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Promoting induced or spontaneous neuronal regeneration in the adult brain

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Tesi di DOTTORATO di RICERCA di Laura Argelich Fernandez

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DECLARATION

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All the results presented here were obtained by myself, except for:

1. ***LV viral vectors production and purification*** *which were performed by: Dr.* ***Serena Giannelli,*** *Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy*

2. ***AAV viral vectors production and purification*** *which were performed by: Dr.* ***Serena Giannelli****, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy*

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterised by the loss of dopaminergic neurons in the *substantia nigra pars compacta*. Currently available treatments alleviate the symptoms temporarily but do not represent a lasting solution besides inducing some severe side effects. Conversion of non-neuronal cells into induced neurons *in situ* represents a promising therapy for brain injuries and neurodegenerative disorders. The following thesis aims to better understand which characteristics of the recipient cells and the local environment affect the process of direct neuronal reprogramming. For this purpose, we examined the capacity of ASCL1 to convert mouse astrocytes into neurons *in vitro* and *in vivo*. This potent transcription factor, which was able to convert primary astrocytes into mature neurons of GABAergic-like identity on the dish, was later used to assess its capacity of conversion *in vivo.* Using *Aldh1l1*-Cre^{ERT2}/Ai9 early-postnatal (P2) and adult mice, together with a stringent LV expressing ASCL1, we obtained a highly efficient astrocyte-to-neuron reprogramming of both immature and mature mouse striatal astrocytes. These newly formed neurons were mainly converted into MSN-like cells, expressing a broad range of MSN characteristic markers.

Nonetheless, direct reprogramming would not represent a disease-modifying approach. The absence of good biomarkers and the long presymptomatic phase of the disorder make it very difficult to prevent the development of neurodegeneration. In this project, we aimed to shed some light on the mechanisms underlying this stage of the disease by exploring if spared dopaminergic neurons undergo axonal remodelling to compensate for the neuronal loss. In order to examine this possibility, we locally denervated the dorsal striatum of mice with 6-OHDA injections. At moderate levels of neuronal loss, a time-dependent reinnervation process of the striatum was observed. To identify the origin of these new projections, two types of signalling methods were used, a genetically inducible AAV encoding for a fluorophore and retrograde fluorescent beads. Both systems raised the possibility that VTA dopaminergic neurons could be implicated in this regenerating event, an unexpected finding given that these neurons are anatomically and functionally different from the ones in the SNpc. To further investigate this event in different levels of neuronal loss, we developed a genetically inducible neurodegenerative method which could substitute 6-OHDA in future experiments. Identifying the factors that induce dopaminergic neurons to become plastic could represent a way to sustain dopamine signalling and delay disease progression at the early stages of the disease.

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Acronyms and abbreviations

6-OHDA	6-hydroxydopamine
AAV	Adeno-associated virus
ABC	Avidin/biotin complex
Aldh1l1	Aldehyde dehydrogenase family 1 member L1
AP	Anteroposterior
ASCL1	Achaete-scute homolog 1
ATACseq	Assay for transposase-accessible chromatin using sequencing
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CBA	Chicken β-actin
CNS	Central nervous system
CP	Caudate putamen
Cre	Cyclic recombinase
CTIP2	COUP TF1-interacting protein 2
DAB	3,3'-diaminobenzidine
DAPI	4 ',6-diamidino-2-phenylindole
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32kDa
DAT	Dopamine transporter
DBS	Deep brain stimulation
DCX	Doublecortin
DDCI	Dopa-decarboxylase inhibitor
DMEM	Dulbecco Modified Eagle Medium
Doxy	Doxycycline
DV	Dorsoventral
EF1α	Human elongation factor 1 alpha
FBS	Fetal bovine serum
FLEX	Flip-excision
FOXP1	Forkhead box P1
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent Protein
GPi	Internal segment of the globus pallidus
HBSS	Hank's Buffered Salt Solution without Ca^{2+} and Mg^{2+}

hESCs	Human embryonic stem cells
IBA1	Ionised calcium binding adaptor molecule 1
i.p.	Intraperitoneal
iDANs	Induced dopaminergic neurons
iNs	Induced neurons
iPSCs	Induced pluripotent stem cells
LMX1A	LIM Homeobox Transcription Factor 1 Alpha
LV	Lentivirus
MAP2	Microtubule-associated protein 2
MEFs	Mouse embryonic fibroblasts
MFB	Medial forebrain bundle
ML	Mediolateral
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NEUN	Neuronal nuclear protein
Neurog2	Neurogenin 2
NPC	Neural progenitor cells
Nurr1	Nuclear receptor 4a2
ORF	Open reading frame
PBS	Phosphate buffer saline
PD	Parkinson's Disease
PEI	Polyethylenimine
PFA	Paraformaldehyde
REM	Rapid eye movement
ROS	Reactive oxygen species
rpm	Revolutions per minute
rtTa	Reverse tetracycline-controlled transactivator protein
SEM	Standard Error of the Mean
shRNA	Short hairpin RNA
SNpc	Substantia nigra pars compacta
SOX9	SRY-Box Transcription Factor 9
STN	Subthalamic nucleus
TF	Transcription factor
TH	Tyrosine hydroxylase
TuJ1	β-tubulin III

VTA	Ventral tegmental area
w.p.i.	Weeks post injection

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1 INTRODUCTION

1.1 Parkinson's disease overview

Parkinson's disease (PD) is a clinicopathological heterogeneous disorder affecting more than 8,5 million people worldwide. Diagnosis often comes after the manifestation of main clinical motor symptoms such as bradykinesia, resting tremors, and rigidity (Kalia & Lang, 2015; Parmar et al., 2020). The average age of onset is around 55 years old and its prevalence is about 0,3% in developed countries, being twice as high in men than in women. However, PD tends to progress more rapidly and severely in women, being their symptoms and their response to treatments significantly different (Cerri et al., 2019). Like many neurological disorders, PD's incidence increases with age, reaching a plateau after 80 years old (Hayes, 2019). Given the ageing of the global population, neurodegenerative diseases, which are strongly age-related, are gaining incidence (Bloem et al., 2021). To avoid a public health challenge, new biomarkers and disease-modifying strategies must be found (Toulouse & Sullivan, 2008). Nowadays, most available treatments for PD are symptom-alleviating, mainly focusing on motor symptoms despite what usually causes a dramatic deterioration in the quality of life in long-term patients are the nonmotor manifestations. These symptoms include anxiety, depression, sleep disturbances and cognitive decline, among others (Bloem et al., 2021). Even though some are common at the late stages of the disease, few of them are believed to be present even before the appearance of the motor symptoms. Olfactory dysfunction, present in ~90% of PD patients, REM behaviour sleep disorder and constipation are the first symptoms to emerge (Langston, 2006). Identification and careful follow-up of subjects who display these symptoms, therefore being candidates to develop PD, would be very useful to understand better the disease progression while providing us with new biomarkers (Tolosa et al., 2006).

1.2 Neuropathology of Parkinson's disease

PD is classically characterised by the progressive and irreversible loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). This neurodegeneration of the nigrostriatal pathway causes a dopamine deficiency in the striatum leading to the appearance of motor symptoms. Nonetheless, diverse pathways involving other neurotransmitters, including the noradrenergic, cholinergic,

serotonergic, and autonomic systems, are also altered or dysfunctional during the disease, giving rise to nonmotor symptoms. One of the most common features is dementia, affecting around 40% of idiopathic PD patients, especially older individuals. It is mainly caused by the degeneration of the hippocampus and the cholinergic system, mostly leading to executive dysfunctions (Dauer & Przedborski, 2003a; Emre, 2003; Hayes, 2019).

The main pathological hallmark of PD is the presence of inclusions in both somas and neurites of the presynaptic protein α-synuclein, forming Lewy bodies and neurites, respectively (Day & Mullin, 2021). These aggregates, whose composition is mainly α-synuclein, ubiquitin, and neurofilaments, have been observed in post-mortem tissue from both idiopathic and familial cases, revealing their crucial role in the development of the disease. α-synuclein's contribution to PD pathogenesis was first studied after the association of mutations in *SNCA*, the gene encoding for this protein, with familial cases of PD (Polymeropoulos et al., 1997). This 140 amino acid protein, whose role in vesicle trafficking and synaptic transmission is still being debated, is natively unfolded, adopting different conformations depending on its interactors. Under disease conditions, α-synuclein misfolds and polymerises forming fibrils, which then aggregate leading to neurodegeneration. Even though it is clear the direct and crucial implication of this protein's toxic inclusions in PD pathogenesis, if these aggregates are the cause or a consequence of the disease is still to be understood (V. M. Y. Lee & Trojanowski, 2006; Obeso et al., 2010; Spillantini et al., 1997; Stefanis, 2012).

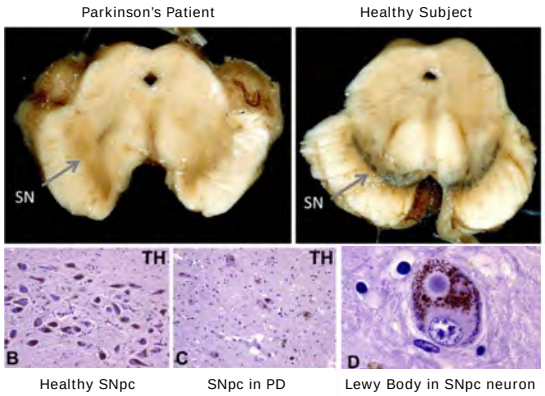

Figure 1.1. Neurodegeneration in PD. Post-mortem tissue of healthy and PD individuals showing the loss of neuromelanin-containing neurons in the SNpc (A). Loss of TH^+ neurons in the SNpc of diseased subjects (B-C). Detail of inclusions of ***α****-synuclein inside a dopaminergic neuron forming Lewy bodies (D). Adapted from* (Mandel et al., 2010).

In 2003, Braak and colleagues divided PD progression into six stages following a meticulous examination of post-mortem brains of control and PD individuals (Braak

et al., 2003). Patients included in the analysis presented different degrees of symptomatology. Authors assumed that those with the mildest manifestations were closer to the onset than those with a more severe phenotype. In this study, they established a correlation between the topographical localisation of Lewy bodies and neurites and the stage of the disease. Findings revealed an ascending pattern of α-synuclein spread from the olfactory structures to the neocortical areas going through the *substantia nigra*. Even though not all subjects suffering from PD present this progression, 51-83% of them do, reinforcing the theory that α-synuclein acts in a prion-like manner, being able to spread from one cell to another (Irwin et al., 2013; Lau et al., 2020; Rietdijk et al., 2017). This idea was further reinforced in 2008 when two different studies (Kordower et al., 2008; Li et al., 2008) showed that brains from individuals who were transplanted with dopaminergic neurons from foetal midbrains more than a decade before, contained Lewy bodies in the grafted cells. According to this theory, α-synuclein gets released in the extracellular matrix by both living and dying cells from where surrounding cells take it up. Then, the internalised α-synuclein in an oligomeric form, serves as a nucleation point for the aggregation of the endogenous α-synuclein, thus causing the formation of new inclusions (Brundin et al., 2008).

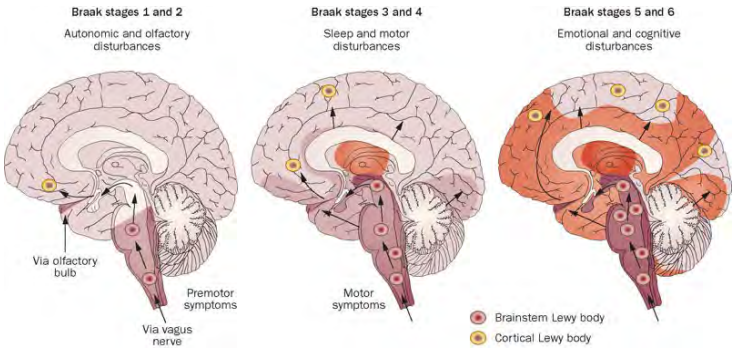

Figure 1.2 Braak stages in Parkinson's disease. Adapted from (Doty, 2012)

Still, there is some debate as if the bottom-up propagation theory describes accurately the dissemination of the disease. In Braak's stages, the neocortex is the last structure affected by toxic inclusions. There is a current of thinking that sustains that this structure could be affected even before the appearance of the motor symptoms. Initial motor manifestations often affect one part of the body and from there the disease spreads to other body regions. This particularly localised affectation hints that it must be due to a dysfunction of a highly organised structure, a pattern incompatible with the loss of extremely arborised dopaminergic neurons (Foffani &

Obeso, 2018). Also, at onset, the level of denervation observed in the striatum (~80%) is higher than the actual degeneration of the dopaminergic neurons in the SNpc (~60%) (Kordower et al., 2013), indicating that the neurodegeneration could be due to a “dying-back” process (Dauer & Przedborski, 2003a).

Unfortunately, the aetiology of the disease is still unknown, although it is often attributed to an interplay between genetic and environmental factors, and lifestyle (Schapira, 2009).

1.2.1 Genetic factors of PD

Even though most PD forms are idiopathic (~85%), due to the implementation of sequencing techniques in clinical medicine, many gene mutations have been associated with the development of the disease (Klein & Westenberger, 2012). While genetic forms represent a very low percentage of late-onset cases (2-3%), they are accountable for around 50% of early-onset PD cases (Obeso et al., 2010). However, monogenic forms are very rare. The most common genes linked to PD are: *SNCA* (α-synuclein), *PARK2* (parkin), *UCH-L1* (ubiquitin C-terminal hydrolase like 1), *PINK1*, *LRRK2* and *PARK7* (DJ-1) (Davie, 2008).

The first mutations associated with the development of PD were found in the *SNCA* gene (Polymeropoulos et al., 1997). This gene, as previously mentioned, encodes for the pre-synaptic protein α-synuclein, which plays a central role in disease pathogenesis. Individuals carrying a pathogenic variant start presenting motor symptoms very early (<50 years), which are usually accompanied by cognitive impairment and dementia after a brief period. So far, only 3 missense mutations have been identified, being p.A53T the most frequent one. Duplications and triplications of the gene, which cause an exacerbated phenotype, have also been reported but with a lower frequency (Fuchs et al., 2007; Klein & Westenberger, 2012). Fortunately, very few people have these more severe mutations, being those most common, the ones with higher penetrance.

Prevalent genetic mutations found in patients with late-onset or sporadic PD are located in *LRRK2* (Kalia & Lang, 2015). This gene encodes for a very large cytosolic protein containing a kinase domain in its C-terminal, region that holds almost all its pathogenic variants, all with low penetrance. The most frequent one is p.G2019S, which generates a phenotype resembling the one observed in idiopathic PD subjects.

This similarity is of extreme interest since it represents an opportunity to identify and study the underlying mechanisms causing idiopathic PD. Many studies link the toxicity of mutations in this gene to increased kinase activity. Still, the role of this protein in PD pathogenesis is not yet understood (Gupta et al., 2008; Tolosa et al., 2020).

Other common genetic variants are in genes associated with autosomal recessive forms of PD (*PARK2*, *PINK1* and *PARK7*). Individuals carrying these mutations, mainly associated with early-onset PD, present comparable features independently on the gene affected, being gliosis and neurodegeneration the main traits. *PARK2* mutations are the most common, representing between 10-20% of early-onset patients, and around 77% of those where the onset is before 30 years of age (Lücking et al., 2000). This gene encodes for the E3 ubiquitin ligase Parkin, which is essential for targeting proteins for their degradation. Dysfunction of the protein due to a loss-of-function mutation results in an accumulation of non-degraded proteins, which in turn is toxic to the cell (Klein & Lohmann-Hedrich, 2007). Mutations in *PINK1* and *PARK7*, reveal an implication of mitochondria in the disorder. *PINK1* encodes for a mitochondrial kinase, which function seems to be related to mitochondrial quality control. Genetic variants in this gene, which are usually missense or nonsense, cause the impairment of mitophagy, thus leading to the accumulation of malfunctioning mitochondria in the cytosol. On the other hand, DJ-1 has been identified as a key component to control oxidative stress. Even though it is not an antioxidant enzyme, in the presence of stress, it induces the expression of antioxidant proteins (Guzman et al., 2010). Therefore, toxicity due to pathogenic variants in this gene could be explained by the unregulated formation of reactive oxygen species (ROS), even if many other functions have been associated with this protein (Gupta et al., 2008; Klein & Westenberger, 2012).

These gene-disease associations are key to unravel molecular pathways involved in the disease pathogenesis. Most of the genes identified so far suggest mitochondrial and lysosomal implications. Understanding the contribution of these mutated proteins in the progression of the disorder will help identify possible targets for new therapies.

1.3 Dopaminergic neurons

Dopaminergic neurons are a heterogeneous group of cells involved in multiple brain functions identified by the staining for tyrosine hydroxylase (TH). They mainly

reside in the ventral part of the mesencephalon (~90%). From there, three different pathways arise: the nigrostriatal, the mesolimbic, and the mesocortical (Chinta & Andersen, 2005). The first originates from dopaminergic neurons present in the SNpc. Their axons project into the dorsal part of the striatum, known as caudate putamen, and are essential in the control of motor movement. Mesolimbic and mesocortical systems, on the other hand, originate from neurons residing in the ventral tegmental area (VTA). These pathways play an important role in behavioural processes such as reward and addiction (Brichta & Greengard, 2014; Chinta & Andersen, 2005). Even though the system mainly affected by PD is the nigrostriatal, causing distinctive motor symptoms, post-mortem studies revealed a loss of dopaminergic neurons also in the VTA of the brain (Damier et al., 1999; Uhl et al., 1985).

Interestingly, although insults derived from genetic mutations and environmental factors are not restricted to the nigrostriatal system, dopaminergic neurons in the SNpc are more vulnerable to cell death than other subtypes. VTA- and SNpc-dopaminergic neurons, although resemblant in many aspects, such as brain location and neurotransmitter released, present very different susceptibilities regarding neuronal death. Diverse studies examining post-mortem tissue of individuals with advanced idiopathic PD revealed that, while neuronal loss in the SNpc is around 80% compared to controls, for VTA is around 50% (Brichta & Greengard, 2014). Also, diverse subgroups within the SNpc present distinct susceptibilities, being those in the ventrolateral area the ones more vulnerable. These observations indicate that molecular differences between dopaminergic neurons influence their susceptibility to cell death. Expression profiles of VTA and SNpc dopaminergic populations extracted from rodents revealed that only 1-3% of the genes were differentially expressed (Greene et al., 2005; Grimm et al., 2004). Many of the upregulated genes in the VTA were involved in cell survival, synaptic plasticity and axon guidance (Grimm et al., 2004). However, it is more reasonable to think that other intrinsic and extrinsic factors are also contributing to the susceptibility divergence between VTA- and SNpc-DANs (Brichta & Greengard, 2014).

One possible contributor to this effect may be the high energetic demands of SNpc-DANs to maintain their extensive axonal arbours. Dopaminergic neurons in the nigrostriatal pathway send their unmyelinated projections to the dorsal striatum, where they establish a massive number of synapses (Bolam & Pissadaki, 2012). In 2009, Matsuda and colleagues estimated the number of striatal neurons innervated

by a single neuron from the SNpc to be around 75.000 in rats (Matsuda et al., 2009). Only the maintenance of such a huge axonal bush, *per se*, requires an enormous amount of energy. Mitochondrial dysfunction is a common feature in patients with idiopathic PD (Lin & Beal, 2006). Even though it affects multiple brain regions, the elevated energetic demands of the dopaminergic neurons could make them more susceptible to oxidative stress and, subsequently, to cell death (Bolam & Pissadaki, 2012).

A recent publication in Science further investigates this correlation between mitochondrial impairment and dopaminergic vulnerability (Burbulla et al., 2017). In that study, authors use iPSCs-derived dopaminergic neurons from idiopathic and familial PD patients, as well as from healthy individuals. Subjects with a genetic cause had mutations in *PARK7*, that as previously mentioned, plays an important role in the control of oxidative stress. Analysis performed at long time points revealed an increment in mitochondrial oxidative stress, which in turn resulted in high levels of oxidised dopamine. This product is a major component of neuromelanin, a pigment characteristic of dopaminergic neurons. The oxidative environment induces the formation of neuromelanin inclusions, which are associated with an impairment of the lysosomal enzyme glucocerebrosidase leading to lysosomal dysfunction. This context of high levels of ROS, and mitochondrial and lysosomal impairment, creates the perfect conditions for the accumulation and aggregation of α-synuclein. These findings, besides linking oxidative stress with mitochondrial and lysosomal impairment, features commonly observed in all patients, provide another possible explanation for dopaminergic vulnerability in PD (Burbulla et al., 2017).

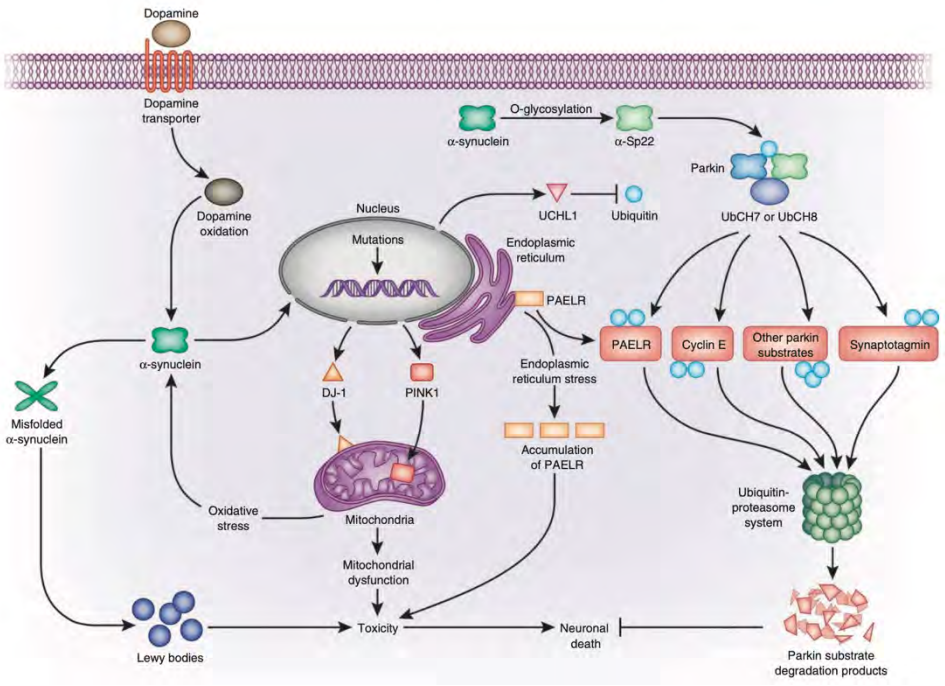

Figure 1.3 Graphical summary of recognised pathogenic mechanisms occurring in SNpc-dopaminergic neurons during PD. Adapted from (Obeso et al., 2010). a 22-kilodalton glycosylated form of α-synuclein (α-Sp22), parkin-associated endothelin receptor-like receptor (PAELR), ubiquitin-conjugating enzyme 7 (UbCH7); ubiquitin-conjugating enzyme 8 (UbCH8), ubiquitin carboxy-terminal hydrolase L1 (UCHL1).

1.4 Animal models of PD

Animal models have been used for many decades to study the pathogenic mechanisms underlying diseases and to test possible treatments to cure human disorders (Betarbet et al., 2002). PD was the first brain disease to ever be modelled. Given the heterogeneity of its symptomatology, comprising both motor and non-motor manifestations, and its uncertain aetiology, multiple disease models have been used to study the diverse features (Konnova & Swanberg, 2018). Depending on the scope of the study, some models will be more suitable than others. They can be mainly categorised between genetic and neurotoxin-based. While one provides a disease progression similar to that observed in patients, the latter provides quick and strong neurodegeneration, mimicking the neuronal loss observed in affected individuals as well as some of the symptoms they present (Blandini & Armentero, 2012).

1.4.1 Neurotoxin-based models

1.4.1.1 6-hydroxydopamine (6-OHDA)

It was the first compound used to model PD (Ungerstedt, 1968). This neurotoxin is an analogue of norepinephrine and dopamine able to enter cells through monoamine transporters. Due to its incapacity to cross the blood-brain barrier (BBB), its administration must be intraparenchymal, targeting the striatum, the SNpc or the medial forebrain bundle (MFB) (Dauer & Przedborski, 2003a). To specifically target the dopaminergic system while protecting noradrenergic neurons, before the 6-OHDA injection, an inhibitor of the norepinephrine transporter must be injected (Konnova & Swanberg, 2018).

6-OHDA accumulates in the mitochondria blocking the mitochondrial complex I, generating reactive oxygen species (ROS) which end up causing cell death (Glinka & Youdim, 1995). The severity of the lesion depends on the concentration of toxin infused and on the targeted brain area. Injections directly in the MFB or the SNpc cause an almost complete loss of dopaminergic neurons (90-100%) in just a few hours (12-24h). However, if the injection is performed in the striatum, the neurodegenerative process occurs in a retrograde fashion, taking around 1-3 weeks and achieving a neuronal depletion of 50-70% (Dauer & Przedborski, 2003b; Konnova & Swanberg, 2018). Usually, lesions are performed unilaterally, so to avoid a high death rate of the injected mice. This protocol is useful to perform some behavioural studies such as apomorphine or amphetamine-induced rotations, to assess the efficacy of a treatment through the evaluation of the gait of lesioned animals (Iancu et al., 2005). Even if this model does not replicate the disease progression with the α-synuclein inclusions and multisystem effects observed in affected individuals, it is still very useful to study the main clinical motor symptoms and to test potential new treatments (Betarbet et al., 2002; Blandini & Armentero, 2012; Konnova & Swanberg, 2018).

1.4.1.2 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP is a lipophilic neurotoxin widely used to model PD. Contrary to 6-OHDA, it can be administered systemically due to its ability to cross the BBB. Once in the brain, astrocytes oxidise it generating its toxic form 1-methyl-4-phenylpyridinium (MPP+), which is then released and taken up by dopaminergic neurons through the dopamine transporter (DAT), accumulating in the cytosol. MPP+, as well as 6-OHDA, enters the

mitochondria and blocks its complex I inducing oxidative stress and, consequently, causing cell death. This model is, therefore, useful to understand the interplay between mitochondrial impairment and ROS generation, which end up leading to neuronal death and inflammation (Dauer & Przedborski, 2003b).

MPTP is typically given through intraperitoneal (i.p.) injections, causing different levels of neurodegeneration depending on the dosage. Acute injections of MPTP induce a quick degeneration of dopaminergic neurons and the appearance of motor symptoms. However, these manifestations do not persist for a long time, and in some cases, animals can recover from the motor deficits. The same neurotoxin administered regularly at lower doses causes a more progressive neuronal loss and triggers the accumulation of α-synuclein, resembling the neurodegenerative process observed in patients. Nonetheless, this prolonged dosing does not induce motor manifestations (Konnova & Swanberg, 2018).

1.4.2 Genetic models

Several genes have been associated with the development of the disease. Mutations in *SNCA* and *LRRK2* display an autosomal dominant form, while those found in Parkin, PINK1 and DJ-1 present an autosomal recessive inheritance (Kalia & Lang, 2015). Transgenic animals carrying the most common pathogenic mutations in these genes have been generated and used to study the mechanisms underlying the disease (Y. Lee et al., 2012). However, all of them present limitations in recapitulating salient points observable in patients.

Transgenic rodents for LRRK2 fail to show neurodegeneration or α-synuclein aggregation, not displaying any motor symptoms. Some success has been achieved through the injection of viral vectors enclosing the most common mutated forms of the gene. Even though this model does not replicate many of the features of PD, it is useful to study the function of the gene and its potential role during disease onset and progression.

The same happens with knockout mice for autosomal recessive genes. Even if some of them show alterations in the dopaminergic system and the mitochondrial activity, none of them shows dopaminergic cell loss or related motor symptoms. Interestingly, it has been observed that these mice are more susceptible to

neurotoxins such as MPTP, evidencing the strong interplay in PD of both genetic and environmental factors (Konnova & Swanberg, 2018).

1.4.2.1 α-synuclein-based models

Since *SNCA* was the first gene to be associated with PD (Polymeropoulos et al., 1997) and given the critical role of α-synuclein in disease progression, many models have been developed trying to unveil its pathogenic contribution (Visanji et al., 2016).

Several strains of transgenic mice have been created for this purpose (Y. Lee et al., 2012). Even though not all present phenotypical resemblance to what is observed in patients, they provide an insight of the possible altered mechanisms during disease. In addition, these models are useful to study the interplay between environmental factors, which are known to play an important role in the disease onset, and genetic variants (Betarbet et al., 2002). One of the most popular transgenic strains is the one bearing the A53T mutated human form of α-synuclein. These mice present a robust neurodegenerative phenotype, displaying LB-like inclusions and mitochondrial impairment, which causes motor deficits leading to paralysis and death (Giasson et al., 2002; Martin et al., 2006).

Viral vectors overexpressing α-synuclein

Injection of viral vectors overexpressing α-synuclein in its WT form or one of the known pathogenic mutated forms, has been widely exploited. This model presents some advantages, since it can be induced at adult stages and the resulting neurodegeneration happens progressively due to the protein accumulation, similarly to what is observed in affected individuals. In rodents, exogenous expression of α-synuclein in the SNpc leads to the appearance of protein inclusions both in axons and somas, causing neurodegeneration and motor deficits (Blesa & Przedborski, 2014).

Preformed Fibrils (PFF)

α-synuclein misfolding is believed to be one of the triggers to PD (Chung et al., 2020; Rietdijk et al., 2017). This protein, soluble in normal conditions, adopts a β-sheet conformation under a pathogenic environment, tending to aggregate and form oligomers. These oligomeric forms, together with other proteins, are the major components of Lewy bodies (Spillantini et al., 1997; Stefanis, 2012). The direct injection of these oligomers is, therefore, a way to induce a quick formation of the

inclusions and cause subsequent neuronal loss. This model mimics in an accelerated way what occurs in PD patients, causing some of the molecular alterations and motor manifestations that can be observed in affected individuals. It is better than the model overexpressing the protein since with this strategy, the levels of α-synuclein are not increased, being more similar to physiological conditions (Chung et al., 2020). This model is useful to study how these inclusions create, propagate, and overall affect disease pathogenesis.

1.5 Therapeutic approaches

1.5.1 Currently available treatments

As previously mentioned, most available treatments are symptom-alleviating, being levodopa (L-DOPA) the most popular one. This dopamine precursor can cross the BBB and convert into dopamine, hence restoring its levels. It is usually administered together with dopa-decarboxylase inhibitors (DDCI) to avoid its peripheral conversion, increasing its half-time life and reducing its side effects. Even though it has been used for more than 50 years, it remains the most potent drug to treat PD. Initially, daily doses of 300-600 mg are effective and sufficient to control the symptoms but progressively, the duration of the effects shortens. In addition, sustained levodopa administration causes dyskinesias, hallucinations, and other psychiatric disorders (Parmar et al., 2020; Salat & Tolosa, 2013).

Another approach to tackle the symptoms of the disease is deep brain stimulation (DBS). This therapy, which can be used in combination with pharmacological treatment, consists in the stimulation of different brain regions with electrodes providing a reduction of motor symptoms. Clinical outcomes differ depending on the brain area that receives the stimuli. The internal segment of the globus pallidus (GPi) or the subthalamic nucleus (STN) are the most suitable areas to be targeted since they reduce most of the classical motor symptoms in PD. STN stimulation, in addition, allows a decrease in the drug dosage, reducing their related side effects. Even though the clinical benefits of DBS-treated patients do not differ much from those observed after drug-based treatments, it has some important advantages. Besides the aforementioned potential to reduce the intake of medication also improving its subsequent adverse effects, DBS lessens the wearing-off period in between doses, which can be truly incapacitating. Unfortunately, DBS is not suitable for all patients,

especially for those individuals with strong cognitive decline, and it may also cause some important side effects (Davie, 2008; Perlmutter & Mink, 2006).

These strategies, yet effective in improving patients' quality of life, do not slow nor halt disease progression. Thus, new disease-modifying treatments are required (Wei & Shetty, 2021).

1.5.2 New avenues of treatment

The vast heterogeneity between cases unveils a complex interplay between factors such as age, environment, genetic mutations, and lifestyle among others (Kalia & Lang, 2015). This fact increases the difficulty of appropriately classifying patients to develop tools and protocols for early diagnosis, making it hard to discover potential treatments (Irwin et al., 2013).

Around 60% of dopaminergic neurons in the SNpc are believed to be lost at the onset of symptoms (Cheng et al., 2010). Given the vast neurodegeneration at the time of diagnosis, the only way to rescue the disease is by restoring the neuronal population. Unfortunately, after development, the central nervous system (CNS) largely loses the capacity for self-repair and although neurogenic niches are still present in the adult brain, neurons arising from them are incapable to restore the disrupted circuits due to their restricted distribution and function (Taupin, 2006). New therapies attempting this replacement can be mainly divided between those aiming for cell transplantation, and those relying on direct reprogramming.

1.5.2.1 Cell replacement therapies

PD is a suitable candidate to treat with cell replacement therapies since the neurodegeneration is, at least initially, mainly restricted to dopaminergic neurons in the SNpc. Multiple cell sources have been used in the past for this aim (Pfisterer et al., 2011; Riaz et al., 2002). In seminal studies performed in the nineties, dopaminergic neurons coming from ventral midbrain tissue of human foetuses were implanted into the striatum of PD patients (Spencer et al., 1992). Although the outcome of this study was controversial, it provided the proof of concept about the feasibility of this approach as a therapy against PD and other brain diseases. The main drawbacks to using foetal tissue are the limited availability and the difficulty of standardisation, therefore new sources must be found. Many other cell sources have

been tested since that study (Evans et al., 2012; Grealish et al., 2014; Hallett et al., 2015), although none of them resulted in significant improvements to the patients.

More recently, studies have focused on the use of human embryonic stem cells (hESCs) (Grealish et al., 2014) or induced pluripotent stem cells (iPSCs) (Swistowski et al., 2010). These cells can be easily expanded *in vitro*, therefore solving the problem of availability. Moreover, the differentiation protocols of the stem cell-derived neurons can be strictly controlled and standardised to always obtain the same product. Currently, iPSCs are the most used source since it would consist in an autologous transplantation, preventing a possible immune rejection while also avoiding the ethical concerns presented by the usage of human embryos. The versatility of iPSCs, which can potentially differentiate into any cell type, makes them a promising source to treat diseases where multiple cell populations are affected. So far, numerous preclinical studies have successfully transplanted induced dopaminergic neurons in PD animal models without alarming side effects. However, several concerns arise in these therapies, the most worrying being the high risk of tumorigenesis. Despite grafted cells undergo sorting processes before transplantation, undifferentiated cells can remain within the bulk and end up forming tumours. Another possibility is that once in the host tissue, some transplanted cells dedifferentiate, becoming tumorigenic. For this reason, it is essential to meticulously control the homogeneity and stability of the injected cells. Successful transplants could represent an important improvement in patients' quality of life since, potentially, implanted cells would release dopamine normally, avoiding pharmacological-induced side effects. However, grafted dopaminergic neurons would mitigate mainly the motor symptoms of the disease, having limited to no effect on nonmotor symptoms (Parmar et al., 2020).

1.5.2.2 Direct reprogramming

Alternatively, the transdifferentiation of somatic cells represents another potential strategy to treat PD. This approach consists in the conversion of differentiated cells into another cell type without going through a pluripotency state. It offers some advantages compared to cell therapy such as no risk of tumorigenesis, due to the evasion of the pluripotency stage. Moreover, conversion occurs fast, opposite to long differentiation protocols, and because it is patient-specific, no immune reaction or ethical concerns arise (Sekiryu & Matsuda, 2021). Many studies have exploited this approach in the last thirty years. The first evidence of direct reprogramming was

obtained in 1989 when Weintraub and colleagues achieved the reprogramming of mouse embryonic fibroblasts (MEFs) into myoblasts with only the overexpression of the transcription factor (TF) MyoD (Weintraub et al., 1989). Since then, the identification of other TFs that could result in transdifferentiation has been key to applying this technique to other cell types. In most cases, the activation of one or a few master genes is sufficient to push the target cell into a specific cell fate. This induction is usually due to the overexpression of TFs involved in the specific lineage of the desired cell population (Kelaini et al., 2014).

For this approach to be successful, many factors must be considered beforehand, such as the target cell, the most suitable vector type, and the reprogramming factors to be used (H. Wang et al., 2021).

The starting cell type is the most crucial element in the conversion process since many features are determined by it, such as the epigenetic landscape or the metabolic state. When trying to convert into neurons, metabolism is one of the big hurdles to overcome since, opposite to proliferating cells or astrocytes, neurons rely on oxidative phosphorylation to obtain energy. This metabolic shift may generate ROS which in turn, may induce ferroptosis and lead to cell death. Therefore, cell death is one of the limiting factors of direct conversion to neurons (Gascón et al., 2016). Reprogramming, same as differentiation protocols, take as a baseline the natural developmental course, thus the target population will be chosen considering the lineage proximity to the desired cell type. To obtain neurons, most studies have targeted astrocytes, given the fact that they arise from the same progenitors and that they are vastly available in the brain tissue. This cell population is characterised by a large heterogeneity in gene expression and chromatin structure depending on the brain region and the age of the astrocyte (Boisvert et al., 2018; Herrero-Navarro et al., 2021; Lattke et al., 2021). These factors must be considered as they could affect the outcome of the transdifferentiation.

To deliver the reprogramming factors to the target cells, most studies take advantage of viral vectors. Depending on the tissue and the cell population that is targeted, the length of the insert, and the desired expression of the transgene, one vector will be more suitable than others. The most used viral types for gene delivery in the CNS are lentiviruses (LVs) and adeno-associated viruses (AAVs). LVs have the capacity to enter the nucleus and integrate the transgene into the host genome of both proliferating and non-proliferating cells, leading to a stable expression even in

dividing cells (Naldini et al., 1996). Furthermore, they display a high transduction efficiency and can hold up to 10kb of foreign DNA (Thomas et al., 2003). AAVs, on the contrary, have a very limited capacity (~5,2kb) and are unable to integrate into the host genome, not being suitable when a lasting expression in dividing cells is desired. Multiple serotypes of AAVs have been identified, eight of them endemic to humans, which present different tropisms and infection efficacies depending on the target cell population. These two types of viral vectors are widely used in preclinical and clinical studies due also to their low immunogenicity (Davidson & Breakefield, 2003). Nonetheless, an immune response can still be induced following the transgene expression (Robbins & Ghivizzani, 1998; D. Wang et al., 2019).

As for the reprogramming factors, some are common in almost all differentiation protocols since they are key to obtaining cells from a particular lineage. For neurons, for example, achaete-scute homolog 1 (*ASCL1*) and neurogenin 2 (*NEUROG2*) represent the main proneural factors. These highly conserved TFs are central regulators of neurogenesis during development, and although they alone can push the fate of many cell types into induced neurons (iNs), the obtained subtypes can differ (Masserdotti et al., 2016). In cultured astrocytes, while *Neurog2* pushes towards a glutamatergic fate, *Ascl1* induces a GABAergic subtype (Heinrich et al., 2010, 2011; Masserdotti et al., 2015). Interestingly, when overexpressed in MEFs, *Ascl1* triggers mainly a glutamatergic fate while *Neurog2* is unable to induce any conversion, evidencing the importance and the effects on the outcome of the cellular context (Gascón et al., 2017). Another important advantage presented by transdifferentiation is the fact that, contrary to what happens in iPSCs differentiation, it can be successfully performed also on quiescent cells (Fishman et al., 2015). That is the case for many cell types such as astrocytes or pericytes. However, even if much more is known regarding the molecular cascades inducing specific neuronal subtypes, there is still much to be unravelled to generate iNs with a specific phenotype through direct reprogramming strategies.

Moreover, post-translational modifications of the overexpressed TFs may also influence the capacity of the reprogramming factors to do their function. Taking as an example the two master regulators previously mentioned, ASCL1 and NEUROG2, only in their dephosphorylated forms they are able to induce the expression of the target genes. In proliferating cells, cyclin-dependent kinases restrain the proneural activity of these TF by preventing the recruitment of chromatin remodellers, therefore

not allowing them to access their target genes (Ali et al., 2014; Gascón et al., 2017; Hardwick & Philpott, 2015).

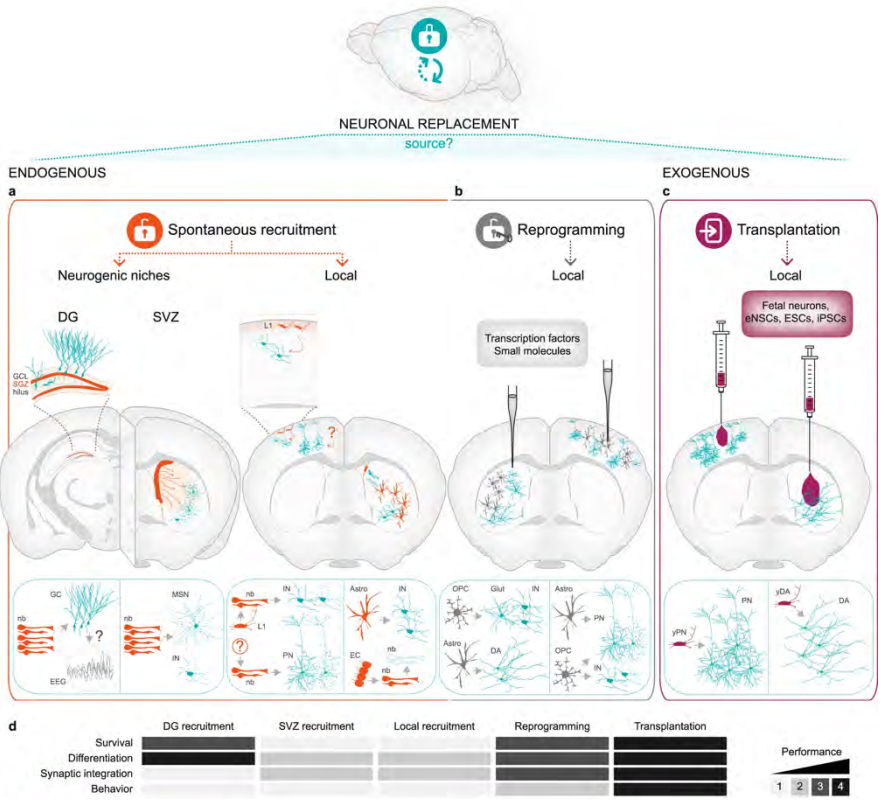

Figure 1.4 Neuronal replacement strategies. Schematic representation of the cell sources for neuronal replacement in the brain and the outcomes achieved with the different approaches. Adapted from (Grade & Götz, 2017). *Abbreviations: Astrocytes (Astro), dopaminergic neurons (DA), dentate gyrus (DG), ependymal cells (EC), electroencephalogram (EEG), embryonic neural stem cells (eNSCs), granule cells (GC), granule cell layer (GCL), glutamatergic neurons (Glut), Huntington's disease (HD), interneurons (IN), neocortex layer 1 (L1), medium spiny neurons (MSN), neuroblasts (nb), neuronal ceroid lipofuscinoses (NCLs), oligodendrocyte progenitor cells (OPC), projection neurons (PN), subgranular zone (SGZ), subventricular zone (SVZ), temporal lobe epilepsy (TLE), young dopaminergic neurons (yDA), young projection neurons (yPN).*

1.5.2.3 Neuronal direct reprogramming in vivo

The possibility to convert non-neuronal cells to neurons *in situ* represents a very powerful approach to tackling brain injuries and neurodegenerative diseases. However, while many studies *in vitro* succeeded in converting non-neuronal cells into

functioning iNs (Berninger et al., 2007; Caiazzo et al., 2011; Heinrich et al., 2010), to do so in an *in vivo* setting has proven more challenging. This is partly due to the local environment and the heterogeneity of the cell populations depending on the targeted brain area. Therefore, understanding the pathways involved in the differentiation to specific lineages, and the influences that the brain structure has on the outcome of the conversion is crucial to optimise this strategy (Barker et al., 2018).

Diseased or injured brain present high levels of astrogliosis and neuro-inflammation. During these processes, glial cells proliferate and become more plastic, which may facilitate their conversion into neurons, although they are highly heterogeneous. However, the alteration of the brain compartment induces a high production of ROS, generating a very oxidative situation which may limit neuronal reprogramming by causing cell death (Gascón et al., 2017). This fact could be one of the reasons behind the disparity on the yield of conversion between *in vitro* and *in vivo* studies.

As aforementioned, one of the most popular cell types to be targeted when trying to achieve transdifferentiation into neurons is astrocytes. They are a suitable population since they are the most abundant cell type in the brain parenchyma, ubiquitously distributed, and share a common origin with neurons (Haydon & Carmignoto, 2006). All these characteristics may ease their transdifferentiation into that specific cell type. In fact, multiple studies have reported astrocyte-to-neuron conversion to happen successfully *in vivo* (Guo et al., 2014; Mattugini et al., 2019; Rivetti Di Val Cervo et al., 2017). Astrocytes' characteristics differ depending on a series of factors such as age, brain region or maturation state. Understanding their heterogeneity could provide us with a better comprehension of the outcome disparities observed in different studies. Recent publications have been focusing on unveiling the chromatin structure and gene expression profiles of the astrocytes from various brain regions and ages (Boisvert et al., 2018; Herrero-Navarro et al., 2021; Lattke et al., 2021). These studies identified region-specific signature transcriptomes of astroglial cells, besides describing extensive changes occurring during maturation and ageing. This diversity must be considered since it could affect the outcome of the transdifferentiation process.

One of the diseases which could benefit from treatments based on direct reprogramming approaches is PD. Several studies have been published in the last

decade exploring the possibility to convert different glial types into induced dopaminergic neurons (iDANs) in order to counteract the motor symptoms presented by affected individuals (Qian et al., 2020; Rivetti Di Val Cervo et al., 2017; Torper et al., 2015). In 2015, the combination of TFs *Ascl1*, *Lmx1a* and *Nurr1* managed to convert striatal oligodendrocyte precursor (NG2) cells and astrocytes into iNs (Torper et al., 2015). The effect of these TFs, which were encoded in flip-excision (FLEX) adeno-associated viruses (AAV), was analysed through a reporter GFP virus co-injected in NG2-Cre, targeting oligodendrocyte precursors, or in GFAP-Cre mice, targeting astrocytes. Analysis of GFAP-Cre mice performed 6 weeks post-injection (w.p.i.) showed how these three TFs, which were successful in converting mouse fibroblasts into iDANs *in vitro* (Caiazzo et al., 2011), were unable to do so in the proposed animal models. Newly formed neurons mainly converted into GABAergic and glutamatergic neurons (Torper et al., 2015). In a follow-up paper by the same lab, besides corroborating their findings through electrophysiological recordings, they assessed the influence of the targeted brain region on the iNs neuronal subtype by injecting their reprogramming factors in the SNpc of NG2-Cre mice. Even though plenty of cells expressing their TFs were detected, none of them was co-expressing tyrosine hydroxylase (TH), a common marker for DA neurons, obtaining therefore, similar results to those observed for striatal NG2 cells (Pereira et al., 2017). That same year, a lab in Sweden proposed the use of another combination of factors to reprogram astrocytes into iDANs. On this occasion, they were injecting a LV encoding for NEUROD1, ASCL1, LMX1A and miR218, into the striatum of GFAP-tTa mice previously lesioned with 6-OHDA. With this combination, they succeeded in converting astrocytes into iDANs after 13 weeks. However, the yield of the reprogramming was quite low (Rivetti Di Val Cervo et al., 2017).

Even though the overexpression of TFs is the most common approach to achieve direct reprogramming, other molecules or constructs can also succeed in doing so. That is the case of the short hairpin RNA (shRNA) against *Ptbp1*, which downregulates the expression of the RNA-binding protein PTB in astrocytes inducing a neuronal fate (Qian et al., 2020). Ten weeks after the injection in the SNpc of an AAV encoding for this silencer, 80% of infected cells showed a neuron-like phenotype, expressing the mature neuronal marker NEUN besides multiple neuronal subtype markers including TH, which accounted for the 30-35% of iNs after 12 weeks. Authors partly ascribe this ease to become iDANs to the higher expression of genes related to the dopaminergic fate in astrocytes from that specific area, as even though the number

of iNs generated after PTB silencing in different brain areas was similar, TH-positive cells were only observed in mice injected in the SNpc (Qian et al., 2020).

However, obtained results in this field of study must be thoroughly controlled, as ectopic expression of the transgenes in endogenous neurons could generate misleading results (Götz & Bocchi, 2021). As aforementioned, AAVs are one of the most used vectors to drive the expression of the reprogramming factors in transdifferentiation studies. For brain analyses, since the targeted region is dependent on the disease or neuronal population that is being studied, reprogramming factors are usually injected intraparenchymal (Davidson & Breakefield, 2003). Because of this, the tropism of the different serotypes is not important and, therefore, it could seem that the election of the serotype is almost not relevant. The most common serotypes used in transdifferentiation studies targeting the brain are rAAV2 and rAAV5. Nonetheless, rAAV2 preferentially transduces neurons while rAAV5 is able to do so both in neurons but also in astrocytes, even if less efficiently in the latter one (Davidson et al., 2000; Götz & Bocchi, 2021; Petrosyan et al., 2014; Xu et al., 2001). In most studies a specific population of cells is targeted by means of cell-specific promoters or by combining FLEX-AAVs with transgenic models expressing a recombinase in a particular cell type. These systems should restrict transgene expression to the target population, nonetheless many studies have reported leakiness in other cell types, especially when using high titre AAVs. Comparison of the specificity of the most used serotypes by GFAP-GFP expression analysis, revealed that even if at shorter timepoints AAVs may seem specific, off-target expression can be observed already after two weeks (L. L. Wang et al., 2021). Recombinant-dependent AAVs can also present an ectopic expression of the transgene due to a recombination during plasmid production or to a low expression from the open reading frame (ORF) (Fischer et al., 2019). Therefore, establishing robust models and vectors is crucial to distinguish truly reprogrammed cells from endogenous neurons.

In fact, several papers published in the last year failed to replicate some of the outstanding results describing astrocyte-to-neuron conversion (Chen et al., 2022; Hoang et al., 2022; Leib et al., 2022; L.-L. Wang et al., 2021; Xie et al., 2022). A recent study published in Cell put in doubt most of the previously published work by using a strict genetic tracing of the targeted cell population, in that case, astrocytes (L.-L. Wang et al., 2021). To that end, authors used *Aldh1l1*-Cre^{ERT2} mice (Srinivasan et al., 2016), which selectively targets astrocytes, crossed with Cre-reporter lines

R26R-tdTomato (Madisen et al., 2010) or R26R-YFP (Srinivas et al., 2001) in order to test previously reported results. With this system they claim to fail in obtaining iNs with the overexpression of *Neurod1* or by downregulating of PTB, as all cells positive for the reporter and neuronal markers did not co-express the fluorophore of the strain. Authors report that promotor-specific or FLEX-AAVs used for these experiments were leaky, and that this could have led to generate misleading results (L. L. Wang et al., 2021). This paper got the rebuttal of some of the authors, that attributed these findings to the fact that they were using very high titres of the viruses, therefore observing artifacts (Xiang et al., 2021). However, many other papers arguing against previously described results have been published, especially reporting the incapacity to achieve conversion with PTB downregulation (Chen et al., 2022; Hoang et al., 2022; Leib et al., 2022). Therefore, it is essential to have very stringent controls so to verify that iNs are indeed newly converted neurons and not endogenous neural cells.

2 AIM OF THE WORK

Even though huge efforts have been made to find alternative therapies to tackle Parkinson's disease, currently available treatments still rely on drug intake and aim only at alleviating the main motor symptoms. Direct reprogramming offers the possibility to treat PD patients by replenishing the dopaminergic population, mitigating the motor symptoms and the need of pharmacological treatments. However, after a thorough literature research, it becomes evident that the mechanisms underlying this process are still poorly understood. In this work, we use *Aldh1l1*-Cre^{ERT2}/Ai9 mice to test the capacity of the single proneural factor *Ascl1* to reprogram *in vivo* mouse striatal astrocytes. With this robust system in combination with a stringent LV vector, we aim to establish strong grounds on astrocyte-to-neuron conversion *in vivo* given the large amount of conflicting literature. Furthermore, we intent to determine if immature cells are more amenable to neuronal conversion than adult astrocytes while assessing environmental influences on the outcome. The goal is to better understand which characteristics of the recipient cells affect the process of neuronal reprogramming to optimise the approach towards obtaining induced dopaminergic neurons.

Still, this approach would not halt nor slow disease progression. Due to the long presymptomatic phase of PD, diagnosis often occurs when most dopaminergic neurons are already lost and, therefore, most proposed approaches are directed to reconstitute this neuronal population. Multiple compensatory mechanisms are believed to converge to temporarily preserve the homeostasis during this disease stage. In this project, we explore if one of the contributors in this event is the remodelling of the axonal dopaminergic bushes from spared neurons. To examine if reinnervation is stimulated after a partial lesion of the nigrostriatal system, we combine a local injection in the dorsal striatum of the neurotoxin 6-OHDA with various labelling methods to identify the responsible neuronal population. First, with a strong genetically inducible AAV and then, with a fluorescent retrograde tracing method, we plan to unravel if, and if so, which subtype and how dopaminergic neurons adapt to progressive neurodegeneration. To robustly obtain different levels of neuronal loss we plan to develop a genetically inducible neurodegenerative method based on the overexpression of the Caspase-3, which could, in the future substitute the 6-OHDA. The identification of signals allowing such process could represent an alternative therapeutic target to delay disease progression at early stages of the disease.

3 RESULTS

3.1 *In vitro* conversion of astrocytes into iNs after ALiN and Ascl1 expression

3.1.1 ALiN generates transdifferentiated iNs with a GABAergic phenotype

In our first try at astrocyte-to-neuron conversion, mouse primary astrocytes were infected with a multicistronic inducible LV containing three TFs crucial to induce neuronal development and differentiation towards a dopaminergic fate. *Ascl1*, *Lmx1a* and *Nurr1* were cloned under a tetO promoter (LV-tetO-ALiN), which provides a very strong expression of the transgenes only in the presence of doxycycline (doxy). We wanted to assess if this combination of TFs, successful in converting fibroblasts into iDANs (Caiazzo et al., 2011), was also able to transdifferentiate cultured primary astrocytes into that specific neuronal subtype. Postnatal WT mice from P0 to P3 were used to obtain pure cortical astrocytic cultures, as shown by the absence of the early neuronal marker β-tubulin III (Tuj1) in an initial characterisation of our culture, and the colocalization between DAPI with astrocytic markers SOX9 and GFAP (Fig. 3.1 A). These cells were then infected with the LV-tetO-ALiN together with an LV-rtTa and left in culture for 10 and 20 days. During this time, cells were kept in a neuronal induction medium, which contains doxy, and small molecules and supplements that help the conversion and the maintenance of neurons (Fig. 3.1 A). The success and efficiency of the infection were followed through the staining against LMX1A, one of the TF encoded in our LV. After 20 days, most cells expressing the virus were effectively converted into iNs, as demonstrated by the colocalization between LMX1A and Tuj1 91,2±6,1% (Fig. 3.1 B). Quantification of the cells positive for Tuj1 revealed that around 30,6±6,4% of astrocytes successfully converted into iNs. Remarkably, 25±4,16% were positive for the microtubule-associated protein 2 (MAP2), a marker of mature neurons (Fig. 3.1 B, C).

After demonstrating that ALiN was successful in reprogramming astrocytes into iNs, we wanted to examine if they were acquiring any subtype identity. Of the three TFs encoded in the LV, two (*Lmx1a* and *Nurr1*) are well-known for their involvement in the differentiation of dopaminergic neurons during development (Andersson et al., 2006; Deng et al., 2011; Jankovic et al., 2005; Zetterström et

al., 1997). Therefore, and given the capacity of these factors to reprogram non-neuronal cells into iDANs in the past (Caiazzo et al., 2011), staining for dopaminergic markers was performed. Unfortunately, immunofluorescence analysis showed an almost complete absence of TH expression in our cultures after 20 days (1,5±0,4%), indicating that ALiN is not able to push astrocytes towards a dopaminergic fate. On the contrary, immunostaining against GABA revealed that iNs mainly acquired a GABAergic neuron-like phenotype (83±8,4%) (Fig. 3.1 B, D). Overall, these results suggest that our multicistronic LV containing *Ascl1*, *Nurr1* and *Lmx1a* is effective in transdifferentiating primary mouse astrocytes into iNs, although newly converted neurons seem to mainly become GABAergic neurons.

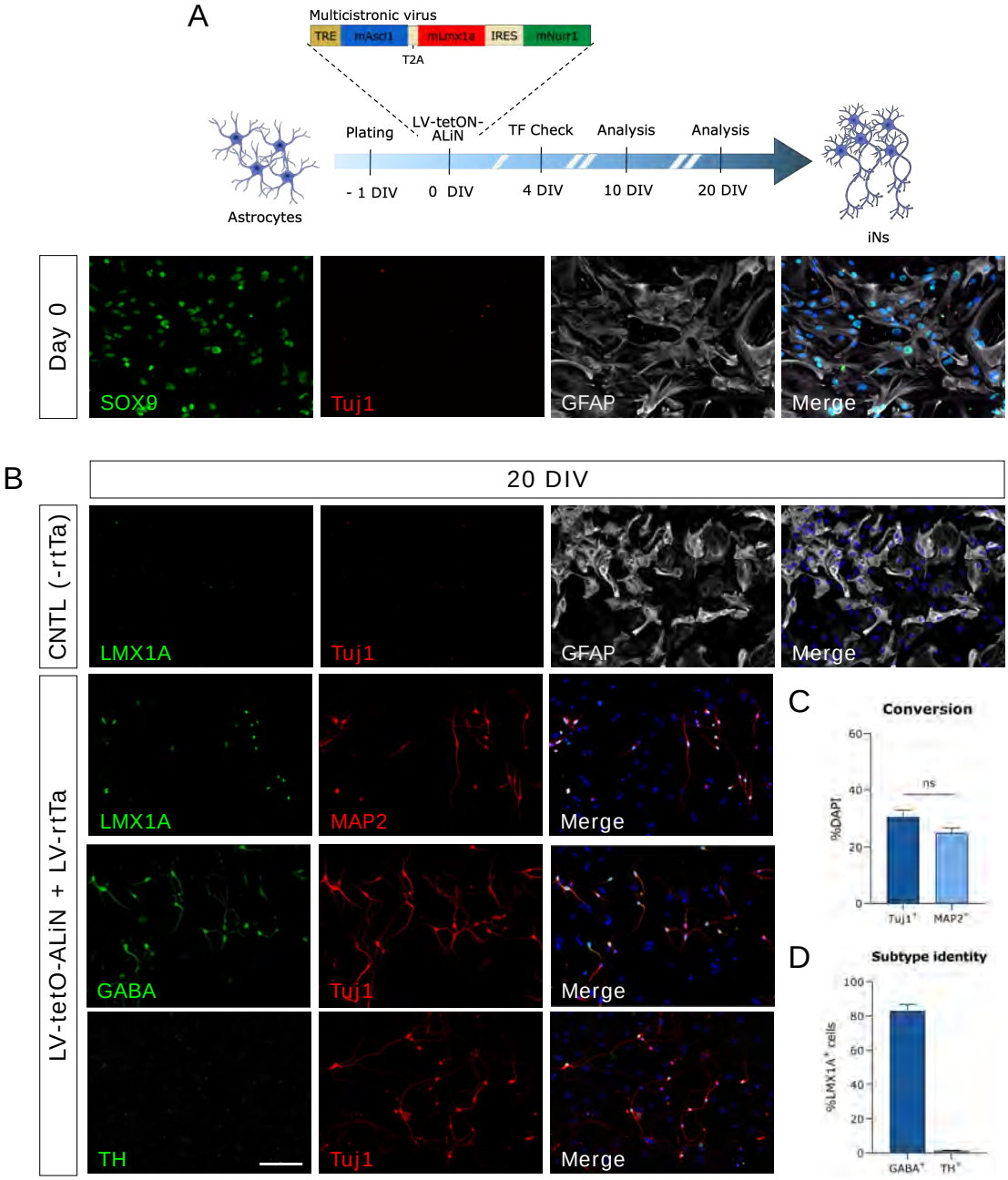

*Figure 3.1. ALiN is able to convert astrocytes into iNs but not iDANs. Schematic representation of the experimental design. Characterisation of the starting culture through immunostaining against astroglial markers (A). Immunofluorescence images showing astrocyte transdifferentiation upon ALiN infection, becoming iNs positive for several neuronal markers (TuJ1 30,6±6,4%; MAP2 25±4,1%) (B-C). Newly formed neurons tend to become GABAergic-like cells (GABA 81,2±3,1%; TH 1,45±0,4%) (GABA/TH in green, TuJ1 in red, scale bar 250µm) (B,D). n=3 independent experiments. Error bars represent ± SEM. (****p<0.0001) Statistical test: Unpaired Student t-test.*

3.1.2 *Ascl1 alone is enough to efficiently convert cultured mouse astrocytes into iNs*

Given that the identity of the newly reprogrammed cells obtained after using dopaminergic-specific TFs was not dopaminergic, we decided to focus on the process of reprogramming itself. *Ascl1* has been identified as a master regulator in the differentiation program to become a neuron (Bertrand et al., 2002). Because of this, many differentiation protocols, and strategies to induce direct reprogramming have been using it in their mixes of factors (Karow et al., 2012; Pang et al., 2011). It has been demonstrated that *Ascl1* alone is able to generate iNs from human and mouse ESCs and fibroblasts (Chanda et al., 2014). We wanted to examine the capacity of conversion presented by this TF when reprogramming mouse primary astrocytes and which outcome we obtained on this occasion. Since from the start we were planning on conducting experiments *in vivo*, we designed a construct which could specifically target astrocytes in that experimental setting. To be able to compare the results without having any added variables in our system, we decided to use the same tools for the experiments on cultured astrocytes. Our construct, LV-FLEX-*Ascl1*-V5-GFP, encodes ASCL1 under the strong constitutive promoter human elongation factor 1 alpha (EF1α), allowing the expression of the transgene only in Cre-expressing cells, as shown by the absence of GFP signal in control cells non-infected with the LV encoding for the recombinase (Fig. 3.2 A). For this set of experiments, astrocytes were co-infected with LV-FLEX-*Ascl1*-V5-GFP and LV-Cre and kept in culture for 10 and 20 days with the same neuronal induction medium used for the experiments with LV-tetO-ALiN. Twenty days after the infection, almost all the cells expressing the virus (GFP^+) had efficiently converted into iNs (91,6±3,9%) (Fig. 3.2 A), achieving a rate of conversion of around 40% (37,6±5,7%) as shown by $Tuj1^+$ cells (Fig. 3.2 A, B). This result resembles the ones observed in other studies where *Ascl1* alone was able to differentiate mouse astroglial cells into iNs (Berninger et al., 2007; Heinrich et al., 2010). These newly converted neurons, which acquired a more mature phenotype overtime ($MAP2^+$), in most cases also displayed a GABAergic marker (83±8,4%), indicating that, similar to what we observed in astrocytes treated with LV-tetO-ALiN, they tend to adopt an inhibitory-like phenotype (Fig. 3.2 A, D). Immunostaining for TH revealed that none of the iNs generated with this construct acquired a dopaminergic fate.

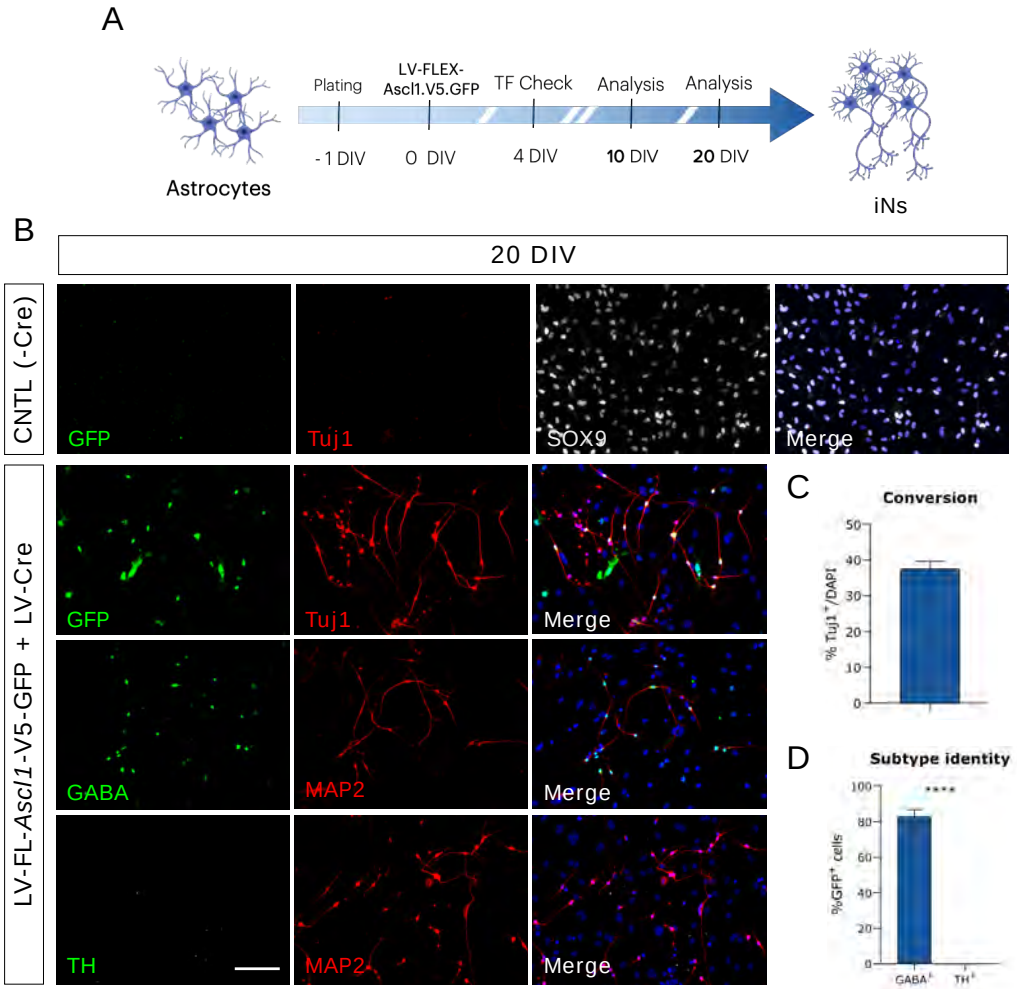

*Figure 3.2. Ascl1 expression induces iNs, mainly of GABAergic identity. Experimental time line (A). Immunofluorescence analysis reveal an efficient conversion of cells expressing Ascl1 towards a neuronal fate (B). Quantification of the cells positive for neuronal marker Tuj1⁺ (37,6±5,7%) (C). iNs adopt mainly a GABAergic identity, as shown by the expression of GABA and the absence of dopaminergic marker TH (D). Scale bar 250µm. n=3 independent experiments. Error bars represent ± SEM. (****p<0.0001) Statistical test: Unpaired Student t-test.*

These observations indicate that *Ascl1* is the main driver for both the conversion and the subtype identity iNs acquire after its overexpression in primary astrocytes. This outcome is in line with some published results reporting that the addition of *Lmx1a* and *Nurr1* in the mix of factors to differentiate mouse or human ESCs into iDANs, did not increase the percentage of iDANs obtained nor the tendency to become one when compared to iNs obtained after the infection with only *Ascl1* (Ng et al., 2021).

3.2 *Ascl1* efficiently converts astrocytes to iNs in early postnatal mice

After successfully converting mouse primary astrocytes into iNs *in vitro,* we decided to move into an *in vivo* setting. However, significant differences have been observed both in the outcome and the yield when attempting to translate the procedure *in vivo*. Since the gene expression profile of astrocytes suffers significant changes with maturation and age (Boisvert et al., 2018; Lattke et al., 2021), we thought that one of the reasons underlying these differences could be the maturation state of the astrocytes. For this purpose, we decided to examine the transdifferentiation process in a system that resembled as much as possible the *in vitro* one but having the influence of the local environment. To that end, postnatal mice of two days of age (P2) were stereotaxically injected in the striatum with our reprogramming factors. For these and all *in vivo* experiments regarding direct reprogramming, we used a mouse model that allowed us to target specifically all astrocytes. *Aldh1l1*-Cre^{ERT2} mice (Srinivasan et al., 2016), which express an inducible Cre recombinase under a pan-astrocytic promoter, were crossed with a ROSA26-loxSTOPlox-tdTomato (Ai9) reporter line (Madisen et al., 2010), so to be able to follow the fate of the reprogrammed astrocytes without relying on the expression of a reporter virus. This strain is the most stringent model available to target astrocytes, as demonstrated by the immunofluorescence analysis of *Aldh1l1*-Cre^{ERT2}/Ai9 brain sections showing the colocalization between tdTomato and the astrocytic markers SOX9 and GFAP (Fig. 3.3). In addition, to verify that the Cre is restricted only in astrocytes, we injected a reporter GFP virus encoded in a FLEX LV like the one containing *Ascl1*. Immunofluorescence images confirm the specificity of the system in targeting only astrocytes as GFP expression is restricted to tdTomato^{+} cells (Fig. 3.3).

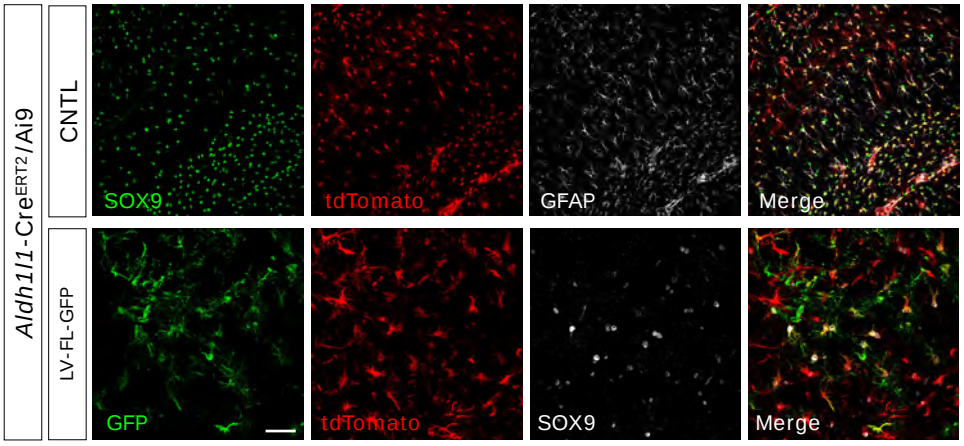

Figure 3.3. Aldh1l1-Cre^{ERT2}/Ai9 express the inducible Cre specifically in astrocytes
Representative immunofluorescence images showing co-localisation between tdTomato from the mouse strain with the astrocytic markers GFAP and SOX9 (SOX9 in green, tdTomato in red, GFAP in grey). Images evidencing the restricted expression of GFP in astrocytes after injection with LV-FL-GFP (GFP in green, tdTomato in red, SOX9 in grey, scale bar 50µm).

Since the results obtained *in vitro* using LV-tetO-ALiN, did not differ much from those presented by LV-FL-*Ascl1*-V5-GFP infected astrocytes, we decided to move on with this second construct, as *Ascl1* alone is able to push astrocytes into a neuronal fate in an efficient manner. Thus, we questioned if *Ascl1* maintains *in vivo* the same capacity to generate iNs with an efficiency comparable to the one observed *in vitro*. As previously mentioned, P2 *Aldh1l1*-Cre^{ERT2}/Ai9 were injected in the striatum with LV-FLEX-*Ascl1*-V5-GFP. After the surgery, tamoxifen was administered for three consecutive days to induce the expression of the Cre recombinase and therefore, of our transgene (Fig 3.4 A).

Immunofluorescence analyses performed after 2, 4 and 6 weeks revealed a progressive conversion of infected astrocytes into iNs, confirming the results observed in cultured cells. Two weeks after the injection, a few GFP$^+$/tdTomato$^+$ cells also expressed the neuronal progenitor marker doublecortin (DCX$^+$) (Fig 3.4 B). At 4 w.p.i. though, tdTomato$^+$ cells infected with our construct (GFP$^+$) displayed significant morphological changes. We found that of these, none of them was positive for DCX, instead, 50,7±10% were expressing the neuronal nuclear protein (NEUN), a mature neuronal marker, indicating a successful and highly efficient conversion of astrocytes into iNs (Fig 3.4 C-D). This percentage increased over time, reaching a 75,8±5,2% of NEUN$^+$ after 6 w.p.i.(Fig. 3.4 E-F).

Next, we examined if newly formed neurons were expressing some subtype-specific marker. As previously said in the introduction, astrocytes present region-specificity, displaying different gene expression profiles and chromatin states depending on the brain area they are in (Boisvert et al., 2018; Herrero-Navarro et al., 2021; Qian et al., 2020). The shared origin of astrocytes with neurons creates a bias on the outcome of reprogramming, as when converting into neurons, they tend to acquire the specific subtype present in that brain region (Herrero-Navarro et al., 2021). In the striatum, the most abundant cell population is medium spiny neurons (MSN), representing around 90% of the total neurons in that area (Arlotta et al., 2008; Precious et al., 2016). Therefore, to assess if these iNs were acquiring an MSN identity, we stained for COUP TF1-interactingprotein 2 (CTIP2), a TF crucial in the early differentiation of MSNs (Arlotta et al., 2008). Results from the immunofluorescence analysis show a high percentage of CTIP2 expressing cells which increase over time being 73,1±6,7% at 4 w.p.i. (Fig. 3.4 C-D) and 80,5±6,9% at 6 w.p.i (Fig. 3.4 E-F).

Overall, these results indicate that overexpression of *Ascl1* in striatal immature astrocytes leads them to reprogram into adopting an identity resembling MSNs.

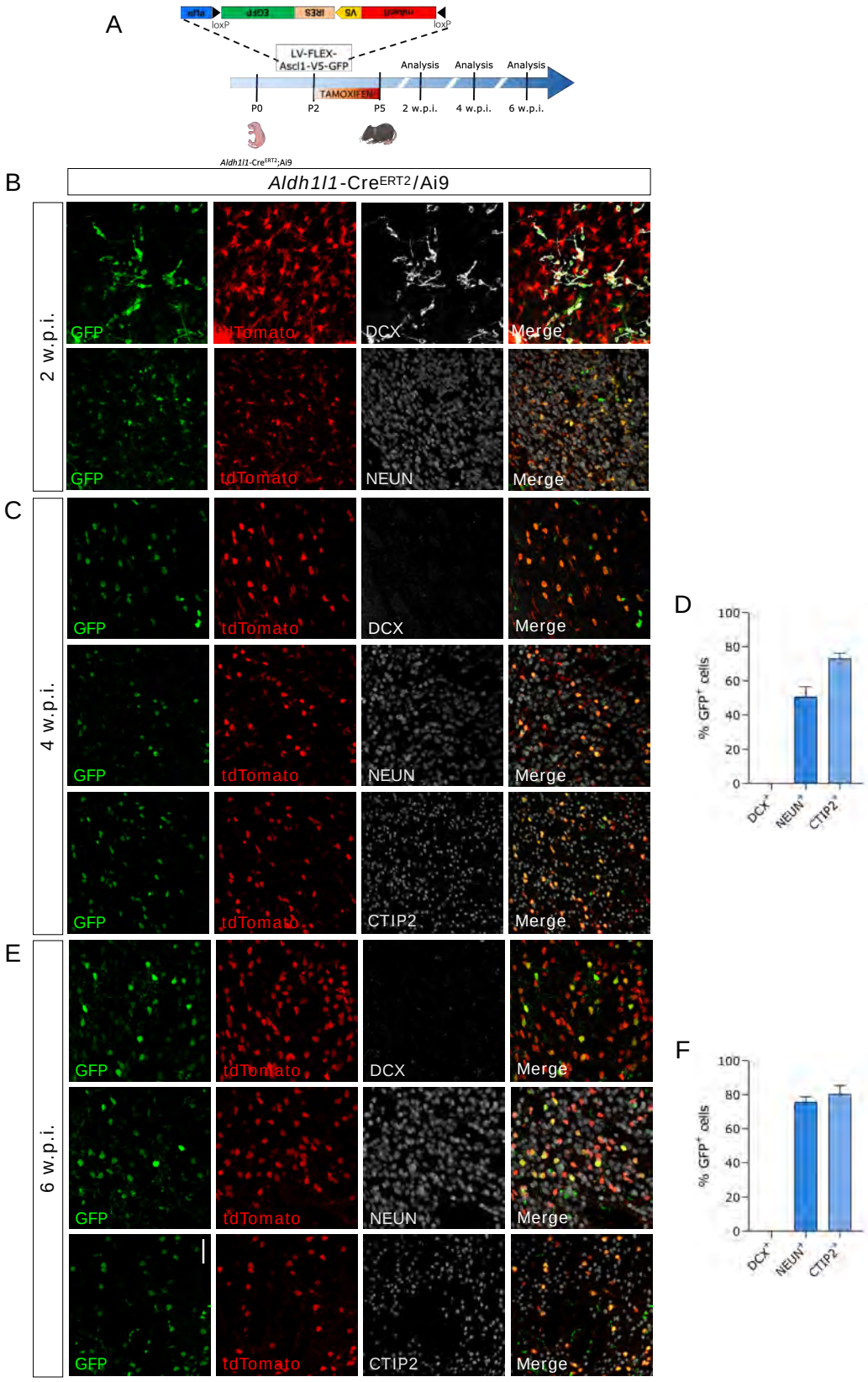

Figure 3.4. Immature astrocytes gradually convert into iNs upon Ascl1 expression.
Immunofluorescence images of striatal sections from mice injected at P2 with LV-FL-Ascl1-V5-GFP performed at several time points show an efficient conversion of astrocytes into mature neurons (NEUN$^+$/DCX$^-$) (B,C,E). Quantification of the percentage of GFP$^+$ positive cells expressing DCX and NEUN (D,F). iNs acquire a MSN-like identity, as shown by CTIP2 expression (C-F). Scale bar, 50μm. (n=3 mice) Error bars represent ± SEM.

3.3 Mature striatal astrocytes become MSN-like cells following *Ascl1* expression

After demonstrating that *Ascl1* alone is able to efficiently convert early postnatal astrocytes into iNs *in vivo*, we wanted to repeat the same experiments but this time targeting adult astrocytes to assess the role of the maturation state of the astrocyte in the reprogramming process. To do that, *Aldh1l1*-Cre^{ERT2}/Ai9 mice of 2-3 months of age were injected stereotaxically in the striatum with the FLEX LV encoding for ASCL1 (LV-FLEX-*Ascl1*-V5-GFP). As previously mentioned, this mouse model is very specific for targeting astrocytes. This line, in addition, due to the expression of the tdTomato in all astrocytes, makes it easier to follow the fate of the infected astrocytes, since they will become GFP$^+$/tdTomato$^+$.

To be able to compare the transdifferentiation process with the one observed in early postnatal mice, we decided to perform the analyses after 2, 4 and 6 w.p.i. like in that experimental setting. We noticed that infected astrocytes undergo gradual morphological alterations in a time-dependent manner, highlighting the progressive transition through increasing maturation states (Fig. 3.5).

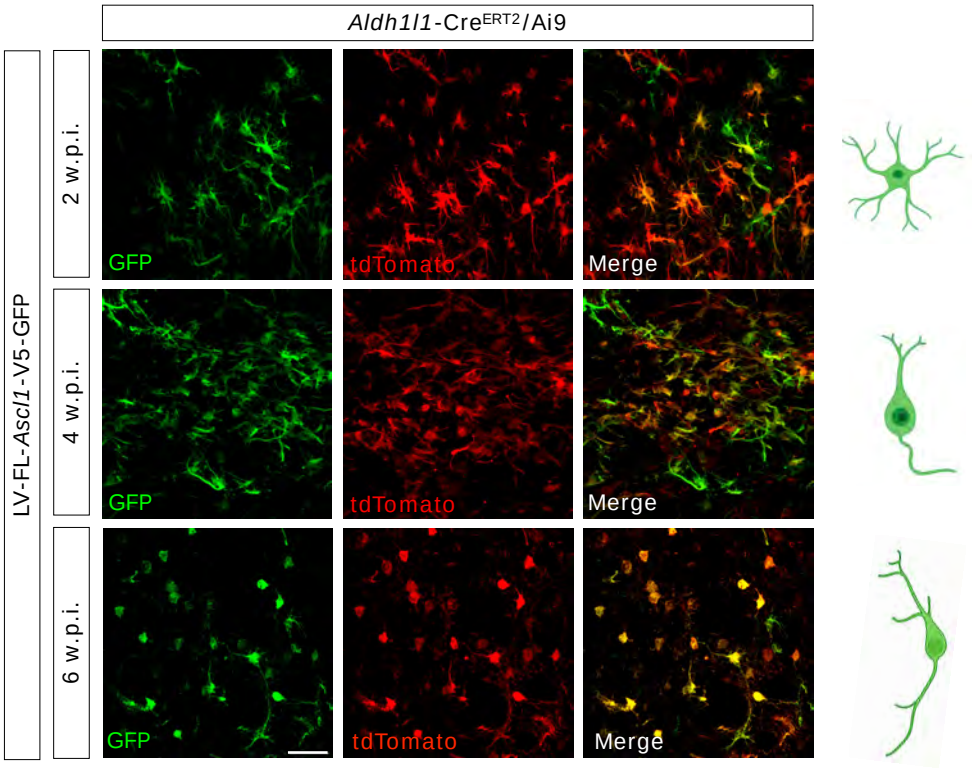

Figure 3.5. Morphological changes of infected striatal astrocytes. Representative immunofluorescence images showing the morphological changes undergone by infected astrocytes throughout the transdifferentiation process (GFP in green, tdTomato in red, scale bar 50µm).

Taking into account the results obtained in P2-injected mice, we decided to perform all our analyses at 6 w.p.i.. We first assessed if GFP$^+$/tdTomato$^+$ cells were still expressing astrocytic markers, such as SOX9. Quantification of the immunofluorescence analysis revealed that only 40% of the cells that received the virus in *Aldh1l1*-Cre^{ERT2}/Ai9 mice also expressed SOX9, while in non-injected animals all tdTomato$^+$ were also positive for SOX9 (Fig. 3.6). This indicates that a significant amount of infected cells had lost the glial properties upon *Ascl1* overexpression at 6 w.p.i.. Whether they were in an intermediate state, expressing both neuronal and glial markers, or instead were still astrocytes could not be assessed due to the limited availability of channels.

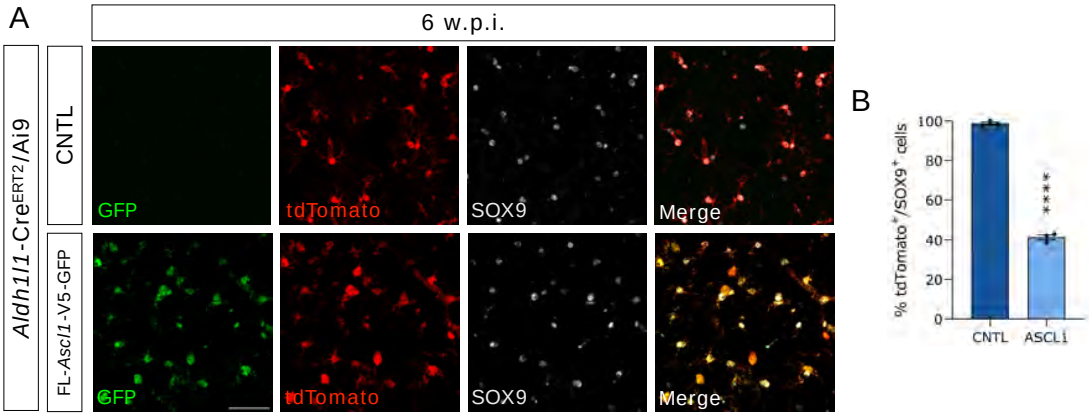

*Figure 3.6. Infected cells lose the expression of glial marker SOX9 at 6 w.p.i. Immunofluorescence images of control and injected animals (GFP in green, tdTomato in red, SOX9 in grey, scale bar 50µm). Quantification of the percentage of tdTomato$^+$ cells co-expressing the astrocytic marker SOX9 in injected (ASCL1) and non-injected (CNTL) conditions (B). (n=3) Error bars represent ± SEM. (****p<0.0001) Statistical test: Unpaired Student t-test.*

Six weeks after the infection with the LV, many GFP$^+$ cells, which were also tdTomato$^+$, were observed in the striatum of these mice. Of them, none were expressing the neuronal progenitor marker doublecortin (DCX). Instead, many were positive for the mature neuronal marker NEUN (64,6±9,5%), indicating that infected cells successfully converted into neurons (Fig. 3.7). To further characterise the fate of these newly converted neurons, we stained for dopaminergic (TH) and medium spiny neurons (DARPP-32) markers. As expected, *Ascl1* was not able to generate TH-positive iNs (Fig. 3.8 A-B). This TF, besides being a central regulator of neurogenesis in the early stages of development, has been associated with the differentiation of GABAergic neurons (Imayoshi & Kageyama, 2014). Nonetheless, in line with what we observed in P2-injected mice

after a few weeks, around 30% of GFP$^+$ cells reprogrammed into MSN-like neurons, as shown by the DARPP-32 positive cells (31±1,8%) (Fig. 3.8 A-B). DARPP-32 is a central protein in the process of dopamine transmission, being expressed exclusively in mature MSNs (Scheggi et al., 2018). The expression of this protein in iNs suggests that they are acquiring an MSN identity.

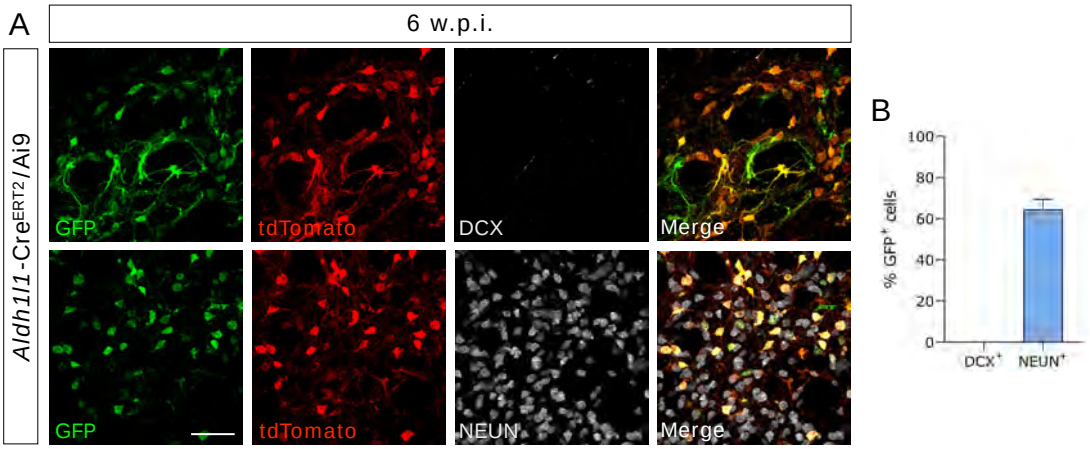

Figure 3.7. Converted striatal astrocytes express mature neuronal marker at 6 w.p.i..
Representative immunofluorescence analysis of striatal sections from adult mice performed 6 weeks after Ascl1 injection show astrocytes efficiently converted into mature neurons (NEUN$^+$/DCX$^-$) (A). Quantification of GFP$^+$ positive cells expressing DCX and NEUN (NEUN: 64,6±9,5%) (B). (n=4 mice) Scale bar, 50µm. Error bars represent ± SEM.

To further confirm the MSN-like phenotype of the newly converted neurons, we performed a staining against forkhead box P1 (FOXP1), a TF crucial for MSN maturity (Precious et al., 2016), and CTIP2, as aforementioned, a TF central to MSN differentiation and development (Arlotta et al., 2008). In accordance with the results obtained in the immunofluorescence against DARPP-32, many of the reprogrammed cells were also positive for CTIP2 (68±10,2%) and FOXP1 (49±10,8%) (Fig. 3.8 A-C). These results are in line with the differentiation process of MSNs, as FOXP1 and CTIP2, present since earlier differentiation and maintained through adulthood, are expressed in higher levels while only a part of them express the mature marker DARPP-32 at 6 w.p.i..

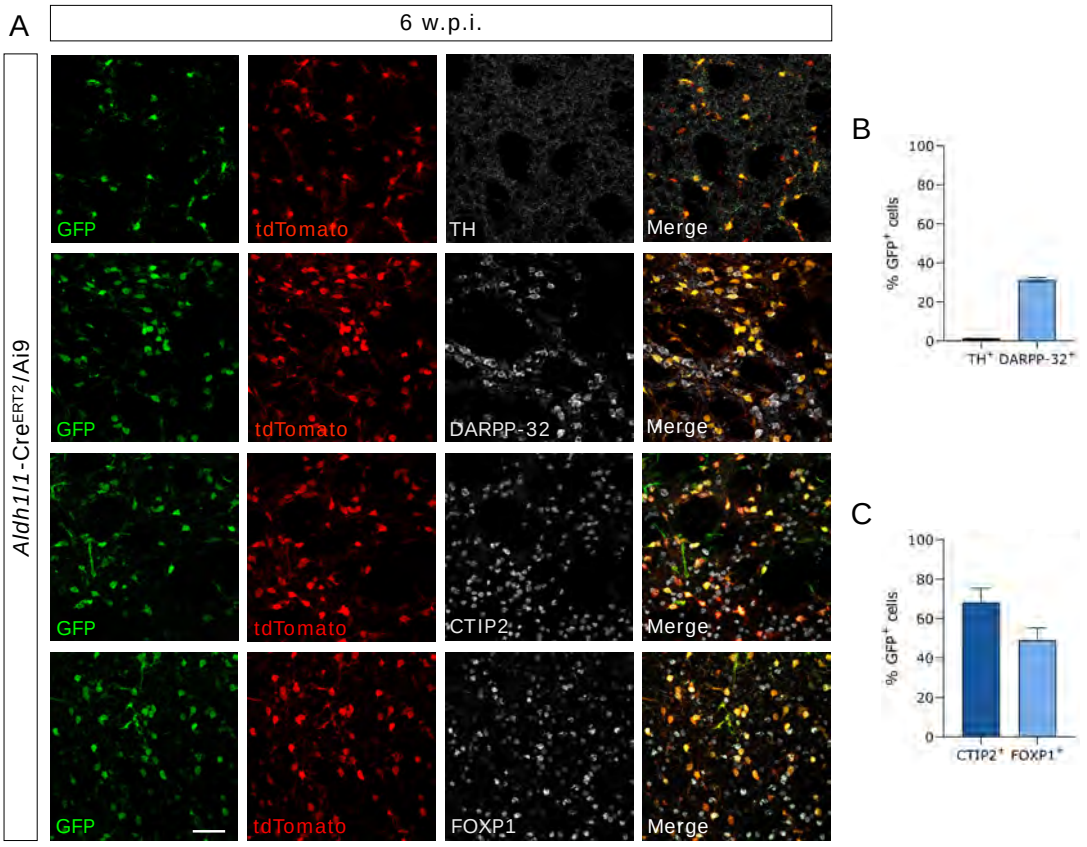

Figure 3.8. Subtype characterisation of iNs at 6 w.p.i..
Representative immunofluorescence images of striatal sections from adult Aldh1l1-Cre^{ERT2}/Ai9 mice analysed 6 weeks after Ascl1 injection show newly converted neurons adopting an MSN-like identity, as shown by DARPP-32^{+} cells (31±1,8%) and the absence of TH^{+} iNs (A-B). Labelling for other MSN markers, CTIP2^{+} (68±10,2%) and FOXP1^{+} (49±10,8%) corroborated the fate of newly converted astrocytes (A,C). (n=3 mice) Scale bar 50µm. Error bars represent ± SEM.

Taken together, these results indicate that the overexpression of *Ascl1* in striatal astrocytes is able to convert them into iNs, mainly acquiring an MSN-like phenotype. These findings are consistent with what is reported in the literature about the region-specificity of astrocytes and how this type of glial cells, when pushed into a neuronal fate, tend to adopt the identity of the sub-population most abundant in the targeted brain area (Herrero-Navarro et al., 2021).

3.4 Astrocyte maturation does not influence the outcome or yield of the reprogramming upon ASCL1 expression

After providing clear evidence that *Ascl1* alone can successfully convert both immature and mature striatal astrocytes into iNs, we wanted to assess if, as hypothesised, there were some differences between the two types. One of the aspects that we were examining in our analyses was if the maturation state of the target astroglia had an impact on the propensity to being reprogrammed. As shown in the previous sections, *Ascl1* can efficiently convert both sets of cells into iNs without any significant differences after 6 weeks. However, we observed how in immature astrocytes, the transdifferentiation process happens faster than in adult.

Regarding the outcome, we assessed if the differences in gene expression from both sets of cell populations and the overall diverse local environment could influence the outcome of the converted cells. Nonetheless, the collected results provide extensive evidence that when injected in the striatum of mice both early-postnatal and adults, *Ascl1* drives iNs towards an MSN-like identity without noteworthy differences.

3.5 Conversion by *Ascl1* does not alter tissue homeostasis

Astrocytes play a very important role in the maintenance of the brain homeostasis, supporting neurons through the release of trophic factors and substrates for their correct function besides controlling the blood flow in the parenchyma (Guttenplan & Liddelow, 2019; Haydon & Carmignoto, 2006; Nicola J. Allen & Ben A. Barres, 2009). Because of this, we wondered if by reprogramming part of the resident astrocytes the tissue homeostasis was altered. To assess the state of the striatum, we checked the expression of GFAP, a marker of reactive astrocytes (Escartin et al., 2021). Immunofluorescence images of mock and *Ascl1* injected brains at 6 w.p.i. show comparable astrogliosis, therefore it being most likely attributable to the injection itself rather than to the conversion process (Fig. 3.9 A-B).

In addition, to evaluate the inflammatory response following astrocyte-to-neuron conversion, we performed an immunofluorescence against IBA1, a

microglial marker whose level of expression depends on its activation (Ito et al., 1998). After 6 weeks, both mock and *Ascl1* injected present no noteworthy differences (Fig. 3.9 C-D), indicating that by that time, tissue homeostasis was almost completely restored.

Since astroglia are a proliferating cell type, even if transiently the population was reduced due to the conversion of part of them, the progressive transdifferentiation process allows for the restoration of initial levels, therefore reinstating homeostasis.

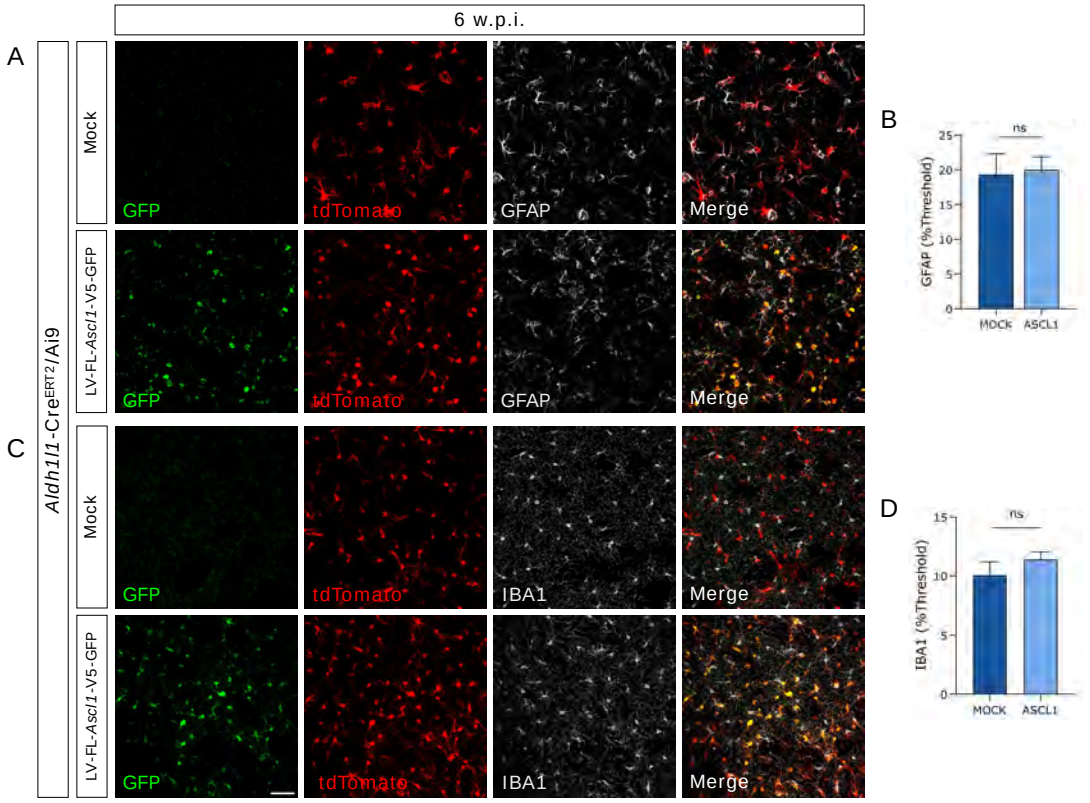

Figure 3.9. Gliosis after transdifferentiation of resident astrocytes
Immunofluorescence images of mock and Ascl1 injected Aldh1l1-CreERT2/Ai9 brains showing no major inflammatory events following reprogramming of astrocytes after 6 weeks (GFP in green, tdTomato in red, GFAP and IBA1 in grey, scale bar 50µm) (A,C). Level of GFAP and IBA1 signal measured as the fraction of the total area occupied by these astrocytic and microglial markers using a fixed threshold (B,D). (n=3 mice) Error bars represent ± SEM. Statistical analysis: Unpaired Student t-test.

3.6 DAN axonal remodelling after 6-OHDA injection in the dorsal striatum

To better understand the adaptive response of dopaminergic neurons to neurodegeneration, we developed a model with local 6-OHDA injection in the dorsal striatum. As aforesaid in the introduction, PD patients start showing clinical motor symptoms when around 60-70% of the dopaminergic neurons in the SNpc are already lost (Dauer & Przedborski, 2003b; Lang & Lozano, 1998). This fact unveils a compensation process whose underlying mechanisms are still far from being understood. Past studies suggest that one of the processes contributing in this counteraction might be the structural remodelling of the dopaminergic projections (Dravid et al., 1984; Finkelstein et al., 2000; Schmitz et al., 2013).

In order to shed some light on this matter, WT adult mice were lesioned with a 6-OHDA injection in the dorsal striatum to damage only the axons involved in the nigrostriatal pathway, therefore coming from the dopaminergic neurons in the SNpc (Schmitz et al., 2013). This neurotoxin, when infused in the striatum, causes retrograde neurodegeneration in a few days (Stott & Barker, 2014). For this purpose, we analysed the injected mice after 2, 5 and 7 weeks to explore if there was any fibre remodelling process. Immunohistochemical analysis of dopamine transporter (DAT) revealed that 2 weeks post-lesion, dopaminergic fibres in the dorsal striatum of these mice were severely reduced. Interestingly, the same area after 5 and 7 weeks displayed newly formed projections, showing a significant reinnervation process (Fig. 3.10 A). To corroborate this result, we performed immunohistochemistry against TH which resulted in the same gradual reinnervation process observed for DAT. Assessment of the optical density of the striatum divided between dorsal, medial and ventral areas, revealed an ascending reinnervation pattern (Fig. 3.10 C). In addition, quantification of the total number of nigral DA neurons in injected mice was evaluated by TH staining and unbiased stereology at 2, 5 and 7 weeks after 6-OHDA lesion. Neurodegeneration was steady at around 45% relative to control mice at all time points, further corroborating that the change in the pattern of striatal TH and DAT staining is due to a reinnervation rather than a restoration of expression (Fig. 3.10 D).

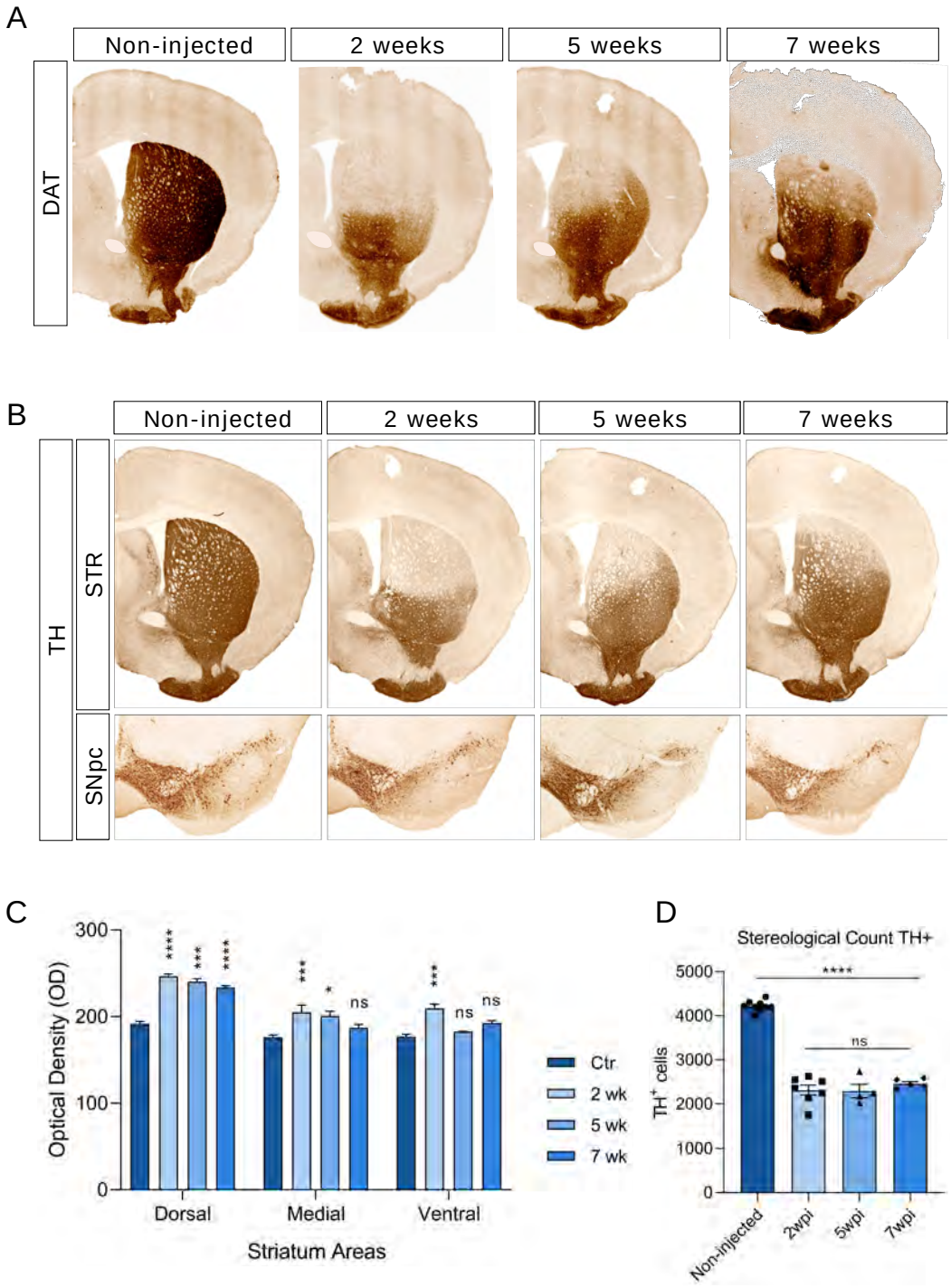

*Figure 3.10. DAN axonal remodelling after severe lesion. Immunohistochemistry analysis of DAT and TH at 2-, 5- and 7 w.p.i. revealed a significant reinnervation process in the striata of 6-OHDA lesioned WT mice (A-B). Optical density measurements of the TH signal in the different parts of the striatum (n=4 mice) (C). Stereological count of TH⁺ cells revealed no differences between time-points (D). Error bars represent ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus non-injected mice. Scale bar: 1mm. Statistical analysis for the OD measurements: two-way ANOVA followed by Dunnett's post hoc correction. Statistical analysis for the stereological count: one-way ANOVA followed by Tukey's post hoc correction.*

3.7 Myr-mCherry construct selectively labels DA projections

Once demonstrated a remodelling of the dopaminergic fibres, we wanted to identify the cell population responsible for this regenerative event. In order to do so, we thought about using a labelling system that allowed us to observe both the soma and projections of DANs, which can be challenging given the large distance between both parts. We exploited the N-myristoylation process to obtain a suitable tool that meet our needs. This co-translational modification, that consists in the covalent attachment of a myristate group to the N-terminal of the acceptor protein, drives acceptor proteins to the plasma membrane. For our purpose, we generated a Cre-inducible lentivirus expressing a myristoylated (myr) mCherry reporter (LV-FLEX-myr-mCherry) and validated it in DAT-Cre transgenic mice (Fig. 3.11 A). Immunofluorescence analysis performed two weeks after the injection of the virus in the SNpc revealed how the RFP signal is specific to DA neurons. Moreover, with this approach, axonal terminals of DA neurons can be clearly observed at the striatum as shown by the mCherry signal (Fig. 3.11 B). To further corroborate the dopaminergic origin of the axons, we stained for DARPP-32, a common marker of medium spiny neurons, with whom dopaminergic neurons synapse in the striatum (Scheggi et al., 2018) (Fig. 3.11 B). Furthermore, precise injection of this virus allows the specific labelling of a subset of dopaminergic neurons. When injected in the SNpc, only the dorsal striatum resulted stained, while if injected in the VTA, the signal was observed solely in the nucleus accumbens (NAc) and the ventral part of the striatum (Fig. 3.12 B).

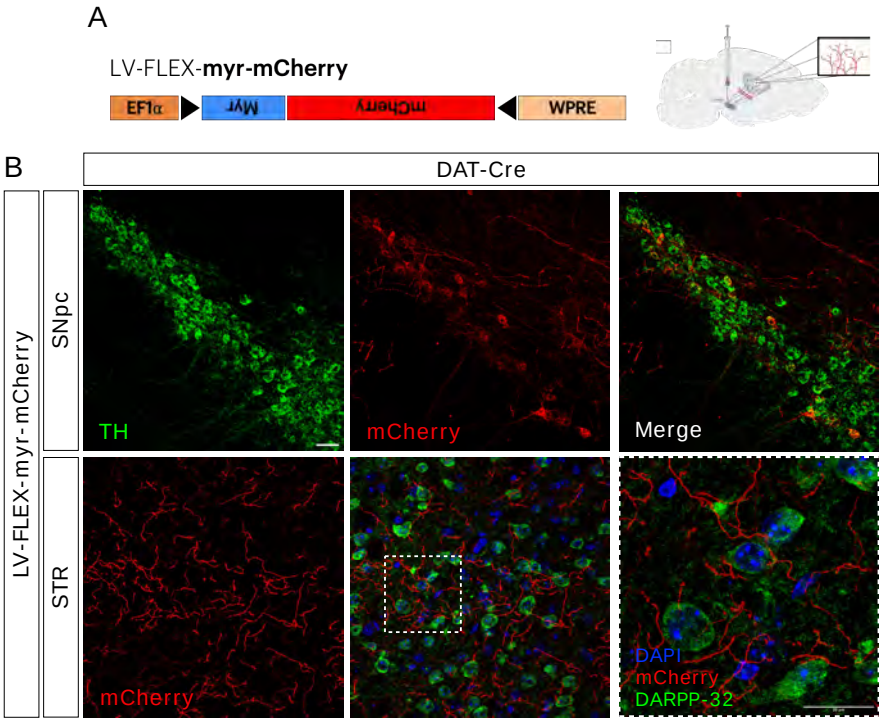

Figure 3.11. LV-FLEX-myr-mCherry specifically labels dopaminergic projections.
Representation of the construct and the injection strategy used to label DANs (A). Immunofluorescence images of substantia nigra slices from adult mice 2 weeks after the injection with LV-FLEX-myr-mCherry show an mCherry restricted expression in dopaminergic neurons (TH in green, mCherry in red, scale bar: 50µm). Images of striatal sections from injected mice displaying dopaminergic terminals in red and MSNs in green (DARPP-32 in green, mCherry in red, DAPI in blue). Scale bar on the high magnification image: 20µm (B).

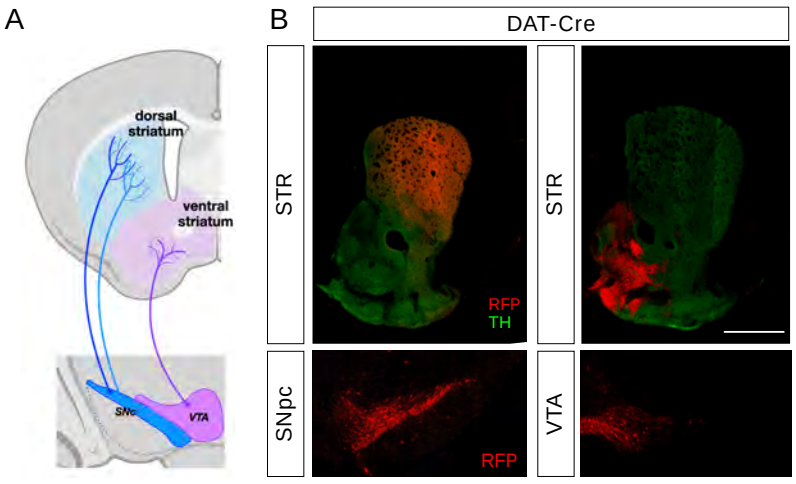

Figure 3.12. Precise injection of LV-FLEX-myr-mCherry labels a specific population of dopaminergic neurons.
Schematic design of the areas innervated by the dopaminergic populations of the SNpc and VTA (A). Immunofluorescence images showing the specific labeled projections depending on the population targeted, (RFP in red, TH in green, scale bar 1mm).

3.8 Newly formed axons arise from VTA-DANs

Our first attempt to identify the neurons reinnervating the depleted striatum was to combine both the labelling system using the LV-FLEX-myr-mCherry with a 6-OHDA lesion. As described in the introduction (see 1.3), even though both VTA and SNc dopaminergic neurons generate, store and release dopamine, their brain function and projection area differ. They also exhibit significantly different susceptibility to degeneration. Neurons in the VTA are much more resistant to neuronal death than SNpc ones (Brichta & Greengard, 2014; Dauer & Przedborski, 2003b), leading us to believe that they are more likely to be involved in the reinnervation process. In order to prove this assumption, we labelled dopaminergic neurons in the VTA by injecting the LV-FLEX-myr-mCherry, consequently observing dopaminergic fibres in the nucleus accumbens and the ventral striatum. These same mice received 6-OHDA injections in the dorsal striatum, to degenerate the dopaminergic fibres in that area. At 5 weeks post-injection, newly formed mCherry^{+} projections appeared in the previously devoid striatum, indicating that they arose from VTA-DANs (Fig. 3.13 A).

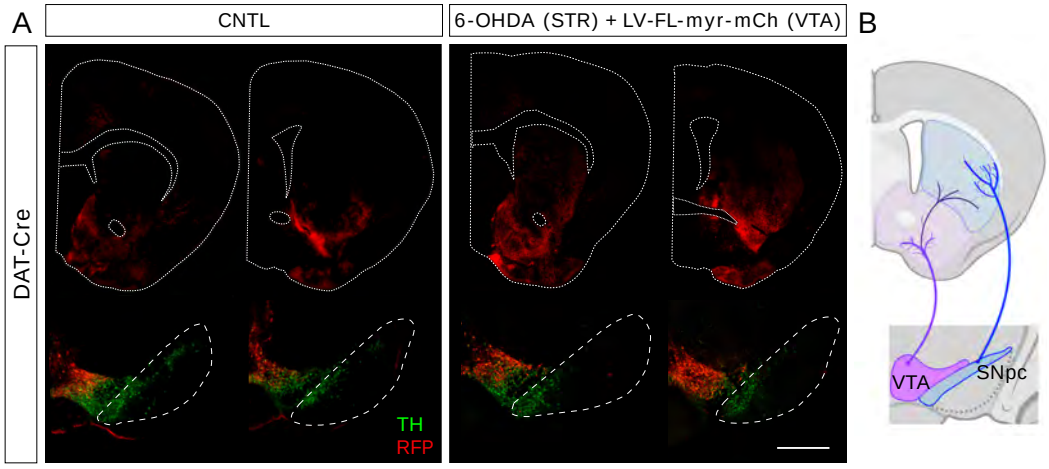

Figure 3.13. LV-FL-myr-mCherry as a tracer of newly formed axons. Immunofluorescence images of striatum and midbrain sections of mouse brains injected with only LV-FL-myr-mCherry in VTA (CNTL) and with both 6-OHDA in the striatum and the labelling virus in the VTA (TH in green, mCherry in red, scale bar: 1mm) (A). Schematic design of a possible reinnervation of the STR by VTA-residing dopaminergic neurons (B).

However, due to the close proximity of the VTA with the SNpc, and given the highly arborised morphology of dopaminergic neurons (Matsuda et al., 2009), it is risky to assume that newly formed axons are coming from VTA-residing neurons and not from labelled SNpc neurons. To corroborate these preliminary

findings and better examine this event, we decided to change the system to a more suitable one.

3.9 Retrograde tracing to identify the origin of newly formed projections

Preliminary results obtained by combining 6-OHDA-mediated lesion and the myr-mCherry reporter virus, raised the possibility that at a moderate neuronal loss, neurons from the VTA send collateral projections to the dorsal striatum to compensate for the death of dopaminergic neurons residing in the SNpc. To confirm this result, we used commercially available fluorescent latex microspheres (Retrobeads™ IX) in combination with the 6-OHDA model of neurodegeneration. Wild-type mice were first injected with 6-OHDA in the dorsal striatum, causing a significant depletion of dopaminergic fibres as previously shown (see 3.6). Five weeks after the lesion with the neurotoxin, newly formed projections had already arisen in the previously depleted area. At that time point, Retrobeads™ were injected in the same coordinates as the 6-OHDA in order to try to avoid reaching the undamaged area (Fig. 3.14 B). These beads are taken up by the synaptic terminals and are able to travel retrogradely to the soma of the neurons. While in control mice, non-injected with 6-OHDA, beads are located inside many SNpc dopaminergic neurons, in those mice that received first, a 6-OHDA injection and then, the Retrobeads™ infusion, much fewer neurons were labelled, some of them located in the VTA (Fig. 3.14 C). This observation is unexpected, given that, as shown by the labelling with the myr-mCherry, VTA neurons do not project in that brain area and because they are involved in very different brain functions. Overall, these results indicate that fibre remodelling is one of the mechanisms operating during the presymptomatic phase of PD.

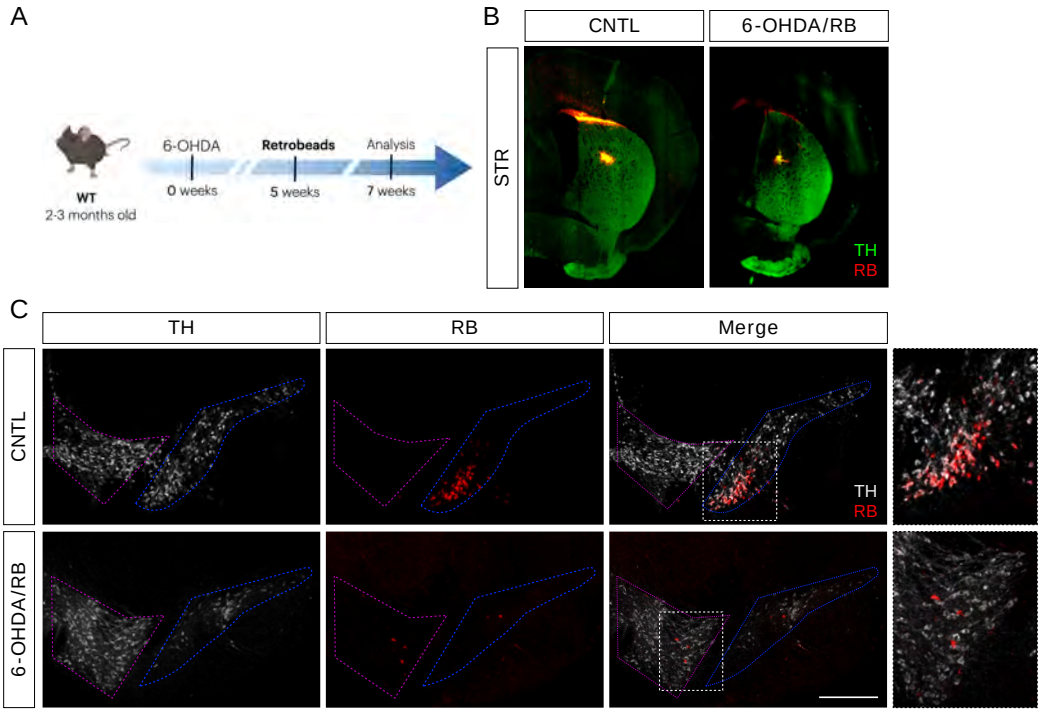

Figure 3.14. Retrobeads™ trace the origin of newly formed projections to VTA-residing dopaminergic neurons.
Timeline of the experimental procedure (A). Immunofluorescence images of striatum slices from mice injected only with Retrobeads™ (CNTL) or with 6-OHDA prior to Retrobeads™ injection (6-OHDA/RB) (RB in red, TH in green) (B). Midbrain sections of mouse brains injected with only Retrobeads (CNTL) and with 6-OHDA and the Retrobeads (6-OHDA/RB) (RB in red, TH in grey, scale bar: 1mm) (C).

3.10 Injection of activated Caspase3 leads to selective and controlled nigral neuronal loss

Even though the compensatory mechanisms active during the presymptomatic phase of PD are still unknown, it seems evident that one of the central factors affecting the capacity of regeneration is the extent of neuronal loss in the SNpc. Commonly used neurodegenerative models hardly allow to obtain intermediate stages of neuronal depletion and can give variable effects. To overcome these limitations, we generated a Cre-dependent virus overexpressing the activated form of Caspase-3, which causes cell-autonomous neuronal death. This construct encodes for an engineered Caspase-3 constitutively active only in Cre-expressing cells (Yang et al., 2013), since it is cloned in a FLEX AAV under the strong constitutive promoter chicken β-actin (CBA) (pH-FL-Casp3). We wanted to explore if with this system we could tune the neurodegeneration obtained by modelling the injected virus titre. To this end, we injected the pH-FL-Casp3 in the SNpc of DAT-Cre transgenic mice to specifically degenerate the dopaminergic neurons.

Immunohistochemistry analysis against TH performed 2 w.p.i. showed a neuronal loss strictly linked to the titre injected, causing different patterns of TH signal in the striatum (Fig. 3.15 A). Stereological count of the TH-positive nigral cells in the SNpc of DAT-Cre mice at the same time point, revealed how DANs loss can be tightly regulated, both spatially and quantitatively, by tailoring the viral dose administered (Fig. 3.15 B). With this genetic tool, we will be able to study the different stages of the neurodegenerative process in a highly reliable and reproducible manner and search for the threshold of neurodeg. eneration that allows this reinnervation event to take place.

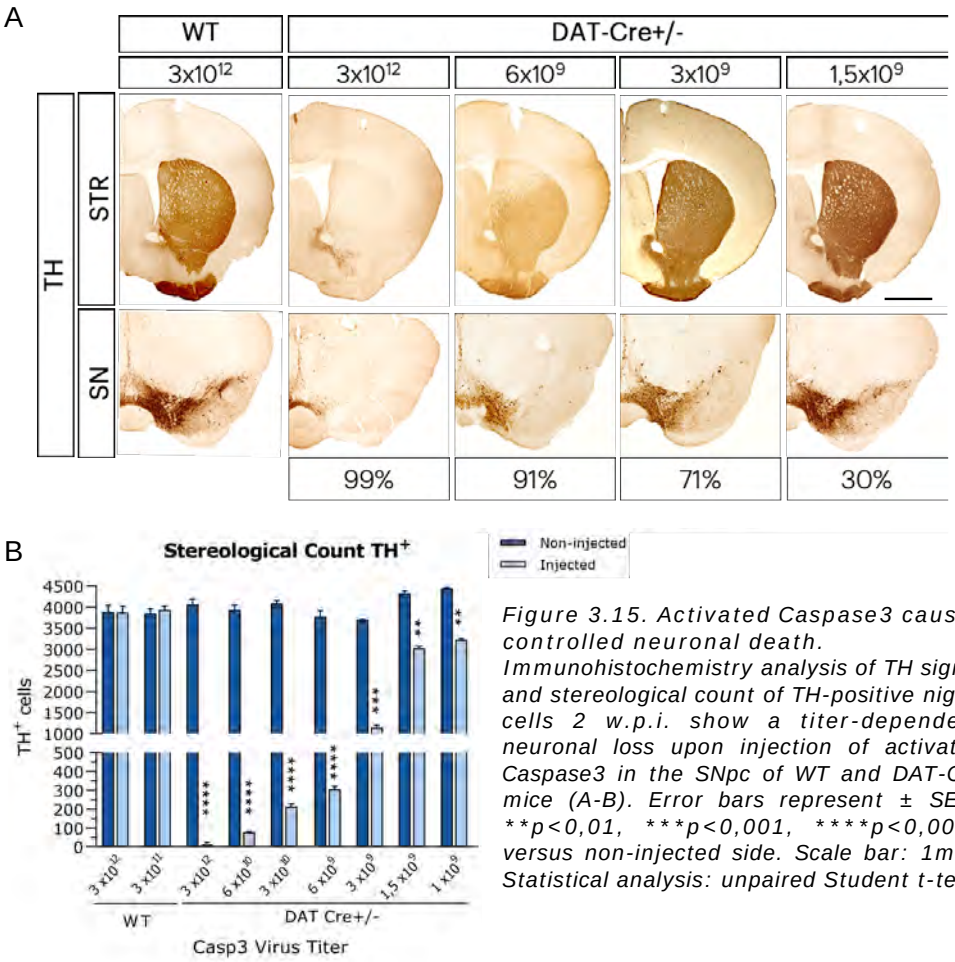

*Figure 3.15. Activated Caspase3 causes controlled neuronal death. Immunohistochemistry analysis of TH signal and stereological count of TH-positive nigral cells 2 w.p.i. show a titer-dependent neuronal loss upon injection of activated Caspase3 in the SNpc of WT and DAT-Cre mice (A-B). Error bars represent ± SEM. **p<0,01, ***p<0,001, ****p<0,0001 versus non-injected side. Scale bar: 1mm. Statistical analysis: unpaired Student t-test*

4 DISCUSSION

Direct neuronal reprogramming represents a potential therapeutic strategy to treat brain injuries and neurodegenerative disorders which, given the ageing of the world's population, stand as a growing challenge for healthcare systems worldwide. Even though much progress has been done since its discovery (Weintraub et al., 1989), the underlying mechanisms directing the transdifferentiation of cells are still not well understood. Several *in vitro* studies have successfully converted various non-neuronal cells into iNs of diverse subtypes. However, most results are not translatable *in vivo*, observing significant differences both in the outcome and the yield. That is because the local environment and the particular characteristics that cells have in their tissues are altered when cultured in the dish (Lange et al., 2012). Given their abundance in the CNS and their shared origin with neuronal cells, astrocytes have been the target of many of the studies aiming to replenish a neuronal population after injury or disease (Wei & Shetty, 2021). In this work, we intended to reprogram striatal astrocytes into induced dopaminergic neurons (iDANs). In our first attempts, we performed experiments with cultured mouse primary astrocytes, to observe the outcome of the transdifferentiation process under controlled conditions. Since we were aiming at obtaining iDANs, we used a viral vector encoding for three TFs with proven capacity to convert non-neuronal cells into iNs of that specific subtype (Caiazzo et al., 2011; Theka et al., 2013). Our multicistronic virus encodes for ASCL1, LMX1A and NURR1 (ALiN), TFs with recognised proneural activity and dopaminergic fate determinants. This combination of factors, even though successful in the conversion of astrocytes to mature neurons (30%), was unable to convert them into iDANs, contrary to what had been observed for other cell types (Caiazzo et al., 2011; Theka et al., 2013). Instead, iNs mainly converted into GABAergic interneurons-like cells. These results are unexpected giving that two of the TF encoded (*Lmx1a* and *Nurr1*) are involved in the differentiation of midbrain dopaminergic neurons during development (Hong et al., 2014) and therefore should guide the fate of the iNs into a dopaminergic identity. In light of these results, we believed that the outcome obtained could be due to the strong influence of *Ascl1*, which has been associated to the generation of GABAergic neurons (Fode et al., 2000; Imayoshi & Kageyama, 2014; Parras et al., 2002).

To better understand the reprogramming process and to assess if the outcome

that we obtained with LV-tetO-ALiN was mainly due to an effect of *Ascl1*, we decided to perform a new set of experiments using a virus encoding only for this TF. As we were planning on performing subsequent experiments *in vivo,* to be able to compare the results obtained in both systems without introducing other variables, we decided to use the same tools for the experiments in cultured astrocytes. We repeated the analyses performed for LV-tetO-ALiN, but this time using the LV-FLEX-*Ascl1*-V5-GFP. After coinfecting the cells with both LV-Cre and the LV-FLEX-*Ascl1*-V5-GFP, 38% of the cells converted into iNs, yield in line with previously published results reporting between a 30-40% of reprogramming (Berninger et al., 2007; Heinrich et al., 2010; Masserdotti et al., 2015). Similar to what we observed for ALiN, *Ascl1* alone mainly generated GABAergic-like cells, suggesting that the effect we observed in the previous set of experiments could be due to the prevalent influence of *Ascl1*. These results are coherent with the observations made in other studies where the expression of *Ascl1* in cultured astroglia induced a GABAergic phenotype (Heinrich et al., 2010; Masserdotti et al., 2015). Indeed, a recent publication described that the presence of additional TFs did not influence the outcome of the reprogramming of ESCs when used in combination with *Ascl1* (Ng et al., 2021), suggesting that *Ascl1* is the main driver for both the conversion and the subtype identity iNs acquire.

Once assessed *in vitro* the capacity of our constructs to efficiently generate iNs, we decided to move to an *in vivo* setting, as usually the local environment limits the plasticity of astrocytes to acquire a particular lineage (Guillemot & Hassan, 2017). Because the results obtained with the multicistronic virus were not significantly different than the ones obtained with just *Ascl1*, we decided to do all subsequent studies expressing only this potent proneural TF. Most *in vivo* studies attempting astrocyte-to-neuron conversion published until now, based their experiments on the use of GFAP models (Mattugini et al., 2019; Qian et al., 2020; Rivetti Di Val Cervo et al., 2017; Torper et al., 2015). However, these models present several limitations since GFAP, even though it is expressed in different brain regions and for many years has been considered a broad astrocytic marker, it is not expressed in all astrocytes. In addition, several publications reported some expression leakiness in neurons driven by the isolated GFAP promoter, which is likely to give misleading results in this particular setting (Fujita et al., 2014; Guttenplan & Liddelow, 2019). Given this hurdle, we opted for our *in vivo* experiments to use the *Aldh1l1*-Cre^{ERT2} mice (Srinivasan et al., 2016), that represents the most specific mouse model currently available to target astrocytes.

This mouse line, in combination with the Ai9 reporter strain (Madisen et al., 2010), creates a robust model to study astrocyte-to-neuron conversion, since even when the astrocytes convert into neurons, the tdTomato expression remains detectable, enabling to trace the cellular evolution of the astrocytes originally targeted. This model, as demonstrated in several publications, as well as by the results I obtained when verifying its specificity, expresses the Cre recombinase in the majority of astrocytes but never in neuronal cells (Cahoy et al., 2008; Srinivasan et al., 2016). Another possible source of leakiness is the use of non-stringent viral vectors. As already mentioned in the introduction, the viral vector used to drive the expression of the transgenes will depend on the requirements and the aim of the experiments. Because direct reprogramming is proposed as a possible therapeutic approach, most used viral types are AAVs and LVs due to their low immunogenicity. However, AAVs can present off-target expression of the transgene, especially when used at high titres (Fischer et al., 2019). Having had this experience in the past, we decided to use LVs as viral platform to express *Ascl1*, which we proved to sustain a restricted expression only in astrocytes. Not many studies attempting astrocyte-to-neuron conversion have used LVs as the viral vector to deliver their transgenes, even if they have proved efficient in infecting astrocytes both *in vitro* and *in vivo* (Humbel et al., 2020). In the first description of *in vivo* transdifferentiation in the brain tissue FLEX-LVs encoding for *Ascl1, Brn2a* and *Mytl1* were injected in the striatum of GFAP-Cre mice achieving a conversion into neurons of less than 10% (Torper et al., 2013).

One of the aims of this study was to assess if immature astrocytes are more prone to reprogramming than mature astrocytes. Recently, it has been described that the astrocytic gene expression profile and chromatin structure is severely altered during maturation (Lattke et al., 2021). We hypothesised that the disparity of results commonly observed when applying the same strategy *in vitro* and *in vivo*, could be partly due to the maturation state of the astrocytes, since cultured astrocytes are obtained from early postnatal mice. In fact, an assay for transposase-accessible chromatin using sequencing (ATACseq) of astrocytes from P4 and 2-month-old mice revealed significant differences in chromatin accessibility between both groups (Lattke et al., 2021). We thought that these differences might be one of the reasons behind the discrepancies between results in the two settings. To assess this variable, we injected our construct overexpressing *Ascl1* (LV-FLEX-*Ascl1*-V5-GFP) in the striatum of P2 and adult

Aldh1l1-Cre^{ERT2}/Ai9 mice. ASCL1 demonstrated the capacity to convert immature astroglia into mature neurons with high efficiency (75%). This capacity was maintained also for adult astrocytes, which showed a comparable level of reprogramming after 6 weeks (65%), indicating that ASCL1 is able to overcome the different molecular features associated to the diverse states of cell maturation. These findings may be explained by the fact that ASCL1 is an on-target pioneer factor, being able to bind to its target genes and induce their expression even when they are repressed by a closed chromatin structure, besides making other genes available to downstream TFs (Aydin et al., 2019; Guillemot & Hassan, 2017; Wapinski et al., 2013). Contrarily, in a study that targeted striatal oligodendrocyte precursor cells (NG2), ASCL1 was not able to efficiently reprogram them into iNs (Torper et al., 2015). This difference might be due to distinctive chromatin features and the presence of other cofactors that influence the capacity of binding of this TF in that particular cell type (Guillemot & Hassan, 2017; Wapinski et al., 2013). However, because the number of cells or the percentage obtained is not specified, it is difficult to make a comparison between both cell types. In that same study, authors also use *Ascl1*, *Lmx1a* and *Nurr1* cloned in single AAVs together with a FLEX synapsin GFP reporter virus to follow the fate of converted cells by GFP expression. With this system, they achieve around a 50% of conversion to iNs after 12 weeks. Still, this combination of factors is unable to convert neither NG2 nor astrocytes to iDANs, in consonance with the results we obtained *in vitro* with the LV-tetO-ALiN. Immunofluorescence and functional analyses identified iNs mainly as parvalbumin-like cells (Pereira et al., 2017; Torper et al., 2015). Recently, Wu and colleagues reported that the expression of *Neurod1* and *Dlx2* in astrocytes achieved a 59% conversion into neurons after 8 w.p.i., similar to the conversion yield we obtained at 6 w.p.i. (Wu et al., 2020). Interestingly, in this work they reported an intermediate state after 4 weeks similar to the one we observed during our experiments at the same time point, when infected cells do not express neither astrocytic nor neuronal markers. These results suggest that *Neurod1* might function similarly to *Ascl1* in astrocytes (Pataskar et al., 2016). *Neurod1*, same as *Ascl1* and *Neurog2*, is a proneural basic helix-loop-helix (bHLH) TF with demonstrated capacity to reprogram several non-neuronal cells into iNs. In fact, many studies have successfully used *Neurod1* in their factors mix to reprogram non-neuronal cells into neurons (Guo et al., 2014; Rivetti Di Val Cervo et al., 2017; Wu et al., 2020).

After demonstrating the capacity of *Ascl1* to convert both mature and

immature astrocytes into iNs *in vivo*, we wanted to assess the neuronal subtype these cells adopted. Recently published studies described significant differences between the transcriptome of the various subsets of astrocytes depending on the region they reside or even within the same brain area (Boisvert et al., 2018; Herrero-Navarro et al., 2021; Qian et al., 2020). Since astroglia and neurons arise from the same progenitors during development, this signature transgene expression is shared with the neurons from the same domain. This fact creates a bias when aiming to obtain iNs from astrocytes, since they tend to acquire the subtype identity of the neuronal population present in that brain area (Herrero-Navarro et al., 2021; Qian et al., 2020). In the striatum, the most common neuronal population are MSNs, accounting for about 90% of the total neurons (Arlotta et al., 2008; Precious et al., 2016). Our results, from immature and mature astrocytes, are coherent with the concept that regional molecular identity strongly influence the subtype specification of the reprogrammed iNs, which in our experimental setting is proven by the acquisition of an MSN phenotype. In fact, obtained iNs were positive for various MSN markers, including CTIP2 and FOXP1, TFs expressed since early differentiation until adulthood, and DARPP-32, the most popular mature marker for MSNs (Arlotta et al., 2008; Precious et al., 2016). Similar results were obtained when striatal astrocytes were converted after expressing *Neurod1* and *Dlx1*, obtaining a transdifferentiation into MSNs of around 61% (Wu et al., 2020). These results further reinforce the idea that ASCL1 and NEUROD1 act in a similar way in astrocytes, even if ASCL1 alone is more efficient in converting astrocytes to iNs (65%) when compared to NEUROD1 alone (35%) (Wu et al., 2020).

Collectively, our study demonstrates through the use of a robust experimental model the capacity of *Ascl1* to convert very efficiently into iNs parenchymal striatal astrocytes, both in a mature and immature state. These newly formed neurons display a coherent range of markers which suggest the acquirement of a canonical MSN identity. The acquisition of this neuronal subtype seems to be driven by region-specific gene expression which is shared for both neurons and astrocytes of the same domain. In order to explore if this process replicates with the same logic in other brain regions, we are conducting follow-up experiments to assess if cortical astrocytes are converted into GABAergic neurons following *Ascl1* overexpression. Moreover, to further confirm the MSN fate at 6 w.p.i. and to observe if iNs function like their endogenous counterparts, we will conduct

electrophysiological recordings.

Still, our main aim is to achieve the conversion of astrocytes into iDANs to, one day, propose direct reprogramming as a feasible therapeutic alternative for PD. Obtained results serve us as a strong baseline for future studies in order to unravel a combination of factors able to overcome the bias presented by regional molecular identity, allowing us to obtain a high conversion yield into iDANs.

Direct reprogramming, however, would not be a disease-modifying strategy against PD, and it would only be applicable after diagnosis as a way to alleviate the motor symptoms and reduce or avoid the uptake of drugs. Due to the absence of biomarkers, neuroprotective strategies still do not represent a realistic strategy for PD since, besides genetically predisposed subjects, which account for the minority of PD patients, disease is hardly detected due to its long presymptomatic stage (Bloem et al., 2021; Kalia & Lang, 2015). The slow disease progression and ensuing cell loss occurring in this disorder allows for compensatory mechanisms enacted by the remaining neurons (Bezard et al., 2003; Brotchie & Fitzer-Attas, 2009). Notably, the biological and molecular basis of this potent, yet transient endogenous protective response are unknown. Compensatory mechanisms interfere in mainly two events: dopamine availability in the striatum and the activity of the indirect pathway (Brotchie & Fitzer-Attas, 2009). Several studies report a remodelling of dopaminergic projections after partial lesion of SNpc-dopaminergic neurons (Finkelstein et al., 2000; Schmitz et al., 2013). We hypothesise that during the presymptomatic stage of PD, cell intrinsic changes in either the spared dopaminergic neurons and/or the striatal target tissue, control morphological and functional plasticity of dopaminergic axonal arbours and connectivity in the striatum. The remaining dopaminergic neurons may undergo profound axonal remodelling to establish ectopic connections that temporarily preserve homeostasis of the nigrostriatal circuit.

In order to test this hypothesis and further understand this compensatory process, we injected the neurotoxin 6-OHDA in the dorsal striatum of adult mice, causing a depletion of dopaminergic fibres in the striatum, and a partial loss of the dopaminergic neurons in the SNpc (Schmitz et al., 2013). Immunohisto-chemistry analyses against TH and DAT performed at several time points revealed a progressive reinnervation of the depleted striatum in a time-dependent manner.

This event, unusual in the adult brain, was not due to a rescue of the expression but to axonal sprouting of the spared neurons, as the number of TH^+ cells in the SNpc was stable in all time points and same results were observed after DAT staining.

Trying to elucidate the origin of these newly formed projections, we generated a labelling system based on the N-myristoylation of the mCherry fluorescent protein. This system allowed us to observe both the soma and the axonal projections of dopaminergic neurons with a strong signal-to-noise ratio. Combination of both the 6-OHDA lesion model with the FLEX-myr-mCherry reporter hinted that these new axons could partly arise from VTA dopaminergic neurons. However, we realised that this system could be misleading due to the highly arborised morphology of dopaminergic neurons and the close proximity of the VTA and the SNpc. In order to overcome this problem, we used retrograde fluorescent beads which were injected in the same coordinates as the 6-OHDA lesion to avoid reaching intact projections. Results following this procedure revealed that while in control mice many dopaminergic neurons mostly residing in the SNpc took them up, in mice previously lesioned with 6-OHDA fewer cells were positive for the RB, some of them located in the VTA. In a previously published study, authors used this same tracing method to assess if dopaminergic neurons that expressed vGlut2 were more prone to axonal sprouting after lesioning the dorsal striatum (Kouwenhoven et al., 2020). Their results are in accordance with the ones we obtained regarding the origin of the collaterals, as they also claim to observe beads in the VTA-residing dopaminergic neurons. Nonetheless, as it was not the aim of their study, they did not inquire further. In another publication reporting axonal sprouting from spared neurons after a severe lesion of the dorsal striatum, authors imply that the reinnervation could come from the neurons projecting to the ventral striatum due to the ascending fashion of this process, but no assessments were performed in order to elucidate this point (Schmitz et al., 2013). Studies performed in rats also observed this reinnervation following a moderate lesion, although in this occasion both the neurotoxin and the tracer were injected in the SNpc. They observed sprouting of the dopaminergic neurons in the SNpc, but could not assess if there was a contribution also from VTA-dopaminergic neurons as they did not receive the anterograde tracer (Finkelstein et al., 2000). Further studies should be performed to elucidate if VTA neurons support SNpc-DANs in the compensation process branching out their axons, or if only dopaminergic neurons of the nigrostriatal

pathway have this capacity. If the involvement of VTA dopaminergic neurons in the reinnervation process is confirmed, it would be an exceptional event as these neurons, even though dopaminergic, are not involved in the same pathway and brain functions than the ones residing in the SNpc.

Overall, these results suggest that reinnervation is indeed a contributor to the compensation process operating in the presymptomatic phase of PD. Despite these compelling observations, the anatomical and physiological bases of DAN plasticity are largely unknown. Identifying the molecular pathways inducing axonal sprouting and reinnervation would disclose new therapeutic targets which could be exploited to delay the appearance of symptoms in PD patients.

One central question that remains unresolved is the threshold of neurodegeneration after which the system fails to adjust. In a previous study, authors define a correlation between the percentage of neuronal loss and the number of varicosities and branches of the collaterals (Finkelstein et al., 2000). In that study performed in rats, they observed how up until 75% of neurodegeneration of the SNpc, spared neurons expand their branches to counteract for the lost neurons. In an effort to set the threshold in mice and evaluate the conditions that permit this regenerative event to occur, we proposed an alternative neurodegenerative model, able to degenerate specifically dopaminergic neurons without altering much the local environment and the surrounding cells. This genetic tool, consistent of the overexpression of the activated pro-apoptotic protein Caspase-3, will replace 6-OHDA with the advantage of controlling cell death through a robust genetic inducible system. With it, we will be able to assess the changes undergone by the spared neurons at different neurodegenerative stages while analysing the alterations of the striatum environment.

Considering all the facts, these results indicate that restorative and adaptive responses in the CNS may be more frequent than previously recognised and suggest that the reparative potential displayed by specific brain circuits could be leveraged to promote functional recovery in response to neurodegenerative diseases or brain injuries (Bezard et al., 2003). For PD patients, the unravelling of this compensatory processes could potentially represent a disease-modifying therapy, causing a delayed appearance of the symptoms and an increased response to pharmacological treatments (Brotchie & Fitzer-Attas, 2009).

5 MATERIALS AND METHODS

5.1 Animals

Mice were bred and maintained at San Raffaele Scientific Institute animal facility under a 12-hour light-dark cycle, with food and water *ad libitum.* All procedures had prior approval of the internal Institutional Animal Care and Use Committee (IACUC 995, Ospedale San Raffaele, Milan, Italy) and reported to the Italian Ministry of Health according to the European Commission Council Directive 2010/63/EU.

The strains used in the experiments described in this thesis are:

Given name	
Aldh1l1-cre/ERT2	B6;FVB-Tg(Aldh1l1-cre/ERT2)1Khakh/J
Ai9	B6;129S6-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J
DAT-Cre	Slc6a3$^{tm1(Cre)Xz}$/J

Table 5.1 Transgenic mouse strains

5.2 Molecular cloning

The plasmids LV-FLEX-*Ascl1*-V5-IRES-GFP, LV-FLEX-myr-mCherry and LV-FLEX-GFP were generated by amplifying through PCR the cassettes *Ascl1*-V5-IRES-GFP, myr-Cherry and GFP adding the loxP and lox2272 and the restriction sites for BamHI and SalI at the 5'- or 3'-end. Subsequently, purified amplicons were cloned into the lentiviral backbone LV-EF1α-GFP, kindly donated by Dr. Naldini's group, and were digested using the same enzymes.

Regarding the plasmid LV-tetO-ALiN, it was generated by cloning of three transcription factors *Ascl1*, *Nurr1* and *Lmx1a* in a single multicistronic LV vector.

Finally, the plasmid AAV-PHP.B-CBA-FLEX-Casp3-TEVp was cloned by amplifying through PCR the cassette FLEX-Casp3-TEVp adding the loxP and lox2272 sites as well as the restriction sites for EcoRI and XhoI at the 5'- or 3'-end. Then, the amplicons were subsequently cloned in the AAV-CBA-GFP (Luoni et al., 2020) digested with the same restriction enzymes.

5.3 Virus production and purification

5.3.1 Lentivirus

To produce lentiviral replication-incompetent vectors, we start by plating HEK-293T cells in 150 mm dishes. The following day, 2-3 hours before the transfection, the medium was changed for Iscove Dulbecco's modified medium complemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin/streptomycin (Pen/Strep), 1% pyruvate solution and 1% non-essential amino acids. Then, cells were transfected with a mix containing 30µg of vector and packaging following a conventional calcium phosphate protocol. Thirty hours later, the medium was collected, filtered through a 0,44µm cellulose acetate filter, and centrifuged at 2×10^4 rpm in the ultracentrifuge for 2 hours at 20ºC. Then, the pellet containing the concentrated virus was resuspended in PBS and aliquoted in small volumes to store at -80ºC until used.

5.3.2 AAVs

To generate AAV replication-incompetent vectors, recombinant viral particles were produced in 293T cells, cultured in Dulbecco Modified Eagle Medium (DMEM) – high glucose containing 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine and 1% Pen/Strep (Luoni et al., 2020). Cells were split every 3-4 days using trypsin 0,25%. Replication-incompetent, recombinant viral particles were produced in 293T cells by polyethylenimine (PEI) co-transfection of three different plasmids: transgene-containing plasmid, packaging plasmid for rep and cap genes and pHelper for the three adenoviral helper genes. The cells and supernatant were harvested after 120 hours. Cells were lysed in hypertonic buffer (40 mM Tris, 500 mM NaCl, 2 mM $MgCl_2$, pH=8) containing 100U/mL Salt Active Nuclease (SAN) for 1 hour at 37°C, whereas the viral particles present in the supernatant were concentrated by precipitation with 8% polyethylene glycol 8000 (PEG8000) and then added to supernatant for an additional incubation of 30 min at 37°C. In order to clarify the lysate cellular debris were separated by centrifugation at 4×10^3 g for 30min. The viral phase was isolated by iodixanol step gradient (15%, 25%, 40%, 60% OptiPrep™) in the 40% fraction and concentrated in phosphate buffer saline (PBS) with 100K cut-off concentrator (Amicon Ultra-15). Virus titers were determined using AAVpro© Titration Kit Ver.2.

5.4 Primary astrocytic cultures (P0-P3)

For this procedure, wild-type mice of 0 to 3 days of age were used. First, after dissecting the head, it was transferred into a Petri dish containing HBSS (Hank's Buffered
Salt Solution without Ca^{2+} and Mg^{2+}) medium. Then, with a pair of tweezers, the skin and skull were carefully removed. Once the brain was extracted, under the microscope, the cortex was separated from the midbrain and the hippocampus, and the meninges were delicately removed. All cortex were then cut in small pieces and collected in a falcon tube containing 5 mL of clean HBSS medium. Before enzymatic dissociation through a 20 min incubation at 37ºC in 5 mL of 0,25% Trypsin, tissue disgregation was achieved by pipetting several times with first, p1000 and then p200 pipette tips. Subsequently, to inactivate the trypsin, 10mL of C+ medium (DMEM, FBS 10%, glutamine 1%, Pen/Strep 1%, Sodium Pyruvate 1% and non-essential AA 1%) was added containing 100µL of DNAse 100x. After a 10-minute centrifuge at $1,2x10^4$ rpm, the whole volume was filtered using a 70µm cell strainer to eliminate any remaining debris. Finally, 10 mL of fresh medium was used to clean the filter so to collect all the cells. Finally, cells were counted, and plated on Matrigel® coated 24-well plates in C+ medium.

5.5 *In vitro* differentiation of primary astrocytes into iNs

Primary astrocytes at low passages were plated on a 24-well plate previously coated with Matrigel® GFR at a density of 6×10^4 cells/well. Plating and infection were done using C+ medium. Then, all wells except control ones received the appropriate combination of LV. Those infected with LV-FLEX-*Ascl1*-V5-GFP were also infected with a LV-EF1α-Cre, while those infected with LV-tetO-ALiN received also an LV-rtTa. The day after the infection, cells were shifted to a neuronal induction medium which consists of DMEM-F12 complemented with Pen/Strep 1%, B27 2%, 20ng/mL BDNF, Forskolin 10µM, glucose 45% and doxycycline 2µg/mL, when required. Half of the medium was changed every other day. Cells were kept 10 and 20 days post infection before analysing them.

5.6 Stereotaxic injections

Mice between 8 and 12 weeks of age were used for most of the experiments. As a standard procedure, mice were initially anesthetised with 4% isoflurane, fixed in a Kopf stereotaxic frame where it kept receiving 2% isoflurane throughout all the procedure, and injected in different coordinates and with a different solution depending on the aim of the experiment. After surgery, animals were left on a heating pad for some time until fully recovered before reintroducing them in the cage.

5.6.1 Intrastriatal P2 injections

Aldh1l1-Cre/ERT2/Ai9 mice of 2 days of age were anesthetised by hypothermia, putting them in crushed ice for five minutes. Once the pups were unresponsive, they were placed on the neonatal mouse adaptor of a Stoelting stereotaxic frame to begin the surgery. After sterilising the head by wiping it with 70% ethanol solution, animals were injected intrastriatally with 200 nL of LV-FLEX-*Ascl1*-V5-GFP at a rate of 100 nL/min. For neonatal P2 mice, the coordinates were the following (mm): anteroposterior (AP): +2,4; mediolateral (ML): ±1; dorsoventral (DV): -1,7 from the skull (relative to lambda). After the injection, the needle was slowly removed so to avoid the reflux of the virus. Last, mice were kept on a heating mat until they recovered completely. For three days following the viral injection, animals were injected with 50µL of 1mg/mL tamoxifen solution to induce the expression of the Cre recombinase. Given their small size, the administration was performed intra-stomach.

5.6.2 Intrastriatal Ascl1 injections in adult mice

8-12-weeks-old *Aldh1l1*-Cre^{ERT2}/Ai9 mice were injected unilaterally with LV-FLEX-*Ascl1*-V5-GFP at a high titre. Each mouse was infused 2µL of the virus at a rate of 0,25µL/min at the coordinates (mm): AP: +0,5; ML: ±1,8; DV: -3,3 from the skull (relative to bregma). After, the needle was left in place for 4 min before removing it. Following surgery, mice were injected i.p. for three consecutive days with 100µL of tamoxifen solution (75mg/kg). They were finally sacrificed at 2, 4 and 6 w.p.i. to perform the subsequent immunofluorescence analyses.

5.6.3 6-OHDA lesions

For depletion of dopaminergic terminals in the dorsal striatum, adult wild-type mice were injected unilaterally or bilaterally with 6-hydroxydopamine-HCl (6-OHDA)

at a concentration of 5µg free-base/µL. 6-OHDA solution was prepared fresh before every surgery by dissolving the powder in the appropriate volume of 0,02% ascorbic acid in 0,9% saline solution to avoid its oxidation. Half an hour prior to undergo surgery, mice were injected i.p. with desipramine solution (25mg/kg) to protect the noradrenergic system from the neurotoxic effect. They were then anaesthetized with 4% isoflurane, fixed in the stereotaxic frame, and injected with 1,5µL of the 6-OHDA solution in the dorsal striatum at the following coordinates (mm): AP: +0,9; ML: ±2; DV: -2,4 from the skull (relative to bregma).

5.6.4 Retrobeads™

Red fluorescent microspheres, Retrobeads™ IX, were purchased from Lumafluor to perform retrograde tracing analysis. Once tested, different volumes of beads diluted and non-diluted were injected in the striatum of WT C57BL6 mice. For the experiments following 6-OHDA lesion, mice were injected unilaterally with 0,8µL of diluted (1:4) beads solution following the same coordinates as for the neurotoxin injection. The needle was left in the injection site for 5 min after the delivery, and then it was slowly removed so to avoid the microspheres reflux.

5.6.5 Activated Caspase3 injections

Adult DAT-Cre and wild-type C57BL6 mice received unilateral injections in the SNpc of pH-FLEX-Casp3-TEVp at different titres (original titre: $3x10^{12}$ vg/mL). Animals were infused 1µL of virus at a rate of 0,2µL/min in the coordinates (mm): AP: -2,9; ML: ±1,2; DV: -4,4 from the skull (relative to bregma).

5.6.6 Myr-mCherry labelling

For labelling the SNpc dopaminergic neurons, 8-12 weeks-old DAT-Cre mice received 1µL injections of LV-FLEX-myr-mCherry infused at a rate of 0,2µL/min in the coordinates (mm): AP: -2,9; ML: ±1,2; DV: -4,4 from the skull (relative to bregma).

To specifically label VTA dopaminergic neurons, DAT-Cre mice received 2 injections of 0,5 µL at the following coordinates (mm): AP: -2,9; ML: ±0,5; DV: -4,3 | AP: -3,4; ML: ±0,3; DV: -3,49 from the skull (relative to bregma).

5.7 Immunohistochemistry

Free-floating brain slices from transcardially perfused mice contained in PBS-Azide 0,1% were selected considering the area of interest. Then, to block endogenous peroxidases, slices were incubated for ten minutes in a 3% hydrogen peroxide solution. After that, three washes in PBS 1x were performed followed by an hour of incubation at room temperature (RT) in a 0,3% triton, 3% bovine serum albumin (BSA) blocking solution. Next, slices were kept overnight at 4ºC in a 0,3% triton, 1% BSA solution containing the primary antibody. The following day, after 3 washes in PBS 1x to remove any trace of primary antibody, incubation with the appropriate biotinylated secondary antibody was performed. After one hour and four washes, slices were moved into a solution containing avidin/biotin complex (ABC) reagent and kept there for another hour. Last, to visualise the target proteins, 3,3'-diaminobenzidine (DAB) substrate kit was used. Slices were then arranged on gelatinated slides and, once dry, inserted into a glass tower containing 50% chloroform, 50% ethanol solution and left overnight to dehydrate. The next day, they were immersed in xylene for thirty minutes prior to mounting with Eukitt® medium.

5.8 Immunofluorescence

5.8.1 Primary cultures

Primary cortical astrocytes derived from P0-P3 mice were fixed for thirty minutes with ice-cold paraformaldehyde (PFA) 4% solution. They were then washed three times with PBS 1x and blocked with a 10% donkey serum (DS), 0,3% triton solution for an hour at RT before an overnight incubation with the primary antibody solution (10% DS, 0,1% triton). The next day, after three washes with PBS 1x, solution containing secondary antibodies (10% DS, 0,1% triton) was added to the cells. After that, they were washed twice and mounted with a fluorescent mounting medium.

5.8.2 Brain slices

Free-floating coronal sections from transcardially perfused mice, flashed frozen in ice-cold 2-Methylbutane, contained in PBS-Azide 0,1% were selected considering the area of interest. Then, a ten-minute incubation with 10% methanol, 3% hydrogen peroxide solution was performed to quench endogenous peroxidase activity. After three PBS 1x washes, 2% Triton solution was used to permeabilise the sections, improving antibody penetration. Next, to saturate unspecific binding sites, slices were left for one hour at room temperature in a 3% BSA, 0,1% Tween solution, prior to incubate them overnight at 4ºC with the primary antibody solution (1% BSA, 0,1%

Tween). Thereafter, following three PBS 1x washes, slices were immersed for one hour in a 1% BSA, 0,1% Tween solution containing the suitable secondary antibodies. Last, few washes were performed to get rid of the excess of antibodies and slices were mounted into slides using a fluorescent mounting medium.

(Antibodies used for this study are detailed in Table 5.2 at the end of the section)

5.9 Stereological cell count

The unbiased quantification of dopaminergic neurons was performed following the stereological count of TH-positive cells with Stereo Investigator software (MFB Bioscience) coupled to an automated Leica DM400B microscope. Five brain sections of 50µm of thickness containing the SNpc were random sampled by the software every 200µm. Then, using a x40 magnification objective cells were counted. The estimated total number was obtained using the optical fractionator stereological probe.

5.10 Acquisition and quantification of immunofluorescent images

Immunofluorescence images of the *in vitro* direct reprogramming experiments were acquired with a Nikon Eclipse upright microscope using a x20 magnification objective. Immunofluorescence images of brain slices were usually taken with a Leica TCS SP8 confocal microscope using a 40x magnification objective. The fluorescent signal of gliosis markers was quantified by setting a threshold to obtain unbiased results. MAVIG TS-G4 with a x20 magnification objective was used to acquire large area fluorescent images. All the images were processed using ImageJ software.

For immunohistochemical images, multiple pictures of every slice were taken with the Nikon Eclipse upright microscope using a x4 magnification objective. Subsequently, all images were blended with Adobe Photoshop and analysed with ImageJ software.

5.11 Statistics

Values are expressed as mean ± s.e.m. as indicated. All the statistical analyses were performed with Prism 8 (GraphPad software) using one-way followed by Tukey's post-hoc correction and two-way ANOVA followed by Dunnett's post-hoc correction. For experimental designs with less than three groups and one variable we used the unpaired Student T-test.

Primary	Species	Dilution	Provider	Cat. Number
CTIP2	Rat	1:500	Abcam	ab18465
DARPP-32	Rabbit	1:250	Thermo Fisher Scientific	PA5-85787
DAT	Rat	1:2000	Millipore	MAB369
DCX	Rabbit	1:500	Abcam	ab18723
FOXP1	Rabbit	1:250	Abcam	ab16645
GABA	Rabbit	1:500	Sigma-Aldrich	A2052
GFAP	Chicken	1:1000	Abcam	ab4674
GFAP	Rabbit	1:1000	Dako	GA524
GFP	Chicken	1:1000	Thermo Fisher Scientific	A10262
GFP	Rabbit	1:500	Thermo Fisher Scientific	A6455
IBA1	Rabbit	1:1000	Wako	019-19741
LMX1A	Rabbit	1:500	Abcam	ab139726
MAP2	Chicken	1:1000	Abcam	ab92434
MAP2	Mouse	1:500	Immunological Sci.	MAB-10334
mCherry	Chicken	1:1000	Abcam	ab205402
NeuN	Rabbit	1:500	Abcam	EPR12763
RFP	Rabbit	1:500	MBL	PM005
TH	Chicken	1:1000	Abcam	AB76442
TH	Rabbit	1:500	Immunological Sci.	AB-10312
TuJ1	Rabbit	1:500	Biolegend	802001
TuJ1	Mouse	1:500	Biolegend	801201
Secondary	Species	Dilution	Provider	Cat. Number
488	Chicken	1:1000	Thermo Fisher Scientific	A11039
488	Mouse	1:1000	Thermo Fisher Scientific	A21202
488	Rabbit	1:1000	Thermo Fisher Scientific	A21206
488	Rat	1:1000	Thermo Fisher Scientific	A11006
546	Mouse	1:1000	Thermo Fisher Scientific	A10036
546	Rabbit	1:1000	Thermo Fisher Scientific	A10040
594	Chicken	1:1000	Thermo Fisher Scientific	A11040
647	Chicken	1:1000	Thermo Fisher Scientific	A21449
647	Mouse	1:1000	Thermo Fisher Scientific	A31571
647	Rabbit	1:1000	Thermo Fisher Scientific	A31573
647	Rat	1:1000	Thermo Fisher Scientific	A21247
Biotinylated	Chicken	1:1000	Vector Laboratories	BA-9010
Biotinylated	Rat	-	Vector Laboratories	BP-9400

Table 5.2. Antibodies

Reagent	Provider	Cat. Number
2-Methylbutane	Sigma-Aldrich	277258
6-Hydroxydopamine hydrochloride	Sigma-Aldrich	H4381
AAVpro© Titration Kit Ver.2	TaKaRa Bio	6233
Amicon Ultra-15	Millipore	UFC910096
B-27™ Supplement	Thermo Fisher Scientific	17504044
BDNF	PeproTech	450-02
Bovine Serum Albumin Fraction V	Roche	10735086001
Chloroform	Aldrich	366927
Corn oil	Sigma-Aldrich	C8267
DAB/Ni Peroxidase Substrate Kit	Vector Laboratories	SK-4100-NB
Desipramine hydrochloride	Sigma-Aldrich	D3900
DMEM-F12	Sigma-Aldrich	D8437
DNAse I	Sigma-Aldrich	11284932001
Dulbecco's Modified Eagle Medium, high glucose	Thermo Fisher Scientific	11965092
Eukitt® Quick-hardening mounting medium	Sigma-Aldrich	3989
Fetal Bovine Serum	Sigma-Aldrich	F7524
Fluorescence Mounting Medium	Agilent technologies	S3023
Forskolin	Sigma-Aldrich	F6886
Glutamine	Sigma-Aldrich	G7513
Hank's Buffered Salt Solution without Ca^{2+} and Mg^{2+}	Euroclone	ECB4007L
Hydrogen peroxide solution 30%	Sigma-Aldrich	H1009
Iscove's Modified Dulbecco's Medium	Sigma-Aldrich	I3390
Iso-Vet	Piramal	
Matrigel GFR	Corning	354230
MEM Non-essential amino acid solution (100×)	Sigma-Aldrich	M7145
Methanol	Sigma-Aldrich	34860
Neurobasal	Thermo Scientific	21103049
OptiPrep™ Density Gradient Medium	Sigma-Aldrich	D1556
Paraformaldehyde	Sigma-Aldrich	441244
Penicillin-Streptomycin	Sigma-Aldrich	P0781
Polyethylene glycol 8000	Sigma-Aldrich	89510
Polyethylenimine	Polyscience	17938
Salt active nuclease	Sigma-Aldrich	SRE0015
Sodium azide	Sigma-Aldrich	S2002
Sodium Pyruvate Solution	Sigma-Aldrich	S8636
Sucrose	Sigma-Aldrich	S7903
Tamoxifen	Sigma-Aldrich	T5648

Triton™ X-100	Sigma-Aldrich	T8787
Trypsin 0,25% EDTA	Sigma-Aldrich	T4049
Tween® 20	Sigma-Aldrich	P7949
Xylene	VWR Chemicals	28975

Table 5.3. Reagents

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