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Cellular and molecular aspects of cell therapy in mouse models of Parkinson's disease and traumatic brain injury

Sarah Mantash

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THESE

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**Cellular and molecular aspects of cell therapy in mouse models
of Parkinson's disease and traumatic brain injury**

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Soutenue le 28 Juin 2019

Devant la Commission d'Examen

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*To my life-mentor and spiritual father,
You told me to never stop no matter what!
For that, I owe it all to you...*

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Foreword

Pathologies of the central nervous system (CNS), whether traumatic (such as traumatic brain injury (TBI)), vascular or degenerative (such as Parkinson disease (PD)) lead to neural cell death and are often associated with functional deficits that may be irreversible depending on the degree or location of the brain damage. Whilst there is no effective treatment to cure neural cell death, several strategies have emerged mainly based on disease modifying treatments including anti-inflammatory and anti-oxidant agents, or symptomatic treatments that fail to restore lost neurons or reverse the denervated neural processes (Kiaei, 2013). Moreover, taking into account the complexity of the lesioned structures and the precision of lost projection patterns, to repair the damaged circuitry is indeed considered a complex task (Gaspard & Vanderhaeghen, 2011). Based on that, cell-based therapies have gained a remarkable interest as a potential alternative treatment. The principle in cell replacement strategy is either to substitute the lost and degenerated neurons with an exogenous cell transplant, or to induce the migration of endogenous stem cells present in specific regions of the brain to the damaged area (Tajiri et al., 2014).

In the present study, we investigated some cellular and molecular aspects of the reparative capacity of cell replacement in lesioned mouse models of PD (as detailed in Chapter I) and TBI (detailed in Chapter II).

Part I of Chapter I presents the involvement of the axon guidance molecule Semaphorin7A (Sema7A), in the development of the nigrostriatal pathway that is degenerated in PD. Part II describes our study on fetal cell therapy in absence of Sema7A in a toxic mouse model of PD. Finally in part III, a non-targeted proteomic investigation associated to fetal cell transplantation in the same mouse model is presented. This latter work is compiled as an article in preparation.

Chapter II is dedicated to the presentation of the cellular aspects of neural stem cells transplantation and the effect of combining a polyunsaturated fatty acid with the grafted cells in the cortex of a mild controlled cortical impact mouse model of TBI. This work is presented in two published articles and one review.

Avant-propos

Les pathologies du système nerveux central (SNC), qu'elles soient traumatiques (comme les lésions traumatiques cérébrales (LTC)), vasculaires ou dégénératives (comme la maladie de Parkinson (MP)), entraînent la mort de populations de neurones et sont associées à des déficits fonctionnels pouvant être irréversibles en fonction du degré ou de la localisation des lésions cérébrales. Bien qu'il n'existe aucun traitement efficace pour empêcher et traiter la mort neuronale, plusieurs stratégies reposent principalement sur l'utilisation d'agents anti-inflammatoires et anti-oxydants, ou sur des traitements symptomatiques qui ne permettent pas de restaurer les neurones perdus ou de contrer les processus de dégénérescence (Kiaei, 2013). En outre, compte tenu de la complexité des structures dégénérées et de leur projections, la réparation des circuits endommagés est en effet considérée comme une tâche (Gaspard & Vanderhaeghen, 2011). De ce fait, les thérapies cellulaires sont d'un intérêt considérable. En effet, la stratégie de remplacement cellulaire est soit de remplacer les neurones perdus et dégénérés par une greffe de cellules exogènes, soit d'induire la migration de cellules souches endogènes présentes dans des régions spécifiques du cerveau vers la région endommagée (Tajiri et al., 2014).

Dans ce travail, nous avons étudié certains aspects cellulaires et moléculaires des capacités réparatrices des cellules greffées dans des modèles murins de la MP (comme détaillé au chapitre I) et des LTC (détaillés au chapitre II).

La première partie du chapitre I présente l'implication de la molécule de guidage axonal Sémaphorine7A (Sema7A) dans le développement de la voie nigrostriée dégénérée dans la MP. Dans la partie II, est détaillée l'étude de l'efficacité d'une thérapie cellulaire fœtale en l'absence de Sema7A dans un modèle de MP toxique chez la souris. Enfin, la troisième partie concerne une étude protéomique non ciblée associée à une greffe de cellules fœtales dans le même modèle murin. Ce dernier travail est présenté dans un article en préparation.

Le chapitre II est consacré à la présentation des aspects cellulaires de la greffe de cellules souches neuronales et de l'effet d'un acide gras polyinsaturé combiné aux cellules greffées dans le cortex d'un modèle murin de souris LTC. Ce travail est présenté dans deux articles publiés et une revue.

Abstract

Neurodegenerative diseases such as Parkinson's disease (PD) and traumatic brain injuries (TBI) are the most common cause of neural cell death and constitute a serious public health concern with hundreds of thousand people affected each year.

In this way, many efforts are developed to understand the molecular and cellular bases of neurodegenerative processes and to devise effective therapies. Indeed, several surgery- or drug-based treatments have been postulated as therapeutic avenues for TBI and PD. For example, deep brain stimulation aims to relieve symptomatic complications in patients with PD and decompressive craniectomy can reduce the intracranial pressure after TBI. Pharmaceutical interventions in TBI include drugs that target secondary injury mechanisms. In PD, using dopamine agonists such as L-DOPA aims to compensate the dopaminergic (DA) loss in the striatum. However, these treatments are most often symptomatologic and do not completely re-establish the degenerated pathways or lost neurons. In this way, cell therapy is a promising avenue to restore disrupted pathways and replace lost neurons in PD and TBI.

In this work, we studied molecular and cellular mechanisms of a cell therapy in mouse models of PD and TBI.

In PD, fetal cell transplantations have been long considered as a promising therapeutic approach to restore the disrupted nigrostriatal dopaminergic connectivity which is initially established under the influence of multiple axon guidance cues. We and others previously showed that intranigral transplantation in adult mice model of PD, of fetal ventral mesencephalon cells collected from E12.5 mice embryos functionally and anatomically restores the motor deficits. In addition, we previously showed that Semaphorin7A (Sema7A) axon guidance molecule may be more particularly involved in this restoration since its expression increases after transplantation in several regions surrounding the nigrostriatal pathway. By this mean, we investigated the influence of Sema7A on the nigrostriatal pathway reconstruction in a mouse model of PD, toxically induced with 6-hydroxydopamine and after grafting with E12.5 ventral mesencephalon fetal cells. We also tested the influence of this axon guidance molecule in the establishment of this pathway during embryogenesis and in intact adult brain using Sema7A knock-out mice. We showed that Sema7A is necessary for the development of the DA neurons in the ventral mesencephalon during embryogenesis and is differentially expressed in the adult striatum, suggesting its implication in the mesostriatal DA topography. After fetal cell therapy

in a mouse model of PD, we showed that Sema7A may play a role in the neuroinflammation processes described to decrease the survival of the grafted cells. These results bring new insights on the role of Sema7A in the development of DA neurons and for protective strategies of grafted cells in cell therapy approaches. Finally, we explored the whole proteome in nigrostriatal pathway-related regions in adults to test the influence of fetal cell transplantation on protein expression.

Cortical brain injuries are also candidates for cell replacement strategies to ameliorate functional recovery and anatomical repair. Thus, as presented in the second part of this work, we assessed the effects of neural cell transplantation in a mild controlled cortical impact mouse model of TBI. We observed that implantation of embryonic neural stem cells or of differentiated cells decreases neuroinflammation and enhances neurogenesis following grafting. Moreover, association of neural stem cell transplantation with docosahexaenoic acid injections significantly attenuated TBI-induced motor function deficits and promoted neurogenesis.

In conclusion, considering a combined therapeutic strategy by integrating cell transplantation protocols and targeting specific molecules involved in normal functioning of the neuronal circuitry, could ameliorate recovery in patients with PD and TBI.

Keywords: Parkinson's disease, Semaphorin7A, cell therapy, traumatic cortical injury, neural stem cells, neurogenesis, neuroinflammation.

Résumé

Les maladies neurodégénératives telles que la maladie de Parkinson (MP), et les lésions traumatiques corticales (LTC) sont la cause la plus courante de mort neuronale et constituent un problème majeur de santé publique puisque des centaines de milliers de personnes sont touchées chaque année dans le monde.

De ce fait, de nombreux efforts sont développés pour comprendre les bases moléculaires et cellulaires des processus neurodégénératifs afin de mettre au point des thérapies efficaces. En effet, de nombreux traitements chirurgicaux et pharmacologiques ont été développés, néanmoins ils sont le plus souvent symptomatiques et ne rétablissent pas complètement les voies en dégénérescence. Ainsi, la thérapie cellulaire est une approche thérapeutique prometteuse pour restaurer les voies endommagées et remplacer les neurones perdus dans la MP et les LTC.

Dans ce travail, nous avons étudié les mécanismes moléculaires et cellulaires après thérapie cellulaire dans des modèles murins de la MP et de LTC.

Dans la MP, les transplantations de cellules fœtales ont longtemps été considérées comme une approche thérapeutique attrayante pour restaurer la connectivité dopaminergique (DA) nigrostriatale endommagée et qui est initialement mise en place sous l'influence de signaux de guidage axonaux. Notre équipe et d'autres avons montré que la transplantation intranigrale de cellules de mésencéphale ventral fœtal chez des souris adultes modèles de la MP, rétablissait les déficits moteurs. De plus, nous avons précédemment montré que la molécule de guidage axonal Sémaphorine7A (Sema7A) pourrait être plus particulièrement impliquée dans cette restauration car son expression varie après la greffe dans plusieurs régions de la voie nigrostriée. Nous avons ainsi étudié l'influence de Sema7A sur la reconstruction de la voie nigrostriée dans un modèle murin toxique de la MP, et après greffe de cellules de mésencéphale ventral fœtal. Nous avons également testé l'influence de cette molécule sur l'établissement de la voie au cours de l'embryogenèse et dans le cerveau adulte intact en utilisant des souris déficientes en Sema7A. Nous avons montré que Sema7A est nécessaire au développement des neurones DA dans le mésencéphale ventral au cours de l'embryogenèse et qu'elle est exprimée de manière différentielle dans le striatum adulte, ce qui suggère son implication dans la topographie DA nigrostriatale. L'étude après thérapie cellulaire dans un modèle murin de la MP, nous a permis de suggérer un rôle de Sema7A dans les processus de neuroinflammation connus pour diminuer

la survie des cellules greffées. Ces résultats apportent de nouvelles connaissances sur la fonction de Sema7A dans le développement des neurones DA et sur les stratégies de protection des cellules greffées dans les approches de thérapie cellulaire. Enfin, nous avons exploré le protéome des régions liées à la voie nigrostriée chez l'adulte après greffe de cellules fœtales.

Les lésions corticales sont également des candidats aux stratégies de remplacement cellulaire afin d'améliorer la récupération fonctionnelle et la réparation anatomique. Ainsi, comme présenté dans la deuxième partie de ce travail, nous avons évalué les effets de la greffe de cellules neurales dans un modèle murin de LTC. Nous avons observé que l'implantation de cellules souches neurales embryonnaires ou de cellules différenciées diminue la neuroinflammation et améliore la neurogenèse après la greffe. De plus, l'association d'une greffe de cellules souches neurales à des injections d'acide gras polyinsaturé (l'acide docosahexaénoïque ou DHA) atténue de manière significative les déficits de la fonction motrice induite par le LTC et favorise la neurogenèse.

En conclusion, envisager une stratégie thérapeutique combinant des protocoles de greffe de cellules et ciblant des molécules spécifiques impliquées dans le fonctionnement normal des circuits neuronaux pourrait améliorer la récupération chez les patients atteints de la MP et de LTC.

Mots-clefs : maladie de Parkinson, Sémaphorine7A, thérapie cellulaire, traumatisme cortical, cellules souches neurales, neurogénèse, neuroinflammation

Table of Contents

List of abbreviations

List of figures

Chapter I: Cellular and molecular aspects of cell therapy in a mouse model of Parkinson's disease.....	1
Résumé.....	2
Introduction.....	8
I. Parkinson's disease.....	10
II. The mesencephalic dopaminergic pathways III.....	37
III. Axon guidance molecules in the adult mesostriatal pathway.....	53
IV. Aim of the project.....	57
Part I: Involvement of Sema7A in the development of the Mesostriatal pathway and in the intact adult brain.....	59
I. Introduction.....	61
II. Experimental approaches.....	62
III. Results.....	71
IV. Discussion & Prospects.....	83
Part II: Involvement of Sema7A in the reconstruction of the nigrostriatal pathway after cell therapy in a mouse model of PD.....	86
I. Introduction.....	88
II. Experimental approaches.....	89
III. Results.....	97
IV. Discussion & Prospects.....	111
Part III: The Proteome associated with cell therapy in a mouse model of Parkinson's disease (Article in preparation).....	116
Chapter II: Repairing TBI with stem cell therapy.....	146
Résumé.....	147
Introduction and aim.....	152
- Transplantation of embryonic neural stem cells and differentiated cells in a controlled cortical impact (CCI) model of adult mouse somatosensory cortex.....	163
- Docosahexaenoic acid (DHA) enhances the therapeutic potential of neonatal neural stem cell transplantation post-Traumatic brain injury.....	164
- Stem cells and combination therapy for the treatment of traumatic brain injury.....	165
- Discussion.....	166
General Conclusion.....	171
References.....	174

List of abbreviations

CNS: central nervous system

CPu: caudate putamen

DA: dopaminergic

DAT: Dopamine active transporter

DBS: Deep brain stimulation

DCC: deleted in colorectal cancer

EGFP: green fluorescent protein

ESC: embryonic stem cells

GFP: green fluorescent protein

Girk2: G protein coupled inward rectifying current potassium channel type 2

GPI: glycosylphosphatidylinositol

iPSCs: induced pluripotent stem cells

mDA: midbrain dopaminergic

MFB: medial forebrain bundle

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NAcc: Nucleus accumbens

Nrp: neuropilins

6-OHDA: 6-hydroxydopamine

PD: Parkinson's disease

Sema: semaphorin

Sema7AKO: semaphorin 7A knockout

SNpc: substantia nigra *pars compacta*

SNr: substantia nigra *pars reticulata*

TBI: traumatic brain injury

TH: tyrosine hydroxylase

Thal: thalamus

VM: ventral mesencephalon

VTA: ventral tegmental area

WT: wildtype

List of figures

Figure 1. Neuropathology of Parkinson’s disease.....	13
Figure 2. Pathogenesis of Parkinson’s disease.....	14
Figure 3: Mechanisms of action of neurotoxic models of PD.....	22
Figure 4. Mechanism of 6-OHDA toxicity on DA neurons.....	24
Figure 5. Schematic overview of mDA cell sources used in restorative cell therapy for PD.....	30
Figure 6: Dopaminergic neuron cell groups in the developing mouse brain sagittal view.....	37
Figure 7: Illustration of the mDA neuron migratory phases during development.....	38
Figure 8. The structure of the growth cone.....	39
Figure 9: Axonal guidance mode of action.....	41
Figure 10: The four conserved axon guidance cues families and their associated receptors.....	42
Figure 11: Schematic presentation of Sema7A and its receptors.....	48
Figure 12: Representation of the of mDA axons rostral orientation in the midbrain under the effect of several guidance cues.....	49
Figure 13: 3D sagittal illustration of a mouse E13.5 mDA system showing the formation of the MFB.....	50
Figure 14: extension of the mDA axons longitudinally at the level of the caudal hypothalamus.....	51
Figure 15: Genotype of Sema7A mutant embryos.....	63
Figure 16: Coronal section from the mouse brain atlas as an illustration for quantification.....	70
Figure 17: Sagittal and coronal sections of mouse brain atlas at E16.5 and E18.5 retained for quantifications.....	70
Figure 18: Western blotting analysis of Sema7A protein expression in brain tissue from WT and Sema7AKO mice.....	72
Figure 19: Expression of TH in sagittal sections of WT embryos taken at different developmental stages.....	73.
Figure 20: Example of coronal and sagittal sections from WT E16.5 embryos.....	74
Figure 21: Number of the TH+ cells in the SN of WT and Sema7AKO embryos at different developmental stages.....	75
Figure 22: Number of the TH+ somas in the VTA of embryonic WT and Sema7AKO embryos at different developmental stages.....	76
Figure 23: Example of striatal expression of TH+ projections on coronal sections of E18.5 Sema7AKO embryo.....	77

Figure 24: Quantification of TH+ projections in the striatum of WT and Sema7AKO embryos.....	78
Figure 25: Expression of Sema7A in the mesostriatal pathway in intact adult mice.....	79
Figure 26: Sagittal sections in WT and Sema7AKO mice showing the midbrain TH+ neurons and their projections.....	80
Figure 27: Coronal and sagittal sections from WT intact adult brains showing quantified areas with TH expression.....	81
Figure 28: Mean (\pm SEM) of TH+ somas number in intact WT and Sema7AKO SN and VTA.....	81
Figure 29: Quantification of TH+ projections in the striatum of WT and Sema7AKO mice.....	82
Figure 30: Schematic illustration showing the experimental setup for grafting WT and Sema7AKO mice.....	91
Figure 31: The apomorphine-induced rotation test.....	92
Figure 32: Sagittal section from the mouse brain atlas as an example for quantification.....	96
Figure 33: Asymmetric turning behavior in response to apomorphine injection following 6-OHDA lesion of the SNc.....	97
Figure 34: GFP+ cells within the transplant in SN.....	99
Figure 35: TH+ cells within the transplant in SN.....	99
Figure 36: Representation of the TH+ cells percentage relative to GFP+ cells in the transplant of WT and Sema7AKO mice.....	100
Figure 37: Coronal section from TH-EGFP grafted Sema7AKO mouse showing GFP expressing cells in the graft.....	101
Figure 38: Asymmetric turning behavior in response to apomorphine injection 3 weeks following 6-OHDA lesion of the SNc.....	102
Figure 39: GFP+ and TH+ cells within the transplant in sagittal sections of a WT mouse brain.....	103
Figure 40: GFP+, TH+, Girk2+ cells within the transplant.....	104
Figure 41: Quantification of TH+ and Girk2+ and TH+ cells in Actin-EGFP grafted WT and Sema7AKO mice.....	105
Figure 42: GFP+, TH+, Girk2+, and Calbindin+ cells within the transplant.....	106
Figure 43: Quantification of TH+, Girk2+/TH+, and Calb+/TH+ cells in TH-EGFP grafted WT and Sema7AKO mice.....	107
Figure 44: Sagittal section from Sema7A KO mouse grafted with 7 TH-EGFP VMs.....	108
Figure 45: Sagittal section from Sema7A KO mouse grafted with 7 Actin-EGFP fetal VMs.....	109
Figure 46: Quantification of striatal projections in Actin-EGFP grafted WT and Sema7AKO mice.....	110
Figure 47: Quantification of striatal projections in TH-EGFP grafted WT and Sema7AKO mice.....	111

Figure 48: Laminar organization of the cortex.....153

Figure 49: Anatomical reconstruction of the damaged pathways post-transplantation of embryonic motor cortex tissue into damaged motor cortex of the adult mouse.....156

List of Tables

Table 1. Characteristics of transgenic and neurotoxic models.....19

Table 2. Transgenic mouse models of autosomal-dominant PD.....20

Table 3. Transgenic autosomal-recessive mouse models of PD.....21

Table 4. Number of quantified sections containing SN and VTA per each developmental stage in WT and Sema7AKO embryos.....68

Table 5. Quantity of TH-EGFP or Actin-EGFP VM transplanted in WT and Sema7AKO mice.....201

Chapter I

**Cellular and molecular aspects of cell therapy in
a mouse model of Parkinson's disease**

Résumé

Dans ce chapitre, nous avons étudié l'influence d'une molécule de guidage axonal, la Sémaphorine 7A (Séma7A) dans l'établissement de la voie mésostriée pendant l'embryogénèse et après thérapie cellulaire dans un modèle animal de la maladie de Parkinson (MP). Nous avons mis en évidence le rôle de la Séma7A d'une part dans la production des cellules dopaminergiques du mésencéphale ventral en développement et d'autre part dans les processus inflammatoires post-greffes. Enfin, nous avons étudié le protéome des régions associées à la voie nigrostriée chez l'adulte après greffe de cellules fœtales.

Parmi les troubles neurodégénératifs, la MP a été identifiée comme une maladie multifactorielle complexe englobant un large éventail de complications motrices et cognitives. La MP est une maladie neurodégénérative progressive et près de 5 millions de personnes sont atteintes dans le monde (Dorsey et al., 2007). Ce chiffre pourrait doubler d'ici 2030. En France, 200 000 personnes ont déclaré la MP et environ 25000 nouveaux cas sont diagnostiqués chaque année. Rare avant l'âge de 50 ans, la MP est diagnostiquée en moyenne autour de 60 ans et la prévalence hommes - femmes reste controversée ((Twelves et al., 2003 ; (Poewe et al., 2017)). La MP est due à la dégénérescence de la voie nigrostriée c'est-à-dire des neurones dopaminergiques de la substance noire (SN, structure du mésencéphale ventral) qui établissent des connections sur le striatum (structure télencéphalique impliquée dans les fonctions motrices). La dégénérescence induit un déficit en dopamine dans le striatum et par conséquent une perturbation de fonctions motrices et cognitives. Les traitements actuels contre la MP sont symptomatiques, ce sont des traitements pharmacologiques à long terme qui permettent de palier au déficit dopaminergique mais dont l'efficacité baisse progressivement et donne lieu à des effets secondaires indésirables. De ce fait, d'autres approches thérapeutiques comme la thérapie cellulaire régénérative sont envisagées pour rétablir la voie neuronale nigrostriée endommagée et favoriser la récupération fonctionnelle.

Des expériences menées sur des modèles animaux de la MP, tels que des rongeurs lésés à la 6-hydroxydopamine (6-OHDA) ou des primates non-humains lésés au 1-méthyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) ont montré que des cellules provenant de mésencéphale ventral fœtal et greffées de manière ectopique dans le striatum pouvaient survivre, innover le striatum, et compenser les déficits comportementaux liés à la lésion (Winkler et al., 2000). Chez les patients atteints de la MP, des essais cliniques impliquant la transplantation de cellules issues de mésencéphale ventral de fœtus humains, ont montré que les neurones peuvent survivre, se différencier en neurones dopaminergiques et innover le striatum assurant une libération de dopamine et pour certains patients des bénéfices cliniques majeurs (Piccini et al., 1999). Néanmoins, de plus larges essais cliniques ont aussi montré que la greffe de cellules dans le striatum n'avait pas d'effet bénéfique: dans certains cas la greffe pouvait induire des dyskinésies (Olanow et al., 2003) et plusieurs années après la transplantation, une faible proportion des cellules greffées contenaient des inclusions similaires aux corps de Lewy, caractéristiques de la pathologie. Une partie de ces effets indésirables pourrait être due à l'emplacement ectopique des cellules greffées dans le striatum, et non dans la SN (Cooper et al., 2009; Mendez et al., 2008). Depuis, notre équipe (A. Gaillard et al., 2009; A. Gaillard & Jaber, 2011) et d'autres (Thompson et al., 2009) ont montré que la greffe de cellules de mésencéphale ventral issu d'embryon de souris d'âge E12.5 dans un modèle animal de la MP, permet la reconstruction anatomique et fonctionnelle de la voie nigrostriée. Ceci suggère que les axones des neurones greffés sont guidés de manière appropriée depuis le greffon intranigral, vers leur cible, dans le striatum. Les axones des cellules fœtales sont donc capables de répondre à l'environnement moléculaire adulte, probablement par le biais de molécules de guidage axonal connues pour être impliquées dans l'établissement de la voie mésostriée pendant le développement normal embryonnaire, telles que les éphrines, la nétrine et les sémaphorines (Pour revue, Prestoz, Jaber, & Gaillard, 2012). En effet, nous avons montré que l'expression de

la Séma7A varie lors des différentes étapes de reconstruction de la voie nigrostriée dans les structures environnant cette voie dans ce modèle animal de la MP (Kalaani et al., 2016). D'autres travaux ont montré que la Séma7A et son récepteur Plexin-C1 sont exprimés dans certaines populations de neurones mésencéphaliques chez l'adulte (Pasterkamp et al., 2007) et seraient importants pour la ségrégation et le guidage des neurones dopaminergiques mésencéphaliques pendant le développement (Chabrat et al., 2017).

Dans cette étude, nous avons testé l'implication de la Sema7A dans l'établissement de la voie mésostriée pendant le développement embryonnaire et dans la réparation anatomique et fonctionnelle de la voie nigrostriée chez l'adulte après greffe. Grâce à des approches de neuroanatomie et de stéréologie réalisées chez des souris déficientes en Séma7A, nous avons quantifié le nombre de neurones dopaminergiques du mésencéphale ventral aux différents stades embryonnaires de développement de la voie nigrostriée : E12.5, E14.5, E16.5 et E18.5. Nous avons mis en évidence une diminution du nombre de cellules dopaminergiques dans la SN et l'aire tegmentale ventrale des embryons en absence de Séma7A et la restauration du nombre de neurones chez l'adulte. Ces résultats suggèrent que la Séma7A est nécessaire au développement des neurones dopaminergiques dans le mésencéphale ventral et pourrait notamment augmenter leur survie. Ceci corrobore une étude récente montrant un effet bénéfique de la Séma7A sur la vulnérabilité des neurones dopaminergiques (Pacelli et al., 2015). Malgré une diminution du nombre de neurones dopaminergiques détectés dans le mésencéphale ventral en absence de Séma7A, nous n'avons pas détecté d'anomalies de connections des axones dopaminergiques sur le striatum. La Séma7A n'est en effet pas la seule molécule impliquée dans la connectivité nigrostriée lors de l'embryogénèse. La Nétrine-1 par exemple est essentielle pour la connexion des neurones dopaminergiques sur le striatum. En effet, plusieurs altérations ont été détectées chez la souris déficiente en DCC, récepteur de la

Netrin-1, suggérant un rôle pour la Netrin-1 dans la formation de la voie dopaminergique pendant le développement (Xu et al., 2010).

Grâce au même type d'approche de neuroanatomie, nous avons également montré que l'absence de la Sema7A avait un impact sur le nombre de cellules greffées après thérapie cellulaire dans un modèle animal de la MP. En effet, le nombre de cellules greffées augmente en absence de Sema7A, et plus particulièrement le nombre de neurones dopaminergiques du mésencéphale ventral. La Sema7A a été décrite dans de nombreuses études comme un modulateur des processus inflammatoires et de l'immunité. La Sema7A active les monocytes *in vitro* et peut stimuler la production de plusieurs molécules proinflammatoires, notamment IL-1, TNF- α et IL-6 (Holmes et al., 2002). Elle est exprimée par les lymphocytes T activés (Mine et al., 2000) et dans une moindre mesure par les lymphocytes B (Gan et al., 2011). Les macrophages qui expriment la β 1-intégrine sont activés par les cellules T exprimant la Sema7A. Par conséquent, la Sema7A induit une chimiotaxie des macrophages, déclenche la production de cytokines inflammatoires et module le fonctionnement des lymphocytes T. De plus, la Sema7A est également impliquée dans la neuroinflammation dans certains troubles du SNC, tels que les lésions de la moelle épinière et la sclérose en plaques ((Kopp et al., 2010; Gutiérrez-Franco et al., 2017). Ainsi, dans notre modèle expérimental, la Sema7A pourrait participer aux événements inflammatoires qui ont un impact négatif sur la survie des cellules greffées. Ces résultats sont d'un intérêt crucial si l'on veut limiter la mort des cellules greffées dans les approches de thérapie cellulaire en bloquant par exemple l'action de cette molécule.

D'une manière plus globale, nous avons étudié le protéome, par spectrométrie de masse par chromatographie en phase liquide (LC-MS/MS), dans les 5 principales régions associées à la voie nigrostriée après thérapie cellulaire dans ce même modèle murin de la MP. Pour cela, des souris adultes ont été lésées unilatéralement avec une injection de 6-OHDA dans la SN afin de tuer spécifiquement les cellules DA. Les souris ont ensuite été transplantées avec des cellules

de mésencéphale ventral (VM) d'embryons d'âge E12.5 dans la SN et les 5 régions cérébrales associées à la voie nigrostriée (mésencéphale ventral, faisceau médian du télencéphale, thalamus, noyau accumbens, caudoputamen) ont été collectées 1 et 7 jours après la transplantation. Nos résultats ont montré que des protéines étaient exprimées de manière différentielle après greffe par rapport au cas contrôles non-greffés. Ces protéines sont particulièrement impliquées dans les réactions inflammatoires locales, le stress oxydatif et les voies métaboliques mitochondriales. De plus, nous avons observé une diminution progressive, entre 1 jour et 7 jours après la transplantation, de l'expression de protéines impliquées dans l'endocytose des récepteurs à la dopamine comme la tétraspanine⁷ et dans le recyclage de la dopamine, comme l'endophiline A1. Ceci suggère que de manière concomitante à la croissance axonale des neurones greffés en direction de leur cible terminale, la transmission dopaminergique est favorisée par le biais de la diminution de l'expression de gènes régulant négativement la libération de dopamine.

Au total, cette étude nous a permis d'apporter de nouvelles connaissances concernant le rôle de la molécule de guidage axonal, Sémaphorine^{7A}, dans la production et la survie des neurones DA que ce soit au cours du développement ou dans un modèle de pathologie comme la MP après thérapie cellulaire. Dans ce même modèle, nous avons identifié des protéines qui pourraient être utilisées comme cibles afin d'améliorer l'efficacité de la thérapie cellulaire dans la MP.

Introduction

Introduction

I. Parkinson's disease.....	10
1. General background.....	10
1.1. <i>Epidemiology</i>	10
1.2. <i>Symptoms and diagnosis</i>	11
1.3. <i>Histopathology</i>	12
1.4. <i>Pathogenesis</i>	14
1.5. <i>Animal models of Parkinson's disease</i>	18
2. Treatments and potential therapeutic strategies.....	26
2.1. <i>Pharmacological targets</i>	27
2.2. <i>Surgical treatment</i>	28
2.3. <i>Developing restorative cell therapy approaches</i>	29
2.3.1. <i>Fetal Cell transplantation</i>	31
2.3.2. <i>ESC-derived DA cells as another potential cell source</i>	32
2.3.3. <i>iPSC-derived DA cells</i>	34
II. The mesencephalic dopaminergic pathways.....	37
1. Emergence of mDA neurons during embryogenesis.....	37
2. Establishment of the dopaminergic mesencephalic pathway.....	39
2.1 <i>Axon guidance signals that mediate axonal steering</i>	39
2.1.1 <i>Netrins and DCC</i>	42
2.1.2 <i>Ephrins and Ephs</i>	44
2.1.3 <i>Slits and Robos</i>	45
2.1.4 <i>Semaphorins, neuropilins and plexins</i>	46
2.2. <i>mDA axon midbrain</i>	48
2.3. <i>Progression of mDA axons in navigation outside the the medial forebrain bundle (MFB)</i>	50
2.4 <i>Arrival of mDA axons to their striatal targets</i>	51
III. Axon guidance molecules in the adult mesostriatal pathway.....	53
1. Axon guidance molecules in intact adult mesostriatal pathway.....	53
2. Axon guidance molecules in altered adult mesostriatal pathway.....	55
IV. Aim of the project.....	57

I. Parkinson's disease

1. General background

Parkinson disease (PD) is a progressive neurodegenerative disorder first described in 1817 by an English doctor, James Parkinson who offered a clinical trial entitled 'An Essay on the Shaking Palsy'(Parkinson, 1817). The study described a detailed disabling progressive condition of six patients with rest tremors, posture and walking disorders, muscle rigidity and slow voluntary movement (Lees et al., 2009). This disease was later renamed in his honor "Parkinson Disease" by French neurologist Jean-Martin Charcot (Charcot and Vulpian, 1862; reviewed by Engelhardt & Gomes, 2017). Although PD has long been described simply as a neurodegenerative disorder distinguished by a prominent dopaminergic neuronal loss in the substantia nigra *pars compacta* (SNpc), it is now commonly recognized as a multisystem neurological disorder associated with many motor and non-motor dysfunctions, where deficiency in striatal dopamine levels is one of several remarkable features (reviewed by Lang & Espay, 2018). The standard neuropathological characteristics for diagnosis of PD are the buildup of proteinaceous intracellular inclusions, the Lewy bodies, containing aggregates of α -synuclein and the deterioration of the nigrostriatal pathway (reviewed in Kalia & Lang, 2015). Hence, patients with PD experience motor, cognitive, and neuropsychiatric complications that often heavily impact their health status and life quality to a greater extent (Lelos et al., 2016).

1.1. Epidemiology

PD is a progressive neurodegenerative disorder with a doubling mortality compared with the general population after 15 to 20 years of the disease onset (reviewed by Lang & Espay, 2018). The incidence rate of PD worldwide is 5 to >35 per 100,000 individuals each year and more frequently affecting individuals >50 years of age (Twelves et al., 2003). Between 2005 and 2030 the number of PD patients is expected to double and as the population ages worldwide,

the financial, societal disease associated burden is estimated to increase progressively in the future (Dorsey et al., 2007).

Rare before the age of 50, PD is diagnosed on average around 60 years of age and is reported to be twice as prevalent in men as in women (reviewed in Twelves et al., 2003) but in some studies, including one study from Japan, no difference in PD prevalence was observed between different genders (reviewed in Poewe et al., 2017).

1.2. Symptoms and diagnosis

Due to the complexity of PD, early and accurate diagnosis of the disease is associated with many challenges (Kalia & Lang, 2015). Diagnosis of PD is established when patients exhibit a combination of cardinal features as well as exclusive and positive criteria that support and improve the accuracy of the clinical examination (Postuma et al., 2015). PD is clinically characterized by its prominent motor symptoms whose onset is unilateral and persists with the advancement of the disease (Alcalay et al., 2010). However the presence of other significant non-motor and neuropsychiatric complications changes the perception of PD to a disorder of movement with a heterogeneous symptomatology (Marras et al., 2013).

The classical motor symptoms of the PD include muscular rigidity, bradykinesia, rest tremor, postural instability and gait impairment (reviewed by Kalia & Lang, 2015). The classical motor symptoms of PD have been recognized since James Parkinson's initial description and later rephrased by Jean-Martin Charcot (Goetz, 2011). These symptoms include bradykinesia (slow movement), muscular rigidity, rest tremors and bradykinesia, postural instability and gait impairment (Jankovic, 2008). It has been found that these motor symptoms vary among the patients, which has led to attempts to classify PD into subtypes (Marras et al., 2013) but no consensus has yet been established. Nevertheless, empirical clinical observations propose two main subgroups: PD subtype with tremor as the dominant symptom (where other motor symptoms are absent), and PD with non-tremor dominant symptoms. Disease progression and

prognosis differ between these two subgroups, with the former often associated with lower disability and slower progression rate than the latter (Jankovic et al., 1990).

Patients with PD also experience non-motor symptoms some of which are present before the onset of classic motor symptoms by more than a decade (Chaudhuri & Schapira, 2009). The non-motor features include cognitive symptoms involving hallucinosis, dementia, memory retrieval deficits, as well as executive dysfunction syndrome where the patient loses the ability to sustain an adaptive response while executing tasks on time (Chaudhuri & Schapira, 2009; Poewe et al., 2017). Patients with PD also experience several autonomic dysfunctioning mainly postural hypotension, constipation, urogenital impairment, excessive hydrosis, pain, sensory symptoms (mainly olfactory dysfunction) as well as fatigue (reviewed by Poewe et al., 2017). In addition to that, neuropsychiatric symptoms are associated with the disease, for instance, several cases of depression have been described in 20 to 40% of patients in early stages of PD (Reviewed by Aarsland et al., 2017).

These features exert a significant effect on the health-related quality of the patients, which deteriorates with the progression of the disease (reviewed in Poewe et al., 2017).

1.3. Histopathology

The pathological hallmarks for the diagnosis of PD at the post-mortem examination was described to be a dopaminergic neuronal loss in the SNpc and Lewy body pathology.

In the ventrolateral tier of the SNpc, the dopamine-producing neurons which innervates the dorsal striatum are degenerated. Moreover, other extranigral regions are affected by the neuronal degeneration and these regions include the isthmic nuclei, the raphe nuclei, and the dorsal motor nuclei of the vagal and hypoglossal nerves, in addition to the olfactory bulb (Braak et al., 2003).

The Lewy body pathology was described over a century ago by Dr. Friedrich Lewy and named Lewy bodies or Lewy neurites by Dr. Konstantin Tretiakoff in 1919 (reviewed by Lees et al.,

2008; Goedert et al., 2013). This hallmark consists of the abnormal accumulation of α -synuclein protein inclusions located in neuronal perikarya referred to as Lewy bodies, and neuronal processes that are known as Lewy neurites (reviewed by Kalia & Lang, 2015) (Figure 1).

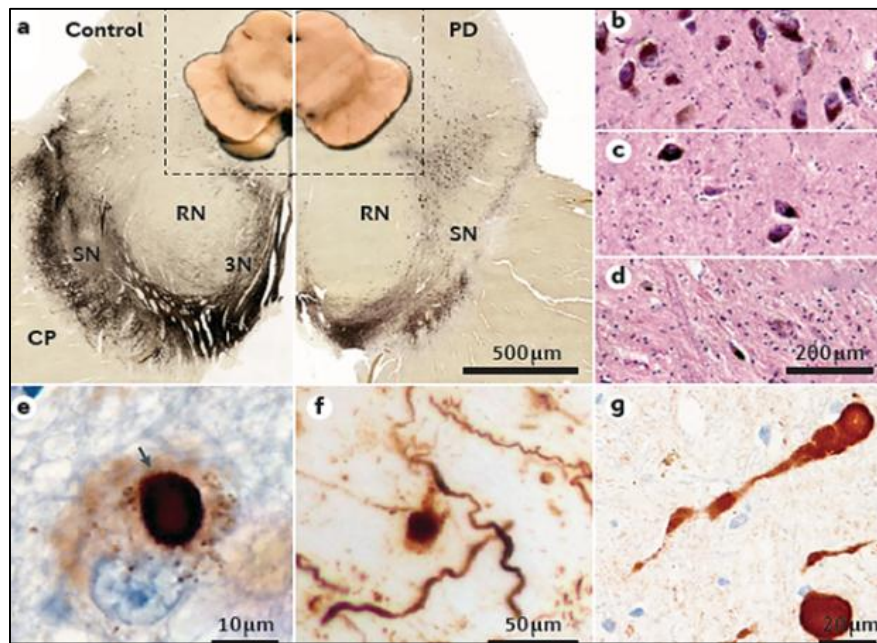


Figure 1: Neuropathology of Parkinson's disease. (a). Immunohistochemical staining in midbrain sections for tyrosine hydroxylase showing the loss of the dopaminergic (DA) neurons and depigmentation in the ventrolateral tier of the SN region of PD patient, in the right panel compared with control (left panel). (b–d) Haematoxylin and eosin staining of a control SN showing a normal distribution of pigmented neurons in (b) and moderate (c) or severe (d) cell degeneration in PD. (e–g) α -synuclein staining showing the intraneuronal Lewy bodies (e), the α -synuclein aggregates in the neuronal processes (f), axonal α -synuclein spheroids (g). *RN*: red nucleus; *SN*: substantia nigra; *CP*: cerebral peduncle; *3N*: third nerve fibers. (Adapted from Poewe et al., 2017).

It has been found that these α -synuclein inclusions initially occur in the monoaminergic and cholinergic brainstem neurons as well as in neurons in the olfactory system, but with disease progression these aggregates are also found in neocortical and limbic brain regions (reviewed by Poewe et al., 2017). Several pathological studies indicate a correlation between the Lewy

pathology and the cognitive symptoms associated with the PD (Yang, Tang, & Guo, 2016). After the discovery of mutations in its encoding gene, SNCA, α -synuclein protein was manifested as a major player in the pathological hallmarks of PD (reviewed by Kalia & Lang, 2015).

1.4. Pathogenesis

Pathological advances along with other epidemiological and genetic observations helped in exploring the underlying mechanisms and pathways involved in the pathogenesis of PD. These mechanisms include impairments in α -synuclein protein accumulation, mitochondrial abnormalities, dysfunction in the intracellular trafficking, oxidative stress, neuroinflammation (Figure 2) and aging (reviewed by Poewe et al., 2017)

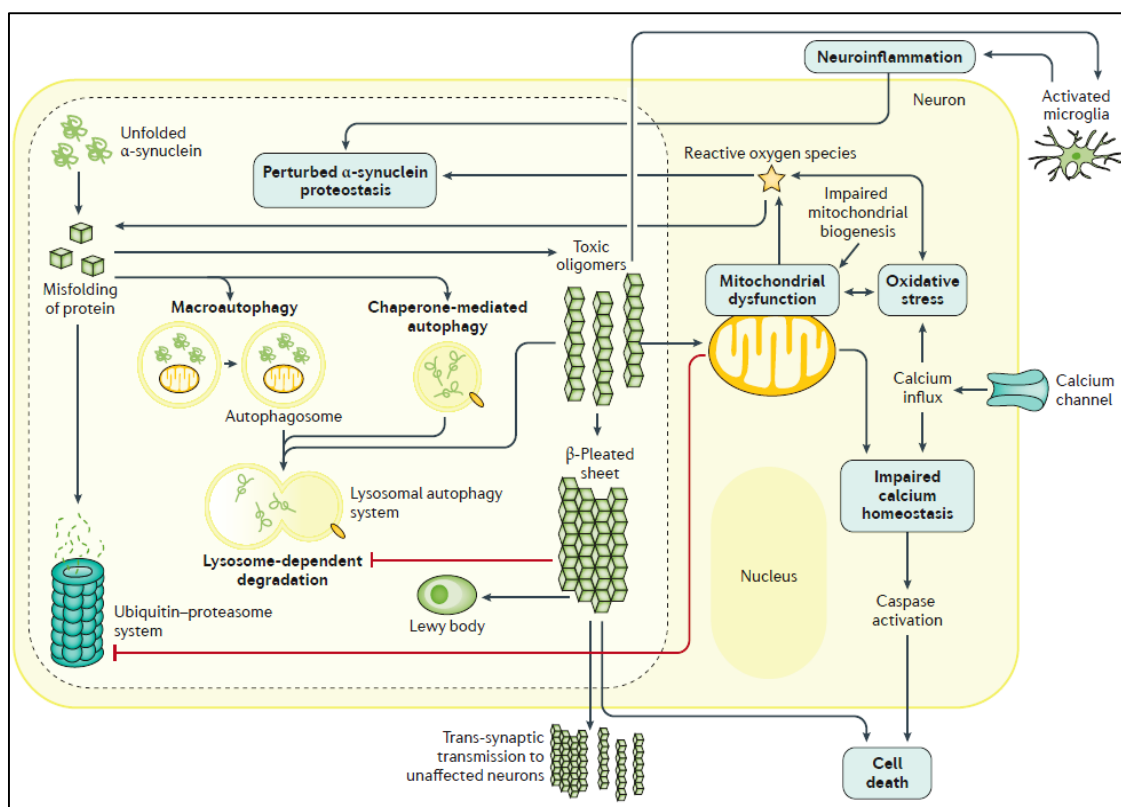


Figure 2: Pathogenesis of Parkinson’s disease. Schematic representation showing major molecular mechanisms involved in the pathogenesis of PD such as α -synuclein proteostasis, oxidative stress, mitochondrial function, calcium homeostasis, and neuroinflammation (Adapted from Poewe et al., 2017).

Indeed, several neurotoxic pathogenic alterations trigger the soluble α -synuclein monomers to undergo structural changes and aggregate into toxic α -synuclein oligomers. These oligomers then polymerize as small protofibrils and eventually large, insoluble α -synuclein fibrils that make up Lewy pathology (C. Kim & Lee, 2008). Subsequently, the accumulation of α -synuclein disrupts several proteasomal and lysosomal degradation mechanisms such as the chaperon-mediated autophagy and macroautophagy, as well as the ubiquitin–proteasome systems (Emmanouilidou et al., 2010; Tanik et al., 2013). Eventually, the presence of the Lewy bodies in the neuronal cell body contributes to mitochondrial dysfunctioning, oxidative stress, aberrations in calcium homeostasis, and neuroinflammation leading to neuronal cell death (Poewe et al., 2017).

In view of the remarkable PD symptoms parallels seen in mitochondrial and α synuclein dysfunctions, many studies have unpinned the underlying molecular pathways of PD and some of which are of genetic basis where certain mutations have been implicated with the onset of PD. For instance, previous studies already identified six genes that were associated with autosomal dominant parkinsonism: SNCA, LRRK2, VPS35, EIF4G1, DNAJC13, and CHCHD2 (Corti et al., 2011) and among which SNCA was the first identified (Polymeropoulos et al., 1997). Mutations in the SNCA gene coding for the α -synuclein leads to the intracellular aggregation of misfolded form of this protein within the neuronal cell body forming Lewy bodies and neuronal processes forming Lewy neuritis (Goedert et al., 2013). Several other genes are associated now with either autosomal recessive (such as PARK2/Parkin, PINK1, and DJ-1) or dominant inheritance (such as SNCA, LRRK2) (Verstraeten et al., 2015). Mutations in PD associated LRRK2 (encoding for leucine-rich repeat kinase 2) are linked to autosomal-dominant parkinsonism (Zimprich et al., 2004) and are associated with deficits in the mitochondrial functioning such which lead to mitochondrial malfunctioning along with other impairments in the autophagy system as mentioned before (Bose & Beal, 2016). Proteins

encoded by parkin (an E3 ubiquitin ligase), DJ-1, and PINK1 (encodes a mitochondrial serine-threonine protein kinase) genes that are involved in the degrading damaged mitochondria through mitophagy, are autosomal recessive in PD and thus reported to also contribute to the mitochondrial dysfunctioning in PD (Pickrell & Youle, 2015). Mutations in PINK1 and DJ-1 are less common causes of autosomal recessive PD compared to parkin mutations which are the most common (Singleton et al., 2013). DJ-1 is implicated in mitochondrial regulation, transcriptional regulation, and protecting mitochondria against the oxidative stress (Ariga et al., 2013).

In Brief, mutations in some of these genes have been mostly linked to α -synuclein accumulation and disrupted mitochondrial functioning, consequently leading to neuronal cell death.

Regarding the α -synuclein proteostasis, both, the ubiquitin–proteasome system (UPS) and the lysosomal autophagy system (LAS) comprise the intracellular homeostasis of α -synuclein protein (Poewe et al., 2017). Within the LAS, macroautophagy encompasses the formation of autophagosomes whereas chaperone-mediated autophagy comprises specific chaperones that target certain proteins to lysosomes. Several studies suggest that suppression of either system leads to increased levels of α -synuclein (Alvarez-Erviti et al., 2010). Several observations reported a reduction of several markers of chaperone-mediated autophagy (Alvarez-Erviti et al., 2010) as well as a decrease in the levels of lysosomal enzymes (Chu et al., 2009) and aggregation of autophagosome (Poewe et al., 2017), in the substantia nigra of PD patients. Another important factor in considering the pathogenic role of α -synuclein in PD is aging which contributes to the disruption of function in both LAS and UPS, (reviewed in Poewe et al., 2017).

In PD, mutations are the most common genetic risk factor and of which some coupled to impairments in α synuclein degradation systems. In familial forms of PD, malfunctioning of LAS is linked to mutations in the gene encoding LRRK2 that that impacts the autophagy lysosomal pathways and more specifically leading to a deficit in α synuclein degradation and

subsequently to the accumulation of α synuclein in the dopaminergic neurons (Volpicelli-Daley et al., 2016). Other mutations may contribute to a reduced LAS functioning such as the heterozygous GBA mutations targeting the glucocerebrosidase gene encoding the lysosomal enzyme GBA (Volpicelli-Daley et al., 2016).

Mitochondrial dysfunctions have been also reported as in normal individuals, where α -synuclein protein is present at low levels inside the mitochondria, however in PD patients α -synuclein levels elevates affecting the mitochondrial normal functioning. The activity of the mitochondrial complex I, a component of the electron transport chain, is found to be altered and reduced due to the aggregation of α -synuclein protein (Devi et al., 2008). In addition, the mitochondrial electron transport chain is impaired in the dopaminergic neurons of the SN due to a deficit in the mitochondrial transcription factor A activity which is selectively reduced in the DA neurons of mice, eventually leading to neuronal degeneration later in adulthood (Ekstrand et al., 2007). As a consequence of mitochondrial malfunctioning, oxidative stress is elevated in the nigral dopaminergic neurons of patients with PD consequently together leading to deficits in the autophagy lysosomal pathways (Dias et al., 2013).

Finally, several studies reported that neuroinflammation is a prominent silent feature also contributing to the pathogenesis of PD. Evidence from patients and experimental studies suggest that both adaptive and innate immune systems in PD play a role in the propagation of the α -synuclein pathology (Valdinocci et al., 2017). Neurodegeneration is subsequently driven by the triggered inflammatory responses such as phagocytosis, activation of many chemokines and cytokines (reviewed in Lang & Espay, 2018). Nevertheless, the activated immune cells such as microglia contribute to the clearance of α -synuclein aggregates suggesting that the activated immune system is not linked only to the deterioration of the disease (reviewed by Poewe et al., 2017).

1.5. Animal models of Parkinson's disease

Several animal models have been generated to elucidate the mechanisms underlying the development and progression of PD with an aim to develop an improved and efficient therapy to fully recover the patients and cure PD.

Ideally, these models should specifically mimic the etiology and progressive neuroanatomical and pathological deficits of the disease in an irreversible and reproducible manner. Thus, animal models should establish Lewy pathology in consistence with the disease. In addition, a progressive and specific degeneration of the target SNpc dopamine neurons, should be developed. This degeneration should lead to a progressive loss of striatal DA innervation accompanied with deletion in the striatal DA levels for the appearance of motor symptoms. Up to date, the different models developed still lack certain neuropathological and behavioral features of PD and thus do not phenocopy the disease (reviewed by Blesa & Przedborski, 2014). Transgenic and neurotoxic models are basically the two prominent animal models used to replicate the disease pathology (Blesa et al., 2012). Several neurotoxic models provide information about the pathology of the disease and are suitable for therapeutic approaches however genetic models reflect the pathogenic mechanisms as well as the aetiology of the disease (Dauer & Przedborski, 2003). Transgenic models were developed based on the genetic causes and targeting the genes associated with the onset of the disease such as α -synuclein, LRRK2, PINK1/Parkin (Table 1).

	Animal model	Motor behavior	SNc neuron loss	Striatal DA loss	Lewy body/Syn pathology
Toxin-based	MPTP Mice	Reduced locomotion, bradykinesia	↑↑↑	↑↑↑	NO
	MPTP Monkeys	Reduced locomotion, altered behavior, tremor, and rigidity	↑↑↑	↑↑↑	NO
	6-OHDA rat	Reduced locomotion, altered behavior	↑↑↑	↑↑↑	NO
	Rotenone	Reduced locomotion	↑↑	↑↑↑	YES
	Paraquat/maneb	Reduced locomotion	↑↑	↑↑↑	YES
	MET/MDMA	Reduced locomotion	↑↑	↑↑↑	NO
Genetic mutations*	α -Synuclein	Altered behavior, reduced or increased motor activity	↑ Not consistent	↑	↑ (in old animals)
	LRKK2	Mild behavioral alteration	NO	NO	NO
	PINK1	No obvious alterations or reduced locomotion	NO	NO	NO
	PARKIN	No obvious locomotion or reduced locomotion	NO	↑	NO
	DJ-1	Decreased locomotor activity	NO	NO	NO

Table 1: Characteristics of transgenic and neurotoxic models. ↑↑↑ corresponds to severe loss; ↑↑ to moderate loss; ↑ to mild loss (adapted from Blesa & Przedborski, 2014).

Only the α -synuclein model exhibits altered motor behavior with mild loss of DA neuron and presence of Lewy bodies. More precisely, two mutations associated with α -synuclein gene, A53T and A30P, were linked to cause a dominantly inherited form of the disease (Krüger et al., 1998) and was used to generate transgenic mice for PD (Table 2).

Promoter	Transgene	Cell death	Motor deficits/ nigrostriatal dysfunction	Cellular abnormalities	References
PDGF- β	WT α -syn	No neurodegeneration	↓ Rotarod performance (12 mo)/↓ TH and fiber density (STR)	Inclusion bodies (Ub, Syn +)	Masliah et al. 2000
mThy-1	A30P α -syn	Sensorimotor neuronal loss (brainstem and SC)	Severe leading to paralysis (8~12 mo)/normal DA and metabolite levels	LB-like inclusion (fibril)	Neumann et al. 2002
mPrP	A53T α -syn	Non-dopaminergic neuronal loss (red nuclei, brainstem, and SC)	Severe (12 mo)/normal DA levels, normal TH morphology	LB-like inclusion (fibril), mitochondrial dysfunction	Giasson et al. 2002; Lee et al. 2002; Gispert et al. 2003
Rat TH (9 kb)	A30P/A53T α -syn	Progressive DA neuronal loss	↓ Locomotor activity (13–23 mo)/↓ DA and metabolites, TH axons beaded	No inclusion bodies, diffuse synuclein in DA neurons, ↑ presynaptic DAT	Richfield et al. 2002
CamKII-tTA (tet-off)	WT α -syn, A53T α -syn	Trend for TH ⁺ cell loss, degenerating cells in SN and hippocampus (WT), non-DA neuronal loss (A53T)	↓ Rotarod performance and motor learning (WT)/normal DA levels (STR)	Condensed mitochondria and lipid droplets	Nuber et al. 2008
BAC	LRRK2 R1441G	No	↓ Rearing (12 mo)/↓ DA transmission (STR), ↓ fiber density (SNr), neurite fragmentation (STR)	↑ Tau and phosphorylated tau	Li et al. 2009
CamKII-tTA (tet-off)	LRRK2-WT/GS/KD	No	Normal	Golgi structure fragmentation	Wang et al. 2008; Lin et al. 2009

Table 2 : Transgenic mouse models of autosomal-dominant PD. *TH*, tyrosine hydroxylase; *STR*, striatum; *Ub*, ubiquitin; *SC*, spinal cord; *DA*, dopamine; *DAT*, dopamine transporter; *SNr*, substantia nigra reticulata; *KD*, kinase dead. (Modified from Lee et al., 2012).

In mice, A53T mutations lead to the formation of Lewy body-like inclusions with no loss in the DA neurons of the SN and the animals usually demonstrate severe motor complications that lead to paralysis and death (Giasson et al., 2002; Masliah et al., 2000). In A30P transgenic mice this phenotype is not obtained and the neurodegeneration of the DA neurons is not affected by the loss of α -synuclein protein in these animals (Blesa et al., 2012). However, in a knock-in

model in *Drosophila*, the expression of the mutant α -synuclein resulted in formation of intracellular inclusions, motor deficits, and DA neurodegeneration (Feany & Bender, 2000).

Unlike α -synuclein transgenic mice models, LRRK2 mouse model doesn't show significant DA loss in the SN where only negligible levels of neurodegeneration is obtained (H. Li & Guo, 2009). As for other target genes in transgenic models, even though in PINK1 knockout mice models the striatal DA level diminished, this model in addition to the PARK1 and DJ-1 transgenic models did not reproduce the principal features of PD (Moore & Dawson, 2008) (Table 3).

PD gene	Motor deficit	Electrophysiological/ neurochemical dysfunction	Biochemical changes	References
Parkin	Declines in voluntary and amphetamine-induced activity, beam traversing, acoustic startle response	↑ DA (STR, limbic); ↓ NE (OB, SC), ↓ striatal neuron excitability	↓ DAT, VMAT2; ↑ reduced glutathione (STR); ↑ parkin substrates (STR, Vmb); ↓ mitochondrial and antioxidant proteins (Vmb)	Goldberg et al. 2003; Itier et al. 2003; Von Coelln et al. 2004
DJ-1	Age-dependent declines in locomotor activity, rearing, and tape removal test (11 mo)	↑ DA re-uptake, DA content, and ↑ stimulated DA release (STR)	Normal amounts of TH, DAT, VMAT2 (STR), and oxidized proteins; ↓ mitochondrial peroxidase activity	Chen et al. 2005; Goldberg et al. 2005; Kim et al. 2005; Andres-Mateos et al. 2007
PINK1	Age-dependent impairment of spontaneous activity	↓ Evoked DA release; ↓ DA content (STR)	↑ Large mitochondria, mitochondrial respiration, ATP generation; ↓ aconitase activity (STR)	Gautier et al. 2008; Gispert et al. 2009; Kitada et al. 2009

Table 3: Transgenic autosomal-recessive mouse models of PD. *NE*, Norepinephrine; *OB*, olfactory bulb; *VMAT*, vesicular monoamine transporter; *Vmb*, ventral midbrain. (Modified from (Y. Lee et al., 2012))

However, degeneration of the DA neurons in the SN is obtained after knocking out Parkin at adult stage in conditional knockout mouse model (Shin et al., 2011).

Even though most transgenic animal models don't replicate the cardinal features of PD they are still useful for understanding the etiology and some of the pathogenic mechanisms of the disease.

Toxin-based models exhibit altered motor behavior and DA neuron loss (Table 1). They are based on local or systemic administration of neurotoxic and pharmacological agents that target the catecholaminergic system and selectively trigger the degeneration of DA neurons of the SN such as the 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat and rotenone (Blandini & Armentero, 2012; Le et al., 2014). These toxins function through several mechanisms generally leading to mitochondrial impairments, oxidative stress, and ATP depletion which eventually trigger the neuronal cell death (Figure 3) (Cabezas et al., 2013).

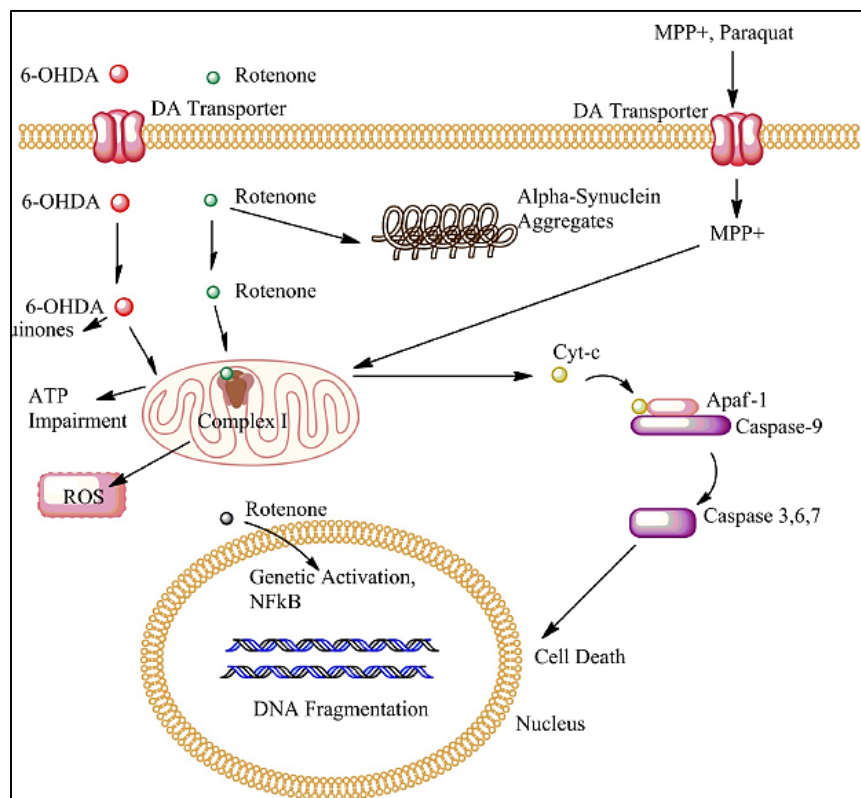


Figure 3: Mechanisms of action of neurotoxic models of PD. 6-OHDA, MPTP, and paraquat enter the neuron cell membrane through the dopamine transporter (DA) whereas rotenone which is extremely hydrophobic can easily cross the cell membrane. Common mechanisms by which these four toxic agents cause neuronal cell death, are α -synuclein aggregation, impairment of the mitochondrial complex I, followed by consequent accumulation of oxidative stress and depletion in ATP inside the cell. Moreover, rotenone can trigger cell apoptosis by nuclear translocation of NF- κ B and by activation of downstream proapoptotic molecules such as cytochrome c that induces several caspase players leading to cell death (taken from Cabezas et al., 2013). *MPP*: 1-methyl-4-phenylpyridinium; *ROS*: reactive oxygen species

The MPTP neurotoxin was initially discovered through cases of chemically induced parkinsonism (reviewed Schober, 2004). MPTP can easily cross the blood-brain barrier to be metabolized in the glial cells into 1-methyl-2,3-dihydropyridinium, this intermediate product is then oxidized into 1-methyl-4-phenylpyridinium (MPP⁺) which is the toxic metabolite entering the dopaminergic neurons through the dopaminergic transporter (DAT). Within the mitochondria, MPP⁺ aggregates and targets the mitochondrial complexes I, III, and IV eventually leading to an increase in the oxidative stress and depletion in the ATP levels within the neuronal cell (H.C.H.M Philippens, 2018). MPTP toxic models have gained particular interest in PD due to their ability to reproduce specific aspects of Parkinson's degeneration, including alterations in the mitochondria and lysosomes ((Bové et al., 2014; Dauer & Przedborski, 2003). MPTP is commonly used as a model for PD in mice more than in rats which are resistant to moderate doses of MPTP and intolerable to higher doses (Giovanni et al., 1994). There are three types of MPTP models, the acute, subacute, and chronic (Selvakumar et al., 2014; Zhang et al., 2017) and depending on the administration of each, different pathological and behavioral outcomes are obtained. Unlike chronic MPTP models, acute and subacute MPTP administration manifests temporal and reversible neurotoxicity with regard to DA neuronal loss in mice (Petroske, Meredith, Callen, Totterdell, & Lau, 2001). Chronic MPTP treatment in mice results in an increase in the oxidative stress, loss of DA neurons and depletion in striatal levels in addition to motor behavioral impairments (Selvakumar et al., 2014). In contrary, in subacute MPTP mice models although MPTP administration succeeds in triggering dopaminergic neurodegeneration within the nigrostriatal system and decrement in striatal DA levels, yet in terms of functional performance, the motor behavior is not impaired (Q. S. Zhang et al., 2017). Therefore, among the MPTP models, chronic MPTP administration is by far the most representative as a model for PD.

Regarding other pathological features associated with this model, a study has shown a form of synucleinopathy in primate animals treated with MPTP with the presence of immunoreactive inclusions for α -synuclein (Kowall et al., 2000). However, there is not enough evidence or real characterization of the α -synuclein accumulation, therefore no conclusion can be drawn as to the existence of an MPTP-induced aggregation of α -synuclein.

On the other hand, 6-OHDA-injected rats were the first animal model of PD developed by Ungerstedt to target SNpc dopaminergic neurons degeneration (Urban Ungerstedt, 1968). Indeed, due to its structural homology with dopamine, this hydroxylated form of noradrenaline and dopamine has a strong affinity for the membrane transporter dopamine (Jonsson & Sachs, 1970). Through an auto-oxidation phenomenon, 6-OHDA accumulates in the cytosol where it is oxidized causing highly toxic oxidative stress, hence inducing neuronal death with gliosis (Figure 4) (Saner & Thoenen, 1971).

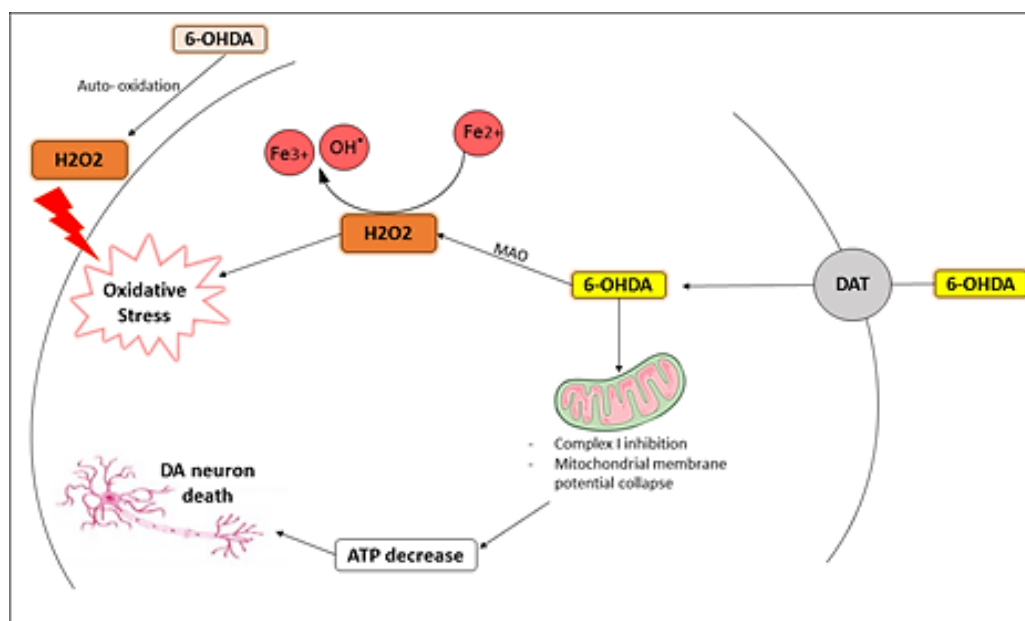


Figure 4. Mechanism of 6-OHDA toxicity on DA neurons. 6-OHDA could trigger DA cell death by three main mechanisms: H₂O₂ formation under the action of monoamine oxidase (MAO) activity, generation of ROS by auto-oxidation, or mitochondrial respiratory chain direct inhibition. ROS formation is augmented by elevated iron levels and may result from two separate mechanisms, intra or extracellular auto-oxidation and deamination by MAO following 6-OHDA administration. 6-OHDA is

a substrate for MAO and this enzymatic reaction catalyzed by MAO, leads to the production of H₂O₂. Moreover, 6-OHDA is able to directly target the mitochondrial respiratory chain by inhibiting the mitochondrial complex I and triggering the mitochondrial membrane potential to collapse through in a ROS-dependent manner. Consequently, these events lead to a decrease in cellular ATP levels and a robust oxidative stress inducing the nigrostriatal DA neurons degeneration. *DAT: Dopamine active transporter; 6-OHDA: 6-hydroxydopamine, H₂O₂: Hydrogen peroxide; MAO: monoamine oxidase; ROS: reactive oxygen species* (modified from Blum et al., 2001).

Because 6-OHDA does not cross the blood-brain barrier, it is usually injected directly into the SN, at the medial forebrain bundle (MFB) or into the dorsal striatum by stereotaxic injection. In rodents, injection of 6-OHDA into the dorsal striatum induces progressive and partial degeneration of SNpc DA neurons (Sauer & Oertel, 1994). On the other hand, an injection in the MFB or SN results in a rapid, and almost complete anterograde degeneration of DA neurons within 12 to 24 hours following the injection (Jeon et al., 1995), leading to a significant decrease in DA terminals (80-100%) and depletion in striatal dopamine level (Przedborski et al., 1995). Bilateral injections at the SN level have shown to cause severe aphagia, akinesia, and adipsia which may lead to death. On the contrary, unilateral injections are well tolerated by the animal where they develop postural asymmetry, contralateral motor deficits, and akinesia thus creating a sustainable and feasible animal model of PD (Ungerstedt, 1971).

In rodents, several behavioral tests can be used to assess the degree of unilateral 6-OHDA lesions which can be quantified in comparison to the intact contralateral side, used as internal control (reviewed by Dunnett & Lelos, 2010). Example on these behavioral tests are the drug-induced rotations such as apomorphine and amphetamine (Ungerstedt & Arbuthnott, 1970). Apomorphine is a DA agonist that binds to post-synaptic DA receptors. These receptors are overexpressed in the striatum after lesion to compensate the loss of dopamine. This hypersensitivity induces contralateral rotations when the loss of DA neurons is significant.

Amphetamine, on the opposite hand, binds to DAT transporters at the pre-synaptic level and promotes the release of dopamine, thus inducing rotation of the animal ipsilateral to the side of lesion. These rotations are visible even with moderate damage to the DA neurons of the SNpc (reviewed by Dunnett & Lelos, 2010).

Although the 6-OHDA model is not a complete model of PD, due to the absence of Lewy bodies and non-motor symptoms, it nevertheless allows the acquisition of a relatively reproducible and cheap hemi-Parkinsonian model. Moreover, it helps to assess the replacement of lost DA neurons and restoration of DA supply to the striatum which is one of the most common current therapeutic strategy.

Other environmental toxic agents can trigger neurotoxic actions leading to development of PD. Exposure to the herbicide paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride) has been shown to trigger the SN-DA cell loss when administered in animals (Brooks et al., 1999; Brown et al., 2006). Yet, the idea of considering paraquat as a candidate etiological factor of PD in humans is still ambiguous and not clear (Berry et al., 2010).

Rotenone is an isoflavonoid naturally present in the roots of some tropical legumes and it is a component used in several pesticides and insecticides and functions as high-affinity specific inhibitor of mitochondrial complex I, therefore influencing the ATP production (Betarbet et al., 2000). Rotenone has also been used to develop PD models in rats (Betarbet et al., 2000) and mice (Inden et al., 2011). However, due to lack of conclusive evidence it is indecisive whether rotenone develop PD in humans (Blesa et al., 2012).

2. Treatments and potential therapeutic strategies

Different therapeutic approaches have been developed to treat patients with PD and improve their living conditions. However, the implementation of an efficient and successful treatment should aim for the repair of the motor complications taking into consideration all pathological and functional aspects of the disease. At the present time, treatments are mostly symptomatic

based on pharmacologic products to improve the motor deficits in the patients (reviewed in Pires et al., 2017).

2.1. Pharmacological targets

An understanding of the pathophysiology of PD has led to the development of a targeted drug strategy limited to the symptomatological treatment. Although the disorders of the disease cannot be summarized by the sole degeneration of the nigrostriatal pathway, medical treatment aims to compensate for the dopaminergic deficit.

For instance, the administration of the L-3, 4-dihydroxyphenylalanine (L-DOPA), a dopamine precursor amino acid to replace the striatal dopamine depletion and treat the motor symptoms was considered as an advanced breakthrough in the treatment of PD (Gray et al., 2014; LeWitt & Fahn, 2016). Despite being the most effective treatment for PD motor symptoms, L-DOPA induces several motor and non-motor fluctuations after several years of drug administration narrowing the therapeutic window for PD patients. The variability in blood–brain barrier transport and the short half-life of L-DOPA makes the drug delivery discontinuous and during this period, the patients experience motor response oscillations, and dyskinesia or what is known as hyperkinetic involuntary movement behavior (reviewed by Poewe et al., 2015). Moreover, non-motor complications are also often accompanied with L-DOPA treatments disease progress. These include autonomic dysfunction, neuropsychiatric problems, and sensory symptoms (Aquino & Fox, 2015). This has led to the administration of other substitutes that have longer half-life than L-DOPA that can treat motor signs with less dyskinesia such as monoamine oxidase type B inhibitors (MAOBIs), dopamine agonists and catechol-O-methyl transferase inhibitors (COMT inhibitors) (Maranis, et al., 2011). However, the use of these substitutes is also limited by their wide spectrum of adverse side effects including sleep disordered breathing (Valko et al., 2014). Moreover, the use of COMT inhibitors is also associated with undesirable

side effects such as dizziness, insomnia, hepatic toxicity, and diarrhea (reviewed by Stayte & Vissel, 2014).

2.2. Surgical treatment

Another therapeutic axis that can be used to reduce motor symptoms in Parkinson's patients is the restoration of functional activity of basal ganglion structures. Following the striatal DA deprivation induced by the death of DA neurons, the entire basal ganglion system is altered. One of the therapeutic strategies to restore the physiological activity of these structures is therefore a functional surgical approach using high frequency stimulation (HFS). Carried out for the first time by Benabid and his colleagues in 1987, HFS resulted in a significant relief of tremor of PD patients when stimulating the ventral intermediate thalamic nucleus (Benabid et al., 1987). In 1993, the subthalamic nucleus was recognized as a novel target for deep brain stimulation (DBS) with the development of the basal ganglia circuitry new concepts (reviewed by Poewe et al., 2017). Since then, this technique was further refined and enhanced with the amelioration of neurophysiological recording and imaging techniques. This technique mainly involves the implantation of an electrode into the brain tissue targeting specific brain target with finding the high-frequency (100– 200 Hz) electrical stimulation of that brain region without damaging the brain tissue. Best candidates with the criteria fulfilled for DBS test are the patients with young onset PD who also respond very well to L-DOPA but due to long-term treatment, have some motor disturbances. Unlikely, patients who no longer respond to the dopaminergic treatment do not respond either to the DBS treatment (Bronstein et al., 2011). Although this strategy can help to relieve severe motor symptoms, still it is not adequate to ensure long term total abolition of the motor features and for all patients with PD. Moreover, in addition to its invasive nature, DBS is a complex therapy that may lead to intracranial bleedings, infections and side effects, particularly psychiatric following the surgery. This surgery cannot therefore be performed on patients who are too old or with an uncertain diagnosis. Strict selection criteria

were then put in place reducing the application of this procedure to only 10 to 15% of patients (reviewed by Walter & Vitek, 2004).

2.3. Developing restorative cell therapy approaches

The fact that therapeutic efficiency of several pharmacological treatments is becoming limited overtime and triggering undesirable side effects such as dyskinesia in most patients, nominates PD as a suitable candidate for cell therapy to restore the motor improvement and degenerated pathway.

The main principle of cell replacement therapy in PD is manifested by the ability of the transplanted midbrain dopaminergic neurons (mDA) neurons to renovate the dopaminergic neurotransmission within the disrupted nigrostriatal pathway and enhance the behavioral performance in the PD patients.

Initial trials of cell transplantation were developed in the 1970s, and different cell sources have been considered as alternatives for dopaminergic cells including the carotid body, adrenal medullary, retinal, and porcine fetal cells (Barker et al., 2015; Bjorklund & Kordower, 2013; Lindvall, 2016). Restorative therapy has been progressively optimized through various cell replacement sources such as cells derived from 6 to 9 weeks-old ventral mesencephalon aborted embryo (fVM), pluripotent human embryonic stem cells (hESC) derived by somatic cell nuclear transfer (SCNT) or from fertilized eggs, and pluripotent stem cells (iPSC) derived from syngeneic donor tissue originating from the patient, and differentiated into dopaminergic cells (Figure 5).

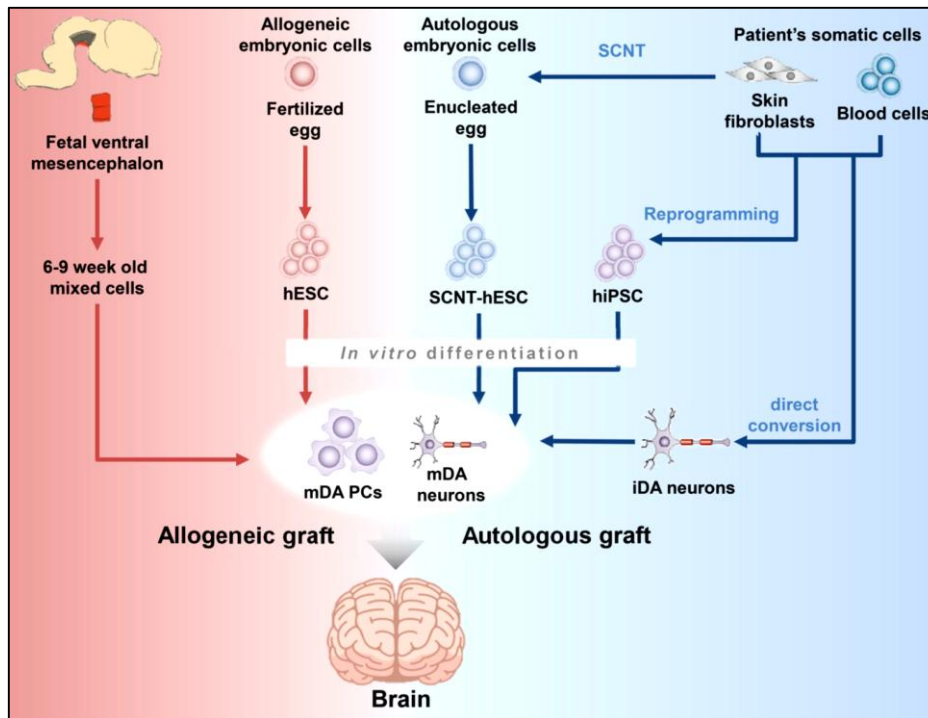


Figure 5: Schematic overview of mDA cell sources used in restorative cell therapy for PD.

Autologous grafts are highlighted in blue, whereas allogeneic grafts in red. *fVM*: Fetal ventral mesencephalon; *SCNT*: somatic cell nuclear transfer; *hiPSC*: human induced pluripotent stem cells; *hESC*: human embryonic stem cells; *iDA*: induced dopaminergic neurons; *mDA PCs*: midbrain dopaminergic neurons progenitor cells (Adapted from Sonntag et al., 2018).

Heterogeneous population of allogeneic fVM cells are obtained from aborted 6 to 9 weeks-old embryos. Another route to obtain mDA progenitor cells is the generation of hESC from donor fertilized eggs resulting in allogeneic grafts. Both allogeneic fVM and hESC grafts demand immunosuppression following transplantation. However, there is an alternative way to establish autologous hESCs by SCNT which is an alternative way based on implanting a donor nucleus from a patient's somatic cell into a recipient enucleated egg therefore avoiding administration of immune-suppressive measurements. On the other side, patient's somatic tissues can be reprogrammed to provide autologous iPSCs or directly converted into induced dopaminergic cells which are then differentiated to mDA neurons and mDA PCs.

2.3.1. Fetal Cell transplantation

Several fetal cell transplantation studies were conducted and considered as an encouraging therapeutic approaches for PD. Embryonic VM grafts were placed in form of tissue ectopically in a dorsal cortical cavity or in the lateral ventricle and in both studies improved functional and histological outcomes were obtained (Björklund & Stenevi, 1979; Perlow et al., 1979). After these studies, cell suspensions were prepared from the developing VM and used as source for transplantation in the denervated striatum (Anders Bjorklund & Kordower, 2013). Moreover, cell transplants from fVM intrastrially grafted in rodent models reported integration and survival of the transplants within the host striatum which enhanced the functional and behavioral outcomes in these animal (Bjorklund et l., 1980; Dunnett et al., 1981). In fact, various clinical studies reported that using DA neurons from fVM of aborted 6.5-9 week weeks-old human embryos for intrastriatal transplantation can improve the functional deficits and reinnervate the striatum in immunosuppressed 6-OHDA rat model (Brundin et al., 1986). Even though previous studies provided proof that in 6-OHDA rodent's models of PD, ectopic intrastriatal transplants physiologically succeeded to supply DA and enhance asymmetric rotational behavior, yet these approaches failed to completely re-establish the sensorimotor behavioral deficits after DA loss and maintain graft regulation by the host. In regards to this matter, several double transplantation approaches were later conducted by transplanting simultaneous intranigral and intrastriatal fVM cells into 6-OHDA lesioned rat model. These studies reported re-establishment of the nigrostriatal circuitry and host-graft connectivity, in addition to amelioration in behavioral performance after the double transplantation(I Mendez, Sadi, & Hong, 1996; Ivar Mendez & Hong, 1997).

Following these early promising outcomes, cell therapy of PD has advanced with the initiation of two double-blind studies performed in 2001 and 2003 with 40 and 37 patients respectively (Freed et al., 2001; Olanow et al., 2003) in the USA. Disappointingly, these sham-controlled

trials established no significant improvements and reported appearance of Lewy bodies in the transplant and development of dyskinesia induced by the graft (J.-Y. Li et al., 2008). The negative results in these approaches are correlated to several clinical factors related to cell preparation and transplantation protocols, immune suppression, variable foetal tissue composition, and patient selection (reviewed by Gaillard & Jaber, 2011). Following these trials, cell therapy was drawn back for more than a decade.

In the previous recent years and with the development of reprogramming protocols and clinical improvements, the clinical approaches of cell therapy has been revived. Moreover, several studies reported robust enhancement in some motor and non-motor defects as well as anatomical and functional restoration of the DA neurotransmission in 6-OHDA lesioned rodent models after fVM nigral transplantation (A. Gaillard et al., 2009; Lelos et al., 2016). Few years ago, more than 400 patients have received fetal cell transplantations worldwide and some of which have displayed significant symptomatic improvements (Nishimura & Takahashi, 2013). In some patients, the transplanted cells were able to restore the nigrostriatal pathway connectivity through re-innervating the striatum and even in some individuals, the motor improving the behavioral impairments associated with PD (Kefalopoulou et al., 2014). Despite the encouraging evidences elucidated by several experimental studies and clinical trials, the use of human fetal cells remains a controversial subject dealing with central practical, ethical, and clinical complications driving lead to the development of alternative sources with the capability to differentiate into multiple cell lineages such as the embryonic stem cells and induced pluripotent stem cells that received a positive impact as potential substitutes for restorative therapy (reviewed by Sonntag et al., 2018)

2.3.2. ESC-derived DA cells as another potential cell source

From the very start following the early challenges attached to the fVM cells, alternative cell sources were being examined to generate renewable DA neuron cells with larger numbers under

safe measurements. Indeed, wide range of different stem cell sources have been identified since then with the human embryonic stem cells (hESCs) showing the most promise (Barker et al., 2013). ESC are pluripotent stem cells derived from the inner cell mass of the early-stage blastocyst and have the potential to produce an unlimited supply of cells. These cells can differentiate into mDA neurons (Kirkeby et al., 2012) and have the capacity to deliver a comparable potential to the fVM transplants in terms of integration and survival within the transplant in the preclinical approaches (Grealish et al., 2014).

Due to their pluripotency, unlimited renewability, and wide range reproducibility mouse ESCs (mESCs), first isolated in 1981, have appealed particular attention and participated in the advancement of the transplantation therapeutic approaches (Evans & Kaufman, 1981; Martin, 1981). Several protocols indeed succeeded in producing cell populations representing the A9-mDA neuronal phenotype from mouse embryonic stem cells (mESCs) and the first DA neurons expressing TH and established from mESCs were generated in 2000 by Lee et al (Lee et al., 2000). Björklund et al. in 6-OHDA rat model. In this study, a restoration of striatal dopamine levels was observed by positron-emission tomography (PET) and functional magnetic resonance imaging (MRI) in addition to improvements in behavioral performance which was analyzed by amphetamine demonstrated in 2002 that DA precursors derived from intrastriatal grafted mouse ESCs were able to differentiate into mature DA neurons, survive, and restore motor deficits following transplantation rotation test. The obtained differentiation protocols and studies for establishing mESC-derived dopaminergic neurons can be extrapolated to human pluripotent stem cells (PSCs) approaches due to the similarities between the human and mouse DA neurons in terms of responsiveness to external similar factors as well as developmental steps (Kai-Christian Sonntag, Simantov, & Isacson, 2005).

The development of the hESCs-derived DA neurons (Grealish et al., 2014) was generated with the help of previous acquired knowledge using the mESC differentiation protocols in

transplantation studies (Bjorklund et al., 2002; Kim et al., 2002). In 2011, Kriks et al. showed in a 6-OHDA rat model a robust survival of intrastriatal grafted mDA neurons derived from hESCs 5 months after transplantation with complete restoration of the behavioral deficits in the amphetamine-induced rotation test (Kriks et al., 2011). More recently established study manifested that after transplantation into a rat model of PD, the intrastriatal grafted hESC-derived mDA neurons were able to establish a functional, morphological and behavioral effects similar to that of intrastriatal grafted human fVM cells (Grealish et al., 2014).

These outcomes, on the whole, support the sustainability of using the hESCs-derived mDA cells as a feasible alternative source in restorative medicine. Yet, hESCs cannot be examined as an optimal therapeutic alternative due to several limitations since only very small proportions of the TH expressing DA neurons generated from the hESCs differentiation protocols expressed the FOXA2 and LMX1A transcription factors that are important mDA markers which could affect the efficacy of these cells after transplantation (Barker et al., 2015). Regardless of the mentioned barriers, mDA cells derived from hESCs are being established for clinical trial Europe and USA where they are presently considered as a feasible standard of the human pluripotent stem cells (Stoker et al., 2017). Finally, the use of both ESC is associated with the risk of tumor formation. Therefore, more extensive investigation is needed to develop efficient and safe differentiation protocols to generate large numbers of mDA neurons while avoiding the formation of undifferentiated tumor entities.

2.3.3. iPSC-derived DA cells

Dramatic progress has been made towards the development of the stem cell therapy technology for PD through generating iPSCs resulting in generation of unlimited certain stem cell sources and establishing any specific cell type by differentiating these cells. This approach offers a plausible opportunity to unveil various disease mechanisms through also providing indefinite autologous stem cell sources (reviewed by Sonntag et al., 2018).

iPSCs are obtained by using specific factors to revert the adult somatic cells into an ESC-like iPSCs (K. Takahashi et al., 2007) and thus provide an unlimited number of cells. In 2006 a groundbreaking study was accomplished by Yamanaka and his team when they validated the generation of iPSCs from adult human fibroblast using viral transduction of four transcription factors (SOX2, OCT4, c-MYC, KLF4) (K. Takahashi & Yamanaka, 2006). Following this revolutionary study, human iPSCs (hiPSCs) were obtained by two additional groups of researchers who succeeded to reprogram hiPSCs from human somatic cells using similar sets of methods (Park et al., 2008; K. Takahashi et al., 2007). This approach has led to much hope that especially that the iPSC technology allows the elimination of immunogenicity and ethical restrictions of using aborted embryos that were addressed before with fetal cell transplantation studies (Sonntag, 2018). Moreover, mature DA neurons can also be obtained from differentiating iPSCs by following similar methods and protocols used with hESCs (Soldner et al., 2009).

More recently, studies revealed that neural cell populations derived from human PSCs can be better characterized and selected based on expressing specific mDA markers after differentiation using sorting techniques such as FACS-sorting by mDA markers and its worth to mention that these PSC-derived neural cells can improve motor behavior in PD rodent models (Lehnen et al., 2017).

As for in vivo studies, iPSCs derived DA neurons were used in several transplantation strategies in animal models of PD. In 2010, two studies conducted on 6-OHDA rat models showed improved behavioral performance after intrastriatal transplantation of hiPSCs-derived DA neurons (Cai et al., 2010; Hargus et al., 2010). Moreover, in 2011 Kikuchi et al showed that transplanted DA neurons derived from hiPSCs in MPTP monkey model, can survive within the animals' brains for at least 6 months after transplantation (Kikuchi et al., 2011). Furthermore, in another study conducted in 2015, grafted hiPSC-derived neural stem cells were able to

integrate within the host grafted striatum and differentiate into DA neural cells in 6-OHDA rat model of PD. Animals displayed an ameliorated motor recovery with the apomorphine-induced rotation test up to 16 weeks post-transplantation (Han et al., 2015). These outcomes rise as an evidence for using hiPSCs in future clinical trials for PD.

Another study manifesting the promising therapeutic potential of transplantation using iPSC was conducted in nonhuman primates using autologous iPSC to generate midbrain-like DA neurons (Hallett et al., 2015). Indeed, Hallett and his team demonstrated that transplantation using iPSC-derived DA neurons could manifest several therapeutic outcomes similar to that obtained with fetal transplantations: they observed long term graft survival, and functional recovery following transplantation using monkeys lesioned by MPTP that received autologous transplantation of iPSC-derived DA neurons into the putamen. The animals showed enhanced motor behavior, complete re-innervation of the denervated putamen and graft survival over two years following transplantation.

More recently, a human clinical trial to treat PD patients is conducted using DA neurons generated from iPSCs which were initially obtained from a donor with HLA loci matching that of the patient who will still receive immunosuppressants. The trial which started in August 2018, is headed by Takahashi and his team in Kyoto University Hospital and is still in progress and will be followed for two years from now (Stoddard-Bennett & Reijo Pera, 2019).

Despite this promising potential, iPSC approach is still in its initial stages where efficacy and long-term safety and other critical measurements need to be pursued to fully advantage of these cell types in cell-based therapy for PD. Due to the heterogeneous nature of the multisystem PD, a single addressed cell based therapy may not be alone sufficient, but rather a group of approaches combined and synchronized all together to target several aspects of the disease to ease the tremendous burden carried by the patients with PD.

II. The mesencephalic dopaminergic pathways

1. Emergence of mDA neurons during embryogenesis

DA neurons residing in the ventral mesodiencephalon (mDA), during embryogenesis, are divided into three main subsets (Figure 6): (i) A8 neurons (in the retrorubral field; RRF), (ii) A9 neurons (in the SNc), and (iii) A10 neurons in the ventral tegmental area (VTA) (Bjorklund and Dunnett, 2007). SNc mDA neurons are involved in monitoring voluntary movement whereas VTA mDA neurons are critical for motivation behavior, controlling emotions, decision making, and monitoring working memory (reviewed by (Morales & Margolis, 2017).

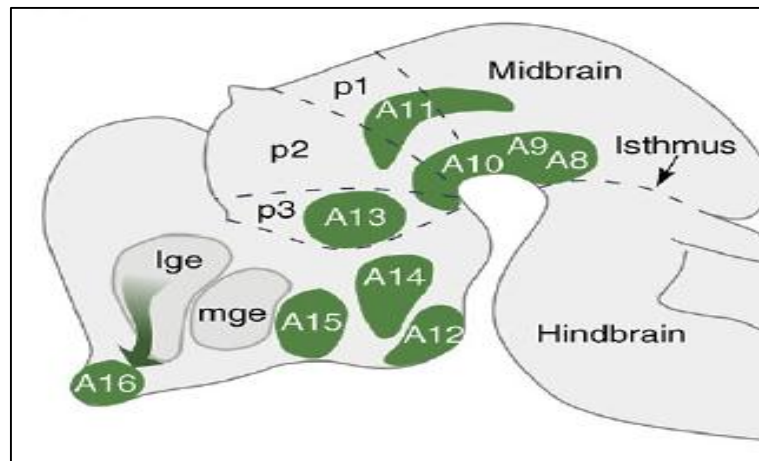


Figure 6: Dopaminergic neuron cell groups in the developing mouse brain sagittal view. The cell subsets classification from A8 to A16 was presented in 1964 by Dahlstrom and Fuxe. *lge*: lateral ganglionic eminence; *mge*: medial ganglionic eminence; *p1–p3*: prosomeres 1–3 (Modified from Bjorklund & Dunnett, 2007).

In mice, the mDA neurons emerge along the ventral midline of the floor plate (FP) of the midbrain ventricular zone (Ono et al., 2007), and around E10.5 mDA subsets of the SNc are born whereas the VTA mDA neuron's neurogenesis takes place later around E11.5 (Bye et al., 2012). In the lateral and medial zones of the FP, the mDA progenitors are divided into two subsets giving rise to the SNc mDA and VTA mDA neurons and as manifested in previous studies, the mapping of the mDA neurons is influenced by the spatiotemporal patterning of

Sonic hedgehog (Shh) expression in the FP (Blaess et al., 2011; Bodea & Blaess, 2015). Around E8.5-E9.5, the medial progenitors exclusively express Shh whereas at E11.5 this expression is restricted to the lateral zone of the FP (Blaess et al., 2011). Following neurogenesis the post-mitotic mDA neurons undergoes two phases of radial and tangential migration through the FP to reach the marginal zone (MZ) which is their final destination (Figure 7).

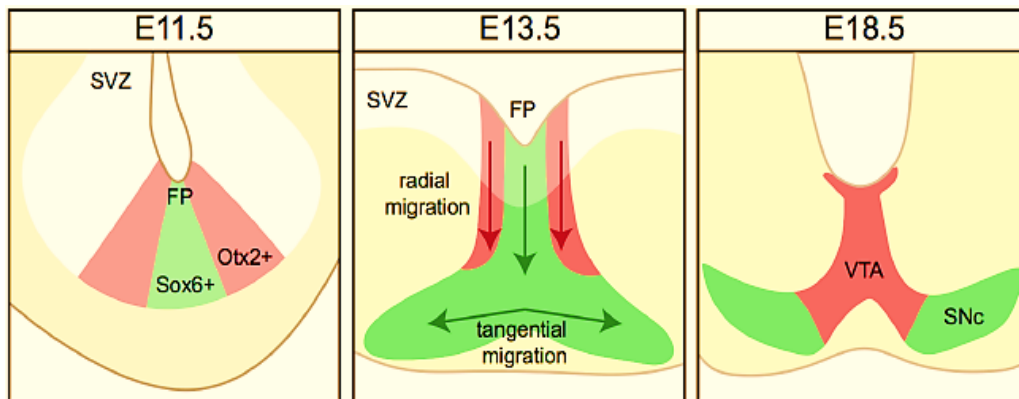


Figure 7: Illustration of the mDA neuron migratory phases during development. SVZ: subventricular zone; FP: medial floor plate; VTA: ventral tegmental area; SNc: substantia nigra pars compacta (Brignani & Pasterkamp, 2017).

Both SNc and VTA neurons move radially in the first migratory phase, following this phase only SNc mDA neurons move tangentially during the second migratory phase towards the lateral MZ. The tangential migration leads to the formation of the two wings-structure of the SNc which extend towards the rostro-lateral sides of the midbrain in early postnatal stages and adult brain (reviewed in Brignani & Pasterkamp, 2017)

Once mDA neurons migrated to constitute the VTA and SN, they extend their axons to their final targets allowing the establishment of the mesostriatal, mesolimbic, and mesocortical pathways. More precisely, neurons in the VTA extend projections to the prefrontal cortex and ventral striatum forming the mesocortical and mesolimbic systems respectively. These pathways are involved in controlling emotions and reward and are linked to drug addiction,

schizophrenia, and depression (Van den Heuvel & Pasterkamp, 2008). On the other side, the mDA neurons of the SN send projection innervating the dorsal striatum and forming the mesostriatal system which is mainly involved in regulating motor functioning (Van den Heuvel & Pasterkamp, 2008).

2. Establishment of the dopaminergic mesencephalic pathway

2.1. Axon guidance signals that mediate axonal steering

The mDA pathway establishes during embryogenesis. Growing axons leave their mesencephalic location move forward and ventrally through the hypothalamus in the diencephalon to reach their appropriate targets in the striatum. To find their route, axons are tipped with a dynamic sensitive structure; the growth cone (Figure 8). This flattened structure is guided by intermediate environmental cues acting as signals to direct axonal growth.

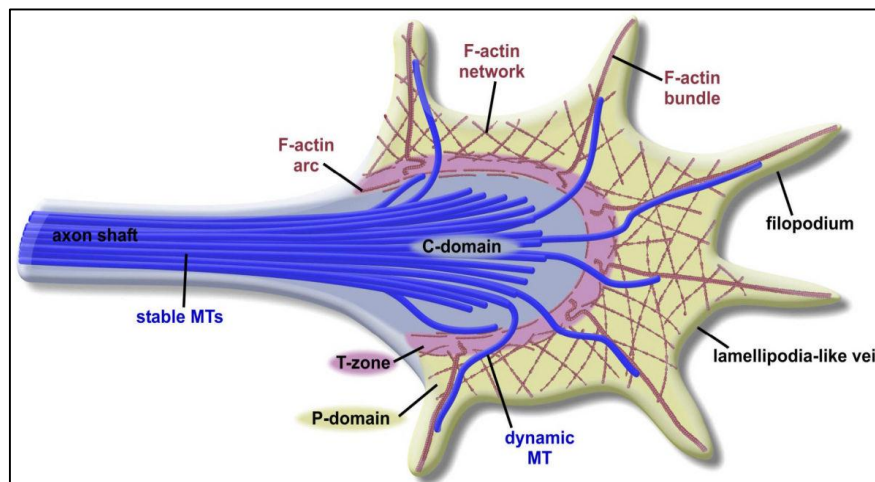


Figure 8: The structure of the growth cone. The cytoskeleton of the growth cone is composed of F-actin bundles which protrude to form finger-like structure; the filopodia separated by a peripheral branched network of actin filaments, the lamellipodia. The peripheral domain is associated with a central core structure containing microtubules extending from the axon shaft and membranous organelles. The transition zone is formed of actomyosin structures and it resides between the borders of the peripheral and central domains. *MTs: Microtubules; T-zone: transition zone; P-domain: peripheral domain; C-domain: central domain* (Modified from Lowery & Van Vactor, 2009).

The cytoskeleton of the growth cone is mainly composed of peripheral lamellipodia composed of branched network of actin filaments (F-actin network), and long protruding filopodia made of bundled actin filaments (F-actin bundles). This peripheral actin network is associated with bundled microtubules projecting from the axon shaft forming the central region of the growth cone. These dynamic filamentous players of the cytoplasm regulate the motility of the growth cone by controlling the expansion and retraction processes during its navigation towards its target zone (Huber et al., 2003). During this journey, the growth cones navigate along specific routes and respond to several intermediate cues present in the extracellular environment through a set of surface receptors. The result is a heterogeneous dynamic complex of guidance cues interfering and interacting with the growth cone to regulate the axonal navigation and steering. These guidance signals can either be diffusible agents that can operate over long distances, or membrane-associated cues acting in a at short range dependent manner. On the top of that, these guiding cues can be chemoattractive or chemorepulsive (Huber et al., 2003) while providing specific information for selective axonal navigation needed by distinct neuronal populations (Figure 9).

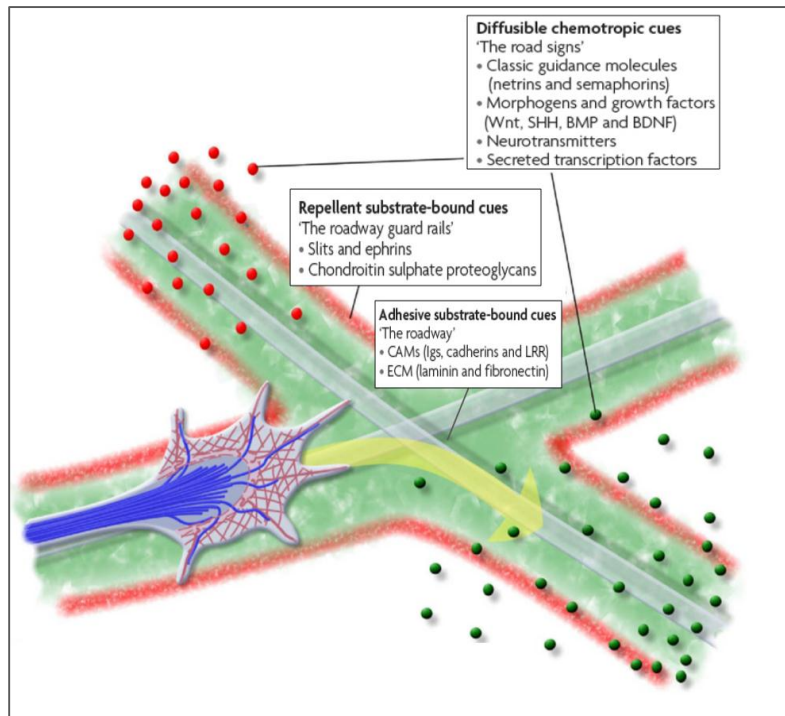


Figure 9: Axonal guidance mode of action. Through its navigation, the growth cone responds to several extracellular environmental cues. This navigation can be compared to the road traffic. The growth cone is guided by “the road signs” that are the diffusible chemotropic cues (either attractive (in green) or repulsive cues (in red)). The growth cone progress on the highway thanks to the “guard rails” that are repellent substrate-bound cues preventing the growth cone to go out from the pathway and adhesive substrate-bound cues that are represented by “the roadway” to keep the growth cone on the appropriate direction. *BMP*: bone morphogenetic protein; *BDNF*: brain-derived neurotrophic factor; *CAM*: cell adhesion molecules; *ECM*: extracellular matrix; *LRR*: Leucine rich repeats; *SHH*: sonic hedgehog (Modified from Lowery & Van Vactor, 2009).

Secreted guidance molecules operating at long distances can either direct the axonal steering towards a specific target or exert a growth limiting function to prohibit axons from navigating through inappropriate regions. These molecules can be: (i) classical axon guidance molecules (see below figure 10), (ii) morphogens and growth factors, (iii) neurotransmitters or (iv) secreted transcription factors. Adhesive molecules present within the extracellular matrix (as fibronectin and laminins) or surface bound to neighboring cells (like the transmembrane cell

adhesion molecules) represent a supportive roadway that promotes axonal growth and steering, whereas bound anti-adhesive cues (such as ephrins and chondroitin sulfate proteoglycans) act as the roadway borders and restrict the growth cone navigation (Lowery & Vactor, 2009).

Axon guidance molecules are conserved families of molecules and their cognate receptors have been well characterized. Prominent among these, are the four conserved families of netrins, ephrins, semaphorins, and Slits along with their associated receptors that are also involved in axon guidance within the dopaminergic system (Figure 10) (Chilton, 2006).

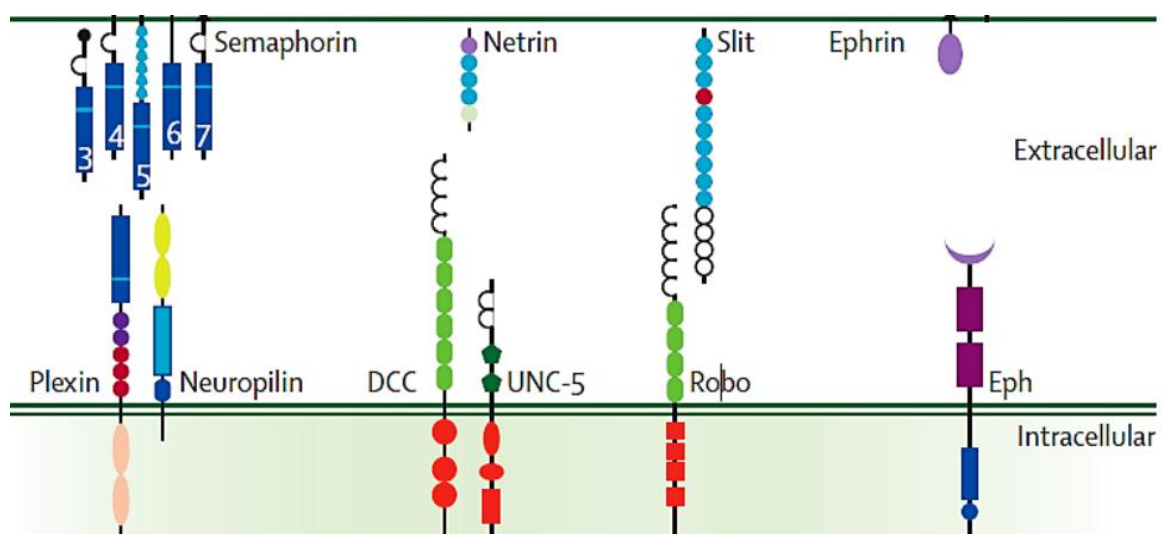


Figure 10: The four conserved axon guidance cues families and their associated receptors. Netrins bind to their deleted in colorectal cancer receptors (DCC) and UNC5 receptors, semaphorins bind the neuropilin and plexin receptors, slits bind the Robo receptors, and Ephrins family bind to the Eph receptors (Modified from Van Battum, Brignani, & Pasterkamp, 2015).

2.1.1. Netrins and DCC

Netrins, whose name refer to “the one who guides” in the Sanskrit terms, were the first conserved guidance cue initially identified when Hedgecock et al. found that *unc-6* gene mediates the dorso-ventral axonal guidance in *C. elegans* (Hedgecock et al., 1990) and this gene was shown to encode a netrin molecule two years later (Ishii et al., 1992). Netrins can be either secreted or membrane-bound proteins, knowing that in mammals three netrins, Netrins-1, 3,

and 4, were identified to be secreted and two other members, Netrins-G1 and G2, were glycosylphosphatidylinositol (GPI)-linked membrane proteins. Although netrins were first identified to be chemoattractant, they can be bifunctional and also act as chemorepellents (Colamarino & Tessier-Lavigne, 1995). DCC (Deleted in Colorectal Cancer) which is the vertebrate homologue of UNC-40 and UNC-5 with its three mammalian homologues function as a receptors for the netrins (Leonardo et al., 1997). The chemoattractive effects of netrins to promote axonal growth are mediated in all species by the DCC receptors, whereas the chemorepellent effects are mediated by both the DCC and Unc5 receptors (separately or together) (Round & Stein, 2007).

Netrin-1, which is secreted by the floor plate cells of the embryonic neural tube, displays a chemoattractive and growth promoting effect on the axons of the spinal commissures in vertebrates (Serafini et al., 1996). Moreover, Netrin-1 by its axonal growth promoting effect, insures appropriate thalamocortical projection (Braisted et al., 2000). During mDA system development, Netrin-1 is expressed in cortical neurons as well as in mdDA projection regions such as the striatum (Hamasaki et al., 2001). DCC receptor is also shown to be expressed in the striatum and in cultured DA neurons from the SN and VTA (Hamasaki et al., 2001; Lin et al., 2005).

This matching expression of Netrin-1 and its DCC receptor in the DA neurons and their targets during development supports the idea that Netrin-1 could be involved in wiring the mDA neurons axons (Lin et al., 2005). This function was later validated in 2014 where Li et al showed that Netrin-1 is involved in targeting the DA neurons to the striatum and more specifically contributing to the topographic patterning of the mDA neurons within the striatum (Lin et al., 2005).

2.1.2. Ephrins and Ephs

Ephrins and their tyrosine kinase receptors are cell-surface signaling molecules that display several roles in many developmental events. Their name comes from the expression of Eph in a human Hepatocellular carcinoma cell line producing Erythropoietin (Hirai et al., 1987). The ephrin ligands are divided into two sub-classes: (i) the five class ephrin-As (A1 to A5) which are GPI-anchored to cell surface and (ii) the three class ephrin-Bs (ephrins B1 to B3) which are transmembrane molecules (Kullander & Klein, 2002). Eph receptors are classified into EphAs that bind to ephrin-A ligands and contains eight members (EphA1 to EphA8), and a B subclass that bind to ephrin-Bs and contains five members (EphB1 to EphB4, EphB6). The primary exception is the EphA4 and EphB2 receptors, which can interact with members of both classes A and B ephrin ligands. Eph receptors are composed of a cytoplasmic domain divided into four functional units and an extracellular domain containing the ligand-binding domain (Kullander & Klein, 2002). Ephrins are all membrane-associated that function at short-range distances where they cluster at the cell-cell contact site to interact with their cognate receptors. Ephrins-Eph complexes transmit signals in a bidirectional manner where forward signaling is triggered by the Eph receptors upon their ephrin ligand binding, and reverse signaling is induced by the ephrin ligands when they signal via their corresponding receptors (Palmer et al., 2002). Moreover, ephrin-Eph complex activation is mostly associated with contraction of the growth cone and repulsion of neighboring cells, but in some cases it may be attraction (Kullander & Klein, 2002).

The role of ephrins and Ephs in mDA axon guidance was first manifested by Yue *et al.* in 1999 when he showed a high level of EphB1 receptor in the SN unlike in the VTA, in addition to strong expression of EphrinB2 ligand- EphB1 in the dorsal striatum when compared to the ventral part suggesting a potential role in establishment of the mesostriatal axons projections(Yue et al., 1999). However, this idea was contradicted in 2007 by Richards *et al.*

who didn't observe any deficits in the mesostriatal projections in EphB1 mutant mice knowing that in his study, from E18 and onward, EphB1 expression was not detected in the SN of normal animals (Richards et al., 2007). Furthermore, it has been shown that ephrins are involved in the retinotectal topographic mapping, mainly ephrinA- EphA molecules are implicated in regulating the retinal axon's mapping along the anterior-posterior axis of the tectum and superior colliculus by repulsion and EphrinB-EphB molecules regulates the topographic mapping in the retinotectal system along the dorsoventral tectal axis (Brown et al., 2000; Mann et al., 2002). Several studies investigated the role of ephrin-Eph complex as a guiding cue for the commissural axons in the brain and spinal cord (Palmer et al., 2002; Yokoyama et al., 2001). In the mDA system, EphA5 molecule has been reported to influence the dopaminergic connectivity where a disturbance in the EphA/ephrin-A signaling resulted in deficits in the dopaminergic innervation (Sieber et al., 2004) therefore supporting a role for EphrinA5 in guiding the mDA projections (Deschamps et al., 2009; Sieber et al., 2004).

2.1.3. Slits and Robos

Slit are secreted proteins, first detected in invertebrates when they were implicated in axonal repulsion along the midline of developing CNS in *Drosophila* (Rothberg et al., 1988). In mammals, three Slit genes have been identified to exhibit about 60% homology (Slit1, Slit2 and Slit3). Repulsive action of Slits is mediated by the roundabout (Robo) receptors family, of which three were detected in mammals (Robo1, Robo2, and Robo3/Rig1). Robo receptors possess large unstructured cytosolic domains that resemble the ectodomains of the cell adhesion molecules (Dickson & Gilestro, 2006).

Remarkably, in the mDA neurons some of the Robo receptors are expressed during development whereas none of the Slits members is detected during embryogenesis in these neurons. Particularly Robo1 and Robo2 members are differentially expressed in the mDA neurons of the VM where Robo2 expression is restricted to the DA neurons subset of the SN,

while Robo1 labels both of the SN and VTA mDA subsets (Marillat et al., 2002). On the contrary, Slit1 expression is not identified in the SN and VTA mDA subsets until P10, but it's strongly detected in the striatum during embryogenesis although this expression decreases towards adulthood. Moreover, Slit3 expression is first observed at P5 in the striatum and increases as development advances, while Slit2 can only be detected in the neurons of the adult SN (Marillat et al., 2002). This structured expression of Slits and Robos proposes a critical role for these proteins in maintaining proper development and guidance of the mDA neurons during early and late developmental events especially after showing that Slit2 proteins can prohibit the mDA axons growth in vitro (Lin et al., 2005).

2.1.4. Semaphorins, neuropilins and plexins

Semaphorins, comprises a large family of secreted and membrane-bound proteins that were initially identified in 1992 in the invertebrates (Kolodkin et al., 1993) and in 1993 in the vertebrates, where one of the semaphorin family members (Sema3A) was extracted from a chicken brain and shown to trigger the collapse of the growth cone of the dorsal root ganglion (Luo et al., 1993). The semaphorin family members are divided according to the Semaphorin Nomenclature Committee into eight classes (Semaphorin Nomenclature Committee.,” 1999). In vertebrates, five classes of semaphorins are expressed (classes 3, 4, 5, 6, 7) excluding sema-5c member of class 5 which along with classes 1 and 2 are identified in the invertebrates; the last semaphorins class (V) comprises is expressed in viral genome. Among the mammalian semaphorins, classes 4-7 are membrane-associated and class 3 contains secreted proteins (Pasterkamp & Kolodkin, 2003). Semaphorins are involved in modulating mechanisms of several neuronal networks formation such as synapse formation and plasticity, axonal growth, guidance and pruning (Pasterkamp & Kolodkin, 2003). For example, Sema3A is involved in guiding the mDA neurons axons during development by repelling the ipsilateral MFB

projections along the midline to maintain their appropriate pattern of mDA trajectory (Kawano et al., 2003).

Plexins and neuropilins are the two main receptors for the semaphorin family. So far, over twenty semaphorins, two neuropilins, and nine plexins have been characterized. Nrp1 and Nrp2 are the two neuropilins identified in the vertebrates, whereas the nine plexins identified in the vertebrates (plexinA1-A4, B1-B3, C1, D1) are classified based on their structure into four major classes (Plexin A-B-C-D) and two other classes that are found in the invertebrates (PlexinA and B) (Alto & Terman, 2017; Hota & Buck, 2012).

The membrane-bound semaphorins corresponding to classes 4–7 bind directly the plexins receptors, while the secreted class 3 semaphorins (except Semaphorin 3E) use a co-receptor proteins functioning as a ligand-binding subunit; the neuropilins (Nrp) and interact indirectly to plexins (Pasterkamp & Kolodkin, 2003). In addition to that, several co-receptor molecules such as the off-track (OTK), cell adhesion molecules, and others can interact with the semaphorin-receptor complexes to modulate its signaling (Pasterkamp, 2012).

Semaphorin7A (Sema7A) is the only GPI-anchored semaphorin and is expressed with its receptor plexinC1 in the mDA system. It has been shown that Sema7A participates in cooperation with plexinC1 receptor in maintaining mDA neuronal network connectivity (Pasterkamp et al., 2007; Van den Heuvel and Pasterkamp, 2008). Nevertheless, Sema7A can also function independently of plexins receptors in some signaling events while binding to another set of receptor family; the integrins (Figure 11) (Pasterkamp et al., 2003).

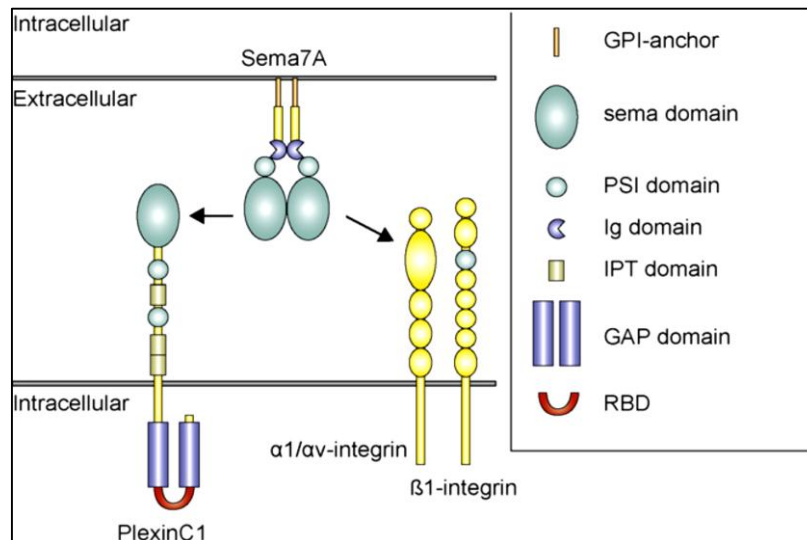


Figure 11: Schematic presentation of Sema7A and its receptors. Sema7A is a GPI-anchored guidance protein, composed of an extracellular conserved “sema domain”, PSI domain, and an immunoglobulin domain. Ig and sema domains can form homodimers at the cell surface. PlexinC1 and $\beta 1$ integrin proteins are the two receptors modulating the signaling of Sema7A. *PSI: plexins, semaphorins and integrins domain ; sema: semaphorin domain; Ig: immunoglobulin.* (Adapted from Jongbloets, Ramakers, & Pasterkamp, 2013).

Overall, netrins, slits, ephrins and semaphorins play the role of short- and long-range signaling molecules that guide the projecting mDA axons in the midbrain to innervate their final target in the striatum. To reach their final targets, the mDA axons have to grow out of the midbrain, to navigate through the medial forebrain bundle in the diencephalon and finally reach the striatal targets.

2.2. mDA axon navigation outside the midbrain

The mDA axons start to extend neurites around E11.5 in mice and follow a rostral orientation that is driven by axon guidance molecules expressed by the cells of the midbrain–hindbrain organizer (MHO) (Raible & Brand, 2004). The fibroblast growth factor 8 (FGF8), which is expressed by the MHO, induces the expression of Semaphorin3F (Sema3F) (Yamauchi et al.,

2009) that interacts with its receptor Nrp2 expressed by mDA neurons to suppress the mDA axonal outgrowth at early developmental stages *in vitro* (Kolk et al., 2009; Yamauchi et al., 2009). Therefore, Semaphorin 3F (Sema3F), which is expressed in dorsal and caudal regions of the mDA system, is induced by the MHO to create a non-permissive environment and repel the mDA axons rostrally (Yamauchi et al., 2009). As observed by Kolk et al., 2009, the mDA axons display a caudal orientation towards the MHO when the Nrp2 receptor is removed from the mDA neurons by gene targeting. Another diffusible chemotropic cue such as Wnt7b has an attractive role and promotes the growth of the mDA axons, whereas Wnt5a is repellent. Fzd3 receptor which is expressed by mDA axons interacts with the Wnt pathway to influence the rostral orientation of the mDA axons at this stage (Figure 12).

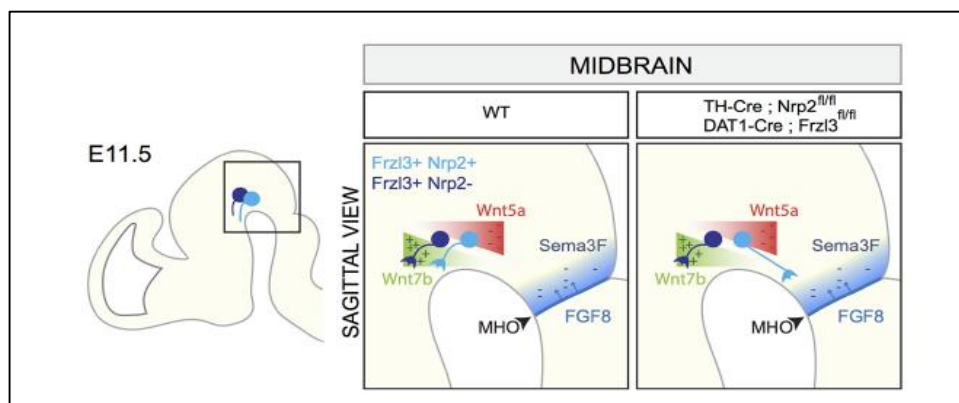


Figure 12: Representation of the of mDA axons rostral orientation (in blue) in the midbrain under the effect of several guidance cues. *FGF8*: fibroblast growth factor 8; *Wnt*: Wingless-related integration site; *MHO*: midbrain–hindbrain organizer (Modified from Brignani & Pasterkamp, 2017).

In vitro studies suggest also an important role for Slit-, expressed in the caudal mesencephalon, in preventing the axons from extending to the caudal midbrain (Lin et al., 2005; Marillat et al., 2002). In addition, Slit-1, expressed in the dorsal midbrain, mediates the repulsion of the mDA neurons axons via its Robo receptors (Prasad & Pasterkamp, 2009).

Finally, during rat embryonic development at E14.5, Netrin-1, expressed in the VM, is likely to display an influence on the mDA neurons axons expressing the DCC receptor to follow a ventral orientation (Lin et al., 2005; Vitalis et al., 2000).

2.3. Progression of mDA axons in the medial forebrain bundle (MFB)

In the ventral diencephalon the axons progress towards the diencephalon-telencephalon border and reorient into a rostral direction (Gates et al., 2004; Nakamura et al., 2000) towards their targets in the telencephalon. They form two fasciculated ipsilateral tracts of the MFB along which the mesostriatal and mesocorticolimbic pathways migrate before separating to reach their final targets in the forebrain (Figure 13).

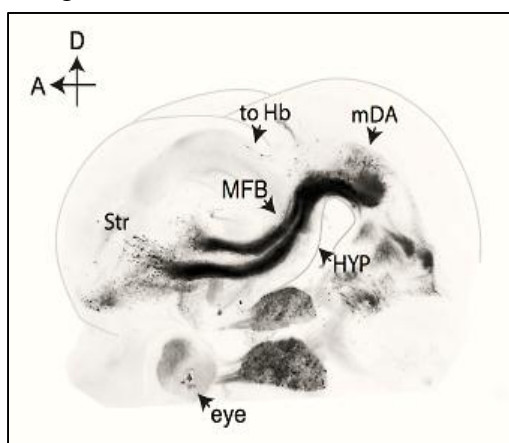


Figure 13: 3D sagittal illustration of a mouse E13.5 mDA system showing the formation of the MFB. The brain is immunostained for TH and cleared using the 3DISCO method. *Str*, striatum; *MFB*, medial forebrain bundle; *to Hb*, represents mDA fibers growing toward the habenula; *HYP*, hypothalamus (from Brignani & Pasterkamp, 2017).

Several different guidance cues and receptors play a role in preventing the mDA axons from diverging dorsally or ventrally and are involved in the fasciculation of these axons in the MFB. *In vitro* studies indicate a potential repelling action by Sema3F exerted on the mDA axons expressing Nrp2 receptor (Kolk et al., 2009; Yamauchi et al., 2009). Indeed, Sema3F is expressed in the VM and other regions surrounding the MFB and the Nrp2 receptor is expressed on the mDA axons of the ventral MFB around E12.5 onward (Kolk et al., 2009; Torigoe et al., 2013; Torre et al., 2010). Moreover, Sema3A which is expressed in the midline may also be

implicated in maintaining the ipsilateral trajectory of axons via the repulsive interaction with its receptors plexin-A1, plexin-A3, Npn-1, and Npn-2 (Kawano et al., 2003; Torre et al., 2010). The caudal hypothalamic region expresses Slit1, Slit2, and Netrin-1. The mDA axons at this intermediate target extend longitudinally along with other axons. In DCC knock-out (KO), Nrp2 KO, Slit1/Slit2 double KO, Netrin-1 KO, and KO Robo1 and Robo2 mice, a subset of mDA axons move ventrally after exiting the MFB and then reach the contralateral brain regions while crossing the ventral midline (Bagri et al., 2002; Xu et al., 2010; Torigoe et al., 2013; Li et al., 2014) (Figure 14).

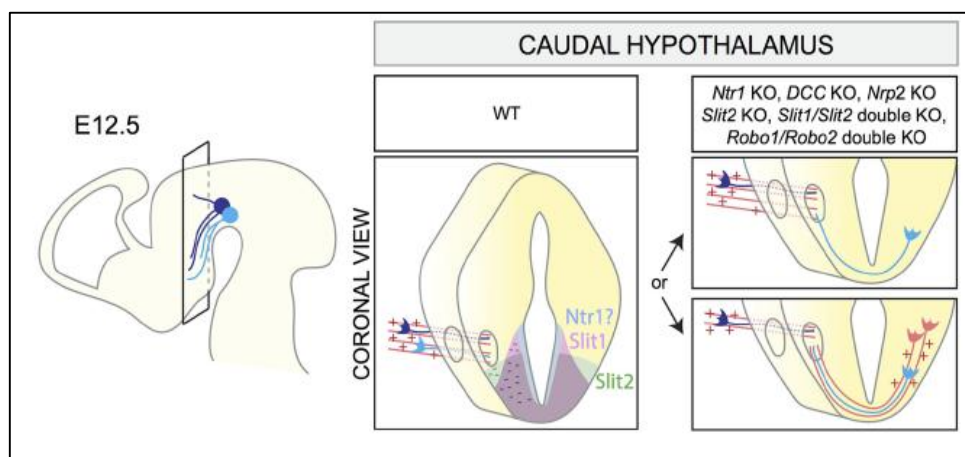


Figure 14: mDA axons extend longitudinally along with other axons at the level of the caudal hypothalamus where repellent guidance cues (Netrin-1, Slit1, and Slit2) are expressed. Blue lines represent the mDA axons whereas the red lines symbolize other pre-existing axons. (Modified from Brignani & Pasterkamp, 2017).

2.4. Arrival of mDA axons to their striatal targets

Around E13.5 in mice, the mDA axons of the MFB arrive at the developing ganglionic eminences then after a waiting period and around E14.5 the mDA axons ventrolaterally innervate first the CPu of the developing striatum and proceed towards the dorsolateral striatum to form later the mDA neurons subsets that will give the SNpc and the VTA. During early development the mDA axon collaterals originating from the embryonic SNc and VTA (E15 and

E17) show no preference for the dorsal or ventral striatum and therefore innervate overlapping striatal areas (Smidt & Burbach, 2007) whereas at late embryonic stages and early postnatal development, the topographical specificity is achieved through the selective elimination of SNc and VTA axon collaterals innervating the ventral and dorsal striatum, respectively (Hu et al., 2004).

At the molecular level several guidance molecules contribute to the DA innervation of the striatum. For instance, Netrin-1 which interacts with DCC receptor contributes to the establishment of the axonal connectivity in the striatum and is known to induce axonal elongation of the mDA neurons and attraction (Xu et al., 2010). Moreover, Netrin-1 displays a differential chemoattractive effect on DA neurons of the SNc and VTA. It has been shown that low level of Netrin-1 expression attracts DA neurons axons of the SNc to innervate the dorso-medial striatum; whereas high levels of Netrin-1 expression directs the VTA DA axons towards the ventral striatum (Li et al., 2014). By this means, in Netrin-1 KO mice SNc axons accumulate in the ventral striatum after failing to innervate the dorsal striatum.

The topography of the mDA axons in the striatum is also modulated by a gradient expression of ephrin-A5. mDA axons that respond to high striatal ephrin-A5 expression have a preference to connect to the dorsal striatum whereas low-responding ephrin-A5 mDA projections are oriented towards the ventral striatum. It is interesting to mention that a group of mDA neurons are guided to the cortical targets, these neurons are not sensitive to ephrin-A5 (Deschamps et al., 2009).

Furthermore, semaphorins can display important role in guiding the mDA axons projections into the striatum. As investigated by several studies, expression of Sema3A in the striatum induces the growth of the mDA axons in the striatum (Tamariz et al., 2010). On the other side, the guidance of the mDA axons towards the medial prefrontal cortex is mediated by Sema3F and its receptor Npn-2 (Hernandez-Montiel et al., 2008; Kolk et al., 2009). Moreover, along the

pathway where the axons of the mDA neurons extend towards the forebrain, *Sema3C* is expressed and suggested to attract the mDA neurons towards the striatum (Hernandez-Montiel et al., 2008). Interestingly, *Sema7A* is also expressed in the striatum and in the cortex. *PlexinC1*; a receptor of *Sema7A* is expressed in the VTA at E15.5 in rat, and due to this expression pattern *Sema7A* was suggested to exhibit a possible role in promoting the DA neurons projections to the telencephalon (Pasterkamp et al., 2007). As demonstrated by previous studies, the differential expression of *plexinC1* in the VTA and *Sema7A* in the striatum, postulates a potential role of these two molecules in guiding the mDA neurons (Prasad & Pasterkamp, 2009; Prestoz et al., 2012). Moreover, *Sema7A* may be involved in the final targeting of VTA axons onto the ventral striatum (Chabrat et al., 2017).

III. Axon guidance molecules in the adult mesostriatal pathway

1. Axon guidance molecules in intact adult mesostriatal pathway

Throughout the embryonic, postnatal and, adult stages the location and level of expression of axon guidance molecules could remain the same or totally modified. In adulthood, the persistence in expression of certain guidance proteins may be beneficial in terms of supporting plasticity and connectivity events (for review Prestoz et al., 2012).

Netrins and their DCC receptors which are expressed during development, are similarly displayed in adults. For instance, *Netrin-1* and *DCC* are both expressed by neurons present in the striatum and in the adult VM where *Netrin-1* displays a predominant expression in the SN while *DCC* is detected at higher levels in the VTA suggesting a remarkable role for these two proteins in DA axonal connectivity and plasticity (Livesey & Hunt, 1997; Osborne et al., 2005). As manifested in another study, mDA circuits in *DCC* mutant mice were disturbed and the mice exhibited defective behavioral reactions. It is therefore plausible that in adults, *DCC* normal

functioning is requested for proper dopaminergic pathway connectivity and functioning (Flores et al., 2005).

Interestingly, several netrin receptors or binding proteins in addition to the DCC such as integrins and Unc5 receptors are also present on the mDA neurons or their targets (Zhang et al., 2004). More recently, further investigations verified that Netrin-1 concentration is higher in the latero-ventral part of the striatum and this high expression is favored by the VTA axons. This study submits a significant role of Netrin-1 in topographic segregation of the mDA axonal innervation into the striatum (Chabrat et al., 2017).

Several proteins of the ephrins family and their receptors are present in the adult brain where they modulate synaptic plasticity and neuronal projections (Goldshmit et al., 2006). EphA5 is detected in the striatum, the VTA, and in the SN (Cooper et al., 2009). Moreover, EphB1 is also present in the striatal matrix compartments, and in the nucleus accumbens (Xiao et al., 2006).

The distribution of the Robo/Slit family guidance molecules, is well characterized in the adult brain. As reported by Marillat et al. (2002), the pattern of Robo1 and Robo2 receptors expression is similar in the developing and adult mDA neurons. By this means, in the SN both Robo1 and Robo2 molecules are expressed however, in the VTA only Robo1 is distinguished (Marillat et al., 2002). In the contrary, some members of the slits family reveal a different expression in adulthood. For example, Slit1 which was not expressed in the mDA neurons of the VTA and SN during embryogenesis, is detected at progressed postnatal stages (P10). In the striatum, Slit1 expression during adulthood is lower in comparison to the strong embryonic levels of expression. On the other side, Slit3 striatal expression level, that is first detected at P5 displays an increase in the adulthood. In the neurons of the SN, Slit2 molecule is exclusively expressed in adults and is detected in other regions such as the thalamus and the hypothalamus (Marillat et al., 2002).

Semaphorin family guidance molecules are well investigated in the adulthood where they are distributed in several brain regions including those within the nigrostriatal pathway. *Sema7A* expression is detected to increase in the mature brain unlike its plexin-C1 receptor levels which tend to decrease (Jongbloets et al., 2013). In a recent study, Chabrat et al. (2017) investigated the impact of *Sema7A* on the topographical innervation of the striatum. They showed that the loss of *Sema7A* altered the projections of the VTA mDA neurons within the dorsal striatum. As remarked, the restricted expression of plexin-C1 to the VTA DA neurons deviated the projections of the mDA neurons towards the ventral striatum and nucleus accumbens. This suggests that *Sema7A* which is abundantly detected in the dorsal striatum exerts a non-permissive effect on the VTA mDA axons expressing plexin-C1. Therefore, *Sema7A* may be a strong candidate required for proper striatal mDA innervation (Chabrat et al., 2017).

2. Axon guidance molecules in altered adult mesostriatal pathway

Complex cognitive skills or even simple motor movements are basically affected by the mDA neurons innervating their appropriate target structures and any alteration in the connectivity of these neurons is associated with several neuronal complications or disorders (Robinson & Kolb, 2004). Moreover, accumulating evidence indicates that changes in the expression and function of the guidance cues may trigger loss of neuronal connectivity and eventually result in the onset and progression of several neurodegenerative disorders including PD. For instance, in patients with PD gene expression profiling of the SN reported variation in the expression levels of several axon guidance proteins (Van Battum et al., 2015). Indeed, this variability in expression is associated with deficits in the nigrostriatal pathway connectivity which increases the risk of early onset PD (Lesnick et al., 2007).

For example, mutations in genes encoding the *Sema5A* protein and DCC receptor, are linked with developing PD (Maraganore et al., 2005). Furthermore, alterations in the expression of Netrin-G1 and Netrin-G2 proteins result in changes in the DA circuitry contributes to the

pathogenesis of PD (Lin et al., 2009). Similarly, Netrin-1 has a fundamental role in maintaining the structural organization and dopaminergic topography within the striatum (Chabrat et al., 2017), therefore variabilities in Netrin-1 could increase the susceptibility to develop PD since deficits in the secretion and functionality of this molecule are correlated with impaired dopaminergic connectivity and transmission (Lin et al., 2009). The dopaminergic circuitry is also regulated by the Ephrins-Eph signaling, and it's probable that alteration in some of the axon guidance molecules within this family is associated with the onset of some neurodegenerative disorders and may predict the outcomes of PD (Lin et al., 2009).

In line with previous studies, repulsive guidance molecule member a (RGMa) is another guiding cue whose variation is linked with PD (Korecka et al., 2017). In PD patients, RGMa expression was upregulated within the SN of the patients. Complication resembling the neuropathological features of PD were obtained after upregulating the expression of RGMa in mice SN. In these mice, the substantia nigral DA neurons were selectively degenerated, DA level within the striatum was depleted, and impaired motor behavior was observed. These outcomes reflect a potential interference of RGMa in the pathology of PD (Korecka et al., 2017).

In brief, the deleterious effects resulting from alterations in axon guidance molecules highlight an important function manifested by these molecules in supporting normal anatomical and functional neuronal circuitry. In the future, targeting guidance molecules may be applied as a potential disease-modifying therapy to repair and enhance neuronal connectivity within the degenerated nigrostriatal pathway.

IV. Aim of the project

In animal models of PD, previous therapeutic approaches using fetal DA neurons from VM as a source of future substantia nigral DA neurons, validated the reparative efficacy of these cells to rewire the degenerated nigrostriatal circuitry.

Especially intranigral transplants, that were ontogenically introduced, displayed a remarkable ability to reconnect the striatum by extending DA axonal projections and subsequently reducing fine motor deficits in the lesioned animals (A. Gaillard et al., 2009; Thompson et al., 2009).

The successful outcomes obtained by these studies support a strong evidence that fetal-derived DA neurons exhibit a promising therapeutic potential in terms of integration and reparation within the injured nigrostriatal circuitry and that the growth and guidance of the grafted fetal DA axons is tolerated by the host tissue where certain directional guidance cues may be involved in this repair.

Indeed, it has been shown that after transplantation of DA fetal cells many axon guidance molecules were differentially expressed in the brain regions surrounding the nigrostriatal pathway and in particular highlighting a possible role of Sema7A in guiding the grafted DA projections to their striatal targets (Kalaani et al., 2016).

Given the fact that certain environmental guidance molecules persist in the adult brain and can still affect the axonal projections of the grafted cells, further therapeutic strategies may be conducted to use these guiding cues and direct the transplanted cells to better rewire their target regions and repair the degenerated pathways.

In this context, the aim of this project was to determine a possible role of Sema7A in the reconstruction of the nigrostriatal pathway after fVM cells intranigral transplantation. As Sema7A could also be involved in the establishment of the pathway during embryogenesis (Chabrat et al., 2017; Kalaani et al., 2016), we also studied the effect of a Sema7A deficiency during the mesostriatal pathway establishment. On another aspect, we aimed to check for the

impact of the transplanted fetal cells on the protein expression in regions surrounding the nigrostriatal pathway in a mouse model of PD.

To achieve this, we first studied the impact of Sema7A on the establishment of the mouse mesostriatal pathway during embryonic development and in intact adult brains. More precisely, we characterized the mesostriatal pathway in WT and Sema7AKO embryos taken at different developmental stages at E12.5, E14.5, E16.5, and E18.5 and of adult mouse brains to explore the effect of the lack of Sema7A on the establishment of the pathway. This study is presented in Part I of this chapter.

Second, we investigated the capacity of the grafted fVM cells to establish striatal connectivity in absence of Sema7A. For this, we studied at the functional level the integration of grafted mDA neurons in the pathway by motor behavioral tests. We quantified the number of the mDA cells within the grafts and analyzed the capacity of the transplanted DA fibers to reach the denervated striatum following transplantation in animal models of PD that were expressing or not Sema7A. The results are discussed in Part II of this chapter.

Third, in 6-OHDA lesioned and transplanted mice with fVM cells we conducted a non-targeted proteomic study after 1 and 7 days of transplantation to explore the whole proteome. The results are presented as an article in preparation in Part III of this chapter.

Part I

Involvement of Semaphorin7A in the development of the mesostriatal pathway and in the intact adult brain

Part I: Involvement of Semaphorin7A in the development of the mesostriatal pathway and in the intact adult brain

I. Introduction.....	61
II. Experimental approach.....	62
1. <i>Animals.....</i>	<i>62</i>
2. <i>Mice genotyping.....</i>	<i>62</i>
3. <i>Western blotting.....</i>	<i>64</i>
4. <i>Tissue processing and Immunohistochemistry on developing brain.....</i>	<i>65</i>
5. <i>Tissue processing and Immunohistochemistry on adult brain.....</i>	<i>66</i>
6. <i>Image acquisition and analysis.....</i>	<i>67</i>
7. <i>Statistical analysis.....</i>	<i>71</i>
III. Results.....	71
1. <i>Validation of Sema7AKO mice.....</i>	<i>71</i>
2. <i>Development of the DA mesostriatal pathway in WT and Sema7AKO animals.....</i>	<i>72</i>
2.1. <i>Less mDA cells are produced during embryonic development in the absence of Sema7A.....</i>	<i>74</i>
2.2. <i>Lack of Sema7A did not show a significant impact on the TH+ projections in striatum primordium of embryos.....</i>	<i>77</i>
3. <i>Investigation of the mesostriatal pathway in adult WT and Sema7AKO animals.....</i>	<i>78</i>
3.1. <i>Sema7A is variably expressed in regions surrounding the mesostriatal pathway in adult.....</i>	<i>78</i>
3.2. <i>The mesostriatal pathway is similar in WT and Sema7AKO mice.....</i>	<i>79</i>
3.3. <i>The number of mDA cells is unchanged in the absence of Sema7A.....</i>	<i>80</i>
3.4. <i>Lack of Sema7A did not show a significant impact on the TH+ projections in striatum.....</i>	<i>82</i>
IV. Discussion and future prospects.....	83

I. Introduction

During development, correct DA neural connectivity is essential for normal cognitive and motor functioning in adulthood. *Sema7A*, together with other guiding cues, has gained much interest regarding its prospective contribution in the establishment of the mesostriatal circuitry during embryogenesis. In 2007, Pasterkamp et al. addressed an innovative potential role for *Sema7A* and one of its receptors during the development of the mesostriatal pathway in rats. *Sema7A* was defined in mDA neurons of SN and interestingly this expression was detected in the projection target regions of these neurons such as the developing striatum. Findings of this study intimates that *Sema7A* may participate in promoting the projection of the mDA axons towards the telencephalon during embryogenesis (Pasterkamp et al., 2007; Prestoz et al., 2012). In adulthood, *Sema7A* expression is detected in brain regions of the nigrostriatal pathway (Kalaani et al., 2016; Pasterkamp et al., 2007). As reported by Kalaani et al. (2016) *Sema7A* mRNA expression is more precisely observed in the caudate-putamen (CPU), the nucleus accumbens (NAcc), the medial forebrain bundle (MFB), the thalamus (THAL), and the VM in adult mice. Moreover, a recent study published in 2017 by Chabrat et al. demonstrated that *Sema7A* is especially highly expressed in the dorsal striatum and that one of its receptors; *PlexinC1* is detected in the VTA (Chabrat et al., 2017). In addition, when *Sema7A* is deficient in the adult brain, it may result in an alteration of the topography of the mDA axons in the striatum. These data suggest a potential role for *Sema7A* and its receptor in the topographical mapping of the mesostriatal system occurring during development.

In this study we aimed to examine the effect of a lack of *Sema7A* in the establishment of the mesostriatal pathway during embryogenesis and in intact adult brain.

We first explored the influence of Sema7A on the development of the mesostriatal pathway at various embryonic stages: at E12.5, when axons are growing out from the midbrain, between E12.5 and E14.5, when axons progress in the diencephalon and from E14.5 onward, when mDA axons reach the striatum (for review Prestoz et al, 2012). We used Sema7A knock-out (Sema7AKO) mice and quantified the number of mDA tyrosine hydroxylase-positive (TH+) cells in the VM as well as their striatal projections.

We also explored the nigrostriatal pathway in intact adult brains in absence of Sema7A. Using immunohistochemistry against TH, we determined the number of mDA cells and we examined the striatal projections in adult Sema7AKO mice compared to wild type (WT).

II. Experimental approach

1. Animals

Housing of the animals and all animal experimental procedures were performed in accordance with the institutional guidelines of the French Agriculture and Forestry Ministry (decree 87849) and were conducted in agreement with the European Communities Council Directive (86/609/EEC). Adult (4 to 6 months old) Sema7AKO (-/-) mice (that were kindly provided by Pasterkamp Laboratory; Netherlands) and WT mice obtained from the same littermate were used for the experimental procedures. For developmental studies embryonic Sema7AKO and WT from the same littermate of different developmental stages (E12.5, E14.5, E16.5, and E18.5) were used. E0.5 was determined as the plug date, and P0 was considered as the date of birth.

2. Mice genotyping

We obtained genomic mice DNA from tail fragments of embryonic and adult animals to determine the mice genotype as described in Pasterkamp et al., 2003. For DNA extraction, the

tissue was digested at 94°C for 1hr in an alkaline lysis solution composed of 25 mM NaOH and 0.2 mM EDTA (pH = 12) and protected with mineral oil solution added on the top of the lysis solution. When the extraction was over, the lysate containing the genomic DNA was neutralized with 40 mM Tris-HCl (pH5).

Sema7A gene were amplified using PCR: amplification step was accomplished using the Invitrogen Kit AccuPrime Taq DNA Polymerase System, and the following primers were used to perform the PCR genotyping: P1, 5'-GGCCACAGGATTCAGTGCAGGCC-3'; P2, 5' CCCACAGACCCAGACATACTGAGC-3'; and P3, 5' CCCGCGTGCCAGCAGAGCTCGC-3'; to generate PCR products of 358 bp (WT) and 513 bp (KO) (Pasterkamp et al., 2003). The initial step of DNA denaturation was carried out for 5 min at 94°C followed by an amplification step performed for 40 cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 1 min. At last, a final cycle for stabilization was performed at 72°C for 10 min followed by a 10°C cooling step. To distinguish between the wild-type Sema7A (+/+) animals (one PCR product at 358 bp) from the mutated animals, homozygous Sema7A (-/-) (one PCR product at 513 bp) and heterozygous Sema7A (+/-) (both PCR products at 358 and 513 bp) a separation by gel electrophoresis was carried out on a 1.6% agarose gels (Figure 15) using a 100bp DNA ladder (Promega) to estimate the PCR fragments size.

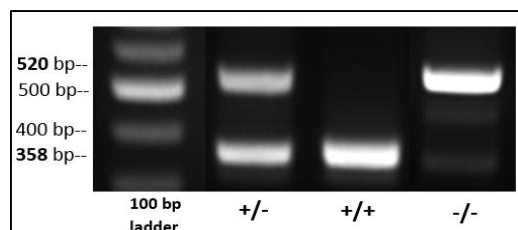


Figure 15: Genotype of Sema7A mutant embryos. The separation on a 1.6% agarose gel electrophoresis shows the amplified Sema7A alleles in wild type (+/+), Sema7A mutant (-/-), and heterozygous (+/-) adult mice.

3. Western blotting

We used western-blotting, first to check for the absence of Sema7A protein in Sema7AKO mice compared to WT brains and second to examine the expression of Sema7A protein in the regions surrounding the nigrostriatal pathway in intact adult WT mouse brain.

For this, a fresh brain tissue frozen on dry ice was dissected from wild type and Sema7A mutant mice. To analyze the expression of Sema7A in the vicinity of the nigrostriatal pathway, brains from adult mice were frozen directly on dry ice and cut using cryostat into 200 μ m thick sagittal sections as described in Kalaani et al., 2016. Briefly, punches were extracted from 5 regions surrounding the nigrostriatal pathway: the CPU, NAcc, MFB, THAL, and the VM. Punches were collected using a 1mm-diameter to collect all tissues except for the VM which was collected using a 2mm-diameter punch (Palkovits, 1973). After tissue collection, sections were labeled with Nissl stain and punched regions were validated according to the adult mouse brain atlas (Franklin& Paxinos, 2008).

The obtained tissues were lysed in lysis buffer containing 0.1M PBS, 1mM NaF (sodium fluoride), 1mM Na₃VO₄ (sodium orthovanadate), 1% SDS (Sodium Dodecyl Sulfate), and 1% protease inhibitor to prevent from protein degradation. Protein lysate was obtained after several rounds of sonication for 2-10 sec followed by a centrifugation at 13,000 r.p.m. for 15 min at RT. Protein quantification was performed using a standard curve of known bovine serum albumin (BSA) standard concentrations in a 1:5 diluted protein assay dye reagent (Bio-Rad Laboratories) and concentrations were obtained by ELIZA plate reader at 595nm wavelength. The lysed proteins were separated using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Briefly, 10% running and 5% stacking polyacrylamide gels were prepared in gel chambers to polymerize. Following polymerization step, proteins were prepared in lamelli (2x) added with 1M dithiothreitol (DTT) then heated for 3 min at 95°C to be loaded into gels. In

each well, 50ug of proteins were loaded for migration and separation. After separation on gels, proteins were transferred into nitrocellulose membrane. Then visualized with Red Ponceau Staining (Sigma) to validate the transfer process. Nitrocellulose membranes were blocked with 5% milk prepared in PBS1X-Tween 0.1% for 1hr30min to prevent non-specific binding, and incubated with primary antibody overnight at 4°C. After washing the membranes with PBS1X-Tween 0.1%, secondary antibody conjugated to horse-radish peroxidase was added for 1h. The following antibodies were used: goat anti-Sema7A (1:1000; AF1835 R&D), mouse anti-GAPDH (1:5000; MAB374 Millipore), secondary anti-goat (1: 10000; HRP705.036.147 Jackson immunoresearch), and anti-mouse, (1:50000; HRP715.036.151 Jackson immunoresearch). After incubation with primary and secondary antibodies, membrane blots were exposed to an enhance chemiluminescence (Luminata-Forte West HRP substrate, Millipore) reagent for 2 min in the dark and finally detected by an automated gel and blot imaging system for revelation (PXi, Syngene). Band intensity was quantified with the use of Genetool software (syngene).

4. Tissue processing and Immunohistochemistry on developing brain

Tissue processing:

Pregnant mice were cervically dislocated at several developmental stages, embryos of different developmental stages E12.5, E14.5, E16.5, and E18.5 were harvested and a tail fragment was cut for genotyping. Embryo heads were then fixed in 4% paraformaldehyde (PFA) overnight at 4°C and cryoprotected with 30% sucrose in 0.1 M phosphate buffered (PB) at 4°C until they sink. After that, the heads were embedded in gelatin solution (7.5% gelatin and 15% sucrose prepared in 0.1 M phosphate buffered saline (PBS)), the embedded heads were frozen in cold isopentane after cooling down. The brains were then cut using a cryostat (CRYOSTAR NX70) into 20µm coronal and sagittal sections and stored at -80°C until immunohistochemistry.

Immunohistochemistry:

2-3h before immunohistochemistry, the sections were kept at room temperature (RT) and rehydrated with 0.1 M PBS. Post-fixation was then performed with 4% PFA for 30min at RT. After several washes with 0.1 M PBS, the non-specific antigenic sites were blocked in 0.1M PBS containing 3% bovine serum albumin (H2B) and 0.3% Triton X-100 for 3hrs at RT. The sections were then incubated with the primary rabbit anti-tyrosine hydroxylase (TH) antibody (Millipore AB152) which was diluted at 1:1000 in a blocking solution, overnight in a wet chamber at 4 °C. Following anti-TH antibody incubation, sections were washed with 0.1 M PBS and incubated at RT for 1h with biotinylated secondary antibody (goat anti-rabbit; 1/200; Vector Laboratories). To quench the endogenous peroxidases, sections were washed with Tris-HCl buffer (0.01M pH7.6), and incubated with 0.3% hydrogen peroxide (Sigma) for 30 minutes. Following that, sections were incubated with an avidin–biotin peroxidase complex (ABC reagent, Vectastain® ABC Kit Vector Laboratories, Burlingame, CA) at RT for 1hr. The sections were subsequently rinsed in Tris-HCl buffer then treated with 0.01M Tris-HCl (pH 7.6) buffer solution containing 0.5 mg/ml 3,3'-Diaminobenzidine (DAB; Sigma Aldrich), and 0.003% hydrogen peroxide (H₂O₂; Sigma Aldrich). The reaction was stopped using 0.1M PBS when a visible brown staining was detected on the tissues. Slides were then dehydrated through several graded concentrations of ethanol (70%, 95%, and 100%) cleared in toluene, then mounted and covered with DePeX mounting medium (VWR).

5. Tissue processing and Immunohistochemistry on adult brain

Tissue processing:

WT and Sema7AKO mice were anesthetized with a lethal intra-peritoneal injection of Dolethal (Vetoquinol) and transcardially perfused with 150mL of 0.9% NaCl followed by 200ml of ice-cold PFA, 4% in 0.1M PB (pH 7.4). A tail fragment was cut for genotyping and brains were

removed, post-fixed overnight in 4% PFA, then cryoprotected in 30% sucrose (prepared in 0.1 M PB; pH 7.4) for 24 hrs. Brains were frozen in isopentane at -50°C, then sectioned into 40µm thick coronal free floating sections and collected into 4 series using a microtome (Microm HM450, Thermo Scientific). The obtained floating sections were stored in a cryoprotective solution (20% glucose, 40% ethylene glycol, 0.025% sodium azide, and 0.05 M phosphate buffer, pH 7.4) at -20°C.

Immunohistochemistry:

After several washes with 0,1M PBS, the free floating brain sections were incubated for 1hr in a blocking solution containing 0,1M PBS, 3% bovine serum albumin (H2B), and 0.3% Triton X-100. Primary rabbit anti-tyrosine hydroxylase (TH) antibody (Millipore) was diluted at 1:1000 in a blocking solution and applied overnight at +4 °C. After several washes with 0.1 M PBS solution, the secondary biotin-conjugated antibody was applied for 1hr min at RT. A goat anti-rabbit antibody (Vector Laboratories) was diluted at 1:200. The sections were then washed with Tris-HCl (0.01M pH7.6) and treated with 0.3% hydrogen peroxide (Sigma) to block the endogenous peroxidases. After that, a reaction with the avidin–biotin peroxidase complex (Vectastain® ABC Kit Vector Laboratories) was carried out for 1hr at RT. Finally, the brain sections were rinsed in Tris-HCl (pH 7.6) buffer solution then treated with 0.5 mg/ml 3-3-diaminobenzidine (DAB; Sigma) and 0.003% hydrogen peroxide prepared in 0.01M Tris-HCl (pH 7.6) solution. Finally, the sections were mounted on slides and dehydrated in successive baths of 70°, 95°, 100° ethanol then cleared in toluene before being cover slipped with DePeX mounting medium (VWR).

6. Image acquisition and analysis

mDA cell quantifications :

In embryos, we used design-based stereological methods to quantify the number of TH+ mDA cells in the SN and VTA in WT and Sema7AKO animals at the following developmental

embryonic stages: E12.5, E14.5, E16.5, and E18.5. In coronal and sagittal sections, areas containing SN and VTA were contoured according to Schambra (2008) (Schambra, 2008) at low magnification objective (2.5X) and cell counting was performed at a high magnification (40X). In coronal sections, the mean of total TH+ cells counted in right and left SN and VTA was calculated per each animal. In sagittal sections, number of mDA TH+ cells was quantified on one side of the brain. According to the size of the developing brain, brains were cut in 1, 2 or 3 series and stereological analysis was performed over 7-13 sections.

Details of the number of sections counted per structure, per stages and per type of mice (WT or Sema7AKO) are presented in the Table 4.

Coronal sections	Total number of sections counted	Number of sections containing SN	Number of sections containing VTA	Number of series cut	Animals used per stage
E12.5	8	8	8	1	2WT+1Sema7AKO
E14.5	12	8	12	1	4WT+4Sema7AKO
E16.5	12	8	12	2	4WT+4Sema7AKO
E18.5	10	10	10	3	3WT+3Sema7AKO
Sagittal sections	Total number of sections counted	Number of sections containing SN	Number of sections containing VTA	Number of series cut	Number of animals
E12.5	7	7	5	1	4WT+4Sema7AKO
E14.5	11	11	7	1	3WT+4Sema7AKO
E16.5	13	13	7	2	3WT+4Sema7AKO
E18.5	11	11	7	3	1WT+1Sema7AKO

Table 4: Number of quantified sections containing SN and VTA per each developmental stage in WT and Sema7AKO embryos. Brains were cut into 1, 2 or 3 series in coronal or sagittal axis.

In WT and Sema7AKO adult brains, VTA and SN containing TH+ mDA cells were delimited according to Franklin and Paxinos (2008) on coronal sections. SN was defined antero-posteriorly (AP) at -2.46 mm to -3.80 mm from bregma whereas VTA was delineated from AP=-2.80 mm to -3.80 mm from bregma. TH+ cells were quantified in sections containing right

and left SN and VTA. Stereological analysis was performed over a total of 8-10 coronal sections/animal and sampling was performed randomly on every 4th section.

For embryos and adult brains of WT and Sema7AKO animals, total number of somas in VTA and SN of each mouse was counted by means of the optical fractionator principle on the following formula: $N = \Sigma Q^{-} \times 1 / asf \times 1 / ssf \times t/h$ (West, 2002; West et al., 1991), where N is the total number of cells estimated, ΣQ^{-} is the total number of cells actually counted, asf is the area sampling fraction, ssf is the fraction of the sections sampled, and t/h is the actual thickness of the section divided by the height of the dissector. All stereological sampling was performed using the Mercator software coupled to a Leica DM5500B microscope with a motorized XYZ stage (Märzhäuser, Wetzlar, Germany).

Striatal projection quantifications:

To quantify the striatal projections in adult and embryos brains of WT and Sema7AKO mice, microscopic acquisitions were carried out on coronal or sagittal sections using mosaic acquisition ZEN software (Zeiss) with a Zeiss Axio Imager.M2 Apotome microscope at x20. ImageJ software v.1.46r (National Institutes of Health; Bethesda, MD) was used to determine the integrated density of TH staining in the striatum. All background intensity was eliminated from the immunohistochemical stained area to quantify only TH immunoreactivity.

More precisely, in adult striatum, the integrated density of TH+ fibers was determined in 10 coronal sections/animal from AP=+1.18 mm to AP=-0.22 mm from bregma. Dorsal and ventral striatum were delineated according to Franklin and Paxinos (2008) (Figure 16). A total of 8 animals per condition (WT or Sema7AKO) was analyzed.

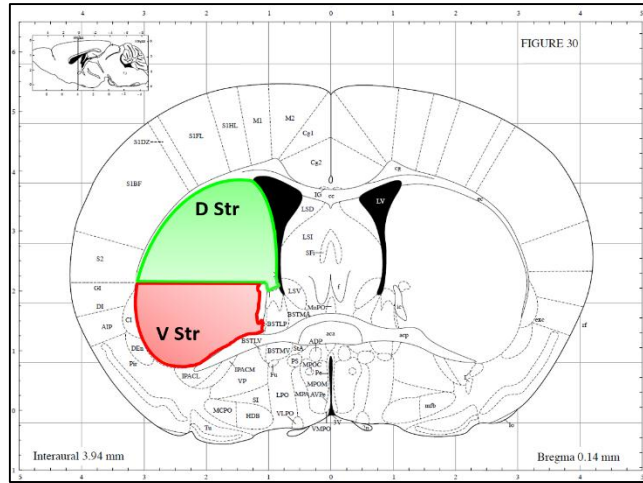


Figure 16: Coronal section from the mouse brain atlas as an illustration for quantification. The dorsal striatum (*D Str*) and ventral striatum (*V Str*) delineated in green and red respectively (adapted from Franklin& Paxinos, 2008).

In embryos, the integrated density of TH staining in the striatum was determined in 9-10 sections/animal using the Schambra atlas (2008). According to the developmental stage, TH staining was quantified either on sagittal sections for E14.5 and E16.5 embryos or on coronal sections for E18.5 aged-animals (Figure 17).

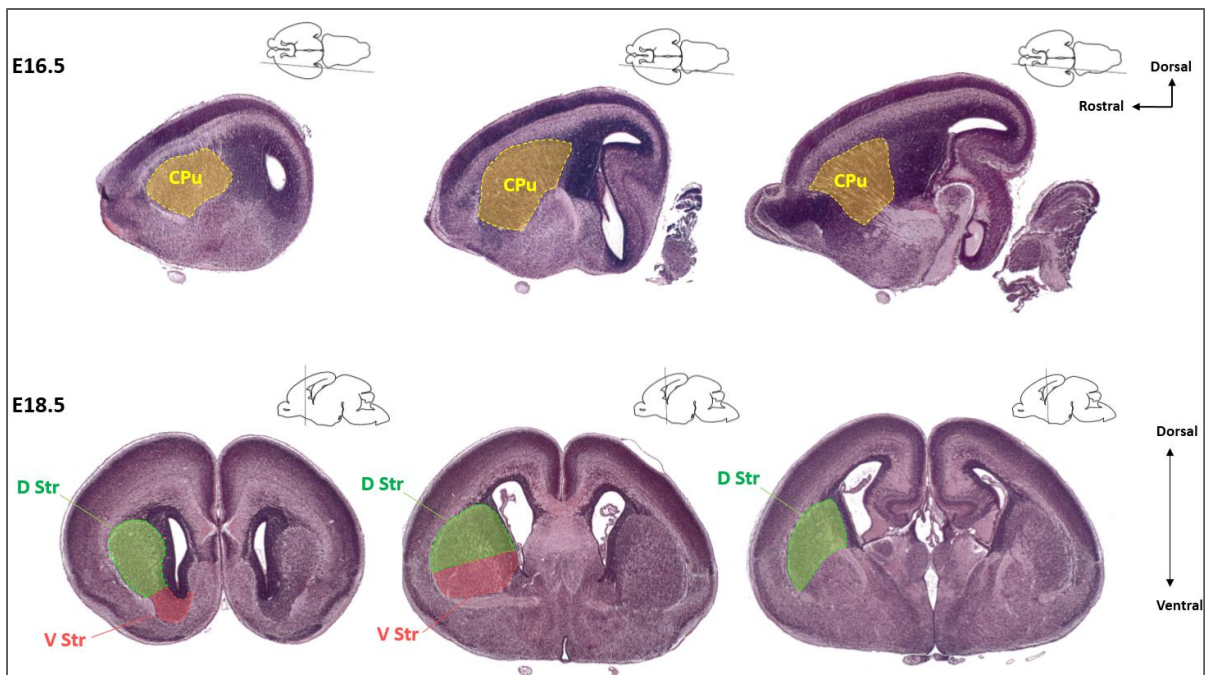


Figure 17: Sagittal and coronal sections of mouse brain atlas at E16.5 and E18.5 respectively retained for quantifications (Adapted from Schambra, 2008). In sagittal sections (E16.5), CPu is

delineated in yellow; in coronal sections (E18.5) the dorsal and ventral striatum are contoured in green and red respectively. *CPu*: caudate putamen, *Str*: striatum, *LGE*: lateral ganglionic eminence.

A total of 3 to 4 animals per condition (WT or *Sema7AKO*) and per embryonic stage were analyzed, except for E12.5 embryos in which no fibers were visualized at this stage in the striatum primordium.

7. Statistical analysis

We used GraphPad Prism software (version 5.0d) for statistical analysis. Data were analyzed by a non-parametric Kruskal-Wallis analysis followed by Dunn's post-hoc test for pairwise multiple comparisons. Results were expressed as the median with interquartile range. For comparisons between two groups, t-test or Mann-Whitney test were used. Results were expressed as the mean \pm sem. $P < 0.05$ values were accepted as statistically significant.

III. Results

1. Validation of *Sema7AKO* mice

We checked the absence of *Sema7A* protein expression in *Sema7AKO* mice in comparison to WT mice. Expression of *Sema7A* protein (75 kDa) was normalized to GAPDH (36 kDa) expression. Protein electrophoresis showed a band of *Sema7A* protein expression in heterozygote (HE +/-) in comparison to WT mouse whereas in *Sema7AKO* (-/-) no band of *Sema7A* expression was detected (Figure 18-A). Quantification of *Sema7A* protein level was higher in WT in comparison with HE with respective ratios of 1.141 and 0.68 relative to

GAPDH expression. In Sema7AKO (-/-) mice, expression of Sema7A was diminished with a ratio of 0.008 relative to GAPDH (Figure 18-B).

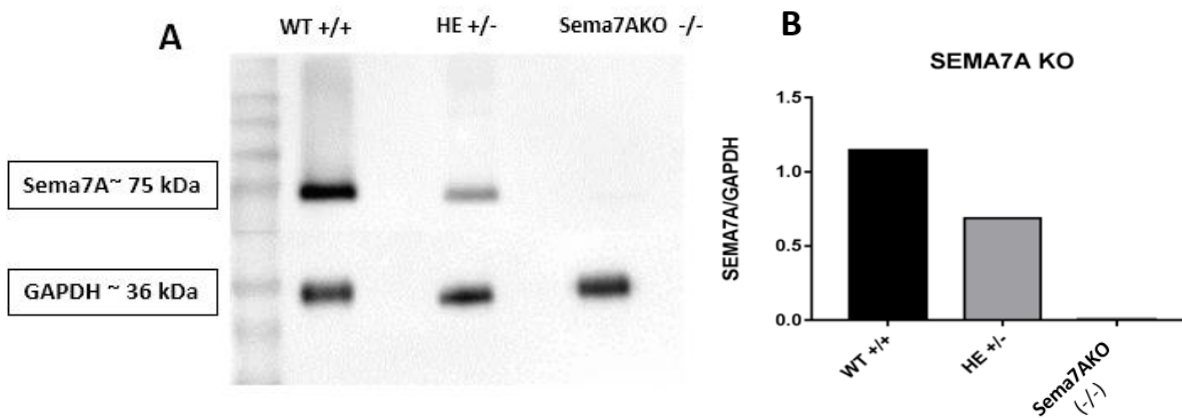


Figure 18: Western blotting analysis of Sema7A protein expression in brain tissue from WT and Sema7AKO mice. (A) Protein electrophoresis and blotting. (B) Expression of Sema7A protein relative to GAPDH in the WT and Sema7AKO animals (n=2 for each).

2. Development of the DA mesostriatal pathway in WT and Sema7AKO animals

We identified the expression of TH during embryogenesis on sagittal or coronal sections in WT and Sema7AKO animals at four developmental stages: E12.5; E14.5; E16.5; and E18.5 in the mesostriatal pathway (Figure 19). TH⁺ cells reside within the VM and the DA axons extend ventrally through the diencephalon to form the MFB at E12.5. Between E14.5 and E16.5, TH⁺ axons migrate rostrally to reach the developing LGE/striatum and at E16.5 the DA projections innervate the striatum. We observed that at E18.5 the DA projections are more localized in the ventral striatum in comparison to the dorsal in both WT and Sema7AKO animals.

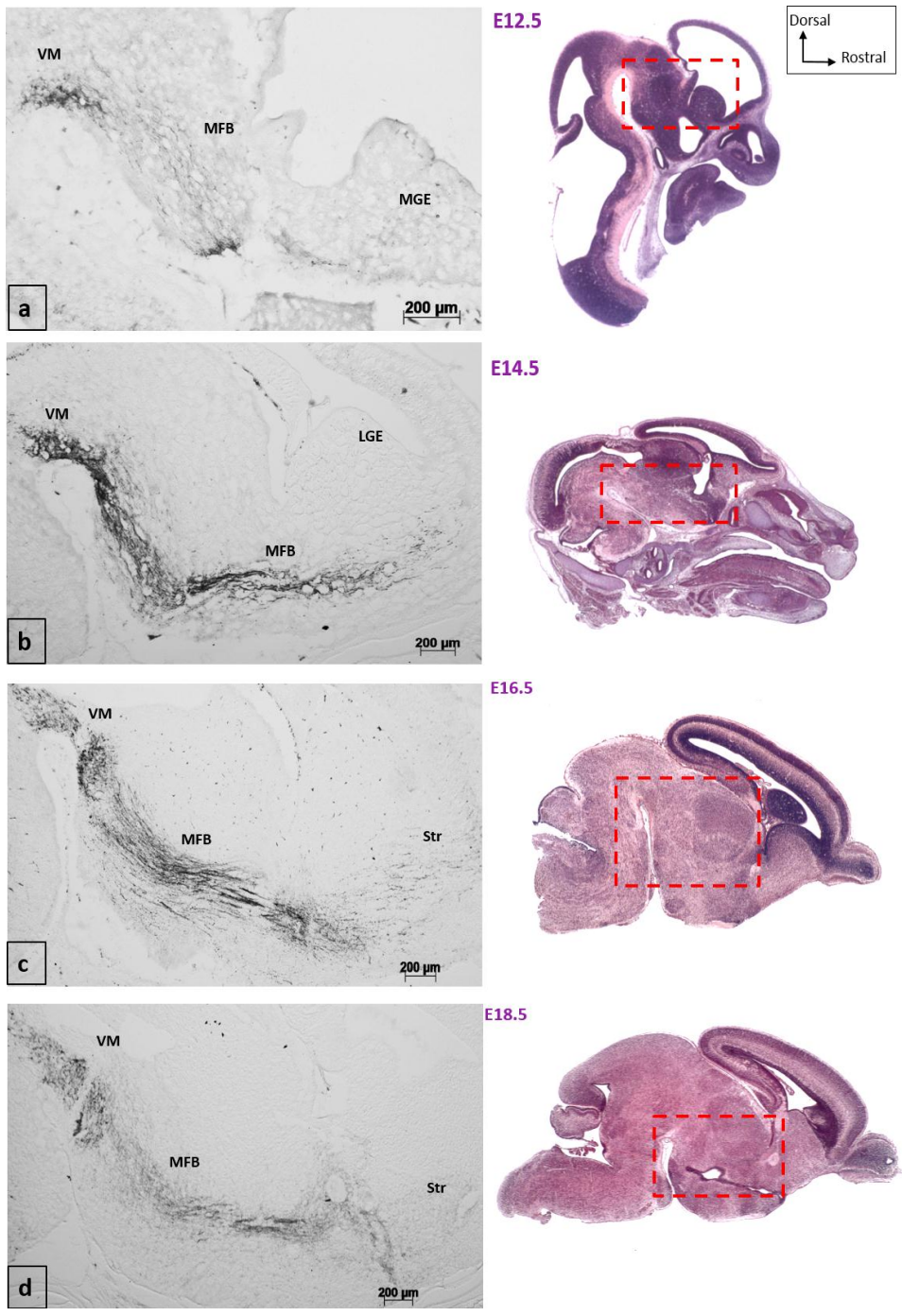


Figure 19: Expression of TH in sagittal sections of WT embryos taken at different developmental stages; E12.5 (a), E14.5 (b), E16.5 (c), E18.5 (d). VTA: ventral mesencephalon. Str: striatum; LGE: lateral ganglionic eminence; VM: ventral mesencephalon; MFB: medial forebrain bundle.

2.1. Less mDA cells are produced during embryonic development in the absence of *Sema7A*

To understand the influence of *Sema7A* on the establishment of the mesostriatal pathway during embryogenesis, we counted the number of mDA neurons within the VM from E12.5 to E18.5 on coronal and sagittal sections treated with DAB against TH. TH⁺ cells were quantified in the SN (delimited in red) and VTA regions (delimited in yellow) in WT and *Sema7A*KO mice as shown for E16.5 embryo in Figure 20.

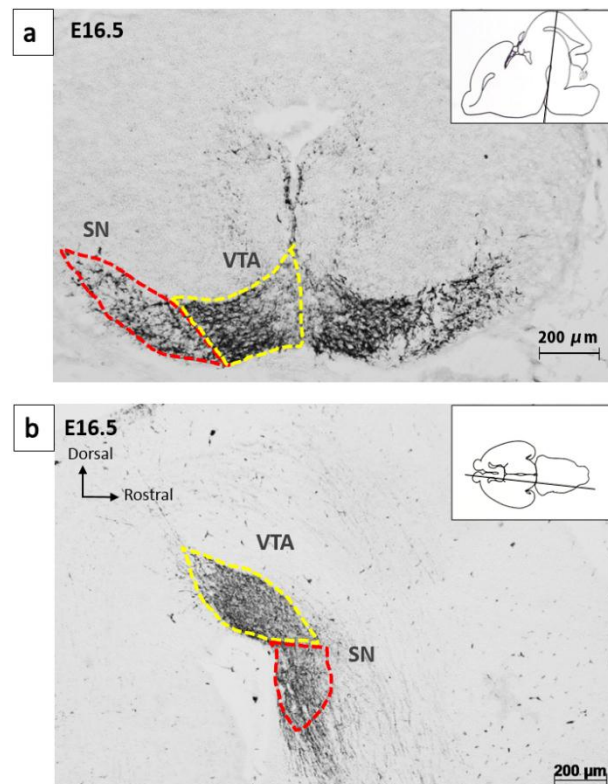


Figure 20: Example of coronal (a) and sagittal (b) sections from WT E16.5 embryos. TH⁺ cell expression is detected in the SN and VTA which are delimited by red and yellow lines respectively. *SN*: substantia nigra; *VTA*: ventral tegmental area.

The number of mDA neurons was lower in the absence of *Sema7A* compared to WT at all developmental stages in the SN and in the VTA.

More precisely, in the SN, the mean number of TH⁺ cells in sagittal sections was 513 ± 64.45 in *Sema7A*KO and 763 ± 66.73 in WT for E16.5 embryos (Figure 21-a). Similarly, on coronal

sections TH+ cells were more numerous from E12.5 to E18.5 in WT than in Sema7AKO with a significant difference at E18.5 where number of TH+ cells was 2615 ± 246.2 in WT versus 2367.5 ± 245.7 in Sema7AKO embryos (figure 21-c).

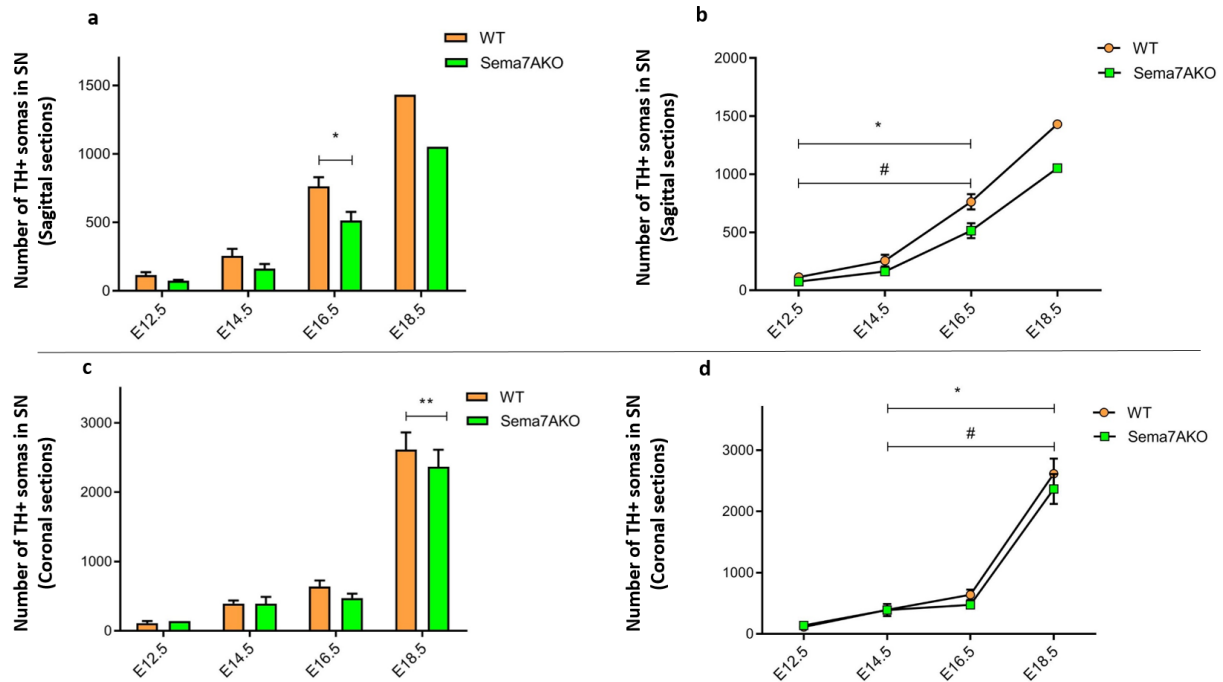


Figure 21: Number of the TH+ cells in the SN of WT and Sema7AKO embryos at different developmental stages. a,b. Mean number of the DA cells expressing TH in the SN from sagittal sections. c,d. Mean number of the DA cells expressing TH in the SN from coronal sections. Significant differences are shown in (a) $p=0.0457$ and (c) $p=0.0044$, (b) $*p=0.0181$; $\#p=0.0137$. (d) $*p=0.0163$; $\#p=0.0346$. *TH*: tyrosine hydroxylase; *SN*: substantia nigra.

We also compared the number of the mDA cells in the SN according to the developmental stages. The number of TH+ cells raise with a particularly more rapid increase from E14.5, E16.5. This increase was significant between E12.5 and E16.5 in both WT and Sema7AKO ($*p=0.0181$; $\#p=0.0137$ respectively) (figure 21-b) when quantified on sagittal sections. On coronal sections, number of TH+ cells was significantly higher in E18.5 embryos with respect to E14.5 stage in both WT and Sema7AKO embryos ($*p=0.0163$; $\#p=0.0346$ respectively) (figure 21-d).

In the VTA, the variation of TH+ cells number between WT and Sema7AKO was similar to those in SN, where in sagittal and coronal sections at all developmental stages the absence of Sema7A was accompanied by a decrease in TH+ cell number (figure 22- a,c). We observed a significant decrease in TH+ cells in sagittal sections at E12.5 in Sema7AKO (107 ± 11.11) compared to WT (195.8 ± 25.08) and at E14.5 where the number of TH+ cells was 507.3 ± 35.49 in Sema7AKO versus 632.7 ± 28.06 in WT (figure 22-a).

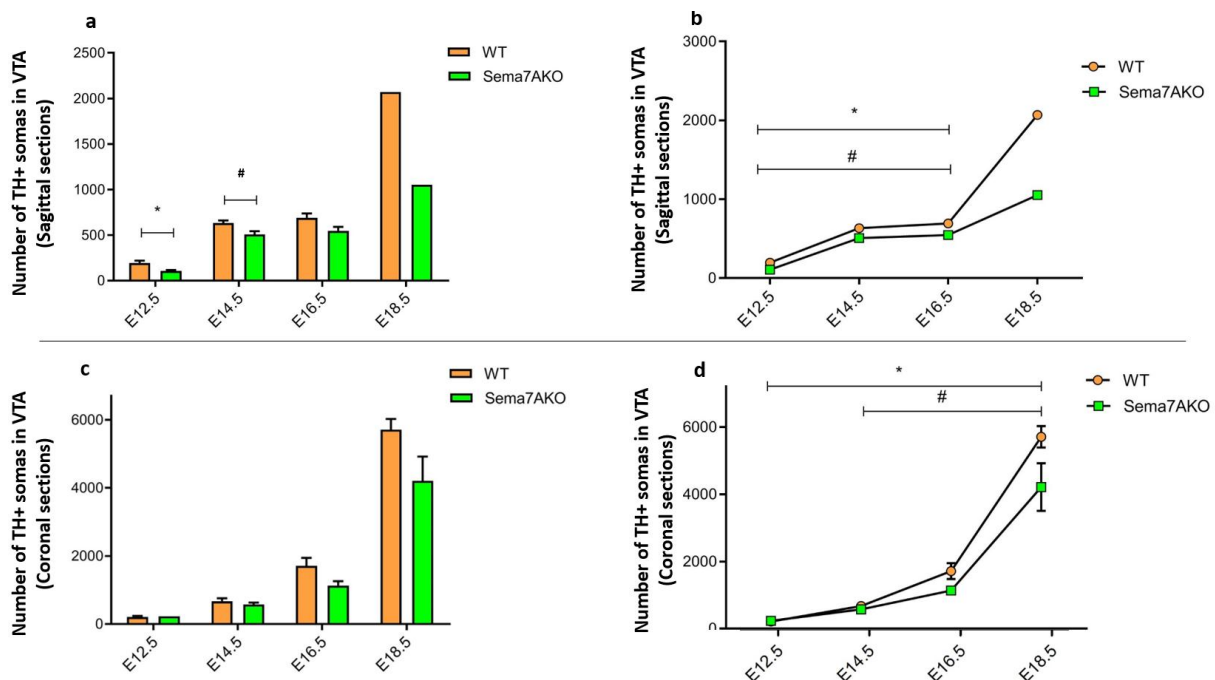


Figure 22: Number of the TH+ somas in the VTA of embryonic WT and Sema7AKO embryos at different developmental stages. a,b. Mean number of the dopamine cells expressing TH in the VTA from sagittal sections. c,d. Mean number of the DA cells expressing TH in the VTA from coronal sections. Statistical analysis was done using Student t-test for 2-groups comparisons with (a) * $P=0.018$; # $P=0.0479$ and using Kruskal-wallis test followed by Dunn's post-hoc for multiple comparisons: (b) * $P=0.0302$; # $P=0.0231$; (d): * $P=0.0107$; # $P=0.0116$. *TH*: tyrosine hydroxylase; *VTA*: ventral tegmental area.

In VTA, quantification of TH+ cells in coronal and sagittal sections similarly revealed an increased number of TH+ cells in WT and Sema7AKO from E12.5 to E18.5. Between E12.5

and E16.5, the mean number of TH+ cells significantly increased in sagittal sections in both of WT and *Sema7AKO* (* $p=0.03202$; # $p=0.0231$ respectively) (figure 22-b). In coronal sections the rise in TH+ cell number was significant between E12.5 and E18.5 in WT embryos (* $p=0.0107$) and between E14.5 and E18.5 in *Sema7AKO* (# $p=0.0116$) (figure 22-d).

2.2. Lack of *Sema7A* did not show a significant impact on the TH+ projections in striatum primordium of embryos

To investigate the effect of lack of *Sema7A* on the TH+ projections in the striatum primordium of embryos, we identified and quantified the TH immunolabeled fibers on sagittal sections at E14.5 and E16.5 in WT and *Sema7AKO* embryos. In E18.5 animals, we detected TH+ fibers on coronal sections in the dorsal and ventral striatum in the two groups of animals as exemplified in figure 23.

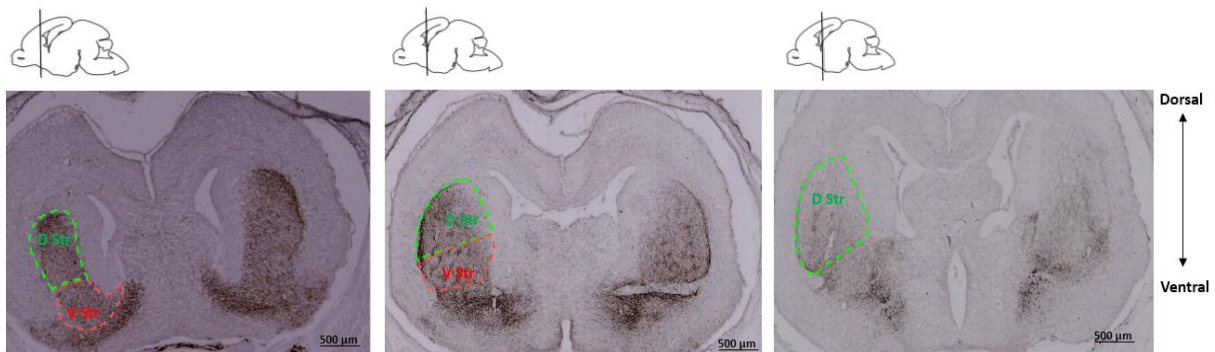
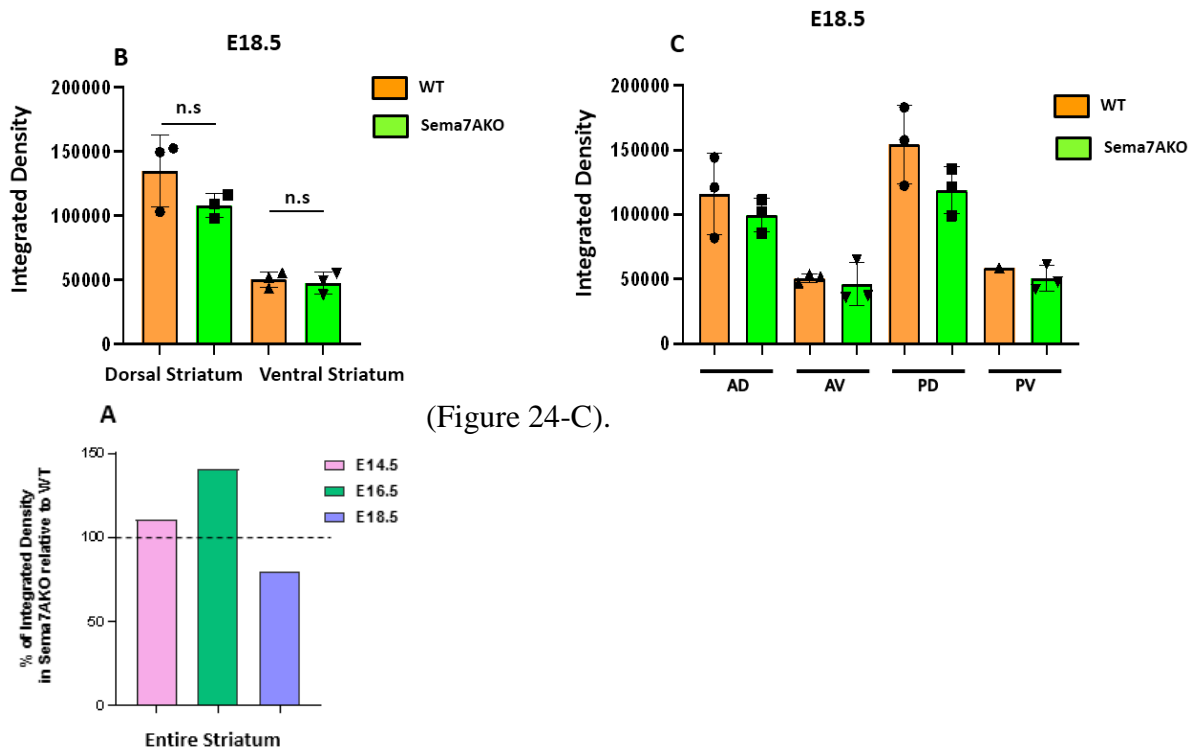


Figure 23: Example of striatal expression of TH+ projections on coronal sections of E18.5 *Sema7AKO* embryo. TH+ fibers are detected in the striatum that is delineated as D Str in green and V Str in red. *D Str*: dorsal striatum; *V Str*: ventral striatum.

Comparison of the integrated density of striatal projections in WT and *Sema7AKO* embryos did not show any significant difference between the groups. Indeed, on the whole striatum the integrated density of TH labeling in E16.5 *Sema7AKO* animals was very similar to WT (110% of the WT density). In the same way in E16.5 and E18.5 embryos the density was not significantly different from the WT, respectively 141% and 79% of WT values (Figure 24-A).

In E18.5 embryos, the decrease of TH density staining observed in the striatum was not significantly dependent on the dorso-ventral (Figure 24-B) nor on the antero-posterior axis



(Figure 24-C).

Figure 24: Quantification of TH+ projections in the striatum of WT and Sema7AKO embryos. Quantification of TH labeling in the entire striatum at E14.5, E16.5, and E18.5 (A), in the dorsal and ventral striatal compartments at E18.5 (B), and in the anterior and posterior parts of the striatum at E18.5 (C). Mann-Whitney test did not show a significant difference: A: E14.5 $p=0.857$; E16.5 $p=0.1$; E18.5 $p=0.4$, B: dorsal striatum $p=0.4$; ventral striatum $p=0.7$, C: AD $p=0.7$; AV $p=0.7$; PD $p=0.2$. AD: anterodorsal, AV: anteroventral, PD: posterodorsal; PV: posteroventral.

3. Investigation of the mesostriatal pathway in adult WT and Sema7AKO animals

3.1. Sema7A is variably expressed in regions surrounding the mesostriatal pathway in adult

Using western blot, we examined the expression of Sema7A protein in the CPu, NAcc, Thal, MFB, and VM (Figure 25-A). Quantification of Sema7A protein level was normalized to GAPDH expression level, and our data showed a variability in expression of Sema7A in regions surrounding the pathway (Figure 25-B).

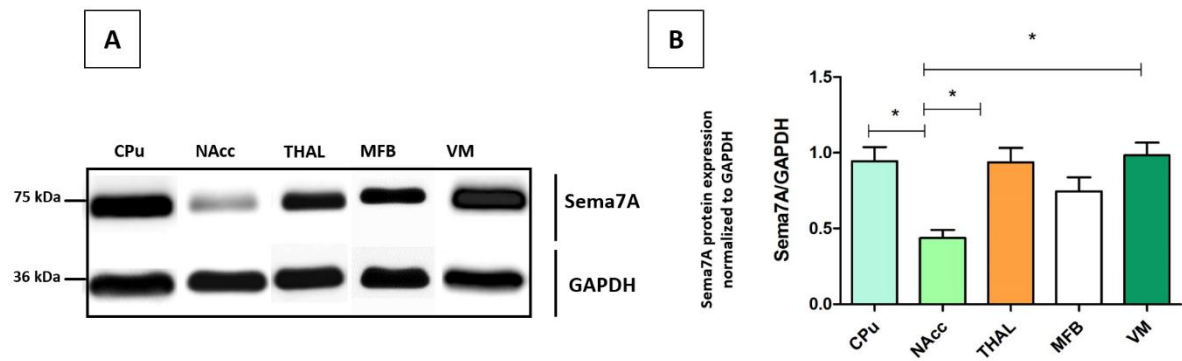


Figure 25: Expression of Sema7A in the mesostriatal pathway in intact adult mice. Western blot (A) showing the expression of Sema7A in CPu, NAcc, THAL, MFB, and VM of an intact adult mouse brain. (B) Quantification of Sema7A protein expression normalized to GAPDH. Significant differences were showed using Kruskal-wallis test followed by Dunn’s post-hoc for multiple comparisons with n=6 per region. *p<0.05. *CPu*: caudate putamen; *NAcc*: nucleus accumbens; *THAL*: thalamus; *MFB*: medial forebrain bundle; *VM*: ventral mesencephalon.

The level of Sema7A expression was significantly reduced in the NAcc compared to other structures, excepted in the MFB where the difference was not statistically different. In the NAcc the level of expression was of 0.436 ± 0.054 relative to GAPDH and was of 0.944 ± 0.0942 , 0.936 ± 0.095 and 0.9835 ± 0.0828 in the CPu, THAL and VM respectively.

3.2. The mesostriatal pathway is similar in WT and Sema7AKO mice

We investigated the topography of the mesostriatal pathway in WT and Sema7AKO mice using TH immunoreactivity. The distribution of mDA neurons within the SN and VTA and the DA pathway was similar in WT and Sema7AKO adult mice as illustrated in figure 26.

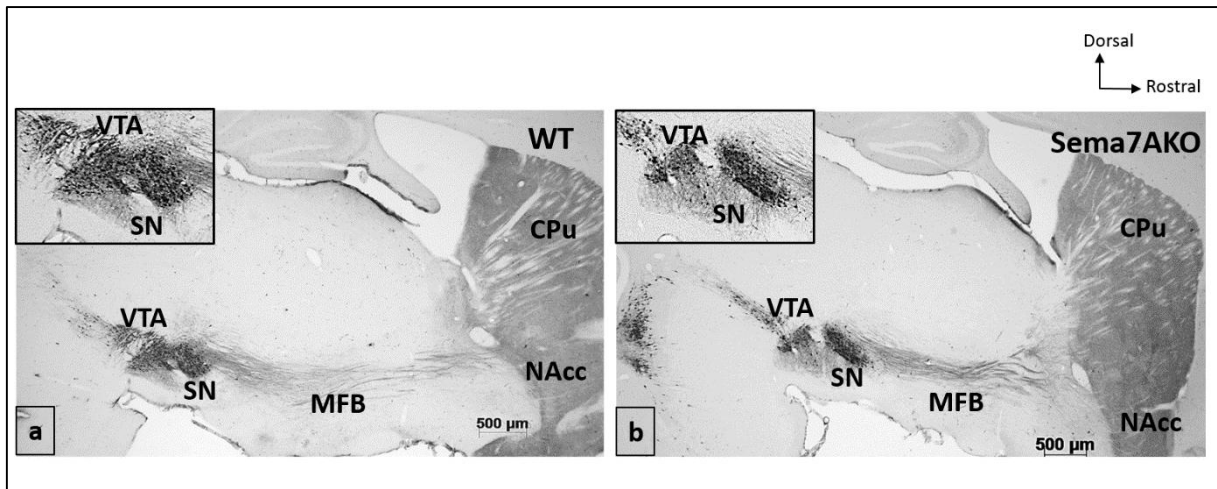


Figure 26: Sagittal sections (Medio-lateral; ML=1.20 mm from the midline); in WT (a) and Sema7AKO (b) mice showing the midbrain TH+ neurons and their projections. *Cpu*: caudate putamen; *nsp*, nigrostriatal pathway; *SN*: substantia nigra pars reticulata, *VTA*: ventral tegmental area; *Nacc*: nucleus accumbens; *MFB*: medial forebrain bundle.

In both WT and Sema7AKO mice we observed TH+ labeled somas within the VM (SN and VTA). As the TH+ neurons exit the VM they project rostro-ventrally as fasciculated axonal tracts forming the MFB to innervate the CPu (dorsal striatum), the NAcc (ventral striatum), and extend to the cortex.

3.3. The number of mDA cells is unchanged in the absence of Sema7A

We compared the number of SN and VTA TH+ cells between WT and Sema7AKO mice. Quantification was performed on sagittal or coronal sections and regions were identified as shown in figure 27.

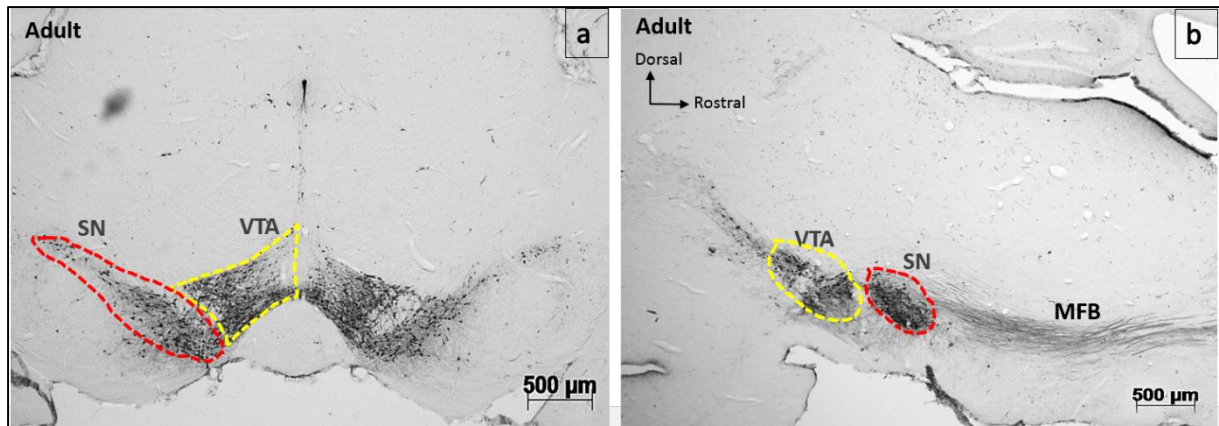


Figure 27: Coronal section at -3.52 mm from bregma (a) and sagittal section at 1.68 mm from the midline (b) from WT intact adult brains. TH+ cell expression is detected in the SN and VTA which are delimited by red and yellow lines respectively. SN: substantia nigra; VTA: ventral tegmental area; MFB: medial forebrain bundle.

Stereological analysis showed no significant differences between WT and Sema7AKO in the total number of TH+ cells counted in left and right SN and VTA (Figure 28).

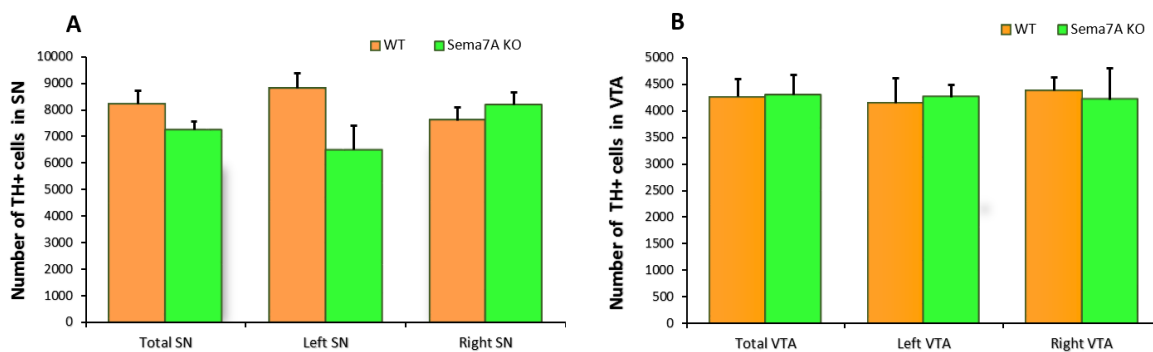


Figure 28: Mean (\pm SEM) of TH+ somas number in intact WT and Sema7AKO SN (A) and VTA (B). n=5 for each of left SN and left VTA; n=5 for each of right SN and VTA; n=6 for each of the right SN and VTA groups. SN: substantia nigra; VTA: ventral tegmental area; MFB: medial forebrain bundle. Statistical analysis were done using Kruskal-wallis test followed by Dunn's post-hoc for multiple comparisons.

3.4. Lack of *Sema7A* did not show a significant impact on the TH+ projections in striatum

To investigate the influence of *Sema7A* on the distribution of TH+ projections in the striatum, we compared the striatal TH labeling in WT and *Sema7AKO* mice.

On the whole striatum, TH labeling tend to decrease in the absence of *Sema7A* but this was not statistically significant (Figure 29-A). Interestingly, this decrease seemed to be more drastic in the dorsal striatum than in the ventral part (Figure 29-B) even if the values were not statistically significant. Indeed, the ratio WT/*Sema7AKO* of the integrated density of TH labeling was 1.48 in the dorsal striatum compared to 1.14 in the ventral striatum. Moreover, there was no effect of the antero-posterior location on striatal TH labeling (Figure 29-C).

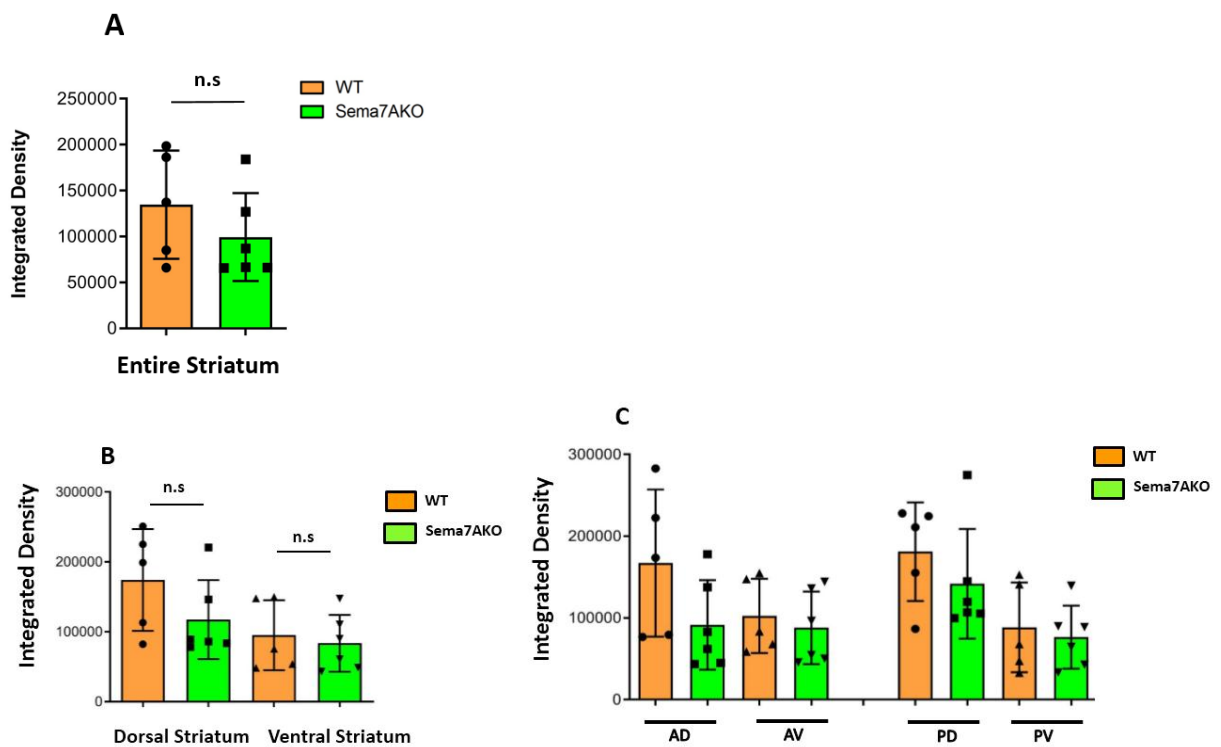


Figure 29: Quantification of TH+ projections in the striatum of WT and *Sema7AKO* mice. Quantification of TH labeling in the entire striatum (A), in the dorsal and ventral striatal compartments (B) and in the anterior and posterior parts of the striatum (C). Comparison between the groups was carried out using Mann–Whitney test: A ($p=0.329$) and using Kruskal-wallis test followed by Dunn’s post-hoc for multiple comparisons: B ($p=0.15$); C ($p=0.07$). AD: anterodorsal, AV: anteroventral, PD: posterodorsal; PV: posteroventral.

IV. Discussion and future prospects

In the present chapter, we investigated the establishment of the mesostriatal pathway in absence of *Sema7A* during development and adulthood. Microscopic observation in embryos and adults showed that formation of mesostriatal pathway is normal in *Sema7AKO* animals and that the striatal density of DA fibers is not significantly altered with the loss of *Sema7A*. Nevertheless, we observed a diminution in the number of DA neurons in the SN and the VTA in *Sema7AKO* embryos that was restored in adults. Overall, these results highlight a new role for *Sema7A* in the early development of mDA neurons.

The developmental time course of the mesostriatal pathway is well described during embryogenesis: between E12.5 and E14.5 the mDA neurons project DA fibers ventro-rostrally along the developing MFB. At E14.5, the DA axons arrive near the ventrolateral region of the developing striatum to finally innervate the striatum at E16.5 (Prestoz et al., 2012 for review). In our study we observed a similar timing of the mesostriatal pathway formation in WT and *Sema7AKO* mice: At E12.5 the descending TH⁺ fibers grow out from the VM to form the MFB and no staining is detected in the striatum primordium at this stage. At E14.5 TH⁺ fibers progress in the telencephalon towards the striatum and starting from E16.5 we observed dense DA fibers innervating the ventrolateral part of the striatum in a similar way in WT and *Sema7AKO* embryos. At E18.5, the lack of *Sema7A* did not have any impact on the detected TH⁺ fibers density in the striatum. Then in adults, the ventral and dorsal parts of the striatum were densely innervated by TH⁺ fibers and surprisingly no differences were observed between the WT and *Sema7AKO* mice. This is even more surprising because first, we showed that *Sema7A* protein is twice more expressed in the CPu (dorsal striatum) than in the NAcc (ventral part of the striatum) as also confirmed by Chabrat et al., (2017), second *Sema7A* has a chemorepellent effect on the axons of the VTA neurons but not on SN subtype in vitro and

third, in adult *Sema7A*KO mice, the mDA axons projecting from the VTA extended more into the dorsal striatum instead of the ventral part (Chabrat et al., 2017). This latter observation was conducted using a retrograde adeno-associated viral vector (AAV-Retro-GFP) directly injected in the different parts of the striatum to visualize the number of corresponding neurons in the SN and VTA. This method is more precise and sensitive to detect differences in fibers density contrary to the one used in our study and this is probably why we could not detect a significant difference in TH+ striatal projections. Another quantification method that could be used is a stereological approach that allows to determine the total length of fibers correlated to the density of fiber projections as used in Vitrac et al. (2014) to quantify cortical projections (Vitrac et al., 2014). With this method it will be also interesting to check for the fibers density in the MFB knowing that *Sema7A* is expressed in this structure. Finally, to have an integrated view of the TH+ connections in absence of *Sema7A* in the whole pathway, it would be useful to use the 3-DISCO clearing technique which stands for the 3D imaging of solvent-cleared organs (Ertürk & Bradke, 2013; Ertürk et al., 2014). In any case, *Sema7A* is obviously not the only cue guiding TH+ fibers towards their targets. For instance, *Netrin-1* is well-known for its crucial role in the establishment of the mesostriatal pathway and terminal connections onto the striatum. Indeed, several defects in DA system were detected in the *Netrin-1* receptor DCC null newborn mice including distortion in the DA innervation of the ventral striatum and defects in DA cell migration (Xu et al., 2010) strongly suggesting a role for *Netrin-1* in the formation of the DA pathway during development. Moreover, in the adults, a gradient of *Netrin-1* is present in the striatum with a lowest concentration in the medio-dorsal striatum and highest in the latero-ventral part. Accordingly, null mice for *Netrin-1* show a loss of innervation in the dorsal striatum (Li et al., 2014) suggesting its involvement in the topographic mapping of the mesostriatal pathway. Thus, even though all mDA neurons express the receptor DCC, VTA axons prefer a higher *Netrin-1* concentration than the SN axon (Li et al. 2014) strengthening

the idea of differentially guided axons growing out from the SN and the VTA. Moreover, ephrinA5 was also speculated to contribute to the establishment of the mesostriatal pathway based on its chemorepulsive effect on mDA axons in vitro and its expression gradient in the developing striatum (Deschamps et al., 2009).

On another aspect, when counting the TH⁺ somas in the VM we detected during embryogenesis either in coronal or sagittal sections a decrease in the number of somas in the SN and VTA in *Sema7A*KO mice that was restored in adults. This decrease seemed to emerge in early development in the VTA (E12.5 and E14.5) and later in the SN (E16.5 and E18.5). These developmental stages correspond to the radial and tangential migration phases that VTA mDA neurons and SN mDA neurons respectively undergo in the VM (Brignani & Pasterkamp, 2017). At this stages mDA neurons are thought to be post-mitotic (Bissonette & Roesch, 2016) then it is unlikely that *Sema7A* modulates the proliferation of mDA neurons. Nevertheless, it is more plausible that *Sema7A* promotes the survival of mDA cells as it has been recently shown that *Sema7A* could modify the arborization of mDA neurons thus decreasing their vulnerability (Pacelli et al., 2015). Moreover, *Sema7A* was found to be a substrate for caspase-9 when linked to survival mechanisms of olfactory bulb neurons (Ohsawa et al., 2009). A survival action of *Sema7A* on mDA cells is an interesting point if we aim to increase the survival of these cells in pathological conditions.

Part II

Involvement of Semaphorin7A in the reconstruction of the nigrostriatal pathway after cell therapy in a mouse model of PD

Part II: Involvement of Semaphorin7A in the reconstruction of the nigrostriatal pathway after cell therapy in a mouse model of PD

I. Introduction.....	88
II. Experimental approach.....	89
1. <i>Animals.....</i>	<i>89</i>
2. <i>6-OHDA lesion and transplantation.....</i>	<i>90</i>
3. <i>Apomorphine induced rotation test.....</i>	<i>91</i>
4. <i>Tissue processing and Immunohistochemistry on adult grafted brain sections.....</i>	<i>92</i>
5. <i>Image acquisition and quantification.....</i>	<i>94</i>
6. <i>Statistical analysis.....</i>	<i>96</i>
III. Results.....	97
1. <i>First set of transplantations.....</i>	<i>97</i>
1.1. <i>Behavioral performance after lesion and transplantation.....</i>	<i>97</i>
1.2. <i>The lack of Sema7A increases the number of grafted cells.....</i>	<i>98</i>
1.3. <i>The grafted cells do not grow out of the transplant in WT nor in Sema7AKO animals.....</i>	<i>100</i>
2. <i>Second set of transplantation.....</i>	<i>101</i>
2.1. <i>Behavioral performance after lesion and transplantation.....</i>	<i>101</i>
2.2. <i>The lack of Sema7A increases the number of Actin-EGFP or TH-EGFP grafted cells.....</i>	<i>103</i>
2.3. <i>In both WT and Sema7AKO animals, grafted GFP+ projections grow out of the transplant, follow the nigrostriatal pathway and reach the striatum.....</i>	<i>107</i>
2.4. <i>The lack of Sema7A does not modify the mDA striatal projections after transplantation.....</i>	<i>109</i>
IV. Discussion and future prospects.....	111

I. Introduction

Previous studies, including ours (A. Gaillard & Jaber, 2011; Thompson et al., 2009) showed that grafting of fetal VM cells in the lesioned adult mouse SN (model of PD) leads to anatomical and functional recovery of the nigrostriatal circuitry. Indeed, axonal projections of the grafted DA fetal cells navigate through intermediate targets in the diencephalon to innervate the striatum in the telencephalon therefore regenerating the damaged DA network. This supports the concept that the host brain tissue is permissive to long axonal navigation and implies that certain molecules residing in the regions surrounding the nigrostriatal pathway, orchestrate the axonal steering of the grafted mDA neurons to ensure proper DA connectivity and enhanced motor performance after transplantation in the host brain (Gaillard et al., 2009; Jaber et al., 2013).

Interestingly, we also observed that expression of several axon guiding cues within the nigrostriatal pathway was modified after grafting in this PD mouse model. Among the axon guidance molecules, semaphorins were significantly modulated at the molecular level by the presence of the grafted fetal VM cells. More particularly, Sema7A guiding molecule has been reported to manifest remarkable variation in expression throughout the lesioned pathway after grafting (Kalaani et al., 2016). This implies that following transplantation, Sema7A may be involved in reconnecting the grafted DA cells to their targets within the nigrostriatal system.

The aim of this work was therefore to understand how this guiding cue could modulate or be involved in the reconstruction of the nigrostriatal pathway after transplantation.

We used Sema7A deficient mice to assess the capacity of the grafted DA cells to repair the pathway in absence of Sema7A. Therefore, we first lesioned the SN of Sema7AKO and WT mice using 6-OHDA injection, then into the lesioned SN we grafted fetal VM cells collected from E12.5 embryos overexpressing green fluorescent protein (GFP) under the control of β -actin (Actin-EGFP) or tyrosine hydroxylase (TH) promoter (TH-EGFP). We conducted two

sets of experiments: in the first set we used fetal cells dissected from VM of TH-EGFP embryos to transplant the lesioned WT and Sema7AKO mice using a protocol similar to that proposed by Gaillard et al. (2009) with the same amount of grafted cells. In the second set of experiments, we grafted different quantities of cells from two different sources: either fetal VM cells from TH-EGFP E12.5 embryos or fetal VM cells from Actin-EGFP E12.5 embryos.

In order to investigate the integration of the grafted mDA neurons we evaluated at the functional level the motor performance of the animals using the apomorphine-induced rotation test 8 weeks after transplantation compared to their performance before grafting 3 weeks after the SN lesion. Then, we carried out an anatomical study of the nigrostriatal pathway using immunohistochemical approach to visualize the grafted mDA neurons and to quantify the number of mDA cells within the grafts in Sema7AKO and WT mice. We also studied the topography of the transplanted DA fibers and evaluated their capacity to innervate the striatal targets in absence of Sema7A.

II. Experimental approach

1. Animals

All animal experimental procedures were performed in agreement with the guidelines of the French Agriculture and Forestry Ministry (decree 87849) and approved by the European Communities Council Directive ((86/609/EEC). All efforts were made to reduce the number of animals used and their suffering. Sema7AKO mice were kindly provided by Pasterkamp Laboratory; Netherlands and wild type (WT) mice from the same littermate were used for the experimental procedures. Ventral mesencephalons (VM) of E12.5 embryos from C57/B16 transgenic mice overexpressing enhanced green fluorescent protein under the control of the

tyrosine-hydroxylase promoter (C57BL/6JRj TH-EGFP) or actin promoter (C57BL/6JRj Actin-EGFP) were used as a source of mDA cells for transplantation.

2. 6-OHDA lesion and transplantation

Adult WT and Sema7AKO female mice were lesioned unilaterally with 6-OHDA injection in the SN to kill specifically dopaminergic cells as described previously (A. Gaillard et al., 2009). Briefly, after being anesthetized by an intraperitoneal injection of Ketamine/Xylazine (100mg/kg and 10mg/kg respectively) the animals were injected using a Hamilton syringe by a 1µl of 6-OHDA (8 µg/µl, Sigma) dissolved in sterile saline solution containing 0.1% ascorbic acid into the left SN pars compacta at the following coordinates AP: -3.2 mm (from bregma), ML=1.4 mm (from midline), DV= -3.8 mm (from dura). 3 weeks after lesion, VMs from TH-EGFP or Actin-EGFP E12.5 transgenic embryos were dissociated in 0.6% glucose saline solution. Different quantities of cell suspension were injected into the left lesioned SN.

Two sets of transplantation experiments were performed: first, 20 Sema7AKO and 19 WT mice were lesioned and 12 out of each group were each transplanted with 150,000 cells/1.5µL from TH-EGFP embryos at E12.5. For the second set of transplantation, 9 Sema7AKO and 9 WT mice were lesioned and of which 8 Sema7AKO and 7 WT mice were used for transplantation where different quantities of VMs from either TH-EGFP or Actin-EGFP E12.5 transgenic embryos were used for grafting as follows: 3WT and 4Sema7AKO mice received the equivalent of 5 or 7 Actin-EGFP VMs, and in another group, 4WT and 4Sema7AKO mice received the equivalent of 2 or 7 TH-EGFP VMs (Figure 30).

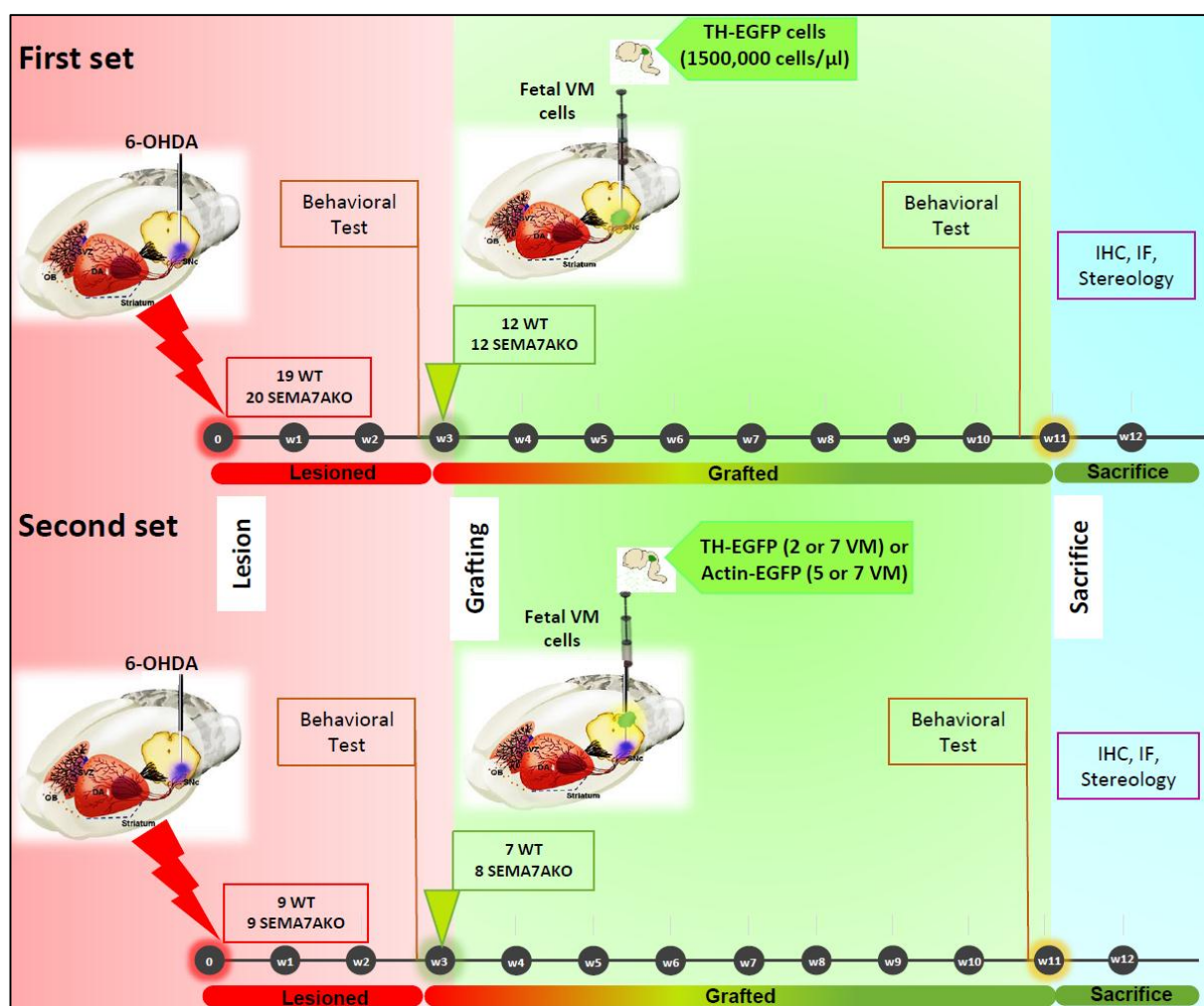


Figure 30: Schematic illustration showing the experimental setup for grafting WT and Sema7AKO mice of in the first and second sets of transplantation. *VM*: ventral mesencephalon, *IHC*: immunohistochemistry, *IF*: immunofluorescence.

3. Apomorphine induced rotation test

The levels of DA receptors elevate after SN lesion to compensate for the dopamine loss in the striatum. Apomorphine is a D1 and D2 dopamine receptor agonist, therefore following apomorphine injection the ipsilateral denervated striatum is activated by apomorphine and the mice are compelled to rotate in the contralateral direction (Torres & Dunnett, 2011). In this study, to assess the functional effect of lesion and transplantation on the behavioral performance in mice, apomorphine induced rotation test was performed 3 weeks after lesion and 8 weeks

following transplantation. Apomorphine was injected subcutaneously at a dose of 0.1 mg/Kg then mice were placed in automated “rotometer” bowls (Imetronic-France) to record clockwise rotations (CW) corresponding to the contralateral full body turns and counter clockwise rotations (CCW) corresponding to the ipsilateral full body turns using the automated rotometer system over a 60 min session (Figure 31)(Torres & Dunnett, 2011; Ungerstedt & Arbuthnott 1970). More precisely, the net rotation was calculated by: CW-CCW (contralateral body turns minus ipsilateral body turns) and mice with positive net rotational scores were considered lesioned.



Figure 31: The apomorphine-induced rotation test. Following 6-OHDA lesion in the left SN, apomorphine injection triggers a rotation behavior in an opposite direction to the lesioned side. The mouse in the bowl is attached to a rotometer head by a harness so that ipsilateral and contralateral turns are assessed by the automated rotometer system.

4. Tissue processing and Immunohistochemistry on adult grafted brain sections

Tissue processing:

8 weeks after transplantation, TH-EGFP or Actin-EGFP fetal VM cell-grafted WT and Sema7AKO mice were anesthetized with a lethal intra-peritoneal injection of Dolethal (Vetoquinol) and transcardially perfused with 150mL of 0.9% NaCl followed by 200ml of ice-

cold PFA, 4% in 0.1M PB (pH 7.4). Then brains were dissected and post-fixed in 4% PFA overnight. Following cryoprotection in sucrose (30% in 0.1 M PB) and freezing in isopentane, 40µm-thick free floating sections were carried out in parasagittal and coronal axis and collected into 4 and 6 series respectively using a cryostat (CRYOSTAR NX70). The obtained floating sections were stored in a cryoprotective solution (20% glucose, 40% ethylene glycol, 0.025% sodium azide, and 0.05 M phosphate buffer, pH 7.4) at -20°C.

Immunohistochemistry:

The phenotype of grafted cells was visualized using immunofluorescent staining and IHC with DAB/nickel staining was used to visualize grafted cells and their striatal projections.

IHC was performed on free-floating sections. Briefly, sections were washed in 0.1M, pH7.4 phosphate buffered saline (PBS) and non-specific binding sites were blocked by 3% bovine serum albumin (H2B group) and 0.3% Triton X-100 for 1h. Primary antibodies diluted in a blocking solution were then applied overnight at 4 °C. The primary antibodies used were as follows: goat anti-GFP (1:1000; Abcam), mouse anti-TH (1:500; ImmunoStar), rabbit anti-TH antibody (1:1000; Millipore), rabbit anti-GirK2 (1:200, CovaLabs), mouse anti-calbindin (1:1000, Swant300). After several 0.1 M PBS washes, secondary biotinylated or coupled to fluorochromes (Alexa Fluor) antibodies were applied for 1hr min at RT. The following secondary antibodies were used: biotinylated donkey anti-goat (1:200; Vector Laboratories), biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories), donkey anti-goat Alexa488 (1:500; Invitrogen), donkey anti-rabbit Alexa568 (1:500; Life technologies), donkey anti-mouse Alexa647 (1:500; Invitrogen).

In order to carry out DAB or DAB/nickel staining, sections were washed with Tris-HCl (0.01M pH7.6) buffer and incubated with 0.3% hydrogen peroxide (Sigma Aldrich) for 30 min to

quench endogenous peroxidases. Subsequently, the sections were reacted with avidin–biotin peroxidase complex (Vectastain® ABC Kit Vector Laboratories) for 1h at RT.

Sections were either stained with 0.01M Tris-HCl (pH 7.6) solution containing 0.5 mg/ml 3-3-diaminobenzidine (DAB; Sigma), or 0.1M sodium acetate (pH=6) solution containing 15.8 mg/ml nickel ammonium sulfate, and 0.5 mg/ml DAB mixed with 0.003% hydrogen peroxide until a visible brown/grey staining was detected on the tissues.

After washing in 0.1M PBS, the sections were mounted on slides, dehydrated in graded concentrations of 70°, 95°, and 100° ethanol, and cleared in toluene before being coverslip with DePeX mounting media (VWR).

To perform immunofluorescent labeling, secondary antibodies used were coupled to fluorochromes, and after several washing, sections were cleared directly in toluene and covered with DePeX mounting media.

5. Image acquisition and quantification

Quantification of cell somas in the grafts:

In the first set of experiment, the total number of grafted cells in the graft was quantified using unbiased stereology on DAB treated sections. In WT and Sema7AKO mice receiving TH-EGFP cell transplants, GFP+ and TH+ cells were counted within the total area covered by the graft in coronal sections. Quantification was done by means of the optical fractionator principle based on the following formula: $N = \Sigma Q^- \times 1 / asf \times 1 / ssf \times t/h$ (West et al., 1991; West, 2002), where N is the estimate of the total number of cells, ΣQ^- is the total number of cells actually counted, asf is the area sampling fraction, ssf is the fraction of the sections sampled, and t/h is the actual thickness of the section divided by the height of the dissector. A systematic randomized sampling of total number of sections containing the graft was performed on every sixth section.

All stereological sampling was performed using the Mercator software coupled to a Leica DM5500B microscope with a motorized XYZ stage (Märzhäuser, Wetzlar, Germany).

Quantifications of number of grafted cells in the second set of the experiments was done on sections labeled by immunofluorescence in mice transplanted by either TH-EGFP or Actin-EGFP cells. For each animal, quantification was performed over the area covered by the entire graft using Axio Imager. M2 Apotome microscope (Carl Zeiss). Counting was done on every 4th or 6th section of the total number of sections cut in sagittal or coronal axis respectively. More precisely, GFP+ cells, Girk2+/GFP+ and Calbindin+/GFP+ cells were counted within the graft in mice receiving TH-EGFP transplant. On the other hand, in mice receiving Actin-EGFP graft, TH+/GFP+ and Girk2+/TH+ were quantified within the graft. The total volume of the transplant was obtained according to the following formula for two truncated cones: $V \text{ (mm}^3\text{)} = [(A1+A2)/2] * d$; where A= surface area (mm²) and d= distance between two sections (mm) as described in Péron et al., (2017).

Evaluation of striatal projections:

To quantify the striatal projections in grafted adults of WT and Sema7AKO mice, microscopic acquisitions were carried out on sagittal sections using mosaic acquisition ZEN software (Zeiss) with a Zeiss Axio Imager.M2 Apotome microscope at x20. ImageJ software v.1.46r (National Institutes of Health; Bethesda, MD) was used to determine the integrated density of TH staining in the striatum as described in Reyes-Corona et al., (2017) and Kells et al., (2010). All background intensity was eliminated from the immunohistochemical stained area to quantify only TH immunoreactivity.

More precisely, in adult striatum, the integrated density of TH+ fibers was determined in 9 sagittal sections/animal from 2.40 mm to 0.84 mm lateral from the midline. Dorsal and ventral

striatum were delineated according to Franklin and Paxinos (2008) as shown in the example of figure 32. A total of 6 WT and 7 Sema7AKO mice were analyzed.

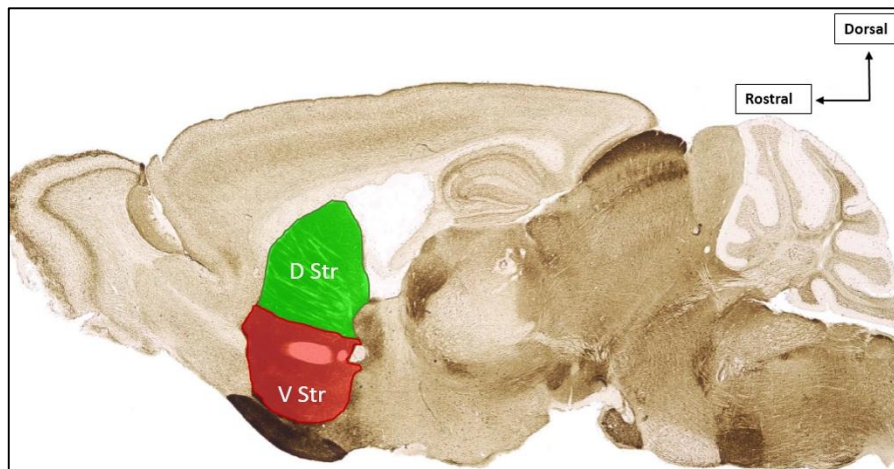


Figure 32: Sagittal section from the mouse brain atlas at 0.96 mm lateral from midline as an example for quantification. The dorsal striatum (*D Str*) and ventral striatum (*V Str*) delineated in green and red respectively (adapted from Franklin& Paxinos, 2008).

6. Statistical analysis

We used GraphPad Prism software (version 5.0d) for statistical analysis. Comparisons between two groups were analyzed using either Student t-test or Mann-Whitney test and Kruskal-Wallis was performed for multiple comparisons. Results were expressed as the mean \pm SEM. $P < 0.05$ values were accepted as statistically significant.

III. Results

1. First set of transplantations

1.1. Behavioral performance after lesion and transplantation

Apomorphine-induced rotational test was performed 3 weeks and 11 weeks after 6-OHDA lesion in the SN to assess the efficacy of the lesion in the first set of WT and Sema7AKO mice (Figure 33-A). Based on the rotation behavior of the lesioned mice in response to apomorphine challenge, a part of WT and Sema7AKO mice established asymmetric turning behavior 3 weeks after lesion (Figure 27-A) where the mean number of turns was 62.57 ± 31.73 and 34.25 ± 34.55 for WT and Sema7KO mice respectively. This asymmetric behavior persisted 11 weeks after the lesion in these mice (Figure 33-A) with a mean = 59.29 ± 38.55 for WT; and 36.88 ± 19.97 for Sema7AKO. There was no significant difference between 3 and 11 weeks after the lesion in WT ($p=0.8741$) nor in Sema7AKO mice ($p=0.7409$). Moreover, no differences were observed between the groups at the same stage after lesion ($p>0.999$). The representation of individual rotation behaviour showed a widespread distribution with differences in the degree of lesion between animals of the same group.

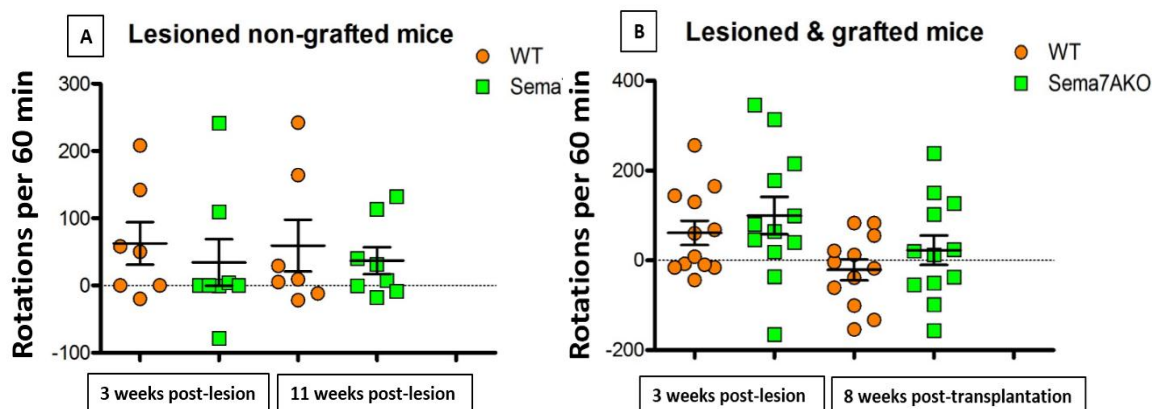


Figure 33: Asymmetric turning behavior in response to apomorphine injection following 6-OHDA lesion of the SNc (WT n=7; Sema7AKO, n=8) (A) and 8 weeks after transplantation (n=12 for all groups) (B). Mann-Whitney was used to compare before and after transplantation in the same group of animals WT or Sema7AKO mice.

In another separate set of animals (Figure 33-B), WT and Sema7AKO were challenged against apomorphine injections 3 weeks after lesion and 8 weeks after transplantation. 3 weeks post-lesion, the mean number of rotations displayed by both groups was 61.42 ± 26.97 for WT and 99.75 ± 41.75 for Sema7AKO although not all mice were lesioned. 8 weeks after transplantation, the mean rotations in WT mice decreased to -21.33 ± 22.88 and to 22.67 ± 32.79 in Sema7AKO mice (Figure 33-B). The difference of rotation scores before and after transplantation was not significant in WT with $p=0.0754$, nor in Sema7AKO mice with $p=0.16$. Moreover, no differences were observed between the groups at the same stage after lesion or transplantation ($p>0.999$). The representation of individual rotation behaviour showed a widespread distribution with differences in the degree of lesion and recovery after transplantation between animals.

1.2. The lack of Sema7A increases the number of grafted cells

3 weeks following SN lesion, WT and Sema7AKO mice were each intranigrally grafted by 150,000 cells/1.5 μ l of fetal VM cells collected from TH-EGFP E12.5 embryos. 8 weeks after transplantation the graft was present in coronal section at AP: -2.46 to -4.48 mm from Bregma. It was integrated within the host SN spreading dorsally in the mesencephalon as shown in a DAB treated coronal section showing GFP expression in Figure 34-A. GFP+ cells were mostly distributed at the periphery of the transplant (red arrows in Figure 34-B) and stereological analysis showed a significantly higher number of GFP+ cells in Sema7AKO mice (mean= 1915 ± 216.5) compared to the WT mice (mean= 973.4 ± 269.1) with $p=0.0068$ (Figure 34-C).

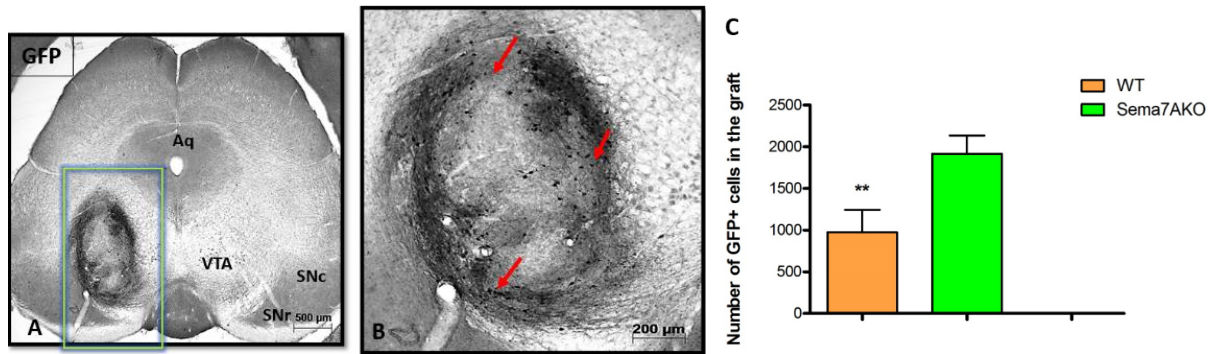


Figure 34: GFP+ cells within the transplant in SN. A: Representation of grafted GFP+ cells in a coronal section of Sema7AKO mouse (AP:-3.64 from bregma) 8 weeks after transplantation. B: Higher magnification of the transplant. Red arrows show GFP+ cells and fibers within the graft. C: Quantification of GFP+ cells in the graft in WT and Sema7AKO mice. Comparison between groups was performed using Mann–Whitney test (**p=0.0068). VTA: *ventral tegmental area*; SNr: *substantia nigra pars reticulata*; SNc: *substantia nigra pars compacta*; Aq: *aqueduct*.

We also compared the number of TH+ cells in the graft of WT and Sema7AKO 8 weeks post-transplantation (Figure 35-A).

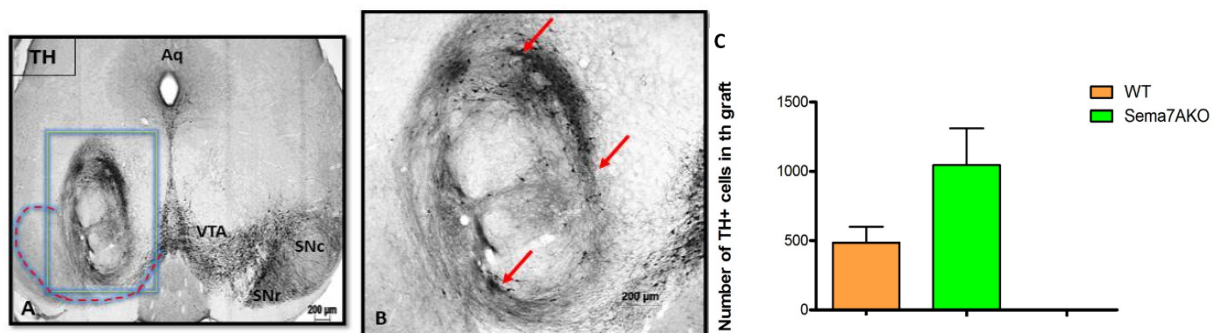


Figure 35: TH+ cells within the transplant in SN. A: Representation of TH+ cells in the graft in a coronal section of a Sema7AKO mouse (AP=-3.64 from bregma) 8 weeks after transplantation. B: Higher magnification of the transplant. Red arrows show TH+ cells and the fibers within the graft. C: Quantification of TH+ cells in the graft in WT and Sema7AKO mice. Comparison between groups was performed using Mann–Whitney test (p=0.155). VTA: *ventral tegmental area*; SNr: *substantia nigra pars reticulata*; SNc: *substantia nigra pars compacta*; Aq: *aqueduct*.

TH+ cells and few TH+ DA fibers were observed throughout the transplant and mostly at the periphery (indicated by red arrows in Figure 35-B). A higher number of TH+ cells was detected

in the grafts of Sema7AKO mice (mean=1045.08 \pm 265.8) in comparison to WT (mean=485.091 \pm 115.9) grafts although this difference was not significant (p= 0.155) (Figure 35-C).

Then, we examined the proportion of TH+ cells relative to GFP+ cells in both groups. Data showed similar percentage of TH+ cells relative to GFP+ cells in the graft of WT (mean=72.8 \pm 20.62) and Sema7AKO mice (mean=65.50 \pm 2.04) (Figure 36).

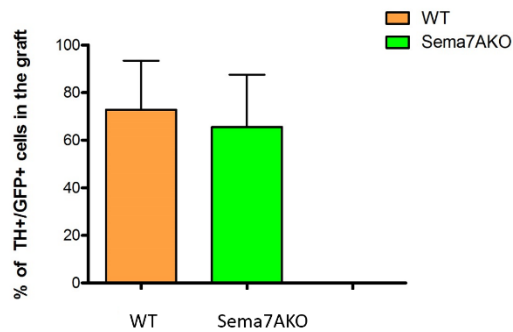


Figure 36: Representation of the TH+ cells percentage relative to GFP+ cells in the transplant of WT and Sema7AKO mice. Comparison between the groups was carried out using Mann–Whitney test (p=0.6922).

1.3. The grafted cells do not grow out of the transplant in WT nor in Sema7AKO animals

Every brain section of each animal, either WT or Sema7AKO, did not show any grafted fibers growing out of the transplant in the VM (Figure 37). No fibers were visible neither along the nigrostriatal pathway, in the MFB nor in the striatum.

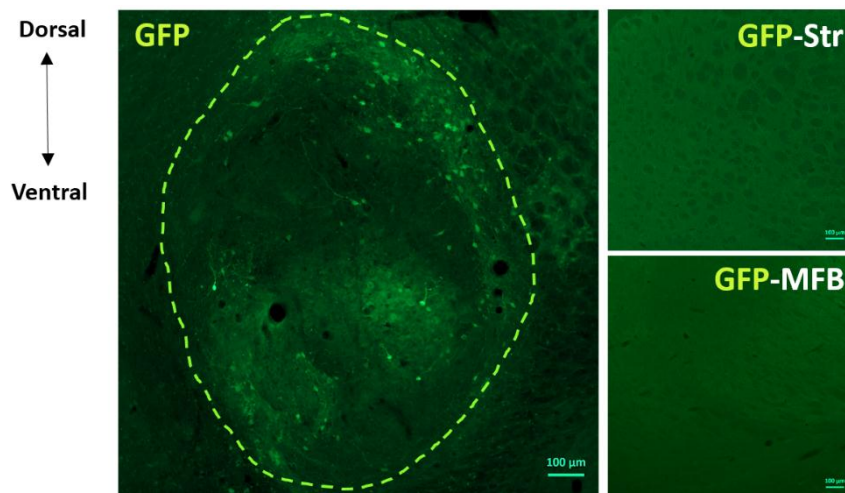


Figure 37: Coronal section (AP: -3.64 from Bregma) from TH-EGFP grafted Sema7AKO mouse showing GFP expressing cells in the graft. Absence of GFP expressing fibers in the MFB and striatum; graft is delaminated in green. *Str*: striatum; *MFB*: medial forebrain bundle.

2. Second set of transplantation

In this second set of transplantation, WT and Sema7AKO mice lesioned in the SN, received cells from 2 to 7 VMs from TH-EGFP or Actin-EGFP E12.5 embryos as indicated in the table below (Table 5).

	2 VMs	5 VMs	7 VMs
TH-EGFP grafted mice	3 WT + 3 Sema7AKO	-	1 WT + 1 Sema7AKO
Actin-EGFP grafted mice	-	2 WT + 2 Sema7AKO	1 WT + 2 Sema7AKO

Table 5: Quantity of TH-EGFP or Actin-EGFP VM transplanted in WT and Sema7AKO mice.

2.1. Behavioral performance after lesion and transplantation

The behavioral changes were examined in both the 6-OHDA-lesioned and transplanted animals by apomorphine induced rotation test 3 weeks after the lesion and 8 weeks following

transplantation of different amounts of Actin-EGFP or TH-EGFP VMs to examine the functional outcome of the implanted cells.

In WT mice, 3 weeks after lesion the mean number of turns was -2.11 ± 8.357 , and 8 weeks after transplantation, the net rotation scores decreased insignificantly to reach a mean of -9 ± 8.197 rotations per 60 minutes ($p=0.4224$) (Figure 38). The three most severely lesioned WT mice (circles 1, 2, 3) exhibited respectively 32, 17 and 15 turns per 60 minutes 3 weeks after lesion and -24, -29 and -17 turns per 60 minutes after transplantation. WT mice 1 and 2 were transplanted with 5 VMs and mouse 3 with 2 VMs. In the *Sema7AKO* group, the mean number of rotations insignificantly decreased from 29.35 ± 15.39 3 weeks after lesion to 9.375 ± 12.62 turns per 60 minutes 8 weeks after transplantation ($p=0.8148$). The two most severely lesioned *SEMA7AKO* mice (squares 1 and 2) exhibiting 131 and 67 turns per 60 minutes 3 weeks after lesion, were transplanted with 5 and 2 VMs respectively and exhibited 34 and 27 turns per 60 minutes 8 weeks after transplantation.

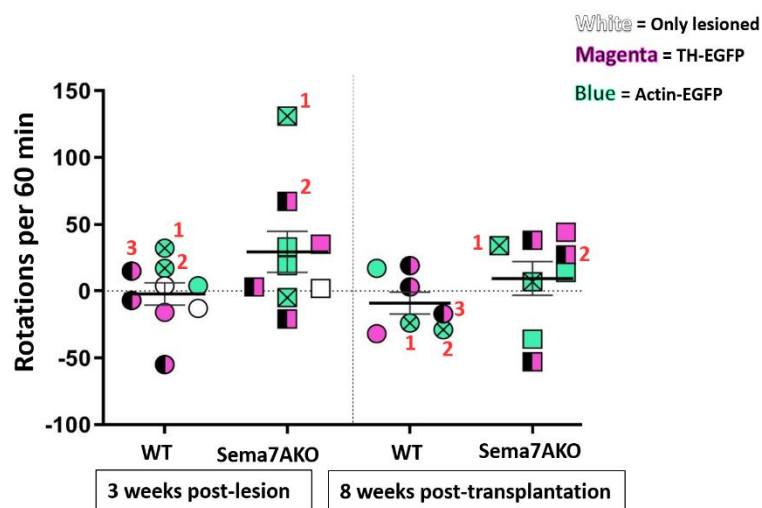


Figure 38: Asymmetric turning behavior in response to apomorphine injection 3 weeks following 6-OHDA lesion of the SNc (WT n=9; *Sema7AKO*, n=9) and 8 weeks after transplantation (WT n=7 and *Sema7AKO* n=8). Mann-Whitney test did not show any difference after and before transplantation in both WT and *Sema7AKO* groups (respectively $p=0.4224$ and $p=0.8148$). Animals represented in white are only lesioned; animals in blue are to be grafted with Actin-EGFP VMs; animals in magenta

are to be grafted with TH-EGFP VMs. Half colored legends: 2VM grafted; crossed legends: 5VM grafted; completely colored legends: 7VM grafted.

2.2. The lack of Sema7A increases the number of Actin-EGFP or TH-EGFP grafted cells

Mice were intranigrally transplanted with different quantities of TH-EGFP+ or Actin-EGFP+ VM cells. 8 weeks after transplantation grafted cells were visualized using GFP and TH (a marker for dopaminergic neurons) immunolabeling. The cells integrated within the VM of WT and Sema7AKO mice in a similar way and few of them were detected along the injection tract. Transplants were located in the SN and spread dorsally into the mesencephalon and were observed from 2.4 to 0.12 mm from the midline on sagittal sections as shown in Figure 39 for a WT mouse grafted with 2VMs of TH-EGFP embryos.

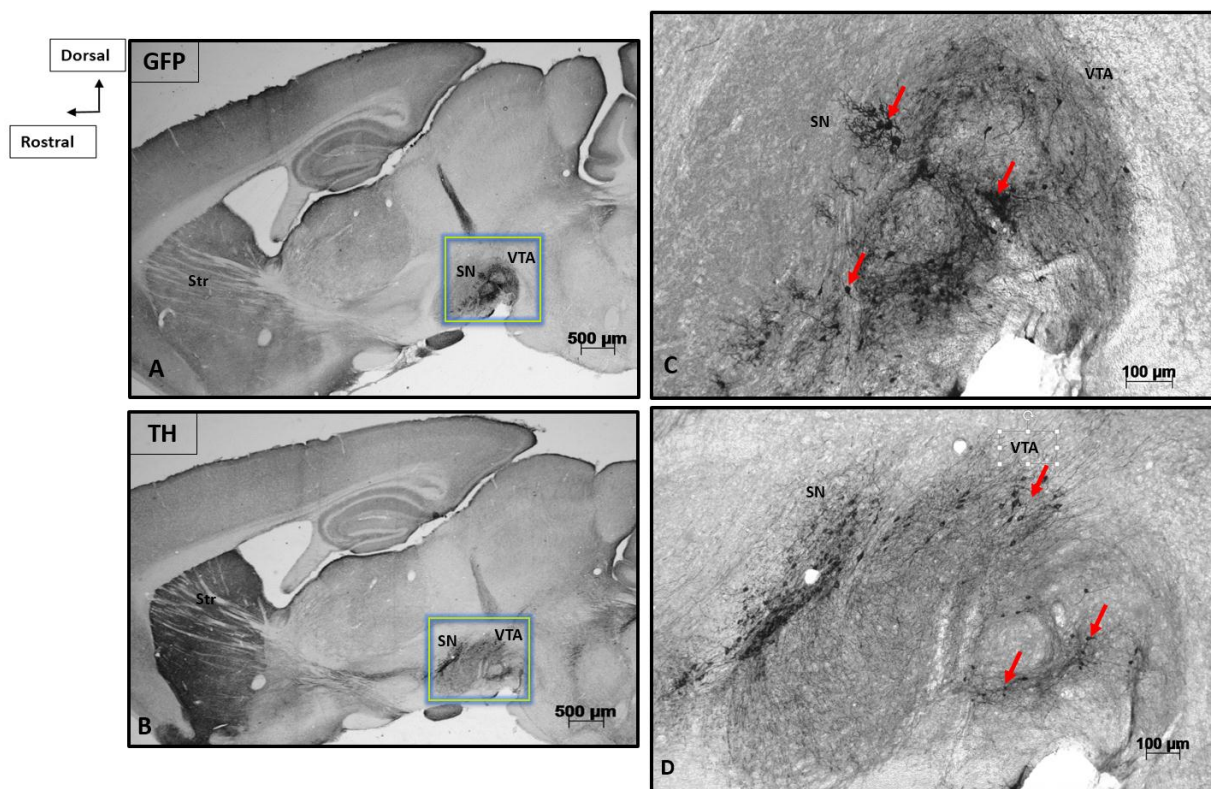


Figure 39: GFP+ (A) and TH+ (B) cells within the transplant in sagittal sections of a WT mouse brain (ML=1.56 mm) 8 weeks after transplantation of 2VMS from TH-GFP embryos. C, D: Higher magnification of transplants. Red arrows represent the GFP+ and TH+ cells and fibers within the graft. VTA: ventral tegmental area; SN: substantia nigra.

The grafted cell phenotype was examined using Girk2 (G protein coupled inward rectifying current potassium channel type 2) that is exclusively expressed by the A9 DA neurons of SNc subtype, and Calbindin (calcium binding protein) which is expressed by the majority of A10 DA cells of VTA subtype.

In Actin-EGFP grafted animals, GFP+ cells of the graft were immunolabeled by TH and Girk2 (Figure 40). TH + cells co-expressing Girk2 were located mostly at the periphery of the graft and had angular cell bodies.

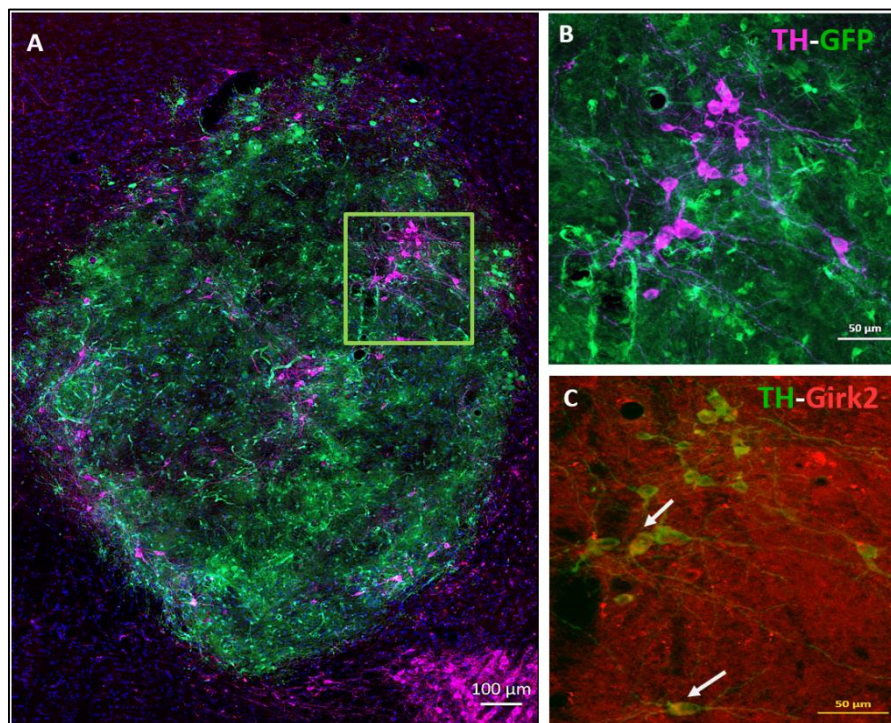


Figure 40: GFP+, TH+, Girk2+ cells within the transplant. Sagittal section at ML= 1.32 mm from midline in a WT mouse grafted with 5 Actin-EGFP VMs showing the graft in the VM (A). Section at higher magnification showing the GFP grafted cells co-labeled by TH (B) and Girk2 (C) as indicated by the arrows. *TH*: tyrosine hydroxylase.

The number of TH+ and Girk2+ cells tend to increase in Sema7AKO brains whatever the number of VM cells grafted. Indeed, TH+ cell number was higher (mean= 21228±5837) in the

transplant of *Sema7AKO* compared to WT mice (mean= 4330±633.1). Within the graft in *Sema7AKO* mice, 38-40% of the TH+ DA cells were *Girk2+* (mean=8241±2379) whereas in WT mice, 29-31% were positive for *Girk2* (mean=1316±198.4) (Figure 41).

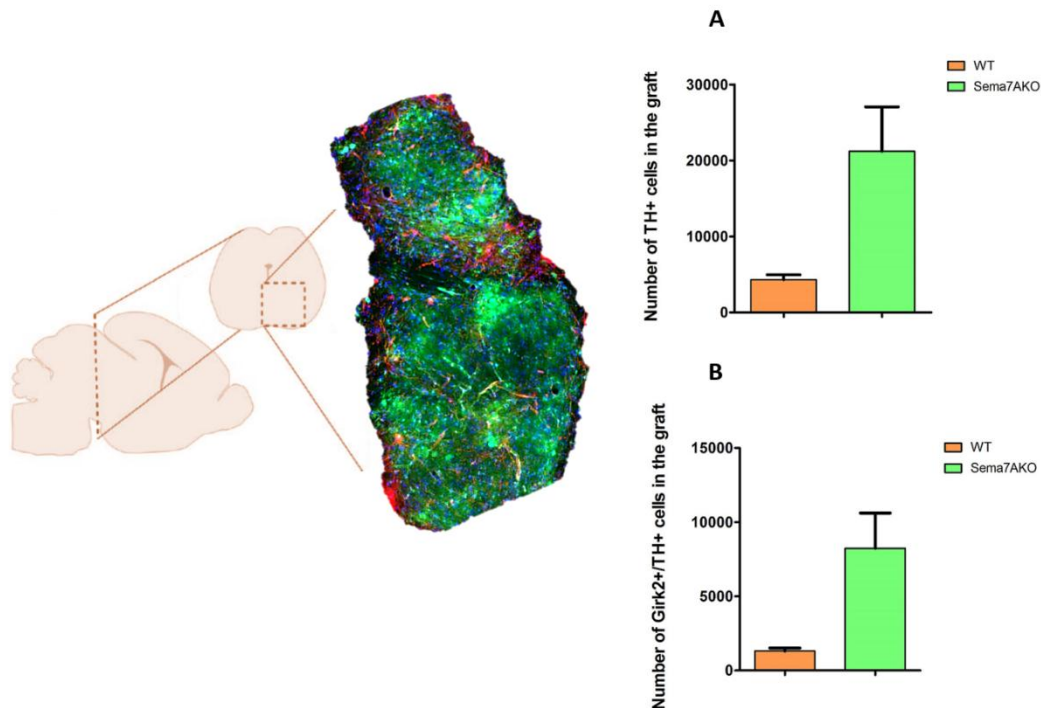


Figure 41: Quantification of TH+ and *Girk2+* and TH+ cells in Actin-EGFP grafted WT and *Sema7AKO* mice. Mean (\pm SEM) of TH+ (A) and *Girk2+* and TH+ (B) cells within the graft in sagittal and coronal sections; WT (n=3) and *Sema7AKO* mice (n=4). The cell quantification was extrapolated to the volume of the transplant as described in the methods and the difference was not statistically different (Student t-test: A: P=0.0587; B: P=0.0575).

Similarly, in TH-EGFP grafted mice, the grafted cells were immunolabeled by TH, *Girk2*, and Calbindin (Figure 42). Calbindin+/TH+ cells were round, small, and preferentially observed at the center in comparison to *Girk2+*/TH+ cells within the transplant.

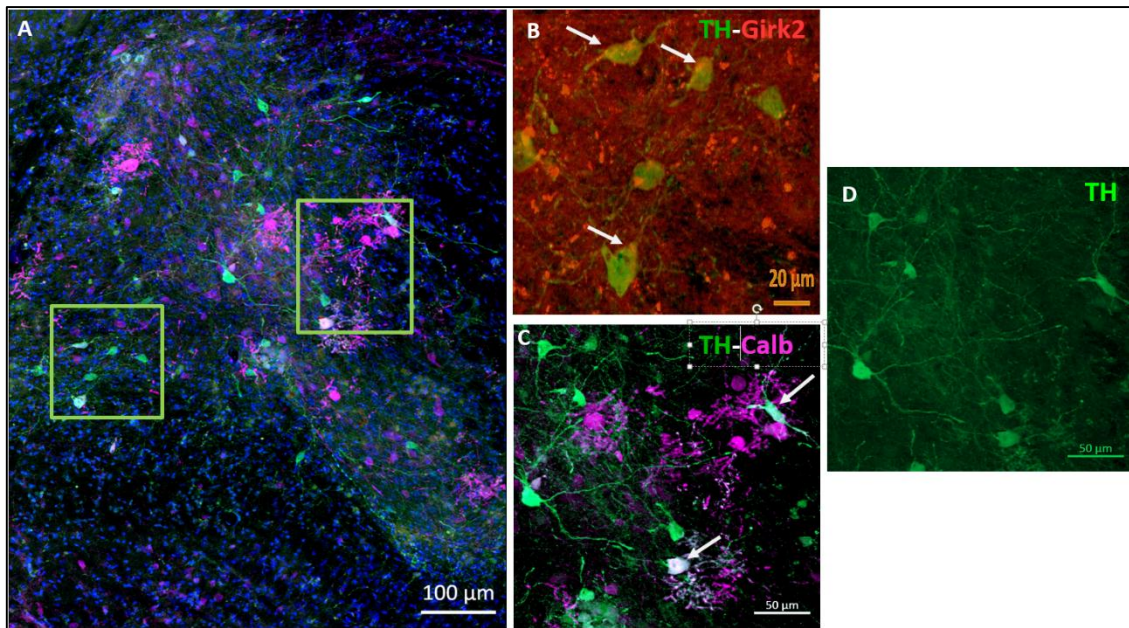


Figure 42: GFP+, TH+, Girk2+, and Calbindin+ cells within the transplant. Sagittal section at (ML= 0.36 mm from midline) from a WT mouse grafted with 2 TH-EGFP VMs showing the graft in the VM (A). Section at a higher magnification showing the grafted cells that are co-labeled by Girk2 (B), Calbindin (C), and TH (D) indicated by the arrows. *TH*: tyrosine hydroxylase; *Calb*: Calbindin.

In TH-EGFP transplanted mice the number of TH+ cells was significantly higher in *Sema7AKO* (mean= 30726±3773) in comparison to WT mice (mean= 15268±2190) whatever the number of VM cells grafted (Figure 43). The mean number of Girk2+ and TH+ cells within the graft also revealed same tendency although not significant and was 1426±459.6 in WT versus 2871±778.6 in *Sema7AKO* mice. In *Sema7AKO* the mean of Calbindin+ and TH+ cells was 7593±1750 compared to WT mice which was 4386±1212. In WT animals 7-10% of TH+ cells in the graft were Girk2+ and 24-32% were Calbindin+ whereas in *Sema7AKO* mice, 8-11% out of the counted TH+ cells were also Girk2+ and 22-27% were Calbindin+.

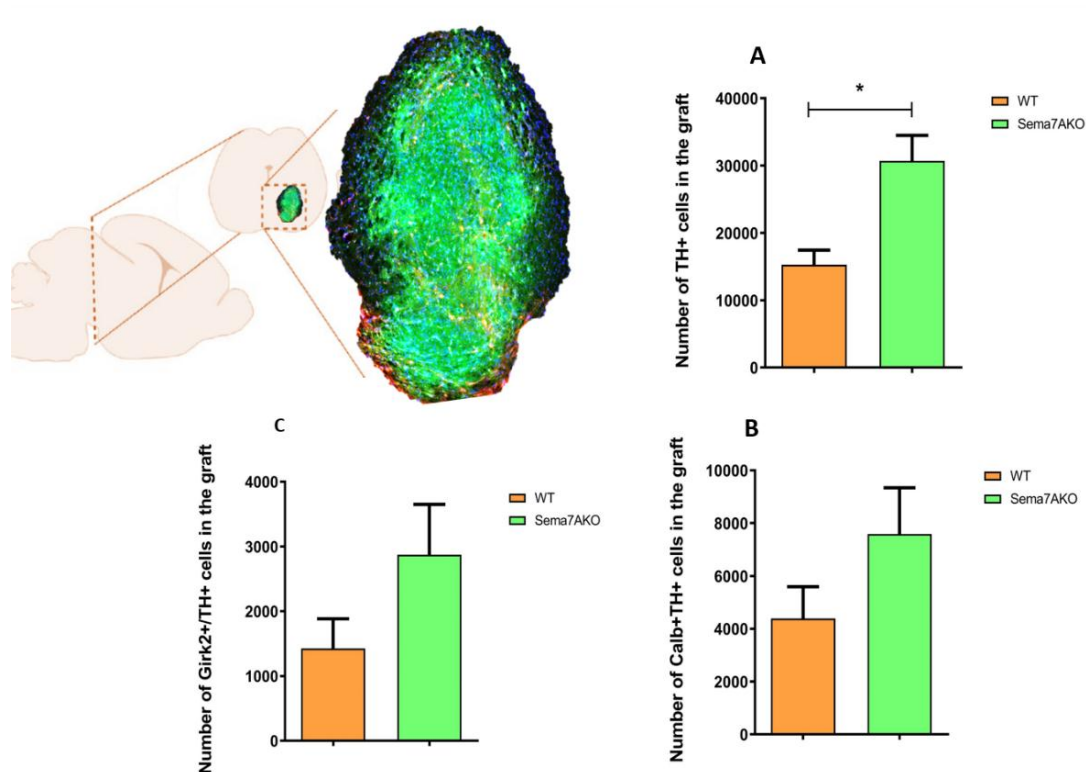


Figure 43: Quantification of TH+, Girk2+/TH+, and Calb+/TH+ cells in TH-EGFP grafted WT and Sema7AKO mice. Mean (\pm SEM) of TH+ (A), Girk2+ (B), and Calbindin+ (C) cells within the graft in sagittal sections of WT (n=3 in A, n=4 in B and C) and Sema7AKO mice (n=4 in all) grafted with TH-EGFP cells. The quantification of the cells was extrapolated to the volume of the transplant and statistical analysis was done using unpaired Student t-test for 2-groups comparisons (A: *P=0.0239; B: p=0.1610; C: p=0.1826).

2.3. In both WT and Sema7AKO animals, grafted GFP+ projections grow out of the transplant, follow the nigrostriatal pathway and reach the striatum

We examined the projections extending from the TH-EGFP or Actin-EGFP grafts in the WT and Sema7AKO mice along the nigrostriatal pathway towards the striatum.

In WT and Sema7AKO TH-GFP grafted mice, 8 weeks after transplantation, few GFP+ fibers were detected leaving the transplant (A), projecting along the MFB region (B), proceeding towards the globus pallidus (C) to innervate the striatum (D) as shown in Figure 44.

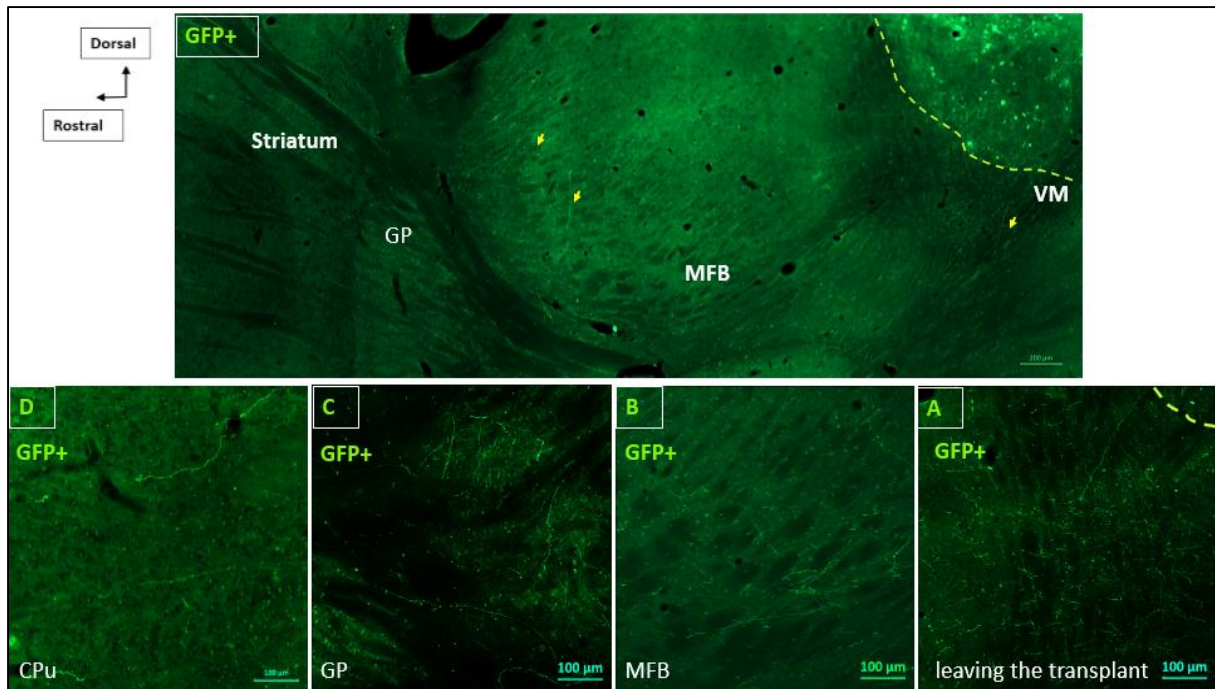


Figure 44: Sagittal section (ML: 0.96mm) from Sema7A KO mouse grafted with 7 TH-EGFP VMs. Immunolabeling with GFP shows few GFP+ fibers leaving the graft (A), through the MFB (B), reaching rostrally the GP(C), and entering the CPu (D). The arrows indicate the GFP+ fibers. *MFB: medial forebrain bundle; GP: globus pallidus; CPu: caudate putamen.*

In Actin-EGFP transplanted mice a higher density of GFP+TH+ fibers were extending outside the graft (A) and through MFB (B) in Actin-EGFP transplanted mice (Figure 45). Rostrally, GFP+TH+ fibers were able to project through the globus pallidus (C) to innervate the striatum (D) in larger clusters in comparison to the TH-EGFP grafted mice.

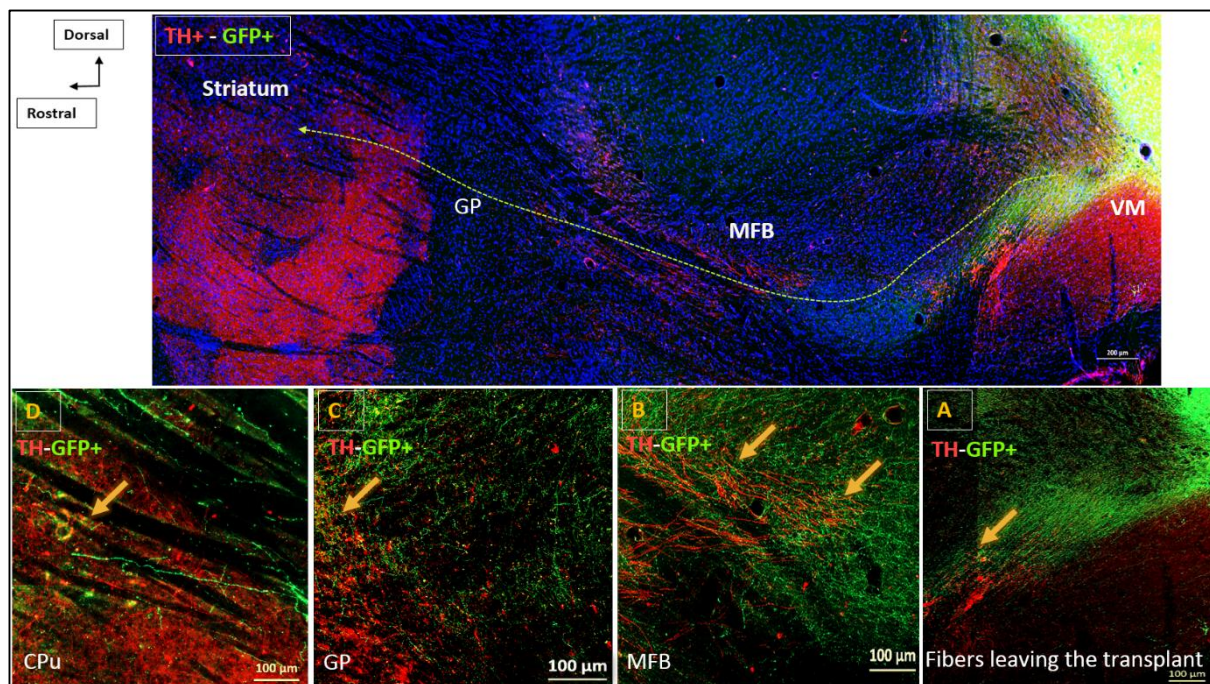


Figure 45: Sagittal section (ML: 0.96mm) from Sema7A KO mouse grafted with 7 Actin-EGFP fetal VMs. Immunolabeling with GFP shows dense GFP+ fibers (in green) projecting outside the graft (A), extending through the MFB (B), proceeding towards the GP (C), and innervating the CPu rostrally (D). These fibers were clearly detected in most of WT and *sema7A*KO mice which were transplanted with either 5 or 7 fetal VMs. TH+ fibers (in red) were also detected leaving the transplant (A), in the MFB (B), GP (C), and innervating the striatum (D). Yellow arrows indicate the GFP+ fibers co-labeled with TH. *MFB*: medial forebrain bundle; *GP*: globus pallidus; *CPu*: caudate putamen.

2.4. The lack of *Sema7A* does not modify the mDA striatal projections after transplantation

To investigate the influence of *Sema7A* on the presence of grafted TH+ fibers in the striatum, we examined the TH labeling within the striatum in the Actin-EGFP and TH-EGFP grafted mice. We measured the integrated density of TH labeling either in the entire striatum or in the ventral and dorsal parts at lateral (L), mediolateral (ML) or medial (M) levels of the striatum after TH immunolabeling in Actin-EGFP grafted mice and GFP immunolabeling in TH-EGFP grafted mice.

In Actin-EGFP grafted animals, striatal TH labeling showed no significant difference between WT (mean=220871) and *Sema7A*KO (mean=268754) grafted mice (Figure 46-A). There was

no significant difference in the expression of TH in the dorsal or ventral compartments of the striatum between the two groups (Figure 46-B) whatever their mediolateral position (Figure 46-C).

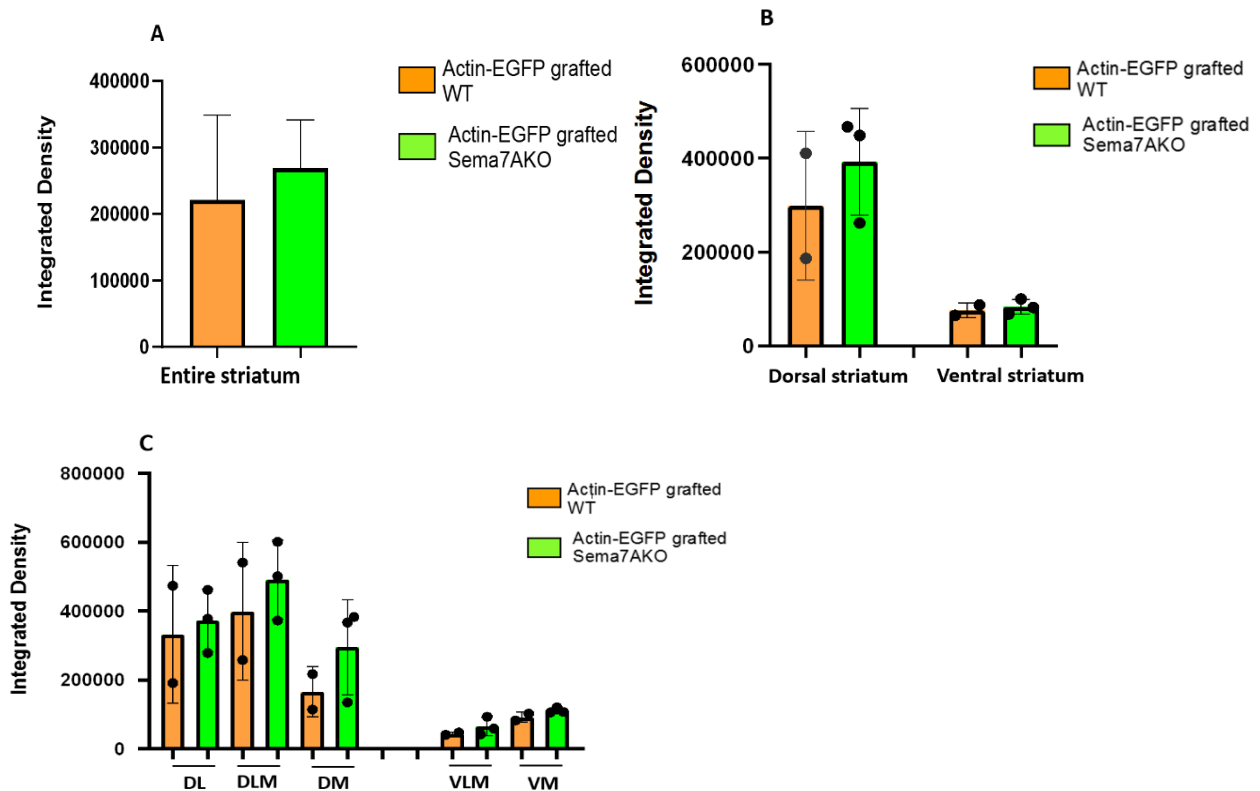


Figure 46: Quantification of striatal projections in Actin-EGFP grafted WT and Sema7AKO mice. Quantification of TH labeling in the striatum (A), in the dorsal and ventral striatal compartments (B) and in the medial and lateral parts of the dorsal and ventral striatum (C). Mann-Whitney test did not show any significant difference: A: $p=0.8$; B: dorsal striatum: $p=0.4$, ventral striatum: $p=0.8$; C: $p>0.99$). *DL*: dorsolateral; *DLM*: dorsolateromedial; *DM*: dorsomedial; *VLM*: ventrolateromedial; *VM*: ventromedial.

Similarly in TH-EGFP grafted mice, there was no significant difference between GFP labeling in the WT and Sema7AKO striatum (Figure 47-A) neither in the dorsal or ventral parts (Figure 47-B) nor at different mediolateral locations (Figure 47-C).

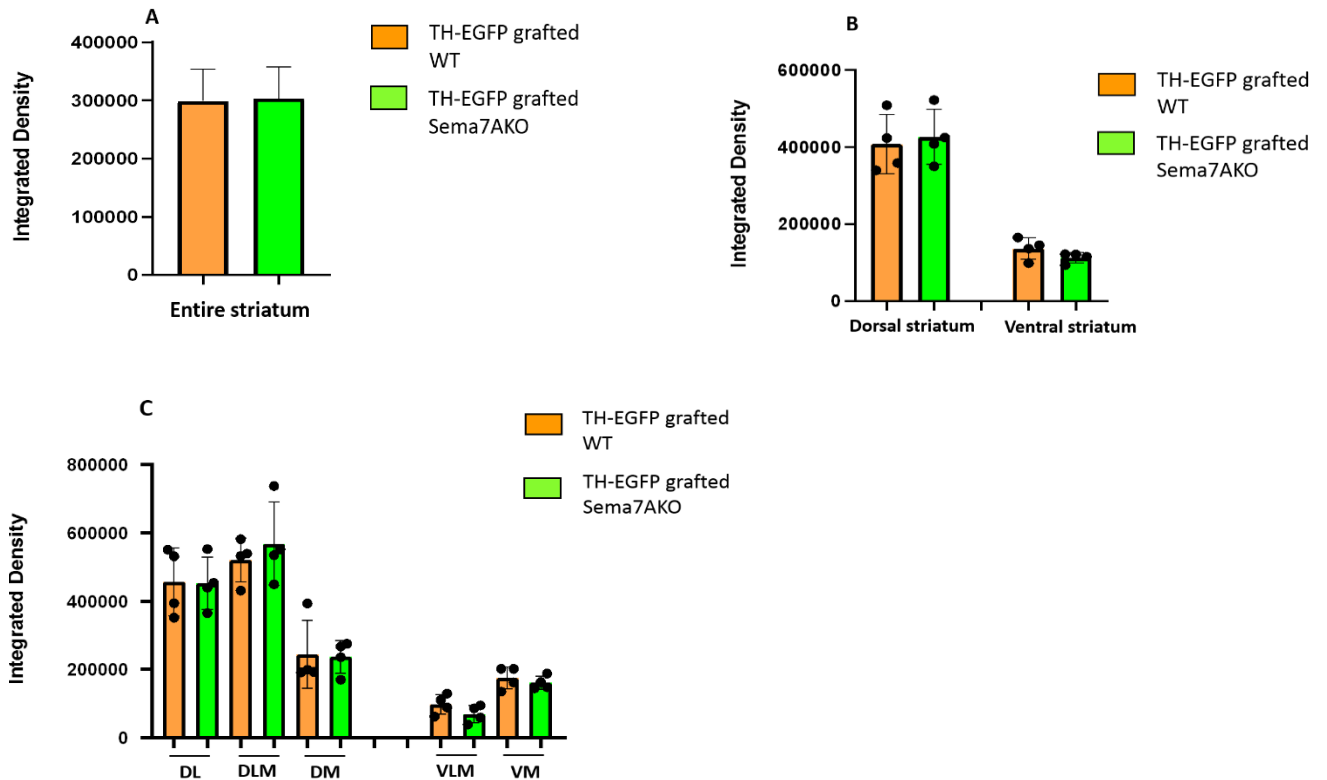


Figure 47: Quantification of striatal projections in TH-EGFP grafted WT and Sema7AKO mice. Quantification of GFP labeling in the entire striatum (A), in the dorsal and ventral striatal compartments (B) and in the medial and lateral parts of the striatum (C). *Mann-Whitney test did not show any significant difference: A: $p=0.8$; B: dorsal striatum: $p>0.99$, ventral striatum: $p>0.99$; C: $p>0.99$). DL: dorsolateral; DLM: dorsolateromedial; DM: dorsomedial; VLM: ventrolateromedial; VM: ventromedial.*

IV. Discussion and prospects

We conducted here a pilot study to investigate the role of Sema7A in the reconstruction of the mesostriatal pathway following fetal cell transplantation in a PD mouse model. We used VMs from transgenic Actin-EGFP or TH-EGFP embryos to graft in adult SN of WT and Sema7AKO animals. These preliminary data show that the absence of Sema7A does not affect the reconstruction of the mesostriatal pathway after cell therapy. Nevertheless and interestingly our

neuroanatomical study revealed a higher number of grafted TH⁺ cells in the transplant in absence of Sema7A, suggesting a deleterious effect of Sema7A on these cells.

Behavioral evaluation of the degree of lesion before transplantation and of a possible repair induced by grafted cells was carried out using the rotacount device after apomorphine injection. We observed that the presence of grafted cells similarly reduced the number of contralateral rotations after apomorphine injection in lesioned WT and in Sema7AKO animals. We did not show any statistical differences between groups possibly because of the important interindividual discrepancies. Indeed, 3 weeks after lesion some mice exhibited a strong rotation behavior whereas others did not. This could be explained by the fact that first, intranigral 6-OHDA lesions show high variability in mice in a way that under the same experimental conditions, only some of the injected mice are well lesioned (Grealish et al., 2010). Second, apomorphine upregulates the hypersensitive D1 and D2 receptors on the lesioned side when at least 90% of the DA connections are damaged (Iancu et al., 2005). Therefore, the severity of the lesion in some animals probably did not lead to 90% of dopaminergic loss. Moreover, in a previous study, Iancu et al. (2005) examined several behavioral tests to assess motor impairments following unilateral 6-OHDA lesion in mice. According to their investigation, less than 1% of functional deficits were uniquely predicted by apomorphine rotation test. Remarkably in the same study, in mice groups with more than 70 % of DA cell loss, some mice responded to apomorphine injection by rotating vigorously whereas other mice did not rotate. This case was also reported in a study conducted in 1993 by Hudson et al. where stereotypic abnormal behaviors were observed and suggested to be the reason behind the difference in responses to apomorphine (Hudson et al., 1993). Remarkably, amphetamine is another dopamine agonist that induces the intact side of the brain and triggers ipsilateral rotation in lesioned mice (Torres & Dunnett, 2011). The use of amphetamine may be more reliable to detect nigral cell loss because it showed a stronger correlation with DA cell loss in the SN in

comparison to apomorphine test in 6-OHDA lesioned mice (Iancu et al., 2005). Therefore, the use of amphetamine could be preferable for assessment of lesion and repair in the transplanted mice. In addition to amphetamine-induced rotation test, we may consider also using spontaneous motor tests such as the cylinder test that is based on examining the forelimb asymmetry in lesioned animals (Grealish et al., 2010). Similarly, the rotarod test which is usually used to evaluate neurological abnormalities following brain injuries or even in drug-based treatments can also be used to assess 6-OHDA lesions in rodents (Iancu et al., 2005; Lane et al., 2011).

Our neuroanatomical study confirmed the presence of two different populations of DA cells in the transplant: the DA neurons of SN and VTA subtypes which were specifically distributed in the periphery and near the center of the graft respectively. These findings were in accordance to previous studies done by Thompson et al. (2009) and Gaillard et al. (2009). Moreover, we observed that grafted DA neurons were mostly distributed in the outer limits of the transplant which could be explained by possible selective DA cell death at the center as described by several previous investigations (Emgard et al., 2003; Emgard et al., 1999). Interestingly, in absence of *Sema7A* and whatever the number or the type of cells used for transplantation (TH-GFP or Actin-GFP fetal cells), we observed a significant increased number of GFP⁺ and TH⁺ cells in the transplant that could also lead to an increase of Girk2⁺ and Calbindin⁺ cells. This means that this increase is not phenotype-specific and suggests that *Sema7A* may have a deleterious effect on these cells. In fact, a major obstacle in neural transplantation approaches is the severe loss of neurons in grafts soon after implantation due to adverse mechanisms triggered before or after transplantation. Several postmortem studies reported the presence of proinflammatory markers around the grafts in immunosuppressed patients with PD (Li et al., 2008; Ivar Mendez et al., 2005). Moreover, in experimental animal models of PD the majority of nigral grafted cells did not survive during the first week of transplantation (Emgard et al.,

2003). Indeed, due to presence of pro-inflammatory cytokines as well as toxic molecules triggered by the host environment surrounding the graft, transplanted DA neurons become more vulnerable for degeneration (Emgard et al., 2003). On another matter, cytotoxicity of 6-OHDA may be correlated to several inflammatory events as it has been reported to induce microglial activation (Marinova-Mutafchieva et al., 2009) as well as astrocytosis following injection (Rodrigues et al., 2001; Wachter et al., 2010). In our study, following transplantation survival of the grafted cells may possibly be influenced by proinflammatory responses modulated by Sema7A. Indeed, Sema7A has been described in many studies as a modulator of inflammatory process and as a player in innate and adaptive immunity. Sema7A that activates monocytes in vitro may stimulates the production of several proinflammatory molecules including IL-1, TNF- α , and IL-6 (Holmes et al., 2002). It is expressed by activated T lymphocytes (Mine et al., 2000) and to a less extent by B lymphocytes (Gan et al., 2011). Macrophages that express β 1-integrin, are activated by the Sema7A-expressing T cells. Therefore, Sema7A induces chemotaxis of macrophages, triggers production of inflammatory cytokines, and modulates T cell functioning. Moreover, Sema7A is also implicated in promoting neuroinflammation in some CNS disorders such as in spinal cord injury and multiple sclerosis (Gutiérrez-Franco et al., 2017; Kopp et al., 2010). Thus, in our experimental model, Sema7A may trigger inflammatory events which could negatively impact the survival of the grafted cells. To test this hypothesis, we aim to investigate neuroinflammation after 6-OHDA lesion and cell transplantation in absence of Sema7A. More precisely, we could check for pro-inflammatory markers expression and assess neuroinflammation by testing the expression of TNF- α inflammatory cytokine, GFAP as indicator of astrocytosis, and IL-6 and CD86 as markers for the activated M1 macrophages to consider microglial activation. If Sema7A is effectively involved in the neuroinflammatory processes following transplantation, it could be targeted in the aim to increase the grafted cell survival. Nevertheless, one have to be aware that to

neutralize *Sema7A* could be a double-edge sword since it has recently been shown that *Sema7A* could modify the arborization of mDA neurons thus decreasing their vulnerability (Pacelli et al., 2015).

Finally, despite of a highest number of grafted cells in absence of *Sema7A*, we did not detect a significant difference of connection of grafted fibers on the striatum. It can be hypothesized that either these cells do not extend their axon towards the striatum or that we were not technically able to detect the difference of fibers density in absence or presence of *Sema7A* on the striatum. This latter case is the most plausible given the higher density of fibers observed in the dorsal compartments of the striatum in *Sema7AKO* compared to WT mice, although this was not significant probably because of the low number of individuals. Indeed, this is in accordance with the study of Chabrat et al. (2017), showing a possible alteration of the route of the mDA projections in *Sema7AKO* mice. In absence of *Sema7A* which is normally highly expressed in the dorsal striatum in comparison to the ventral part (as showed in Chapter I Part I), mDA fibers could be misrouted towards the dorsal part of the striatum because of the lack of *Sema7A* repulsive influence. This is then more likely that in our model, in absence of *Sema7A* mDA fibers extend axons towards the striatum but may be misrouted. To refine our study, we need first to increase the number of individuals and second, to use a stereological quantification method that consists in evaluating the length of the fibers, which is correlated to the fibers' density (reviewed in Mark J West, 2018). This method could allow us to detect finer differences of striatal connections contrary to the ImageJ integrated density calculation we used.

Part III

The Proteome associated with cell therapy in a mouse
model of Parkinson's disease

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Abstract

Advances in large scale analysis is becoming very useful in understanding health and disease. In this study, a mouse model of Parkinson disease (PD) was used in which C57BL/6 mice were lesioned with an injection of 6-hydroxydopamine (6-OHDA) in the substantia nigra (SN) to specifically kill dopaminergic cells. Mice were then transplanted with ventral mesencephalon (VM) of E12.5 embryos and protein were extracted from 5 nigrostriatal-related brain regions 1- or 7-days post-transplantation. Proteins were then subjected to high throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify differentially expressed proteins between various regions and time-points of transplanted mice. Differentially expressed proteins were identified on which advanced systems biology and enrichment analyses were performed. A total of 57 proteins were regulated after transplantation in which 5 proteins were especially down-regulated after grafting in comparison to non-transplanted cases. Interestingly, these proteins are known to be involved in the inhibition of neurotransmission via the activation of endocytic pathways. This suggests that 7 days after transplantation when the first grafted axon reach their targets, DA neurotransmission may be promoted through a decrease of DA and D2 DA receptors endocytosis.

Key words: Parkinson disease, Proteome, cell therapy, mesostriatal pathway

I. Introduction

Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by the degeneration of the nigrostriatal system, affecting 10 million people worldwide (Delenclos et al., 2016). The major neuropathological features of PD are the loss of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc) and deposition of α -synuclein aggregates, the Lewy bodies, within the cytoplasm of DA neurons (Kalia and Lang., 2015). Several studies have elucidated some underlying mechanisms associated with the onset and progression of PD, which include four major events: α -synuclein protein accumulation, mitochondrial deficits, oxidative stress, and impairments in the intracellular trafficking (Poewe et al., 2017). Toxic forms of α synuclein leads to their aggregation into insoluble fibrils constituting the Lewy pathology (Kim et al., 2008), interrupting several proteasomal and lysosomal degradation pathways including macro-autophagy, chaperon-mediated autophagy and ubiquitin-proteasome systems (Xilouri et al., 2009; Emmanouilidou et al., 2010; Tanik et al., 2013). In addition, Lewy bodies increase oxidative stress and inflammatory pathways leading to neuronal cell death (Poewe et al., 2017). Moreover, mitochondrial defects are associated with the pathogenesis of PD (Franco-Iborra et al., 2016, Schapira, et al., 2007; Bose et al., 2016). For instance, the mitochondrial serine-threonine protein kinase PINK-1, parkin (E3 ubiquitin ligase), and the deglycase and chaperone protein DJ-1, are implicated in the degradation of impaired mitochondria to maintain cell survival through mitophagy. Mutations in these genes are highly associated to mitochondrial dysfunction in PD (Pickrell et al., 2015). Consequently, oxidative damage occurs in the nigral DA neurons of PD patients (Jenner, 2003) leading to prominent deficits in the autophagy lysosomal pathways (Dias et al., 2013). Several transcriptional studies were performed to identify the molecular pathways implicated in PD initiation and development. However, differential expression at the RNA level may not reflect the true functional aspect defined by the proteins within a cell. Recently, the

development of new proteomics tools, such as high throughput mass-spectrometry, is improving our understanding of diseases by accelerating the discovery of new candidate targets and revealing the functional pathways within a tissue or a disease. Several proteomic approaches were undertaken in animal and cellular models of PD to better examine the proteomic changes linked to this disease (Kasap et al., 2017). Indeed, various proteins associated with changes in SN were examined in PD patients which detected some alterations in proteins modulating iron and redox mechanisms (Werner et al., 2008). Moreover, other proteomic studies identified modulation in the expression of several proteins including calmodulin, cytochrome C, and cytochrome C oxidase in brain samples of hemiparkinsonian rats (Pierson et al., 2004). Another proteomic study reported a differential expression of beta-actin and alpha enolase proteins in the SNpc of 6-OHDA lesioned mice (De luliis et al., 2005). Regardless of advances in the understanding of PD pathology, it remains challenging to extrapolate this progress into therapeutic strategies. Innovative approaches are still needed to establish effective treatments for PD patients. Proteomics studies conducted in PD may offer a new strategy to better identify novel diagnostic biomarkers as well as therapeutic approaches affiliated with the development and progression of the disease. Previous studies, including ours (Thompson et al., 2009; Gaillard et al., 2009) showed that grafting of fetal VM cells in the lesioned adult mouse SN leads to anatomical and functional recovery of the nigrostriatal circuitry. Indeed, axonal projections of the grafted DA fetal cells navigate through intermediate targets in the diencephalon to innervate the striatum in the telencephalon, therefore regenerating the damaged DA network. This supports the concept that the host brain tissue is permissive to long axonal navigation and implies that certain molecules residing in the regions surrounding the nigrostriatal pathway, orchestrate the axonal steering of the grafted mDA neurons to ensure proper DA connectivity and enhance motor performance after transplantation in the host brain (Gaillard et al., 2009; Jaber et al., 2013).

In this study, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to reveal the proteome of a mouse model of PD after fetal cell therapy. C57BL/6 mice were first lesioned unilaterally with an injection of 6-hydroxydopamine (6-OHDA) in the substantia nigra (SN) to specifically kill DA cells. Mice were then transplanted with ventral mesencephalon (VM) of E12.5 embryos and 5 nigrostriatal-related brain regions were collected 1- or 7-days post-transplantation. Our results showed that differentially expressed proteins were involved in pathways implicated in local inflammatory reactions, oxidative stress and mitochondrial metabolic pathways. In addition, we observed a down regulation of genes involved in the neurotransmission of DA synapses.

II. Materials and methods

1. Animals

All animal experimental procedures were performed in agreement with the guidelines of the French Agriculture and Forestry Ministry (Decree 87849) and approved by the European Communities Council Directive (86/609/EEC). All procedures were approved by the local ethical committee and all efforts were made to reduce the number of animals used and their suffering.

Adults (4 to 6 months old) C57BL/6 mice were used as well as ventral mesencephalon (VM) of E12.5 embryos from C57/Bl6 transgenic mice overexpressing enhanced green fluorescent protein (EGFP) under the control of the tyrosine-hydroxylase promoter (C57BL/6JRj TH-EGFP). VMs of embryos were used as a source of cells for transplantation as described in Gaillard et al. (2009).

2. Lesion with 6-OHDA and transplantation

C57BL/6 wild type mice (n=9) were lesioned unilaterally with an injection of 6-hydroxydopamine (6-OHDA) in the SN to specifically kill dopaminergic cells as described previously (Gaillard et al., 2009), with slight modifications. Briefly, mice were first anesthetized by an intraperitoneal injection of Ketamine/Xylazine (100mg/kg and 10mg/kg respectively). They were then injected, using a Hamilton syringe, with a 1 μ l 6-OHDA (8 μ g/ μ l, Sigma) dissolved in sterile saline solution containing 0.1% ascorbic acid. The injection was performed into the left SN pars compacta at the following coordinates AP: -3.2 mm (from bregma), ML=1.4 mm (from midline), DL=3.8 mm (from dura). One week following the lesion, VMs from TH-EGFP E12.5 transgenic embryos were dissociated in 0.6% glucose saline solution, and a cell suspension of 1.5 μ l containing 150,000 cells was injected into the lesioned SN mice. The sham grafted groups received only a vehicle solution.

3. Tissue processing and sampling

Animals were sacrificed by cervical dislocation 1 day (LSNTD1, n=3) or 7 days (LSNTD7, n=3) after transplantation. Sham transplanted mice were sacrificed 7 days after the surgery (Sham, n=3). The brains were instantly collected and frozen on dry ice and cut into 200 μ m sagittal sections using a cryostat. For each animal, 5 brain regions were collected: the caudate putamen (CPU), the nucleus accumbens (Nacc), the medial forebrain bundle (MFB), the thalamus (Thal), and the ventral mesencephalon (VM). Regions were collected using a 1mm diameter puncher for the CPU, Nacc, MFB and Thal while a 2 mm-diameter puncher was used for the VM region, as illustrated in **Figure 1**.

4. Protein Extraction and Digestion

Tissue samples were washed with 50mM ABC buffer. Beads beater (Beadbug microtube homogenizer, Benchmark Scientific, Edison, NJ) was applied to perform cell lysis. Tissues were mixed with 5% sodium deoxycholate (SDC) (Sigma-Aldrich, St. Louis, MO) solution and 3 mm zirconium beads (OPS Diagnostics, Lebanon, NJ) in a 2 mL microtube. The solution of 5% SDC was added for efficient protein extraction. The samples were homogenized at 4 °C at 4,000 rpm for 30 seconds, followed by a 30 second-pause. This step was repeated for 6 times. Next, tissue lysate was sonicated for 30 minutes in 0 °C ice-water bath to enhance the protein dissolving. After centrifuging at 21,100 g for 10 minutes, the supernatant was collected and diluted 10 times by adding 50 mM ammonium bicarbonate (ABC) buffer. Protein concentration was determined by BCA protein assay kit (Thermo Scientific/Pierce, Rockford, IL) following the manufacture's instruction.

A 25- μ g aliquot of extracted proteins of each sample were then subjected to reduction, alkylation and tryptic digestion. ABC (50 mM)-SDC (0.5%) solution was first added to samples to maintain a volume of 50 μ L. Proteins were thermally denatured at 80 °C for 10 minutes. The reduction of proteins was performed by adding a 1.25- μ L aliquot of 200 mM dithiothreitol (DTT) solution and incubating at 60 °C for 45 minutes. The reduced proteins were then alkylated by adding a 5- μ L aliquot of 200 mM iodoacetamide (IAA) solution and incubation at 37 °C in the dark for 45 minutes. To quench the excessive IAA, a 1.25- μ L aliquot of DTT solution was added again and samples were incubated at 37 °C for 30 minutes. Following the reduction and alkylation of proteins, trypsin (Promega, Madison, WI) was added at a ratio of 1:25 (enzyme: proteins, w/w) into samples and incubated at 37 °C for 18 hours. After incubation, formic acid was added at a final concentration of 0.5% (v/v) for the purposes of both quenching the enzymatic reaction and removing the SDC detergent. Samples were then mixed thoroughly and centrifuged at 21,100 g for 10 min. The supernatant was collected, speed-

vac dried and resuspended in aqueous solution containing 2% acetonitrile (ACN) and 0.1% formic acid (FA) prior to LC-MS/MS analysis.

5. LC-MS/MS measurement

Aliquots of 1 μg tryptic digests were subjected to the untargeted proteomic analysis. A Dionex 3000 Ultimate nano-LC (Dionex, Sunnyvale, CA) interfaced to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA) that is equipped with ESI source was used for the analysis. Tryptic digests were first loaded to an Acclaim PepMap100 C18 guard column (3 μm , 100 \AA , Dionex) at a flow rate of 3 $\mu\text{L}/\text{min}$ for on-line desalting. Next, the separation of peptides was achieved using an Acclaim PepMap100 C18 capillary column (75 μm id \times 150 mm, 2 μm , 100 \AA , Dionex) at 0.35 $\mu\text{L}/\text{min}$ in 120 min. The mobile phase A contained 2% ACN, 0.1% FA and 97.9% water, while mobile phase B contained 0.1% formic acid in ACN. The LC gradient was as follow: Solvent B was kept at 5% for the first 10 min, increased from 5% to 20% over 55 min, 20-30% over 25 min, 30-50% over 20 min, 50%-80% over 1 min, kept at 80% for 4 min, decreased from 80% to 5% over 1 min and finally maintained at 5% over 4 min. The LTQ Orbitrap Velos was used in data dependent acquisition mode. The scan events were set as a full MS scan of m/z 400-2000 at a mass resolution of 15000, followed by CID MS/MS scan repeated on the 10 most intense ions selected from the previous full MS scan with an isolation window of m/z 3.0. The normalized collision energy was set to 35% with an activation Q value of 0.25 and activation time of 10 ms. The dynamic exclusion was enabled with repeat count of 2, repeat duration of 30 seconds and exclusion duration of 90 seconds.

6. LC-MS/MS Data Analysis

The raw data obtained from LC-MS/MS analysis were processed with the MaxQuant software version 1.6.3.4. Database search was performed against UniProtKB/Swiss-Prot mouse

database. The search included cysteine carbamidomethylation as a fixed modification and variable modifications, including methionine oxidation and acetylation of protein N-terminal. Trypsin was specified as the proteolysis enzyme and a maximum of 2 missed cleavages were allowed. For identification, the peptide precursor mass tolerance was 20 pm in the first search and 4.5 pm in the main search. As for fragments matching, a deviation of 0.5 Da was allowed. Only peptides with a minimum length of 7 amino acids were considered for identification. The false discovery rate (FDR) was set to be 0.01 at both peptide and protein levels. The minimum ratio count was set as two to determine the intensities of proteins for label-free quantification (LFQ). Both unique and razor peptides were considered for quantification.

7. Bioinformatics, Subnetwork Enrichment Pathway Analyses and statistical testing

Pairwise comparisons were performed using student t-test. Principle component analysis (PCA) and hierarchical clustering were applied on samples based on their scaled expression level using the R/Bioconductor package ade4 package (Thioulouse *et al.*, 2007). Gene Set Enrichment Analysis (GSEA) was performed using default parameters (Subramanian *et al.*, 2005). Enrichment analysis on gene ontology biological processes and KEGG pathways was conducted in R environment using BioConductor's clusterProfiler package (Yu *et al.*, 2012). Protein-protein interaction network was created using STRING-db web tool (Szklarczyk *et al.*, 2015).

III. Results

1. Pathways contributing to variance among conditions

Principal component analysis (PCA) was performed based on the expression level of all proteins in the dataset to extract proteins contributing to variance among samples. In fact, most

of the variance between samples could be attributed to differences in tissue of origin (Axis 1), followed by time of transplantation (Axis 2) (**Figure 2A**). The contribution of proteins to the variance in axis 1 and 2 among samples was extracted and used for GSEA analysis to extract top pathways contributing among tissues and duration of transplantation. Indeed, the top 30 proteins contributing to the variance among samples were determined (**Figure 2B**). Interestingly, among top pathways involved in tissue-specific response to transplantation were those related to mitochondrial metabolism, protein folding, oxidative phosphorylation, Huntington disease and L1 cell adhesion molecule signaling (**Figure 2C and Table 1**).

2. Differentially expressed proteins, PCA and clustering

Pairwise comparison was performed to identify differentially expressed proteins in each tissue at days 1 and 7 post-transplantation, compared to sham. VM, CPU, Thal, MFB and Nacc tissues showed, respectively, a total of 71, 59, 54, 46 and 44 proteins whose expression were specifically altered at day1 post-transplantation, compared to sham (**Figure 3A**). On the other hand, a total of 70, 68, 61, 52 and 46 proteins were differentially expressed in VM, CPU, Thal, MFB and Nacc tissues, respectively, at day 7 post-transplantation, compared to sham (**Figure 3B**).

PCA analysis using tissue-specific protein expression showed that these proteins could differentiate between tissues on the first axis with highest variance (Axis 1) but also can differentiate lesioned and transplanted samples from sham on the second axis (Axis 2) (**Figure 3C**).

Clustering analysis showed that most proteins were down-regulated between day 1 and day 7 after transplantation, compared to sham. However, few genes showed inconsistent changes between days 1 and 7, as shown in the heatmaps of the various tissues (**Figure 4**).

3. Enrichment analysis and Tissue-specific proteins and pathways

Enrichment analysis showed that most tissues share common downregulated pathways, both at day 1 and day 7 post-transplantation, that are related to neurodegeneration such as Parkinson and Alzheimer diseases (**Figure 5A**), but also other common pathways such as oxidative phosphorylation and thermogenesis (**Figure 5A**). The proteins associated to these pathways were mostly downregulated in the MFB and the Thal after grafting. Interestingly, proteins associated with Parkinson and Alzheimer diseases were only upregulated in VM tissue at both day 1 and day 7 as well as proteins associated with dopaminergic synapses, 7 days after grafting in the VM. Similarly, dopaminergic synapses and tight junctions were only upregulated in VM tissue at day 7 post-transplantation, compared to sham. Importantly, similar pathways were extracted when using the GO pathways (**Figure 5B**).

4. Common pathways among tissues, Gene Ontology and functional networks associated with differentially expressed proteins

Since the different tissues involved in this study shared common differentially regulated pathways, we then aimed to identify differentially expressed proteins in at least 3 tissues, at day 1 or day 7. Results showed that a total of 57 extracted common proteins clearly differentiated between transplanted samples, in comparison to sham (Axis 1), with higher divergence at day 7 than at day 1 (**Figure 6A**). Gene ontology analysis for biological processes showed that these proteins are involved in autophagy and cell adhesion (**Figure 5B**), oxidative metabolism and ROS production (**Figure 6C**), as well as negative regulation of protein phosphorylation and axonal sheaths (**Supplementary Figure S1**),

Network analysis of these proteins identified 3 main modules of genes with 3 main hub proteins corresponding to Cox6a1, Arpc5 and Hnrnpa1 (**Figure 6D**). Correlation analysis identified two main clusters within common proteins among 3 or more tissues (**Figure 6E**). On the other hand,

enrichment analysis for the larger cluster, using Enrichr web tool, identified candidate drugs that have been experimentally associated with these proteins, with the top hits corresponding to Tamoxifen, Carbamazepine and Phenelzine (**Table 2**).

5. Unique Proteins per tissues

Only one protein, Pip4k2a, was found to be commonly altered among all tissues at day 1. However, 4 proteins were commonly deregulated in all tissues at day 7 including Actc1, Atp6v1e1, Sh3gl2 and Tspan7 (**Figure 3A and 3B**). All these proteins are downregulated at day 1 or day 7, compared to sham (**Figure 7**).

IV. Discussion

In this study we aimed to identify differentially expressed proteins in 5 regions related to the nigrostriatal pathway after intranigral transplantation of fetal VM cells that were shown to restore the lesioned nigrostriatal pathway in a mouse model of PD (Gaillard et al., 2009; Thompson et al., 2009). For this we used non-targeted proteomic analysis based on mass spectrometry approach. Among top cellular pathways involved in tissue-specific response to transplantation, we identified pathways related to oxidative phosphorylation, mitochondrial metabolism and protein folding, which are cellular processes well-known to be involved in the degenerative process of PD. Moreover, we identified pathways associated with L1 cell adhesion molecule signaling where it is worth noting that L1 functions as a signal transducing receptor providing neurons with cues from their environment for axonal growth and guidance. In all 5 regions surrounding the nigrostriatal pathway, expression of proteins varied between 1- and 7-days after transplantation, in comparison to non-grafted mice, showing that the presence of fetal cells may interfere with the host cellular functioning.

Proteins associated with PD pathway and oxidative phosphorylation were particularly down-regulated in the MFB and Thalamus after transplantation, suggesting the presence of fibers in the MFB and the reconstruction of the pathway. Moreover, proteins associated with Parkinson's pathway and the dopaminergic synapses were upregulated exclusively in the VM where the somas of grafted neurons are located and when DA grafted neurons begin to reach the striatum (Gaillard et al., 2009).

PCA analysis showed 57 regulated proteins after transplantation in which 5 proteins were especially down-regulated after grafting. This decrease of expression was progressive from 1 day to 7 days after transplantation in comparison to non-grafted animals. As mentioned above, these time-points correspond to the period of growth of the grafted axons from the SN towards their telencephalic target, the striatum. Our analysis showed that during this time the following 5 proteins were down-regulated: the alpha cardiac muscle 1 (ACTC1), which expresses 1 of 6 actin families implicated in cell motility and ATP6V1E1, an ATPase H⁺ transporter. Both of them are known to be prognosis markers in gliomas as they are involved in invasion and motility of cancer cells (Ohtaki et al., 2017; Yang et al., 2012). Moreover, PIP4K2A expression was down regulated between 1 and 7 days after transplantation. This protein is known to be involved in the biosynthesis of phosphatidylinositol-4, 5-bisphosphate which regulates exocytotic fusion of synaptic vesicles (glutamate and dopamine) with the plasma membrane. Polymorphisms in this gene are observed in Schizophrenia (Kaur et al., 2014) and our results suggest that this gene could also be linked to PD. Moreover, in our model of cell therapy, other genes involved in DA endocytosis may be shut down during the navigation of the grafted fibers towards the striatum. Indeed, SH3GL2 gene, coding for endophilin A1, was down regulated. Endophilin A1 is known as a risk factor for PD and is involved in the synaptic vesicle retrieval following neurotransmission. Mutations in SH3GL2 has been found to be associated with Parkinsonism.

It implicates defective synaptic vesicle endocytosis as a contributor to the degeneration of midbrain dopaminergic neurons in patients (Nguyen et al, 2019).

Finally, and surprisingly, Tspan7 expression was found to decrease with the growth of DA fibers towards their targets although this protein is known to mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility. This protein is a cell surface glycoprotein that may also have a role in the control of neurite outgrowth and is known to complex with integrins (Usardi et al., 2017). Integrins are known to be linked to ECM proteins and axonal guidance molecules to promote axonal growth and navigation. For example, Semaphorin7A axon guidance molecule promotes dendrite growth, complexity and spine development through β 1-subunit-containing integrin receptors in the adult hippocampal dentate gyrus (Jongbloets et al., 2017). However, a new role for TSPAN7 has been described recently as a regulator of D2 dopamine receptors (Lee et al., 2017). The authors showed that TSPAN7 is associated with D2 dopamine receptors and reduces their plasma membrane expression by enhancing internalization process. Immunocytochemical analysis revealed also that TSPAN7 that resides in the membrane of early and late endosomes promotes internalization of D2 dopamine receptors and their location to endosomal compartments. Furthermore, TSPAN7 deficiency increases surface location of D2 dopamine receptors concomitantly with a decrease of its endocytosis. Finally, TSPAN7 negatively affects D2 dopamine receptor-mediated signaling. Overall, the decrease of TSPAN7 expression observed after transplantation, associated with endophilin A1 down regulation, suggest that up to 7 days after transplantation these genes may be shut down to promote DA transmission through a decrease of DA and D2 dopamine receptors endocytosis.

In the future, it would be of a crucial interest to determine the proteome of these nigrostriatal-related regions later after transplantation when motor function is recovered in animal models. These proteins may be used as targets to enhance cell therapy efficiency in PD patients.

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List of figures

Figure 1: Punched regions on a sagittal section of adult mouse brain labeled with Nissl stain. CPU, NAcc, MFB, and Thal regions were collected using a 1mm-diameter punch whereas VM was collected using a 2mm-diameter punch. (Scale bar: 2mm). CPu: caudate putamen; NAcc: nucleus accumbens; Thal: thalamus; MFB: medial forebrain bundle; VM: ventral mesencephalon. (Adapted from Kalaani et al., 2016).

Figure 2: Top variance-contributing genes.

(A) PCA analysis on all data set including all measured proteins.

(B) Top 30 proteins contributing to the variance in samples from axis1 and axis 2.

(C) Top canonical gene sets from gene set enrichment analysis (GSEA) performed on all proteins ranked by their contribution to variance from axis 1.

Figure 3: Differential protein expression.

(A) Venn diagram showing the number of proteins differentially expressed between SNLTD1 and Sham.

(B) Venn diagram showing the number of proteins differentially expressed between SNLTD7 and Sham.

(C) PCA analysis using tissue-specific protein expression.

Figure 4: Clustering analysis and Heat maps.

Figure 5: Enrichment analysis and Gene Ontology

(A) Overrepresented KEGG pathways associated with differentially expressed proteins in each tissue.

(B) Overrepresented Gene Ontology (GO) pathways associated with differentially expressed proteins in each tissue

Figure 6: Common proteins between tissues.

(A) PCA analysis on proteins differentially expressed between each of SNLTD1 and SNLTD7, compared to Sham that are common among 3 or more tissues.

(B) Gene ontologies that are overrepresented and associated with common proteins.

(C) Top enriched gene ontology pathways for common differentially expressed proteins among 3 or more tissues shown by Circus plot.

(D) Network analysis of the common proteins among 3 or more tissues.

(E) Correlation matrix of common differentially expressed proteins among 3 or more tissues.

Figure 7: Common differentially expressed proteins among all tissues.

Figure 1:

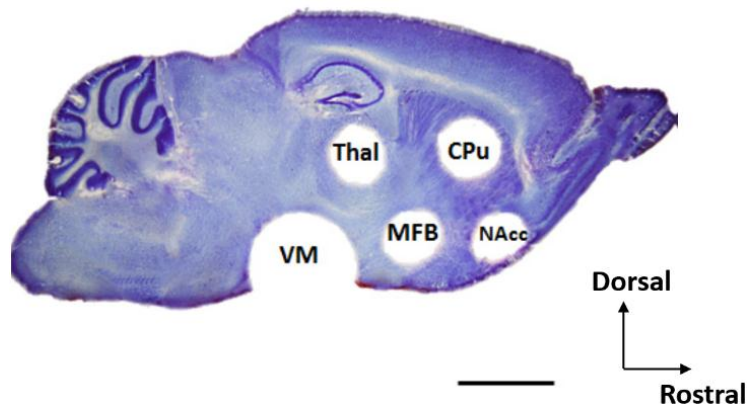


Figure 2:

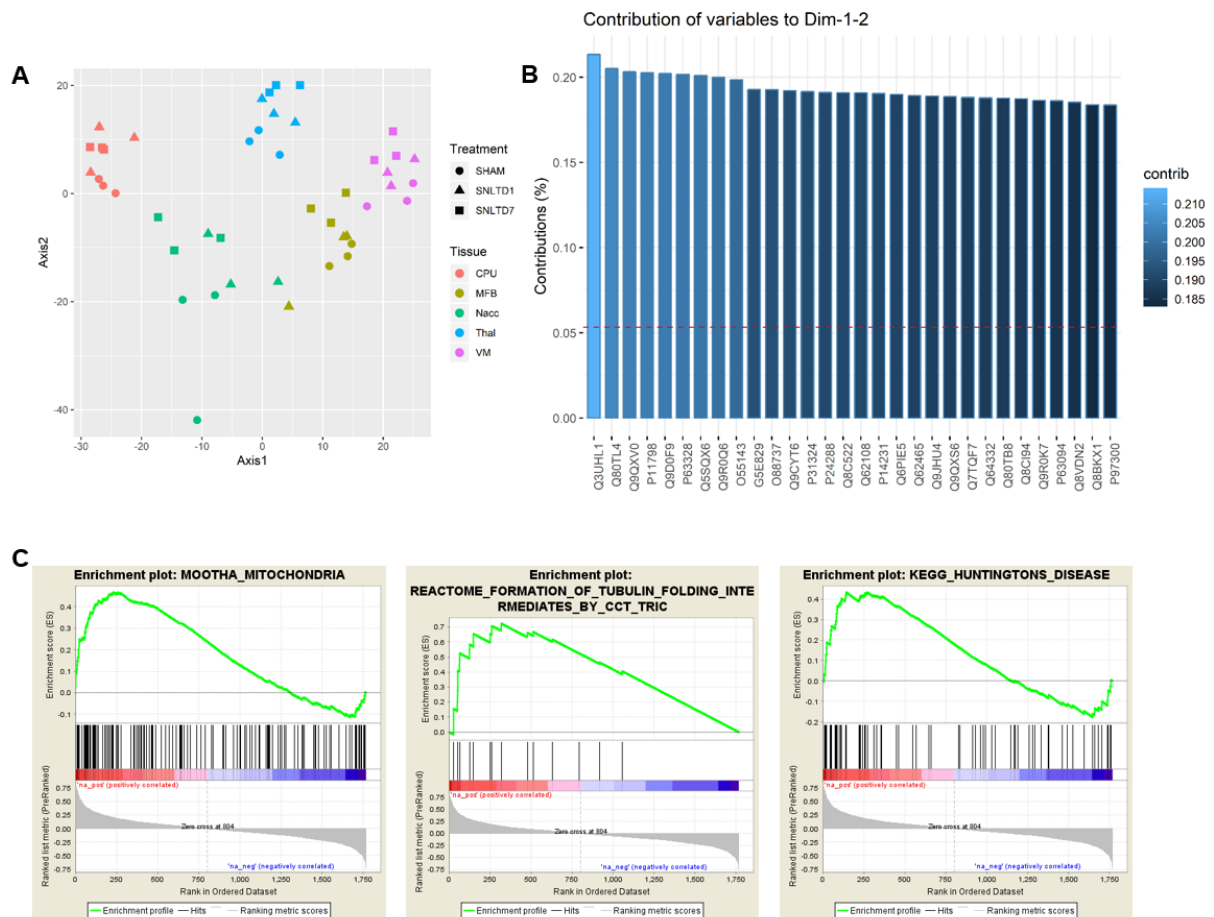


Table 1: Pathways contributing to variance between samples.

NAME	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX
MOOTHA_HUMAN_MITODB_6_2002	156	0.479	2.150	0.000	0.012	0.017	232
MOOTHA_MITOCHONDRIA	146	0.468	2.059	0.000	0.021	0.056	231
REACTOME_FORMATION_OF_TUBULIN_FOLDING_INTERMEDIATES_BY_CCTRIC	15	0.723	1.977	0.002	0.047	0.176	319
REACTOME_PROTEIN_FOLDING	18	0.685	1.943	0.002	0.055	0.258	319
REACTOME_PREFOLDIN_MEDIATED_TRANSPORT_OF_SUBSTRATE_TO_CCTRIC	16	0.667	1.901	0.002	0.074	0.394	319
WONG_MITOCHONDRIA_GENE_MODULE	96	0.445	1.868	0.000	0.092	0.531	304
BROWNE_HCMV_INFECTION_18HR_UP	18	0.608	1.757	0.006	0.250	0.911	376
WAKABAYASHI_ADIPOGENESIS_PPAR_G_RXRA_BOUND_WITH_H4K20ME1_MARK	30	0.527	1.742	0.007	0.253	0.937	123
KEGG_HUNTINGTONS_DISEASE	73	0.433	1.718	0.002	0.278	0.966	272
MOOTHA_VOXPPOS	43	0.462	1.694	0.004	0.307	0.986	140
REACTOME_SIGNAL_TRANSDUCTION_BY_L1	15	0.605	1.678	0.012	0.323	0.992	272
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	18	0.556	1.626	0.018	0.457	1.000	223

Figure 3:

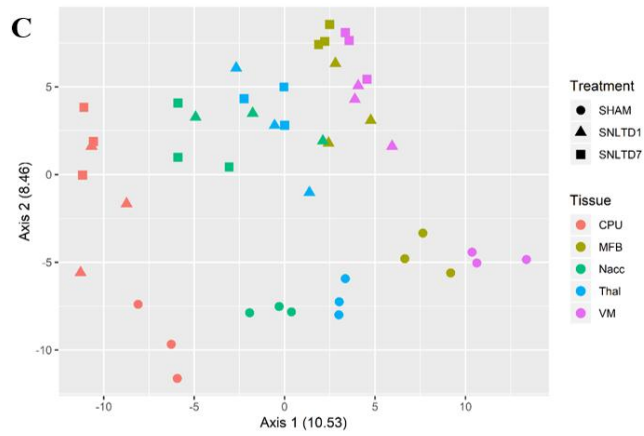
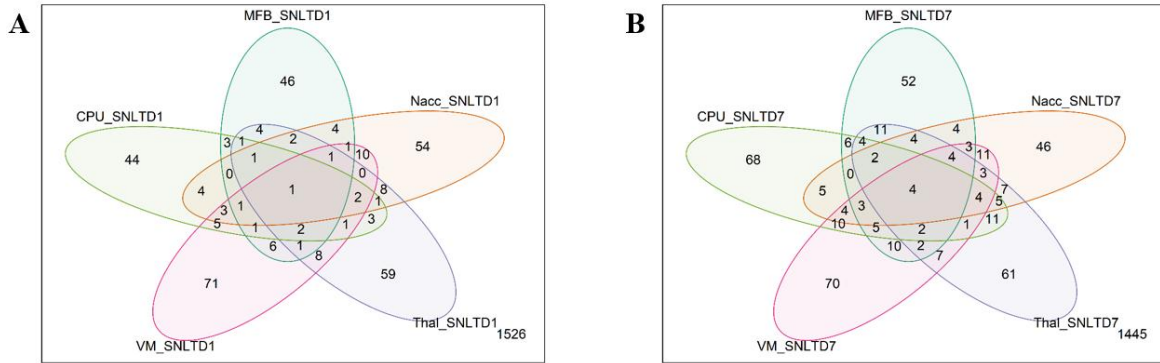


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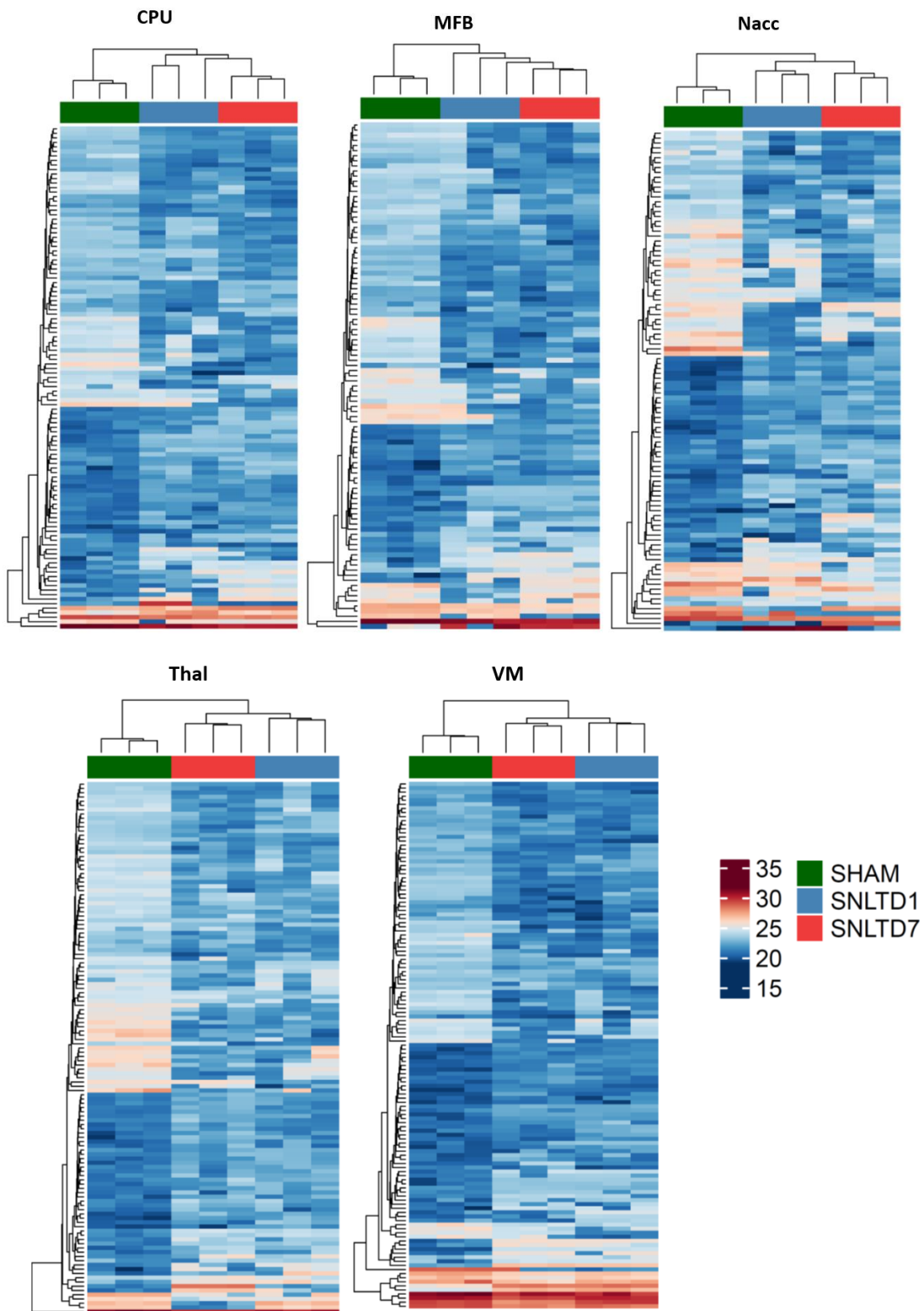
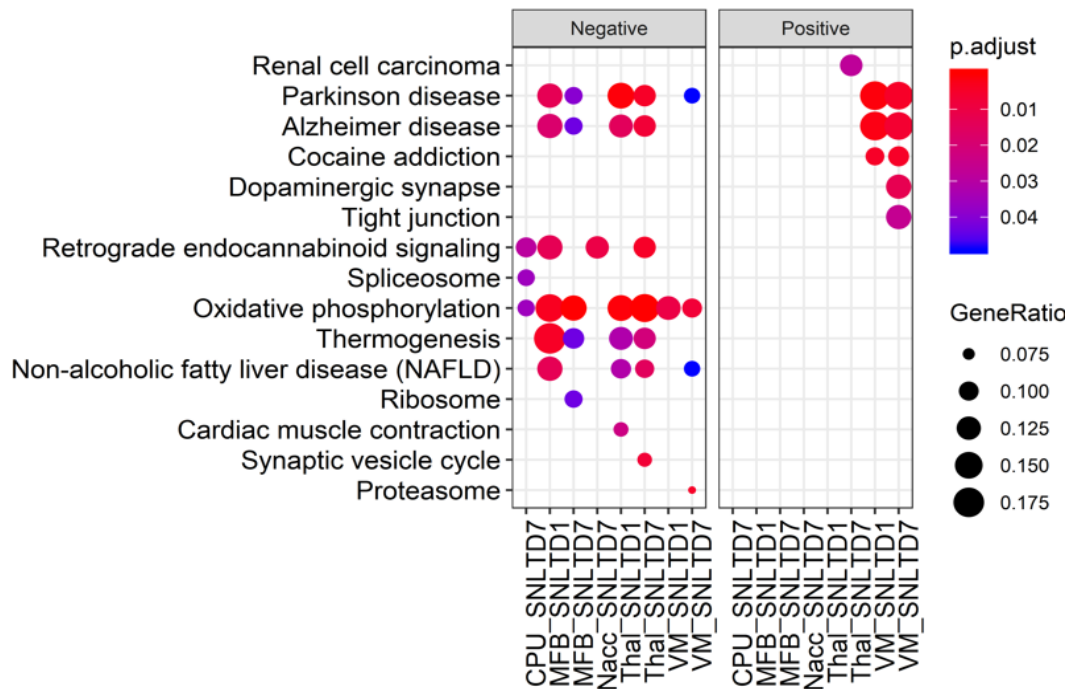


Figure 5:

A



B

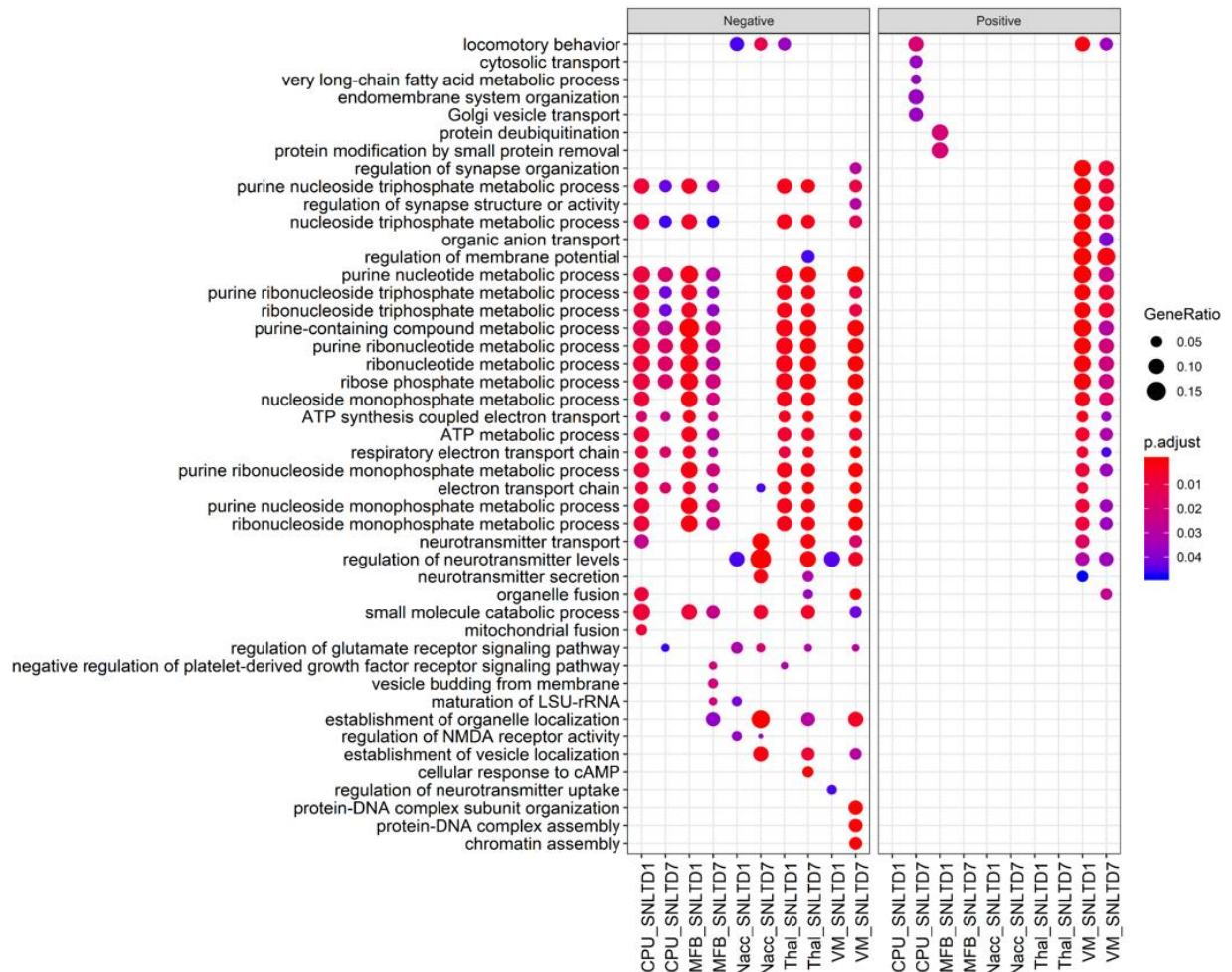


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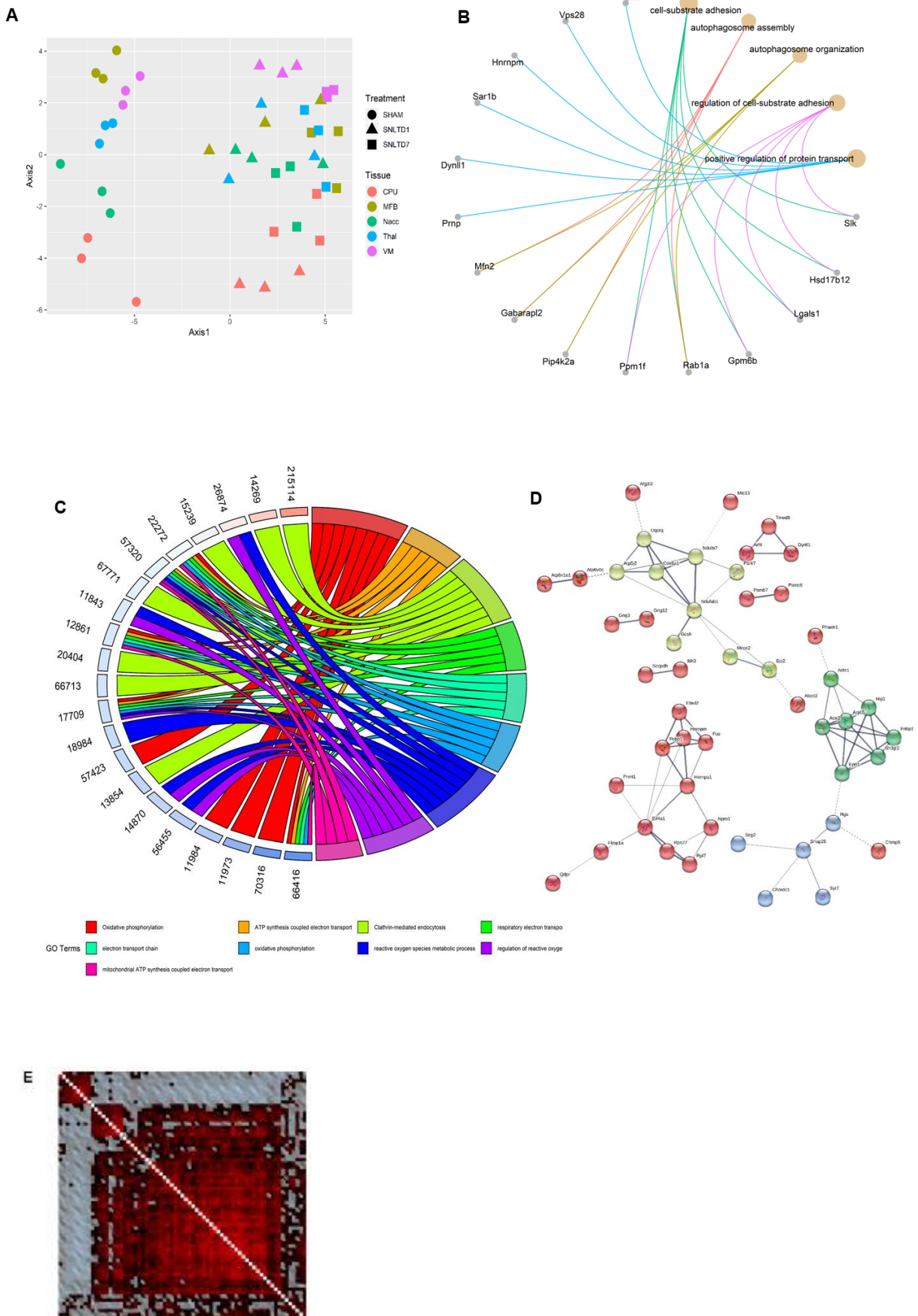


Table 2: Top enriched drugs. Over-represented drug gene expression signatures for common differentially expressed genes among 3 or more tissues.

Term	Overlap with gene set	P-value	Adjusted P-value	Genes
Tamoxifen-64_mg/kg_in_Corn_Oil-Rat-Liver-1d-up	10/305	2.26E-08	2.62E-05	PSMB7;LYPLA2;ARF4;NPM1;TMED9;ITIH3;SCCPDH;SAR1B;MAL2;DYNLL1
Carbamazepine-490_mg/kg_in_CMC-Rat-Liver-3d-up	10/306	2.33E-08	2.62E-05	FKBP1A;PSMB7;EIF4A1;AFG3L2;NPM1;TMED9;SCCPDH;SAR1B;FTH1;MAL2
Phenelzine-27_mg/kg_in_Water-Rat-Brain-3d-dn	10/315	3.06E-08	2.62E-05	FKBP1A;PSMB7;ITIH3;GNG3;SCCPDH;FTH1;MAL2;PARK7;DYNLL1;GPM6B
Genistein-375_mg/kg_in_Corn_Oil-Rat-Liver-5d-up	10/313	2.88E-08	2.62E-05	PSMB7;LYPLA2;EIF4A1;NPM1;ITIH3;SCCPDH;SAR1B;MAL2;PARK7;DYNLL1
Sulfadiazine-1170_mg/kg_in_Water-Rat-Liver-3d-dn	10/296	1.7E-08	2.62E-05	PSMB7;EIF4A1;AFG3L2;TMED9;ITIH3;SAR1B;FTH1;MAL2;PARK7;DYNLL1
Estriol-313_mg/kg_in_CMC-Rat-Liver-5d-up	10/313	2.88E-08	2.62E-05	FKBP1A;PSMB7;EIF4A1;ARF4;AFG3L2;NPM1;TMED9;SCCPDH;FTH1;MAL2
Hydroxyurea-400_mg/kg_in_Saline-Rat-Liver-5d-up	10/306	2.33E-08	2.62E-05	FKBP1A;PSMB7;AFG3L2;NPM1;TMED9;ITIH3;SCCPDH;SAR1B;FTH1;DYNLL1
Dexamethasone-1_mg/kg_in_Corn_Oil-Rat-Liver-3d-up	10/290	1.4E-08	2.62E-05	PSMB7;EIF4A1;ARF4;NPM1;TMED9;ITIH3;SAR1B;FTH1;MAL2;PARK7
Dexamethasone-1_mg/kg_in_Corn_Oil-Rat-Liver-1d-up	10/306	2.33E-08	2.62E-05	FKBP1A;PSMB7;EIF4A1;NPM1;TMED9;ITIH3;FTH1;MAL2;PARK7;DYNLL1
44'-Diethylaminoethoxyhexestrol-20_uM_in_DMSO-Rat-Primary_rat_hepatocytes-1d-dn	10/321	3.65E-08	2.71E-05	PSMB7;LYPLA2;ARF4;TMED9;ITIH3;SAR1B;FTH1;MAL2;PARK7;DYNLL1

Figure 7 :

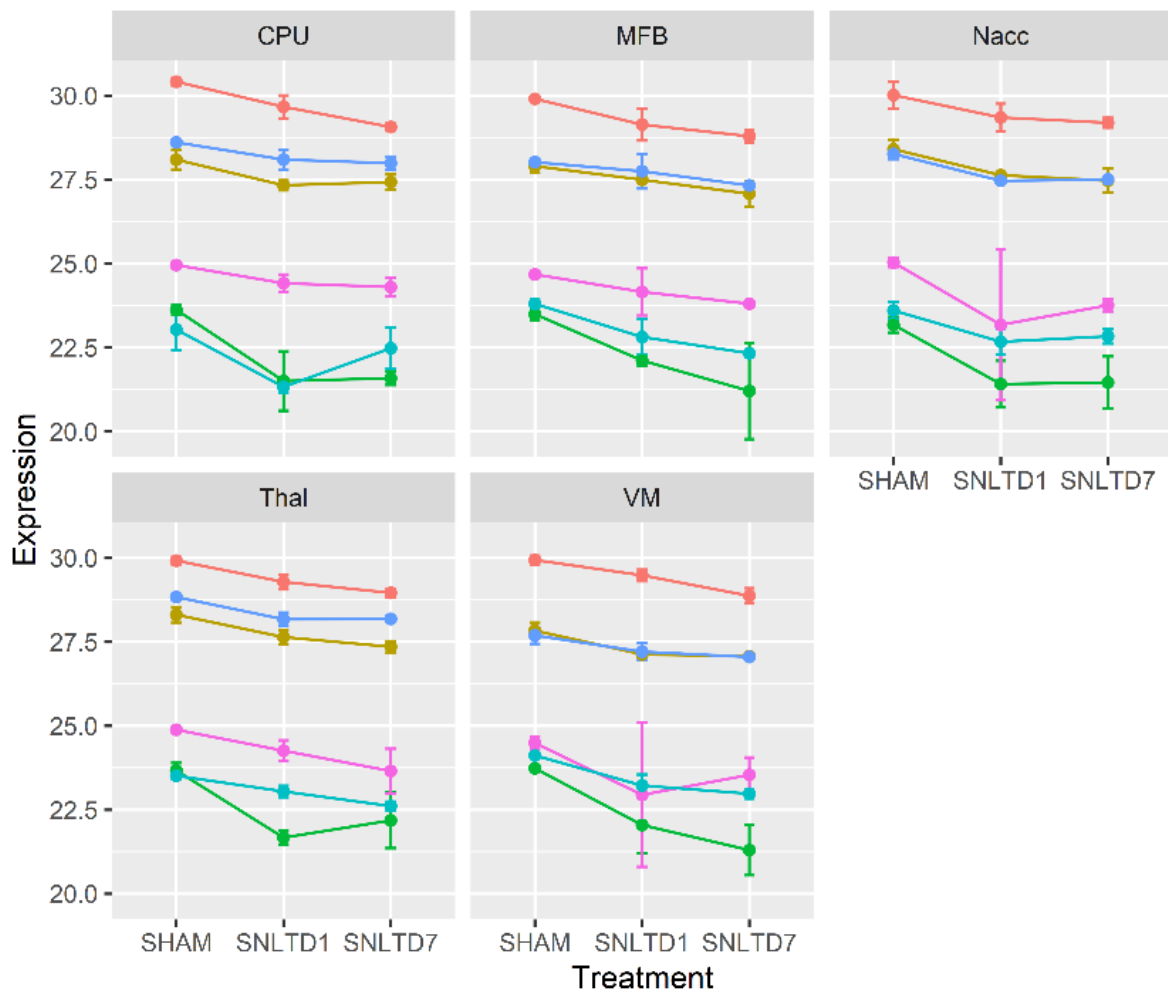
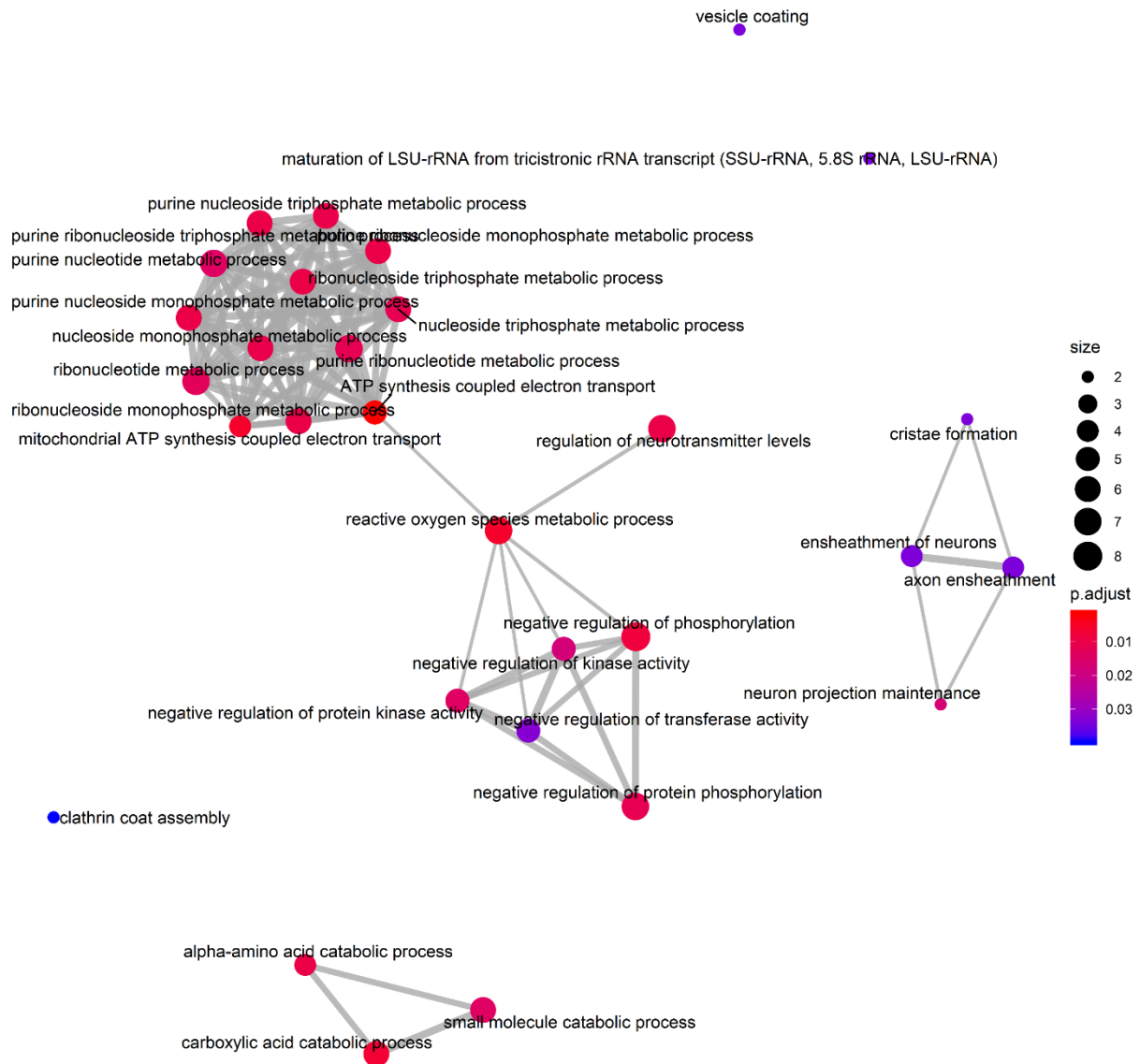


Figure S1: Network showing interconnection between enriched gene ontology pathways for common differentially expressed genes among 3 or more tissues.



Chapter II:

Repairing TBI with stem cell therapy

Résumé

Les lésions traumatiques cérébrales (LTC) sont considérées comme un problème majeur de santé publique dans le monde, avec une incidence annuelle de 150 à 200 cas pour 100 000 personnes (Shear & Tortella, 2013). Elles représentent la principale cause d'invalidité et de mortalité chez les moins de 45 ans (Faul & Coronado, 2015), avec un taux de mortalité 3,5 fois supérieur à celui du cancer et des maladies cardiaques combinées (Rostami et al., 2016). La LTC se manifeste généralement par un ou plusieurs signes cliniques, notamment une perte ou une altération du niveau de conscience, une amnésie, avec ou sans déficits neurologiques. La classification des LTC se fait selon leur sévérité et est basée sur le score de Glasgow (GCS) : LTC légère (GCS 13-15), modérée (GCS 9-12), ou sévère (GCS 3-8). La LTC est généralement suivie d'une lésion secondaire caractérisée par une cytotoxicité, un œdème cérébral, un stress oxydatif, une apoptose, un dysfonctionnement mitochondrial et une inflammation due à la rupture de la barrière hémato-encéphalique (McGinn & Povlishock, 2016). Après une LTC, la perméabilité membranaire augmente en raison de la défaillance des pompes dépendantes de l'ATP. Cela conduit à une dépolarisation de la membrane et à une libération excessive de glutamate, induisant une perturbation de l'homéostasie des électrolytes et l'influx élevé de Ca^{2+} dans la mitochondrie favorise alors la production d'espèces réactives de l'oxygène (ROS). Le stress causé par le Ca^{2+} active également les caspases et les calpaïnes qui déclenchent respectivement des voies apoptotiques et nécrotiques, conduisant à la mort cellulaire immédiate et à l'accumulation de débris. De plus, la signalisation liée au TNF entraîne une activation de l'expression de l'aquaporine, AQP4. Cette protéine favorise la formation d'œdème et l'augmentation de la pression intracrânienne et une dégénérescence des neurones. Elle favorise également la formation de la cicatrice gliale. De nombreuses études ont montré une récupération fonctionnelle spontanée après une LTC mais celle-ci reste limitée (Darling et al., 2011). En outre, il n'existe pas de traitement pharmacologique efficace spécifique aux LTC (Shear et al., 2013). Ainsi, la thérapie cellulaire est considérée comme une approche prometteuse pour

remplacer les neurones dégénérés et restaurer les voies corticales endommagées. Son efficacité dépend de la capacité des cellules greffées à développer des populations cellulaires appropriées et à s'intégrer dans les circuits neuronaux préexistants de l'hôte. Différents types de cellules peuvent être destinés à la greffe. Par exemple, la transplantation de cellules souches humaines différenciées dans des cerveaux de rats modèles de LTC assure la survie des neurones au niveau de la zone lésée, ainsi qu'une réduction de l'astrogliose et un renforcement de l'angiogenèse (Skardelly et al., 2011). De plus, la transplantation de neurones corticaux embryonnaires, immédiatement après une lésion dans le cortex moteur adulte, permet la reconstruction anatomique des voies motrices lésées et le développement de projections sur des cibles hôtes corticales et sous-corticales appropriées (A. Gaillard et al., 2007).

Récemment, davantage de recherches ont été consacrées à l'augmentation de l'efficacité de la greffe par la production d'un microenvironnement permissif à la survie et à la prolifération de ces cellules greffées (Aligholi et al., 2016; Haus et al., 2016; He et al., 2016; Koutsoudaki et al., 2016). Pour cela différents supports combinés aux cellules destinées à la greffe peuvent être utilisés. Parmi ces agents, l'acide docosahexaénoïque (DHA, lipide polyinsaturé du système nerveux central) est connu pour favoriser la différenciation neuronale, la croissance des neurites et la formation des synapses (Green & Yavin, 1998; Kawakita et al., 2006). En outre, le DHA a un rôle de support des cellules neurales *in vitro* (Katakura et al., 2013) et favorise le maintien des neurosphères primaires et tertiaires (Kawakita et al., 2006).

Ainsi, le travail présenté dans ce chapitre sous forme de deux articles publiés, concerne d'une part la greffe de cellules souches neurales dans un modèle d'impact cortical modéré et d'autre part la greffe de cellules souches neurales combinées au DHA dans ce même modèle.

Dans la première étude nous avons utilisé un modèle d'impact cortical modéré ciblant la région corticale somatosensorielle primaire de souris, et nous avons étudié les conséquences de la transplantation de cellules souches neurales provenant d'éminence ganglionnaire d'embryons

de souris au stade de développement E14.5 ou de cellules différenciées, 7 jours après le traumatisme. Nous avons observé une activation microgliale qui persiste 1 et 4 semaines après la greffe. De manière intéressante, nous avons montré que la greffe de cellules souches neurales différenciées ou non, permet une réduction significative de l'astrogliose et une augmentation de la neurogénèse probablement via l'augmentation de la prolifération de progéniteurs neuraux. Dans la deuxième étude de transplantation cellulaire, nous avons utilisé des cellules souches neurales combinées à des injections de DHA. Pour cela, des cellules souches neurales dérivées de la SVZ de rats nouveau-nés ont été cultivées en neurosphères et transplantées dans le cortex d'un modèle murin d'impact cortical modéré. Les déficits moteurs ont été évalués à l'aide des tests du *pole climbing* et du *rotarod*. Par immunohistochimie, nous avons montré que la greffe de cellules souches neurales combinée aux injections de DHA atténue de manière significative les déficits moteurs, favorise la neurogénèse et augmente la réactivité gliale au niveau du site de lésion. De plus, il a été observé que le nombre de neurones exprimant la tyrosine hydroxylase augmente dans l'aire tegmentale ventrale et la substance noire chez les animaux ayant reçu le DHA. Enfin, une analyse de la fragmentation de la GFAP et de l' α -II-spectrine, connue pour être une signature des lésions corticales, a montré que la transplantation de cellules souches neurales associées au DHA réduit les produits de dégradation de la GFAP et de la spectrine. Ceci suggère une diminution de l'activation de la calpaïne/caspase chez ces animaux.

En conclusion, ces études montrent que les cellules souches neurales permettraient d'augmenter la neurogénèse et de réduire l'inflammation après greffe dans un modèle murin de LTC. De plus, un traitement préalable au DHA pourrait être une stratégie d'amélioration de l'efficacité thérapeutique de la transplantation de cellules souches neurales dans les lésions.

Enfin, la dernière partie de ce chapitre présente une revue dans laquelle sont exposées les connaissances récentes sur les LTC concernant la pathophysiologie, les options

thérapeutiques actuelles et notamment l'utilisation de différents types de cellules pour la thérapie cellulaire (cellules souches mésenchymateuses, embryonnaires ou induites) ainsi que la combinaison de divers traitements. La thérapie cellulaire est ainsi une approche thérapeutique très prometteuse pour les LTC si l'on a au préalable déterminé la capacité de différenciation et de prolifération des cellules greffées, leur potentiel tumorigène, leurs voies d'administration et leurs éventuels effets secondaires.

I. Introduction

1. The cerebral cortex

The cerebral adult cortex is a complex structure in terms of projections and cell specificity; it is separated into several distinct areas that are anatomically and physiologically different. The neocortex is a six-layered structure residing within the peripheral part of the cerebral hemispheres and is composed of hundreds of different types of neurons, the interneurons and glial cells. Several unique and cognitive functions such as sensory perception, language, spatial reasoning, conscious activity, or voluntary motor commands are controlled by the neocortex (Heimer, 1994; Finlay & Darlington, 1995).

The cerebral cortex is organized into six layers (Figure 48) distinguished by their unique cellular composition and numbered I to VI from the surface of the brain (Heimer, 1994). Layer I is known as the plexiform or the molecular layer which receives thalamic afferents that transmits information from the basal ganglia. This layer consists of glial cells, nerve fibers, star-shaped neurons, and Cajal-Retzius cells (Kuramoto et al., 2009). Layer II (outer granular layer) and layer III (outer pyramidal layer) which emit dendrites into layer I, are made up of small granular neurons and pyramidal cells; respectively (Petreanu et al., 2009). These two layers also send projections to the striatum (Reiner et al., 2003), the cortico-spinal neurons of layer V (Schubert et al., 2006), as well as towards the contralateral cortex (for review, Molyneaux et al., 2007). Among the layers of the primary motor cortex M1, Layer IV (inner granular layer) is the thinnest and which contains pyramidal and starry neurons. These neurons project into all cortical layers, especially layers II and III (Lubke et al., 2000). Layer V (inner pyramidal layer) is composed of large pyramidal or projection neurons, which send projections towards subcortical targets, such as the striatum and pyramidal tract (Reiner et al., 2003). As for Layer VI (polymorphic layer), it contains pyramidal neurons that project towards the thalamus as well

as into other cortical regions and it receives afferents from thalamus and other cortical regions (Zhang & Deschenes, 1997, 1998).

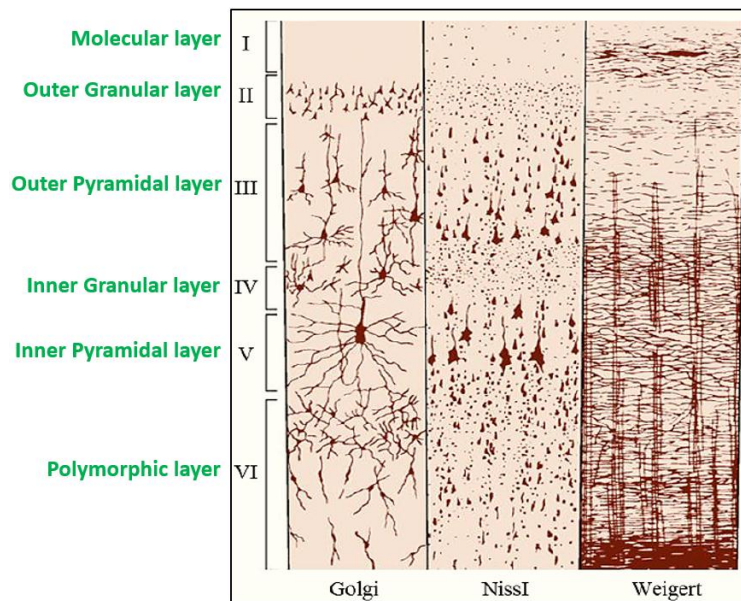


Figure 48: Laminar organization of the cortex. The 6 cortical layers are visualized and delimited using 3 staining techniques. Golgi staining shows the cellular bodies of neurons and dendrites. Nissl staining reveals cellular bodies and proximal dendrites whereas Weigert staining visualizes axonal distribution (adapted from Heimer, 1994).

At the cellular level, the cerebral cortex is composed of two cell types, neurons and glial cells. Around 80% of neurons are excitatory glutamatergic pyramidal neurons while 20% are non-pyramidal inhibitory interneurons that use glutamate as a neurotransmitter (Hendry et al., 1987). A small proportion of cortical neurons are the Cajal-Retzius neurons that inhabit cortical layer I and regulate the laminar positioning of cortical neurons during development (reviewed by Tissir & Goffinet, 2003). Glial cell populations include oligodendrocytes that contribute to the formation of myelin sheath, astrocytes which provide structural, metabolic and trophic support for motor neurons and regulate certain physiological functions of the brain, and microglia that are the immune-competent cells of the CNS (Burda & Sofroniew, 2014; Molofsky et al., 2012; Sloan & Barres, 2014).

2. Traumatic Brain Injury

Traumatic brain injury (TBI) is a major cause of death and disability in patients under the age of 45 (Faul et al., 2015). Annually, around 200 people per 100,000 cases suffer from TBI (Carrion et al., 2005). TBI results in physiological and morphological alterations; therefore, disturbing homeostasis within the brain and altering cellular functionality and integrity (Maxwell, 2013). According to the neuropathological features of TBI, and given the complexity and the heterogeneous characteristic of the brain structure, TBI encompasses cascades of primary and secondary injuries. Primary injury is the result of mechanical forces at the time of injury period that is followed by a secondary phase, which is a determining factor in morbidity of patients, characterized by oxidative stress, cytotoxicity, mitochondrial deficits, apoptosis, and inflammation (reviewed by McGinn et al., 2016; Reis et al., 2017). Based on the lesion severity, TBI can be classified into mild, moderate, or severe (Corrigan et al., 2015). The most common type of TBI is the mild traumatic brain injury (mTBI) (McInnes et al., 2017) which is described as a mild concussion to the head leading to cognitive impairments, short-term confusion, and transient unconsciousness (Desai et al., 2014).

Several animal models have been established to assess lesions in the cerebral cortex and develop restorative strategies. Among these animal models are those of (TBI) such as the open-head Controlled Cortical Impact (CCI). The CCI model is based on inducing a deformation in the cortical layer after a craniectomy by exerting an external physical force on the animal brain using a pneumatic impact device. The velocity and the depth of the cortical impact are the two main parameters used to characterize the severity of the injury. In comparison to moderate and severe TBI reproduced by this model, a mild TBI is characterized by a lower cortical lesion depth and velocity associated to a mild damage mostly confined to the cortical tissue (Reviewed by Siebold et al., 2018).

3. Restoration of damaged pathways by neurotransplantation

Due to the limited capacity of the adult brain to repair or replace degenerated neural cells, currently, there are no effective pharmacological treatments for TBI (Shear et al., 2013). Indeed, established TBI pharmacotherapies using glutamate antagonists, steroids, free radical scavengers, progesterone, anticonvulsants and others either had no effect on patients or failed to reach clinical trials (reviewed by Narayan et al., 2002; Stein, 2015). To this end, neurotherapeutic approaches by cell transplantation hold great potential to enhance recovery post-TBI. Indeed, several experimental studies have been investigated to replace damaged neurons and re-establish the degenerated pathways (Ballout et al., 2016; Gaillard et al., 2004; Peron et al., 2017) The efficacy of neuronal transplantation strategies is dependent on the reparative capacity of the transplanted cells. In cell transplantation strategies, the ultimate purpose is to establish specific neuronal cell populations capable of surviving and integrating within the host neuronal circuitry and to reconnect damaged pathways; in addition to enhancing behavior deficits post-TBI (Ballout et al., 2016; Peron et al., 2017).

Several attempts have emerged in neural transplantation procedures using embryonic neural stem cells (ENSCs) (Hentze et al., 2007), mesenchymal stem cells (MSCs) (Nichols et al., 2013), neural stem cells (NSCs) (Walker et al., 2009), and induced pluripotent stem cells (iPSCs) (Kobayashi et al., 2012).

In 2007, Gaillard et al. transplanted a fragment of motor cortical tissue from E14 embryos into lesioned mouse cortex. Several weeks after transplantation, the grafted neurons were able to innervate appropriate cortical and subcortical targets of the host, and over long distances reaching the striatum or spinal cord with a topographic organization similar to that of the intact motor cortex (Gaillard et al., 2007). In addition, the host neurons also established synaptic contacts with the transplant. Therefore, the outcomes of this study demonstrated the permissive capacity of the adult brain to axonal regeneration by the grafted embryonic neurons and the

ability to reconstruct damaged motor circuitry while respecting the topographic organization found in the intact host cortex (Figure 49).

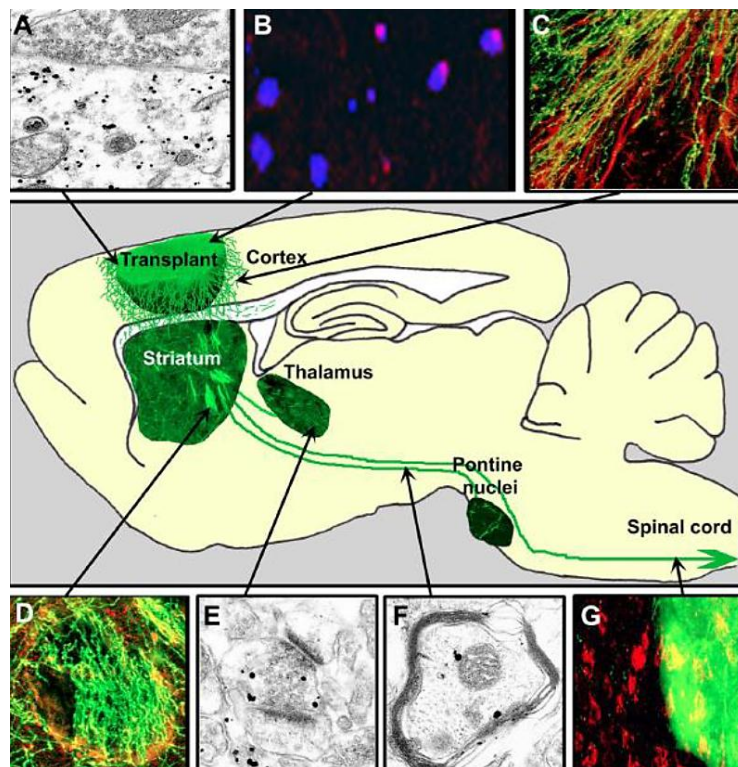


Figure 49: Anatomical reconstruction of the damaged pathways post-transplantation of embryonic motor cortex tissue into damaged motor cortex of the adult mouse. Electron microscopy detection of synaptic contacts established between the host and transplanted GFP neurons in the cortex (A), thalamus (E) and internal capsule (F); several axons are myelinated. The absence of cell fusion between the transplanted cells (blue) and the host cells containing the Y chromosome (pink) (B). The GFP+ axons leave the transplant and innervate the adjacent cortical and striatal areas. (G) GFP+ axons contact targets over long distances (C, D). Cells marked by a retrograde tracer injected into the spinal cord are present in the transplant (yellow) and in the adjacent motor cortex (red) (Adapted from Gaillard et al., 2007).

Moreover, transplantation of pre-differentiated human ENSCs into lesioned cortex in rats supported angiogenesis and neuronal survival within the lesioned area and on another aspect astrogliosis was diminished and motor behavior was ameliorated (Skardelly et al., 2011, 2014). Recent studies have reported that transplantation of ESC-derived visual cortical neurons into lesioned visual cortex successfully restored the damaged cortical projections and synaptic connections (Michelsen et al., 2015). Moreover, proper graft integration and specific

anatomical organization were achieved following grafting of cortical embryonic progenitors into damaged cortex (Falkner et al., 2016).

On another aspect, several combined cell therapies using molecules with neuroprotective properties were proposed to ameliorate neural repair and functional recovery in TBI studies. For instance, the use of progesterone in combination with embryonic NSCs showed to manifest remarkable behavioral ameliorations in comparison to either treatment alone (Nudi et al., 2015). Moreover, growth factors has been considered as candidate targets in multiple TBI treatments as demonstrated by Chen et al. who reported improvements in behavioral performance as well as in survival of the graft when transplanting modified NSCs expressing brain-derived neurotrophic factor (BDNF) into lesioned rat brains (Chen et al., 2017). Similarly, docosahexaenoic acid (DHA) which is a polyunsaturated lipid prevailing in the CNS is known to promote neurite growth, neural differentiation, and synapse formation within the brain (Green & Yavin, 1998; Ikemoto et al., 1999). In this matter, supplementation of DHA diminished neuroinflammation and neuronal damage after fluid percussion injury (FPI) in rats, nominating DHA as a potential viable candidate to mitigate the detrimental outcomes of TBI (Zhu et al., 2017).

Therefore, integrating appropriate strategies of cell transplantation and drug based therapies would present a promising therapeutic perspective to optimize neural restoration and repair in damaged brain cortex.

4. Cellular mediators of neuroinflammation in response to TBI

The brain is an immune privileged organ protected by the blood-brain barrier (BBB), and therefore has relatively limited neuroimmunological exchanges (for review, (Muldoon et al., 2013). However, upon injury or entry of an infectious agent into the CNS, it can react either by activating glial cells (astrocytes, microglia, and oligodendrocytes) or through central inflammation, which could be accompanied by an invasion of peripheral immune cells. In this

way, the ruptured BBB following TBI, allows the circulation of the hematopoietic cells into the site of injury. Neutrophils are the first to enter the nervous tissue followed by monocytes and lymphocytes (Kato & Walz, 2000).

4.1. Astrocytes

Astrocytes belong to the macroglia family and represent the most numerous glial cells in the CNS. For a long time, they were considered only as passive support cells but their role in the formation and functioning of synapses has been highlighted over the past decade (for review (Clarke & Barres, 2013). Astrocytes respond to CNS infections and brain damage by hypertrophy, contributing to astrogliosis which is marked by increased expression of the glial fibrillary acidic protein (GFAP) (G Raivich et al., 1999). It is now well established that astrocytes play a critical role in modulating neuroinflammation by produce a wide range of pro-inflammatory molecules such as TNF- α in the injured CNS (Kipp et al., 2008).

In damaged cortex, activated astrocytes assemble around the lesion forming a physiochemical barrier called a "glial scar" which is considered as a major restrain to regeneration after neural damage (Buffo et al., 2010). Indeed, reactive astrocytes acquire a hypertrophic morphology in the vicinity of the injured area 3 days after cortical lesion and astrogliosis may persist from 7 days to 2 months following lesion (Villapol et al., 2014). In this regard, astrogliosis displays antagonistic effects where activated astrocytic cells can augment neurotoxicity and neuronal degeneration. For instance, secretory astrocytes release pro-inflammatory cytokines as TNF α therefor obstructing neurite growth as well as reactive oxygen species and nitric oxide consequently exacerbating the initial injury (reviewed by (Buffo et al., 2010).

Nevertheless, the molecular composition of the glial scar changes over time following TBI, acquiring axonal growth permissive and protective properties (Gaillard & Jaber, 2007). This notion is consolidated by ability of activated astrocytes to reduce the spreading of the neurotoxic substances released from degenerated cells by isolating the lesioned area from its surrounding

(Rolls et al., 2009). Moreover, reactive astrocytes may promote neuronal survival by eradicating neurotoxic components (Buffo et al., 2010), resealing the disrupted BBB (Faulkner et al., 2004), and secreting growth factors such as VEGF (Rosenstein & Krum, 2004). In fact, it has been recently suggested that the astrocytic neurotoxic or neuroprotective phenotype can be triggered by microglial cells (Liddel et al., 2017).

In light of these facts, the dynamics of astrocyte functioning in response to lesion is essential in pronouncing the neuropathological outcomes following brain injuries.

4.2. Microglia

Immunocompetent microglia are the resident form of CNS macrophages among other glial cells. Microglial cells constantly monitor the microenvironment and respond to foreign antigens by phagocytosis and secreting pro-inflammatory cytokines (Xing, et al., 2012). In the absence of foreign bodies, microglial cells are in a "resting" state and do not have either phagocytic capacity or a migration profile (Chung et al., 2010). During inflammation, "active" microglia are characterized by the retraction of their branches, the hypertrophy of the cellular body and adaptation of an amoeboid morphology, similar to that of macrophages, facilitating their migration to the lesion site (Raivich, 2005). Following neuronal death, activated microglia proliferate and secrete pro- and anti-inflammatory cytokines and chemokines (Xing et al., 2012).

Microglial activation may amplify neurodegeneration (H.-M. Gao & Hong, 2008); therefore, a dual neuroprotective and neurotoxic functions are attributed to them. Upon continuous activation, microglia produce pro-inflammatory cytokines and reactive oxygen species (ROS) that can target and kill surrounding pathogens. However, these molecules also affect viability and neural functions since chronic exposure to pro-inflammatory signals promotes an exaggerated microglial response and subsequently lead to neuronal deterioration (Perry et al., 2007). On the other hand, microglia are suggested to have a protective role by secreting anti-

inflammatory cytokines (Masliah et al., 2005) and neurotrophic molecules involved in tissue repair and neuronal survival (Elkabes et al., 1996; Batchelor et al., 2002). Indeed, the microglia can be polarized into two different phenotypes, the classical M1 pro-inflammatory phenotype and the alternative pro-inflammatory M2 phenotype (Chhor et al., 2013; Kumar et al., 2013). However, when microglia are exposed to IL-4 and IL-13 cytokines, they are oriented towards an M2 (alternative) phenotype (Chhor et al., 2013).

Previous studies have described a peak in M2-phenotype microglial activation up to 7 days after lesion which is then characterized by activation of M1-like microglial cells (Jin et al., 2012; Wang et al., 2013).

Indeed, activated microglia may promote neuronal survival by providing protective factors and remove damaged cells (Wang et al., 2013). However, microglial cells can also proliferate and migrate to the site of injury where they contribute to cell damage by releasing cytotoxic mediators such as pro-inflammatory cytokines, nitrogen species, and excitatory neurotransmitters (reviewed by (Donat et al., 2017).

Therefore, microglial activation in response to TBI contributing to neuroprotective or neurotoxic priming states is differential depending on the stage and severity of the injury (reviewed by Donat et al., 2017).

4.3. Oligodendrocytes

Oligodendrocytes, similar to astrocytes, belong to the microglial cell family. Oligodendrocytes are locally present in the cortex and striatum (for review (Zhang et al., 2013). They are responsible for myelin production and have been shown to contribute to the maintenance of axonal integrity and neuronal plasticity in the adult brain (reviewed by (Zhang et al., 2013).

Due to their limited ability to manage oxidative stress, mature oligodendrocytes are vulnerable to stroke insults. Several studies support the concept that oligodendrocytes experience a range of responses to brain damage (Shi et al., 2015). In fact, the number of oligodendrocytes present

in the white matter decreases during the first week after a lesion by fluid percussion injury (FPI) of rat brains (Lotocki et al., 2011). In addition, a rapid increase in the number of oligodendrocytes progenitor cells has been reported in white and grey substances in the vicinity of the lesion site (Buffo et al., 2005; Dent et al., 2015). These cells persist until the 14th day after the lesion (Hampton et al., 2004). Moreover, acute brain injuries are associated with demyelination which is characterized by oligodendrocyte cell death and loss of myelin sheath. Long-term cognitive and sensorimotor deficits are attributed to demyelination following brain injuries due to the limited capacity of the adult brain to regenerate oligodendrocytes and remyelinate axons (Shi et al., 2015). Thus, modulation of oligodendrocyte activation may be an important factor in the design of therapeutic strategies to promote axonal myelination of neurons.

Overall, inflammatory processes may indeed interfere with the survival of the grafted cells within the host and their ability to repair damaged pathways which is of crucial importance to consider while developing therapeutic strategies for TBI. The success of stem-cell therapy after cortical injuries critically depends on the integration and survival of the transplant to maintain anatomical and functional recovery. In this regard, investigating neuroinflammation and neurogenesis following TBI-neural cell transplantation would increase the success of functional recovery and regeneration within the injured cortex in future TBI studies.

To this end, in this chapter we first aimed to assess the effect of transplantation on neuroinflammation and neurogenesis, following TBI in a mild controlled cortical impact (mCCI) mouse model, using embryonic neural stem cells (ENSC) and differentiated cells (DCs). For this reason, we transplanted ENSC or DC into lesioned mouse cortex, 7 days after lesion. We then examined the effect of transplanted cells on neurogenesis as well as

neuroinflammation, 1 or 4 weeks following grafting. The results of this study are addressed in the first article of this chapter.

Second, given the neuroprotective properties of DHA upon neural damage, neural stem cells (NSCs) originating from the subventricular zone (SVZ) of neonatal mice were grafted in combination with DHA administration to assess the functional and cellular outcomes of transplantation in a mild TBI mouse model. This study is presented as a second article in this chapter and it was mainly conducted by Hussein Ghazale, the lead author.

(Article 1)

Transplantation of Embryonic Neural Stem Cells and Differentiated Cells in a Controlled Cortical Impact (CCI) Model of Adult Mouse Somatosensory Cortex



Transplantation of Embryonic Neural Stem Cells and Differentiated Cells in a Controlled Cortical Impact (CCI) Model of Adult Mouse Somatosensory Cortex

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Traumatic brain injury (TBI) is a major cause of death worldwide. Depending on the severity of the injury, TBI can reflect a broad range of consequences such as speech impairment, memory disturbances, and premature death. In this study, embryonic neural stem cells (ENSC) were isolated from E14 mouse embryos and cultured to produce neurospheres which were induced to generate differentiated cells (DC). As a cell replacement treatment option, we aimed to transplant ENSC or DC into the adult injured C57BL/6 mouse cortex controlled cortical impact (CCI) model, 7 days post-trauma, in comparison to saline injection (control). The effect of grafted cells on neuroinflammation and neurogenesis was investigated at 1 and 4 weeks post-transplantation. Results showed that microglia were activated following mild CCI, but not enhanced after engraftment of ENSC or DC. Indeed, ipsilateral lesioned somatosensory area expressed high levels of Iba-1+ microglia within the different groups after 1 and 4 weeks. On the other hand, treatment with ENSC or DC demonstrated a significant reduction in astrogliosis. The levels of GFAP expressing astrocytes started decreasing early (1 week) in the ENSC group and then were similarly low at 4 weeks in both ENSC and DC. Moreover, neurogenesis was significantly enhanced in ENSC and DC groups. Indeed, a significant increase in the number of DCX expressing progenitor cells was observed at 1 week in the ENSC group, and in DC and ENSC groups at 4 weeks. Furthermore, the number of mature neuronal cells (NeuN+) significantly increased in DC group at 4 weeks whereas they decreased in ENSC group at 1 week. Therefore, injection of ENSC or DC post-CCI caused decreased astrogliosis and suggested an increased neurogenesis via inducing neural progenitor proliferation and expression rather than neuronal maturation. Thus, ENSC may play a role in replacing lost cells and brain repair following TBI by improving neurogenesis and reducing neuroinflammation, reflecting an optimal environment for transplanted and newly born cells.

Keywords: TBI, NSC, neurosphere, astrocyte, neuron, lesion, transplantation, cortex

INTRODUCTION

The complexity of the cerebral cortex in terms of cell specificity and projections is revealed by the difficulty to repair damaged areas caused by injuries or diseases within the central nervous system (CNS). Indeed, the loss of cortical neurons is a common characteristic to many neuropathological conditions such as lesions (trauma, stroke) or neurodegenerative diseases (amyotrophic lateral sclerosis, Huntington's disease) and is associated with irreversible functional deficits and cell death (1, 2). Traumatic brain injury (TBI) is a worldwide silent epidemic problem leading to the disability and sometimes the death of patients (3). Annually, one out of 200 people suffers from TBI (4) and an estimated 1.5 million people lose their lives due to TBI (5). Mild TBI, which accounts for ~75% of all head injuries (6), is a concussion that causes neurobiological modifications in the CNS (7), short-term confusion, cognitive impairments, and transient unconsciousness (8). According to pathology, TBI causes primary injury due to mechanical forces at the time of the incident followed by a secondary injury. The latter is characterized by cytotoxicity, cerebral edema, oxidative stress, apoptosis, mitochondrial dysfunction, and inflammation (9–11). After brain injury, the blood-brain barrier (BBB) is disrupted allowing the migration of immune cells to the site of injury associated with long-lasting neuronal dysfunction and cell death (12).

Many studies have shown spontaneous functional recovery after TBI (13); however, it remains limited (14). Animal models mimicking patients TBI have been established to better understand TBI pathophysiology and to develop potential treatments (15–19). Several preclinical studies have targeted secondary injury events in animal TBI models to test the therapeutic efficacy of drugs such as calcium channel blockers, inhibitors of lipid peroxidation, excitatory amino acid inhibitors and others (20). Except for few limited treatments relieving specific symptoms, especially in acute animal models, available TBI therapies were either ineffective on patients or didn't reach clinical trials (21–24). The role of stem cells in tissue reconstruction, neuroprotection and trophic support to the host tissue makes them a potential therapeutic option (25). Recently, neural transplantation as a prospective therapy for TBI has emerged using mesenchymal stem cells (MSCs) (26), neural stem cells (NSCs) (27–30), induced pluripotent stem cells (iPSCs) (31) and embryonic neural stem cells (ENSCs) (32).

In response to TBI, the capacities of axonal regrowth and spontaneous regeneration of the CNS becomes limited. ENSCs are the optimal cell source for neural transplantation owing to their unlimited capacity of self-renewal and plasticity. When transplanted into damaged brain areas, these cells are capable of differentiation, migration, and innervation (32). A recent study confirmed that transplantation of pre-differentiated human

ENSCs increased angiogenesis and reduced astrogliosis along with improving long-term motor function (33, 34). Another study established that transplantation of differentiated human ENSCs into severely injured controlled cortical impact (CCI) rat brains resulted in neuronal survival at the lesioned area, reduction in astrogliosis and enhancement in angiogenesis (33). Moreover, it was shown that transplantation of embryonic cortical neurons, immediately after injury in the adult motor cortex, allows the anatomical reconstruction of injured motor pathways and the development of projections to appropriate cortical and subcortical host targets (35, 36). Furthermore, it was demonstrated that cortical progenitors are capable to develop into specific neuronal phenotypes and to express specific transcription factors according to their developmental program (37). Neuronal transplantation appears as a promising therapeutic strategy to replace neurons and damaged pathways. Its effectiveness depends on the capacity of grafted cells to develop appropriate cellular populations and to integrate into host preexisting neuronal circuitry. Understanding the spatiotemporal development of different cell populations within the graft, and their axonal outgrowth, is of crucial importance.

In fact, TBI can lead to both sensorimotor and cognitive deficits (38). It could be hypothesized that many of the deficits observed after TBI, whether cognitive or sensory, are aggravated by damages in sensory processing (39). Multiple studies used different models of trauma or ischemia in the somatosensory cortex and aimed to determine the reproducibility of these models for behavioral and functional consequences after the injury (40, 41). In the present study, we aimed to establish a mCCI model targeting the primary somatosensory cortical area followed by studying the consequences of transplantation of embryonic neural stem cells (ENSCs) or differentiated cells (DCs) on inflammation and neurogenesis.

In this study, following mCCI, we hypothesized that cell therapy by transplantation of ENSCs or differentiated cells (DCs) can reduce neuroinflammation and promote neurogenesis in the adult injured cortex. Therefore, a mCCI was performed in the cortical somatosensory area of adult C57BL/6 mice followed by transplantation, 7 days post-injury, with either an injection of ENSCs, DCs, or saline. Using specific markers, the capacity of transplanted cells to improve injured cortical area, replace lost cells and decrease neuroinflammation was then investigated after CCI. Furthermore, a time-course analysis was performed at 1 and 4 weeks post-transplantation comparing the response to the transplantation of ENSCs and DCs after mCCI.

METHODS

Animals

This study was approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (AUB). All animal experimental procedures and housing were carried out in accordance with the guidelines of the Agriculture Ministry and the European Communities Council Directive (2010/63/EU). All experiments were conducted in compliance with current Good Clinical Practice standards and in accordance with relevant guidelines and regulations and the

Abbreviations: ENSC, embryonic neural stem cells; DC, differentiated cells; BBB, blood-brain barrier; CCI, controlled cortical impact; IF, immunofluorescence; GE, Ganglionic eminences; GFAP, glial fibrillary acidic protein; DCX, doublecortin; NeuN, neuronal nuclei; PBS, Phosphate-buffered saline; Iba-1, ionized Ca binding adaptor molecule; H&E, Hematoxylin and Eosin; SVZ, subventricular zone; DG, dentate gyrus.

principles set forth under the Declaration of Helsinki (42). All efforts were made to reduce the number of animals used and their suffering. Female mice were coupled with males for one night before removal of the male. Females were then tested for vaginal plug and positive mice were considered pregnant. A total of 56 mice were used in this study: 8 C57BL/6 pregnant mice at E14 were used as donors of ENSCs and DCs, 16 mice were used as controls (TBI/injected with saline) and divided into 2 time points (1 and 4 weeks, $n = 8$ each), 16 mice were transplanted with ENSCs after TBI and divided into 2 time points (1 and 4 weeks, $n = 8$ each), and 16 mice were transplanted with DCs after TBI and divided into 2 time points (1 and 4 weeks, $n = 8$ each).

Establishment of the Experimental CCI *in-vivo* Model

A CCI device (Leica Angle Two System, Leica microsystem, USA) was used to produce TBI in mice as described previously (43). Briefly, adult (6–8 weeks old) C57BL/6 mice (~20 g, $n = 48$, Jaxon laboratories, Maine, USA) were anesthetized with a mixture of xylazine (90 mg/kg, Panpharma) and ketamine (10 mg/kg, Interchemie), injected intra-peritoneally. The animal was held in the instrument U frame with the head at the closed end of the "U." Animals were supported in a stereotactic frame in a prone position and secured by ear and incisor bars. To secure the animal during surgery, the ear bars, nose clamp, and incisor bar were attached to the U frame. Thereafter, a midline scalp incision was made and the skin was retracted from the skull surface. Using the software associated with the device, a target site between bregma and lambda was set, where craniotomy was made. A unilateral (ipsilateral to the site of impact) craniotomy (2 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda (ML: 1.88 mm, AP: -0.57 mm, DV: -1.58 mm). To produce mild CCI in the somatosensory area, an impact was induced by an impactor of 1 mm diameter with an impact velocity of 4 m/sec, a depth of 1 mm and a dwell time of 150 ms, after which the surgical site was sutured. CCI was always performed at 7 days prior to cell injection. ENSCs were injected after 14 days of cell culture (P2) whereas DCs were injected 21 ± 3 days thereafter.

Experimental Design

A total of 48 mice, divided into 6 groups of 8 mice each, were used in this study. After 7 days of CCI lesion establishment, two groups of 8 mice each received a single injection of either saline, embryonic stem cells (ENSC, 1.5×10^5 cells) or differentiated cells (DCs, 1.5×10^5 cells). Injection of cells or saline was performed using a Hamilton syringe in a total volume of 3 μ l. One group of mice was sacrificed after 7 days of injection (1 week) while the second group was left for 28 days (4 weeks). A total of 4 mice from each group and time point were used for immunofluorescence (IF). Ipsilateral and contralateral cortical or hippocampal brain tissue samples were then harvested and kept at -80°C for further analysis.

Harvesting ENSCs From E14 Embryos

ENSCs were isolated from E14 embryos after anesthesia of pregnant females. Briefly, heads were separated from the embryos

at the level of cervical spinal cord, a horizontal cut made at the level of the eyes and then midline from the forehead toward the back of the head. Brains were removed by pushing the edges of the cut section, held steady and then a cut was performed through the cortex of each hemisphere, originating from the olfactory bulbs to the back of the hemisphere. Ganglionic eminences (GE) containing the ENSCs were then exposed and micro-dissected.

Neurosphere Assay

The harvested GE tissue is dissociated by gentle pipetting in 3 ml complete media (F12-DMEM, Sigma) supplemented with 3% B27 (50X), 0.5% N2 (100X), 1% ABAM, 1% glutamax and 1% BSA (all from Gibco). The mixture was then centrifuged for 5 min at 110 g and the pellet was resuspended in 1 ml complete media. After counting the cells using a hemacytometer, a total of 200,000 cells were seeded in T25 flasks containing 5 ml of complete media supplemented with 10 μ l EGF (20 ng/ml) and incubated at 37°C and 5% CO₂. At this stage, the obtained cells (Passage 0/Day 0, P0/D0) would grow into primary neurospheres, which takes 6–7 days to observe in the flask. After 3 days (D3) of culture, 1–2 ml complete media supplemented with 2 μ l EGF (20 ng/ml) were added. At D 6–7, neurospheres were observed and were ready for the first passage. The suspensions of neurospheres were then collected, centrifuged at 110 g for 5 min. A total of 1 ml trypsin-EDTA (0.05%) was then added on the neurosphere pellet for about 2–3 min at 37°C and then its activity was stopped using 2 ml DMEM. The suspension of dissociated cells was then centrifuged at 110 g for 5 min, re-suspended in 1 ml complete media, and counted using a hemacytometer. A total of 4×10^5 cells were then distributed in T25 flasks containing 5 ml of complete media supplemented with 10 μ l EGF (20 ng/ml), and incubated at 37°C and 5% CO₂. At this stage, the obtained cells (P1/D7) would grow into secondary neurospheres. This time point (D7) is the day of performing CCI. Three days after the first passage (D10), 1–2 ml complete media supplemented with 2 μ l EGF (20 ng/ml) were added to each flask. Seven days after their passage (D14), secondary neurospheres were ready for injection.

Neuroblast Assay (NBA)

To produce differentiated cells (DCs), the neuroblast assay was used which is divided into 2 parts: the proliferation stage and the differentiation stage. During the proliferation stage (3–4 days), cells obtained from secondary neurospheres (P2) were plated at a concentration of 4×10^5 cells/ml in the above ENSC complete media supplemented with 20 ng/ml EGF and 5% heat-inactivated FCS (Sigma). Culture flasks were then incubated in a 37°C humidified incubator with 5% CO₂. The obtained cells proliferated and grew in a monolayer attached to the substrate. During the differentiation stage (3–4 days), when cells became 90% confluent, the media of each flask was switched to ENSC complete media supplemented with 5% FCS, without any growth factors. Culture flasks were again incubated in a 37°C humidified incubator with 5% CO₂. Neuronal progenitors appeared on top of an astrocytic monolayer. These neuronal progenitors proliferated and produced colonies of immature neuronal cells.

Immunocytochemistry (ICC) of Neurospheres

Secondary neurospheres were fixed with 4% PFA for 20 min and then washed twice in PBS (1X) for 5 min. Cells were then resuspended and washed in PBST (0.5% Triton-X in PBS 1X) 3 times for 10 min each. Thereafter, neurospheres were blocked (3% BSA+ 10% HS in PBST) for 20 min at room temperature and then rinsed again with PBST 3 times for 10 min each. Cells were then incubated for 2 h with primary antibodies against glial fibrillary acidic protein GFAP (rabbit anti-mouse polyclonal antibody PAb, Abcam, 1/1,000) and Nestin (goat anti-mouse polyclonal antibody, Abcam, 1/3,000), washed 3 times in PBST for 10 min each and incubated with appropriate secondary antibodies (goat anti-rabbit, Cruz Fluor 594, 1/1,000 and rabbit anti-goat, Alexa Fluor 488, Abcam, 1/500) for 1 h at room temperature. The nuclei were counter stained with Hoechst for 10 min and washed 3 times in PBST for 10 min each. Finally, stained neurospheres were mounted on microscopic slides and revealed by a fluorescent microscope (Axio Observer Inverted Microscope, Carl Zeiss, Germany).

Immunocytochemistry (ICC) of Differentiated Cells (DCs)

Cells in NBA cultures were validated by a double immunostaining assay using glial and neuronal specific markers. Cells were first fixed with 4% PFA for 25 min and then washed twice in PBS (1X) for 5 min. DCs were permeabilized in PBST (0.1% Triton-X in PBS 1X) 3 times for 10 min each then blocked (5% FBS in PBST 1X) for 30 min at room temperature. DCs were then rinsed with PBST 3 times for 10 min each, followed by subsequent midnight incubation with primary antibodies against GFAP (mouse anti-GFAP, Abcam, 1/1,000), TuJ1 (rabbit anti-mouse PAb to beta-Tubulin III, Abcam, 1/2,000) and Neuronal Nuclei NeuN (rabbit anti-mouse PAb to NeuN, Abcam, 1/1,000). Appropriate secondary antibodies (donkey anti-mouse PAb, Alexa-fluor 488, 1/1,000 and goat anti-rabbit, Alexa Fluor 488, 1/1,000, Abcam) were added for 1 h at RT followed by washing the cells in PBST (3x, 10 min each). Hoechst was then added for 5 min to counter stain the DCs nuclei which were then washed in PBST (2x for 10 min each). Finally, DCs were mounted on microscopic slides and revealed by a fluorescent microscope (Axio Observer Inverted Microscope, Carl Zeiss, Germany).

Transplantation

After cell culture, ENSCs and DCs were ready to be injected in mouse brains at the somatosensory area injury site. After cell counting, a total of 1.5×10^5 cells were prepared in 3 μ L DMEM in a Hamilton syringe for injection into each mouse. Adult (6–8 weeks old) C57BL/6 mice ($n = 48$, Jaxon laboratories, Maine, USA) were used as recipients after establishing TBI lesion. The origin of cortical donor cells was from embryonic day 14 C57BL/6 mice ($n = 8$) embryos. Briefly, the mouse was anesthetized, placed in the stereotaxic frame and the CCI machine impactor was replaced with the Hamilton syringe containing the 3 μ L suspension of cells. A midline incision was made to expose the skull, then Bregma and Lambda points were located manually

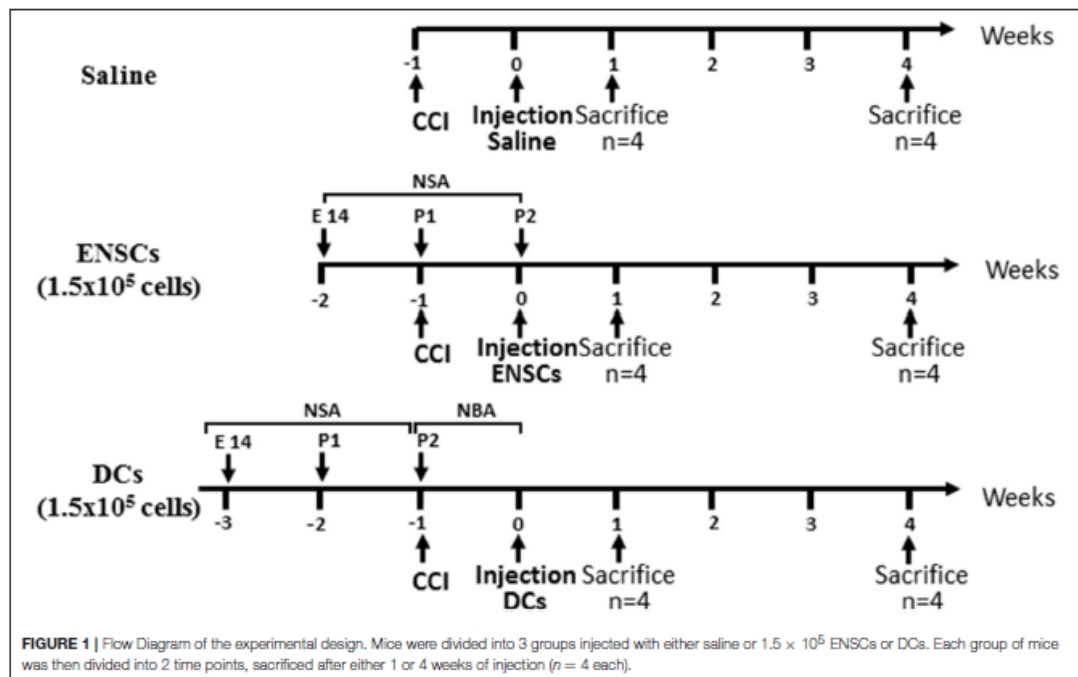
using the Hamilton syringe. The latter is then moved to “zero” in the instruments mediolateral (ML) and anteroposterior (AP) coordinates; at this point, it is right above the target site. Using the dorsoventral (DV) drive, the syringe is lowered until it reaches the lesion site (coordinates; ML: 1.88 mm, AP: -0.57 mm, DV: -1.58 mm). This is followed by lowering the DV axis by 1 mm to perform the injection at the lesion core. Following the establishment of CCI lesion, mice were maintained for 1 week and then divided into 3 groups ($n = 8$ each) which received a single injection of either saline, ENSCs or DCs (at 1.5×10^5 cells). Each group of mice was then divided into 2 time points ($n = 4$ each), sacrificed after either 1 or 4 weeks of injection (Figure 1).

Perfusion and Brain Tissue Preparation

Control mice without transplantation (CCI), and mice at different time points after transplantation of either ENSCs (CCI+ENSC) or DCs (CCI+DC) (day 7 and day 28) were anesthetized and their brains perfused, removed and sectioned into brain slices. Briefly, mice were injected intra-peritoneally with a mixture of xylazine (90 mg/kg, Panpharma) and ketamine (10 mg/kg, Interchemie), and perfused transcardially with 20 ml PBS (1X) followed by 30 ml of ice-cold paraformaldehyde (PFA, 4%). Brains were then removed and fixed in 4% PFA overnight. They were cut into 40 μ m coronal sections with a microtome (Leica Microsystems, USA) and stored at 4°C in 0.01% sodium azide (97.5 mg of NaN₃ in 100 mL PBS 1X). Brain sections were used to perform histological observations such as immunofluorescence (IF), Cresyl violet (Nissl stain) and Haematoxylin and Eosin staining (H&E).

Immunofluorescence

This was performed as previously described (44). Briefly, brain slices were first washed twice with PBST (0.1% Triton in PBS, 1X) for 5 min each, at room temperature (RT) and on a shaker. A blocking solution (5% FBS in PBST) was then added (1 mL/well) for 1 h with continuous shaking before adding the primary antibody (500 μ L/well), which was left overnight at 4°C. Polyclonal antibodies used were: rabbit anti-mouse GFAP (1:1,000, Abcam), goat anti-rabbit doublecortin (DCX, 1:500, Santa Cruz), a microtubule-associated protein localized in somata and processes of migrating and differentiating neurons, rabbit anti-mouse ionized Ca binding adaptor molecule (Iba-1, 1/1,000, Abcam) and rabbit anti-mouse NeuN (1/1,000, Abcam). After incubation, sections were washed for 3 times with PBST (15 min each, RT, on a shaker), and then incubated for 1 h at RT in the appropriate secondary antibody (500 μ L), previously diluted in blocking solution. The secondary antibodies used were either goat anti-rabbit PAb (Alexa-Fluor 488, 1/1,000, Abcam) or rabbit anti-goat PAb (Alexa Fluor 488, 1/500, Abcam) specific for primary antibodies. Hoechst stain was then added (1 ml) for 5 min and sections were washed twice for 5 min each at RT and on a shaker. Slices were then mounted on microscope slides (star frost) using a mounting solution (2–3 drops) and a cover slip. Slides were then evaluated and photographed using a fluorescent microscope (Axio Observer Inverted Microscope, Carl Zeiss, Germany). Appropriate negative controls were performed in each assay.



Hematoxylin and Eosin (H&E) Stain

Brain tissues were transferred into coated slides (star frost) and organized from anterior to posterior. Slices were stuck on the slide using soaked filter paper in PBS (1X) or the slide is left on the bench for 1 day until it dries. The prepared slides were then placed in distilled water (dH₂O) for 3 min for hydration then transferred to hematoxylin for up to 1 min. The slides were then placed in running tap water to provide the necessary alkalinity for “bluing” process. The slides were then immersed in eosin for up to 1 min. Slides were then placed in 95% ethanol for 2 times, 3 min each and then immersed in 100% ethanol for 5 min to prevent cell lysis. Finally, slides were placed in Xylol for 1 min, which acts as a clearing solvent to remove alcohol from tissues. Slides were then mounted and visualized on the microscope.

Nissl Stain

Brain tissue slides were first prepared, placed in distilled water (dH₂O) for 3 min and then transferred to Cresyl violet (0.5%) for up to 2 min. The slides were then placed in running tap water and then immersed in distilled water. Slides were then placed in 75% ethanol for 1min, 95% ethanol for 2min, and then immersed in 100% ethanol for 3 min. Finally, slides were placed in xylol for 2 min and were mounted and visualized on the microscope.

Data Acquisition and Quantification

For each mouse, images were acquired with a LSM710 confocal microscope, Carl Zeiss, Germany. At least four

sections corresponding to the areas of interest were used for quantifications using Image J program (NIH). For Iba-1 and GFAP expression, the total area covered by these markers at the lesioned site was quantified with respect to the total injury area. For DCX marker, the area covered by the DCX+ cells throughout the lateral SVZ was quantified with respect to the total length of the SVZ. For NeuN quantification, the total NeuN+ area in the hippocampus was quantified on both contralateral and ipsilateral sides among different groups.

Statistical Analysis

All results are expressed as mean \pm SEM. For data concerning IF, within each experimental group, statistical significance was evaluated using two-way analysis of variance (ANOVA) having the group (control, transplantation) as a parameter. The *p*-value was determined and values for $p < 0.05$, $p < 0.001$, $p < 0.0001$ (*, **, *** respectively within the same group and #, ##, ### respectively among groups) were considered significant.

RESULTS

Establishment and Validation of Mild CCI Model, Neurosphere, and Neuroblast Assays

CCI lesion was established as described in the materials and methods section. CCI was then validated using H&E staining on histological sections of the mouse brain (Figure 2). Indeed, the

expected morphological changes such as damaged parenchymal tissues and reduction in the cytoplasm of the injured cortex were observed in the lesioned somatosensory region (Figure 2). On the other hand, no significant malformations in the hippocampal region were observed. Moreover, Nissl stain confirmed that neurons residing in the ipsilateral cortex were highly injured (Supplementary Figure 1).

In parallel, ENSCs were harvested from E14 embryos and cultured using the neurosphere assay. They showed microspikes morphology, characteristic of neurospheres, at 5–6 days of culture (Figure 3A). On the other hand, the three types of differentiated cells (DCs): flat astrocytic cells, round neuronal progenitor cells, and mature neurons were obtained using the neuroblast assay (NBA), after proliferation and differentiation stages (Figure 3B). The phenotype of cells was validated using immunocytochemistry where neurospheres showed nestin and GFAP staining, thus reflecting the undifferentiated state of stem cells whereas differentiated cells were positive for Tuj-1, GFAP, and NeuN markers (Figure 3C).

Microglia Is Activated Following CCI, but Not Enhanced After Injection of ENSCs or DCs

Iba-1 protein, a microglial specific marker, is usually used as a measure of neuroinflammation and neuroprotection during different phases of CCI. Microglial activation using Iba-1 was assessed by IF on the 6 groups of CCI mice ($n = 4$ each) injected with either saline, ENSCs or DCs and maintained for 1- or 4-weeks after injection (Figure 4A and Supplementary Figure 2). Quantification of IF data demonstrated a significant increase ($*p < 0.05$) in Iba-1 expression post-CCI in saline and DCs groups, but not in ENSC group, at 4 weeks in comparison to 1 week (Figure 4B). In addition, similar morphological changes were observed in different areas of the injured cortical region among the 6 groups of mice. Indeed, bushy hypertrophied Iba-1+ microglia were found to be highly expressed around the CCI region whereas resting microglia of amoeboid morphology were observed at distant places from the CCI zone (Figure 4C). Taken together, ipsilateral lesioned cortex expresses high levels of Iba-1+ microglia after 1 and 4 weeks of injection within the different groups (Saline, ENSCs, or DCs).

Astrogliosis Is Significantly Reduced by Treatment With ENSCs at 1 Week or DCs at 4 Weeks Post-transplantation

Previous studies have shown that acute CCI induces inflammation, glial cell reactivity and a dramatic increase in the number of activated astrocytes, with hypertrophic cellular processes, leading eventually to neuronal apoptosis and “reactive astrogliosis” (45). The latter is characterized by GFAP protein, upregulated in the activated astrocytes, and occurs mostly in the ipsilateral cortex of the injured brain, as demonstrated by immunofluorescence (Figure 5A). When compared to the saline group, our data demonstrated a significant reduction in the number of GFAP expressing astrocytes in the ENSCs group, at 1 and 4 weeks post-injection ($**p < 0.01$, Figure 5B). This

reduction was also highly significant in the ENSCs group at 1 week, in comparison to DCs ($***p < 0.001$). In addition, the DCs group also showed a highly significant reduction in GFAP expression at 4 weeks, in comparison to controls ($***p < 0.001$, Figure 5B). Taken together, treatment with ENSCs or DCs (glial and neuronal cells) following mild CCI demonstrated a reduction in astrogliosis, especially 4 weeks post-injection, as shown by a decrease in GFAP expression. This decrease in GFAP levels started very early in the ENSCs group (1 week), in comparison to DCs group, which was comparable in both groups at 4 weeks.

Neurogenesis Is Significantly Enhanced at the Progenitor Level in ENSCs Before DCs Groups

Previous studies reported an activation of neurogenesis and cellular proliferation in the subventricular zone (SVZ) and dentate gyrus (DG), following CCI (46, 47). Protein expression of DCX, a marker of late neuronal progenitors, was used to demonstrate the effect of injecting ENSCs or DCs, following CCI, on neurogenesis in the SVZ region (Figure 6A and Supplementary Figure 3). Our data demonstrated a significant increase in the number of DCX expressing progenitor cells at 1 week in the ENSCs group, in comparison to control or DCs groups ($***p < 0.001$) (Figure 6B). In addition, a significant increase in DCX was also obtained in DCs and ENSCs groups at 4 weeks post-injection, in comparison to controls ($***p < 0.001$ and $*p < 0.05$; respectively) (Figure 6B). In summary, treatment with ENSCs or DCs after CCI increased the expression of DCX, therefore enhancing cellular proliferation, progenitor cell survival and neurogenesis at 1 and 4 weeks post-injection. Unlike DCs, ENSC showed an enhancement in neurogenesis 1 week after transplantation as well as reduced astrogliosis. In contrast, DCs treatment is less effective at this time point.

Expression of Mature Neurons

Protein expression of NeuN, a marker of mature neurons, was used to demonstrate the effect of injecting ENSCs or DCs, following CCI, on neuronal maturation in the hippocampus region (Figure 7A). Results showed a significant increase in the number of neuronal cells in DCs group at 4 weeks post-injection, in comparison to controls ($**p < 0.01$, Figure 7B). On the other hand, our data demonstrated a significant decrease in NeuN expressing cells in control mice at 4 weeks post-saline injection, in comparison to 1 week, but also in ENSC group at 1 week, in comparison to controls ($*p < 0.05$, Figure 7B). Finally, no significant increase in NeuN levels was observed in the other groups and time points (Supplementary Figure 4). Taken together, injection of ENSCs or DCs post-CCI suggests that it may increase neurogenesis via inducing neural progenitor's proliferation and expression rather than neuronal maturation.

DISCUSSION

Traumatic brain injury, an intracranial injury, has been recognized among the major causes of death worldwide with

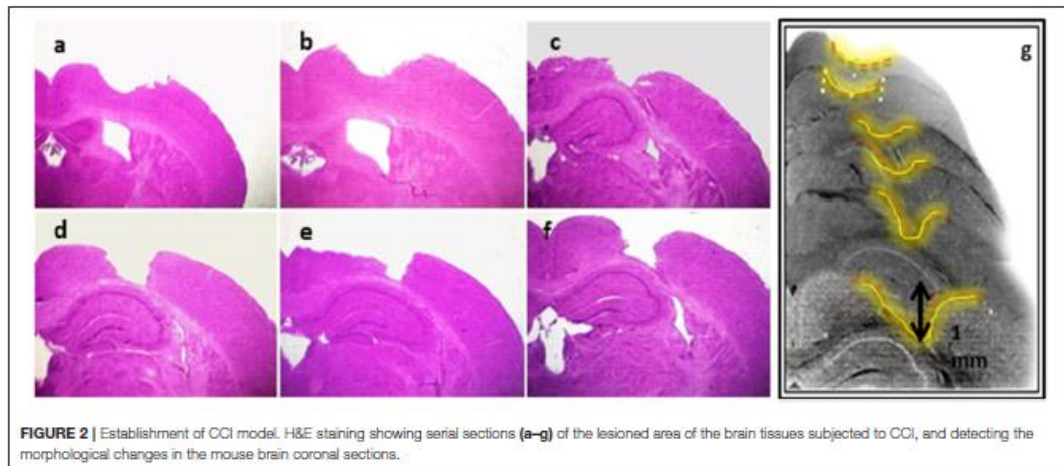


FIGURE 2 | Establishment of CCI model. H&E staining showing serial sections (a–g) of the lesioned area of the brain tissues subjected to CCI, and detecting the morphological changes in the mouse brain coronal sections.

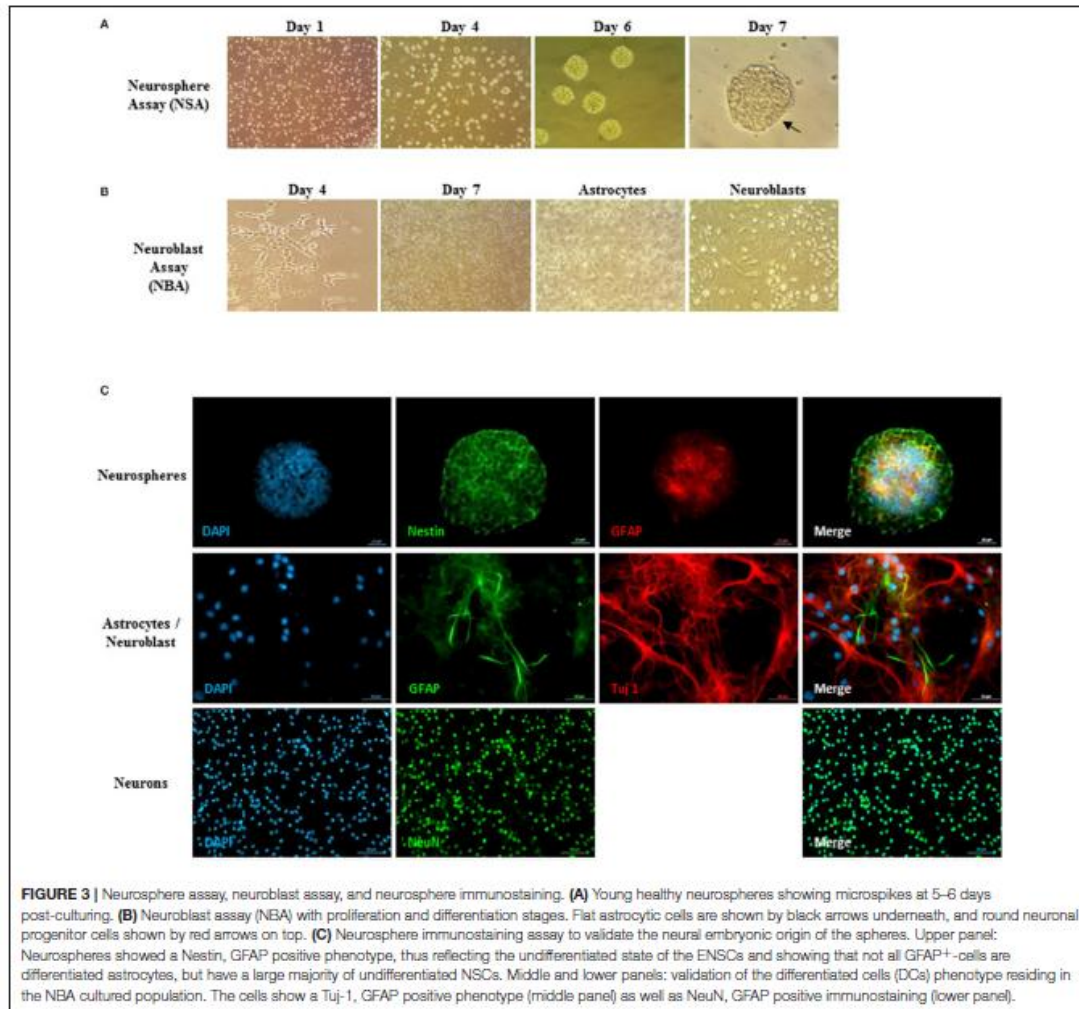
broad devastating symptoms and disabilities. TBI causes primary injury due to mechanical forces at the time of the incident followed by a secondary injury. The latter is characterized by cytotoxicity, cerebral edema, oxidative stress, necrosis, apoptosis, mitochondrial dysfunction, and inflammation (9–11). These neuropathological sequelae happen progressively, aggravating the neural injury and inducing neuropsychological and motor deficits. Thus, several options have been proposed as a prospective therapy for TBI including stem cell transplantation using mesenchymal stem cells (MSCs) (26, 48), neural stem cells (NSCs) (27–30), induced pluripotent stem cells (iPSCs) (31) and embryonic neural stem cells (ENSCs) (32). Thus, the aim of our study was to investigate the effect of grafting different cell types (ENSCs or DCs) on neuroinflammation and neurogenesis after mild CCI. This study describes the environment surrounding the injury after transplanting ENSCs or DCs in the same conditions.

Neuroinflammation is considered as a potential therapeutic target due to its correlation with neurological symptoms and pathology (49). In fact, inflammatory response post-TBI is signaled by glial cell activation, peripheral leukocyte recruitment via disturbed BBB and rapid rise in the levels of inflammatory mediators such as cytokines and chemokines (50, 51). Mediators and cellular events activate the immune cells and induce their migration toward the injured area (52). For instance, neutrophils are the first cells infiltrating the injured brain followed by migration of microglia and activation of astrocytes (52). Neuroinflammation can be either harmful by inducing oxidative damage and neuronal death or beneficial by promoting the clearance of debris and tissue remodeling (53).

Our data showed an up-regulation of microglial activation which is in accordance with a recent study in moderate TBI, using the CCI model, demonstrating that microglial activation occurred 1-week post-CCI, which significantly increased 5 weeks after injury and persisted for 1 year (54). Since there is an interaction between astrocytes and microglia post-CCI,

DCs which contain populations of astrocytes, but not ENSCs, affect the microglial expression more significantly at 1 week. It was previously demonstrated within the cortex of a moderate diffuse TBI mouse model that the expression of tumor necrosis factor alpha (TNF α) and IL-6 peaked at 3–9 h post-TBI (55). Similarly, increased microglial activation was shown at 6 h post-TBI in pigs, indicating an association between the induction of an inflammatory response and brain pathology (56). Microglial activation is well established as one of the secondary biochemical changes following TBI, providing an evidence of neuroinflammation post-TBI (54). Indeed, microglial activation is characterized by morphological as well as functional modifications where the resting ramified microglia transforms into hypertrophic bushy cells involved in secretion of pro- and anti-inflammatory molecules (54). In fact, M1-like microglial activation is implicated in the deterioration of injury due to the release of cytotoxic mediators (57, 58) whereas M2-like microglial activation contributes to injury recovery and repair due to the release of neurotrophic and immunomodulatory factors (59, 60). Several TBI studies reported a peak in M2 microglia during the first 7 days after injury; however, this phenotype shifts to M1 microglia thereafter (61, 62). In another model of stroke, the M2 marker CD206 was highly expressed 3 days after ischemia whereas the M1 marker MHCII was found highly expressed at day 7 post-ischemia (63). Our observation of microglial up-regulation following mild CCI suggests that induced neuroinflammation may last for a long time after the initial brain insult, increasing the risk of persisting neurodegeneration and therefore the need of targeting inflammatory events using new therapies. Chronic microglial activation is associated with up-regulation of pro-inflammatory cytokines such as IL-1 β and TNF α (64) contributing to long-term neurodegeneration following TBI.

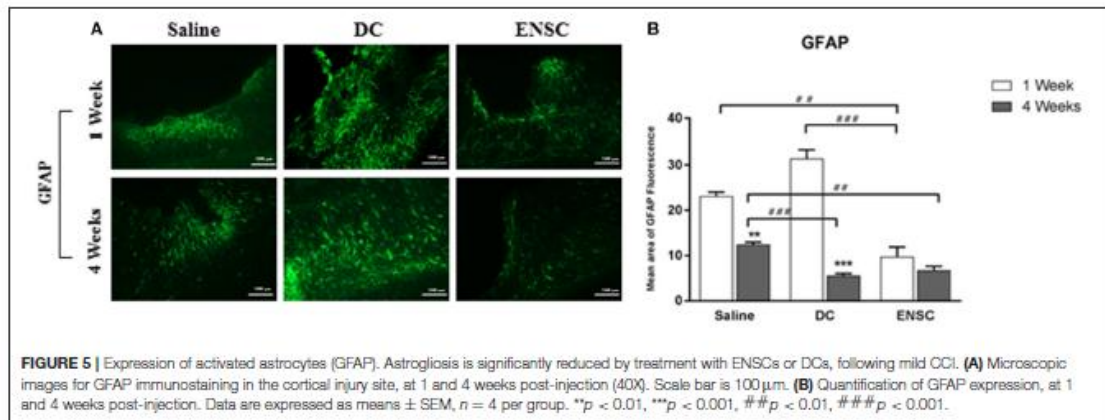
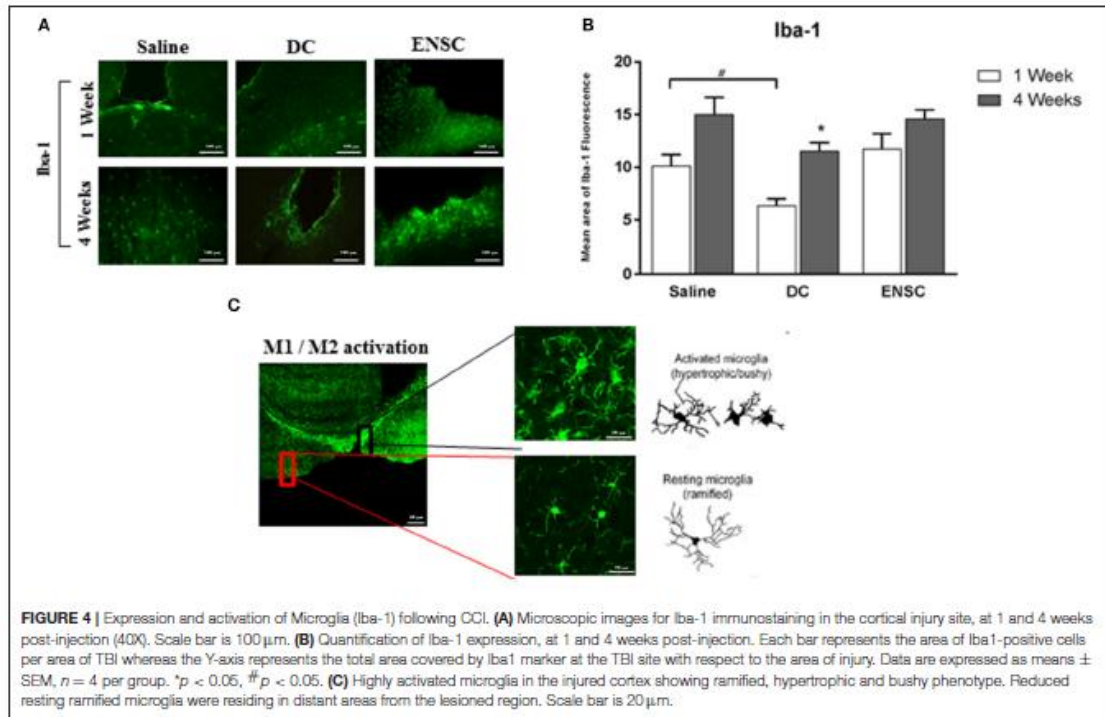
On the other hand, our data demonstrated a significant reduction in the number of GFAP expressing astrocytes in



control and DC groups at 4 weeks post-injection, in comparison to 1 week. In addition, GFAP decreased in the ENSC group at 1 week, in comparison to control or DC groups, which was maintained at 4 weeks. Previous studies have shown that TBI causes a dramatic increase in the number of activated astrocytes with hypertrophic cellular processes resulting in “reactive astrogliosis” (45). In fact, astrocytes affect many essential neural functions in normal CNS by maintaining the extracellular balance of ions and neurotransmitters, regulating the blood flow and influencing synaptic activity and plasticity (65). Therefore, dysfunction in the mechanisms underlying scar formation and reactive astrogliosis cause detrimental effects on the CNS (65). During the acute phase of TBI,

inflammation and glial cell reactivity are induced due to the alteration in the BBB leading eventually to neuronal apoptosis (45). Our results demonstrated that following mild CCI, treatment with ENSCs or DCs (glial and neuronal cells) caused a reduction in astrogliosis, suggesting that our cell therapy at 1 and 4 weeks’ time intervals provide a neuroprotective (anti-inflammatory) role which helps in recovery post-CCI.

One of the major results of this study was that Doublecortin (DCX) expression increased significantly in the CCI groups after transplantation. In fact, our data showed a significant up-regulation in the number of DCX expressing progenitor cells in DCs and ENSCs groups at 4 weeks post-injection, in



comparison to controls, in addition to a significant increase at 1 week in the ENSCs group, in comparison to control and DC groups. Indeed, it is well known that the subgranular zone (SGZ) of the DG in the hippocampus, in addition to the SVZ region, is a site of neurogenesis in adults where there is a continuous regeneration of neuronal cells (66). Recent evidence showed that neurogenesis occurs near the damaged brain regions after TBI in humans, where the newly produced

cells differentiate into mature neurons after migrating into the boundary zone of the lesioned cerebral cortex (67). In addition, spontaneous behavioral recovery is initiated after activation of endogenous neurogenesis following TBI (68). Despite this initial cellular proliferation and neurogenesis after trauma, survival and maturation of cells are not maintained since this early endogenous recovery is not sufficient to sustain the progenitor cell population (69). It was shown that only 20% of the newly

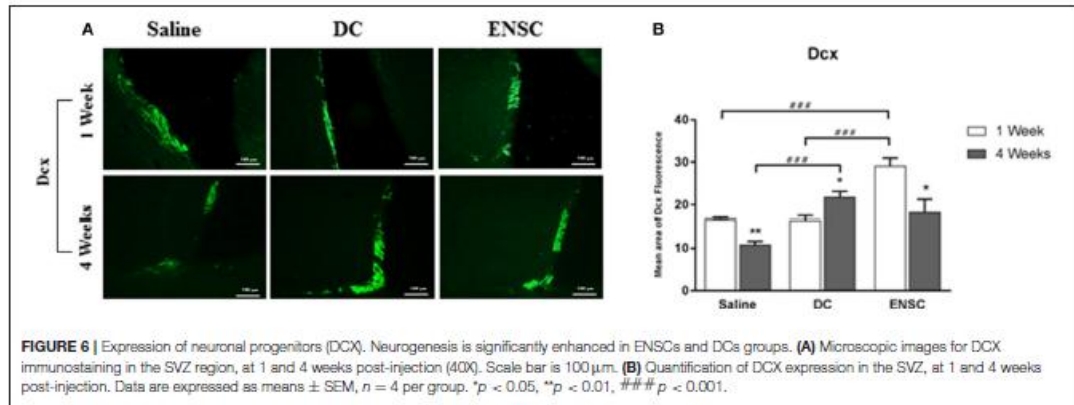


FIGURE 6 | Expression of neuronal progenitors (DCX). Neurogenesis is significantly enhanced in ENSCs and DCs groups. **(A)** Microscopic images for DCX immunostaining in the SVZ region, at 1 and 4 weeks post-injection (40X). Scale bar is 100 μ m. **(B)** Quantification of DCX expression in the SVZ, at 1 and 4 weeks post-injection. Data are expressed as means \pm SEM, $n = 4$ per group. * $p < 0.05$, ** $p < 0.01$, ### $p < 0.001$.

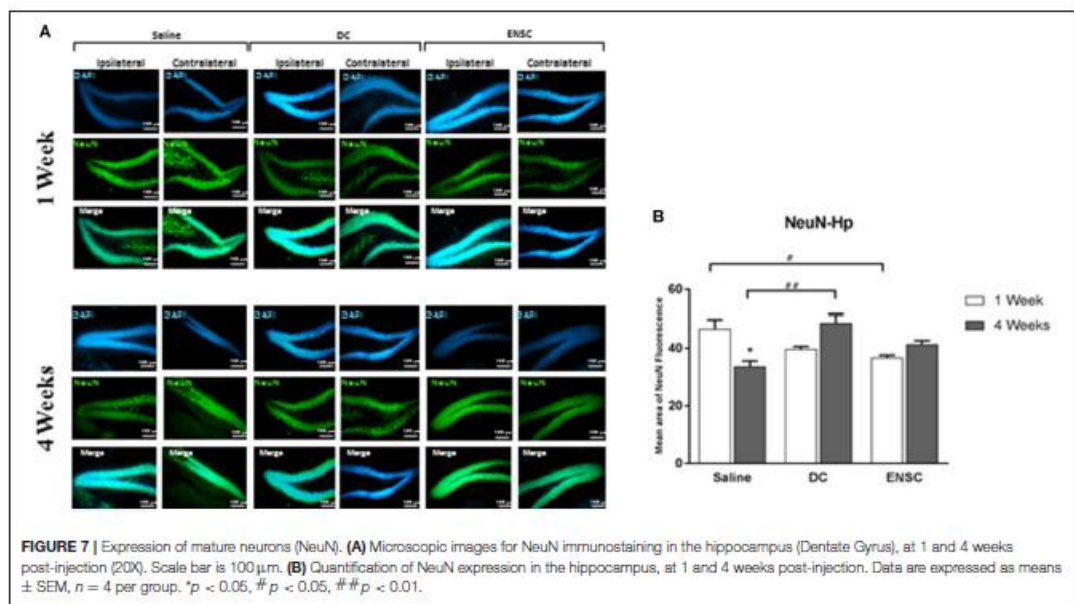


FIGURE 7 | Expression of mature neurons (NeuN). **(A)** Microscopic images for NeuN immunostaining in the hippocampus (Dentate Gyrus), at 1 and 4 weeks post-injection (20X). Scale bar is 100 μ m. **(B)** Quantification of NeuN expression in the hippocampus, at 1 and 4 weeks post-injection. Data are expressed as means \pm SEM, $n = 4$ per group. * $p < 0.05$, # $p < 0.05$, ## $p < 0.01$.

born striatal neurons survived for 2 weeks following middle cerebral artery occlusion in rats (70). Similarly, after intracerebral hemorrhage, most newly generated cells, observed between 72 h and 1 week, did not survive more than 3 weeks (71). It's important to note that it takes 4 weeks for the new progenitors to become functionally mature neurons (72). Moreover, the generated immature neurons are sensitive to environmental factors such as the release of inflammatory mediators by activated microglia and astrocytes which affect the newly produced progenitor cells, suppressing long-term neurogenesis after TBI (69). Interestingly, targeting the inflammatory events after brain injury may augment neurogenesis and maintain its activation.

Several studies have demonstrated the complex role of microglia in induced neurogenesis following TBI (20).

Using NeuN as a marker of mature neurons, we validated the effect of cell transplantation on neurogenesis. Although it takes 4 weeks to obtain mature and functional neurons, the control group showed a decrease in NeuN expression after 4 weeks of injury. On the other hand, the groups receiving DCs demonstrated a significant increase in NeuN expression.

One limitation in our study, however, was the incapacity to distinguish engrafted neurons and their axons from host tissue. In fact, it was not possible to characterize the transplanted neurons, to study their survival and migration and to assess

possible developed projections of the engrafted cells. Alternatives to overcome this limitation would be to use GFP mice (73) or fluorescently labeled cells for transplantation (74). As for the survival of the engrafted cells, it is affected by the reduced perfusion, i.e., low vascularization and oxygen levels, and the persisting neuroinflammation in the lesion core. The inflammatory response seems to impact neuronal survival depending on a balance between pro- and anti-inflammatory mechanisms induced by different factors. Chronic neuroinflammation induced after injury increases the risk of persisting neurodegeneration and therefore reduces the survival of engrafted cells. Meanwhile, in another model of lesion, it was shown that a 1-week delay between the cortical lesion and embryonic neurons transplantation can significantly enhance graft vascularization, cell proliferation, survival and density of projections developed by grafted neurons, leading to a beneficial impact on functional repair and recovery (75).

Taken altogether, 4 weeks after injection of ENSC or DCs, our data revealed no preference for the transplantation of one cell type over the other. In fact, microglial activation was enhanced in both groups whereas astrogliosis was reduced, which also suggests that ENSCs and DCs may play a critical role in targeting the inflammatory events and in enhancing early progenitor cell survival. Moreover, transplantation of both cell types, ENSCs or DCs, enhanced neurogenesis. Hence, to repair the damaged brain, concomitant use of both ENSC and DCs, targeting post-injury neuroinflammation and enhancing the survival of newly generated cells by supporting an optimal environment for the cells, may provide a new perspective for cell therapy.

AUTHOR CONTRIBUTIONS

MN, SM, FB, FN, NR, KZ, and FK carried out the experiments. MN, NB, KZ, and JS analyzed data. KZ and FK designed and supervised the study. FK, MN, and KZ wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2018.00895/full#supplementary-material>

Supplementary Figure 1 | Nissl staining. Brain tissue sections of CCI mice injected with saline, DCs or ENSC, respectively, 1 week post-CCI. Cortex at CCI region (ipsilateral) with 10x magnification showing the injured area depth of 1mm. Higher magnification confirmed damaged parenchymal tissues in the cortex post-CCI (data not shown).

Supplementary Figure 2 | Expression of Microglia (Iba-1) in the cortex following transplantation. (A,B) Microscopic images for Iba-1 immunostaining, at 1 and 4 weeks post-injection. Scale bar is 100 μ m. (C) High magnification images from regions of interest showing immunolabeled microglia. Scale bar is 20 μ m.

Supplementary Figure 3 | Expression of neuronal progenitors (DCX) following injection. Microscopic images for DCX immunostaining, in (A) saline, (B) DCs, and (C) ENSCs groups, at 1-week post-injection. Scale bar is 100 μ m.

Supplementary Figure 4 | Expression of mature neurons. (A) Microscopic images for NeuN immunostaining, at 1 and 4 weeks post-injection. Scale bar is 100 μ m. (B) Quantification of NeuN expression in the Cortex, at 1 and 4 weeks post-injection. Data are expressed as means \pm SEM, $n = 4$ per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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(Article 2)

Docosahexaenoic acid (DHA) enhances the therapeutic
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Neural stem cell transplantation post—Traumatic brain
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Research report

Docosahexaenoic acid (DHA) enhances the therapeutic potential of neonatal neural stem cell transplantation post—Traumatic brain injury



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ABSTRACT

Traumatic Brain Injury (TBI) is a major cause of death and disability worldwide with 1.5 million people inflicted yearly. Several neurotherapeutic interventions have been proposed including drug administration as well as cellular therapy involving neural stem cells (NSCs). Among the proposed drugs is docosahexaenoic acid (DHA), a polyunsaturated fatty acid, exhibiting neuroprotective properties. In this study, we utilized an innovative intervention of neonatal NSCs transplantation in combination with DHA injections in order to ameliorate brain damage and promote functional recovery in an experimental model of TBI. Thus, NSCs derived from the sub-ventricular zone of neonatal pups were cultured into neurospheres and transplanted in the cortex of an experimentally controlled cortical impact mouse model of TBI. The effect of NSC transplantation was assessed alone and/or in combination with DHA administration. Motor deficits were evaluated using pole climbing and rotarod tests. Using immunohistochemistry, the effect of transplanted NSCs and DHA treatment was used to assess astrocytic (Glial fibrillary acidic protein, GFAP) and microglial (ionized calcium binding adaptor molecule-1, IBA-1) activity. In addition, we quantified neuroblasts (doublecortin; DCX) and dopaminergic neurons (tyrosine hydroxylase; TH) expression levels. Combined NSC transplantation and DHA injections significantly attenuated TBI-induced motor function deficits (pole climbing test), promoted neurogenesis, coupled with an increase in glial reactivity at the cortical site of injury. In addition, the number of tyrosine hydroxylase positive neurons was found to increase markedly in the ventral tegmental area and substantia nigra in the combination therapy group. Immunoblotting analysis indicated that DHA + NSCs treated animals showed decreased levels of 38 kDa GFAP-BDP (breakdown product) and 145 kDa α II-spectrin SBDP indicative of attenuated calpain/caspase activation. These data demonstrate that prior treatment with DHA may be a desirable strategy to improve the therapeutic efficacy of NSC transplantation in TBI.

1. Introduction

Traumatic brain injury (TBI) is considered a major health concern worldwide with an annual incidence of 150–200 cases per 100,000 individuals [1–3]. TBI represents a leading cause of disability and

mortality among individuals under the age of 45 [4]. TBI is defined as the damage of the brain tissues and structures caused by mechanical forces that strike the head such as car accidents, falls, and or due to a sudden hit by a moving or stationary object [5]. TBI is characterized by both morphological and physiological changes, where it can cause

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disturbances at the level of cellular integrity and/or affect the homeostasis of the brain tissues and cell functionality; thus, causing altered cellular dysfunction [6]. Given the heterogeneous nature of brain neural structure and the complexity of brain trauma, TBI pathophysiology involves cascades of concatenated primary and secondary phases of injury. Controlled cortical impact (CCI) is among the widely used injury models of TBI [7–11] and involves long-term functional deficits of motor function as well as a decline in cognitive status [12].

Despite the wealth of information available on the molecular and cellular basis of TBI, there exists no specific effective pharmacological drug treatment for TBI [2]. Thus, effective neurotherapeutic drug intervention holds great potential to modulate TBI-induced pathological cascades in an attempt to salvage neural substrates required for neurological functional recovery and to enhance the rehabilitative capacity of surviving neural tissue [13]. To this end, several neurotherapeutic strategies—both clinical trials and experimental studies—have been proposed to ameliorate TBI outcomes, including drug administration and/or cellular interventions [2,14,15]. Among these experimental approaches, neural stem cells (NSCs) of various origins have been proposed as a next-generation neurotherapeutic targets in TBI [15–26]. These cells represent a potential therapeutic adjuvant for regenerative therapy post-TBI, due to their ability to generate mature, functional neural cells able to replace degenerated ones [24]. NSCs are mostly derived from embryonic ganglionic eminence or subventricular zone (SVZ). Transplanted cells at different locations of an injured brain show migration to the site of injury, a long-term survival post-transplantation, and differentiation into neurons capable of functionally integrating with the host tissue [27–29]. This has resulted in cognitive and functional improvement in rodents [15,30]. Recently, there has been more research devoted to increase the efficacy of NSCs, and provide a suitable microenvironment for the survival and proliferation of these transplanted cells [17,31–33].

Along the same line, Docosahexaenoic acid (DHA, 22:6n-3) is among the prevalent polyunsaturated structural lipids in the central nervous system [34]. DHA promotes brain development by promoting neuronal differentiation, neurite growth, and synapse formation [34–36]. DHA supplementation improves memory [37,38] and cognition [39,40] regulating the expression of different neurotransmitters, such as glutamate. On the other hand, it is shown that a DHA-depleted brain impedes neurite growth and synaptogenesis [41]. In addition, other studies have shown that DHA has a direct neuro-supportive effect on neural cells *in vitro* [42]. Studies investigating of DHA effect on neurogenesis using neurosphere assays have indicated that DHA promotes the maintenance of both primary neurospheres that are neurogenic NSCs and tertiary neurospheres that are gliogenic NSCs [35]. DHA treatment; however, leads to the neuronal differentiation of gliogenic NSCs but not neurogenic NSCs [43–45].

In the present study, we aimed to investigate the effect of transplanted NSCs derived from neonatal mice SVZ with or without DHA pretreatment on neural regeneration in a murine TBI model assessing neural cellular changes along with behavioral outcomes. In addition, other markers including the astrocytic (Glial fibrillary acidic protein, GFAP) and microglial (ionized calcium binding adaptor molecule-1, IBA-1) activity were assessed in the DHA/NSCs treatment to evaluate altered cellular changes. This was coupled with the quantification of neuroblasts (doublecortin; DCX) and dopaminergic neurons (tyrosine hydroxylase; TH) expression in relation to the treatment of DHA. Combined NSC transplantation and DHA injections significantly attenuated TBI-induced motor function deficits (pole climbing test), promoted neurogenesis, coupled with an increase in glial reactivity at the cortical site of injury. In addition, the number of tyrosine hydroxylase positive neurons was found to increase markedly in the ventral tegmental area and substantia nigra in the combination therapy group.

We hypothesize that DHA treatment upstream the transplantation of NSCs at the site of the injured ipsilateral cortex would be a novel strategy for NSCs survival leading to amelioration of injury severity and

promoting functional recovery. Data from this work demonstrated that combined DHA injections and neonatal NSC transplantation significantly attenuated TBI-induced motor function deficits (pole climbing results) and promoted neurogenesis. These changes were accompanied by an increase in the number of dopaminergic neurons in the ventral tegmental area and the substantia nigra upon DHA-NSCs co-treatment.

2. Material and methods

2.1. Animals

The study was carried out at the Animal Care Facility of the American University of Beirut (AUB) and all animal experiments were performed in compliance with the AUB Institutional Animal Care and Use Committee (IACUC) guidelines with the reference number: (15-1-326). C57BL6 mice were obtained from Charles Rivers Laboratories and housed in a controlled environment (12 h light/dark cycles, 22 ± 2 °C). All animals were handled under pathogen-free conditions and fed chow diet *ad libitum*. A total of 31 male mice aged 7–8 weeks were subjected to TBI. One day post TBI mice were injected intraperitoneally with DMSO or DHA with a dosage of 12 mg/kg/day given on a daily basis for a period of 20 days (until sacrificed). Mice were divided into four main groups: group 1A- DMSO-injected [TBI + DMSO] ($n = 12$); group 1B-DHA-injected [TBI + DHA] ($n = 12$); group 2A-injected with DMSO and NSC transplanted [TBI + NSCs] ($n = 12$), group 2B-injected with DHA and NSCs [TBI + NSCs + DHA] ($n = 12$). Animals were randomly attributed to experimental groups in a blinded manner; in addition, the microscopic examinations were performed by two independent students in a blinded manner. Animals that showed weak health post-surgery and exhibited any disease condition were not included in the study as per the IACUC regulations. These animals were not subjected to the behavioral testing. Each group had a total number of 12 mice/group aimed at the different experiments. We had ~3 animals eliminated from each group with approximately of 14% mortality rate. We have used 9 animals per group for each behavioral testing.

2.2. Controlled cortical impact injury (CCI) model

An open head injury was performed to induce TBI using the electromagnetic controlled cortical impact device (Leica Impact One Angle with Leica Angle Two™ Stereotaxic Instrument, Biosystems, Buffalo Grove, IL, USA). Mice were anesthetized by intramuscular injection of Ketamine/Xylazine (90 and 10 mg/kg, respectively) and positioned in a stereotaxic frame. The target region was set parasagittally between Bregma and Lambda (somatosensory area of the parietal cortex) with standard coordinates (+1.0 mm AP, +1.5 mm ML, and –2 mm DV). After removal of the bone using a drill, unilateral mild injury of 1 mm depth was induced by activating a piston positioned vertically above the cortical tissue. For the device injury parameter, the impactor speed was adjusted to 2 m/s while the dwell time was set at 150 ms. Mice were then rapidly removed from the stereotaxic frame, sutured and kept in a holding cage until recovery from anesthesia. The tip of the impactor was 1 mm in diameter. The size of the bone flap was 1.7 mm in diameter removed using a manual trephine. TBI was performed on day 17 of cell culture in a way that the cells are ready for transplantation after 1 week of inducing TBI (Fig. 1).

2.3. Hematoxylin and eosin stain (H&E)

Fifteen weeks post-transplantation, animals were euthanized and transcardially perfused with 10 ml 0.1 M phosphate-buffered saline (PBS) followed by 20 ml 4% paraformaldehyde. Brains were removed, post-fixed in paraformaldehyde overnight at 4 °C, and cut into 50 μ m coronal sections on a vibratome. Tissue sections were mounted on slides

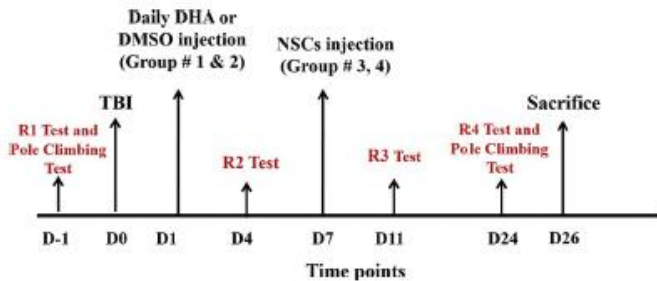


Fig. 1. A timeline representing experimental approach.

Mice were divided into four groups. Pole climbing test was performed one day before TBI and at day 24 post-TBI. Rotarod tests (R1, R2, R3 and R4) were performed at one day before TBI and at day 4 (R2), day 11 (R3) and day 24 (R4) post-TBI. The animals were sacrificed on day 26.

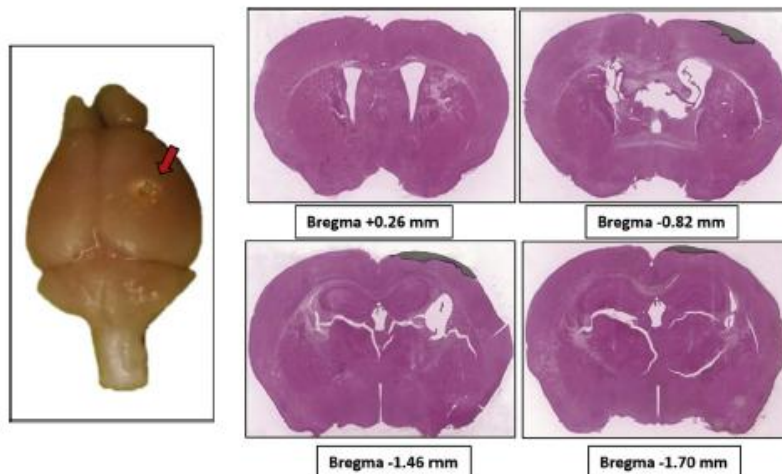


Fig. 2. Controlled cortical impact (CCI) mainly targets the somatosensory area of the parietal cortex. Mice brain sacrificed 26 days post mild-CCI on the ipsilateral side of the parietal cortex shows the existence of a cavity in the targeted region (red arrow). Representative Hematoxylin & Eosin stained coronal brain sections are marked with their coordinates to the Bregma through the brain of an injured mouse, it shows a 1 mm maximum depth CCI injury. The black area in each section shows the contralateral hemisphere superimposed on top of the lesioned hemisphere to visualize the brain damage regions. The lesion covered the ipsilateral parietal cortex targeting mainly the somatosensory area and the secondary visual cortex V2.

and washed with distilled water for 3 min, followed by placing them in hematoxylin for 50 s to 1 min. After hematoxylin, slides were placed under running tap water and then rinsed with eosin for 50 s to 1 min. Subsequently, slides were immersed in 95% and 100% ethanol for 3 min and 5 min, respectively. Afterwards, slides were washed with xylol for 1 min. Finally, 2–3 drops of mounting solution (Aqueous Mounting Medium [sigma F4680-25ML]) were added to the slides to be visualized on the microscope (Fig. 2).

2.4. Isolation of neural stem cells (NSC)

A total of ten pups (aged 1–7 days) were used for harvesting NSCs for group 2; these are taken from C57BL6 pregnant mice. NSCs were isolated from subventricular zone (SVZ) of the lateral wall of the ventricle. This zone, on coronal brain sections, was dissected under a microscope. Tissues were collected in a tube containing DMEM/F12 medium (Gibco 31331-028) placed on ice and dissociated by adding trypsin solution (NaCl 8 g/l; KCl 0.4 g/l; glucose 1 g/l; NaHCO₃ 0.85 g/l, 0.5 g/l; trypsin 2 g/l) at 37 °C. Deactivation of dissociation was induced by DMEM/F12, 10% Fetal Bovine Serum (FBS), and DNase (20 µg/ml). Cells were centrifuged (5 min, 1000 rpm) and maintained in a complete medium (DMEM/F12; insulin 50 mg/ml; B27; N2; glucose 16.25Mm; HEPES 1M; Penicillin/Streptomycin). Cells were counted on hemacytometer using trypan blue then seeded in a total of 100,000 cells per T25 flask at 37 °C, 5% CO₂. At this stage, primary neurospheres were obtained. EGF (20 ng/ml) and FGF (20 ng/ml) were added every 3rd, 5th, and 6th day of cell culture to maintain the cells as proliferating neurospheres that were passaged twice every 7–10 days to obtain secondary and tertiary neurospheres.

2.5. Transplantation of neural stem cells

One week post-CCI injury, all mice were anesthetized using ketamine/xylazine (90 and 10 mg/kg, respectively) and placed in a stereotaxic device. Tertiary neurospheres were co-labeled with Hoechst 33342 (10 µg/L) and cultured at 37 °C for 30 min in a humidified incubator containing 5% CO₂ prior to transplantation. Cells were dissociated into single cells and a total of 100,000 cells were stereotactically transplanted into the cortex at the injury site (coordinates: AP + 1.0 mm, ML + 1.5 mm from bregma, DV 2.5 mm from dura) via a 10 µl Hamilton syringe (Fig. 1). Cells were injected with 3 µl of complete medium using a syringe pump, which was calibrated to achieve a constant flow rate of 0.25 µl/min. Following all injections, the needle was kept in place for 5 min before being slowly withdrawn.

2.6. Behavioral tests

2.6.1. Rotarod performance test

The rotarod performance test was used to assess balance, grip strength and motor coordination of the mouse [5,46], especially post-TBI utilizing an automated rotarod instrument (UgoBasile, 47750 – Rat Rota-Rod NG, Comerio, Italy). The mouse was placed on a rotating rod under continuous acceleration and the time it took the mouse to fall was recorded. Rotarod test was performed four times for each group: 4 days before TBI (R0), 3 days after TBI (R1), 3 days after stem cell injection (R2), and 10 days after stem cell injection (R3) (Fig. 1). Each test was performed for 4 consecutive days, starting with 3 trials of 5 min each with an acceleration speed of 4–40 rpm in the first three days with an inter-trial time of 15 min. The fourth day (challenging

session) consisted of one trial of 8 min with an acceleration speed of 4–60 rpm.

2.6.2. Pole climbing test

Fourteen days after NSC injection, pole climbing test was performed to study motor coordination of the mice (Fig. 1) assessing motor performance and coordination [47,48]. Each mouse was trained for three consecutive trials followed by 5 consecutive experimental trials. The mouse was placed on the tip of a vertical rod (height = 60 cm, diameter = 1 cm) and the time at which the mouse reached the bottom (t-total) was recorded; in addition the t-turn (time at which the mouse turned on the rod to head down), the t-half (the time at which the mouse reached half of the rod) and the t-stop (the time at which the mouse stopped on the rod), were also recorded.

2.7. Immunofluorescence

Brain floating sections (50 μ m thick) were washed with PBS and PBST (0.1% Triton in PBS), then incubated for 2 h in a solution of 10% heat-inactivated fetal bovine serum (FBS) in PBST. Thereafter, tissues were incubated with primary antibodies diluted in PBST-1% FBS overnight at 4 °C. The following antibodies were used: GFAP (1/1000; Abcam 7260) for astrocytes; DCX (1/500; Santa Cruz sc-8066) for neuroblasts; IBA1 (1/1000; Wako 091-19741) for microglia and macrophages. Tyrosine hydroxylase (TH) (mouse monoclonal antibody, 1/1000; Millipore MAB318) was used to assess the number of dopaminergic neurons at SN and VTA. Then sections were rinsed in PBST, and incubated with the appropriate fluorochrome-conjugated secondary Ab for 1 h at room temperature, followed by three washes of 20 min each in PBST. All samples were counterstained in 1 μ g/ml Hoechst (Sigma) in PBS, and mounted in Fluoromount (F4680-25ML).

2.8. SDS-polyacrylamide gel electrophoresis and immunoblotting technique

Immunoblotting technique was performed according to standard procedures using ECL detection as performed in our laboratory. In summary, Tissue samples (20 μ g) samples were run on SDS-PAGE (4–20% acrylamide) with a Tris-glycine running buffer system and transferred onto a PVDF membrane using a wet transfer unit (Bio-Rad) at 30 V overnight. The blots were probed with the primary antibodies used including monoclonal mouse anti α -tubulin (BML-FG6090-0500, ENZO, 1/4000), rabbit monoclonal anti GFAP (Abcam #7260, 1/10000). Uneven loading of samples onto different lanes might occur despite careful protein concentration determination and careful sample handling and gel loading (20 μ g per lane). B3-tubulin (monoclonal #sc-58888; Santa Cruz Biotechnology, 1/2000) was used as protein loading evenness control. The blots are washed with TBST and exposed to HRP coupled secondary antibodies then results revealed using the Chemidoc machine (Bio-Rad, Ca). The molecular weights of intact proteins and their potential BDPs were assessed by running alongside all blue molecular markers (#161-0373/BIO-RAD).

2.9. Quantification and imaging

All the quantification was done using the NIH-Image J program. DCX-positive area was quantified along the lateral wall of the SVZ with respect to the total length of the SVZ. IBA-1 and GFAP quantifications were performed by determining the total area covered by these markers at the TBI site with respect to the area of the injury. Quantification of dopaminergic neurons at the SN and VTA was determined by counting individual TH-positive cells. At least three sections per mouse were used for quantification. Microscopic imaging was done using Zeiss LSM 710 confocal microscope. Images were acquired as tile scans with 40 X oil objectives and analyzed using the Zeiss ZEN 2009 image-analysis software. Images for the different experimental interventions were acquired under the same laser and microscopic parameters for the

purpose of consistency.

2.10. Statistical analysis

Statistical analysis was conducted using Statistical Package of Social Science SPSS 22.0 software (SPSS Inc., Chicago, IL, USA), and GraphPad Prism 6. The measurement data are expressed as mean \pm SEM and were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple group comparisons. When two groups were compared independent Student's t-test was applied. Pole climbing and rotarod data were analyzed using two-way repeated measures ANOVA (RM ANOVA) followed by post hoc contrasts or pairwise comparisons (with Bonferroni adjustment for multiple comparisons).

Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Establishing the CCI model of mild injury targeting the somatosensory area of the parietal cortex

All mice in the 4 groups sustained the same injury with the same coordinates (Fig. 2). Hematoxylin and eosin (H&E) staining of coronal brain sections from CCI animals revealed a cortical cavity spanning from the lateral ventricles to the beginning of the hippocampus. The impact produced a 1 mm depth tissue deformation, which is considered a mild TBI of the first layers of the parietotemporal cortex. The lesion occurred mainly in the primary somatosensory cortex (S1) and the second visual cortex (V2) and was associated with cortical swelling and herniation as documented in previous studies [49].

3.2. Effects of NSC transplantation and DHA daily injection on motor coordination

To monitor the effect of DHA and NSC treatments on motor impairment in injured mice, pole climbing and accelerating rotarod tests were performed. Pole-climbing test consisted of placing mice at the top of a vertical rod, head upwards, where time-to-turn and time-to-descend the rod are recorded (Fig. 3). Prior to TBI, all the mice in the 4 different groups exhibited similar behavioral results, with the total time needed to cross the rod (T-total) of \sim 18 s. Three weeks post injury (TBI), a significant difference in the performance was detected in T-total among the 4 tested groups. The TBI group exhibited a motor

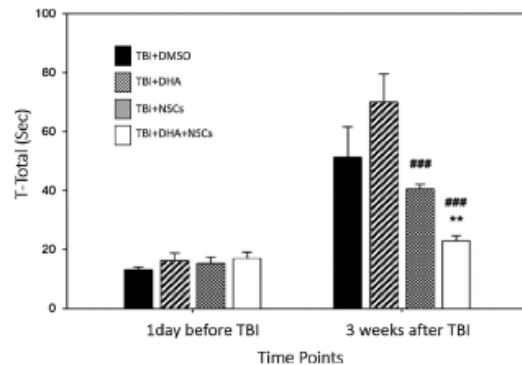


Fig. 3. Significant improvement in motor coordination was observed between groups tested on pole climbing. Motor balance assessment using pole climbing test was measured 1 day before TBI and 3 weeks after TBI. Data are presented as means \pm S.E.M. Significant differences between the groups were evaluated using two-way ANOVA. Symbols indicate the statistical difference between NSCs + DHA group and the DMSO group, (***) $p < 0.01$.

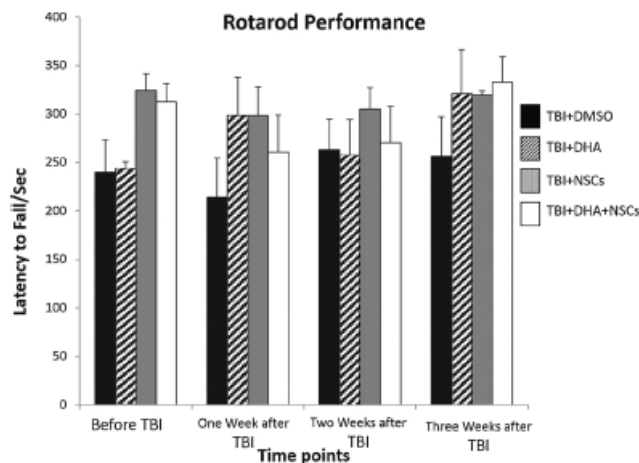


Fig. 4. Rotarod Data on Motor Coordination among the Different Groups. Rotarod performance was measured using the accelerating rotarod test (4–60 rpm/min) in the 4 groups of mice. The latency to fall is indicated. Mice were tested once a week before TBI and for 3 weeks following TBI. No significant improvement in motor coordination was observed among the groups tested on rotarod. Data represent means \pm S.E.M. of each group. Statistical analysis was performed using independent student *t*-test.

deficit with an increased T-total (51 ± 10). Similarly, both the (TBI + DHA) and the (TBI + NSCs) group showed a similar deficit (70 ± 9 ; 40 ± 2 , respectively). On the other hand, the (TBI + DHA + NSCs) group showed a significant decrease in the T-total (23 s) compared to DMSO group ($p < 0.001$), indicative of improved motor coordination. Lastly, utilizing an accelerating rotarod test, no significant statistical difference in motor coordination was detected among all groups as shown in Fig. 4.

3.3. Increased neurogenesis in the lateral SVZ after NSC transplantation coupled with DHA injections

It has been shown that, after injury, neuroblasts migrate from the subventricular zone (SVZ) to the lesion site. The level of neurogenesis in the SVZ was assessed by quantifying DCX positive cells, a specific marker of neuroblasts (Fig. 5A). Quantification of DCX positive cells in the lateral SVZ (Fig. 5B) showed no significant increase in neurogenesis in TBI + DHA (12.74 ± 1) compared to DMSO injected group (11.78 ± 4). While a significant increase of DCX positive cells was observed in the TBI + NSCs group (18.73 ± 0.6) compared to DMSO injected and this increase was higher in the TBI + DHA + NSCs group (26.06 ± 2.32 ; $p < 0.05$).

3.4. Increased glial reactivity at the cortical site of injury after NSC transplantation coupled with DHA injections

Our results have shown that there is an increased inflammatory response apparent by an elevation in the number of glial cells, including astrocytes and microglia. Astroglial reactivity was assessed by glial fibrillary acidic protein (GFAP) immunolabeling, an astrocytic marker known to be upregulated in reactive astrocytes (Fig. 6A). GFAP, on the other hand, increased in the TBI + DHA + NSCs group. This observation was reflected by the quantification of GFAP positive cells in the ipsilateral cortex over the intact cortical area compared to the control group (Fig. 6B). GFAP quantification showed similar levels of astrogliosis in both TBI + DHA (0.06 ± 0.003) and TBI + NSCs (0.06 ± 0.009) groups compared to the DMSO group (0.084 ± 0.016). While in the TBI + DHA + NSCs group, astrogliosis increased significantly (0.10 ± 0.013 ; $p < 0.05$) compared to the control group (0.084 ± 0.016). Similarly, reactive astrogliosis due to brain damage is associated with microglial activation. We examined the expression of IBA1, a microglial marker at the cortical site of injury (Fig. 7A). Quantification of the IBA1 positive cells in the injured cortical

area (Fig. 7B) showed a similar level of microgliosis in TBI + DHA group (0.081 ± 0.019) and TBI + NSCs group (0.098 ± 0.014) compared to the DMSO group (0.069 ± 0.014). On the other hand, a significant expansion in microglial cells was observed in the TBI + DHA + NSCs group (0.13 ± 0.008 ; $p < 0.05$).

3.5. Quantification of GFAP and α II-spectrin breakdown products (BDPs)

In addition, we assessed for both GFAP fragmentation and α II-spectrin proteolysis as they have been considered biomarkers of TBI as discussed later. Both GFAP and α II-spectrin proteins have been shown to be vulnerable to calpain-mediated truncation resulting in molecular signature breakdown products (BDPs) of 38 kDa (GFAP-38 kDa); this 38 kDa GFAP breakdown product has been recently validated as a potential biomarker of (necrotic) calpain-mediated neural injury and which has been assessed in both clinical and experimental brain injury studies [50–59]. Similarly, this 38 kDa (GFAP-38 kDa) has been compared to the α II-spectrin BDPs of 120, 145 and 150 kDa (α II-spectrin SBDPs) [56,59–64]. Analysis of these BDPs, the 38 kDa GFAP BDP as well as the SBDP-150 levels were significantly higher in TBI + DMSO and TBI + DHA compared to the TBI + NSCs + DMSO and TBI + NSCs + DHA (Fig. 8A&B). The 145 and 120 kDa SBDPs showed no significant difference among the groups (Fig. 8A&B). Similarly, the 38 kDa calpain associated GFAP-BDP was detected in the TBI groups with the lowest level detection in the TBI + NSCs + DHA (Fig. 8A&B). It is well established that these specific markers (BDPs) are useful biomarkers indicative of the level and mechanism of TBI (50–54, 60–64). The presence of GFAP-38 kDa and 150 kDa SBDP evident in the TBI groups (indicative of calpain activation) conversely was minimally detected in the TBI + NSCs + DHA group. These findings demonstrate that calpain activation was ameliorated in the combined DHA + NSCs treatment.

3.6. Increased TH positive neurons in the midbrain after NSC transplantation coupled with DHA injections

It is well-established that, in addition to the initial cortical damage, TBI induces a series of secondary insults, including a rapid loss of the dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra (SN). For this reason, we examined the effect of NSC transplantation and DHA injection on TH-positive (TH+) neurons in the SN and VTA (Fig. 9A). TBI + DHA group and TBI + NSCs group showed comparable numbers of TH+ neurons (447 ± 40 and 482 ± 58 , respectively) compared to DMSO group (363 ± 80)

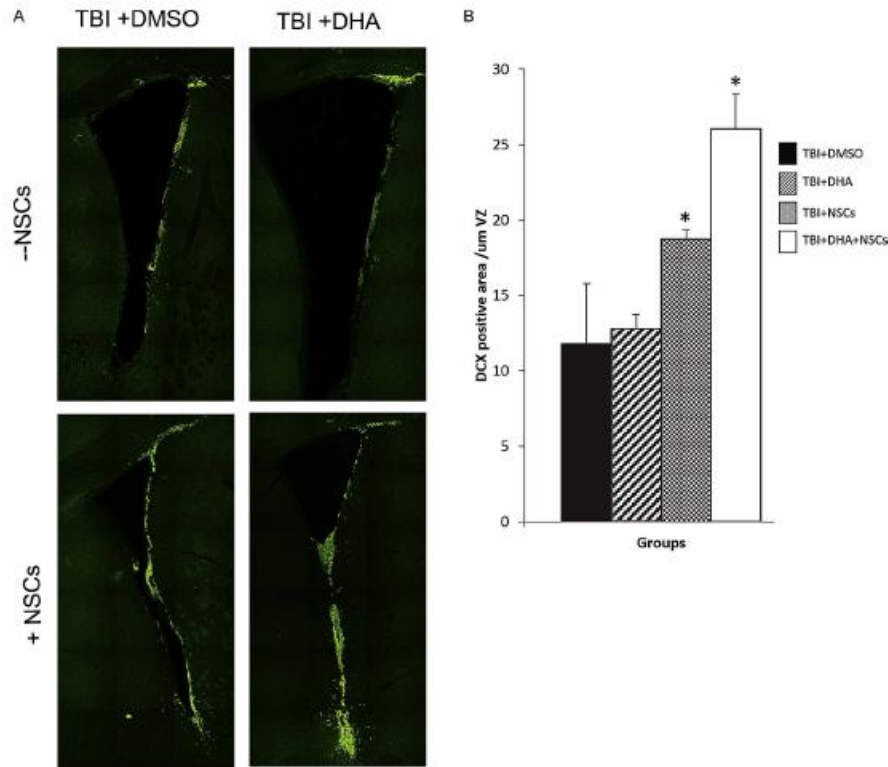


Fig. 5. Increased DCX-positive neuroblasts at the lateral SVZ in mice given a dual neurotherapeutic treatment of NSCs and DHA. (A) In the lateral SVZ, DCX labeling reflects neuroblast production. Representative confocal tile scan images (40 X oil objective) of 40 μm mice brain coronal sections immunostained with anti-DCX antibody (green) and counterstained with Hoechst (blue) show the area of DCX positive cells. Scale bars 100 μm. (B) Quantification of DCX-positive area is shown in the histogram. No difference in DCX expression is observed between DMSO and DHA groups. NSC transplantation induces expansion of DCX expression in the SVZ (NSC + DMSO group). This expansion is increased when NSC transplantation is combined with daily DHA injection (NSCs + DHA group). Each bar represents the area of DCX-positive cells per VZ length (mean ± S.E.M., $n = 3$ in each cohort). Symbols indicate statistical differences between groups vs. the DMSO group (*) $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 9B). on the other hand, TBI + DHA + NSCs group showed a synergistic effect on the number of nigrostriatal dopaminergic neurons, leading to a significant increase of TH+ neurons (905 ± 67), compared to the other groups.

4. Discussion

In this work, we hypothesized that the co-treatment of transplanted NSCs derived from the neonatal mice SVZ with DHA pretreatment would carry neurotherapeutic potential in an experimental model of chronic mild TBI. For this purpose, NSCs were transplanted in the injured cortical region 1 week post injury based on the best available literature evidence demonstrating that the 7-day time frame would be optimal for blood vessel vascularization along with enhanced survival and proliferation of grafted cells [65,66]. Although mild CCI was induced, in some animals a modest herniation was observed further damaging the surrounding brain tissue. This particular time point of 7 days would provide a better environmental “niche” compared to early time points (1 day, 2 days and 5 days) where primary and secondary brain injury phases are still active [67]. These unfavorable survival conditions would not be ideal for NSC survival and axonal sprouting due to the lack of significant blood supply coupled with an active pro-cell death machinery. Data from this work demonstrated that combined

DHA injections and neonatal NSCs transplantation significantly attenuated TBI-induced motor function deficits (pole climbing results) and promoted neurogenesis which was accompanied by an increase in the number of dopaminergic neurons as discussed below.

4.1. Behavioral recovery

Rotarod and pole climbing tests were used to study the effect of stem cells transplantation and DHA injection on motor recovery. Rotarod test is widely used to assess motor and balance coordination in studies including various TBI mouse models and treatments [68–70]. Climbing tests are used to monitor sensorimotor impairments associated with hypothalamic and nigrostriatal motivational systems in rats and mice [71]. In our study, rotarod test did not show any significant changes in the motor behavior pre- and post-TBI induction with or without DHA treatment (Fig. 4). However, pole climbing reflected high sensitivity enabling us to detect motor deficits and motor recovery post-treatment. This discrepancy in results could be related to the nature of the CCI injury level where mild cortical injury was performed (a mild injury of 1 mm depth). The use of pole climbing test may represent an ideal marker to detect behavioral deficit recovery in assessing mild severity of TBI used in our study. In addition, it is possible that the mild injury may have not led to sufficient neuronal loss that could generate

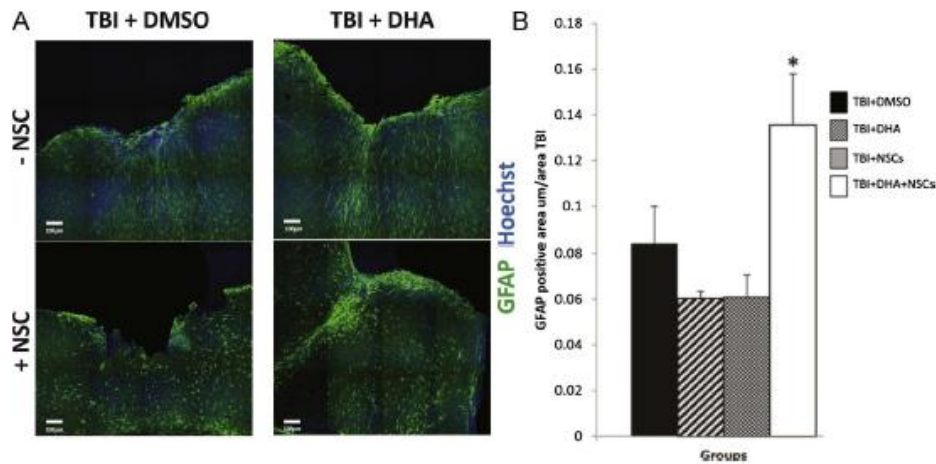


Fig. 6. Increased glial reactivity at the TBI region in mice given a dual neurotherapeutic treatment of NSC and DHA.

(A) Representative confocal tile scan images (40 X-oil objective) of 40 μm mice brain coronal sections immunostained with anti-GFAP antibody (green) and counterstained with Hoechst (blue) show an increased number of GFAP+ cells in the NSCs + DHA group compared to the other 3 groups. Scale bars 100 μm. (B) Quantification of GFAP-positive area is shown in the histogram. No difference in GFAP expression is observed among the DMSO, DHA and NSC + DMSO groups. NSC transplantation coupled with daily DHA injection (NSCs + DHA group) increases the GFAP positive cell area in the TBI region. Each bar represents the area of GFAP-positive cells per area of TBI (mean ± S.E.M., n = 3 in each cohort). Symbols indicate statistical differences between the NSCs + DHA group and the NSC + DMSO group (*) $p < 0.05$.

motor deficits that are detectable with the Rotarod testing.

4.2. Neurogenesis mechanism

Recently, a number of studies have utilized different modes of neural stem cell therapy as a treatment of TBI [15,27,29,31,32,72]. These studies have mainly used stem cells derived from embryonic tissue or fetal stem cell lines [15,72–77]. Cells were engrafted at different locations in the brain, in the ventricles or at the cortical site of injury. Varying degrees of cell survival after transplantation into the injured brain have been reported and results indicate that transplanted cells were able to differentiate into neurons, astrocytes and oligodendrocytes precursors. Sun et al., had successfully used adult-derived

NSCs that were transplanted into experimental injured rat brain [78]. Many cells migrated out of the injection site into surrounding areas expressing markers for mature astrocytes or oligodendrocytes. Electrophysiological studies have shown that the transplanted cells possessed typical mature glial cell properties demonstrating that the adult-derived NSCs became region-specific functional cells [78]. In our work, we utilized neonatal NSCs, which have advantages over embryonic stem cells given their “limited clinical translatability” due to ethical and technical obstacles. Injected NSCs with DHA treatment showed a marked neurogenesis both in the SVZ region as being assessed by DCX immunostaining (Figs. 5 A and 4 B). The exact fate of transplanted NSCs remains unknown in our study. While some of these cells may have died, our data suggest that NSCs are still alive and may have

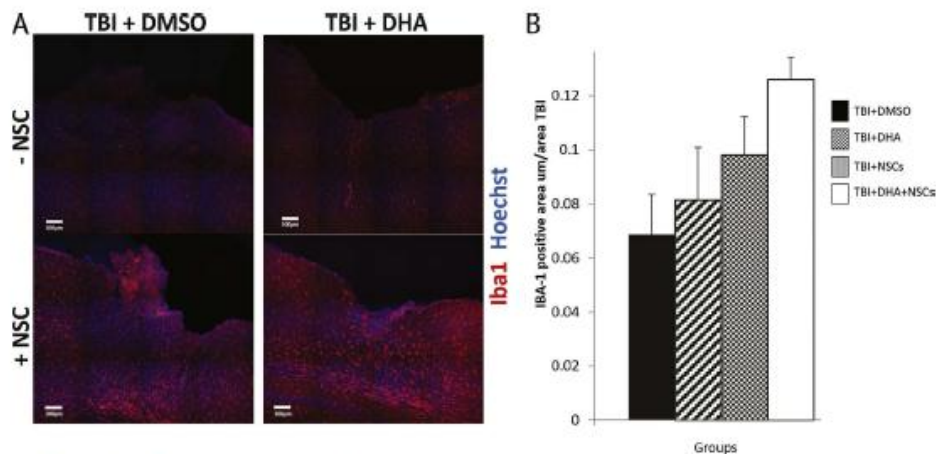


Fig. 7. Increased microglial reactivity at the TBI region in mice given dual neurotherapeutic treatment of NSC and DHA.

(A) Representative confocal tile scan images (40 X-oil objective) of 40 μm mice brain coronal sections immunostained with anti-IBA1 antibody (red) and counterstained with Hoechst (blue) show an increased number of IBA+ cells in the NSCs + DHA group compared to the other 3 groups. Scale bars 100 μm. (B) Quantification of the IBA1-positive area is shown in the histogram. No difference in IBA1 expression is observed between DMSO, DHA and NSC + DMSO groups. NSC transplantation coupled with DHA daily injection (NSC + DHA group) increases the IBA1 positive cell area in the TBI region. Each bar represents the area of IBA1 positive cells per area of TBI (mean ± S.E.M., n = 3 in each cohort). Symbols indicate statistical differences between NSCs + DHA group vs. DMSO group (*) $P < 0.05$.

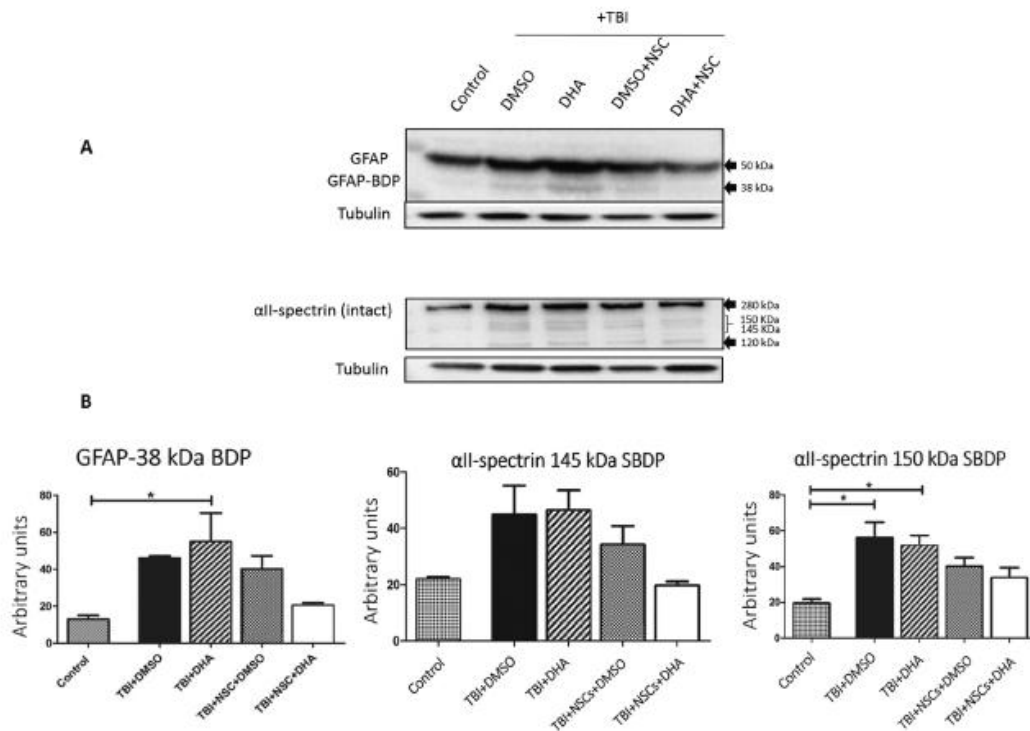


Fig. 8. Evaluation of TBI Biomarkers: GFAP 38-BDP, SBDP145 and SBDP150.
A: Assessment of Western blot analysis of the GFAP 38-BDP, SBDP145 and SBDP150. Shown is the Western blot analysis of intact GFAP 38-BDP (1), SBDP145 (2) and SBDP150 (3). These blots show altered protein abundance among the different treatment of TBI treated with DHA/NSCs. Analysis of these BDPs, SBDP-150 levels were significantly higher in the TBI + DMSO and TBI + DHA compared to the TBI + NCS + DMSO and TBI + NCS + DHA. The 145 and 120 kDa SBDPs showed no significant difference among the groups. Similarly, the 38 kDa calpain associated GFAP-BDP was detected in the TBI groups with the lowest level detection in the TBI + NCS + DHA.
B: Bar graph of mean \pm SEM of the GFAP 38-BDP (1), SBDP145 (2) and SBDP150 (3) as a function of injury condition and treatment. Shown are the bar graph of mean \pm SEM of the different BDPs associated with GFAP and all-spectrin proteins. (1) The 38 kDa GFAP-BDP was higher in TBI + DHA groups compared to control group ($p < 0.05$), while the levels were not significantly elevated in TBI + DMSO, TBI + NCS + DMSO and TBI + NCS + DHA animals ($n = 3$). (2) Levels of SBDP145 were not significantly different across treatments and compared to controls, (3) while SBDP150 levels were significantly elevated in TBI + DMSO and TBI + DHA animals compared to controls but TBI + NCS + DMSO and TBI + NCS + DHA animals did not exhibit significant differences ($n = 3$). Values represent arbitrary densitometry units means. *: $p < 0.05$.

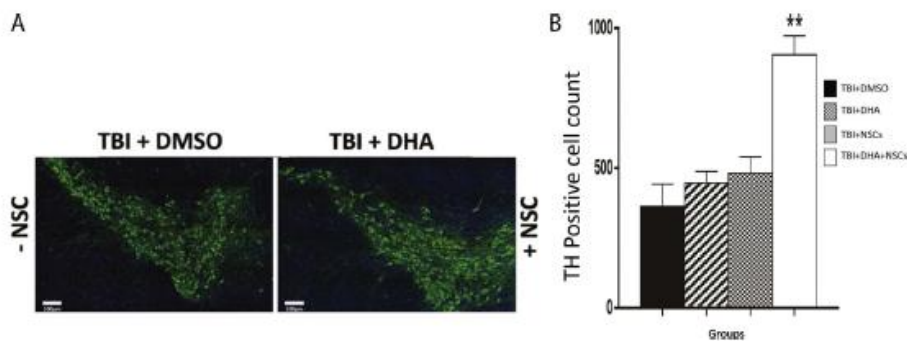


Fig. 9. Increased Tyrosine hydroxylase positive neurons in the midbrain in mice given a dual neurotherapeutic treatment of NSCs and DHA. (A) Representative confocal tile scan images (40 X oil objective) of 40 μ m mice brain coronal sections immunostained with anti-TH antibody (green) and counterstained with Hoechst (blue) show an increased number of TH + cells in the NSCs + DHA group compared to the other 3 groups (shown is a representative image of TBI + DMSO vs. the NSCs + DHA group). Scale bars 100 μ m. (B) Quantification of TH-positive cells is shown in the histogram. No difference in TH+ numbers is observed among DMSO, DHA and NSC + DMSO groups. NSC transplantation coupled with daily DHA injection (NSCs + DHA group) increase the TH-positive cell number in the SN and VTA. Each bar represents the mean of TH-positive cell number in 2–9 sections per mouse brain (mean \pm S.E.M., $n = 3$ for DMSO group NSC + DHA group, $n = 3$ for DHA group and NSC + DMSO group). Symbols indicate statistical differences between the NSC + DHA group and the DMSO group (***) $P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentiated into mature cells. However, it is unknown whether these cells have functionally integrated into the neural circuit and how DHA may have contributed to the therapeutic outcome warrant further investigation.

Based on previous studies, Katakura et al. reported that DHA-induced neuronal differentiation by decreasing Hes1 expression and increasing p27 expression, which caused cell cycle arrest in NSCs [45]. In addition, Sakayori et al. demonstrated that DHA increased the neuronal differentiation of gliogenic tertiary NSCs *in vitro* [43]. DHA has been shown to maintain NSCs during embryogenesis. It is also essential for maintenance and proliferation of NSCs in adult rat hippocampi and fat-1 transgenic mice. Furthermore, DHA depletion in the brain by chronic omega-3 fatty acid deficiency exacerbates injury levels and impedes the functional recovery. DHA has been shown to promote neurite outgrowth, synaptogenesis and glutamatergic neurotransmission by increasing the glutamate receptor subunit.

In their study, Kawakita et al., explored whether DHA affects NSCs' potential to differentiate into neurons. Rat neurospheres were cultured under differential conditions with or without DHA. DHA promoted differentiation into neural stem cells and increased newborn neurons modulating the hippocampal function through enhancement of neurogenesis [35]. Several lines of evidence have indicated that DHA modulates the expression of the brain plasticity-related protein BDNF (Brain-derived neurotrophic factor) and have associated CREB (cAMP response element binding protein)- (a transcription factor involved in learning and memory)-and synapsin 1 with reduced oxidative damage and increased neural plasticity [79,80]. Could DHA facilitate the survival, growth, and differentiation into neurons of these transplanted NSCs *in vivo*? In our study, we hypothesize that these neuroprotective data and rehabilitative effects observed post stem cell transplantation/DHA co-treatment arise from multiple events as discussed later.

In addition, several studies have been able to confirm that brain injury by itself is capable of inducing endogenous neurogenesis in different brain regions, including SVZ, dentate gyrus and hippocampus [81–83]. This injury-induced neurogenesis contributes considerably to post-injury recovery [84,85]. Neuroblasts can migrate from the SVZ to the injured striatum and cortex in an attempt to enhance the outcomes of injury recovery and to replace damaged neurons [86]. Similar results to ours have been reported in other studies showing that stem cell transplantation can affect endogenous stem/precursors neurogenesis [87,88], increase endogenous neurogenesis in the hippocampus [89,90] and provide a neuroprotective environment for cell population at the site of transplantation [91,92]. In our study, this observed increase in endogenous neurogenesis is enhanced for the DHA-treated NSCs group, which is translated in enhancing motor recovery post-TBI. These results complement different studies that show that either DHA or NSCs would induce neural repair and behavioral recovery [16,22,33,35,37,41,44,45,79,93–97]. To the best of knowledge, this is the first study to utilize such dual treatment of DHA and NSCs for neural repair and functional outcomes. In our work, the only neurotherapeutic outcome was observed when NSCs and DHA were simultaneously rather than separately applied as a treatment.

We postulate that the higher number of DCX positive cells in the group receiving the dual therapy compared to those with DHA or stem cells transplantation could be attributed to the ability of DHA to influence positively the survival and proliferation of the exogenous transplanted stem cells and promote their survival by modulating the microenvironment via neurotrophic factor secretion.

Studies have documented that endogenous stem cells interact with the transplanted NSCs and modulate the microenvironment of the injured brain by secreting different chemokines, and growth factors [98]. These, in turn, may enable the transplanted stem cells to survive and migrate to the damaged brain part where they settle near blood vessels and interact with GFAP and IBA-1 positive cells [99]. Alternatively, neurogenesis and behavioral recovery have been attributed to neurotrophic factors triggering endogenous repair on the premise that the

percentage of stem cells capable to differentiate into neurons is believed to be negligible [100,101].

4.3. Microglial and astroglial activation

TBI induces reactive gliosis characterized by hypertrophic and proliferating astrocytes, proliferating microglia and NG2-positive oligodendrocytes, which eventually form a bordering glial scar around the damaged area [102]. This general reactive gliosis can exert protective functions essential for neuronal survival during the acute stress-handling phase of neurotrauma [103]. However, these benefits seem to be counterbalanced by restricted regenerative potential at a later stage [104,105]. This neuroprotective or neurotoxic ability depends on specific molecular stimuli, resulting in containment or aggravation of disease progression [106]. Along the same line, the exact role of microglia has been under investigation due to its diverse roles. Although microglial cells were considered to impede injury-induced neurogenesis [107], recent studies have shown that microglia can enhance neurogenesis upon activation by IL-4 and IFN-gamma [108]; while under chronic activation, microglia was shown to adopt an anti-inflammatory response [109]. In an elegant study by Deierborg et al., the role of microglia in the proliferation of neural stem/progenitor cells (NSPCs) harvested from the SVZ region 1–2 weeks was investigated following excitotoxic striatal model of brain injury [20]. It was found that there was a direct relation between microglia from the injured brain and the increased proliferation of NSPCs along with an increased ability of NSPCs to differentiate into neurons and oligodendrocytes rather than astrocytes. This has been attributed to secreted microglial trophic factors [20]. In addition, microglial-induced NSPCs neurogenesis was observed *in vivo* as well, which highlights the importance of microglial temporal activation on the dual role that microglia perform (Fig. 7). Similarly, we have also observed an increased astrocytosis in the injured cortical vicinity in the DHA-NSCs treated groups. This upregulation in GFAP positive cells has been suggested to induce blastogenesis giving rise to NSCs that can differentiate into neurons and glia (Fig. 6). [110–112]. This is of high significance to us that –along with the elevated DCX positivity- astrogliosis was also detected. Our study doesn't answer whether these astrocytes may have undergone blastogenesis differentiating into functional neuronal cells. Further investigation is needed to assess the exact role exerted by the GFAP positive reactivity.

In addition, it was shown that transplanted NSCs in an injured brain can interact with microglia and reactive astrocytes secreting neuroprotective substances to rescue host neurons [91,92], and can also increase endogenous neurogenesis. In one study, Harvey et al. assessed DHA for inflammatory indices in a rat CCI model. DHA was administered intraperitoneally post-TBI for 3–21 days [93]. It was shown that DHA ameliorated neuronal endoplasmic reticulum stress and interfered with the microglial conversion from the TBI-observed activated amoeboid shape to the surveillance state expressing the anti-inflammatory marker CD206 [93]. In our study, neither DHA nor transplanted NSCs administered separately resulted in an increase in reactive gliosis at the site of injury. We hypothesize that the increased gliosis observed upon NSC transplantation coupled with DHA treatment, result in a crosstalk between glial cells and NSCs which may be mediated via secreted factors (cytokines and/or trophic factors) [98]. Thus, neurogenesis, along with the observed behavioral recovery, may highlight a neuroprotective role of microglial activation which may be mediated by DHA supportive environment. Definitely, further studies are needed to assess the exact function(s) of such an activation may have.

4.4. Tyrosine hydroxylase cell enhancement & neural injury markers

In addition to the above events, we have observed that the dual treatment has increased the nigrostriatal TH-positive neuronal cells in the ventral tegmental area (VTA) and substantia nigra 3 weeks post

-treatment compared to either treatment (Fig. 9A and B). It has been previously shown that there is a marked decrease in the dopaminergic neuron survival in rats raised on n-3 PUFA-deficient diet which has been attributed to a decrease in dopaminergic cell number, as well as function [113]. Loss of dopaminergic innervating fibers from the ventral tegmental area and substantia nigra alter the synaptic structure and dendritic complexity within the striatum and frontal cortex [114,115]. Similarly, other reports have indicated that NSCs are potentially capable of differentiating into dopaminergic neurons [116,117]. In one study by Chang et al., the effect of DHA treatment on Induced pluripotent stem (iPS) cells indicated that DHA treatment facilitated iPS differentiation into TH positive neurons both in vitro and in vivo. This was coupled with an enhanced functional recovery along with an increase in dopamine release in case of Parkinson's disease [94]. This TH conversion co-occurred with upregulation in the expression of neuro-protective pro-survival genes, including Bcl-2, Bcl-xl, BDNF and glial cell-derived neurotrophic factor [94]. We hypothesize that the combined effect of DHA and NSC transplantation has contributed to the elevated numbers of TH-positive neurons of the VTA and the SN (Fig. 9).

Along the same line, we have quantified TBI-associated biomarkers that are indicative of calpain/caspase activation assessed via GFAP and α -I-spectrin proteolysis resulting in defined BDPs. It is well established that these specific BDPs are useful biomarkers indicative of the injury level and mechanism of TBI (50–54, 60–64). In this work, the presence of the GFAP-38 kDa and the 150 kDa SBDP in the TBI groups (indicative of calpain activation) was minimally detected in the TBI + NSC + DHA group (Fig. 8). These results indicate that calpain activation was ameliorated in the combined DHA + NSCs treatment resulting in a decreased neural injury. Thus, this combination of DHA would represent a desirable strategy to improve the therapeutic efficacy of NSC transplantation post-TBI.

4.5. Limitation and future directions

This work builds on previous and current recent studies from our and other labs that have assessed the capabilities of neural stem cells in ameliorating neural injury post-TBI [118,119]. Implanted NSCs would modulate existing pathological conditions replacing injured neurons via proliferation and/or inducing trophic effects. To the best of the knowledge, this is the first study to assess the role of combined treatment of DHA and NSCs injection in an experimental model of TBI. We planned our study based on the complementary neurotherapeutic roles and potential incremental effect of DHA and NSCs on promoting cellular repair and behavioral recovery. Data from this work demonstrated that NPC-DHA treatment would promote cellular neurogenesis and facilitate functional recovery with no overt behavioral or morphological abnormalities. However, one should be cautious in interpreting the data as they carry a number of limitations that future work needs to address.

First, this research utilized mild TBI model of CCI (1 mm) which does not cause pronounced cellular injury and behavioral/cognitive deficits as compared to moderate or severe experimental brain injury models. This model carries a number of inherent limitations where a greater part of the injury will be due to the herniation that naturally occurs; thus, imposing variation and inconsistency in the injury level. The exact effect of NSCs implementation with DHA would be better assessed in much severe CCI model rather than the mild TBI which may be too mild to fully assess our hypothesis explaining the unaltered rotarod behavioral testing among the groups. Second, this work would have benefitted from the use of GFP-transgenic mice where implemented NSCs are traced depicting better assessment for proliferation and neurogenesis as well as comparing the M1 M2 microglial population to precisely determine their neuroprotective or detrimental roles. Third, our work did not include a sham surgery or naive surgery cohorts with the treatments alone as they may offer better analysis on the

obtained data. The sham surgery itself with DHA would assess the role of DHA and/or NSCs treatment on naive animals; this needs to be addressed in future work as it will highlight on the cellular and molecular mechanisms of DHA/NSCs treatment. Finally, a major concern is raised concerning the time frame of NSC transplantation; the literature is rich in different studies and clinical trials with conflicting optimal time point for NSCs implementation; each with its own rationale which needs to be addressed in future work. Our work has relied on previous work showing that NSC-transplanted at 7 days would result in enhanced cellular response and improved cognitive outcomes and would be clinically more relevant. Several researchers would debate that this time point has resulted in the observed gliosis/astroglia data which warrants further optimization. In conclusion, our humble work demonstrated encouraging results for NSCs complemented with DHA treatment in terms of behavioral and molecular endpoints. Future work is required to answer further gaps raised in this work.

Competing financial interests statement

The authors declare no financial or any other conflicts of interest pertaining to this work.

Author contribution

WAA, JS and FK conceived the study design, obtained funding for the study, and revised and edited the manuscript. JS, HG & NR harvested and assessed the neural stem cells used for the experiments. JS, HG, NR, & SM, conducted the experimental design of the TBI. WAK, JS, FK, KZ, FS and HD wrote the manuscript. FK, JS, SM and WAA assisted in the study design. FK, RMB, JS and WAA helped analyze the output data. SS, NR, HG performed the behavioral testing. All authors assisted in the final assessment of data and reviewed the manuscript. All authors have read and approved the final manuscript.

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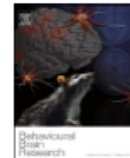
(Review)

Stem cells and combination therapy for the treatment of
traumatic brain injury



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Research report

Stem cells and combination therapy for the treatment of traumatic brain injury

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HIGHLIGHTS

- This review covers existing treatments in TBI therapy, with a focus on their potential applications to improve the functional outcomes of TBI
- Complex pathophysiology and molecular mechanisms of TBI are reviewed.
- Stem cell therapy as an optimal solution to treat TBIs reviewed, including endogenous or exogenous stem cells from embryonic, induced pluripotent, mesenchymal, and neural origin.
- Combination therapy is discussed as a novel method to treat TBI. Two approaches are highlighted, growth factors and ROCK inhibitors.
- Emphasis is given for improvements in motor and cognitive functions after stem cell therapy.

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ABSTRACT

TBI is a nondegenerative, noncongenital insult to the brain from an external mechanical force; for instance a violent blow in a car accident. It is a complex injury with a broad spectrum of symptoms and has become a major cause of death and disability in addition to being a burden on public health and societies worldwide. As such, finding a therapy for TBI has become a major health concern for many countries, which has led to the emergence of many monotherapies that have shown promising effects in animal models of TBI, but have not yet proven any significant efficacy in clinical trials. In this paper, we will review existing and novel TBI treatment options. We will first shed light on the complex pathophysiology and molecular mechanisms of this disorder, understanding of which is a necessity for launching any treatment option. We will then review most of the currently available treatments for TBI including the recent approaches in the field of stem cell therapy as an optimal solution to treat TBI. Therapy using endogenous stem cells will be reviewed, followed by therapies utilizing exogenous stem cells from embryonic, induced pluripotent, mesenchymal, and neural origin.

Abbreviations: AIDS, acquired immuno deficiency syndrome; ATP, adenosine triphosphate; AOC, alteration of consciousness; AQP4, aquaporin 4; Kv4.2, A-type voltage-gated potassium channel; bFGF, basic fibroblast growth factor; BBB, blood brain barrier; BM, bone marrow; BDNF, brain-derived neurotrophic factor; Ca²⁺, calcium; CBF, cerebral blood flow; CNS, central nervous system; CD, cluster of differentiation; CCI, controlled cortical injury; DG, dentate gyrus; DNA, deoxyribonucleic acid; ESCs, embryonic stem cells; EphA5, ephrin type A receptor 5; EGFR, epidermal growth factor receptor; FGF2, fibroblast growth factor 2; FDA, food and drug administration; GCS, Glasgow Coma Score; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GEF, guanine nucleotide – exchange factor; hESC, human embryonic stem cell; hEGF, human epidermal growth factor; HIV, human immunodeficiency virus; HBOT, hyperbaric oxygen therapy; iPSCs, induced pluripotent stem cells; IGF-1, insulin-like growth factor-1; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin - 1 beta; IL-10, interleukin-10; IL-6, interleukin-6; ICP, intracranial pressure; KLF-4, Kruppel-like factor-4; LOC, loss of consciousness; MSCs, mesenchymal stem cells; NGF, nerve growth factor; NPCs, neural progenitor cells; NSCs, neural stem cells; NMDA, N-methyl-D-aspartate; Oct-4, octamer binding protein 4; P-selectin, platelet-selectin; PEG-SOD, polyethylene glycol superoxide dismutase; PTA, post traumatic amnesia; K⁺, potassium; RhoA, Ras homolog gene family member A; ROS, reactive oxygen species; RA, retrograde amnesia; ROCK, Rho GTPase and Rho serine/threonine kinases; RBD, RhoA binding domain; Sox-2, SRY (sex determining region Y)-box-2; SVZ, subventricular zone; TGFβ, transforming growth factor beta; TBI, traumatic brain injury; TNFR1, tumor necrosis factor receptor 1; TNFα, tumour necrosis factor alpha; VNS, vagal nerve stimulation; VCAM-1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor; VDH, vitamin D hormone; WJ-MSCs, Wharton's jelly derived mesenchymal stem cells; HA-1077, 1-(5-isquinolinesulfonyl) homopiperazine; Y-27632, (1)(R)-trans-4-(1-aminoethyl)-N-(4 pyridyl) cyclohexanecarboxamidehydrochloride.

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1. Introduction

Traumatic brain injury (TBI) is a clinical condition characterized by brain dysfunction experienced upon an insult to the brain by an external mechanical force applied to the head, such as a blow to the head, as seen in car accidents [1]. Nevertheless, there is actually a narrow distinction between an injury being considered a typical brain injury or a TBI. In fact, injury is considered as TBI when the initiating force would affect brain functioning, usually manifested by one or more clinical signs including a loss, or altered level of consciousness, amnesia, with or without neurologic deficits (Table 1). In humans, the CDC classification of TBI is in terms of severity and based on the Glasgow Coma Scale (GCS) score, as either mild (GCS 13–15), moderate (GCS 9–12), or severe (GCS 3–8). Recently, TBI has been categorized based on the length of loss of consciousness (LOC), alteration of consciousness (AOC), and post-traumatic amnesia (PTA) [2]. TBI stands as the leading cause of death in young adults in industrialized countries, with a death rate 3.5 times higher than that of cancer and heart diseases combined [3]. Moreover, with an incidence rate of 1.7 million new head injury victims per year, affecting all age groups from both genders, TBI has a higher incidence rate than any other disease, including breast cancer, AIDS, Parkinson's disease and multiple sclerosis [4,5]. Despite substantial investments in the field, TBI is still one of the major brain disorders that lack defined pharmacotherapy and treatment options [6]. In order to discover new therapeutics, it's mandatory to understand the complex pathophysiology of TBI and to explore the molecular mechanisms implicated in the initiation of the disease.

1.1. TBI pathophysiology and molecular mechanisms

Ever since TBI was described by Masel and DeWitt in 2010 as a process rather than an event, understanding the multidimensional cascades of primary and secondary brain damage was considered the basis to novel treatments development or refinement of established treatment strategies [7]. TBI is a challenging disease because of its heterogeneity in terms of injury severity as well as related modality, in other words penetrating or focal injury differs from diffused one [8]. Furthermore, TBI phases add to its heterogeneity as they are neuropathologically classified as either primary or secondary. Primary injury occurs at the moment of exposure to

the external force and involves immediate mechanical disruption of brain tissue [9]. The complex cascades of metabolic, cellular and molecular events initiated by the primary injury collectively constitute the secondary injury; these may evolve over hours to months post the primary injury [10]. An illustration of these secondary injuries was described in the "Walk and die" or "Talk and deteriorate" patients who initially showed no clinical signs but later developed serious intracranial complications [11]. Most TBI treatments target the secondary insults since those arising from the primary injury cannot be influenced therapeutically [8]. In fact, with respect to time post-injury, secondary damage is classified into three overlapping phases: early, intermediate, and late [12]. In the early phase, usually 24 h following the trauma, an interruption of normal local blood flow often leads to an ischemic cascade that begins with an accumulation of lactic acid due to a metabolic switch from the usual aerobic process to anaerobic fermentation [13]. Since the latter is not enough to preserve cellular energy homeostasis, ATP stores are depleted causing an increase in membrane permeability due to failure of ATP-dependent ion pumps [8]. This would lead to terminal membrane depolarization along with excessive release of the excitatory glutamate neurotransmitter, which binds to glutamate receptors and induces a perturbation in calcium, potassium, and sodium homeostasis [14]. This is manifested by an elevated Ca^{2+} influx into the mitochondria [4,15] promoting the production of reactive oxygen species (ROS) which cause additional damage [12]. Radical formation after TBI can be measured directly and also indirectly via lipid peroxidation, protein nitration and DNA oxidation [4]. Moreover, this Ca^{2+} stress activates caspases and calpains that trigger apoptotic and necrotic pathways, respectively, leading to immediate cell death and debris accumulation [16] (Fig. 1). In the intermediate phase, a multitude of immunological/inflammatory tissue responses are induced [12]. During this phase, the brain immune privilege is violated where the blood brain barrier (BBB) is broken down and the brain is not only influenced by, but also contributes to, inflammatory responses [17]. This is demonstrated by activation of resident immune cells that release a number of cellular mediators including pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF α , coupled to anti-inflammatory cytokines production, mainly IL-10 and TGF β , along with prostaglandins, free radicals, chemokines, and complement activation [12,18]. There is also leukocyte infiltration facilitated by upregulation of adhesion molecules such as P-selectin, ICAM-1, and VCAM-1 proteins [8]. In fact, inflammation seems to have dual roles in the brain, a beneficial role promoting regeneration and a detrimental one inducing further damage. In the latter, TNF α signaling via TNFR1 is positively linked to the upregulation of the aquaporin protein, AQP4, that belongs to the family of membrane water channels. This protein enhances edema formation that in turn contributes to an elevated intracranial pressure (ICP) in brain tissues and the cerebrospinal fluid (CSF), accompanied with an increased degeneration of neurons. In addition, AQP4 promotes microfilaments production by astrocytes, inducing the synthesis of scar tissue [8,12]. As for the late stage, the most prominent feature is an increased susceptibility to seizures and, in more severe cases, epilepsy which is positively correlated with

Table 1
Symptoms of mild and severe TBI.

TBI symptoms	
Mild	Severe
Headache & confusion	Accentuation of symptoms of mild TBI
Dizziness & blurred vision	Persistent worsening headache
Sleepiness & fatigue	Slurred speech
Change in sleep habits	Seizures
Change in behavior & mood	Loss of coordination
Cognitive problems	Enlargement of the pupil
Photophobia & hyperacusis	Numbness/tingling of extremities
Nausea or vomiting	Loss of consciousness (few mins to hrs)
Loss of consciousness (few sec to mins)	

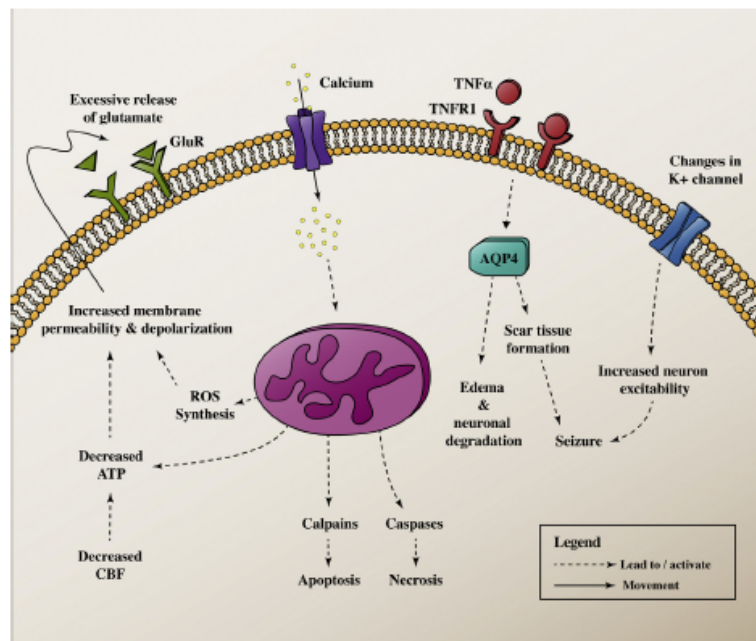


Fig. 1. Pathophysiology of TBI.

Following TBI there is an increase in membrane permeability due to failure of ATP-dependent ion pumps. This leads to terminal membrane depolarization and excessive release of the excitatory glutamate neurotransmitter, which binds to glutamate receptors inducing a perturbation in electrolytes homeostasis: the elevated Ca^{2+} influx into the mitochondria promotes the production of reactive oxygen species (ROS). Ca^{2+} stress also activates caspases and calpains that trigger apoptotic and necrotic pathways, respectively, leading to immediate cell death and debris accumulation.

TNF α signaling via TNFR1 is positively linked to the upregulation of the aquaporin protein, AQP4. This protein enhances edema formation contributing to an elevated intracranial pressure (ICP) and increased degeneration of neurons. It also induces the production of microfilaments by astrocytes, promoting the synthesis of scar tissue. In the late stage, there is an increased susceptibility to seizures, which is thought to be the result of decreased neuronal excitation threshold caused by TBI-induced reduction in the potassium channel Kv4.2 protein expression.

the nature and severity of the trauma [19]. It has been reported that excitotoxicity is a contributing factor in the development of seizures; the TBI-induced reduction in the potassium channel Kv4.2 protein expression makes the neurons more excitable and consequently more subject to seizures (Fig. 1). This channel is an A-type voltage-gated K^+ channel that plays a crucial role in returning the depolarized cell to a resting state and current. Moreover, TBI has been reported to contribute to seizure activity via a reduction of inhibitory currents [12].

1.2. Treatment options

In order to better understand the underlying pathophysiology and to explore potential treatments for TBI, several animal models that mimic TBI patterns similar to those experienced in humans have been developed [10]. Despite anatomical and physiological differences between humans and rodents, and challenges relevant to difficulties in extrapolating neurobehavioral outcomes measured in rodents in comparison to humans, these animal models offer the ability to perceive the events that follow TBI and to translate them into clinical contexts [3]. The most commonly used TBI models can be classified as either "penetrating" or "non-penetrating" injury. The penetrating injury involves direct brain deformation due to injuries caused by lateral fluid percussion, controlled cortical impact (CCI), or focal cortical compression. On the other hand, non-penetrating injury includes injuries caused by impact acceleration and weight drop models [10,20–23].

Current TBI treatments have been developed to target each of the three distinct pathological stages of TBI, with some treatments targeting more than one stage (Table 2). Several preclinical studies have tested the therapeutic efficacy of drugs in animal TBI models by targeting secondary injury mechanisms belonging to the early stage. These include calcium channel blockers such as *Nimodipine* which had mild influence in TBI patients and did not provide convincing evidence as a potential therapeutic strategy, regardless of proving effective in rodents [24,25]. In addition, *Tirilazad*, an inhibitor of lipid peroxidation, initially used to treat brain edema associated with brain tumors, has shown neuro- and vaso-protective abilities in multiple animal models of acute TBI. Based on these results, clinical trials were performed to reveal *Tirilazad* capacity to improve neurological recovery in moderately and severely injured closed TBI patients [26–28]. *Tirilazad* failed to show much improvement over placebo in phase III TBI clinical trials, however, *Tirilazad* is now an approved drug, in European countries, to treat aneurysmal subarachnoid hemorrhage (aSAH) [26–28].

Moreover, excitatory amino acid inhibitors such as *Selfotel*, an NMDA receptor antagonist and the first glutamate antagonist to go into phase III clinical trials, were shown to induce psychomimetic/psychoactive behavioral effects on TBI patients and as a result their TBI clinical trials were discontinued [29,30]. Similarly, free radical scavengers like *PEG-SOD*, were shown to prevent secondary injury in experimental TBI models and phase I trials but showed no significant difference in neurological outcome or mortality in phase III trials [31]. On the other hand, *Cyclosporine*

Table 2
Existing TBI treatment options and their corresponding modes of action.

Target Stage	Drug	Mode of Action	Ref #
Early	Nimodipine	Calcium channel blocker	[24,25]
	Selfotel	NMDA receptor antagonist	[29,30]
	Cyclosporine	Temporary inhibition of calcium entry into the mitochondria	[32]
	Hypothermia Intervention	Temperature reduction in affected tissue	[33]
	Hyperbaric Oxygen Therapy (HBOT)	Normalization of Cerebral Metabolism	[12,40–42]
Early & Intermediate	Tirilazad	Inhibition of lipid peroxidation	[26–28]
	PEG-SOD	Free radical scavenger	[31]
Intermediate	Hyperosmolar agents	Reduction of Intracranial Pressure	[43–47]
Late	Antiepileptics	Prophylaxis against post-traumatic seizures	[12,48,49]
All stages	Statins, Erythropoietin	Anti-inflammatory, anti-apoptotic and antioxidant roles	[50–52]
	Progesterone	Neuroprotective Role	[53–55]

was found to be neuroprotective in animal models and temporarily prohibits Ca^{2+} from entering into the mitochondria, yet not in TBI patients [32]. *Hypothermia Intervention* is another treatment option that aims to reduce temperature in the brain tissue before or soon after the early ischemia-precipitating event. This treatment showed promising positive results in experimental animal models of TBI as well as in clinical trials in specific patient populations where it significantly improved behavioral and histological outcomes [33]. However, many factors concerning injury severity, age, sex, therapeutic window of hypothermic treatment, duration of cooling and rewarming pose critical questions about the efficacy of this treatment intervention [34–39].

Hyperbaric Oxygen Therapy (HBOT) is a different, yet similar, treatment that aims at pushing cerebral metabolism toward its normal, aerobic state through providing increased oxygen saturation in the brain. It consists of the inhalation of pure oxygen in a hyperbaric chamber whereby increasing oxygen concentration in the blood and, consequently, in cerebral metabolism is expected to attenuate brain tissue hypoxia and neuronal cell death following TBI [12,40]. In fact, HBOT has shown neuroprotective effects in a variety of TBI models as well as in clinical studies; however, adverse effects and complications such as damage to the lungs and sinuses and changes in vision have raised doubts about its utility as an ultimate treatment option [40–42]. *Hyperosmolar agents such as Mannitol and hypertonic saline* along with a less supported treatment route, where a skull flap is removed to allow space for cerebral expansion, are currently used to reduce elevated ICP caused by inflammation, the hallmark of the intermediate phase [43–47]. Furthermore, *antiepileptic drugs*, such as *levetiracetam*, *phenytoin*, and *vagal nerve stimulation (VNS)* are used to relieve TBI associated seizures or to reduce the probability of post-traumatic seizures of the late stages following TBI [12,48,49].

In summary, the currently available treatment options for TBI either did not reach clinical trials yet or were ineffective, except for a few that are only limited to the alleviation of related symptoms. This is probably due to the fact that available treatments are directed to inhibit the secondary injury cascade by targeting a single event post TBI, while in reality many pathophysiological events contribute to secondary injury.

Of these mono-therapies that showed promising results, *Statins* have been seen to play anti-inflammatory, anti-apoptotic, and antioxidant roles in preclinical models of TBI [50]. Despite clinical data showing their efficacy, there are still issues concerning their adverse effects, timing, duration of administration, and choice of a particular statin [51]. *Erythropoietin* has similar effects in animal models but recent clinical studies failed to support its beneficial neurologic effect in TBI patients [52]. Likewise, *Progesterone*, which had positive effects in animal TBI models and two phase II randomized, controlled clinical trials, also failed to show any clinical benefit in patients with severe TBI in a large prospective, phase III randomized clinical trial [53–55].

To date, there is no FDA approved pharmacologic agent up that proved to improve the outcomes following TBI. This justifies the need for a treatment that targets multiple stages along the cascade [56]. Therefore, combination therapy among the previously mentioned drugs might seem beneficial if designed to target multiple events post TBI.

Recently, the use of stem cells (SCs) as a potent treatment option for TBI has emerged. Given their neuroprotective abilities, the role of SCs in tissue reconstruction and their capabilities in preventing cell death make them a suitable treatment option for TBI [57]. On this basis, the current review focuses on the use of stem cell therapy and combination therapy in TBI treatment. This review details the treatment of TBI with endogenous neural stem cells, as well as exogenous stem cells such as embryonic (ESCs), induced pluripotent (iPS), mesenchymal (MSCs), and neural stem cells (NSCs). This is coupled with combination therapy including the use of growth factors, lipids, and ROCK inhibitors. Finally, the route of administration for these two main treatment options will be presented in terms of the consequent improvements in motor and cognitive functions following treatment.

2. Endogenous stem cells in adults

The traditional concept that mature mammalian brain is an organ with no regenerative capacity has changed since the discovery of multipotent NSCs, that can give rise to new neurons and glial cells [58]. Two regions of the adult CNS have been confirmed to exhibit ongoing neurogenesis; the subventricular zone (SVZ) surrounding the lateral ventricle and the dentate gyrus (DG) of the hippocampus. Cytogenesis occurring in these regions has been quantified in many studies and clearly shows that large numbers of new cells are regularly generated by stem cells [59].

Stem cells are defined by two characteristics: self-renewal and differentiation into multiple cell types. Adult tissue stem cells share these features and constantly replace lost and senescent cells by proliferation and differentiation [60]. Indeed, adult born neurons in the hippocampus play a critical role in many important functions in normal adult brain. Moreover, newly generated granular cells in the adult DG of hippocampus have the ability to become mature DG neurons generating action potentials and functional synapses [61]. Therefore, endogenous adult stem cells residing in the neurogenic niche would be beneficial for brain repair. It's important to note that the functional role of these new cells is dependent upon a significant number of cells being generated, and their differentiation, survival and integration into existing neuronal circuitry.

2.1. Response of endogenous NSCs to brain injury and their role in brain repair

Studies have shown that TBI significantly increases cell proliferation in the SVZ and DG of adult injured mice and rats [58]. Indeed,

the regenerative capacity of stem cells residing in these zones is of particular interest since adult-generated neurons within these regions have important functional roles and can integrate into the brain circuitry when added into an injured brain area. Therefore, the use of endogenous NSC populations is an attractive strategy to repair the injured brain by regenerating injured brain cells [58].

In a recent study in the cortex of injured human brains, an increased number of cells were found to express endogenous NSCs/neural precursor cells (NPCs) markers, indicating the reparative attempts of the brain after injury [62]. Neurogenesis can be enhanced, particularly in the hippocampus, by using the endogenous NSCs as a therapeutic target for boosting the endogenous repair response after TBI. For instance, biochemical factors such as serotonin, glucocorticoids, and growth factors strongly regulate the proliferation and maturation of cells within the SVZ and DG [58,59]. In addition, the intraventricular administration of basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF), which enhances endogenous neurogenesis, drastically enhance TBI-induced cell proliferation in the SVZ and the hippocampus, and significantly improve cognitive functional recovery [63]. Further understanding of the pathways that drive endogenous neurogenesis is necessary for better intervention at the time of injury. For example, activating the signals that drive endogenous NSCs towards glial and neuronal fates will help in the use of these cells for repair. In this respect, restoring the regenerative potential via upregulation of Sonic Hedgehog signaling is considered a promising strategy contributing to NSC activation and proliferation following TBI [64]. More recently it has been shown that neural reprogramming can be facilitated in an environment of low oxidative stress and Bcl-2 overexpression, which was accompanied by glial-to-neuron conversion after TBI *in vivo* [65].

In a recent study, the regenerative responses to a focal brain lesion showed that cell proliferation in the dorsolateral SVZ doubled by 48 h in the injured immature animals in comparison to an undetectable proliferative response in the adult brain [66]. Likewise, precursors from the injured immature SVZ formed twice the amount of neurospheres, and divided more rapidly, in comparison to those from uninjured brains of the same age [66]. Nonetheless, this reparative response did not result in significant neuronal regeneration, indicating that new neuroprotective strategies need to be implemented to combat brain injuries. Targeting endogenous neurogenesis via activation of NSCs is not simple considering that endogenous stem cells cannot be equally stimulated because of the complex environment surrounding each cell [67].

Other studies showed that neuron formation and integration into an existing network takes approximately 10–14 days [58]. Finally, it has been estimated that less than 1% of astrocytes within the SVZ were undergoing cell division in normal humans. Although these endogenous NSCs can be expanded in culture, their response to injury in patients has not been studied [68].

Some drugs and behavioral changes may increase endogenous neurogenesis. Recently, metformin was found to increase endogenous neurogenesis in a hypoxia-ischemia injury model. Its administration activated endogenous NPCs in the injured neonatal brain expanding the size of NPC pool and promoting their migration and differentiation [69]. In fact, primary neurospheres derived from neonatal pups produced more oligodendrocytes and neurons in the presence of metformin, in comparison to control groups [69]. In addition, metformin promoted the migration of endogenous NPCs into the surrounding parenchyma and improved sensory-motor functions [69].

Extensive studies have shown the potential of endogenous stem cell therapy to successfully repair the injured brain [58], however, many challenges still exist. One major challenge is the generation of sufficient mature neurons capable of integrating into injured brain neural circuitry whereas another obstacle is the focal microenvi-

ronment of the injury site. Primary brain damage together with loss of the secondary tissue induced by oxidative stress, inflammation and ischemia create a hostile environment following TBI, which antagonize the survival and integration of transplanted cells. Therefore, other sources of inexhaustible, self-propagating alternative precursors should be employed [58].

3. Exogenous stem cells

Due to the limited capacity of the injured brain to repair the damaged neurons endogenously, neural transplantation is a promising therapy for TBI. In fact, transplanted cells have the capacity to replace lost cells in the injured brain by differentiation into region-specific cells and providing trophic support. Studies have implemented a wide array of cell types for neural transplantation including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stromal cells (MSCs) or patient-derived adult Neural stem/precursor cells (NSPCs) [70].

3.1. Embryonic stem cells (ESCs)

Adult stem cells have limited differentiation ability into only the cell types of their tissue of origin. In contrast, ES cells are an alternative pluripotent stem cell therapy option due to their ability to differentiate into derivatives of all of the three germ layers in addition to their ability of indefinite self-renewal *in vitro* [71]. ES cells can be harvested from the ganglionic eminences of mouse embryos at day 14 of gestation in addition to their isolation from areas of active neurogenesis in the SVZ of lateral ventricle [72,73]. Due to their high plasticity, they are an ideal cell source for neural transplantation since they can differentiate, migrate and make innervations to different layers of the brain when transplanted into normal or damaged CNS [74]. On the other hand, ESCs have the drawbacks of being ethically debated, prone to transplant rejection and may create teratomas when administered *in vivo*.

ES cells derived from human or mice fetal brains have been used as a source for cell transplantation in different animal models of TBI where they restored motor and cognitive functions (Table 3). For instance, Wennersten et al. have transplanted NSCs derived from ES cells of human fetal brain where they survived and differentiated into neurons and astrocytes after migrating to the contralateral cortex of the injured brain [73]. Recently, it was shown that transplanting differentiated human fetal ES cells into the injured rat brain following a severe controlled cortical injury (CCI) resulted in a transient elevation in angiogenesis and an increase in neuronal survival in the lesion area with a reduction in astrogliosis and brain injury lesion volume [72]. Moreover, NSCs derived from mice fetal brain grafted into injured brain of adult mice showed significant improvement in motor and spatial learning functions, as these cells differentiated into neurons and glial cells that helped in tissue repair [72].

Stem cells have emerged as a potential cell therapy for degenerative diseases and many traumatic injuries due to their ability of differentiation and integration into the neural parenchyma of the host. However, a recent model has been developed suggesting that the paracrine actions of these cells may also contribute to their functional benefits [75]. Indeed, stem cells can secrete at the site of injury a broad spectrum of chemokines, cytokines, growth factors, immunosuppressive molecules and other trophic factors that actively contribute to their environment and to induce responses from host residing cells leading to healing effects and neuroprotective properties. For instance, glial cell- and brain- derived neurotrophic factors (GDNF and BDNF) can be secreted by human NSCs, thus protecting the motor neurons from dysfunctioning and prolonging the lifespan of the animal in models of amyotrophic

Table 3

References for studies on TBI treatment using ESCs and iPSCs. ENSCs, embryonic neural stem cells; iPSCs, induced pluripotent stem cells.

Stem Cells	Author	Content	Year	Ref#
ENSCs	Hoane M.R	Transplantation of neuronal and glial precursors dramatically improves sensorimotor function but not cognitive function in the traumatically injured brain	2004	[163]
	Riess P	Embryonic stem cell transplantation after experimental TBI dramatically improves neurological outcome, but may cause tumors	2007	[161]
	Dobrowolski	Stem cells in TBI	2013	[57]
	Rolfe A	Implications for Repair and Regeneration of Injured Brain in Experimental TBI Models using stem cell therapy	2015	[58]
iPSCs	Kobayashi Y	Pre-evaluated safe human iPSC-derived neural stem cells promote functional recovery after spinal cord injury in common marmoset without tumorigenicity	2012	[79]
	Dobrowolski	Stem cells in traumatic brain injury	2013	[57]
	Dunkerson J	Combining enriched environment and induced pluripotent stem cell therapy results in improved cognitive and motor function following traumatic brain injury	2014	[164]
	Rolfe A	Implications for Repair and Regeneration of Injured Brain in Experimental TBI Models using stem cell therapy	2015	[58]

lateral sclerosis (ALS) transplantation [75]. More recently, transplanting modified ES cells, over-expressing growth factors such as BDNF, into TBI animals showed both motor and cognitive improvement in the injured animals concomitant with better graft survival and neuronal differentiation [73]. On the other hand, further studies are needed to improve the survival of ESCs and their functional neural replacement and integration into the host neural network by modifying the injured host environment.

NSCs have been used in clinical trials for the treatment of motor neuron diseases such as amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative condition with unknown causes and no effective treatment to date. ALS leads to paralysis and ultimately to death with a life expectancy of 2–5 years. The NSCs, called human spinal stem cells (HSSC), were engineered from the spinal cord of a single fetus electively aborted after 8 weeks of gestation. In this trial, the stem cells are transplanted into the ALS patient's spinal cord after laminectomy [clinical trial ID# NCT01348451]. Moreover, in a recent Phase I trial, the safety of human spinal cord derived NSC transplantation for the treatment of chronic spinal cord injury (SCI) is being tested. Patients within this study will receive spinal cord injections of HSSC through a period of 6 months, and will be followed for additional 54 months during which safety assessments will be conducted [clinical trial ID# NCT01772810].

There is a lot of variety in the sources of original ES cells and the differentiation protocols, with different degrees of success, make it hard to draw any conclusion at the moment about the efficacy of the use of stem cells for TBI treatment. Drawbacks of variation in the lines used, also the healing effect of stem cells is due to integration in the neural network plus secretion of neuroprotective trophic factors.

Although ESCs offer new means of treatments, it still raises intense ethical controversies since it involves the destruction of human embryos. Human ESC research raises some complex ethical restrictions that must be discussed along with scientific challenges to ensure that the potential therapies offered by stem cell research is carried out in an ethically appropriate manner.

3.2. Induced pluripotent stem cells (iPS)

It is now well established that reprogramming of differentiated somatic cells into pluripotent ES cells can be accomplished by the ectopic expression of four transcription factors: Oct4, Sox2, c-Myc, and Klf4 [76]. The obtained iPS cells can self-renew and differentiate into various cell types, similar to ES cells. The discovery of iPS cells gives new hopes to stem cell therapy (Table 3). The two main advantages of iPS cells are to avoid the use of ESCs for ethical reasons and the ability to be generated from the patients themselves, thus

allowing iPS cells to be transplanted without any immunological rejection [77].

A recent study confirmed that iPS cells, derived from an 82-year-old patient with amyotrophic lateral sclerosis (ALS), could differentiate *in vitro* into motor neurons and into all various body cell types [78]. Another study described a protocol for using an acutely harvested dura mater, from a 60-year-old patient suffering severe cognitive impairment, for the generation of iPS cells using fibroblasts transduced with non-integrating Sendai virus [78]. Dura mater can be easily taken during most cranial neurosurgical operations, which makes it an efficient source for generation of iPS cells. The iPS clones were shown to express some ESC markers like Nanog, the transcription factors SOX2 and OCT-4 in addition to having a normal chromosomal metaphase spread and karyotype [78]. Therefore, these iPS cells are candidates for auto-transplantation that can be efficiently used in targeted differentiation applications and protocols.

In a spinal cord injury (SCI) model in adult marmosets, transplanted human iPS cells survived and differentiated into all three neural lineages without becoming tumorigenic, enhancing axonal regeneration and preventing demyelination after SCI [79]. The undifferentiated NSCs/NPCs residing in the injured spinal cord could secrete trophic factors that prevent neural cell death and glial scar formation, thus enhancing endogenous remyelination. The transplanted iPS cells not only promoted regeneration but also exerted immunomodulatory and neuroprotective effects in order to prevent tissue damage [79].

Furthermore, iPS cells are also potential stem cell replacement therapy for human cellular models of Huntington's disease (HD), a neurodegenerative irreversible disorder resulting in massive cell death in the striatum of patients who suffer from psychological problems, cognitive deficits and continuous motor dysfunction. Surprisingly, the phenotype of diseased cells could be reversed in a study using corrected iPS cells derived from a HD patient. The corrected cells retained pluripotent characteristics and were successfully reprogrammed and differentiated into striatal neurons [80]. In addition, cadherin and TGF- β signaling alterations and mitochondrial deficits associated with HD were also retained. Besides, NSCs derived from these corrected cells could repopulate the striatum in a HD mouse model [80].

On the other hand, iPS technology has some major limitations. [71]. In fact, the risk of tumor formation is present due to the use of viral infections and a low efficiency of reprogramming during the production of these cells. It is critical to carefully assess safety concerns prior to the use of iPS cells in the clinic since the produced cells are of unknown genetic and epigenetic background [74]. Further

knowledge of iPS cells is urgently requested for the understanding and application of these cells in cell based therapies.

3.3. Mesenchymal stem cells (MSCs)

These are adult stem cells with multipotent capacity that are found in the bone marrow (BM) and other adult tissues [81]. MSCs normally express various surface molecules such as CD105, CD73, and CD90, whereas they lack the expression of hematopoietic markers including CD45, CD19, and CD34 [82,83]. They can be easily isolated from rodents and humans, cultured, and manipulated *in vitro* and *in vivo*. They have significant plasticity, high self-renewal, proliferative, and differentiation capacities. These characteristics allow MSCs to exhibit a therapeutic potential for a wide range of diseases including neurological injuries such as TBI [81,84] (Table 4). The cultured MSCs can be administered intravenously or injected directly into the site of injury, where they will secrete growth factors, aid in tissue repair and induce angiogenesis [82]. When administered intravenously, MSCs were shown to penetrate the BBB and at the same time to induce increased levels of trophic factors in rat brain after TBI [85]. The most known secreted factors are Brain-Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Vascular Endothelial Growth Factor (VEGF), and Glial Cell-Line Derived Neurotrophic Factor (GDNF). Owing to their plastic-adherent characteristic and their ability to differentiate into different cell lineages, MSCs have been proposed as candidates to treat TBI. Indeed, neural cells, expressing neural markers such as beta-tubulin III and glial fibrillary acidic protein (GFAP), can be derived from MSCs when they are cultured with bFGF and hEGF. Following TBI, transplantation of MSCs showed migration, survival, and differentiation of these cells in the injured brain where they participated in neuroprotection, neural repair, and motor function improvements due to the formation of new astrocytes and neurons [83,86]. However, intravenous administration of MSCs into rat TBI models only showed neuroprotection and neural repair abilities, with no significant amelioration in motor recovery [87].

The effect of MSCs on brain stroke is assessed in different experiments. Recently, Cheng et al. revealed that inflammatory cytokines such as TNF- α , IL-17, and IL-23 can be attenuated by MSCs thus indirectly modulating inflammation [88]. Moreover, Mert et al. revealed that the levels of IL-6 and IL-1 β were suppressed by the locally transplanted MSCs which enhanced IL-10 levels [89]. On the other hand, a new study has investigated the effect of MSCs and NSCs combination therapy on the neurological functioning following brain stroke. Groups receiving the combination therapy showed a better anti-inflammatory, anti-oxidant, neuroprotection, secreted neurotrophic cytokines and less brain lesion [90]. These effects make the surrounding microenvironment more suitable for the reparative activity of transplanted NSCs. After brain stroke both MSCs and NSCs, separately, have the potential to reduce brain damage and enhance the neuronal regeneration. However, their combination is more efficient and more significant in recovery after brain stroke [90]. Finally, neural differentiation is enhanced robustly after reducing the inflammation, which develops following brain stroke [91].

MSCs are the most promising stem cell therapy for TBI. Indeed, a recent clinical trial aimed to use autologous BM mononuclear cells (MNCs) intravenously (*i.v.*) for treatment of adult severe TBI and whether these cells could retain the structure of the white matter (WM) as well as gray matter (GM) volume and integrity [NCT02525432]. Pre-clinical and Phase I clinical progenitor cell therapies have shown promise in TBI by promoting the CNS structural preservation, and reducing neuroinflammatory response to injury. The trial is a blinded, randomized, placebo-controlled study for adults with severe acute TBI and is a follow-up from a previ-

ously performed Phase 1–2a trial where 40 additional participants will be enrolled yielding outcome data for 65 adult TBI subjects over the 4 year term of the study. After 48 h from the initial injury, eligible subjects will receive a single infusion of either autologous BM MNCs or placebo. Subjects will be monitored for infusion related toxicity while also receiving the usual standard care for TBI [NCT02525432].

In another recent clinical study, the efficacy, safety and tolerability of intracranial administration of SB623 cells' will be evaluated in patients suffering from chronic motor deficit after TBI [NCT02416492]. SB623 cells were derived from adult BM and protected neurons by producing trophic factors in models of ischemic insult. Significant improvement of motor function was noticed after the implantation of these cells around the injury area in a rat TBI contusion model. Further, no adverse effects, dose-limiting toxicities, laboratory, histological or clinical abnormalities were found after the safety evaluation of the animal models receiving SB623 cells. To date, the study has shown significant motor function improvements according to the European Stroke Scale (ESS), the National Institute of Health Stroke Scale (NIHSS) and the Fugl-Meyer scale.

3.4. Adult stem cells (ASCs)

Embryonic or fetal tissue stem cells are mainly exploited in cell therapy areas, however, their use is complicated by many technical and ethical issues, the risk of tumor formation, and immunological rejection [92]. To overcome these complications, adult stem cells (ASCs) are considered as a promising potential source for TBI therapy (Table 4). In adult brains, both humans and rodents, ASCs are harvested from and are confined to the SVZ region surrounding the lateral ventricle. These cells are multipotent stem cells that have a decreased potential of self-renewal, unlike ESCs, and normally, for the purpose of repair, differentiate into only one cell lineage of the tissue where they reside (unipotent). ASCs can differentiate into neuronal cells and hence their use as a possible therapeutic option in TBI.

ASCs showed an improved survival capacity for a long period when transplanted into the rodent injured brain and could mature into region specific functional cells [93]. When rat or human ASCs were injected into injured brain, they survived and restored the anatomy and function of the injured areas [83,93,94]. In fact, these cells migrate away from the site of injury towards surrounding areas where they express markers of mature glial cells, such as astrocytes and oligodendrocytes, indicating that the transplanted cells were able to differentiate into both neurons and glia [72,77]. Another study showed that NSCs from human spinal cord can graft successfully in adult rat spinal cord and that the grafts can show substantial neuronal differentiation in the injured adult rat spinal cord with good potential of integration into host neural circuits. In addition, excellent neuronal survival and differentiation of human NSC grafts was achieved in normal rats [95].

Several clinical studies involve the use of neural stem cell approaches in patients. For instance, purified human ASCs have been transplanted into patients with chronic spinal cord injury in a phase I/II clinical trial by *Stem Cells Inc* [77]. Another example in the NSCs therapy domain is *Neuralstem's* phase I clinical trials for ALS patients and *ReNeuron's* trial for disabled stroke patients [96].

The discovery that transplanted exogenous neural cells can integrate into the neural parenchyma facilitates the ability to use cell based therapy in traumatic diseases of the nervous system. The use of stem cells with different differentiation pathways is sometimes needed. For instance, diffuse TBI which involves axonal damage due to demyelination, unlike focal TBI, may need to introduce combination therapy of SCs that include cells that are in the neu-

Table 4

References for studies on TBI treatment using adult stem cells. ASCs, adult stem cells; MSCs, mesenchymal stem cells; BMSCs, bone marrow stem cells; NSCs, neural stem cells.

Stem Cells	Author	Content	Year	Ref #
ASCs	Walker P.A	The barriers and opportunities of progenitor cell therapies for TBI	2009	[83]
	Dobrowolski	Stem cells in TBI	2013	[57]
	Rolfe A	Implications for Repair and Regeneration of Injured Brain in Experimental TBI Models using stem cell therapy	2015	[58]
MSCs	Tajiri N	Intravenous transplants of human adipose-derived stem cell protect the brain from TBI-induced neurodegeneration and motor and cognitive impairments	2014	[159]
BMSCs	Mahmood A	Intracranial bone marrow transplantation after TBI improving functional outcome in adult rats	2001	[160]
	Mahmood A	Treatment of TBI in adult rats with IV administration of human BMSCs	2003	[156]
	Mahmood A	Long-term recovery after bone marrow stromal cell treatment of TBI in rats	2006	[86]
	Mahmood A	Treatment of TBI with a combination therapy of marrow stromal cells and atorvastatin in rats	2007	[103]
	Mahmood A	Long-lasting benefits after treatment of TBI in rats with combination therapy of marrow stromal cells and simvastatin	2008	[104]
	Li L	Transplantation of marrow stromal cells restores cerebral blood flow and reduces cerebral atrophy in rats with traumatic brain injury TBI: in vivo MRI study	2011	[157]
	Anbari F	Intravenous transplantation of bone marrow mesenchymal stem cells promotes neural regeneration after TBI	2014	[158]
	CBs	Harting M.T	Intravenous mesenchymal stem cell therapy for TBI	2009
Walker P.A		The barriers and opportunities of progenitor cell therapies for TBI	2009	[83]
Dobrowolski		Stem cells in TBI	2013	[57]
Gutierrez-Fernandez		Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke	2013	[82]
Lam P.K		Transplantation of mesenchymal stem cells to the brain by topical application in an experimental TBI model	2013	[141]
Donega V		Intranasal mesenchymal stem cell treatment for neonatal brain damage; long-term cognitive and sensorimotor improvement	2013	[142]
Mouhieddine T.H Hosseini S.M		Stem cells in neuroinjury and neurodegenerative disorders Combination cell therapy with MSCs and NSCs for brain stroke in rats	2014 2015	[106] [90]
NSCs/NPCs	Wennersten A	Proliferation, migration, and differentiation of human neural stem progenitor cells after transplantation into a rat model of TBI	2004	[73]
	Bakshi A	Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following TBI	2006	[162]
	Yan J	Extensive neuronal differentiation of human NSC grafts in adult rat spinal cord	2007	[95]
	Walker P.A	The barriers and opportunities of progenitor cell therapies for TBI	2009	[83]
	Wallenquist U	Grafted neural progenitors migrate and form neurons after experimental TBI	2009	[92]
	Barkho B.Z	Response of adult neural stem cells to stroke injury and their potential role in therapeutic applications	2011	[77]
	Skardelly M	Long-term benefit of human fetal neuronal progenitor cell transplantation in a clinically adapted model after TBI	2011	[72]
	Sun D	Sustained survival and maturation of adult neural stem/progenitor cells after transplantation into the injured brain	2011	[93]
	Donega, M	Systemic injection of neural stem progenitor cells in mice with chronic EAE	2014	[70]
	Hosseini S.M	Combination cell therapy with MSCs and NSCs for brain stroke in rats	2015	[90]
	Rolfe A	Implications for Repair and Regeneration of Injured Brain in Experimental TBI Models using stem cell therapy	2015	[58]

ronal differentiation pathway and others that are differentiated into oligodendrocytes.

4. Combination therapy

A strong rationale for the use of combination therapies is brought upon by the complexity of the TBI insult. Moreover, the success of this approach for other medical conditions presents another driving force for exploring combination therapies in TBI [97]. A number of studies have shown the efficacy of combining promising single therapies with other treatments for treating medical conditions such as HIV/AIDS, Crohn's disease and Alzheimer's [98–101].

Indeed, a number of combination therapies were undertaken in TBI with positive effects. For instance, the use of erythropoietin (EPO) combined with granulocyte-colony stimulating factor (G-CSF) was recently linked to an improvement in functional recovery and brain nerve cell regeneration after TBI in rats [102]. Moreover, it has been shown that when administered in combination with MSCs, atorvastatin and simvastatin improve functional outcomes after TBI in rats [103,104]. However, no clear evidence of the clinical value of either combination therapies can be found in humans. On the other hand, the use of progesterone in combination with 1,25-dihydroxyvitamin D(3) (VDH) has been clinically proven to have a much profound ability at reducing neuro-inflammation compared

to progesterone and VDH administered separately at 24 h following TBI [105].

Given the fact that growth factors may have a promising role in enhancing cell proliferation and survival during early differentiation of MSCs to neural progenitor-like cells *in vitro* and *in vivo* in addition to the role of ROCK inhibitors in axonal regeneration in the adult CNS injury, we choose to focus in the following discussion on the use of stem cells in combination with growth factors on one hand and stem cells along with ROCK inhibitors on the other [106,107].

4.1. Growth factors

Growth factors have been linked to TBI (Table 5), for instance, human epidermal growth factor receptor (EGFR) has been shown to decrease oligodendroglia death and enhance the generation of new oligodendrocytes from progenitor cells [108]. In addition, in an ischemic adult rat model, treatment with vascular endothelial growth factor (VEGF), which promotes blood vessel growth and is upregulated following brain injury, has been linked to improved neurological deficit scores and an inhibited apoptosis in the basal ganglia and cortex [109,110]. Moreover, Glial cell line-derived neurotrophic factor (GDNF), a sequence-related factor of the transforming growth factor-beta (TGF- β) family, has been shown to be a potent neurotrophic factor for a variety of neuronal cell populations and to protect damaged cortical neurons in rats [111,112]. In addition, the expression of insulin-like growth factor-1 (IGF-1) significantly increases following TBI. Its administration alone resulted in a significant decrease in cell injury markers and improved the "motor activity" of injured rats. On the other hand, its co-administration with growth hormone produces sustained improvement in metabolic and nutritional endpoints after moderate to severe acute TBI [113–115]. Moreover, IGF-1 has a crucial role in the support of proliferation and differentiation of MSCs into the neuronal lineage [116]. Other growth factors such as neural growth factor (NGF) significantly improves the outcome after TBI. In fact, NGF was shown not only to protect neurons after TBI in rats but also to confer on NGF-transduced NSCs the capacity to improve cognitive and neuromotor functions [117,118]. Recent studies on rodents have shown that transfection of human umbilical SCs with VEGF and fibroblast growth factor 2 (FGF2) leads to the transformation of these stem cells into astrocytes and contributes to the survival of damaged neurons. Finally, genetically modifying NSCs to express brain-derived neurotrophic factor (BDNF) demonstrated a more potent neuronal differentiation and higher survivability as well as a significant improvement in motor functions in rat models of TBI [119]. This is not surprising given the roles of BDNF in promoting growth and survival of neurons [120]. A most recent combination therapy study uses propranolol, a β -adrenergic receptor blocker, in combination with MSCs to treat TBI. In this study the combination of propranolol and MSCs improved cognitive and memory functions more significantly than either treatment alone [121]. Therefore, growth factors are promising candidates for combination therapy with stem cells.

Recently, it was shown that only one among six preclinical studies of combination therapies for TBI in adult and immature male rats reported significant improvements in long-term outcomes across a battery of behavioral tests [122].

4.2. ROCK inhibitors

Rho GTPase and Rho associated serine/threonine kinases (ROCKs) are known to have a disrupted function in TBI. It has been shown that the activation of RhoA and its effectors Rho kinases (ROCK) is a key element for axonal growth inhibition and thus blockade of the Rho-ROCK pathway promotes axonal

regeneration *in vivo* [123,124]. This in turn renders ROCK inhibition a promising candidate for the treatment of TBI. Rho and ROCK are downstream effectors in the Ephrin signalling pathway that is initiated when a membrane-bound Ephrin type A receptor 5 ligand (EphA5) binds to an EphA receptor. This binding activates signaling *via* the kinase domain of the EphA receptor. The activated EphA receptor kinase then leads to the consequent recruitment and clustering of several signaling molecules into a complex that in turn activates one of two GEFs, either Ephexin or Vms-Rho. The activated GEF further activates RhoA, which in turn stimulates a downstream ROCK signaling pathway by binding to the RhoA binding domain (RBD) of ROCK, ultimately leading to a block of nerve fiber growth (Fig. 2). Among ROCK inhibitors are the isoquinoline derivative *Fasudil* (HA-1077), which stands as one of the first identified small-molecule ROCK inhibitors. Y-27632, which belongs to the 4-aminopyridine series, is another small-molecule ROCK inhibitor [125]. While *Fasudil* has been experimentally proven to have anti-inflammatory, anti-oxidative, and anti-dementia effects, both drugs demonstrated anti-apoptotic roles and neuroregenerative capacity [126–132]. Most importantly, and concerning the combination therapy using ROCK inhibitors, *Fasudil* has been recently shown to have the ability to stimulate neurite outgrowth and differentiation in the stem-like neural progenitors C17.2 cells [133]. *Fasudil* appeared to play a role in driving mobilization of adult NSCs after hypoxia/reoxygenation injury in mice [133,134]. Moreover, priming Wharton's jelly derived MSCs (WJ-MSCs) with *Fasudil* represents a possible application for enhanced cell therapy in stroke given its effect on morphology as well as expression of neuronal markers *in vitro* and on functional performance *in vivo* [135]. Furthermore, separate experiments have shown that Y-27632 promotes stem cell differentiation into cortical and basal telencephalic progenitors, increases the survivability and recovery of cryopreserved hESCs, and could inhibit the anti-neurogenesis/neurodifferentiation effect of chondroitin sulfate proteoglycan, found in glial scar tissue, on MSCs [136–138]. Taken together, ROCK inhibitors are considered as promising candidates for combination therapy following TBI.

5. Route of administration

3 Since the brain is the most complex organ in a vertebrate's body, "effectiveness" and "minimal invasiveness" are two major issues that must be considered when choosing stem cell therapy. In fact, the route of delivery of stem cells has a profound impact on the above two characteristics. Among the many routes of administration, the intravenous, intra-arterial and the direct routes are the most relevant [83]. The intravenous route is a minimally invasive procedure; however, it has been shown that a very modest percentage (1.48–3.69%) of infused MSCs reach the arterial circulation and even less of the infused cells (0.0005%) make their way to, and remain at, the site of injury in the brain [87]. This 'diameter-induced sequestration' drawback is a result of the pulmonary first-pass effect brought upon by the pulmonary microvasculature. The effect of this drawback can be diminished by pre-treatment with *sodium nitroprusside* [83]. However, there are issues associated with the intravenous delivery route, mainly cell homing where stem cells appear to scatter among different organs in the body, which requires the infusion of a large number of stem cells [139]. To overcome this problem, intra-arterial injection is used; similarly, this is a minimally invasive approach that bypasses the pulmonary first-pass effect. It involves injecting the cells directly into the carotid artery ipsilateral to the side of the ischemic lesion. Although previous data have linked this route to an increased risk of damage caused by decreased cerebral blood flow (CBF) and inflammation, new data have shown that intra-

Table 5
Effect of growth factors on the progression of TBI. Growth factors post TBI protects and enhances the survival of neural cells.

Growth Factor	Effect on TBI upon administration	Ref #
EGFR	Promotes survival and regeneration of oligodendrocytes	[106]
VEGF	Stimulates angiogenesis post-TBI; inhibits apoptosis in basal ganglia and cortex	[107,108]
GDNF	Protects cortical neurons	[109,110]
IGF-1	Decreases cell-injury markers; improves motor activity and metabolism after TBI; promotes the proliferation and differentiation of MSCs into neuronal lineages	[111-114]
NGF	Protects neurons after TBI; enhances cognitive and neuromotor functions	[115,116]
BDNF	Promote survival and growth of neurons; prevents neuronal dysfunction; improves cognitive and motor functions	[117,118]

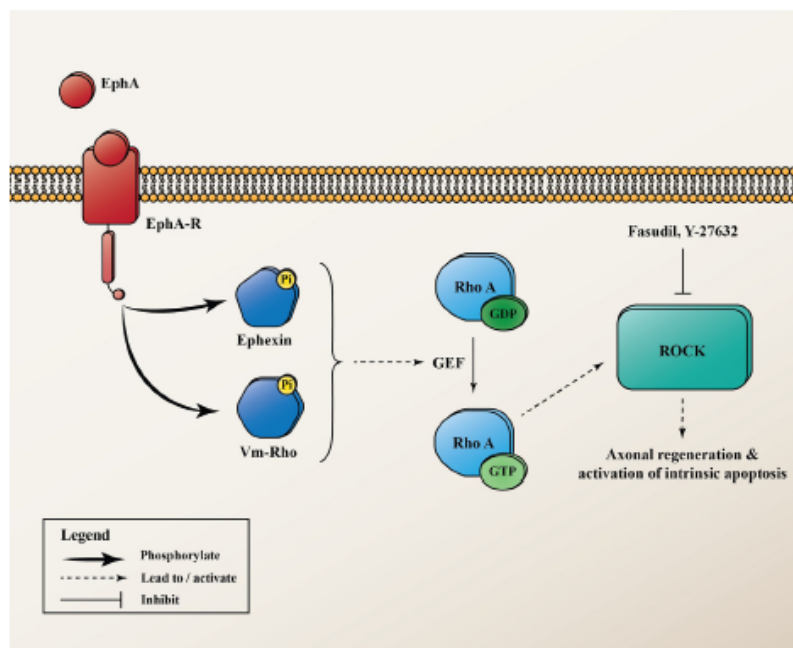


Fig. 2. EphA5 pathway. EphA5 ligand binds to an EphA receptor (EphA-R) thus activating signaling via the kinase domain of the EphA-R. The activated EphA receptor kinase then leads to activation of one of two GEFs, either Ephexin or Vms-Rho. The activated GEF further activates RhoA, which in turn stimulates a downstream ROCK signaling pathway ultimately leading to a block of nerve fiber growth.

arterial injection of NSCs using a micro-needle technique does not cause microembolic strokes [140]. Finally, direct stem cell implantation, though invasive, it is promising and maximizes the amount of stem cells at the site of injury. In fact, studies have shown that MSCs topically applied to the brain surface can migrate to a TBI site in a rat model and show a higher proliferation rate as compared to intravenous administration [83,141]. Moreover, transplanting NSCs into a nearby uninjured site rather than directly at the site of injury seems to provide a much better opportunity for the transplanted stem cells to proliferate away from the inflammation at the TBI site [106]. It's worth noting that intranasal administration of MSCs in a mouse model of neonatal ischemic brain injury proved successful in delivering stem cells to the site of injury [142]. In summary, the best route of administration is yet to be determined since each route presents its own advantages and disadvantages. As such, future comparative studies concerning the fate of cells after administration would aid in selecting the most appropriate route

of administration with the least side effects, minimal costs, and highest efficiency.

6. Improvements in motor and cognitive functions after SC therapy

Motor and cognitive functions have been shown to be compromised following mild, moderate and severe TBI in patients. Animal experimental TBI models including blast and impact acceleration injury, lateral and midline fluid percussion injury, and controlled cortical impact (CCI) injury produced cognitive and motor deficits similar to those observed in humans [10,143-145]. For instance, in order to characterize impairments in memory in animal models of TBI, Zhou and Riccio found that TBI is associated with retrograde amnesia (RA) which is the lack of ability to remember events occurring minutes to years prior to injury, as well as working memory and anterograde memory, the loss of the ability to create new

memories after the TBI event [145–149]. Similarly, Williams et al., presented several studies reporting an impairment of motor functions after TBI in humans [145,150–154]. Moreover, Hamm and colleagues showed motor deficits following mild and moderate central fluid percussion brain injury and an impaired ability of animals to perform the rotarod task [155]. Data showing improvements in motor and cognitive functions following exogenous stem cell therapy in TBI are numerous. In a rat TBI model, treatment with hMSCs significantly improved neurological function in the injured brain, as evaluated with the rotarod test and neurological severity score. Moreover, their intravenous administration contributed to cognitive improvement in spatial learning and memory using a Morris water maze test [156,157]. Also, in another rat model, motor deficits improved significantly in the group transplanted with MSCs, as compared with TBI group [158]. More recently, MSCs derived from adipose tissue and their secretome were found to play a significant role in amelioration of motor and cognitive functions [159]. In another study, bone marrow transplantation improved motor functions after TBI [160]. Other improvements included the attenuation of motor dysfunction brought about by the transplantation of immortalized NSCs, derived from GDNF secreting foetuses. These NSCs survive and differentiate into neurons and/or glia when transplanted into peri-injured cortex. Cognitive improvement was observed when these same NSCs were transplanted into the hippocampus [161,162]. In addition, a large motor but not cognitive improvement was detected when ESCs, pre-differentiated into neuronal and glial precursors, were used [163]. In a separate study, injections of ESCs after experimental TBI dramatically improved the neurological outcome on a rotarod task and a Composite Neuroscore test [161]. Recently, Dunkerson and partners linked iPSCs treatment with improved cognitive and motor performance following TBI in rats [164].

7. Conclusions and future directions

Despite the expansion of research on TBI diagnosis, treatment, and prevention, a major question remains unanswered: What is the most effective method for promoting recovery of brain function after TBI? Given the complex genetic variability, a definitive answer would be far from easy, but it is not surprising if the answer lies within our own bodies: our stem cells. Whether exogenous or endogenous, in combination with growth factors or ROCK inhibitors, stem cells clearly present good candidates for TBI treatment, and consequently a hope for many TBI survivors. Various stem cell types have been investigated, however, further development is required to improve their use in TBI transplantation therapy. In addition, the limitations and controversies associated with stem cell therapies (MSCs, iPSCs, etc.) should be explored carefully in quest for solutions. For example, the ethical debate of using ESCs, the differentiation and proliferation capacity of the stem cells, their routes of administration and their potential side effects should all be considered. Therefore, although stem cell treatment is a promising option for TBI, further research is mandatory in order to translate the research outcomes into a clinical level by exploring the fate of these cells, their tumorigenic potential, and their differentiation status and proliferation capacity. Mode and site of administration, patient history and age should also be taken into account before establishment of stem cell therapy for TBI.

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Discussion

Neuronal loss leads to several neurological sequelae following TBI resulting in pathophysiological and behavioral deficits (McGinn & Povlishock, 2016). Despite the available information and experimental approaches in progress, there is no approved pharmacological treatment specific for TBI (Diaz-Arrastia et al., 2014). Therefore, neurotherapeutic have been proposed to moderate the consequences of TBI including cell replacement therapies and combinational drug administration which aim to retrieve neuronal survival and functional recovery.

In the present chapter we outlined the effects of transplanting ENSCs and DCs as therapeutic adjuvants for stem cell therapy in a murine mCCI model. Moreover, the administration of neural stem cells combined with DHA was investigated as a means to enhance recovery following brain injury.

In our first study, we mainly grafted ENSCs or DCs into mouse model of mCCI and investigated their effect on neuroinflammation and neurogenesis one or four weeks following grafting.

Previous evidences suggest that neuroinflammation triggered after brain injury, may result in either detrimental outcomes such as upregulated oxidative stress and neuronal cell apoptosis or in injury recovery and tissue repair (Kumar et al., 2013). In fact, several induced immune cells infiltrate the damaged site following brain injury including the neutrophils, microglia and astrocytes (Gyoneva & Ransohoff, 2015). Therefore, activation of microglial cells is recognized as one of the secondary biochemical markers of neuroinflammation following TBI(Loane t al., 2014).

We demonstrated in our results an elevation in microglial activation more significantly at one week interval in the DCs grafted group. In fact, several morphological and functional modulations emerge in parallel with microglial activation. These modifications include the

activation of the ramified resting microglia into bushy hypertrophic cells secreting anti- and pro-inflammatory mediators (Loane et al., 2014). Hence, two types of microglial cells are distinguished based on their phenotype and mode of action; the M1 and M2 populations. As demonstrated in several studies on TBI, during the first week of injury active M2-like microglial cells are upregulated then subsequently the M1-like microglial phenotype prevails (Jin et al., 2012; Wang et al., 2013). As a matter of fact, activation of M2 microglial cells ameliorates repair and recovery following injury (Loane & Byrnes, 2010) in contrary to M1 microglial activation which contributes to cytotoxicity and injury deterioration (Vexler & Yenari, 2009). In this context, astrocytes also undergo morphological changes in response to brain injury and become activated cells with hypertrophic cellular ends inducing reactive astrogliosis (Babae et al., 2015). Our data showed a significant diminution in astrogliosis at one week in ENSCs in comparison to control and DC injected groups. This reduction was also demonstrated at four weeks in the DC and control groups when compared to one week post-injection. Actually, due to disruption in the blood brain barrier (BBB) during the acute phase of TBI, glial cells reactivity and neuroinflammation are upregulated which triggers neuronal cell death (Babae et al., 2015). Our data verified that reduced astrogliosis was associated with ENSCs or DCs administration into the lesioned cortex, therefore our cell treatment may have a neuroprotective aspect at one and four week's intervals post-injection.

Interestingly, expression of DCX was significantly elevated after grafting in ENSCs and DCs treated animals with respect to control group at four weeks post-injection. In humans, following TBI the newly produced endogenous cells migrate near to the damaged zone and become mature neurons (Zheng et al., 2013). Yet, taking into consideration the inflammatory processes triggered by astrocytes and microglia, the maturation and survival of the newly generated progenitors are not maintained subsequently restraining long term endogenous repair (Bregy et al., 2012).

Knowing the fact that neurons need four weeks to become mature and functional, our data showed a significant upregulation in NeuN expression in DCs injected group but not in control group after four weeks post-treatment.

Overall, in the present study we did not find a preference for transplantation of ENSCs over DCs or the opposite. Activation of microglia and diminution in astrogliosis were obtained after treatment with both cell types. Despite technical limitations in our study, ENSCs and DCs (glial and neuronal cells) may have an impact on neuroinflammation as well as neurogenesis to support recovery and repair following brain injury.

On another aspect, the use of multiple complimentary therapies has been introduced to be efficient in several therapeutic approaches for treating certain medical conditions and TBI studies (Margulies & Hicks, 2009).

In the second article introduced in this chapter, the effect of transplanting NSCs and DHA pre-treatment on neural regeneration and functionality was assessed in mild TBI mouse model.

For instance, NSCs obtained from the SVZ of neonatal mice were transplanted into the cortex of lesioned mice pre-treated or not with DHA.

Data obtained demonstrated a significant enhancement in motor behavior following NSC transplantation and DHA treatment in the lesioned mice. Moreover, an increase in DCX expression was detected in animals treated with NSCs and DHA in comparison to those receiving treatments separately, suggesting that DHA could enhance the survival of transplanted cells which consequently could explain the enhanced neurogenesis in these groups. Interestingly, NSC treatment coupled with DHA injection resulted in elevated microglial reactivity and astrogliosis in the vicinity of the injured area along with an increase in DCX expression. By this means, there exists an evidence that upregulated astrocytes may induce the production of NSCs through blastogenesis which in turn can give rise to mature glial cells and

neurons (Itoh et al., 2013). On the other side, in separately DHA injected or NSCs treated mice astrogliosis was not upregulated. Therefore, co-treatment of NSCs with DHA may induce a cross talk between NSCs and glial cells providing a potential neuroprotection after mild TBI.

Taken altogether, both studies demonstrated upregulated microglial activation and possible enhanced neurogenesis upon administration of combined NSCs/DHA or ENSC/or DCs treatment in mild TBI mouse model. In the first study, astrogliosis was reduced in animals receiving ENSC or DC transplantation, where as in the second study astrogliosis was not affected by the single administration of NSCs or DHA in contrary, it was elevated upon dual administration of both treatments together. Therefore, stem cell transplantation using ENSCs or NSCs complemented with DHA treatment may provide a promising perspective in cell therapy for TBI.

General Conclusion

Developmental studies are essential to understand cellular and molecular mechanisms involved in pathological conditions and to develop therapeutic strategies, especially in degenerating brain. For example, the development of the mesencephalic dopaminergic circuitry is dependent on several factors and ordered events including the growth and steering of neurites of the residing mDA neurons in response to axon guiding cues. Interestingly, Sema7A is an axon guidance molecule required for promoting and maintaining the survival of the mDA neurons during early development as demonstrated in our study. In this regard, a better understanding of the mechanisms by which Sema7A impacts the presence of the mDA cells in the VM during development is essential from a clinical perspective if we aim to increase the survival of these cells in transplantation procedures for neurodegenerative conditions as in PD.

Indeed, cell therapy has already gained a great consideration in brain repair strategies since it may allow to re-establish a functional neural circuitry that is damaged or degenerated in neurological disorder. However, this approach is still a major challenge since it deals with immunological and inflammatory responses, proliferation, differentiation and survival of the grafted cells that represent as many challenges to therapeutic strategies. Indeed, neuroinflammation is one of the deleterious events that can reduce the survival of grafted cells. In this way, our work suggests that blocking Sema7A after cell therapy in a mouse model of PD, may reduce inflammatory mechanisms and may reinforce the survival of the grafted mDA neurons. Moreover, several combined strategies may help to overcome the substantial death of grafted cells. For example, co-administration of neuronal cell transplantation and agents such as DHA would be beneficial in enhancing neurogenesis and survival of the grafted cells following brain trauma as observed in our studies. Furthermore, the outcome of cell replacement procedures in neuropathological disorders is also influenced by the proper choice of cells and the presence of supportive microenvironment. Thus, replacing the lost neurons within a damaged cortex can also be achieved by transplanting neural stem cells produced from

ganglionic eminence of E14.5 embryos as we showed that these cells integrate and ameliorate the neurogenesis of neuronal progenitors subsequently promoting neural recovery within the host following transplantation. Actually, the use of fetal or embryonic stem cells is being hindered by several obstacles, such as ethical concerns, tumorigenicity and immunogenicity. Rather, induced pluripotent stem cells (iPSCs) that are reprogrammed from patient somatic cells, possess unique properties of self-renewal and differentiation without immunogenic barriers. In this way, these cells constitute a tremendous potential for cell therapy for lesioned brain in TBI or PD. In vivo reprogrammed iPSCs from reactive glial cells could also be induced to endogenously repair as it has been shown in a mouse model of cortical lesion (Gao et al., 2016). Among therapies to treat PD, promising clinical trials using iPSCs are currently developed in several countries (Cyranoski, 2018; Morizane, 2019; Takahashi, 2019).

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