Understanding the regulation of adult-neurogenesis following acute and neurodegenerative brain injury

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Abstract

Adult-neurogenesis is a physiological event occurring under normal conditions, which produces new neurons in specific brain regions and may have significant potential as a target for brain repair. Neurogenesis is impaired in certain neurological conditions including neurodegenerative disorders like Parkinson’s disease or acute injuries like stroke. In the last 50 years, tremendous effort has been put into understanding adult neurogenesis and how it is affected and can be manipulated in disease, with the hope to develop regenerative approaches for brain-repair. However, while extensive progress has been made, the clear mechanisms underlying its regulation remain to be further characterised. The focus of this thesis is to comprehend the potential and to define the limitations for applications related to neuronal replacement therapies based on ‘endogenous repair’. We investigated two different disease models: a progressive injury, Parkinson’s disease and an acute injury, stroke.

In a rat model of Parkinson’s disease (PD), we investigated the relative importance of the dopaminergic and noradrenergic systems in regulation of hippocampal neurogenesis. We demonstrated that, contrary to existing literature, the dopaminergic system does not project directly to the neurogenic dentate gyrus, which receives a rich noradrenergic innervation. Although studies have concluded that dopaminergic projection to the dentate gyrus regulates hippocampal neurogenesis, we further showed that the depletion of these two systems had no impact on hippocampal neurogenesis, suggesting mechanisms independent of dopamine and noradrenaline loss may underlie reduced neurogenesis in PD. Furthermore, we presented a new tool for the study of noradrenergic neuronal transplantation, the DBH-eGFP reporter mouse.

To assess the regenerative capacity of the brain after an acute injury, we used a model of ischemia using the vasoconstrictor endothelin-1 (ET-1). The results contribute to a mixed literature where there has been conjecture as to whether the adult brain is capable of generation region-specific neuronal phenotype in
response to injury. We found that after ischemic injury to the striatum of adult rats, the proliferative response does not include the production of new projection neurons. Interestingly, we also found that even in the early postnatal brain, where striatal projection neurons are being actively generated, stroke does not impact on the rate of neuronal production. This gives cause to rethink the brain’s capacity for self-repair.

This thesis comes at a time where interest for the development of regenerative medicine is very high and where the use of endogenous stem cells as a therapeutic approach is very attractive. The work presented here showed that while the brain may have potential for self-repair, there are clear limitations in its regenerative potential and the use of endogenous stem cells for therapeutics is still a long way away.
Declaration

This is to certify that:

(i) this thesis comprises of only my original work towards the PhD;

(ii) due acknowledgement has been made in the text to all other material used;

(iii) this thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Charlotte Ermine
Preface

In accordance with the regulations set by the University of Melbourne, I acknowledge that some of the work presented in this thesis was a result of collaboration. I therefore assess my contribution to this thesis to be as follows:

Chapter 3: >80% - published work. Co-authored with Dr. Jordan Wright, who provided technical assistance and with Dr. Davor Stanic, A/Prof Clare Parish and Dr. Lachlan Thompson who were involved with the study design, data interpretation and manuscript preparation.

Chapter 4: 90% - with technical assistance provided by Dr. Jordan Wright, Dr. Jessica Kauhausen and Mr. Stefano Frausin.

Chapter 5: 100%

Chapter 6: >80% - with technical assistance provided by Dr. Jordan Wright

Appendix 1: 10% - published work. I provided technical assistance to this paper.

My overall contribution to the work presented in this thesis is 90%.
Publications, Presentations & Awards

Publications:


Presentations:


C.M. Ermine, J.L. Wright, C.L. Parish & L.H. Thompson. Catecholamine innervation of the hippocampus and the relevance to adult neurogenesis and dementia in Parkinson’s Disease. Federation of European Neuroscience Societies, Milan, Italy (5th-9th July 2014). Poster presentation.

Awards:

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2015 – Lois Browne Memorial travel award

2014 – Keystone Symposia travel scholarship

2014 – Melbourne award travelling scholarship

2013-2016 – Melbourne International Engagement Award
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<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<td>Acc</td>
<td>nucleus accumbens</td>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<td>BAC</td>
<td>bacterial artificial chromosomes</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>BL</td>
<td>basal lamina</td>
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<td>BrdU</td>
<td>5-bromo-2′-deoxyuridine</td>
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<tr>
<td>BV</td>
<td>blood vessel</td>
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<tr>
<td>CC</td>
<td>corpus callosum</td>
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<td>ChAT</td>
<td>choline acetyltransferase</td>
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<td>CPu</td>
<td>striatum</td>
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<td>CR</td>
<td>calretinrin</td>
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<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>Darpp32</td>
<td>cyclic-AMP-regulated phosphoprotein of molecular weight 32,000</td>
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<td>DBH</td>
<td>dopamine-β-hydroxylase</td>
</tr>
<tr>
<td>DCX</td>
<td>doublecortin</td>
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<td>DG</td>
<td>dentate gyrus</td>
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<tr>
<td>DSP-4</td>
<td>N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>ET-1</td>
<td>endothelin-1</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FG</td>
<td>fluorogold</td>
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<td>FGF-2</td>
<td>basic fibroblast growth factor</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>Glu</td>
<td>glutamate</td>
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<td>GPi</td>
<td>globus pallidus intern</td>
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<td>HBSS</td>
<td>Hank's buffered salt solution</td>
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<tr>
<td>HD</td>
<td>Huntington's disease</td>
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<td>HP</td>
<td>hippocampus</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>i.c.v.</td>
<td>Intra cerebral ventricle</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
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<td>L-DOPA</td>
<td>L-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LV</td>
<td>lateral ventricle</td>
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<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
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<td>mDA</td>
<td>midbrain dopamine</td>
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<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>NA</td>
<td>noradrenaline</td>
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<tr>
<td>NET</td>
<td>noradrenergic transporter</td>
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<td>OB</td>
<td>olfactory bulb</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PD</td>
<td>Parkinson's disease</td>
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<td>Prox1</td>
<td>Prospero homeobox 1</td>
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<tr>
<td>RMN</td>
<td>Raphe nucleus magnus</td>
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<tr>
<td>RMS</td>
<td>rostral migratory stream</td>
</tr>
<tr>
<td>SERT</td>
<td>serotonin transporter</td>
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<tr>
<td>SGL</td>
<td>sub-granular layer</td>
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<td>SGZ</td>
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<td>SNpc</td>
<td>substantia nigra pars compacta</td>
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<td>SVZ</td>
<td>subventricular zone</td>
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<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
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<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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1.1. Adult neurogenesis

It was long thought that neurons were only produced during developmental stages and that the brain was ‘set’ at birth. But evolution of technologies and techniques have shown that neurons can be produced in adulthood. Indeed, the development of autoradiography allowed Altman to present in 1963 the first evidence of adult neurogenesis in rodents and cats (Altman 1963). This finding was later confirmed, by Nottebohm who studied song birds and identified adult born neurons in the vocal centre of the neostriatum (Kirm, Alvarez-Buylla, and Nottebohm 1991). A few years later, adult neurogenesis was shown to occur in human brain thanks to an in vitro study of adult human temporal lobe cultures autoradiographed with 3H-thymidine (Kirschbaum et al 1994) and to a human post-mortem immunostaining analysis with the proliferating cell marker 5-bromo-’2’-deoxyuridine (BrdU) (Eriksson et al. 1998). Furthermore, the phenotypic analysis of BrdU proliferating cells in aged rodents, has confirmed that adult neurogenesis was occurring throughout adulthood with an age-related decline (Kuhn, Dickinson-Anson, and Gage 1996). More recently, Frisen’s team used a novel technique to date human cells: the C14 released in the atmosphere during nuclear bomb testing in the cold war (Spalding et al. 2013). They compared the proportion of C14 contained in the genomic DNA of different human brain cells, with the proportion of C14 contained in the atmosphere in the birth year for each individual. They identified a discrepancy in hippocampal DNA that led to the conclusion that the hippocampal cells were born during the adulthood of the individual. They described a 1.75% annual neuronal turnover in the hippocampus.

Today two regions in the adult mammalian brain are known to be neurogenic niches: the subgranular zone (SGZ) in the dentate gyrus and the subventricular zone (SVZ) surrounding the lateral ventricle (Gage 2000).
1.1.1. Subgranular zone

The subgranular zone is a germinal layer located between the dentate gyrus and the hilus of the hippocampus. Three cell types have been characterised in the SGZ: dividing astrocytes, that give rise to immature neuroblasts (type D cells) and the granule cells which are mature neurons (Doetsch 2003). The progenitor cells, type D, migrate to the granular layer and mature into granule neurons (type G cell) (Gould 2007; Alvarez-Buylla and Lim 2004) (Figure 1.1).

Figure 1.1: Adult SGZ neurogenesis, image from (Alvarez-Buylla & Lim 2004). A) Coronal section of the adult mice brain showing the DG. B) Cellular organisation of the SGZ, with the astrocytes (As, in blue), immature neuroblasts (D cells, in orange) and granule neurons (G cell, in red). C) SGZ lineage. Abbreviations: DG, Dentate gyrus; HP, Hippocampus; BL, Basal lamina; BV, Blood vessel.

Since the discovery of adult neurogenesis, a lot of effort has been put towards understanding its physiological and functional role, but it is still not completely understood. Some progress has however been made in this regard in recent years, especially the role played by DG neurogenesis in hippocampal functions. Indeed, since the hippocampus receives major projections from the DG, it was suggested that the DG plays an important role in hippocampal functions like learning and memory.
Nottebohm's study in song birds supports this hypothesis (Kirn, Alvarez-Buylla, and Nottebohm 1991). They used a cell proliferation marker [3H] thymine and a retrograde tracer, fluorogold, to identify new progenitor cells and their destination. The new neurons were sending projections to the part of the brain that controls song learning in birds and their survival rate was correlated with seasonal song modification. Additional evidence supporting this role in learning and memory was presented by Gould et al. (1999) who reported that hippocampal neurogenesis is regulated positively by hippocampal-dependent learning (Gould, Beylin, et al. 1999). In their study, they developed a series of behavioural tasks involving hippocampal learning (spatial navigation learning in Morris water maze) or its absence (cue training in Morris water maze) and showed an increase in cellular proliferation in the hippocampal learning task only. It should however be noted that the interaction between hippocampal neurogenesis and learning seems complex. Indeed, Olariu et al. (2005), have shown that one day of training for an associative learning task increases the survival of new progenitor cells, while two days of training had the opposite effect (Olariu et al. 2005). Additionally, an eye-blink conditioning study has shown that the elimination of new neurons generated in the DG, induces a reduction of hippocampal-dependent learning, suggesting the implication of hippocampal neurogenesis in memory formation (Shors et al. 2001). They further estimated the cells to be about 1-2 weeks of age when they are involved in memory acquisition.

1.1.2. Subventricular zone

The subventricular zone is located along the lateral wall of the lateral ventricle. The SVZ is organised in four different cell types: type B, type C, type A and ependymal cells. The ependymal cells form a thin layer of cells along the lateral ventricle wall. Type B cells are astrocytes, which differentiate into type C cells, transit-amplifying neural precursor cells. The type C cells give rise to migrating neuroblasts, classed as type A cells. The type A cells migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they mature into granule and periglomerular cells, the two major types of interneurons (Doetsch 2003; Luskin 1993) (Figure 1.2, Alvarez-Buylla & Lim, 2004).
While in rodents and primates the new neurons are shown to preferentially migrate to the OB (Lois and Alarez-Buylla 1994; Carleton et al. 2003; Kornack and Rakic 2001) human studies have revealed that the SVZ mature neurons are not all going to the olfactory bulb in the human brain, but have a rather shared destination to the striatum and the prefrontal cortex (Sanai et al. 2011; Ernst et al. 2014).

Figure 1.2: Adult SVZ neurogenesis, image from (Alvarez-Buylla & Lim 2004). A) Coronal section of mice brain at the level of the SVZ. B) Cellular organisation of the SVZ, with the four cell types, migrating neuroblasts (type A, in red), astrocytes (type B, in blue), transit-amplifying neural precursors (type C, in green) and ependymal (in grey). C) SVZ lineage. **Abbreviations:** LV, Lateral ventricle; BL, Basal lamina; BV, Blood vessel.

The SVZ has been implicated in odor discrimination in rodents (Gheusi et al. 2000). In adult mice, an odor-enriched environment was shown to increase the survival of new born cells in the OB and to increase olfactory memory (Rochefort et al. 2002), further confirmed by an olfactory sensation deprivation study (Petreanu and Alvarez-Buylla 2002). Cecchi et al. (2000) have developed a computational model of the OB and suggest an adaptive mechanism of neurogenesis activity to respond to environmental changes (Cecchi et al. 2000).
1.1.3. Adult neurogenesis and brain injury

Adult neurogenesis has been the focus of many research studies in the last two decades with the aim to identify its physiological function, but also its potential involvement in brain injury and disease. A reduction of neurogenesis levels is observed in patients suffering from neurodegenerative diseases like Parkinson’s disease (PD), Huntington’s disease (HD) and Alzheimer’s disease (AD); and from psychiatric diseases like depression and anxiety. For example, different studies have reported a decrease in hippocampal proliferation in rodents models of HD (Lazic et al. 2004), AD (Verret et al. 2007) and PD (Beate Winner et al. 2008) and anti-depressants have been described as pro-neurogenic in the DG (Malberg et al. 2000). In addition, the inflammation associated with epilepsy has also been shown to be detrimental for neurogenesis (Ekdahl et al. 2003). Moreover, an increase in neurogenesis activity has been shown to be detrimental in patients suffering from epilepsy. Indeed seizures have been reported to increase proliferation in both the SVZ and the SGZ (Jessberger and Parent 2015). However, increased neurogenesis seen both in the SVZ and SGZ after acute injuries such as Stroke and ischemia, might have potential therapeutic benefits (Kokaia and Lindvall 2003).

In this thesis we will focus on understanding: 1) the regulation of adult hippocampal neurogenesis using an example of neurodegenerative disease, Parkinson’s disease and 2) the potential for brain repair using an example of acute injury, striatal ischemia.

1.2. Neurogenesis in neurodegenerative disease: Parkinson’s disease

1.2.1. Parkinson’s disease

James Parkinson was the first to describe PD in his essay on the shaking palsy written in 1817 (Parkinson, 2002). PD is the second most common neurodegenerative disease and affects around 1% of individuals older than 60 years (Samii, Nutt, and
PD is mainly described as a movement disorder. Its major motor symptoms, which are often referred to as the triad, are: resting tremor, rigidity and bradykinesia (Samii, Nutt, and Ransom 2004). Although the motor symptoms in this disorder are the most commonly described and studied, PD patients also express non-motor symptoms. Thus PD is sometimes described as an iceberg, where the motor symptoms are the visible part and the non-motor symptoms are the submerged part of it (Barbosa 2013) – i.e. the motor symptoms are the conspicuous indicator of a much broader pathology. The non-motor symptoms are diverse and often include: neuropsychiatric problems (depression or cognitive disorder), sleeping disorder, autonomic dysfunction (constipation, postural hypotension, miccional disorder), fatigue and pain (Barbosa 2013). It was reported in a study involving 112 PD patients that 96.4% experienced at least one non-motor symptom (Khedr et al. 2013).

According to the pioneering study from Hinnell et al. (2012) the non-motor symptoms have a bigger negative impact on a patient’s quality of life than motor symptoms, with depression considered the most disabling. Non-motor symptoms usually arise at early onset of the disease, before the first motor dysfunctions and before the patient is diagnosed with PD. One of the first non-motor symptoms is olfactory impairment, with more non-motor symptoms developing as the disease progresses (Breen and Drutyte 2013; Cosentino, Nuñez, and Torres 2013).

The pathology of PD is associated with a major loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) (Greenfield and Bosanquet 1953). The diagnosis is often made very late as the motor symptoms arise when 75% of dopaminergic neurons have already degenerated (Lees 2007). The brains of PD patients are characterised by the presence of inclusions, called Lewy bodies. They represent aggregates of mis-folded proteins; of which α-synuclein is the most predominant (Wakabayashi et al. 2013). The role of the Lewy bodies as neuroprotective or neurotoxic inclusions is still unknown (Wakabayashi et al. 2013). The most extensively examined structure with neuronal loss in PD is the SNpc; however, an autopsy study revealed that Lewy bodies are not confined to the SNpc, but are spread out throughout the brain (Braak and Del Tredici 2008). In PD various regions are undergoing degeneration, including the locus coeruleus (LC) (Braak and
Del Tredici 2008). Braak et al. (2008) were the first to suggest a caudo-rostral progression of the disease, whereby early pathology may be relatively confined to the enteric nervous system and spread rostrally to progressively incorporate the brain stem (including the LC) and midbrain (Braak and Del Tredici 2008).

While currently there is no curative treatment for PD, some treatments are effectively used to relieve the motor symptoms and two approaches are predominant. The first one is a pharmacologic approach based on dopamine (DA) replacement, where the most popular and effective compound is a dopamine precursor: L-dihydroxyphenylalanine (L-DOPA) (Birkmayer and Hornykiewicz 1998). The second approach performed, which has been shown to successfully relieve motor symptoms is a surgical intervention, that involves high frequency deep brain stimulation of the subthalamic or globus pallidus interna (Rascol et al. 2011).

As mentioned above, current treatments merely treat the motor symptoms in PD; nevertheless, the non-motor symptoms are the most disabling for the patient. Therefore, it is necessary to study their origins in order to identify new targets for therapeutic treatments of non-motor symptoms.

### 1.2.2. Parkinson’s disease & adult neurogenesis

#### 1.2.2.1. Subventricular zone

Adult neurogenesis is thought to be impaired in the SVZ of Parkinsonian patients. Indeed, it is suggested by human post-mortem studies, that SVZ neurogenesis is reduced in patients suffering from Parkinson’s disease compared to aged matched controls (Höglinger et al. 2004). Höglinger et al. (2004) have shown a 30% reduction in SVZ proliferation, using the number of proliferating cell nuclear antigen (PCNA) positive cells, which is a proliferation marker, present in the subependymal zone. Olfactory impairment has been reported to be among the first symptoms in PD. Since SVZ neurogenesis seems to play an important role in olfactory memory and discrimination, another group has indirectly suggested a decrease in SVZ neurogenesis, by reporting a significant loss of volume of the OB in PD patients.
(Brodoehl et al. 2012). However, while those studies have suggested an impairment of SVZ neurogenesis, one post-mortem analysis reported no difference in proliferation between PD patients, controls and Lewy body disease patients (Van Den Berge et al. 2011).

The reduced SVZ neurogenesis observed in PD patients was further suggested in rodent models of DA depletion, often used as a simplified model of PD; however, the literature here is mixed. For example, using the 6-hydroxydopamine (6-OHDA) toxin in rodents to reproduce the DA depletion in PD, some groups have shown a reduction in cellular proliferation (Baker, Baker, and Hagg 2004; Beate Winner et al. 2006) while others have reported an increase in proliferation (Aponso, Faull, and Connor 2008; B. fang Liu et al. 2006). Similarly in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model, one group reported a reduction in proliferation and neurogenesis (Höglinger et al. 2004), while another group reported no difference in proliferation level (Van Den Berge et al., 2011). The use of DA receptor agonists have either been reported to increase proliferation in rats (Beate Winner et al. 2009; Van Kampen and Eckman 2010) and mice (Lao, Lu, and Chen 2013) or show no effect in mice (Baker, Baker, and Hagg 2005) or murine and human midbrain cell culture (Milosevic et al. 2007). Furthermore, PD-induced reduction of SVZ neurogenesis was further supported by transgenic mouse studies. One example is the transgenic mice model of PD expressing human α-synuclein, which has shown a decrease in SVZ neurogenesis possibly caused by a reduction of survival of newly generated neurons (Nuber et al. 2008; Marxreiter et al. 2009) and by an impaired neuronal migration to the OB (Tani et al. 2010). Table 1.1 presents an overview of the literature, which has revealed mixed findings on the effect of DA denervation on proliferation in the SVZ.
Table 1.1: Summary of studies that investigated the role of DA on SVZ proliferation and their conclusion. Studies in green have reported a positive effect of DA on proliferation, studies in red have reported a negative effect and studies in white have reported no effect of DA on SVZ proliferation.

<table>
<thead>
<tr>
<th>Study</th>
<th>Experimental design</th>
<th>Concluded role of DA on proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker et al. 2004</td>
<td>6-OHDA, mice</td>
<td>↑</td>
</tr>
<tr>
<td>Van Kampen et al. 2004</td>
<td>D3 agonist, rats</td>
<td>↑</td>
</tr>
<tr>
<td>Höglinger et al. 2004</td>
<td>MPTP, mice</td>
<td>↑</td>
</tr>
<tr>
<td>Baker et al. 2005</td>
<td>D3 agonist, mice</td>
<td>No effect</td>
</tr>
<tr>
<td>Liu et al. 2006</td>
<td>6-OHDA, rats</td>
<td>↓</td>
</tr>
<tr>
<td>Winner et al. 2006</td>
<td>6-OHDA, rats</td>
<td>↑</td>
</tr>
<tr>
<td>Milosevic et al. 2007</td>
<td>D2/D3 agonist, in vitro</td>
<td>No effect</td>
</tr>
<tr>
<td>Aponso et al. 2008</td>
<td>6-OHDA, rats</td>
<td>↓</td>
</tr>
<tr>
<td>Winner et al. 2009</td>
<td>DA agonist, rats</td>
<td>↑</td>
</tr>
<tr>
<td>Van Den Berge et al. 2011</td>
<td>MPTP, mice</td>
<td>No effect</td>
</tr>
<tr>
<td>Lao et al. 2013</td>
<td>D3 agonist, mice</td>
<td>↑</td>
</tr>
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1.2.2.2. Subgranular zone

Neurogenesis in the SGZ is also thought to be impaired in PD patients. This was indirectly shown by measurements of hippocampi from PD patients with and without dementia, where a progressive hippocampal volume loss was reported in PD, with PD with dementia having a larger atrophy than PD alone (Laakso et al. 1996; Camicioli et al. 2003). More directly, a post-mortem study has revealed a reduction in number of neural precursor cells in the SGZ of patients suffering from PD (Höglinger et al. 2004).

In rodent models of PD, a reduction of neurogenesis was observed in the DG when the animals were injected with low dose of MPTP, as reported by the reduced number of PCNA+ cells (Höglinger et al. 2004); however only a transient increase in
proliferation and neurogenesis was observed in the DG when the animals were injected with high dose of MPTP (Park and Enikolopov 2010). In another study, rats injected with 6-OHDA in the SNpc, presented an enhanced proliferation and survival of new neurons in DG compare with control rats (Suzuki et al. 2010). Transgenic mouse models of PD over-expressing the human α-synuclein have reported a reduction of proliferation and survival in the SGZ in one study (Crews et al. 2008) and no difference in proliferation, but with a reduction of survival in two other studies (Beate Winner et al. 2004; Nuber et al. 2008). In addition, in another transgenic mice model presenting a mutation found in familial cases of PD, LRRK2, a reduction in proliferation and survival was reported in the SGZ and also in the SVZ (B Winner et al. 2011).

1.2.3. Adult hippocampal neurogenesis and the origins of cognitive symptoms

The hippocampal neurogenesis contributes to cognitive functions, including memory and learning ((Gould, Tanapat, Hastings, & Shors, 1999; Kirn et al., 1991, and reviewed in (Oomen et al., 2014)), which are symptoms observed in PD. It was suggested that the non-motor symptoms of PD correlate with an hippocampal neurogenesis impairment (Regensburger, Prots, and Winner 2014; Marxreiter, Regensburger, and Winkler 2013). Many studies have therefore focused on identifying the origins of this dysfunction of neurogenesis and many have focused on the hippocampal regulators that are known to be degenerating in PD, i.e. the dopaminergic system and the noradrenergic system (Höglinger et al. 2004; Suzuki et al. 2010; Schlachetzki et al. 2016; Langston and Ballard 1984; Park and Enikolopov 2010; Jhaveri et al. 2010; Kulkarni, Jha, and Vaidya 2002).
1.2.3.1. The dopaminergic hypothesis

The different studies presented below, have suggested that the dopaminergic system is implicated in hippocampal function, including memory processes. These studies conclude that a key regulator of hippocampal neurogenesis is the dopaminergic system.

It was first suggested that the hippocampus and ventral tegmental area (VTA), a suggested source of DA in the midbrain, are part of a loop that is responsible for long-term memory formation (Lisman and Grace 2005). Pharmacological studies using antagonists of dopamine receptors have shown that hippocampal dopamine acts through D₁ dopamine receptors to mediate long-term memory formation (Carroll et al. 2006) and persistence (Rossato et al. 2009). In addition whole cell patch clamping of the granule cells of the DG from both rat and humans, has shown that dopamine release can predispose the induction of long-term potentiation, an essential electrical process for synaptic plasticity, by those cells (Hamilton et al. 2010). The reward mechanism, which involves DA release, induces an increase in learning rate. A recent study has investigated the effects of bilateral lesion of the nigrostriatal dopaminergic pathway on synaptic plasticity and cognitive performance in mice (Bonito-Oliva et al. 2013). The authors have shown that total SNpc lesion and partial LC lesion caused a reduction of LTP in the DG. Using pharmacological D₁ dopamine receptor agonist and L-DOPA they discriminate the factor as being DA and not noradrenaline (NA). However, NA cannot be totally excluded from the potential factors as they are comparing a total SN lesion and a partial LC lesion, thus the NA neurons left may compensate for the ones missing. Furthermore some studies have associated dopaminergic degeneration with impairment of adult-neurogenesis in the SVZ and the SGZ in PD (Höglinger et al. 2004; Suzuki et al. 2010), thus suggesting that DA depletion in PD is the main factor responsible for the cognitive symptoms. However, both studies appear to have underestimated the importance of the potential role of NA depletion in regulating hippocampal neurogenesis. Indeed, some evidences suggest a bigger role of a non-dopaminergic pathway. Firstly an argument against the dopaminergic hypothesis is: DA replacement therapy does not alleviate the cognitive symptoms (Deak and Bishop 2011). Secondly DA fibres are not projecting to the DG
but to the CA3 layer of the hippocampus, suggesting that other neurotransmitters may more directly play important roles in regulating neurogenesis (Amaral and Lavenex 2007).

These reports suggest that while DA is important for intact cognitive functions, it might not be the major regulator of hippocampal neurogenesis and thus another mechanism may be involved in PD associated cognitive symptoms.

### 1.2.3.2. The noradrenergic hypothesis

It was in 1980 that dense noradrenergic projections to the DG were identified (Loy et al. 1980), but it was only a decade later that a relationship between the LC-NA system and the hippocampus was first suggested by Berridge and Foote (Berridge and Foote 1991). Using a cholinergic agonist to activate LC activity they showed changes in hippocampal activity. They further supported their finding, by looking at changes in hippocampal activity when the LC was inactivated by clonidine (Berridge et al. 1993).

Later studies focused on the possible role of NA on hippocampal functions and neurogenesis. First it was identified that a lesion of the LC with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) decreases cell proliferation in the dentate gyrus in rats, but had no effect on survival of cell progenitors born prior to the LC lesion (Kulkarni, Jha, and Vaidya 2002). Recently a neurosphere assay was performed to further identify factors necessary for hippocampal neurogenesis. The result of this experiment showed a stimulation of cellular proliferation when NA was added to the media, thus supporting the hypothesis of a relationship between LC and hippocampal neurogenesis (Jhaveri et al. 2010).

Together these findings strongly suggest a regulation of the hippocampal neurogenesis by the LC-NA system. However, the evidence remains indirect and thus future research should focus on direct links to establish the nature of the regulation of the LC on the DG.
1.3. **Acute injury: Striatal ischemia**

1.3.1. **Striatum**

The striatum is an important brain region located in the forebrain. In rodents it can be divided into dorsal striatum comprising the caudate nucleus and putamen; and the ventral striatum which is composed of nucleus accumbens and olfactory tubercle. The striatum is physically and functionally segregated, indeed the ventral striatum is implicated in reward cognition, while the dorsal striatum is implicated in motor functions (Kreitzer 2009). The striatal cellular structure is composed at 90-95% of γ-Aminobutyric acid (GABA) medium spiny neurons, which are identified by the markers dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000 (Darpp32) and calbindin (F. C. Liu and Graybiel 1992; Ouimet, Langley-Gullion, and Greengard 1998) and at 5-10% of interneurons which contain the markers parvalbumin, somatostatin, calretinin (CR), choline acetyltransferase (ChAT) (Kawaguchi et al. 1995; Marin, Anderson, and Rubenstein 2000). The striatum is part of basal ganglia circuitry, which can be divided in two pathways: a direct or Striatonigral pathway, which arises from the putamen and project directly to the globus pallidus intern (GPi) and SN; and an indirect or Striatopallidal pathway, which arises from the pallidum and projects to the GPi/SN through the globus pallidus extern and the subthalamic nucleus (Kreitzer 2009; DeLong 1990). A schematic representation of the basal ganglia circuit is presented in Figure 1.3 (image from Fino & Venance, 2010). The striatum receives mainly glutamatergic projections from the cortex (Reep, Cheatwood, and Corwin 2003) and dopaminergic projections from the midbrain, where the VTA innervates the ventral striatum and SN innervates the dorsal striatum (Van den Heuvel and Pasterkamp 2008).
1.3.2. Striatal ischemia

Ischemic injury is the disruption of blood flow to the brain, which leads to low oxygen levels inducing a cerebral hypoxia and cellular death. Commonly in Stroke, the middle cerebral artery (MCA) is occluded leading to decrease of blood supply to the basal ganglia and cerebral cortex (Hacke et al. 1996). Stroke is a leading cause of disability that affects one in six people according to the Australian Stroke Foundation. It is therefore a very highly researched area and different animal models have been created to mimic ischemic stroke. For example, in adults a common model is the physical ligation of the MCA to block cerebral blood flow, called the middle cerebral artery occlusion (MCAO) model (Belayev et al. 1996; El-Sabban et al. 1994). Modelling of stroke in newborns has been achieved using a hypoxic ischemia model, in this
model seven days old pups receive a carotid artery ligation in addition to be exposed for 3.5h to an hypoxic environment (8% O2) (Rice, Vannucci, and Brierley 1981). While these models are very efficient and widely used to model striatal ischemia, a new model arose in the early 90s involving the administration of endothelin-1 (ET-1), a vasoconstrictor peptide, directly in the area of interest (Sharkey, Ritchie, and Kelly 1993; Robinson et al. 1990). This model has the advantage of requiring a smaller surgical intervention compared to the MCAO model, while generating a highly reproducible locus of focal ischemic damage.

1.3.3. Striatal neurogenesis after ischemia

In humans suffering from stroke, biopsy sections from the ischemic infarct have revealed the presence of neuroblasts in the ischemic penumbra (Jin et al. 2006). In addition, many studies have used stroke models, mainly the MCAO model, and reported an increase in proliferation and neurogenesis after injury (Arvidsson et al. 2002; Parent et al. 2002; Teramoto et al. 2003; Thored et al. 2006; Yamashita et al. 2006; Zhang et al. 2001). The proliferation is described as a short-term increase, with a peak at seven days after ischemia (X. S. Liu et al. 2013; Lichtenwalner and Parent 2006) and the level of proliferation is supposed to go back to normal six weeks after stroke (Thored et al. 2006). However, the new progenitor cells marked by doublecortin (DCX), a marker for neuroblasts, were increased in number until four months after ischemia (Thored et al. 2006), suggesting a long lasting alteration of the SVZ neurogenesis. Although SVZ neurogenesis is increased long term, the survival of these new neurons has been described as very poor – Arvidsson et al. (2002) have estimated that more than 80% of the newborn neurons die.

Interestingly, the new neurons formed were not migrating to their normal destination, the OB, but were rather redirected towards the injury site. Indeed, immunohistochemistry for DCX+ progenitor cells, has revealed the formation of a chain of neuroblasts from the SVZ to the injury site (Jin et al. 2003; Parent et al. 2002) suggesting a migration of neuroblasts form the SVZ to the injury site, which was further confirmed by green fluorescent protein (GFP) transgenic mice (Yamashita et
al. 2006). Thored et al. (2006) have suggested that the migration was through cell-derived factor-1a and its receptor, CXCR4 (Thored et al. 2006). This redirected migration seems to occur at the expense of OB neurogenesis. Very importantly, it was shown that those newborn neurons are functionally integrating in the neuronal circuitry by forming synapses and firing action potentials, however it is still unclear if the appropriate connections are made (Hou et al. 2008; Yamashita et al. 2006).

Arvidsson et al. (2002) and Parent et al. (2002) went even further and suggested that neuroblasts coming from the SVZ were changing their fate and were capable of replacing neurons lost after the focal ischemia (Arvidsson et al. 2002; Parent et al. 2002). They identified cells expressing the mitotic marker, BrdU and a marker of medium spiny neurons, Darpp32 (Figure 1.4).

**Figure 1.4: New medium spiny neurons generated after MCAO in adult rats.** Immunohistochemistry representing Darpp32+ (1.A) and BrdU+ cell (1.B) and merged image (1.C and 2.D) found in the striatum. 1) is an image adapted from Arvidsson et al. (2002), where they induced a striatal injury to Wistar rats using MCAO and labelled proliferating cells using BrdU twice daily for two days. Five weeks after the injury, they identified a number of BrdU+/Darpp32+ cells corresponding to 42% of the BrdU+/NeuN+ cells. 2) is an image adapted from Parent et al. (2002). They used MCAO to injure the striatum of Sprague Dawley rats and used BrdU (twice daily for three days) to label the proliferating cells. They reported five weeks after injury, that 70% of the BrdU+ cells were also Darpp32+. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; Darpp32, dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000; MCAO, Middle cerebral artery occlusion.
With neuronal survival and the rate of neurogenesis being so low, increasing investigation has focused on enhancing neuronal production, with the ultimate goal of functionally repairing the brain. One approach is to use neurotrophic factors, which will be further detailed in the paragraph 1.4. Recently, a study has investigated the potential of the neurotrophic factor meteorin on striatal neurogenesis following ischemia (Wright et al. 2016). Surprisingly, this study failed to identify any BrdU+/Darpp32+ cells after striatal injury, but rather only found BrdU+/CR+ a marker of interneurons. These results were however supported by a similar study published in the Journal of Neuroscience. In this study, Liu et al. (2009) induced MCAO in rats and were also not able to identify any BrdU+/Darpp32+ cells, but instead have identified that almost all the new cells were BrdU+/CR+ (F. Liu et al. 2009). Their results were similar to another study that suggested that the SVZ neurons are only capable of generating a small subset of neurons and can not generate the type of neurons lost in the striatum after ischemia (Yang, You, and Levison 2008).

In summary, it is well documented that SVZ neurogenesis is increased after a brain injury, such as focal ischemia. In addition, it is suggested that the brain is attempting to repair itself by redirecting the new neurons produced to the injury site, however whether the neurons can adopt the identity of the ones lost is unclear, but appears unlikely. The rate of neurogenesis is limited after striatal injury and the functional integration remains uncertain. Thus the major question still remains, is the human brain capable of self-repair?

1.4. Brain regeneration potential after injury

From acute to degenerative injuries, the human brain suffers from extensive neuronal loss and associated functional deficits. Neuroscientists around the globe are focusing on repairing the brain for physical and cognitive recovery. A lot of emphasis has been placed on the use of stem cells to repair the damage caused after the injury. Currently there are two different approaches using stem cells: cell replacement therapy, which introduces exogenous stem cells directly into or near the injury site,
and enhancement of neurogenesis, which aims to initiate or increase the production of endogenous neurons for brain ‘self-repair’.

Exogenous cell replacement therapy is a very promising approach for brain repair and has been extensively studied as a treatment for PD. Extensive work in rodents has shown that grafted DA neurons could restore motor functions diminished in parkinsonian rat model (Bjorklund and Stenevi 1979). This work has led to several clinical trials, but while the first one was positive with patients experiencing functional improvement with the DA graft, the following trials were unfortunately not conclusive, showing limited or no effect of the graft and in some cases graft-associated side effects ((Freed et al. 2001; Olanow et al. 2003) and reviewed in (Winkler, Kirik, and Björklund 2005)). While cell replacement therapy is a very encouraging approach for brain repair, it faces many challenges including: increasing the survival of the grafted cells, and standardisation of the therapeutic outcome.

The ‘re-discovery’ of adult neurogenesis in the early 2000’s lead to the exploration of another approach for brain repair: enhancement of endogenous neurogenesis, also called endogenous cell therapy. Because neurogenesis levels after injury is very poor (Arvidsson et al. 2002), researchers have investigated the capacity of growth factors to manipulate cell survival, neuronal differentiation and overall neurogenesis in an attempt to reach a meaningful level for brain repair. Growth factors are very interesting because of their well-established role in development and in the adult brain under physiological conditions. For example, basic fibroblast growth factor (FGF-2) was shown to be required for proliferation and neurogenesis in the developmental cortex (Raballo et al. 2000) and also to increase proliferation in the SVZ (Kuhn et al. 1997) and proliferation and neurogenesis in DG and SVZ in the adult brain (Wagner, Black, and DiCicco-Bloom 1999; Rai, Hattiangady, and Shetty 2007). Therefore, many studies have studied the impact of over-expression of different growth factors on brain repair and have commonly used stroke and traumatic brain injury (TBI) animal models (Patel and Sun 2016; Guerra-Crespo 2012). In the diseased brain, FGF2 was shown to increase proliferation and the number of neuroblasts in the striatum after ischemia (Wada et al. 2003; Baldauf and Reymann 2005). In stroke animal models, other neurotrophic factors have been reported to: increase
proliferation, like epidermal growth factor (EGF) (Kuhn et al. 1997; Teramoto et al. 2003); to increase neurogenesis and angiogenesis, like VEGF (Thau-Zuchman et al. 2010; Sun et al. 2003); and to increase neuronal survival and recruitment to the injury site, like glial cell line-derived neurotrophic factor (GDNF) (Kobayashi et al. 2006). Another factor, brain-derived neurotrophic factor (BDNF) was particularly exciting for its capacity to increase proliferation, neurogenesis and migration of neurons to the injured striatum (Benraiss et al. 2012; Gustafsson et al. 2003; Schäbitz et al. 2007) and also for its impact hippocampal neurogenesis under physiological conditions, by supporting neuronal survival and dendritic development (Gao and Chen 2009; Gao, Smith, and Chen 2009).

In the hippocampus, two other factors were reported as having an impact on neurogenesis, the insulin-like growth factor 1 (IGF-1) which increases proliferation and neurogenesis (Aberg et al. 2000; Anderson et al. 2002) and the neurotrophic factor S100β which enhances proliferation and improved cognitive functions (Kleindienst et al. 2005). The therapeutical benefits of these neurotrophic factors were however variable upon delivery methods. The use of osmotic pumps (Baldauf and Reymann 2005), adeno-associated virus (Kells et al. 2004; Jorgensen et al. 2011) and also exogenous cell grafting (Emerich et al. 1997; Bachoud-Levi et al. 2000) allowed for controlled and long-term delivery. Thus the concept of endogenous cell therapy is promising; however, important questions still remain to be answered. What is a sufficient level for therapeutic benefits? How to redirect the fate of the new born neurons to the correct neuronal identity? Are the new born neurons functionally integrating in the neuronal circuitry?

1.5. **Summary and thesis aims**

The last 50 years has seen major gains in understanding of adult neurogenesis and its involvement in diseases including PD and stroke. However, despite the significant progress, a complete understanding of neurogenic mechanisms and its regulation under physiological and specific pathological conditions remains to be further characterised. The focus of this thesis is to understand the potential and limitations
for development of neuronal replacement therapies in both acute (stroke) and progressive (PD) brain injury.

In the first part of this thesis, a rodent model of Parkinson’s disease was used as an example of progressive disease, to identify the effect of specific neural systems on hippocampal neurogenesis. To this aim, we first clarified the nature of hippocampal inputs received from the DA midbrain compared to the NA hindbrain. We further investigated the impact of NA, DA and acetylcholine (ACh) degeneration on hippocampal neurogenesis. Finally, we described a novel tool for advancement of NA cell transplantation.

In the last part of this thesis, a striatal ischemia model was used as an example of acute injury, to understand the potential of the post-natal brain for self-repair. To this aim, we compared striatal neurogenesis after striatal ischemia in the post-natal and the neonatal brain.
1.6. References


Liu, Bing fang, Er jing Gao, Xian zhi Zeng, Man Ji, Qing Cai, Qiang Lu, Hui Yang, and Qun yuan Xu. 2006. “Proliferation of Neural Precursors in the Subventricular Zone after Chemical Lesions of the Nigrostriatal Pathway in Rat Brain.” Brain Research 1106 (1): 30–39.


Chapter 2
General Methods

2.1. Animals

All experiments were conducted in accordance with the Australian National Health and Medical Research Council’s published code of practice for use of animals in research and were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee. All animals were housed in individually ventilated cages under a 12h light/dark cycle with *ad libitum* access to food and water. For this work we used different aged rats and various strains of mice. As a rule in the lab, females are used over males for housing purposes, when there is no evidence supporting any sex-effect on the outcome of the experiment. The details of the animal species and strains used are summarised in Table 2.1.

A Green Fluorescent Protein (GFP) reporter mouse, under the dopamine-β-hydroxylase (DBH) promoter was used in Chapter 5, which is the enzyme that synthesises noradrenaline. This construct marks all the noradrenergic cells with the fluorescent marker GFP. We used these animals to visualise noradrenergic cells implanted into the hippocampus, to examine integration and survival of the grafted cells in the hippocampus and to characterise the connectivity established within the host brain. The mouse strain STOCK Tg(Dbh-EGFP)FI99Gsat/Mmucd was obtained from the Mutant Mouse Regional Resource Center, a NIH funded strain repository, and was donated to the MMRRC by the NINDS funded GENSAT BAC transgenic project.


<table>
<thead>
<tr>
<th>Chapter</th>
<th>Animals used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 3</td>
<td>Adult female rats Sprague Dawley (n= 10)</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Adult female rats Sprague Dawley (n= 8 per experimental groups)</td>
</tr>
</tbody>
</table>
| Chapter 5 | - Transgenic mice DBH-eGFP with a FVB-N/Swiss background  
- adult mice C57/Bl6 and Swiss (n= 22) |
| Chapter 6 | - 1 day old Sprague Dawley rats (n= 13)  
- adult female Sprague Dawley rats (n= 4, 5, 8 per experimental groups)  
- adult female athymic (‘nude’) rats (n= 6) |

Table 2.1: Details of animals used in this thesis.

2.2. Embryonic tissue dissection

Transgenic male mice for DBH-eGFP were crossed with wild-type C57/Bl6 females. The embryos were harvested at embryonic day 12.5 and 13.5 in order to dissect the locus coeruleus using the GFP+ noradrenergic neurons as a guide for the dissection parameters. Briefly the mother was sacrificed by cervical dislocation and the uterus was collected by caesarean and placed in L15 media for conservation. Each embryo was removed from its embryonic sac while in L15 media. The embryonic brain and the mesencephalon were opened from the top (dorsal) and each side was pinned down in order to reveal the GFP fluorescence. The locus coeruleus (LC) was identified and dissected out bilaterally as two pieces (Figure 2.1).

Two different cell dissociation techniques were tested for preparation of the noradrenaline (NA) cells before transplantation and are summarised below:

a) We followed a well-established protocol for embryonic donor cell preparation based on dissection of ventral mesencephalon (L. H. Thompson and Parish 2013) and used DNase (0.1%) during cell dissociation in a calcium and magnesium-free Hank’s buffered salt solution (HBSS). The viable cell number was then estimated by diluting 5µl of cell suspension with trypan blue (1:10) and the viable cells were counted using a hemocytometer. The cell stock was then centrifuged at 500xG at 4°C for 5min and the pellet was resuspended in the appropriate amount of HBSS media to obtain a density
of 150,000 cells/µl. While the survival rate was correct during the hemocytometer count, we noticed that the cell survival was poor after transplantation. The cells seem to be very sensitive to centrifugation and DNase addition, which resulted in failed grafts.

b) We therefore trialled cell dissociation without centrifugation. The pieces of embryonic hindbrain were dissociated by trituration in HBSS-Ca²⁺, -Mg²⁺ media to obtain a cell suspension solution.

The cells were then maintained on ice until transplantation.

Figure 2.1: Dissection of LC from embryonic mice at day E13.5. A) Dashed line indicates the first cut made through the dorsal midline. B) The boxed area indicates a top-down view of the approximate location of the LC. C) Overlay of bright-field and GFP+ cells illustrating the LC. Each LC piece was dissected separately.

2.3. Surgical procedure

The surgical procedures were done through stereotaxic surgery and were used to: a) induce discrete lesions of a specific neuronal population (Chapters 4 and 6); b) inject retrograde tracer to label neuronal projections (Chapter 3) and c) deliver cells (Chapter 5).

All surgeries were done using a stereotaxic frame (Kopf, Germany). Prior to surgery the animals were anaesthetised with isoflurane (5% at 1L/min) and kept under anaesthesia for the duration of the surgery (2% at 1L/min. All animals were administered intraperitoneally (i.p.) with an analgesic, meloxicam (3mg/kg). The
toxin, tracer or cells were delivered via a borosilicate glass capillary (Harvard instruments) connected to a Hamilton microsyringe to insure discrete delivery to the targeted brain region while minimising physical damage. To prevent backflow, the administration rate was set at 1 µl/min and the glass capillary was left in place for 5 minutes.

### 2.3.1. Stereotaxic lesion

The toxins used and their injection locations are summarised in Table 2.2.

DBH-saporin, 192-IgG-saporin, anti-SERT-saporin and endothelin-1 were prepared in saline (0.9% NaCl) to the desired concentration. All toxins were prepared on the day of the surgery and kept on ice for preservation.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Injection location</th>
<th>Injection coordinates</th>
<th>Concentration</th>
<th>Volume</th>
<th>Source</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OHDA</td>
<td>Medial forebrain bundle (MFB)</td>
<td>AP -4.2, ML -1.2, DV -7.8</td>
<td>3.5 µg/µl</td>
<td>4 µl</td>
<td>Tocris</td>
<td>4</td>
</tr>
<tr>
<td>DBH-saporin</td>
<td>Intra cerebral ventricle (i.c.v.)</td>
<td>AP -0.6, ML -1.5, DV -3.0</td>
<td>0.4 µg/µl</td>
<td>2.5 µl</td>
<td>Advanced targeting systems</td>
<td>4</td>
</tr>
<tr>
<td>192-IgG-saporin</td>
<td>i.c.v.</td>
<td>AP -0.6, ML -1.5, DV -3.0</td>
<td>1 µg/µl</td>
<td>5 µl</td>
<td>Advanced targeting systems</td>
<td>4</td>
</tr>
<tr>
<td>Endothelin 1, adult rats</td>
<td>Striatum (CPu)</td>
<td>AP +0.5, ML -3.0, DV -5.0</td>
<td>800 pmol/µl</td>
<td>1 µl</td>
<td>Auspep</td>
<td>6</td>
</tr>
<tr>
<td>Endothelin 1, neonate rats</td>
<td>CPu</td>
<td>AP +0.7, ML -2.3, DV -2.9</td>
<td>800 pmol/µl</td>
<td>1 µl</td>
<td>Auspep</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2.2: Toxins and injection sites used in this thesis

36
2.3.1.1. Dopamine lesion

In order to lesion the dopaminergic system we use the neurotoxin 6-hydroxydopamine (6-OHDA) (Breese and Traylor 1970). Because this toxin is selective for all catecholamines and can ablate both dopamine (DA) and NA neurons, to protect the latter, we injected the rats 30 min prior to 6-OHDA administration with 20mg/kg (i.p.) desipramine (sigma), which is a noradrenergic transporter (NET) blocker.

6-OHDA (Tocris) was prepared in 0.02% ascorbic acid (Sigma) in saline (0.9% NaCl) to prevent oxidation.

As part of the experimental design, we intended to reproduce results from a study by Suzuki et al. (2010) and thus used the same stereotaxic co-ordinates for 6OHDA lesion. However, we found this to be inconsistent with accurate targeting and thus producing poor lesioning in our own hands (Figure 2.2). At the beginning of the study we aimed at reproducing the results from Suzuki et al. and validate our hypothesis that the reduction of hippocampal neurogenesis they measured is due to destruction of both DA and NA neurons and not only of DA neurons. We therefore changed our protocol and used a well-defined injection site for the 6-OHDA delivery, the medial forebrain bundle (MFB) (L. H. Thompson and Parish 2013).

A summary of the different injection sites and 6-OHDA dosages tested to lesion the dopaminergic system is presented table 2.3.

![Figure 2.2: DA lesion using Suzuki coordinates](image-url), scale bar = 1mm
<table>
<thead>
<tr>
<th>Toxins</th>
<th>Injection sites</th>
<th>Injection coordinates</th>
<th>Concentrations</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OHDA</td>
<td>Substantia nigra (SN)</td>
<td>AP -4.8, ML -1.5, DV -7.8</td>
<td>3 µg/µl</td>
<td>2 µl</td>
</tr>
<tr>
<td></td>
<td>MFB</td>
<td>AP -4.2, ML -1.2, DV -7.8</td>
<td>3.5 µg/µl</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

Table 2.3: Summary of injection sites and 6-OHDA dosages tested during pilot study to lesion the DA system. The emboldened font in the table indicates the lesion paradigm selected for the thesis.

### 2.3.1.2. Noradrenaline lesion

To lesion the NA system we tested 2 toxins - 6-OHDA and DBH-saporin, which is a ribosome inactivator attached to an anti-DBH antibody. As previously mentioned 6-OHDA is selective for catecholaminergic neurons and can be used to lesion NA neurons. We tested its efficacy when injected in the LC fibre bundles, but we did not observe a sufficient NA cell loss. We therefore pursued the anti-DBH-saporin toxin (Advanced Targeting Systems). A thorough pilot experiment was performed to identify the best injection site and dosage (Table 2.4).
<table>
<thead>
<tr>
<th>Toxins</th>
<th>Injection site</th>
<th>Injection coordinates</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OHDA</td>
<td>LC fibre bundles</td>
<td>AP -8.2, ML -1.6, DV -6.2</td>
<td>0.2 µg/µl, 5 µg/µl, 0.2 µg/µl</td>
<td>0.5 µl, 2 µl, 1 µl</td>
</tr>
<tr>
<td>DBH-saporin</td>
<td>LC</td>
<td>AP -9.8, ML -1.3, DV -6.2</td>
<td>0.2 µg/µl</td>
<td>1 µl</td>
</tr>
<tr>
<td></td>
<td>LC fibre bundles</td>
<td>AP -5.7, ML -1, DV -6.2</td>
<td>0.1 µg/µl, 0.2 µg/µl, 3 µg/µl</td>
<td>2 µl, 0.5 µl - 1 µl - 2 µl, 1 µl</td>
</tr>
<tr>
<td></td>
<td>i.c.v.</td>
<td>AP -0.6, ML -1.5, DV -3.0</td>
<td>2 µg/µl, 0.4 µg/µl</td>
<td>2.5 µl, 2.4 µl - 5 µl</td>
</tr>
</tbody>
</table>

Table 2.4: Summary of toxins and dosages tested during pilot study to lesion the NA system. The emboldened font in the table indicates the lesion paradigm selected for the thesis.

2.3.1.3. Acetylcholine lesion

To lesion the cholinergic system, we used the 192-IgG-saporin toxin (ATS). This toxin is directed against a cell-surface antigen, p75NTR, located on cholinergic neurons only. During pilot studies we tested different dosages for this toxin and validated an injection of 5 µl at 1 µg/µl in the i.c.v. (Table 2.5) as being most effective.
Toxin | Injection site | Injection coordinates | Concentration | Volume |
--- | --- | --- | --- | --- |
192-lgG-saporin | i.c.v. | AP -0.6, ML -1.5, DV -3.0 | 0.7 µg/µl, 0.3 µg/µl, 1 µg/µl | 7 µl, 7 µl, 5 µl |

Table 2.5: Summary of 192-lgG-saporin dosages tested during pilot study to lesion the cholinergic system. The emboldened font in the table indicates the lesion paradigm selected for the thesis.

### 2.3.1.4. Serotonin lesion

To lesion the serotoninergic system, we used the serotonin transporter (SERT)-saporin (ATS) which is a ribosome inactivator attached to an anti-SERT antibody (serotonin transporter). While we tested this toxin at high concentration and at 2 injection sites we fail to lesion the serotoninergic system. The parameters used during the pilot study are summarised in *Table 2.6*.

| Toxin | Injection site | Injection coordinates | Concentration | Volume |
--- | --- | --- | --- | --- |
anti-SERT-saporin | i.c.v. | AP -0.6, ML -1.5, DV -3.0 | 0.4 µg/µl, 1 µg/µl | 2.5 µl, 8 µl |

Table 2.6: Summary of injection sites and SERT-saporin dosages tested during pilot study to lesion the serotoninergic system.
### 2.3.1.5. Striatal ischemia

To induce a striatal ischemia in Chapter 6, we used endothelin 1 (ET-1) which is a vasoconstrictor and a commonly used to model focal ischemia (Figure 2.3) (Robinson et al. 1990; Sharkey, Ritchie, and Kelly 1993). We injected the ET-1 peptide to the striatum of both adult and neonatal rats, to induce an acute striatal ischemic injury.

![Figure 2.3: Representation of focal ischemia induced by injection of ET-1 in neonatal striatum.](image)

### 2.3.2. Stereotaxic cellular transplantation

Cells collected from DBH-eGFP embryos at different embryonic age E12.5, E13.5 and 14.5 were injected bilaterally directly into the hippocampi of C57/Bl6 and Swiss mice at the following coordinates: AP-1.75, ML+/-2.3 and DV-1.8. The cells were either injected at a known density of 150,000 cells/µl or to increase our cell survival by avoiding cell centrifugation, we injected a total 6-8 pieces of LC from 3-4 embryos in 10-15µl of media. The cells were resuspended before each injection and kept on ice for the duration of the surgery.

The mice were sacrificed 2 weeks after surgery.
2.3.3. Stereotaxic retrograde tracing

To assess the capacity for afferent innervation by midbrain DA neurons to the dentate gyrus (DG), we used a retrograde labelling technique via direct injection of the retrograde tracer Fluorogold into the DG of adult rats.

We first optimised the retrograde tracing by selecting the best retrograde tracer and the optimal coordinates to target the DG. We tested 2 different tracers, fluorescent beads and fluorogold (FG; Fluorochrome). The beads were very diffuse even at small injection volumes and were not only found in the DG, but also widely in the surrounding areas. The FG injection however, was considerably more discrete and limited to the DG. We therefore selected FG as our retrograde tracer for this study in conjunction with injection using a ‘Neuros’ Hamilton syringe, which allowed precise injection of small volumes. We further optimised the retrograde tracing by testing different injection coordinates using injection of dye and we selected the following: AP -3.8, ML -1.8 and DV -3.3.

Finally, 10 rats were injected with 0.1 µl of 2 % FG via the Neuros Hamilton Syringe into the DG. The animals were sacrificed 5-7 days later.

2.4. BrdU intra-peritoneal injections

The 5-bromo-2'-deoxyuridine (BrdU) solution was used to mark newly generated cells. BrdU is a thymidine analogue that incorporates in the DNA of replicating cells. To allow consistency in BrdU concentration injected among all the animals, a stock solution was made fresh before every BrdU session and stored at -20°C between injections. BrdU was made up at 20mg/ml in saline solution and the pH was controlled to be between 7.2-7.4. The concentration and regime were as follows: in the Parkinson’s disease modelling (Chapter 4) the adult rats were injected 4 weeks after surgery with 50mg/kg, twice daily for 1 week; in the striatal ischemia modelling (Chapter 6) the adult rats were injected 1 day after surgery with 100mg/kg, daily for 1 week, and the neonatal rats were injected twice daily with 50mg/kg for 1 week.
2.5. Tissue collection and preparation

The animals were sacrificed by lethal injection of pentobarbitone (100mg/kg; Virbac, Peakhurst, Australia). The brains were then collected after a transcardial perfusion process which consists of injection of a Tyrode solution to clear blood from the brain (lg/ml), followed by injection of a fixation solution (4% paraformaldehyde, phosphate buffer 0.4M and 7% picric acid). The brains were then collected and post-fixed for 2 hours in the fixation media, followed by 1-2 days equilibration in 20% sucrose PBS solution. The brains were then sectioned coronally at 40µm using a microtome (Leica, Wetzlar, Germany).

2.6. Immunohistochemistry

Free-floating immuno-histochemistry was performed on 1:12 series as previously described (L. Thompson 2005). The antibodies used are summarised in Table 2.7.

2.6.1. Chromogenic immunohistochemistry

For the chromogenic immunohistochemistry the tissue sections were washed 3 times for 5 minutes in phosphate buffered saline (PBS) to remove any trace of cryoprotectant. A quenching step was performed to remove the endogenous peroxidase activity, the sections were incubated in 3% H₂O₂, 10% methanol in PBS media for 20 minutes. After washing the sections 3 times with PBS, they were incubated overnight with the primary antibody diluted in blocking solution (5% donkey serum, 0.5% of Triton X and PBS). The following day the sections were incubated in blocking solution for 45-60 minutes, incubated for 2 hours with a biotinylated secondary antibody diluted in blocking solution, followed by 3 PBS washes and incubation with streptavidin–horseradish peroxidase complex (ABC Elite kit, Vectastain; Vector Laboratories, Burlingame, CA) for 1 h. The tissue was further washed in PBS and exposed to di-amino-benzidine (0.5 mg/mL, DAB, Sigma) for 2 minutes. The precipitation of the chromophore was catalysed by the addition of 1% H₂O₂. The brain sections were washed and mounted onto gelatinised glass slides. After
dehydratation in increasing concentration of alcohol (50%, 70%, 90% and 2x 100%) and in xylene (OilChem Pty Ltd, Australia), the tissue was coverslipped with DePex mounting media (BDH Chemicals, Poole, UK).

2.6.2. Fluorescent immunohistochemistry

For the fluorescent immunostaining, the sections were washed 3 times for 5 minutes in PBS and incubated overnight with primary antibodies diluted in blocking solution (5% donkey serum, 0.5% of Triton X and PBS). After 3 x 5 min washes the sections were incubated for 45-60 minutes in a blocking solution. The fluorophore-conjugated secondary antibodies were diluted in the blocking solution and applied for 2 hours on the sections. The sections were mounted on glass slides and directly coverslipped using DAKO (USA).
<table>
<thead>
<tr>
<th>Species</th>
<th>Antigen</th>
<th>Origin</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>Tyrosine hydroxylase (TH)</td>
<td>Immunostar, 22941</td>
<td>1:1000</td>
</tr>
<tr>
<td>sheep</td>
<td>TH</td>
<td>Pel-freeze, P60101-0</td>
<td>1:800</td>
</tr>
<tr>
<td>rabbit</td>
<td>TH</td>
<td>Pel-freeze, P40101-0</td>
<td>1:1000</td>
</tr>
<tr>
<td>mouse</td>
<td>Dopamine-β-hydroxylase (DBH)</td>
<td>Millipore, MAB308</td>
<td>1:5000</td>
</tr>
<tr>
<td>goat</td>
<td>Choline acetyltransferase (ChAT)</td>
<td>Millipore, MAB144</td>
<td>1:100</td>
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<tr>
<td>rabbit</td>
<td>γ-aminobutyric acid (GABA)</td>
<td>Sigma, A2052</td>
<td>1:1000</td>
</tr>
<tr>
<td>rabbit</td>
<td>fluoro gold</td>
<td>Millipore, MAB153</td>
<td>1:1000</td>
</tr>
<tr>
<td>rabbit</td>
<td>Prox1</td>
<td>Millipore ABN278</td>
<td>1:2000</td>
</tr>
<tr>
<td>goat</td>
<td>Doublecortin (DCX)</td>
<td>Santa Cruz sc-8066</td>
<td>1:1000</td>
</tr>
<tr>
<td>rat</td>
<td>Dopamine transporter (DAT)</td>
<td>Millipore, MAB369</td>
<td>1:3000</td>
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<td>sheep</td>
<td>BrdU</td>
<td>Exalpha, A205P</td>
<td>1:1000</td>
</tr>
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<td>rabbit</td>
<td>Ki67</td>
<td>Thermo Fisher LBVRM-9106-S1</td>
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<td>mouse</td>
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<td>Millipore, MAB377</td>
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<tr>
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<td>GFP</td>
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<table>
<thead>
<tr>
<th>Secondary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
</tr>
<tr>
<td>Dylight fluorophore 488 anti-mouse</td>
</tr>
<tr>
<td>Dylight fluorophore 488 anti-rabbit</td>
</tr>
<tr>
<td>Dylight fluorophore 488 anti-sheep</td>
</tr>
<tr>
<td>Dylight fluorophore 549 anti-rabbit</td>
</tr>
<tr>
<td>Antibody</td>
</tr>
<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>Dylight fluorophore 549 anti-sheep</td>
</tr>
<tr>
<td>Dylight fluorophore 549 anti-goat</td>
</tr>
<tr>
<td>Dylight fluorophore 549 anti-mouse</td>
</tr>
<tr>
<td>Dylight fluorophore 647 anti-goat</td>
</tr>
<tr>
<td>Dylight fluorophore 647 anti-rabbit</td>
</tr>
<tr>
<td>Horseradish peroxidase anti-rat</td>
</tr>
<tr>
<td>Tyramide-fluorescein 488</td>
</tr>
<tr>
<td>Biotinylated donkey anti-mouse</td>
</tr>
<tr>
<td>Biotinylated donkey anti-rabbit</td>
</tr>
<tr>
<td>Biotinylated donkey anti-sheep</td>
</tr>
<tr>
<td>Streptavidine fluorophore 550</td>
</tr>
</tbody>
</table>

Table 2.7: Primary and secondary antibodies used for immunohistochemistry.

2.7. High performance liquid chromatography

To validate the DA and NA lesions, we assessed DA and NA levels in the brain using high performance liquid chromatography (HPLC). Reversed-phase high-performance liquid chromatography (RP-HPLC) separates molecules bases upon their hydrophobicity. Hydrophobic molecules in the polar (aqueous) mobile phase tend to adsorb to the hydrophobic stationary phase (the column), while hydrophilic molecules in the mobile phase pass through the column and are thereby eluted first. The DA and
NA levels were assessed at the level of terminal fields, in the striatum and in the hippocampus, respectively. The HPLC protocol was done according to Parish et al. (Parish et al. 2001).

In brief, to collect the brain tissue, animals were decapitated and their brain's rapidly dissected on a cold plate to collect striatum and hippocampi. The brain tissues were weighed and stored at -80°C until analysis. At that time, the tissue samples were homogenized in an extraction buffer containing 0.4M perchloric acid, 7.9mM sodium metabisulphite, 1.34mM disodium ethylenediaminetetra-acetic acid (EDTA) in distilled water. In order to rupture the vesicular membranes, samples were sonicated for 15s and were centrifuged for at 10,000g (3x5min), reserving only the supernatant. The resultant supernatant was transferred to HPLC vials and placed in an autosampler for injection onto the HPLC. The HPLC consisted of a LC-20AT pump (Shimadzu), SIL-20A Autosampler (Shimadzu) and C18 reverse phase column (Bio-Rad, Hercules, USA). Detection was via a 3mm VT-03 flow cell with glassy carbon working electrode (Antec Leyden) and Decade II Electrochemical Detector (Antec Leyden). The mobile phase consisted of 17% v/v methanol in purified deionized water containing 70mM KH2PO4 (Merck), 0.5mM EDTA (Merck) and 8.0mM sulfonic acid (Merck), pH 3.0 and was run at a flow rate of 0.5ml/min.

2.8. Genotyping protocol

To identify the genetically modified mice carrying the DBH-eGFP mutation in Chapter 5, we extracted DNA from tissue samples from each animal and performed a polymerase chain reaction (PCR) to identify the presence of the transgene.

The DNA was obtained from a small cut made to the tail of neonatal mice. We extracted the DNA using DNA crude extraction technique, incubating the samples overnight at 55°C in extraction buffer (0.44g Tris HCL, 0.87g Tris base, 1ml 500mM EDTA pH8, 2ml 10% SDS, 5ml 4M NaCl and topped up to 100ml with sterile water). The following day the samples were centrifuged in proteinase K (20mg/ml, promega) at 15,000 g for 20 minutes, and in 70% ethanol for another 20 minutes. The DNA was then resuspended in nuclease free water and stored at 4°C.
The PCR protocol was adapted from the guidelines developed by MMRRC at University of California, Davis and the details of primers used, reagents used and PCR cycle are presented in Table 2.8, 2.9 and 2.10 respectively. We used as an internal control the housekeeping gene interleukine 2.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DBH(11771) F1</td>
<td>AATGGGAGATGAGGGTTGGG</td>
</tr>
<tr>
<td>2. GFP R2</td>
<td>TAGCGGCTGAACGACACTGCA</td>
</tr>
<tr>
<td>3. IL2-42</td>
<td>CTAGGCCACAGTGAAATGATCT</td>
</tr>
<tr>
<td>4. IL2-43</td>
<td>GATGGTCTAATTCTAGCATCATCC</td>
</tr>
</tbody>
</table>

Table 2.8: Primers used for DBH-eGFP genotyping.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq polymerase mix</td>
<td>6</td>
</tr>
<tr>
<td>Primer 1 (20 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>3</td>
</tr>
<tr>
<td>DNA</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.9: Reagents used for DBH-eGFP genotyping. The Taq polymerase master mix (Promega, M7123) contains Taq DNA polymerase, dNTPs, MgCl2 and a green dye.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initiation/Melting</td>
<td>94</td>
<td>5:00</td>
<td>1</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>94</td>
<td>0:15</td>
<td>40 times, in sequence 2-3-4</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>65</td>
<td>0:30</td>
<td></td>
</tr>
<tr>
<td>4. Elongation</td>
<td>72</td>
<td>0:40</td>
<td></td>
</tr>
<tr>
<td>5. Amplification</td>
<td>72</td>
<td>5:00</td>
<td>1</td>
</tr>
<tr>
<td>6. Finish</td>
<td>4</td>
<td>hold</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 2.10: PCR protocol used to genotype DBH-eGFP mice. We tested different annealing temperatures from 65 to 55°C and selected 65°C. PCR machine was Takara PCR Thermal Cycler Dice.
The PCR outcome was read using electrophoresis technique with a 5% agarose gel, where the DBH transgenic band was located at 300bp and the internal control band was located at 330bp. Since the band sizes are similar, we performed 2 separate amplification PCRs, one for each gene. Figure 2.4 represents an example of genotyping results for 12 samples #1-12.

Figure 2.4: Example of genotyping results. A) Detection of transgenic DBH gene, by identification of the corresponding band after UV exposure. The samples #1, 2, 6, 9, 12 were identified as transgenic. B) Detection of housekeeping gene interleukine 2 used as a positive control.

2.9. Analysis

2.9.1. Stereology

The number of BrdU cells in the striatum (Chapter 6) was estimated using a stereology counting technique. We used the optical dissector method from the Stereoinvestigator V7 software to examine the BrdU+ cells over 4 consecutive striatal sections from a 1:12 series (separated by 480µm between sections) and approximately
located at the following distances relative to bregma +1.70, +1.20, +0.70, +0.20. The striatal boundaries were limited to the corpus callosum dorsally and laterally and to the lateral ventricle medially (Figure 2.5). The rostral boundary was defined using the distance separating the midline and the lateral ventricle, defined in figure 2.4 as ‘X’. The rostral and ventral limits of the striatum were determined as ‘X/2 cm’ away from the midline and the ventral edges of the brain. The nucleus accumbens and the injury site, which has a high level of non-specific signal, were excluded from the region to be quantified. We used a randomly positioned sampling grid of 150x200 µm and a counting frame of 40x40 µm, to count the BrdU+ cells at a magnification of 100X by gradually assessing every focal plane for each counting frame. By using the number of sections counted (4), their thickness (40µm) and the series (1:12) the estimated volume was calculated. We used the Cavalieri principle to determine the volume corresponding to the number of BrdU+ cells counted and thus to obtain a BrdU density per unit of tissue. The Cavalieri principle allows the estimation of a volume of a solid with known cross-section areas (Kern and Bland 1938).

**Figure 2.5: Representation of the striatum borders used for stereology counts of BrdU+ cells.** The red region represents the striatum delineated dorsally and laterally by the corpus callosum, medially by the lateral ventricle and ventrally by the distance ‘X/2’, with ‘X’ defined as the distance between the midline and the lateral ventricle. The injury area represented here is an example of injury we observed, the area may vary in size between the different brain sections. **Abbreviations:** Acc, Nucleus accumbens; CC, Corpus callosum; Cpu, striatum.
2.9.2. Cellular counting

All cellular counting was performed blinded for Chapter 4 and Chapter 6.

2.9.2.1. Light microscopy

In Chapter 4, the BrdU+ and Ki67+ cells in the DG and in the SVZ were counted using a Leica DM6000 microscope at magnification 20X. The number of BrdU+ cells and Ki67+ cells in the SGZ were quantified across 6 consecutive sections from a 1:6 series located at approximately -2.6mm, -2.84mm, -3.08mm, -3.32mm, -3.56mm and -3.8mm from bregma. The number of Ki67+ cells in the SVZ was quantified across 3 sections located at approximately 1.7mm, 1.20mm and 0.70mm from bregma. For the counting delineation purposes, the SGZ counts included the granular layer of the dentate gyrus.

2.9.2.2. Fluorescent microscopy

In Chapter 4, the proportion of BrdU/Prox1 double positive cells was quantified from 80 BrdU+ cells identified in the SGZ of sections ranging from 2.6mm to 3.8mm relative to bregma. Double-labeling was confirmed using orthogonal imaging with a Zeiss Meta confocal microscope (LMS 780).

In Chapter 6, the number of striatal BrdU+/NeuN+/Darpp32+ cells was quantified across 3 sections located at approximately 1.7mm, 1.20mm and 0.70mm from bregma. A representative photomontage of the entire striatum in X, Y and Z planes was acquired with the confocal microscope at magnification 20x and we used the Zen lite software for manual quantification. All BrdU+ cells were inspected and those identified as NeuN+ and/or Darpp32+ were examined at magnification 40X to validate co-expression on the Z-axis using the orthogonal analysis tool. Using the same protocol, we determined also the number of BrdU+/CR+ cells and BrdU+/CR+/NeuN+ cells. Using the Cavalieri principle, we estimated the density in BrdU+/NeuN+, BrdU+/NeuN+/Darpp32+, BrdU+/CR+ and BrdU+/CR+/NeuN+, like previously described in section 2.9.1.
2.10. References


Chapter 3

Combined immunohistochemical and retrograde tracing reveals little evidence of innervation of the rat dentate gyrus by midbrain dopamine neurons

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3.1. Abstract

Although the functional neuroanatomy of the midbrain dopamine (mDA) system has been well characterized, the literature regarding its capacity to innervate the hippocampal formation has been inconsistent. The lack of expression of definitive markers for dopaminergic fibers, such as the dopamine transporter, in the hippocampus has complicated studies in this area. Here we have used immunohistochemical techniques to characterize the tyrosine hydroxylase expressing fiber network in the rat hippocampus, combined with retrograde tracing from the dentate gyrus to assess the capacity for afferent innervation by mDA neurons. The results indicate that virtually all tyrosine hydroxylase fibers throughout the hippocampus are of a noradrenergic phenotype, while the overlying cortex contains both dopaminergic and noradrenergic fiber networks. Furthermore, retrograde tracing from the dentate gyrus robustly labels tyrosine hydroxylase-immunoreactive noradrenergic neurons in the locus coeruleus but not mDA neurons.
3.2. Introduction

Recent studies have reported that loss of midbrain dopamine (mDA) neurons in the rodent brain results in a reduction in hippocampal cell proliferation and neurogenesis (Suzuki et al. 2010), and can also impair cognitive function (Wisman et al. 2008). Interestingly, the rate of hippocampal neurogenesis has been shown to decline sharply with age (Ben Abdallah et al. 2010; Spalding et al. 2013) with a concomitant cognitive decline, and this is thought to be part of the pathophysiological basis of age-related dementia (Drapeau et al. 2003; Small et al. 2012; Seib et al. 2013). It might also be a point of convergence for other pathological changes and cognitive decline in neurodegenerative disease. For example, in Parkinson’s disease (PD), in addition to the well-characterized motor dysfunction resulting from the loss of mDA neurons, a subset of patients develops dementia – particularly during the end-stages of the disease (Levy et al. 2002; Emre 2003).

Studies investigating the role of dopaminergic signaling in adult neurogenesis have primarily focused on the sub-ventricular zone (SVZ). The SVZ is in close proximity to the rich dopaminergic innervation of the striatum and removal of the dopamine (DA) neurons has been shown to reduce precursor cell proliferation in the SVZ in PD patients and animal models of dopamine depletion (Baker, Baker, and Hagg 2004; Höglinger et al. 2004; Freundlieb et al. 2006) – reviewed in Regensburger et al. (2014). The potential impact on hippocampal neurogenesis in the sub-granular zone of the dentate gyrus is less well understood and it is unclear whether the dentate gyrus receives any significant projections from the mDA projection system.

The mDA system is one of the most well characterized projection systems in the rodent brain (Björklund and Dunnett 2007). However, there has been lack of consensus in the literature regarding its capacity to innervate the hippocampus. Early studies highlighted very low levels of DA in the hippocampus compared with other well-characterized target structures, such as the striatum (Anders Bjorklund 1978), and led to the view that the hippocampus did not receive an afferent dopaminergic input. In contrast, subsequent work reported that hippocampal DA levels were sensitive to selective lesions of the mDA system (Bischoff, Scatton, and Korf 1979; Scatton et al.
Tract tracing studies have reported either the presence (Swanson 1982; Gasbarri et al. 1994) or absence (Reymann et al. 1983; Pohle, Ott, and Muller-Welde 1984) of retrograde labeled mDA neurons after injections of tracer into different hippocampal regions. Further complicating the scenario is the presence of DA receptors in the hippocampus (Dubois et al. 1986), but absence of the dopamine transporter (DAT) on putative pre-synaptic DA fibers, leading to the idea that the lack of DAT expression is a specialized feature of the DA innervation of the hippocampus. On the other hand, there has also been recent work suggesting that the noradrenergic (NA) hippocampal innervation from the locus coeruleus (LC) acts as the primary, and perhaps exclusive, source of hippocampal DA signaling (Borgkvist et al. 2012; Smith and Greene 2012).

Here we sought to investigate the presence of midbrain-derived dopaminergic projections in the septal hippocampus through microinjection of the retrograde tracer fluorogold (FG) into the dentate gyrus. As a basis for comparison, we also describe the retrograde labeling of NA neurons in the LC, which are known to densely innervate the hippocampus, particularly the sub-granular region of the dentate gyrus.

### 3.3. Materials and Methods

#### 3.3.1. Animals

Adult female Sprague Dawley rats were used for retrograde tracing studies, with all experiments approved by the Florey Institute for Neuroscience and Mental Health animal ethics committee. All animals were housed in individually ventilated cages under a 12h light/dark cycle with ad libitum access to food and water.

#### 3.3.2. Surgical procedures and tissue preparation

Rats were deeply anesthetized with isoflurane (induction at 5%, maintenance at 2%) and placed in a stereotaxic frame (Kopf, Germany). A small burr-hole was drilled in the skull 3.8mm caudal and 1.8mm lateral to bregma, and 0.1µL of 2% FG (Fluorochrome, Denver, CO, USA) was microinjected into the hilus of the dentate
gyrus using a ‘Neuros’ Hamilton syringe over a period of 1 min. The cannula was left in place a further 5 min to limit reflux along the injection tract. After 5–7 days the animals were killed by lethal injection of pentobarbitone (100mg/kg; Virbac, Peakhurst, Australia) and were transcardially perfused with Tyrode solution (1mL/1g), followed by 4% paraformaldehyde in 0.4M phosphate buffer with 0.2% picric acid. The brain was then collected and post-fixed for 2 h in the fixation media followed by cryoprotection in 20% sucrose PBS solution for 1–2 days. All brains were frozen on dry ice and sectioned in the coronal plane in to 40µm sections using a freezing-microtome (Leica, Wetzlar, Germany).

3.3.3. Immunohistochemistry

Free-floating immunohistochemistry was performed on a 1:12 series as previously described (L. Thompson 2005). Briefly the tissue sections were incubated overnight with primary antibodies diluted in blocking solution (5% donkey serum and 0.5% Triton X in PBS) and subsequently for 2 h with fluorophore-conjugated secondary-antibodies diluted in the same blocking solution. The sections were mounted on glass slides and cover-slipped using DAKO mounting media (USA). The primary antibodies used were: mouse anti-tyrosine hydroxylase (Immunostar; 22941, 1:1000), mouse anti-dopamine-β-hydroxylase (Millipore; MAB308, 1:5000); goat anti-choline acetyltransferase (Millipore; MAB144, 1:100); rabbit anti-γ aminobutyric acid (GABA, Sigma; A2052, 1:1000); rabbit anti-5-hydroxytryptamine (Immunostar, 20080, 1:5000); rabbit anti-fluorogold (Millipore; MAB153 1:1000); rabbit anti-Prox1 (Millipore, ABN278, 1:2000); goat anti-doublecortin (Santa Cruz sc-8066, 1:1000) rat anti-dopamine transporter (Millipore, MAB369; 1:3000). The secondary antibodies used were conjugated to Dylight Fluorophores 488, 549, or 647 anti-mouse, anti-rabbit, anti-sheep, or anti-goat (Jackson ImmunoResearch, West Grove, PA, USA; 1:400). For amplified detection of DAT, a horseradish peroxidase (HRP)-conjugated secondary antibody was used (swine anti-rat, Jackson ImmunoResearch, West Grove, PA, USA, 1:500) followed by incubation with biotinylated tyramide-fluoroscein for 10 min using a commercial kit (TSA+, NEN Life Science Products, Inc., Boston, MA) as previously described (Sui, Horne, and Stanić 2012).
3.3.4. Analysis

All microscopic analysis was performed using a Zeiss inverted microscope (AxioObserver.Z1) or Zeiss Meta confocal microscope (LSM 780). The presence of double-labeled TH and FG immunoreactive cells within the midbrain or LC was verified using confocal microscopy. Schematic overviews of the distribution of FG labeled cells in these areas were generated using Inkscape software. Cell counts were performed twice to ensure accuracy.

3.4. Results

3.4.1. Immunohistochemical detection of dopaminergic and noradrenergic fibers in the hippocampus.

The hippocampus of the adult rat brain is richly innervated by tyrosine hydroxylase (TH)-expressing fibers (Figure 3.1 A-E). This includes a dense TH+ fiber network in and around the dentate gyrus, particularly the sub-granular layer (SGL) where newborn doublecortin-expressing neurons are generated before migrating into the overlying granule layer as Prox1+ interneurons (Figure 3.1). Immunohistochemistry for dopamine-beta-hydroxylase (DBH), expressed exclusively in NA fibers, revealed a similarly dense innervation of the SGL and surrounding dentate gyrus (Figure 3.1 F-J), while DAT labeling did not reveal the presence of any DAT fibers (Figure 3.1 K-O) throughout the hippocampus. Double-labeling for TH and DBH revealed a complete overlap of expression within the dentate gyrus (Figure 3.1 P-S). Careful inspection via confocal microscopy showed good penetration of both antibodies throughout the 40µm tissue sections and we were unable to find examples of TH+ fibers that were not immunoreactive against DBH. This was also the case when looking at other areas receiving prominent TH+ innervation, including the molecular layer of the dentate gyrus and CA3 (Figure 3.2 A, B, C), and, indeed, throughout the entire hippocampus (not shown). However, in the overlying cingulate cortex (CC), discrete patches of TH+/DAT+ fibers were clearly distinguishable amongst the more diffuse network of TH+/DBH+ fibers (Figure 3.2 A, D).
Figure 3.1: Immunohistochemical detection of dopamine and noradrenaline fibers in the rat dentate gyrus. The dentate gyrus contains a fiber network that can be labeled with antibodies against tyrosine hydroxylase (A) or dopamine-β-hydroxylase (F) but not the dopamine transporter (K). Enlargements of the boxed areas in A and F show that TH (B-E) and DBH (G-J) fiber innervation is particularly dense in and around the sub-granular and granular layers, identified by doublecortin and Prox1 respectively, while there is a complete absence of DAT expression (L-O). Triple-labeling for TH, DBH, and DAT (P-S) shows that all TH+ fibers express DBH, while none co-label with DAT. Scale bars: A, F, and K, 200 µm and B-E, G-J, L-O, and P-S, 50 µm. Abbreviations: DAT, Dopamine transporter; DBH, Dopamine-beta-hydroxylase; DCX, Doublecortin; Prox1, Prospero homeobox 1; TH, Tyrosine hydroxylase.
Figure 3.2: Overview of dopamine and noradrenergic fibers in the septal hippocampus and overlying cortex. A) Photomontage of immunohistochemical detection of TH, DBH, and DAT in the rat hippocampus and cortex. Boxed areas are shown as enlarged views of hippocampal areas with prominent TH fiber labeling, including the sub-granular zone and molecular layer of the dentate gyrus (B) and CA3 (C) and also the overlying cortex (D) where TH+/DAT+ fibers (arrows) were intermingled with TH/DBH fibers. Scale bars: A, 200 µm and B-D, 50 µm. Abbreviations: CA1, Cornus Ammonis 1; CA3, Cornus Ammonis 3; DGmol, Dentate gyrus molecular layer.
3.4.2. Injection of fluorogold into the dentate gyrus retrogradely labels neurons in the ventral midbrain and the locus coeruleus.

Detection of FG, using immunohistochemistry, allowed for verification of the location and extent of delivery to the dentate gyrus. In 10 animals used for the analysis of retrograde labeling patterns, FG immunoreactivity was confined to a discrete area on the medial dentate gyrus in the septal hippocampus, including the ventral and dorsal blades, and adjacent hilus, and in some animals, additional labeling of the overlying molecular layer and CA1 area (Figure 3.3 A). FG was retrogradely transported to neurons in a number of nuclei throughout the brain, including those with well-characterized projections to the hippocampus such as the cholinergic neurons in the septum and nucleus basalis and serotonergic neurons of the raphe nucleus (Supplemental Figure 3.1).

In the ventral midbrain, FG+ neurons were consistently found distributed in and around midline nuclei, including the paranigral, parabrachial pigmented, interfascicular, interpeduncular, and rostral linear raphe nuclei, and throughout the ventral tegmental area (VTA) (Figure 3.3 B & 3.4 A). In all animals, there was prominent labeling of cells closely associated with the optic tract, including the medial and lateral terminal nuclei of the accessory optic tract (Figure 3.3 B).

Close inspection using confocal microscopy of midbrain sections labeled for TH in order to identify mDA neurons, showed that, overwhelmingly, the FG+ neurons did not co-label with TH (Figure 3.3 C-E). However, we did identify a total of three DA neurons across two of the 10 animals that were unambiguously co-labeled with TH (Figure 3.3 G-I). Notably, in both of these animals detection of FG showed labeling in the cortex overlying the dentate gyrus target site (Figure 3.3 F). A total of 605 FG+ cells were identified through the examination of three coronal sections spanning most of the rostro-caudal extent of the territory covered by the A9 and A10 DA cell groups in the 10 animals. Figure 3.4 shows the cumulative spatial distribution of these FG cells (red circles), and the three FG+/TH+ cells (yellow circles), in the representative sections. Retrograde labeling was more prominent in the rostral sections and approximately 8% of the FG+ cells were located in the midbrain contralateral to the injected hippocampus. Further immunohistochemical characterization showed that
some of the FG+ cells had a GABAergic phenotype (Figure 3.4 B-D). Poor penetration of the GABA antibody prevented a conclusive assessment of the GABA-phenotype across the majority of FG+ cells at a more quantitative level.

**Figure 3.3:** Retrograde labeling of midbrain neurons 5–7 days following injection of fluorogold into the dentate gyrus. Immunolabeling of fluorogold in a representative animal at the injection site (A) showing distribution in the dentate gyrus and some labeling in the overlying molecular layer and CA1, and in the midbrain (B), showing retrogradely labeled neurons with TH labeling (green) shown for anatomical reference. (C-E) Merged image and individual color channels showing fluorogold-labeled cells (arrows) interspersed amongst, but
not overlapping with TH+ DA neurons in the VTA. (F) Representative fluorogold labeling at the injection site in one of two animals where fluorogold labeling was found along the injection tract in the cortex. (G-I) In these animals a total of three fluorogold-labeled TH+ DA neurons were identified in the midbrain. Scale bars: A, B, F, 200 µm; C-E and G-I, 50 µm.

Abbreviations: FG, Fluorogold; TH, Tyrosine hydroxylase.

Figure 3.4: Cumulative spatial representation of fluorogold labeling in the midbrain of 10 animals 5–7 days after injection of fluorogold into the dentate gyrus. A) Schematic overview of three representative coronal levels spanning the A9-A10 cell groups illustrating the location of all 605 fluorogold-labeled cells, indicated as red circles for FG+/TH- cells and yellow circles for FG+/TH+ cells. The black arrow represents the ipsilateral side injected with fluorogold. B-D) Co-labeling for GABA showed that some of the FG+ neurons innervating the hippocampus were GABAergic projection neurons. Scale bars: B-D, 50 µm. Abbreviations: FG, Fluorogold; fr, fasciculus retroflexus; GABA, γ aminobutyric acid; IPC, Interpeduncular nucleus central; MT, Medial terminal nucleus accessory optic tract; SNr, Substantia nigra reticular; VTA, Ventral tegmental area.
Inspection of the LC, as identified by TH+ cell bodies, showed the presence of FG+ cells in all animals that received hippocampal FG injections (Figure 3.5 A-C). In all cases, cells were found in the ipsilateral LC, with a smaller contribution of cells found in the contralateral LC (Figure 3.5 D). Cells were also identified bilaterally in the adjacent central grey pons (PCG). Figure 3.5 D illustrates the cumulative distribution of FG+ cells in 10 animals across three coronal sections spanning the rostro-caudal extent of the LC. Confocal analysis showed that the vast majority of the FG+ cells were NA neurons based on the co-expression of TH (Figure 3.5 A-C).
Figure 3.5: Retrograde labeling of noradrenergic neurons in the locus coeruleus 5–7 days after injection of fluorogold into the dentate gyrus. A-C) Immunolabeling of fluorogold and TH showed extensive overlap in the locus coeruleus (arrowheads) as well as a minor population of FG+/TH- cells (arrows). D) Schematic overview of three representative coronal levels spanning the locus coeruleus where the location of fluorogold labeled cells are indicated as red circles for FG+/TH- cells and yellow circles for FG+/TH+ cells. The black arrow represents the ipsilateral side injected with fluorogold. **Scale bars:** A-C, 200 µm. **Abbreviations:** FG, fluorogold; TH, tyrosine hydroxylase; PCG, pontine central gray; PDTg, posterodorsal tegmental nucleus.
3.5. Discussion

These results indicate that the adult rat dentate gyrus receives very little, if any, afferent input from mDA neurons. Tyramide-based amplification allowed for the robust detection of DAT+ fibers in the brain with a resolution of single fibers, as seen in the CC. Although it has previously been suggested that DA fibers in the hippocampus do not express DAT, detailed confocal analysis showed that TH+ fibers throughout the hippocampal formation universally co-labeled with DBH, indicating they were derived from NA projections from the LC. Therefore, it is unlikely that the hippocampus contains DA terminals that do not express DAT.

The absence of definitive histological markers for DA terminals in the hippocampus has necessitated indirect means to infer their presence. For example, the presence of hippocampal DA receptors (Dubois et al. 1986), biochemical detection of low levels of hippocampal DA that can be partly reduced by chemical or electrolytic lesioning of the midbrain (Bischoff, Scatton, and Korf 1979; Scatton et al. 1980), and the persistence of DA and DOPAC levels (Bischoff, Scatton, and Korf 1979; Scatton et al. 1980) or TH+ fibers (Verney et al. 1985) in rats with lesions of the NA system have all been interpreted as evidence for a hippocampal DA terminal network (for review see Gasbarri et al. (1997). However, there are caveats to these interpretations. For example, lesioning of the NA system using 6-hydroxydopamine (6-OHDA) or N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) are often incomplete, as was the case in the study by Verney et al. (1985), and thus one cannot unequivocally exclude the possibility that the remaining TH+ fibers or DA metabolites originate from spared NA neurons. Similarly, 6OHDA or electrolytic lesioning of the mDA system can also damage NA neurons. In the study by Scatton et al. (1980), lesioning of the NA system produced a partial reduction in hippocampal DA content, that was further reduced by electrolytic lesioning targeted at the VTA. It cannot be completely excluded that the severity of an initially incomplete NA lesion is increased by subsequent electrolytic damage to NA axons passing through the midbrain.

Interestingly, studies performed more recently have provided convincing evidence that the NA terminal network serves as the source of DA signaling in the
Microdialysis studies measuring DA levels in the hippocampus have shown that DA is cleared by the NA transporter and that antagonism of NA presynaptic (α2) receptors, but not DA D2 autoreceptors, increased hippocampal DA (Borgkvist et al. 2012). Similarly, Smith & Greene (2012) have reported that amphetamine-induced stimulation of DA receptors in the CA1 field is not affected by pharmacological blockade of DAT to prevent amphetamine uptake into DA terminals, or by selective inhibition of VTA input through siRNA knockdown of TH, but is inhibited by blockade of the NA transporter or knockdown of TH in the LC. These findings highlight the important point that a lack of a direct midbrain dopaminergic projection to the hippocampus does not preclude a role for dopamine in modulating hippocampal activity and provide an interesting point of consideration for the neuroanatomical basis of changes in hippocampal dopamine levels in experimental work in rodents (e.g. Kwon et al. 2008). Additionally, it is important to recognize that the lack of a direct projection does not preclude mDA modulation of hippocampal activity through indirect, multi-synaptic pathways. For example, mDA neurons in the VTA are known to innervate the locus coeruleus (for review see Samuels and Szabadi 2008) and thus modulation of hippocampal neuronal firing through activation of the VTA (Rosen, Cheung, and Siegelbaum 2015) may well occur through this pathway.

Another important approach for characterizing the afferent input to the hippocampus has been through retrograde tracing studies. Early studies in this area confirmed the presence of hippocampal projections originating in the midbrain, but not their neurochemical identity (Meibach and Siegel 1977; Schwab, Javoy-Agid, and Agid 1978; Simon, Le Moal, and Calas 1979; Wyss, Swanson, and Cowan 1979). These studies showed similar patterns of cell labeling to those reported here, with the majority of labeled cells contained within the VTA and adjacent nuclei, close to the midline. Later studies combined retrograde labeling with fluorescence immunohistochemistry in order to further identify these neurons. Studies by Reymann et al. (1983) and Pohle et al. (1984) report that ‘not one neuron’ identified as containing biogenic amine in the A9-A10 cell groups was retrogradely labeled, while other studies report 6–18% are dopaminergic (Swanson 1982; Gasbarri et al. 1994). Here we report that following FG injection into the dentate gyrus of the septal hippocampus, only
three of 605 FG+ cells in the ventral midbrain (0.5%) were TH+. Notably, these cells were found in two animals with some reflux of FG into the overlying cortex, where, unlike the hippocampus, we noted the presence of DAT+/TH+ fibers. Given that retrograde labeling studies targeting the hippocampus necessitates that the injection cannula passes through the cortex, one cannot completely exclude ‘off-target’ labeling of neurons projecting to the cortex. Gasbarri et al., 1994 reported a greater proportion of DA neurons when retrogradely labeling from the temporal (15–18%) compared with septal (10–12%) hippocampus. Here, we limited the retrograde tracing studies to the septal dentate gyrus. We found the absence of DAT and the complete overlap between DBH and TH to be consistent throughout all regions of the hippocampus. On this basis, we would not predict a greater propensity for labeling mDA neurons when injecting the tracer into other hippocampal regions. Co-labeling with markers for other neurotransmitter phenotypes showed at least some of the midbrain hippocampal projections to be GABA-containing neurons. Although it is known that the VTA consists of up to 30% GABA neurons, including interneurons, as well as projections to areas such as cortex and nucleus accumbens (Carr and Sesack 2000; for review see Creed et al. 2014), projections to the hippocampus have not been well characterized. In order to obtain additional detailed information on the identity of the FG+ cells, including the quantity and topographical distribution of specific phenotypes, further investigation is certainly warranted.

FG injection into the dentate gyrus also retrogradely labeled TH+ neurons in the dorsal segment of the LC, both ipsilateral and, to a lesser extent, contralateral to the injection. This is consistent with retrograde tracing studies in both rats (Loy et al. 1980) and monkeys (D. G. Amaral and Cowan 1980) showing that NA neurons project bilaterally to the hippocampus. Work by Loughlin et al. 1986 also showed that projections to the hippocampus are located in the dorsal segment of the LC, while the ventral LC contains neurons projecting to the spinal cord. We did not set out to perform a detailed characterization of the NA innervation of the hippocampus, but rather describe the results from retrograde labeling as a basis for comparison to the labeling in the midbrain. Compared with the mDA system, the functional neuroanatomy of afferent input to the hippocampus from NA neurons in the LC has
been relatively more straightforward to characterize – for reviews see (Harley 2007; Samuels and Szabadi 2008; Szabadi 2013). The development of new tools, such as viral-genetic tracing approaches (Schwarz and Luo 2015), has allowed for even further refinement of our understanding of central NA connectivity and their application may help to further characterize and clarify connectivity of other systems, including the mDA system.

In summary, using immunohistochemical and retrograde tracing techniques combined with confocal microscopy, we have found that the TH+ fiber innervation of the rat dentate gyrus is overwhelmingly, and perhaps exclusively, accounted for by NA projections from the LC. mDA neurons appear to provide little, if any, afferent input to the hippocampus, particularly the dentate gyrus. This has implications for how we may interpret data from studies that look at the impact of mDA loss at the level of the hippocampus. For example, reduced neurogenesis (Suzuki et al. 2010) or deficits in cognitive function (Wisman et al. 2008) after 6OHDA lesioning may reflect mechanisms that are more indirect than loss of a direct mDA-hippocampus pathway – i.e., changes in hippocampal signaling at a broader systems level involving dopaminergic innervation of other nuclei that feed forward to the hippocampus. Both the nucleus accumbens and amygdala may be interesting targets to investigate further in this context. Both receive innervation from mDA neurons and both structures are involved in associative learning tasks through connectivity with the hippocampus (Ito et al. 2008; Sara 2009; J. I. Broussard et al. 2016).
Supplemental Figure 3.1: Immunohistochemistry shows retrograde labeling of ChAT+ cholinergic neurons in the septum (A-D) and 5HT+ serotonergic neurons in the raphe nucleus (E-H) 5-7 days after injection of fluorogold into the dentate gyrus. Scale bars: A, 200 µm; B-D, 50 µm; E, 100 µm and F-H, 25 µm. Abbreviations: 5-HT, 5-hydroxytryptamine; ChAT, choline acetyltransferase; FG, fluorogold.
3.7. References


Chapter 4

Modelling the dopamine and noradrenergic cell loss that occurs in Parkinson’s disease and the impact on hippocampal neurogenesis

4.1. Abstract

Key pathological features of Parkinson’s Disease (PD) include the progressive degeneration of midbrain dopaminergic neurons and hindbrain noradrenergic neurons. The loss of dopaminergic neurons has been extensively studied and is the main cause of motor dysfunction. Importantly, however, there are a range of ‘non-movement’ related features of PD including cognitive dysfunction, sleep disturbances and mood disorders. The origins for those non-motor symptoms are still unclear, but some evidence has implicated reduced adult-hippocampal neurogenesis as a possible substrate for cognitive decline and it is also reported to be impaired in PD. The mechanisms regulating hippocampal neurogenesis under physiological conditions and the mechanisms underlying reduced neurogenesis in PD, are not well-established. With this study we aimed to test the hypothesis that noradrenaline (NA) and dopamine (DA) depletion, as occurs in PD, results in reduced hippocampal neurogenesis. We used 6-hydroxydopamine and the immunotoxin dopamine-β-hydroxylase (DBH)-saporin to selectively lesion the DA and the NA neurons in adult Sprague Dawley rats and assessed hippocampal neurogenesis through phenotyping of cells birth-dated using 5-bromo-2’-deoxyuridine (BrdU). The results showed there was no difference in proliferation, survival or differentiation of newborn cells in the subgranular zone (SGZ) after NA or DA lesion. This suggests that impairment of hippocampal neurogenesis in PD likely results from mechanisms independent of degeneration of DA and NA neurons.
4.2. Introduction

Parkinson’s disease is a neurodegenerative disease that affects individuals over sixty years old. The pathology of PD is most often described as a degeneration of DA neurons of the substantia nigra pars compacta (SNpc), which leads to motor impairments that include rigidity, tremor and bradykinesia. However, PD is a multi-system disorder and the neuronal degeneration is not limited to DA neurons, but is a progressive pathology that impacts various nuclei including degeneration within the locus coeruleus (LC) (Zweig et al. 1993; G. Halliday 2012), basal forebrain (Nakano and Hirano 1984; Hall et al. 2014) olfactory structures (Pearce, Hawkes, and Daniel 1995) and raphe nuclei (G. M. Halliday et al. 1990). Braak et al. (2008) used immuno-marked Lewy bodies, a hallmark of PD, to identify the areas affected in the disease and developed a theory for the ‘spread’ of pathology, characterised by a caudal-to-rostral progression (Braak and Del Tredici 2008). While PD is often described as a motor disease, PD patients also exhibit a wide range of non-motor symptoms, including olfactory impairment, depression, and mild cognitive impairments progressing to dementia in late-stage disease (Barbosa 2013). Exploring the relationship between non-motor symptoms and patterns of pathology in specific brain regions is becoming an increasingly important area in PD research.

Hippocampal neurogenesis has been linked to cognitive function and is reportedly reduced in parallel with the onset of age-related cognitive decline (Small et al. 2012). Interestingly, hippocampal neurogenesis has been suggested to be deficient in parkinsonian patients, especially through MRI studies that have shown that PD patients with dementia had smaller hippocampi compared with PD patients without dementia (Camicioli et al. 2003; Laakso et al. 1996). Höglinger et al. (2004) also investigated hippocampal neurogenesis level in PD patients and reported a reduction in the number of neuronal precursors. Since these reports on impairment of hippocampal neurogenesis in PD and its potential role in non-motor symptoms, a number of studies have focused on identifying the factors responsible for reduced neurogenesis in order to identify therapeutic targets (Geraerts et al. 2007; Vaidya, Vadodaria, and Jha 2007; Kotani et al. 2008; Meneghini et al. 2014; Malberg et al. 2000). The DA degeneration has been suggested to be a key factor underlying a
decrease in neurogenesis in the hippocampus (Höglinger et al. 2004; Suzuki et al. 2010; Park and Enikolopov 2010). Indeed, Suzuki et al. (2010) used 6-hydroxydopamine (6-OHDA) to remove DA neurons and observed a reduction in hippocampal proliferation in the SGZ, which has also been reported using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin (Höglinger et al. 2004). However, both 6-OHDA and MPTP are not only DA specific. 6-OHDA is a toxin that targets all catecholamines (Breese and Traylor 1970) and MPTP has been reported to also kill NA and serotonin (5HT) neurons (Namura et al. 1987; Gupta, Felten, and Gash 1984). It is possible that in these previous studies, NA depletion was underestimated and lead us to hypothesise that the NA depletion may play an important role in the reduced hippocampal neurogenesis reported. Supporting this hypothesis, the LC, which is the primary source of NA in the brain, is thought to play a major role in cognitive functions, including arousal, sleep-wake cycle, response to stress, memory processing and maintenance of attention (Bouret and Sara 2005; Neuman and Harley 1983; Cummings et al. 1983; Bourgin et al. 2000; Aston-Jones and Bloom 1981). There is also support for this hypothesis. We recently published a study where we have shown that the dentate gyrus (DG) receives a dense innervation from NA neurons originating in the LC, but completely lacks innervation from DA neurons from the midbrain (Ermine et al. 2016). Finally, it has been shown that NA neuronal degeneration occurs before DA neuronal degeneration and that non-motor symptoms arise before the motor symptoms, which at least indirectly supports a link between NA loss and non-motor symptoms. In addition, the most commonly used treatment to alleviate the symptoms, L-DOPA does not have any effect on the non-motor symptoms.

We therefore hypothesise that the NA loss in PD is responsible for hippocampal neurogenesis impairment, which leads to cognitive deficits and dementia. To test this hypothesis, we compared the effect of specific DA depletion and NA depletion alone and together on hippocampal neurogenesis in adult rats.
4.3. Materials and methods

4.3.1. Animals

Adult female Sprague Dawley rats were used in this study. The experimental design and procedures were approved by the Florey Institute for Neuroscience and Mental Health. All animals were housed in individually ventilated cages under a 12h light/dark cycle with ad libitum access to food and water.

4.3.2. Surgical procedures

The procedures for lesioning of the DA and NA systems through stereotaxic delivery of toxins are as described under Methods, Chapter 2, section 2.3.1. Briefly, we anesthesied the animals prior to the surgery with isoflurane (5% at 1L/min), they were maintained under anaesthesia for the duration of the surgery (2%, 1L/min). The animals were placed in a stereotaxic frame (Kopf, Germany) and the different toxins were administered at a rate of 1µl/min. The rats were divided into 5 treatment groups:

1) DA lesion, by microinjection of 14µg of 6-OHDA into the medial forebrain bundle (MFB) with i.p. injection of desipramine (20mg/kg) 30 min prior to surgery, to protect NA neurons (n= 8 rats) (Chapter 2, section 2.3.1.1);

2) NA lesion, by intra cerebral ventricle (i.c.v.) injection of 1ug of dopamine-β-hydroxylase saporin (DBH-saporin) (n= 8 rats) (Chapter 2, section 2.3.1.2);

3) double-lesion of DA & NA, by microinjection of 6-OHDA and DBH-saporin using the same parameters for the single lesions (n= 8 rats);

4) acetylcholine (ACh) lesion, by microinjection of 5ug of 192-IgG-saporin as described in (n= 8 rats) (Chapter 2, section 2.3.1.3).

5) controls, by saline injection in MFB or i.c.v. to control for DA, NA and ACh lesion, and by saline injection in MFB and i.c.v. to control for the double lesion group. The injection volumes were matched with the corresponding lesion group (n= 8 rats per control group).
The animals were sacrificed at either four or nine weeks post lesion in order to assess the impact of the treatments on hippocampal cell proliferation and neurogenesis at different time-windows. The shorter time-point, four weeks, was chosen for its relevance to study designs in the existing literature (Höglinger et al. 2004; Suzuki et al. 2010; Baker, Baker, and Hagg 2004). The longer time point, nine weeks was chosen to investigate a long term effect of the lesions on neurogenesis, nine weeks allow enough time for the new cells to differentiate into their neuronal phenotype.

4.3.3. Lesion validation

High performance liquid chromatography (HPLC) was performed on 8 animals from each treatment group, four weeks after lesion in order to measure the amount of DA and NA remaining in the hippocampus and the striatum relative to saline injected control animals. The HPLC was performed according to standard methods previously described (Parish et al. 2001) and is detailed in Chapter 2, section 2.7.

4.3.4. BrdU treatment

Four weeks post-surgery, 56 animals across the different treatment groups were injected with 5-bromo-2’-deoxyuridine (BrdU) (50mg/kg, i.p.) every 12h for 7 days in order to label all cells dividing over a 1 week period. For further details, see Chapter 2, section 2.4. The remaining 24 animals were sacrificed for histology.

4.3.5. Tissue collection and Immunohistochemistry

Animals were sacrificed by terminal dose of pentobarbitone (100mg/kg; Virbac, Peakhurst, Australia) and were transcardially perfused with paraformaldehyde media (PFA, 4% in 0.4M phosphate buffer with 0.2% picric acid). The brains were collected and further post-fixed for 2h in PFA media, followed by cryo-protection in 20% sucrose PBS solution for 1-2 days. After freezing the brains with dry ice, coronal
sections of 40 µm were collected using a freezing-microtome (Leica, Wetzlar, Germany) - see Chapter 2, section 2.5.

Free-floating immunohistochemistry for tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), choline acetyltransferase (ChAT), BrdU, Ki67 and Prox1 were performed on a 1:12 and 1:6 series as described in Chapter 2, section 2.6. Tissues used for BrdU labeling were pre-treated by incubation in Omnipur deionized formamide (Merck Millipore) at 65°C for 2h, in 2M HCL at 27°C for 30 min and in Borate buffer at room temperature for 20 min. Primary antibodies and dilutions used were: sheep anti-BrdU (Exalpha, A205P, 1:1000); mouse anti-DBH (Millipore, MAB308, 1:5000); rabbit anti-TH (Pel-freeze, P40101-0, 1:1000); rabbit anti-Ki67 (Thermo Fisher, LBVRM-9106-S1, 1:1000); rabbit anti-prox1 (Millipore, ABN278, 1:2000); goat anti-ChAT (Millipore, MAB144, 1:100). Secondary antibodies and dilution factors were: anti-rabbit and anti-sheep conjugated to Dylight Fluorophores 488 and 549 (Jackson ImmunoResearch, 1:200) for fluorescent staining and anti-mouse, anti-rabbit, anti-goat and anti-sheep biotin-conjugated (Jackson ImmunoResearch, 1:400) for chromogenic staining.

4.3.6. Analysis

Fluorescent microscopic analysis and images were performed using a Zeiss Meta confocal microscope (LSM 780) and chromogenic images were captured using a Leica DM6000 microscope.

The number of BrdU+ cells and Ki67+ cells in the SGZ were quantified across six sections located at approximately 2.6mm, 2.84mm, 3.08mm, 3.32mm, 3.56mm and 3.8mm caudal to bregma. The number of BrdU/Prox1 double positive cells was quantified among a total of 80 BrdU+ cells located in the SGZ across all sections and double-labelling was verified through orthogonal reconstruction using the confocal microscope.

Statistical analysis was performed using an unpaired t-test with Welch’s correction to assess the difference of DA and NA levels at their terminal fields between the lesioned and saline control groups. The difference among the groups in BrdU+ cells, Ki67+ cells and BrdU+/Prox1+ double positive cells in the DG was assessed via one-way
ANOVA, with a Dunnett’s multiple comparison test. The difference between the groups in the number of BrdU+ cells and Ki67+ cells in the SVZ was assessed via unpaired t-test.

4.4. Results

4.4.1. Unilateral injection of DBH-saporin and 6-OHDA toxins ablates the NA and DA systems respectively.

To test whether the dopaminergic system and/or the noradrenergic system are functionally involved in the regulation of adult hippocampal neurogenesis, we performed three different lesions: 1) a selective ablation of NA neurons from the LC using the toxin DBH-saporin; 2) a selective ablation of DA neurons from the SN using 6-OHDA and desipramine; and 3) a double lesion of DA and NA neurons from SN and LC respectively using 6-OHDA and DBH-saporin.

Examination of the density of NA and DA neurons and fibres, using immunohistochemistry for DBH or TH, enzymes that synthesize NA and DA respectively, allowed initial validation of both the NA and DA lesion. The number of NA cell bodies in the LC and fibres in the hippocampus were clearly reduced in the NA lesion group compared to saline injected controls (Figure 4.1 A-D). Similarly, TH staining in the SN and in the striatum, showed a reduction in number of DA cell bodies and DA fibres respectively, in the DA lesion group (Figure 4.1 E-H).

Both DA and NA lesion quality were further validated by measurements of DA level in the striatum and NA level in the hippocampus using HPLC (Figure 4.1 I, J). The level of NA in the hippocampus of the NA lesion group was significantly reduced by 13-fold compared to the control group (NA lesion: mean ± SEM = 0.2290 ± 0.036 nM/mg of tissue, n = 8; control: mean ± SEM = 2.998 ± 0.2475 nM/mg of tissue, n = 15). The DA level in the striatum of animals who received 6-OHDA lesion, was significantly reduced by 8.8-fold compared to control (DA lesion: mean ± SEM = 53.76 ± 21.88 nM/mg of tissue, n = 8; control: mean ± SEM = 218.8 ± 6.621 nM/mg of tissue, n = 16).

Thus robust DA and NA lesions were validated both at neuroanatomical and biochemical levels.
Figure 4.1: DBH-saporin and 6-OHDA successfully lesion the NA and DA system respectively in adult SD rats. Immunohistochemical detection of DBH using a brightfield microscope, reveals a reduction in the number of NA cell bodies in the LC of animals lesioned with DBH-saporin (A) compared with control animals (B). Darkfield imaging of chromogenically labelled DBH in the hippocampus shows an almost complete ablation of NA fibres of lesioned animals (C) compared with the intact NA system in the control animals (D). The analysis of TH staining shows an almost complete ablation of DA cell bodies in the right SN (E) and of the axon terminals in the striatum (G) of the animals lesioned with 6-OHDA, compared with control (F, H). The HPLC further confirm the success of both lesions by showing a 13-fold reduction in NA levels in the hippocampus of NA-lesioned animals (I) and an 8.8-fold reduction of DA levels in the striatum of DA-lesioned animals (J) compared with their respective control groups. The immunohistochemical analysis was completed in n=8 rats per groups and all rats demonstrated a similar pattern of staining. Statistical analysis: unpaired t-test with Welch’s correction, **** p ≤0.0001 (I, J), error bars= Standard deviation, for control groups n=16, for lesion groups n=8. Scale bars: A,B,F,G,H,I, 500 µm and C,D 200 µm. Abbreviations: DBH, dopamine-β-hydroxylase; NA, noradrenaline; DA, dopamine; LC, locus coeruleus; HPLC, high performance liquid chromatography; SN, substantia nigra.
4.4.2. Cell proliferation and hippocampal neurogenesis remains unchanged after NA and/or DA lesion.

To determine the number of proliferating cells in the SGZ and their phenotypic fate, we injected the adult rats with BrdU four weeks after lesioning, and performed histological analysis four weeks later. We observed no significance difference in the number of BrdU+ cells in the SGZ between all treatment groups (control: mean ± SEM = 182 ± 16.96, n = 15; NA lesion: mean ± SEM = 178.5 ± 18.44, n = 8; DA lesion: mean ± SEM = 177.4 ± 18.0, n = 8; double lesion: mean ± SEM = 181.3 ± 24.96, n = 7) (Figure 4.2 A, B).

To assess the effect of each lesion on the differentiation of the newborn cells in the SGZ, we labelled the SGZ cells with Prox1, a neuronal marker, and identified the number of newborn cells that were Prox1+. The number of double positive BrdU+/Prox1+ cells in the SGZ did not reveal any difference between the groups (control: mean ± SEM = 61.07 ± 1.54, n = 15; NA lesion: mean ± SEM = 63.63 ± 1.51, n = 8; DA lesion: mean ± SEM = 60.43 ± 2.26, n = 7; double lesion: mean ± SEM = 62.86 ± 1.94, n = 7) (Figure 4.2 C-G).

Because the number of BrdU cells at nine weeks will be a reflection of both proliferation and survival, we also quantified the number of Ki67+ cells to assess the number of actively dividing cells in each group as a more precise measure of proliferation. The number of Ki67+ cells in the SGZ across six sections was not different between all the groups (control: mean ± SEM = 57.53 ± 4.80, n = 15; NA lesion: mean ± SEM = 58.38 ± 10.512, n = 8; DA lesion: mean ± SEM = 65.63 ± 5.558, n = 8; double lesion: mean ± SEM = 66.25 ± 16.0, n = 8) (Figure 4.2 H, I).
Figure 4.2: Proliferation and neurogenesis in the SGZ is unchanged following DA-lesion, NA-lesion and combined double lesions in adult rats. Immunohistochemical analysis of BrdU allowed manual counting of BrdU+ cells in the SGZ across 6 sections (A), which did not reveal any difference between the groups (B). Phenotypic analysis revealed no significant difference in hippocampal neurogenesis as shown by immunohistochemistry for cells double positive for BrdU and Prox1, (C-G). Double labelled cells for BrdU/Prox1 were counted across 80 BrdU+ cells in the SGZ (C), D-F represents a high magnification example of
a BrdU+/Prox1+ cell. Proliferation of cells in the SGZ was characterised by immunohistochemistry for Ki67. The number of Ki67+ cells was counted in the SGZ across 6 sections (H) and was not different between all groups (I). The immunohistochemical analysis was completed in n=8 rats per lesion groups and n=16 rats for the control group, and all rats demonstrated a similar pattern of staining. **Statistical analysis:** (B,G) One-way ANOVA with Dunnett’s test for multiple comparison, $F_{3,34} = 0.013$ and $p =0.9979$ (B), $F_{3,32} = 0.6291$ and $p =0.6016$ (G), $F_{3,35} = 0.2696$ and $p =0.8468$ (I). Error bars= Standard deviation, for control groups n=16, for lesion groups n=8. **Scale bars:** A,B,F,G,H,I, 500 µm and C,D 200 µm. **Abbreviations:** BrdU, 5-bromo-2'-deoxyuridine; NA, noradrenaline; DA, dopamine; SGZ, sub-granular zone.

4.4.3. 6-OHDA injury does not affect cell proliferation in the SVZ.

While a recent study has reported a reduction in hippocampal neurogenesis after lesioning of midbrain DA neurons (Suzuki et al. 2010) it has been more widely reported that dopamine deficiency results in a reduction in SVZ proliferation (Höglinger et al. 2004; Baker et al. 2004; Winner et al. 2006). In this study we wanted to confirm this finding with our experimental design, to have a kind of ‘internal control’ to contextualize findings from analysis of the hippocampus. Therefore, the number of BrdU+ cells and Ki67+ cells were counted in the SVZ across three sections and compared between the DA lesion group and the control group. Given the almost complete absence of NA innervation of the striatum and SVZ we did not include the NA lesion group for assessment. Surprisingly, 6-OHDA lesion did not affect the number of BrdU+ cells (Figure 4.3 A, B) (control: mean ± SEM = 808 ± 30.98, n = 8; DA lesion: mean ± SEM = 808.3 ± 49.88, n = 8) or Ki67+ cells (Figure 4.3 C, D) (control: mean ± SEM = 1430 ± 120.8, n = 8; DA lesion: mean ± SEM = 1513 ± 91.54, n = 8).
Figure 4.3: Proliferation in the SVZ remains unchanged after 6-OHDA induced DA lesion in adult rats. Immunohistochemical analysis of BrdU (A-B) and Ki67 (C-D) in the SVZ revealed no difference in proliferation rate after DA lesion. The immunohistochemical analysis was completed in n=7-8 rats per groups and all rats demonstrated a similar pattern of staining. **Statistical analysis:** (B,D) unpaired t-test, p =0.9968 (B) p =0.5926 (D), error bars= Standard deviation, for all groups n=8. **Scale bars:** A and B, 200 µm. **Abbreviations:** BrdU, 5-bromo-2'-deoxyuridine; SVZ, sub-ventricular zone.

4.4.4. Cell proliferation in the SGZ and the SVZ remains unchanged four weeks after DA and NA lesion.

We were surprised at the lack of impact of DA lesion on SGZ and SVZ cell proliferation and looked closely at the existing literature to determine possible experimental design elements underlying the conflicting results. One conspicuous
difference was that studies reporting reduced neurogenesis had often looked at shorter survival times after lesioning – e.g. four weeks. We hypothesized that the dopaminergic or noradrenergic effect on neurogenesis may be transient and not seen at the nine week time-point we used. Therefore, NA and DA ablation were performed in a new cohort of animals, and sacrificed at four weeks post lesion. As seen previously, immunohistochemistry for DBH, showed a robust reduction in the number of cell bodies in the LC and in the number of fibres in the hippocampus of the NA lesion group compared to control, confirming that DBH-saporin injection had successfully removed the NA neurons (Figure 4.4 A-D). Similarly, 6-OHDA injection resulted in a unilateral lesion of DA neurons, as shown by the reduction of TH+ cells in the SN and TH+ fibres in the striatum of DA lesion group compared to control (Figure 4.4 E-H).

Proliferation was assessed comparing the number of Ki67+ cells in the SGZ between NA-lesion, DA-lesion and control; and the number of Ki67+ cells in the SVZ between DA lesion and control (Figure 4.4 I, J). In the SGZ, the results showed no difference in the number of Ki67+ cells between NA lesion and DA lesion compared to control (Control: mean ± SEM = 100 ± 10.38, n = 8; NA lesion: mean ± SEM = 112.4 ± 15.22, n = 7; DA lesion: mean ± SEM = 114.9 ± 20.15, n = 8). Similarly, there was no significant impact of DA lesion on the number of Ki67+ cells in the SVZ (Control: mean ± SEM = 1598 ± 121.2, n = 7; DA lesion: mean ± SEM = 1673 ± 82.25, n = 7).
Figure 4.4: Lesion of the dopaminergic or noradrenergic systems does not affect proliferation in the SVZ or SGZ of adult rats four weeks post lesion. Injection of DBH-saporin toxin in the i.c.v. robustly ablates NA neurons as shown by DBH labelling in the LC and hippocampus (A, C), compared to sham lesion (B, D). TH labelling shows a near complete reduction in the number of DA neurons in the SN and DA fibres in the striatum in the 6-OHDA lesion group (E, G) compared to controls (F, H). Analysis of the number of Ki67+ cells revealed no difference in proliferation in the SGZ between the NA lesion, DA lesion and control groups (I) or in the SVZ between the DA lesion and controls (J). The immunohistochemical analysis was completed in n=7-8 rats per groups and all rats demonstrated a similar pattern of staining. Statistical analysis: (I) one-way ANOVA, $F_{2, 20} = 0.2614, p = 0.7726$; (J) unpaired t-test, $p = 0.6199$, error bars= Standard deviation. Scale bars: A - H, 500 µm. Abbreviations: DBH, dopamine-β-hydroxylase; LC, locus coeruleus; TH, tyrosine hydroxylase; SGZ, sub-granular zone; SN, substantia nigra; SVZ, sub-ventricular zone.
4.4.5. Acetylcholine lesion results in a reduction in cell proliferation, but no change in overall hippocampal neurogenesis.

Based on these largely negative results, we became further interested to investigate the impact of other neurotransmitters such as ACh and 5HT, on adult hippocampal neurogenesis. Unfortunately, we failed to find a toxin capable of successfully ablating 5HT neurons in the raphe nucleus and we therefore could not investigate the impact of 5HT on hippocampal neurogenesis.

To explore the effect of ACh on hippocampal neurogenesis, the cholinergic system was lesioned using the toxin 192-IgG-saporin. This is in fact one of the first ‘immunotoxins’ developed for neuroscience research and has been used extensively for selective lesioning of cholinergic neurons (Leanza 1996; Jeong et al. 2011). Nine weeks after i.c.v. delivery, immunohistochemistry for ChAT showed reduction of ChAT+ cells present in the septum (Figure 4.5 A-B’) and in the nucleus basalis of the ACh lesion group compared to control (Figure 4.5 C-D’).

We assessed the survival and differentiation in the SGZ after ACh lesion and found no significant change in the number of BrdU+ cells (Control: mean ± SEM = 250.8 ± 29.95, n = 6; ACh lesion: mean ± SEM = 204.8 ± 17.79, n = 8; p = 0.1871) (Figure 4.5 E) or in the number of BrdU+/Prox1+ cells (Control: mean ± SEM = 60 ± 1.528, n = 6; ACh lesion: mean ± SEM = 57 ± 3.338, n = 8; p = 0.4569) (Figure 4.5 F). However, there was a significant reduction in the number of Ki67+ cells (Control: mean ± SEM = 71.67 ± 10.05, n = 6; ACh lesion: mean ± SEM = 35.13 ± 7.496, n = 8; p = 0.014) (Figure 4.5 G).
Figure 4.5: Acetylcholine lesion induces a reduction in cell proliferation but no change in hippocampal neurogenesis in adult rats nine weeks post-lesion. Immunohistochemical analysis of ChAT showed a robust reduction in the number of ChAT+ neurons in the septum (A, A’) and nucleus basalis (C, C’) of rats injected with 192-IgG-saporin, compared with control (B, B’, D, D’). A’, B’ and C’, D’ are high magnification of ChAT+ cells in the septum and nucleus basalis respectively. The analysis of the number of BrdU+ cells (E), and the number of double positive cells for BrdU+/Prox1+ (F) in the SGZ, showed no significant difference between lesion and control groups. Proliferation in the SGZ was reduced in the ACh lesion group compared with control based on reduction in the number of Ki67+ cells (G). The immunohistochemical analysis was completed in n=6-8 rats per groups and all rats demonstrated a similar pattern of staining. Statistical analysis: (E-G) unpaired t-test, p = 0.1871 (E), p = 0.4569 (F) * p = 0.0114 (G), error bars= Standard deviation. Scale bars: A – D’, 500 µm. Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; SGZ, subgranular zone.
4.5. Discussion

These results show that the NA and the DA systems do not impact hippocampal adult neurogenesis when ablated alone or together. Thus if cognitive decline in PD is in fact related to reduced neurogenesis it may be through mechanisms unrelated to NA and DA neuronal degeneration. Alternatively, DA and NA loss may cause cognitive decline independently of any interaction with hippocampal neurogenesis impairment previously suggested in parkinsonian patients (Höglinger et al. 2004; Laakso et al. 1996; Camicioli et al. 2003).

In our experimental model, a unilateral lesion of DA neurons or a lesion of NA neurons had no effect on proliferation, survival or differentiation in the SGZ. The fact that DA and NA share a synthesis pathway, where DA is the precursor to NA, lead us to suspect that there might exist a close redundancy mechanism between the two systems, that allowed one system to functionally compensate the other one if ablated. Therefore, we also included a double lesion group, where both DA and NA systems were lesioned to account for such a scenario. However, the double lesion group was no different from the control or the single lesion groups - we observed no change in hippocampal neurogenesis. Our results are in clear contrast to current literature, where DA is described as a regulator of adult hippocampal neurogenesis (Suzuki et al. 2010; Höglinger et al. 2004; Broussard 2012; Wisman et al. 2008; Mu, Zhao, and Gage 2011), although the actual effect on neurogenesis still seems to be controversial. Indeed different doses of MPTP used to lesion DA neurons in rodent models of PD, have been reported to decrease proliferation at low doses and to increase proliferation at higher doses (Höglinger et al. 2004; Park and Enikolopov 2010). The effect of DA on SGZ neurogenesis has also been studied by pharmacological replacement. The use of the D2 agonist haloperidol has shown no effect (Malberg et al. 2000), a decrease (Wakade et al. 2002) and an increase (Keilhoff, Grecksch, and Becker 2010) of SGZ proliferation levels. Haloperidol has also been reported to have no effect (Halim et al. 2004) and to increase (Keilhoff et al. 2010) survival in the DG. Thus there is a mixed literature in this area.
Since the role of DA on SVZ neurogenesis seemed to be better established in the literature, we assessed the effect of our 6-OHDA lesion on SVZ proliferation as an ‘internal control’. Surprisingly, our results revealed no change in the number of Ki67+ cell or BrdU+ cell in the SVZ following 6-OHDA lesion, suggesting no effect of DA ablation on proliferation or survival in the SVZ. These results are contradictory to three studies that have reported a reduction in SVZ proliferation after DA ablation (Höglinger et al. 2004; O’Keeffe et al. 2009; Baker, Baker, and Hagg 2004). After closely comparing our experimental design, we identified a major difference in the survival time of the animal after lesion. While other studies assessed the neurogenesis level at four weeks post lesion, we waited nine weeks in order to ensure a chronically neurotransmitter-deficient environment. To verify if the change in proliferation observed in the previous studies, was a transient effect due to the short survival time, we reproduced our protocol, but sacrificed the animals at four weeks post-lesion. The analysis of proliferation in both SGZ and SVZ did not reveal a significance difference in the number of Ki67+ cells in DA lesion animals or NA lesion animals compared with controls. A more thorough literature analysis reveals that in fact the role of DA in SVZ neurogenesis is still controversial. Indeed, we found that DA was variously reported to increase SVZ neurogenesis in seven studies (Baker, Baker, and Hagg 2004; Van Kampen et al. 2004; Höglinger et al. 2004; Winner et al. 2006; Winner et al. 2009; Lao, Lu, and Chen 2013; O’Keeffe et al. 2009), reduce it in two studies (Liu et al. 2006; Aponso, Faull, and Connor 2008) and had no effect in three studies (Baker, Baker, and Hagg 2005; Milosevic et al. 2007; Van Den Berge et al. 2011). It is not clear why there is such a difference in observation. In those studies, different systems have been used to manipulate the DA system: toxins (6-OHDA and MPTP); and pharmacological agonists. However, the same toxin was shown to present different results in different studies, so this seems unlikely to be the only factor.
Table 4.1: Summary of studies that investigated the role of DA on SVZ proliferation and their conclusion. Studies in green have reported a positive effect of DA on proliferation, studies in red have reported a negative effect and studies in white have reported no effect of DA on SVZ proliferation.

<table>
<thead>
<tr>
<th>Study</th>
<th>Experimental design</th>
<th>Concluded role of DA on proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker et al. 2004</td>
<td>6-OHDA, mice</td>
<td>↑</td>
</tr>
<tr>
<td>Van Kampen et al. 2004</td>
<td>D₃ agonist, rats</td>
<td>↑</td>
</tr>
<tr>
<td>Hoglinger et al. 2004</td>
<td>MPTP, mice</td>
<td>↑</td>
</tr>
<tr>
<td>Baker et al. 2005</td>
<td>D₃ agonist, mice</td>
<td>No effect</td>
</tr>
<tr>
<td>Liu et al. 2006</td>
<td>6-OHDA, rats</td>
<td>↓</td>
</tr>
<tr>
<td>Winner et al. 2006</td>
<td>6-OHDA, rats</td>
<td>↑</td>
</tr>
<tr>
<td>Milosevic et al. 2007</td>
<td>D₂/D₃ agonist, <em>in vitro</em></td>
<td>No effect</td>
</tr>
<tr>
<td>Aponso et al. 2008</td>
<td>6-OHDA, rats</td>
<td>↓</td>
</tr>
<tr>
<td>Winner et al. 2009</td>
<td>DA agonist, rats</td>
<td>↑</td>
</tr>
<tr>
<td>O’Keefe et al. 2009</td>
<td>6-OHDA, rats</td>
<td>↑</td>
</tr>
<tr>
<td>Van Den Berge et al. 2011</td>
<td>MPTP, mice</td>
<td>No effect</td>
</tr>
<tr>
<td>Lao et al. 2013</td>
<td>D₃ agonist, mice</td>
<td>↑</td>
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It should also be noted that we found an ACh lesion had a limited effect on proliferation in the SGZ, while the number of progenitor cells was increased, they did not survive as shown by a similar number of BrdU⁺ cells in both ACh lesion and control group. However in the literature, ACh was reported to have an effect on proliferation, survival and overall neurogenesis in the SGZ (Cooper-Kuhn, Winkler, and Kuhn 2004; Mohapel et al. 2005).

The regulation of hippocampal neurogenesis by neurotransmitter input may well be a complex balance between multiple systems and the direct impact of one neurotransmitter system may be difficult to isolate from compensation from other
systems, including those not studied here such as 5HT. Indeed, there are a number of studies that show the impact of 5HT on hippocampal neurogenesis (Klempin et al. 2013; Benninghoff et al. 2010; Brezun and Daszuta 1999), although the serotonin system is not known to be a major site of neurodegeneration in PD.

Parkinson's disease is a multi-systems disorder, where many brain regions degenerate at the same time and it is a loss of this complex interaction between systems that leads to the different symptoms experienced by patients. Although we explored the idea that hippocampal neurogenesis may be involved, we suggest that DA and NA degeneration in PD might have a more direct impact on non-motor symptoms, such as dementia. Indeed, both NA and DA have been shown to be directly related to cognition (Bonito-Oliva et al. 2013; Bouret and Sara 2004; Aston-Jones and Bloom 1981). It can also be hypothesised that hippocampal neurogenesis in PD might be impaired due to the presence of α-synuclein in the hippocampus, which in terms leads to memory impairments observed in PD patients (Schlachetzki et al. 2016).

In conclusion, our data suggest that the reduction of hippocampal neurogenesis observed in PD is independent of direct DA and NA input to the hippocampus. In fact we recently reported also that the hippocampus lacks a direct DA input (Ermine et al. 2016). Therapeutic drugs to improve neurogenesis in PD should not only focus on DA or NA replacement, although such drugs might be beneficial for the non-motor symptoms associated with the DA and NA loss through other mechanisms.
4.6. References


Liu, Bing fang, Er jing Gao, Xian zhi Zeng, Man Ji, Qing Cai, Qiang Lu, Hui Yang, and Qun yuan Xu. 2006. “Proliferation of Neural Precursors in the Subventricular Zone after Chemical Lesions of the Nigrostriatal Pathway in Rat Brain.” *Brain Research* 1106 (1): 30–39.


Chapter 5

Initial characterisation of DBH-eGFP mice: a new tool for transplantation studies

5.1. Abstract

The Locus coeruleus (LC)/noradrenaline (NA) system is very important for normal cognitive functions, including arousal, attention, memory and stress control. Post-mortem studies have revealed that the LC degenerates in many diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Down syndrome and Korsakoff’s psychosis. Therefore, NA cell replacement therapies could be a beneficial treatment for these diseases. Previous studies have focused on developing a protocol for NA embryonic cell transplantation in the rat hippocampus. This protocol was extensively used to study seizures in a rat model of epilepsy. Furthermore, dopaminergic cell transplantation therapy in PD is a very promising treatment for patients. The basic science underlying its refinement has been greatly aided by the use of green fluorescent protein (GFP) reporter mouse for tyrosine hydroxylase (TH), the enzyme synthesising dopamine (DA), called TH-GFP reporter mouse. The use of reporter mice in transplantation studies presents the advantage of distinguishing the graft versus host cells and fibres, which allows a better appreciation of graft survival and graft integration in the host circuitry. In this study we aim at describing a new tool for transplantation: the DBH-eGFP mouse. This approach was used to optimise NA transplantation in rodents. We transplanted hindbrain dissections containing the developing LC from mouse embryos at embryonic days E12.5, E13.5 and E14.5 into hippocampus of adult mice. The cell preparation was performed with or without centrifugation and the host and donor tissues were strain matched or non-strain matched. This study is ongoing, however preliminary results showed that the most GFP+ fibres were observed in grafts from mice at E12.5 when the cells were not centrifuged and when the strains were matching. Developing and improving NA transplantation in rodents is a very critical step towards the development of NA cell replacement therapies that could be beneficial in humans suffering from PD or AD for example.
5.2. Introduction

The locus cœruleus is a nucleus located in the pontine tegmentum of the brainstem. Even though LC neurons contain different neurotransmitters including galanin and neuropeptide Y, they are most prominently known for being the major source of NA in the brain (Benarroch 2009; Moore and Bloom 1979). NA is a catecholamine and is synthesised from DA by the enzyme dopamine-β-hydroxylase (DBH). The LC/NA system is important for many brain functions, including: maintenance of attention (Mair et al. 2005; Bouret and Sara 2005), arousal via thalamocortical circuits (Aston-Jones and Bloom 1981), response to stress (Cassens et al. 1981; Cummings et al. 1983) and finally memory processing (Neuman and Harley 1983; Stanton and Sarvey 1985). The LC/NA system is impaired in many diseases including those involving memory deficit, like AD and Korsakoff’s psychosis (W J McEntee and Mair 1978; Adolfsson et al. 1979; Willian J. McEntee and Mair 1980; Zarow et al. 2003). It was shown in post-mortem studies that the LC was degenerating in AD brains and in pharmacology replacement studies that clonidine, an α-noradrenergic agonist, improves the memory deficit observed (Adolfsson et al. 1979; Willian J. McEntee and Mair 1980). But the LC/NA system is also impaired in brains of patients suffering from PD and Down syndrome, as shown by a major cell loss in LC observed in disease brains (German et al. 1992).

Various tools have been used in gain and loss of function approaches to study the noradrenergic system, including: 1) pharmacologic injection of a non-natural NA precursor L-Threo-dihydroxyphenylserine (L-threo-DOPS) and adrenergic agonists or antagonists; 2) Lesion of LC/NA, using DSP4 (Ross and Stenfors 2014) or DBH-saporin (Vadodaria and Jessberger 2014); 3) Knock-out mice: DBH -/-, noradrenaline transporter (NET) -/- (Perona, Waters, and Hall 2008; Weinshenker et al. 2002); 4) in vitro studies of brain slices (Kuo et al. 2016) and cell culture of LC neurons (Masuko et al. 1986). Additionally, Bjorklund et al. (1979) developed a cell transplantation paradigm, where LC from embryonic rats are implanted in adult rat hippocampus previously deprived of noradrenergic inputs (Anders Bjorklund, Segal, and Stenevi 1979). They observed that the cells transplanted from the LC of rats at embryonic day E16-E17, was successfully reinnervating the hippocampus at three to six months post
transplantation and the innervation was persisting at fourteen months. The same group further optimised the protocol for the embryonic noradrenergic cell preparation, by testing for example the use of trypsin, which is a proteolytic enzyme (A Bjorklund, Nornes, and Gage 1986). Other studies used their protocol, to graft NA neurons in the hippocampus of a rat model of epilepsy, in order to rescue the hypersensibility to hippocampal kindling observed in the disease model (Barry et al. 1987; Clough et al. 1994; J. Bengzon et al. 1990; Johan Bengzon et al. 1991; Kokaia et al. 1994). Furthermore, NA embryonic transplantation in the hippocampus was used to study the deficient memory performance related to aging and successfully improved the performance to a learning task (Collier, Gash, and Sladek 1988). Alternatively, the embryonic neurons from the LC were transplanted in transected rat spinal cord to restore the spinal cord functional activity after an injury (Commissiong 1984). One group performed NA cell transplantation in mice hippocampus, and reported that glial cell lined derived neurotrophic factor (GDNF) was necessary for survival of the NA inputs to the hippocampus (Quintero et al. 2004).

Here we aim at improving the characterisation of NA cells transplanted and we describe DBH-enhanced green fluorescent protein (eGFP) reporter mice as a new tool that may be particularly useful for transplantation studies using embryonic LC.

Cell replacement therapy for Parkinson’s disease is an experimental treatment that has shown promise for some patients (Kordover et al. 1995; Hagell et al. 1999; Piccini et al. 1999). Some of the basic science behind the approach has been greatly aided by the use of reporter mice for DA neurons, particularly the TH-GFP mice (Sawamoto et al. 2001). In 1997, Okabe et al. created a “green mice” by introducing the green fluorescent protein (GFP) in the genome of mice, to express ubiquitinously the GFP gene in the all organism. GFP is found in the jelly fish *aequorea Victoria*. Since then, many different GFP mice have been created to express GFP in specific population types, by introduction of GFP under the control of a specific promoter, including TH-GFP mice. TH-GFP mice were used successfully for DA neurons grafting in animal models of DA depletion (L. H. Thompson et al. 2009; J. Kauhausen, Thompson, and Parish 2013; Neto et al. 2012). This technique has allowed the detailed description of DA neuron graft features, by characterising neuronal survival, anatomical integration
and functional connectivity with the host tissue (Tønnesen et al. 2011; L. Thompson 2005; Denham et al. 2012; Somaa et al. 2015). On-going pre-clinical studies using such tools are important for identifying critical parameters for successful transplantation, including donor age and neuronal subpopulation identity. Notable advantages of reporter mice expressing GFP under specific reporters, are to identify and to create a pure population for transplantation thanks to fluorescence-activated cell sorting (FACS) technology (Bye et al. 2015; Sawamoto et al. 2001) and also unambiguously identify the grafted cells after transplantation within the host and study their integration and projections (L. H. Thompson et al. 2009; J. A. Kauhausen, Thompson, and Parish 2015).

In this preliminary study we aim at describing a new GFP reporter mouse, DBH-eGFP mice, where NA neurons selectively express GFP. Reconstruction of the NA system through transplantation experiments in rodents would be a very critical step towards the identification of parameters for successful NA cell therapy in humans. Noradrenergic cell replacement therapy could be a very beneficial treatment for a wide range of diseases and age dependent cognitive deficits, such as AD and PD.

### 5.3. Materials and methods

#### 5.3.1. Animals

The experimental design and procedures were approved by the Florey Institute for Neuroscience and Mental Health. All animals were housed in individually ventilated cages under a 12h light/dark cycle with ad libitum access to food and water.

In this study we used transgenic mice, DBH-eGFP mice (embryos and adults), obtained from the Mutant Mouse Regional Resource Center (MMRRC) as well as C57/Bl6 and Swiss wild type mice. The DBH-eGFP mice, also called Tg(Dbh-EGFP)Fl99Gsat, was generated in the Gene expression nervous system atlas (GENSAT) bacterial artificial chromosomes (BAC) transgenic project (Gong et al. 2003). The eGFP reporter gene, followed by a polyadenylation sequence, was inserted at the first exon
of the DBH gene into a BAC clone. The resulting BAC DNA was injected in oocytes of FVB/Swiss mice.

5.3.2. Genotyping

The genotype for colony maintenance was performed as described in Chapter 2, section 2.8. Briefly the genotypes of littermates from DBH-eGFP males x C57/Bl6 or Swiss females were obtained by polymerase chain reaction (PCR) protocol for the presence of the eGFP transgenic gene. The primers used were: 1) Forward: 5’-AATGGCAGAGTGGGGTTGGG - 3’ and 2) Reverse: 5’- TAGCGGCTGAAGCACTGCA - 3’.

5.3.3. Embryo dissection and transplantation

The embryos were obtained by time-mating of DBH-eGFP male with C57/Bl6 or Swiss female mice. The DBH-eGFP embryos were harvested on embryonic days 12.5, 13.5 and 14.5 and the brains were dissected out. Guided by the GFP+ NA neurons, the LC was dissected as described in Chapter 2 section 2.2. Approximately six to eight tissue pieces were pooled together and the cell preparation was prepared as described in Chapter 2, section 2.2. We did not include trypsin based on earlier reports that showed that LC neurons were sensitive to trypsin treatment (A Bjorklund, Nornes, and Gage 1986). To further characterise the LC neurons sensitivity, we also compared with and without a centrifugation step. We also compared the graft success for strain matching and strain non-matching of the host and donor tissues.

The cells were stored on ice and 1.5µl of the cell suspension were transplanted into the dorsal hippocampus of intact adult C57/Bl6 and Swiss mice, at the following coordinates: 1.75mm posterior from bregma, 2.3mm lateral from bregma and 1.8mm below the dura (see Chapter 2, section 2.3.2.). The total number of animal grafted was 22 mice.
5.3.4. Tissue processing and immunohistochemistry

Grafted animals were sacrificed two weeks after transplantation by transcardial perfusion as described in Chapter 2 section 2.5.

DBH-eGFP embryonic brains were obtained from E13.5, were post fixed for 3 hours in 4%PFA, and embedded in OCT Compound (Tissue-Tek), before being stored at -20°C. They were cut sagittally using a cryostat (Leica, CM 1950) at 16 µm, in a 1:10 series. Adult DBH-eGFP brains were cut coronally, horizontally and sagittally using a microtome at 40µm, in a 1:12 series (Leica, Wetzlar, Germany).

Immunohistochemistry on free-floating and slide mounted sections were performed as described in Chapter 2 section 2.6. The primary antibodies used were: rabbit-TH (Pelfreeze, 1:1000) and rabbit-GFP (Abcam, AB290, 1:1000). The secondary antibody used was a biotinylated anti-rabbit (Jackson ImmunoResearch, 1:400). Detection of the antibody-complex was via precipitation of the DAB chromogen as described in Chapter 2, 2.6.

5.4. Results

5.4.1. DBH-eGFP in the embryonic brain

Embryonic mice were harvested at embryonic days E12.5, E13.5 and E14.5. The embryonic brain and the mesencephalon were opened dorsally to reveal the GFP fluorescence, which helped guiding the LC dissection (Figure 5.1 A-C). The GFP fluorescence at embryonic day E12.5 was very limited (data not shown), however robust GFP staining was observed at embryonic days E13.5 and E14.5 (Figure 5.1 C).

Immunohistochemistry for GFP showed that the DBH expression has reported by the GFP is seen at embryonic day E13.5 (Fig.5.1 D-E). The GFP+ cells and fibres are mainly observed in the tissue deriving from rhombomere 1, which gives rise to most NA neurons from the LC and subcoeruleus dorsal. Few GFP+ fibres were also seen in the tissue deriving from rhombomeres 3 and 4 which gives rise to the ventral subcoeruleus in the pons.
Figure 5.1. Dissection of LC from embryonic mice at day E13.5 and representation of the GFP+ cells in the LC of a sagittal section at E13.5. A) Dashed line indicates the first cut made through the dorsal midline. B) The boxed area indicates a top-down view of the approximate location of the LC. C) Overlay of bright-field and GFP+ cells illustrating the LC. Each LC piece was dissected separately. D) Immunohistochemistry for GFP shows the location of NA neurons discretely located in the hindbrain at the level of the tissue deriving from rhombomere 1 mainly, but also in the tissue deriving from rhombomeres 3 and 4. Boxed area is shown as enlarged view of the rhombomere region (E). The immunohistochemical analysis was completed in n=2 embryos and they demonstrated a similar pattern of staining. Scale bars: D 500 µm; E 100 µm. Abbreviations: GFP, green fluorescent protein; LC, locus coeruleus.

5.4.2. DBH-eGFP in the adult brain.

To validate that the GFP is only expressed in the LC, we used chromogenic staining for GFP in the adult brain in the sagittal, horizontal and coronal plans (Figure 5.2 A-C). The immunohistochemistry for GFP in adult transgenic DBH-eGFP mice, revealed a single and discrete staining in the LC, located next to the fourth ventricle.
We further validated the phenotypic identity of the GFP+ cells, by looking at the expression of TH in the LC and in the midbrain. The double fluorescent staining for GFP and TH in the LC reveals that all the GFP+ cells were also TH+. However, it should be noted that while a few GFP+/TH+ cells had an intense GFP staining (Figure 5.2 E'-E''), most were expressing lower GFP fluorescence (Figure 5.2 F'-F''). Furthermore, the difference in GFP fluorescence intensity made difficult the phenotypic characterisation of the TH+ cells in the LC; however, the immunoanalysis suggests that the majority of the TH+ cells were also GFP+. On the contrary, in the midbrain all the cells were GFP-/TH+ (Figure 5.2 G). GFP+/TH- cells were observed around the rostral linear nucleus (Figure 5.2 H'-H'').

**Figure 5.2: Representation of DBH-eGFP adult mice brain.** Immunohistochemistry for GFP shows the NA neurons in the LC on a sagittal (A), horizontal (B) and coronal plan (C). Fluorescent immunohistochemistry for TH and GFP shows that the GFP+ cells are also TH+ in the LC (D-F''). E'-E'' are high magnification images of two bright GFP+ cells, also TH+. F'-F'' represents other GFP+/TH+ cells where the GFP staining was less intense. Arrow heads are showing GFP+/TH+ cells. The double fluorescence staining for GFP and TH in the midbrain shows the absence of GFP+/TH+ (G-H''). H'-H'' are high magnification images of the rostral linear nucleus region, showing GFP+/TH- cells (white arrows) and GFP-/TH+ cells. The immunohistochemical analysis was completed in n=2 mice and they demonstrated a similar pattern of staining. **Scale bars:** A-C and G, 500 µm; D-F'', 100 µm and H'-H'', 200 µm. **Abbreviation:** GFP, green fluorescent protein; LC, Locus caeruleus; TH, Tyrosine hydroxylase.
5.4.3. Pilot studies on transplantation of embryonic LC from DBH-eGFP mice.

Cell suspension obtained from DBH-eGFP+ embryos were transplanted into adult C57/Bl6 or Swiss mice. The outcome of transplantation was assessed histologically two weeks later.

Grafts obtained from E13.5 and E14.5 embryos were not observed in the hippocampus, when the cell preparation was performed with a centrifugation step (n=8; data not shown). However, when the cells were only acutely dissociated to obtain a suspension with minimal handling, we observed a few GFP+ cells and associated GFP+ fibres in the hippocampus of C57/Bl6 mice, with E13.5 graft (Figure 5.3 A, B). When the strains between the female donor and the host were matching, i.e. both C57/Bl6 mice, we observed a higher concentration of GFP+ fibres, suggesting that the strain matching is important for the graft survival (Figure 5.3 C, D). The graft from E12.5 embryos expressed a slightly higher density of GFP+ fibres, compared with graft from E13.5 embryos, however it is hard to draw a definitive conclusion as we have only one animal per group. This work is very much on-going.

Figure 5.3. Hippocampal cell transplantation of DBH-eGFP+ cells. Staining for GFP reveals few GFP+ fibres (A) and one GFP+ cell (B) after transplantation of DBH-eGFP cells from E13.5 embryos with a Swiss background, into the hippocampus of C57/Bl6 mice. Transplantation of DBH-eGFP cells from E12.5 (C) and E13.5 (D) embryos with a C57/Bl6 background into the hippocampus of C57/Bl6 mice shows the survival of slightly more GFP+ fibres. A’, A”, B’, C’ and D’ are high magnification images of the corresponding boxed area. Scale bars: A-D 100 µm. Abbreviations: GFP, green fluorescent protein.
5.5. Discussion

This preliminary study provides a first promising validation and description of a new tool to study NA cell replacement therapy. The ultimate goal is to create a standardised protocol for NA cell preparations for grafting and to study the potential therapeutic benefits for cell-replacement therapy, for example in PD. While the TH-transgenic mouse could be used to study NA cell replacement therapy, the DBH transgenic model provides a greater specificity for neuronal phenotypes.

Characterisation of the GFP pattern in the embryonic brain (Fig. 5.1) and the adult brain (Fig 5.2) have allowed validation of the DBH-eGFP transgene. Indeed, the GFP+ cells were observed predominantly in the LC in both embryonic and adult brains (Fig 5.1 C-E). Furthermore, in the adult brain a double fluorescent analysis has shown that virtually all GFP+ cells were also TH+ in the LC, but importantly the midbrain TH+ cells were GFP-. In addition, we only observed a limited number of GFP+ cells in the LC at E13.5, which can be explain by the fact that DBH is a mature marker, thus the NA progenitor cells might only express the DBH and GFP later in the development stages.

Our data suggest that the GFP+ cells are sensitive to centrifugation, indeed the cell survival measured at the end of the cell preparation and the graft survival was very poor. This was further confirmed when we observed GFP+ fibres in the hippocampus after transplantation of cells that were not centrifuged (Fig.5.3 A, B). This observation confirms an earlier report by Bjorklund et al. (1986), which omitted trypsin and also suggested ‘sensitivity’ of the LC cells (A Bjorklund, Nornes, and Gage 1986).

Our results also highlight the importance of strain matching between donor and host. When the donor tissue was issued from a C57/Bl6 female and the host was also C57/Bl6, we saw a greater GFP+ fibre density in the hippocampus. This is in line with recent findings by Somaa et al. (manuscript in preparation) showing that strain matching between the graft and host had a significant impact on graft survival. Somaa et al. showed that the host immune response was higher when the donor and host were mismatched strains and was therefore detrimental for graft survival. In addition,
a matching donor study is more relevant and comparable to therapeutical studies where major histocompatibility complex classes are matching.

We tested three different embryo ages for the donor tissue, E12.5, E13.5 and E14.5. We observed GFP+ fibres in the hippocampus for E12.5 and E13.5, however since we were testing and adjusting different parameters including donor-host strain-matching, and because we have a very limited number of animals grafted, our results are not sufficient to conclude on the best donor age at this stage. The early indication, however, is that embryonic day E12.5 might be the best age for graft survival as we observe more fibres in the hippocampus (Fig. 5.3 C, D).

In the present study we have been grafting the NA cells in the hippocampus naturally rich in NA inputs. However, it was suggested that the host environment plays a major role in the survival of the transplanted cells. Studies have shown that transplanting stem cells in a denervated environment, such as the striatum, increases the survival and migration of the transplanted cells, compared to the transplantation in an intact environment (Behrstock et al. 2008; Watts and Dunnett 1998; Gage and Bjorklund 1986). In addition, the successful NA grafting in rats was performed in a noradrenaline-deprived environment (Anders Bjorklund, Segal, and Stenevi 1979; A Bjorklund, Nornes, and Gage 1986; Barry et al. 1987; Clough et al. 1994; J. Bengzon et al. 1990; Johan Bengzon et al. 1991). Therefore, transplanting the DBH-eGFP+ cells after induction of a NA lesion in the LC, might also improve the graft survival in the hippocampus.

In summary, while these studies are preliminary, the DBH-eGFP reporter mouse appears to be a promising tool to study NA transplantation, for being able to identify and characterise the capacity of grafted NA cells to survive and integrate and to distinguish fibre patterns from host fibres. Furthermore, a standard protocol for cell preparation should be created and specify the best embryonic age for the donor tissue. The aim of the on-going work is to optimise the number of surviving NA neurons after transplantation. One strategy that may allow for enrichment of the NA contribution to the final grafts is cell-sorting. The use of FACS technology, which uses fluorescent light to separate different cell populations, would allow the purification of the GFP+ NA progenitors, although given DBH marks relatively mature cells, it is possible that
the cell that survives transplantation is in fact a DBH- progenitor. In addition, the fluorescent reporter affords the possibility to assess the functional integration of the grafted cells in host circuitry, for example by using electrophysiology. Finally, the cognitive benefits of the graft would be interesting to assess and compare to anatomical integration based on GFP+ fibre patterns, for example through Morris water maze or touch-screen tests. If the NA graft is successful, it may be an interesting strategy to restore cognitive levels reduced in PD.
5.6. References


Chapter 6

Striatal projection neurons are not replaced after ischemic damage

6.1. Abstract

Neurogenesis from the subventricular zone (SVZ) is increased after striatal ischemia in both adult and neonatal rats. Under normal conditions the adult SVZ generates interneurons that migrate to the olfactory bulb, while the neonatal SVZ generates both interneurons and medium spiny neurons, which is the main neuronal subtype affected after striatal ischemia. In addition, it has been reported that the adult brain is capable of redirecting SVZ progenitors to the injury site and also reprogram their fate to become medium spiny neurons. These results suggested an attempt of the brain to repair itself and stimulated research into successful ‘endogenous cell therapies’. However, a few years later conflicting results reported that the adult brain was in fact not capable of generating medium spiny neurons after ischemia, thus reconsidering the real potential of endogenous cell therapies. In this study, we aimed at comparing the adult and neonatal brain after striatal ischemia, by investigating proliferation, neurogenesis levels and progenitors’ phenotypes. The results showed that while the neonatal brain was producing medium spiny neurons, the rate of production was not increased by the injury. In addition, the results showed that the adult brain only generates interneurons in response to injury. This suggests that while endogenous cell replacement therapies are attractive, many factors including rate of neurogenesis and neuronal fate, need to be manipulated in order to have a successful treatment for brain injury such as stroke.
6.2. Introduction

Stroke is one of the main causes of death and a leading cause of disability in Australia. With an aging population the incidence of stroke is increasing and the number of people living with the effects of stroke is thought to reach 709,000 by 2032 according to the Australian Stroke Foundation (www.strokefoundation.com.au/about-stroke/facts-and-figures-about-stroke). Stroke can be classified in two categories: haemorrhagic stroke, which happens in 20% of cases and is caused by rupture of a cerebral blood vessel; and ischemic stroke, which happens in 80% of cases and is due to an occlusion of a cerebral artery (Thrift et al. 2001; Donnan et al. 2008).

Treatments for stroke are still very limited, however, the discovery of the persistence of neurogenesis in the adult brain has opened up new avenues for brain therapies after injury such as stroke. Indeed, it is now well established that neurogenesis persists in adulthood and two regions have been identified as neurogenic niches: the sub-granular zone in the dentate gyrus and the SVZ surrounding the lateral ventricle (Gage 2000). Various studies have shown that following injury to the striatum, proliferating neuroblasts in the SVZ are able to migrate to the injury site and mature into neurons (Arvidsson et al. 2002; Ong et al. 2005; Parent et al. 2002; Jin et al. 2001; Jin et al. 2003; Jin et al. 2006). Furthermore, Arvidsson et al. 2002 and Parent et al. 2002, have characterised the phenotype of the new neurons and identified neurons positive for the protein, dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000 (Darpp32), which is a marker for medium spiny neurons, also called striatal projection neurons, which are the main neurons injured by ischemia. Their results suggested that the adult brain was attempting to repair itself, with however a poor survival rate of the new neurons. Their results stimulated the search for strategies to increase the neurogenesis rate to levels that could be therapeutically relevant. Specifically, studies have focused on using growth factors such as BDNF (Benraiss et al. 2012), GDNF (Kobayashi et al. 2006) or meteorin (J. L. Wright et al. 2016). More recently, Liu et al. 2009 have contradicted the finding that the adult brain can generate medium spiny neurons after striatal injury. They suggested on the contrary that the injury does not influence the predetermined differentiation of neuroblasts, which nonetheless can migrate to the injury site and
give rise to interneurons (Liu et al. 2009). These results have proposed that the capacity of the adult brain for self-repair is more limited than we originally thought.

We considered that, while the adult brain may not be capable of generating new striatal projection neurons, it may be possible in the early postnatal brain. A recent study by Wright et al. 2013, has shown the capacity of the neonatal rat brain to generate not only interneurons but also striatal projection neurons until five days after birth. Thus we aimed at characterising the capacity of the neonatal brain to respond to a striatal injury by increasing the number of striatal projection neurons generated. We used the vasoconstrictor endothelin-1 (ET-1), to induce striatal ischemia in neonatal rats, at postnatal day 0 and as a comparison in adult rats.

In other attempts to identify possible factors that can negatively affect the injury-induced neurogenesis in the adult brain, we tested the hypothesis that the immune system and inflammation might have a negative impact of striatal neurogenesis following injury and describe results from a pilot experiment involving ischemic injury using immuno-compromised athymic, rats - also called 'nude' rats.

6.3. Materials and methods

6.3.1. Animals

Adult female Sprague Dawley rats, adult female athymic rats and neonatal 1 day old Sprague Dawley rats were used in this study. The experimental design and procedures were approved by the Florey Institute for Neuroscience and Mental Health. All animals were housed in individually ventilated cages under a 12h light/dark cycle with ad libitum access to food and water. The experimental timeline is represented in Figure 6.1.
Figure 6.1: Experimental design. At day 0 all the groups underwent stereotaxic surgery for injection of ET-1 or saline in the striatum. One day after surgery, all the groups were injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU) for 7 days, once daily at 100mg/kg for the adult groups (A-B) and twice daily at 50mg/kg for the neonate group (C). The animals were sacrificed at 4 weeks (A) or 12 weeks after surgery (B-C).

6.3.2. Stereotaxic lesion

Striatal ischemia was induced by ET-1 injection to the lateral part of the anterior head of the striatum. The stereotaxic procedure is described in methods Chapter 2, section 2.3.

Prior to the surgery the adult animals were anaesthetised with isoflurane (5% at 1L/min) and placed in a stereotaxic frame (Kopf, Germany) where the anaesthesia was maintained for the duration of the surgery (2% at 1L/min). The neonatal rats were anaesthetised through hypothermia, induced by placing the pup in ice for 5 minutes. The pups were placed in a Cunningham adaptor stage (Stoelting, Germany) fitted onto the stereotaxic frame, and the anaesthesia was maintained with dry ice added to absolute ethanol held in the reservoir of the Cunningham adaptor.

The ET-1 toxin for the lesion animals or saline for the controls was injected in the striatum at the following coordinates: 0.5 mm rostral and 3.0 mm lateral to bregma and 5.0 mm below the surface of the brain for the adult rats, and 0.7 mm rostral and 2.3 mm lateral to bregma and 2.9 mm below the surface of the brain for the neonatal rats.
The animals were sacrificed at either four or twelve weeks post-lesion. In the first study, we investigated the adult’s capacity to respond to injury and chose 4 weeks as the survival time. However, with the negative results observed for the “adult 4w”, we hypothesised that 4 weeks was not sufficient to observe the differentiation of newborn neurons into Darpp32+ cells following injury, thus we added the adult 12 week group. In addition, in the second study we included the neonatal group, which was also sacrificed at 12 weeks for the same reason.

6.3.3. BrdU treatment

To label the dividing cells, we used the marker 5-bromo-2′-deoxyuridine (BrdU). One day after surgery, BrdU was injected intraperitoneally at 100mg/kg once a day for seven days for the adult groups and at 50mg/kg, twice daily for 7 days for the neonatal groups (Figure 6.1). The dosage was the same between the adult and neonatal groups, however the regime was adapted to each group to allow for injection volumes in the smaller animals.

The BrdU preparation is further detailed in Chapter 2, section 2.4.

6.3.4. Tissue collection and immunohistochemistry

Animals were sacrificed by terminal dose of pentobarbitone (100mg/kg; Virbac, Peakhurst, Australia). They were further transcardially perfused with paraformaldehyde media (PFA, 4% in 0.4M phosphate buffer with 0.2% picric acid). The brains were collected and additionally post-fixed in PFA media for 2 hours, followed by equilibration in 20% sucrose PBS solution for 1-2 days. The brains were snap frozen with dry ice to collect coronal sections of 40 µm using a freezing-microtome (Leica, Wetzlar, Germany) - see Chapter 2, section 2.5.

Free-floating immunohistochemistry was performed on a 1:12 series for chromogenic staining of BrdU and fluorescent staining of BrdU, NeuN, Darpp32 and Calretinin (CR), as described in Chapter 2, section 2.6. Tissue stained for BrdU was pre-treated by incubation in Omnipur deionized formamide (Merck Millipore) at 65°C.
for 2 hours, in 2M HCL at 27°C for 30 minutes and in Borate buffer at room temperature for 20 minutes. Primary antibodies and dilutions used were: sheep anti-BrdU (Exalpha, A205P, 1:1000); mouse anti-NeuN (Millipore, MAB377, 1:1000); rabbit anti-CR (SWANT # CG1, 1:4000) and rabbit anti-Darpp32 (Millipore, AB10518, 1:500). Secondary antibodies and dilutions used were: anti-sheep and anti-rabbit conjugated Dylight Fluorophores 488 and 647 (Jackson ImmunoResearch,1:200) for fluorescent staining and anti-sheep biotin conjugated (Jackson ImmunoResearch,1:400) for chromogenic staining. Fluorescent staining for NeuN was amplified using the secondary antibody anti-mouse biotin-conjugated and streptavidine-550 (Abcam, 134348, 1:400). The sections used for BrdU density quantifications were counter stained with neutral red, to allow better identification of the physical limits of the striatum.

6.3.5. Analysis

Fluorescent microscopic analysis and images were performed using a Zeiss Meta confocal microscope (LSM 780), chromogenic microscopic analyses were performed using an Olympus Bx51 microscope and chromogenic images were captured using a Leica DM6000 microscope.

The number of BrdU+ cells were estimated using a stereological counting technique, as described in Chapter 2, section 2.9.1. Briefly, we counted the BrdU+ cells of four striatal consecutive sections (approximately located at +1.70 mm, +1.20 mm, +0.70 mm, +0.20 mm relative to bregma) using the optical dissector from the software Stereoinvestigator V7. We randomly assessed the number of BrdU+ cells at 100x magnification, using a sampling grid of 150x200 µm and counting frame of 40x40 µm. The Cavalieri principle was used to estimate the BrdU density per unit of tissue in the striatum.

To determine the number of BrdU+/NeuN+; BrdU+/NeuN+/Darpp32+; BrdU+/CR+ and BrdU+/CR+/NeuN+ cells, photomontages of the striatum on the X, Y, Z planes were acquired using a Zeiss Meta confocal microscope, as described in Chapter 2, section 2.9.2.2. The BrdU+ cells were carefully inspected using Zen lite
software for NeuN, Darpp32 and/or CR identity, which was further confirmed at 40x and 63x magnification. The frequency of each cell type was determined and density was then estimated using Cavalieri’s principle.

Statistical analysis was performed using one-way ANOVA to assess the difference between ET-1 lesion 1, ET-1 lesion 2 and saline subgroups of the “adult 4w” group, in the densities of BrdU+ cells, BrdU+/NeuN+ cells, BrdU+/CR+ cells and BrdU+/CR+/NeuN+ cells. For the “adult 12w” group, an unpaired t-test was used to analysis the difference between the ET-1 lesion and saline subgroups in the density of BrdU+ cells, BrdU+/NeuN+ cells, BrdU+/NeuN+/Darpp32+ cells and BrdU+/CR+/NeuN+ cells. An unpaired t-test with Welch’s correction was used to assess the difference between the “nude 4w” and the “SD 4w” in BrdU+/NeuN+, BrdU+/CR+ and BrdU+/CR+/NeuN+ densities; the difference in BrdU+/CR+ density between the ET-1 lesion and saline subgroups of both “adult 12w” and “neonate 12w” groups; and the difference in BrdU+/CR+/NeuN+ density between the ET-1 lesion and saline of “neonate 12w” group.

6.4. Results

In this study, we defined four main groups according to the age of the animals and the survival time for analysis:

1) Adult rats at 4 weeks kill point, labelled “Adult 4w”, which contains 3 subgroups, a control group injected with saline, and two ET-1 lesion groups. The two lesion groups were generated as two separate experimental cohorts, with the second group being established to increase animal numbers. They are labelled “adult 4w_control” (n= 4 rats), “adult 4w_ET-1_1” (n= 6 rats) and “adult 4w_ET-1_2” (n= 4 rats).

2) Adult rats killed at 12 weeks time point, labelled “Adult 12w”, which contains a control (“adult 12w_control”, n= 4 rats) and an ET-1 group (“adult 12w_ET-1”, n= 8 rats). This group was created in response to negative results from the Adult
4w experiments with the view that failure to detect new Darpp32+ neurons may be due to insufficient time for terminal differentiation.

3) Neonatal rats killed at 12 weeks time point, labelled “Neonate 12w”, which contains a control (“neonate 12w_control”, n= 7 rats) and a ET-1 lesion group (“neonate 12w_ET-1”, n= 6 rats).

4) Athymic rats, called “adult 4w_ET-1_nude” (n= 6 rats).

6.4.1. ET-1 delivery to the striatum induces ischemic striatal injury.

To induce striatal ischemia, we injected the vasoconstrictor ET-1 in the striatum and we assessed the extent of the injury by immunostaining for the neuronal marker, NeuN. Figure 6.2 shows representative sections illustrating injuries for all the groups. While all the ET-1 lesion groups express a reduction of NeuN staining in the striatum, it is important to note that the extent of the injury was different between the groups. The two “adult 4w” lesion groups have a very different injury pattern. The first group called “adult 4w_ET1_1” presented a robust injury incorporating large areas of the striatum and the adjacent cortex, as shown by the absence of NeuN staining in these regions (Figure 6.2 B). In comparison, the second group, labelled “adult 4w_ET1_2” present a more discrete injury, localised in the centre of the striatum and extending to the overlying cortex (Figure 6.2 C). This injury pattern is similar to the injury induced in the “adult 12w” groups, as shown on Figure 6.2 E. The “neonate 12w_ET-1” group present a very different injury pattern, the NeuN staining is not reduced in the striatum or in the cortex, however the lateral ventricle is enlarged and the striatum is approximately half of the size of the control group (Figure 6.2 F, G).
Figure 6.2. ET-1 injection induces striatal ischemia in adult and neonatal SD rats. Immunohistochemical detection of the neuronal marker NeuN shows a reduction of cell labelled in the striatum and adjacent cortex for the “adult 4w_ET1_1” (B), in the striatum and above cortex for the “adult 4w_ET1_2” (C), compared to the control group “adult 4w_control” (A). Twelve weeks after the ET-1 delivery, the NeuN staining reveals a discrete reduction of NeuN cells marked in the striatum of “adult 12w_ET1” (E), and no change in the NeuN staining in the striatum of “neonate 12w_ET1” but enlarged ventricle and reduced size of the striatum (G), compared with their control (D) and (F) respectively. The immunohistochemical analysis was completed in n= 4-8 rats and all rats demonstrated a similar pattern of staining. Scale bars: A-G, 500 µm.
6.4.2. Estimation of BrdU density shows an increase in proliferation in the striatum twelve weeks post lesion in adult and neonatal brain, but not four weeks post ischemia.

The proliferation and the phenotypic fate of the new born cells in the SVZ were assessed via the thymidine-analogue BrdU, which was injected one day post-surgery for seven days. The number of BrdU+ cells in the striatum was estimated using stereology at four and twelve weeks post ET-1 injection. As shown in Figure 6.3 K we quantified BrdU throughout the striatum but excluded the damaged region where we found a high degree of non-specific signal associated with immunohistochemical labelling procedures. The stereological analysis in the striatum of “adult 4w” groups did not show any statistical difference between the control and the two lesion groups (ET-1_1: mean ± SEM = 6561.822 ± 1639.567 cells/mm³, n = 5; ET-1_2: mean ± SEM = 5510.665 ± 1741.456 cells/mm³, n = 4; Control: mean ± SEM = 2098.312 ± 247.764 cells/mm³, n= 3) (Figure 6.3 A-D). It should however be noted that the ET-1 lesion group present 2-3 times more BrdU+ cells compared with the control despite no statistical significance. In contrast the estimation of BrdU+ cells in the striatum of “adult 12w” reveals a significant increase, by almost two fold, of the BrdU+ cell density compared with the control group (ET-1: mean ± SEM = 2350 ± 230.8 cells/mm³, n=6; Control: mean ± SEM = 1447 ± 173 cells/mm³ n=3) (Figure 6.3 E-G). Similarly, in the neonatal group an ET-1 lesion robustly increases the density of BrdU+ cells in the striatum (ET-1: mean ± SEM = 12487 ± 480.1 cells/mm³, n= 5; Control: mean ± SEM = 9069 ± 472.1 cells/mm³ n=7) (Figure 6.3 H-J). Interestingly, the “neontate 12w” group presents almost ten fold more BrdU cells compared with the “adult 4w” and “adult 12w”.

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Figure 6.3. Proliferation in the SVZ is increased in the adult and neonatal brain twelve weeks after injection of ET-1 in the striatum, but remains unchanged four weeks post injection. Immunohistochemical analysis of BrdU allowed estimation of total number and density of BrdU+ cells in the striatum. Stereological analysis of BrdU staining in the striatum of “adult 4w_control” (A), “adult 4w_ET-1_1” (B) and “adult 4w_ET-1_2” (C) did not show any difference in BrdU+ cell density between the groups (D). Immunohistochemistry for BrdU in the striatum of “adult 12w_control” (E) and “adult 12w_ET-1” (F) reveals an increase of proliferation after ET-1 lesion (G). Similarly, BrdU staining in the striatum of “neonate 12w_control” (I) and “neonate 12w_ET-1” shows a significant increase in the density of BrdU+ cells after lesion (J). K, is a representation of the striatal delineation used for the stereological counts, where the red region represents the region included for the counting. The striatum was delineated using the corpus callosum dorsally and laterally, and the lateral ventricle medially. The ventral limits were defined using the distance X/2, where X is the distance between the midline and the lateral ventricle. It should be noted that the injury site itself, defined by accumulation of BrdU+ cells, was excluded. The immunohistochemical analysis was completed in n= 4-8 rats and all rats demonstrated a similar pattern of staining. Statistical analysis: (D) one-way ANOVA F_{2,9} = 1.908, P = 0.2037; (G) unpaired t-test control, P = 0.0389; (J) unpaired t-test control, P = 0.0006; error bars= standard deviation. Scale bars: A-C, E-F and H-I, 100 µm. Abbreviations: Acc, Nucleus accumbens; BrdU, 5-bromo-2’-deoxyuridine; CC, Corpus calosum; Cpu, Striatum.
6.4.3. **Striatal neurogenesis is unchanged after ET-1 lesion in the striatum of adult or neonatal rats.**

To determine the effect of striatal ischemia in the adult and neonatal brain on striatal neurogenesis, we used the mature neuronal marker NeuN and the proliferation marker BrdU to identify the number of newborn neurons in the striatum. The number of BrdU+/NeuN+ cells was counted manually in three striatal sections at a 1:12 interval between 1.7 mm and 0.7 mm relative to Bregma and the density of BrdU+/NeuN+ cells was estimated in the all striatum using Cavalieri’s principle.

A representation of the location of BrdU+/NeuN+ cells in the striatum for the ischemia groups is presented Figure 6.4 A-C. The double positive cells in both adult groups were found mainly in the dorsal part of the striatum, however a pattern could not be clearly identified due to the limited number of cells. Indeed, the density of BrdU+/NeuN+ cells in the striatum of adult brains were very low, they were ranging from 0 to 3 cells/section which equated to 0 to 4 double positive cells per mm³ (Figure 6.3 D, E). However, in the neonatal brain the density of BrdU+/NeuN+ cells was greater and was ranging from 200 to 700 cells per mm³ (Figure 6.4 F). The double positive cells were found throughout the striatum, with a higher concentration of cells located in the ventral part of the lateral ventricle (Figure 6.4 C). Statistical analysis did not reveal a difference in the density of double positive cells between the groups of “adult 4w” (ET-1_1: mean ± SEM = 1.896 ± 0.891 cells/mm³, n= 5; ET-1_2: mean ± SEM = 0.1718 ± 0.17 cells/mm³, n= 4; Control: mean ± SEM = 0.3693 ± 0.214 cells/mm³, n= 4) (Figure 6.4 D) or between the groups of “adult 12w” (ET-1: mean ± SEM = 1.073 ± 0.7059 cells/mm³, n= 8; Control: mean ± SEM = 0.5294 ± 0.3332 cells/mm³ n=4) (Figure 6.4 E). Similarly, in the “neonate 12w”, the striatal ischemia induced with ET-1 did not significantly change the density of BrdU+/NeuN+ cells compared with control (ET-1: mean ± SEM = 487.5 ± 79.96 cells/mm³, n= 5; Control: mean ± SEM = 376.8 ± 37.96 cells/mm³, n=7) (Figure 6.4 F).
Figure 6.4. Striatal neurogenesis remains unchanged after ischemic injury in the adult and neonatal brain of rats. Neurogenesis was assessed using immunohistochemical detection of cells double labelled for BrdU and NeuN. Representative striatal sections for the lesion groups of “adult 4w” (A), “adult 12w” (B) and “neonate 12w” (C). Statistical analysis of BrdU+/NeuN+ cell density did not reveal any difference between the lesion and control groups for “adult 4w” (D), “adult 12w” (E) or “neonate 12w” (F). G-J is an orthogonal representation of an example of BrdU+/NeuN+ double labelled cell at high magnification. Arrow heads are pointing at double labelled cells for BrdU/NeuN. The immunohistochemical analysis was completed in n= 4-8 rats and all rats demonstrated a similar pattern of staining. Statistical analysis: (D) one-way ANOVA F$_{2,10} = 2.448$, P = 0.1363.; (E) unpaired t-test, P = 0.2980; (F) unpaired t-test, P = 0.1987; error bars = standard deviation. Scale bars: A-C, 500 µm; G, 50 µm and H-J, 25 µm. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine.
6.4.4. Striatal ischemia does not increase the density of BrdU+/NeuN+/Darpp32+ cells in the neonatal brain and fails to produce any BrdU+/NeuN+/Darpp32+ cells in the adult brain.

To determine the phenotypic identity of the new neurons, we first investigated if the new neurons can adopt a medium spiny neurons phenotype by looking at the marker Darpp32. The BrdU+/NeuN+ cells identified in each group were further investigated to identify triple positive cells for BrdU+/NeuN+/Darpp32. Figure 6.5 represents an example of striatal section from the “neonate 4w_ET-1” group, where triple positive cells have been observed in the striatum, with a high concentration located ventral to the lateral ventricle. Statistical analysis did not reveal any difference in BrdU+/NeuN+/Darpp32+ density between the ET-1 lesion group and the control (ET-1: mean ± SEM = 230.3 ± 51.81 cells/mm³, n= 5; Control: mean ± SEM = 200.8 ± 22.97 cells/mm³, n=7) (Figure 6.5 G). In the adult brain groups “adult 4w” and “adult 12w”, all the BrdU+/NeuN+ cells were carefully analysed on the confocal microscope at high magnification but no triple BrdU+/NeuN+/Darpp32+ cell was identified (Figure 6.5 H-L).
Figure 6.5. Striatal ischemia does not affect the number of medium spiny neurons produced in the striatum of neonatal rats and fails to induce production in the adult rat brain. Immunohistochemical detection of triple labelled cells for BrdU/NeuN/Darpp32 has revealed the presence of BrdU+/NeuN+/Darpp32+ cells in the striatum of brains that received ischemic injury during the neonatal period, with a representative image of “neonate 12w ET-1” as an example (A). Arrow heads are pointing at the BrdU+/NeuN+/Darpp32+ cells. B-F, represents an orthogonal image of a BrdU+/NeuN+/Darpp32+ cell at high magnification. The density of triple positive cells was not different between lesion and control group in the neonatal brain (G). H-L, represents an orthogonal image of a BrdU+/NeuN+/Darpp32- cell found in the adult brain. The immunohistochemical analysis was completed in n= 5-7 rats and all rats demonstrated a similar pattern of staining. **Statistical analysis:** unpaired t-test, P= 0.0.5759. error bars = standard deviation. **Scale bars:** A, 500 µm; B-F and H-L, 50 µm. **Abbreviations:** BrdU, 5-bromo-2’-deoxyuridine; Darpp32, dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000.
6.4.5. Striatal ischemia does not increase the number of BrdU+/NeuN+/CR+ cells in the neonatal or adult brains.

With the aim to characterise the possible identity of the BrdU+ and the BrdU+/NeuN+ cells identified, we immuno-stained for calretinin (CR) a marker for interneurons. Immunohistochemical detection, reveals the presence of BrdU+/CR+ and BrdU+/CR+/NeuN+ cells in all the experimental groups (Figure 6.6 A-C).

The statistical analysis for the “adult 4w” group, did not reveal any difference between the lesion and control groups in the density of BrdU+/CR+ cells (ET-1_1: mean ± SEM = 1.789 ± 0.662 cells/mm³, n = 5; ET-1_2: mean ± SEM = 4.017 ± 0.734 cells/mm³, n=4 ; Control: mean ± SEM = 5.130 ± 1.933 cells/mm³, n=4 ) (Figure 6.6 N) or of BrdU+/CR+/NeuN+ cells (ET-1_1: mean ± SEM = 0.156 ± 0.156 cells/mm³, n = 5; ET-1_2: mean ± SEM = 0.1764 ± 0.1764 cells/mm³, n=4 ; Control: mean ± SEM = 0.4035 ± 0.233 cells/mm³, n=4) (Figure 6.6 Q). Interestingly, very few of the BrdU+/CR+ cells were also positive for NeuN, virtually less than 10%. Similar results were observed for the “adult 12w” group, where no statistical significance was observed between the groups in the density of BrdU+/CR+ cells (ET-1: mean ± SEM = 1.785 ± 0.3719 cells/mm³, n=8; Control: mean ± SEM = 4.078 ± 1.576 cells/mm³, n=4) (Figure 6.6 O) and the density of BrdU+/CR+/NeuN+ cells (ET-1: mean ± SEM = 0.5078 ± 0.2185 cells/mm³, n=8; Control: mean ± SEM = 0.3565 ± 0.2060 cells/mm³, n=4) (Figure 6.6 R). The neonatal groups presented almost five times more BrdU+/CR+ compared with the adult groups, but the analysis did not reveal any statistical difference in BrdU+/CR+ cell density between the lesion and control group (ET-1: mean ± SEM = 36.56 ± 14.43 cells/mm³, n=5; Control: mean ± SEM = 21.59 ±1.594 cells/mm³, n=7) (Figure 6.6 P). In addition, there was no statistical difference between the groups in the density of BrdU+/CR+/NeuN+ cells (ET-1: mean ± SEM = 20.32 ± 7.722 cells/mm³, n=5; Control: mean ± SEM = 10.23 ±1.354 cells/mm³, n=7) (Figure 6.6 S).
Figure 6.6. Striatal ischemia does not affect the number of interneurons produced in the striatum of neonatal and adult rats. Immunohistochemical detection for BrdU+/CR+, shown by arrow heads and BrdU+/CR+/NeuN+ cells, shown by arrows in the striatum of “adult 4w_ET-1” (A), “adult 12w_ET-1” (B) and “neonate 12w_ET-1” (C). D-H and I-M represents an orthogonal image at high magnification of BrdU+/CR+/NeuN- and BrdU+/CR+/NeuN+ cells respectively. Statistical analysis of BrdU+/CR+ cell density in the lesion compared with the control group, has revealed no difference for all three groups: “adult 4w” (N), “adult 12w” (O) and “neonate 12w” (P). Similar results were observed for BrdU+/CR+/NeuN+ cell density for “adult 4w” (Q), “adult 12w” (R) and “neonate 12w” (S). The immunohistochemical analysis was completed in n= 4-8 rats and all rats demonstrated a similar pattern of staining. Statistical analysis: (N) one-way ANOVA F_{2,10} = 2.193, P= 0.1623; (O) unpaired t-test with Welch’s correction, P= 0.2429; (P) unpaired t-test with Welch’s correction, P= 0.3595; (Q) one-way ANOVA F_{2,10} = 0.5224, P= 0.6084; (R) unpaired t-test, P= 0.6706; (S) unpaired t-test with Welch’s correction, P= 0.2636; error bars = standard deviation. Scale bars: A-C, 500 µm and D-M, 50 µm. Abbreviations: BrdU, 5-bromo-2’-deoxyuridine; CR, calretinin.

6.4.6. Investigation of the effect of immune system on striatal neurogenesis after injury.

To determine if the immune system played a role in the poor neurogenesis observed in the adult animals, an immune suppressed group was introduced into the existing experimental paradigm. This was essentially a pilot experiment to assess the potential for further investigation. We used athymic (‘nude’) rats and compared the result of ET-1 lesion with the same injury in SD rats (group, “adult 4w_ET-1_SD” from above) which acted as a control for the effect of the immune system. These animals were sacrificed four weeks after injury and are labelled “adult 4w_ET-1_Nude”.

The ET-1 lesion induced in the nude animals was large in the striatum and extended to the adjacent cortex, as shown by the NeuN staining (Figure 6.7 A). The BrdU density analysis did not reveal any significant difference between the “adult 4w_ET-1_Nude” group and the control group “adult 4w_ET-1_SD” (SD: mean ± SEM = 6962 ± 1640 cells/mm³, n=5; Nude: mean ± SEM = 5927 ± 818.9 cells/mm³, n= 5) (Figure 6.7 B, C). Looking at striatal neurogenesis after ET-1 lesion in the nude animals, the density of BrdU+/NeuN+ cells was low and was below 5 cells per mm³ for 5 out of the 6 animals and peaked at 18 cells per mm³ for the last animal. Overall BrdU+/NeuN+ cell density was similar to that of the “adult 4w_ET-1_SD” group (SD: mean ± SEM = 1.896...
± 0.891 cells/mm³, n=5; Nude: mean ± SEM = 5.540 ± 2.657 cells/mm³, n= 6) (Figure 6.7 D, F). To characterise the BrdU+ cells identity, we investigated at high magnification the co-labelling of BrdU+/NeuN+ cells for the medium spiny neuron marker, Darpp32 (data not shown). Similarly, to the other two adult groups (“adult 4w” and “adult 12w”) we only identified BrdU+/NeuN+/Darpp32- cells. To further characterise the BrdU+ cells, we looked at the interneuron marker, CR. The density of BrdU+/CR+ and BrdU+/CR+/NeuN+ cells was variable within the “adult 4w_ET-1_Nude” group and was ranging from 1 to 10 cells per mm³ and 0 to 5 cells per mm³ respectively. In comparison with the “adult 4w_ET-1_SD” group there was no significant difference in both BrdU+/CR+ cell density (SD: mean ± SEM = 1.789 ± 0.662 cells/mm³, n=5; Nude: mean ± SEM = 5.077 ± 2.104 cells/mm³, n= 5) and BrdU+/CR+/NeuN+ cell density (SD: mean ± SEM = 0.1563 ± 0.1563 cells/mm³, n=5; Nude: mean ± SEM = 2.029 ± 1.069 cells/mm³, n= 5) (Figure 6.7 E, G, H).
Figure 6.7. Effect of the immune system on striatal neurogenesis after striatal ischemia is limited. Immunohistochemical detection of NeuN allowed visualisation of the lesion area, which included the dorsal and lateral part of the striatum and the adjacent lateral cortex (A). BrdU staining (B) and statistical analysis of the BrdU density in the striatum (C) did not reveal any difference between the “adult 4w_ET-1_Nude” and the “adult 4w_ET-1_SD”. Striatal neurogenesis through identification of BrdU+/NeuN+ double positive cells (D) did not reveal any difference between the two groups (F). Further characterisation of the identity of the BrdU+ cells reveals the presence of BrdU+/CR+ double positive cells, shown by the arrow head and BrdU+/CR+/NeuN+, shown by the arrow (E). However, statistical analysis did not reveal any difference in the densities of BrdU+/CR+ cells (G) or BrdU+/CR+/NeuN+ cells (H) between the nude and SD animals. The immunohistochemical analysis was completed in n= 5 rats and all rats demonstrated a similar pattern of staining. Statistical analysis: (C) unpaired t-test, P= 0.7381; (F) unpaired t-test with Welch’s correction, P= 0.2405; (G) unpaired t-test with Welch’s correction, P= 0.1988; (H) unpaired t-test with Welch’s correction, P= 0.1552. Scale bars: A, B, D, E, 500 µm. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CR, calretinin.

6.5. Discussion

This data suggests that the adult brain is not capable of replacing striatal projection neurons lost after striatal ischemia. Even in the neonatal brain, where medium spiny neurons are actively being produced under physiological conditions (J. Wright, Stanic, and Thompson 2013), striatal ischemia is not sufficient to induce an increase in production for brain repair.

In this study, we used the vasoconstrictor ET-1 to induce striatal ischemia in both adult and neonatal rats, in order to investigate the potential of the brain for self-repair after injury. The ET-1 injection was successful in inducing an injury to the striatum, as shown by labelling for the neuronal marker NeuN (Figure 6.2). However, the severity of the striatal damage was limited and variable within both the “adult 4w_ET-1_2” and the “adult 12w_ET-1” group, where the injury was limited to a discrete region in the centre of the striatum. The ET-1 used for these two groups was from a different batch than for the other groups and we hypothesised that its efficiency was limited either due to a deficient batch or resuspension issues experienced with the latter batch. In addition, it should be noted that the striatum of “neonatal 12w_ET-1” was reduced, which is consistent with the established literature that shows a brain atrophy following ischemic injury (Hara, Harada, and Sukamoto 1993). It is however not
surprising to fail to observe this phenomenon for the “adult 4w_ET-1” group as the time post-lesion was too short or for the “adult 12 w_ET_1” group as the lesion was very mild. However, it should be noted that we did not considered the potential direct effect of ET-1 on neurons.

Looking at striatal neurogenesis, we observed an increase of proliferation after striatal ischemia, based on staining for the proliferation marker BrdU for both “adult 12w” and “neonate 12w”. However, for all the groups killed four weeks after injury, i.e.: “adult 4w_ET-1_1”, “adult 4w_ET-1_2” and “adult 4w_ET-1_nude”, the proliferation was unchanged. Nonetheless, a tendency was observed for an increase of BrdU density after injury, but the low number of animals per group and the variability within each group, prevented reaching statistical significance. The increase in proliferation in the 12 week groups, is in accordance with the literature, where it is well-established that injury induces a cellular proliferation response (Cavanagh 1970; Nait-Oumesmar et al. 1999). Interestingly, a more recent study by Thored et al, (2006) observed the proliferation strictly in the SVZ, where they reported an increase in BrdU+ cells after medial cerebral artery occlusion (MCAO) four hours after injury, but a return to baseline levels at six weeks post-lesion (Thored et al. 2006). This could suggest that the increase in proliferating cells in the SVZ is transient and happens shortly after injury, the progenitors then migrate away from the SVZ, most likely to the injury site. It is unclear why our results are different, maybe the nature of the injury is responsible for the discrepancy, they induced the ischemia through MCAO while we used ET-1 which could have a longer-term effect.

The striatal neurogenesis rising after ischemia has also been well documented, especially after injury induced by MCAO in adult (Arvidsson et al. 2002; Teramoto et al. 2003; Parent et al. 2002; Plane et al. 2008) or hypoxic-ischemia in neonates (Ong et al. 2005; Plane et al. 2004). Surprisingly, our results did not show an increase of neurogenesis in any of the groups, measured by the number of BrdU+/NeuN+ cells. We hypothesised that result was due to the low number of animals in each group, and also due to the variability of the lesion severity in the adult groups. Indeed, it was previously shown that the lesion severity is an important factor that can influence the neurogenic response (Thored et al. 2006). Additionally, in our study the neonatal
brain did not present an increase in neurogenesis after injury. However, while we failed to observe an increase in neurogenesis, it is possible that any small increase in BrdU+/NeuN+ cells was masked by physiologically already high neurogenesis levels in the neonatal brain as shown here and also as previously reported (J. Wright, Stanic, and Thompson 2013).

In order to identify the specific phenotypes of these progenitor cells in both the adult and the neonatal brains, we used the medium spiny projection neuron marker, Darpp32 and the interneuron marker CR. Our data has confirmed the previous finding from Liu et al. 2009 and Wright et al. 2016, supporting the idea that the adult brain is incapable of changing the progenitor fate into new medium spiny neurons to repopulate the injury site, but rather maintain their differentiation potential as interneurons (Liu et al. 2009; J. L. Wright et al. 2016). However, the injury did not induce a change in the number of BrdU+/CR+ interneurons generated in the adult brain, which contradicts Yang et al., 2008 who showed an increase in the CR+ cells in the striatum after injury (Yang, You, and Levison 2008). This difference might be explained by a different phenotype identity used for counting the interneurons, indeed while we specifically counted the co-labelled cells BrdU+/CR+, Yang et al. counted single cells for CR+, therefore it is possible that a high number of already mature interneurons were recruited to the injury site. In addition, as mentioned previously the ET-1 lesion we induced was very heterogeneous within groups, which might further be responsible for this discrepancy. It is important to note that the CR+ neurons do not count for all the BrdU+/NeuN+ cells, the remaining neurons might be interneurons expressing other markers such as parvalbumin or neuropeptide Y (Collin et al. 2005).

It is well-established that under normal conditions in the adult, the SVZ only provides interneurons to the olfactory bulb (Lois and Alarez-Buylla 1994) and it was recently established that the neonatal brain not only generates interneurons but also striatal projection neurons that express the marker Darpp32 (J. Wright, Stanic, and Thompson 2013). Therefore, since the neonatal brain already generates striatal projector neurons, the neurons lost in striatal ischemia, we aimed to determine if the brain at a more immature stage is capable of self-repair. Our results suggested that a
striatal injury such as ischemia is not sufficient to increase the levels of medium spiny neurons produced. We also identified some of the BrdU+ cells adopting an interneuron phenotype, by expressing CR. However, the number of CR+ interneurons generated after ischemia was unchanged, suggesting that while the progenitor cells seems to be redirected to the injury site, and that striatal proliferation is increased, the progenitors remained undifferentiated or have a poor survival rate.

These findings suggest that the capacity for spontaneous recovery of the postnatal brain after injury, such as ischemia, is very limited. Early reports that identified production of striatal projector neurons after MCAO, gave hope to restorative therapies through endogenous cell replacement (Arvidsson et al. 2002; Parent et al. 2002). The main issue at the time seemed to be a low neurogenesis rate. Therefore, a strong research focus was orientated towards increasing neurogenesis through growth factors, with a success for example with infusion of GDNF (Kobayashi et al. 2006). However, our findings suggest that the neurons produced after injury in the adult brain are not adopting the correct phenotype and that while the neonatal brain generates the correct neurons for repair, the striatal neurogenesis rate is unchanged and therefore not therapeutically relevant. Therefore, while in the neonatal brain, increasing the neurogenesis rate might be sufficient for brain repair, it is not for the adult brain.

Furthermore, in this study we tested the hypothesis that the immune system might be impeding the neurogenesis rate after injury and also the production of Darpp32+ cells in the adult. However, our results showed that in an immune suppressed animal, the injury-induced neurogenesis is similar to that of a normal animal, suggesting that the immune system is not the limiting factor.

In summary, our data suggest that the adult brain is not capable of replacing neurons lost to striatal ischemia and we further showed that the neonatal brain which generates striatal projection neurons under normal conditions, does not attempt to repair the injury by producing more Darpp32+ cells. It is important to properly set the ground rules of both the behaviour of a healthy adult brain and an injured adult brain, in order to clearly understand where the research should focus to identify possible therapies for brain injuries, such as stroke. Endogenous cell replacement therapies are
attractive, but their correct application is still a long way away. They imply mastering all the process for a proper integration of the correct neurons, which include: 1) increased neurogenesis, 2) migration to the injury site, 3) redirection of the fate into the correct phenotype, 4) survival of new neurons and 5) functional integration in the neuronal circuitry.
6.6. References


Chapter 7
Conclusions & future directions

7.1. Summary of the current findings

Since the ‘rediscovery’ of adult neurogenesis in the mammalian brain in the early 90s, substantial advances have been made in understanding its functions under physiological and also pathological conditions, such as Parkinson’s disease (PD) and Stroke. However, an extensive understanding of the neurogenic mechanisms involved in regulation of meaningful and proper integration of the newborn neurons in the circuitry is still unclear. The aim of this thesis was to understand the potential and limitations for neuronal replacement therapies in two models of diseases: acute injury (Stroke) and neurodegenerative injuries (Parkinson’s disease).

In Chapter 3, we aimed at characterising the extent of dopaminergic inputs to the dentate gyrus (DG). We combined retrograde tracing and immunohistochemistry to identify the phenotype of fibre innervation of the DG. The results showed that the midbrain projecting fibres are non-dopaminergic and that the tyrosine hydroxylase (TH) fibres observed in the DG are noradrenergic fibres coming from the locus coeruleus (LC). These data suggest that any influence from the dopaminergic (DA) system to the DG, is likely to be through an indirect pathway rather than a direct through a DA-hippocampus pathway. This finding is of importance in interpreting studies that investigate the DA neuronal loss on hippocampal functions like neurogenesis (Suzuki et al. 2010) or cognitive functions (Wisman et al. 2008).

Going further, in Chapter 4 we aimed to test the hypothesis that in PD the noradrenergic (NA) and/or DA loss induce a reduction in hippocampal neurogenesis. We compared the effect of a NA depletion, a DA depletion and a combined NA & DA depletion on hippocampal neurogenesis. To our surprise neither NA and DA depletion alone nor together induced a change in hippocampal neurogenesis. Furthermore, the DA depletion did not impact subventricular (SVZ) neurogenesis. These findings contribute to a very mixed literature on the regulation of neurogenesis by
catecholamines such as DA and NA (Höglinger et al. 2004; Park and Enikolopov 2010; Malberg et al. 2000; Wakade et al. 2002; Keilhoff, Grecksch, and Becker 2010; Halim et al. 2004; Suzuki et al. 2010). Our conclusion is that any decrease in hippocampal neurogenesis in PD would be independent from a direct DA and NA hippocampal deficiency. Both hippocampal neurogenesis and catecholaminergic degeneration might act through indirect, systems level mechanisms to affect neurogenesis in PD and other non-movement related phenomenon, contradictory to previously thought (Suzuki et al. 2010; Höglinger et al. 2004).

While NA degeneration does not seem to impact hippocampal neurogenesis in PD, it is well established that the LC/NA system has a strong impact on cognition, and might be in part responsible for the apparition of non-motor symptoms like depression or attention deficits observed in PD (Aston-Jones and Bloom 1981; Bouret and Sara 2004). Therefore, in Chapter 5 we aimed at describing a new tool for NA cell replacement therapies: the reporter mice DBH-eGFP. In the late 80’s NA cell transplantation was developed in rats by Bjorklund et al. (1986). The use of GFP reporter mice in basic research has already been proven to be very beneficial for the development and improvement of cell therapy for PD – for example using the TH-GFP mice (Kauhausen, Thompson, and Parish 2013; Neto et al. 2012; Thompson et al. 2009). The reporter mouse has the advantage of unambiguously differentiating between host and grafted cells, thanks to the GFP marker. This allows robust analysis of the graft survival, integration and when combined with cell sorting approaches, can successfully purify the cells to be grafted as the correct phenotype. This ongoing study has shown so far that the DBH-eGFP mice correctly and selectively express the GFP marker in NA cells of the LC. Furthermore, grafts from LC mouse embryos at E12.5 transplanted into the hippocampus of strain matched adult mice, show the presence of GFP+ fibres two weeks after the implantation. The limited number of animals and the very much ongoing work aiming at improving the cell preparation did not allow the creation of standardised protocol of NA cell transplantation as of yet, this work however is a very promising and requires further testing to develop such protocol.

In this thesis we also explored the effect of an acute injury like Stroke on neurogenesis. In Chapter 6, we investigated the capacity of the adult and neonatal
brain to regenerate the medium spiny neurons loss to striatal ischemia. Our results confirmed previous studies from Liu et al. (2009) and Wright et al. (2013), showing that striatal ischemia in the adult does not redirect the newborn neuron phenotype to a medium spiny neuronal fate. Furthermore, our results showed that even in the neonatal brain, where the newborn neurons already differentiate into medium spiny neurons, striatal ischemia is insufficient to enhance their production. Our data helped to better grasp the actual potential of the brain for self-repair, contrary to popular thinking in this field, we suggest we are still a long way away from functional recovery through neurogenesis.

7.2. **Future directions**

It has been previously reported that patients suffering from Parkinson’s disease have impaired hippocampal neurogenesis (Laakso et al. 1996; Camicioli et al. 2003; Höglinger et al. 2004). In this thesis we have concluded that any decrease in neurogenesis is likely to be independent from any DA and NA depletion observed in PD. It is therefore important to understand the origin of neurogenesis impairment in order to develop appropriate treatments.

One hallmark of PD is the presence in the brain of Lewy bodies, which are aggregates of misfolded protein, with the major component being α-synuclein. Interest in α-synuclein has increased since a link with PD development has been suggested (Breydo, Wu, and Uversky 2012; Olanow and Brundin 2013; Lin and Scott 2013; Recchia et al. 2004), in addition it was reported that Lewy bodies are present in the hippocampus (Hall et al. 2014; Flores-Cuadrado et al. 2016). Therefore, we hypothesise that neurogenesis impairment is at least in part due to the presence of the α-synuclein in the hippocampus. Further work needs to be done to validate this hypothesis, one way could be to administered the mutated α-synuclein directly in the hippocampus via direct injections (Paumier et al. 2015) or viral vectors (Van der Perren et al. 2016), and to investigate hippocampal neurogenesis levels in addition to behavioural performance n cognitive tasks.
In addition, it is known that DA and NA degenerate in PD and it was suggested to be responsible for onset of some non-motor symptoms (Delaville et al. 2012; Murai et al. 2001; Chaudhuri and Schapira 2009; Vazey and Aston-Jones 2012; Espay, Lewitt, and Kaufmann 2014). Our data suggest that the effect of the DA and NA degeneration on the onset of the non-motor symptoms is independent to the level of hippocampal neurogenesis. Therefore further investigation needs to be performed to identify the mechanism underlying the development of non-motor symptoms after DA and NA depletion. We hypothesise that the mode of action might be a more direct mechanism, but behavioural experiments will need to be performed to support his hypothesis. For example, following different DA and NA depletion conditions, one could test: cognition, using Morris Water maze and touchscreens cognitive tests; depression, with the forced swim test and anxiety, with the elevated plus maze. The DBH-eGFP reporter mice will also be a great tool to identify effects of NA neuronal replacement after depletion at the cellular and functional level. Furthermore, newly developed technologies such as optogenetics or Designer Receptors Exclusively Activated by Designer Drugs (Dreadd), that can control the activation of a certain neuronal type by light or synthetic ligand, could be used for a NA neuronal cell line transplanted in the hippocampus or LC, to confirm the functional impact of a NA graft.

In the striatum, neurogenesis following ischemia seems to be more limited than previously thought, even in the neonatal brain, the medium spiny neurons produced are not increased after the injury. The neonatal brain is still capable of generation medium spiny neurons (DARPP32+), therefore further experiment should identify the factors that direct the phenotype to DARPP32+ neurons and test the possibility to redirect the newborn neurons in the adult brain. Recent in vitro studies successfully generated DARPP32+ neurons using a combination of factors including FOXP2, GSX2 and CTIP2, where CTIP2 seems to be the key to DARPP32 expression (Delli Carri et al. 2013; Arber et al. 2015). In addition, a CTIP2 deficient mouse model presents a reduction in the number of DARPP32+ neurons in the striatum (Arlotta et al. 2008). One could investigate if CTIP2 infused in the striatum of adult brain after ischemia is sufficient to induce the production of medium spiny neurons. Alternatively, a gene
expression and transcriptome analysis of the neonatal SVZ compared with the adult SVZ could identify other potential targets to redirect neuronal phenotype to medium spiny neurons and increase neurogenesis levels in the adult. Moreover, a recently developed technique, called parabiosis, where mice of different age are parabiotic paired to share a circulatory system, could allow the identification of any systemic factors (Conboy et al. 2005).

7.3. Concluding remarks

A new area of research has intensely questioned the possibility of using neurogenesis to repair the brain after injury. Indeed, the human brain is capable of some plasticity, as shown by stroke patients that relearn to speak (Pedersen et al. 1995) or by the symptoms arising in PD only when more than 80% of the DA neurons are lost (Zigmond et al. 1990). However, the potential of adult neurogenesis for functional self-repair in the brain have clear limitations. Evolution provided the mammalian brain with anatomical complexity, which came with a reduction of the reparative potential of adult neurogenesis compared to other non-mammalian vertebrates, which is seen by some as an “evolutionary constraint” (Bonfanti 2011; Weil et al. 2008). Therefore, maybe the rodent brain is not the best model to study the mechanism of regeneration as it is very limited. Studying the brain of amphibians like the salamander might provide insight on identification of the pathways activated for recovery after injury and provide potential translations to mammalian brains.
7.4. References


Appendix 1

Over-expression of Meteorin drives Gliogenesis following Striatal Injury

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A1.1. Abstract

A number of studies have shown that damage to brain structures adjacent to neurogenic regions can result in migration of new neurons from neurogenic zones into the damaged tissue. The number of differentiated neurons that survive is low, however, and this has led to the idea that the introduction of extrinsic signaling factors, particularly neurotrophic proteins, may augment the neurogenic response to a level that would be therapeutically relevant. Here we report on the impact of the relatively newly described neurotrophic factor, Meteorin, when over-expressed in the striatum following excitotoxic injury. Birth-dating studies using bromo-deoxy-uridine (BrdU) showed that Meteorin did not enhance injury-induced striatal neurogenesis but significantly increased the proportion of new cells with astroglial and oligodendroglial features. As a basis for comparison we found under the same conditions, glial derived neurotrophic factor significantly enhanced neurogenesis but did not effect gliogenesis. The results highlight the specificity of action of different neurotrophic factors in modulating the proliferative response to injury. Meteorin may be an interesting candidate in pathological settings involving damage to white matter, for example after stroke or neonatal brain injury.
A1.2. Introduction

Meteorin is a member of a newly described family of secreted proteins that also includes the related protein, Meteorin-like. It is highly expressed in the mammalian brain, throughout mid- to late-development and is maintained at lower levels in the post-natal brain (Jorgensen et al., 2009). In adult rodents, Meteorin protein is most conspicuously expressed by bergmann glia in the cerebellum, but also more diffusely at lower levels in glia cells throughout the brain. It was first characterized in vitro for its ability to promote astrocyte differentiation and axon growth in neurosphere cultures and dorsal root ganglion explants respectively (Nishino et al., 2004). Later studies established that Meteorin’s pro-gliogenic functionality acted on the Jak-STAT3 pathway (Lee et al., 2010; Sun et al., 2014), although to date, the receptor it functions through remains unknown.

In vivo studies in rodents demonstrated that after excitotoxic injury to the striatum, endogenous Meteorin is up-regulated in striatal s100β+ astrocytes (Jorgensen et al., 2011), and has also been observed in neurons after ischaemic damage (Wang et al., 2012). To explore its functional impact in this setting, subsequent studies demonstrated that over-expression of Meteorin via viral (Jorgensen et al., 2011) or encapsulated cell (Tornoe et al., 2012) delivery prior to excitotoxic injury resulted in significantly reduced levels of striatal damage (Jorgensen et al., 2011; Tornoe et al., 2012). More recently, studies have also begun to investigate the possibility that, in addition to neuroprotection of existing circuitry, Meteorin might also act as a survival factor for newborn neurons generated in response to striatal injury (Wang et al., 2012).

A number of studies have shown that, following striatal injury resulting in extensive cell loss, neuroblasts constitutively generated in the sub-ventricular zone (SVZ) can migrate into the damaged striatal parenchyma and differentiate into mature neurons (Arvidsson et al., 2001; Jin et al., 2001; Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003; Jin et al., 2006). The overall survival of these new neurons is notably poor and well below levels likely to be required for therapeutic replacement of neurons lost to the injury. This has motivated studies that investigate strategies to improve the survival of newborn neurons, for example through delivery of trophic
factors. Topical delivery of soluble proteins known to influence cell survival, neural stem cell proliferation and neurogenesis in other disease models have proven to be robust regulators of these cellular processes in the context of forebrain injury. Examples of such factors are Glia-derived neurotrophic factor (GDNF) (Kobayashi et al., 2006), Vascular endothelial growth factor (VEGF) (Sun et al., 2003), along with Noggin and Brain-derived neurotrophic factor (BDNF) (Benraiss et al., 2012) which have all been shown to positively regulate neurogenesis in the damaged striatum. The expression of Meteorin during neural development and in the adult striatum makes it an interesting candidate in this context. Recently, Wang Z. et al. (Wang et al., 2012) demonstrated that chronic infusion of Meteorin increased SVZ proliferation and neuroblast migration into the damaged striatum after medial cerebral artery occlusion in rats, resulting in increased numbers of surviving newborn neurons.

Here we sought to characterize the phenotype of new cells generated in the presence of Meteorin after striatal damage – including the identity of neuronal and glial subtypes. Following excitotoxic damage, we found that stable expression of Meteorin using a lenti-virus resulted in a small increase in the survival of new neurons which did not reach statistical significance. To contextualize this impact on neuronal survival, we also present results from GDNF expression in the same experimental paradigm, where there was a much larger effect on neurogenesis in response to injury. The major impact of Meteorin was in fact to drive gliogenesis, where birth-dating studies using Bromodeoxyuridine (BrdU) showed that there was a significant increase in the proportion of new s100β+ astrocytes and Oligodendrocyte transcription factor 2 (Olig2+) oligodendrocyte progenitors in response to damage to the adult striatum.

A1.3. Materials and Methods

A1.3.1. Ethical approval

All experimental procedures carried out in this study conform to the rules set out by the Australian National Health and Medical Research Council published code of practice and experiments were approved by the Florey Neuroscience Institutes animal ethics committee (no. 09-105).
A1.3.2. In vivo procedures

Adult Sprague Dawley rats were housed under a 12-hour light/dark cycle with ad libitum access to food and water. Surgery was performed at 6 weeks of age using age matched animals. Prior to surgery, rats were deeply anaesthetized with isoflurane (5% at 1 L/min) and kept under anaesthesia (2-3% at 1 L/min) for the duration of surgery.

All surgical procedures were performed using a stereotaxic frame (Kopf, Germany) and intra-striatal delivery was performed using a fine glass capillary fitted to a 5 μl microsyringe (SGE Analytical Sciences, Australia). Under deep anaesthesia, all animals received 2 μl of quinolinic acid (QA) solution (100 nmol/μl in 0.9% saline) injected over 2 minutes into the striatum (1.2 mm anterior and 3 mm lateral to bregma and 4 mm below the dural surface). The cannula was kept in place for 5 minutes after injection to minimize back-flow.

Twenty four hours post-injection, all animals underwent a second round of surgery (setup as previously described) for intra-striatal lentivirus delivery constructed as previously described (Jorgensen et al., 2011). Three treatment groups were compared within this study. Animals received one of either lentivirus encoding Meteorin (Jorgensen et al., 2011), GDNF (pHsCXW, NsGene) and GFP (pHsCXW, NsGene) for control animals via a 1.5 μl solution (1.2 × 10^5 transforming units total) over 2 minutes into the striatum (1.2 mm anterior and 3.2 mm lateral to bregma and 4 mm below the dural surface). All vectors were under the control of the woodchuck post-regulatory element for constitutive transgene expression.

To label dividing cells, animals received BrdU (50 mg/kg i.p. using a 20 mg/ml solution in 0.9% saline) every 12 hours for 2 weeks as previously described (Arvidsson et al., 2002), beginning the day of lentiviral delivery. Animals were perfused for histological assessment 4 weeks after BrdU treatment (6 weeks after QA lesion).

A1.3.3. Tissue preparation

At 6 weeks after surgery, animals under deep anaesthesia induced with isoflurane (5% at 1 L/min), subsequently received a lethal dose of pentobarbitone. Animals were
then transcardially perfused with 50 ml saline (0.9% w/v) followed by 200–250 ml paraformaldehyde (PFA; 4% w/v in 0.1 M PBS). The brains were removed, post-fixed a further 2 hours in PFA and cryoprotected in sucrose (20% w/v in 0.1 M PBS). Brains were sectioned in the coronal plane in a 1:12 series at a thickness of 30 μm on a freezing microtome (Leica, Germany).

A1.3.4. Immunohistochemistry

Primary antibodies were diluted in a solution of 0.1 M PBS containing 5% normal serum and 0.5% Triton X-100 (Amereso, USA) and incubated with sections overnight at room temperature. Prior to the addition of the secondary antibody solution, the sections were blocked with 5% normal serum for 0.5-1 hour. Secondary antibodies were diluted in a solution of 0.1 M PBS containing 2% normal serum and 0.5% Triton X-100 and incubated for 2 hours at room temperature. For florescent analysis, secondary antibodies were conjugated to Dylight Florophores 488, 549 or 647 and slide-mounted sections were cover-slipped with florescent mounting medium (DAKO, USA). For chromogenic analysis, secondary antibodies were conjugated to biotin and visualized using the Avidin/Biotin enzyme complex (VECTASTAIN ABC system, Vector Labs) coupled with peroxidase-driven precipitation of diaminobenzidine (DAB). DAB-labelled sections were dehydrated in alcohol and xylene, and cover-slipped with DePex mounting medium (BDH Chemicals, UK). For BrdU labelling, free-floating sections were incubated for 2 hours at 65°C in 50% de-ionized formamide in 0.1 M PBS. The tissue was rinsed 3 x 5 minute washes in 0.1M PBS and acid treated with 2 M HCL for 30 minutes at 37°C. The tissue was then washed in 0.1 M Sodium Borate (pH 8.5) for 15 minutes and finally rinsed with 3 x 5 minute washes in 0.1 M PBS.

Primary antibodies and dilution factors were as follows: mouse anti-APC (1:200; #OPT80, Calbiochem, Germany), rat anti-BrdU (1:300; #BTO030, Axyll Laboratories, USA), mouse anti-Calretinin (1:1000; #7697, Swant, Switzerland), rabbit anti-Darpp32 (1:500; #AB15518, Millipore, Germany), goat anti-GDNF (1:200; #AB212NA, R&D, USA), chicken anti-GFP (1:1000; AB13970, AbCam, USA), rabbit anti-GFP (1:20,000; #AB13970,
AbCam, USA), rabbit anti-Iba1 (1:200; #01919741, Wako, Japan), goat anti-Meteorin (1:400; #AF3475, R&D, USA), mouse anti-NeuN (1:200; #MAB377, Millipore, USA), rabbit anti-Olig2 (1:200; #AB9610, Millipore, USA), rabbit anti-s100β (1:200; #HPA015768, Sigma-Aldrich, USA).

A1.3.5. Imaging

Images of representative co-labelled cells were acquired via 40x or 63x PL-APO oil immersion objectives (Zeiss) using a Zeiss 780 laser-scanning confocal upright microscope. Representative overview images of DAB stained sections were acquired on a Leica DM6000B microscope using a HCX PL 5x/0.5 objective and tiled images aligned using Leica application suite v3.8 software. NeuN, BrdU, Iba1, Meteorin, GDNF and GFP immuno-labelled DAB representative images were acquired on the same microscope using HCX PL 20x/0.5 and HCX FL PLAN 40x/0.65 objectives (Leica). The brightness and contrast of individual images was adjusted using the photo enhancing software Photoshop v7.0 (Adobe) to optimally represent the immunohistochemistry observed through the microscope.

A1.3.6. Stereology and BrdU+ cell density estimation

Stereological estimation was used to determine the number of BrdU+ cells residing in the anterior head of the striatum across the three treatment groups. For all animals, 30 µm coronal sections each 360 µm apart (1:12 series) were immuno-labelled for BrdU. To establish the region of interest, the rostral boundary of striatum was defined at 1.70 mm anterior to bregma and the caudal boundary defined at 0.26 mm anterior to bregma (spanning 0-1440 µm of the anterior striatum) and delineated by standard anatomical boundaries (Paxinos and Watson, 1998). Estimates of BrdU-labelled cells were determined using a fractionator sampling design according to optical dissector rules (Gundersen et al., 1988; Mayhew, 1991). Counting frame grid dimensions and fractionator x,y coordinates were determined using the grid overlay program Stereoinvestigator v7.0 (MicroBrightField, Williston, VT, used on a
microscope, Leica, with a 40x objective, Zeiss). Guard zones were set at 1 µm (top and bottom) and BrdU-labelled nuclei quantified within the counting frame (dimensions used were 90 µm x 90 µm) at periodic intervals (x = 300 µm, y = 350 µm) in the delineated region of interest.

To determine BrdU density, volumetric analysis was conducted to determine the volume of measured striatum. Striatal volumetric measurements for each brain were achieved via Cavalieri estimation using the program Stereoinvestigator v7.0 (MicroBrightField, Williston, VT). The resultant volume was used to determine the BrdU+ cell density in the striatum based on the stereological estimation of BrdU+ cells and reported as BrdU+/mm3. The accuracy of the stereological estimations was determined by the coefficients of error and coefficients of variance. Estimations were deemed acceptable if coefficients were >0.1 (West et al., 1991).

A1.3.7. Cell quantification

In order to assess the phenotype of newborn cells post-lesion in the striatum, sections from animals sacrificed at six weeks post-lesion were double or triple labelled using florescent immunohistochemical techniques. Sections were stained with either BrdU/NeuN/Darpp32, BrdU/NeuN/Calretinin, BrdU/s100β or BrdU/Olig2/APC. Due to the relatively low number of double and triple labelled cells in the striatum, cells were manually counted across 3 sections spanning the anterior head of the striatum (1.70 mm, 1.34 mm and 0.98 mm anterior to bregma). An additional rostral striatal section was analysed at 0.62mm from bregma for quantification of BrdU+/s100β+ co-localization.

Using a confocal microscope (Zeiss) and a 20x/0.8 PL-APO objective (Zeiss), z-stacking and x-y tile scanning features were used to capture large 3-dimensional volumes of the entire striatum for each tissue section. ZEN digital imaging software (Zeiss) was then used to unambiguously identify all double/triple labelled BrdU+ cells. When delineating the striatal volume for analysis, we did not include the highly damaged area immediately in the vicinity of the QA delivery, which we found to have
a high level of brightly florescent non-specifically labelled cells. All BrdU+ cells initially identified as co-labelled, were verified by inspection with a 63x PL-APO oil immersion objective (Zeiss) and orthogonal reconstruction on the z-axis.

The total number of cells counted across the quantified striatum (1.70 mm – 0.98 mm anterior to bregma) was estimated by extrapolation based on the series interval (1:12) and the number of sections counted (3) across the series. In addition to reporting total cell counts, total cell counts were normalized to BrdU+ cell density to account for any variability in these parameters across animals. This normalized result is reported in the text as a percentage of BrdU+ cell density (BrdU+/mm3).

For newborn Iba1+ microglia quantification, sections were stained with BrdU/Iba1. Due to the abundance of BrdU+/Iba1+ cells, one section per animal across treatment groups (n = 3 per group) was used for quantification (1.34mm anterior to bregma). Striatal sections were systematically analysed from the lateral ventricle to the site of injury across all animals for consistency. BrdU+ cells and BrdU+/Iba1+ cells were quantified in this manner. The resultant BrdU+/Iba1+ cell counts were normalized to the BrdU+ cell counts obtained from the same experiment and represented as a percentage.

A1.3.8. Statistics

Statistical analysis was conducted using Graphpad Prism v6.0 software. BrdU+ cell density, total cell counts and normalized cell counts were compared across the three experimental treatment groups using one-way ANOVA with post-hoc correction (Bonferroni) for multiple comparison. In this study the data across experimental treatment groups is reported as the mean ± standard error of the mean (SEM).
A1.4. Results

A1.4.1. Neuronal cell loss and local proliferation after excitotoxic lesioning.

Immunohistochemical labelling of the mature neuronal marker NeuN showed that injection of quinolinic acid into the anterior head of the striatum resulted in a robust loss of neurons (Fig. 1A-B). In some cases we also observed neuronal loss in the overlying cortex, likely resulting from reflux along the injection tract (not shown). Labelling for BrdU showed a robust proliferative response during the first two weeks after the lesion (Fig. 1C-D). The BrdU+ cells were distributed densely in the immediate vicinity of the QA injection site, along with what appeared to be some degree of non-specific labelling, and also more diffusely throughout the entire striatum. The excitotoxic lesion also resulted in an inflammatory response that was persistent at six weeks, as demonstrated by a large increase in the density of striatal Iba1+ microglia with a reactive, amoeboid morphology (Fig. 1E-F). Some degree of microglial activation was also observed along the needle tract in the overlying cortex. Anatomically, the distribution of microglial activation matched well with the area of NeuN+ cell loss and increased BrdU+ labelling.
Figure A1.1: Assessment of lesioning, proliferation and microglial response 6 weeks after intra-striatal injection of quinolinic acid. Representative striatal sections of intact and lesioned brains 6 weeks post-lesion (arrow indicates injection site of QA lesion). The boxed area on each section corresponds to the adjacent 20x image detailing the immunolabelling. (A) NeuN labelling in intact striatum and (B) 6 weeks post-lesion, detailing loss of neurons in adjacent 20x image. (C) BrdU labelling of an intact adult striatal section and (D) 6 weeks post lesion. (E) Iba1 staining in intact sections with adjacent 20x image detailing ramified microglia in the striatum and at (F) 6 weeks post-lesion with reactive microglia illustrated in the adjacent 20x image. Scale bar: 1mm brain sections (A-F), 200μm boxed images (A-F).

A1.4.2. Meteorin is highly diffusible within the striatum when over-expressed using a lentiviral vector.

Transduction of the striatum using lentiviral vectors carrying either GFP (lvGFP), GDNF (lvGDNF) or Meteorin (lvMeteorin) resulted in robust expression of the transgenes (Fig. 2A-F). At six weeks, immunohistochemical analysis showed the expected cytoplasmic pattern of GFP expression (Fig. 2B). The morphology of the GFP+
cells showed that predominantly astrocytes were transduced with the vector, although we observed a smaller population of GFP+ neurons also. Injection of either lvMeteorin or lvGDNF resulted in a robust expression throughout the entire striatum (Fig. 2C and E). In both cases, the profile of expression was consistent with that expected for a secreted protein, including a diffuse labelling pattern that reduced in intensity with distance from the injection site (Fig. 2D and F).

![Image of immunohistochemical analysis](image)

**Figure A1.2:** Validation of lentiviral expression 6 weeks post-lesion. Immunohistochemical analysis at 6 weeks post-injury in animals administered corresponding lentiviral over-expression constructs across three representative sections (arrow indicates injection site of lentivirus) (A, C, E) corresponding to 1.70mm, 1.34mm and 0.98mm from bregma, left to right. (A) lvGFP treated animals with GFP immuno-labelling with (B) 40x representative image of GFP+ astrocytic cells in the striatum. (C) lvMeteorin and (E) lvGDNF treated animals with Meteorin and GDNF immuno-labelling respectively. (D) tiled 40x image of lvMeteorin treated animals and (F) lvGDNF treated animals illustrate diffuse labelling within the striatum. **Scale bar:** 1mm (A) analogous for (C and E), 0.1mm (B, D and F).
A1.4.3. Over-expression of Meteorin did not increase the number of new neurons in the striatum after excitotoxic damage.

To assess the impact of Meteorin on neurogenesis in response to injury, animals were administered BrdU twice daily for two weeks to label newborn cells immediately after excitotoxic damage to the striatum. Animals were perfused for histology four weeks later to allow for cell migration and differentiation of BrdU-labelled cells. Stereological estimation of the total number of newborn BrdU$^+$ cells in the striatum six weeks after injury showed no significant difference in the density of BrdU$^+$ cells in the striatum between the lvGFP control (1.8 ± 0.3 x 10$^4$ BrdU$^+$ cells/mm$^3$), lvMeteorin (1.2 ± 0.1 x 10$^4$ BrdU$^+$ cells/mm$^3$ ) and lvGDNF (2.7 ± 0.9 x 10$^4$ BrdU$^+$ cells/mm$^3$) groups (p = 0.67, lvGFP v lvGDNF; p = 0.95, lvGFP v lvMeteorin, one-way ANOVA, Bonferroni post-hoc), although the number of BrdU$^+$ cells was notably variable in the lvGDNF group.

To estimate the number of new neurons generated during the two week BrdU-labelling period after striatal injury, cells unambiguously immuno-labelled for both BrdU and NeuN were counted in three coronal sections from each animal 4 weeks after the final BrdU injection (6 weeks after injury). This involved a first screening whereby every BrdU$^+$ cell was inspected for evidence of double labelling, followed by a second level of screening where double labelling was confirmed by confocal analysis, including orthogonal reconstruction on the z-axis (Fig. 3E and F).

Comparison of the total BrdU$^+$/NeuN$^+$ cell counts revealed that the lvGFP control group (86.6 ± 48.4 cells) was not significantly different in newborn mature neurons compared to lvMeteorin treated animals (88.5 ± 32.6 cells), but a significant increase was detected in the lvGDNF group (696 ± 116.2 cells, p = <1 x 10$^{-4}$, one-way ANOVA, Bonferroni post-hoc). To account for variability in BrdU dosage and striatal volume between animals and to assess the proportion of newborn cells within the striatum acquiring a mature neuronal phenotype, we normalized the total number of BrdU$^+$/NeuN$^+$ cells to BrdU$^+$ cell density (BrdU$^+$ cells/mm$^3$) to acquire a percentage (Fig. 3B). In lvGFP-treated controls a low percentage of newborn cells post-lesion obtained a NeuN$^+$ phenotype (5.3 ± 0.2 x 10$^{-2}$ % of BrdU$^+$ cells/mm$^3$). This was not
significantly different to lvMeteorin-treated animals (8.5 ± 0.4 x 10^-2 % of BrdU+ cells/mm³) but in the lvGDNF-treated group, an 11-fold increase in the percentage of BrdU+/NeuN+ cells was detected (47.1 ± 0.7 x 10^-2 % of BrdU+ cells/mm³, p = <1 x 10^-4, one-way ANOVA, Bonferroni post-hoc) (see Fig. 3E-F for examples of immunohistochemistry).

Immunohistochemical co-labelling of BrdU+/NeuN+ cells with markers for striatal projection neurons (Darpp32) or certain interneuron subtypes (Calretinin) allowed us to assess the impact of Meteorin or GDNF over-expression on the phenotype of newly generated neurons following injury. Although previous studies have indicated a proportion of new neurons generated after injury acquire a Darpp32+ identity at (Arvidsson et al., 2002; Parent et al., 2002), we did not observe any Darpp32+/BrdU+ cells across the three treatment groups. Some of the BrdU+/NeuN+ cells were found to express Calretinin. Looking at BrdU+/NeuN+/Calretinin+ cells as a percentage of total BrdU+ cells/mm³, there was no significant difference across the three treatment groups although there was a trend towards a greater proportion in the lvGDNF group (6.5 ± 0.2 x 10^-2 % of BrdU+ cells/mm³) compared to the lvGFP control group (2.4 ± 0.1 x 10^-2 % of BrdU+ cells/mm³; p = 0.069, one-way ANOVA, Bonferroni post-hoc) – Fig. 3C and G. Similarly, as a percentage of total newborn neurons (BrdU+/NeuN+ cells) we observed similar proportions of cells obtaining Calretinin+ phenotype between the lvGFP (20.8 ± 12.7 % of cells), lvMeteorin (19.6 ± 7.7 % of cells) and lvGDNF groups (16.7 ± 6.4 % of cells) – Fig. 3D. We also observed across all groups numerous examples of BrdU+/Calretinin+ cells with aspiny neurites that did not express NeuN within the damaged striatum (Fig. 3H). This may be indicative of a more immature population of newborn interneurons.
Figure A1.3: Birth-dating of striatal neurons after quinolinic acid lesion to assess the effect of GDNF and Meteorin on these populations. (A) Quantification of BrdU+ cell density across treatment groups revealed no significant change in newborn cells in the striatum. (B) Quantification of BrdU+ cells co-expressing NeuN across treatment groups normalized to BrdU+ cell density revealed lvGDNF increased the number of new neurons in the ipsilateral striatum, with lvMeteorin having no effect compared to lvGFP controls. (C) Quantification of BrdU+/NeuN+/CalR+ cells normalized to BrdU+ cell density identified lvGDNF treatment trended towards an increase in this population. (D) CalR+ cells normalized to total BrdU+/NeuN+ cells indicated differentiation into this phenotype is altered
across all newborn neurons. Overview image of BrdU/NeuN/CalR staining (E) and representative orthogonal z-stack confocal images of immuno-labelled (F) BrdU+/NeuN+/Darrp32- neurons and (G) BrdU+/NeuN+/CalR+ interneurons (arrow). (H) NeuN negative interneurons were also observed as a primary subset of BrdU+/Calretinin+ cells (arrows). Data represents mean ± SEM, **** P < 1 x 10^-4). lvGFP n = 7; lvMeteorin n = 6; lvGDNF n = 5. Scale bars: 100 µm. cc: corpus callosum, lv: lateral ventricle, ac: anterior commissure, CalR: Calretinin.

**A1.4.4. Meteorin increases gliogenesis following striatal damage.**

Co-labelling of BrdU+ cells with s100β or Olig2 showed that Meteorin over-expression significantly increased the proportion of BrdU+ cells with astrocyte and oligodendroglial progenitor phenotypes, respectively.

Six weeks after injury, quantification of BrdU+/s100β+ cells in the lvGFP control group showed that, as a fraction of BrdU+ cell density, 1.3 ± 0.3 % were identified as s100β+ astrocytes (Fig. 4A and C). One-way ANOVA showed that the average fraction of BrdU+ cells co-labelled with s100β was not significantly different in the lvGDNF group (0.9 ± 0.3 %) but was significantly greater in animals treated with lvMeteorin (4.6 ± 1.5 %; p = 0.048, one-way ANOVA, Bonferroni post-hoc) – Fig. 4C.

To look at the effects of treatment on oligodendrocyte lineage cells, cells were co-labelled for BrdU+ with the pan oligodendrocyte marker Olig2 and the post-mitotic oligodendrocyte marker APC. In all treatment groups we observed examples of BrdU+/Olig2+ cells that were both positive and negative for APC expression, indicative of oligodendrocyte progenitors (BrdU+/Olig2+/APC-) and more mature oligodendrocytes (BrdU+/Olig2+/APC+), respectively (Fig. 4B). As a percentage of BrdU+ cell density, lvMeteorin significantly increased the fraction of Olig2+/APC- oligodendrocyte progenitors (8.1 ± 0.8 %) compared to lvGFP-treated animals (3.3 ± 1 %; p = 0.04, one-way ANOVA, Bonferroni post-hoc), while there was no significant change between the lvGFP control group and the lvGDNF group (5.1 ± 2 %) – Fig. 4D. This equated to a significantly greater total number of BrdU+/Olig2+/APC- oligodendrocyte progenitors in the anterior striatum of lvMeteorin treated animals (8.3 ± 0.8 x 10^3 cells), compared to the lvGFP (3.1 ± 0.9 x 10^3 cells) and lvGDNF groups.
(6.2 ± 2.2 x 10^3 cells; lvGFP v lvMeteorin, p = 0.03, one-way ANOVA, Bonferroni post-hoc).

We then assessed whether the Meteorin mediated increase in newborn oligodendrocyte progenitors translated to a greater increase in differentiation towards post-mitotic (APC+) oligodendrocytes. To do this, we assessed the fraction of BrdU+/Olig2+ cells that adopted an APC+ phenotype. In the lvGFP group, an average of 13.4 ± 4.3 % of total BrdU+/Olig2+ cells co-expressed APC. Comparison between treatment groups revealed that there was no significant difference between lvGFP animals and lvMeteorin (8.0 ± 1.5 %) or lvGDNF animals (9.9 ± 3.1 %; p = 0.71, lvGFP v lvMeteorin; one-way ANOVA, Bonferroni post-hoc) – Fig. 4E. This result was consistent when comparing the average total BrdU+/Olig2+/APC+ cell number between groups (n.s., p = 0.55, data not shown; lvGFP v lvMeteorin, one-way ANOVA, Bonferroni post-hoc).

We assessed the effects of Meteorin treatment on microglia proliferation using the microglia marker Iba1 in conjunction with BrdU (Fig. 4F). In the lvGFP treatment group, 36.8 ± 1.8 % of BrdU+ cells were Iba1+, indicating that this is the predominant phenotype of newborn cells post-injury (Fig. 4G). To determine whether Meteorin or GDNF treatment influenced microglia proliferation after striatal injury, Iba1/BrdU quantification was conducted across these groups. Compared to the lvGFP control group, no significant difference was observed in the percentage of Iba1+/BrdU+ cells in the lvMeteorin groups (49.3 ± 8.2 % of BrdU+ cells; p = 0.27) and the lvGDNF group (31.1 ± 5 % of BrdU+ cells; p = 0.72; one-way ANOVA, Bonferroni post-hoc) – Fig. 4G indicating that these factors did not influence microglia proliferation post-striatal injury.
Figure A1.4: Birth-dating of glial cells in the striatum after quinolinic acid lesion and assessment of the effect of Meteorin on these populations. (A) Confocal orthogonal representation of cells in the striatum ipsilateral to lentiviral delivery and lesion confirm double labelling of BrdU+ cells co-stained for either s100β to identify new born astrocytes or (B) Olig2 and APC to identify newborn OPCs (BrdU+/Olig2+/APC- cells) and mature oligodendrocytes (BrdU+/Olig2+/APC+ cells; boxed insets detail double and triple labelling of the cells). Newborn glial cells were quantified and represented as a percentage of total BrdU+ cell density. (C) Cell quantification of BrdU+/s100β+ cells normalized to BrdU+ cell density revealed lvMeteorin-treated animals had significantly more newborn s100β+ astrocytes in the ipsilateral striatum compared to lvGFP controls. For Oligodendroglial lineages, (D) BrdU+/Olig2+/APC- (OPCs) revealed a significant increase in this population in lvMeteorin-treated animals compared to lvGFP-treated controls. (E) The percentage of BrdU+/Olig2+ cells that express APC (mature OLs) was assessed and was not significant between treatment groups. Oligodendrocyte lineage cell quantification was normalized to BrdU+ cell density. (F)
Representative BrdU/Iba1 stain with example BrdU+/Iba1+ cell (boxed) and (G) quantification of BrdU+/Iba1+ cells as a percentage of total BrdU showed no significant difference between treatment groups. Data represents mean ± SEM, lvGFP n = 7; lvMeteorin n = 6; lvGDNF n = 5 for astrocyte and oligodendrocyte quantification and n = 3 for microglia cell counts across all groups. Scale bar: 100μm. OPC: Oligodendrocyte precursor cell, OL: Oligodendrocyte.

A1.5. Discussion

These results show that lentiviral delivery of Meteorin to the damaged striatum leads to robust over-expression of the diffusible protein resulting in increased gliogenesis but not neurogenesis in response to striatal injury. Cell proliferation is a well-established response to acute brain injury (Cavanagh, 1970; Nait-Oumesmar et al., 1999) and it has been convincingly demonstrated that the majority of the newborn cells are local microglia (Marty et al., 1991; Amat et al., 1996) which we have shown accounts for 40% of newborn cells post-injury. Importantly, it has also been shown that, following damage to the striatum, a small proportion of the newborn cells at the site of injury are neurons that migrate from the adjacent SVZ (Arvidsson et al., 2001; Parent et al., 2002). This has led to the idea that augmentation of this neurogenic response might lead to regenerative therapies for brain repair.

A number of studies have significantly improved the level of injury-induced striatal neurogenesis through delivery of growth factors, either as protein infusions (Pencea et al., 2001; Kobayashi et al., 2006; Ninomiya et al., 2006) or using viral vectors (Chmielnicki et al., 2004; Benraiss et al., 2012; Yu et al., 2013). Here we chose to investigate Meteorin in this context based on previous work showing that it is up-regulated in the striatum following injury (Jorgensen et al., 2011; Wang et al., 2012; Lee et al., 2015), can protect striatal neurons from excitotoxic death (Jorgensen et al., 2011) and is a potent chemokinetic regulator of migratory doublecortin+ neuroblasts in the striatum after middle cerebral artery occlusion (MCAO) (Wang et al., 2012). Although immunohistochemical analysis showed robust and diffuse over-expression of Meteorin throughout the lesioned striatum, there was no significant impact on the number of new striatal neurons generated in the first week after injury relative to the lvGFP control group. This is in contrast to a similar study recently reporting that Meteorin
infused as a protein over two weeks following medial cerebral artery occlusion significantly increases the number of newborn neurons in the striatum by around 70% (Wang et al., 2012). The conflicting findings might be at least partly explained by experimental variables, including differences in the timing and dose of Meteorin delivery and also the severity of striatal damage. Notably, however, over-expression of GDNF using the same lentiviral construct delivered at the same time relative to injury resulted in a significant increase in the number of new neurons compared to the Meteorin and control groups.

We included a GDNF group in order to contextualize the impact of Meteorin. GDNF is well established as a neurotrophic factor in models of CNS injury and has previously been shown to increase the number of newborn cells that survive as mature neurons in the striatum after striatal injury (Kobayashi et al., 2006). We observed an 11-fold increase in the number of mature striatal neurons after striatal injury in GDNF treated animals, but no significant difference in Meteorin treated animals under the same experimental conditions. Collectively, the results suggest that the neurogenic impact of Meteorin following striatal damage is modest and may be relatively more sensitive to factors related to timing and dose as well as the nature of the injury. Understanding the efficacy of neurotrophic proteins in specific injury models is an important challenge for developing regenerative gene-based therapies for CNS injury.

Another potential role for neurotrophic factors in modulating the neurogenic response to injury is through impact on the differentiation of newborn cells towards therapeutically relevant cell types - for example striatal projection neurons that can replace those lost after damage. Based on immunohistochemistry for Darppp32 six weeks after lesioning, no examples of new striatal projection neurons were found across any of the three treatment groups. However, a number of BrdU+/NeuN+ cells were found to acquire a Calretinin+ interneuron phenotype. These findings are consistent with a recent study showing that striatal injury can facilitate migration of nearby SVZ neuroblasts to the site of injury but does not influence their original, interneuron differentiation potential (De Marchis et al., 2007). Neither GDNF nor Meteorin influenced the fraction of BrdU+/NeuN+ cells to adopt a Calretinin+ identity. Thus the mechanism underlying greater neuronal numbers in the GDNF group is
likely to be related in increased survival rather than an influence on differentiation potential. We also observed many BrdU+/Calretinin+ cells that did not express NeuN. This may represent a different Calretinin+ phenotype or reflect an immature status of these cells, as has been shown in studies on staging of protein expression in hippocampal neurogenesis (Brandt et al., 2003). It is important to note that the majority of BrdU+/NeuN+ cells did not express Calretinin or Darpp32. This may reflect an immature phenotype within the newborn population of striatal neurons. However, previous studies have shown that insult-mediated neurogenesis in the striatum produces other interneuron phenotypes characterized by the markers parvalbumin and neuropeptide Y (Collin et al., 2005) which may also account for the remaining newborn neurons.

The most prominent impact of Meteorin over-expression on newborn cells generated after injury was a significant increase in the proportion of glial cell types, including s100β+ mature astrocytes. This is consistent with previous in vitro findings using primary cultures, where Meteorin has been shown to act through the Jak-STAT3 pathway to increase astrocyte differentiation (Nishino et al., 2004; Lee et al., 2010; Sun et al., 2014). We have previously reported that Meteorin is up-regulated in s100β cells at the site of injury after excitotoxic lesioning of the striatum. Taken together, these data suggest that Meteorin may act as a paracrine signalling molecule to drive gliosis via differentiation in response to brain damage as opposed to acting as a trophic factor for these cells. We also looked at microglial phenotype amongst newborn cells. Iba1+ microglia accounted for ~40% of all newborn BrdU+ cells post injury. This is in line with previous literature which describes an early and rapid proliferative response of microglia after acute injury in the CNS (Morioka et al., 1993; Topper et al., 1993; Thored et al., 2009; Taylor and Sansing, 2013). The lack of impact on the proportion of BrdU+ microglial cells suggests a specific function for Meteorin in astrocyte differentiation rather than a broad exacerbation of inflammatory response to injury.

Interestingly, Meteorin also increased the number of oligodendrocyte progenitor cells (OPCs). Here we report that Meteorin significantly increases the number of Olig2+ OPCs in the striatum following excitotoxic damage. This reveals a novel biological function for Meteorin in vivo and indicates therapeutic potential in
pathological settings involving loss of oligodendrocytes - for example the
demyelination of neurons resulting from loss of oligodendrocytes after ischemic stroke
(Pantoni et al., 1996; Dewar et al., 2003). Notably, while the OPC pool increased in
Meteorin treated animals, this did not translate to an increase in mature APC+
oligodendrocytes. This may reflect that additional signalling components are required
for maturation in the lesioned striatum and/or an unfavourable environment for
differentiation. In future studies it will be valuable to assess and compare the
oligogenic effects of Meteorin with other molecules reported to drive
oligodendrogliogenesis after injury, such as Erythropoietin (Zhang et al., 2010) and
Noggin (Irvin et al., 2008) as well as the mechanism driving this increase in OPCs to
determine whether Meteorin influences the proliferation and differentiation of this
population or offers trophic support to OPCs. Assessment of the timing and
combinatorial delivery of these molecules may reveal an optimal approach for
increasing both the number of oligodendrocytes and their capacity for myelination
following de-myelinating injuries. Other valuable future studies to extend on the
present findings would include lineage tracing work aimed at identifying the cells that
respond to MTRN in order to drive gliogenesis. There is strong evidence to suggest
that the neurogenic response in a variety of striatal injury models, including MCAO
(Arvidsson et al., 2002), QA lesioning (Collin et al., 2005) and traumatic brain injury
(Richardson et al., 2007) is largely provided by the nearby pool of neurogenic
precursors in the SVZ. The origin of the gliogenic response is less well characterised
but may include both an SVZ component – for example OPCs can be generated by SVZ
precursors and migrate into the striatum following stroke (Li et al., 2010) – as well as
the proliferation of local parenchymal progenitors or differentiated glia at the site of
injury.

In summary, we demonstrated that Meteorin was able to increase astrogenesis in
the striatum after acute injury characterized by widespread neuronal cell loss. We also
revealed a novel function for Meteorin in significantly increasing the number of OPCs
post-injury. Interestingly, Meteorin did not influence the maturation of these cells into
mature oligodendrocytes, suggesting a discrete function on the OPC population.
Meteorin was not effective for augmentation of the neurogenic response to injury.
These findings highlight that development of strategies for brain-repair based on trophic-factor delivery will likely require combinatorial approaches, where specific proteins are targeted to affect the survival and differentiation of specific cell types.

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A1.6. References


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