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Study of the pair Netrin-1/DCC in Parkinson's diseases

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Etude de Netrin-1 et de son récepteur DCC en tant que nouvelles pistes thérapeutiques contre la maladie de Parkinson

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Ce mémoire de doctorat intitulé « Etude de Netrin-1 et de son récepteur DCC en tant que nouvelles pistes thérapeutiques contre la maladie de Parkinson » est une thèse sur article qui s'appuie sur la reproduction d'un article en préparation, fruit d'un travail de collaboration entre les laboratoires de Patrick Mehlen, Mart Saarma et Keqiang Ye, que je signe en 1^{er} auteur. Le mémoire est rédigé en anglais et sera présenté publiquement le 12 décembre 2018 en vue de l'obtention du grade de Docteur.

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Enjoy your reading, *bonne lecture,*

Mélissa Jasmin
Lyon, le 03/12/2018

RESUME DETAILLE DU TRAVAIL DE RECHERCHE

La maladie de Parkinson est une maladie neurodégénérative caractérisée par la perte progressive et sélective des neurones dopaminergiques de la substance noire. Ces neurones émettent des projections axonales dans le striatum et forme la voie nigrostriée ; un circuit de neurones impliqué dans le contrôle de la motricité. La perte de ces neurones est responsable des troubles moteurs caractéristiques de la maladie. Bien qu'il existe des traitements symptomatiques permettant d'améliorer la qualité de vie des patients, aucun traitement ne permet d'arrêter l'évolution de la maladie. Freiner la dégénérescence de ces neurones aurait un impact médical, économique et de santé publique considérable compte tenu du vieillissement croissant de la population. Dans ce but, beaucoup d'études ont été menées sur des facteurs neurotrophiques [1], [2], en particulier le GDNF (glial cell-derived neurotrophic factor), afin de favoriser la survie des neurones et la croissance des axones. Plusieurs essais cliniques ont été conduits mais l'efficacité thérapeutique de ces facteurs reste controversée [3], [4]. Trouver des molécules plus spécifiques des neurones dopaminergiques, impliquées dans leur développement, leur survie ou leur fonction, et qui pourraient éventuellement agir de façon complémentaire avec les facteurs neurotrophiques déjà identifiés offrirait des perspectives thérapeutiques nouvelles.

Nous avons pensé que netrin-1 pouvait être un candidat intéressant. En effet, cette protéine de guidage axonal, par l'intermédiaire de son récepteur DCC (deleted in colorectal cancer), favorise la pousse et l'orientation des axones, la migration des neurones [5], [6] et participe à la formation et au maintien des synapses [7], [8], [9]. Ce couple ligand/récepteur joue un rôle majeur dans l'établissement et le maintien de connexions entre les neurones et participe notamment à la formation de la voie nigrostriée [10]. Si le rôle de netrin-1 au cours du développement est largement documenté, sa fonction dans le cerveau adulte est peu connue. De façon intéressante, Livesey and Hunt montrent que la substance noire est la région du cerveau adulte contenant le niveau le plus élevé de transcrits de netrin-1 [11]. Netrin-1 est également exprimée dans les zones du striatum où projettent les fibres dopaminergiques des neurones de la substance noire *pars compacta*, neurones qui, par ailleurs, expriment très fortement DCC [12]–[13]. Cette expression conjointe de netrine-1 et du récepteur DCC suggère un rôle dans le maintien, la survie voire la fonction des neurones dopaminergiques de la substance noire. Par ailleurs, certains polymorphismes du gène DCC sont associés à différents aspects de la maladie de Parkinson (susceptibilité de développer la maladie, l'âge d'apparition des symptômes, sévérité de la progression de la maladie) [14], [15]. Ces différentes observations indiquent que netrin-1 et DCC pourraient réguler le maintien ou la survie des neurones dopaminergiques et donc participer au développement et/ou à la progression de la maladie de Parkinson. De plus, les travaux du laboratoire ont montré que netrin-1 était une molécule de survie. En son absence, DCC est capable d'activer une signalisation sensibilisant la cellule et entraînant la mort de celle-ci [16], [17]. L'ajout de netrin-1 permet d'inhiber cette signalisation pro-apoptotique.

En raison du rôle de netrin-1 sur la croissance des axones, le maintien des synapses et la survie cellulaire nous avons choisi d'étudier son effet sur la survie des neurones dopaminergiques de la substance noire dans des modèles *in vivo*.

Pour cela, nous avons étudié l'effet de l'injection de netrin-1 sur la dégénérescence des neurones dopaminergiques de la substance noire dans un modèle animal de référence de la maladie de Parkinson, le modèle de rat lésé à la 6-hydroxydopamine (6-OHDA) [18]. Cette molécule, similaire à la dopamine, est injectée dans le striatum en unilatéral (côté droit) et, captée par les transporteurs à la dopamine, induit un stress oxydant et mitochondrial conduisant à la dégénérescence rétrograde et progressive des neurones dopaminergiques de la substance noire. Deux semaines après la lésion, les rats ont été traités (stéréotaxie) avec : un contrôle positif (GDNF 10 μ g) ; un contrôle négatif (PBS) ; différentes doses de netrin-1 (1, 5, 10, 20 μ g). Nous avons suivi la progression de la lésion et l'effet du traitement toutes les deux semaines pendant trois mois à l'aide de tests moteurs (tests de rotation ipsilatérales), puis les animaux ont été sacrifiés et les cerveaux récupérés pour des analyses histologiques. Les résultats obtenus démontrent un effet protecteur clair de netrin-1 (10 μ g) à la fois sur le comportement moteur, sur la densité des fibres dopaminergiques dans le striatum et le nombre de neurones dans la substance noire, révélant le potentiel thérapeutique de netrin-1. L'effet protecteur de netrin-1 a ensuite été confirmée dans des souris transgéniques exprimant netrin-1 de façon inductible deux semaines après lésion à la 6-OHDA.

D'autre part, des travaux menés en collaboration avec le laboratoire de Keqiang Ye (Emory institut, Etats-Unis) ont montré que la suppression de netrin-1 entraînait la perte des neurones dopaminergiques de la substance noire *in vivo*, en partie à cause de l'activité pro-apoptotique de son récepteur DCC. Afin de corroborer ces résultats, nous avons testé la résistance des neurones dopaminergiques, de souris mutées pour l'activité pro-apoptotique de DCC, à la toxicité induite par la 6-OHDA. Les résultats préliminaires semblent indiquer que le blocage de l'activité apoptotique de DCC retarde la dégénérescence des neurones dopaminergiques de la substance noire.

Nos travaux indiquent donc que netrin-1 et DCC jouent un rôle important pour la survie des neurones dopaminergiques de la substance noire. Netrin-1 apparaît ainsi comme une cible thérapeutique d'intérêt contre la maladie de Parkinson.

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**NETRIN-1, A NOVEL CANDIDATE FOR NEURORESTORATION IN
PARKINSON'S DISEASE**

LIST OF ABBREVIATIONS

3-MT	3-methoxytyramine
3-OMD	3-O-methyl-dopa
6-OHDA	6-hydroxydopamine
AAADC	aromatic L-amino acid decarboxylase
AD	Alzheimer's disease
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APP	amyloid precursor protein
BBB	blood-brain-barrier
Bcl-2	B-cell lymphoma-2
BSA	bovine serum albumin
CamKII	calmoduline kinase II
CBLN4	cerebellin 4
CDNF	cerebral dopaminergic neurotrophic factor
CED	convection-enhanced delivery
CNS	central nervous system
COMT	catechol-O-methyltransferase
DA	dopamine
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DAT	dopamine transporter
DBS	deep brain stimulation
DCC	deleted in colorectal cancer
DJ-1	Parkinsonism associated deglycase
DOPAC	dihydroxyphenylacetic acid
DR	dependence receptor
DSCAM	down-syndrome cell adhesion molecule
ECM	extracellular matrix
EGF	epidermal growth factor

eIF4E	eukaryotic initiation factor
EphA4	ephrin type A receptor 4
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FLRT	Fibronectin leucine-rich repeat transmembrane protein
FNIII	fibronectin type III domains
GAP43	growth associated protein 43
GDNF	glial cell line- derived neurotrophic factor
GLP-1	Glucagon-like peptide-1
Gpe	globus pallidus external segment
Gpi	globus pallidus internal segment
GPI	Glycophosphatidylinositol
GWAS	Genome Wide Association Studies
HSPCs	heparin sulphate proteoglycans
HVA	Homovanillic acid
IAP	Inhibitor of apoptosis
IDP	intrinsically disordered protein
Ig	Immunoglobulin
IGF-1R	insulin-like growth factor 1 receptor
IP3	inositol 1,4,5-triphosphate
IR	insulin receptor
L-DOPA	L-dihydroxyphenylalanine
LRRK2	leucine-rich repeat kinase 2
LTP	long-term potentiation
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO-B	monoamine oxidase B
MFB	medium forebrain bundle
mPFC	medial prefrontal cortex
MPP	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-propion-oxypiperidine
MPTP	1,2,3,6-methyl-phenyl-tetrahydropyridine

NDS	normal donkey serum
NET	norepinephrine transporter
NFAT	Nuclear factor of activated T-cell
NGF	Nerve growth factor
NPF	nucleation promoting factors
NTF	neurotrophic factors
NTRN	neurturin
p75NTR	Neurotrophin receptor p75
PARK7	Parkinsonism associated deglycase
PBS	Phosphate buffer saline
PD	Parkinson's disease
PI3K	PI-3 kinase
PIKE-L	phosphoinositide-3 kinase enhancer L
PINK1	PTEN-induced putative kinase protein 1
PKA	protein kinase A
PKC	Protein Kinase C
PKG	protein Kinase G
PLC γ	phospholipase C γ
PITP α	phosphatidylinositol transfer protein- α
PRKN	parkin
PTCH-1	patched-1
PTEN	Phosphatase and tensin homolog
RBD	rapid eye movement sleep behavioural disorder
RET	rearranged during transfection
RGL	radial-glial-like cells
RGMa	repulsive guidance molecule
ROS	reactive oxygen species
SHH	sonic hedgehog
SN	substantia nigra
SNc	substantia nigra <i>pars compacta</i>
SNCA	alpha-synuclein

SNr	substantia nigra <i>pars reticulata</i>
STN	subthalamic nucleus
T2D	Type 2 diabetes
TH	tyrosine hydroxylase
TIMPS	tissue inhibitor of metalloproteinase
TrkA	tropomyosin receptor kinase A
TrkC	tropomyosin receptor kinase C
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UNC-6	uncoordinated-6
UPR	unfolded protein response
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area

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INTRODUCTION

Re-wiring the brain, the neurorestorative challenge

The human brain is a fascinating multifaceted organ that defines us as “human being” as it is the seat of identity/personality and the great orchestrator of physiological functions. Through a complex network of approximately 100 billion interconnected neurons and at least as many glial cells, the brain constantly integrates information from various sources and controls accordingly numerous functions (cognition, motricity, adjustment of body homeostasis etc) which enable adaptation and interaction with the environment. Structural and functional maintenance of this network is crucial for proper brain function.

Brain wiring takes place mainly during foetal development where neurons extend their axons, long cytoplasmic protrusions, to reach appropriate target cells according to specific patterns, a process called axon pathfinding or axon navigation. This requires dynamic interactions between axons and their environment which are largely controlled by the interplay between axon membrane-bound receptors and attractive and repulsive guidance molecules whose spatially and temporally regulated expression guides the outgrowth of axons and determines their target sites. The remodelling of the cytoskeleton at the end of developing axons into a specialized and highly motile structure, termed “growth cone”, allows the detection of these guidance cues and subsequent directional growth of the axon. Axon growth not only depends on these guidance cues but also on the substrate they are migrating on. They can migrate while adhering to other axons, a process called fasciculation, which enables the migration of groups or “bundles” of axons following similar path, or to the extracellular matrix (ECM). Again, composition of the ECM can affect axonal growth. Some matrix proteins can be growth-permissive such as laminins, while others, like chondroitin sulphate proteoglycans, act as growth inhibitors, but this may also vary according to the matrix and microenvironment composition. Thus, axon growth is a fine-tuned process depending on the dynamic interplay of various elements from the cell and the extracellular environment. When axons reach their targets, functional connections between neurons, synapses, are then formed. These key structures are the support of neural communication and therefore, brain function. Neurons that fail to reach proper targets are eliminated by apoptotic programmed cell death (Stiles and Jernigan, 2010).

Neuronal circuits formed during foetal development and childhood mature and refine during adolescence, then are maintained stable throughout adult life. However, adult neuronal connectivity is not fully rigid. Indeed, some restricted forms of structural flexibility persist on a smaller scale to enable plastic adaptations to environmental changes. Particularly, synapses are highly dynamic and tightly regulated structures where balance between stability and plasticity (loss, formation, strengthening of connections) is key to the brain’s functions, especially learning. Recent evidence indicates that guidance molecules not only play a role during brain development but also participate to the maintenance and plasticity of adult neuronal circuits. Non-permissive guidance molecules, which are generally up-regulated in the mature brain, are likely to participate to synaptic stabilization and limitation of neuronal growth in adulthood while growth-promoting cues would favour synaptic changes and neuronal repair.

Despite evidence of restricted adult plasticity, the adult brain, composed of post-mitotic neurons, has a limited regenerative capacity. This is due to a non-permissive local environment favouring stability of connections, lack of adult axonal growth and poor production of new neurons (neurogenesis). Thus, injuries and diseases that damage or destroy axons or synapses eventually lead to the disruption of neuronal networks, which typically results in permanent neurological deficits. To date, no drug or treatment can functionally and stably replace or restore damaged neurons and synapses. Due to global aging, this issue is becoming a major medical and public health concern as the incidence of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease (PD) rises rapidly.

Neurodegenerative diseases represent a wide range of neurological disorders which are characterised by the progressive loss of neuron function and structure (neurodegeneration) of specific subsets of neurons in specific functional anatomic systems, which lead to distinct clinical and pathological expressions. Neurodegeneration is generally accompanied by commonly shared features such as reactive gliosis, inflammation, intra- or extracellular proteinaceous aggregates and apoptotic cell death. Nevertheless, it is not clear yet to what extent these features participate in the neurodegenerative process and underlie clinical symptoms. Indeed, despite the considerable progress made over the past three decades to better characterise neurodegeneration, the causes and mechanisms initiating neurodegeneration remain poorly understood. Therefore, in the absence of clearly identified causes, finding effective neurorestorative¹ treatments that could stop degeneration and promote regeneration is even more challenging. An ideal neurorestorative strategy would combine approaches that could i) support neuron survival, ii) replace damaged neurons and axons or promote regeneration (growth) and importantly, iii) maintain functional integrity of residual (and restored) neurons and connections. Thus, mimicking, or reactivating processes at play during brain wiring (from axon growth to synapses formation and stabilization) may represent a relevant approach to promote regeneration. However, contrary to axon growth in the developing brain, regeneration in the adult brain may face two major hurdles: a non-permissive environment (glial scar, inhibitory ECM, inflammation) and the energy cost related to the dynamic process of axon navigation over longer distances and synapse formation. Neurorestoration is thus a complex task.

The present work comes within the scope of neurodegeneration and brain repair since it aims at studying the neurorestorative potential of a guidance and survival cue, netrin-1, in an experimental model of Parkinson's disease, the second most frequent neurodegenerative disease.

¹ In this dissertation, the notion of "neurorestoration" will refer to any intervention, occurring during the neurodegenerative process, that mitigates, stops, or reverses neuronal demise, and hence improves neuronal function and clinical outcome or associated phenotype in patients or experimental models of neurodegeneration. By contrast, "neuroprotection" will refer to any intervention, preceding neurodegeneration, that prevents or delays the initiation of the disease in patients, and neuron loss and associated phenotype in experimental models.

1 NETRIN-1, A PLEIOTROPIC MOLECULE: IMPLICATIONS FOR NEURODEGENERATIVE DISEASES

In the 1990s, almost a century after Santiago Ramón y Cajal expressed the pioneer idea that growing axons might be guided over long distances by diffusible signals from target cells, Marc Tessier Lavigne's laboratory identified two factors that promoted guidance and axon outgrowth in embryonic rat spinal cord explants. These factors, purified from the chick brain, were named netrin-1 and netrin-2 after the root "*netr*" from Sanskrit meaning "one who guides" (Serafini et al., 1994). Subsequent studies confirmed that this family of proteins, homologous to the first described uncoordinated-6 (UNC-6) in *C. elegans* (Hedgecock et al., 1990; Ishii et al., 1992), were indeed guidance cues for axons and migrating neurons. Since then, netrin-1, the best characterised member of the netrin family, has been depicted in virtually all neurosciences textbooks as the paragon of the guidance cue.

Over and above axon guidance, netrin-1 biology is incredibly rich. Since the last decade netrin-1 has been emerging as a multifunctional cue with roles both inside and outside the nervous system, both in development and in adulthood, and in a range of biological processes as diverse as organogenesis, modulation of inflammation, tumour cell survival and neural repair.

This chapter will review various aspects of netrin-1 biology including structure, function and signalling in an attempt to reconcile its seemingly distinct roles, with a particular attention on features that may be relevant for neurorestoration, notably for neurodegenerative diseases treatment. A special focus will be given on the cell survival/death facet of netrin-1 signalling, through its dependence receptors, which has long been central to the research activity of Patrick Mehlen's laboratory.

1.1 WHAT IS NETRIN-1?

1.1.1 A prototype of the evolutionary conserved netrin family ...

1.1.1.1 Insights from evolution

Since the identification of UNC-6, the first reported member of the netrin family, in *C.elegans*, follow-up studies in vertebrates and invertebrates have all demonstrated a highly conserved role for netrins **in directing migrating cells and growing axons** in the developing central nervous system (CNS). More precisely, netrin-1's orthologues have been identified in all bilaterians studied so far, with mouse and rat netrin-1 sharing 99% amino acids with the human netrin-1. The invalidation of netrin-1 in mice further demonstrated its crucial role in wiring the developing brain since knock-out (KO) mice showed severe neurodevelopmental defects, notably the disruption of multiple CNS commissures and died rapidly after birth (Bin et al., 2015; Serafini et al., 1996). Altogether these observations suggest that netrin-1 has specialised during evolution to shape bilateral symmetry, mainly guiding axons crossing the midline. Interestingly, netrin is found in the sea anemone *N. vectensis* a model organism exhibiting early bilaterians features (Rajasekharan and Kennedy, 2009). Centralised nervous system (neurons grouped into nerves with polarised conduction, coordinating various parts of the body) is a trait of bilaterian. Sponge and radially symmetrical animals generally exhibit a nerve net where neurons are diffusely dispersed with no apparent pattern or neural territories. Although *N. vectensis* does not possess a centralised nervous system, its neural morphology is patterned and exhibits distinct neural territories along the oral–aboral axis (Marlow et al., 2009). Thus, beyond shaping bilateral symmetry, netrin might have specialised to regulate **neural patterning**, guiding axons and cells to the appropriate targets and territories. Furthermore, netrins, and especially netrin-1, might have been co-opted later in evolution, notably in vertebrates, to influence **branched morphogenesis** outside the nervous system.

1.1.1.2 Insights from structure

Netrin-1 is a member of the netrin family of **laminin-related proteins**, a class of protein mainly involved in axon guidance. In mammals, this family of extracellular proteins is composed, of six members: four secreted netrins — netrin-1, netrin-3, netrin-4, netrin-5 — and two glycoposphatidylinositol (GPI)-membrane-tethered netrins — netrin G1 and G2. Beside a conserved role in axon guidance, netrins display high degree conservation in protein secondary structure (Sun et al., 2011).

Netrins ~600 amino acids protein structure consists of two sequences homologous to N-termini of laminins and a basic C-terminal domain, coined the Netrin (NTR) module (Figure 1). More precisely, netrins N-terminal sequence contains the **laminin IV** domain and the **laminin V** domain which consists of three EGF repeats. These domains are similar to the laminin gamma chain, in netrin-1 and 3, and to the laminin beta chain in netrin- 4, G1 and G2. Netrin-5, by contrast, lacks the laminin IV domain and contains only two EGF repeats (Yamagishi et al., 2015). Laminin-

like N-terminal sequences are necessary for netrin-1 binding to its canonical transmembrane receptors, deleted in colorectal cancer (DCC) and UNC-5B. Interestingly, like laminins, netrin-1 can associate with fibronectins, collagens and integrins, however, these interactions with ECM and cell surface components are likely to be mediated also by the **NTR module** (Sun et al., 2011; Yebra et al., 2003). This module, not homologous to any laminin domains, is rich in basic amino acid residues and was shown to bind heparin (Rajasekharan and Kennedy, 2009). Therefore, this domain may account for reported netrin-1 “sticky” association with the ECM and cell surface and low diffusion capacity. In addition, netrin-1 C domain exhibits sequence similarity to the tissue inhibitor of metalloproteinase (TIMPS) and contains RGD motifs allowing binding to integrins (Bányai and Patthy, 1999). Although the functional significance of this module is still uncertain, it may contribute to presenting secreted netrins to receptors on cell surfaces or concentrating them locally in the ECM, thus modulating netrin signalling according to the matrix composition.

According to these structural characteristics, netrin-1 can be defined as a **secreted extracellular matrix protein** interacting both with ECM proteins and cell surface proteins. These features indicate that netrin-1 may participate to the dialog between cells and their environment regulating cell-matrix or cell-cell interactions, which is important for dynamic processes like guidance.

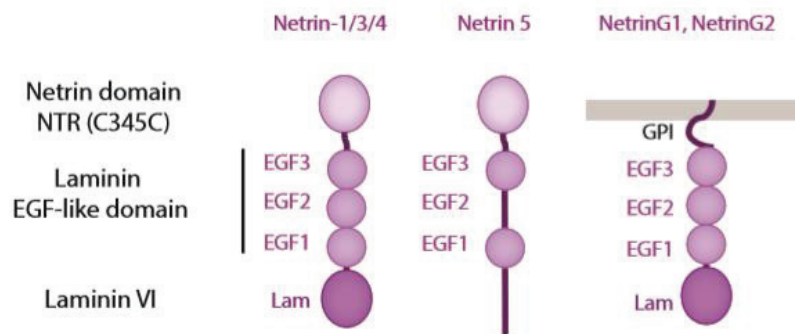


Figure 1 Structure of netrin proteins

1.1.2 ...with diverse functions

1.1.2.1 Insights from tissue expression

Netrin-1 is expressed in various structures and tissues both inside and outside the nervous system and both in development and adulthood. Indeed, expression pattern analysis, especially in mice, shows that netrin-1 is expressed in various regions of the central nervous system such as the optic disc, the spinal cord and the forebrain in the development; and the midbrain, the cerebellum, and the spinal cord in the adult brain (Deiner et al., 1997; Kennedy et al., 1994; Livesey and Hunt, 1997). Netrin-1 is also expressed in the adult peripheral nervous system in Schwann cells and its

expression is up-regulated after peripheral nerve transection injury (Dun and Parkinson, 2017; Madison et al., 2000).

Moreover, netrin-1 is highly expressed outside the nervous system in various developing organs such as the lung, the pancreas, and the mammary gland (Liu et al., 2004b; Srinivasan et al., 2003; Yebra et al., 2003). In the adult, netrin-1 is also expressed in various tissues such as the kidney, the liver, the heart, ovaries, skeletal muscles (Kennedy et al., 1994; Meyerhardt et al., 1999) and was shown to be increased in context of tissue injury and pathological conditions such as cancer and inflammation (Fitamant et al., 2008; Lahlali et al., 2016; Löw et al., 2008; Tsuchiya et al., 2007).

The broad expression of netrin-1 indicates that netrin-1 is not only a developmental cue dedicated to the guidance of axons and neurons but that its function may encompass a wider range of actions.

1.1.2.2 Reported roles of netrin-1

Controlling neural architecture in the nervous system

Netrin-1 function was extensively investigated during CNS development and was shown to be key to neural modelling and wiring (Bin et al., 2015; Hedgecock et al., 1990; Ishii et al., 1992). Indeed, netrin-1 is a bimodal chemotropic cue (Finci et al., 2014) promoting the guided **migration** of various subsets of neurons including midbrain dopaminergic neurons, inferior olive, and pontine neurons, to proper neural territories mediating both attractive and repulsive signals (Bloch-Gallego et al., 1999; Hegarty et al., 2013; Yee et al., 1999). In addition, experiments in netrin-1 KO mice showed that pontine and olivary neurons were not incorrectly targeted, but actually missing (Bloch-Gallego et al., 1999; Yee et al., 1999) indicating that beside guidance, netrin-1 is also involved in **survival**. This was later demonstrated by Llambi and colleagues (2001). Similarly, netrin-1 also controls **attraction, repulsion, and elongation** of axons to proper target sites and further participates in sculpting neuronal architecture and wiring the brain through the regulation of **branching** events and subsequent establishment of connections: **synaptogenesis** (Colon-Ramos et al., 2007; Hegarty et al., 2013; Keleman and Dickson, 2001; Stavoe and Colón-Ramos, 2012; Xu et al., 2010).

Surprisingly, the role of netrin-1 in the adult brain has received less attention, probably due to technical limitations (KO mice die shortly after birth) and is so far poorly known. Nevertheless, emerging evidence demonstrates a critical role of netrin-1 in the regulation of **synaptic stabilisation and plasticity** (Bayat et al., 2012; Glasgow et al., 2018; Goldman et al., 2013; Horn et al., 2013).

Beside influencing the formation of neuronal circuits, netrin-1 regulates myelination and blood-brain-barrier (BBB) stability. Indeed, netrin-1 controls oligodendrocyte progenitor cells migration and proliferation, oligodendrocyte extension and branching and is required for the maintenance of axo-oligodendroglial paranodal junctions in mature myelin (Jarjour et al., 2008; Manitt et al., 2001). Netrin-1 also promotes endothelial cell-cell interaction and **adhesion**, thus

stabilising and controlling BBB integrity (Podjaski et al., 2015). Interestingly, after brain injury, netrin-1 is upregulated in neurons and endothelial cells, while its receptors are upregulated in astrocyte feet, endothelial cells, and neurons, suggesting a role in repair (He et al., 2018; Podjaski et al., 2015; Wang et al., 2013).

Netrin-1 functions in the nervous system strikingly illustrates the many faces of netrin-1 signalling. Indeed, netrin-1 can both promote attraction and repulsion, motility (migration) and stability (synapse formation), as well as long range function (axon attraction) and short-range function (cell-cell interaction). These various facets of netrin-1 signalling are also particularly noticeable when examining netrin-1 function outside the nervous system, in physiological and pathological conditions.

Morphogenesis of branched structures

Reminiscent of its role in the nervous system, netrin-1 was shown to participate to branched organ morphogenesis.

During lung branching morphogenesis, netrin-1 shapes epithelial buds, both acting on distal epithelial cells to promote the **outgrowth** of buds and on proximal epithelial cells to limit inappropriate side branching and ectopic bud formation through differential localisation of receptors (Liu et al., 2004b).

In mammary gland however, netrin-1 is required for proper **branching** morphogenesis and structural **maintenance** through the control of **cell adhesion** (Srinivasan et al., 2003). In addition, netrin-1 may facilitate functional **differentiation** of mammary epithelial cells during lactation (Strizzi et al., 2008).

Another insight into the role of netrin-1 in branched organs comes from studies of the role of netrin-1 in pancreatic morphogenesis. Indeed, *in vitro* assays have reported a role for netrin-1 in the **adhesion** and **migration** of pancreatic cells thus contributing to pancreatic morphogenesis. Particularly, Yebra et al 2003 showed that netrin-1 is expressed in a discrete population of epithelial cells, localizes to basal membranes, and specifically associates with elements of the extracellular matrix such as collagen IV and fibronectin. Of interest, interactions between netrin-1 and $\alpha 6\beta 4$ integrin mediates pancreatic epithelial **cell adhesion**, whereas interactions with $\alpha 6\beta 4$ and $\alpha 3\beta 1$ regulate the **migration** of putative pancreatic progenitors.

The vascular system shares many striking similarities with the nervous system as many molecular players originally uncovered as regulators of axon pathfinding have also been found to affect branching and development of blood vessels: angiogenesis (Carmeliet and Tessier-Lavigne, 2005). Like in axon guidance, netrin-1 can have a **bi-functional** role in angiogenesis. In some cases, netrin-1 is pro-angiogenic, with the ability to promote **proliferation**, **migration**, and **adhesion** of endothelial cells and to induce vascular **sprouting** and **branching** (Navankasattusas et al., 2008; Park et al., 2004; Wilson et al., 2006). In other cases, netrin-1 is reported to be anti-angiogenic, inhibiting the migration of endothelial cells and new vessel formation (Larrivée et al., 2007; Lu et al., 2004). Through its role on endothelial cells adhesion and regulation of vascularisation in tissues, netrin-1 may also impact oxidative stress (through nitrite oxide

production) but also inflammation, since cytokines, chemokines and immune cells circulate in blood vessels (Bongo and Peng, 2014).

Cellular plasticity in pathological contexts...

Along this line, netrin-1 was shown to have immunomodulatory functions (Mirakaj and Rosenberger, 2017). It has protective **anti-inflammatory roles** in several pathological conditions among which acute kidney injury (Ranganathan et al., 2014; Wang et al., 2008), peritonitis (Mirakaj et al., 2011), cardiac ischemia-reperfusion injury (Mao et al., 2014), and acute lung injury (Mutz et al., 2010). This role is supported by the **inhibition of immune cell infiltration** (Aherne et al., 2012; Ly et al., 2005; Mao et al., 2014), the induction of macrophage **polarization** (Ranganathan et al., 2013), and the reduction of the levels of proinflammatory cytokines and chemokines (Tadagavadi et al., 2010). In some cases, like in atherosclerotic plaques, netrin-1 can inhibit **emigration of macrophages** from the lesions and promotes macrophage **survival** (van Gils et al., 2012; Ramkhelawon et al., 2013) and thus promote inflammation.

Moreover, netrin-1 was shown to be upregulated in certain cancers (Delloye-Bourgeois et al., 2009a, 2009b; Fitamant et al., 2008) and promote **proliferation, migration, and survival** of tumour cells (Chen et al., 2017; Fitamant et al., 2008; Ylivinkka et al., 2016).

Throughout the organism and in various contexts, netrin-1 appears to be implicated in a number of processes involving migration, growth, adhesion, differentiation, proliferation, and survival, in other words, **plasticity**. Indeed, all these require sensing the environment through cell-cell or cell-matrix interplay and subsequent morphological **remodelling**. Along this line, survival, or rather some cases of programmed cell death (extrinsic apoptosis and anoikis for example), can be viewed as a consequence of loss of signal (loss of cell contact with other cells or the matrix, loss of trophic support) and thus a plastic adaptation to the environment.

However, how a single cue can control such diverse and sometimes opposite functions? For example, switching from controlling motility to adhesion. This versatility is supported by a range of receptors, some of them exhibiting a dual signalling, and signal modulators...

1.2 NETRIN-1 SIGNALLING: BUILDING, GUIDING, ELIMINATING...

The DCC family of receptors and UNC-5 homologues are the canonical receptors of netrin-1. But netrin-1 was shown to bind many other putative receptors and co-receptors. This repertoire of receptors underlies netrin-1 diverse functions, although the exact signalling mechanisms are generally poorly deciphered. Netrin-1 signalling via each receptor induces a specific signalling that can be modulated by other receptors and the cellular environment leading to a spectrum of effects ranging from cell adhesion to migration, and cell survival to cell death.

1.2.1 Netrin-1 receptors

All netrin-1 canonical receptors are single pass transmembrane proteins from the immunoglobulin (Ig) superfamily. Classically, DCC family of receptors mediate cell and axon attraction and cell-cell adhesion, while UNC-5 homologues signal chemorepulsion (Sun et al., 2011). Moreover, these receptors are dependence receptors, a class of receptors inducing programmed cell death when unbound to their ligand (section 1.2.3, p32). In addition, to these classical receptors, netrin-1 was shown to interact with several other receptors, such as Down-syndrome cell adhesion molecule (DSCAM) (Rajasekharan and Kennedy, 2009), the amyloid precursor protein (APP) (Rama et al., 2012), the adenosine receptor A2b (Corset et al., 2000), as well as cell surface and matrix proteins (Höpker et al., 1999; Nikolopoulos and Giancotti, 2005). But, barring DCC, UNC-5 and neogenin which are extensively and robustly described, netrin-1 binding with other receptors is either less documented, poorly described, or controversial and would thus require additional studies to confirm to what extent they can be considered classical receptors. Below, a brief description of selected receptors:

DCC

In humans, **DCC** was initially described as a candidate tumour suppressor linked with an allelic deletion of chromosome 18q21 in colon cancer (Fearon and Vogelstein, 1990) and later identified as a key mediator of axonal migration in response to netrin-1 (Keino-Masu et al., 1996). The most studied members of the DCC family comprise DCC and neogenin in mammals, UNC-40 in *C. elegans* and Frazzled in *D. melanogaster*.

DCC extracellular domain is composed of four Ig domains followed by six fibronectin type III domains (FNIII), while the intracellular domain contains three highly conserved sequences, named the P1-3 motifs (Keino-Masu et al., 1996) (Figure 2). DCC extracellular domain binds to laminin domains VI and V of netrin-1 via the FNIII domains (Finci et al., 2014; Xu et al., 2014), while the intracellular domain is thought to be responsible of the dimerization of the receptor in response to netrin-1 (Stein et al., 2001) and recruitment of intracellular signalling molecules.

DCC signalling in response to netrin-1 is best studied in the nervous system where it controls axon guidance (Colamarino and Tessier-Lavigne, 1995; Deiner et al., 1997; Keino-Masu et al., 1996), cell migration (Ding, 2005; Junge et al., 2016), synapse formation and plasticity (Glasgow et al., 2018; Goldman et al., 2013; Horn et al., 2013), branching (Rajasekharan et al.,

2009; Xu et al., 2010), and myelin maintenance (Jarjour et al., 2008; Low et al., 2008). Outside the nervous system, DCC has been linked with cancer (Castets et al., 2011).

In addition to binding netrin-1, DCC also binds netrin-3 (Wang et al., 1999), netrin-4 (Qin et al., 2007), draxin (Ahmed et al., 2011), and cerebellin 4 (Haddick et al., 2014) which might thus modulate netrin-1/DCC signalling.

Neogenin shares ~50% amino acid identity with DCC and thus similar domain structure (Vielmetter, 1994). Although not as well-studied than DCC, neogenin was shown to act as an attractive axon guidance receptor when bound to netrin-1. However, neogenin acts also as a repellent receptor when bound to repulsive guidance molecule (RGMA), a ligand that does not belong to the netrin family (Matsunaga et al., 2004).

Beside their roles in axon guidance, both DCC and neogenin regulate cell-cell adhesion and tissue organization (Bin et al., 2015; Park et al., 2004; Srinivasan et al., 2003).

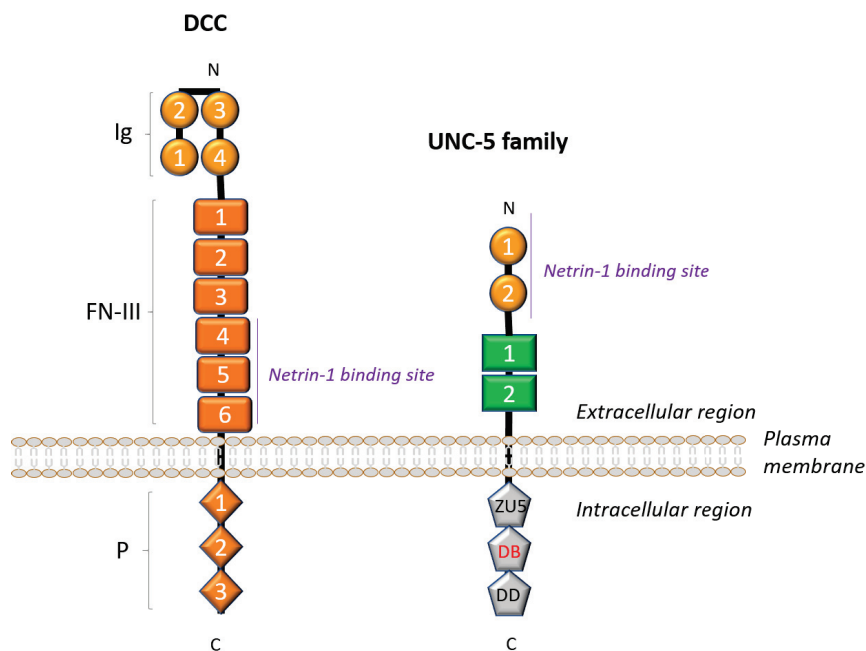


Figure 2 Netrin-1 canonical receptors

UNC-5 homologues

The **UNC-5** family is composed of four single pass type I transmembrane receptors belonging to the Ig superfamily: UNC-5 A-D. Their extracellular portion consists of two Ig domains and two thrombospondin type 1 domains while the cytosolic portion contains a ZU5 domain, a DCC binding motif (DB), and a death domain (DD) (Figure 2). Netrin-1 binds to these receptors via their Ig repeats (Xu et al., 2014).

In the developing brain, netrin-1 binds to the Ig domains of UNC-5 to mediate chemorepulsion (Leonardo et al., 1997). Interestingly, DCC is required for UNC-5 mediated long-

range repulsion whereas UNC-5 alone has been shown to mediate short-range repulsion (Keleman and Dickson, 2001).

Functions of netrin-1/UNC-5 signalling include axon guidance (Hong et al., 1999; Killeen, 2009), cell migration (Jarjour et al., 2003), angiogenesis (Lu et al., 2004), and cell survival (Castets et al., 2009; Tang et al., 2008).

UNC-5 receptors have also been shown to bind netrin-3 (Wang et al., 1999), Robo4 (Koch et al., 2011), draxin (Ahmed et al., 2011), and FLRT2 and 3 (Yamagishi et al., 2011).

A2b

A2b is a G-protein coupled receptor belonging to the adenosine receptor family. Several studies have shown that netrin-1 can activate A2b receptors (Corset et al., 2000; Mirakaj et al., 2011); however, only one study has provided evidence that activation is via direct binding to netrin-1 (Corset et al., 2000). Thus, its role as a netrin-1 receptor remains controversial (Stein et al., 2001). Netrin-1/A2b signalling has been primarily implicated in inhibiting inflammation (Mirakaj et al., 2011).

Integrins

Netrin-1 can bind and activate some of the integrin receptors, including $\alpha 6\beta 4$ and $\alpha 3\beta 1$ (Yebra et al., 2003). These interactions have been shown to mediate epithelial cell adhesion, cell migration, axon guidance, and protection from apoptosis following hypoxia (Lemons et al., 2013; Stanco et al., 2009; Yebra et al., 2003). So far, it is the only netrin-1-receptor interaction known to require the C-domain of netrin-1 (Yebra et al., 2003).

Extracellular matrix protein

As mentioned, secreted netrins also interact with extracellular matrix proteins such as heparin, raising the possibility that they may bound to cell surfaces through heparin sulphate proteoglycans (HSPCs) (Rajasekharan and Kennedy, 2009).

1.2.2 Netrin-1 signalling

The precise signalling mechanisms by which netrin-1 mediates its diverse functions are still poorly deciphered, especially outside the nervous system and in chemorepulsion. Globally, upon binding, classical netrin-1 receptors, which do not bear any catalytic domain, dimerise, and recruit several signalling proteins that will presumably trigger the remodelling of the actin cytoskeleton and protein synthesis. Netrin-1 signalling through DCC in axon guidance is best characterised and will serve here to illustrate how netrin-1 can mediate its functions and be so versatile.

Additionally, DCC KO mice phenocopy very closely Netrin-1 KO mice, indicating that DCC underlies essential actions of netrin-1 (Fazeli et al., 1997). Indeed, netrin-1/DCC signalling has been shown to play a role in numerous neuronal processes including axon guidance, the formation of cell-cell and cell-matrix adhesions, synaptogenesis, synaptic plasticity, and SNARE-mediated exocytosis (Cotrufo et al., 2011; Deiner et al., 1997; Horn et al., 2013; Jarjour et al., 2008; Zylbersztejn and Galli, 2011). Besides, netrin-1/DCC signalling will eventually be

interrogated in my thesis work, thus we will report here what is known about Netrin-1/DCC signalling according to its description in the vertebrate developing nervous system.

1.2.2.1 The example of netrin-1/DCC receptor signalling in axon guidance

Several axon tracts in the spinal cord and the brain express DCC and the loss of DCC expression in mice causes defects in the formation of spinal and cerebral commissures that are comparable to those observed in netrin-1-deficient mice (Bin et al., 2015; Fazeli et al., 1997; Keino-Masu et al., 1996; Serafini et al., 1996).

Following netrin-1 binding, several signalling proteins are activated and recruited to DCC. The signalling cascade initiated by the activation of DCC induce protein and lipid synthesis, as well as cytoskeletal remodelling to promote guidance. This signalling pathway is described below and illustrated in Figure 3.

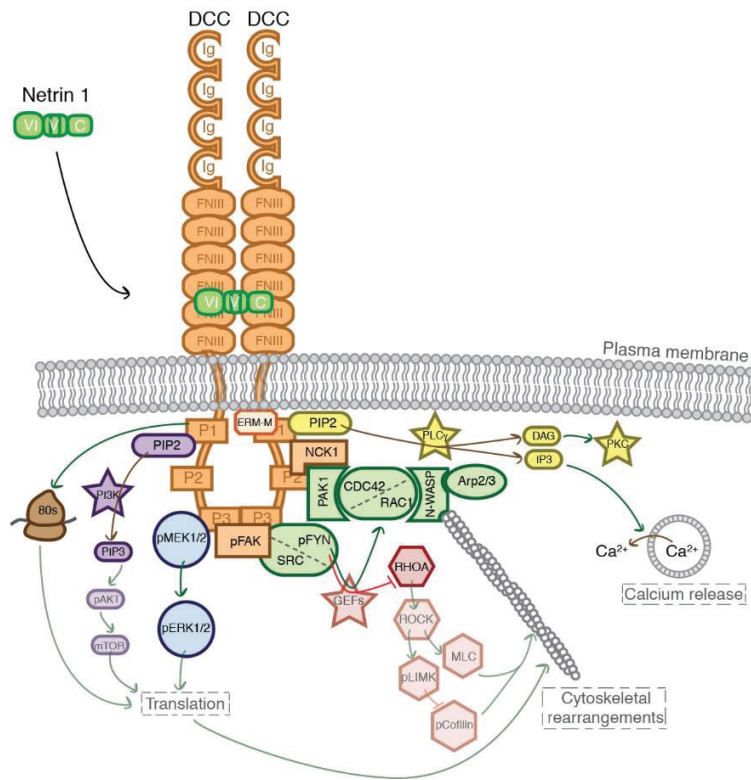


Figure 3 Netrin-1/DCC chemoattractive signalling (Sun et al., 2011)

Activation of DCC by tyrosine kinases

The binding of netrin-1 induces the dimerization of DCC via the P3 domain, as well as the phosphorylation of tyrosine residues on the receptor (Meriane et al., 2004; Stein et al., 2001) which contribute to the activation of DCC and the initiation of netrin-1/DCC signalling. Phosphorylation on other residues (serine and threonine) has also been described but the functional relevance of these remains unclear (Meriane et al., 2004). Indeed, DCC constitutively binds the adaptor Nck1

and the tyrosine kinase FAK (Li et al., 2004). Upon netrin-1 binding, FAK activation recruits and activates Src family kinases and both tyrosine kinases phosphorylate DCC on tyrosine residues further contributing to the activation of DCC which in turn recruits and activates additional FAK and Src proteins (Liu et al., 2004a; Ren et al., 2008). The tyrosine kinase Fyn is also activated and recruited to DCC and initiates the phosphorylation of the receptor on tyrosine residues (Li et al., 2004; Meriane et al., 2004).

Regulation of Rho GTPases and control of the cytoskeleton dynamics

Upon netrin-1 binding and DCC activation, Nck1 recruits the kinase PAK1, the Rho GTPases Cdc42 and Rac1 (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005), both of which regulate **F-actin**. Moreover, Fyn phosphorylates the Rac GEF Trio and further mediates the activation of Rac1 (Briaçon-Marjollet et al., 2008; Meriane et al., 2004). N-WASp, part of the WASP family of actin nucleation promoting factors, is also recruited to this intracellular signalling complex via PAK1 and Cdc-42 (Shekarabi et al., 2005). Cdc42 and N-WASp interaction leads to Arp2/3-dependent **actin nucleation** which induces cytoskeletal rearrangements downstream of netrin-1 binding to DCC. In parallel to the positive regulation of Rac1 and Cdc42 activity, the activity of RhoA, another RhoGTPase, is inhibited downstream of netrin-1/DCC signalling further promoting axon outgrowth and guidance (Moore et al., 2008). Thus, the regulation of Rho GTPases downstream of netrin-1/DCC signalling mediates the changes in growth cone dynamics and cytoskeleton rearrangement by increasing actin polymerization and F-actin content within the growth cone.

Additionally, the microtubule dynamics is also regulated by netrin-1 binding to DCC through the recruitment of the β -tubulin isoform TUBB3 to DCC and the regulation of the microtubule-binding activity of MAP1B (Del Río et al., 2004; Qu et al., 2013).

Regulation of second messengers

Netrin-1 induces the activation of phosphatidylinositol transfer protein- α (PITP α) and the hydrolysis of PIP2 by phospholipase C γ (PLC γ), a process that generates the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) and increases intracellular Ca²⁺ levels (Ming et al., 1999; Xie et al., 2006). Ca²⁺ effectors CamKII, calcineurin and Protein Kinase C (PKC) converge to the remodelling of the cytoskeleton through the modulation of microtubule dynamics, further activation of Rho GTPases, and the translocation of NFAT transcription factors. Indeed, studies on cultured spinal neurons from *Xenopus* suggested that there are two groups of guidance cues in function of their dependence on second messengers, notably the intracellular Ca²⁺ levels. In the group dependant on intracellular Ca²⁺, the response to guidance molecules requires the coactivation of PI-3kinase (PI3K) and PLC γ . Moreover, the type of response depends of the levels of cAMP and on the activation of protein kinase A (PKA). In the other group, the response is independent of Ca²⁺ and PI3K and is regulated by cGMP and protein Kinase G (PKG) (Song and Poo, 1999). Netrin-1 hence, belongs to the first group since an increase in the levels of intracellular Ca²⁺ and cAMP as well as the activity PKA are required for netrin-1-dependent chemoattraction in *Xenopus* spinal neurons (Hong et al., 2000; Wang and Poo, 2005). In this model, netrin-1-induced

attraction is thus abolished in response to decreased extracellular Ca^{2+} level or the blockage of calcium channels (Hong et al., 2000). Intriguingly, netrin-1 does not alter cAMP concentration or PKA activity in rat DRG neurons or spinal commissural neurons (Moore et al., 2008). Thus, the requirement for Ca^{2+} /cAMP signalling downstream of netrin-1 and DCC might be cell-specific and should be assessed further.

Regulation of ERK activity and protein synthesis

Activation of local protein synthesis in growth cones enable axon outgrowth (Campbell and Holt, 2001; Tcherkezian et al., 2010; Welshhans and Bassell, 2011). Upon binding of netrin-1, the extracellular signal-regulated kinases (ERK) 1 and 2 are activated and recruited to DCC via the P1 domain (Campbell and Holt, 2001; Forcet et al., 2002). Netrin-1-stimulated ERK1/2 activity **promotes transcription and protein synthesis** by activating proteins such as the transcription factor Elk-1 and the eukaryotic initiation factor (eIF4E), respectively (Campbell and Holt, 2001; Forcet et al., 2002). *In vitro* assays in non-neuronal cells have demonstrated that the P1 domain interacts with the ribosomal protein L5 and that netrin-1 promotes the dissociation of translational components from DCC (Tcherkezian et al., 2010). This suggests that netrin-1 promotes local protein synthesis in growth cones by releasing the translation machinery from DCC-mediated inhibition. Along this line, DCC was shown to constitutively interact with the translation machinery, including ribosomal subunits and initiation factors of the eIF protein family in the spinal cord (Tcherkezian et al., 2010).

1.2.2.2 Modulation of DCC signalling

The modulation of netrin-1/DCC signal transduction is achieved by different molecular mechanisms. Proteins expressed in the extracellular environment have been shown to regulate the response of neurons to netrin-1. The ECM component heparin can bind to both netrin-1 and DCC and may thus modulate attraction. Also, heparan sulphate produced by cell and found in the ECM, favours netrin-1/DCC-mediated axon guidance in the spinal cord (Matsumoto et al., 2007; Serafini et al., 1994). On the other hand, in *Xenopus* spinal neurons, netrin-1/DCC-mediated attraction can be converted to repulsion in the presence of laminin-1 in the basement membrane (Höpker et al., 1999). In addition, netrin-1/DCC signalling may be modulated by several other mechanisms.

Co-receptors

Several receptors, but not only netrin-1 receptors, are known to regulate netrin-1/DCC-mediated signal transduction. For instance, heterodimerization of DCC and UNC-5 mediates netrin-1-dependent long range chemorepulsion. The adenosine A2b receptor identified as a DCC co-receptor is involved but not required in axon outgrowth but was shown to regulate the response of axons to netrin-1 by controlling cell surface levels of UNC-5A receptors (Corset et al., 2000; McKenna et al., 2008; Stein et al., 2001). Additionally, netrin-1/DCC induced chemoattraction can be silenced by Slit receptor Robo1 when associated with DCC (Stein and Tessier-Lavigne, 2001).

In contrast, Robo3 and APP were each identified as co-receptor that can potentiate netrin-1-dependent chemoattraction in commissural neurons (Rama et al., 2012; Zelina et al., 2014).

Membrane receptor levels

The modulation of a receptor's expression levels or cell surface presentation is a common mechanism for tuning responses to extracellular ligands. For example, endocytic internalisation of UNC-5 converts repulsive netrin-1 responses to attraction (Bartoe et al., 2006). Netrin-1 exposure or loss can readily participate to modulate DCC levels and thus the cell response. Lipid rafts regulate the distribution of netrin-1 and DCC at the cell surface and are required for netrin-1/DCC signal transduction (Hérincs et al., 2005; Petrie et al., 2009). In *Xenopus*, spinal neuron chemoattraction is modulated by phases of desensitisation and re-sensitisation to netrin-1, which are believed to be essential for long-range chemotaxis (Ming et al., 2002). This phenomenon called adaptation is mediated by the fast endocytosis (desensitisation) and the slower protein synthesis (re-sensitisation) of DCC. Studies in embryonic cortical neurons supported this observation showing that DCC was ubiquitinated, internalised and then degraded rapidly after netrin-1 exposure (Kim et al., 2005). DeGeer et al., 2013 also demonstrated that netrin-1 induces a transient decrease of DCC surface expression in growth cones relative to axon shafts in cortical neurons (DeGeer et al., 2013). Other showed that DCC proteolysis was required for guidance and that DCC cytoplasmic cleavage by gamma-secretase produces a DCC ICD fragment that may serve as a **transcriptional coactivator** in vertebrates (Bai and Pfaff, 2011; Neuhaus-Follini and Bashaw, 2015). Along this line, netrin-1 KO mice exhibit increased levels of DCC (Bin et al., 2015). Inversely, netrin-1 exposure was also showed to increase DCC localisation to the plasma membrane (Matsumoto and Nagashima, 2010). Thus, DCC levels may be dynamically controlled by netrin-1.

Netrin-1 concentration

Axon guidance or outgrowth experiments often show a bimodal response of axons with increasing netrin-1 concentrations (Moore and Kennedy, 2006; Serafini et al., 1994) indicating that netrin-1 concentration determine which receptors, and thus signalling pathways, are activated. Along this line, it has been demonstrated that netrin-1 binds to DCC with higher affinity than UNC-5-H (Xu et al., 2014). Hence, at relatively low concentration netrin-1 will preferably bind to DCC, and at higher concentration UNC-5, probably in heterodimer with DCC (Finci et al., 2014; Xu et al., 2014). Furthermore, above a certain threshold, netrin-1 may saturate receptors preventing their dimerization and thus abolishing signal transduction. Similarly, under a certain threshold, receptors would be unbound and thus at a monomeric state. Accordingly, netrin-1 display an “inverted U” dose response. In some cases, poor levels but also extremely elevated levels of netrin-1 are associated with no or toxic effects. Indeed, in a monomeric state, netrin-1 receptors DCC and UNC-5 can be cleaved by caspases and trigger cell death: it is the dependence receptor hypothesis (Goldschneider and Mehlen, 2010; Negulescu and Mehlen, 2018).

1.2.3 Netrin-1, between creation and destruction: the dependence receptor hypothesis

As mentioned earlier, during CNS development, netrin-1 acts not only as an axon guidance molecule, but also as a survival factor (Llambi et al., 2001). Its survival function is mediated by the binding to its receptors DCC and UNC-5. Indeed, in the absence of netrin-1, cells expressing DCC or UNC-5 trigger a programmed cell death signalling leading to cell elimination (Mehlen et al., 1998; Tang et al., 2008). This mechanism would also allow tumour cells whether mutated for DCC or UNC-5 or overexpressing netrin-1, to escape apoptosis and thereby proliferate and migrate contributing to tumour progression (Goldschneider and Mehlen, 2010). Additionally, this process may contribute to the elimination of neurons or axons migrating out of their normal (netrin-1-rich) territory and thus ensuring proper patterning during CNS development. This control of cell death thus represents an important mechanism in the setting up of neuronal circuits in the developing CNS.

1.2.3.1 Apoptotic cell death and activation of caspases

Cell death is defined as the phenomenon that causes **irreversible** permeabilization of the cell membrane or complete fragmentation of the cell. Cell death can be subdivided into two groups: accidental and programmed cell death. Accidental cell death occurs instantly and remains unaffected by any pharmacological or genetic intervention. In contrast, programmed cell death involves genetically regulated molecular pathways. By targeting the different components of these pathways, the kinetics of cell death **can be modulated**. More importantly, this programmed cell death is not only activated by external signal from the microenvironment but also by developmental, homeostatic, or immunological programs (Galluzzi et al., 2007, 2018).

Apoptosis is a programmed cell death, occurring widely during development, and throughout life of multicellular organisms to maintain cell homeostasis. In short, apoptosis can be initiated through two main pathways: the intrinsic pathway, or mitochondrial pathway, which is a response to internal damage and stress; and the extrinsic pathway that is activated in response to extracellular signals. These two pathways are, however, not strictly distinct since external signals can also activate the intrinsic pathway of apoptosis. In all cases, these pathways converge to the activation of cell death through caspases (Galluzzi et al., 2018). Indeed, in both pathways, initiator caspases are activated, which in turn activate effector caspases, which then carry out cell death with minimal effect on surrounding tissues by indiscriminately degrading proteins. Morphological features comprise cell shrinkage, chromatin condensation, nucleus fragmentation, irregular blebbing of the plasma membrane, and apoptotic bodies that will be engulfed by neighbouring cells (Majno and Joris, 1995). The Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) enables the detection of DNA fragmentation by labelling the 3'-hydroxyl termini in the double-strand DNA breaks which are generated during apoptosis.

Apoptosis is an essential process during brain development since it enables to control the size and shape of structures and thus proper neural patterning. In the adult brain, apoptotic cell

death can be a feature of both acute and chronic neurologic diseases. In acute injury, such as ischemia, apoptosis occurs in areas surrounding the core of the lesion, while the core undergoes necrosis (MacManus et al., 1993). Apoptotic death is also a component of the lesion that appears after brain or spinal cord injury (Crowe et al., 1997; Rink et al., 1995). In chronic neurodegenerative diseases, it is allegedly the predominant form of cell death (Smale et al., 1995; Thomas et al., 1995).

Caspases belong to a family of cysteine-dependent proteases playing essential roles in programmed cell death (including apoptosis, pyroptosis and necroptosis) and inflammation. They are synthesised as inactive zymogens (pro-caspases) then dimerise and are cleaved upon apoptotic stimuli, leading to their activation. This post-translational level of control allows rapid and tight regulation of the enzyme. Additionally, modulators of apoptosis such as Inhibitors of Apoptosis (IAPs) which can bind directly to caspase or ubiquitinylate them, can contribute to the control of active caspase intracellular levels. Once activated, caspases cleave cytoplasmic proteins that contain caspase cleavage sites contributing, depending on the substrate, to activate a cleavage-dependent-activity, or to the degradation of the substrate (Shalini et al., 2015). These cleavage sites infallibly contain an aspartic acid residue. Caspases are classified into three subgroups according to their function:

- Apoptosis initiator caspases: caspases 2, 8, 9, 10.
- Apoptosis effector caspases: caspases 3, 6, 7, cleaved caspase 3/7 are often used as a marker of apoptosis
- Inflammatory caspases: 1, 4, 5, 11 and 12 which are associated with the execution of programmed cell death named pyroptosis.

However, caspases are abusively associated with apoptosis, or more generally, cell death. Indeed, caspases are also involved in a range of functions such as proliferation, differentiation, migration. In neurons especially, caspases are increasingly associated with remodelling events such as axon growth, pruning and synaptic plasticity (for review (Mukherjee and Williams, 2017)).

1.2.3.2 The dependence receptor paradigm

Described twenty years ago, the dependence receptors hypothesis revolutionised the view that membrane receptors remained inactive in absence of external signal by their ligand. Indeed, according to the dependence receptor paradigm, some receptors exhibit a dual signalling, a so-called “positive” signalling when bound to their ligand, and a “negative” but active signalling inducing cell death when unbound to their ligand. Therefore, dependence receptors are a functional family of receptor, united by their ability to induce cell death in the absence of ligand. They do not share specific structural traits (Negulescu and Mehlen, 2018). The first dependence receptor identified was p75NTR, which binds to the neurotrophin NGF (Nerve Growth factor); the death induced by this receptor in neuronal cells could be saved by the presence of NGF (Rabizadeh et al., 1993). But it was only later that Patrick Mehlen proposed the first definition of DR when characterizing the cell death function of DCC *in vitro* (Mehlen et al., 1998). In addition to DCC and p75NTR, more than twenty DR have been identified to date, among which netrin-1 receptors

UNC-5 H, neogenin, receptors to the morphogen sonic hedgehog (SHH), patched-1 (PTCH-1), the Semaphorin-3E receptor, Plexin D1, receptors with tyrosine kinase activity, rearranged during transfection (RET), tropomyosin receptor kinase A and C (TrkA and TrkC), ephrin type A receptor 4 EphA4), insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R), some integrins and more recently Kremen-1, Notch3 and the GPCR receptor Latrophilin (Negulescu and Mehlen 2018).

Shared features of cell death signalling

The precise mechanisms of DR activation and signalling are not fully characterised in part because they vary between receptors, especially in the signal transduction and effectors lying downstream DR cleavage. When unbound to their ligand, DR, in a monomeric state, are commonly cleaved in their intracellular domain by caspases. The cleavage of DR is thought to unmask a proapoptotic domain and allows the recruitment and activation of pro-apoptotic caspases or partners, which amplify the apoptotic signal (Goldschneider and Mehlen, 2010). To sum, the common features and conditions for DR-induced cell death are:

- Monomeric state (that is to say, unbound)
- Cleavage of the receptor
- Activation of caspases
- Induction of cell death

1.2.3.3 DCC pro-apoptotic signalling

In the section 1.2.1 we reported that DCC was initially identified as a tumour suppressor and later described as a receptor of netrin-1 implicated in chemoattraction. The identification of DCC as a DR reconciles these two facets: in addition to its positive signalling in response to netrin-1, DCC actively induces cell death when unbound to netrin-1 (Mehlen et al., 1998) (Figure 4).

The pro-apoptotic signalling of DCC in the absence of netrin-1 is only partially elucidated. When unbound to netrin-1, DCC monomers are presumably cleaved by caspase 3 in its intracellular domain. Interestingly, the procaspase-3 has a basal proteolytic activity (Yang et al., 1998) and interacts with DCC in presence of netrin-1. This interaction might allow rapid and tight regulation of the activation of DCC pro-apoptotic signalling upon decreased level of ligand (Forcet et al., 2001). Following caspase cleavage DCC would undergo an intracellular conformational change that allows it to directly recruit caspase-9 and subsequently activate further caspase-3 independently of the extrinsic and intrinsic pathways of apoptosis (Forcet et al., 2001) contributing to the amplification of DCC cleavage and caspase activation. Caspase inhibitors are able to block DCC-induced cell death. The mutation of aspartic acid (D) 1290 from DCC intracellular domain (a putative caspase cleavage site) into asparagine (N) also inhibits cell death induced by this receptor (Mehlen et al., 1998). Of note, the corresponding caspase cleavage site is conserved in all mammalian species but is absent in UNC40 of *C.elegans* or in Frazzled of *D.melanogaster*. It is thus tempting to speculate that the survival activity of netrin-1/DCC signalling has been acquired in complex organisms and is a relatively late event in the evolution of DCC. This would be

consistent with the greater plasticity of the mammalian nervous system compared with those of invertebrates (Furne et al., 2008).

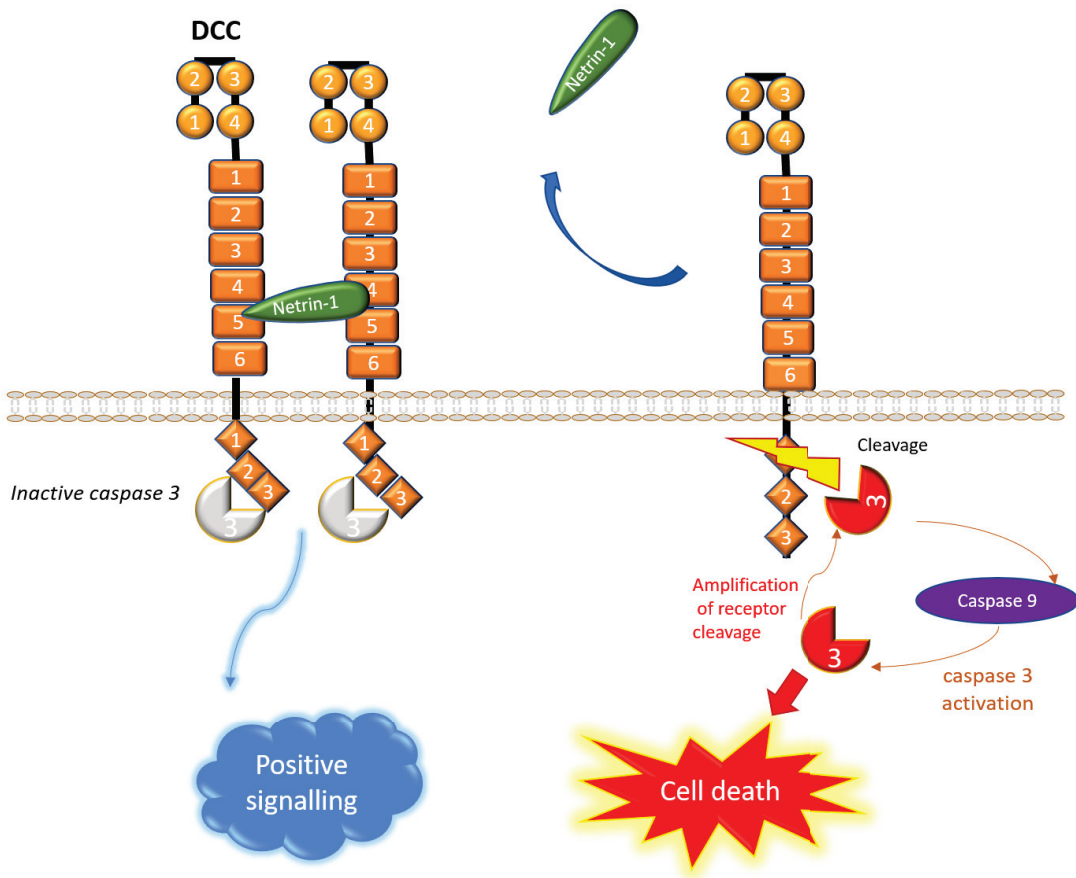


Figure 4 DCC dependence receptor dual signalling

Due to their ability to induce cell death in the absence of the ligand, dependence receptors may be involved in tissue homeostasis to limit the number of cells. In such hypothesis, we would expect that DCC mutant mice would be associated to more survival of commissural neurons however, DCC inactivation phenocopies commissural defects observed in netrin-1 mutant mice (Fazeli et al., 1997). This may be explained by the concurrent loss of netrin-1 positive signalling. However, the decreased expression of the DCC receptor in several cancers such as cancers of the stomach, prostate, endometrium, ovary, breast, colon, testes, or neuroblastomas (Mehlen and Fearon, 2004) reinforce the DR hypothesis. In addition, this loss of expression is a factor of poor prognosis and favours metastases in colon cancer (Saito et al., 1999). The mutation of DCC caspase cleavage site (D1290) induces intestinal tumours and increases the number of intestinal tumours in a mouse model, mutated for the APC protein, predisposed to colon cancer (Castets et al., 2011) further indicating that DR-apoptotic activity plays a role in the control of tissue homeostasis.

1.2.3.4 *Netrin-1 a survival factor for neurons?*

According to the DR theory, netrin-1 would have a survival role, in addition to its guidance role, by inhibiting apoptosis induced by its dependence receptors. In this sense, netrin-1 can be defined as a neurotrophic factor. However, unlike neurotrophins which actively trigger survival pathways activating for example PI3K or MAPK-dependent pathways, netrin-1 would generate a cell survival dependent state by inhibiting proapoptotic pathways (Reichardt, 2006).

In line with the DR theory, a decrease in the number of olivary and pontine neurons has been described in the netrin-1 KO mutant mice (Bloch-Gallego et al., 1999, Yee et al., 1999). Netrin-1 was also reported to be a survival factor for commissural neurons of the spinal cord and brainstem (Furne et al., 2008; Llambi, 2001; Tang et al., 2008). However, contradictory observations came from studies that failed to demonstrate an elevation of neuronal death in complete Netrin-1 KO mice (Bin et al., 2015; Yung et al., 2015). Thus, the role of netrin-1 in neuronal apoptosis remains to be confirmed.

Binding to various receptors, netrin-1 mediates diverse functions. Some receptors like DCC, mediates various function: migration and adhesion; others like DCC and neogenin are involved in similar roles: cell adhesion and chemoattraction, while others like DCC and UNC-5 display opposite roles: chemoattraction and chemorepulsion respectively. Receptors of netrin-1 exhibit different affinity to netrin-1 and can also bind together or even with other (non-netrin) receptors contributing to the modulation of netrin-1 signalling. Additionally, netrin-1 primary receptors, DCC and UNC-5 are dependence receptors and hence exhibit a dual signalling. Of importance, they can induce cell death in absence of netrin-1.

Taken together, netrin-1 signalling seems to be highly flexible. This is consistent with its guidance role in axon navigation and cell migration during development for example. Indeed, growth cones and cells may need to readily integrate spatiotemporal information from their environment in order to grow and migrate to the proper target sites.

1.3 ...AND RESTORING?

In the CNS development, DCC or UNC-5 DR-signalling may participate as a “quality control” mechanism to rapidly correct errors of guidance and control the number of neurons. **Could this mechanism play a role in the mature brain?** The answer is likely no, since the adult brain is fully wired, has poor regenerative capacity, and only exhibit restricted plasticity. The reactivation of such a mechanism may even have harmful consequences, possibly leading to aberrant cell death. What about the opposite? Could the activation of the positive pathway impact on damaged neurons in neurodegenerative diseases or following brain injury promoting survival, growth or synapse formation and maintenance? It might depend on the tissue environment and notably receptors ratio, levels, and localisation.

Among the four classic guidance family of proteins, Netrins, Slits, Ephrins and Semaphorins, netrin-1 has the strongest chemoattractive ability to promote axon extension (Pinato et al., 2012; Sun et al., 2011). Furthermore, netrin-1 expression is often upregulated following cerebellar and spinal cord injury and brain ischemia, indicating that netrin-1 may have an effect on lesion (Löv et al., 2008; Tsuchiya et al., 2007; Wehrle et al., 2005). However, studies addressing the role of netrin-1 in neuron repair or maintenance are only starting to emerge. A rapid overview:

1.3.1 Insights from nerve regeneration

In the adult spinal cord and optic nerve, netrin-1 is allegedly an inhibitory molecule contributing to axon regeneration failure after injury due to its tight association with myelin which is a known inhibitor for repair (Löv et al., 2008). However, the inhibitory role of netrin-1 on nerve regeneration is so far poorly demonstrated. Of note, the ratio DCC:UNC-5-H and receptor pattern along and across the nerve may affect netrin-1 regenerative properties since these receptors may mediate distinct, and even opposite, signals. In the peripheral nervous system, netrin-1 is expressed in Schwann cells and its expression is up-regulated after peripheral nerve transection injury. Recent studies indicated that netrin-1 plays a positive role in promoting peripheral nerve regeneration, Schwann cell proliferation and migration, and that this role is tightly **dependant on the concentration** of netrin-1 (Dun and Parkinson, 2017).

1.3.2 Insights from models of acute brain injury

Growing studies in models of brain injury, mostly models of stroke (ischemic or haemorrhagic), demonstrate an upregulation of netrin-1 and DCC that may be associated with neuroprotection (Tsuchiya et al., 2007; Wang et al., 2013). Following, focal cerebral ischemia reperfusion ischemia, netrin-1 localises in neuronal cells and around small vessels while DCC increased expression localises in membranes and protrusions of neurons and astrocytes, which could indicate that netrin-1/DCC plays important roles in neurite outgrowth, glial cell network reconstruction, and vascular regeneration after cerebral ischemia (Wang et al., 2013). Indeed, netrin-1 overexpression **inhibits neuronal apoptosis** during ischemia reperfusion injury (Wu et al., 2008) and after middle cerebral artery occlusion (MCAO) (Lu et al., 2011). In addition, netrin-1 overexpression after MCAO was shown to promote **axonal regeneration** and **synaptic**

formation (Zheng et al., 2018). Following global ischemia, intrahippocampal injection of netrin-1 in rat significantly improved **synaptic plasticity** (Bayat et al., 2012).

In these studies, however, it is not clear whether netrin-1 neuroprotection is a direct effect of netrin-1 action on neurons or an indirect effect due to netrin-1 action on other cells. Since these results are reported from ischemic models, we can also speculate that netrin-1 injection or overexpression may participate in vascular reconstruction after injury.

In addition, netrin-1 overexpression after focal cerebral ischemia was shown to promote **neural stem cells migration** (Lu et al., 2016) and white matter repairing and remodelling (He et al., 2013). Furthermore, several studies point that netrin-1 **mitigates inflammation** in the brain following injury by regulating astrocyte activation but also blood-brain-barrier stability (He et al., 2018; Podjaski et al., 2015; Xie et al., 2018).

These findings indicate that netrin-1 may achieve recovery after injury both promoting neuron survival, repair, and function, and, acting on the microenvironment (glial cells and endothelial cells).

1.3.3 Insights from models of neurodegenerative diseases

Netrin-1 signalling has been studied in several models of neurodegenerative diseases.

In animal models of Alzheimer's disease (AD), netrin-1 interacts with amyloid precursor protein and inhibits amyloid-beta amplification (Bredesen, 2009; Lourenço et al., 2009). Netrin-1 brain administration was also associated with **cognitive improvement**. In accordance with these observations, other investigators showed that netrin-1 improved memory and biomarkers of AD in a mouse model of AD (Spilman et al., 2012).

Moreover, it was recently reported that netrin-1 pathway is dysregulated in Huntington's disease (HD). DCC and UNC-5D, are increased in human HD Neural Stem Cells (HDNSC) derived from HD patient-induced-pluripotent cells and that addition of netrin-1 is **neuroprotective** in HD Stem Cell Model (Ring et al., 2015).

In PD research, netrin-1 axon guidance properties were assessed in a neuro-transplantation study in order to reconstruct the nigrostriatal pathway. Similar to glial cell derived neurotrophic factor (GDNF), netrin-1 injection in rat brain showed significant **increase in axonal outgrowth** from transplanted dopaminergic cells, and combination of GDNF and netrin 1 supported robust growth of axons from transplants over long distance.

Although these approaches are quite different, they all point that netrin-1 exhibit potential neuroprotective and neurorestorative properties.

Following injury, netrin-1 expression is usually reactivated suggesting that it might contribute to repair. Indeed, overexpression or exogenous application of netrin-1, in various pathological models, appears to have a range of effects — especially axon growth, synapses formation and maintenance, inflammation, and survival — particularly interesting for neurorestoration studies.

2 PARKINSON'S DISEASE: PATHOLOGY AND THERAPY

Parkinson's disease (PD) is an age-related neurodegenerative movement disorder characterised by the progressive and selective loss of dopaminergic neurons of the substantia nigra. The disease was named after James Parkinson who first described the clinical features of the disease in "*An Essay on the Shaking Palsy*" (1817). Based on the description of six patient cases, Parkinson defined the then-called shaking palsy (or *paralysis agitans*) as follows: "*Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.*"

Early descriptions of symptoms resembling those of PD, namely tremor, drooling, lack of movements, were already reported in ancient Egypt and India several centuries B.C and at the beginning of our era with Galen. Following Parkinson's essay, different neurobiologists, and most remarkably Charcot, who popularised the term "Parkinson's disease" initially coined by William Sanders (1865), contributed to better describe and refine the panel of clinical symptoms characteristic of the disease. However, little was then known about the physiopathology of the disease. Hence, treatments were empiric (surgery and anticholinergic drugs) and showed modest effects.

In the 20th century, successive breakthroughs participated to revolutionise the understanding and the management of the disease. Indeed, in 1912 Frederic Lewy described a pathologic feature present in patient's brains, later called "Lewy body". Then, Konstantin Tretiakoff, in 1919, and Rolf Hassler, in 1939, identified the anatomic structure of the brain mainly affected by the disease: the substantia nigra. In the 1950s, Arvid Carlsson's seminal work on dopamine led to the discovery that dopamine deficit was responsible for PD motor symptoms and that compensating dopamine deficit with L-DOPA could alleviate those symptoms. This work opened the way to dopamine replacement-based therapies for PD, which highly improved patients' quality of life, and was eventually awarded the Nobel Prize in Physiology or Medicine 2000. More recently, genetic studies on familial cases of PD led to the identification of various gene mutated in families (SNCA, PRKN, DJ-1, PINK1, LRRK2), providing additional insight into PD molecular pathogenesis.

Despite the tremendous progress made in the characterisation of the disease over the past two centuries, the initiating events and mechanisms leading to PD remain a conundrum. Also, current therapies do not cure the disease and lack long term efficacy. With global population ageing, finding effective therapies that could slow or stop the progression of the disease is becoming a major health concern, as PD incidence rises. In France, for example, the number of patients with PD doubled between 1993 and 2018.

This chapter will provide a more detailed review, although not exhaustive, on the disease and existing treatments in order to highlight current research challenges and clinical needs in the field.

2.1 THE PATHOLOGY

2.1.1 Clinical description and statistics

Parkinson's disease (PD) is the second most frequent neurodegenerative disease worldwide. It is usually diagnosed between the ages of 45 and 70 years. Accordingly, PD prevalence, which is of 0.3% in the general population, markedly increases with age reaching 1 to 2% in the population over 65 years and 4 to 5% in the elderly over 85 years. Familial/genetic forms of the disease account for less than 10% of PD cases.

PD diagnosis is based on the presence of cardinal motor symptoms: **bradykinesia**, **resting tremor** and **rigidity**, the so-called parkinsonian triad. Patients can also experience non-motor symptoms such as sleep disturbance, autonomic dysfunction, cognitive and psychiatric disorders. Motor symptoms and non-motor symptoms increase with age and PD progression, and both have a major impact on the quality of life for patients with PD (Lees et al., 2009). To date, the management of patients with PD has mainly been addressing the motor symptoms.

At the onset of motor symptoms, neurodegeneration is already advanced (cf. section 2.1.3.2, p44). Therefore, efforts have been made to find early PD (clinical, biochemical, imaging, genetic) biomarkers to improve the management of patients with early PD. For example, hyposmia, constipation, and rapid eye movement sleep behavioural disorder (RBD) have been identified as prodromal signs of PD (Doty, 2012; Edwards et al., 1991; Pfeiffer, 2011; Postuma et al., 2015). However, these signs taken individually can easily go unnoticed and are not predictive enough to diagnose early PD. Combining different classes of biomarkers for PD (biochemical, clinical, imaging) and examining possible co-occurrence of risk factors in patients could help defining people particularly at risk for PD (and promote neuroprotective interventions) and increase the predictability of disease progression in patients (which can be useful to assess clinical trials benefit).

2.1.2 Aetiology and risk factors

Despite 200 years of research and studies, **PD aetiology remains obscure**. The late onset and slowly-progressing nature of the disease suggest that PD may be driven by chronic environmental insults in combination with ageing and genetic susceptibility. Possibly, different initiating events may lead, through convergent pathogenic mechanisms, to dopaminergic neuron degeneration. Below, a rapid overview of several factors that have been linked with PD and that might help to better understand PD pathogenesis.

2.1.2.1 Genetic factors

Most of PD cases are sporadic, however, the discovery of genes linked with relatively rare familial forms of the disease and subsequent studies in transgenic animal models have provided some insights into molecular mechanisms possibly involved in PD pathogenesis. Currently approximately 24 genetic loci have been identified as being associated with parkinsonism, and mutations of the alpha-synuclein (SNCA), leucine-rich repeat kinase 2 (LRRK2), parkin (PRKN),

PTEN-induced putative kinase protein 1 (PINK1), and Parkinsonism associated deglycase (PARK7 or DJ-1) genes can be listed as proven genetic factors.

SNCA

Alpha-synuclein is a soluble intrinsically disordered protein (IDP) abundantly found in the mammalian brain and especially in presynaptic terminals (Iwai et al., 1995; van Rooijen et al., 2009). It exists in various conformations (monomers, oligomers, fibrils) in a dynamic equilibrium that can be modulated by several intracellular factors, including oxidative stress, post-translational modifications and concentrations of phospholipids and metal ions. Although its physiological role is not fully understood, studies suggest that alpha-synuclein is involved in vesicular trafficking and axonal transport (Burre et al., 2010; Cooper, 2006). Alpha-synuclein aggregates are observed both in familial and in sporadic forms of PD. They are the major components of intracytoplasmic protein aggregates called **Lewy bodies**, a key histopathologic hallmark of the disease, but are also found outside Lewy bodies. The presence of these aggregates in all PD patients suggests that alpha-synuclein proteostasis is impaired in PD (Baba et al., 1998; Chartier-Harlin et al., 2004). However, it is still unclear what causes their aggregation and to what extent it is responsible for the disease progression.

PRKN and PINK1

Mutations in the PRKN or PINK1 genes are the leading cause of the autosomal recessive form of Parkinson's disease (Arkinson and Walden, 2018). These genes are involved in the **mitochondrial quality control**. Parkin is a E3 ubiquitin ligase that particularly directs the degradation of mitochondria outer membrane proteins upon cellular stress mediating the clearance of damaged mitochondria via mitophagy. PINK1 (PTEN-induced putative kinase protein 1) is a serine/threonine kinase with a mitochondrial targeting domain. In stress conditions, PINK1 stabilizes on the mitochondria outer membrane and, through its kinase activity, targets Parkin recruitment and activation which eventually leads to the elimination of damaged mitochondria (Kang et al., 2018). Loss of this pathway leads to the accumulation of impaired mitochondria, increased reactive oxygen species (ROS) and neuronal cell death.

DJ-1

DJ-1 function is not precisely characterised, it is a redox sensitive protein ubiquitously expressed in various tissues and implicated in several processes such as response to cell stress, cell cycle regulation, control of gene transcription (da Costa, 2007). In neurodegeneration studies, DJ-1 was shown to protect against **oxidative stress**, in part through an action on mitochondria (Cookson, 2010). It was also shown to associate with synaptic membrane (Usami et al., 2011) and to inhibit alpha-synuclein aggregation (Shendelman et al., 2004).

LRRK2

LRRK2 is a protein kinase, mainly cytoplasmic that was also shown to associate with the mitochondria. It is involved in various functions such as regulation of cytoskeleton dynamics, modulation of synaptic vesicle recycling and autophagy contributing to neurite outgrowth and synaptic plasticity (Webber and West, 2009). Mutations in LRRK2 are the most common causes

of familial PD in the Western World (Li et al., 2014b). Curiously, some PD cases associated with LRRK2 mutations lack Lewy bodies, a common hallmark of the disease.

These top mutated genes in familial forms of PD strongly implicate mitochondria, oxidant stress and synaptic/axonal dynamics in PD pathogenesis. Genetic susceptibility is not the only factor for PD, differences in prevalence between identical ethnic groups in different countries support the role of environmental factors in PD pathogenesis (Chaudhuri et al., 2000; Richards and Chaudhuri, 1996; Stoessl, 1999).

2.1.2.2 Environmental factors

Pesticides and other industrial chemicals – mitochondrial stress

A hint for an environmental factor in PD came from the observation of parkinsonian syndrome in drug addicts after having used 1-methyl-4-phenyl-propion-oxypiperidine (MPPP) as a synthetic opioid (Davis et al., 1979; Langston et al., 1983). In those cases, MPPP contained the toxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) as a major by-product impurity. MPTP can cross blood-brain barrier and, once inside the brain, be metabolised by glial enzyme monoamine oxidase B (MAO-B) into the neurotoxic cation 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ interferes with **complex I** of the mitochondrial electron transport chain which leads to cell death and generates free radicals (Przedborski et al., 2000). Similarly, the pesticides rotenone and paraquat were shown to increase the risk to develop PD, inhibiting complex I mitochondria respiratory chain. Along this line, epidemiological studies indicate a link between residence in a rural environment (and associated exposure to pesticides) and elevated risk of PD (Tanner et al., 2011). In France, PD is recognised as an occupational disease for farmers. Nevertheless, humans are exposed to numerous pesticides and toxins in nature, yet not everyone develops sporadic PD, suggesting that the factors required for disease development are more complex.

Infection- inflammation

Some observations put forward the possibility that occurrence of inflammation in the brain, because of traumatic brain injury or exposure to infectious agents, especially in early-life, may play a role in the pathogenesis of PD (Logroscino, 2005). Along this line, the alleged protective role of ibuprofen in PD has been discussed in several studies (Bornebroek et al., 2007; Gao et al., 2011). In both cases, epidemiologic studies are inconclusive. However, the presence of **activated microglia** and proinflammatory cytokines are a common feature of PD. Nevertheless, it is not clear whether inflammation is a result of neurodegeneration or an initiating event in the disease. Inflammation is also associated with a broad spectrum of neurodegenerative diseases, including AD, synucleinopathies, amyotrophic lateral sclerosis, Creutzfeldt–Jakob disease, Huntington's disease (McGeer and McGeer, 2004; Griffin, 2006; Kim and Joh, 2006;).

Lifestyle- Insulin resistance

Growing evidence indicates that Type 2 diabetes (T2D) is a risk factor for developing PD and that common pathophysiological features including oxidative stress, inflammation, insulin resistance, abnormal protein processing, and cognitive decline exist between T2D and PD

suggesting common underlying pathological mechanisms (Santiago and Potashkin, 2014; Yang et al., 2017). Also, many non-diabetic PD patients show features of insulin resistance (Dunn et al., 2014). Insulin can participate to several processes in the brain including regulating neuronal survival and growth, dopaminergic transmission, maintenance of synapses raising the possibility that defective insulin signalling pathways may contribute to the development of the pathological features of PD, and thereby suggests that the insulin signalling pathway may be a target for disease modification. Glucagon-like peptide-1 (GLP-1)-based antidiabetic drugs have shown neurotrophic and neuroprotective effects in numerous *in vitro* and *in vivo* preclinical studies. Several double-blind clinical trials of GLP-1R agonists are ongoing (Hölscher, 2018; Kim et al., 2017). These elements suggest that PD might also be a **metabolic disease**. Therefore lifestyle (food, physical exercise) may influence the progression of the disease.

2.1.3 Neuropathology

PD is defined by two key neuropathological hallmarks; the loss of dopaminergic neurons of the substantia nigra *pars compacta* (SNc) and Lewy body cytoplasmic inclusions.

2.1.3.1 Lewy pathology (LP)

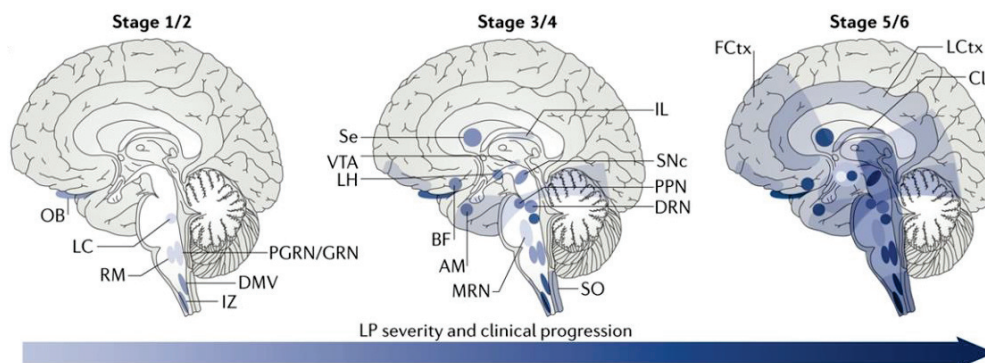


Figure 5 Distribution of Lewy bodies and PD progression (Surmeier et al., 2017)

The anatomical progression of the disease through the brain increases over time (from left to right), and the darker the colour the more LP is present in each region at a given stage. **BF**, magnocellular nuclei of the basal forebrain; **Cl**, claustrum; **cp**, cerebral peduncle; **DMV**, dorsal motor nucleus of the vagus; **DRN**, dorsal raphe nucleus; **FCtx**, frontal cortex; **IL**, intralaminar nuclei of the thalamus; **IZ**, intermediate reticular zone; **LC**, locus coeruleus and subcoeruleus; **LCtx**, limbic cortex; **MRN**, median raphe nucleus; **OB**, olfactory bulb; **opt**, optic tract; **PGRN/GRN**, paragigantocellular and gigantocellular reticular nucleus; **PPN**, pedunculopontine nucleus; **RM**, raphe magnus; **Se**, septum; **SNc**, substantia nigra pars compacta; **SO**, solitary tract nuclei; **VTA**, ventral tegmental area

In 1912 Lewy described for the first time intraneuronal inclusion bodies in the nucleus basalis of Parkinson's patients. Later, Tretiakoff (1919) observed the same type of inclusion in the SNc of parkinsonian patients and named them Lewy bodies. These bodies are spherical eosinophilic cytoplasmic inclusions with a dense centre and a peripheral light halo, composed of neurofilaments, alpha-synuclein, parkin, ubiquitin, and tubulin (Forno, 1996; Spillantini et al., 1998). In PD, they are commonly present in neurons of various but precise brain structures beside

the substantia nigra *pars compacta*. Braak and others have argued that Lewy bodies distribution is not random and develops over time and in a staged-manner (from the lower brainstem to rostral regions of the brain) that matches with the progression of clinical symptoms (Figure 5) (Braak et al., 2003). Although some observations supported this model in some PD patients, it is still not known how Lewy bodies spreads in the brain and how they contribute to the neurodegenerative process. Also, despite most patients with PD commonly have Lewy body inclusions, this is not always the case. Besides, Lewy bodies are also found in patient with no apparent PD symptoms, and in neurons that do not degenerate in PD (Rietdijk et al., 2017; Surmeier et al., 2017). Thus, the functional consequences of Lewy pathology in the disease is still uncertain.

2.1.3.2 Loss of dopaminergic neurons

Dopaminergic (DA) neurons of the SNc preferentially degenerates in PD. These midbrain dopaminergic neurons are involved in the control of motricity supplying the dorsal striatum (putamen) with dopamine through long axonal projections forming the nigrostriatal pathway. Post-mortem analysis of patient with PD shows a noticeable loss of these neuromelanin-containing neurons in the SNc.

Degeneration time course and specificity

Animal studies demonstrated that the degeneration of dopaminergic neurons is retrograde. It starts from axon terminals in the dorsal striatum and is followed by axon and cell body demise (Björklund et al., 1997). Compelling evidence indicate that degeneration in human may similarly follow a “**dying-back**” process. Indeed, the loss of DA striatal terminals significantly overweighs the loss of dopaminergic cells in the SNc; at the onset of motor symptoms, there is a 30-50% loss of dopaminergic neurons in the SNc whereas there is a drop of 60% to 80% in the dopamine content of the striatum (putamen) (Burke and O'Malley, 2013). Several years can pass between the loss of striatal dopamine and the onset of motor symptoms. This delay is due to endogenous compensatory mechanisms exerted by remaining healthy neurons (Reetz et al., 2009). There are both dopaminergic compensatory mechanisms, enhancing the effects of existing dopamine, and non-dopaminergic mechanisms, which reduce the activity of the indirect striatal output pathway (cf section 2.2.3,p50).

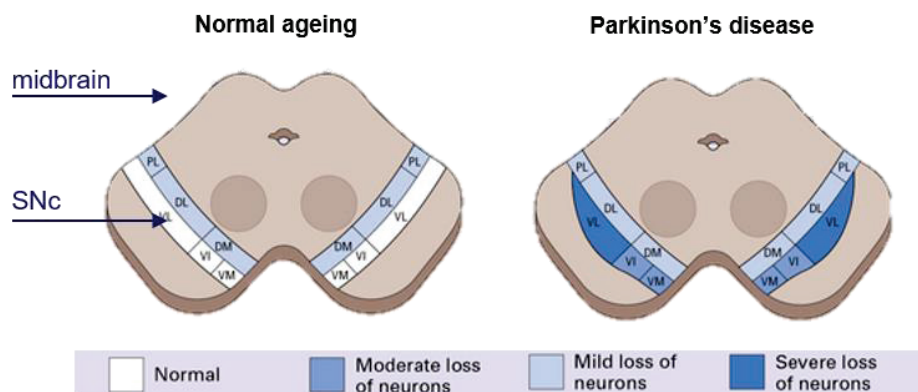


Figure 6 Regional selectivity to cell death within SNc
adapted from <https://clinicalgate.com/parkinsonism-and-akinetic-rigid-disorders>

Within the substantia nigra there is also a regional selectivity to cell death (Figure 6). The SNc can be divided into ventral and dorsal tiers, which project to different brain areas, and each tier can be further subdivided into regions (medial to lateral). In normal ageing, the estimated rate of cell loss from the dorsal tier (DT) of the substantia nigra is 7% per decade, leading to 40–50% cell loss by 65 years of age while the ventral tier is unaffected. In PD, cell loss is greatest in the ventral tier (VT). Typically, in the ventrolateral tier, 70–90% cells have been lost by the time a patient dies. Cell loss from the dorsal tier is not significantly different from that in normal ageing (Fearnley and Lees, 1991).

The other midbrain dopaminergic neurons are less vulnerable to degeneration in the disease. The loss of dopaminergic neurons from the ventral tegmental area (VTA) and the peri- and retrorubral tegmental area is modest whereas other DA neurons such as those in the periaqueductal region (Hirsch et al., 1988) or the hypothalamus (Matzuk and Saper, 1985) remain unaffected.

Features of degeneration

As mentioned earlier, degeneration of dopaminergic neurons of the SNc is strongly associated with increased **alpha-synuclein aggregates**, **mitochondrial dysfunction**, and **inflammation** in the substantia nigra. In addition, various cellular system involved in the maintenance of homeostasis accompany degeneration such as **autophagy**, **unfold protein response (UPR)** and **endoplasmic reticulum (ER) stress** (Cerri and Blandini, 2018; Mercado et al., 2016; Varma and Sen, 2015). A recurrent question is about the sequence of these events, especially what comes first? And what initially triggers these processes? Those are still a matter of debate. It is however interesting to note that mitochondria dysfunction, microglial activation and alpha-synuclein aggregates are not confined to dopaminergic neurons of the substantia nigra and are found in other brain areas, and even in other tissues. Nevertheless, dopaminergic neurons of the substantia nigra are the one consistently lost. The eventual loss of these neurons is presumably due to the activation of programmed cell death as a consequence of an accumulation of stressing events. Indeed, mitochondria dysfunction, ER stress, oxidative stress, and autophagy are known activators of programmed cell death (Levy et al., 2009). Studies in genetic and toxin-based animal models of PD showed that **apoptotic cell death** accounted for the cell loss in the substantia nigra. Although it is difficult to evaluate cell death in human post mortem tissue, because of the slowly progressive nature of the disease (only a small number of neurons dies at a given time and dead cells are rapidly cleared), TUNEL staining and increased activated caspase-3 immunostainings tend to confirm a role of apoptotic cell death in PD (Venderova and Park, 2012). Nevertheless other types of cell death have been reported (Venderova and Park, 2012).

2.1.3.3 Non-dopaminergic cell loss

Many neurons from non-DA systems in the brain (Figure 7) and peripheral organs are affected in the disease, although in a lesser extent and at later stages (Arendt et al., 1983; Chan-Palay and Asan, 1989; Thannickal et al., 2007) (Figure 7). Degeneration of these systems is inconsistent between patients and is thought to be responsible for the secondary symptoms of PD such as dementia, depression, sleep disorders and dysfunctions of the autonomic nervous system.

It may also account for motor symptoms that are resistant to DA treatments. Interestingly neuronal loss in PD (Figure 7) follows a different pattern than does Lewy body inclusions (Figure 5).

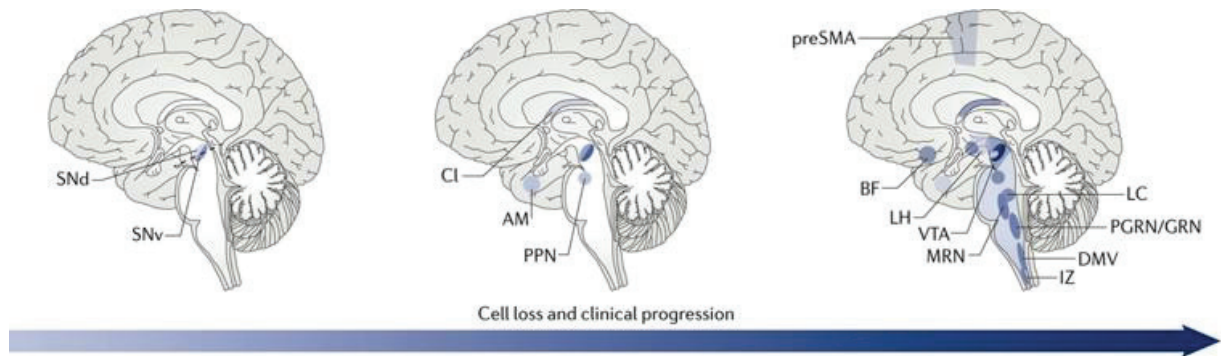


Figure 7 Staging of degeneration in (sporadic) PD (Surmeier et al., 2017)

The schematics represent the progression of neuronal cell loss following the onset of PD. The anatomical distribution of neuronal loss increases with time, and the darker the colour, the more neuronal loss evident in each region. **AM**, amygdala; **BF**, magnocellular nuclei of the basal forebrain; **Cl**, claustrum; **cp**, cerebral peduncle; **DMV**, dorsal motor nucleus of the vagus; **IZ**, intermediate reticular zone; **LC**, locus coeruleus and sub-coeruleus; **LH**, lateral hypothalamus; **MRN**, median raphe nucleus; **PGRN/GRN**, paragigantocellular and gigantocellular reticular nucleus; **PPN**, pedunculopontine nucleus; **preSMA**, presupplementary motor area; **R**, red nucleus; **SNd**, dorsal tier of the SNc; **SNv**, ventral tier of the SNc.

In summary, PD pathogenesis is a complex process in which both genetic features and environmental stressors **converge** and compromise, over time, neuron function and viability by affecting the cellular systems dedicated to the maintenance of homeostasis: mitochondria and protein quality control systems particularly. PD thus appears as a **multifactorial disease**, resulting from different combination of events. In this sense, there might be different subtypes of PD. Although several aspects of the disease remain obscure or inconsistent, the selective degeneration of nigral dopaminergic neurons is **invariably** linked with the motor symptoms which are the prime criterion for PD clinical diagnosis and the main cause of disability in patients.

2.2 ZOOM ON THE DOPAMINERGIC NEURONS OF THE SUBSTANTIA NIGRA

Loss of dopaminergic neurons of the substantia nigra is the main hallmark of Parkinson's disease. What are these neurons and what is their role? Most importantly, why are they selectively vulnerable in the disease? An overview of their function, activity, and morphology may bring relevant insights into their selective vulnerability and may help identifying therapeutic strategies that could prevent their loss and support their function to develop efficient treatments for PD.

2.2.1 Dopamine producing neurons: dopamine metabolism

Dopamine (Hornykiewicz, 1966) is a neurotransmitter involved in many brain functions including motor control, emotions, cognition, and endocrine regulation.

As a catecholamine, like noradrenaline and adrenaline, it is synthesized from the conversion of the amino acid tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme **tyrosine hydroxylase (TH)**. As illustrated in Figure 8 L-DOPA is converted to dopamine (DA) by aromatic L-amino acid decarboxylase (AAADC). Dopamine is then transported by vesicular monoamine transporter 2 (VMAT2) and stored into vesicles at the presynaptic terminals of dopaminergic neurons or converted by monoamine oxidase (MAO) to dihydroxyphenylacetic acid (DOPAC).

When an action potential reaches the dopaminergic axon terminal, dopamine is released from storage vesicles into the synaptic cleft in a calcium-dependent manner. Dopamine is then uptaken by the postsynaptic neuron and glial cells where it is metabolised while remaining extracellular dopamine is recaptured by presynaptic **dopamine transporter (DAT)** and stored in vesicles via VMAT2. In postsynaptic neurons and glial cells, DA is converted into 3-methoxytyramine (3-MT) through catechol-O-methyltransferase (COMT) or to DOPA through MAO. Homovanillic acid (HVA), often viewed as the final metabolite of dopamine, is metabolized from DOPAC or 3-MT by COMT or MAO respectively (Figure 8). The primary metabolites of dopamine, in terms of amount, are **HVA** and **DOPAC**. Thus, dosage of DA, HVA and DOPAC can be used to assess the activity/functionality of a given dopamine pathway.

Most of the dopamine released in the synaptic cleft is rapidly recaptured by membrane DAT at the presynaptic terminal and stored. Indeed, when not stored into vesicles, cytosolic dopamine can easily **auto-oxidize** to form toxic reactive oxygen species: hydroxyl radicals, superoxide anions, and dopamine–quinone species (Graham et al., 1978; Muñoz et al., 2012). These reactive oxygen species can oxidize protein, lipids and, DNA impacting cellular homeostasis and generating cellular stress.

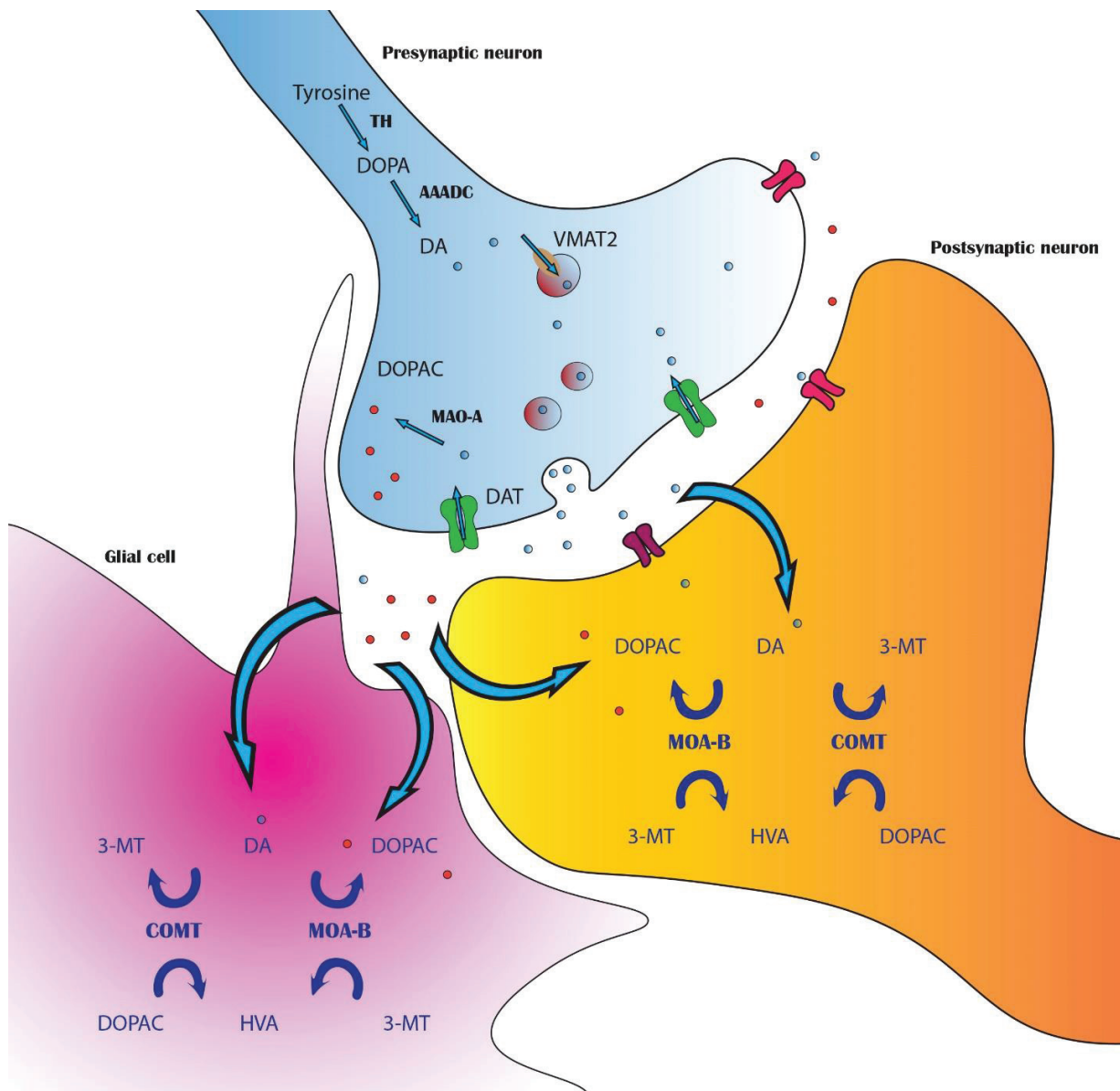


Figure 8 Dopamine synthesis and metabolism
● DA ● DOPAC

2.2.2 Unique morphological and physiological traits

There are three major dopaminergic pathways in the brain, the nigrostriatal, the mesolimbic/mesocortical and the tuberohypophyseal pathway. Dopaminergic neurons of the substantia nigra form the so-called **nigrostriatal** pathway; their cell bodies are located in the substantia **nigra** pars compacta in the ventral midbrain and their axons project mainly to the dorsal part of the **striatum** (caudate/putamen) (Figure 9). This dopamine pathway is involved in the control of voluntary movements, posture, the acquisition of motor programs and habit formation

through the release of dopamine in the striatum. This pathway alone accounts for 75% of the dopamine in the brain (and more than 90% of striatal dopamine) (German and Manaye, 1993).

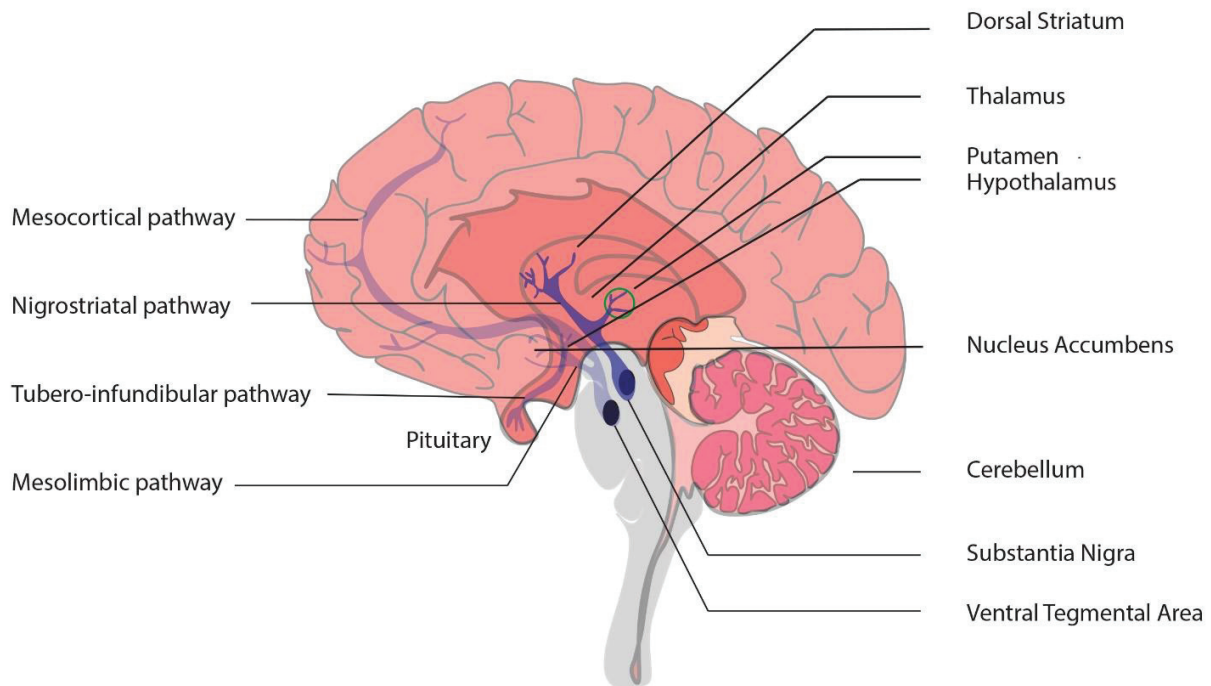


Figure 9 The nigrostriatal pathway and other dopaminergic pathways in the brain

To reach the dorsal striatum (which is a distant and voluminous structure) and provide it with dopamine, the dopaminergic neurons of the substantia nigra extend **long axonal projections**, among the longest in the brain, and diffusely display a **massive axonal arborisation** throughout the dorsal striatum (Bolam and Pissadaki, 2012; Matsuda et al., 2009). These highly branched axons establish hundreds of thousands of synapses, which is orders of magnitude greater than other neurons (Moss and Bolam, 2008; Moss and Paul Bolam, 2009). These morphological features may put these neurons under a high energy demand not only to maintain cell architecture and basal homeostasis but also function, particularly action potential propagation and synaptic activity (Pissadaki and Bolam, 2013). Furthermore, these dopaminergic neurons have a tonic spiking activity at rest: they are **autonomous pacemakers** (Guzman et al., 2009). This is associated with large oscillations in intracellular Ca^{2+} concentration enabling oscillation in membrane potential and resulting in a continuous release of dopamine in a “drip feed” manner maintaining dopamine concentration rather constant (nanomolar levels) in the striatum (Goto et al., 2007). Contrary to other dopaminergic neurons, notably VTA neurons, dopaminergic neurons of the substantia nigra possess bigger voltage-dependent Cav1 Ca^{2+} channels and **poor intrinsic Ca^{2+} buffering proteins** that favour elevated intracellular Ca^{2+} levels presenting advantages but also disadvantages for the cell (Philippart et al., 2016; Surmeier et al., 2017). Unbuffered cytosolic Ca^{2+} fluctuations promote mitochondrial ATP production, hence ensuring energy for the cell. But high calcium loads can

promote mitochondrial oxidant stress and increase generation of reactive oxygen species that can be toxic for neurons. Supporting this hypothesis, isradipine, a blocker of voltage-dependent calcium channels, prevents mitochondrial stress, thus inducing protection of dopaminergic neurons in animal models of PD (Guzman et al., 2018; Wang et al., 2017).

2.2.3 A neuromodulatory role in the basal ganglia

In a bigger scale, dopaminergic neurons of the substantia nigra form part of a functional group of subcortical nuclei interconnecting several brain structures, called the basal ganglia. The basal ganglia control a variety of processes such as procedural learning, cognition, and emotion and importantly, control of **voluntary movements**. The main components of the basal ganglia involved in the control of movements are the dorsal striatum (caudate nucleus and putamen), the internal and external segments of the globus pallidus (GPi and GPe respectively), the substantia nigra (SN) and the subthalamic nucleus (STN) (Lanciego et al., 2012).

The striatum is the core component of these circuits, it receives input from the midbrain and the cortex to modulate movements. Medium spiny neurons are GABAergic (inhibitory) neurons constituting more than 90% of the cell bodies in the striatum, the remaining cells are either cholinergic or GABAergic interneurons which regulate the excitability of the medium spiny neurons. There are two main subtypes of medium spiny neurons: medium spiny neurons that express D1 dopamine receptors and contain substance P (D1 type) and neurons that express D2 dopamine receptors and contain enkephalin (D2 type). Dopamine receptors D1 and D2 are part of a G proteins coupled receptor family and differs in that D1 receptor activates G proteins that stimulate cAMP-synthesizing adenylyl-cyclase whereas D2 receptor activates adenylyl-cyclase inhibitory G proteins (Surmeier et al., 2007).

In response to cortical excitation, D1-type medium spiny neuron efferences contacting the GPi and the substantia nigra *pars reticulata* (SNr) are part of a “direct pathway” that leads to the suppression of thalamic neurons tonic inhibition thus activating cortical motor neurons. This direct pathway facilitates the initiation and timing of voluntary movements. To reinforce the suppression of inappropriate motor actions, the “indirect pathway” consisting of striatal D2 medium spiny neurons inhibiting the GPe, results in disinhibition of the GPi which is then free to inhibit the thalamus. Hence, the indirect pathway antagonizes the activity of the direct pathway, both pathways functioning together in the fine modulation of initiation and termination of motor responses (Figure 10, left panel). Dopamine, through dopaminergic neurons of the substantia nigra *pars compacta* significantly impacts these pathways directly modulating medium spiny neuron response to cortical influx. Indeed, dopaminergic synapses with medium spiny neurons are localised on medium spiny neuron dendrites receiving cortical afferences. This setting suggests that dopamine acts on medium spiny neurons reinforcing excitatory output, through D1 receptors, or limiting them through D2 receptors. This balance is slightly in favour of the direct pathway, that facilitates activation of thalamic and cortical neurons (Lanciego et al., 2012). In PD, loss of dopamine leads to a shift of the balance to the indirect pathway (Albin et al., 1989) leading to a greater inhibition of glutamatergic thalamocortical neurons that are thus less likely to be timely

activated (Figure 10, right panel). Upregulation of corticostriatal glutamatergic synaptic transmission is evident in PD (Blandini et al., 2000). These modifications result in decreased facilitation of movements which favour many traits of rigidity and bradykinesia in PD.

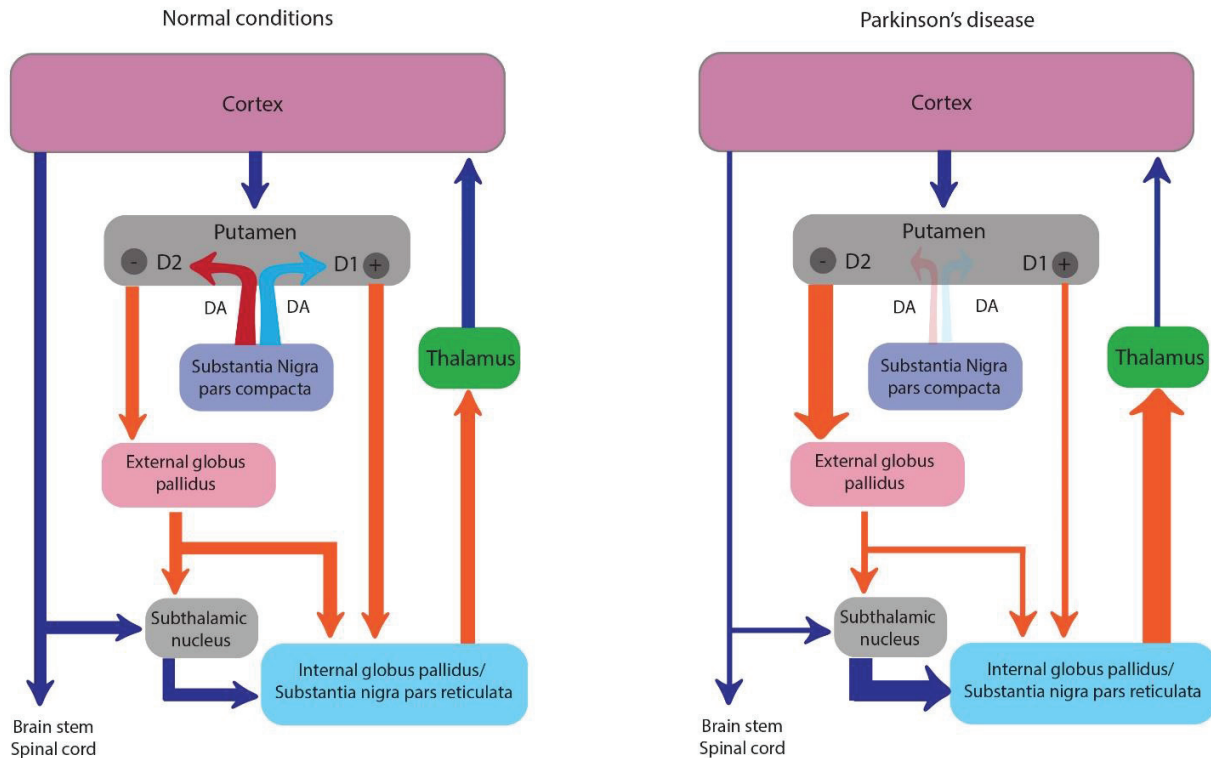


Figure 10 Functional changes of the basal ganglia circuitry in Parkinson's disease

Dopaminergic neurons of the substantia nigra possess long and highly branched axons, make hundreds of thousands of synapses and exhibit an autonomous pacemaker activity continually releasing dopamine. This unique morphology and basal activity may be **highly energy demanding**. As long as energy demand does not exceed the supply, these neurons may do well. However, any situation that would perturb the balance may significantly impact these neurons more than other neurons. Additionally, these neurons show a sustained mitochondrial oxidant stress and are continuously immersed in a dopamine-rich milieu that can easily generate ROS. Therefore, it appears that these neurons are dealing with challenging conditions and may be constantly “**on the edge**”. When coupled with cellular ageing, chronic exposure to toxins, genetic susceptibility or alpha-synuclein aggregation, these features may make these neurons more **vulnerable** to cell death and accelerate their demise, compared to other neurons. Loss of these neurons impacts the control of movement by the basal ganglia and is responsible for the motor symptoms in PD. Replacing missing dopamine or regulating other actors of this circuitry may compensate for the loss of dopaminergic cells and alleviate symptoms... at least, for a time.

2.3 CURRENT AND FUTURE THERAPIES FOR PD

2.3.1 Dopamine replacement-based treatments

Levodopa (L-Dopa) has been the treatment of choice for PD since its introduction in the late 1960s, due to rapid and spectacular effects on motor symptoms (bradykinesia, rigidity and resting tremor) (Birkmayer and Hornykiewicz, 1961). Levodopa is a dopamine precursor mainly metabolized by AAADC (also known as dopa decarboxylase (DDC)) which converts levodopa to dopamine, or by COMT that converts levodopa to 3-O-methyl-dopa (3-OMD) (Khor and Hsu, 2007). Since levodopa can cross the blood brain barrier, it can have both central and peripheral effects. To minimise peripheral effect and promote central effect, thus improving treatment efficacy and limiting adverse side effects, levodopa is usually co-administered with AAADC or COMT inhibitors that cannot cross the blood brain barrier (Leegwater-Kim and Waters, 2007; Ovallath and Sulthana, 2017). However, long-term levodopa treatment induces disabling motor side-effects (“on/off” fluctuations and dyskinesia) after a three to five years period of maximal benefit. Beside levodopa, other dopamine replacement-based treatments exist.

Dopamine agonists can be used as first line monotherapy therapy before levodopa supplementation or as an adjunct to levodopa. However, they usually show more adverse effects than levodopa, in particular nausea, sedation and psychiatric effects, and less effects on motor symptoms notably resting tremor. One reason evocated for this failure is that agonist treatments are often abandoned before the optimal posology is determined, otherwise they would achieve effects comparable to levodopa (Schapira, 2005).

MAO-B inhibitors used to enhance striatal dopaminergic activity also show beneficial effects in early disease as a monotherapy or combined with levodopa in more advanced stage of the disease. These inhibitors block MAO-B, the predominant isoform of MAO in the brain implicated in the metabolism of dopamine and offer effective relief of motor symptoms with a minimal risk of motor complications (Schapira, 2011). However, like dopamine agonists, these treatments are not as potent as levodopa.

2.3.2 Non-dopaminergic drugs

Advances in the understanding of the functional system of the basal ganglia have enabled the development of non-dopaminergic therapeutic targets.

2.3.2.1 Anticholinergic drugs

Anticholinergics were the first pharmacological drugs used in PD, even before the neuroanatomic basis of PD pathology was discovered (Charvin et al., 2018). Cholinergic neurons and interneurons innervate the nigrostriatal pathway but also the cortico-striatal-loop and may thus modulate dopaminergic activity in these systems and hence motor symptoms. Additionally, some cholinergic systems, like the basalis nucleus, are affected in PD, showing Lewy bodies inclusion but also cell death, and may be associated with secondary symptoms (Bohnen and Albin, 2011). Anticholinergic are used both as monotherapy and as part of combination therapy. Although anticholinergic drugs do produce limited beneficial effects on PD symptoms (mostly on tremor)

they are associated with adverse parasympatholytic (dry mouth, constipation, blurred vision) and cognitive effects (Katzenschlager et al., 2003).

2.3.2.2 Targeting GABAergic and glutamatergic neurons

Adenosine A_{2A} receptors are relatively selectively expressed in the striatum. Activation of these receptors may enhance GABA release in the GPe and contribute to the overactivity in the indirect pathway in PD. Several A_{2A} receptors antagonists (Jenner, 2005) improved motor impairments in PD pre-clinical models however they showed **inconsistent efficacy** in clinical trials (Fernandez et al., 2010). Moreover, it likely increases risk for AD (see above).

Glutamate antagonists, especially amantadine, showed beneficial effects on motor symptoms. Amantadine, although less potent than levodopa is sometimes used in early stages of the disease or as an adjunct to levodopa (Schwab, 1969). Glutamate antagonists are useful to alleviate secondary symptoms: they improve cognition and decrease levodopa induced-dyskinesia (Charvin et al., 2018).

2.3.2.3 Deep brain stimulation

In 1987, Benabid and co-workers introduced chronic deep brain stimulation (DBS) of the thalamic nucleus as a therapeutic strategy in PD. With an increase in the understanding of the pathophysiology of the disease, including the involvement of the basal ganglia, the sub-thalamic nucleus (STN) has become the main surgical target, in the 1990s. DBS inhibits the activity of STN neurons (Benabid, 2003). Since its discovery, STN DBS has become a standard alternative for the symptomatic treatment of advanced PD, and non-responders to classical medications.

2.3.3 Neurorestorative strategies: the future for PD?

All current treatments for PD are symptomatic, do not provide long lasting effect and often lead to incapacitating adverse effects such as dyskinesia. Indeed, these therapies do not prevent or delay the continuous loss of dopaminergic neurons which is the cardinal feature of the disease. Neurorestorative approaches are attractive therapeutic strategies that may meet these clinical needs.

2.3.3.1 Neurotrophic factors delivery

Extensive pre-clinical studies have provided convincing evidence for the neurorestorative properties of several growth factors, i.e. paracrine messengers that modulate cellular proliferation, death and/or differentiation through specific membrane receptors displaying tyrosine kinase activity on their cytoplasmic end. Exogenously administered in animal models such as rats, primates and porcine PD models, these endogenous messengers consistently showed robust effects, restoring morphology and function of degenerated dopamine neurons. To date, four have entered clinical trials in PD: **glial cell line- derived growth factor (GDNF)**, **neurturin (NTRN)**, **platelet-derived growth factor** and more recently **cerebral dopaminergic neurotrophic factor (CDNF)**(Sullivan and O'Keeffe, 2016). So far, the clinical outcome of these trials has been disappointing regarding the effects on symptoms and the development of adverse side effects. Although difficult to ascertain, technical aspects may account for these results, such as inadequate dosing, patient population selection, inappropriate protein administration methods and treatment time window. Gene therapy, used to induce endogenous production of trophic factor, is an

emerging method for the delivery of such factors. This method may provide better access to target cells, long term expression of the transgene, and less immunologic reactions, and thus better results (Hutchinson et al., 2007; Paul and Sullivan, 2018; Torres et al., 2017).

The neurorestorative effect of a novel neurotrophic factor CDNF (Lindholm et al., 2007; Voutilainen et al., 2011) is currently assessed by Herantis in a first-in-human double-blind, placebo-controlled clinical study in patients with PD. (www.treatER.com; ClinicalTrials.gov Identifier: NCT03295786). First results are expected by the end of 2020.

The example of GDNF

GDNF shows robust effects in various pre-clinical models of PD and has been the first neurotrophic factor to enter clinical trials for disease modification (neurorestoration) in PD. However, first results were disappointing: the intraventricular GDNF infusion resulted in no therapeutic benefit and produced serious adverse effects. Promising results then emerged from two open-label trials, which used direct intraputaminally infusion of recombinant human GDNF in PD patients (Gill et al., 2003; Slevin et al., 2005). But, subsequent placebo-controlled trial showed no significant motor improvements (Lang et al., 2006). Reasons for these discrepancies may lie in variations in patient selection, placebo effect, poor diffusion of GDNF throughout the caudate-putamen. To overcome this latter issue, ongoing clinical trials are using new delivery methods. A phase 1 clinical trial for advanced PD is being implemented using AAV-mediated delivery of GDNF using convection-enhanced delivery (CED) (Richardson et al., 2011) (NIH trial No. NCT01621581). A second clinical trial conducted by the Bristol group using a CED infusion protocol (UK CRN 12085) enrolled 42 patients and is now concluded. The study had an open-labelled extension for another 9 months after first results reported no significant difference in motor score compared to the placebo group at the first endpoint of 9 months (<http://medgenesis.com/news.htm>).

2.3.3.2 Transplant-based therapy

Neurorestoration also includes the repopulation of dopamine neurons using cell transplantation or through the simulation of endogenous neural progenitor cells. The idea is to inhibit disease progression by replacing damaged neurons by cells that would not only be able to restore DA levels, but that would also secrete neurotrophic factors and immunomodulatory factors and stimulate neuroplasticity (de Munter et al., 2014). Transplant approach has been tested in clinical trials since the 80's and produce so far modest results which are accompanied by severe side effect, notably violent dyskinesia related to ingestion of L Dopa. Grafts remain largely experimental, the major unmet questions remain: the source of graft (which cells should be used?) and which site should be targeted (the putamen, the substantia nigra or the subventricular zone?). Teams are currently involved in developing new kinds of implantable cells like mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) (Torres et al., 2017).

To date, PD treatments are **symptomatic** and are limited by the lack of long-term efficacy and apparition of adverse effects. Current treatments only counterbalance the loss of dopamine in the basal ganglia but do not halt neurodegeneration. The more the disease progresses, the harder it is to maintain this balance. **Neurorestorative therapies** aiming at maintaining, restoring, or replacing dopaminergic neurons showed promising outcome in pre-clinical models of PD but so far, the clinical benefits are inconsistent. Hence, there is a need for the identification of new targets or new neurorestorative strategies.

3 RATIONAL AND AIM OF THE STUDY

In Parkinson's disease, dopaminergic neurons of the substantia nigra are progressively lost and current therapies fail to stop or slow the neurodegenerative process. We reported, in the first section of this dissertation, netrin-1 neurorestorative potential for neuron repair and neurodegenerative diseases. **Thus, we asked whether netrin-1 could be a relevant candidate for SNc dopaminergic neuron restoration in PD.**

3.1 THE PAIR NETRIN-1/DCC AND SNc DOPAMINERGIC NEURON MAINTENANCE

3.1.1 Netrin-1 in the formation of the nigrostriatal pathway

As previously described (section 1.1.2.2, p22), in the developing brain, netrin-1 signalling regulates axon and neuron migration, axon branching and establishment of neuronal connections. This is particularly the case for dopaminergic neurons of the ventral midbrain.

In the developing midbrain, DCC is expressed by ventral midbrain dopaminergic neurons during their migration from the floor plate to their final destination territory, the marginal zone. (Brignani and Pasterkamp, 2017; Hegarty et al., 2013; Xu et al., 2010). Netrin-1 is expressed in the ventral midbrain, both in the floor plate, notably by radial-glia-like cells (RGL), and in TH⁺ nuclei in the marginal zone. Evidence from KO mice indicate that netrin-1/DCC signalling controls **the migration of post-mitotic midbrain dopaminergic neurons** since DCC-KO and Netrin1-KO mice show ectopically (dorsally) positioned midbrain dopaminergic neurons (Brignani and Pasterkamp, 2017; Li et al., 2014a; Xu et al., 2010). However, it is unclear yet how netrin-1/DCC signalling controls the migration of these neurons.

Subsequently, netrin-1 induces, via DCC, axon attraction (Li et al., 2014a), elongation (Zhang et al., 2013), and branching (Xu et al., 2010) of ventral midbrain dopaminergic neurons in their target region, the striatum and prefrontal cortex. Netrin-1 and DCC are indeed expressed in a complementary fashion in the ventral midbrain, and in dopaminergic neuron target regions such as the striatum (Livesey and Hunt, 1997; Shatzmiller et al., 2008). Interestingly, midbrain dopaminergic **axon attraction and elongation** are induced at lower netrin-1 concentrations in SNc explants as compared to VTA explants (Li et al., 2014a). Additionally, nigral dopaminergic axons do not respond to high netrin-1 levels (Li et al., 2014a). In netrin-1 KO mice, nigral dopaminergic axons fail to innervate the dorsal striatum and accumulate in the ventral striatum (Li et al., 2014) while in DCC null mutants, ventral striatal innervation is ectopically shifted in a more dorsal location whereas prefrontal cortex innervation is significantly reduced (Xu et al., 2010). Netrin-1 was also shown to promote nigral dopaminergic neuron **arborization** (Xu et al., 2010).

Altogether, these observations indicate that netrin-1 signalling plays a critical role in the formation of the nigrostriatal pathway directing nigral dopaminergic neurons and axons to proper target territories. **Adult SNc dopaminergic neurons may thus be still responsive to endogenous or exogenous netrin-1.**

3.1.2 Netrin-1 and DCC in the adult nigrostriatal pathway: an altered signalling in PD?

To determine whether netrin-1 could be a relevant neurorestorative candidate for SNc dopaminergic neurons degenerating in PD, we were interested to know if netrin-1 receptors were expressed by mature SNc dopaminergic neurons. From the literature, DCC was the netrin-1 receptor whose expression and localisation were the most reported and robustly characterised in the substantia nigra *pars compacta*.

Indeed, DCC was shown to be expressed in the adult ventral midbrain and to colocalise with dopaminergic neurons of the VTA and substantia nigra (Osborne et al., 2005; Reyes et al., 2013). More precisely, DCC is present both in the cell bodies and in striatal axon terminals of adult SNc dopaminergic neurons (Osborne et al., 2005; Volenec et al., 1998). Particularly, ventral tier dopaminergic neurons of the substantia nigra *pars compacta*, which are the most vulnerable to cell death in PD, are the subpopulation of midbrain dopaminergic neurons exhibiting the **highest level of DCC** (Osborne et al., 2005; Reyes et al., 2013). Whether DCC expression is causally linked with the selective vulnerability of these neurons in PD is not known. However, **Genome Wide Association Studies (GWAS) reported association between DCC single nucleotide polymorphisms and several aspects of PD**, such as age at onset, severity, and progression of the disease (Lesnick et al., 2008; Lin et al., 2009). Thus, DCC may be intrinsically linked with nigral neurons function or maintenance. Given DCC reported dependence receptor apoptotic activity, we hypothesised that DCC may be linked with SNc dopaminergic neuron selective vulnerability in PD, further reinforcing the idea to test netrin-1 effect in PD.

Interestingly, netrin-1 expression in the adult substantia nigra was shown to be **the highest** of the mature brain (Livesey and Hunt, 1997). The adult striatum though, shows moderate and graded expression of netrin-1; netrin-1 expression being restricted to a small population of neurons densely located in the ventral striatum and less present dorsally (the SNc dopaminergic neuron projection target) (Shatzmiller et al., 2008). Nevertheless, because netrin-1 immunostaining has proved difficult due to its secreted and extracellular matrix interacting nature, these observations were not formally demonstrated and further characterised at the protein level.

Beside its key role in the guidance of dopaminergic neurons of the substantia nigra in the developing brain, netrin-1 may continue to influence nigrostriatal function or structure in the adult brain. Therefore, in addition to **testing netrin-1 effect** on degenerating SNc dopaminergic neurons in PD, we were curious to **assess netrin-1 signalling status in PD**.

Given netrin-1 neurorestorative potential and the need to find disease-modifying therapies in PD, we sought **to investigate the effect of netrin-1 on SNc dopaminergic neuron degeneration in PD.**

These neurons still express DCC in the mature brain and are thus likely to respond to a netrin-1 treatment. Additionally, because the dependence receptor DCC was shown to be a marker of dopaminergic neurons which are the most susceptible to degeneration in PD and that polymorphisms in DCC gene are associated with PD, we were interested to **study the role of the pair netrin-1/DCC in SNc dopaminergic neuron survival** and test whether an aberrant dependence receptor mechanism may be at play in PD.

In this assumption, netrin-1 may favour neurorestoration not only by its positive signalling, promoting growth or maintenance of connections, but also as a survival factor blocking DCC induced-cell death.

In other words, two aspects will be explored:

- a **“therapeutic” aspect**, aiming at assessing netrin-1 neurorestorative properties in PD.
- a **“pathophysiologic” aspect**, aiming at exploring the role of the pair netrin-1 and DCC in SNc dopaminergic neuron survival and thus, PD pathogenesis.

4 EXPERIMENTAL STRATEGY

Parkinson's disease and neurorestoration are quite new research topics in Patrick Mehlen's laboratory, which has mainly been working on cancer and development. Hence, this project required to establish collaborations, to implement new tools and to carefully choose an appropriate experimental model, simple to implement and that could generate preliminary results relatively rapidly. Especially, we needed a PD model reproducing SNc dopaminergic neuron degeneration and where both cellular/morphological and functional/behavioural analyses could be done.

4.1 PD ANIMAL MODELS AND MODEL SELECTION TO STUDY NEURORESTORATION

Since no naturally-occurring animal forms of PD are known, the development of animal models of the disease has proved indispensable to study the pathology but also therapy for PD. The most extensively used and best characterised idiopathic animal models of PD are: mice and monkeys intoxicated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the 6-hydroxydopamine (6-OHDA) lesioned rats. Transgenic models have also been developed, based on genetic mutations observed in family forms of PD. Although every model has limits, it is about choosing the most appropriate one according to the aim of the study, here neurorestoration.

Suitable disease models should, as much as possible, be based on the aetiology of the disease, that is, causative genes or risk factors of human disease, and exhibit a significant degree of behavioural signs or anatomic pathology that parallels the human disease. As, by definition, models do not fully mimic human condition, they are evaluated according to two main criteria: **reliability** and **validity**. Reliability refers to the ability of the model to provide consistent results under different conditions. Validity comprises the ability to recapitulate disease initiating mechanisms (**construct validity**), to replicate the disease symptoms including anatomic or behavioural features (**face validity**) and to predict patient's response to treatments (**predictive validity**).

4.1.1 The rat 6-OHDA lesioned model of PD

6-OHDA is hydroxylated dopamine that is specifically captured by catecholamine transporters: dopamine transporter (DAT) and norepinephrine transporter (NET). Once in the cell, 6-OHDA auto-oxidizes and causes structural and functional alterations leading to the sensitization of the cell and eventually cell death. 6-OHDA is thus used to specifically induce degeneration of catecholamine neurones and was the first neurotoxic agent used to reproduce PD in animals (Ungerstedt, 1968). To favour degeneration of dopaminergic neurons over noradrenergic neurons, noradrenergic transport blockers can be used. Since it does not cross the blood-brain barrier, 6-OHDA has to be directly injected into the brain by stereotaxic surgery.

4.1.1.1 Dopaminergic neurons degeneration strategies

Different degrees of dopamine nigral neuron degeneration can be achieved depending on the 6-OHDA injection site in the nigrostriatal pathway. Injection in the substantia nigra and medium forebrain bundle (MFB) leads to a massive (>90%) and rapid cell death (within 24h) of dopaminergic neurons. Striatal injection induces a more progressive and moderate degeneration, starting at the axon terminals and gradually reaching the cell bodies in the substantia nigra *pars compacta* (Penttinen et al., 2016; Sauer and Oertel, 1994) and thus mimics PD better. This retrograde degeneration progresses generally over one to four weeks depending on the dose of 6-OHDA injected. Indeed, the extent of degeneration is dependent on the amount of 6-OHDA administered, the side of injection (Kirik et al., 1998) and also sensitivity between animal strain (or species). Another plus of the intrastriatal injection is the possibility to perform a topographic lesion of the nigrostriatal neurons. Usually the dorsolateral, sensorimotor, striatal region is targeted to achieve selective nerve loss comparable to what is observed in PD patients.

6-OHDA can be injected bilaterally (into both hemisphere) or unilaterally into the striatum (Figure 11). Bilateral lesions result in significant hypokinesia, postural abnormalities at rest and reduced capacity to maintain balance. However, animals also exhibit aphagic behaviour which requires force-feeding and increases mortality risk (Ungerstedt, 1968). Unilateral lesion has the advantage to provide an internal control of degeneration which is the contralateral (intact) side. Also, it does not impact animal well-being and mortality risk. The counterpart is that behaviour changes are subtle unless the lesion is severe. Typically, unilateral lesions cause postural asymmetry (torsion of the head), unilateral akinesia and contralateral sensorimotor deficits which are noticeable when the level of intrastriatal dopamine is decreased by at least 70% (Hefti et al., 1980). This sensorimotor asymmetry has been used to develop simple and objective tests for monitoring the effects of symptomatic or neurorestorative treatments.

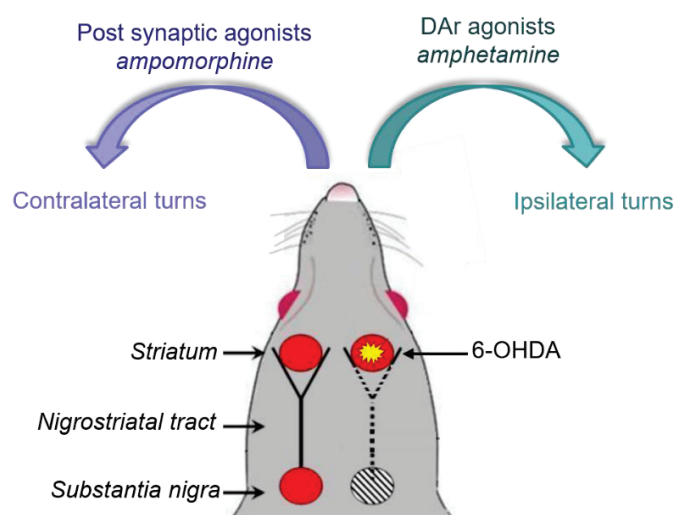


Figure from M.H.Voutilainen

Figure 11 The 6-OHDA-induced hemi-parkinsonian rat model

4.1.1.2 *Read-outs*

The unilateral and intrastriatal injection of 6-OHDA result in a dopaminergic imbalance between the two hemispheres which can be exacerbated with drugs acting on the dopamine system (Blandini et al., 2008) (Figure 11). Challenged with these drugs, animals exhibit an asymmetric rotational behaviour that is more or less pronounced depending on the extent of the nigrostriatal lesion (Ungerstedt and Arbuthnott, 1970). Apomorphine, a direct post-synaptic dopamine agonist induce rotation contralateral to the lesioned side due to the stimulation of dopaminergic receptor upregulated after 6-OHDA lesion (to compensate for dopamine loss). On the contrary, dopamine-releasing agents, such as amphetamine, reinforce the dopaminergic imbalance in favour of the non-lesioned side and hence produce a rotational behaviour on the ipsilateral side to the lesion. Amphetamine produce rotational behaviour after moderate lesion (>30-50% dopamine neuron loss) it is thus a more sensitive tool than apomorphine which induces animal rotation only when the loss of dopamine neurons is massive (>90% cell loss) (Dunnett and Lelos, 2010; Hudson et al., 1993; Kirik et al., 1998). Above these sensibility threshold, asymmetrical rotation correlates with the extent of the lesion. Thus, asymmetric circling behaviour can be used as a read-out to estimate the severity of degeneration, which can be a valuable advantage to assess anti-parkinsonian properties of tested drugs. There are also tests to evaluate more subtle motor changes, in absence of dopaminergic drugs, such as limb asymmetry (cylinder test) and forelimb placing test (Glajch et al., 2012).

4.1.1.3 *Mechanism of neurotoxicity*

The exact mechanism underlying 6-OHDA-induced neurodegeneration is not well defined but oxidative stress was invariably shown to participate to 6-OHDA-induced neurotoxic process. In the cell, 6-OHDA auto-oxidises and generates highly toxic reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, superoxide radicals and quinones (Dauer and Przedborski, 2003; Glinka et al., 1997). It has also been suggested that 6-OHDA would also have a deleterious role via inhibition of the mitochondrial respiratory chain complex I independently of ROS production (Blum et al., 2001; Glinka et al., 1996). All these processes are known to converge to the activation of programmed cell death. Along this line, 6-OHDA was shown to trigger apoptotic cell death but also necrotic cell death (Hanrott et al., 2006; Ochu et al., 1998; Woodgate et al., 1999). The site of injection (at axon terminals *vs* cell bodies) and the dose administrated may determine the type of cell death triggered in neurons (acute degeneration is more likely to induce necrosis while a more progressive degeneration, apoptosis).

4.1.2 **MPTP**

The MPTP was first shown to cause severe PD-like symptoms in drug addicts (cf section 2.1.2.2, p42). Since then MPTP intoxication has been used in many species including mice, cats, dogs, nematodes, and monkeys (Langston and Ballard, 1984; Przedborski et al., 2000). The sensitivity

of these species to MPTP varies. For example, rodents are less sensitive than primates and require about 30 times more MPTP than the amount used to intoxicate monkeys. Particularly, rats are insensitive to peripherally administered MPTP, this is why MPTP is usually administered directly into the striatum in rats (Schmidt and Ferger, 2001). The MPTP intoxication model is however more often used in mice and monkeys.

4.1.2.1 Mode of action

MPTP can cross the blood-brain barrier and is metabolized in astrocytes to its active metabolite MPP⁺ (cf section 2.1.2.2, p42) by MAO-B. MPP⁺ is selectively taken up into dopaminergic neurons via its affinity for DAT and is thus selective for dopamine neurons. Once in the cell, MPP⁺ blocks mitochondrial respiration by binding to mitochondrial enzyme complex I (Przedborski et al., 2000; Tipton and Singer, 1993) thus decreasing ATP cellular levels and eventually leading to cell death. But MPTP effect is probably not limited to mitochondrial stress (Rojas et al., 2000).

4.1.2.2 Degeneration pattern

MPTP damages the dopaminergic pathway in a topographical pattern similar to that seen in PD, including greater loss of neurons in the substantia nigra pars compacta than the VTA and preferential loss in the ventral tier (Varastet et al., 1994). Besides, alpha-synuclein aggregates are sometimes observed. But contrary to PD progression, this drug induces an acute and non-progressive degeneration of dopaminergic neurons. In contrast to human and non-human primates, dopamine terminals are destroyed only transiently in mice exposed to a single dose of MPTP and spontaneous recovery occurs weeks to months after lesioning (Date et al., 1993; Mitsumoto et al., 1998)

4.1.2.3 MPTP administration to non-human primate

Clinically, the MPTP intoxicated monkey exhibits almost all the cardinal symptoms of the disease, including rigidity, akinesia, bradykinesia, as well as postural disorders with a characteristic "arched" posture. Tremor is less consistent. Animals also develop non-motor symptoms such as cognitive symptoms (Schneider and Kovelowski, 1990; Schneider and Pope-Coleman, 1995) and sleep disorders that appear, as in Parkinson's patients, before the onset of motor symptoms (Barraud et al., 2009; Belaid et al., 2014) which make the MPTP-lesioned monkey a valuable pre-clinical model for PD.

4.1.3 Genetic models

Based on the identification of human gene mutations linked with PD, transgenic mice models have been created in which those genes are mutated or in which the functional consequence of human PD-linked mutations are recapitulated. Alpha-synuclein, LRRK2, DJ-1, Parkin and PINK1 models have thus been generated (Lee et al., 2012). Although these models are instructive for studying molecular mechanisms by which these mutations may cause PD and despite some subtle functional abnormalities in the nigrostriatal system and neurodegeneration in other anatomical circuits may be present, most of these models fail to reproduce nigral dopaminergic cell loss (or at best not before several (>10) months after generation) .

4.1.4 Which model for neurorestoration studies?

None of these preclinical models of PD fully recapitulates features of construct validity, face validity, and predictive validity together. Regarding face validity, administration of MPTP to non-human primates reproduces PD symptoms and neuropathological hallmark the most remarkably. Although not optimal, the rat intrastriatal 6-OHDA-lesioned model do replicate the selective and relatively progressive death of nigrostriatal neurons and show motor deficits. This model appears more relevant than MPTP rodent models that exhibit a massive and acute degeneration. However, in terms of construct validity these PD toxin-based models are rather “artificial”, forcing neurodegeneration. Indeed, it is not clear how well MPTP and 6-OHDA-induced toxicity corresponds to the neurodegeneration observed in sporadic PD. Regarding feasibility and ethical constraints, the rat 6-OHDA looked more appropriate than monkey MPTP model for **initial screening** for neurorestorative molecules. Genetic models, on the other hand, meet the best the construct validity but usually fail to reproduce neurodegeneration and/or motor symptoms. Besides, they are relatively expensive and time-consuming—and so may not be the most suitable models for neurorestoration pilot or proof of concept studies.

Ideally, any potential treatments should be tested in both toxin-based and alpha-synuclein models, until an animal model reuniting both PD hallmarks emerges. Nevertheless, **the rat 6-OHDA model** appears to be a useful and appropriate starting model to screen new molecules with neurorestorative potential. This model presents also high predictive validity since it has demonstrated efficacy of virtually all antiparkinsonian drugs currently used in clinic. We thus selected this model to test netrin-1 neurorestorative potential for Parkinson’s disease.

4.2 STUDY DESIGN USING LOW-DOSE PARTIAL 6-OHDA LESION IN RODENT

To test the potential neurorestorative effect of netrin-1 on dopaminergic neurons in PD, we implemented in Patrick Mehlen’s laboratory a new low-dose 6-OHDA rat model of PD as described in (Penttinen et al., 2016) with the help of Prof. Mart Saarma’s laboratory and Dr. Merja H Voutilainen (Biotechnology Institute of Helsinki, Finland).

This model consists in the unilateral injection of low dose 6-OHDA into three sites of the striatum (*cf.* material and methods chapter for coordinates and injected doses) and leads to a retrograde and progressive degeneration of nigral dopaminergic neurons that is stably maintained over time. Compared to single site injection, injection into three sites of the dorso-lateral striatum induces an extensive and reproducible degeneration of dopaminergic neurons with lower doses of 6-OHDA needed. When challenged with intraperitoneal (ip) injection of d-amphetamine (2,5mg/kg), this model exhibits a reliable and reproducible ipsilateral circling behaviour that correlates with the extent of the lesion. As assessed by histochemical analysis of TH-positive and DAT positive axons and TH-positive cell bodies, this model achieves an approximately 70% loss of dopaminergic axons in the striatum and 60 to 70 % dopaminergic neuronal loss in the substantia nigra, which resemble PD stage at diagnosis.

Because intrastrially injected 6-OHDA induces a retrograde degeneration progressing over two to four weeks, it provides a useful time window to test the effect of a given molecule on the neurodegeneration process. Intrastriatal 6-OHDA-induced degeneration progresses in a dynamic way as follows (Björklund et al., 1997):

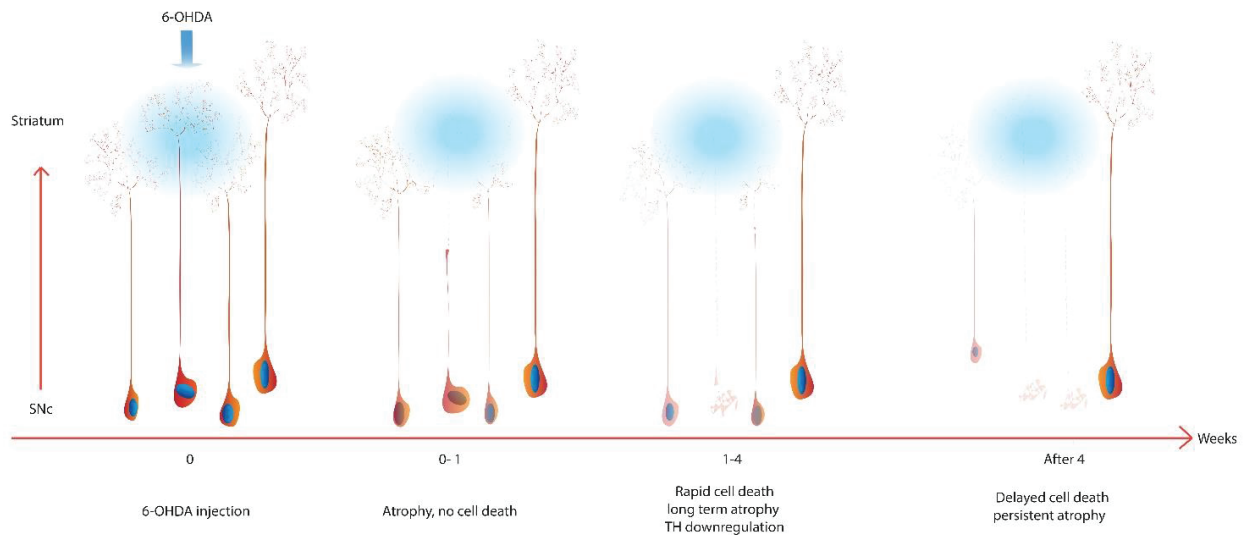


Figure 12 6-OHDA-induced degeneration over time (adapted from Björklund et al., 1997)

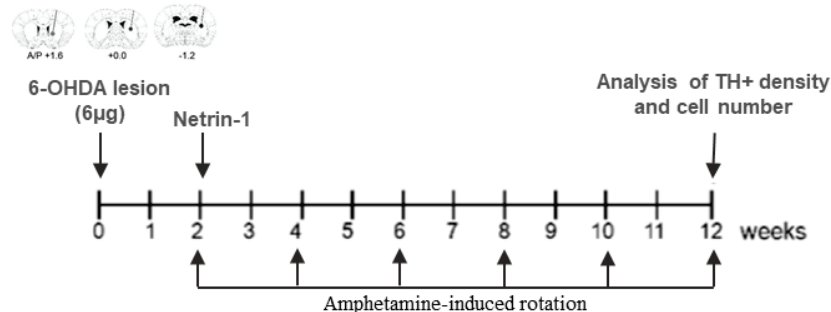
- Day 0 to day 7: 6-OHDA induces dopaminergic axon atrophy of sensitized neurons
- After 1 week: Atrophy progresses, most axons are shrinking, and cell bodies start to die
- After 2 weeks: Peak of cell death and persistent atrophy
- After 4 weeks: Persistent atrophy

Since we hypothesised that netrin-1 could prevent dopaminergic cell death and may promote regeneration, we decided to test netrin-1 effect, at a time point when cell death is ongoing and when cell bodies and damaged axons are still present to support regeneration. Additionally, this time point had to be clinically relevant and thus correspond, at the earliest, to the disease stage when PD is diagnosed. Two weeks after 6-OHDA injection thus seemed an appropriate time for netrin-1 treatment.

Regarding the injection site, we chose to inject netrin-1 into the same three striatal sites where 6-OHDA was injected so that it could act directly on degenerating neurons. As netrin-1 receptor DCC is present on nigral dopaminergic neuron axons and cell bodies, both the striatum and the substantia nigra represented possible injection sites for netrin-1 delivery. However, we assumed that injecting netrin-1 into the striatum, thus into dopaminergic neurons target site, might be more relevant than an intranigral injection given netrin-1 reported role as a guidance cue.

Finally, to assess the effect of netrin-1 on 6-OHDA-induced SNc dopaminergic neuron degeneration we performed amphetamine-induced ipsilateral rotation tests every two weeks for ten weeks and then sacrificed rats to performed immunohistochemical studies to evaluate the extent of

dopaminergic neuron loss. Vehicle (PBS) and GDNF were used, respectively, as negative, and positive control of neurorestoration.



We have also more generally addressed the role of netrin-1 signalling on dopaminergic neuron survival and PD progression, in part through a collaboration with Dr. Keqiang Ye laboratory (Emory University School of Medicine, United States). Given the preferential expression of DCC in ventral tier dopaminergic neurons and genetic studies implicating DCC in PD pathogenesis, an important question we wanted to address was the role of DCC in PD. Particularly, in the scope of the laboratory research activity, we were interested to test the implication of DCC pro-apoptotic signalling in PD.

To this end, we took advantage of a transgenic mouse line available in the laboratory in which DCC is point-mutated (D1290N) on its caspase cleavage site thus preventing its apoptotic activity (see methods). Hence, DCC D1290N mutated cells should be more resistant to cell death. In this model, we performed a neuroprotection study to check if DCC pro-apoptotic activity could be involved in dopaminergic neuron death in PD. DCC mutated ($DCC^{m/m}$) and wild type littermates (WT) were unilaterally and intrastrially lesioned with 6-OHDA. Then ipsilateral rotation tests were assessed two weeks and six weeks (one month) after lesion, then animals were sacrificed, and brain removed for nigral dopaminergic neurons quantification analysis to compare the two groups.

However, prior to that, it was important to determine whether netrin-1 or DCC levels were modulated in PD pathogenesis or progression (and in our PD animal model), toward a low ligand availability setting, to assess whether a dependence receptor-induced cell death may be at play in the disease. Results and methods are presented in the following chapter.

5 RESULTS

The results obtained during my thesis are part of a collaborative project with the laboratories of Mart Saarma and Merja Voutilainen (Institute of Biotechnology, Helsinki, Finland) and Keqiang Ye's laboratory (Emory University School of Medicine, Atlanta, USA). An article is in preparation and the results will be presented accordingly. The missing results are expected by the end of December.

Authors contribution

Patrick Mehlen and Keqiang Ye: Developed rationale and designed experiments related to the characterisation of the role of netrin-1 and its dependence receptor DCC on dopaminergic neurons maintenance (Figure 1, 2, 3 and 4).

- Experiments related to the characterisation of netrin-1 levels in patient brain lysates (Figure 1f) and to netrin-1 conditional knock-out in *netrin-1^{fl/fl}* mice using Cre virus (Figures 2a-f and 3a-f, and supplementary figures 1a-c and 2a-b) were performed and analysed by Keqiang Ye's team (Ahn Eun Hee, Seong Su Kang and Keqiang Ye).
- Experiments related to the characterisation of netrin-1 status in the nigrostriatal pathway in physiological conditions and in PD (Figure 1a-e), and DCC1290N neuroprotective experiments (Figure 4) were performed, and analysed by Patrick Mehlen's team (Mélissa Jasmin, Joanna Fombonne, Catherine Guix).

Patrick Mehlen, Merja Voutilainen and Mart Saarma: Developed rationale and designed "neurorestoration" experiments (Figure 5 and supplementary figures 3,4).

- Experiments testing netrin-1 neurorestorative properties in mice and rats (Figure 5a-i) and netrin-1 effects on primary dopaminergic neurons were performed and analysed by Patrick Mehlen's team (Mélissa Jasmin, Joanna Fombonne, Catherine Guix).
- Netrin-1 distribution experiments (Supplementary figure 3a-b) was performed and analysed by Mart Saarma and Merja Voutilainen teams.

Mélissa Jasmin: Contributed to develop rationale, design experiments (Figure 1a-e, Figure 4a-e, Figure 5a-i and supplementary figures 3 and 4). Performed and analysed experiments (Figure 1a-e, Figure 4a-e, Figure 5a-i, supplementary figure 3c and figure 4a-c) and prepared corresponding figures. Wrote the entire manuscript and organised all figures (revised by Patrick Mehlen and Joanna Fombonne).

The pair netrin-1/DCC regulates dopaminergic neuron maintenance and impacts on Parkinson's disease

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Conflict of Interests

The authors have declared that no conflict of interest exists.

Abstract

The secreted protein netrin-1 has diverse functions among which guidance and survival of neurons when bound to its dependence receptor DCC. In the absence of netrin-1, DCC is cleaved and actively triggers cell death in various contexts. DCC is highly present in a subset of mature neurons that typically degenerates in Parkinson's disease, dopaminergic neurons of the substantia nigra. Besides, polymorphisms of DCC gene are associated with the disease. Thus, netrin-1 signalling might be implicated in Parkinson's disease pathogenesis. Here we show, in samples from Parkinson's disease patient brains, a reduction of netrin-1 levels associated with an increase of DCC cleavage. Specific deletion of netrin-1 in the substantia nigra induces DCC cleavage and leads to the loss of dopamine neurons and motor deficits in mice while both injection or overexpression of netrin-1 restores dopamine neurons and improves motor behaviour in rodent models of the disease. These results highlight the therapeutic potential of targeting netrin-1 signalling in Parkinson's disease.

Parkinson's disease (PD) is an age-related neurodegenerative disease characterised by the preferential degeneration of dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc) leading to debilitating motor symptoms. Indeed, these neurons are involved in the control of motricity supplying the dorsal striatum with dopamine through long axonal projections forming the nigrostriatal pathway. Understanding what makes these neurons particularly vulnerable to degeneration may open the way to the identification of new targets for the treatment of the disease. Following this approach, studies in mice and human reported that netrin-1 receptor deleted in colorectal cancer (DCC) was highly expressed in nigral neurons which are more vulnerable to degeneration^{1,2}. Moreover, genetic studies showed that single nucleotide polymorphisms found in the DCC gene are associated with the susceptibility to develop PD^{3,4}. These findings led us to investigate whether DCC and its ligand netrin-1 may influence the development and progression of PD.

Netrin-1, initially identified as an axon guidance molecule^{5,6}, is now emerging as a multifunctional secreted molecule implicated both during tissue patterning and adult pathologies⁷. In the developing and adult central nervous system, netrin-1 regulates various biological processes, including axonal growth^{8,9}, synaptic plasticity^{10,11} and inflammation^{12,13} which are of relevance for neurodegeneration. Importantly in this scope, netrin-1 main receptors, DCC and UNC-5B, belong to the class of dependence receptors that is a functional family of receptors shown to actively trigger cell death in settings of poor ligand availability^{14,15}. Hence, these receptors exhibit a dual signalling depending on the cellular context. Typically, when unbound to their ligands, dependence receptors undergo proteolysis which in turn activates cell death mainly by apoptosis whereas binding to their ligands induces a "positive" signalling ensuring cell survival but also the activation of the ligand's canonical pathway (proliferation, migration, differentiation for instance)¹⁶. Along this line, netrin-1 was shown to act as a neuronal survival cue in various contexts^{17,18}. Given DCC expression in adult dopaminergic nigral neurons and its reported role in cell death as a dependence receptor, we sought to characterise netrin-1 expression in adult nigral neurons, in physiological conditions and in PD, to next determine if targeting netrin-1 levels could modulate neurodegeneration.

Results

Netrin-1 is expressed in the substantia nigra and is decreased in PD

To explore the role of netrin-1 in PD, we interrogated netrin-1 status in the adult nigrostriatal pathway in control conditions and in the pathology. First, we looked at netrin-1 expression profile in different human brain structures using the Allen Human Brain Atlas data bank. Netrin-1 expression in the adult brain was globally low except in some structures of the brainstem. Notably, the substantia nigra was one of the structures exhibiting the highest expression levels of netrin-1 (Fig.1a), suggesting that netrin-1 may play a role in the maintenance or function of this structure in the adult brain. Using a gene reporter strategy, we found that netrin-1 expression in the mouse brain was, like in the human brain, particularly strong in the substantia nigra (Fig1.b) as previously reported¹⁹. Since netrin-1 is a secreted protein, its protein localisation may differ from its expression pattern. We thus sought to look at netrin-1 protein localisation in the nigrostriatal pathway. We showed that netrin-1 was present in the substantia nigra and largely colocalised with dopaminergic (TH positive) ventral tier neurons which are the most vulnerable to degeneration²⁰ reinforcing the idea that netrin-1 may support the function or survival of these neurons (Fig.1c). We then asked whether netrin-1 could be secreted along dopaminergic projections (DAT staining) in the striatum. Yet, netrin-1 staining did not colocalised with DAT diffuse immunoreactivity (Fig.1d) but was restricted to a small population of netrin-1-expressing cells mainly located ventrally and laterally in the striatum, presumably interneurons²¹ (Fig.1d).

Then we addressed the status of netrin-1 in PD. Interrogating different databases, we found that netrin-1 gene expression levels in the substantia nigra were significantly decreased in PD patients (Fig.1e). Netrin-1 reduction in PD was confirmed at the protein level in nigral samples from PD patients and age-matched control patients (Fig.1f). Interestingly, we showed that several PD models also recapitulated the loss of netrin-1 (Supplementary Fig. 1 a-d). Particularly, in human alpha-synuclein (SNCA) transgenic mice, netrin-1 levels gradually decreased whereas DCC levels progressively increased in an age-dependent manner (Supplementary Fig. 1b-c). The loss of netrin-1 was generally accompanied by increased DCC and UNC-5B levels and cleavage (Fig.1f). Although netrin-1 reduction might be a consequence of the loss of netrin-1-expressing cells in PD, the concomitant increase of netrin-1 receptors cleavage may indicate that an altered netrin-1 signalling might be at play during PD.

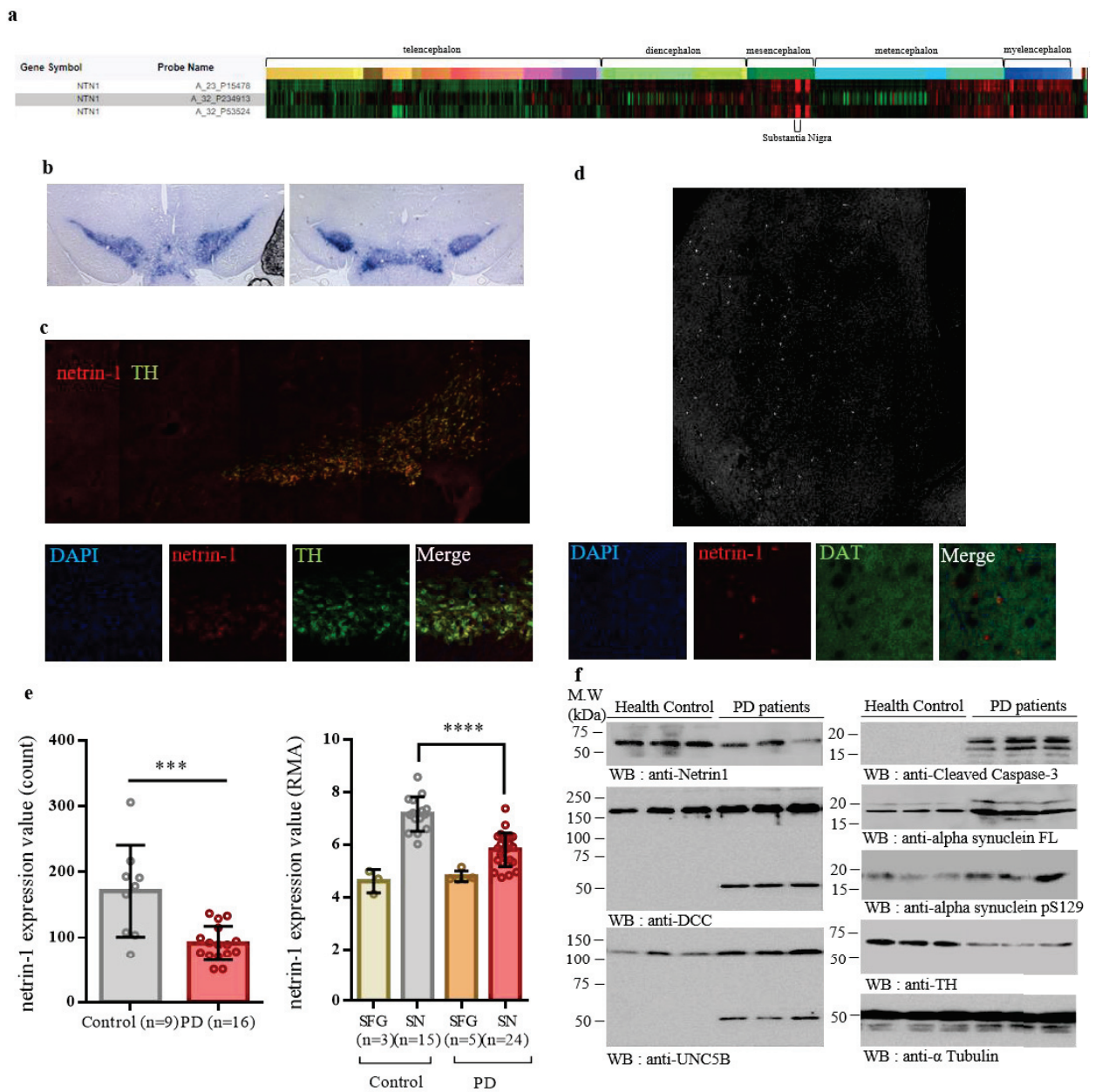


Figure 1 : Netrin-1 is highly expressed in the substantia nigra and is decreased in PD. **a**, NTN1 gene expression profiling from 6 adult control brains using microarray data from the Allen Human Brain Atlas. **b**, Reporter gene expression in the substantia nigra of netrin-1 +/- lacZ+ adult (P60) mice incubated with S-Gal. **c-d**, Netrin-1 detection by immunofluorescence in the substantia nigra pars (c) and in the striatum (d) of adult rat. **e**, Netrin-1 gene expression profiling by array of PD substantiae nigrae from datasets GDS2821 (left) and GDS3129 (right). Unpaired t-test, *** $p < 0.0005$; **** $p < 0.0001$, Means + SD are shown. **f**, Immunoblot of Netrin-1, DCC, UNC5B, active caspase-3, alpha synuclein FL, alpha synuclein pS129 and Tyrosine Hydroxylase levels in Parkinson's disease patients brain tissue samples compared to age-matched health control groups.

Netrin-1 loss induces nigral dopaminergic cell death

To determine whether netrin-1 reduction may effectively contribute to PD, we tested the effect of netrin-1 silencing on the survival of dopaminergic neurons of the substantia nigra. Netrin-1 depletion in the substantia nigra was achieved using adult netrin-1^{fl/fl} mice with the combined unilateral injection of Cre-virus (2 μ g) into the ventral midbrain and was validated by western blot analysis (Fig 2.f). Nigral dopaminergic neurons population and mice motor behaviour were monitored six weeks after netrin-1 conditional deletion. Loss of TH positive phenotype, in nigral cell bodies (Fig.2a-b) and in striatal fibres (Fig.2c), coupled with a massive increase of TUNEL reactivity in the substantia nigra (Fig.2d) and caspase-3 cleavage (Fig 2.f) demonstrated that the conditional depletion of netrin-1 (Cre-virus) elicited the death of dopaminergic neurons. Consistently, netrin-1 reduction eventually led to motor impairment as evaluated by rotarod, cylinder and grid motor behaviour tests (Fig.2e). Moreover, because SN dopaminergic neuron degeneration in PD is characterised by the accumulation of alpha-synuclein aggregates, we looked at the consequence of netrin-1 depletion on serine 129 (S129) phosphorylation of alpha-synuclein, which is thought to lead to toxic insoluble aggregates formation²². Immunoblot analyses demonstrated that conditional netrin-1^{fl/fl} mice exhibited increased alpha-synuclein S129-phosphorylation levels (Fig2.f). Since the loss of dopaminergic neurons and alpha-synuclein aggregation are the key features of PD, altogether these results suggest that decreased netrin-1 levels may participate to PD pathogenesis or may worsen its progression. Also, it is interesting to note that, as observed in PD patients (Fig1.f), netrin-1 silencing led to an increase in DCC and UNC-5 receptors levels (Fig2.f and Supp Fig2.a) and cleavage (Fig2.f).

Unbound DCC triggers dopaminergic cell death

To investigate whether netrin-1 dependence receptors could actively mediate dopaminergic cell loss in the absence of netrin-1, we co-injected Cre virus with virus expressing DCC shRNA or UNC-5B shRNA into the ventral midbrain of netrin^{fl/fl} mice. Immunofluorescence analysis demonstrated that DCC silencing in the substantia nigra partially protected dopaminergic neurons in the absence of netrin-1 (Fig.3a-d). By contrast, UNC-5B silencing did not prevent dopaminergic cell death (Fig3.a-d). Same observations were made from SN lysates: DCC silencing, and at a much lesser extent UNC-5B, prevented the loss of TH levels and strongly suppressed caspase-3 activation (Fig.3e, left panels) indicating that DCC, at least in part, mediates apoptotic dopaminergic cell death in the absence of netrin-1. Motor behaviour tests demonstrated that suppressing either DCC or UNC-5B attenuated motor deficits induced by netrin-1 conditional depletion in the SN (Fig.3f). However, DCC depletion exhibited more prominent protective effect compared to UNC-5B. An explanation might be that, unlike DCC, UNC-5B is only poorly expressed in the substantia nigra therefore, changes in UNC-5B levels have less impact on nigral neurons compared to DCC.

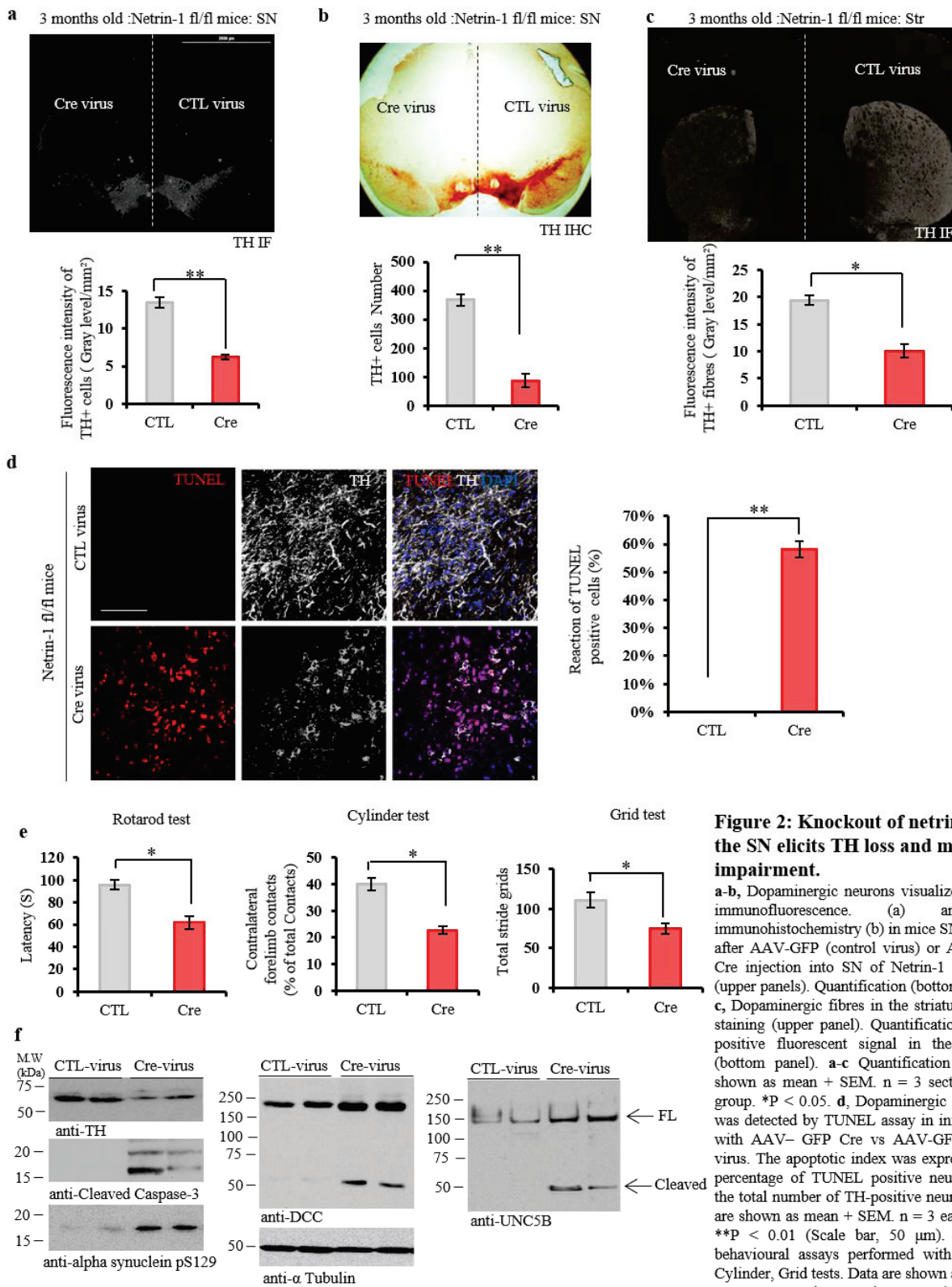
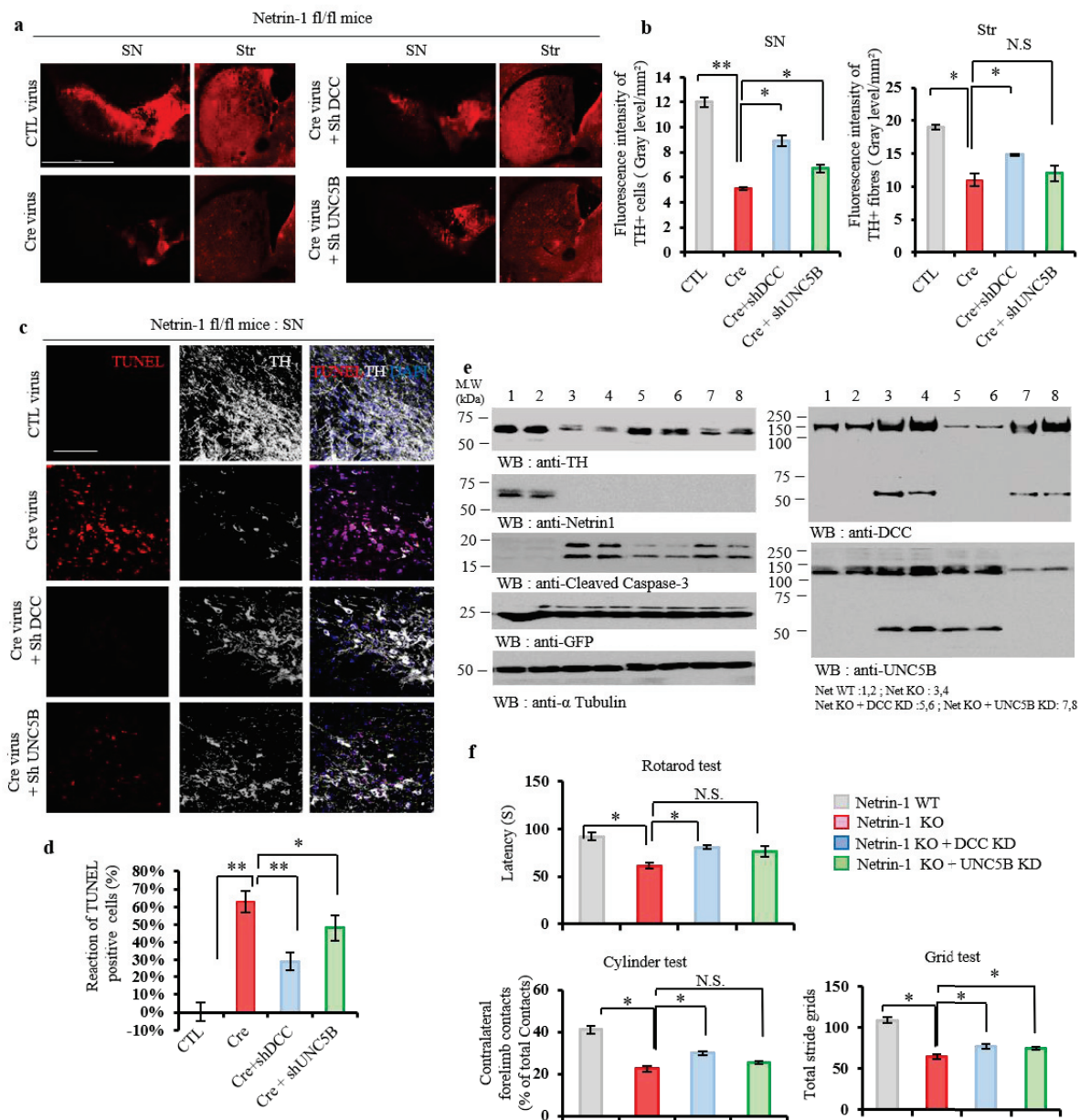


Figure 2: Knockout of netrin-1 in the SN elicits TH loss and motor impairment.

a-b, Dopaminergic neurons visualized by TH immunofluorescence. (a) and (b) by immunohistochemistry (b) in mice SN 6 weeks after AAV-GFP (control virus) or AAV-GFP Cre injection into SN of Netrin-1 fl/fl mice (upper panels). Quantification (bottom panels). c, Dopaminergic fibres in the striatum by TH staining (upper panel). Quantification of TH-positive fluorescent signal in the striatum (bottom panel). a-c Quantification data are shown as mean + SEM. n = 3 sections each group. *P < 0.05. d, Dopaminergic cell death was detected by TUNEL assay in infected SN with AAV-GFP Cre vs AAV-GFP control virus. The apoptotic index was expressed as a percentage of TUNEL positive neurons over the total number of TH-positive neurons. Data are shown as mean + SEM. n = 3 each group. ***P < 0.01 (Scale bar, 50 µm). e, Motor behavioural assays performed with Rotarod, Cylinder, Grid tests. Data are shown as mean + SEM. n = 8 each group. *P < 0.05, **P < 0.01. f, Immunoblot using the indicated antibodies was performed on SN protein lysates.



To assess whether DCC cleavage could contribute to dopaminergic neuron loss, we next used a mice model in which DCC pro-apoptotic activity is silenced via a point mutation in its caspase cleavage site^{23,24}. In this mice model, DCC is functional for its positive signalling but is not able to trigger cell death when unbound by netrin-1. We elicited dopaminergic neurons degeneration upon 6-OHDA intrastratial and unilateral injection and monitored mice asymmetrical ipsilateral rotation in response to amphetamine 2 weeks and 6 weeks after 6-OHDA lesion to follow dopaminergic degeneration in D1290N mutant mice ($DCC^{m/m}$) compared to WT littermates (Fig.4a). We observed that ipsilateral rotational behaviour was significantly reduced in $DCC^{m/m}$ mice compared to control mice 2 weeks after 6-OHDA lesion. However, at 6 weeks, the ipsilateral rotational behaviour of $DCC^{m/m}$ mice was not significantly different from rotational behaviour of control mice suggesting that preventing DCC cleavage by caspases delayed dopaminergic neurons degeneration in $DCC^{m/m}$ mice compared to WT mice (Fig4.b). (Immunohistochemical analyses are ongoing and may confirm motor behaviour observations) (Fig4c-d). DCC cleavage may sensitize dopaminergic neurons to degeneration.

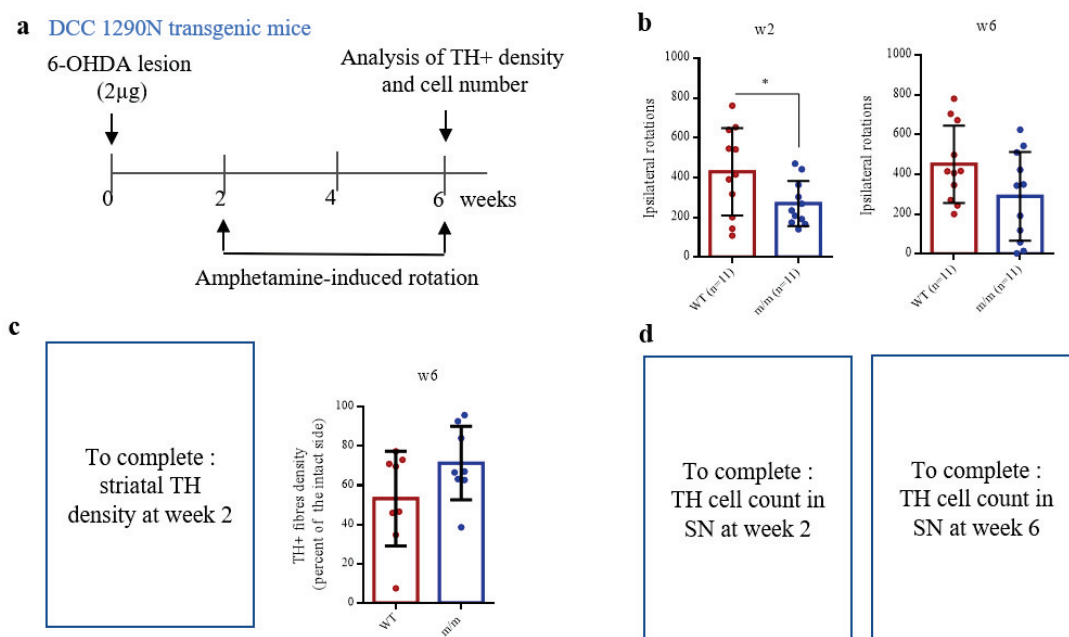


Figure 4: Preventing the cleavage of DCC by caspases is neuroprotective in a mouse 6-OHDA model of PD. **a**, Experimental design. Homozygous *DCC* wild type (WT) or *DCC* mutant ($DCC^{m/m}$) mice were lesioned with 6-OHDA in two sites of the right striatum ($2 \times 1 \mu\text{g}$). Amphetamine-induced behaviour was assessed at week 2 and week 6 post lesion. At week 6 brains were taken to perform immunohistological analyses. **b**, Amphetamine-induced rotations at two weeks and 6 weeks after lesion. Data are shown as individual values and mean + SD. $n = 10-11$. Tukey post hoc analysis after two-way ANOVA, $*p < 0.05$ compared to vehicle group. **c**, Density of TH-positive fibres in the striatum. Individual values and mean + SD are shown, $n = 8$ in each group. Unpaired t-test. **d**, TH-positive cell bodies count in the SN.

Netrin-1 is neurorestorative in 6-OHDA models of PD

Then we assessed the effect of netrin-1 overexpression on dopaminergic neurons degeneration in netrin-1 inducible transgenic mice injected with 6-OHDA as mice model of PD. CAG:Cre;Rosa26-LSL-Netrin-1 mice received intra-peritoneal (i-p) injection of tamoxifen to induce netrin-1 overexpression (O/E) two weeks after 6-OHDA intrastriatal injection. To monitor the effect of netrin-1 induction on the progression of the lesion, ipsilateral rotation assays were performed right before and 1 month after tamoxifen injection (Fig5.a). We found that ipsilateral rotational behaviour was markedly reduced following netrin-1 induction (Fig5.b). Immunohistochemical analyses showed that TH-positive striatal fibres density was significantly higher in the netrin-1 overexpressing (O/E) group than in netrin-1 WT littermates and (results in SN to come) (Fig5.c-d).

Finally, we explored the therapeutic potential of exogenous netrin-1 administration using the 6-OHDA partial lesion rat model of PD described in Penttinen et al 2016²⁵. Rats were lesioned with 6-OHDA (6µg) distributed evenly in three sites of the striatum. Two weeks after, rats received intrastriatal infusion of human recombinant netrin-1 (10µg), or GDNF as a positive control (10µg), or vehicle (PBS 1X) into those same three sites. The effect of netrin-1 treatment on the progression of the lesion was monitored measuring ipsilateral rotational behaviour every two weeks for ten weeks in response to amphetamine (Fig5.e). Whereas the ipsilateral rotational behaviour of vehicle group significantly increased over time, rats treated with netrin-1 exhibited a stable rotational behaviour (Fig5.f) that was significantly lower than rats treated with vehicle (Fig5.g). Immunohistochemical analyses showed that TH-positive striatal fibres density was significantly higher in the netrin-1 treated group and GDNF treated group than in the vehicle treated group (Fig5.h) three months after 6-OHDA lesion. The number of TH-positive cells in the substantia nigra was higher, although not significant, in the netrin-1 group compared with vehicle group (Fig5.i). Globally, GDNF treatment showed stronger effect than netrin-1 treatment when compared to vehicle group. To better characterise netrin-1 neurorestorative action, we performed transportation and distribution experiments using iodinated netrin-1 (125I-netrin-1) and GDNF (125I-GDNF). We observed, 24h after injection, that netrin-1 was not actively transported to the substantia nigra (Supplementary Fig3.a) contrary to GDNF but was detectable in different regions of the striatum and in frontal brain region including the cortex. We also checked netrin-1 distribution by immunofluorescence 24h after intrastriatal injection of his-tagged netrin-1 (Supplementary Fig3.c) and found no signal in the substantia nigra, consistent with 125I-netrin-1 transportation and distribution experiments, but neither in the frontal part of the brain. His staining was restricted to the striatum (Supplementary Fig3.c). Given the different distribution profiles of netrin-1 and GDNF, these results suggest that netrin-1 might have a different mode of action than GDNF and may act locally on dopaminergic axon terminals or other targets. Using primary culture of mouse ventral midbrain primary neurons, we showed that netrin-1 was not only able to promote dopaminergic neurons survival but also neurites outgrowth as respectively indicated by increased proportion of dopaminergic cells in culture (Supplementary Fig4.a) and increased TH surface area

(Supplementary Fig4.b). Thus netrin-1 neurorestorative effect might result from a direct effect on dopaminergic neurons survival and, possibly, growth.

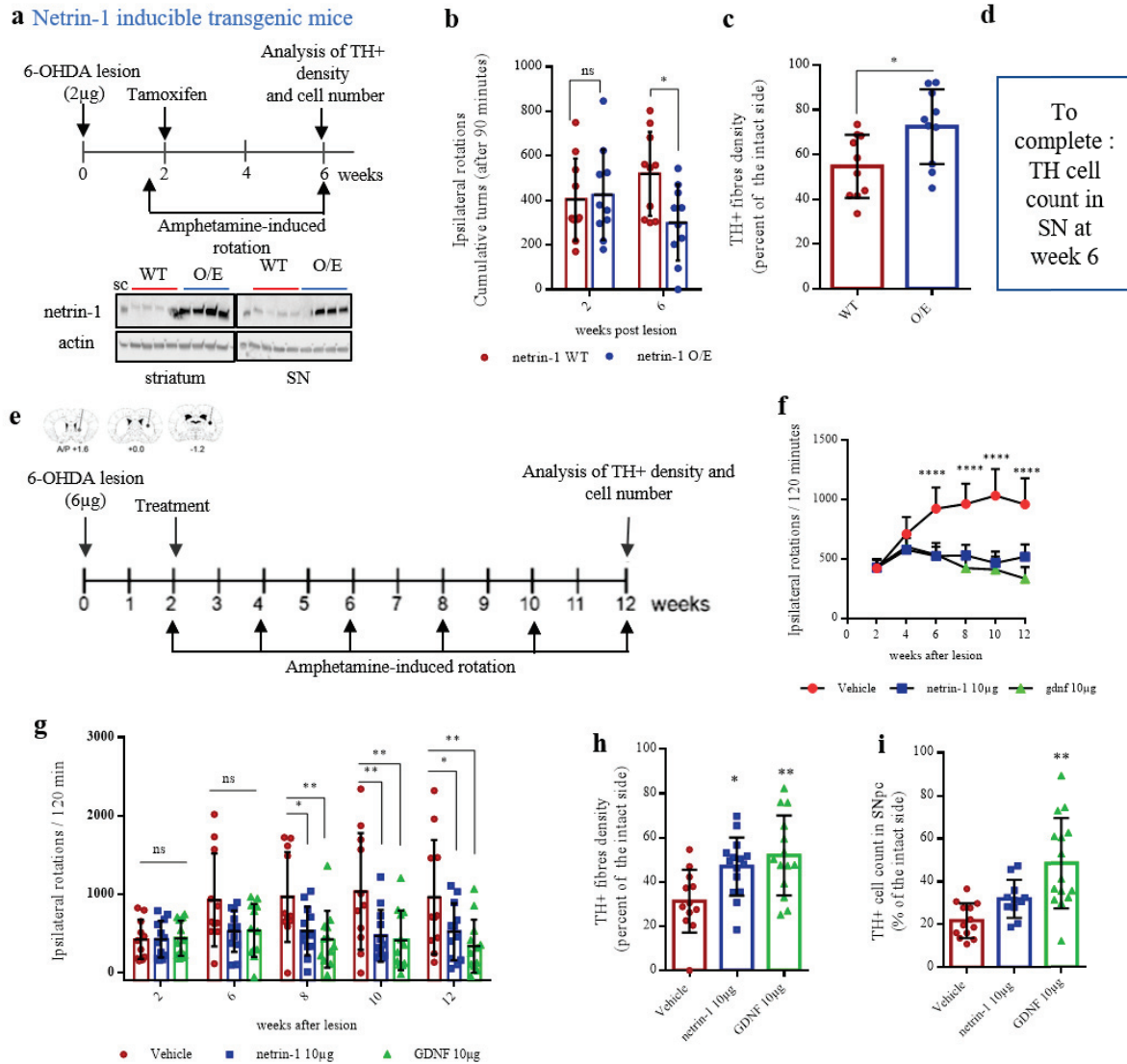


Figure 5 : Netrin-1 is neurorestorative in the 6-OHDA model of PD.

a, Experimental design. Netrin-1 inducible mice were lesioned with 6-OHDA in two sites of the right striatum (2 \times 1 μ g). After two weeks, mice received i-p injections of tamoxifen to induce netrin-1 expression. Amphetamine-induced behaviour was assessed at week 2 and week 6 post lesion then brains were taken to perform IHC analyses. Validation of netrin-1 overexpression (O/E) by western blot. Spinal cord (SC) lysates from E11.5 mice embryo were used as a positive control. **b**, Amphetamine-induced rotations at 2 and 6 weeks after lesion. Individual values and Mean + SD are shown, n=10 in each group. Tukey post hoc analysis after two-way RM ANOVA, *p < 0.05 compared to WT **c**, Density of TH-positive fibres in the striatum. **d**, TH-positive cell bodies in the SN. **c,d** Individual values and mean+ SD, n= 10, unpaired t test. **e**, Experimental design. **f**, Cumulative amphetamine-induced ipsilateral rotations. Means + SEM are shown, n=11–12 in each group. Tukey post hoc analysis after two-way RM ANOVA, ****p < 0.0001 compared to the initial rotation score at week 2. **g**, Cumulative amphetamine-induced ipsilateral rotations. Individual values and means + SD are shown, n=11–12 in each group. Tukey post hoc analysis after two-way RM ANOVA, *p < 0.05, **p < 0.01 compared to vehicle group. **h**, Density of TH-positive fibres in the striatum. **i**, TH-positive cell bodies in the SN. **h,i** Individual values and Means + SD are shown, n > 10 in each group. Tukey post hoc analysis after one-way ANOVA, *p < 0.05, **p < 0.01 compared to vehicle group.

Discussion

Toward developing netrin-1 mimetics in PD?

Our results indicate that netrin-1 plays a key role in the maintenance of SN dopaminergic neurons. In the adult brain, netrin-1 is mainly expressed in the brainstem and notably in the substantia nigra. These structures are predominantly affected by Lewy pathology and degeneration in PD²⁶. Specially, SNc DA neurons are specifically susceptible to degeneration. Targeting features underlying DA neurons selective vulnerability might represent a therapeutic approach to slow or halt degeneration. Studies reported, both in human and mouse midbrain, that ventral SNc DA neurons exhibited a stronger signal for DCC than other midbrain DA neurons (from dorsal SNc and the VTA)^{1,2}, suggesting that DCC protein level may be a marker for DA neurons that are most vulnerable to degeneration. However, it was unclear whether the expression pattern of DCC was causally linked with the selective DA neuron vulnerability and how DCC may modulate the susceptibility of DA neurons to degeneration. We showed that removing endogenous netrin-1 in the adult substantia nigra induced dopaminergic cell loss indicating that endogenous netrin-1 plays a key role for the maintenance of these neurons. Simultaneous deletion of DCC partly prevented dopaminergic cell loss. Thus, netrin-1 receptor DCC might be a double-edged sword for SNc DA neurons depending on netrin-1 availability: promoting survival in healthy conditions and cell death in conditions of low trophic supports. As SNc DA neurons are thought to be prone to oxidative stress and inflammation due to their unique morphology and pacemaker activity²⁶, DCC dual signalling could be a safeguard mechanism eliminating damaged or “diseased” neurons protecting healthy neurons from additional harm. Further studies on the role of endogenous netrin-1 on adult SNc DA neurons and how netrin-1 is lost in PD might bring new understandings on SNc DA neurons selective vulnerability and PD pathogenesis.

Beside dopaminergic neuron loss, the other key hallmark of PD is Lewy pathology. Although we centred our study on the first hallmark, we also showed that netrin-1 silencing was associated with increased S129-alpha synuclein phosphorylation. Moreover, we observed in SNCA transgenic mice, a decrease of netrin-1 expression and protein levels in an age-dependent manner in the adult substantia nigra. Hence, future studies addressing the effect of netrin-1 levels on alpha-synuclein aggregates, might provide additional insights into the therapeutic potential of netrin-1.

Even though further preclinical work is required, the present work shed light on the therapeutic relevance of targeting netrin-1 signalling in PD. We demonstrated *in vivo*, using netrin-1 conditional knockout mice, that netrin-1 reduction could trigger dopaminergic neurons demise and motor deficits, in part through DCC-induced cell death, and thus that its loss could contribute to PD development or progression. Inversely, both the administration of netrin-1 recombinant protein in 6-OHDA-lesioned rats and the inducible overexpression of netrin-1 in 6-OHDA-lesioned transgenic mice, showed significant neurorestorative effect on mature dopaminergic neurons. Thus, netrin-1 mimetics might represent a promising disease-modifying treatment for PD.

Material and Methods

Key resources Table

Used in experiments in figures : Fig.1c,d Fig.4 and Fig.5 and supplemental figure 3 and 4:

Antibodies	Dilution ratio	Source	Manufacturers	Reference
TH	1:500	Chicken	Abcam	ab76442
DAT	1:500	Rabbit	Abcam	ab184451
HIS-tag	1:500	Mouse	Abcam	ab18184
Netrin-1	1:500	Rabbit	Abcam	ab126729

Used in experiments in figures : Fig.1f, Fig.2 and Fig.3 and supplemental figure 1c and 2:

Antibodies	Dilution ratio	Source	Manufacturers	Reference
α -Synuclein FL	1:1000	Mouse	Santa Cruz	SC-9977
α -Synuclein-pS129	1:1000	Rabbit	LS Bio	LS-C380861-1
TH	1:750	Mouse	Santa Cruz	SC-25269
TH	1:750	Rabbit	Abcam	ab112
DCC	1:500	Mouse	Santa Cruz	SC-515834
Netrin-1	1:1000	Rabbit	Santa Cruz	SC-20786
UNC5B	1:500	Mouse	Santa Cruz	SC-5570
Cleaved caspase-3	1:500	Rabbit	Cell signaling	#9661

Human tissue samples

Post-mortem brain samples were dissected from frozen brains of PD patients and aged-match non-demented controls from the Emory Alzheimer's Disease Research Center. The study was approved by the Biospecimen Committee. PD was diagnosed according to the criteria of the Consortium to

Establish a Registry for PD and the National Institute on Aging. Informed consent was obtained from all cases.

Animals

Experimental procedures involving animals were approved by the Committee for Animal Experiments of the Rhône-Alpes Region (CE015), France (authorization n°: APAFIS#1565), the Committee for Animal Experiments of the University of Helsinki and the chief veterinarian of the County Administrative Board.

All animals were housed in filter-topped cages under a 12h light/dark cycle at an ambient temperature of 22°C. Females and males were kept separately. Tap water and chows were available ad libitum.

Rats

Six-week-old male Wistar rats were purchased from Janvier Labs (Genest Saint-Isle, France). After one-week acclimatization followed by a one-week habituation period, rats (two-month-old, 250g body weight) were lesioned with 6-OHDA for neurorestorative experiments.

Transgenic models

DCC D1290N mutant mice

To test whether DCC pro-apoptotic activity may influence dopaminergic neuron survival we took advantage of a mouse line available in the laboratory. These mice were generated as described previously²⁴. In this model, netrin-1 receptor DCC is point mutated at the caspase cleavage site D1290 to prevent the cleavage of DCC by caspases, thus blocking DCC pro-apoptotic activity. This mutation does not impair netrin-1/DCC positive signalling as assessed by phospho-ERK and neurite outgrowth experiments. This mouse line is viable, fertile and do not exhibit obvious central nervous system anatomic defects. However, DCC-D1290N homozygous mice (DCC^{m/m}) present a 10% increase of olivary neurons but no alteration of the inferior olivary structure. Although behavioural analysis did not demonstrate major behavioural defects, females tend to show decreased locomotor activity (not published). We thus chose, to exclude DCC^{m/m} females from the study.

Two months old (25-30g body weight) male mutant mice (DCC^{m/m}) and control wild-type littermates (WT) were used for the experiments.

Inducible netrin-1 transgenic mice CAG:Cre;Rosa26-LSL-Netrin-1

Our laboratory generated a netrin-1 inducible transgenic line (tgNetrin-1) containing the human NTN1 cDNA (huNetrin-1) preceded by a lox-stop-lox cassette (LSL) inserted in the Rosa locus as described in²⁷. This mouse line is viable and fertile. When tgNetrin-1 mice are bred with CAG:Cre-ERT2 mice²⁸, tamoxifen-inducible Cre-mediated recombination can be activated in the offspring resulting in the deletion of the stop sequence and thus ubiquitous netrin-1 overexpression.

Therefore, CAG:CRE;Rosa26-LSL-huNetrin-1^{+/+} (referred as CAG:CRE;Netrin^{+/+}) will overexpress netrin-1 in response to tamoxifen injection (O/E) whereas CAG:CRE;Rosa26-LSL-

huNetrin-1^{-/-} (referred as CAG:CRE;Netrin^{wt/wt}) mice express endogenous levels of netrin-1 (WT). Two months old (20-25g body weight) CAG:CRE;Netrin^{+/+} and control littermates were used for the experiments.

Netrin-1^{fl/fl} mice (Keqiang Ye's laboratory): did not provide the description yet

Salmon gal staining

Netrin-1⁺/LacZ generation and salmon gal staining

Netrin-1⁺/lacZ mice were generated as described²⁹. P60 mice were euthanised (pentobarbital 90mg/kg, ip) and fixed (formaldehyde 4%) through perfusion. Brain were removed, post fixed for 4h and embedded in paraffin. Coronal sections of 10µm thickness were stored at -80°C until salmon gal staining.

For salmon gal staining, brain cryosections were incubated overnight in pre-stain solution without substrate (potassium ferrocyanide 200 mM, MgCl₂ 4 mM, NP40 0,04% in PBS) at 37 °C to reduce endogenous β-galactosidase activity. Cryosections were then washed three times in PBS and incubated for 1 h at 37 °C in the staining solution (1 mg/mL Salmon-Gal, 0.33 mg/mL NBT, MgCl₂ 2 mM, NP40 0,04% in PBS) then rinsed in PBS.

Oral gavage of rotenone treatment (Keqiang Ye's laboratory)

Mice were treated with 0.1 ml/25 grams of vehicle containing 1% methylcellulose (Sigma Cat# M0512) and 1.25% chloroform (Sigma Cat# 2432) or a solution containing 0.625 mg/ml of rotenone (ULTRA Scientific Cat# PST-890) with 1% methylcellulose and 1.25% chloroform. Rotenone solution was solubilized in chloroform and then diluted into 1% methylcellulose solution while mixing vigorously. Rotenone or vehicle control treatment was orally administrated with a 1.2 x 60 mm gavage (Unimed, Switzerland) once a day for 5 days a week, consecutively for 3 months.

Immunoblotting (Keqiang Ye's laboratory)

Mouse and human brain tissue samples were lysed in lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na₃VO₄, 50 mM NaF, 10 mM sodium pyrophosphate and 10 mM sodium β-glycerophosphate, supplemented with a cocktail of protease inhibitors), and centrifuged for 15 min at 4 °C 15000 rpm for cell lysate or ultra-centrifuged for 20 min at 4 °C 40000 rpm for mouse brain tissues and human tissues. The supernatant was boiled in SDS loading buffer. After SDS-PAGE electrophoresis, the samples were transferred to a nitrocellulose membrane. Primary antibodies to the following targets were used: α-Syn FL (Santa Cruz, Cat# SC9977) α-Syn pS129 (LS bio, Cat# LS-C380861-1)); Tyrosine Hydroxylase (Santa Cruz, SC-25269; Abcam, Cat# ab112); Netrin-1(Santa Cruz, Cat# SC20786); netrin-1 (Abcam,

ab126729); UNC-5B (Santa Cruz, Cat# SC5570); DCC (Santa Cruz, Cat# SC515834) and cleaved caspase-3 (Cell signaling Cat# 9661).

Ventral midbrain primary culture

Dopaminergic primary neurons were prepared from the ventral midbrain of embryonic mice on day 13.5 of gestation. Ventral midbrain was isolated and dissected in ice-cold Dulbecco's medium + 0.2% BSA. Fragments were dissected into tiny pieces and collected in 2 ml vials. Then pieces were washed three times in HBSS (Ca²⁺ and Mg²⁺ free) medium. Pieces were dissociated in HBSS containing 0.01% trypsin for 20 min at 37°C. Cells were dissociated by soft trituration in FBS medium containing 1µg/ml of DNase I. After trituration, cells were washed in DA neuron culture medium (DMEM F12, N2 1X, 0.36% D-(+)-Glucose (wt/vol), primocin 100µg/ml) and plated on 96-well plates coated with poly-Lornithine at a density of 5.0×10^4 cells/well and let to adhere for 1hour. Then, cells were treated with netrin-1 or GDNF. The treatment was renewed every two days during four days. Finally, cells were fixed with PFA and kept in PBS 1X at 4°C for immunostaining.

TUNEL assay (Keqiang Ye's laboratory)

To measure the effects of Netrin-1 gene knockout, GFP Cre; GFP Cre + Sh-DCC and GFP Cre + Sh-UNC-5B viruses were injected into the substantia nigra of Netrin-1 *f/f* mice. 6 weeks after viral infection, the mouse brains were fixed in 4% formaldehyde, permeabilized and immune-stained with anti-TH antibody. The dopaminergic cell death effect by Netrin-1 depletion was detected with an *in situ* cell death detection kit TMR Red (Roche, Cat# 12156792910). The apoptotic index was expressed as a percentage of TUNEL positive neurons out of the total number of TH-positive neurons.

Cells quantification (Keqiang Ye's laboratory)

TH-positive cells in the substantia nigra and striatum were estimated using fluorescence intensity by Image J software. For each animal, 8 consecutive sections of the substantia nigra and striatum were analysed. For quantification of positive cells, the stained colour was selected and set at the proper threshold for the binarization of the selected colour image. The total number of immune-reactive neurons were analysed using the same threshold (Image J). The conditions of the analysis were blinded to the investigator.

Stereotaxic injection

Cre-virus injection in Netrin-1^{f/f} mice (Keqiang Ye's laboratory)

Three months old Netrin-1^{f/f} mice were anesthetized with isoflurane (Piramal Healthcare). Meloxicam (2 mg/kg) was injected subcutaneously for analgesics (Loxicom, Norbrook). Unilateral stereotaxic injection of PFFs was performed at coordinates corresponding to the substantia nigra:

anteroposterior (A/P): ± 3.1 mm, mediolateral (M/L): ± 1.2 mm from Bregma, and dorsoventral (D/V): -4.3 mm from dura surface. Each site received $2\mu\text{l}$ of viruses: AAV6-Cre (1×10^{12}); Lenti-Sh UNC-5B (1×10^{10}) or Lenti-Sh DCC (1×10^{10}) at a rate of $0.25\mu\text{l}/\text{min}$, using $10\mu\text{l}$ Hamilton syringe. The needle was kept in place for 5 min after the injection was completed and gently removed. Mice were placed on a heating pad until they recover from the anaesthesia.

6-OHDA lesioned animals

All stereotaxic and surgical procedures were performed while animals were under isoflurane anaesthesia, as described in^{30,31}. Meloxicam ($2\text{mg}/\text{kg}$) was used for analgesia and lidocaine-adrenaline solution ($10\text{mg}/\text{ml}$ lidocaine, $0.005\text{mg}/\text{ml}$ adrenaline) for local anaesthesia before surgery. 6-OHDA (Sigma Aldrich, H4381) was injected at the rate of $0.5\mu\text{l}/\text{min}$ at different coordinates, according to the mouse brain atlas and rat brain atlas of Paxinos and Watson (2005) using a 33G, $10\mu\text{l}$, outer diameter 0.21mm blunt needle (NanoFil, World Precision Instruments, NF33BL-2).

In mice, $2\mu\text{g}$ of 6-OHDA ($2 \times 1\mu\text{g}$; $1\mu\text{l}/\text{site}$ of $1\mu\text{g}/\mu\text{l}$ solution) were injected unilaterally into two sites of the dorsal striatum (coordinates relative to Bregma and dura: A/P: $+1$, M/L: $+2.2$, D/V: -3mm and A/P: 0 , M/L: $+2.2$, D/V: -3mm).

In rats, $6\mu\text{g}$ of 6-OHDA ($3 \times 2\mu\text{g}$; $1.5\mu\text{l}/\text{site}$ of a $1.33\mu\text{g}/\mu\text{l}$ solution) were injected unilaterally into three sites of the striatum (A/P: $+1.6$, M/L: $+2.8$, D/V: -5.5 ; A/P: 0 , M/L: $+4.1$, D/V: -6 and A/P: -1.2 , M/L: 4.5 , D/V: -6). The dose of 6-OHDA was calculated as a free base and dissolved in degassed saline with 0.1% sodium metabisulfite to prevent oxidation. After each injection, the needle was kept in place for 4min to minimize backflow of the solution. For neurorestorative experiments, $10\mu\text{g}$ ($1.5\mu\text{l}/\text{site}$ of a $2.22\mu\text{g}/\mu\text{l}$ solution) of recombinant human netrin-1 (R&D, 6419-N1-025/CF) or $10\mu\text{g}$ ($1.5\mu\text{l}/\text{site}$) of recombinant human GDNF (eurobio, PCYT-305) or $1.5\mu\text{l}/\text{site}$ of 1X PBS Vehicle (Gibco) were injected two weeks after 6-OHDA lesion into the same three sites. For distribution studies, $10\mu\text{g}$ ($1.5\mu\text{l}/\text{site}$) of iodinated ^{125}I -netrin-1 (R&D, 6419-N1-025/CF) or ^{125}I -GDNF were injected similarly.

Perfusion and tissue processing for immunofluorescence and immunohistochemistry

Mice and rats were anesthetized with an overdose of sodium pentobarbital ($90\text{ mg}/\text{kg}$, i.p.) and perfused intracardially with PBS followed by 4% paraformaldehyde in a 0.1M sodium phosphate buffer, pH 7.4 . Brains were removed, post fixed overnight and stored in sodium phosphate buffer containing 30% sucrose at 4°C . Serial coronal frozen sections of $40\mu\text{m}$ thickness were made using a cryostat. Six sets of sections were collected in a cryoprotectant solution (0.1M phosphate buffer, pH 7.4 , 20% glycerol and 2% dimethyl sulfoxide) and stored at -20°C until immunohistochemical or immunofluorescence processing.

Immunofluorescence staining

Free-floating slices were rinsed in PBS then permeabilized and blocked with PBS-BT (50 mM Tris-HCL, 150 mM NaCl, 3% bovine serum albumin (BSA), 0.1% Triton-X100, pH7.4) blocking solution for 1h. Afterwards, the sections were incubated with primary antibodies (see key resource table) in a 2% normal donkey serum (NDS) and 0,3% Triton-X100 PBS solution on a shaker overnight at 4°C. The next day, sections were rinsed and incubated with corresponding secondary antibodies directly conjugated with fluorophores (1:500, Cy3- and Alexa Fluor 488 conjugate from Jackson ImmunoResearch) for 2h at room temperature. Finally, slices were rinsed in PBS and mounted (Sigma Aldrich, F4680).

Ventral midbrain primary neurons

Primary neurons in 96-well plate were fixed with 4% paraformaldehyde for 15min and permeabilized with PBS solution containing and 0,2% Triton-X100 for 20min. Then, rinsed and incubated 1h with a PBS blocking solution containing 2%NDS. Cells were next incubated with TH (Abcam, primary antibody ab76442) primary antibody overnight at 4°C. The next day, cells were rinsed and incubated for 1h with donkey anti-chicken fluorescent secondary antibody, then rinsed and kept at 4°C in PBS.

Immunohistochemistry

Free-floating sections were processed for tyrosine hydroxylase (TH) immunohistochemistry. Endogenous peroxidase activity was quenched for 5 min in a 3% H₂O₂, 10% methanol, PBS-1X (PBS) solution, and then washed with PBS. Sections were next preincubated with 2% normal donkey serum (Jackson ImmunoResearch, 017-000-121), 0.3% Triton X-100 in PBS to block non-specific staining. Then, sections were incubated overnight at room temperature with a 1:500 dilution of chicken anti-Tyrosine hydroxylase antibody (Abcam, ab76442), rinsed, and incubated 2h with a 1:300 dilution of biotinylated donkey anti-chicken antibody (Jackson ImmunoResearch, 703-065-155) before 1h incubation with the avidin–biotin peroxidase complex using the Elite ABC Vectastain kit (Vector Laboratories). Then TH staining was revealed using 3,3'-diaminobenzidine (DAB) as a chromogen. Sections were then put onto glass microscope slides, dried overnight at room temperature, dehydrated, and mounted in a xylene-containing medium.

Tamoxifen injection in netrin-1 inducible mice

Two weeks after 6-OHDA unilateral and intrastriatal lesioning, CAG:CRE;Netrin^{wr/wt} and CAG:CRE;Netrin^{+/+} mice received 100µl intraperitoneal (ip) injection of fresh tamoxifen solution (Sigma Aldrich, T5648) once every other day for five days (total of three injections). Tamoxifen

solution for injection was dissolved in corn oil (Sigma Aldrich, C8267) at a concentration of 10mg/ml.

Behavioural tests

Netrin-1^{fl/fl} mice (Keqiang Ye)

Loss of motor function was tested 6 weeks following virus (Cre, Cre + Sh-DCC, Cre + Sh-UNC-5B) injection. Behavioural test included the rotarod test, cylinder test, and grid tests. Rotarod test: Mice were trained for 3 days using the Rotarod (San Diego Instruments) at a slow rotational speed (5 rpm) for a maximum of 10 minutes. The rotational speed and latency to fall from the accelerating Rotarod were recorded, after three test trials on the test day. The rotational speed of Rotarod was modulated from 0 rpm to a maximum 40 rpm. It was gradually increased during the trial at a rate of 0.1 rpm/second. Cylinder test: Mice were placed individually into a glass cylinder (12 cm diameter x 22 cm height) and recorded by video camera. The recorded files were analysed by a blinded observer. Between 20 and 30 wall touches per animal (contacts with fully extended digits executed with the forelimb ipsilateral and contralateral to the lesion) were counted. Grid test: the grid was inverted, so the mice were hanging upside down by their paws. Animals were recorded for up to 60s, and then 10s-segments scored for the number of grids crossed at each step.

Amphetamine-induced ipsilateral rotations in 6-OHDA lesioned rodents

Amphetamine-induced rotational activity was monitored with the Rotometer system from Omnitech electronics, Inc, comprising a cylindrical test chamber (30cm x 30cm), rat or mice harnesses and sensors for circling data collection. Animals were placed in the test chamber and, after a habituation period of 15 min, a single dose of d-amphetamine (Tocris; CAS-51-63-8) was injected intraperitoneally. The number of full (360°) clockwise and counter clockwise turns was recorded for a period of 120min for rats and 90 min for mice. Net ipsilateral turns were automatically calculated by the Fusion software (Omnitech electronics) subtracting the turns to the left from the turns to the right side.

In rats, rotation tests were done 2 to 3 days before, and 2, 4, 6, 8 and 10 weeks after treatment (netrin-1, GDNF or vehicle injection) upon d-amphetamine (2,5mg/kg). Results are shown as the total (cumulative) of net ipsilateral turns performed over 120min

In mice, rotation tests were done 2 and 6 weeks after 6-OHDA lesion, upon d-amphetamine (5mg/kg). Results are shown as the total of net ipsilateral turns performed over 90min.

Rats and mice that did less than 100 ipsilateral turns at the first rotation test, 2 weeks after 6-OHDA lesion, were excluded from the experimental procedure. Indeed, according to Bäck and colleagues³², rats exhibiting less than 100 ipsilateral amphetamine-induced rotations /120min at 2 weeks after 6-OHDA lesion can be considered as not successfully lesioned. We decided to apply the same threshold for mice.

Estimation of striatal fibres optical density

The optical densities of the TH-positive fibres in the striatum were determined from three coronal striatal sections. Every sixth section between A/P: +1.6 and A/P: -0.20 for rats and between A/P: +1 and A/P: 0 for mice was cut on a cryostat and processed for TH immunohistochemistry. Sections were scanned with an automated scanner (Axio Sacn.Z1, service provided by the Ciqle imaging platform, Lyon). The images were converted to 16-bit gray scale and the optical density of striatal fibres from control and lesioned side were measured using the integrated densities divided by area in ImageJ (NIH). The corpus callosum which is devoid of TH signal, was used as an internal control for nonspecific background staining. Data are presented as percentage of the intact side.

TH-positive cell counting

The number of TH-immunoreactive cells in the SNpc was determined with Matlab (RRID: SCR_001622, MathWorks, Kista, Sweden) as previously described²⁵, by a blinded observer. Images taken with whole slide scanner (Pannoramic 250 Flash II, 3D Histech, Budapest, Hungary, with 20x objective) from six adjacent nigral sections were analysed. Data are presented as a percentage of the intact side.

Preparation and use of ¹²⁵I-labeled recombinant protein

Recombinant human netrin-1 (R&D) and GDNF (amgen) were iodinated with ¹²⁵I-Na using the lactoperoxidase method. Proteins were dissolved in 30 µl of 0.25 M phosphate buffer, pH 7.5, and mixed with ¹²⁵I-Na (1 mCi 37 mBq; GE Healthcare). The reaction was started by adding lactoperoxidase 10 µl of 50 µg/ml and 0.05% H₂O₂. The mixture was incubated at room temperature for 20 min and the reaction was stopped by adding 3 vol of 0.1 M phosphate buffer, pH 7.5, containing 0.1 M NaI, 0.42 M NaCl, and 25 µl of 2.5% BSA. Free iodine and iodinated growth factor were separated by Sephadex G-25 columns (PD10; GE Healthcare). For column equilibrium and elution, 0.1 M phosphate buffer, pH 7.5, with 1% BSA was used. The iodinated proteins were concentrated by using YM-10 Centricon columns (Millipore). The specific activity of ¹²⁵I-labeled netrin-1 and GDNF was >108cpm/µg protein.

Gamma counting and autoradiographic analyses

Portions of brain tissue were used for gamma counting, and the remainder was sectioned for autoradiography.

Autoradiographic analysis of the distribution of ¹²⁵I-proteins.

Rats that received intrastriatal injection of ¹²⁵I-netrin-1 or ¹²⁵I-GDNF were perfused 24 h after stereotaxic injections. Coronal paraffin sections (7µm thick) were juxtaposed against an autoradiography film (Kodak Biomax MS) for 4 weeks.

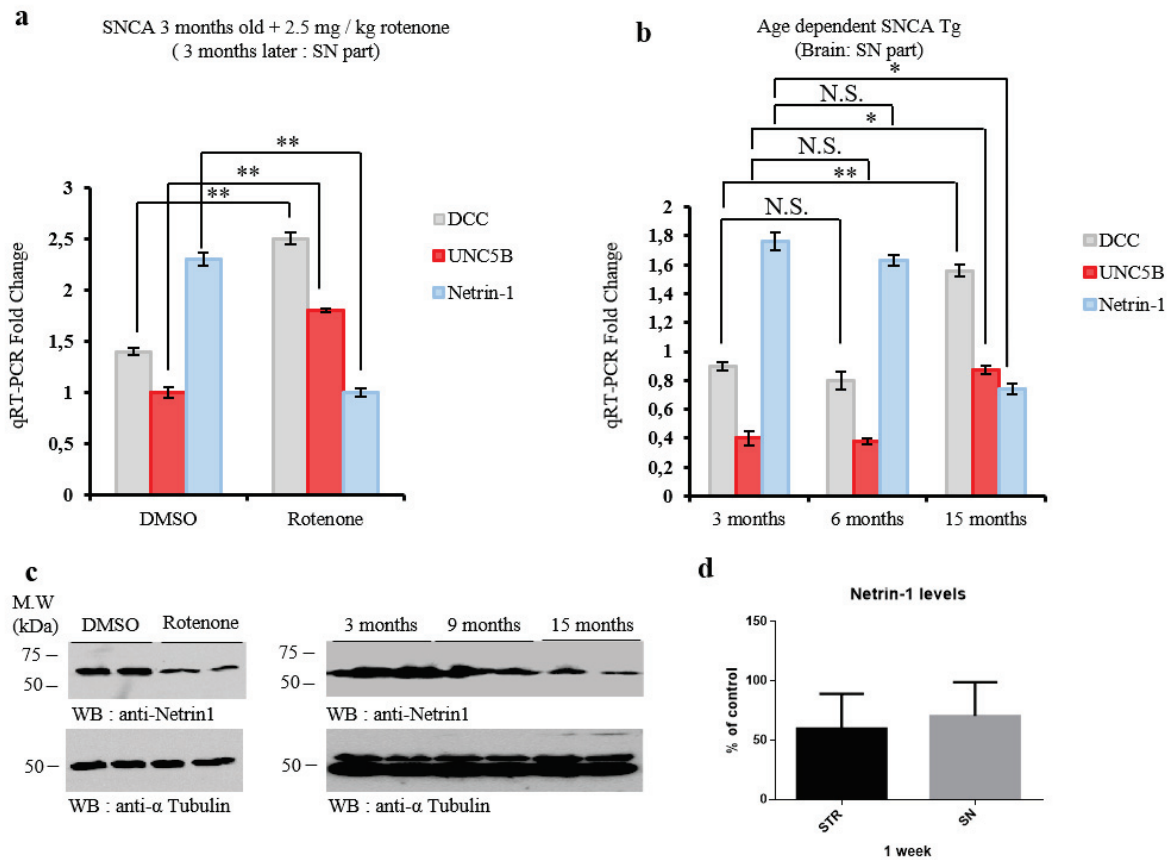
Quantification of ¹²⁵I-proteins in brain tissues after intrastriatal injections

The amount of intrastriatally administered proteins in different brain structures was determined after perfusions. The brain was removed from the skull and the hippocampus, substantia nigra, striatum, and cortex were dissected out, and the wet tissue was weighed. Results are expressed as counts per minute per milligram of wet weight.

Statistical analysis

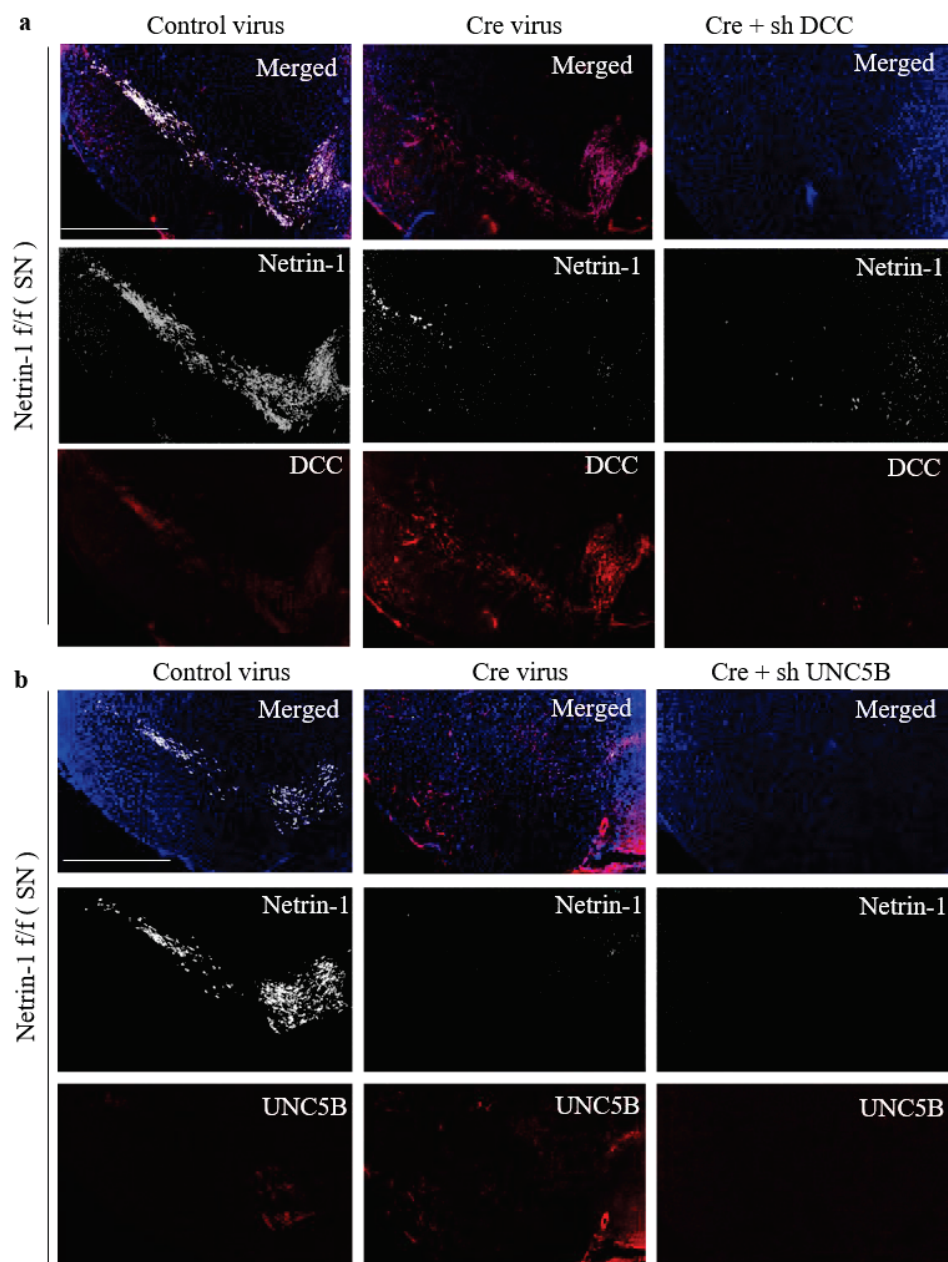
Statistical analysis of results was performed using GraphPad (Prism). All data were tested for normal distribution in order to analyse results accordingly using parametric or non-parametric tests. To compare results between two groups, Student's unpaired t-test was used. When more than two groups were compared, One-way ANOVA followed by Tukey post hoc test was applied. For repeated measures, a Repeated-measures (RM) ANOVA or 2way ANOVA test was performed followed by Tukey multiple comparisons post hoc test. A value of $p < 0.05$ was considered to be statistically significant.

Supplementary



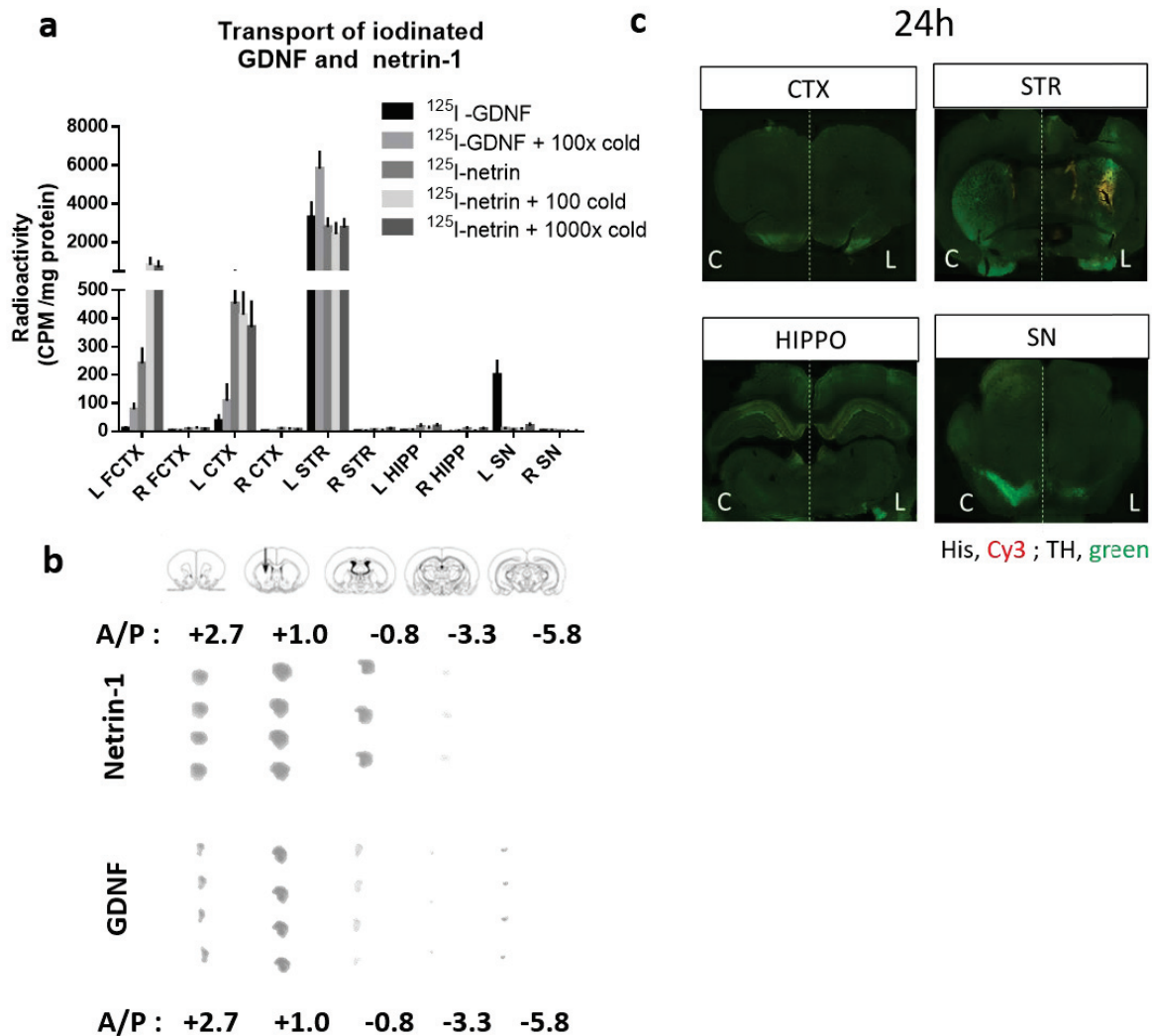
Supplemental Fig 1. Netrin-1 is reduced in PD mouse models.

a-b. Quantitative RT-PCR analysis of Netrin-1, DCC and UNC5B mRNA levels in rotenone orally injected mouse PD model (**a**), and different ages of SNCA transgenic PD mouse model (**b**). Data are shown as mean + SEM. $n = 3$ each group (**a-b**). * $P < 0.05$, ** $P < 0.01$. **c.** Netrin-1 is reduced upon rotenone treatment in aged SNCA Tg mice. Western blotting with rotenone-treated PD animal model (left), and age-dependent SNCA transgenic mice (right). **d.** Netrin-1 levels, assessed by western blot, in the striatum (STR) and the substantia nigra (SN) of mice, 1 week after 6-OHDA injection.

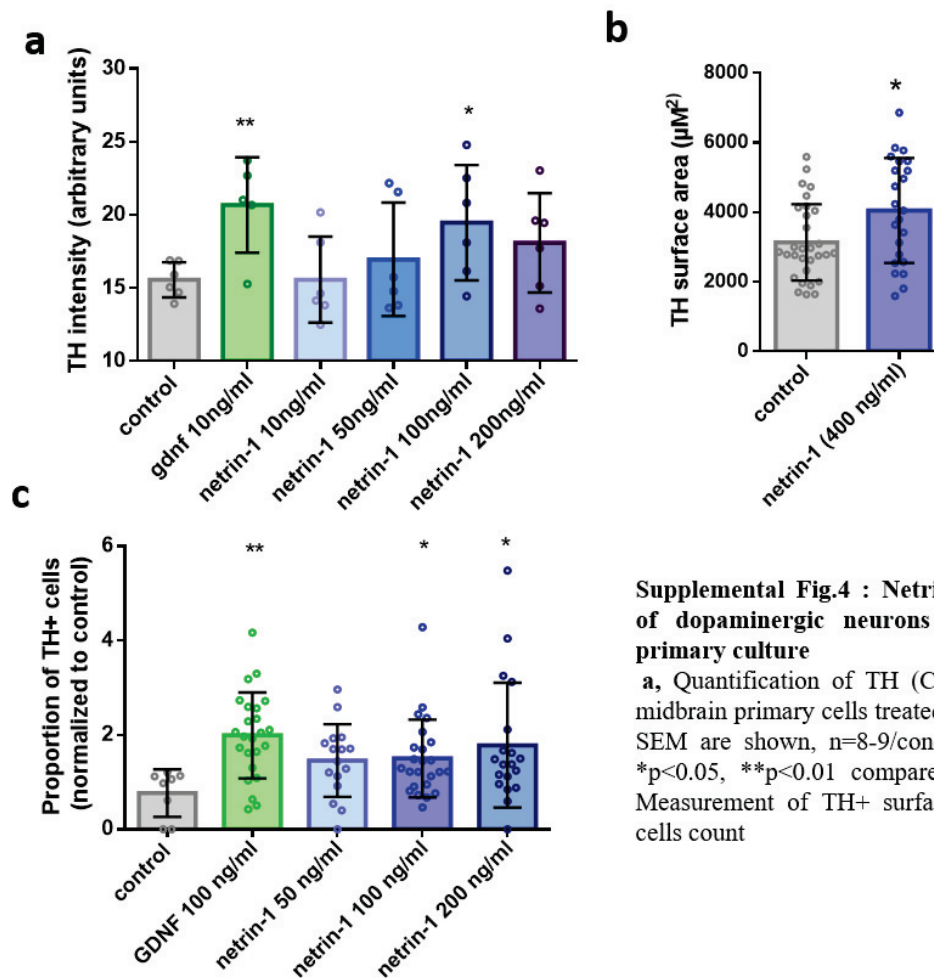


Supplemental Fig 2. Netrin-1 depletion upregulates DCC and UNC5B receptors.

a & b. Immunofluorescent staining of netrin, DCC or UNC5B on the brain sections depleted with netrin-1 Cre virus in the presence of sh-DCC or UNC5B: netrin (Cy5), DCC or UNC5B (Red), DAPI (blue) (Scale bar, 500 μ m).



Supplemental Fig.3 : Distribution of netrin-1 and GDNF after a single striatal injection. a,b, Distribution of ^{125}I -netrin-1 and ^{125}I -gdnf after intrastriatal injection. **a,** Radioactivity of ^{125}I -labelled proteins in different brain areas.Means +SEM are shown, n=10 in each group. **b,** Photomicrograph of an autoradiographic film after intrastriatal injections of ^{125}I -netrin-1 and ^{125}I -GDNF. Brain slice figures modified from the rat brain atlas of Paxinos and Watson (1997). **c** Diffusion of rhnetrin-1 6-His-tagged 24h after injection. **c** Fluorescence microscopy images of rhnetrin-1 His-tagged (His, Red) in cortex (CTX), striatum (STR), hippocampus (HIPPO) and substantia nigra (SN);“C”: control side, “L” : Lesioned side, Green : TH staining for dopaminergic (and catecholaminergic neurons).



Supplemental Fig.4 : Netrin-1 promotes survival of dopaminergic neurons in ventral midbrain primary culture

a, Quantification of TH (Cy3) intensity of ventral midbrain primary cells treated with netrin-1. Means + SEM are shown, n=8-9/conditions. Unpaired t-test, *p<0.05, **p<0.01 compared to control group. **b**, Measurement of TH+ surface area. **c**, TH-positive cells count

Table 1. Correlation between behavioural and morphological measures assessed by Spearman correlation test.

Model	DCC D1290N	CAGS:CRE-Netrin	Rat	Rat
Parameters tested	Behaviour x STR	Behaviour x STR	Behaviour x STR	Behaviour x SN
Spearman coefficient and p value	r=-0.4421 p<0.0888	r=-0.7564 p<0.003	r=-0.6148 p<0.0001	r=-0.6724 p<0.0001
significance	ns	***	***	***

Correlation between rotation score (behaviour) and TH+ fibres density in the striatum (STR) or cell number in the substantia nigra (SN)

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6 DISCUSSION

The present work shed light on the therapeutic relevance of targeting netrin-1 signalling in PD. We demonstrated *in vivo*, using netrin-1 conditional knockout mice, that netrin-1 silencing could trigger dopaminergic neurons demise and motor deficits. Inversely, both administration of netrin-1 recombinant protein in 6-OHDA-lesioned rats and overexpression of netrin-1 in 6-OHDA-lesioned transgenic mice showed significant neurorestorative effect on mature dopaminergic neurons. Taken together, these findings indicate that netrin-1 signalling plays a significant role on the survival of nigral dopaminergic neurons, which raises two major questions:

- I. Pathophysiological role of netrin-1 signalling: What is netrin-1 physiological role in the adult substantia nigra? and why is it decreased in PD?
- II. Therapeutic potential of netrin-1: What underlies netrin-1 neurorestorative effect (target cells, receptors, signal transduction)? Is a netrin-1-base therapeutic strategy suitable for PD?

6.1 PART I: PATHOPHYSIOLOGICAL ASPECT

6.1.1 Netrin-1 in the adult nigrostriatal pathway: localisation and function?

We showed that netrin-1 was strongly expressed in the adult substantia nigra in human and rodents, co-localised with dopaminergic neurons of the substantia nigra and was preferentially located in the ventral tier of the *pars compacta*. Netrin-1 localisation profile strongly resembles DCC reported localisation profile that was also shown to be highly present in ventral SNc dopaminergic neurons (figure 13).

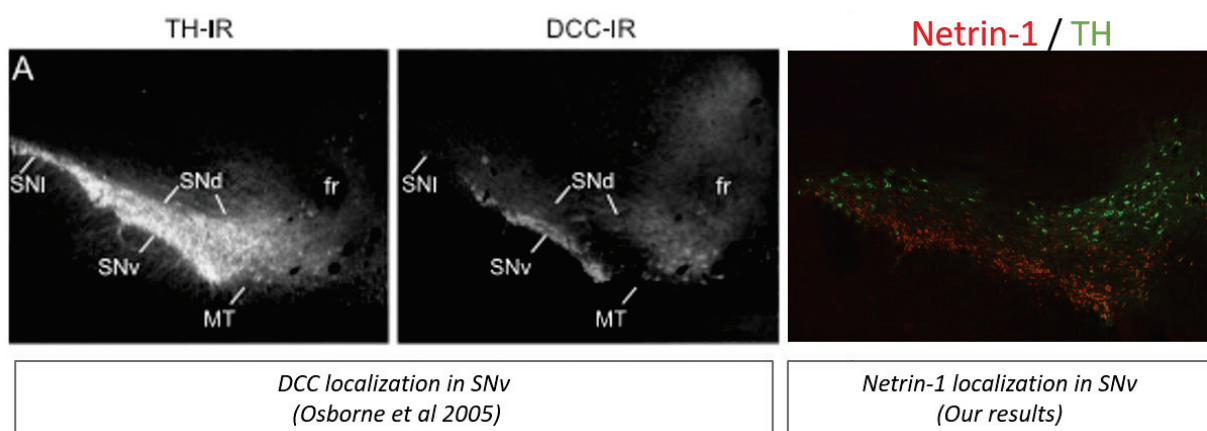


Figure 13 DCC and netrin-1 in the SNc

SNC dopaminergic neurons also strongly express DCC in striatal axon terminals in the dorsal striatum. *What about netrin-1?* Since netrin-1 is highly expressed in SNC dopaminergic cell bodies, we hypothesised that netrin-1 may be secreted along axons and at axon terminals.

Netrin-1 staining in coronal slices of the adult rodent striatum demonstrated that netrin-1 localised in a discrete population of previously reported netrin-1 expressing cholinergic interneurons (Shatzmiller et al., 2008). These acetylcholine secreting cells fire spontaneously and possess a large axonal arbour projecting throughout the striatum playing a neuromodulatory role in the basal ganglia. Indeed, they contact glutamatergic terminals from the thalamus and the cortex, GABAergic interneurons, medium spiny neurons and nigral dopaminergic terminals and conversely receive inputs from these structures (Bennett and Wilson, 1998; Wilson et al., 1990). Since these neurons secrete netrin-1 and that nigral axon terminals express DCC, it is plausible that these cholinergic interneurons, through netrin-1, induce structural and functional changes in nigral dopaminergic terminals further modulating the basal ganglia activity. Indeed, the balance and interplay between dopaminergic and cholinergic transmission critically influence basal ganglia function so that growing studies highlight the relevance of targeting cholinergic transmission in addition to dopaminergic transmission in PD treatment.

Except in these neurons, we failed to detect any other netrin-1 immunoreactivity in the dorsal striatum, especially netrin-1 signal did not seem to co-localise with DAT signal. Thus netrin-1 might not be secreted by nigral dopaminergic axon terminals or else, netrin-1 is not present in sufficient amount to be clearly detected in the dorsal striatum. Indeed, since netrin-1 is a secreted extracellular matrix protein, low levels and diffuse localisation of the protein may be difficult to detect. The diffuse dopaminergic axonal network may also impede immunostaining efficiency. Indeed, even TH immunostaining, for example, that shows a strong fluorescent signal when the cell bodies are imaged in the substantia nigra, shows much less intense signal when striatal fibres are imaged. Besides, we were able to show by western blot from rodent dorsal striatum lysates, that netrin-1 protein levels decreased after 6-OHDA intrastriatal injection, indicating that, beside cholinergic interneurons, dopaminergic axons may contribute to the pool of netrin-1 in the dorsal striatum (Supplementary Fig 1.d). Secreted netrin-1 levels may thus be gradually decreasing from SNC dopaminergic cell bodies to axon terminals and may have distinct roles depending on its localisation, and thus concentration, in the nigrostriatal pathway. *What could be netrin-1 function?*

Netrin-1 physiological function in the adult brain is poorly known, but growing evidence suggest that it may regulate synaptic plasticity. Indeed, as netrin-1 was shown to regulate cell-cell or cell-matrix adhesive contact during maturation of tissue patterning, it may be that elevated levels of netrin-1 promote connection stabilisation or consolidation whereas limited levels of netrin-1 favour loosely connected interaction and plasticity. This may be additionally modulated by levels and type of netrin-1 receptors expressed. Along this line, DCC was shown to activate several Src family member of kinases and GTPase leading to the regulation of cytoskeleton dynamics. Also, netrin-1 via DCC can control local translation, notably through the activation of mTOR signalling pathway, promoting membrane remodelling and growth (Goldman et al. 2013). Rearrangement of the actin cytoskeleton and growth, in response to netrin-1 signalling, may also be significant in a

reinnervation or regeneration process, through the migration of neural precursor or regulation of sprouting. Intriguingly, the dorsal striatum, characterised by poor levels of netrin-1, is associated with the degeneration of medium spiny neurons in Huntington's disease and SNc dopaminergic projections in PD. On the contrary, the ventral striatum, characterised by higher levels of netrin-1, is associated with striatal (dopamine) hyperfunction (schizophrenia, drug addiction). Could netrin-1 expression reflect the potential for reinnervation of neurons?

As a secreted protein we cannot exclude that netrin-1 may also have a paracrine action on several targets among which not only neurons, but also glial cells or endothelial cells, thus coordinating at a larger scale neuronal homeostasis.

Immunostaining on sagittal brain slices comprising the nigrostriatal tract may help to better characterise netrin-1 localisation profile in this pathway. Additionally, double staining of netrin-1 and receptors may help capture what could be the role of the netrin-1 signalling in the adult nigrostriatal pathway. However, our attempts to stain DCC and UNC-5 receptors were unsuccessful and need further optimization.

So far, we can only assert that netrin-1, like its receptor DCC, is expressed in SNc dopaminergic neuron, particularly in the subset of neurons reported to be prone to degeneration in PD. *Does it mean that netrin-1 and DCC are causally linked with SNc dopaminergic neuron selective vulnerability?*

Three options can be considered:

- 1) No correlation: Netrin-1 and DCC preferential expression in these neurons are not linked with their selective vulnerability.
- 2) Causal link: Netrin-1 and DCC preferential expression contributes to their selective vulnerability.
- 3) Consequent/adaptive link: Netrin-1 and DCC preferential expression are a protective/safeguard mechanism to overwhelm their selective vulnerability.

An approach to answer this question is to compare SNc netrin-1/DCC levels in healthy conditions *versus* pathological conditions, this may additionally provide insight into the role of netrin-1 signalling in SNc dopaminergic neurons.

6.1.2 Decreased netrin-1 gene expression and protein levels in substantia nigra in PD

6.1.2.1 Cause, or consequence?

Gene profile expression analyses from human PD microarrays showed that netrin-1 gene expression was reduced in substantiae nigrae of PD patients compared to healthy controls. Consistently, netrin-1 protein levels are decreased in PD patients compared to healthy patients. However, this latter result in human was shown in three patients only and would be worth to be validated on a bigger number of patients. Additionally, these observations in human and PD animal models do not demonstrate a causal link between netrin-1 loss and PD. Indeed, since SN

dopaminergic neurons were shown to express and produce netrin-1, decrease levels of netrin-1 in PD might just be a consequence of the progressive loss of SN dopaminergic neurons in PD.

Regarding DCC gene expression and protein levels, patient samples and animal models show an increase of DCC levels and proteolysis. Analyses of PD databases did not show significant changes in DCC expression profile in human SN (data not shown). Like netrin-1, DCC-expressing neurons are lost in PD, we would thus expect reduced DCC levels in SN of PD patients, however the opposite is observed. This might indicate that an altered netrin-1 signalling may be causally linked with PD, or that a netrin-1-loss compensatory mechanism may be activated in remaining neurons. Indeed, DCC increased levels might compensate for the reduced netrin-1 levels to maintain neuron function or to promote neuron survival. In prolonged absence of netrin-1 levels, however, DCC might eventually undergo proteolysis to be eliminated or to activate apoptosis, precipitating neural demise.

According to these results we cannot state whether increased DCC and decreased netrin-1 is a cause or consequence of neurodegeneration. To do so, it would be interesting to assess netrin-1 and DCC levels in early/pre-PD SN and striatal samples, just before neuron demise, however this stage is currently difficult, if not impossible, to define and human samples difficult to obtain. Alternatively, kinetic analyses, in toxin-based or genetic animal models of PD may help determining the time window when netrin-1 levels may decrease (or DCC increase) and whether this change precedes the onset of degeneration or accompanies cell death.

However, what is more important to elucidate is whether these changes may impact the neurodegenerative process and next, if targeting netrin-1 or DCC levels may lead to a better outcome.

6.1.2.2 Do decreased levels of netrin-1 contribute to degeneration?

To answer this question, we adopted a loss of function strategy, silencing netrin-1 in WT mice and assessing the maintenance (immunohistochemical analyses) and function (behavioural analyses) of SNc dopaminergic neurons. We observed that netrin-1 silencing triggered SNc dopaminergic neuron loss, suggesting that netrin-1 is a key factor for SN dopaminergic neuron homeostasis and that netrin-1 loss may participate to PD development. However, one should keep in mind that this conditional knock-out strategy is artificial and does not prove that netrin-1 loss is actually an initiating event in the disease. Besides, the sudden deletion of a gene in adult mice may generate artefacts and might not reflect accurately the effect of a likely progressive/chronic process leading to the decrease of netrin-1 levels in the adult SN. Nevertheless, this experiment provides a proof of concept showing that netrin-1 is involved in the maintenance of SN dopaminergic neurons and that its loss can actively contribute to SNc dopaminergic neuron loss in PD.

Indeed, we showed that the co-depletion of netrin-1 receptors, especially DCC, could partially prevent SN dopaminergic cell death in response to netrin-1 silencing, thus suggesting that:

- Netrin-1 receptors actively contributes to SNc dopaminergic neuron loss, according to the dependence receptor paradigm, in the absence of netrin-1.

- Netrin-1 positive signalling may also be involved in dopaminergic neuron maintenance since the deletion of DCC or UNC-5-B prevented *only partly* the loss of SNc dopaminergic neuron in response to netrin-1 silencing. It would have been interesting to test the effect of a double DCC and UNC-5-B deletion in conditional netrin-1 knock-out mice to assess whether dependence-receptor-induced cell death may fully account for the effect of netrin-1 silencing on SN dopaminergic neurons.

The possible implication of a DCC-DR-induced cell death in PD is further strengthened in our work by results showing that SN dopaminergic neuron degeneration may be delayed in mice mutated for DCC apoptotic activity (cf: section 4.2, p63). These results indicate that DCC DR signalling may sensitise neurons to degeneration in PD.

Indeed, in this neuroprotection experiment, 6-OHDA was unilaterally injected into the striatum of DCC D1290N (DCC^{m/m}) mice and WT littermates to test whether DCC DR signalling might contribute to sensitise neurons in a pathologic context, here induced by 6-OHDA neurotoxicity. We checked beforehand that 6-OHDA toxic effect was accompanied by a diminution of netrin-1 levels in the striatum and substantia nigra. DCC^{m/m} mice showed significantly reduced amphetamine-induced rotation two weeks after 6-OHDA lesion compared to WT littermates, but this effect was not significant anymore six weeks after the lesion and, accordingly, quantification of striatal TH⁺ fibre density at six weeks did not show a significant protective effect of the mutation. Analyses of TH⁺ cell number in the SN are ongoing. For now, the results may indicate that DCC mutation may confer resistance to degenerating SN dopaminergic cells, delaying thus the degenerative process. However, this would need to be ascertain by immunohistochemical analyses of TH positive striatal fibres and nigral cell bodies at an earlier time point, to assess the effect of the mutation on the progression of the degeneration.

Other possibility, preventing DCC cleavage by caspases, and thus DCC apoptotic signalling, may indeed prevent cell death but not axon degeneration induced by 6-OHDA: nigral cell bodies might be spared but not dopaminergic projections. Indeed, it was frequently observed that experiments aiming at blocking apoptosis in a variety of neurotoxin models of parkinsonism in adult rodents, succeeded to prevent cell body, but not axonal, degeneration (Cheng et al., 2010; Ries et al., 2008). Thus mechanism underlying degeneration are likely to be independent of cell death/survival pathway. TH cell counting in the substantia nigra at week six will provide elements supporting or not this assumption.

On a more technical aspect, we cannot rule out the possibility that the DCC D1290N mutation may structurally or functionally impact neural circuits controlling movement and/or circuits controlling the dopaminergic activity of the nigrostriatal tract, but that these alterations may be subtle, and thus go unnoticed in normal conditions, or may have been compensated during development. These possible masked defects may account for reduced amphetamine-induced turning behaviour in DCC^{m/m} mice and may explain the moderate correlation score between circling behaviour and TH⁺ striatal density.

Taken together however, our results indicate that netrin-1 reduction in PD may lead to dopaminergic degeneration through a DCC-induced apoptotic signalling.

What could be the role of a dependence receptor-induced cell death in the adult nigrostriatal pathway?

6.1.2.3 Pathophysiological role of a dependence receptor (DR) mechanism in the adult nigrostriatal pathway and general thoughts on the DR hypothesis

In the developing nervous system, netrin-1 was shown to promote neuron and axon guidance and subsequent establishment of synaptic contacts between neurons and their targets. According to the dependence receptor hypothesis, this neuronal patterning may be finely shaped by the elimination of supernumerary or ectopically localised neurons and axons through the activation of cell death by so-called dependence receptors controlled by ligand availability. This is reminiscent of the neurotrophic factor paradigm, well established in the peripheral nervous system, according to which, target tissues produce trophic molecules in limited amounts, and thus only neurons that have successfully established synaptic contacts with proper target cells are able to survive (Davies, 1996). Cell death induced by dependence receptors is likely a tightly regulated mechanism during neural development, restricted spatially and temporally to refine shape and size of neural circuits and connections.

Indeed, to date around 20 pairs of ligand/dependence-receptor have been identified (Negulescu and Mehlen, 2018). We can assume that several of these ligand/receptor pairs may be expressed on a given cell. In this case, according to the dependence receptor hypothesis, a drop in only one of these ligand levels may lead to cell death. Yet, in the scale of an organ or of the whole organism this would have a severe impact. Therefore, the negative signalling activated by dependence receptors in conditions of low ligands is highly likely to be strongly controlled by the cellular environment: trophic factors/survival signals vs cellular stress signals, availability/levels of receptors or co-receptors, levels/availability/subcellular localisation of second messengers, effectors (like caspases) or anti-apoptotic regulators (Bcl2, IAP).

Also, this mechanism, described to activate or amplify caspase signalling, may not necessarily lead to cell death. Indeed, caspase signalling (active caspase 3, 6 and 9, mostly) controls a number of non-apoptotic functions, especially in neurons, such as control of differentiation, proliferation, migration, axon growth or neurite pruning (D'Amelio et al., 2010; Hyman and Yuan, 2012; Mukherjee and Williams, 2017). Along this line, the kinase Akt, the glutamate AMPA receptor (AMPA) subunits, cytoskeleton proteins like actin, tubulin, the microtubule associated protein tau, the growth associated protein GAP43 are key growth/plasticity-associated proteins reported to be caspase substrates (D'Amelio et al., 2010; Hyman and Yuan, 2012; Mukherjee and Williams, 2017). Therefore, dependence receptors may not only function as activators of cell death through the activation of caspases, but more generally as activators of cellular remodelling referred here as a “partial dependence-receptor mechanism”. In situations of local ligand diminution, partial dependence-receptor mechanism, distinct from attraction or repulsion, would lead to cellular or

neuronal atrophy or neurite pruning, in other words: local degeneration. This may for example contribute to directing growth during axon guidance. Compared to kinase/phosphatase pathway, this protease pathway based on cleavage may lead more rapidly to structural changes.

This stated, what might happen in the adult nigrostriatal pathway in PD?

Reactivation of a developmental mechanism:

Netrin-1 and DCC are involved in the formation of the nigrostriatal pathway regulating SNc dopaminergic neuron migration to the ventral midbrain and also axon elongation and branching in the dorsal striatum. Since DCC is expressed by midbrain dopaminergic neurons while netrin-1 is expressed in the midbrain, in the floor plate and the marginal zone, and less expressed in the dorsal striatum compared to the ventral striatum (cf, section 3.1.1, p56), we would expect according to the dependence receptor hypothesis to see supernumerary neurons in the substantia nigra or ectopically located neuron in the midbrain and more dorsally located axons in the dorsal striatum of DCC KO mice. In netrin-1 KO mice conversely, we would expect ectopically located neurons in the midbrain, poor midbrain DA neuron innervation in the dorsal striatum and globally less neurons due to cell death. Although cell death has not been reported in netrin-1 KO mice, these predictions are relatively similar to the actual depicted DCC KO and netrin-1 KO phenotype in mice. Therefore, a dependence-receptor (DCC)-induced cell death mechanism may contribute, in the developing nigrostriatal pathway, to the finely-tuned control of proper cell or axon localisation and number. In the adult brain, we can speculate that a pathological context leading to decreased netrin-1 levels (or increased DCC levels) may aberrantly reactivate a dependence-receptor apoptotic cell death.

An overwhelmed safeguard mechanism:

Another possibility could be that, dependence-receptor mechanism is not a “vestige” from the development but an active mechanism in the adult brain that contributes to punctually and locally clear defective or diseased neurons or neurites to protect healthy neurons or maintain function and homeostasis. In this case, netrin-1 receptor DCC might be a double-edged sword for SN dopaminergic neurons depending on netrin-1 availability: promoting survival in healthy conditions and cell death or local degeneration in conditions of low trophic supports. In PD, maintained and generalised loss of netrin-1 levels, coupled with cellular stress mechanisms, may chronically activate this mechanism, elevating the pool of active caspases, sensitising neurons, and leading to uncontrolled degeneration.

A functional role in synaptic plasticity through a “partial DR mechanism”:

In the same line, more than a simple safeguard mechanism, DCC-dependence receptor activity may be functionally relevant in the nigrostriatal pathway dynamically contributing to the regulation of plastic events.

Indeed, SN dopaminergic neurons, characterised by a massive and loosely connected axonal arborisation, coordinate the activity of basal ganglia neurons which are spatially distributed throughout the striatum. This neuromodulatory role presumably requires flexible and diffuse

connections. Recent evidence suggests that netrin-1 via DCC may be involved in synapse formation and maturation (Goldman et al., 2013; Horn et al., 2013). In the hippocampus, netrin-1 is secreted at dendrites of excitatory neurons and promotes long-term potentiation (LTP) of synaptic transmission through DCC (Glasgow et al., 2018). This is consistent with netrin-1 reported function as a regulator of cell-cell adhesion and interaction in (neural and non-neural) tissue organization. Netrin-1 may thus participate to consolidation of connections through DCC-induced cytoskeleton rearrangement. Interestingly, the dorsal striatum exhibits low levels of netrin-1, except punctually in few cells, presumably cholinergic interneurons. These low levels of netrin-1 may provide a permissive environment for nigral dopaminergic neuron diffuse arborescence and for the flexibility of connections in the mature brain. Indeed, restricted levels of netrin-1 may favour oscillation between transient states of poor netrin-1, promoting synapse weakening through “partial DR mechanism”, and states of sufficient levels of netrin-1, promoting synapse maintenance or strengthening through netrin-1/DCC positive mechanism. DCC may thus play a critical role in the homeostasis of the adult nigrostriatal pathway.

Netrin-1 signalling through DCC in the adult nigrostriatal pathway may contribute to both maintenance and plasticity of SN dopaminergic neurons. Other netrin-1 receptors may be involved in netrin-1 function in the adult nigrostriatal pathway. Studies reported an upregulation of netrin-1 UNC-5-H receptors, notably UNC-5-C, at puberty and onwards in dopamine midbrain neurons from the VTA that projects to the medial prefrontal cortex (mPFC), changing the DCC:UNC-5-H ratio and regulating the maturation of the mesocorticolimbic pathway dopamine projections. Whether UNC-5-H, especially UNC-5-C may be expressed in SNc dopaminergic neurons and modulate their function has not been assessed.

Although we showed that netrin-1 may support the maintenance of adult SNc DA neurons, the positive signalling, including receptors, underlying this effect remains to be characterised.

6.1.2.4 What could be the cause of netrin-1 reduced levels in PD?

As mentioned before, we can speculate that decreased netrin-1 levels may result from the progressive loss of netrin-1-expressing cells and may accelerate the degeneration of remaining neurons. Alternatively, cellular stress or features of degeneration in PD, such as inflammation, alpha-synuclein aggregates, through direct or indirect mechanisms may alter netrin-1 function and/or netrin-1 or receptors expression thus precipitating neuron demise.

Although these assumptions remain to be demonstrated, studies have reported that netrin-1 signalling can be modulated by inflammation. Netrin-1 and receptors UNC-5-A and B are indeed reported transcriptional targets of NF- κ B, and conversely netrin-1 signalling was shown to regulate NF- κ B activation (Chen et al., 2017; Paradisi et al., 2009). It might thus be worth studying the possibility of a defective pro-inflammatory loop between netrin-1 and inflammatory mediators leading to cell loss in PD.

Regarding alpha-synuclein, we showed that netrin-1 levels progressively decreased over time in human SNCA mice. Again, whether netrin-1 progressive loss in this model is an indirect effect of dying netrin-1-expressing neurons, a result of alpha-synuclein aggregation or simply the

effect of age remains to be elucidated. However, we also showed that silencing netrin-1 increased P-S129-alpha-synuclein. These findings are intriguing and may suggest that there is a link between netrin-1 signalling and alpha-synuclein. Indeed, both netrin-1 signalling, through DCC, and alpha-synuclein were reported to play a role in synaptic plasticity and vesicle trafficking in synaptic terminals (Bellani et al., 2010; Busch et al., 2014; Cotrufo et al., 2011; Eisbach and Outeiro, 2013; Zylbersztejn and Galli, 2011). Hence, DCC and alpha-synuclein present similar subcellular location and are involved in similar processes and may thus interact with common partners. Therefore, altered function of one may indirectly impact the activity or the expression of the other. For example, it was reported that phosphoinositide-3 kinase enhancer L (PIKE-L), a putative netrin-1 signalling effector (Tang et al., 2008) was sequestered by alpha-synuclein in Lewy bodies inducing dopaminergic neuron cell death (Kang et al., 2017). It would thus be worth testing the possibility of an interplay between alpha-synuclein aggregates and netrin-1/DCC (or other receptor) signalling both in physiological conditions and in PD.

To finish on the pathophysiological role of netrin-1 in PD, it would be important to assess whether netrin-1 and receptor levels might be modulated with age, since ageing remains the major risk factor for PD.

Conclusion part I: Our results indicate that netrin-1 levels are decreased in PD and that low levels of netrin-1 might contribute to PD progression. Although the cause/s of netrin-1 diminution in PD remain to be determined and whether netrin-1 diminution is an initiating event in SNc dopaminergic neuron degeneration remain to be elucidated, netrin-1 appears to be a key factor for nigral dopaminergic neuron maintenance in the adult nigrostriatal pathway. Its receptor DCC may be central in the regulation of SNc dopaminergic neuron plasticity, through its positive signalling in presence of optimal amounts of netrin-1 and through its (partial) dependence-receptor signalling in response to low levels of netrin-1. However, the latter signalling may contribute to the sensitisation of axons and neurons in conditions of chronic cellular stress, possibly through the elevation of the active caspase pool above a sublethal threshold. Given genetic studies associating DCC gene polymorphisms to PD, exploring the pathophysiological role of netrin-1/DCC signalling in PD may bring new insights into the disease pathogenesis and netrin-1 function in the adult brain.

6.2 PART II: NETRIN-1, A PROMISING NEURORESTORATIVE FACTOR FOR PD TREATMENT?

We next assessed the effect of netrin-1 on degenerating SNc dopaminergic neurons using a partial lesion of the nigrostriatal pathway induced by intrastriatal injection of 6-ODHA and inducing netrin-1 stable overexpression in transgenic mice or injecting human recombinant netrin-1 in rats. Although the 6-OHDA model is not perfect in that it remains a relatively acute neurotoxic model compared to the slowly-progressing course of PD pathology, and in that it does not recapitulate Lewy pathology, it has the advantage to reproduce the preferential degeneration of SN

dopaminergic neurons and associated motor impairments. Additionally, since degeneration progresses over weeks, this model allows addressing not only the ability of a tested molecule to protect dopaminergic neurons from degeneration and cell death, but also their effects on recovery and regeneration. Here the purpose was to test the restorative effects of human netrin-1 in those rodent unilateral partial 6-OHDA PD model using both behavioural (amphetamine-induced rotations) and morphological (immunohistochemistry) analyses.

6.2.1 Netrin-1 neurorestorative properties in 6-OHDA PD model

6.2.1.1 Neurorestorative properties upon stable overexpression in mice:

Netrin-1 overexpression was induced in netrin-1 inducible transgenic mice two weeks after 6-OHDA injection into the striatum. After four weeks, netrin-1 induction significantly increased the density of dopaminergic projections in the striatum and induced behavioural recovery from unilateral lesion by restoring balance of movements in response to amphetamine compared to WT littermates. These behavioural and morphological analyses correlated well (Supplementary, Table 1) indicating that the restorative effect of netrin-1 on dopaminergic terminal density may account largely for the behavioural recovery. The results regarding netrin-1 effects in the SN are awaited and may provide additional information on netrin-1 neurorestorative properties. Interestingly, rapid examination of coronal slices from the *intact* striatum and substantia nigra at four weeks after netrin-1 induction, did not demonstrate obvious structural changes between netrin-1 WT and overexpressing mice, indicating that netrin-1 induction did not impact non-lesioned dopaminergic neurons. This would deserve nevertheless further attention, especially on a longer time period, to determine whether netrin-1 have an effect on healthy dopaminergic neurons (for example, inducing sprouting) and mostly to determine whether a chronic treatment with netrin-1 may have long term side effects. So far, no apparent toxicity of chronic netrin-1 expression was observed in animals.

6.2.1.2 Neurorestorative properties upon single-injection in rats

Human recombinant netrin-1 (10 μ g) was injected into the same three lesion sites in the striatum that were used two weeks before for 6-OHDA injection. Following netrin-1 injection, rats exhibited a stable amphetamine-induced rotational behaviour over time (ten weeks), that was significantly reduced compared to rats in the vehicle group. Morphological analyses at ten weeks after netrin-1 injection demonstrated significant increase in striatal dopaminergic fibre density compared to control animals but no significant increase of dopaminergic cell soma in the SN. In addition, behavioural results correlated well with morphological results in the striatum, indicating that morphological changes induced by netrin-1 injection may account largely for the behavioural effect. Netrin-1 (10 μ g) showed globally weaker efficacy than GDNF (10 μ g), but this may depend on the dose used (the dose of netrin-1 may have been either too low or too high), protein stability, diffusion capacity as well as the injection time window. Indeed, netrin-1 effect may be optimised

by modulating these different parameters. We will discuss this point later in the discussion (see section 6.2.3, p108).

The absence of significant effect in the SN may be explained by statistic power, we are currently counting additional slices to verify this result. If it is confirmed, the discrepancies between netrin-1 effect in the striatum and the substantia nigra may be explained by a local effect, in the striatum, promoting sprouting, or maintaining connections of dopaminergic axon terminals whereas severely atrophied neurons are not rescued. Indeed, distribution assays showed that after injection, netrin-1 diffusion was restricted to the striatum, suggesting a local action. Unlike netrin-1, many neurotrophic factors (NTF), such as GDNF, NRTN and CNDF, share the characteristic to be retrogradely transported from the striatal axon terminals to cell bodies in the substantia nigra (Ito and Enomoto, 2016). This active transport to the substantia nigra may contribute to their neurorestorative effect. Surprisingly, however, injecting GDNF directly in the substantia nigra do not provide efficient neurorestoration of dopaminergic terminals and fails to improve motor behaviour (Kirik et al., 2000). Only intrastriatal injection of GDNF induces functional neurorestoration. This might highlight the importance of local action on axon terminals to achieve functional efficiency. MANF (Mesencephalic astrocyte-derived neurotrophic factor), another neurotrophic factor that was shown to be both neuroprotective and neurorestorative in animal models of PD, is not retrogradely transported in the substantia nigra but transported to frontal cortical areas when injected to the striatum (Voutilainen et al., 2009, 2011). These differences in transport may reflect different mechanisms of action or targets between these different NTF, thus combined injection of netrin-1 and one of these NTFs may show additive effects potentiating neurorestoration. Also, it would be worth testing whether netrin-1 injection in the SN might potentiate intrastriatal netrin-1 neurorestorative properties.

6.2.1.3 What does these approaches tell about netrin-1 neurorestorative effect?

The netrin-1 induction approach, in mice, and the netrin-1 single-injection approach, in rat, although similar in that they both aimed at testing netrin-1 effect in a unilateral partial 6-OHDA PD model, were dissimilar in that the former provided a chronic/long term exposure to netrin-1 and the latter an acute/short term exposure to netrin-1. Also, the netrin-1 induction model enabled the ubiquitous expression of netrin-1 so that netrin-1 may act in several parts of the lesioned nigrostriatal pathway whereas in the injection model, netrin-1 action was restricted to the striatum. Moreover, the level of active protein was certainly not the same in these two models. In the injection model, netrin-1 effects may vary depending on the concentration of the active protein which was probably different over time (since the stability of the protein will eventually decrease) but also spatially (since netrin-1 may be more concentrated at the injection site and more diluted around). Finally, since the stereotaxic surgeries were performed in different settings (different animals, sites of injection and doses of 6-OHDA), the 6-OHDA lesion, and thus the severity of dopaminergic degeneration, was probably different in these models. Indeed, in WT mice we achieved only 40% loss of TH⁺ fibres density whereas in rats we reached a 70% loss. Therefore, it is difficult to compare these two approaches and to assess which treatment method might be the most efficient. Despite these numerous differences, netrin-1 induces in both models a significant

and stable (over four weeks) neurorestorative effect, highlighting its promising therapeutic potential. However, in addition to testing whether netrin-1 may also mitigate alpha-synuclein-induced toxicity, it would be now important to get more insights into what (structural effects, targets, signalling mechanism) underlies netrin-1 neurorestorative effects in order to validate and define a netrin-1 based therapeutic strategy for PD.

6.2.2 Interrogating netrin-1 neurorestorative mechanism

6.2.2.1 *At the cellular and molecular scale*

Netrin-1 neurorestorative action is likely to be directly targeting SN dopaminergic neurons since they express important level of netrin-1 receptor DCC. Moreover, *in vitro* experiments showed that netrin-1 had a direct effect on primary dopaminergic neurons survival and neurites outgrowth indicating that netrin-1 may directly promote dopaminergic neurons survival and possibly sprouting *in vivo*. However, primary dopaminergic neurons (dissected at E13.5) are post mitotic embryonic neurons, they may not fully respond like mature neurons.

Although DCC was shown to mediate cytoskeleton rearrangement and local translation in response to netrin-1, which makes it a potential mediator of plastic events such as regeneration, netrin-1-induced neurorestoration may also be controlled by other receptors. In order to identify the receptor or receptors required for netrin-1 effect, additional studies in the rat 6-OHDA model could combine the injection of netrin-1 with blocking peptides (traps), antagonist antibodies against DCC or UNC-5-H, or silencing strategies. Alternatively, recombinant peptides mimicking netrin-1 but mutated for the binding to one receptor may be used in the same settings. This information may be relevant for the optimisation of netrin-1 based strategies, for example in the design of netrin mimetics minimising possible off targets effects.

Moreover, netrin-1 action on degeneration may not be limited to a direct action on degenerating dopaminergic neurons but also by actions on neighbouring cells that might indirectly favour function, stabilisation, or growth of dopaminergic neurons. Since endogenous netrin-1 in the striatum is produced by cholinergic interneurons, it may indicate that netrin-1 participates to the neuromodulatory function of this neurons and that several cholinergic inputs or outputs might be regulated by netrin-1. Hence, in addition to dopaminergic terminals, netrin-1 may act on glutamatergic and GABAergic cells. Moreover, endothelial cells and astrocytes were shown to upregulate the expression of netrin-1 receptors in conditions of brain injury and reciprocally netrin-1 was shown to be upregulated following brain lesion by neurons but also endothelial cells (He et al., 2018; Podjaski et al., 2015; Tsuchiya et al., 2007; Wang et al., 2013). This might indicate a role of netrin-1 in neural repair and brain homeostasis. Along this line, exogenous administration or netrin-1 overexpression was reported to be neuroprotective in several models of brain lesions, especially stroke, regulating synapse formation (Bayat et al., 2012; Zheng et al., 2018), axon repair (Wang et al., 2018; Zheng et al., 2018), blood-brain barrier function (Podjaski et al., 2015), inflammation (Xie et al., 2018), and white matter remodelling (Cayre et al., 2013; He et al., 2013). Similar mechanisms might underlie netrin-1 neurorestorative effects and would need to be addressed individually to better understand and thus optimize netrin-1 utilisation in a therapeutic

perspective. Indeed, netrin-1 pleiotropic functions may be a plus but also, if not well controlled, a disadvantage (through possible off targets effects) in the scope of a therapeutic use.

6.2.2.2 *At a larger scale, structural consequence on the nigrostriatal pathway: consolidation of connections, sprouting or regeneration?*

The term neurorestoration was used in the present work to define any intervention favourably modulating the course of neurodegeneration providing structural maintenance or repair accompanied with functional benefit. Hence “neurorestoration” encompassed: survival, restoration of phenotype, regeneration, sprouting, synapse formation, and maintenance or consolidation of connections. It can be the result of both the activation of plastic events (growth, cell adhesion, migration) and the activation of survival or trophic support against degeneration. Along this line, it was proposed that the neurorestorative effect of various neurotrophic factors was attributable to inhibition of ER stress, oxidative stress, or mitochondrial damage but also to long-term effects on neural plasticity, however the precise signalling mechanisms underlying those effects are not clearly elucidated. Characterising the neurorestorative effect/s of netrin-1 would provide valuable information for the development of a netrin-1-based therapy, notably to determine which temporal-window would be the more appropriate for netrin-1 administration.

Indeed, in our experiments, when netrin-1 is injected two weeks after 6-OHDA lesion, at the peak of cell death, there is already significant cell loss, most neurons have lost phenotype and synaptic contacts, exhibit axon atrophy and many of them are undergoing programmed cell death. However, several weeks after netrin-1 injection in rats (ten weeks), or netrin-1 induction in mice (four weeks), quantification of TH+ fibres in the striatum demonstrated a significant rescue of SN dopaminergic fibres density in the striatum of 6-OHDA lesioned animals when compared to (negative) control animals. Importantly, this effect was associated with functional benefit as demonstrated by amphetamine induced-behavioural test. What led to the increase of (functional) dopaminergic fibres in the striatum compared to control animals? Several hypothetical scenarios may explain the neurorestorative effect of netrin-1, I gathered them into three main groups (A, B, C) of changes converging to:

The maintenance of existing connections (A):

Netrin-1 may have promoted the structural and functional maintenance or consolidation of relatively spared or mildly atrophied dopaminergic neurons. In other words, it promoted neuronal resistance to the course of degeneration. This may be both the result of direct or indirect actions of netrin-1 on dopaminergic axons through the activation of trophic or survival pathways. Neurons that were too engaged in the degenerative process (synaptic loss, severe atrophy or undergoing cell death) would eventually die or remain in a permanent atrophic state. Therefore, in a clinical perspective, netrin-1 would have to be administered the sooner in order to provide the best functional efficacy.

The restoration of functional connections from degenerating neurons (B1):

Netrin-1 may have promoted the growth of mildly to severely atrophied neurons that had lost synaptic contacts, thus re-establishing functional synapses. This would allow a bigger time-window

for clinical intervention compared to the case A, as patients in late stage may still benefit a little from netrin-1 regenerative effect.

The formation of new connections from spared (non-lesioned) dopaminergic neurons (B2):

In this partial-lesion model, netrin-1 may have induced the sprouting of healthy non-lesioned dopaminergic neurons that may have thus compensate for missing axons. This case is slightly different from the case B1 where netrin-1 would have promoted successful sprouting of atrophied neurons. Indeed, degenerating neurons may be too vulnerable to undergo stable and functional growth, which require energy so that only sprouting from healthy neurons may provide benefits. This may thus restrict the treatment time window, limiting it to early stage of PD. However, we did not detect so far, any sign of sprouting in intact (non-lesioned) striatal slices of netrin-1 overexpressing mice compared to WT mice.

The reinnervation of the striatum through the migration of neural stem cells (C):

Netrin-1 was shown to promote the migration of neural stem cells in situations of injury, arguing for a role of netrin-1 in tissue repair. This would have the largest time-window, and would possibly allow treatment of late stage PD.

6.2.3 Netrin-1 as a potential therapeutic protein?

Further preclinical work has to be done to evaluate the relevance of a netrin-1 based strategy for the treatment of PD. Indeed, several questions would need to be addressed, beside unavoidable efficacy and pharmacokinetic characterisation.

Netrin-1 and alpha-synuclein aggregates and toxicity?

The 6-OHDA model of PD do not reproduce alpha-synuclein aggregates which is the second hallmark of PD. Promoting the survival, the maintenance or the regeneration of degenerating neurons is highly valuable but if these neurons are diseased and continue to accumulate alpha-synuclein it may lead to poor or even worst effects since alpha-synuclein can spread from cell to cell. Therefore, molecules that can regulate alpha-synuclein aggregation and toxicity are highly valuable in the search for therapeutic candidates for PD. GDNF, for example, the most studied NTF in PD is ineffective on alpha-synuclein PD animal models (Decressac et al., 2011). Thus, it would be important to test netrin-1 in an alpha-synuclein model to evaluate its therapeutic significance.

Combinations?

In the same line, testing netrin-1 in combination with other neurorestorative molecules may be relevant since PD is a complex disease. Netrin-1 and NTFs have distinct receptors and signalling in addition to a seemingly different distribution in the brain after injection. It would be worth testing if they might have complementary effects. Also, combining treatments to target various features such as inflammation, cell death, alpha-synuclein aggregates, sugar metabolism may lead to better outcome.

Adverse effects

Netrin-1 was shown to have versatile functions both inside and outside the nervous system, both in physiological context and pathological context, especially in cancer. Indeed, extensive studies have linked netrin-1 expression to cancer so that netrin-1 is abusively considered as an oncogene. Indeed, to date there is no direct demonstration of a role of netrin-1 on tumour initiation. Nonetheless, several studies show that netrin-1, like many developmental cues, is reactivated by tumour cells and contributes to tumour progression through the control of plastic events such as proliferation and migration. Since pre-cancerous lesions can go unnoticed, it is preferable to limit netrin-1 treatment to the selected target treatment site and avoid systemic delivery. In the adult brain, various cells may respond to netrin-1 treatment, especially in context of injury. Therefore, it is important to test the effect of acute and chronic netrin-1 infusion on non-lesioned and lesioned tissue to get insights into potential adverse effects, potentially on glial cells and endothelial cells. So far, we did not notice any adverse effects, toxicity, or damage phenotype in adult mice overexpressing netrin-1. Non-lesioned dopaminergic neurons seemed unaffected. Netrin-1 signalling may thus be tightly controlled in the adult brain and activated only upon physiological stimuli or in settings of injury or stress. However, those mice were sacrificed only four weeks after netrin-1-induced overexpression.

Time window

Future studies assessing netrin-1 effect at different time of intrastriatal injection may provide understanding on netrin-1 action on dopaminergic neurons and help determining if netrin-1 would be a relevant therapy for early or late PD. Indeed, if netrin-1 intrastriatal injection shows better morphological and behavioural results in neuroprotection settings or if injected at the same time or few days after 6-OHDA, it might indicate that netrin-1 is more efficient in protecting neurons or maintaining them than in inducing their regeneration. In this case, netrin-1 might induce stronger neurorestorative effects if injected at an early time point. On the contrary, if netrin-1 still exhibit robust effect when injected four weeks after 6-OHDA, then it would indicate a strong regenerative or reinnervating action and might be used in late stage of PD.

Dose

Since UNC-5-B, that mainly promotes repulsion, and DCC, which mainly promotes attraction, have distinct affinity for netrin-1, and since netrin-1 also binds different receptors such as integrins or neogenin, the concentration of netrin-1 might highly influence netrin-1 signalling as it would determine which receptors may be activated. Netrin-1 often exhibits a bell-shaped dose response (in promoting axon growth or survival), it is thus necessary to test a range of doses to characterise netrin-1 effects at the optimal dose but also to define whether netrin-1 can be deleterious above or under certain thresholds.

Delivery Strategy

Since netrin-1 does not cross blood brain barrier (BBB), it would thus have to be administered directly intracranially. Intraputaminal injection might be the more relevant target site for netrin-1 delivery as it will favour netrin-1 action on nerve terminals, which is important for functional recovery. However, the caudate/putamen (striatum) is a large structure and one reason advanced for the failure of neurotrophic factors in clinical trials so far is poor diffusion throughout the striatum. Moreover, netrin-1 is described as a “sticky” protein with a presumably low diffusion capacity due to its binding to ECM and heparin through domain C (section 1.1.1.2, p20). Therefore, removing domain C could be a strategy to promote netrin-1 diffusion, provided that it does not impact netrin-1 neurorestorative effects. Indeed, studies reported that the deletion of domain C decreased but did not abrogate axon guidance. Alternatively, new delivery methods such as convection-enhanced delivery and gene therapy using viral vector may enhance the tissue coverage of netrin-1. Generation of netrin-1 peptidomimetics that could cross the BBB could be an option, however given netrin-1 multiple targets and pleiotropic roles outside and inside the nervous system, especially on the promotion of tumour development, restricting netrin-1 delivery in space but also in time would be the best option.

Conclusion part II: Our results demonstrate for the first time a potent neurorestorative role of netrin-1 on degenerating SN dopaminergic neurons in an animal model of PD. Both acute and chronic netrin-1 treatment induced significant increase in dopaminergic striatal fibres density and significant behavioural recovery. Strikingly, a single intrastriatal injection of netrin-1 induced the functional neurorestoration of dopaminergic axons that was maintained over months. In addition, chronic netrin-1 overexpression did not display, so far, adverse effects. Although additional pre-clinical characterisation and validation are required, these results are promising and may open the way to the development of netrin-1 as a therapeutic molecule for PD. In this perspective, deciphering the mechanism but mostly the target cells and receptors involved in this effect would help optimizing a netrin-1 based therapeutic strategy limiting potential off target effects.

7 CONCLUSION

In this work, we showed that netrin-1 levels were decreased in PD. To assess whether decreased levels of netrin-1 could contribute to PD progression, we used conditional knock-out mice and evaluated dopaminergic neuron survival and function. We showed that the loss of netrin-1 was sufficient to induce dopaminergic neuronal death in normal conditions, in part through DCC dependence receptor apoptotic activity.

We then sought to evaluate whether netrin-1 could mitigate degeneration in PD. To this end, we tested the effect of netrin-1 injection and overexpression in partial-6OHDA animal model of PD. The results indicate that netrin-1 restored dopaminergic fibres and more importantly, function.

Taken together, these results indicate that modulating netrin-1 levels could impact (directly or indirectly) on SN dopaminergic neurons maintenance and function and thus, possibly PD. Particularly, we demonstrated for the first time, netrin-1 neurorestorative effect in a model of PD. Although, additional validation and further characterisation are needed, netrin-1 appears as a promising candidate for disease modifying-therapy in PD.

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