx1a/b dans les neurones dopaminergiques matures. L'analyse des projections axonales dopaminergiques de souris doubles conditionnelles mutantes (cKO) pour Lmx1a/b a mis en évidence un défaut de guidage axonal confirmant le rôle essentiel de ces deux facteurs de transcription dans la formation des circuits dopaminergiques. Afin d'identifier précisément les molécules impliquées dans la régulation du système dopaminergique des techniques adaptées doivent être développées pour déterminer les principaux acteurs régulés par Lmx1a/b. À cette fin, nous avons mis au point une technique de marquage immunohistochimique rapide de la tyrosine hydroxylase (TH, enzyme nécessaire à la synthèse de la dopamine) sur des sections de mésencéphale de souris afin de délimiter la région d'intérêt. Par la suite, nous utilisons une technique de microdissection au laser afin de spécifiquement récolter les cellules dopaminergiques du mésencéphale pour réaliser un profil d'expression génique. Un premier article de méthodologie a été publié concernant cette technique. Cette procédure menée sur des souris cKO pour Lmx1a et Lmx1b et leurs contrôles associés a permis de mettre en évidence des gènes régulés par Lmx1a et Lmx1b tels que Plxnc1.

Plxnc1 est une protéine de guidage axonal ayant pour ligand la sémaphorine 7a (Sema7a). Afin d'observer si la régulation de Plxnc1 par Lmx1a/b est à l'origine du défaut de guidage axonal observé chez les souris cKO pour Lmx1a/b, nous avons réalisé une analyse *in vitro* de l'effet de la Sema7a sur les axones d'explants mDA. Notre étude a montré un effet chimiorépulsif de la Sema7a pour les axones des

neurones mDA exprimant Plxnc1. De plus, l'étude de souris Sema7a KO montre une augmentation de l'innervation DA dans la partie dorsale du striatum, partie exprimant Sema7a chez des souris contrôles. Ce phénotype met en évidence une chimiorépulsion induite par l'interaction Sema7a/Plxnc1. L'étude de souris surexprimant Plxnc1 a, quant à elle, montré une perte d'innervation DA dans la partie dorsale du striatum. En effet, la majorité des cellules du mésencéphale se mettent à exprimer Plxnc1, les rendant ainsi sensibles à la chimiorépulsion induite par Sema7a. L'ensemble de ces résultats met en évidence l'importance de la régulation de la protéine de guidage axonal Plxnc1 par Lmx1a/b pour l'innervation des cibles du mésencéphale. La répression de Plxnc1 dans les neurones dopaminergiques de la substance noire *pars compacta* (SNpc) semble nécessaire à l'innervation du striatum dorsal riche en Sema7a. Cette étude est la première à identifier les bases moléculaires du guidage axonal expliquant la ségrégation des voies mDA nigrostriée et mésolimbique, et devrait contribuer à améliorer l'efficacité des thérapies cellulaires pour la maladie de Parkinson. Un second article sera soumis prochainement sur le rôle des facteurs de transcription Lmx1a/b dans les neurones dopaminergiques post-mitotiques du mésencéphale.

La principale caractéristique histopathologique de la maladie de Parkinson est la dégénérescence des neurones mDA de la SNpc. La thérapie de remplacement cellulaire utilisant des neurones dopaminergiques nouvellement générés à partir de cellules souches représente une thérapie prometteuse. Cependant, la mauvaise innervation des neurones nouvellement greffés limite le succès des études de transplantation. L'identification de facteurs régulant la connectivité des neurones mDA devient primordiale pour élucider les mécanismes impliqués dans la mise en place du système dopaminergique. C'est pourquoi, dans une derniere partie, afin d'illustrer cette possibilité d'amélioration d'une thérapie de remplacement cellulaire, j'ai réalisé l'implantation de cellules souches différenciées en neurones dopaminergiques dans un modèle de souris lésées à la 6-hydroxydopamine (6OHDA). Les cellules nouvellement réimplantées sont de type SNpc, en raison de l'infection par un vecteur viral induisant l'inhibition de l'expression de Plxnc1.

ABSTRACT

Lmx1a and Lmx1b are transcription factors known for their role in the development of midbrain dopamine neurons (mDA). They were shown as essential for each stage of differentiation from progenitors to mature dopaminergic neurons. Recent studies have also highlighted the importance of these two transcription factors in dopaminergic neurons in adult mice. Lmx1a/b are involved in the regulation of mitochondrial genes and in autophagy. Although some evidence suggest that they could be involved in the formation of mDa circuit formation, their role in post-mitotic mDA neurons remains unknown. The aim of this thesis is to elucidate the role of Lmx1a/b in post-mitotic dopaminergic neurons. Analysis of dopaminergic axonal projections of double conditional mutant (cKO) mice for Lmx1a/b showed an axon guidance defect confirming the essential role of these transcription factors in the formation of dopaminergic circuits. In order to precisely identify the molecules involved in the regulation of the dopamine system, suitable techniques must be developed to identify the main genes that are regulated by Lmx1a/b. To this end we developed a new technique allowing gene profiling of brain sub-population. By combining rapid immunolabeling of mDA neurons with laser capture microdissection we manage to extract RNA from two sub-regions of mDA neurons such as ventral tegmental area (VTA) and substantia nigra *pars compacta* (SNpc). The advantage of this technique is to compare quickly the regulation of genes expression by studying controls and mutant mice. A first methodological article has been published regarding this procedure. We then applied this technique on cKO mice for Lmx1a/b and their associated controls to identify genes regulated by Lmx1a and Lmx1b. Among these genes, we identified Plxnc1, an axon guidance receptor for the semaphorin 7a (Sema7a). In order to verify whether the regulation of Plxnc1 by Lmx1a/b is at the origin of the axon guidance defect observed in double conditional mutant for Lmx1a/b, we have made an *in vitro* analysis of the effect of Sema7a on mDA explants. Our study showed a chemorepulsive effect of Sema7a on Plxnc1 positives axons. In addition, the study of knockout mice for Sema7a shows an increase of DA innervation in the dorsal part of the striatum which is the region expressing Sema7a in control mice. This phenotype reveals a chemorepulsion

induced by Sema7a/Plxnc1 interaction. The study of mice overexpressing Plxnc1 shows a loss of DA innervation in the dorsal striatum. Indeed, by overexpressing Plxnc1, the majority of midbrain cells begin to express this axon guidance protein instead of only mDA neurons from the VTA. Thus, all mDA neurons including neurons from the SNpc express Plxnc1 making them sensitive to Sema7a. This interaction Sema7a/Plxnc1 leads to a chemorepulsion of axons guided away from the dorsal striatum. Overall these results highlight the importance of the regulation of the axon guidance protein Plxnc1 by Lmx1a/b for the innervation of midbrain targets. The repression of Plxnc1 expression in dopaminergic neurons of the SNpc appears necessary for the innervation of dopaminergic axons in the dorsal striatum, rich in Sema7a. This study is the first to identify the molecular basis of the development of the dopaminergic system explaining the segregation of the nigrostriatal and mesolimbic pathways. These results should help to improve the effectiveness of cell therapies for Parkinson's disease. A second article will be submitted soon about the role of Lmx1a/b transciption factors in post-mitotic midbrain dopaminergic neurons.

The main histopathological feature of Parkinson's disease (PD) is the degeneration of SNpc neurons. The cell replacement therapy using newly generated dopaminergic neurons from stem cells represents a promising therapy. However, a poor innervation of the newly grafted neurons limits the success of transplantation studies. The identification of factors regulating neuronal connectivity of mDA neurons becomes essential to elucidate the mechanisms involved in the establishment of the dopaminergic system. Therefore, in a final section of this thesis, I report preliminary study about cell replacement therapy in PD mouse model. I differentiated DA neurons from stem cells, knock-down Plxnc1 expression and performed grafting in 6-hydroxydopamine (6OHDA) mouse model to illustrate the possibility of improving a cell replacement therapy.

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List of abbreviations

6-OHDA	6-hydroxydopamine
AC	adenylate cyclase
ACC	Anterior cingulate cortex
Adcyap1	Adenylate Cyclase Activating Polypeptide 1
AHVsema	herpes virus semaphorin homologs
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1
ALP	autophagic-lysosomal pathway
ALV	autophagic-lysosomal vesicle
Ascl	Achaete-Scute Complex-Like
BDNF	Brain Derived Neurotrophic Factor
BMP	Bone morphogenetic protein
CA	Catecholamine
Cck	Cholecystokinin
Chrna4	Neuronal acetylcholine receptor subunit alpha-4
Clstn2	calsyntenin 2
CNA	Central nucleus of the amygdala
CNS	Central nervous system
cAMP	cyclicadenosine monophosphate
DAT	Dopamine transporter
DBS	Deep brain stimulation
DCC	Deleted in colorectal cancer
DJ-1	Protein deglycase 1
dr	dreher
DS	Dorsal sriatum
EN	Engrailed
ERC	Entorhinal cortex
ES	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
Foxa	Forkhead box A
GDNF	Glial-Derived Neurotrophic Factor
GFP	Green fluorescent protein
Girk2	G-protein-regulated inward-rectifier potassium channel 2
Gli1	GLI-Kruppel family member GLI1
GPCR	G protein–coupled receptors
GPI	glycophosphatidylinositol
GRK	G protein-receptor kinase
Gsg1l	germ cell-specific gene 1-like protein
H	Habenula

HC	Hippocampus
Ig	Immunoglobulin
iPSC	induced Pluripotent stem cell
IPT	immunoglobulin–plexin–transcription
IsO	Isthmic organizer
IZ	Intermediate zone
L-AADC	L-Aromatic amino acid decarboxylase
LC	Locus coeruleus
Ldb1	LIM domain-binding protein 1
L-DOPA	L-dihydroxyphenylalaline
LH	Lateral hypothalamic area
Lmx	LIM-homeodomain transcription factor
LRRK2	Leucin-rich repat kinase 2
LS	Lateral septum
Map2	Microtubule-associated proetein 2
mDA	midbrain dopaminergic neurons
MFB	Medial forebrain bundle
MiRNA	microRNA
MPTP	Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Msx1	Homeobox, msh-like 1
Na	Nucleus accumbens
Neurog2	Neurogenin 2
Nkx2-2	NK2 transciption factor related, locus 2
Nrf1	Nuclear respiratory factor-1
Nr4a2	Nuclear receptor subfamily 4, group A, member 2
NSC	Neural stem cell
Ntf	Nuclear transport factor
Nurr1	Nur-related factor 1
OT	Olfactory tubercle
OTX	Orthodenticle Homeobox
P	Pituitary
Park2	Parkinson protein 2
Park7	Parkinson disease protein 7
Pax2	Paired box gene 2
Pax5	Paired box gene 5
PD	Parkinson’s disease
PFC	Prefrontal cortex
Pink	Putative kinase 1
Pitx	Paired like homeodomain transcription factor
PKA	protein kinase A

PRC	Perirhinal cortex
PSC	Pluripotent stem cell
PSI	plexins, semaphorins and integrins
Raldh	Retinaldehyde dehydrogenase
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RRF	retrorubral field
SAG	Smoothened (Smo) receptor agonist
Satb1	special AT-rich sequence-binding protein-1
Sema	Semaphorin
SHH	Sonic hedgehog
Slc32a1	vesicular inhibitory amino acid transporter
SNCA	α-synuclein
SNpc	*Substantia nigra pars compacta*
TGFβ	Transforming growth factor-β
TH	Tyrosine Hydroxylase
VESPR	Virus-encoded semaphorin protein receptor (VESPR)
Vip	Vasoactive Intestinal Peptide
VM	Ventral midbrain
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral Tegmental Area
VZ	Ventricular Zone
WNT	Wingless-related integration site
Wt	Wild type

A tous ceux qui m'ont soutenue...

Remerciements

Je voudrais tout d'abord remercier mon directeur de thèse Dr. Martin Lévesque pour m'avoir donné l'opportunité de travailler dans son laboratoire sur un super projet. On m'avait dit qu'en travaillant avec lui j'apprendrais tellement de techniques que ce serait comme être à disneyland, et en effet j'ai énormément appris pendant ces années de thèse.

Je remercie également Dr. Armen Saghatelyan et Dr. Paul de Koninck pour leurs remarques et conseils lors des differentes rencontres de comité le long de mon cursus.
Je remercie Dr. Emmanouil Metzakopian pour ses conseils avec les cellules souches.
Je remercie la Fondation de l'Institut Universitaire en Santé Mentale de Québec, et le CTRN pour avoir financé une partie de ma thèse.

Je remercie le laboratoire Lévesque avec une mention spéciale pour Véro qui a été ˋgame˝ pour me suivre lors des gros batchs d'injections de cellules en tenant au coca et barres de céréales. Ayant commencé dans un nouveau laboratoire en tant que première étudiante, je souhaiterais également remercier les équipes Saghatelyan, et Parent qui m'ont accueillie comme électron libre. Je voudrais aussi remercier tous les autres collègues avec qui j'ai pu interagir tout au long de ces années, ainsi que le personnel de l'animalerie, et de la plateforme d'outils moléculaires dont Nizar pour les conversations virales.

Je remercie tous mes amis sans qui je ne serais jamais arrivée jusque là. Que ce soient les amis de l'autre côté de l'océan: les chicas, les amis de l'ESBS, ou les amis rencontrés à Québec. Une mention spéciale pour la COSA et les amis rencontrés à l'institut qui ont toujours été en première ligne pour me soutenir pendant les moments moins drôles du doctorat, mais aussi pour tous les fous-rire, célébrations, jeux de mots interminables, week-ends, chevauchées fantastiques. Je voudrais remercier notre correctrice COSA officielle Emilie pour avoir accepté de passer au travers de ma thèse en un temps record. Un gros merci à PA qui a été présent et m'a toujours

soutenue rendant cette épopée doctorale plus légère (mais attention ce n'est pas pour ça que tu auras la moitié de mon diplôme).

Je remercie bien évidemment ma famille, ma mère et ma soeur qui ont toujours été présentes et m'ont encouragée tout au long de cette aventure.

Enfin, je souhaiterais remercier le jury pour avoir accepté d'évaluer mon travail.

Preface

I followed the neurobiology program from the Laval University and performed all the research for my PhD project at the *Centre de recherche en santé mentale de Québec* (CRIUSMQ) in Dr. Martin Lévesque's laboratory.

This thesis encompasses two papers written during my PhD project: the first one is published, the second one is in review in Nature Cmmunications, and the third one is in progress. This third paper is described in the fourth chapter in which I talk about my ongoing work constituting a proof of concept of the second article. The goal of this last article is to show the reinnervation of newly generated dopaminergic SNpc-like neurons to their targets.

In the first chapter, I introduce the subject of my thesis, developping the role of the transcription factors Lmx1a/b during the early development of dopaminergic neurons from progenitors to mature dopaminergic neurons. I also elaborate on the role of Lmx1a/b in the regulation of mitochondrial genes and autophagy in adult mice. This way I show the importance to work inbetween those stages to understand the role of Lmx1a and Lmx1b in post-mitotic neurons during the dopaminergic circuitry establishment. In chapter 2, I develop an approach to quickly immunolabel midbrain dopaminergic neurons in order to specifically extract RNA from those cells. The region of interest is harvested using laser capture microdissection; the tissue is then processed to extract RNA for gene profiling assay. This approach has been used in the third chapter to find targets for Lmx1a/b in midbrain dopaminergic neurons at postnatal day 1 (P1).

The third chapter is the major paper of my thesis. It represents the core of my project and explains the role of the transcription factors Lmx1a and Lmx1b in post-mitotic midbrain dopaminergic neurons. In this chapter, *via* the gene profiling assay, we determined Plxnc1 as target for Lmx1a/b. Plxnc1 is known as an axon guidance protein showing a new role for Lmx1a/b as regulators of dopaminergic neurons axon guidance. The midbrain is constituted of two main subgroups of mDA neurons, the

substantia nigra *pars compacta* (SNpc) and the ventral tegmental area (VTA), forming the well-known nigrostriatal and mesolimbic pathways innervating the dorsal and the ventral striatal regions respectively. Until now, very little has been known about factors regulating axon projection of mDA sub-populations. Here we show that two transcription factors, Lmx1a an Lmx1b, are required for the appropriate topographical axon innervations of dopamine neurons from the VTA and the SNpc. In absence of Lmx1a/b, axon projections of SNpc neurons are misguided toward the ventral striatal region (VTA target site). The gene expression profiling experiment comparing Lmx1a/b double conditional mutants to control mice identified Plxnc1 as Lmx1a/b target gene. Plxnc1 expression is normally restricted to VTA neurons while in Lmx1a/b mutants, Plxnc1 expression is expanded to mDA neurons of the SNpc. *In vitro* examination of dopaminergic growth cones exposed to Sema7a, Plxnc1 ligand, indicates that Sema7a acts as a chemorepellent cue for VTA neurons. Dorsal striatal region shows strong expression of Sema7a and knockout mice for Sema7a results in an inappropriate innervation of VTA neurons in the dorsal striatal region. Forced expression of Plxnc1 in mDA neurons of transgenic mice results in the same axon guidance defect observed in cKO Lmx1a/b mutants animals. Our results reveal that Lmx1a and Lmx1b act as repressors of Plxnc1 in SNpc neurons, and that Sema7a-Plxnc1 are responsible of the segregation of nigrostriatal and mesolimic dopaminergic pathways. This study would help to increase the efficiency of cell replacement therapy for Parkinson's disease leading to reimplantation of SNpc-like neurons specifically to allow reinnervation of nigrostriatal pathway targets.

The fourth chapter constitutes a proof of concept of the second article showing the possibility of reinnervation of newly generated dopaminergic SNpc-like neurons to their targets. This manuscript is in preparation. I already differentiated embryonic stem cells into midbrain dopaminergic neurons for injection in mice. I did the characterization of those cells using immunostaining and western blotting. I performed 3 stables lines of cells, one repressing Plxnc1 expression using sh Plxnc1, one expressing a sh-scrambled construct, and another one overexpressing Plxnc1. I realized the 6-OHDA (lesionned in the medial forebrain bundle (MFB), and

others in dorsal striatum) mice model for cells injection, and performed behavioral tests (cylinder and open field tests) on those mice.

The fifth chapter is a discussion describing what can be done to decipher in more details the pathways behind the role of Lmx1a and Lmx1b in post-mitotic midbrain dopaminergic neurons. I also discuss my scientific contribution among the other papers published on the same subject, and the perspectives of my thesis project in the actual scientific context.

Finally, the sixth chapter is a brief conclusion of the main points discussed through this manuscript.

Chapter II: RNA Isolation from Cell Specific Subpopulations Using Laser-capture Microdissection Combined with Rapid Immunolabeling.

Chabrat A, Doucet-Beaupré H, Lévesque M.

URL: http://www.jove.com/video/52510

This article has been published the 11th of April 2015 in the Journal of Visual Experiment (JOVE). This article aims to explain step by step a technique of quick staining associated with laser capture microdissection (LCM) for the isolation of specific cells from thin tissue sections with high spatial resolution for RNA extraction. I participated to the elaboration of the technique and wrote the article with Martin Lévesque, except the part about the q-PCR which has been written and performed by H. Doucet-Beaupré. H. Doucet-Beaupré and I participated equally to the video. Dr. Martin Lévesque and I answered the reviewers and corrected the text for the final version.

Chabrat, A., Doucet-Beaupré, H., Lévesque, M. RNA Isolation from Cell Specific Subpopulations Using Laser-capture Microdissection Combined with Rapid Immunolabeling. *J. Vis. Exp.* (98), e52510, doi:10.3791/52510 (2015).

Chapter III: Transcriptional repression of Plxnc1 by Lmx1a and Lmx1b directs topographic dopaminergic circuit formation.

Chabrat A, Metzakopian E., Brisson G., Doucet-Beaupré H, Salesse C., Akitegetse C., Schaan-Profes M., Charest J., Pasterkamp R.J., Ang S.-L., Lévesque M.

This article is in review in Nature Communications. I am the first author of this paper. I conducted the majority of the experiments of this paper. I performed laser capture microdissection (LCM) combined with rapid immunolabeling for TH on mice at P1; cDNA library; performed and analyzed *in situ* hybridization; immunostainings and fluorescence quantification; stereological counting; explants and stripes assay; retrograde and anterograde tracing experiments; primary midbrain dopaminergic neurons culture, transfection; and I characterized the midbrain and striatal phenotype of the following mice at P1: Dat+/+ Lmx1aF/F/bF/F, Datcre/+ Lmx1aF/F/bF/F, Sema7a heterozygote, Sema7aKO, wildtype, and Plxnc1 over-expression mice. M.L. and I wrote the article. G.B. performed and analyzed explants experiments. C.S. performed and analyzed electrophysiological experiment. H.D-B. performed and analyzed qRT-PCR experiments. E.M. H.D-B and M.L. performed and analyzed mRNA sequencing at P1. M.S-P. performed and analyzed western blot experiments, and neuron counting at P15. G.B., J.C., and I performed mDA primary neurons culture experiments. C.A. performed imaging of transparent brains.

Chapter IV: Improvement of cell based replacement therapy for Parkinson's disease implanting newly generated cells repressing Plxnc1 expression.

Chabrat A, Metzakopian E., Rioux V.; Salesse C., Lévesque M.

This article is in preparation. For this article I differentiated embryonic stem cells into midbrain dopaminergic neurons for injection in mice. I did the characterization of those cells using immunostaining and western blotting. I performed 3 stables lines of cells, one repressing Plxnc1 expression using sh Plxnc1, one expressing a sh-scrambled construct, and another one overexpressing Plxnc1. I realized the 6-OHDA mice model for cells injection, and I performed behavioral tests on those mice: cylinder and open field tests.

During my thesis I also obtained a patent: **Methods for producing dopaminergic neurons and uses thereof**; Martin Lévesque, Audrey Chabrat; 2015.
This patent protects the idea of developping newly generated SNpc-like mDA neurons in which Plxnc1 is repressed.

Collaborations

I had the opportunity to collaborate on six different projects in parallel of my own:

The morphological and molecular changes of brain cells exposed to direct current electric field stimulation.

Pelletier S.-J., Lagacé M., St-Amour I., Arsenault D., Cisbani G., **Chabrat A.**, Fecteau S, Lévesque M., Cicchetti F.
DOI:10.1093/ijnp/pyu090

I performed explants used for this study, and wrote the material and method associated with this technique.

(2014). The Morphological and Molecular Changes of Brain Cells Exposed to Direct Current Electric Field Stimulation. *International Journal of Neuropsychopharmacology*, *18*(5), pyu090. http://doi.org/10.1093/ijnp/pyu090

Cystamine/cysteamine rescues the dopaminergic system and shows neurorestorative properties in an animal model of Parkinson's disease.

Cisbani G., Drouin-Ouellet J., Gibrat C., Saint-Pierre M., Lagacé M., Badrinarayanan S., Lavallée-Bourget MH., Charest J., **Chabrat A.**, Boivin L., Lebel M., Bousquet M., Lévesque M., Cicchetti F.
DOI:10.1016/j.nbd.2015.07.012

For this study I performed the entire *in vitro* part using explants. I designed experiments of neurorescue and neurorestoration on explants. I took pictures and analyzed the explants treated with cysteamine, rasagiline, and cysteamine plus rasagiline to test a synergic effect. I also performed explants used for HPLC and western blotting experiments. I wrote the material and methods part about explants experiments.

(2015). Cystamine/cysteamine rescues the dopaminergic system and shows neurorestorative properties in an animal model of Parkinson's disease. *Neurobiology of Disease*, *82*, 430. http://doi.org/10.1016/j.nbd.2015.07.012

Lmx1a and Lmx1b regulate mitochondrial functions and survival of adult midbrain dopaminergic neurons.

Doucet-Beaupré H., Gilbert C., Schaan Profes M., **Chabrat A.**, Pacelli C., Giguere N., Charest J., Rioux V., Laguna A., Deng Q., Perlmann T., Ericson J., Ang S-L., Cicchetti F., Parent M., Trudeau L-E., Lévesque M.
DOI: 10.1073/pnas.1520387113

For this study I did the characterization of the DatcreLmx1a/b mice at P1. I performed *in situ* hybridisation, and laser capture microdissection (LCM) combined with rapid immunolabeling for TH on mice at P1 for the mRNA sequencing. I also did some genotyping, perfusions and brain dissection of adult mice for this project.

Doucet-Beaupré, H. et al., 2016. Lmx1a and Lmx1b regulate mitochondrial functions and survival of adult midbrain dopaminergic neurons. *Proceedings of the National Academy of Sciences*, p.201520387.

Survival of a novel subset of midbrain dopaminergic neurons projecting to the lateral septum is dependent on NeuroD proteins,

Khan S., Stott S., **Chabrat A.,** Truckenbrodt A., Spencer-Dene B., Nave K-A., Guillemot F., Levesque M. and Ang S-L.

This article has been accepted in Journal of Neuroscience on November 30th 2016. For this project, I performed stereotaxic injections of retrograde marker and fluorogold in the lateral septum of Nex1-cre mice to characterize projections of NeuroD6-expressing mDA neurons to the Lateral Septum.

I participated to another paper about SlitRK2/5 in Dr. Lévesque's laboratory: **Salesse C. *et al*., manuscript in preparation**. For this project, I performed genotyping for all Dat+/+ Lmx1aF/F/bF/F, Datcre/+ Lmx1aF/F/bF/F mice in order to select only these mice and no alternative genotype for electrophysiological experiments.

I finally performed ESC-derived dopaminergic neurons for a project investigating the potential neuroprotective effect of GSSE on dopaminergic neurons; paper in collaboration with Dr. Lévesque's laboratory: **Ben Youssef S. *et al*., manuscript in preparation**.

Chapter 1. INTRODUCTION

Mesodiencephalic dopamine neurons (mDA) play crucial roles in the control of a variety of brain functions, including voluntary movement and behavioural processes such as mood, reward and attention (Albin et al. 1989). The dopaminergic system is constituted of DA neurons localized for 75% in the midbrain and innervating several targets throughout the brain (Björklund & Dunnett 2007). The midbrain encompasses two distinct populations of neurons: mDA neurons from the VTA forming the mesolimbic pathway, and mDA neurons from the SNpc constituting the nigrostriatal pathway (Björklund & Dunnett 2007). In order to differentiate in mDA neurons, progenitors go through several steps of differentiation. Each step is regulated by multiple factors like morphogens and transcription factors (Ang 2006; Blaess & Ang 2015; Poulin et al. 2014). Indeed, the specification of these neurons requires the action of multiple transcription factors including LIM-homeodomain transcription factor 1a and 1b (Lmx1a and Lmx1b), and orthodenticle homeobox 2 (Otx2) for example (Doucet-Beaupré & Lévesque 2013; Blaess & Ang 2015). Lmx1a and Lmx1b are well known for their role in mDA neurons differentiation, and recent studies shown their roles in adult mice controlling mitochondrial functions and autophagy (Laguna et al. 2015; Doucet-Beaupré et al. 2016). However, nothing is known about the role of Lmx1a/b in late developmental stages of post-mitotic mDA neurons. Some evidences suggest that Lmx1a and Lmx1b could regulate axon pathfinding but the role of these factors for mDA circuit fromation has not been investigated yet. It is known that the deregulation of the nigrostriatal or mesolimbic pathways leads to several diseases like depression, schizophrenia, and Parkinson's disease (PD) (Blaess & Ang 2015). PD is characterized by both, motor and non-motor symptoms; and by a degenerescence of mDA neurons in the SNpc (Ang 2006). Nowadays, one therapy proposed in PD is a cell-based replacement therapy. Unfortunately, this kind of therapy was not conclusive, because the newly generated mDA neurons from stem cells do not innervate their targets correctly. Lmx1a and Lmx1b transcription factors were shown as factors involved in the regulation of axon guidance in other systems than the dopaminergic system (Kania et al. 2000). Investigating their role in mature mDA neurons could give us a cue about mDA circuit development and help the development of efficient cell-based replacement therapy for PD.

1. Dopaminergic System

The dopaminergic system encompasses four different pathways (See Figure 1): the nigrostriatal, the mesolimbic, the mesocortical, and the tuberoinfundibular pathways (Björklund & Dunnett 2007).

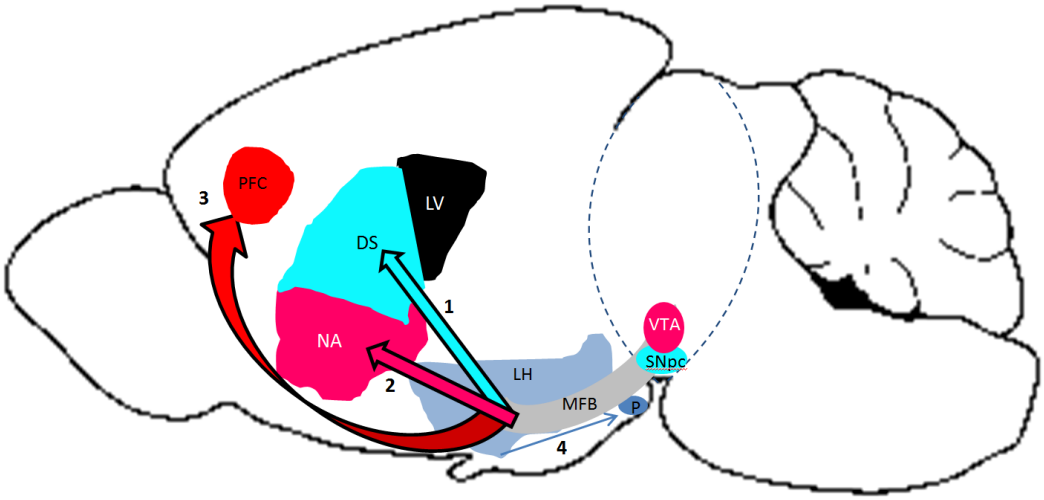

Figure 1 Schematic Representation of the Four Pathways Constituting the Dopaminergic System in the Adult Mouse Brain.
Inbetween the doted lines is the midbrain region. (1) represents the nigrostriatal pathway with neurons from the SNpc innervating the dorsal striatum (DS). (2) is the mesolimbic pathway constituted from VTA neurons innervating the nucleus accumbens (NA). (3) shows a representation of the mesocortical pathway with the prefrontal cortex (PFC) innervated by VTA neurons. (4) represents the tuberoinfundibular pathway showing hypothalamic neurons, from the lateral hypothalamic area (LH), projecting to the pituitary median eminence.

The nigrostriatal pathway is constituted from mDA neurons from the SNpc innervating the dorsal striatum (caudate nucleus and putamen) as shown in Figure 1. This pathway is mostly involved in motor behaviour (Vandenheuvel & Pasterkamp 2008; Björklund & Dunnett 2007).

A second important pathway is the mesolimbic pathway formed by dopaminergic neurons from the VTA projecting to the ventral part of the striatum, the nucleus accumbens (NA). This pathway plays an important role in the reward system, feeding, and olfaction (Dreyer 2010).

The mesocortical pathway encompasses neurons from VTA projecting to the prefrontal cortex (PFC) regulating cognitive, learning, memory, and emotional functions (Gorelova et al. 2011).

The tuberoinfundibular pathway refers to hypothalamic neurons projecting to the pituitary median eminence. This connection, through dopamine release, leads to the secretion of hormones such as prolactin (Stahl 2013).

2. Dopaminergic Neurons

The dopaminergic neurons are part of the catecholamine (CA) neurons. These CA neurons are designated from A1 to A17 according to their localization in the mammalian brain from the medulla oblongata to the retina. Among these groups of dopaminergic neurons, neuronal cell groups from A8 to A17 contain dopamine (Dahlström & Fuxe 1964; Björklund & Dunnett 2007).

Dahlström and Fuxe's study in 1964 gave the numbering of the cell groups from A8 to A16. DA neurons belonging to cell groups 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 2) in the retina (Dahlström & Fuxe 1964; Björklund & Dunnett 2007).

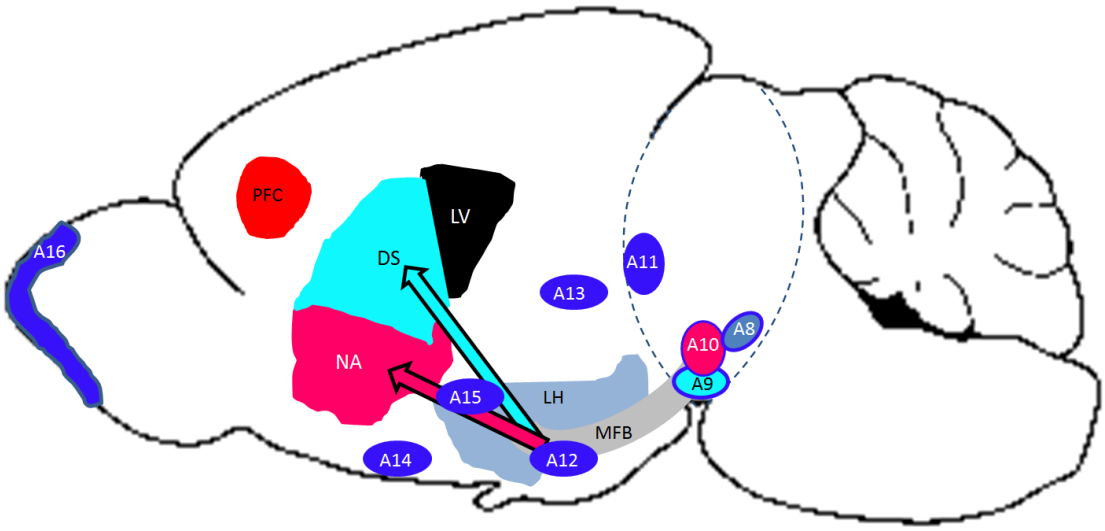

Figure 2 Dopamine-Containing Neuronal Cell Groups Localization through the Mouse Brain.
The dopaminergic neurons in the mammalian brain are localized in nine distinctive cell groups. They are distributed from the mesencephalon to the olfactory bulb, as illustrated schematically in a sagittal view of mouse brain.

2.1 The Dopamine and Its Receptors

2.1.1. Dopamine

The main function of dopaminergic neurons is to synthesize dopamine. Dopamine is an essential neurotransmitter for the transfer of information throughout synapses (Goridis & Rohrer 2002). The substrate for dopamine synthesis is the tyrosine. Two different enzymes, the Tyrosine hydroxylase (TH), and the L-Aromatic amino acid decarboxylase (L-AADC), are involved in the transformation of tyrosine into dopamine. As shown on Figure 3, the TH by hydroxylation of the aromatic cycle leads to L-dihydroxyphenylalaline (L-Dopa) synthesis (Levitt et al. 1965). The L-Dopa is known as one of the most effective treatments for PD until now. Through a second reaction regulated by the L-AADC, the L-Dopa is transformed in dopamine by decarboxylation (Goridis & Rohrer 2002).

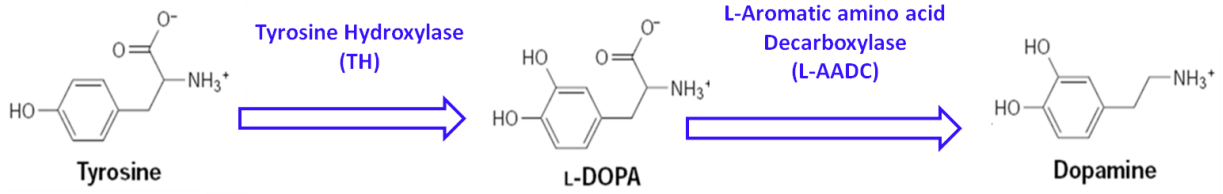

Figure 3 Mechanism for Dopamine Synthesis.
Dopamine is synthesized from Tyrosine through two enzymatic reactions. The two enzymes involved in dopamine synthesis are the Tyrosine hydroxylase (TH), and the L-Aromatic amino acid decarboxylase (L-AADC). The TH by hydroxylation of the aromatic cycle of the tyrosine leads to L-dihydroxyphenylalaline (L-Dopa) synthesis. Then the L-Dopa is transformed into dopamine by decarboxylation *via* L-AADC (modified from Goridis and Rohrer, 2002).

The dopamine synthesized by mDA neurons is concentrated into synaptic vesicles in the cytoplasm of these neurons. These synaptic vesicles are formed through the regulation of the vesicular monoamine transporter type 2 (VMAT2). Once an action potential is generated and arrives at the terminus of the pre-synaptic axon, the dopamine is released to the synaptic cleft. A fusion of the vesicles containing DA with the cytoplasm membrane allows the DA release as schematized on Figure 4. Then, DA can either bind to specific DA receptors on the postsynaptic membrane, be degraded in the synapse, or be recycled using the dopamine transporter DAT for a reuptake into the pre-synaptic terminal (Giros & Caron 1993).

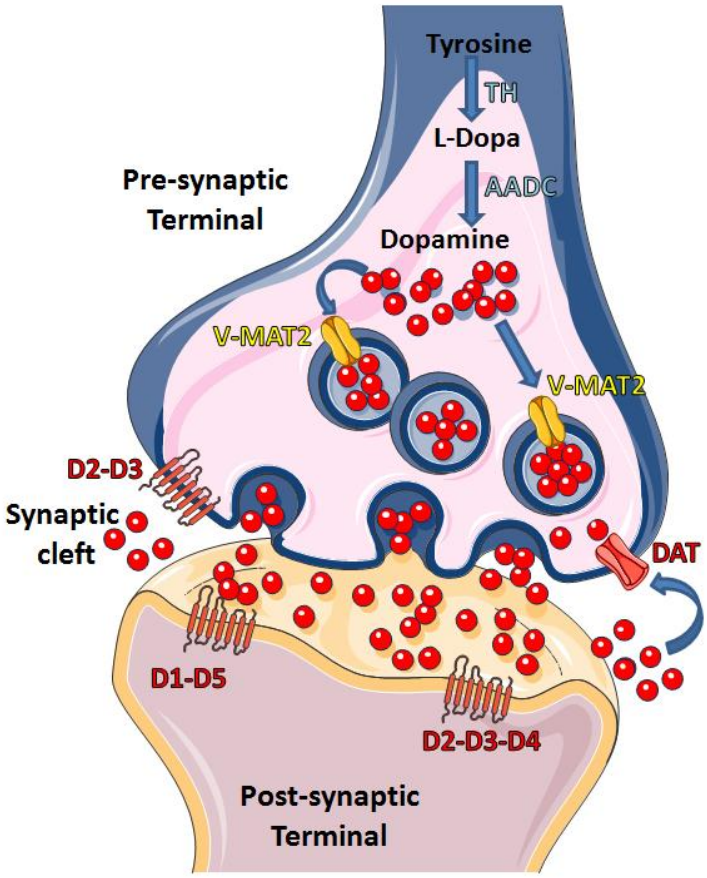

Figure 4 Mechanism of Dopamine Release to the Synaptic Cleft.
The dopamine is synthesized in mDA neurons cytoplasm then concentrated into synaptic vesicles *via* the vesicular monoamine transporter type 2 (VMAT2). A fusion of these vesicles with the cytoplasm membrane when an action potential occurs leads to dopamine release in the synaptic cleft. DA can either bind to specific DA receptors, D1 to D5, on the postsynaptic membrane, be degraded in the synapse, or be recycled using the dopamine transporter DAT for a reuptake into the pre-synaptic terminal (Giros & Caron 1993).

2.1.2. Dopamine Receptors

The dopamine receptors are G protein–coupled receptors (GPCR) with seven-transmembrane domains (Beaulieu et al. 2015). These receptors are divided into two classes: D1 and D2. Each class encompasses multiple groups. The receptors that constitute class D1 are D_1 and D_5 receptors; while receptors constituting class D2 encompass D_2, D_3, and D_4 receptors (Beaulieu & Gainetdinov 2011).

Those GPCR receptors are activated by the G protein leading to the initiation of the adenylate cyclase (AC) and to the production of a second messenger: cyclicadenosine monophosphate (cAMP) represented in Figure 5. The protein kinase A (PKA) is then

regulated according to the presence of cAMP. In a second step, G protein-receptor kinase family (GRK) and βarrestin are recruited to form the receptor/protein complex (GPCR/AP2/βarrestin/clathrin). In a third step this complex is internalized in clathrin coated-pits and disassembled. The fourth step consists in the recycling to the membrane or degradation by proteasome of the complex (Beaulieu et al. 2015). However, the mechanisms can differ and be independent from G protein coupling. These mechanisms can involve ion channels, or receptor tyrosine kinases (Beaulieu et al. 2015). Moreover, D1 receptors stimulate the activity of AC and the production of the second messenger cAMP, inversely to the D2 class receptors which inhibit the production of cAMP (Kebabian 1978; Memo et al. 1984; Beaulieu & Gainetdinov 2011).

Both classes of dopamine receptors D1 and D2 have their specific signalling cascades. These receptors use the βarrestin to regulate specific complexes composed, for D1 receptors, of βarrestin2/Erk/Raf/MEK for the induction of Erk; and, for D2 receptors, of the Akt/βarrestin 2/PP2A complex leading to the inhibition of GSK3 *via* the activation of Akt (Del'Guidice et al., 2011).

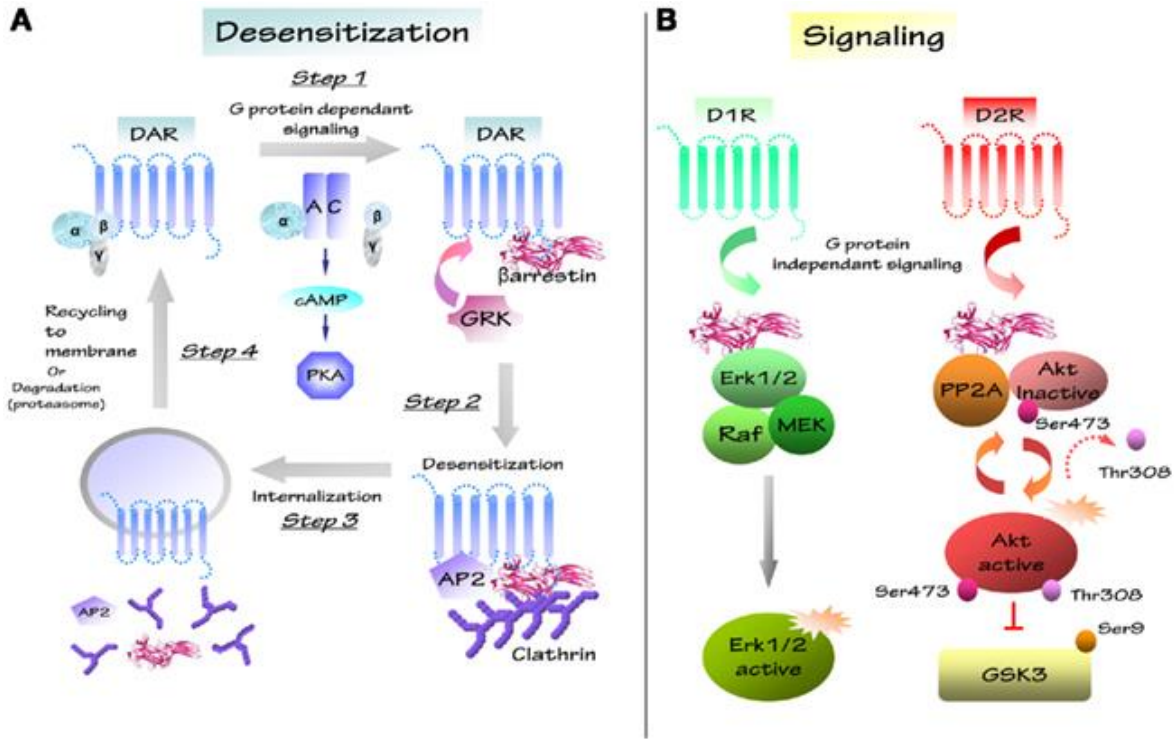

Figure 5 Schematic Representation of Dopamine Receptors.
In (A) are represented the 4 steps following the activation of the dopamine receptors GPCR. In a first step, after G-protein activation several second messengers are recruited: adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA). In a second step, G protein-receptor kinases family (GRK) and βarrestin are recruited

to form the receptor/protein complex (GPCR/AP2/βarrestin/clathrin). In a third step this complex is internalized in clathrin coated-pits and disassembled. The fourth step constitutes in the recycling to the membrane or degradation by proteasome. In (B) the 2 classes of dopamine receptors D1 and D2 are schematized with their specific signalling cascade. Both receptors use the βarrestin to regulate specific complexes composed for D1 receptor of βarrestin2/Erk/Raf/MEK for the induction of Erk; and for D2 receptor of the Akt/βarrestin 2/PP2A complex leading to the inhibition of GSK3 *via* the activation of Akt (Del'Guidice et al., 2011).

It is important to understand those complex dopamine regulation mechanisms in order to develop new therapeutic strategies such as pharmacological targets for diseases involving the dopaminergic system.

2.2 Midbrain dopaminergic neurons

The midbrain dopaminergic neurons constitute about 75% of DA neurons in the adult brain. Those neurons encompass neurons from the retreorubral field (RRF), the SNpc, and the VTA (respectively groups A8 to A10) mDA neurons (Wallén & Perlmann 2003). However, even though those groups seem to be well-defined, new studies start to decipher the heterogeneity in those groups (Roeper 2013; Poulin et al. 2014).

2.2.1. Origin of Midbrain Dopaminergic Neurons: the Floor Plate and Secreted Factors.

Located at the ventral midline of the neural tube, the floor plate is a small group of cells acting as an organizing centre extending from the spinal cord to the posterior diencephalon (Placzek & Briscoe 2005; Blaess & Ang 2015). These specialized sets of cells have an important influence in the development of the vertebrate nervous system (Placzek & Briscoe 2005). The floor plate, by secreting the morphogen sonic hedgehog (SHH) monitors the specification of neuronal and glial identities. By non-prolonging SHH signalling the floor plate keeps its ventral midbrain identity (Ribes et al. 2010; Metzakopian, Lin & Salmon 2012b). The progenitors from the floor plate giving rise to mDA neurons are CORIN-positive and express Lmx1a (Ono et al. 2007). Other important factors for neuronal differentiation are expressed by the floor plate, such as the transforming growth factor-β (TGF-β) superfamily, including bone morphogenetic protein (BMP); the Wingless-related integration site (WNT) family; and the fibroblast growth factors (FGFs) also have important role for neuron specification (Placzek & Briscoe 2005).

Morphogens and transcription factors are expressed at specific times to induce the different steps of differentiation: regionalization, fate specification, proliferation/neurogenesis, and maturation. During a really short window of time at the induction of the midbrain floor plate, the simultaneous expression of FGF8 from the isthmic organizer in the mid/hindbrain boundary and SHH signalling from the FP leads to the induction of mDA neurons [See embryonic day 8 (E8) Figure 6 (Hynes et al. 1995; Ye et al. 1998)]. Prakash *et al*. shown the importance of WNT1 by using WNT1-null mutant mouse model enabled to produce mDA neurons (Prakash et al. 2006). Those evidence show the importance of the presence of secreted molecules at the right place and the right time for mDA neurons production.

A panel of transcription factors associated with the secreted factors previously mentionned are involved, such as Forkhead box A 1 (FOXA1), Forkhead box A 2 (FOXA2), the GLI-Kruppel family member GLI1 (Gli1), the Homeobox msh-like 1 (Msx1), Engrailed 1 and Engrailed 2 (En1/2), LIM-homeodomain transcription factor 1a and 1b (Lmx1a and Lmx1b), orthodenticle homeobox 2 (Otx2), Paired box gene 2 (Pax2), and Paired box gene 5 (Pax5). Foxa2 has multiple roles in the development but can also be involved in tumorigenesis. In the central nervous system (CNS), Foxa2 is involved in the maintenance of the ventral floor plate identity in the midbrain (Metzakopian, Lin & Salmon 2012a). Foxa2 also plays an important role in the different stages of development of mDA neurons. This transcription factor regulates the specification, neurogenesis, and differentiation of mDA neurons as well (Arenas 2008, Blaess & Ang 2015).

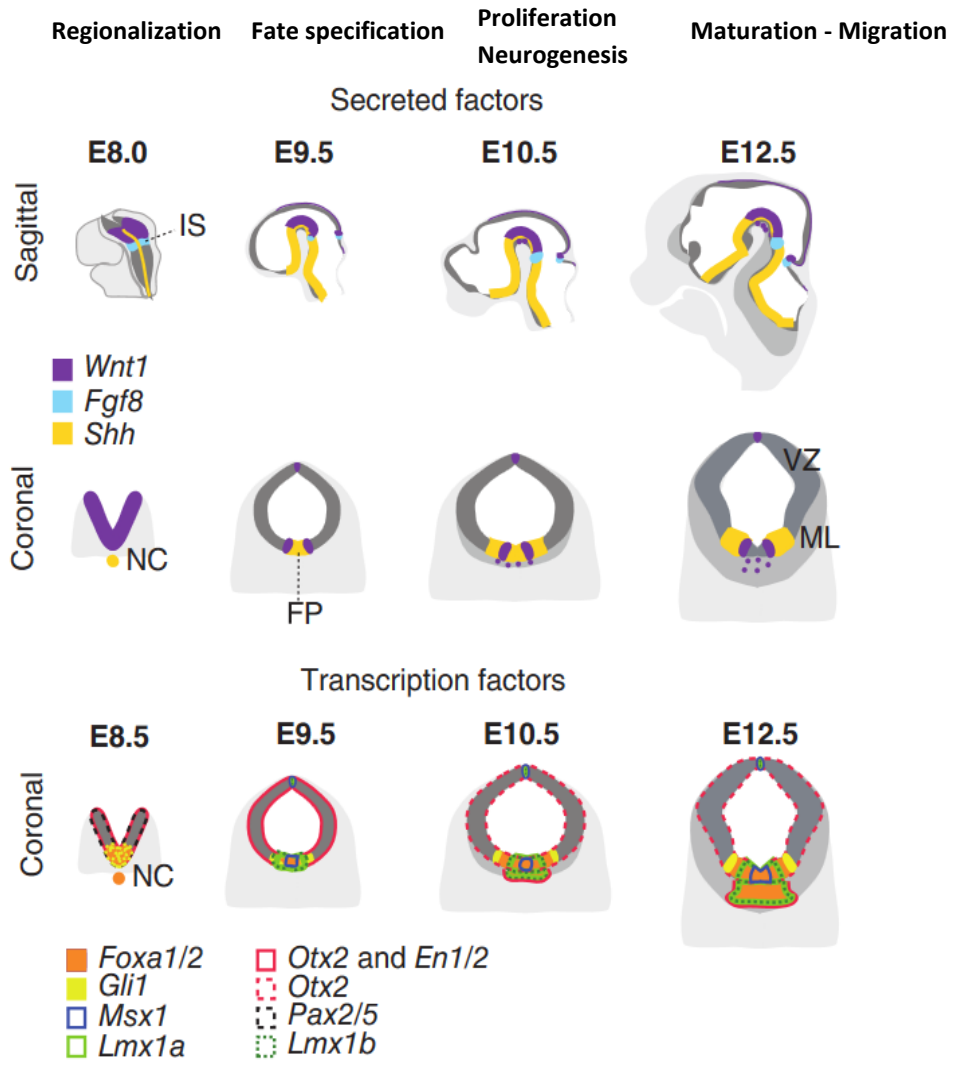

Figure 6 Early Development of mDA Neurons: Secreted and Transcription Factors.
Coronal and sagittal views of secreted and transcription factors expression. The sagittal view allows following the expression of secreted factors along the anterior-posterior mDA progenitor domain. The secreted factors are Wnt1, Fgf8, and Shh. The key transcription factors mentioned are Foxa1/2, Gli1, Msx1, Lmx1a, Lmx1b, Otx2, Pax2/5, and En1/2. FP, floorplate; IS, isthmus; ML, mantle layer; NC, notochord; VZ, ventricular zone, modified from (Blaess & Ang 2015).

A recent study showed the importance of the complex formation with nuclear cofactors during early development. Indeed, transcription factors can interact with nuclear cofactors to form multiprotein complexes important for transcription regulation (S. Kim et al. 2016). A nuclear cofactor, the LIM domain-binding protein 1 (Ldb1) has been shown as functioning cooperatively with Lmx1b. Indeed, a deletion of Ldb1 in mid- and hinbrain development specifically in the Engrailed-1-expressing region leads to similar defects of those observed in mice lacking Lmx1b. Predominant signalling molecules are impaired

like Fgf8, Wnt1, and Shh affecting the isthmic organizer and the ventral midbrain (Kim et al. 2016). Those defects led to the total loss of mDA neurons in Ldb1 mutant (Kim et al. 2016).

Thus, several markers need to be considered during the development of dopaminergic neurons, especially because secreting factors and transcription factors are all linked with nuclear cofactors by multiple regulatory pathways. The investigation of these molecules is of real interest for the identification of mDA neuronal sub-types. Efforts have been put into discovering mDA neurons diversity using single cell gene profiling approach (Poulin et al. 2014; Anderegg et al. 2015). Indeed, Poulin *et al.* (Poulin et al. 2014) used an elegant approach with high-throughput single-cell gene-expression analysis of mDA neurons in order to highlight molecular signatures differences between DA neuron sub-groups (Poulin et al. 2014). Six specific sub-groups of DA neurons with their own molecular signature were identified (Poulin et al. 2014). The two first groups, DA^{1A} and DA^{1B}, harbor similar expression profiles with a high expression of Sox6 and Ndnf, and low expression of Foxa2 and Lmx1a. However, DA^{1A} expresses one more factor than DA^{2B} neurons: Aldh1a1. These factors correspond to the most described in the pattern of expression of mDA neurons until now. DA^{1B} have similarities with the DA^{2C} subtype. However, the DA^{2C} subtype is more difficult to define because it shows molecular similarities to other groups like high levels of expression for Calb1, Cholecystokinin (Cck) and Slc17a6 which correspond to genes characterizing the second cluster. Indeed, more subgroups were defined by other factors: DA^{2A} neurons express a high concentration of the vesicular inhibitory amino acid transporter (Slc32a1) but low concentration of special AT-rich sequence-binding protein-1 (Satb1), germ cell-specific gene 1-like protein (Gsg1l), and calsyntenin 2 (Clstn2); DA^{2B} is the only group coexpressing the lipoprotein lipase (Lpl), the Adenylate Cyclase Activating Polypeptide 1 (Adcyap1), Otx2, and Aldh1a1; DA^{2D} is the only group expressing the Vasoactive Intestinal Peptide (Vip), with low expression of α-synuclein (Snca), Neuronal acetylcholine receptor subunit alpha-4 (Chrna4), and Nuclear transport factor (Ntf) (Poulin et al. 2014).The discovery of these several groups of mDA neurons brings new avenues of research to identify their specific roles and functions in the dopaminergic system.

2.2.2. The Midbrain Dopaminergic Neurons Development

The midbrain dopaminergic neurons are generated from neural progenitor cells in the floor plate in four stages. These stages are defined according to the expression of different molecular factors as described in Figure 7.

The first step of regional specification is characterized by the expression of Otx, Lmx1a and Lmx1b (Lmx1a/b), En1/2, Msx1 and Msx2 (Msx1/2), Aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1), Neurogenin 2 (Neurog2) and Achaete-Scute Complex-Like 1 (Ascl1 or Mash1). This step starts, as described in Figure 6, in the ventricular zone (VZ). First, neuroepithelial cells and subsequently radial glia become proliferative progenitors. During the specification, Foxa2 activates Shh, and is regulated itself by Shh *via* Gli2. At this step, Foxa2 inhibits NK2 transcription factor-related locus 2 (Nkx2-2) to allow FP specification. Foxa2 also regulates the expression of neurogenin 2 (Neurog2), as Msx1 and Wnt1. Finally, Foxa2 leads to the differentiation of DA precursors into DA neurons by regulating the expression of En1/2 and Nur-related factor1 (Nurr1) also known as Nuclear receptor subfamily 4 group A member 2 (Nr4a2) (Arenas 2008; Blaess & Ang 2015).

After an asymmetric division of mDA progenitors lying in the VZ of the floor plate to the intermediate zone (IZ), the proliferative progenitors become postmitotic precursors. Progenitors exit cell cycle and differentiate into immature neurons in the IZ where they start to express Nur-related factor 1 (Nurr1). This second step, called early differentiation or neurogenesis, is regulated by Lmx1a/b, Neurog2, Nurr1, En1/2, Aldh1a1 and βIII-tubulin (Rodríguez-Traver et al. 2015; Arenas 2008). Neurog2 is, indeed, an important transcription factor for the neuronal commitment, cell cycle withdrawal, neuronal differentiation, and survival of mDA neurons (Lacomme et al. 2012). This proneural gene is a crucial basic helix-loop-helix (bHLH) regulator of neurogenesis and subtype specification in many areas of the nervous system (Ang, 2006). In the ventral midbrain, Neurog2 is expressed in mDA progenitors, but also in postmitotic immature mDA neurons at an early stage of immature mDA neurons. At a later stage of this second step of differentiation, Neurog2 would not play a direct role, but will induce the expression of Nurr1+. Furthermore, Neurog2 induces the differentiation of Sox2(+) ventricular zone

progenitors into Nurr1(+) postmitotic dopaminergic neuron precursors in the intermediate zone (Andersson, Tryggvason, Deng & Friling 2006; Kele et al. 2006).

Finally, immature neurons differentiate to emerge in the marginal zone as DA neurons. These neurons acquire the expression of mature mDA neuron markers such as TH, AADC, paired like homeodomain transcription factor 3 (Pitx3), and dopamine transporter (DAT) during the late phase of differentiation while reaching the marginal zone (dark green, Figure 7) (Andersson, Tryggvason, Deng & Friling 2006; Puelles et al. 2003; 2011; Kele et al. 2006; Yan, Levesque, et al. 2011). Neurog2 is required at this step of differentiation (Andersson, Tryggvason, Deng & Friling 2006; Kele et al. 2006). Indeed, Kele et al. using mutant mice for Neurog2, shown an important loss of mDA neurons illustrating the important role of Neurog2 in the development of mDA neurons (Kele et al. 2006).

The knowledge of these transcription factors leads to a better understanding of the specific mDA progenitors and of their further differentiated forms (immature, and mature mDA neurons). Moreover, their identification through these different steps, make them interesting candidate regulators to promote the specification and differentiation of progenitors, as well as the differentiation of stem cells specifically involved into mDA neurons in the case of PD cell replacement therapy (Ang 2006).

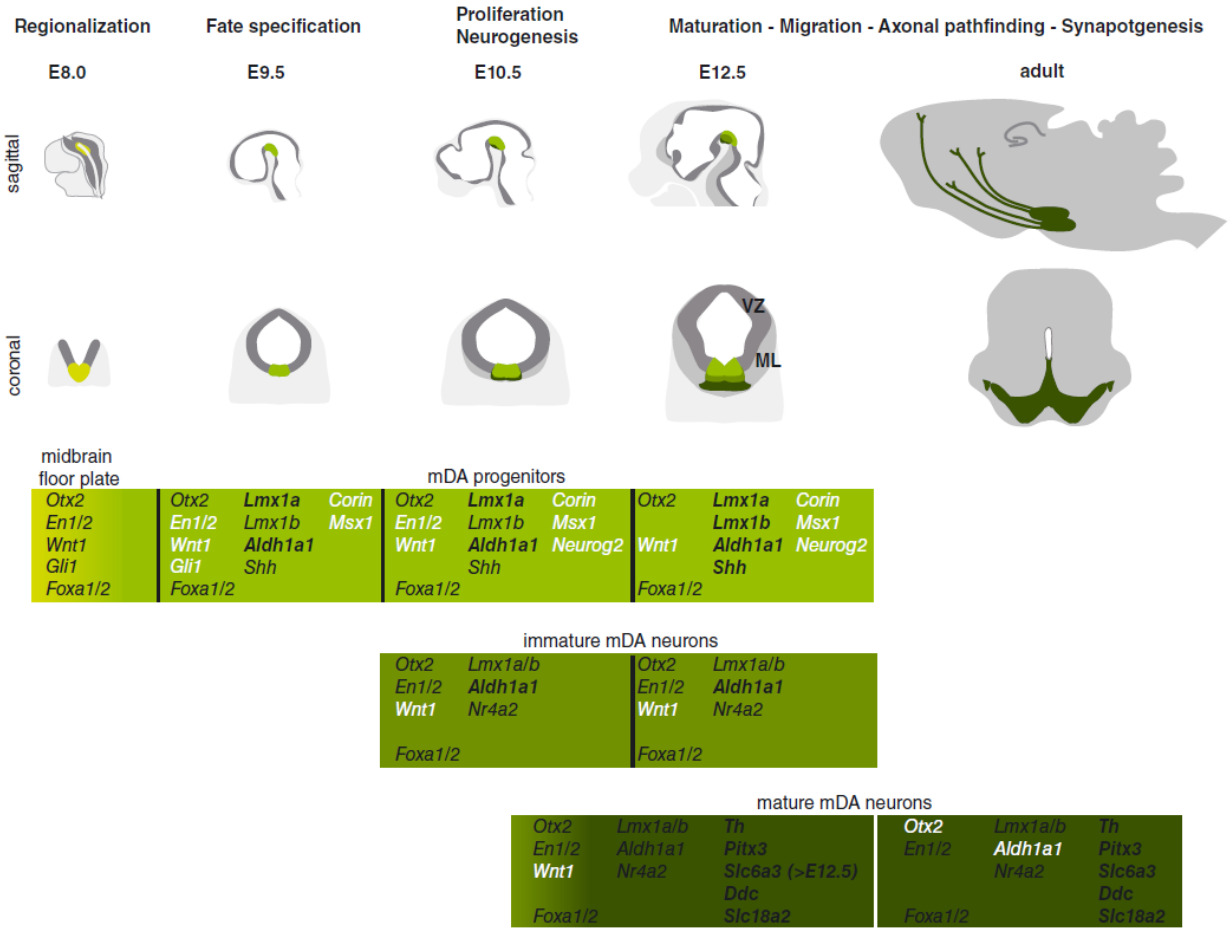

Figure 7 Midbrain Dopaminergic Neurons Differentiation.
Several transcription factors have been identified as necessary for mDA neurons differentiation. In white are the factors expressed in a subtype of mDA progenitors and/or neurons. In black are the factors expressed in all mDA progenitors and/or neurons. mDA progenitors and/or neurons expressed in the ventral midbrain are in bold (Blaess and Ang, 2015).

In addition to morphogens, secreted factors and transcription factors, microRNA (miRNA) have been recognized as important regulators of some of the key factors of mDA differentiation, such as Lmx1b (Blaess & Ang 2015). MiRNAs regulate brain development by monitoring transcription factors and proteins that are required for neurogenesis and development (Follert et al. 2014). As described earlier, the expression of different regulation factors is necessary to allow the expression of a certain type of proteins at the right moment and the right place. By their silencing effect, miRNAs are able to control the expression of the proteins responsible for cell entry into the neurogenic program (Follert et al. 2014). One way to act for miRNAs is by feedback loops with their targets (Follert et al. 2014).

MiR-135a2 is involved in the regulation of morphogens. Anderegg and co-workers uncover that miR-135a2 regulates some morphogens by limiting their expression and function to a specific area at a determined time (Anderegg et al. 2013). In the embryonic midbrain, Wnt1 is an important morphogen which directs proliferation, survival, patterning and neurogenesis (Anderegg et al. 2013). Anderegg et al. shown a regulation in the extent of Wnt1/Wnt signalling and the size of the dopamine progenitor domain induced by an autoregulatory negative feedback loop between the transcription factors Lmx1b and miR135a2 (Anderegg et al. 2013). Conditional gain of function studies revealed that Lmx1b promotes Wnt1/Wnt signalling, and thereby increases midbrain size and dopamine progenitor allocation; and the opposite effect is observed when Lmx1b is conditionally removed as described Figure 8 (Anderegg et al. 2013). The implication of miRNAs in the regulation of the size of dopamine progenitor domain was shown using embryonic stem cells (ESCs) modified with conditional loss of Dicer1 (Anderegg et al. 2013). Indeed, miRNAs no more able to play their repressive role, an expansion of Lmx1a/b+ progenitors was observed; and on the contrary, an overexpression of miR135a2 was observed in Lmx1b conditional knockout during an early short period of time showing an autoregulatory negative feedback loop between the transcription factor Lmx1b and miR135a2 (Anderegg et al. 2013). The expansion of Lmx1a/b+ progenitors is selectively reduced when En1::Cre is used for recombination (Anderegg et al. 2013). All these evidences put together show that the microRNA act to maintain the boundary between the Lmx (red) and Nkx6.1 (green) domains only during an early critical window during the development. Thus, midbrain size and allocation of dopamine progenitors could be determined by the modulation of the Lmx1b/Wnt axis in the early midbrain/isthmus by miRNAs (Anderegg et al. 2013). This study has shown the regulation of the domain of midbrain dopaminergic neurons progenitors, but other important mechanisms for brain development are regulated by miRNAs like myelination.

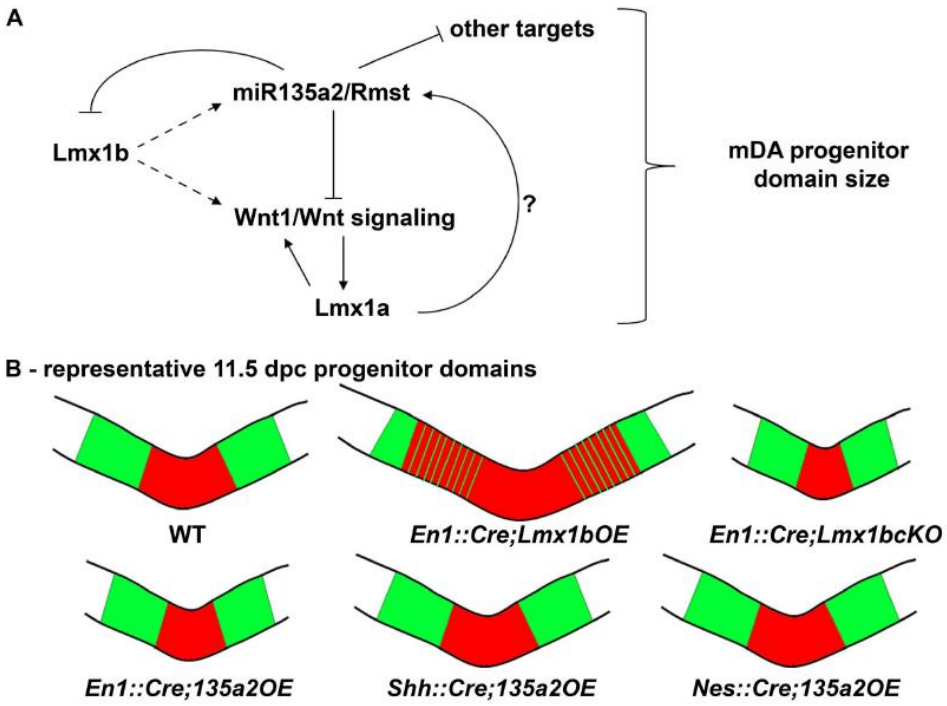

Figure 8 Mir-135a2 Regulates mDA Progenitor Pool.
Mir135a and Lmx1b influence mDA progenitor patterning and expansion in an opposite way (Anderegg *et al*., 2013).

Thus, many complex regulations are controlled by miRNA during brain development. The regulation is dependent of the cellular environment but also of the regulation between several miRNA, and signal transfer through synapses (Follert et al. 2014).

In summary, development of mDA neurons can be divided into four phases termed regionalization, fate specification, proliferation/neurogenesis, and maturation as shown in Figures 6-7. In each of these steps, transcription factors Lmx1a/b are necessary, showing their importance in the differentiation process of midbrain dopaminergic neurons. Knowing their role in the early development of mDA neurons, we would be interested to investigate the role of Lmx1a/b in mature dopmainergic neurons, discover if they have any regulatory role for migration, axonal pathfinding, or synaptogenesis.

2.2.3. Axonal Projections of mDA Neurons

As mentioned previously, mDA neurons are constituted of three *nuclei* of neurons: SNpc neurons named A9, VTA neurons forming the group A10, and neurons from the RRF called A8 group. SNpc and VTA neurons are involved in the formation of the nigrostriatal, and mesocorticolimbic pathways respectively. However, these neurons, with neurons from RRF, also innervate other targets (Figure 9).

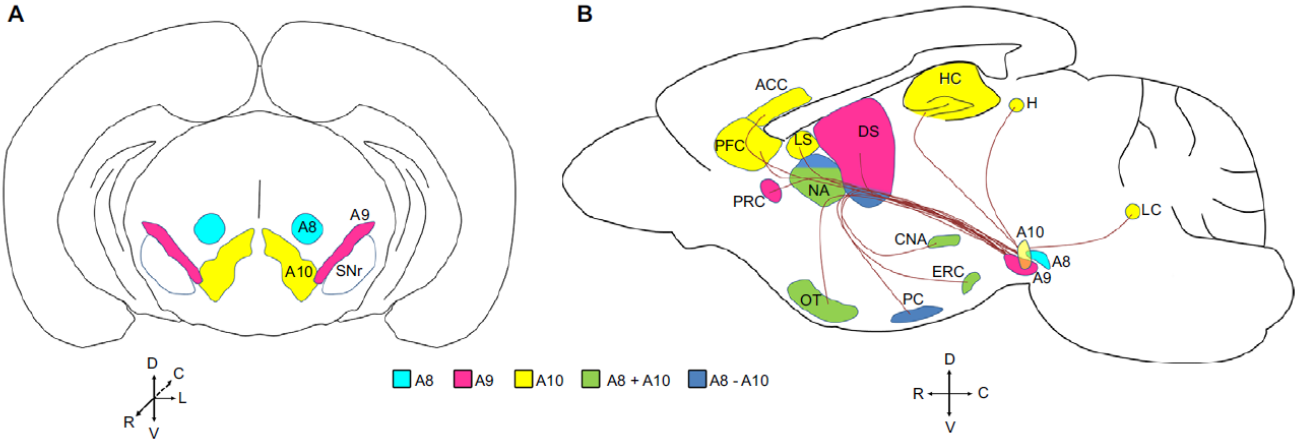

Figure 9 Schematic Representation of mDA Neurons and their Axonal Projections throughout the Adult Mouse Brain.
(A) and (B) represent respectively a coronal and sagittal section of the adult mouse brain. Three nuclei of mDA neurons are shown in the coronal section of the midbrain in (A). The neurons in the A8 cluster develop in the retrorubral field (RRF). Neurons localized in A9 develop in the SNpc, and neurons in A10 develop in the VTA. The sagittal section in (B) shows mDA neurons with their axonal projection. in pink are represented targets of SNc neurons; in yellow targets of VTA neurons; in green targets of VTA and RRF neurons; in blue targets innervated by the three nuclei of mDA neurons. The nigrostriatal pathway is represented by projections from A9 to dorsal striatum (DS). A9 neurons also project to the perirhinal cortex (PRC); and to the pyriform cortex (PC) with A8 and A10 neurons. Projections from A10 to the nucleus accumbens (NA) represent the mesolimbic pathway. The NA is also innervated by neurons from the A8 cluster. Neurons from the VTA and RRF project to the olfactory tubercle (OT), the central nucleus of the amygdala (CNA), and the entorhinal cortex (ERC). VTA neurons projecting to the prefrontal cortex (PFC) form the mesocortical pathway. Neurons from VTA also innervate the latera septum (LS), the habenula (H), the hippocampus (HC), the locus coeruleus (LC), and the anterior cingulate cortex (ACC) (Arenas et al., 2015).

From the SNpc, most neurons project to the dorsal striatum, forming the nigrostriatal pathway, but A9 neurons also project to the perirhinal cortex (PRC). A9 neurons, associated with A8 and A10 neurons innervate the pyriform cortex.

From the VTA, DA neurons project to the ventral striatum and the ventro-medial part of the head of the caudate-putamen in rodents (equivalent to nucleus caudatus in primates). VTA neurons by projecting to the nucleus accumbens (NA) form the mesolimbic pathway. A10 neurons also project to the lateral septum (LS), the olfactory tubercle (OT), the amygdala, the habenula (H) the hippocampus (HC), the locus coeruleus (LC), and the anterior cingulate cortex (ACC) (Arenas et al. 2015; Vandenheuvel & Pasterkamp 2008; A. Björklund & Lindvall 1984). VTA neurons projecting to the prefrontal cortex (PFC) form the mesocortical pathway (Arenas et al. 2015).

The A8 cell group that forms a dorsal and caudal extension of the A10 cell group contains cells that project to both striatal, limbic and cortical areas (A. Björklund & Dunnett 2007). Indeed, the NA is not only innervated by neurons from VTA but also by neurons from the A8 cluster. Neurons from the RRF and VTA also both projects to the olfactory tubercle (OT), the central nucleus of the amygdala (CNA), and the entorhinal cortex (ERC) (Arenas et al. 2015).

The regulation of axonal projections of mDA neurons needs to be understood to identify important players, such as transpritpion factors, in post-mitotic mDA involved in the establishment of the dopaminergic system.

3. Lmx1a/b Transcription Factors

3.1. Structure/Function of Lmx1a/b

LIM proteins are originally named by the initials of the three LIM domain containing transcription factors Lin11, Isl1, and Mec3 (Freyd et al. 1990; Way & Chalfie 1988; Karlsson et al. 1990). LMX1A/B are part of the LIM-homeodomain family of proteins. These proteins contain two N-terminal zinc-binding LIM domains binding each two zinc ions to form two tandemly repeated zinc fingers (Sadler et al. 1992; Hunter & Rhodes 2005). LMX1A/B share highly similar functional domains, and have 64% of homology in

their overall amino acid composition (cysteine-histidine-rich LIM domain has an approximate size of 50–60 amino acids) (Sadler et al. 1992; Hunter & Rhodes 2005). LMX1A/B are constituted of three identical DNA binding homeodomains as described on Figure 10 (Hunter & Rhodes 2005; Hobert & Westphal 2000).

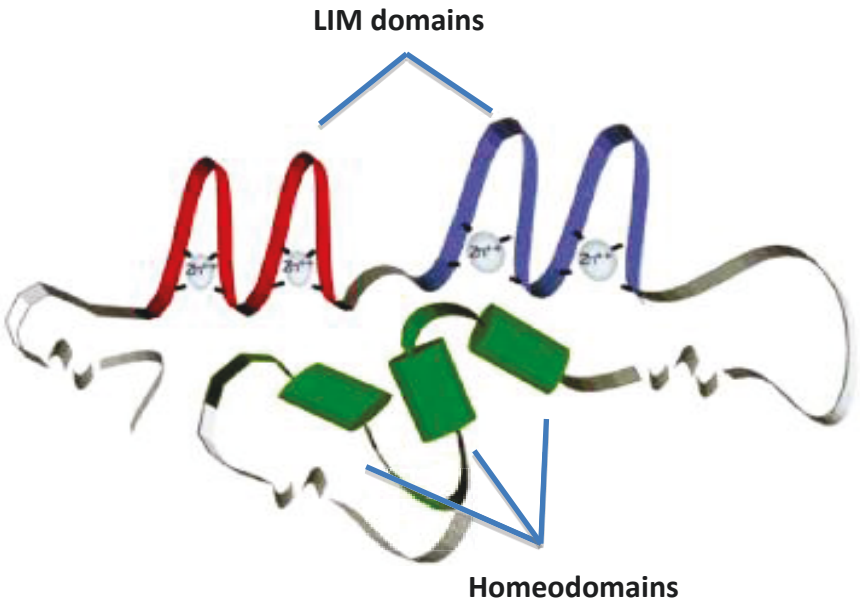

Figure 10 Hypothetical Configuration of LMX Proteins.
LIM-homeodomain family proteins contain two N-terminal zinc-binding LIM domains and three homeodomains (Gill, 1995; Hunter and Rhodes, 2005; Sadler et al., 1992).

Lmx1a/b function as transcription factors involved in the regulation of several tissues. During embryonic development, Lmx1b is expressed in: the IsO and the roof plate as sources of signalling molecules (Chizhikov 2004), the posterior hypothalamus (Asbreuk et al. 2002), the ventral midbrain DA neurons (Smidt et al. 2000), the ventral hindbrain in serotonergic neurons (Ding et al. 2003), the dorsal spinal cord (Ding 2004), the developing ears (Abelló et al. 2007), the anterior segment of the eye (Pressman et al. 2000), the skull and the limbs (Shimokawa et al. 2013; Cygan et al. 1997). Mutations in Lmx1b are associated with the nail-patella syndrome characterized by abnormalities of the arms and legs as well as kidney disease and glaucoma (Dreyer et al. 1998; Dai et al. 2009).

In embryos Lmx1a is expressed on the roof plate along the neuraxis, the basal plate of the hypothalamus, mDA neurons and choroid plexuses, cerebellar anlage, otic vesicles, notochord posterior to the hindlimb level (Millen et al. 2004; Millonig et al. 2000; Andersson, Tryggvason, Deng, Friling, et al. 2006; Failli et al. 2002).
Thus, several homologies appear in the pattern of expression of both transcription factors showing the co-operation between both transcription factors.

3.2. Role of Lmx1a/b in mDA neurons development

Lmx1a and Lmx1b, play important roles in mDA neurons development, and are cooperatively expressed in mDA neuron progenitors in the ventral midbrain during development of mDA neurons (Smidt et al. 2000; 2011; Yan, Levesque, et al. 2011). Lmx1a and Lmx1b start to be expressed in embryos at embryonic day 8.5 (E8.5) and E7.5 respectively (Smidt et al. 2000; Millen et al. 2004; Andersson, Tryggvason, Deng, Friling, et al. 2006; Guo et al. 2006). However, at E11.5, a down-regulation of Lmx1b expression in mDA progenitors is observed (Andersson, Tryggvason, Deng, Friling, et al. 2006). These transcription factors are required for the regulation of proliferation, specification, and differentiation of mDA progenitors (Figure 5) (Yan, Levesque, et al. 2011). For example, both factors activate Msx1 expression leading to a regulation of Neurog2 expression and a repression of Hes1 (Yan, Levesque, et al. 2011). This co-regulation leads to the cell cycle exit through activation of p27Kip1 expression, and Shh down regulation (Yan, Levesque, et al. 2011). Thus, the requirement of both factors is necessary for mDA progenitors to acquire neurogenic potential. Lmx1a/b cooperatively regulate neurogenesis of mDA neurons, and, by regulating the expression of floor plate genes such as Corin and Slit2 are required for the specification, then differentiation of postmitotic mDA neurons (Deng, et al. 2011; Yan, Levesque, et al. 2011). The neurogenic role of Lmx1a/b for mDA neurons has also been shown by gain-of-function studies. Indeed, inducing an ectopic expression of Lmx1a and Lmx1b in embryos or in differentiating embryonic stem cells leads the generation of mDA neurons (Andersson, Tryggvason, Deng & Friling 2006; Lin et al. 2009). In their study using chick embryos, Andersson et al. shown that Lmx1a is both required and sufficient to induce DA neuron generation in the ventral midbrain (Andersson, Tryggvason, Deng & Friling 2006). Indeed, after inducing Lmx1a expression in chick midbrain led to an ectopic proliferation of DA neurons in the ventral midbrain

region. Inversely, repressing the Lmx1a using a siRNA knockdown reduces considerably postmitotic DA neurons.

Lmx1b expression is not only restricted to the ventral midbrain but occupy a specific area reaching from the ventral to the dorsal surface of the mesencephalon (Matsunaga, Katahira & Nakamura 2002b; Alavian et al. 2007; Guo et al. 2006). Lmx1b, located in the isthmic organizer, is essential for patterning of the mid- and hindbrain by controlling the expression of secreted signalling molecules, such as FGF8, Wnt1, En1 and Pax2 (Matsunaga, Katahira & Nakamura 2002a; Alavian et al. 2007; Guo et al. 2006).

Lmx1a expression in the ventral midbrain is required and sufficient for the generation and maintenance of mDA progenitors and neurons. A compensatory role between Lmx1a and Lmx1b has been described (Yan, Levesque, et al. 2011). The inactivation of Lmx1b specifically in mDA progenitors using a ShhCre mouse model shows a normal specification and differentiation of progenitors due to a compensatory effect by Lmx1a (Yan, Levesque, et al. 2011). In the case of a deletion for Lmx1a using spontaneous mouse mutant dreher, a small decrease of mDA neurons is observed, meaning that Lmx1b cannot totally compensate for the loss of Lmx1a in DA neuron generation (Yan, Levesque, et al. 2011).

The existence of a compensatory effect for Lmx1a and Lmx1b leads us to use a double conditional mutant for Lmx1a and Lmx1b in order to understand the role of these transcription factors in mature mDA neurons.

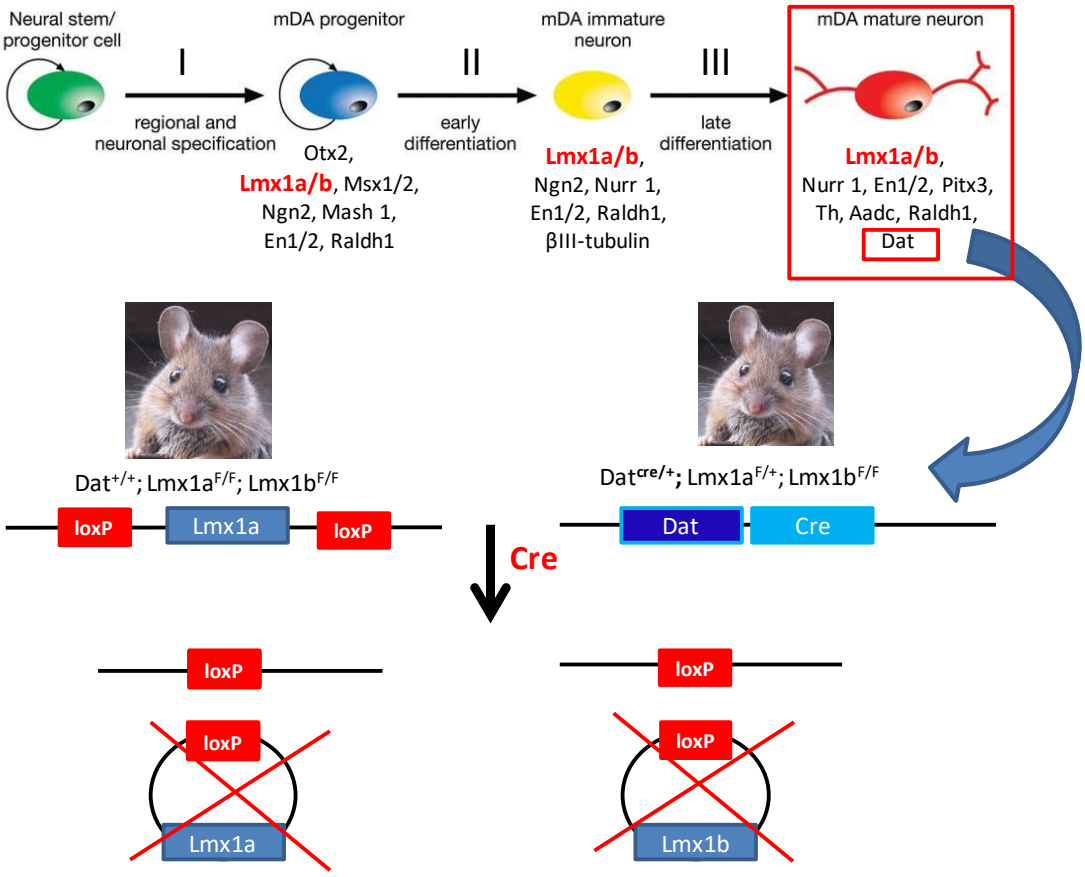

Figure 11 Schematic Representation of the Mouse Model Used for the Study of the Role of Lmx1a/b in Mature mDA Neurons.
A double conditional mutant mouse for Lmx1a and Lmx1a has been used to inactivate Lmx1a/b specifically in mature dopaminergic neurons to avoid any incidence during development. Thus, a Cre recombinase has been integrated under the regulation of the promoter Dat expressed only in mature mDA neurons and both gens Lmx1a and Lmx1b have been floxed.

Our study using double conditional mutant mice for Lmx1a and Lmx1b aims to decipher the role of Lmx1a and Lmx1b in post-mitotic dopaminergic neurons. Indeed, there is a gap in the literature between the role of Lmx1a/b in early development and in adulthood during which Lmx1b and Lmx1a expression is maintained in the SNpc and VTA (Smidt et al. 2000; Doucet-Beaupré et al. 2016).

3.3. Role of Lmx1a/b in the Adult

Two recent studies deciphered roles for Lmx1a/b in adult mice (Laguna et al. 2015; Doucet-Beaupré et al. 2016). In both studies they first used a double conditional mutant mouse model for Lmx1a and Lmx1b (Lmx1a/b cKO) inactivating these transcription factors in post-mitotic neurons for observation of the adult phenotype. However, in order to specifically assess the role of Lmx1a/b in adults they used other strategies to inactivate Lmx1a/b such as double homozygous floxed mice with a copy of the tamoxifen-inducible Cre under the control of DA gene regulatory sequences (Laguna et al. 2015), and double homozygous floxed adult mice in which a virus leading to Cre expression was injected (Doucet-Beaupré et al. 2016).

Laguna et al., shown that Lmx1b regulate the autophagic-lysosomal pathway (ALP) and maintains mDA neurons survival keeping the integrity of dopaminergic nerve terminals (Laguna et al. 2015). This study revealed the presence of abnormal enlarged nerve terminals in the striatum of adult mice in which Lmx1a/b were inactivated by tamoxifen induction. Using electron microscopy, they identified the presence of vacuoles and multilamellar autophagic-lysosomal vesicles (ALVs) in nerve terminals. The presence of ALVs suggests a dysfunction of the ALP. Moreover, in mice lacking Lmx1a/b a decrease in the number of lipofuscin granules was observed in SNpc neurons attesting an abnormal lysosomal function. All those observations are induced only to the loss of Lmx1b as they checked using three different tamoxifen inducible mouse models: cLmx1a/b^{DatCreERT2}, cLmx1b^{DatCreERT2}, and cLmx1a^{DatCreERT2}. In order to confirm this regulation of the ALP by Lmx1b, they injected the rapamycin, a mTOR inhibitor, to compensate for the lack of Lmx1b. mTOR negatively regulate the ALP in normal condition, thus removing this inhibition using rapamycin should stimulate lysosomal biogenesis. Indeed, when using rapamycin in double conditional mutant mice for Lmx1a/b, an important reduction in large boutons of the nerve terminals was observed with a rescue of the reduced striatal innervation (Laguna et al. 2015).

Doucet-Beaupré et al., showed that Lmx1a and Lmx1b regulate the expression of fundamental genes involved in mitochondrial functions (Doucet-Beaupré et al. 2016). This study shown evidences of accumulation of α-synuclein in nerve terminals in the striatum

of adult mice double conditional mutant mice for Lmx1a/b, and the same phenotype was observed in adult mice in which Lmx1a/b were inactivated by stereotaxic injection of an Adeno-Associated Virus mediated Cre. In order to find targets for Lmx1a/b which could explain the formation of α-synuclein aggregates, an experiment of laser capture microdissection was performed on double conditional mutant mice for Lmx1a/b at E15.5, followed by a mRNA sequencing. Results revealed that Lmx1a/b regulate mitochondrial-associated genes. Indeed, the inactivation of Lmx1a/b leads to a decrease of several nuclear-encoded mitochondrial subunits of the respiratory chain and of the transcription factor nuclear respiratory factor-1 (Nrf1). The regulation of Nrf1 by Lmx1a/b was confirmed by overexpression of Lmx1a/b in midbrain primary cell cultures leading to an increase in Nrf1 expression. Moreover, respirometry experiments showed a decrease in mitochondrial respiration in cells from mutant embryos for Lmx1a/b pointing to a mitochondrial dysfunction at the respiratory chain level. This dysfunction was confirmed by an increase in reactive oxygen species (ROS) production, and mitochondrial DNA damages. In order to counteract, mitochondrial dysfunctions induced by the inactivation of Lmx1a/b, a forced expression of Nrf1 was performed in cell cultures rescuing the phenotype of ROS production. The *in vivo* forced expression of Nrf1 in double floxed mice for Lmx1a/b also led to an amelioration of the phenotype harbouring fewer abnormal terminals (Doucet-Beaupré et al. 2016).

Both studies highlight the importance of Lmx1a/b for the maintenance and survival of dopaminergic neurons

3.4. Involvement of Lmx1a/b in Patterning Mechanisms

As described before, Lmx1a/b are both transcription factors known for their role in the early development of mDA neurons, and in maintenance and survival of these neurons in adulthood. Nothing is known yet about their role in post-mitotic mDA neurons, however, looking in other systems away from the dopaminergic system, other roles for Lmx1a/b were described. Indeed, in their study Scweizer et al. shown that Lmx1b plays an important role in dorso-ventral patterning of the vertebrate limb (Schweizer et al. 2004). Myogenic precursors at the origin of limb buds are guided through the dorso-ventral boundary of expression of Lmx1b within the limb (Schweizer et al. 2004). A study of gain

of function of Lmx1b shown bidorsal features of the limb and a loss of ventralization (Vogel et al. 1995). On the contrary, a study of the loss of function of Lmx1b led by Chen et al., shown a total ventralization of the limb (Chen et al. 1998). Thus, both of these experiments of gain and loss of function for Lmx1b highlighted the important role of this transcription for the regulation of the patterning mechanism of migratory pathways of myogenic cells within the limb.

In another study, briefly summarized on Figure 12, Kania et al., demonstrated the cooperative role of two LIM-homeobox genes (Lim1 and Lmx1b) leading to the choice of ventralization and dorsalization for myogenic precursors (Kania et al. 2000). In the wild type both ventral and dorsal part of the limb are specifically innervated by two types of neurons, respectively: motor neurons innervating axial muscles in the lateral motor column in the median division (LMC(m)), and innervating axial muscles in the lateral motor column in the lateral division (LMC(l)). For Lim1 mutant, motor neurons innervate axial muscles located in the lateral motor column in the lateral division (LMC(l)), neurons first project their axons into the dorsal and ventral motor nerve branches within the limb then retract (dashed green lines) from the limb, but no change for axons innervating axial muscles in the lateral motor column in the median division (LMC(m)) is observed (Kania et al. 2000). In Lmx1b mutant axons from those 2 subtypes of neurons project to the dorsal and ventral limb instead of innervating only the ventral or the dorsal part of the limb (Kania et al. 2000).

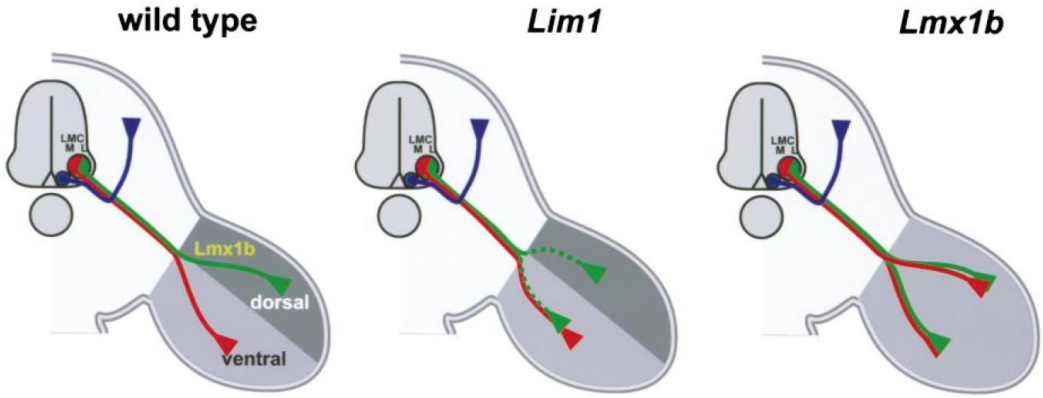

Figure 12 Co-operation of LIM-Homeodomain Proteins for Motor Axon Trajectory Regulation.
In blue is a schematic representation of motor neurons that innervate axial muscles located in the median motor column in the median division (MMC(m)); in red innervating axial muscles in the lateral motor column in the median division (LMC(m)); and in green innervating axial muscles in the lateral motor column in the lateral division (LMC(l)). Wild-type, Lim1, and Lmx1b mutant phenotypes are shown. For Lim1 mutant, LMC(l) neurons first project their axons into the dorsal and ventral motor nerve branches within the limb then retract (dashed green lines) from the limb, but no change for LMC(m) axons is observed. In Lmx1b mutant axons from those 2 subtypes of neurons project to the dorsal and ventral limb (Kania et al. 2000).

This study shows that both homeodomain proteins Lim1 and Lmx1b cooperate to monitor the trajectory of motor axons in the developing mammalian limb deciphering a role for Lmx1b in the regulation of axon guidance.

4. Axon Guidance Molecules Involved in Dopaminergic Circuit Formation

The dopaminergic circuit formation is dictated by multiple axon guidance molecules allowing the correct patterning of the circuitry. Axon guidance molecules can either have chemo repulsive or chemo attractive effect on growing axons. Axons grow towards a predefined pathway according to the sensory of the extracellular environment by the growth cone as schematized Figure 13. The growth cone cell surface harbours receptor proteins able to bind to axon guidance proteins to induce axon steering. Axon guidance molecules can be secreted proteins forming a gradient in the extracellular matrix or be membrane-bound proteins with short-range effects (Van Battum et al. 2015).

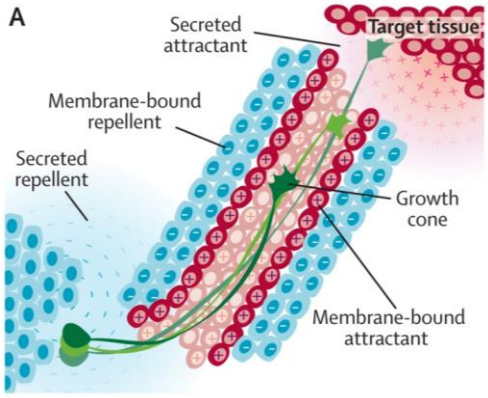

Figure 13 Principal of Axon Guidance.
Axons grow towards a predefined pathway according to the sensory of the extracellular environment by the growth cone schematized in (A). The growth cone cell surface harbours receptor proteins able to bind to axon guidance proteins to induce axon steering. Axon guidance molecules can either have chemo repulsive (blue, -) or chemo attractive (pink, +) effect on growing axons. Axon guidance molecules can be secreted proteins forming a gradient in the extracellular matrix or be membrane-bound proteins with short-range effects, modified from (Van Battum et al. 2015).

4.1. Netrin

Netrin is an axon guidance molecule with two identified receptors: deleted in colorectal cancer (DCC) and UNC-5. Netrin by binding to DCC induce attractive effect. Inversely Netrin/UNC-5 interaction induces a repulsive effect (Van Battum et al. 2015).

DCC is expressed by mDA neurons during development. At later developmental stage and until adulthood, mDA neurons express UNC-5 in ventral mesencephalic nuclei (Xu et al. 2010).

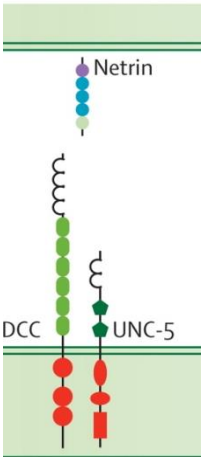

Figure 14 Netrin and its Receptors
Netrin has two identified receptors: DCC and UNC-5. Netrin by binding to DCC induce attractive effect. Inversely Netrin/UNC-5 interaction induces a repulsive effect, modified from (Van Battum et al. 2015).

4.1.1. DCC Regulates DA Axonal Projections

The expression of DCC and UNC-5 by mDA neurons let hypothesized a possible regulatory role of Netrin-1 in the guidance of mDA axons for the formation of the dopaminergic circuitry. Thus, in their study Xu et al. investigated the phenotype of newborn DCC null mice and observed several defects of the dopaminergic system (Xu et al. 2010). Indeed, a fewer number of mDA cells and DA innervation in the cerebellar cortex was observed as well as DA progenitors cell migration anomalies, and malformation in DA innervation of ventral striatum and DA ventral commissure (Xu et al. 2010). Moreover, apoptosis processes with the activation of caspase3 were detected in DA cells migrating to the wrong targets (Xu et al. 2010). In the second part, instead of deleting DCC, they used heterozygote mutant mice for DCC and still observed a loss of mDA neurons. However, no decrease in the innervation of the ventral striatum was observed pointing to an increase in DA innervation also observed by an increase in DA innervation in the prefrontal cortex of adult DCC heterozygotes mice (Xu et al. 2010). Thus, this study deciphered the role of DCC regulating DA progenitor cell migration, and mDA neurons arborizations and gave new cues about the formation of the dopaminergic circuitry.

4.1.2. Netrin-1 Topographically Patterns Midbrain DA Axons

Four years after the study of Xu et al. deciphering the role of the Netrin receptor DCC in the formation of the dopaminergic circuitry, Li et al. deciphered Netrin-1 as a key player for the topographic guidance of dopaminergic axons. Using *in vitro* experiment with a co-culture of mDA explants and COS cells secreting Netrin, Li et al. observed an attraction of DA axons towards Netrin-1. Moreover, the study of the phenotype of Netrin null and heterozygous mice showed a clear orientation of DA axons towards the striatum in heterozygous mice, which is not observed in Netrin null mice. The co-culture of DA explants with striatum explants showed a directed outgrowth of axons towards the striatum which is lost when adding an anti-DCC in the medium (Li et al. 2014). An immunohistochemistry for TH on control and null mice for Netrin-1 shows a loss of innervation in the dorsal striatum associated with the lack of Netrin-1. Netrin-1 expression in the striatum corresponds to 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 show a preference for VTA neurons for the highest Netrin-1 concentration, and SNpc neurons preferring lowest concentrations (Li et al. 2014).
This study provided a new mechanism leading to the appropriate innervation of mDA neurons according to Netrin-1 concentration.

Only few studies investigated the molecules involved in dopaminergic circuitry formation. Here Netrin-1 was shown as an important cue for the establishment of the dopaminergic system, however, as previously described by Prestoz a balance in between several axon guidance cues is necessary alongside the development of the dopaminergic system (Prestoz et al. 2012).

4.2. Plxnc1 an Important Receptor for Topographic Axon Projection of mDA Neurons?

Plexins are a large family of transmembrane glycoprotein receptors for semaphorins (Sema). These proteins are known to regulate axon guidance, cell motility and migration, and can regulate the immune response by binding to its ligand. Plexins' family encompasses four types from A to D (PlxnA1, A2, A3, A4, B1, B2, B3, C1 and D1) (Pasterkamp, Kolk, Hellemons, et al. 2007).

Class A Plxn proteins act in a neuropilin-plexin receptor complex (Timothy & Bargmann 2001; Van Battum et al. 2015). Plxnc1, does not need neuropilin to interact with its ligand (Jongbloets & Ramakers 2013). Plxnc1 is also called after its ligand: Virus-encoded semaphorin protein receptor (VESPR), but also CD232. Indeed, Plxnc1 is the receptor for semaphorin7A (Sema7A), the poxvirus (A39R protein), and herpes virus semaphorin homologs (AHVsema). Plxnc1 is constituted of an extracellular region formed by a sema, PSI, and immunoglobulin–plexin–transcription (IPT) domains followed by a transmembrane domain. Two segments brought together by a Rho-GTPase binding domain (RBD) homologous region form the intracellular domain of Plxnc1. A GAP activity towards R-Ras is observed for two segments together. Plxnc1 harbours similar features between species. The human Plxnc1 shares 85%, 82% and 71% amino acids identity with murine, bovine and opossum Plxnc1, respectively (Gene Cards, human gene database).

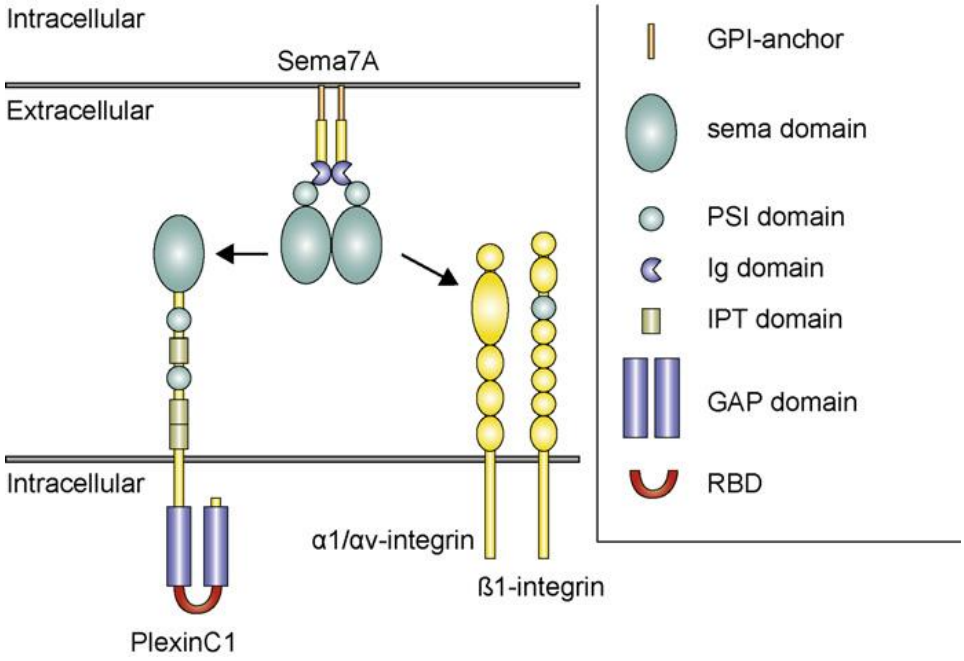

Figure 15 Plxnc1/Sema7A Interaction.
Sema7A has two receptors: Plxnc1 and β1-integrin. Sem7A is constituted of an extracellular semaphorin (sema) domain followed by a "plexins, semaphorins and integrins"` PSI domain and an immunoglobulin (Ig)-domain linked to the cell membrane *via* a glycophosphatidylinositol (GPI) anchor. Interactions between the sema domain and the Ig-domain of two Sema7A can form homodimers. One of the receptor for Sema7A, Plxnc1, is constituted of an extracellular region formed by a sema, PSI, and immunoglobulin–plexin–transcription (IPT) domains followed by a transmembrane domain. Two segments brought together by a Rho-GTPase binding domain (RBD) homologous region form the intracellular domain of Plxnc1. A GAP activity towards R-Ras is observed for two segments together. The other type of Sema7A

receptors, integrins, are composed of a α and β subunit. Until now, only the interaction of Sema7A with integrin receptors containing a α1 or αv subunit in combination with a β1 subunit has been described (Jongbloets & Ramakers 2013).

Plxnc1 is expressed in several types of tissue as neuronal and non-neuronal tissues. This protein is present as well in embryos as in adult structures. The role of Plxnc1 in neuronal development is still not fully understood. However, correlations between the expression pattern for Sema7a and Plxnc1 during rat neuronal development would suggest an implication of Plxnc1/Sema7a interaction for neuronal network organization (Pasterkamp, Kolk & Hellemons 2007b). Moreover, based on the expression of Sema7a and Plxnc1 in the prefrontal cortex and striatum in the rat, Plxnc1/Sema7a interaction seems to play a role in the formation and maintenance of midbrain dopaminergic connections (Pasterkamp, Kolk, Hellemons, et al. 2007).

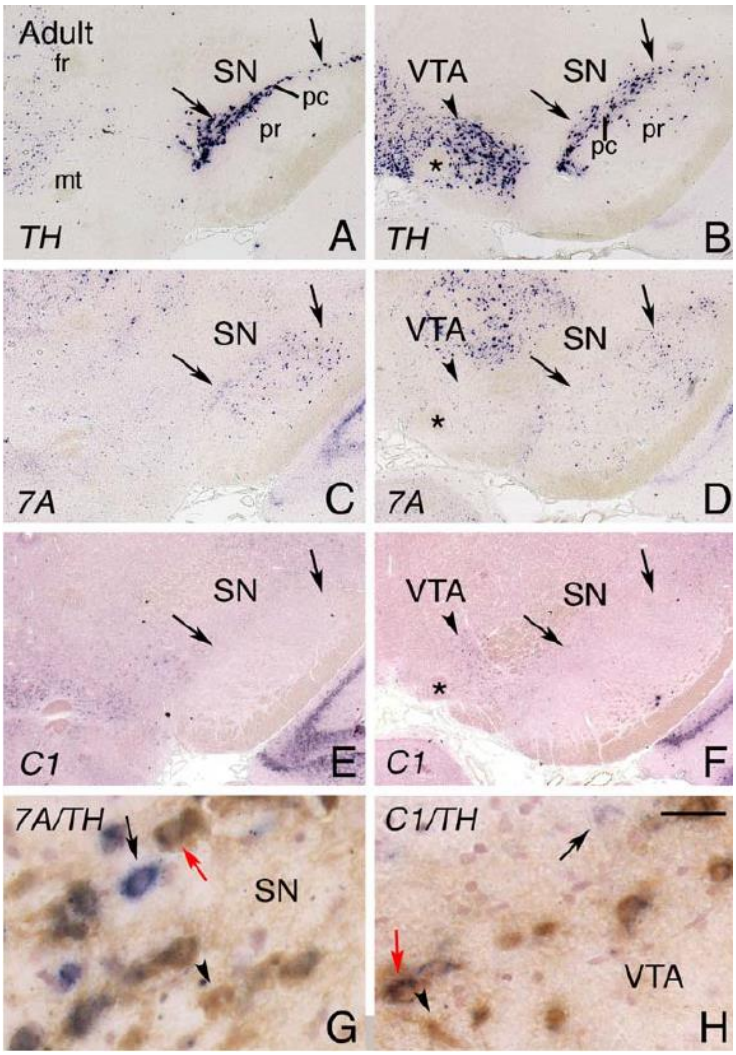

Figure 16 Plxnc1/Sema7a Expression in the Midbrain.
Sema7A (C,D,G) and Plxnc1 (E,F,H) *in situ* hybridization associated with TH (A,B,G,H) *in situ* hybridization on adult midbrain sections show the presence of Sema7A and Plxnc1 in mDA neurons. From A to F arrows point to *substantia nigra* neurons, named SN, whereas in B,D, and F arrowheads point to VTA neurons. These pictures show TH/Sema7A overlap in the SNpc, whereas a TH/Plxnc1 overlap is only seen in the central part of the VTA, but no Plxnc1 expression is detected in the SNpc; fr, fasciculus retroflexus; mt, mammillothalamic tract; pc, pars compacta; pr, pars reticulare. Scale bar 250 µm (A-F), and 65 µm (G, H) (Pasterkamp, Kolk, Hellemons, et al. 2007).

4.3. Sema7a a New Key Player for Topographic Axon Projection of mDA Neurons?

The semaphorin family is composed of more than 20 members among which eight classes of Semaphorins (Sema) have been characterized. Sema1-3 are secreted semaphorins; Sema4-7 are membrane associated semaphorins and the class 8 are virally encoded semaphorins (Pasterkamp 2012; Kumanogoh & Kikutani 2013).

Semaphorin 7a (Sema 7A, CD108) is a membrane-bound semaphorin that associates with cell membranes *via* a glycosylphosphatidylinositol (GPI) linkage: GPI membrane anchor. As described in Figure 8 by Jongbloets et al., Sema7A has two receptors: Plxnc1 and β1-integrin detailed in the previous section. Sema7A is constituted of an extracellular semaphorin (sema) domain followed by a "plexins, semaphorins and integrins"` PSI domain and an immunoglobulin (Ig)-domain. Interactions between the sema domain and the Ig-domain of two Sema7A can form homodimers (Jongbloets & Ramakers 2013).

Sema7a/β1-integrin interaction has been well studied for understanding their role in immune-modulatory responses. Actually, Sema7A promotes axon outgrowth through integrins and MAPKs. This interaction Sema7a/ β1-integrin could appear as a signalling mechanism involved in the development of the nervous and immune systems. However, the role of the interaction Plxnc1/Sema7a remains unclear in nervous system development (Pasterkamp et al. 2003).

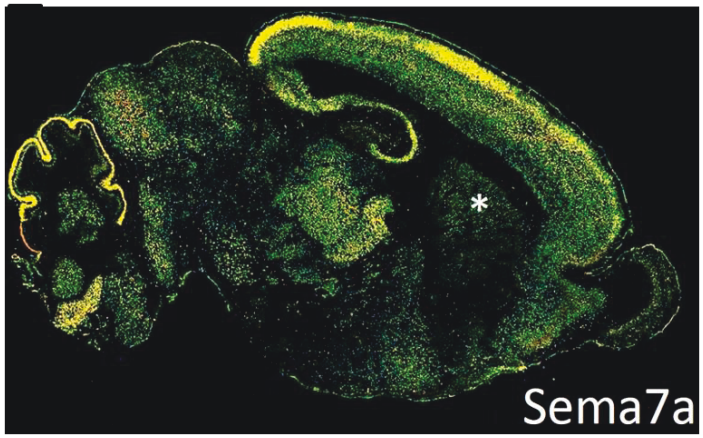

Figure 17 Sema7a Repartition in the Adultl Mouse Brain.
Sema7a RNA expression on a sagittal section of the mouse brain. An increase of Sema7A expression is observed in the dorsal striatum (asterisk) compared to the ventral striatum (Allen Brain Atlas).

Sema7A is an important molecule recognized for axon guidance. However, its role in the dopaminergic system has not been determined yet. RNA expression studies have shown a highest Sema7A expression in the dorsal striatum compared to the ventral striatum (Allen brain Atlas). Thus, by correlation with evidences in other systems, we could hypothesize a role of Sema7A in the segregation of the nigrostriatal and mesolimbic systems.

5. Parkinson's Disease

Age-related diseases are among the most widespread syndromes in the population. Most of them are neurodegenerative diseases such as Alzheimer's or Parkinson's diseases. Parkinson's disease (PD) is a neurodegenerative disorder characterized by a loss of mDA neurons in the Snpc. During the disease onset, dopamine neurons from SNc progressively degenerate, leading to a drastic reduction of dopamine release in the dorsolateral striatum characterized by bradykinesia, rigidity, resting tremor, gait disturbances and postural instability (Lang & Lozano 1998; Lees et al. 2009; Ang 2006; Dunnett & Björklund 1999).

5.1. Characterization of the Disease

First described in 1817 by James Parkinson in "An Essay on the Shaking Palsy", the Parkinson's disease (PD) was characterized by: "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace." (Parkinson 2002). Almost two hundred years later, these symptoms are still considered as the "cardinal symptoms of Parkinson's disease", but other non-motor symptoms need to be added to this list like dementia, psychosis, depression and apathy (Coelho & Ferreira 2012). Those symptoms would be more related to the degeneration of other types of neurons like: locus coeruleus noradrenergic neurons (Hassler 1938), basal forebrain cholinergic neurons (Tagliavini et al. 1984), peptidergic neurons (Agid et al. 1986) and serotonin neurons (Calabresi et al. 2013; Halliday et al. 2011; Chaudhuri & Schapira 2009; Chaudhuri et al. 2006). From this clinical definition, and through several studies, it has been possible to elaborate a definition on a molecular basis. PD is a neurodegenerative disease occurring with a decrease in the number of mDA SNpc neurons as shown on Figure 18.

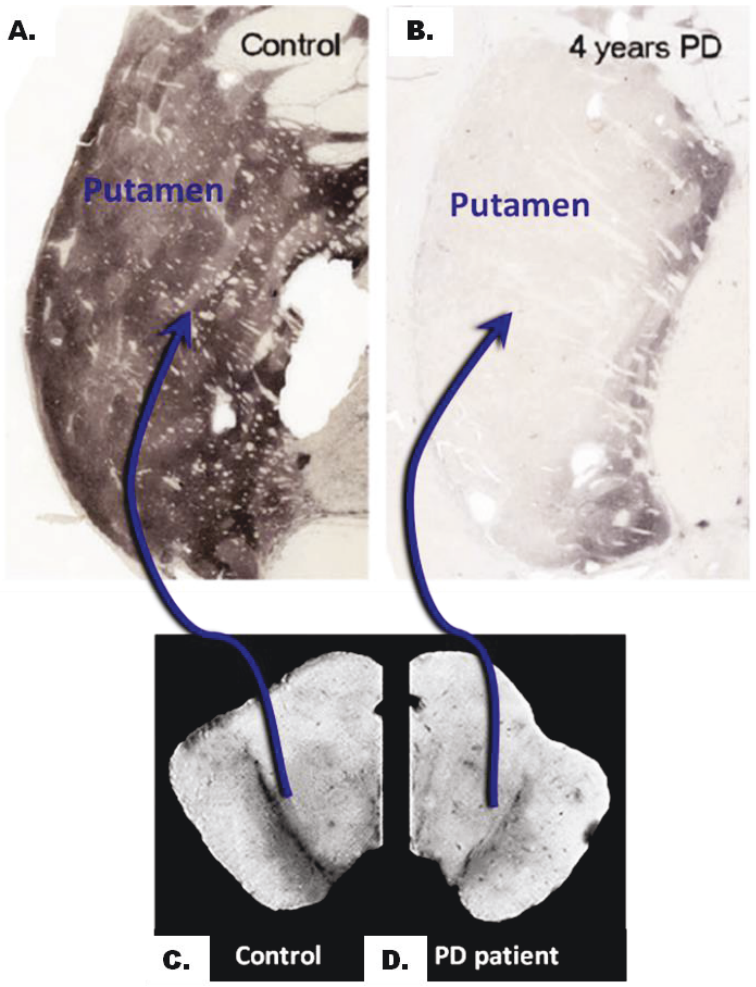

Figure 18 Characterization of PD in Human Brain Sections.
During the disease onset, dopamine neurons from SNc progressively degenerate, leading to a drastic reduction of dopamine release in the dorsolateral striatum. A TH staining was performed from A to D. In A is shown putamen innervation in a control; and in B a loss of innervation of the putamen is observed in a patient diagnosed for PD for 4 years. C and D show mDA neurons of the SNpc. In D the PD patient shows a loss of SNpc neurons (modified from (Alexi et al. 2000; Kordower et al. 2013)).

As mentioned earlier, multiple factors are involved in dopamine synthesis. Figure 19 represents factors involved in different pathways candidates for the neurodegeneration of dopaminergic neurons from SNpc (Venda et al. 2010). Multiple *scenari*os can be taken into account for cell death in SNpc (Venda et al. 2010). For example, whether the amount of dopamine transporter (DAT) increases too much compared to the one of vesicular monoamine transporter 2 (VMAT2), an accumulation of dopamine into the neuron is observed (Miller et al. 1999; Uhl 1998). This accumulation will lead to dopaminergic neurons death because of too high a concentration of toxic reactive oxygen species (ROS)

(Miller et al. 1999; Uhl 1998). Dopamine concentration can also increase because of too important a Ca2+ entry into the neuron (Mosharov et al. 2009). Furthermore, Ca2+ activates the enzyme involved in dopamine synthesis (Mosharov et al. 2009). The last scenario proposed on this scheme is linked to α-synuclein dysfunction (Conway 2001; Volles & Lansbury 2003). This dysfunction would lead to a failure in vesicle docking and recycling (Conway 2001; Volles & Lansbury 2003). So newly synthetized DA could not be exported out of the neurons, leading once more in an increase in cytosolic DA concentration (DAcyt) (Conway 2001; Volles & Lansbury 2003).

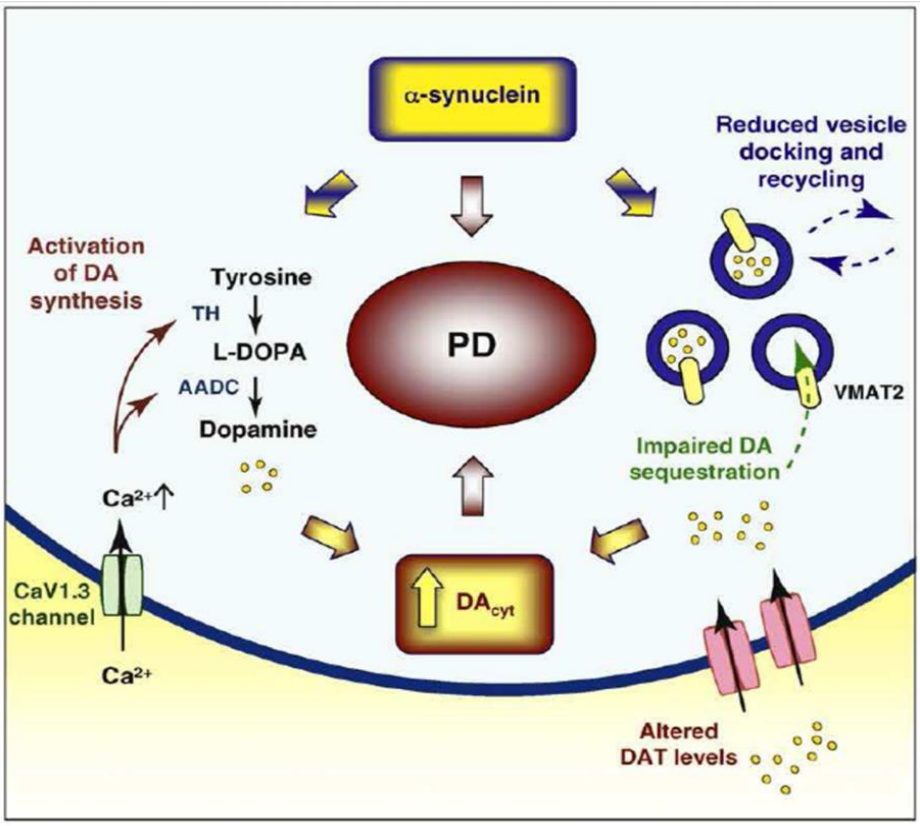

Figure 19 Schematic Representation of Pathways Involved in PD.
This scheme represents factors involved in different pathways candidates for the neurodegeneration of dopaminergic neurons from substantia nigra pars compacta (SNc). Multiple *scenari* can be taken into account for cell death in SNc. For example, whether the amount of dopamine transporter (DAT) increases a lot compared to the one of vesicular monoamine transporter 2 (VMAT2), an accumulation of dopamine into the neuron is observed. This accumulation will lead to dopaminergic neurons death because of a too high concentration of toxic reactive species. Dopamine concentration can also increases because of a too important Ca2+ entry into the neuron. In fact, Ca2+ activates the enzyme involved in dopamine synthesis. The last *scenario* proposed on this scheme, is linked to α-synuclein dysfunction. This dysfunction would lead to a failure in vesicle docking and recycling. So newly synthetized DA could not be exported out of the neurons, leading once more in an increase in cytosolic DA concentration (DAcyt) (Venda et al. 2010).

Recent studies investigated a theory based on the elevated bioenergetic requirements for the neurons leading to highest vulnerability of the cells (Pacelli et al. 2015). In their study, Pacelli et al. showed that SNpc neurons have a higher basal rate of mitochondrial oxidative phosphorylation (OXPHOS), an elevated level of basal oxidative stress, a smaller reserve capacity, and a more complex axonal arborization (Pacelli et al. 2015). A diminution of this axonal arborization was observed using Sema7a as well as a reduction in mitochondrial OXPHOS (Pacelli et al. 2015). An amelioration in the resistance of SNpc neurons to the neurotoxic agents MPP+ (1-methyl-4-phenylpyridinium) and rotenone was also observed (Pacelli et al. 2015). Thus this study highlights the fundamental role of mitochondrial regulation for the survival of SNpc neurons.
Moreover, Doucet-Beaupré et al., showed that the regulation of mitochondrial genes by Lmx1a/b is required for the maintenance and survival of mDA neurons (Doucet-Beaupré et al. 2016). Indeed, in this study mitochondrial dysfunctions are observed following the inactivation of Lmx1a/b. Defects in the respiratory chain activity leads to an increase of oxidative stress, and mitochondrial DNA damages (Doucet-Beaupré et al. 2016). Moreover, these abnormalities would lead to the principal axonal pathology in PD which are α-synuclein inclusions which would precede neuronal loss (Doucet-Beaupré et al. 2016). Interestingly, these data indicate that mitochondrial defect preceed protein aggregation.

In human, multiple genetic mutations are associated with PD. Indeed, mutations in the α-synuclein (SNCA) gene can lead to the development of PD (Polymeropoulos 1997). In this case, the protein aggregation is directly associated to a defective gene and not induced by mitochondrial dysfunctions (Polymeropoulos 1997). The onset of the disease can also be triggered by mutations in the putative kinase 1 (Pink1) (Marini et al. 2003). This mutation characterized an early hereditary form of PD (Valente 2004). A lot of other targets were assessed through the years has being involved in PD such as leucin-rich repat kinase 2 (LRRK2), Parkinson's protein 2 (Park2), Park7, protein deglycase1 (DJ-1) (Bandmann 2004; Brooks et al. 2009; Stephenson 2002).

5.2. Parkinson's Disease Models

No real model exists for Parkinson disease. The most common model used today is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, a prodrug to the neurotoxin MPP+, or the 6-hydroxydopamine (6-OHDA) model. Those drugs can be used as well in mice, rats, as in monkeys, and lead by injection in the striatum, MFB, or directly to the SNpc, to mimic the degeneration characterizing PD.

However, other models exist like transgenic mice expressing SNCA or LRRK2, or losing Pink1 and Park2, all genes involved in genetic forms of PD. Unfortunately, these models do not exhibit neurodegeneration of mDA neurons making them not suitable for a correct mimicking of PD (Westerlund et al. 2009; Bifsha et al. 2014; Y. Li et al. 2005).

Nevertheless, two transgenic mouse models seem to mimic PD in a most complete manner than other transgenic models before: DJ-1-/- mouse model, and $Dat^{cre/+}Lmx1a^{F/F}Lmx1b^{F/F}$ mouse model.

In a mouse and a rat model in which the protein deglycase DJ-1, also known as Parkinson's disease protein 7 (Park7), has been suppressed, DJ-1-/-, a neurodegeneration has been observed showing an interesting role of DJ-1 in the regulation of mDA neurons (Dave et al. 2014; Rousseaux et al. 2012).

Another transgenic model inactivating Lmx1a/b in mature mDA neurons studied in Dr. Lévesque's laboratory appear as a good candidate for a PD model. Indeed, in these mice, important transcription factors for mDA neurons maintenance are inactivated leading to the progressive degeneration of SNpc neurons around 30 days postnatal (Doucet-Beaupré et al. 2016).

From these studies, DJ-1, and Lmx1a/b appear to be primordial players in the maintenance of mDa neurons but moreover, these animals develop a very similar histolpathological feature PD such as SNCA inclusions, elevated ROS level, mitochondrial dysfunction and progressive degeneration of mDA neurons.

5.3. Treatments and Therapies for Parkinson's Disease

Nowadays, no cure exists for this pathology, and only symptomatic treatments mostly pharmalogical are used (Martin & Wieler 2003).

5.3.1. L-DOPA

The L-DOPA treatment represents the most common for PD. The mechanism of the drug, called levodopa, is based on compensating the loss of dopamine induced by SNpc neurodegeneration by providing a dopamine precursor, the L-Dopa (Brichta & Greengard 2016; Brichta et al. 2013).

However, a loss of efficacy of the L-Dopa treatment can be observed, and associated with motor complications like dyskinesias (Stern 2004; Weiner 2004).

5.3.1. Other Pharmacological Agents

Other pharmacological agents using different mechanisms of action but still targeting the dopaminergic system can be used like the catechol-o methyl-transferase (COMT) which is a dopamine agonist; the monoamine oxidase type B (MAOB) inhibitors.

Non-dopaminergic agents can also be administered like antidepressants or cholinesterase inhibitors for dementia (Connolly & Lang 2014).

However, as described with the L-Dopa treatment, the use of dopamine agonists can cause behavioural alterations (Jankovic & Aguilar 2008).

5.3.2. Deep Brain Stimulation

The deep brain stimulation (DBS) is invasive surgery used only in a few numbers of patients who need to answer drastic conditions to be considered as good candidates. This surgery is performed only when medication connot control motor symptoms anymore. The DBS targets the thalamus, the subthalamic nucleus or the globus pallidus, but no mechanism of action is known yet. A proposition of explanation would be the activation of inhibitory neurons desynchronizing tremorogenic pacemakers by blocking depolarization (Jankovic & Poewe 2012).

Although, all of these treatments help control the most visible symptoms of PD, they do not impact the disease progression. In order to reverse mDA neurons loss a cell-based replacement therapy appears like a treatment of choice to restore the innervation of the striatum (Meyer et al. 2010; Arenas et al. 2015; Gao et al. 2013).

6. Cell Replacement Therapy

The cell replacement therapy, consisting in introducing newly generated cells to compensate for a lack of functional cells, is confronted by several challenges. One of them is to choose the localization for cell implantation (Meyer et al. 2010). Indeed, in the case of PD, cells can be implanted in the striatum, where the loss of innervation is observed, or in the midbrain to replace mDA neurons from SNpc which degenerated during PD progression.

Another challenge concerns the ability to choose the correct type of cells for transplantation, to allow a functional integration of the newly generated cells avoiding any tumorigenic effect, or other side-effects (Meyer et al. 2010; Gao et al. 2013). Cell replacement therapy has been used since about thirty years for treatment of leukemia using human umbilical cord blood cells (UCB), and for bone marrow transplantation (Meyer et al. 2010). Thus this kind of therapy has already been used for several years showing patients' symptoms improvement. However, different attempts were performed to use cell replacement therapy for neurodegenerative disorders, but were not conclusive yet. Today, the challenge is to determine the best source of stem cells allowing the restoration of the degenerated cells in neurodegenerative disorders (Meyer et al. 2010).

6.1. Sources of Stem Cells for Cell Replacement Therapy in PD

Stem cells are used for cell replacement therapies, because they are undifferentiated cells able to differentiate into the cell type of interest. These cells can divide to give an enough number of cells for transplantation. In the human organism, three types of stem cells have been characterized: embryonic stem cells (ES) (very early, at blastocyst stage), fetal neural stem cells (NSCs, very early 6 to 9 weeks post-fertilization (Milosevic et al. 2007; Storch et al. 2001; Wegner et al. 2008; Schwarz et al. 2006; Milosevic et al. 2006; Svendsen et al. 1997). Adult stem cells include different subtypes of cells like adult NSCs, and adult multipotent stem cells. Thus, as described in Figure 15, different sources of stem cells could be considered for cell replacement therapy in PD (Meyer et al. 2010). However, stem cells do not represent the only potential cells for cell-based replacement therapy. Indeed, progresses in cell engineering allow reprogramming adult and fibroblast (Zou et al. 2014) cells to differentiate them in cells of interest. As previously mentioned a

lot of cell types seem to be potential candidates for stem cell replacement therapy in PD. Today, the challenge is to determine which type of cells would be the best for a treatment for PD.

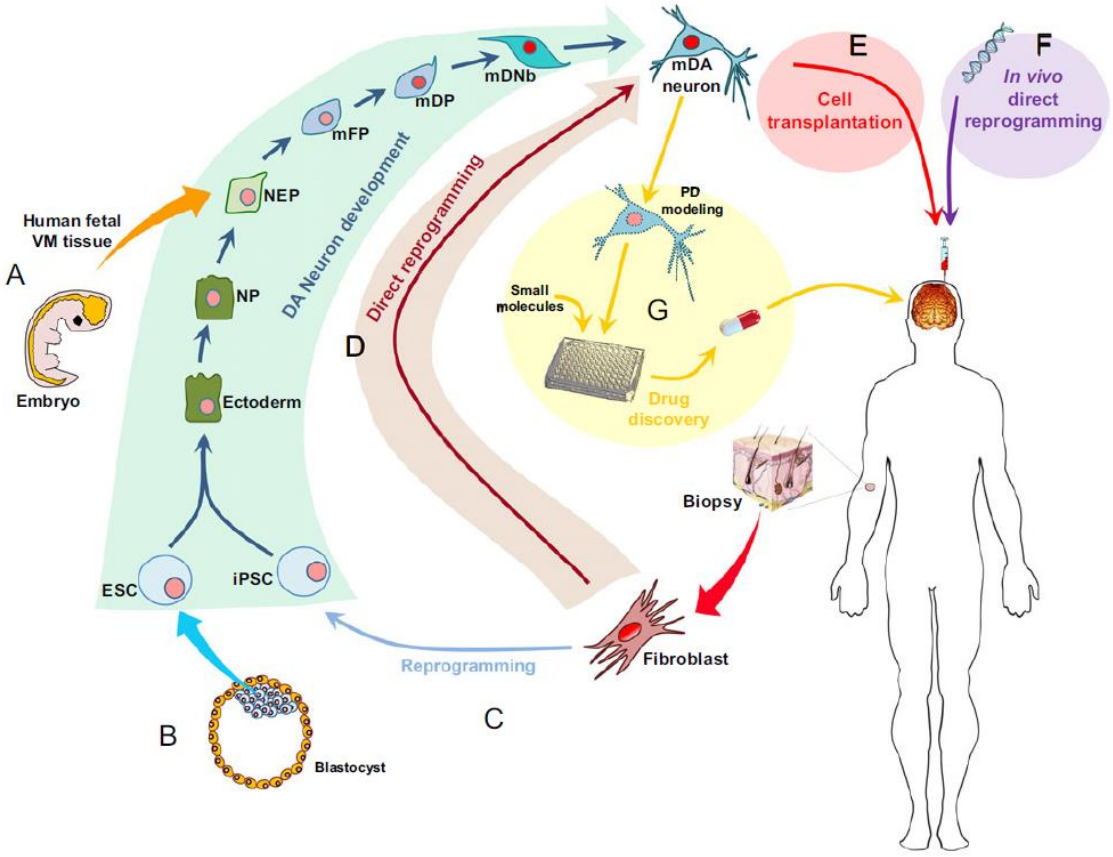

Figure 20 Sources of Stem Cells for Cell Replacement Therapy in PD.
Three different strategies are proposed to obtain mDA neurons: (A) use human fetal ventral midbrain (VM) tissue, (B) mDA neurons derived from human pluripotent stem cells (PSC) or blastocysts (ESCs), (C) mDA neurons derived from human fibroblasts (iPSC). This scheme encompasses some of stem cells sources for cell replacement therapy in PD. (Arenas et al. 2015).

6.1.1. ES Cells

ES cells were the first cells used for cell-replacement therapy because of their properties of self-renwing, pluripotency, and capacity to differentiate in a specific cell type in high quantity (Meyer et al. 2010; Amit et al. 2000; Evans & Kaufman 1981; Hynes & Rosenthal 2000; Bradley et al. 1984). ES cells were obtained by isolation from the inner mass of a blastocyst. Moreover, autologous embryonic stem cells were generated through therapeutic cloning (Amit et al. 2000; Evans & Kaufman 1981; Hynes & Rosenthal 2000;

Bradley et al. 1984). In 2002, Kim and co-workers, reported an efficient differentiation of ES cells into functional DA neurons (Meyer et al. 2010). Unfortunately, evidences have shown a poor rate of cell survival after intrastriatal transplantation, and tumorigenic effects were observed (L. M. Björklund & Sánchez 2002; Lindvall & A. Björklund 1978; Brüstle et al. 1997; Sonntag et al. 2007; Roy et al. 2006; Deacon et al. 1998). Despite their promising discovery, for now, ES cells do not appear as a viable candidate for cell replacement therapy in PD anymore. Indeed, the only way to use them would be managing to counteract the tumorigenic effect induced by transplantation of ES cells. Moreover, ethical issues were raised concerning the use of material able to lead to a human being. According to the country laws are very strict concerning the use of human embryos for research, restricting the access considerably to ES cells. However, today other cells with similar potential, less ethical issues, and less known side-effects seem to represent best candidates.

6.1.2. Neural Stem Cells (NSCs)

NSCs are multipotent cells having the capacity to self-renew, but unlike ES cells, NSCs do not seem to induce tumorigenicity (Arsenijevic et al. 2001; Ling et al. 1998; Kilpatrick & Bartlett 1993; Svendsen et al. 1999). NSCs can differentiate into the main cell-types of the brain: neurons, oligodendrocytes, and astrocytes (Arsenijevic et al. 2001; Ling et al. 1998; Kilpatrick & Bartlett 1993; Svendsen et al. 1999). A real advantage of these cells is that they are already committed to become neural cells (Meyer et al. 2010), thus their differentiation in mDA neurons requires less modifications than using adult multipotent stem cells or iPSCs for example (Meyer et al. 2010).

6.1.2.1. Fetal NSCs

Fetal NSCs, for midbrain studies, were isolated in rodent embryos at embryonic days (E) 14 to E15 (Ling et al. 1998; Potter et al. 1999; Smith & Bagga 2003; Storch et al. 2003), and at 6–9 weeks post-fertilization in human (Milosevic et al. 2007; Storch et al. 2001; Wegner et al. 2008; Schwarz et al. 2006; Milosevic et al. 2006; Svendsen et al. 1997). Grown in good condition, 3% oxygen levels and Nurr1 transcription factors, fetal NSCs were able to expand over a long-term period (Kim et al. 2003; Storch et al. 2001; Maciaczyk et al. 2008; Zhang et al. 2005). Thus, fetal NSCs are multipotent stem cells, which seem to induce only mild immunoreactions, but no tumour formation (Meyer et al.

2010). In the dish, fetal NSCs appear as a good candidate for cell replacement therapy. However, studies have shown that implantation of fetal NSCs led to a poor survival of grafted neurons (Galvin & Jones 2006). Moreover, the use of fetal NSCs raises an ethical issue, as ES cells did, because these cells are isolated from human fetal brain tissue. Another drawback of these cells is that they are allogenetic cells, which could induce high risks of immunological rejection. Thus, even if these cells seem to have a potential for cell replacement therapy, they cannot represent a viable treatment.

6.1.2.2. Adult NSCs

Unlike fetal NSCs, adult NSCs are autologous cells limiting risks of reject by the organism. These cells are present in two regions of the adult mammalian brain known as neurogenic regions: the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles (Ehninger & Kempermann 2008; Gould et al. 1999; Lois & Alvarez 1993; Siebzehnrubl & Blumcke 2008). These multipotent cells are able to differentiate into neural cells. Indeed, one group managed to perform mDA neurons from precursors from the SVZ and white matter using cells overexpressing Nurr1 (Shim et al. 2007). Moreover, Kim and co-workers manage to differentiate adult NSCs using only one of the four factors, Oct4, Sox2, Klf4, and c-Myc, commonly used to induce pluripotency in mouse and human fibroblasts: Oct-4. This study demonstrated that Oct-4 is required and sufficient for inducing pluripotency in adult NSCs (Kim et al. 2009). These multipotent autologous neural stem cells appear like a very interesting candidate for cell replacement therapy in PD because of their original genetic information inducing their differentiation into cells in the brain, and their capacity to integrate correctly the region of interest.

6.1.3. Adult Multipotent Stem Cells

Adult multipotent stem cells, as iPSCs and adult NSCs, have the advantage to be patient-specific (Meyer et al. 2010). One advantage to use these stem cells is that several sources of these cells are available like: umbilical cord blood, bone marrow, adipose-derived, placental and amniotic fluid (Meyer et al. 2010).

Hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs) can be easily isolated from the human umbilical cord blood (UCB), and represent an important pool of cells for cell transplantation (Meyer et al. 2010). These UCB stem cells are able to

differentiate into neurons using pro-neurogenic factors (Zigova et al. 2002). Studies revealed that injection of UCB stem cells, differentiated in mDA neurons, led to an improvement for Parkinsonian rats symptoms (Fu et al. 2006; Willing et al. 2003; Lu et al. 2002; Garbuzova 2003).

Bone marrow stromal cells, also known as mesenchymal stem cells (MSCs), represent another source of adult multipotent stem cells (Jiang et al. 2002). These cells are able to become a source of neural stem cells (NSCs) by bringing the good factors for their conversion (Hermann, Liebau, et al. 2006). Usually lineage restricted, a study from Dezawa and colleagues demonstrated the possibility to differentiate these cells into neuronal-like-cells by the administration of different trophic factors (Dezawa et al. 2004). The same team performed dopaminergic neurons by overexpressing Notch intracellular domain in these cells, and administrating GDNF (Dezawa et al. 2004). The transplantation of these cells into a PD model seems to show protective or regenerative effects on dopaminergic cells (Jin et al. 2008; Levy & Bahat 2008; Bouchez et al. 2008).

Thus, adult multipotent stem cells represent an interesting pool of stem cells for differentiation into mDA neurons. Indeed, the encouraging results obtained in different studies with improvement for motor symptoms in rodents, and their easy supply appear as evidences of the potential of these cells. However, even if protocols for differentiation of these cells into functional neuroectodermal cells were performed without any genetic modification, adult stem cells do not show a high rate of integration after transplantation (Woodbury et al. 2000; Dezawa et al. 2004; Hermann et al. 2004; Tondreau & Dejeneffe 2008; Hermann, Liebau, et al. 2006; Hermann, Maisel, et al. 2006) making it difficult to use them for cell replacement therapy in PD.

6.1.4. Induced Pluripotent Stem Cells (iPSCs)

First generated from adult mouse fibroblast by Yamanaka's group in 2006, then from human dermal fibroblasts in 2007, iPSCs are a recent source of stem cells (Takahashi & Yamanaka 2006; Takahashi et al. 2007). These cells have the capacity to self-renew and can be differentiated into various cell types (Gao et al. 2013). iPSCs can be obtained by therapeutic cloning. This therapeutic cloning consists in reprogramming adult cells, like fibroblasts for example, into ES cells by nuclear transfer to oocytes (Meyer et al. 2010).

iPSCs can also be obtained by fusion of adult cells with ES cells (Meyer et al. 2010). Added to these two techniques for reprogramming cells into pluripotent cells, another method has been developed using a limited set of transgenes including Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi & Yamanaka 2006). These three different techniques for iPSCs production lead to an enough quantity to enable disease investigation and drug development (Gao et al. 2013). In 2008, Wernig and co-workers, managed to differentiate iPSCs into functional mDA neurons. Indeed, the good function of these newly generated mDA neurons was observed by an improvement of Parkinsonian symptoms after implantation of these cells in the striatum of rats (Wernig et al. 2008). iPSCs appear like a very interesting choice for cell replacement therapy because of the possibility to use patient-specific cells to generate new ones decreasing a possible immunological rejection (Takahashi & Yamanaka 2006).

In order to avoid the tumorigenic effect observed with iPSCs replacement therapy, improvements in techniques to generate them were performed. Actually, human somatic cells can be reverted into pluripotent cells using another set of transgenes including Oct4, Sox2, Nanog, and Lin28, without c-Myc possible factor in the induction of tumorigenicity (Yu et al. 2007). These transgenes can be delivered using different methods: retroviral transduction, plasmid transfection, direct reprogramming factor delivery, and mRNA transfection (Gao et al. 2013). Having the choice between these four methods of transgenes delivery is interesting, because even if retroviral transduction is a very effective way to deliver transgenes, retroviral vectors might induce tumorigenicity by integration of the viral DNA into the genome of transduced cells (Okita et al. 2008). Thus, three other approaches which do not involve the same problems can be used.

Moreover, in a publication of Doi and co-workers from March 2014, a new technique to avoid tumorigenic content or inappropriate cell transplantation when using iPSC-derived donor cells for cell replacement therapy has been presented. They have shown an efficient way to isolate specifically human iPSC-derived DA progenitor cells by cell sorting using a floor plate marker, CORIN (Doi et al. 2014). The transplantation of these iPSCS into 6-OHDA-lesioned rats, has shown a survival of CORIN+ cells, and their differentiation into

midbrain DA neurons *in vivo*. These “pure” iPSCs transplantation led to a significant improvement of the motor behaviour, without tumour formation (Doi et al. 2014).

In an ethical point of view, the use of iPSCs is appreciated because human embryos are not involved (Gao et al. 2013; Green 2007). Moreover, studies demonstrated that iPSCs and ES cells properties as pluripotency and self-renewal are similar (Boué et al. 2010). iPSCs also represent an interesting material to study neurodegenerative diseases. Indeed, patient cells can be harvested to be converted into iPSCs, and used to study the disease. Even if these cells acquired ES cells characteristics, they still carry the genetic information of the patient (Inoue 2010), which is of real interest for testing different treatment strategies. However, in a recent study, April 2014, comparing iPSC-derived DA neurons, and primary mDA neurons epigenetic features, Roessler and co-workers observed differences in global gene expression and DNA methylation between both types of cells. Such variances might affect unambiguous long-term functionality and hamper the potential of iPSC-derived DA neurons for *in vitro* disease modelling or cell-based therapy (Roessler et al. 2014). This study by exhibiting differences between original mDA neurons and newly generated ones warns on the possible limitation of these iPSCs for cell replacement therapy. Nonetheless, these variations could be overridden by genetically modifying iPSCs by integration of transgenes inducing a higher expression of the genes characterized as less expressed in the newly generated cells. Even if we still need to increase our knowledge about the features of iPSCs cells, they remain a very interesting cell type for cell replacement therapy in PD.

6.1.5. Fibroblast a New Source of Cells for Cell Replacement Therapy

The current lack of understanding of the reprogramming process generating hiPSCs makes it difficult to substitute them to hESCs for a treatment strategy for the moment (Doi et al. 2014). Thus, other strategies have been developed to produce cell types of interest for drug screening and other clinical applications. These strategies would use an easily accessible source of cells like skin fibroblasts to convert them directly into DA cells, bypassing the pluripotent stage (Doi et al. 2014). In 2011, Caiazzo et al. managed to generate DA neurons from mouse and human fibroblasts using the following transcription factors Lmx1a, Nurr1, and Mash1 (Caiazzo et al. 2011). Three years later, Mitchell and

co-workers, using only the Oct4 factor, manage to perform a direct conversion process of primary adult human fibroblasts (hFib) to neural progenitor cells (NPC). These NPCs present the same features as hNPCs. They are able to proliferate, express neural stem/progenitor markers, and to differentiate into all the three major subtypes of neural cells (Mitchell et al. 2014).

6.2. An Alternative to New Cells Implantation: Endogenous Regeneration

Endogenous regeneration is a natural mechanism observed in the adult mammalian brain (Reynolds & Weiss 1992; Palmer et al. 1999; Palmer et al. 1997; Palmer et al. 1995; Lois & Alvarez 1993; Rietze et al. 2001). In the adult, the subependymal layer represents a pool of cells able to divide, migrate, and differentiate into neurons near to the olfactory bulb (Altman 1969; Lois & Alvarez 1994). For therapeutic use, the endogenous regeneration consists in the use of pharmacological manipulations to induce the proliferation and differentiation of endogenous stem cells into mDA neurons (Meyer et al. 2010). In some cases of brain insults, a rescue mechanism, by the organism itself, has been observed (Meyer et al. 2010). This mechanism was characterized by the generation of new neurons in different parts of the brain: striatum (Jin et al. 2008), cortex (Magavi et al. 2000; W. Jiang et al. 2001), and Snpc (Zhao et al. 2003). The regeneration of the nigrostriatal pathway triggered by endogenous factors appears as a powerful therapeutic avenue to investigate (Meyer et al. 2010). One advantage of this kind of technique is that if we manage to find the right factors inducing the formation of new DA neurons in the midbrain it would be less invasive than implanting new cells (Meyer et al. 2010). However, this technique would not allow us to perform a screening to find therapeutic agents because the lack of availability in disease material (Gao et al. 2013).

7. Aim of Project

The aim of this project is to decipher the role of Lmx1a/b in post-mitotic mDA neurons. What is the role of Lmx1a/b in mature dopaminergic neurons? Are Lmx1a/b regulating axon guidance in dopaminergic system? What incidence has Lmx1a/b expression on the dopaminergic system formation? By characterizing the phenotype of double conditional mutant mice for Lmx1a/b, we identified some molecules involved in axon guidance. Among them, Plxnc1 appeared to be an interesting target to investigate due to the high increase of the expression of this molecule in mice in which Lmx1a/b are inactivated in post-mitotic neurons. Deciphering the mechanistic behind the establishment of the dopaminergic circuitry is a real interest in cell replacement therapy. Indeed, knowing the cues involved in the elaboration of the dopaminergic system would help obtain specific mDA neurons SNpc-like able to compensate for the degenerated neurons in PD. Indeed, recent publications shown that cell based replacement therapy for PD still needs improvements in the specificity of newly injected neurons.

Several studies depicted the role of Lmx1a/b in the early development of mDA neurons. Moreover, recent studies deciphered the role of Lmx1a/b in mDa neurons in adulthood. Thus, we need to understand the role of Lmx1a/b in between these stages, and if they have any. The first hypothesis of this project is that Lmx1a/b play a regulatory role in mature mDA neurons. Thus we characterized the phenotype of Lmx1a/b in mature mDA neurons using double conditional mutant mice for Lmx1a and Lmx1b. We observed a defect in dorsal striatum innervation. We attested this loss of innervation by anterograde and retrograde tracer experiments. The injection of the anterograde viral construct in the SNpc showed a lack of innervation in the dorsal striatum for the mice lacking Lmx1a/b in post-mitotic mDA neurons. The experiment of retrograde tracers, fluorogold, injections in the nucleus accumbens showed an innervation of this area not only by neurons from VTA but also by SNpc neurons when Lmx1a/b are inactivated.

From these evidences we hypothesized that Lmx1a/b regulate dopaminergic circuitry *via* the regulation of axon guidance molecules. In order to answer this question, we looked for targets for Lmx1a/b using LCM-associated with a quick TH staining for RNA extraction. After mRNA sequencing, among all the targets found for Lmx1a/b, Plxnc1 appeared as

an interesting target to investigate. Thus we asked what is the role of Plxnc1 in dopaminergic axon guidance of post-mitotic mDA neurons. To confirm the mRNA sequencing results we performed an in situ hybridization for Plxnc1 on midbrain sections from double conditional mutant mice for Lmx1a/b and their control. We observed that expansion of the domain of expression of Plxnc1 not restricted only in the VTA as previously shown in the literature (Pasterkamp, Kolk & Hellemons 2007a) but also in the SNpc for the mutant. This difference of expression could be correlated with the segregation of the nigrostriatal and mesolimbic pathways. Thus we investigated the pattern of expression of the Plxnc1 ligand, Sema7a. We saw that Sema7a is expressed in the striatum according to a gradient with the highest concentration in the dorsal striatum.

The third hypothesis was that Plxnc1 by interaction with Sema7a regulates axon guidance in the dopaminergic circuit *via* a chemorepulsion. To characterize the effect of Plxnc1/Sema7a interaction on axon guidance in the dopaminergic circuit, in vitro experiments (explants, stripe assay), and transgenic mouse models for Sema7a and Plxnc1 were used.

Our results led to the proposition of a model explaining the role of Lmx1a/b in the regulation of dopaminergic neurons axon guidance with genes regulatory network for nigrostriatal and mesolimbic axons development. Based on our data, Plxnc1 expression is negatively regulated by Lmx1a and Lmx1b while Otx2, another transcription factor expressed by VTA neurons, positively controls Plxnc1. In mutant mice lacking both Lmx1a and Lmx1b, Plxnc1 expression seems to expand to all mDA neurons. This aberrant expression of Plxnc1 by SNpc neurons makes them responsive to Sema7a (expressed in the dorsal striatal region) and causes their axons to grow away from this repellent cue.

From these results we would be able to generate specific SNpc like neurons for cell replacement therapy for PD. So we generated mDA neurons derived from mice ESC in which we repress the expression of Plxnc1 to obtain SNpc-like neurons. We inject these newly generated mice in 6-OHDA lesionned mice to observe the rewiring of the nigrostriatal pathway.

Chapter 2. RNA Isolation from Cell Specific Subpopulations Using Laser-Capture Microdissection Combined with Rapid Immunolabeling

Résumé

La microdissection au laser (LCM) permet d'isoler un type cellulaire spécifique avec une haute résolution spatiale à partir de coupes minces de tissu hétérogène. L'identification de cellules d'intérêt pour la LCM est généralement basée sur des critères morphologiques ou des protéines endogènes fluorescentes. La combinaison de la LCM avec un immunomarquage rapide offre un moyen efficace pour visualiser des types cellulaires spécifiques, et les isoler du tissu environnant. De l'ARN de haute qualité peut alors être extrait d'une population cellulaire pure, puis traité pour des applications ultérieures telles que le séquençage d'ARN messager. Cette approche est déjà connue et décrite brièvement dans la littérature. Le but de cet article est de montrer comment effectuer un immunomarquage rapide d'une population de cellules tout en conservant l'intégrité de l'ARN, et comment isoler ces cellules spécifiques en utilisant la LCM pour des cellules dopaminergiques dans le tissu cérébral de souris.

RNA Isolation from Cell Specific Subpopulations Using Laser-capture Microdissection Combined with Rapid Immunolabeling.

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Disclosures

The authors have nothing to disclose.

Video Link

The video component of this article can be found at : http://www.jove.com/video/52510/

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Abstract

Laser capture microdissection (LCM) allows the isolation of specific cells from thin tissue sections with high spatial resolution. Effective LCM requires precise identification of cells subpopulations from a heterogeneous tissue. Identification of cells of interest for LCM is usually based on morphological criteria or on fluorescent protein reporters. The combination of LCM and rapid immunolabeling offers an alternative and efficient means to visualize specific cell types and to isolate them from surrounding tissue. High-quality RNA can then be extracted from a pure cell population and further processed for downstream applications, including RNA-sequencing, microarray or qRT-PCR. This approach has been previously performed and briefly described in few publications. The goal of this article is to illustrate how to perform rapid immunolabeling of a cell population while keeping RNA integrity, and how to isolate these specific cells using LCM. Herein, we illustrated this multi-step procedure by immunolabeling and capturing dopaminergic cells in brain tissue from one-day-old mice. We highlight key critical steps that deserve special consideration. This protocol can be adapted to a variety of tissues and cells of interest. Researchers from different fields will likely benefit from the demonstration of this approach.

Introduction

The brain is composed of a large variety of different neuron types forming complex networks. These neurons are organized in distinct groups and subgroups according to their morphology, connectivity and gene expression pattern[1]. The development of microarrays, next-generation sequencing, and qRT-PCR offered the possibility to compare gene expression profiles of neuronal populations in different biological contexts[1,2]. These sensitive analyses require the precise identification and isolation of cell types of interest while keeping RNA integrity[2-4]. Fluorescent activated cell sorting (FACS) technique has been widely used to isolate specific cell types based on cell-surface markers and/or morphology. FACS requires a cell dissociation step prior to sorting, which results in a complete loss of spatial resolution[5]. Many neuronal subpopulations are distinguished from one another according to their anatomical distribution in the brain. Laser capture microdissection applied on thin brain sections provides an appropriate option for specific isolation of cells with high spatial resolution[6-8]. A major limitation of LCM has been the need to identify cells of interest based on morphological criteria or on fluorescent protein reporters genetically engineered in animal models. The development of new techniques to perform quick immunolabeling methods that preserved RNA integrity, in combination with LCM, now allows the isolation of cell subpopulations to proceed with gene-profiling experiments.

This approach has been previously performed and briefly described in few publications[1,9-13]. Here, we demonstrate a detailed procedure to obtain high-quality RNA from a specific subset of cells in a complex tissue structure by combining quick immunolabeling with LCM. We show how to perform key critical steps to obtain maximal RNA recovery and avoid RNA degradation as it may significantly impact gene expression profiling.

For the demonstration of this protocol, dopaminergic neurons from one-day-old mouse midbrain were targeted. Dopaminergic neurons can be immunolabeled using an antibody directed against tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis. Following TH immunostaining, individual or groups of dopaminergic neurons can then be isolated using LCM. Microdissected cells are collected in the lysis buffer, and RNA is extracted using a RNA isolation kit. Quality and quantity of extracted RNA are

measured using a bioanalyzer[5]. Further analysis of gene expression using: RNA sequencing, microarray, or qRT-PCR can subsequently be performed[2,4,6]. As an example, a two-step qRT-PCR is demonstrated on the laser captured isolated dopaminergic domains. Relative quantification of expression levels of two dopaminergic neuron marker genes illustrates the selectivity of this protocol.

Protocol

NOTE: The 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.

1. Sample Preparation

NOTE: In this example, we used mouse brains at postnatal day 1.

1. Use hypothermia anesthesia in crushed ice for 3 to 4 min for pups, then dissect brains as quickly as possible in ice-cold L15 medium.
Perform this step within 2 min.

2. Transfer dissected brains in embedding mold (*cf*. list of material) and fill with frozen tissue embedding media taking care to orient the specimen in the desired position. Freeze specimens immediately in liquid nitrogen and store at -80 °C. Perform this step within 30 sec.

NOTE: Specimens can be stored few months without compromising the RNA quality.

2. Sectioning

1. Treat membrane coated glass slides (*cf*. list of material) with surface RNAse decontamination solution to eliminate any trace of RNAses.
Wash the slides in DEPC water and let them dry. Alternatively, bake slides overnight at 200 °C.

2. Transfer frozen samples in a cryostat previously cleaned with a surface RNAse decontamination solution. Set cryostat chamber temperature at -20 °C and slice

specimens at 10 µm thickness. Collect tissue sections on the membrane coated slides and let them dry 10 min before staining. Alternatively, store slides at -80 °C until staining.

3. Preparation of Staining Solutions

1. Prepare all solutions just before the start of the experiment, and use RNAse inhibitor in all staining solutions (except for washing solutions) to prevent RNA degradation.

2. Prepare the buffer solution. Use the same buffer in all solutions (composed of RNAse-free phosphate-buffered saline (PBS) supplemented of 1% BSA, 0.2% triton and 2% RNAse inhibitor). For each slide, prepare 400 µl of buffer solution (200 µl for the primary and 200 µl for the secondary antibodies).

3. Prepare the fixative solution: use 70% ethanol kept at -20 °C. Keep tissue fixation procedure minimal to avoid a drastic reduction in the RNA extraction yield.

NOTE: Alternatively, use 95% ethanol solution supplemented of 5% acetic acid kept at -20 °C.

4. Prepare the primary antibody solution by diluting the primary antibody in the buffer solution. For dopaminergic neurons labeling, use an antibody targeting the tyrosine hydroxylase (TH, rate limiting enzyme for dopamine production). Use a high concentration of antibody to compensate for the quick incubation time. Use TH antibody at a dilution of 1:25.

NOTE: In normal immunofluorescence protocol, this antibody gives a strong signal when incubated overnight at a dilution of 1:1,000. In this protocol, it is used 40X more concentrated due to the short incubation time. Due to the high concentration of antibody used for this method, perform a standard immunostaining in parallel in order to check any increase in non-specific labeling.

5. Prepare the secondary antibody solution by diluting the secondary antibody in the buffer solution. Use a biotinylated secondary antibody at a concentration of 1:100.

NOTE: In this example, we used an anti-rabbit biotinylated antibody.

6. Prepare the avidin-biotin complex (ABC) solution for the detection of biotinylated secondary antibodies. Make ABC solution by adding the compound A and the compound B at a concentration of 1:100 diluted in DEPC PBS supplemented with 2% RNAse inhibitor. Prepare the Avidin-Biotin complex 30 min before adding it to the slides.

4. Staining

1. Thaw one or two slides at the time from -80 °C by flicking them in the air quickly (or use an air blower). Surround sections with a hydrophobic barrier pen and let dry for a few sec.

    1. Put the slides in the cold fixative solution for no more than 5 min at -20°C. Shake once in the air to remove excess of fixative solution then, quickly dip slides in DEPC PBS 6-8 times for washing.

    2. Flick the slides to remove excess of DEPC PBS. Ensure that the slides defrosting step does not exceed 5 min.

2. Place slides on a clean tray (pretreated with a surface RNAse decontamination solution to eliminate any trace of RNAses) and put 200 µl of the primary antibody solution on each slide (rabbit anti-TH) for 10 min at room temperature. Dip slides quickly in DEPC PBS 3 times for washing and flick the slides once to remove the excess of DEPC PBS.

3. Put 200 µl of the secondary antibody solution on each slide for 6 min at room temperature. Dip slides quickly 3 times in DEPC PBS for washing and flick the slides to remove the excess of DEPC PBS.

4. Put 200 µl of the ABC solution on each slide for 4 min at room temperature. Dip slides quickly in DEPC PBS 3 times for washing and flick the slides to remove the excess DEPC PBS.

5. Prepare the DAB (3.3’ diaminobenzidine) solution during the 4 min of incubation in the ABC solution. Use the reagents provided in the kit (DAB Peroxidase Substrate Kit). Mix 1 drop of provided buffer, 1 drop of DAB solution and 1 drop of 30 % H2O2 in 2.5 ml of DEPC water. Mix the solution by inverting.

6. Take 196 µl of the DAB solution and add 4 µl (2%) of RNAse inhibitor solution just before spreading on slides. Place slides on a clean tray (pretreated with a surface RNAse decontamination solution) and put the 200 µl of DAB solution on slides.

NOTE: DAB solution should react within1 or 2 min.

7. Dip slides quickly in DEPC PBS 3 times for washing and put slides for few seconds in absolute ethanol. Dry the slides by flicking them in the air (or use an air blower).

NOTE: Slides can be stored at -80 °C for future use or processed for LCM.

5. Laser Capture Microdissection

1. Defrost slides quickly by flicking them in the air (or use an air blower). Ensure that the slides defrosting step does not exceed 5 min.

2. Proceed to LCM. Place the slide under the microscope with the sample side facing the collecting tube cap. Choose the best magnification for the sample. Adjust the focus to see the cells of interest.

1. Define the cutting area and start cutting with the laser. Collect dissected cells or pieces of tissue in the collecting tube cap filled with 50 µl of lysis buffer (from RNA extraction kit). Ensure that the step of LCM does not exceed 30 min.

3. Extract total RNA from isolated cells using an RNA extraction kit (*cf*. list of materials). Follow the manufacturer's protocol.

1. Incubate collected cells for 30 min at 42 °C to lyse the cells. Pre-condition the RNA purification column by adding 250 µl of conditioning buffer. Load 50 µl of ethanol to lysed cells. Load the 100 µl of lysed cells onto a preconditioned purification column.

2. Centrifuge the column at 16,000 x g for 2 min at room temperature. Wash the column twice with washing buffers. Elute in the minimal recommended volume (11 µl) of RNAse-free water (not DEPC treated as it may interfere with the bioanalyser). Keep 2 µl to test quality.

NOTE : All solutions and reagents are provided in the kit.

6. DNA Synthesis and Quantitative RT-PCR

1. Reverse-transcribe RNA samples from laser captured dopaminergic cells into complementary DNA using reverse transcriptase and oligo(dT).
Follow the manufacturer's protocol (*cf*. list of material).

2. Use 1 µl of cDNA for qRT-PCR amplification with gene-specific primers. Set up PCR reactions in 20 µl volume with a one-component hot start reaction mix for quantitative PCR as recommended by the manufacturer. Carry out each reaction in triplicate.

1. For the experiments described here, use the following primer pairs: Gapdh-F(5'-CCA CCC AGA AGA CTG TGG AT-3')/Gapdh-R (5'- GGA TGC AGG GAT GAT GTT CT-3'); Rpl13a-F (5'-ACA GCC ACT CTG GAG GAG AA-3') / Rpl13a-R (5'-CTG CCT GTT TCC GTA ACC TC-3') ; Th-F (5'-CAG TGG AGG ATG TGT CTC -3') / Th-R (5'-GAA AAT CAC GGG CAG ACA G-3'); Aadc-F (5'- CAT GAG AGC TTC TGC CCT TC -3')/Aadc-R (5'-GGA TGT GGT CCC CAG TGT AG -3').

3. Perform quantitative RT-PCR amplification using the following rapid cycling parameters: 95°C for 5 min followed by 45 cycles of 10 sec 95°C, 10 sec at 60°C and 10 sec at 72°C. Perform melting curve analysis using the default setting of the instrument to determine homogeneous product formation.

4. Analyze the data using the built-in software.

1. Normalize the expression values of DA markers genes Th and Aadc with the expression of the two reference genes Gapdh and Rpl13a to obtain relative expression levels as described previously[14].

Representative Results

This rapid immunolabeling procedure allows visualization of cells of interest while keeping RNA integrity. **Figure 1** illustrates the steps of tissue preparation before RNA extraction. After cryosectioning, dopaminergic neurons are labeled using an antibody directed against the tyrosine hydroxylase (labeled neurons appear in brown). Depending of the experimental question, single cells or larger region of interest can be isolated using LCM (**Figure 1D**-**E**). It is important to assess the quality of RNA extracted, as degraded RNA

will have a considerable impact on the quality of the following analysis. Each sample is thus processed on a microfluidics-based platform bioanalyser for quantification and to check RNA integrity. **Figure 2** shows typical bioanalyser results of degraded and good-quality samples. Both digital gel and electropherograms demonstrate the importance of using RNAse inhibitor in each solution to protect RNA. The expected amount of RNA extracted from 500 neurons is around 1-2 ng. Microdissected cells from several tissue sections can be pooled in the same tube to increase RNA quantity.

Discussion

The most critical point of this method consists in labeling cells of interest while preventing RNA degradation. As RNAses are active in aqueous solutions, decreasing incubation times improves RNA conservation[11,15]. All solutions used must be treated with DEPC to inactivate RNAses. All materials and surfaces need to be treated with a surface RNAse decontamination solution to prevent RNAses contamination. Finally, in these conditions, the addition of RNAse inhibitor in all solutions is effective in preserving RNA from being degraded. The use of high salt conditions applied during antibody incubation has been previously shown to be effective in preserving RNA[9,10]. However, this alternative method remains limited to quick staining using robust antibodies. The thickness of the sections represents another critical point and depends on the size of the cell of interest. Since the average size of the cell body of dopaminergic neurons is around 10µm, we select this section thickness to obtain one layer of cells.

The major limitation of this technique compared to FACS is the low number of cells that can be microdissected. The use of RNA amplification kit is required for gene profiling experiments using RNA-sequencing or microarray[16]. However, qRT-PCR can be performed directly without the amplification step (**Figure 2c**). Qualitative assessment of the staining obtained with the quick immunolabeling protocol should always be performed and should not differ from the staining obtained using the normal immunolabeling protocol.

Until now, laser capture experiments were mostly used to isolate populations of cells from transgenic animals expressing markers like GFP or using quick histological colorations such as Nissl staining. The method presented here allows visualizing cells of interest using immunohistochemistry. Isolating and acquiring the gene expression profile of chemically

identified populations of cells is now possible[17]. The protocol described here can be applied to any tissues and used with any suitable antibodies.

In the brain, neurons can be divided by their geographic localization but most brain regions contain a variety of different subpopulations based on their chemical identity. The gene expression pattern of certain types of neurons can be largely different from another cell type in the same cerebral area. According to the biological or pathological context, gene expression profile of a specific cell population can also change considerably. Using the procedure described here, it is possible to monitor these changes, paving the way to new discoveries[8,18,19].

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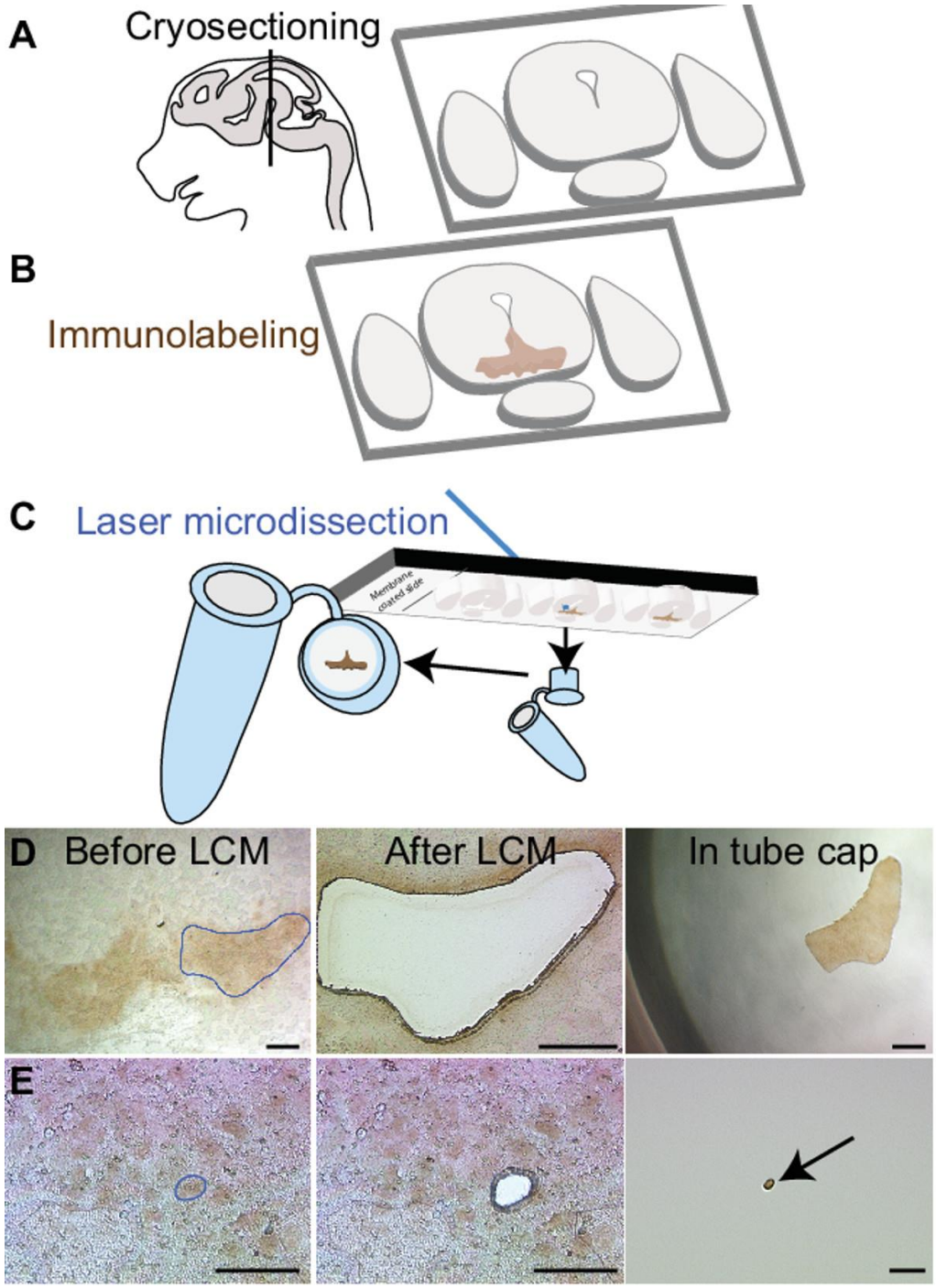
Materials

Tableau 1 Materials List for RNA Isolation from Cell Specific Sub-populations Using Laser-Capture Microdissection Combined with Rapid Immunolabeling.

Name	Company	Catalog Number	Comments
Membrane coated slides	Leica Biosystems	11505158	MembraneSlides PEN-Membrane 2,0 µm; 50 pcs
Surface RNAse decontamination solution	Life technologies	AM9780	RNaseZap
Fixative			70% Ethanol kept at -20 °C
Anti-TH	Pel-Freez	P40101	1:25
RNAse free buffer			DEPC PBS + 1% BSA+0.02% triton
RNAse inhibitor	Promega	N2615	Rnasine Plus Rnase Inhibitor 40 kU/ml (stock)
Anti-rabbit biotinylated	Vector Laboratories	BA-1000	
Vectastain Elite ABC kit Standard	Vector Laboratories	PK-6100	8µl/ml
DAB Peroxidase Substrate Kit	Vector Laboratories	SK-4100	
RNA isolation kit	Life technologies	KIT0204	Arcturus PicoPure RNA Isolation Kit
H_2O_2 30%	Sigma	HC4060	
Ethanol absolute			
Laser microdissection system	Leica microsystems		Model AS-LMD
DEPC	Alfa Aesar	B22753-14	
Bioanalyzer	Agilent		Agilent 2100 Bioanalyzer
Embedding mold	Leica Biosystems	14702218311	6x8mm
Hydrophobic barrier pen	Vector Laboratories	H-4000	
Frozen tissue embedding media	Tissue-Tek	4583	
Bioanalyzer chip	Aligent Technologies	5067-1513	RNA 6000 Pico LabChip used with Agilent 2100 Bioanalyzer
Hot start reaction mix for quantitative PCR	Roche	6402712001	Fast Start Essential DNA Green Master
Reverse transcriptase	Life technologies	11752-050	SuperScript III First-Strand Synthesis SuperMix for qRT-PCR

Figures



Figue1 Rapid Immunolabeling and Laser Capture Microdissection Procedure.

A) Schematic showing sagittal view of a mouse brain at embryonic day 15.5. Black line indicates the location of the anteroposterior regions where midbrain dopamine neurons are located. Thin sections are made using a cryostat and are collected on membrane coated glass slides. Following immunolabeling, midbrain dopaminergic neurons are visible in brown (B). Laser microdissected samples are collected in a tube cap filled with lysis buffer (C). Region of

interest (blue line in D) or individual cell (E) can be dissected and retrieved in the tube cap. Scale bars : (D) 250µm, (E) 50µm. Please click here to view a larger version of this Figure.

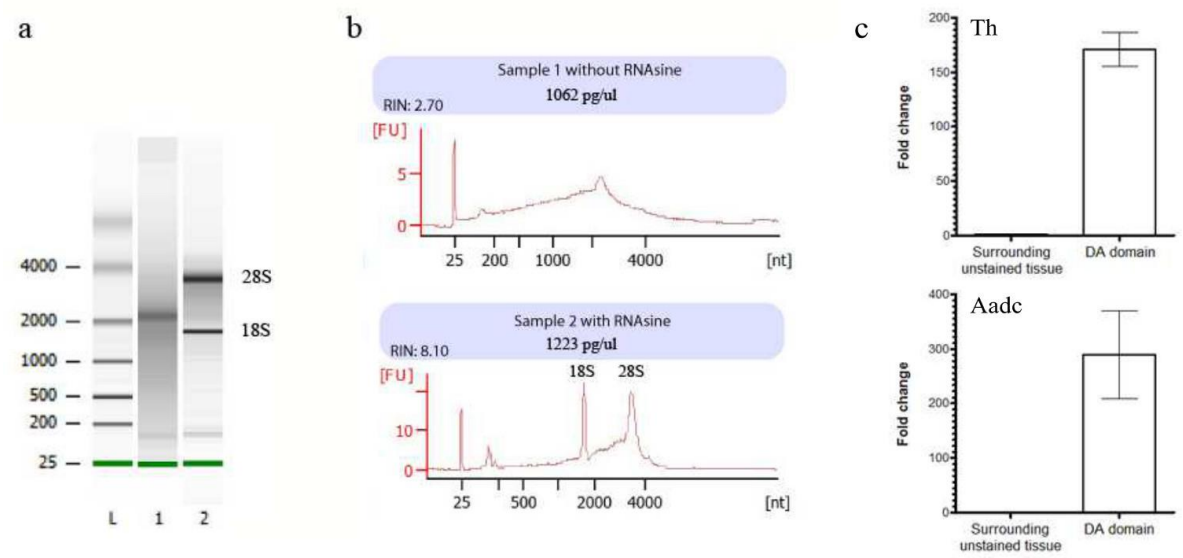


Figure 2 Quality Control of Total RNA Sample Isolated After Laser Capture Microdissection Using a Bioanalyzer Chip Kit.

(A) The digital gel obtained with the bioanalyzer (L, ladder; 1, sample 1 without RNAse inhibitor (RNAsine), sample 2 with RNAsine). (B) Electropherograms of sample 1 (upper panel) showing partially degraded RNA and sample 2 (lower panel) typical of good-quality RNA (RIN >8 is acceptably good) in which the 18S/28S rRNA peaks are clearly visible. (C) Normalized relative expression levels represented in fold change for two DA marker genes (TH and AADC) between surrounding unstained tissue and DA domain.

Chapter 3. Lmx1a and Lmx1b Repression of Plxnc1 Directs Dopaminergic Sub-Circuits Formation

Résumé

Les neurones dopaminergiques du mésencéphale (mDA) jouent un rôle crucial dans le contrôle d'une variété de fonctions cérébrales. Les neurones mDA de la SNpc et de la VTA, forment les voies nigrostriée et mésolimbique innervant respectivement la région dorsale et ventrale du striatum. Jusqu'à présent, les mécanismes impliqués dans la séparation de ces deux voies restent inconnus. Une analyse des projections axonales dopaminergiques de souris double conditionnelles mutantes pour Lmx1a/b a mis en évidence un défaut de guidage axonal confirmant le rôle essentiel de Lmx1a/b dans la formation des circuits dopaminergiques. Un profil d'expression génique comparant des animaux mutants et contrôles a mené à l'identification de Plxnc1 comme cible de Lmx1a/b. Nos résultats ont révélé que Lmx1a/b agissent comme répresseurs de Plxnc1 dans les neurones de la SNpc, et que l'interaction Sema7a/Plxnc1 (Sema7a ligand de Plxnc1) est responsable de la ségrégation des voies nigrostriée et mesolimique.

Lmx1a and Lmx1b repression of Plxnc1 directs dopaminergic sub-circuits formation

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Abstract

Mesodiencephalic dopamine neurons (mDA) play crucial roles in the control of a variety of brain functions, including voluntary movement and behavioural processes such as mood, reward and attention. These functions are deserved by distinct subtypes of mDA neurons located in the subtantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA), which form the nigrostriatal and mesolimbic pathways. Until now, the mechanisms involved in the formation of the dopaminergic sub-circuits remained largely unknown. Here we show that transcription factors, Lmx1a and Lmx1b, control subtype specific mDA neurons and their appropriate axon innervation in the forebrain. Our results revealed that Lmx1a and Lmx1b act as a repressor of Plxnc1, and that Sema7a/Plxnc1 interactions are responsible of the segregation of nigrostriatal and mesolimbic dopaminergic pathways. These finding identify Lmx1a/b and Plxnc1 as determinants of dopaminergic sub-circuits and should help engineering proper mDA neurons capable of regenerate appropriate connections for cell therapy.

Introduction

Subsets of midbrain neurons forming respectively the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNpc), produce a large majority of the dopamine in the central nervous system. Although they share the same neurotransmitter, these neurons innervate different brain regions, deserve different functions, and have a different vulnerability to degeneration. In Parkinson disease (PD), mDA neurons from SNpc are the most affected population whereas VTA neurons degenerate at much later stages of the disease [1]. Dopaminergic axons from SNpc neurons target the dorsal striatum and control motor behaviour. They are often referred as forming the nigrostriatal pathway. Dopaminergic neurons from the VTA innervate numerous brain structures including the ventral striatal region, the nucleus accumbens, and the prefrontal cortex. VTA neurons are involved in variety of behavioural processes such as reward and motivation; they are referred as the mesolimbic and the mesocortical pathways. Although the absence of clear boundaries between these groups of neurons, single axon tracing studies reported that ascending projection from SNpc and VTA are topographically organized [2-5].

Very little is known about the molecules regulating axonal targeting of mDA neurons, and how the different mDA neuronal populations establish their specific circuit is unclear. Recent progresses led to the identification of transcription factors expressed in mDA progenitors and required for their differentiation. Two LIM-homeodomain transcription factors, Lmx1a and Lmx1b (Lmx1a/b), are specifically expressed in mDA progenitors, and this expression persists in post-mitotic and adult mDA neurons. We and others have recently shown that Lmx1a and Lmx1b were required for the survival of adult mDA neurons [6, 7]. Although some evidences suggest that Lmx1a/b might also be involved in axon growth and guidance, no studies have yet determined their role in the formation of the dopaminergic circuits.

Here we show that Lmx1a and Lmx1b are required for the topographical organization of the dopaminergic innervation in the striatum. Our gene expression profiling experiments identified Plxnc1 as an Lmx1a/b target gene. Using *in vivo* and *in vitro* approaches, we found that the interaction of Plxnc1 with its ligand Sema7a segregates the nigrostriatal and mesolimbic pathways. Our finding elucidate central mechanism leading to the

establishment of key ascending circuits in the brain and pave the way to the development a more efficient cell replacement therapy for PD.

Results

Lmx1a and Lmx1b are required for the appropriate mDa axon projections.

During midbrain development, Lmx1a and Lmx1b are among the first transcription factors expressed by mDA precursors [8, 9]. Previous studies showed that Lmx1a/b are required for the specification, proliferation, and differentiation of dopaminergic progenitors [10, 11]. Immuno-fluorescent labeling applied on embryonic and postnatal midbrain sections shows that virtually all post-mitotic mDA neurons continue to express both Lmx1a and Lmx1b (Fig. 1). Given the functional redundancy established between these two factors in mDA progenitors [11], we developed a double conditional mutant mouse line in which Lmx1a and Lmx1b were genetically ablated in mature mDA neurons. To produce this line, we crossed mouse strains that were double homozygous for loxP-flanked alleles of Lmx1a and Lmx1b [10, 12] with mice expressing Cre-recombinase under control of the dopamine transporter gene locus (Dat or Slc6a3) [13]. The generated conditional knock-out (DatCre/+;Lmx1a/bf/f or Lmx1a/b cKO) animals were born at the expected Mendelian frequency, were fertile and morphologically indistinguishable from their control littermates (Dat+/+;Lmx1a/bf/f).

In agreement with previous studies [13-15], Cre expressed from the Dat promoter is efficient in deleting Lmx1a and Lmx1b in the ventral midbrain (Supplementary Fig. 1). Inactivation of Lmx1a and Lmx1b using the Dat-Cre line starts around embryonic day E13.5 [15], and corresponds to the developmental period where mDA neurons initiate their axonal growth toward the forebrain [16]. Histological analysis and stereological neurons counting from Lmx1a/b cKO and control mice did not reveal any significant difference in the total number and the distribution of mDA neurons (Fig. 2e-h). However, analysis of Tyrosine hydroxylase (TH) immunostaining in the striatum of Lmx1a/b cKO mice reveals an obvious lack of DA axons innervating the dorsal and caudal portion of the striatum at postnatal day 1 (P1) and in 2 weeks old mice (Fig. 2a-d and Supplementary Fig.2). Accordingly, quantification of TH positive axonal innervation in the striatum shows a decrease of mDA axon density in the dorso-posterior striatal region (Fig. 2i, j). No obvious

defect was observed in other regions innervated by mDA neurons (Supplementary Fig.4). We also used iDisco brain clearing [17] combined with TH immunostaining and lightsheet whole brain imaging to further analyze the dopaminergic system. The whole brain analysis of TH immunostaining confirmed the lack of DA axons in the dorsal striatum but no other obvious defect could be detected in Lmx1a/b cKO mice brains (Fig. 2n, Supplementary videos).

Since dopamine was shown to induce synapses formation *in vitro* [18], we evaluated the physiological consequences of a lack of DA in dorsal striatum. Postsynaptic currents (PSC) were recorded in the dorsal and ventral striatum of Lmx1a/b cKO mice and control littermates using whole-cell patch-clamp (Fig. 2k-m). Analysis of miniature inhibitory (mIPSC) and excitatory (mEPSC) PSC in the dorsal striatum revealed a decrease in the frequency of both excitatory and inhibitory synaptic inputs (Fig. 2l). Inversely, we found an increase in the frequency of excitatory and inhibitory synaptic inputs in the ventral striatum of Lmx1a/b cKO mice (Fig. 2m). These data are consistent with a lack of DA innervation in the dorsal striatum observed in Lmx1a/b cKO mice. To confirm a potential axon-targeting defect, we examine the specific axon projections of dopaminergic neurons from SNpc and VTA. We performed series of anterograde and retrograde tracing experiments. First, we used Cre recombinase-dependent viral vector expressing GFP for labeling mDA neurons and their axonal projections [19, 20]. Ten days after birth, Lmx1a/b cKO and control (Dat+/+) mice received stereotaxic injections of AAV-FLEx-GFP in SNpc (Fig. 3a-f). Upon Cre-mediated recombination, infected DA neurons express GFP. Histological analyses show that in both, controls and Lmx1a/b cKO mice, SNpc and part of the VTA neurons express GFP following AAV-FLEx-GFP injection (Fig. 3e,f). Analysis of GFP axons innervating the striatum corroborates the axon-targeting defect observed with TH immunostaining in Lmx1a/b cKO mice. In control animals, GFP axons could be found in the entire striatal region whereas in Lmx1a/b cKO mice, GFP axons were absent from the dorsal striatal region (Fig. 3b,c). We next confirm this phenotype by performing stereotaxic injections of a retrograde tracer (Fluorogold) in the nucleus accumbens (Fig. 3g-j). When injected in control animals, backfilled neurons can be observed almost exclusively in the VTA (Fig. 3h). However, injection of Fluorogold in nucleus accumbens of Lmx1a/b cKO mice results in numerous labeled neurons not only in VTA but also in SNpc (Fig. 3i,j).

Lmx1a and Lmx1b regulate Plxnc1 expression

Transcription factors can positively or negatively regulate the expression of hundreds of genes. To identify regulated genes by Lmx1a/b during the axonal development of mDA neurons, we performed gene expression profiling on one-day-old Lmx1a/b cKO mice and control littermates (Fig. 4a,b). We used laser-capture microdissection (LCM) to isolate TH-stained neurons of the SNpc and VTA followed by next-generation RNA-sequencing (RNA-seq) (Fig. 4a) [21]. We found 517 genes differently expressed by at least 2 fold between mutant and control animals (p value ≤ 0,001). Gene Ontology (GO) analysis identified possible enrichment genes in different developmental processes including neuron differentiation, axonogenesis, cell morphogenesis, transmission of nerve impulse, and neuron projection development (Fig. 4b). Among the differently expressed genes, the mRNA for the axon guidance receptor Plxnc1 was found 7 times more abundant in Lmx1a/b cKO mutants than in controls (Fig. 4c). To confirm the mRNA sequencing data, we performed in situ hybridization for Plxnc1on midbrain sections (Fig. 4d). As previously reported in mDA neurons of wild type mice, Plxnc1 expression was found restricted to VTA [22], with no apparent expression detected in SNpc (Fig. 4d). In contrast, mDA neurons from both VTA and SNpc express significantly higher level of Plxnc1 in Lmx1a/b cKO mice (Fig. 4d). Quantification of relative Plxnc1 mRNA transcript level, precisely in SNpc or VTA, reveals that Plxnc1 is respectively 7 and 11 fold more abundant in these two regions in Lmx1a/b mutant mice at P1 (Fig. 4c). Gain of function experiments by overexpressing Lmx1a or Lmx1b in mDA primary neuron cultures also induce a significant decrease in Plxnc1 mRNA levels, confirming the repressive role of Lmx1a/b on Plxnc1 (Fig. 4e).

Sema7a/Plxnc1 interaction mediates repulsion of VTA mDA neurons

Plxnc1 is a known guidance receptor for the membrane-associated GPI (glycosylphosphatidylinositol)- linked semaphorin 7a (Sema7a). Semaphorins are a large family of soluble and membrane bound proteins largely known for their repulsive function on developing axons [23]. *In vitro* studies shown that Sema7a binds directly Plxnc1 [24]. However, the functional significance of Sema7a on Plxnc1 expressed by mDA neurons from VTA remains unknown. Using protein extract from dorsal and ventral striatum, we quantified the abundance of Sema7a protein and found that Sema7a is 3 times more

abundant in the dorsal striatum than in the ventral striatum (Fig. 5). Based on the expression of Plxnc1 in VTA and Sema7a in the dorsal striatum, we hypothesized that Sema7a acts as a repellent cue on mDA axons of the VTA, which target the ventral striatum. To address this hypothesis, we tested in vitro the effect of Sema7a on mDA neurons coming from either VTA or SNpc. We precisely dissected mDA neurons of SNpc and VTA from one-day-old Pitx3-GFP heterozygous mice and cultured them for 2 days. In these mice, all mDA neurons express GFP [25], allowing a more accurate dissection of VTA and SNpc regions. We then exposed these neuronal cultures to Sema7a for 2 hours and measured the axonal and dendritic arborization of mDA neurons. Sholl analysis revealed a decrease in both dendritic and axonal complexity of mDA neurons from VTA but not from SNpc (Fig. 6a-c). We obtained the same results using VTA and SNpc explants cultured in collagen gel matrix and exposed to Sema7a for 2 hours. Both length and complexity of axons from VTA explants were reduced in presence of Sema7a, while Sema7a had no effect on mDA axons from SNpc explants (Fig. 6d,e). Because Sema7a is a membrane-associated GPI-anchored protein and not a diffusible cue, we also tested the axon response of VTA explants grown on alternated stripes of Sema7a (Fig. 6h-k). Control stripes had no effect on mDA axons from VTA and they grew randomly from the explant. In contrast, mDA axons displayed a clear avoidance for stripes when provided with a choice between control and Sema7a substrate (Fig. 6j,k). Interestingly, we also observed a significant enlargement of mDA growth cones following treatment with Sema7a (Fig. 6f,g). Altogether, these experiments show that Sema7a functions as a chemorepulsive and/or non-permissive signal for mDA axons from VTA. The specific expression of Sema7a in dorsal striatum may thus contribute to the dorso-ventral organization of the nigrostriatal and mesolimbic pathways.

Distribution of Plxnc1 containing axons in the striatum is altered in Sema7a KO mice

To further study the role of Sema7a on mDA axons development, we analyzed the axon projections of mDA neurons in absence of Sema7a (Fig. 7a-g). Because only VTA neurons express Plxnc1, the loss of Sema7a should affect mDA axon projections to the ventral striatum and nucleus accumbens. As expected, detailed analysis of Sema7a KO mice revealed that mDA axons expressing Plxnc1 are more widely distributed in the dorsal

striatum than in control mice. Quantification of Plxnc1 axonal density in dorsal and ventral striatal regions shows that Plxnc1+ axons extend more dorsally (Fig. 7g). These data suggest that Sema7a in the dorsal striatum function as repelling factor for Plxnc1 containing axons.

Forced expression of Plxnc1 in mDA neurons recapitulates the Lmx1a/b cKO phenotype

Our *in vitro* and *in vivo* experiments suggest that the interaction of Sema7a on Plxnc1 could be responsible of the topographical segregation of two main dopaminergic circuits, the nigrostriatal and mesolimbic pathways. To confirm that the aberrant Plxnc1 expression observed in SNpc neurons of the Lmx1a/b cKO mice could lead to axon targeting defect, we generated a transgenic mouse in which Plxnc1 is produced by all dopaminergic neurons. These animals were generated by pronuclear injection of a plasmid in which the TH promoter drives Plxnc1 and td-Tomato expression (TH-Plxnc1-Ires-tdTomato; Fig. 7j-o). In these mice, the expression of the reporter gene td-Tomato has been visualized in virtually all mDA neurons (Fig. 7l). We also measured the expression level of Plxnc1 by RT-qPCR from 1mm thick ventral midbrain sections and found that these mice show an increase of more than 5 fold of the normal level of Plxnc1 (Fig. 7n). Stereological counting of mDA neurons labeled for TH and td-Tomato shows no difference in the total mDA neuron number or distribution (Fig. 7k,l). However, analysis of dopaminergic axons projections of these mutants animals revealed a very similar axon-targeting defect than the one observed in Lmx1a/b cKO mice (Fig. 7m). The density of dopaminergic axons innervating the dorsal and posterior striatal region of these mutants was reduced (Fig. 7o) indicating that the nigrostriatal circuits are also defective in these mutants.

Otx2 and Lmx1a/b regulation of Plxnc1 expression in mDA neurons

Among transcription factors expressed by mDA neurons, very few have a restricted localization in either VTA or SNpc [24]. The transcription factor Otx2 is restricted to VTA neurons and has been shown to regulate the subtype identity of mDA neurons [25, 26]. In contrast to Otx2, Lmx1a and Lmx1b are found in both SNpc and VTA. Our results show that Lmx1a/b are repressing Plxnc1, but because Plxnc1 remains expressed in VTA, we wanted to test if Otx2 could contribute to the regulation of Plxnc1 expression in the VTA.

First, we performed double immunolabeling to test if Otx2 could also be ectopically expressed in the SNpc of Lmx1a/b cKO. As reported, Otx2 was restricted to VTA neurons in control mice [27, 28]. In contrast, numerous DA neurons co-expressing Otx2 and TH were found in SNpc of Lmx1a/b cKO mice at P1 (Fig. 8a,b).

Next, using RT-qPCR, we quantified Otx2 expression in VTA and SNpc samples obtained from laser capture microdissection comparing controls and Lmx1a/b cKO mice. Otx2 expression was found significantly increased in SNpc of Lmx1a/b cKO mice, when compared to control animals (Fig. 8c). However, Otx2 transcript level was not significantly change in VTA of Lmx1a/b cKO animals. To understand the mechanisms by which subtype identify is controlled in mDA neurons, we overexpressed either Lmx1a, Lmx1b or Otx2 using transient transfection in primary cultures of mDA neurons and measured Plxnc1 and Otx2 expression by Rt-qPCR. As expected, Lmx1a and Lmx1b overexpression lead to decrease of Plxnc1 (Fig. 4e) but were not sufficient to repress Otx2 (Fig 8e). Inversely, overexpression of Otx2 induced a significant increase of Plxnc1 transcript levels (Fig. 8d). Among transcription factors expressed by mDA neurons, very few have a restricted localization in either VTA or SNpc [26]. The transcription factor Otx2 is restricted to VTA neurons and has been shown to regulate the subtype identity of mDA neurons [27, 28]. In contrast to Otx2, Lmx1a and Lmx1b are found in both SNpc and VTA neurons. Our results show that Lmx1a/b are repressing Plxnc1, but because Plxnc1 remains expressed in VTA, we wanted to test if Otx2 could contribute to the regulation of Plxnc1 expression in the VTA. First, we overexpressed Otx2 using transient transfection in primary cultures of mDA neurons and measured Plxnc1 expression by Rt-qPCR. Overexpression of Otx2 induced a significant increase of Plxnc1 transcript levels (Fig. 8d). Because Otx2 is upregulated in our mRNA sequencing data, we next validated this result by Rt-qPCR. We quantified Otx2 expression in VTA and SNpc samples obtained from laser capture microdissection comparing controls and Lmx1a/b cKO mice. Otx2 expression was found significantly increased in SNpc of Lmx1a/b cKO mice, when compared to control animals (Fig. 8c). However, Otx2 transcript level was not significantly change in VTA of Lmx1a/b cKO animals. Finally, we performed double immunolabeling to test if Otx2 could also be ectopically expressed in the SNpc of Lmx1a/b cKO. As reported, Otx2 was restricted to DA neurons of the VTA in control mice [29, 30]. In contrast,

numerous DA neurons co-expressing Otx2 and TH were found in SNpc of Lmx1a/b cKO mice at P1 (Fig. 8a,b). To understand the mechanisms by which subtype identity is controlled in mDA neurons, we overexpressed Lmx1a and Lmx1b in primary cultures of mDA neurons and measured Plxnc1 and Otx2 expression by Rt-qPCR. As predicted, Lmx1a and Lmx1b overexpression lead to a decrease in Plxnc1 transcripts (Fig. 4e). However, Lmx1a and Lmx1b overexpression were not sufficient to repress Otx2 (Fig 8e). Altogether these results indicate that Plxnc1 expression in VTA is maintained by the transcriptional control of Otx2 while in SNpc, Lmx1a and Lmx1b repress Plxnc1 expression. Consequently, the specific topography of VTA and SNpc axon projections to striatum is established by the presence or the absence of Plxnc1 in mDA axons (Fig. 8f). Altogether these results indicate that Plxnc1 expression in VTA is maintained by the transcriptional control of Otx2 while in SNpc, Lmx1a and Lmx1b repress Plxnc1 expression. Consequently, the segregation between VTA and SNpc axons projecting to striatum is established by the presence or the absence of Plxnc1 in mDA axons (Fig. 8f).

Discussion

The proper functions of the dopaminergic system depend on the precise organization of dopaminergic circuits during development. Here we show that transcriptional regulation of Plxnc1 by Lmx1a/b and Otx2, controls the formation and the topographical organization of the nigrostriatal and mesolimbic pathways. We found that the inactivation of Lmx1a/b in post-mitotic mDA neurons leads to aberrant nigrostriatal axon projections. We also discovered that Lmx1a/b function as transcriptional repressor (directly or indirectly) on Plxnc1 expression. Our *in vitro* data and mutant mice analysis revealed a new mechanism by which the interaction of Sema7a/Plxnc1 regulates the striatal innervation.

Transcriptional control of Plxnc1

In addition to their roles in early development of mDA progenitors [10, 11], we show here that Lmx1a and Lmx1b have other functions in post-mitotic mDA neurons. By controlling Plxnc1 expression, Lmx1a, Lmx1b and Otx2 regulate the proper organization of nigrostriatal and mesolimbic axon projections. In SNpc, Lmx1a and Lmx1b are required and sufficient to repress Plxnc1. Lmx1a and Lmx1b are also required for the repression of Otx2 in SNpc but were not sufficient to repress Otx2 in VTA primary mDA neuron

cultures. In VTA, Lmx1a/b are required and sufficient for Plxnc1 repression but they are not required or sufficient to repress Otx2. Our gain of function experiments have also shown that Otx2 can drive Plxnc1 expression in mDA primary culture. Altogether our data indicate that Plxnc1 expression is precisely controlled in mDA neurons by the coordinate action of Lmx1a, Lmx1b and Otx2. In VTA, all three transcription factors are present. Lmx1a/b repress Plxnc1 but Otx2 promotes and maintains Plxnc1 expression. In SNpc, Otx2 is normally absent and both Lmx1a and Lmx1b repress Plxnc1. Following Lmx1a/b ablation, we found ectopic Otx2 expression in mDA neurons located in SNpc. In addition, Rt-qPCR experiments in Lmx1a/b cKO revealed that Otx2 expression was only increased in SNpc, while in VTA Otx2 expression was unchanged. According to these results, it is likely that another factor, only present in SNpc, cooperatively contributes to Otx2 repression in SNpc.

Axon guidance in the dopaminergic system

Previous anatomical studies described the expression of Sema7a in the striatum and Plxnc1 in the VTA and suggested a possible role of these proteins in the guidance of mDA neurons [16, 22]. We shown here that Sema7a is more abundant in the dorsal striatum than in the ventral striatal region. Our *in vitro* assays revealed that Sema7a functions as a repellent cue for VTA axons expressing Plxnc1. Moreover, in mutant mice for Sema7a, Plxnc1 axons extend more dorsally in the striatum whereas transgenic mice overexpressing Plxnc1 in mDA neurons, lack DA innervation in the dorsal striatum. These *in vivo* data indicate that Sema7a-Plxnc1 interaction controls DA axon targeting in the striatum. The repellent function of Sema7a on mDA axons from VTA reported here represents a major mechanism assuring the proper topographical innervation of the main mDA target regions, the striatum. It is reasonable to think that other axon guidance cues contribute to attract mDA neurons in the striatum. Indeed, gradient of Netrin-1 is present in the striatum. The latero-ventral region of the striatum is rich in Netrin-1 whereas the medio-dorsal striatum is poor in Netrin-1. Although all mDA neurons express the receptor DCC [31], VTA axons prefer higher Netrin-1 concentration than SNpc axon [32]. The mechanism by which Netrin-1 attracts differently VTA and SNpc DA axons is not understood but the convergence of guidance signals could direct trajectory of mDA neurons. A recent study showed that motor growth cones synergistically integrate both

Netrin-1 and Ephrin signals. Receptors for these ligands can form complex that act in synergy on a common downstream effector [33]. In the striatum, Sema7a and Netrin-1 could also synergistically interact at receptor level and control mDA innervation in the striatum. In Lmx1a/b cKO mice, we found DA axons redirected ventrally, but although our electrophysiological recording indicated an increase in DA in the ventral striatum, we did not observe a significant increase in DA axon density in this region. One possible explanation is that SNpc axonal branching is limited by another cue such as high Netrin-1 concentration. It is also possible that although SNpc axons are repelled ventrally, they compete with VTA axons for the synaptic space and thus limit the number of SNpc axons ventrally.

The mechanism regulating DA innervation in the striatum reported here shed a new light about how VTA and SNpc establish their connections with the striatum. However, anatomical and genetic evidences indicate that mDA neurons can be subdivided in more distinct subpopulations [2, 3, 34, 35]. In the striatum, subregions can be divided based on their neurochemical content and two main compartments can be defined, the striosomes and the surrounding matrix [36]. In SNpc, neurons from ventral tier are believed to innervate preferentially the striosomes whereas the dorsal tier preferentially innervate the matrix compartment [34]. In our study, we did not observe change in striosomes and matrix innervation in either Lmx1a/b cKO, Sema7 KO or Plxnc1 overexpressing mice. These data suggest that the specific DA projections to these striatal compartments are regulated by other developmental mechanisms. Based on gene expression, multiple groups of mDA neurons can also be subdivided [35] but the precise axon projections of these subpopulation and the guidance mechanisms regulating these subpopulations remain to be discovered.

Our finding has an important implication for stem cell engineering and transplantation for PD. This chronic progressive neurodegenerative disorder is characterized by the selective loss of DA-containing neurons in the SNpc. The identification of Plxnc1 as a main guidance receptor allowing subset-specific axon targeting of mDA neurons provides a valuable tool to improve the efficiency for reconnection of grafts of newly generated neurons. Indeed, to replace degenerated SNpc mDA neurons that will innervate and

integrate in the dorsal striatum specifically, newly generated neurons should not express Plxnc1. In a recent study, forced expression of Otx2 has been used in human embryonic stem cells (hESC) to generate DA neurons. When grafted in SNpc, these hESC-derived DA neurons were able to innervate the forebrain structures but displayed a strong preference for VTA-specific target regions with few axons innervating the dorsal striatum [37]. According to our results, Otx2 promotes Plxnc1 expression and thus, it is likely that the grafted neurons in this study innervated VTA target regions because they express Plxnc1. Further studies are needed to test the efficiency of a cell replacement therapy using Plxnc1 deficient DA neurons.

In sum, our work shows a new mechanism for Lmx1a and Lmx1b beyond early developmental stages to control the dopaminergic innervation of the striatum. Mechanisms involved in the development of the dopamine circuits still remain largely unknown despite the involvement of these circuits in important physiological functions and in various mental disorders such as schizophrenia. Interestingly, three SNIPs in LMX1A and one in LMX1B were found associated with schizophrenia and were the same as those previously identified in PD [38]. However, further studies will be necessary to evaluate the role of these SNIPs on the Plxnc1 expression and the development of the dopaminergic system.

Methods

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. Lmx1af/f [7, 10], Lmx1bf/f [12], DatCre/+ [13], Pitx3-GFP [25], Sema7a KO [23] mice were genotyped as previously described. Lmx1a/b cKO mice were generated by intercrossing DatCre/+ and Lmx1a/bf/f mouse lines. Plxnc1-Ires-td-Tomato mice were generated by standard procedure using microinjection into the pronucleus of fertilized single-cell mouse embryos as previously described [39]. Transgenes contained the 9Kb TH promoter [40] (gift from Dr. Kazuto Kobayashi), the full coding sequence of mouse Plxnc1 (NM_018797.2) and td-Tomato (Plateforme d'outils moléculaires, IUSMQ). Transgenic mice were identified by PCR with forward primer in

Plxnc1 sequence, 5–GGCTGGAAGAAGCTCAGAAA-3; and reverse primer in IRES sequence, 5-TACGCTTGAGGAGAGCCATT-3.

Tissue analysis. Mice brains at P1 were incubated in 4% paraformaldehyde in PBS at 4°C, followed by cryoprotection in 30% sucrose in PBS, before freezing on dry ice. For mice older than P1 a perfusion using 4% paraformaldehyde in PBS was instead performed. After cryostat sectioning at 60µm, sections were washed in PBS, then blocked with 1% normal donkey serum (NDS) and 0.2% Triton X-100 for at least 30 min. Primary antibodies used in this study were: rabbit anti-TH (Pel-Freez, 1:1000), sheep anti-TH (Millipore, 1:1000), rat anti-DAT (Millipore, 1:500), sheep anti-Plxnc1 (R&D systems, 1:150); rabbit anti-Lmx1a (Millipore, 1:1000); guinea-pig anti-Lmx1b (Gift from Dr Carmen Birchmeier, Max Delbruck Center of Molecular Medicine, Berlin, Germany, 1:100); goat anti-Otx2 (R&D systems, 1:200), mouse anti-Tyrosine tubuline (Sigma, 1:3000), mouse anti-Actin (Millipore, 1:1000). Secondary antibodies used in this study were donkey Alexa-Fluor-488, donkey Alexa-Fluor-555 or donkey Alexa-Fluor-647, were used at 1:200 (Jackson immunoresearch).

Optical density measurements on sections. Striatal sections were co-labeled with TH and Actin or with Plxnc1 and Actin and then incubated with infrared immunofluorescence secondary antibodies (donkey anti-mouse Alexa-Fluor-680, 1:5000; donkey anti-sheep Alexa-Fluor-790, 1:5000; donkey anti-rabbitAlexa-Fluor-790, 1:5000). Sections were then mounted on glass slides, coverslipped with fluorescent mounting medium (Aqua-Poly/Mount, PolysciencesInc.), and scanned using an infrared imaging system (Odyssey CLx; LI-COR biosciences, Lincoln, NE, USA [41]). Optical density measurements were done using Image Studio Lite Ver5.2 software after delimitating the striatal areas with the software freehand tool. The distinction between dorsal versus ventral striatum areas was determined by dividing the striatum in the middle. The ratio of dorsal striatum intensity on ventral striatum intensity was measured at 3 anteroposterior levels corresponding to 2.79mm, 2.43mm, and 2.07mm according to the Atlas of the Developing Mouse Brain at P0 [42].

In situ hybridization. In situ hybridization was performed as previously described [6, 11]. Brains were dissected in diethylpyrocarbonate (DEPC) treated PBS, and fixed in PFA 4%

(DEPC) overnight. After an overnight incubation in sucrose 30% (DEPC), brains were frozen with dry ice and then cryosectioned in 20µm thick coronal sections. Sections were collected on superfrost plus slides (Fisher Scientific). cDNA templates were generated from E18.5 whole brain RNA and used to generate RNA probes for Plxnc1, Lmx1a and Lmx1b using RT-PCR following the protocol described previously [43].

Quick TH staining and LCM. The experiment was performed as described in [21]. Briefly, P1 mouse brains were quickly dissected and snapfrozen in liquid nitrogen. 12µm thick cryostat sections were harvested on membrane-coated glass-slides (Leica Biosystems), allowed drying, before being fixed in 70% ethanol at -20ºC. The fixed sections were quickly stained (20 min) using rabbit anti-TH (Pel-Freez, 1:25) as first antibody, washed with PBS, and then exposed to a biotinylated anti-rabbit secondary antibody (Vector Labs, 1:100). The stained slides were stored frozen at -80ºC. After defrosting, the SNpc and VTA areas were collected using a Laser capture microdissection microscope (Leica).

cDNA library production. Cells from SNpc and VTA respectively were isolated using laser capture microdissection (Leica AS-LMD) from stained frozen section of control and Lmx1a/b cKo mice as described above. Microdissected cells were collected in lysis buffer and total RNA isolation was carried out using RNA Isolation Kit (Arcturus Picopure, Applied Biosystems) according to the manufacturer's instructions. RNA was reverse transcribed using Superscript III (Life technologies) and cDNA obtained was used for RT-qPCR.

RT-qPCR. Quantitative RT-PCR was performed using a cDNA library obtained from LCM experiment. Analysis of expression level of mRNAs was achieved with Platinum SYBR Green Super Mix (Invitrogen) and performed in triplicate using the LightCycler 480 (Roche Diagnostics). Primers for amplification were designed in the 3' region of each gene using online Primer3 tool (http://bioinfo.ut.ee/primer3/). (Primer sequences are available upon request.) Amplifications were performed in 20µl containing 0.5µM of each primer, 0.5µl SYBR Green (Invitrogen) and 2µl 50-fold diluted cDNA, with 40 cycles at 94°C for 15 seconds, 60°C for 1 minute, 72°C for 30 seconds, and 79°C for 5 seconds. Analysis of real-time quantitative RT-PCR triplicate reactions was performed with the LCS 480 software (Roche Applied Science; Version 1.5.0.39); and outliers removed according to

the method described by [44]. "Advanced Relative Quantification" mode of LCS 480 software was used to estimate relative gene expression; and 2−ΔCT formula was used to calculate relative quantifications. Values normalization was realized using the target gene amount over at least two or three reference genes (GAPDH, TBP, and RPL13). In order to confirm homogeneous product formation, melting curve analysis was performed. Data are represented as mean ± s.e.m. of the fold change to the expression at P1 and normalized against reference genes.

RNA sequencing. Total RNA was isolated from SNpc and VTA of 3 controls and 3 Lmx1a/b cKO mice. The Ovation RNA-Seq system (NuGEN) was used for RNA amplification. Following cDNA library, samples were sequenced on an IlluminaGAIIx. CLC Genomics Workbench version1 (CLC bio) was used for RNA-seq libraries analysis. A description of expressed genes with an RPKM (reads per kilobase of transcriptper million reads mapped) of 1 and above was realized, but only genes expressed with a two fold (or more) difference between Lmx1a/b mutant tissue over control tissue were considered. The GO Tool Box (genome.crg.es/GOToolBox), using the MGI identities of the list of genes, was used for GO analysis. Gene lists and GO term results are provided in SI Materials and Methods.

Brain injections. The anterograde virus pAAV-mCBA-FLEX-EGFP-WPRE (9 e12 genome copy/ml; from Plateforme d'Outils Moléculaires, IUSMQ) was injected in the SNpc of DatCre/+ Lmx1a/bf/f or DatCre/+ mice at P10. Mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus (Stoelting, Wood Dale, IL). 200nl of this virus was injected at the following coordinates: anteroposterior (AP) from lambda:+1 mm; mediolateral (ML): 1,1 mm; dorsoventral (DV, taken from the surface of the brain): -3,2 mm [45]. Mice were sacrificed 17 days after surgery for immunohistochemistry analysis. Injection of fluorogold retrograde tracer was also performed in the nucleus accumbens of the DatCre/+ Lmx1a/bf/for Dat+/+ Lmx1a/bf/f mice at P10. 1µl of Fluorogold (2% in 0.9% sodium chloride, Millipore) was injected in the nucleus accumbens of both hemispheres at the following coordinates from the bregma: AP: +0.7 mm; ML: 1 mm; DV: -3,2 [45]. Mice were sacrificed 3 days after surgery for analysis.

Stereological neuron counting. Stereological methods (optical fractionator method, StereoInvestigator, MBF Science) [46-48] were used to compare the number of TH-positive neurons within the ventral midbrain of control and mutant mice at P1 and P15. Contours were traced at 5X magnification to define the area of interest and counting was realized at 40X magnification at 1 in 2 section interval. Coefficients of error (Gundersen, m = 1) were less than 0.08.

Western blot. Striatum from controls Pitx3-GFP mice brains at P1 were dissected by cutting a 1mm coronal slice followed by dissection of samples from dorsal and ventral striatum. These samples were then snap frozen. Sample lysis was performed in radioimmunoprecipitation (RIPA) buffer complemented with protease inhibitor and phosphatase inhibitor cocktails (Roche) (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2mM EDTA, 50mM NaF, pH 7.4). Quantification of protein content in the samples was realized using a DC-protein assay (Bio-Rad). 15µg of protein extracts were separated by SDS- polyacrylamide gel electrophoresis (12% SDS-PAGE Tris-glycine gels). Nitrocellulose membranes (Bio-Rad) were used for the transfer. Further experimental procedures are described in supplementary. Blots were immunostained overnight at 4°C with primary antibodies. The primary antibodies mouse anti-actin (1/10000; Millipore, MAB1501), rabbit anti-Sema7a (1/500; Abcam, ab23578), were diluted in blocking solution containing TBS 1X and 0.01% Tween-20 (Sigma). Blots were washed 3 X 5 min with TBS 1X and 0.01% Tween-20 and immune complexes were detected with species-appropriate secondary antibody conjugated to HRP: Goat anti-rabbit HRP (1/3000; CST 7074), Goat anti-mouse HRP (1/5000; Life Technologies, G-21040). Membranes were covered with ECL for 5 minutes (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.

***In vitro* mDA neurons and explant cultures.** Primary mDA neuron cultures were performed as previously described [6]. In brief, embryonic ventral midbrains were dissected from E18.5 Pitx3-GFP embryos in chilled L15 containing 5% fetal bovine serum (FBS). After 1DIV, cells were transfected using Lipofectamine 2000 (Life technologies)

with either the control plasmid pCMV-mCherry (Plateforme d'Outils Moléculaires, IUSMQ), or plasmid allowing overexpression of Otx2 (Gift from Dr. Siew-Lan Ang), Lmx1a (pCAGGS-Lmx1a-IRES2-nucEGFP, Addgene), Lmx1b (pCMV-Lmx1b-RFP, Applied Biological Materials inc.), or Lmx1a plus Lmx1b. Cells were harvested after 3 DIV and processed for RT-qPCR.

Explants of embryonic ventral midbrain were dissected from E14.5 Pitx3-GFP embryos in chilled L15, 5% FBS. Explants were grown on 12mm diameter glass coverslips coated with 7µl Matrigel™ (BD Bioscience, Mississauga, ON, Canada) in 24 wells plates. Then, explants were each covered by 7µl Matrigel and cultured in 1ml neurobasal medium complemented with B27, PenStrep, Glutamax, sodium pyruvate, and FBS (86,8% neurobasal medium, 5% P/S, 2% B27, 0,2% Glutamax, 1% NaPyruvate, 5% FBS) for two days at 37°C, 5% CO2. After 2DIV, the medium was replaced by the same complemented medium without FBS. Then explants were exposed to Sema7a (0.5µg/ml) for 2 hours, and fixed for 30 minutes at 4°C in fixative solution (3,5% PFA, 4% Sucrose, in PBS1X). The actin was stained by incubating the explants for 40 min at room temperature with a buffer solution containing 1% rhodamine-phalloidin (life technologies) and 1% BSA while TH was immunostained by incubating overnight with rabbit anti-TH (Pel-Freez, 1:1000) in 5% NDS and 0.1% Triton X-100 in PBS. Axonal outgrowth analysis was performed on confocal images of the TH signal using Neurite-J plug-in [49].

Stripe assay. The stripe assay was performed as previously described [50]. Alternating stripes (IgG or Sema7a 100µg/ml, R&D system) were applied to glass coverslips. These stripes were then covered by laminin (20µg/ml). Approximately 3 to 4 explants were seeded on the middle, and at the left and right extremities of each coverslip. These explants were then cultured for 3 days, and fixed with 3,5% PFA, 4% Sucrose, in DPBS1X before immunostaining. For quantification, the number of neurites terminating on control versus Sema7a stripes was counted for each explant.

iDISCO. Mice brain were dissected at P1 then treated through the different steps described in iDISCO method protocol [17]. Briefly, after tissue harvesting and fixation, a pretreatment with methanol was performed before immunostaining. Then, permeabilization and blocking of the tissue were realized before incubation in primary

antibodies at the following concentrations; rabbit anti-TH (Pel-Freez, 1:250) for 7 days. After 1 day of washings in buffer PTwH (100mL PBS 10X, 2mL Tween20, 1mL of 10mg/mL Heparin stock solution), brains were incubated in the secondary antibody Alexa fluor594 donkey anti-rabbit (Life technologies, 1:400) for 6 days. After washings, brains were transferred in clearing solution for further imaging.

Light sheet imaging. Whole hemisphere tridimensional (3D) images were obtained using a two-photon light-sheet microscope. An excitation light sheet was generated by scanning a long thin near infrared (800nm) Bessel beam into the sample through a long working distance objective (Olympus, XLFLUOR4X/340, NA 0.28, WD 29.5mm). The length and the thickness of the beam were decoupled thereby achieving a large field of illumination (25mmx2.5mm) without compromising the axial resolution (2 µm). To produce a light sheet with sufficient energy, a Femtosecond Titanium-Sapphire source laser (Coherent Mira 900) was used with an amplifier (Coherent RegA 9000). The emitted two-photon fluorescence was detected by a scientific CMOS camera (Hamamastu Orca-Flash4.0 V2) through a long working distance multi-immersion objective (Olympus XLPLN10XSVMP, NA 0.6, WD 8mm) placed perpendicular to the scanning plane, achieving an effective field of view of 1.3mm by 1.3mm. The whole brain images were obtained by moving the sample using a motorized stage (Sutter MPC-385), thus generating tridimensional (3D) images. These images were stitched together using a custom Matlab script to form a 3D image of the entire sample.

Electrophysiological recordings. Sagittal whole brain slices (300µm thick) were prepared from DatCre/+Lmx1a/bf/f cKO mice and control littermates (P9–14). Animals were lightly anesthetized with isoflurane and brains were dissected and sliced in ice-cold (0 to 4°C) solution containing (in mM): 250 sucrose, 2 KCl, 1.25 NaH2PO4, 26 NaHCO3, 7 MgSO4, 0.5 CaCl2, and 10 glucose, saturated with 95% O2 and 5% CO2, 340-350 mOsm. Slices were immediately transferred to a heated (34°C) oxygenated solution containing (in mM): 126 NaCl, 2.5 KCl, 1.4 NaH2PO4, 26 NaHCO3, 2.4 MgCl2, 1.2 CaCl2, and 11 glucose (60 minutes, after which they were kept at room temperature until use). During recordings, slices were continuously perfused at 2mL/min with a standard artificial cerebrospinal fluid (ACSF) (126mM NaCl; 2.5mM KCl; 1.4mM NaH2PO4; 25mM

NaHCO3; 1.2mM MgCl2; 2.4mM CaCl2; and 11mM D-glucose; osmolarity adjusted to 295–305 mOsm) saturated with 95% O2 and 5% CO2 at near-physiological temperature (30– 32ºC). Whole-cell voltage-clamp recordings were obtained from visually identified cells. For recordings, 3.5–5 MΩ borosilicate glass pipettes were filled with a 115mM CsMeSO3, 20mM CsCl, 10mM diNa-phosphocreatine, 10mM HEPES, 2.5mM MgCl2, 0.6mM EGTA, 4mM ATP-Mg, 0.4mM GTP-Na (pH 7.25; osmolarity adjusted to 275–285mOsm). Data acquisition (filtered at 2–3 kHz; digitized at 10 kHz) was performed using a Multiclamp 700B amplifier and Clampex 10.6 software (Molecular Devices, Union City, CA, USA). Data were analyzed using Clampfit 10.6 (Molecular Devices). Miniature events were analyzed using the search event algorithm of Clampfit 10.6 and fitted with a double exponential.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPadSoftware, La Jolla, CA, USA) software. Differences between two groups were determined by Student's t test. Explants and mDA cells intersection profiles were analyzed by two-way ANOVA and Sidak test was used for post-hoc comparisons. All data in this manuscript are derived from at least three independently performed experiments. All data are represented as means ± SEM, and significance is defined as $p < 0,05$.

Microscopes. All immunofluorescence images were acquired using a Zeiss LSM5 Pascal confocal microscope ora Zeiss LSM700 confocal microscope, then processed using Zen software and Adobe Photoshop CS4.

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Figures

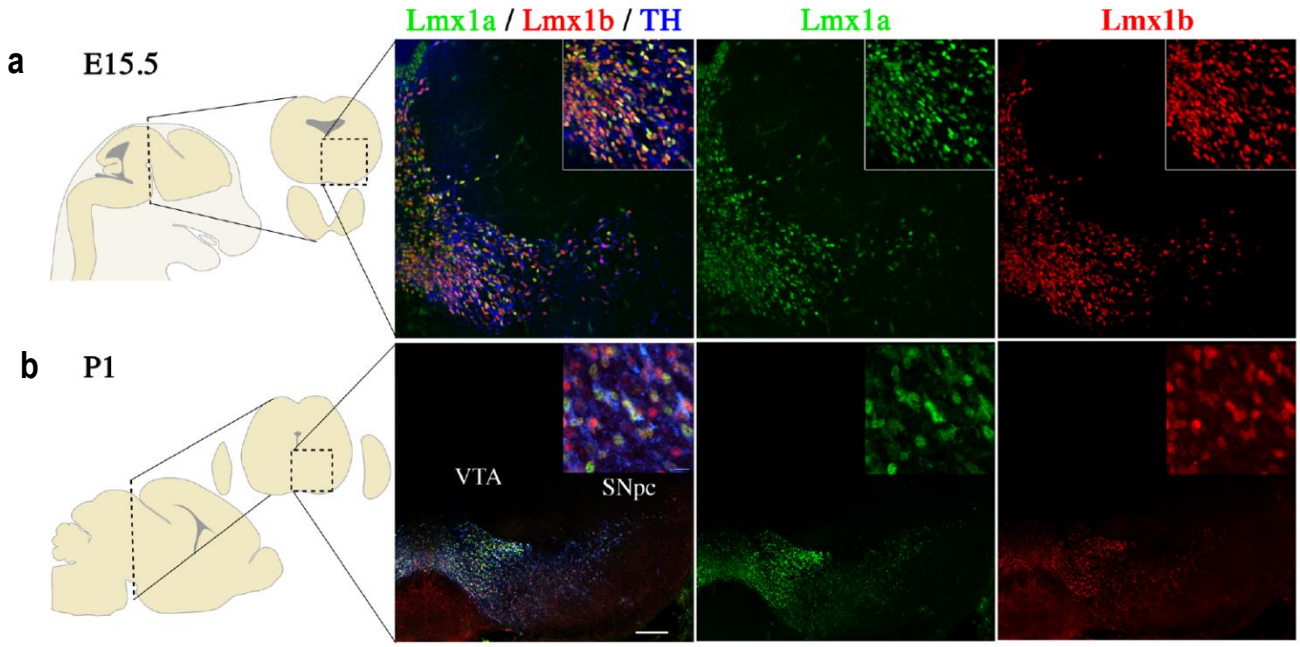


Figure 1: Ventral midbrain expression of transcription factors Lmx1a and Lmx1b at early stage of development. Representative confocal images of immunohistochemical analysis of the transcription factors Lmx1a and Lm1b in TH positive cells of midbrain coronal sections at E15.5 (**a**) and P1 (**b**). Scale bar: 200 µm, and 25 µm for high magnification inserts. Abbreviations: VTA, Ventral tegmental area; SNpc, Substantia nigra pars compacta.

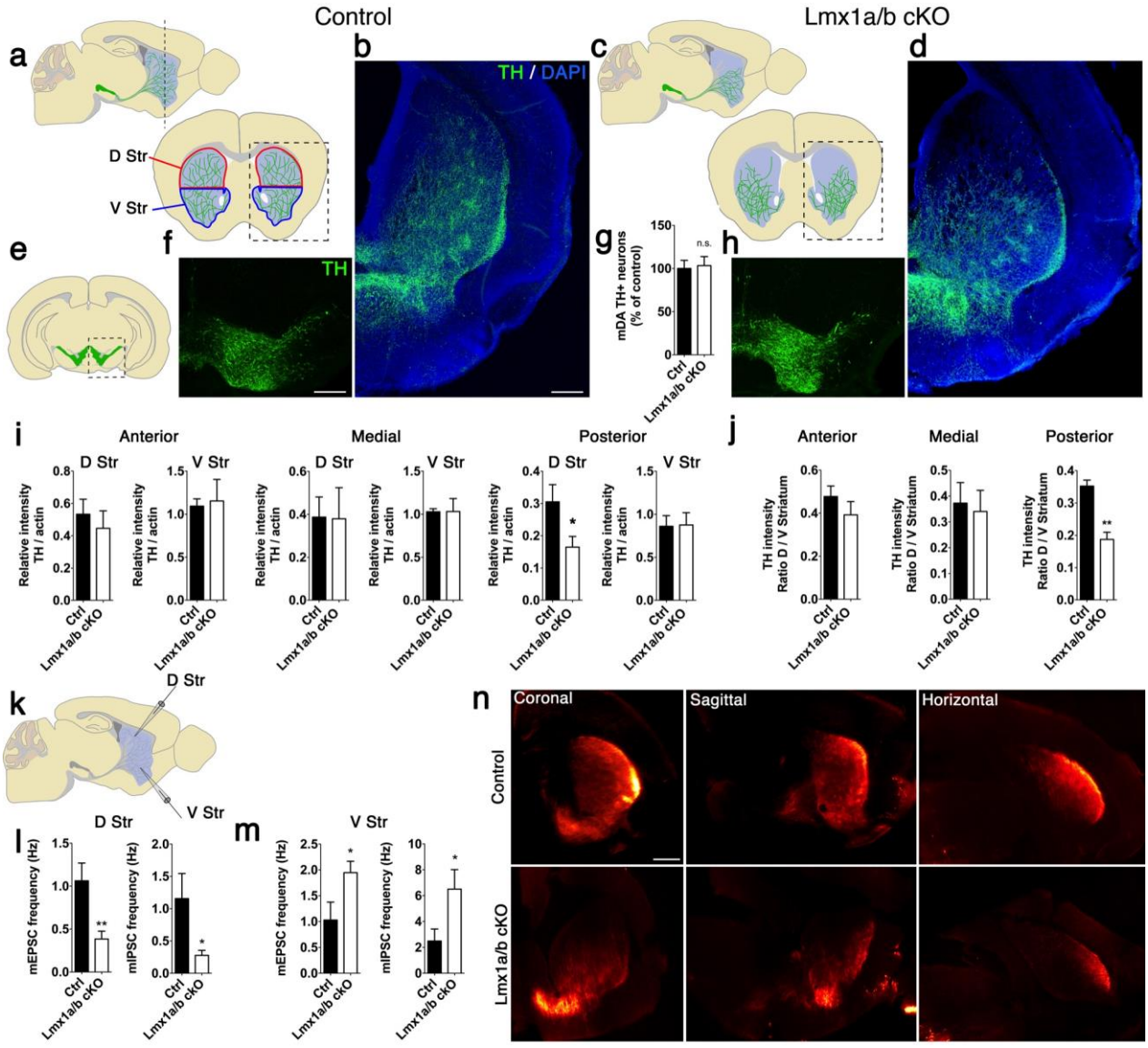


Figure 2: Characterization of the phenotype of Lmx1a and Lmx1b double conditional mutant mice at P1.
(**a** and **c**) Sagittal and coronal schematic representations of axonal innervation in the striatum in control and Lmx1a/b cKO mice brains at postnatal day 1 (P1). (**b** and **d**) Representative confocal images of control and Lmx1a/b cKO mice brains showing a loss of dopaminergic innervation in posterio-dorsal striatum for Lmx1a/b cKO mice (TH in green and DAPI in blue). (**i** and **j**) Optical density measurements of TH axons in the striatum. Graphs in **j** show the ratio of TH intensity in dorsal vs ventral striatum. (**e**) Schematic representation of coronal section of mouse brain at the midbrain level. Dashed line indicates the delimitation of the pictures shown in **f** and h. Distribution (**f** and **h**) and number (**g**) of mDA neurons in the midbrain were not different between controls and Lmx1a/b cKO at P1. (**k**) Schematic representation of the location of electrophysiological recordings in the striatum. (**l**,**m**) Analysis of frequency of miniature excitatory postsynaptic currents (mEPSC) and miniature inhibitory postsynaptic currents (mIPSC) in the dorsal (**l**) and ventral (**m**) striatal regions. Scale bars: 250 µm for **b**,**d**,**f**,**h** and 500 µm for **n**).

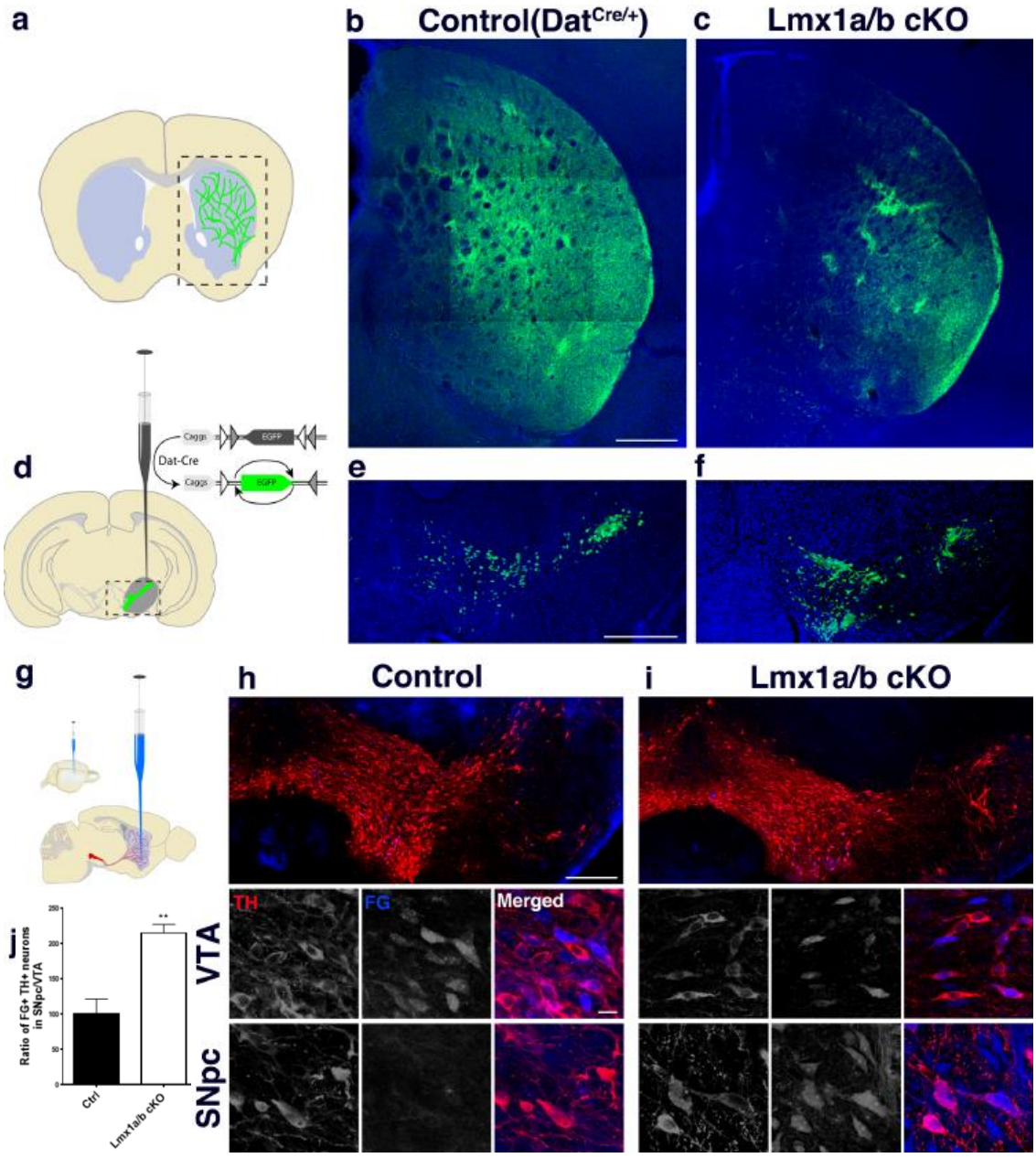


Figure 3: Anterograde and retrograde axonal tracing experiments showing aberrant dopaminergic axonal connections in Lmx1a/b conditional mutants.

(**a** and **d**) Schematic description of the AAV-Flex-eYFP experiment showing the labeling of the axonal projections in the striatum (**a**) following injection of the viral vector in the SNpc (**d**). (**e-f**) Representative confocal images of of the GPF positive cells in the midbrain (AAV-Flex-eYFP in green and DAPI in blue) and the resulting GFP positive axons at striatal level in the control (**b**) and in the Lmx1a/b cKO mutant (**c**). (**g**) Schematic representation of the injection site of the fluorogold retrograde tracer in the Nucleus accumbens. (**h-i**) Representative confocal images of the retrogradly labeled cells in control (**h**) (TH in red, Fluorogold in blue) and in Lmx1a/b cKO mutant mice (**i**). Lower panels show higher magnification in VTA and SNpc. (**j**) Stereological count results representing the ratio of Fluorogold (FG) and tyrosine hydroxylase (TH) positive neurons in the SNpc vs VTA. Scale bars: b-c and e-f, 250µm; h-i upper panel 250µm, lower panels, 15µm.

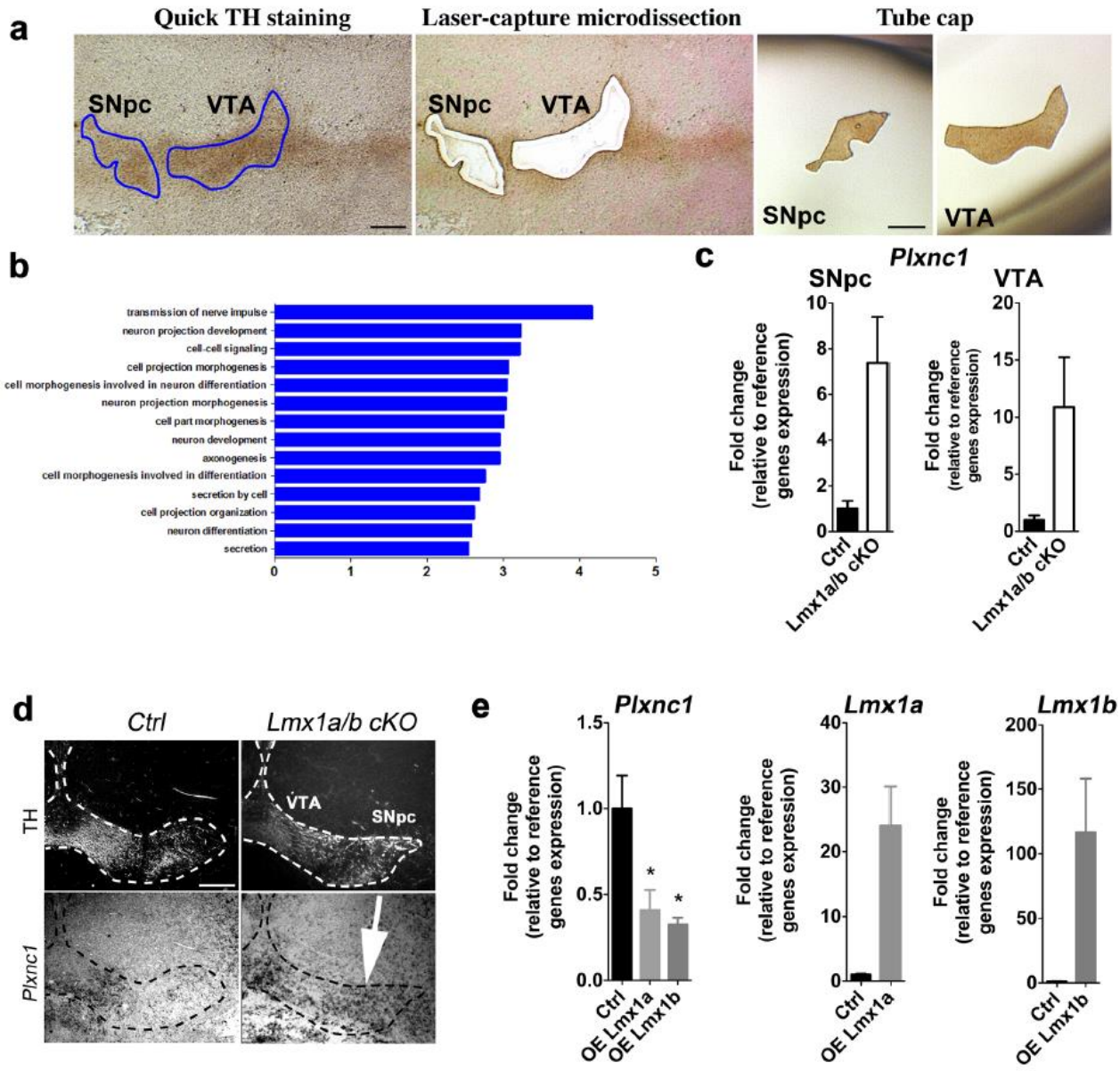


Figure 4: Lmx1a and Lmx1b regulate the expression of the axon guidance receptor.
(**a**) Images of brain tissue section following quick TH staining, before LCM and in the tube cap after LCM. (**b**) GO term enrichment analysis for the identification of Lmx1a/b regulated genes at P1. (**c**) RT-qPCR analysis of *Plxnc1* expression specifically in SNpc and VTA neurons isolated with LCM. (**d**) *In situ* hybridization for *Plxnc1* expression on ventral midbrain coronal sections of control and Lmx1a/b cKO mice. (**e**) RT-qPCR quantification of *Plxnc1* expression after Lmx1a and Lmx1b overexpression in primary mDA neuron cultures. Scale bars: 200µm.

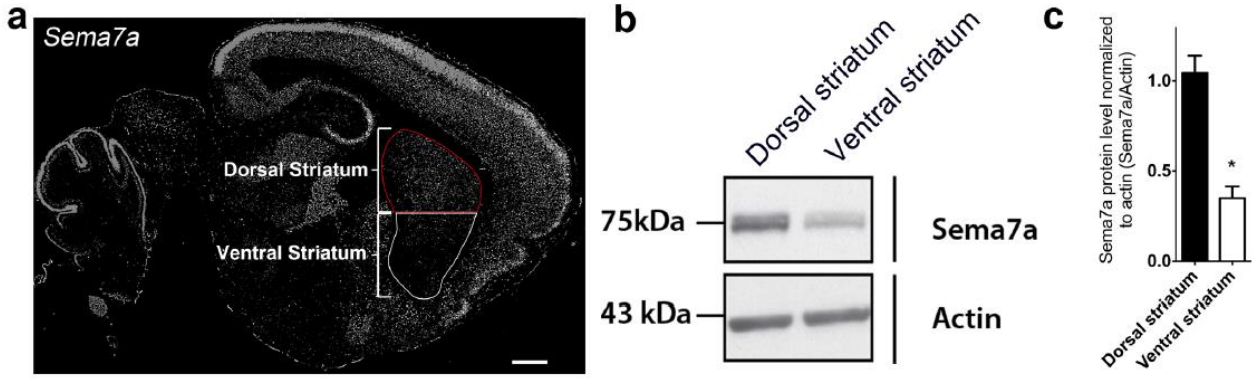


Figure 5: The axonal guidance repellent cue Sema7a expression is localized in the dorsal striatum.
(**a**) In situ hybridization for Sema7a on a sagittal section from postnatal day 4 mouse brain section (from Allen brain atlas). The dorsal striatum (delineated in red) is rich in Sema7a. (**b-c**) Western blot and quantification of Sema7a protein level in the dorsal and ventral striatum at P1. Scale bar: 500 µm.

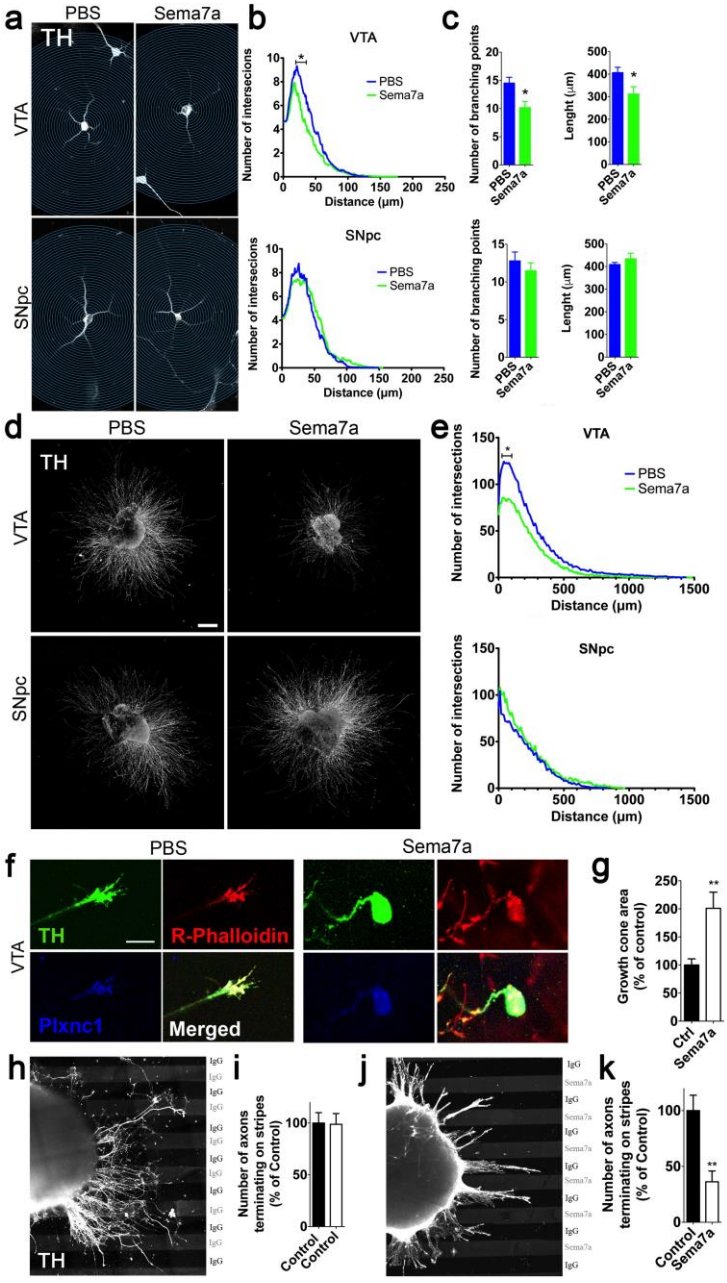


Figure 6: *In vitro* experiments showing the effect of Sema7a on VTA and SNpc neurons.
(**a-c**) Sholl analysis on primary VTA or SNpc neuron cultures exposed to PBS or Sema7a. (**a**) Examples of confocal images of VTA and SNpc neurons with their respective Sholl intersection circle mask. (**b,c**) Quantitative analysis of the VTA and SNpc neurite intersection profiles, number of branching, and neurite length. (**d**) Examples of embryonic ventral midbrain explants from E14.5 Pitx3-GFP embryos grown in collagen matrix then exposed to PBS (Control) or Sema7a. (**e**) Sholl analysis of the neurite intersection profiles for the VTA and SNpc explants exposed to PBS or Sema7a. (**f**) Confocal images of VTA neuron explants growth cones exposed to Sema7a compared to PBS. (**g**) Growth cone size measurements expressed in pourcent of control. (**h-k**) Confocal images of stripe assay and quantification of the number of axons terminating on stripe for VTA explants grown on the control alternating IgG stripes (**h,i**) and the Sema7a stripes alternating with IgG stripes (**j,k**). (N = 4, total of 27 explants for Sema7a and control). Scale bars: a, 20 µm; d,h,j, 250 µm; f, 10 µm.

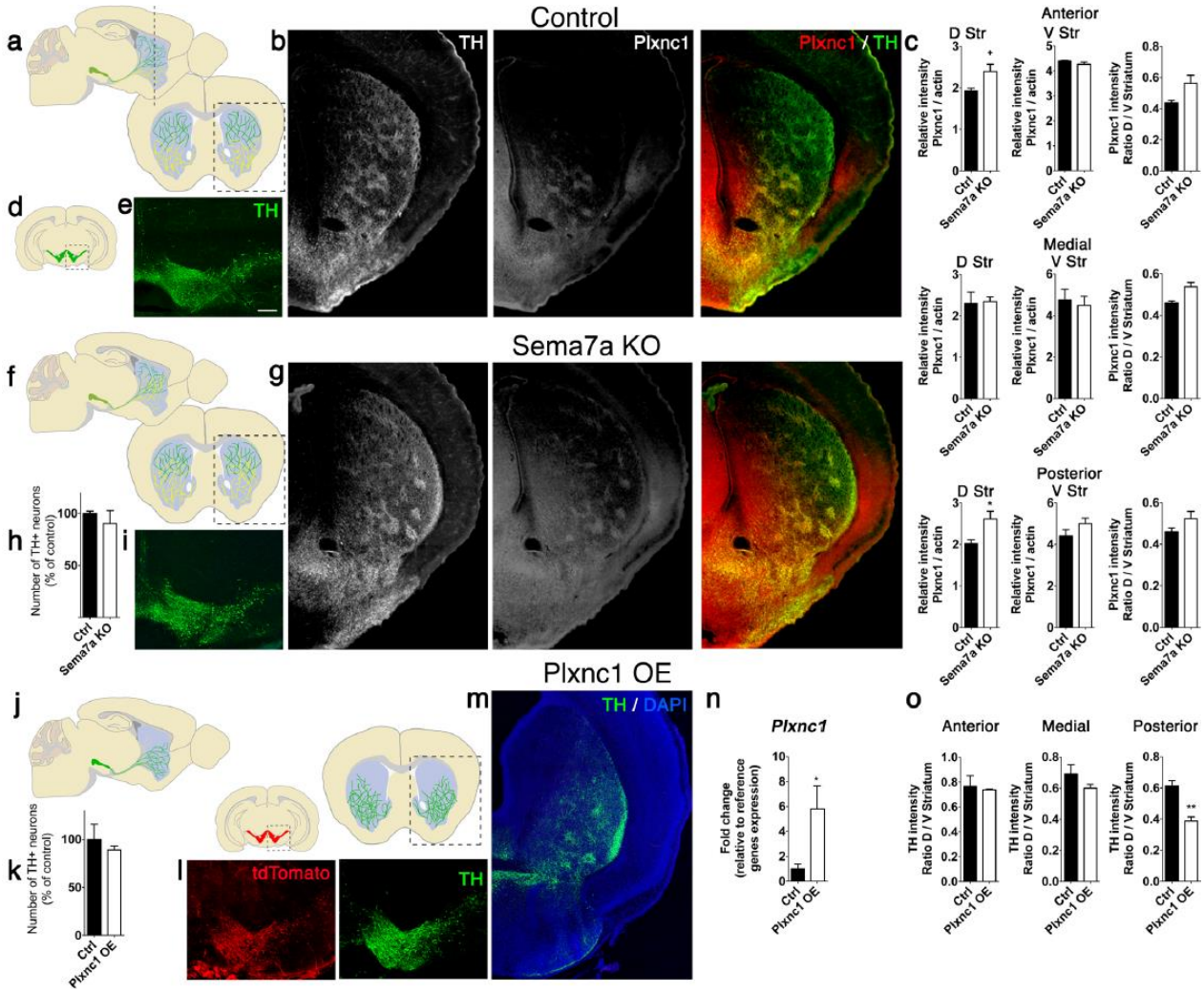


Figure 7: Sema7a/PlexinC1 interaction regulates mDA axon guidance. (**a** and **f**) Schematic of sagittal and coronal views of the axonal projections in the striatum showing that PlexinC1 mDA axons (in yellow) are more numerous in dorso-posterior striatal region of Sema7a KO mice compared to control animals. (**b** and **g**) Confocal images of TH and Plxnc1 immunostained striatal sections from control and Sema7a KO mice. (**c**) Quantification of Plxnc1 relative optical density at different striatum levels. (**d**) Schematic representation of coronal section of mouse brain at the midbrain level. Dashed line indicates the delimitation of the pictures shown in e and i. (**h**) Stereological counts of mDA neurons in control and Sema7a KO mice and representative confocal images of the VTA and SNpc on one hemisphere (**e**,**i**). (**j-m**) Schematic and confocal images of TH labeling in Plxnc1 overexpression mice showing a loss of DA innervation in dorso-posterior striatum. (**k-l**) Stereological counts of mDA neurons in control and Plxnc1 overexpression mice and corresponding representative confocal images. The reported tdTomato in (**l**) indicate that all mDa neurons express the transgene (Plxnc1-ires-tdTomato). (**n**) Rt-pPCR quantification of Plxnc1 from ventral mdbrain section of control and Plxnc1 overexpression mice. (**o**) Optical density measurements of TH axons in the striatum. Graphs show the ratio of TH intensity in dorsal vs ventral striatum at three antero-posterior levels. Scale bars: e,i,l, 200 µm).

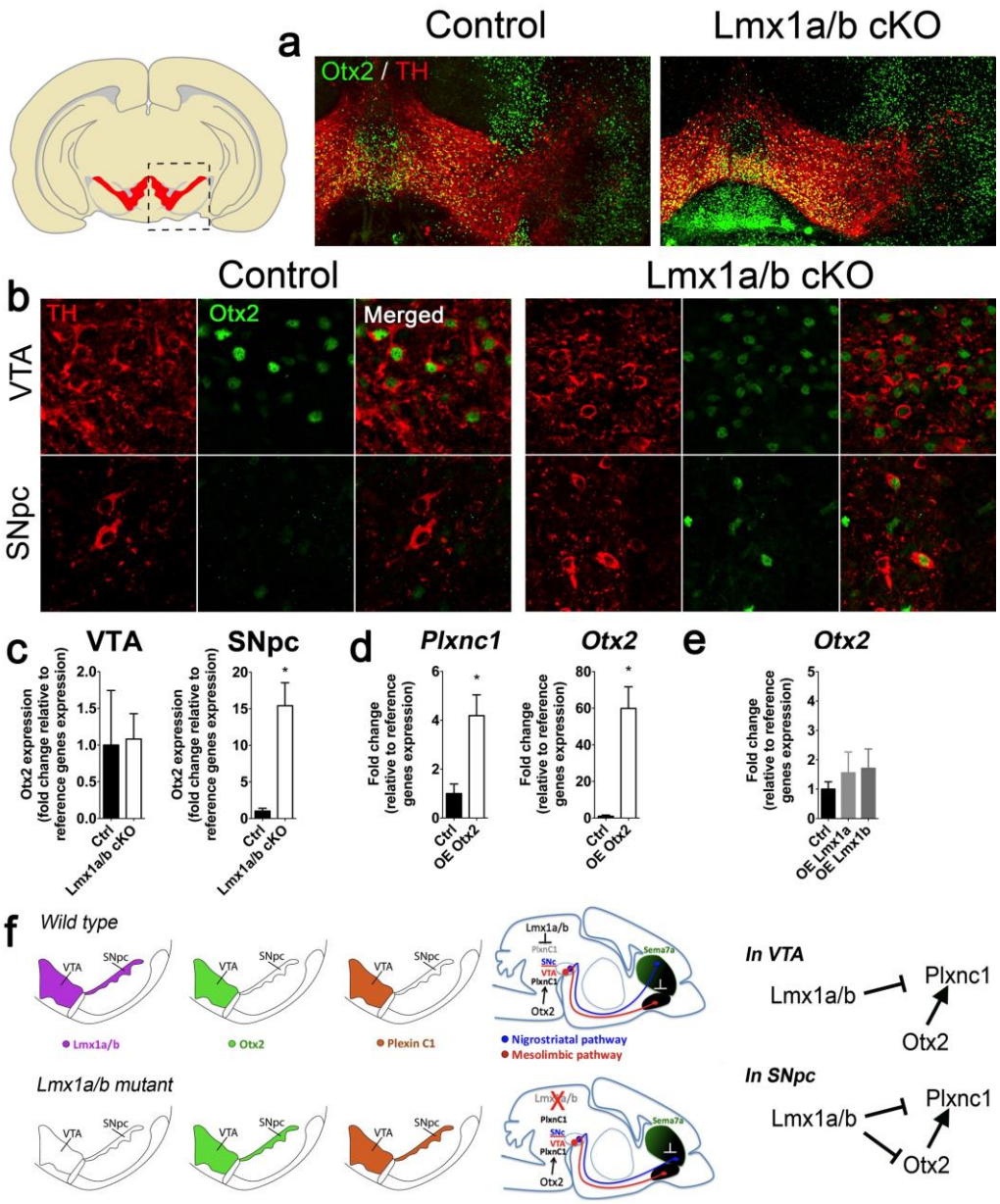
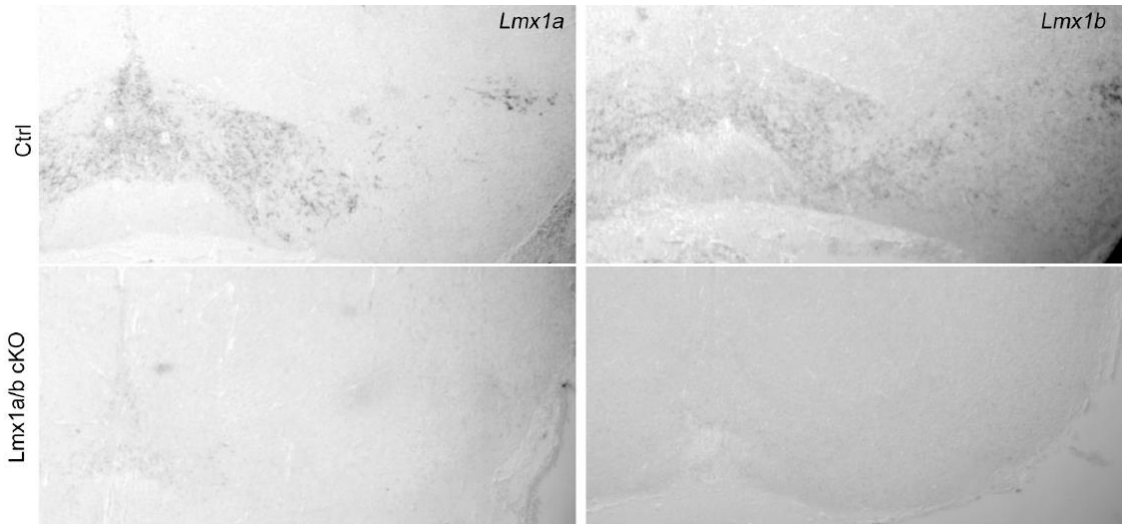
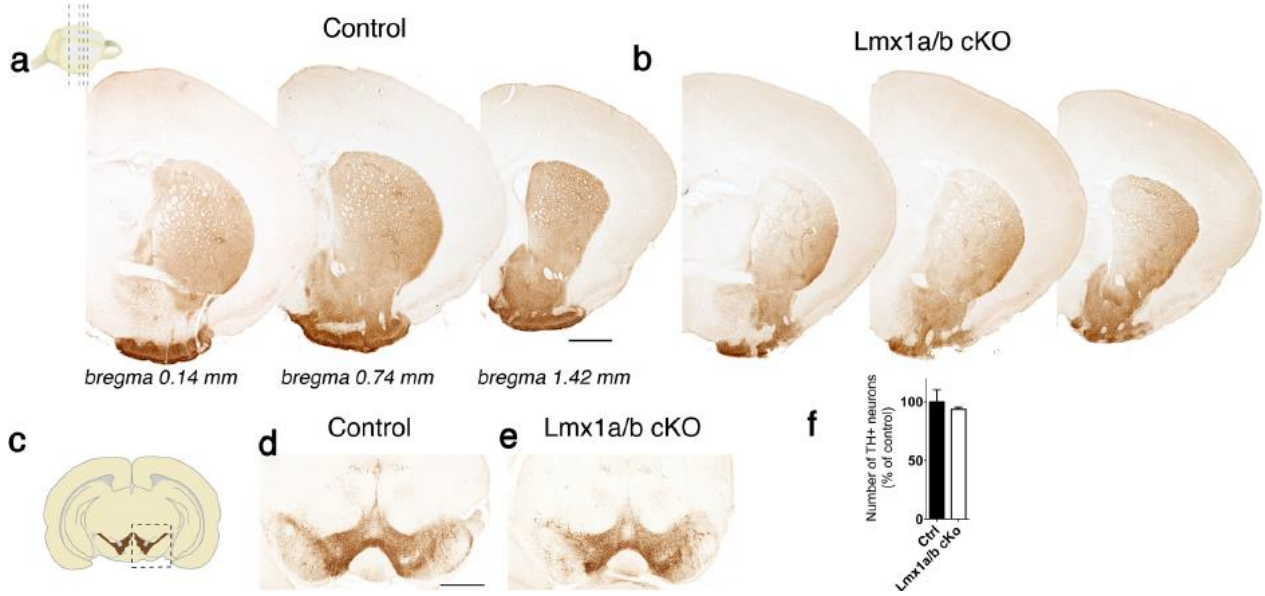


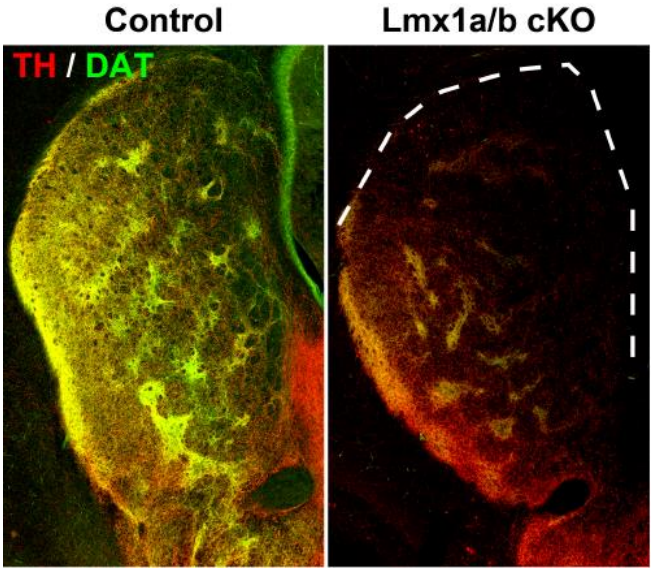
Figure 8: Otx2 controls Plxnc1 expression in the VTA. (**a,b**) Confocal images of Otx2 immunostaining in TH positives cells in coronal mibrain sections of control and Lmx1a/b cKO mutant mice showing ectopic Otx2 expression in SNpc mDA neurons. (**c**) Rt-qPCR quantification of Otx2 in VTA and SNpc isolated from control and Lmx1a/b cKO with LCM. (**d**) Overexpression of Otx2 in mDA primary neurons culture leads to an increase of Plxnc1 expression as quantified by Rt-qPCR. (**e**) Rt-qPCR quantification of Otx2 in mDA primary neurons culture following Lmx1a and Lmx1b overexpression. Scale bars: a, 200µm; b, 10 µm. (**f**) Proposed putative model for genes regulatory network for nigrostriatal and mesolimbic axons development. Based on our data, Plxnc1 expression is negatively regulated by Lmx1a and Lmx1b while Otx2, expressed by VTA neurons, positively controls Plxnc1. In mutant mice lacking Lmx1a and Lmx1b, both VTA and SNpc express Plxnc1 and Otx2. This aberrant expression of Plxnc1 in SNpc neurons makes them responsive to Sema7a (expressed in dorsal striatal region) and causes their axons to grow away from this repellent cue. PlexinC1. In mutant mice lacking both Lmx1a and b, PlexinC1 expression is expanded to all mDA neurons. This aberrant expression by SNc neurons makes them responsive to Sema7a (expressed in dorsal striatal region) and causes their axons to grow away from this repellent cue.



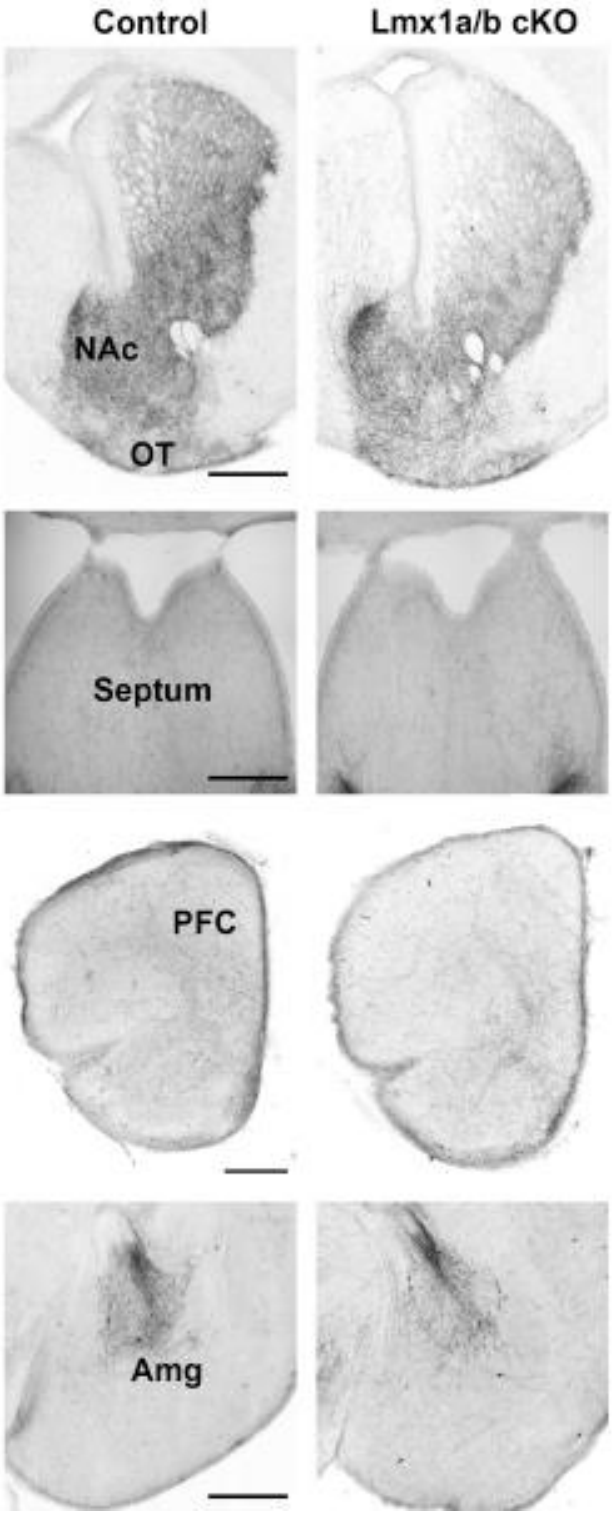
Supplementary Figure 1 : Lmx1a and Lmx1b are deleted in mDA neurons of Lmx1a/b cKO mice. *In situ* hybridization for Lmx1a and Lmx1b on Lmx1a/b cKO and control midbrain sections at P1 showing the absence of Lmx1a and Lmx1b expression in the Lmx1a/b cKO midbrain. Scale bar: 250 µm.



Supplementary Figure 2 : Phenotype analysis of Lmx1a/cKO mutant mice at P15. (**a** and **b**) Representative images of TH immunostaining of coronal striatal sections at P15 in control (**a**) and double conditional mutant mice for Lmx1a/b (**b**) showing a loss of innervation in the dorsal striatum for the mutant. (**c-e**) Schematic and representative images of TH immunostaining of coronal midbrain sections for the control and Lmx1a/b cKO mutant showing no change in the number of mDA cells as quantified by stereological counting of TH-positive cells in (**f**). Scale bar: 250 µm.



Supplementary Figure 3: Lack of TH and DAT expression in Lmx1a/cKO mutant mice. Confocal images of the dopaminergic markers TH (in red) and DAT (in green) in the striatum of control and Lmx 1a/b cKO mutant were performed to attest the loss of dopaminergic innervation using those two specific dopaminergic markers. Scale bar: 200 µm.



Supplementary Figure 4: Extrastriatal targets in Lmx1a/b cKO mutant mice and control.
Representative images of TH stained coronal sections in the extrastriatal dopaminergic targets do not show abnormal DA innervation in Lmx1a/b cKO mice. Abbreviation : nucleus accumbens (NAc), olfactory tubercule (OT), prefrontal cortex (PFC), and amygdala (Amg). Scale bars= 250 µm

Table S1. Differential expression of genes related to axon development identified by RNA sequencing at E15.5

Genes	RNA-seq at E15.5, fold change
Plxnc1	+2.0
Pax6	+2.7
Slitrk5	+2.1
Nefm	-2.1
Bcl11b	+2.1

Chapter 4. Improvement of Cell Based Replacement Therapy for Parkinson's Disease Grafting Neurons Lacking Plxnc1.

Résumé

La thérapie de remplacement cellulaire pour la maladie de Parkinson représente un traitement de choix pour compenser la perte des neurones dopaminergiques de la substance noire (SNpc). L'intégration de neurones dopaminergiques nouvellement générés capables d'innerver spécifiquement les cibles de la SNpc est primordiale pour restaurer la voie nigrostriée. De plus, cette amélioration de la spécificité des neurones implantés permet d'éviter une surexpression de dopamine dans des cibles non lésées pouvant mener à des effets secondaires telle que la dyskinésie. Plxnc1 a été montrée comme une protéine essentielle dans la ségrégation des voies mésolimbique et nigrostriée. La répression de Plxnc1 dans des neurones dopaminergiques différenciés en culture pour implantation permettrait une innervation spécifique des cibles de la SNpc. Notre étude préliminaire a pour but le développement d'une thérapie cellulaire chez un modèle de souris 6OHDA par l'implantation de neurones SNpc-like (répression de l'expression de Plxnc1) au niveau de la SNpc lésée.

Improvement of cell-based replacement therapy for Parkinson's disease implanting newly generated cells repressing Plxnc1 expression.

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Ongoing project

Acknowledgments

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The authors wish to thank Dr Carmen Birchmeier for sharing Lmx1b antibody. We also thank the *plateforme d'outils moléculaires* (https://neurophotonics.ca/fr/pom) for the production of viral vectors.

Abstract

The cell replacement therapy for Parkinson's disease is a treatment of choice to compensate for the loss of dopaminergic neurons of the substantia nigra (SNpc). The integration of new dopaminergic neurons capable of specifically innervate the SNpc targets is essential to regenerate the nigrostriatal pathway. Moreover, this improvement in cells specificity allows avoiding dopamine overexpression in unlesionned targets that may lead to side effects such as dyskinesia. Plxnc1 has been shown as an essential protein in the segregation of the mesolimbic and nigrostriatal pathways. Repression of Plxnc1 in newly generated dopamine neurons for grafting in a 6OHDA mouse model should lead to a specific innervation of SNpc targets.

Introduction

Primary sudies led on cell replacement therapy for Parkinson's disease consisted in fetal ventral mesencephalon grafts injection in the striatum (Lindvall et al. 1989). This strategy was preferred to cells injection in the SNpc, because fetal grafts were not shown as growing axons able to reconstruct the nigrostriatal pathway. However, with improvement of cell sources and tracking methods, more recent research have shown the capacity of mice embryonic stem cells to restore the nigrostriatal pathway in rodents (Gaillard et al. 2009; Thompson et al. 2009).

The last few years focused on developing a new source of cells for grafts. Indeed, human fetal cells represent a very limited source of cells due to the small number of cells possible to obtain from one foetus, and also for ethical reasons. Moreover, evidences shown a poor rate of cell survival after intrastriatal transplantation, and tumorigenic effects were observed (Sonntag et al. 2007; Roy et al. 2006; Deacon et al. 1998; Spiro et al. 1997; Lindvall et al. 2004). Thus, efforts were put together to perform a new pool of cells for transplantation in humans: human embryonic stem (hES) cells differentiated in midbrain dopaminergic neurons. In 2011, Kriks and co-workers shown a good DA survival in parkinsonian Monkeys after hES cells engrafment in the striatum (Kriks et al. 2011). In 2014, a study showed that the efficacy of human ESC-Derived Dopamine Neurons for transplatation in rat model of Parkinson's disease was equivalent to the one observed using fetal neurons when grafting in the SNpc (Grealish et al. 2014).

Grealish et al., demonstrated the ability of ESC-Derived Dopamine Neurons to restore the nigrostrital pathway in rodents (Grealish et al. 2014). However, other targets were also innervated by the newly implanted neurons. Indeed, VTA targets were innervated showing the importance of implanting SNpc-like neurons to avoid any side effect due to a too high release of dopamine in ventral striatum for example. In our study, we aim to show that implantation of neurons in which Plxnc1 expression is repressed, allows targeting more specifically the dorsal striatum. We should be able to have a more efficient innervation of the dorsal striatum in more natural conditions.

Methods

Animals.

The 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. Four month C57Bl6 male mice from Charles Rivers were used for this study.

Tissue Analysis.

Mice were perfused using 4% paraformaldehyde in PBS brains, after dissection mice brains were incubated in 4% paraformaldehyde in PBS at 4°C, followed by cryoprotection in 30% sucrose in PBS, before freezing on dry ice. After cryostat sectioning at 60µm, sections were washed in PBS, then blocked in 1% normal donkey serum (NDS) for at least 30 min. Primary antibodies used in this study were : rabbit anti-TH (Pel-Freez, 1:1000), sheep anti-TH (Millipore, 1:1000), rabbit anti-GFP (Abcam, 1:1000), mouse anti-mCherry (Millipore, 1:1000), Rat anti-DAT (Millipore, 1:500), sheep anti-Plxnc1 (R&D systems, 1:150); rabbit anti-Lmx1a (Millipore, 1:1000); guinea-pig anti-Lmx1b (Gift from Dr Carmen Birchmeier, Max Delbruck Center of Molecular Medicine, Berlin, Germany, 1:100); Goat anti-Otx2 (R&D systems, 1:200), mouse anti-Tyrosine tubuline (Sigma, 1:3000); goat anti Foxa2 (Santa Cruz, 1:100); rabbit anti-Pitx3 (Gift from Jaques Drouin, IRCM, 1:500); mouse anti-calbindin (Sigma, 1:3000); rabbit anti-Girk2 (Alomone Labs, 1:400); rabbit anti-MAP2 (Millipore, 1:1000); guinea-pig anti-Sox6 (Gift from Dr, Perlmann, 1:1000). Secondary antibodies used in this study are donkey Alexa-Fluor-488, donkey Alexa-Fluor-568, or donkey Alexa-Fluor-647, were used at 1:200 (Jackson immunoresearch).

6OHDA Lesion

The 6-OHDA lesion was performed as previously described by Thiele, S.L., et al., 2012 (Thiele et al. 2012).

Solutions preparation

[Desipramine]=2.5 mg/ml; administer 10 ml/kg to the animal.

Ascorbic acid 0.02 % in sterile saline 0.9 %

6OHDA solution, use it at [6OHDA]=6 mg/ml, vortex the solution until all powder is dissolved, be careful keep it in foil on ice.

Procedure

Put mice under anesthesia 4% isoflurane 500 ml/min, inject Desipramine 20 minutes before 6OHDA injection. Correctly fix mouse head with teeth and ear bars. Disinfect injection site by 3 alcohol/chlorhexidine 0.5% washings. The skin was opened, and coordinates of injection were defined from the skull for cells injection in the MFB: AP=-1,2 ML=1.1 DV=-5 (measured at the skull). Fulfill the pipette with 6OHDA solution at the last minute. Inject 250nl at 5nl/sec, let the pipette in place for 5 minutes before removing the pipette. Then suture the skin and administer 1 ml of saline

ESC-Derived DA Neurons Culture.

We generated ESC-derived DA neurons from E14TG2A stem cells obtained from Dr. Metzakopian. Culture and differentiation of those cells in DA neurons was realized as previously described (Jaeger et al. 2011; Metzakopian et al. 2015). Briefly, after expansion of ES cells on FBS in the following medium : 420 ml DMEM high glucose (Invitrogen 41965-039), 15 % FBS (Invtirogen), 10 ml 1 % Penicillin/Streptomycin (Invitrogen 15140-114), 5 ml, 1 % L-glutamine (Invitrogen 25030-024), 5 ml 1 % Non-essential amino acids (Invitrogen 11140-035), 5 ml 1 % Sodium-Pyruvate (Invitrogen 11360-039), 5 ml β-Mercaptoethanol (Sigma), 4 µl LIF (10^7 u/ml) (ESGRO) 55 µl, a new medium (called N2B27) is added to the cells to obtain Epiblasts (100 mL Neurobasal media (Invitrogen, 21,103); 100 mL DMEM/F12 (Invitrogen, 21,331); 2 mL 50X B27 (without vitamin A; Invitrogen, 12587-010); 1 mL 100X N2 (Invitrogen, 17502-048); 200 µL 2-Mercaptoethanol (Invitrogen, 31350-010); 2 mL L-Glutamine P/S (Invitrogen, 10,378) : this medium is also called N2B27 medium, plus 1.2 µL/mL media of 10 µg/mL FGF2 (Peprotech, 110-18 b); 0.8 µL/mL media of 25 µg/mL Activin A (R&D, 338-AC-025)). After at least 2 weeks in this stage, differentiation in DA neurons start on day 0 by using N2B27 medium plus PD0325901 at 1µM final concentration for 2 days. Then cells are passed on precoated wells with poly-L-Lysine and laminin on day 2. Cells are kept until day 5 in N2B27 medium. On day 5, add Shh agonist (SAG, Alexis biochem ALX-270-426-m001) at final concentration 100nM and Fgf8 (R&D 423-F8-025) at 100 ng/mL. On day 9, add

growth factors : BDNF (Peprotech 450-02) at 10 ng/mL, GDNF (Peprotech 450-10) at 10 ng/mL, and ascorbic acid (SIGMA A4544-25G) at 1/1000 from 200 mM stock.
Those newly generated dopaminergic neurons were characterized using immunocytochemistry for well described dopaminergic factors: TH, Lmx1a, Lmx1b, Otx2, Tubuline, Foxa2, Pitx3, Calbindin, Girk2, MAP2, Sox6.

The expression of Plxnc1 in these ESC differentiated in dopaminergic neurons was characterized by western-blotting.

Epiblasts Infection

In order to generate cells of interest in which Plxnc1 is repressed, epiblasts were infected with respectively the following lentiviral constructs from the Plateforme d'outils moléculaire: pHAGE-CBA-tdTomato-WPRE (9098 bp); pHage emCBA Plxnc1 (11,618 bp); pGFP-C-shLenti-Scrambled (5,8E9 TU/mL, TR30021 from Origene). Thus, cells repressing Plxnc1 express Tomato, control cells infected with the sh scrambled construct epress GFP, and the overexpression of Plxnc1 is performed in cells already infected with te sh scrambled construct in order compare cells expressing the sh construct in all cases. Briefly, medium was removed from epiblasts reaching about 50% confluence, and replaced by 1 ml cold medium containing virus at 100 multiplicity of infection (100 MOI) for 2 hours. Then medium containing the virus is removed and replaced by common epiblast medium. The following lentiviral constructs from the Plateforme d'outils moléculaire were used: pHAGE-CBA-tdTomato-WPRE (9098 bp); pHage emCBA Plxnc1 (11,618 bp); pGFP-C-shLenti-Scrambled (5,8E9 TU/mL, TR30021 from Origene).

Western blot

After infection, ESC-Derived DA neurons were characterized by western blotting. Cells were harvested in RIPA complemented with protease inhibitor and phosphatase inhibitor. Harvested cells were kept on ice for 30 min before sonication and centrifugation. Cell lysat was transfered ta a new tube and quantified using Microplate DC Protein Assay. After taking the volume necessary to load 15µg proteins on gel, loading buffer and RIPA were added to the samples for heating 5 minutes at 95°C. The same quantity of proteins and

volumes were loaded on precast gel migration (gel 12%). Following migration, protein transfer was performed on a nitrocellulose membrane 0.45µm using Biorad 10-minute transfer. Membrane was blocked TBS-Tween, 5% milk for 30 to 60 minutes, RT, with shaking. The primary antibodies were added to the membranes: sheep anti-Plxnc1 1:150; mouse anti-Actin 1:20000 for incubation overnight at 4ºC. After washings in TBS-T 1X, RT, with shaking, membranes were incubated with the secondary antibody: HRP-Conjugate Donkey anti-sheep IgG 1:5000 (Santa Cruz); HRP-Conjugate Goat anti-mouse IgG 1:5000 (Life Technologies), for 1 to 2 hours, RT, with shaking. After washings, membranes were incubated for 1 minute in Enhanced Chimioluminescence (ECL, BioRad). Membranes were exposed to Pierce CL-Xposure film (Thermo Scientific). Films were scanned and analyzed using the Image Studio Lite ver5.2.

Behavioral Tests.

Two weeks after 6-OHDA lesion in the medial forebrain bundle, lesion efficacy was assessed using cylinder test. Only mice doing rotations to the ipsireversive side of the lesion were kept for further experiments.

Cylinder and open field tests were performed on mice every 2 weeks after cell or vehicle injections.

Injection of ESC Differentiated in mDA Neurons in 6OHDA Lesioned Mice

Cells preparation

Medium N2B27+BDNF+GDNF+Ascorbic acid without P/S L-Glutamine was prepared for cells used for transplantation. Cells were scratched then counted in trypan blue to accurately determine the number of healthy cells for injection. Then cells were centrifuged 2 min 30 s at 1000 RPM and resuspended in the correct volume to have a concentration of 400,000 cells in 1.5 µl.

Procedure

Mice were under anesthesia 4% isoflurane 500 ml/min air, and the mouse head was correctly fixed to the stereotaxic set up using ear bars. After disinfection of the injection site by 3 alcohol/chlorhexidine 0.5%, the skin was opened, and coordinates of injections were defined in the skull for cell injections in the SNpc: AP=-3 ML(Z)=1.3 DV(X)=-3.8 (measured at the skull). 1.5µl of cells were injected using a motorized machine at

5nl/second. Wait 5 minutes after cells injection before removing pipette, and suture the skin before injecting 1 ml of saline in the animal.

Results

Differentiation of Mice Embryonic Stem Cells into mDA Neurons

We generated ESC-derived DA neurons from E14TG2A stem cells (Jaeger et al. 2011; Metzakopian et al. 2015). After expansion of ES cells, a new medium containing FGF2 and Activin A is added to the cells to obtain Epiblasts. After at least 2 weeks in this stage, differentiation in DA neurons start on day 0 by using N2B27 medium plus PD0325901 at 1µM final concentration for 2 days. Then cells are passed on precoated wells with poly-L-Lysine and laminin on day 2. Cells are kept until day 5 in N2B27 medium. Then, multiple factors identified during mDA development were administered to the cells at different time points. As described Figure 21, from day 5 to day 9 the Shh agonist, SAG, and Fgf8 are added to the medium to induce the differentiation into DA neurons. At day 9, BDNF, GDNF, and ascorbic acid are added to the medium.

Differentiated cells express main factors of mature mDA neurons. The characterization of these cells differentiated into mDA neurons was performed using immunostainings for these factors expressed in mDA neurons: Lmx1a, Lmx1b, Pitx3, Map2, TH, Foxa2, Girk2, Calbindin, Nurr1, TH, Otx2, Tubuline, Foxa2, Sox6.

Generation of SNpc-like Neurons for Reconstruction of the Nigrostriatal Pathway

In chapter 3 we highlighted Plexnc1 as an important axon guidance receptor involved in the segregation of the nigrostriat and mesolimbic pathway. We observed that only neurons in which PlexinC1 expression is repressed are able to target the dorsal striatum containing a high concentration of Sema7a ligand of PlxnC1. Indeed, VTA neurons expressing Plxnc1 target exclusively the ventral part of the striatum due to a chemo repulsion induced by Sema7a/Plxnc1 interaction. Thus, in the case of cell replacement therapy for PD we are trying to reconstruct the nigrostriatal pathway lost following the degeneration of SNpc neurons. We put effort in generating SNpc-like neurons which could replace those degenerated without creating an overload of dopamine in VTA targets. In order to

generate these mDA neurons repressing PlexinC1, we infected cells with a lentivirus containing the construct sh Plxnc1 and a puromycin cassette. We added puromycin in the cell medium in order to culture only cells containing the construct sh Plxnc1. The size of this construct being too big to insert a fluorescent marker, we co-infected these cells with a retrovirus expressing tomato. Then, we used fluorescence-activated cell sorting (FACS) to keep only cells expressing both the sh Plxnc1 construct and the fluorochrome Tomato. Moreover, we generated cells overexpressing Plxnc1 in order to observe the pattern of migration of both cells types. Like for the sk PlxnC1 construct, the construct for PlxnC1 overexperession was too big to integrate a fluorescent marker, thus we infected Epiblast already expressing the sh scrambled construct with the green fluorescent protein (GFP) as a marker.

The expression of Plxnc1 was determined by western blotting in order to be able to compare for next steps with cells repressing and other overexpressing Plxnc1.

Western blotting results from cells infected with the sh-Plxnc1 construct show a significative decrease for Plxnc1 expression. Then, these cells were injected in the SNpc of mice brain as shown Figure 23.

Mice behavior after cells injection

Three different groups of mice were injected: sham (8 mice), mice 6-OHDA lesioned followed 9 weeks later by vehicle injection (7 mice), and mice 6-OHDA lesioned followed 9 weeks later by injection of a mix of ESC differentiated in DA neurons infected with sh-scrambled and sh-Plxnc1 plus Tomato (12 mice).

The characterization of the lesion was done 2 weeks after 6OHDA injection using the cylinder test. Only mice doing 100% ipsilateral rotations were kept for this study. However, the lesion for mice in the batch with vehicle injection seemed less severe, than the other mice used for cells implantation. This observation was confirmed by the open field test as shown in Figure 24. Indeed, we observe a trend showing a better motor activity for the group used as control for vehicle injection. In order to avoid any quick conclusion comparing the group vehicle and the cells injected group I compared the motor activity of cells injected mice between itself at different stages. The Figure 25 shows the evolution of motor behavior for mice injected with cells. However, we can observe that between two

and 9 weeks after injection a natural recovery of motor behavior happens. Unfortunately, the results after cells injection do not show a real improvement for mice due to the cells implantation. We will have to wait until the sacrifice of those mice, 10 weeks after cells injection to see if any nigrostriatal pathway reconstruction happened.

Discussion

This study aims to highlight the importance to differentiate neurons into the right kind of DA neuron in order to increase efficacy of cell replacement therapy for PD. Moreover, by generating specific neuron type we avoid problems due to non-permissive growth territory for outgrowing axons (Nikkhah et al., 1995; Bentlage et al., 1999). Indeed, during development axons are guided by axon guidance molecules having repulsive or attractive effects depending to their concentration or of the kind of receptors present at the growth cones surface membrane. Thus, by generating SNpc like neurons repressing Plxnc1 expression we give the correct cues to axons to innervate the dorsal striatum harbouring a high concentration of Plxnc1 ligand: Sema7a.

Once this proof of concept will be finished in mice using ESC-derived DA cells, the next step would be to do the same study using human cells differentiated from fibroblasts into DA neurons to reimplant in lesioned rats, or in MPTP monkeys. This way we would be able to test the transdifferentiation of fibroblasts into DA neurons and the effect of the repression of Plxnc1 in those cells. This strategy developed for clinical trials would be of real interest decreasing the risk of immunological rejection (Takahashi & Yamanaka 2006). A lot of improvement for the transdifferentiation of fibroblasts into DA neurons was achieved in the past decade. For example, Mitchell and co-workers, succeed in a direct conversion process of primary adult human fibroblasts (hFib) to neural progenitor cells (NPC) using only the Oct4 factor (Mitchell et al. 2014). This strategy would be even more interesting than transdifferentiation, because we could isolate specifically human fibroblast-derived DA progenitor cells by cell sorting using a floor plate marker, CORIN, avoiding tumorigenic content or inappropriate cell transplantation (Doi et al. 2014).

In order to show the reinnervation of newly generated dopaminergic SNpc-like neurons to their targets, we are using 4 months C57Bl6 male mice from Charles Rivers. We

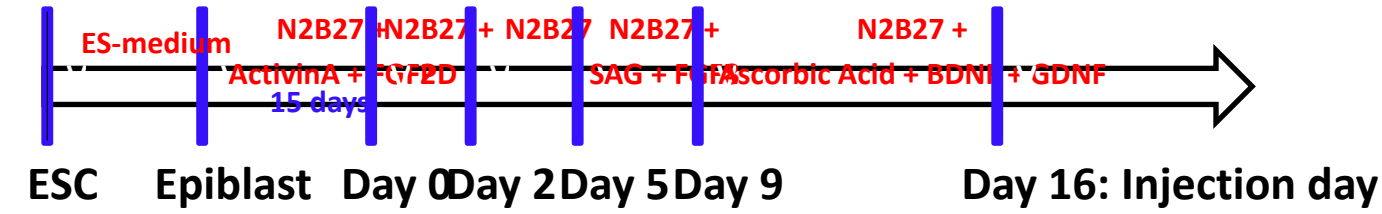
specifically use males to try to avoid hormonal effect and attest that a possible nigrostriatal reconstruction would be only due to the newly implanted cells. Indeed, several studies shown an effect of estrogens in PD patients with an anti-dopaminergic effect of estrogens described in PD patients treated with L-DOPA (Session et al. 1994). In female PD patients showing side effects like dyskinesias due to an increase in medication, those symptoms were worsened few days before their menses corresponding to their lowest serum estradiol levels showing the anti-dopaminergic effect of estrogens (Session et al. 1994). A study showed that especially SNpc mDA neurons are regulated by estrogens in primates (Leranth et al. 2000). In their study, Leranth *et al*., ovariectomized female monkeys and show a loss of over 30% of mDA neurons from SNpc after 30 days, that they were able to restore with estrogen replacement only if performed 10 days after ovariectomy (Leranth et al. 2000). Moreover, in a recent paper Nishimura et al., showed that estradiol, *via* activation of integrin α5β1, leads to a better integration of iPSC-derived DA neurons into striatal neuronal circuits (Nishimura et al. 2016). Although, hormonal therapy has previously been tried in the past for PD, it may be interesting to pursue studies to define a right estradiol concentration to inject with newly generated neurons.

The 6-OHDA lesion model that we used was performed as previously described by Thiele *et al*., by injection in the MFB. This strategy was preferred to lesions in the SNpc or striatum to avoid inflammation at the place of cells grafting in the SNpc, or in the striatum where newly generated axons are supposed to terminate. However, as mentioned by Bagga et al., the reproducibility for MFB lesions in mice is less important than with striatal 6-OHDA injection (Bagga et al. 2015). Moreover the MFB lesion is more intense than the striatal lesion. Indeed, with MFB lesion we observe near to a total loss of innervation in the striatum whereas striatal injection of 6-OHDA is less aggressive innervation in the ventral striatum is still present. For the first attempt of implantation of newly generated DA cells we did not manage to see innervation in the striatum, we hypothesized that difficulties in reinnervation could be due to difficulty for axons to regrow the MFB in an environment where all innervation has been lost. Thus, in case we still do not manage to see axonal growth in our last batch of mice lesioned in the MFB, we decided to start a new batch with striatal 6-OHDA lesion.

As mentioned on Figure 25 an important variability exists between 2 groups of 6OHDA lesioned mice. This variability invalids behavioral tests which could attest of the ongoing reconstruction of the nigrostriatal pathway. Thus, the 6OHDA mouse model may not be the best model, we could try to use transgenic mice model showing a degeneration of SNpc neurons such as double Lmx1a/b cKO mutant mice previously described (Doucet-Beaupré et al. 2016). This way we would overcome these problems of variability in the lesion and need fewer animals.

Figures

A


ES-medium
N2B27 +
ActivinA + FGF2D
15 days
N2B27 +
N2B27 +
SAG + FGF4
N2B27 +
Ascorbic Acid + BDNF + GDNF
ESC
Epiblast
Day 0
Day 2
Day 5
Day 9
Day 16: Injection day

B

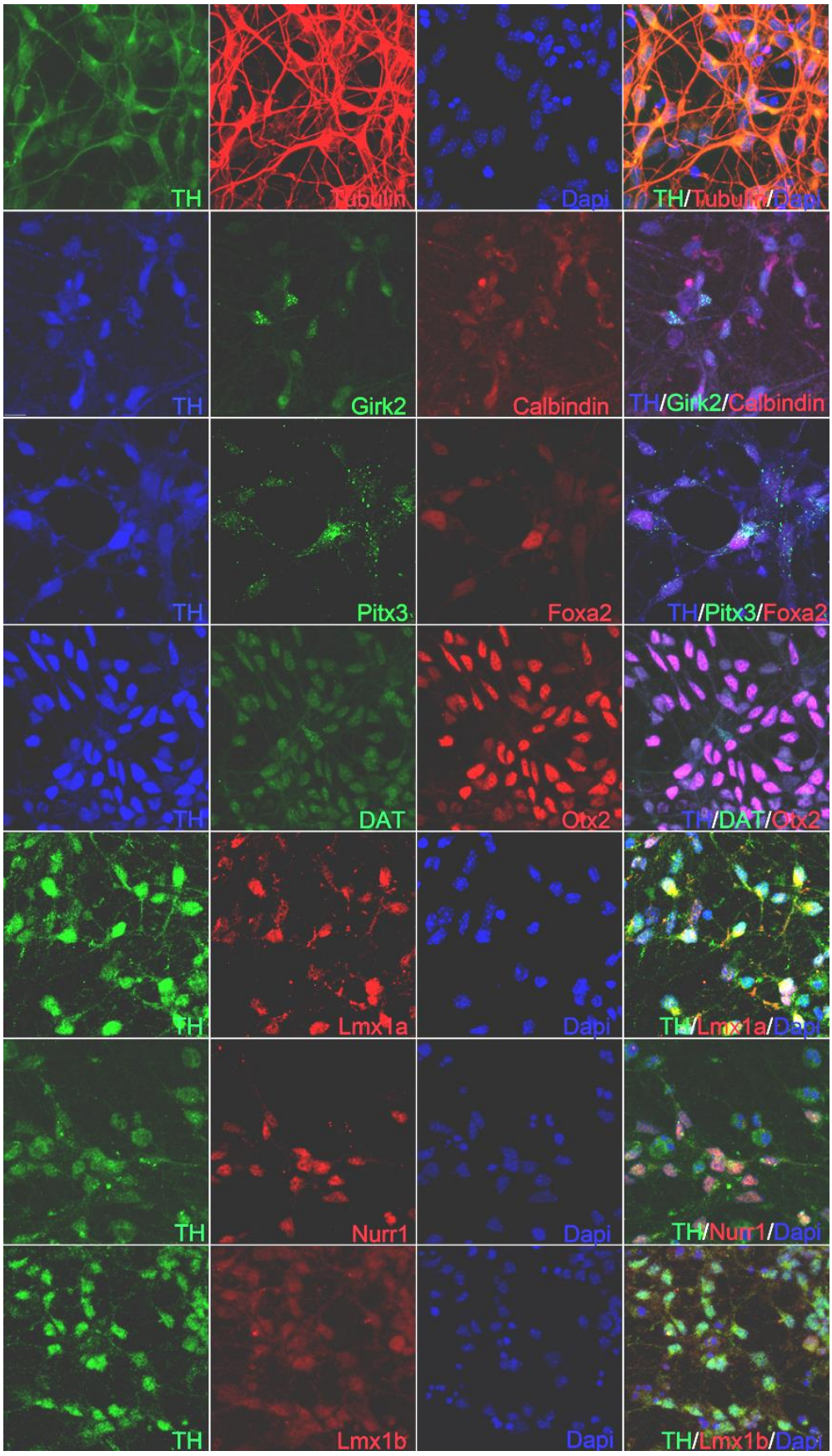

TH
Tubulin
Dapi
TH/Tubulin/Dapi
TH
Girk2
Calbindin
TH/Girk2/Calbindin
TH
Pitx3
Foxa2
TH/Pitx3/Foxa2
TH
DAT
Otx2
TH/DAT/Otx2
TH
Lmx1a
Dapi
TH/Lmx1a/Dapi
TH
Nurr1
Dapi
TH/Nurr1/Dapi
TH
Lmx1b
Dapi
TH/Lmx1b/Dapi

Figure 21 Differentiation of ESC into mDA Neurons.
A represents the different steps for the differentiation of ESC into mDA neurons. B encompasses different immunostainings performed on ESC-Derived DA neurons at day 16 of neuronal differentiation: TH/Tubulin, TH/Girk2/Calbindin, TH/Pitx3/Foxa2, TH/DAT/Otx2, TH/Lmx1a/Dapi, TH/Nurr1/Dapi, TH/Lmx1b/Dapi. These different markers show that not all cells are fully differentiated in DA neurons yet.

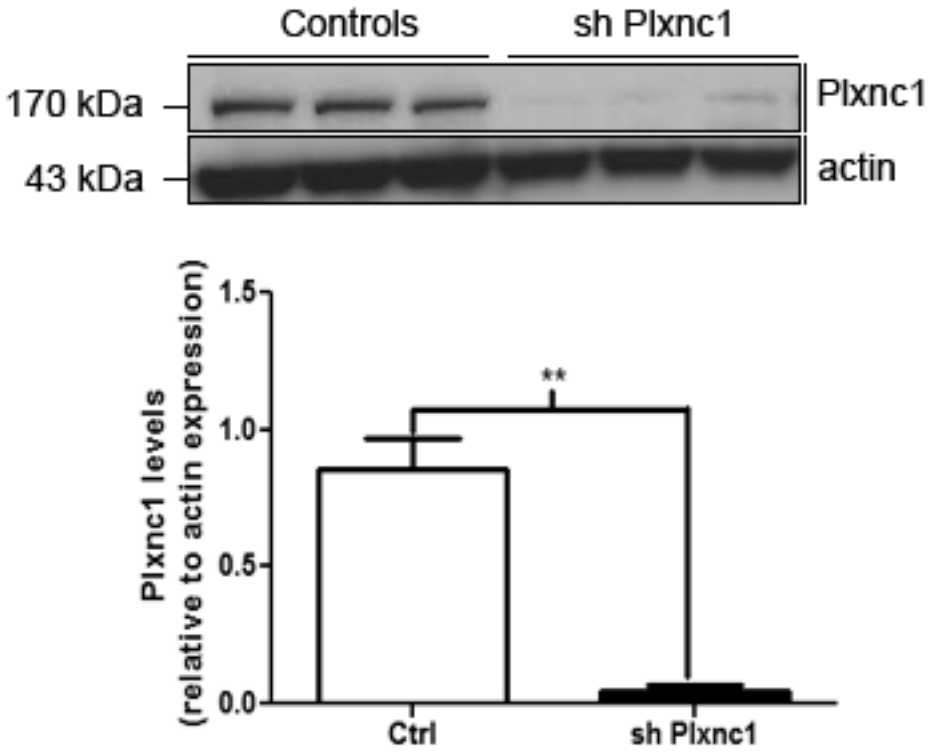


Figure 22 Modulation of Plxnc1 expression in ESC-derived DA neurons.
ESC-derived DA neurons were harvested on day 16 of differentiation for characterization of Plxnc1 expression by western blotting. Mouse-Plxnc1 band is observed at 170kDa.

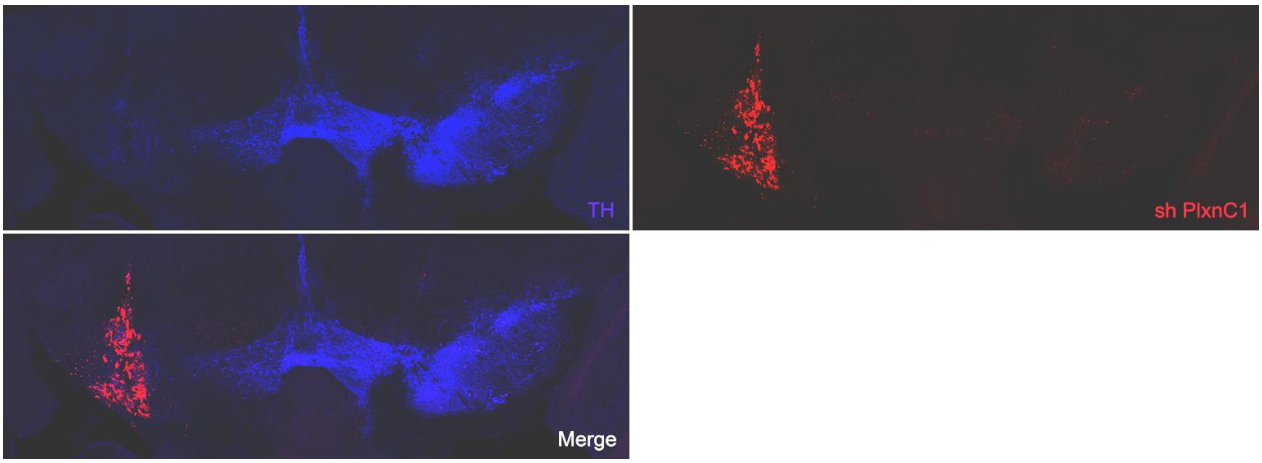


Figure 23 MFB 6-ODA Lesion in Mice.
First batch of MFB lesion in mice leading to a loss of SNpc and VTA neurons as shown with TH immunostaining. A first trial of ESC-derived DA neurons sh Plxnc1 injection was performed in the SNpc as shown in red.

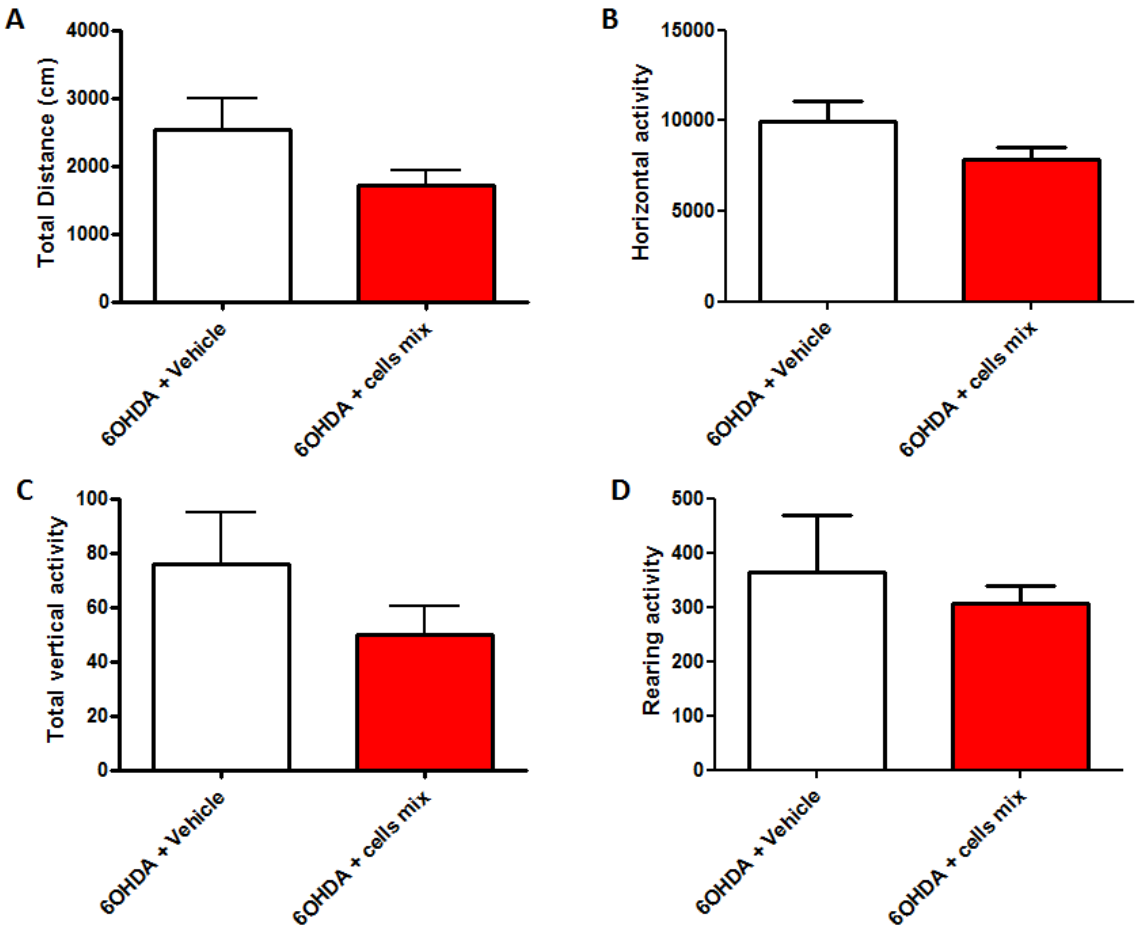


Figure 24 Comparison between 2 groups 6OHDA lesioned mice.
An open field test was performed on 2 groups of mice 2 weeks after 6OHDA injection. The first group has been used 9 weeks after for vehicle injection, and the second one for cells injection. In A is represented the coveredl distance for 1 hour; B represents the horizontal activity of both groups; C shows the vertical activity, and D the rearing activity for each group.

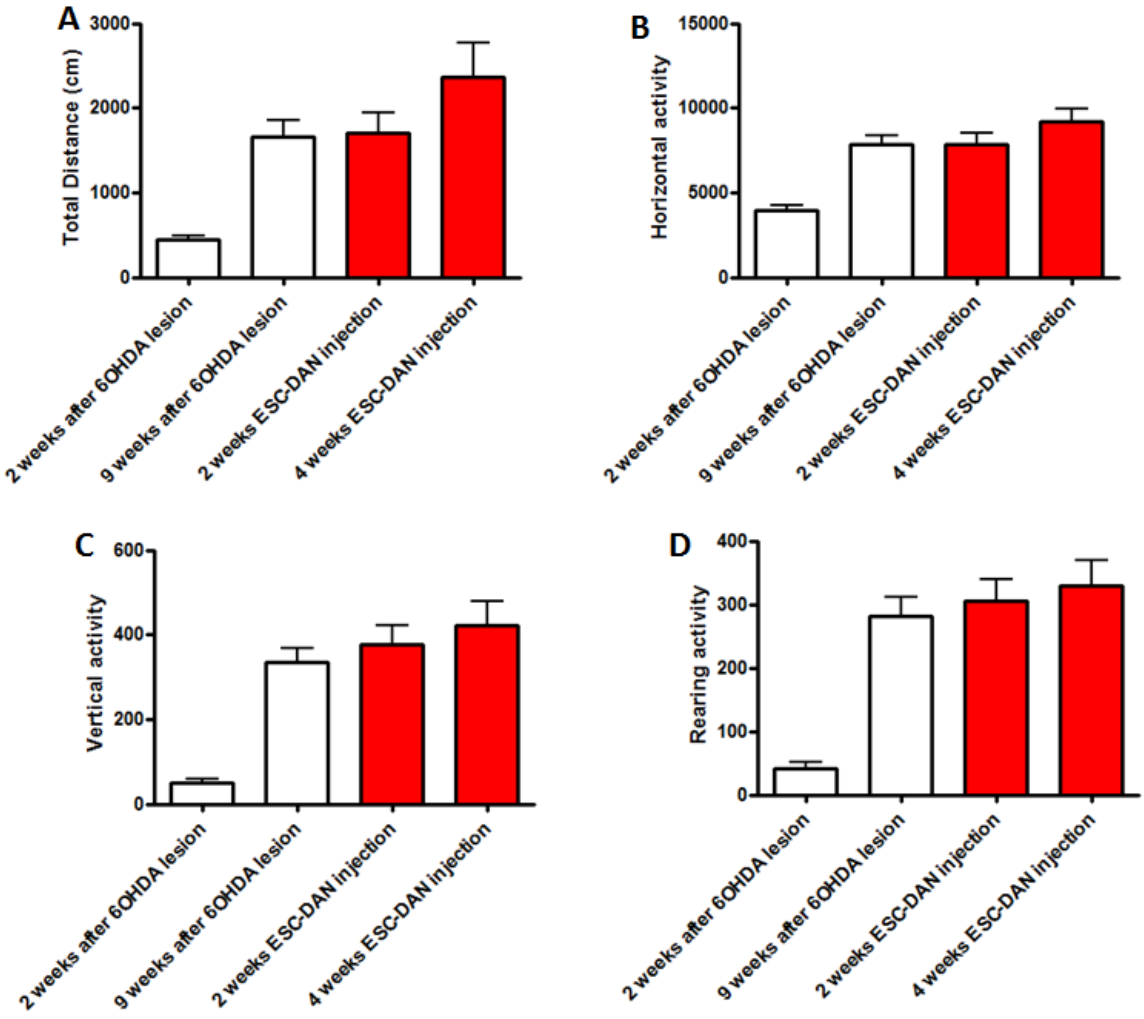


Figure 25 Evolution of motor activity in mice after MFB 6OHDA lesion and cells injection.
In A is represented the coveredl distance for 1 hour; B represents the horizontal activity of both groups; C shows the vertical activity, and D the rearing activity for each group. For all those measurements no significative difference is observed when comoaring to 9 weeks after lesion.

Chapter 5. Discussion

In this study, the role of the transcription factors Lmx1a/b was studied in post-mitotic neurons. Indeed, until now, a lot of studies investigated the role of Lmx1a/b in early development for the differentiation in mDA neurons (Blaess & Ang 2015). The last few years, efforts were enhanced to find new molecules, new regulators for mDA neurons differentiation in order to be able to develop the best cocktail of molecules for transdifferentiation of fibroblasts into mDA neurons, or differentiation of hiPSCs in mDA neurons for cell replacement therapy for PD (Huangfu et al. 2008; Doi et al. 2014; Mitchell et al. 2014). Recent studies also described the role of Lmx1a/b in maintenance and survival of mDA neurons by regulating mitochondrial genes and autophagic-lysosomal processes (Laguna et al. 2015; Doucet-Beaupré et al. 2016). Thus, our study is the first to describe a regulatory role of Lmx1a/b in mDA circuit development, and bring a new view on the functions regulated by Lmx1a/b.

In order to reach conclusions on the role of Lmx1a/b in mature neurons we needed to develop an appropriate technique allowing us to identify targets gene regulated by Lmx1a/b.

5.1. Pros and cons LCM combined with rapid immunolabeling

The second chapter describing LCM combined with rapid TH immunolabeling for RNA extraction from a specific cell subtype was used in this study to extract RNA from SNpc and VTA cells. This technique is powerful because it can be applied to any tissue; just a good antibody is required compared to the necessity to have transgenic mice expressing fluorescent proteins for techniques like FACS. Moreover, the LCM on stained sections allows keeping the spatial organization of cells, which is not possible when using the FACS which requires a step of dissociation. However, the FACS is much more adapted to high-throughput screening experiments (Poulin et al. 2014). Indeed, after identification by their fluorophore, a specific number of cells can directly be loaded on plates for screening. Thus, when the hypothesis for a study is clear due to the observation of a phenotype, and that a good antibody exists to target the region of interest; LCM on stained sections represents the best approach. However, as soon as several cell populations are

concerned, and that the study aims to characterize all molecules expressed by these cells, it is worth it to invest in a transgenic mouse model. The FACS method with transgenic mice will lead to a gain of time and reproducibility due to the accurate number of cells which can be loaded in all wells from a 96 wells plate, for example. However, this method needs to be followed by several stainings to localize the cells according to their molecular signature.

From LCM combined with quick TH staining performed on midbrain sections from mice lacking Lmx1a/b in mature neurons and their associated controls, we identified more than 500 targets for Lmx1a/b. According to the phenotype defect observed in the mutant harbouring a loss of innervation in the dorsal part of the striatum, we looked for targets involved in axon guidance. Plxnc1 a known axonal guidance protein appeared as an interesting target to investigate.

5.2. Plxnc1 expression regulated by Lmx1a/b, a new role for Lmx1a/b in axon guidance of mature neurons

Our study identified Plxnc1 as a regulated target of Lmx1a/b in mature neurons.
We used several *in vitro* experiments to illustrate the chemorepulsive effect of Sema7a on Plxnc1, and we attested our discovery using several transgenic mice models. The study of the phenotype of Sema7aKO and Plxnc1 overexpression mice both corroborate the chemorepulsive effect induced by Sema7a/Plxnc1 interaction. Moreover the phenotype of Plxnc1 overexpression mice showing a loss of innervation in the dorsal striatum totally mimicked the one observed for mice lacking Lmx1a/b in mature neurons.
Thus in our study we highlighted the mechanistic behind the regulation of Plxnc1 by Lmx1a/b and also Otx2. We showed that Plxnc1 is an important factor essential for the appropriate development of the nigrostriatal and mesolimbic pathways.

During postnatal development axon growth is guided through all the brain area according to the expression of several molecules orienting them to their correct targets (Bentlage et al. 1999; Nikkhah et al. 1995). We identified Sema7a/Plxnc1 interaction as an important regulator for striatal patterning. Li *et al*., shown the interaction Netrin1/DCC as a key player as well (Li et al. 2014). Altogether, our and Li studies show that the main DA projection

system to the striatum are guided through attraction by Netrin-1 and chemorepulsion by Sema7a which both occupy their own part of the striatum; Sema7a in dorsal striatum, and Netrin-1 in ventral striatum. However, said this way the story may appear too simple and other axon guidance cues must be involved.

5.3. Others molecules involved in axon targeting of dopaminergic pathways

Several axon guidance molecules others than Sema7a/Plxnc1 and Netrin1/DCC have been described during the late development such as: the Ephrin family with EphrinA2, A3, A5, B2 (Janis et al. 1999), but also Sema3a (Prestoz et al. 2012; Hernández-Montiel & Tamariz 2008). Janis *et al*. shown that EphrinA1 and EphrinA4-binding determine the striatal compartment (Janis et al. 1999). Thus, studies identified cues delineating the all striatal region, and others the dorsal and ventral parts of the striatum. We still need to identify which subtype of neurons innervates specifically the striosomes localized in the dorsal striatum compared to those innervating the striatal matrix. In order to do that, experiments like the one performed by Poulin *et al*., would be of real interest (Poulin et al. 2014). Indeed, defining the molecular signature of the entire midbrain population would help discovering all the cues involved in the establishment of the dopaminergic system. Until now, when talking about axon guidance we mostly emphasize on the receptor and the ligand only, but maybe some complexes can exist inbetween different ligands. In order, to check this possibility immunoprecipitation experiments could be performed targeting for example an Ephrin and then a Semaphorin using a striatal lysat. Recent studies showed the importance in the change of conformation of PlxnA receptors known for their important role in the nervous sytem development (Kong et al. 2016). Thus, having a good knowledge of the structural biology of axon guidance receptors would help in understanding their mode of action. This mechanistic could become new pharmacological targets to play with in order to induce for example migration for regeneration. Moreover with the advances in microscopy we could directly follow changes in conformation using for example the STED microscopy. Indeed, we could observe the formation of rafts with all receptors grouping together answering to a characteristic signal such as their specific ligand or even electrical inputs.

Moreover Lmxa/b could also been involved in post-mitotic mDA neurons regulation by controlling other genes expression than Plxnc1. Indeed, we observe a redundancy comparing the results obtained from the mRNA sequencing of Lmx1a/b cKO mutant mice and controls, with the resulst from Poulin *et al*. (Poulin et al. 2014) identifying molecular signatures for subtypes of mDA neurons. Among these targeted genes for Lmx1a/b, Cck appears as an interesting target to investigate. This peptide hormon is known as regulating pancreatic enzymes release in the gut, but its role in the brain has not been totally defined yet. Studies shown that Cck is involved in drug tolerance to opioids such as morphine and heroin (Kissin et al. 2000). This evidence shows a regulation of the mesolimbic system by Lmx1a/b *via* Cck. In their study, Fukazawa et al. highlighted a pain hypersensitivity associated with spinal Cck receptors during intrathecal morphine analgesia following electroacupuncture stimulation in rats (Fukazawa et al. 2007). More recent studies focused on the role of CCK-8 in the prefrontal cortex which is an area innervated by the dopaminergic sytem. They shown a regulation of apoposis related genes by CCK-8 (Ye et al. 2016). Poulin et al. shown that Cck is part of the molecular signature for the cluster 2, DA^2, of mDA neurons (Poulin et al. 2014). Thus, this specific subtype of neurons, regulated in part by Lmx1a/b, could be involved in pain regulation and would represent an interesting taget to investigate.

In our study, through the analysis of Sema7aKO mice we observed evidence of increasing sprouting in different areas of the brain like the septum and dorsal striatum (data not shown). This sprouting could be interpreted as a consequence of the non-binding of Sema7a to its other known receptor: β1-integrin. We had preliminary results (data not shown) on the study of the role of β1-integrin in axon targeting of dopaminergic neurons using explant experiments. Indeed, we used an antibody against β1-integrin to check whether the effect of decrease in axonal length and increase of growth cone areas in presence of Sema7a was due to its interaction with Plxnc1 or β1-integrin. We obtained the same results with or without this antibody comforting that our observations about axon guidance seem to be due to Sema7a/Plxnc1 interaction. However, it would be interesing to use the CRISPR-Cas9 technology to stop all production of β1-integrin in cell cultures to attest the real effect obtained in the preliminary results. Moreover to investigate

specifically the role of the interaction Sema7a/β1-integrin in the dopaminergic system it would be necessary to use the same strategy, but this time to stop Plxnc1 expression. Thus, studying the role of β1-integrin in the establishment of the dopaminergic system would be pertinent to discover new regulating pathways.

5.4. Plxnc1 repression in newly generated DA cells for the reconstruction of the nigrostriatal pathway

In the fourth chapter I described my ongoing work on differentiation of ESC from mice into DA neurons. I performed 4 stable lines of Epiblast: Epiblasts not infected, Epiblasts infected with a construct sh scrambled, Epiblasts infected with a construct sh Plxnc1 plus Tomato, and the last line of Epiblasts are Epiblasts infected with the sh scrambled construct but also with the Plxnc1 construct in order to overexpress this protein.

The construct sh scrambled contains GFP allowing us to identify DA neurons when injected in mice. However, the construct sh Plxnc1 being too big it was not possible to add a fluorochrome in the construct explaining why I co-infected with a retrovirus expressing tomato. Both sh constructs contain a resistance gene to puromycin attesting that cells present in the wells with medium containing puromycin all express the sh construct. I used the FACS to have a pure line of cells expressing sh Plxnc1 and tomato. Otherwise I would have injected cells in mice that I would not be able to follow.

I injected a mix of both types of cells: sh Plxnc1 and sh scrambled in the SNpc of lesioned mice in the MFB. The aim of this co-injection is to observe cells expressing the sh scrambled in green innervating the ventral striatum due to the chemorepulsion induced by Sema7a in the dorsal striatum, and cells repressing Plxnc1 expression in red innervating the dorsal striatum. I injected these cells directly in the SNpc to try to reconstruct the nigrostriatal pathway in a more natural way than injecting neurons directly in the striatum. This strategy was also preferred than injecting in the striatum to avoid any overload of dopamine release. Indeed, normally only axonal projections of the mDA neurons innervate the striatum, and there are no mDA neurons cell bodies integrated directly in this area. Thus, side effects could be more observed than by injecting newly generated neurons in the SNpc. Moreover, axon guidance molecules like Sema7a and Netrin-1 present during

the establishment of the dopmaniergic system are still present in the adult allowing a reconstruction of the nigrostriatal pathway. Although this strategy consisting in the injection of newly generated neurons in the SNpc seems logical; for the translation to cell replacement therapy in patients, the surgery would be much more complicated than injected in the striatum. Indeed, the SNpc is a small structure quite deep in the brain compared to the striatum. Moreover, even if in mice and rats models a reconstruction of the nigrostriatal pathway has worked we would need more evidence to attest the possibility of a reconstruction of the nigrostriatal pathway in the human brain. Thus, we reach the question of benefit/risk and until now people prefer to target the striatum despite the possibility of side effects like dyskinesia.

5.5. Downstream targets of Plxnc1

In order to decipher more extensively the mechanistic behind the segregation of those two main dopaminergic pathways, the study of downstream targets of Plxnc1 would need to be performed. Our preliminary results (data not shown) pointed to Src kinases as potential targets. Actually, immunochemistry on explants treated with Sema7a shown an increase of Src kinases phosphorylation in growth cones compared to explants not treated with Sema7a. In order to demonstrate this involvement of Src kinases in the downstream mechanism of Sema7a/Plxnc1 binding, we used the well-known inhibitor of Src kinases: PP2 and its negative control PP3 on mDA explants culture. We observed that Src kinases inhibition by PP2 blocks the effect of Sema7a on mDA axons suggesting that Src kinases are necessary for signalization. Src kinases would act as a second messenger downstream of Plxnc1.

Plxnc1 is known to be involved in axon guidance in different pathologies like neuro-inflammation or oncology. As an example, Plxnc1 mediates activation of monocytes and inhibition of dendritic cell and neutrophil phagocytosis binding to A39R.
Plexin C1 is an intracellular suppressor of cytokine signaling-2 (SOCS2), and kinesin family member 21A (KIF21A) on bovine chromosome 5. Intragenic SNPs of the gene PLXNC1 seem to play an important role in the pathogenesis of Bilateral convergent strabismus with exophthalmus (BCSE), a widespread hereditary defect known in many cattle breeds. Indeed, the study of Plexin C1 deletion in mice shown defects in the

partitioning of paraventricular and supraoptic neurons in the hypothalamus (Fink et al. 2012).

A study of Scott *et al.*, has shown Plxnc1 as a tumour suppressor in melanoma progression. As illustrated in Figure 26, Sema7a/Plxnc1 binding leads to cofilin phosphorylation by Lim kinase II (LIMK II) playing an inhibitory signalling on cofilin activation (Fender 2009). Thus, in the absence of Plxnc1, as characterized in human melanoma cell lines, an increase in metastasic melanoma invasion is observed. In this case, Plxnc1, after binding to Sema7a, plays the role of a tumour suppressor by inactivating Cofilin (Fender 2009). Previous data put the light on the role of Sema7a in axon guidance of melanoma cell lines, stimulating their adhesion and dendricity through opposing actions of β1-integrin and Plexin C1 receptors (Fender 2009).

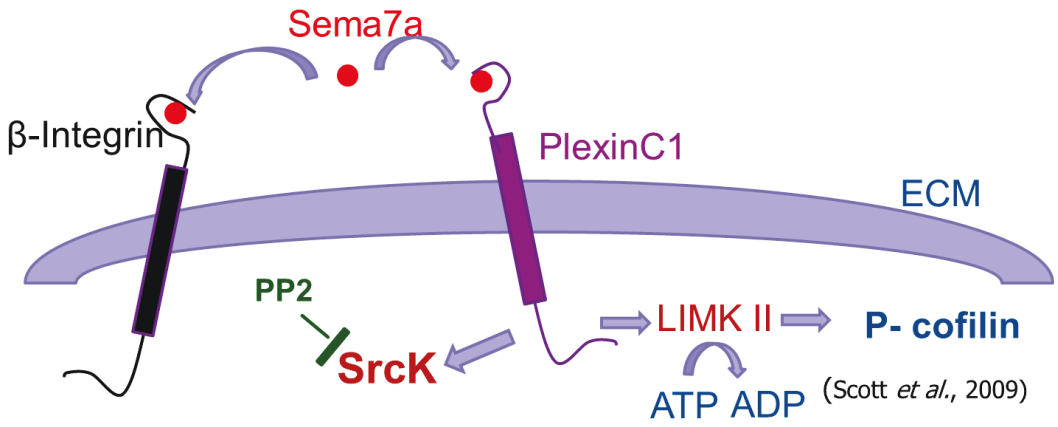


Figure 26 Plxnc1 : a potential tumour suppressor protein.
Observation of signalling intermediates of Sema7A and downstream targets of Plexin C1 in human melanocytes shown Sema7A activated mitogen-activated protein kinase and inactivated cofilin, an actin-binding protein involved in cell migration. When Plexin C1 expression was silenced, Sema7A failed to phosphorylate cofilin, indicating that cofilin is downstream of Plexin C1. Further, Lim kinase II, a protein that phosphorylates cofilin, is upregulated by Sema7A in a Plexin C1-dependent manner. This data identify Plexin C1 as a potential tumour suppressor protein in melanoma progression, and suggest that loss of Plexin C1 expression may promote melanoma invasion and metastasis through loss of inhibitory signalling on cofilin activation.

In the immune system, Plxnc1 seems to play an opposing role on T cell-mediated inflammatory response than Sema7a/β1-integrin binding, like the one observed on Sema 7A-mediated spreading and dendrite formation in melanocytes and described on Figure 27 (Jongbloets, 2013).

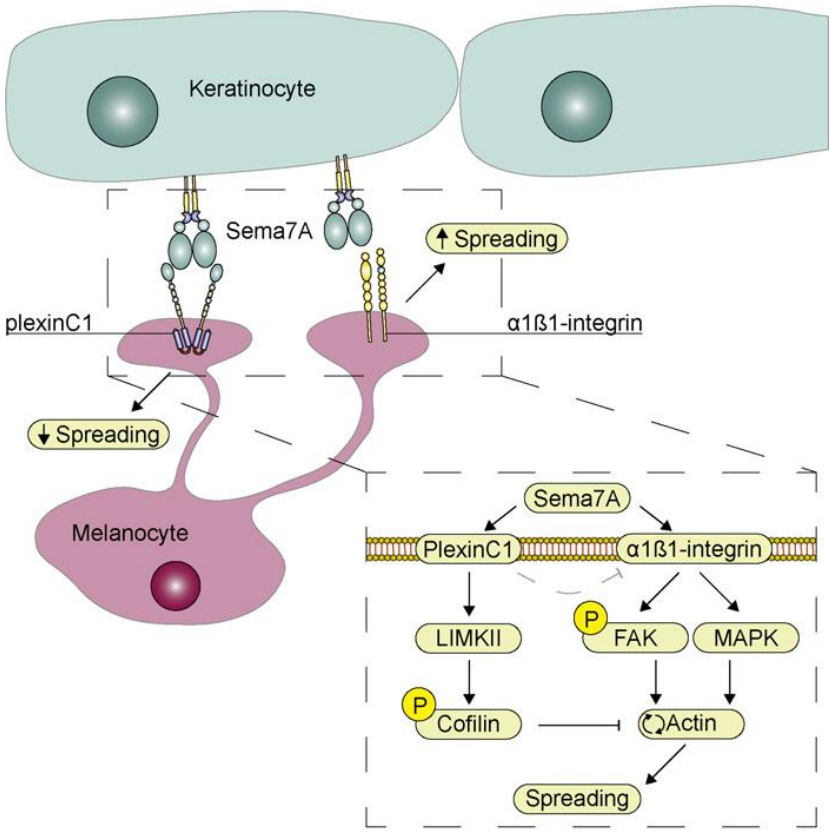


Figure 27 Opposing role of Plxnc1/Sema7a binding to β1-integrin/Sema7a.
Opposing role in the regulation of melanocyte spreading *via* two Sema7a pathways. Sema7A/ β1- integrin binding initiates melanocyte spreading, whereas Sema7A/Plxnc1 binding represses melanocyte spreading by suppressing actin remodeling. Plxnc1/Sema7A binding may directly play an inhibiting role on integrin signalling (Jongbloets, 2013.).

Regarding these studies it seems interesting to look at those downstream targets, LIMKII, Cofilin, FAK, MAPK in the dopaminergic system as well. It could also be interesting to target molecules modulating the tubulin such as the microtubule-associated proteins (MAP), such as tau. Indeed, on Figure 27 we can see an actin regulation by FAK and MAPK, downstream targets of α1β1-integrin, but a modulation of the extent and rate of microtubule also play an important role in axonal growth.

It would be interesting look at these proteins which appear like good downstream candidates for Sema7a-Plxnc1 signaling. Thus, the study of Plxnc1 downstream signaling could represent a nice following study.

5.6. Cell replacement therapy in PD dream or reality?

Cell replacement therapy is a technique that has been already used in different pathologies, like leukemia or multiple myeloma, for about thirty years. Indeed, hematopoietic stem cell transplantation is the most common cell replacement therapy used today with a total of about 50,000 transplants in 2006 (Gratwohl et al. 2010). Different types of cells are used for this therapy like UCB or MSCs (Meyer et al. 2010; Gratwohl et al. 2010). This treatment attests of the feasibility and efficacy of cell replacement therapy. Different clinical trials were performed for cell replacement therapy in neurodegenerative disorders over the past twenty years. More than 350 patients suffering from PD have received human fetal mesencephalic tissue transplants since 1980. Most of these transplantations had a positive effect on motor symptoms of PD patients. Indeed, a restoration of the DA transmission by a successful re-innervation of the striatum was observed (Piccini et al. 1999). However as mentioned before, for fetal NSCs, the cell replacement therapy in PD using human fetal stem cells is not viable. Indeed, the number of functional cells correctly integrating the neural circuitry remains too low, the source of these cells raises ethical issues because of the procurement of tissue from aborted human fetuses, and there is a major risk of rejection of these allogenetic cells by the patient (Gratwohl et al. 2010). Even if these cells do not allow a total recovery of patients with PD, clinical trials led with human fetal stem cells increased our knowledge about all the factors to take into account to permit a translation from animal models to clinical trials. Moreover, the interesting results obtained using fetal SCs would lead to an evolution of the treatment using adult NSCs. Adult NSCs represent an important candidate for cell replacement therapy (Shim et al. 2007). Being able to reimplant its own brain cells to a PD patient will be a real challenge for the next years. Indeed, the implantation of adult NSCs seems promising because of their lack of tumorigenicity, their predisposition to differentiate into brain cells, and the fact that they are autologous cells. However, studies using these cells are at a very early stage, and no technical tests, like a double surgery was reported on

parkinsonian rodents models yet. Many technical aspects are to consider for the use of these cells for cell replacement therapy in PD. The first one is that patients would need to have two surgeries, one to harvest the adult NSCs, and another one to implant the newly generated cells, meaning that only a few patients could have access to this therapy, because not all of them would have the strength to withstand two surgeries (this is also a reason why the deep brain stimulation is a strategy only used on few patients). The harvesting of adult NSCs is also questionable. The region of the brain from which the adult NSC would be harvested need to be determined carefully. Indeed, taking cells at this place should not induce any deficit for the patient. Taking into account all of these limits, the implantation of adult neural stem cells for cell replacement therapy in PD does not appear as a viable treatment. More recent trials performed using in one case the transplantation of embryonic dopamine neurons, and in the other tissue using double blind, placebo-controlled protocols (Olanow et al. 2003; Freed et al. 2001). In both of these trials, the intrastriatal implantation of dopaminergic cells led to their integration in the host striatum (Olanow et al. 2003; Freed et al. 2001). However, this cell replacement was benefic only for a few of the treated patients, and 56% of them showed parkinsonian symptoms relapsing (Hagell et al. 2002; Olanow et al. 2003; Freed et al. 2001). In January 2009, the first clinical trial using an embryonic stem cell-based therapy on humans was started, but unfortunately discontinued in 2011 (for economic reasons). In this trial, embryonic stem cell-derived oligodendrocyte progenitor cells were tested on patients with acute spinal cord injury. Even if this trial was not directly about PD, all cell replacement therapies are important to take into account for the improvement of the technique and the observation of interactions between new implanted cells and the organism. Moreover, obtaining the authorization to lead clinical trials using ES cells is an important step for the scientific community, because studies led in 2011 by Kriks and co-workers have shown a good DA survival in parkinsonian Monkeys after hES cells engraftment (Kriks et al. 2011). Resulting from all the promising studies, a EU-funded research consortium has been formed to promote the optimization of cell replacement therapy using fetal cells for PD (Grealish et al. 2014).

Cell replacement therapy using stem cells differentiated into DA neurons has been shown, in several studies mostly led in rodents (Grealish et al. 2014) but also in clinical trials, as

an efficient way to reduce motor symptoms associated with PD. Indeed, a decrease in the motor handicap, depression, pain, constipation, and sleeping disorders, which ultimately improves quality of life leading to an extent of the overall life span were observed in clinical trials (Lees et al. 2009; Gratwohl et al. 2010). Thus, cell replacement therapy appears like an interesting way of treatment for PD, but the best type of cell to use remains to be determined. Even if studies are only at their beginning, iPSCs appear like good candidates for this treatment therapy, and ES cells are potential candidates especially since the successful study led in monkeys by Kriks *et al*. showing no tumour formation. However, one of the limitations observed with cell replacement therapy is that implanted cells may not only differentiate into mDA neurons which could induce tumorigenicity (Meyer et al. 2010; Gao et al. 2013). Using the new technique developed in Takahashi's laboratory, we would be able to introduce only the cell of interest eliminating the risk of tumorigenicity. Thus, cell replacement therapy in PD represents a real promising treatment, even if pre-clinical studies still need to be performed to assure a successful translation to humans. However, a new strategy to avoid any cells implantation started to be developed. Indeed, in a recent study Torper *et al*. (Torper et al. 2015) described the possibility to reprogram striatal glia into functional neurons (Torper et al. 2015). Thus instead of a cell replacement therapy the new therapy for PD would turn to gene therapy.

5.7. Perspectives

Cell replacement therapy appears like a very interesting strategy for PD treatment. The optimization of the production of new cells types, like iPSCs or neurons from transdifferentiation from fibroblasts led to new hope for the treatment of neurodegenerative disorders. However, even if cell replacement therapy is very attractive there are still a lot of issues to solve. For example, we need to be able to isolate mDA neurons of the Snpc from those of the VTA. Actually, if we implant mDA neurons but only VTA specific we would not be able to restore the nigrostriatal pathway mostly degenerated in PD. Thus, we need to determine a selective factor to differentiate neurons from Snpc to those from VTA. Our work determined Plxnc1 as a possible selective factor. Indeed, differentiated DA cells Plxnc1 negatives would be the one to use for cell replacement for restoring the nigrostriatal pathway. Most of the studies consist in injecting newly generated cells in the striatum, where the loss of innervation is observed, but more studies should be focused on the injection of mDA neurons in the midbrain in order to restore DA neurons from the SNpc which degenerated. A new strategy for cell replacement therapy in PD could be the injection of human iPSC-derived DA cells Plxnc1 positives in the Snpc of patients with PD. However, the origin of the loss of mDA neurons from the Snpc in PD is not known yet. Thus, the cell replacement therapy might not represent a cure for PD. This loss could be due to neurotoxicity for these cells, deregulation by microRNAs or nuclear receptors; thus the newly generated mDA neurons would degenerate like the others (Eendebak et al. 2011; Venda et al. 2010). A way to understand the mechanism of degenerescence would be, like suggested by Gao and co-workers, to work on iPSCs from patients to go through a personalized medicine. Actually, studies on these cells could lead to a better understanding of the disease. Thus, if we managed to find the mutated molecules, we could use the RNAi technology, no tumorigenic, to rectify mutations present in iPSCs from the patient and reimplant them into the midbrain (Gao et al. 2013).

Chapter 6. Conclusion

Throughout this thesis I have shown that Lmx1a/b regulate midbrain dopaminergic axons targeting by repressing Plxnc1 expression in SNpc neurons. This repression of Plxnc1 in SNpc neurons allows the innervation of these neurons in the dorsal striatum. Indeed, the dorsal striatum contains a high concentration of Sema7a which acts as chemo repulsive cue for mDA neurons from VTA expressing Plxnc1. This mechanism would explain the segregation of nigrostriatal and mesolimbic dopaminergic axonal pathways. Today, the graft of dopaminergic neurons newly generated from stem cells represents a promising therapeutic avenue for PD. However, being able to differentiate stem cells into mDA neurons from the SNpc represents a real challenge. Plxnc1 is a cue to take into account for graft of dopaminergic neurons newly generated from stem cells for Parkinson's disease therapy. Data from this study will shed a new light on mechanisms that regulate dopamine neurons connectivity and should help in the effort to understand the molecular factors contributing to the efficiency of cell replacement therapies in PD. After developing a functional cell based replacement therapy, another challenge would be to find biomarkers specific to PD and being able to act before the degeneration occurs.

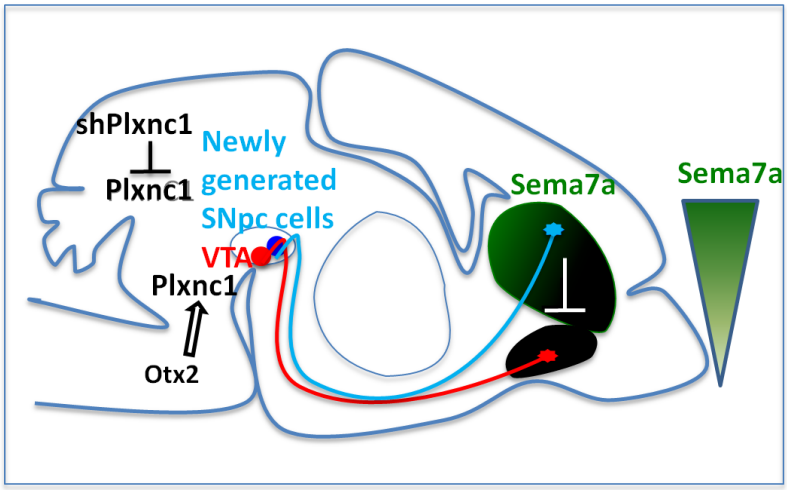


Figure 28 Role of Important Factors for Increasing Efficacy of Cell-Based Replacement Therapy for PD. Lmx1a/b by playing a repressive role in Plxnc1 in SNpc neurons allow the innervation of the dorsal striatum. For a cell replacement therapy for PD newly generated neurons should repress Plxnc1 expression.

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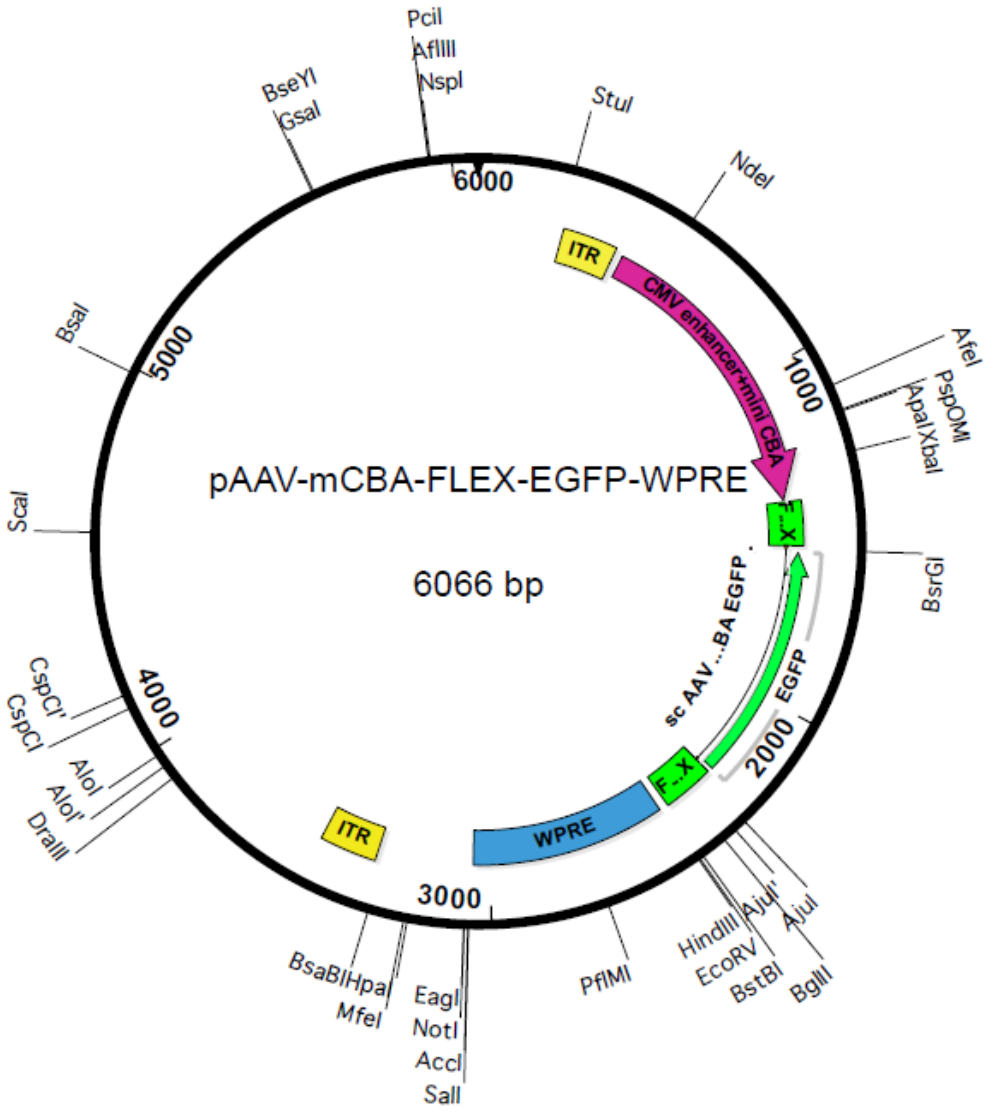
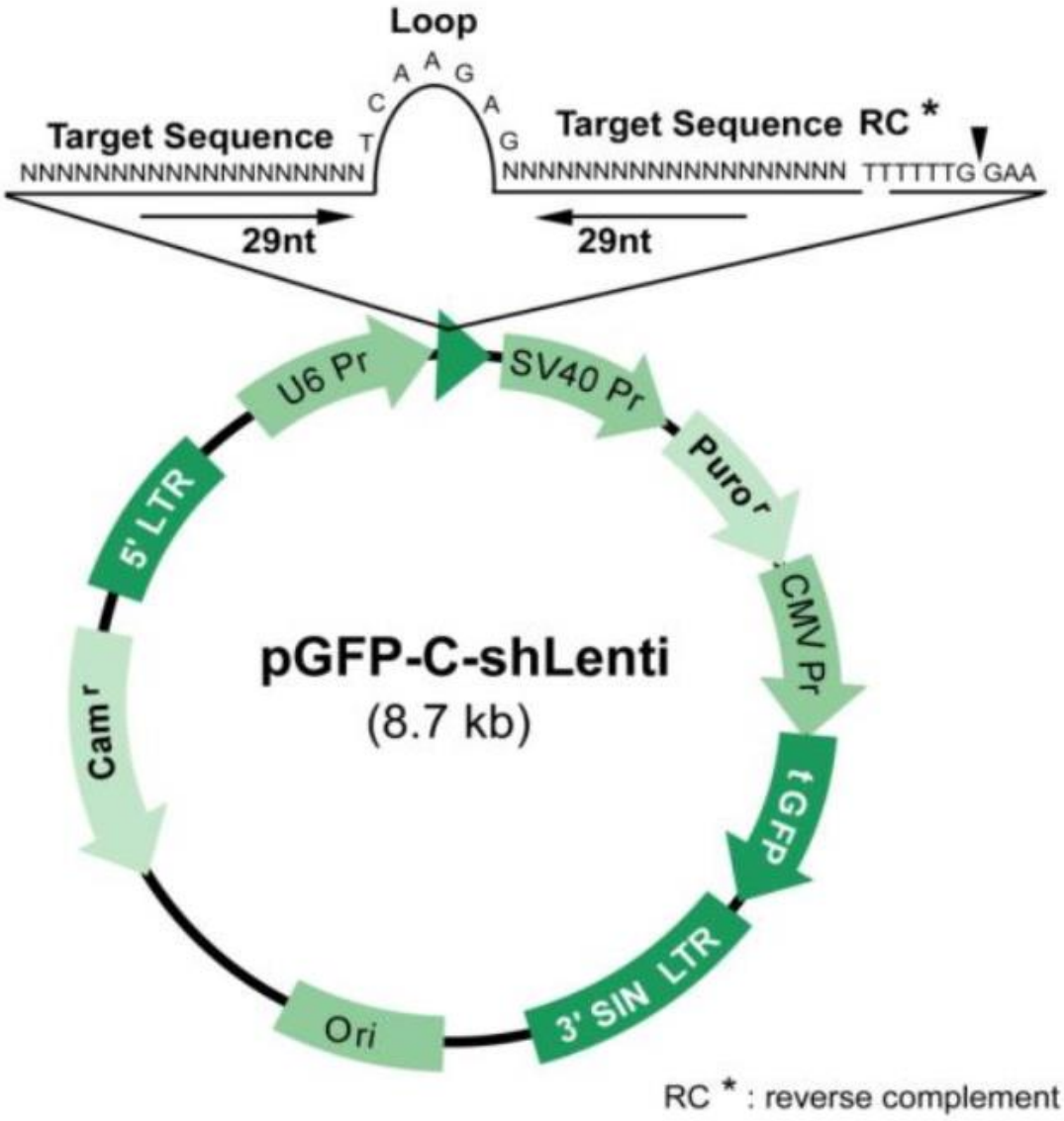
Appendices

Appendix 1: Anterograde virus construct

pAAV-mCBA-FLEX-EGFP-WPRE

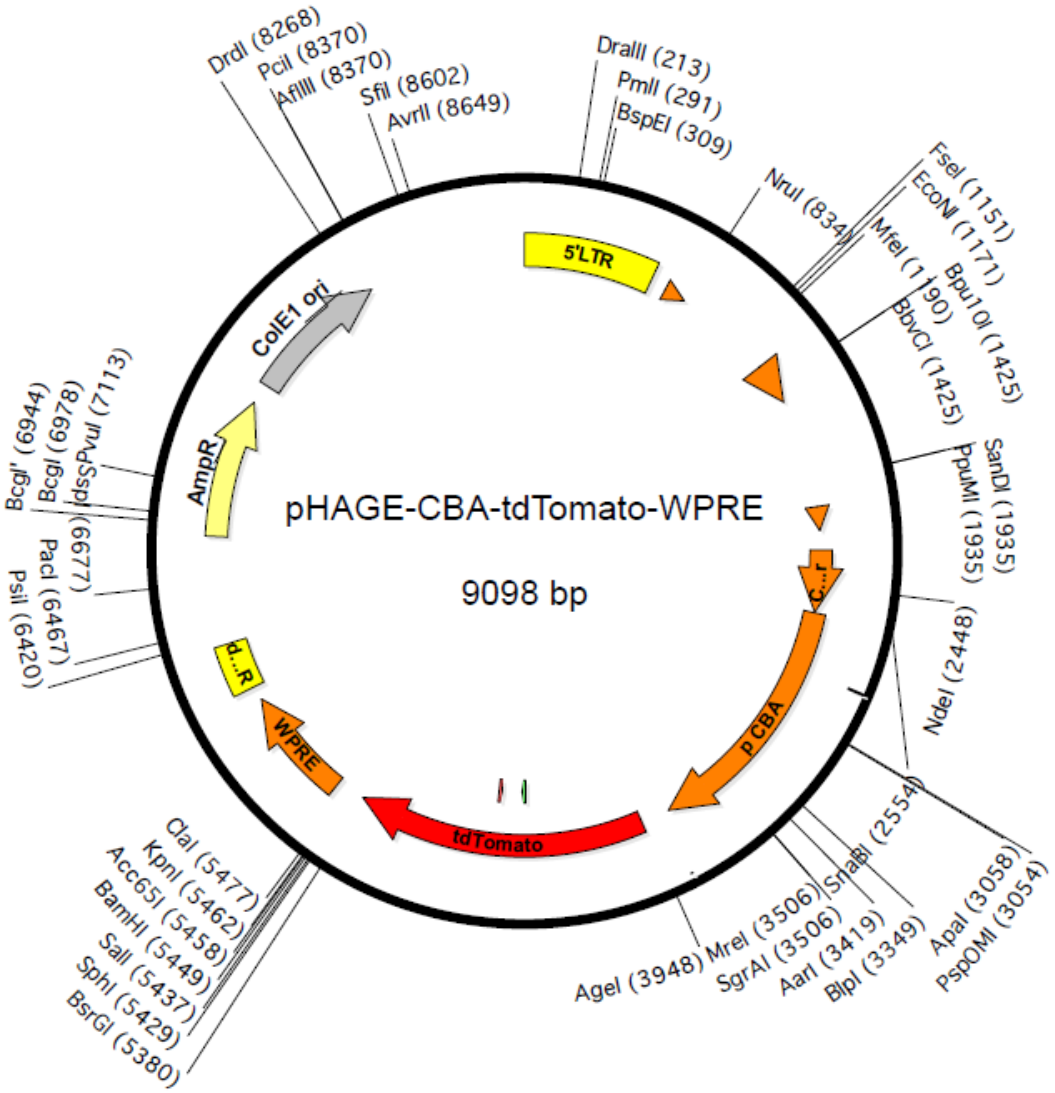


Appendix 2: Plasmid vector sh scrambled

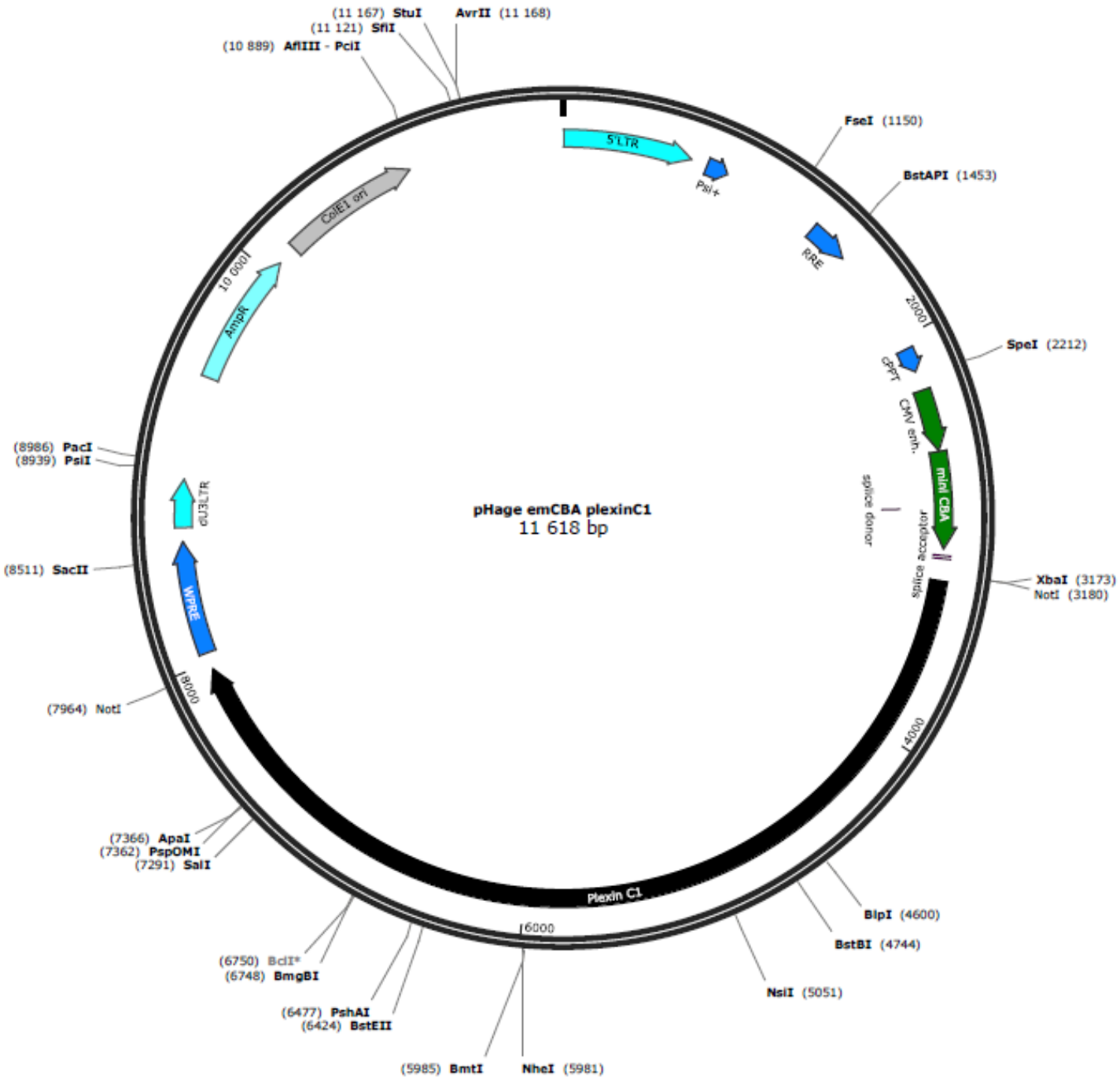


Appendix 3: Plasmid vector tdTomato

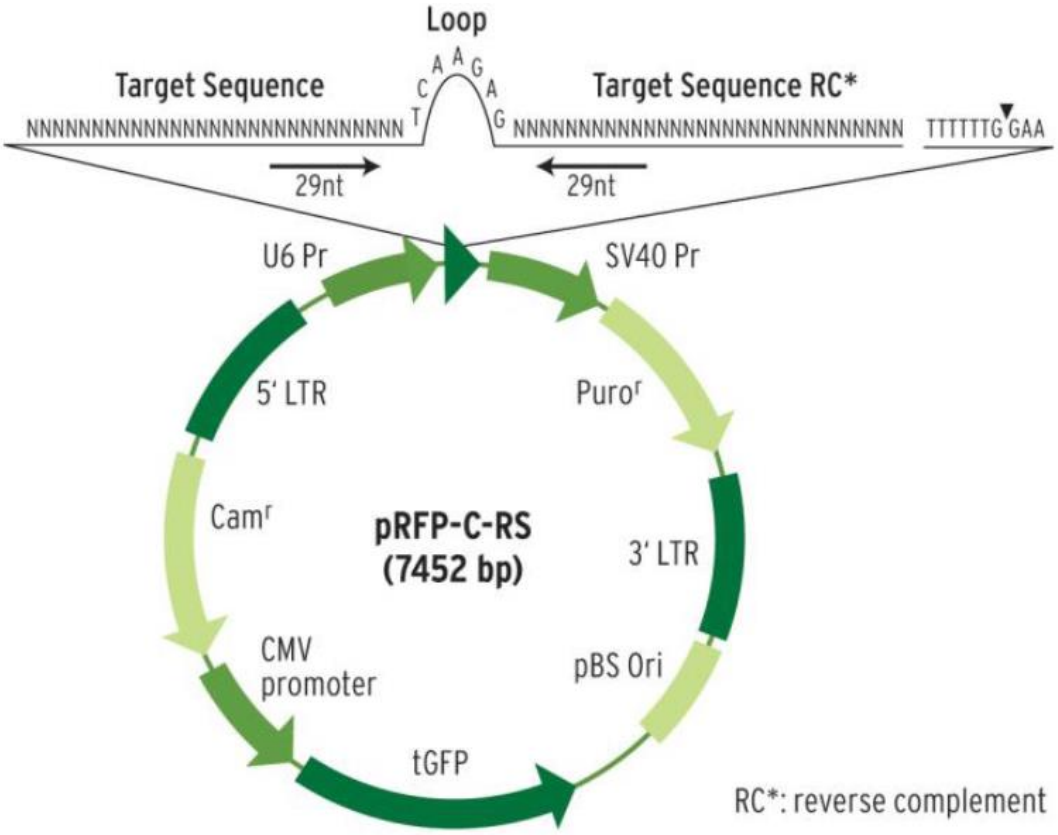
pHAGE-CBA-tdTomato-WPRE



Appendix 4: Plasmid vector OE PlexinC1



Appendix 5: Plasmid vector sh PlexinC1



Plxnc1-Mouse, 29mer shRNA construct in retroviral RFP vector
GACAGTTCCTCTGTGATTCTTGAAGATGG