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ON ASTROCYTES AND NEURAL STEM CELLS: A STUDY OF REACTIVE AND CANONICAL NEUROGENESIS

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Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-447-4

Cover illustration: Sagittal section of the adult mouse brain illustrating sites of canonical neurogenesis. Astrocytes are labelled in red and doublecortin-expressing neuroblasts are in green. All cell nuclei are marked in blue.

On Astrocytes and Neural Stem Cells: A Study of Reactive and Canonical Neurogenesis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Public Defense on **Friday**, **January 14th, 2022** at **09:00**
At **Eva & George Klein Biomedicum Lecture Hall**, Solnavägen 9, Solna

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To Oliver.

I hope you'll find your passions in life.
And as Nonno and Nonna did for me,
I will do everything in my power to help you chase your dreams.

POPULAR SCIENCE SUMMARY OF THE THESIS

The mammalian brain and spinal cord are particularly inefficient at repairing themselves after injury. A traumatic lesion to the brain can lead to extensive death of neurons, which are never replaced. The poor regenerative capacity of these cells is at the basis of the lifelong neurological symptoms seen in patients who suffered brain damage. Thus, the ultimate objective of regenerative medicine approaches is to promote replacement of the cells lost to injury in order to aid recovery of function. Several sources for neuronal cell replacement are currently being explored. Transplantation of cells derived from external sources is showing promising results in pre-clinical settings and is now being tested in patients. This approach, however, comes with risks and warrants research towards attempting to recruit and repurpose cells that are already found in the injured brain. Small populations of neural stem cells that generate new neurons throughout life do exist, but their restricted population size limits the extent to which these cells can be utilized for replacing vast number of lost neurons. Astrocytes represent an attractive alternative for the generation of new neurons after injury because they are abundantly found throughout the brain and share several molecular and functional characteristics with adult neural stem cells. Work presented in this thesis aims at demonstrating that astrocytes can be considered as a latent source of neural stem cells, which can be recruited under the right conditions to replace lost neurons. We, furthermore, provide extensive characterization of neural stem cells and their progeny (i.e., neural progenitors and immature neurons) from the human brain, which will, in the future, represent a valuable resource to identify what cellular mechanisms could be targeted in order to promote more efficient regenerative repair.

ABSTRACT

The mammalian central nervous system has limited regenerative capacity and the long-lasting functional impairment resulting from trauma and neurodegenerative disease derives from a failure to repopulate neuronal cell populations that are lost to injury. The studies included in the present thesis aim at proposing astrocytes as a novel source for achieving widespread replacement of neurons. Central to this work are notions within the field of astrocyte biology, adult neurogenesis, and regenerative medicine, which are discussed in the literature review.

Astrocytes are an abundant cell population that supports neuronal development, survival and activity in health and participate in inflammation and injury resolution in disease. Learning about astrocyte biology allows us to develop tools for selectively targeting these cells or their specific functions. It will also help us learn about heterogeneous subtypes with distinctive functional properties or differential potential for regenerative repair.

Adult neurogenesis is the process through which new neurons are added throughout life to a pre-existing neural circuitry. This process has been extensively explored in model organisms, but it has been challenging to achieve the same level of resolution and detail for the study of human neurogenesis. It is nevertheless important to identify and characterize neurogenic cells in the human brain and investigate their functional impact on cognition, as well as how their dysregulation may be linked to neurodegenerative and psychiatric diseases. In the context of this thesis, learning about the molecular dynamics driving neurogenesis allowed us to demonstrate that astrocytes, once recruited, unfold a process of differentiation like that seen in neural stem cells (**Paper I** and **Paper II**). We, furthermore, profiled the transcriptome of young and adult human hippocampal cells and depicted, for the first time, a comprehensive molecular framework that describes the neurogenic program in the human dentate gyrus (**Paper III**).

Cell replacement therapies are obtaining promising results in pre-clinical settings and are starting to be successfully used in patients to replace specific cell populations depleted due to neurodegeneration or injury. The present thesis discusses the two main strategies to approach cell replacement, namely transplantation and recruitment of endogenous cells with stem cell properties. The latter has gained momentum in recent years to overcome limitations characteristic of cell transplantation. Work within this thesis is aimed in this direction and provides evidence that parenchymal astrocytes can be considered latent neural stem cells and can be recruited to replace neurons after injury (**Paper I** and **Paper II**).

From the methodological point of view, genomics technologies and computational approaches are important concepts to this thesis and have been applied, here, to study canonical and reactive neurogenesis. Published single-cell omics data across organs, developmental times, and species have enabled comprehensive investigations of cell processes and interactions that had remained until now elusive. Here, we leveraged transcriptome-wide analysis to investigate the molecular dynamics underlying astrocyte-mediated neurogenesis in the striatum (**Paper I**) and the cortex (**Paper II**), and to study human hippocampal neurogenesis with an unparalleled

level of detail, which we used to identify markers of neural progenitors and to compare molecular processes across species (**Paper III**).

LIST OF SCIENTIFIC PAPERS

I. Magnusson, J. P., **Zamboni, M.***, Santopolo, G.*, Mold, J. E., Barrientos-Somarribas, M., Talavera-Lopez, C., Andersson, B., & Frisen, J. (2020). Activation of a neural stem cell transcriptional program in parenchymal astrocytes. *Elife*, 9. doi:10.7554/eLife.59733

II. **Zamboni, M.**, Llorens-Bobadilla, E., Magnusson, J. P., & Frisen, J. (2020). A Widespread Neurogenic Potential of Neocortical Astrocytes Is Induced by Injury. *Cell Stem Cell*, 27(4), 605-617. doi:10.1016/j.stem.2020.07.006

III. Dumitru I.*, Paterlini M.*, **Zamboni M.‡**, Ziegenhain C.‡, Tata M., Giatrellis S., Alkass K., Druid H., Sandberg R., & Frisén J. Delineation of a neurogenic cell trajectory in the human hippocampus (*Manuscript*)

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Mold J. E.*, Modolo L.*, Hård J.#, **Zamboni M.**#, Larsson A. J. M., Stenudd M., Eriksson C. J., Durif G., Ståhl P. L., Borgström E., Picelli S., Reinius B., Sandberg R., Réu P., Talavera-Lopez C., Andersson B., Blom K., Sandberg J. K., Picard F., Michaëlsson J., Frisén J. (2021). Divergent clonal differentiation trajectories establish CD8+ memory T cell heterogeneity during acute viral infections in humans. *Cell Rep*, 35(8):109174. doi: 10.1016/j.celrep.2021.109174

II. Kvastad, L., Carlberg, K., Larsson, L., Villacampa, E. G., Stuckey, A., Stenbeck, L., Mollbrink, A., **Zamboni, M.**, Magnusson, J. P., Basmaci, E., Shamikh, A., Prochazka, G., Schaupp, A. L., Borg, A., Fugger, L., Nister, M., & Lundeberg, J. (2021). The spatial RNA integrity number assay for in situ evaluation of transcriptome quality. *Commun Biol*, 4(1), 57. doi:10.1038/s42003-020-01573-1

III. **Zamboni, M.**, Santopolo, G., & Frisen, J. (2020). Induction of Leptomeningeal Cells Modification Via Intracisternal Injection. *J Vis Exp* (159). doi:10.3791/61009

IV. Llorens-Bobadilla, E., Chell, J. M., Le Merre, P., Wu, Y., **Zamboni, M.**, Bergenstrahle, J., Stenudd, M., Sopova, E., Lundeberg, J., Shupliakov, O., Carlen, M., & Frisen, J. (2020). A latent lineage potential in resident neural stem cells enables spinal cord repair. *Science*, 370(6512). doi:10.1126/science.abb8795

CONTENTS

LIST OF ABBREVIATIONS

AAV	Adenoassociated virus
Aqp4	Aquaporin 4
Ascl1	Achaete-Scute Family bHLH Transcription Factor 1
BMP	Bone morphogenic protein
CA1-3	Cornu ammonis
Dlk-1	Delta Like Non-Canonical Notch Ligand 1
Dlx2	Distal-Less Homeobox 2
Egf	Epidermal growth factor
GABA	Gamma-aminobutyric acid
Gfap	Glial fibrillary acidic protein
hESCs	Human embryonic stem cells
Igf1	Insulin-like growth factor 1
Il6	Interleukin 6
iPSCs	Induced pluripotent stem cells
Mki67	Marker of proliferation Ki-67
NeuroD1	Neuronal Differentiation 1
Neurog2	Neurogenin 2
NG2	Neural/glial antigen 2
Pax6	Paired box 6
Pcna	Proliferating Cell Nuclear Antigen
PSA	Polysialic acid
scRNA-seq	Single-cell RNA-sequencing
Shh	Sonic hedgehog
snRNA-seq	Single-nucleus RNA-sequencing
Stat3	Signal Transducer and Activator of Transcription 3
TGFb	Transforming growth factor beta
UMAP	Uniform Manifold Approximation and Projection

1 INTRODUCTION

The mammalian central nervous system has limited regenerative capacity following injury, and damage can lead to functional impairment that is never fully overcome. Populations of specialized astrocytes that act as adult neural stem cells exist in specific regions of the mammalian brain, where they contribute to lifelong addition of new neurons, through a process called adult neurogenesis (Doetsch et al., 1999; Kriegstein and Alvarez-Buylla, 2009). These cells can also respond to injury by increasing their proliferation and migrating towards sites of damage (Arvidsson et al., 2002; Gotts and Chesselet, 2005; Kaneko et al., 2018; Yan et al., 2018). Their contribution to regenerative repair is, however, very limited due to their small population size. Parenchymal astrocytes, on the other hand, make up about half of all neural cells and they are found throughout the brain and spinal cord. Our studies in **Paper I** and **Paper II** have shown that parenchymal astrocytes may act as latent neural stem cells whose neurogenic potential can be unlocked following injury or through genetic manipulation of signaling pathways known to be implicated in neuronal cell fate specification. Our work suggests that astrocytes may be a valuable endogenous resource for replacing neurons lost to injury and that learning the molecular profiles of astrocytes committed to the lineage transition can help us harness a greater regenerative potential. The present thesis discusses the importance of astrocyte biology in health and disease, and it goes on to explore the body of research pertaining to adult neurogenesis. Finally, it reviews current strategies to leverage our knowledge in the field of astrocyte and neural stem cell biology in order to promote regenerative repair in the injured brain.

Astrocytes undertake a wide range of functions in the intact brain that ensure wellbeing and correct activity of neuronal cells (Barres, 2008). Interestingly, there is accumulating evidence suggesting that, similar to the diverse collection of neuronal subtypes, astrocytes, too, are a widely heterogeneous population (Bayraktar et al., 2020; Khakh and Deneen, 2019). This raises questions of whether all astrocytes are equally capable of displaying the full repertoire of glial functions. Particularly relevant for the present research, is the question of whether all astrocytes retain the potential to generate neurons. Interestingly, we have detected some differences in the stimuli required to activate the neurogenic program between subcortical (**Paper I**) and cortical astrocytes (**Paper II**). Nevertheless, we were able to demonstrate a widespread potential to generate neurons, which is preserved in astrocytes from different cortical regions, as well as throughout the cortical layers (**Paper II**). Astrocytes also partake during injury and disease where they undergo morphological changes and gain functions of reactive cells (Sofroniew, 2020). In the context of traumatic injury, astrocytes proliferate and contribute to the formation of a scar to contain damage (Wahane and Sofroniew, 2021). Other aspects of gliosis are, instead, displayed in infections and neurodegenerative diseases, such as the release of neurotoxins that kill the neighboring neurons (Guttenplan et al., 2021; Liddelow et al., 2017). Our analysis explored the molecular profiles of astrocytes as they commit to the neuronal lineage fate and revealed that neurogenesis in the injured cortex unfolds independently of reactive gliosis (**Paper II**). Astrocytes that display reactive morphological and transcriptional

changes are, in fact, rarely associated with the activation of pro-neuronal transcription factors, which suggests that the neurogenic program may be hindered in this cell state.

Neural stem cells are specialized astrocytes (Kriegstein and Alvarez-Buylla, 2009) that exist throughout life but are restricted to two small regions of the mammalian brain, namely the subventricular zone and the dentate gyrus of the hippocampus (Gould, 2007). Stem cells in the subventricular zone give rise to migratory neural progenitors that innervate distinct regions in different mammalian species. Mouse neuroblasts, for instance, target the olfactory bulb (Belluzzi et al., 2003), whereas human neuroblasts mature into striatal interneurons (Ernst et al., 2014). The lower reliance of humans on olfaction may explain why neuroblasts have been redirected to the striatum, a region of the brain important for movement coordination, but also higher cognitive functions and affection regulation. In the hippocampus, adult-born granule neurons that are added to the dentate gyrus are crucial for memory formation and learning (Akers et al., 2014; Clelland et al., 2009). Although adult hippocampal neurogenesis has been shown across mammalian species, and their occurrence in humans validated using several independent measurements, a recent study has raised controversy proposing that human neurogenesis would not endure into adulthood (Sorrells et al., 2018). Demonstration of neurogenesis relied on the ability to detect specific marker proteins expressed in immature neurons. These proteins are, however, quickly degraded with increasing postmortem delay and protocols used to visualize them can be complex, particularly when using specimens from adult brains (Kempermann et al., 2018). Thus, failure of this study to demonstrate the presence of neuroblasts rather reflects the technical challenges characteristic of working with human postmortem tissue and expresses the need for finding additional ways to follow the addition of new neurons to the hippocampus. In **Paper III**, we used single-nucleus RNA-sequencing (snRNA-seq) to profile cells of the human dentate gyrus across ages and provided an in-depth characterization of the neurogenic trajectory. Furthermore, we compared neurogenic programs between human and mouse, to learn the extent to which the molecular profiles of neural progenitors overlap. Our study will provide a resource for better understanding the differentiation trajectory of human neural stem cells and expand the collections of cell type markers to detect the presence of neurogenic cells in the human brain. Importantly, transcriptomics analysis offers a more complete molecular framework that characterize neural stem cells and their lineage commitment. This, in turn, may allow us to identify potential regulators of neurogenesis that could be targeted to unlock the neurogenic potential of facultative stem cells, such as parenchymal astrocytes, to promote regeneration in widespread areas of the brain after injury.

Brain injuries and neurodegenerative diseases display complex and distinct pathophysiological profiles but tend to share aspects of neuronal death that are at the basis of their long-lasting functional impairment. Efforts of regenerative medicine are aimed at replacing cells lost to injury in the hope to achieve recovery of function. Two main strategies are explored towards this end, namely the replacement of neurons through cell transplantation and recruitment of resident cells with regenerative potential. Transplantation approaches have proven promising and are currently being explored in clinical trials (Parmar et al., 2020). There are, however,

limitations and risks associated with these therapies, which have encouraged efforts towards trying to harness the regenerative potential of endogenous cells (Parmar et al., 2020). Our work in **Paper I** and **Paper II** moves in this direction, proposing that astrocytes may be a valuable alternative source of latent stem cells that can generate new neurons in the injured brain. Strategies that leverage endogenous sources for cell replacement overcome some of the shortcomings of cell transplantation, but there is still a lot of work to be done to (1) improve efficiency of the conversion from the original cell; (2) demonstrate integration of the newly generated neurons and functional impact of their contribution; and (3) develop safe and effective therapeutic strategies to recruit the latent stem cells into regenerative programs. Single-cell transcriptomic analysis can be leveraged to explore the molecular dynamics driving lineage fate switch and instruct search for regulators of this phenomenon (Kamimoto et al., 2020). Particularly interesting, in this sense, is the study of cells that fail to turn on the neurogenic program, and comparing these to successfully activated cells can help identifying factors we can target to achieve greater recruitment. In **Paper I**, we show that many striatal astrocytes initiate upregulation of pro-neuronal programs but fail to commit to the transition before entering cell division. Forcing cells into the cell cycle was, however, sufficient to allow them to complete the transition. Gene editing tools, such as the CRISPR-Cas9 system, and virus-mediated delivery of genetic material hold great promises for the development of therapeutic strategies to achieve specific and efficient gene expression manipulation (Dahlman et al., 2015; Zhou et al., 2018). To promote replacement of neurons lost to injury, these technologies can be adapted to drive expression of the regulators of cell fate switch (Liu et al., 2018; Russo et al., 2021), in order to activate regenerative programs in larger numbers of resident cells and, ultimately, achieve greater recovery (Zhou et al., 2020).

2 ASTROCYTES IN HEALTH AND DISEASE

> *"What could hold these tiny, isolated, flexible, very delicate cells, much more delicate and smaller than the nerve cells?"*
>
> — Ramón y Cajal, 1895

The neuroscience community has made enormous progress at characterizing the wide range of physiological functions undertaken by astrocytes in the central nervous system. Originally described as a rather inert, uninteresting cell type that keeps the brain together structurally, as the term 'glia' (from Greek, "glue"), coined by Virchow in 1856, implies, we have now learned that astrocytes are instrumental for survival and correct functioning of the neighboring neurons (Barres, 2008). Early investigations have demonstrated their trophic support of neurons when cultured together (Banker, 1980). Since then, they have also been implicated in synaptic transmission via uptake of glutamate from the synaptic cleft and maintenance of ionic balance, which tunes the neurons' membrane potential (Seifert et al., 2018; Verkhratsky and Nedergaard, 2018; Verkhratsky et al., 2020). Furthermore, they actively participate in neuronal communication, responding to synaptic activity through alterations in cytosolic calcium concentrations (Fischer et al., 2020; Verkhratsky et al., 2012) and release of gliotransmitters to signal neighboring neurons (Araque et al., 2014). Astrocytes are also crucial, during development, for the establishment of connections by providing molecular cues, such as thrombospondins, that drive synaptogenesis, as well as instructing axonal growth and path finding via secretion of cytokines (Molofsky et al., 2012). The dynamic regulation of synaptic connectivity, as well as the ability of specialized astrocytes to generate new neurons, is retained in the adult brain where they contribute, for instance, to hippocampal plasticity (Harris et al., 2021; Lee et al., 2021). With their end-feet enwrapping blood vessels (Foo et al., 2011), astrocytes are also known for regulating blood flow (Beiersdorfer et al., 2019; Otsu et al., 2015) and establishing the blood-brain barrier, which ultimately contributes to a finely regulated metabolic and ionic supply to the parenchymal environment (Abbott et al., 2006). Considering the wide range of astroglial functions that support neurons, it is perhaps not surprising that malfunctioning or reactive astrocytes may also have the power to trigger neurotoxicity and neurodegeneration (Liddelow and Sofroniew, 2019). For instance, astrocytes carrying a mutant form of SOD1, a gene linked to amyotrophic lateral sclerosis, are directly responsible for the degeneration of motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007). Similarly, chimeric mice harboring human glia with Huntingtin mutations have shown that mutant astrocytes alone can impart behavioral phenotypes typical of the disease, whereas healthy glia are able to rescue Huntington's symptoms in diseased mice (Benraiss et al., 2016). As described later on in this thesis, astrocytes that participate in the context of injury change their molecular and structural

profiles (Sofroniew, 2020) and may contribute to either inflammation and neuronal death or to repair, in a context-dependent manner (Hasel et al., 2021; Zamanian et al., 2012).

2.1 ASTROCYTE HETEROGENEITY

Appreciation of the wide range of astroglial functions has raised questions around the extent to which these are shared among astrocytes throughout the central nervous system. Are all astrocytes displaying the same functions? Or are there spatiotemporal differences in their commitment to brain physiology? For instance, there may exist regionally specialized glia whose functions are tailored to supporting specific subtypes of neurons (Kelley et al., 2018; Lanjakornsiripan et al., 2018). Additionally, within each region, astrocytes may be found in contact with, or separated from blood vessels (Foo et al., 2011). The so called juxtavascular astrocytes, with their cell body adjacent to blood vessels, originate from a pool of progenitors that is distinct from that of non-juxtavascular astrocytes (Bribian et al., 2016; Figueres-Onate et al., 2016) and present unique molecular and electrophysiological properties in the intact and injured brains (Gotz et al., 2021). Interestingly, even though they only make up 30% of the total parenchymal astrocytes, they are the major contributors of proliferating astrocytes (Bardehle et al., 2013; Frik et al., 2018; Sirko et al., 2013). Astroglial functions can also differ based on developmental time (Cahoy et al., 2008). Secretion of thrombospondins and semaforins, for example, are crucial for synaptogenesis and axon guidance during development, but they lose relevance as the neural circuit becomes established (Allen and Eroglu, 2017; Christopherson et al., 2005). Importantly, there also exist a subtype of specialized astrocytes that function as adult neural stem cells and generate new neurons that are added to the brain throughout the lifespan. This subtype of glia is discussed more extensively later in this thesis, in the context of adult neurogenesis.

The existence of heterogeneous subtypes within the astrocyte population has been postulated since the seminal work of Ramon y Cajal, who recorded morphological differences of astrocytes across brain structures (Navarrete and Araque, 2014). These differences are, for instance, evident between white and gray matter astrocytes, where glia present fibrous morphologies with few well-defined processes, or a ‘bushy’ appearance with numerous small processes, typical of protoplasmic astrocytes in the gray matter (Kettenmann and Verkhratsky, 2008). Further differences in morphologies are readily observable in the human brain, where gray matter astrocytes also encompass layer I intralaminar and layer V-VI polarized astrocyte subpopulations (Oberheim et al., 2006). Recently, systematic investigations have revealed more minute morphological differences among astrocytes from different cortical and subcortical regions (Lanjakornsiripan et al., 2018; Minge et al., 2021), as well as distinct developmental times (Minge et al., 2021). Paralleling these morphological aspects are also differences in the molecular profiles of astrocyte subtypes spanning the layers of the neocortex (Batiuk et al., 2020; Bayraktar et al., 2020), as well as region-specific differences in transcription factor expression profiles (Huang et al., 2020; Lozzi et al., 2020). Original investigations of astrocyte heterogeneity could report about distinctive morphological and molecular features, but it has been challenging to demonstrate that these phenotypes are

associated with heterogeneity of function. However, recent investigations are beginning to tackle the question of whether there exist functionally distinct subpopulations of glia. For instance, spinal cord astrocytes associated with a subtype of motor neurons express distinct levels of potassium channels, which appear to be necessary for specific contractile muscle behaviors (Kelley et al., 2018). Furthermore, other studies have recorded differences in calcium signaling across brain regions, with hippocampal astrocytes being more receptive to neuronal stimulation than striatal astrocytes (Chai et al., 2017), a property that may facilitate plasticity and synaptic potentiation (Adamsky et al., 2018). Recent development in multiomics technology, which surveys transcriptional and epigenetic profiles with unprecedented, genome-wide resolution and high-throughput, will further advance our understanding of functional diversity of astrocytes in health and disease (Cahoy et al., 2008; Hasel et al., 2021; Zeisel et al., 2018)

It is still unclear where the astrocyte's diversity is originating from. Interestingly, contrary to other glial cell types that are ontologically younger, it is possible to identify astrocyte-like cells in any organism with a central nervous system. Astroglia in the *C.Elegans* and the *Drosophila* express glutamate and gamma aminobutyric acid (GABA) receptors, and have been implicated in axon guidance, suggesting that they may play roles that are analogous to their mammalian counterpart (Rival et al., 2004; Soustelle et al., 2002). In simpler organisms, however, the astroglial population appears to be rather homogeneous. Throughout evolution, astrocyte subtypes have, instead, emerged in concomitance with the expanding neuronal subtype collection that characterizes the brain of higher organisms (Freeman and Rowitch, 2013). In fact, the growing number, complexity and diversity of astroglia that have paralleled the increasing breadth of nerve cell diversification, may have directly contributed to the enhanced cognitive capabilities characterizing the mammalian, and particularly, the human brain (Freeman and Rowitch, 2013). During development, astrocytes are mostly generated from ventricular zone radial glia, although contribution of marginal zone progenitors and neural/glial antigen 2 (NG2) glia has also been recorded (Ge et al., 2012; Huang et al., 2014), and they are first seen in the mouse brain after the neurogenic phase (i.e., around embryonic day 16)(Miller and Gauthier, 2007). The majority of cells populating the adult brain are, however, generated postnatally, from astrocyte precursors that proliferate extensively to give rise to glia that remain in close proximity (Rowitch and Kriegstein, 2010). The astroglial diversity may be encoded by cell autonomous factors, so that different astrocyte subpopulations are, for instance, generated from a diverse population of precursors. In the brain, this could be the case, for instance, for juxtavascular and pial astrocytes, which originate from distinct progenitors (Bribian et al., 2016; Figueres-Onate et al., 2016). In addition, thalamic astrocytes and neurons from distinct sensory nuclei are clonally related and share region-specific signatures, which have been shown to be retained even when astrocytes are reprogrammed into region-appropriate thalamic neurons using the same transcription factor overexpression protocol (Herrero-Navarro et al., 2021). Conversely, their differences may be dictated by surrounding environmental factors (Ben Haim and Rowitch, 2017) and neighboring cells, such as the neuronal subtypes they come in contact with (Kelley et al., 2018; Lanjakornsiripan et al., 2018). Interestingly, spinal cord

astrocyte progenitors are subjected to dorsoventral gradients of secreted factors, which determine their radial patterning and the establishment of subpopulation of white matter astrocytes in the mature spinal cord (Hochstim et al., 2008). Comprehensive fate-mapping studies combined with transcriptional analysis, which are currently being established (Ratz et al., 2021; Weinreb et al., 2020), will let us examine to what extent functional heterogeneity is developmentally determined.

Within the scope of this thesis, the question of astrocyte heterogeneity is relevant for better understanding the extent to which parenchymal astrocytes can be recruited to generate new neurons that contribute to regenerative repair after injury. As briefly introduced earlier and further discussed below, there exist specialized astrocyte populations that act as neural stem cells and produce new neurons throughout life. These cells are, however, rare and only restricted to two small regions of the brain, namely the subventricular zone and the dentate gyrus of the hippocampus (Gage, 2019; Gould, 2007). They can mount reactive responses after injury and increase proliferation (Faiz et al., 2015), but the extent to which they are able to contribute to regenerating neurons lost to injury is limited by their small population size (Arvidsson et al., 2002). By contrast, parenchymal astrocytes are numerous, making up 50% of all neural cells in the brain, and work contained in this thesis, as well as published by other laboratories (Nato et al., 2015), shows that they can be recruited into the neurogenic program (Magnusson et al., 2014; Magnusson et al., 2020; Zamboni et al., 2020). From our work is, however, evident that astrocytes display some differences to the extent to which they can reveal their neurogenic potential. Striatal astrocytes, indeed, readily generate neural progenitors after either exposure to an ischemic stroke or depletion of Notch signaling (Magnusson et al., 2014; Magnusson et al., 2020). By contrast, Notch signaling inhibition in the cortex is not sufficient to drive their cell fate conversion, which is only evident when animals are also subjected to an injury (Zamboni et al., 2020). On the other hand, our work in the cortex suggests that the same stimuli are able to recruit astrocytes beyond a specific cortical region, and that this potential can be displayed by glia across cortical layers (Zamboni et al., 2020). It remains to be established, however, whether the differences we have recorded are based upon cell-intrinsic factors or whether they are determined by the surrounding microenvironment. Seminal transplantation studies (Gage et al., 1995; Herrera et al., 1999) have suggested that the cortex may be a particularly hostile environment for survival of neural progenitors. This may hinder cell fate conversion of Notch-depleted astrocytes, which need instead additional stimulations in order to unlock their neurogenic program. Similarly, increased exposure to trophic blood- and cerebrospinal fluid-borne factors may give striatal astrocytes an advantage in their commitment to the neuronal lineage fate transition (Ihrie and Alvarez-Buylla, 2011; Sirko et al., 2013; Tavazoie et al., 2008).

2.2 REACTIVE GLIOSIS

Astrocytes have been known to respond to injury for over a hundred years, but their contributions have largely been neglected until recently. In their review from 2010, Sofroniew

and Vinters were among the firsts that attempted to offer a broad and comprehensive definition of astrocyte reactivity (Sofroniew and Vinters, 2010). Since then, a vast number of findings have brought considerable progress to the study of reactive astrocyte diversity (Hasel et al., 2021; Moulson et al., 2021; Sofroniew, 2020; Zamanian et al., 2012), the molecular regulators of reactivity (Escartin et al., 2021; Liddelow and Barres, 2017; Liddelow et al., 2017), as well as the functional contribution to the injury microenvironment and surrounding cellular communities (Guttenplan et al., 2021; Han et al., 2021; Sofroniew, 2020). Astrocyte reactivity is, now, considered to be a spectrum of molecular, functional, and structural changes that sometimes lead to cell proliferation (Sofroniew, 2020; Wilhelmsson et al., 2006; Zamanian et al., 2012). Their response to injury is heterogeneous and context-dependent, varying based on type and severity of the insult (Zamanian et al., 2012), as well as along spatiotemporal axes (Hasel et al., 2021; Li et al., 2014; Rakers et al., 2019). Molecular and cellular changes that are common across diseases are the upregulation of intermediate filament proteins, such as glial fibrillary acidic protein (Gfap), Vimentin, and Nestin, the astroglial action on the extracellular milieu, and the interaction with immune cells (Zamanian et al., 2012).

According to Sofroniew (2020), reactive astrocyte responses can be divided into two broad categories based on whether glia are recruited into cell division. Non-proliferative reactive astrocytes are seen in systemic inflammatory diseases and neurodegenerative conditions. They tend to maintain their territorial domain and engagement with the surrounding neuronal and glial cells. Proliferative astrocytes, on the other hand, are seen in traumatic injury and tend to be recruited to form borders against areas of fibrosis and tissue inflammation. The diverse reactive response may in part be attributed to intrinsic determinants, with proliferating astrocytes largely originating from juxtavascular astrocytes (Bardehle et al., 2013; Frik et al., 2018; Lange Canhos et al., 2021) or ependymal cells (Carlen et al., 2009; Sabelstrom et al., 2013). Notably, the lesion bordering response of the proliferative reactive glia has classically been understood as a scarring process with detrimental and anti-reparative characteristics. However, conditionally ablating genes implicated in structural changes of glial morphology, such as Gfap and Vimentin (Li et al., 2008b; Liu et al., 2014) or that regulate proliferation and migration of reactive cells, such as Stat3 (Signal Transducer And Activator Of Transcription 3) and Notch1, show that impaired border formation is linked to increased spread of inflammation and secondary damage, as well as greater neuronal death and functional impairment (Shimada et al., 2011; Wanner et al., 2013; Xie et al., 2020). Furthermore, unlike attenuation of the fibrotic scar, which facilitates regeneration of neuronal processes after spinal cord injury (Dias et al., 2018; Goritz et al., 2011), presence of astrocytes along the borders of the scar tissue does not seem to impede axonogenesis (Anderson et al., 2016; Anderson et al., 2018). In fact, recent studies by Chen and colleagues (2018) and Xie and colleagues (2020) show that increasing proliferation and border formation may actually contribute to enhanced repair after spinal cord injury. Notably, however, another study has shown that neuroblast migration towards the ischemic lesion is impeded by reactive astrocytes in the striatum (Kaneko et al., 2018), reflecting the highly diverse and context-dependent response of reactive glia.

Another prominent classification of reactive astrocytes is based on their pro- or anti-inflammatory action. Once again, different phenotypes arise in a context-dependent manner. Pro-inflammatory, neurotoxic astrocytes ("A1") have, for instance, been recorded in bacterial infection, which is experimentally modelled with systemic administration of lipopolysaccharide (Liddelow et al., 2017), as well as neurodegenerative diseases (Liddelow et al., 2017; Rodriguez-Arellano et al., 2016), and are progressively more prominent in the aging brain, where they may contribute to cognitive decline due to their role in inflammation and synapse disruption (Clarke et al., 2018; Orre et al., 2014; Rodriguez-Arellano et al., 2016). This cell state is characterized by activation of the classical complement cascade (i.e., C1s, C3, and C4) (Zamanian et al., 2012), a process induced by neighboring neuroinflammatory microglia (Liddelow et al., 2017), that may represent a priming factor for neurodegeneration (Clarke et al., 2018; Liddelow et al., 2017). Neuroprotective reactive glia ("A2") are instead a hallmark of traumatic and ischemic lesions and have been shown to secrete cytokines and neurotrophic factors, such as interleukin 6 (Il6) and thrombospondins, that help resolving inflammation and rebuilding synapses (Christopherson et al., 2005; Zamanian et al., 2012). Zamanian and colleagues (2012) have compared the transcriptional profile of these two subsets of astrocytes and found prominent differences in gene expression, reflecting the highly diverse set of functions each group undertakes. The authors, however, warn that the binary classification may actually represent an oversimplification and that reactive gliosis may instead be more diverse than observed in their study (Liddelow and Barres, 2017). Recent developments in single-cell omics and improvements in astrocyte isolation techniques, have allowed researchers to substantially increase the throughput of transcriptional experiments and revealed greater diversity in the reactive gliosis response (Hasel et al., 2021). Accordingly, a recent consensus statement published by major exponents in the field, recommends avoiding binary classifications and focusing, instead, on the diverse molecular and functional responses of reactive astrocytes (Escartin et al., 2021).

Interestingly, reprogramming studies that aim at using parenchymal astrocytes to induce new neurons have shown that efficiency of the astrocyte-to-neuron conversion is enhanced after injury (Robel et al., 2011; Sirko et al., 2013), and have, thus, proposed that reactive gliosis may be necessary in order to unveil the neurogenic potential of astrocytes. In support of this notion is the fact that reactive astrocytes and neurogenic astrocytes from the subventricular zone share several molecular and morphological characteristics (Magnusson and Frisen, 2016). Our analysis of astrocyte-mediated neurogenesis in the injured cortex, however, suggest that initiation of the neurogenic program can occur independent of reactive gliosis and shows, in fact, that reactive astrocytes rarely express the pro-neuronal transcription factor achaete-scute family bHLH transcription factor 1 (Ascl1) (Zamboni et al., 2020).

3 ADULT NEUROGENESIS

> *"It is this potential for plasticity of the relatively stereotyped units of the nervous system that endows each of us with our individuality."*
>
> — Eric R. Kandel, 2000

Neurogenesis is the process through which neural stem cells, which are located in specialized niches across the brain, give rise to new neurons. It was traditionally believed that the brain, once development had ended, would be immutable and that no new neurons could be created and added to the established neural circuits. The seminal work of Altman and Das in the 1960s, shows incorporation of radioactive thymidine in the rat dentate gyrus, suggesting for the first time that new cells are added to the adult brain (Altman and Das, 1965). A decade later, Kaplan and Hinds replicated these results and used electron microscopy to demonstrate that the adult-born cells in the dentate gyrus were in fact neurons (Kaplan and Hinds, 1977). Nottebohm, subsequently, studied the songbird brain and demonstrated the seasonal occurrence of bursts of neurogenesis (Nottebohm, 1989). These initial experiments were followed by other studies confirming that adult-born neurons are present across mammalian species, including humans (Eriksson et al., 1998). For decades, the notion of adult neurogenesis was met with skepticism and intense criticism. Conceptually, the scientific community was concerned that if indeed new neurons were generated and added to the established neural circuit of the mature brain, memories and even the concept of self-identity could not be stable. Since the early 1990s, however, experiments validating adult neurogenesis with independent measures, along with physiological and functional analyses, have accumulated exponentially, and eventually laid the disbelief to rest. Surprisingly though, the recent publication of two papers with contradicting results has reignited the debate. Sorrells and colleagues (2018) suggested that human hippocampal neurogenesis would sharply decrease to undetectable levels shortly after childhood. By contrast, Boldrini and colleagues (2018) support previous evidence and show high numbers of neural progenitor cells throughout the lifespan. Arguably, their findings likely reflect the challenges encountered when working with human postmortem tissue and the need for reliable detection methods to unambiguously identify neural stem cells and young neurons. Indeed, there are several sources of variation that could influence immunostaining experiments and lead to drawing contradictory conclusions, namely the post-mortem delay, tissue preservation and fixation, but also physical and psychological state of the subjects, which is particularly important in the context of hippocampal neurogenesis (van Praag et al., 2000). Rigorous tissue collection and documentation of the subjects' medical history, along with the use of systematic protocols and quantification methods, have led to more convincing results on the persistence of hippocampal neurogenesis throughout life (Moreno-Jimenez et al., 2019) and how this changes in the presence of neurodegenerative diseases (Terreros-Roncal et al., 2021). Moving forward, the use of more sophisticated approaches, such as fate-mapping studies and transcriptional analysis, will lead to a greater understanding of the cellular and molecular

components of adult neurogenesis in the human brain. A few attempts have already been made to identify and characterize neurogenic cells with single-cell transcriptomics in the human (Ayhan et al., 2021; Habib et al., 2017), however studies may have been underpowered or have misclassified other cell types as neural progenitors (Franjic et al., 2021; Sorrells et al., 2021). A recent publication leveraged snRNA-seq across species to show that human dentate gyrus cells rarely co-cluster with neural progenitors from mouse, pig, or monkey (Franjic et al., 2021). In our study (Dumitru, *manuscript*), we identified neural stem and progenitor cells from young human brains (aged 0 to 5 years old), learned their transcriptional profile, and used machine learning algorithms to determine the minimum marker combination to define each state along the neurogenic program. These molecular signatures were, subsequently, used to reveal cells of adult dentate gyrus datasets, with transcriptional profiles indicative of a neurogenic cell state.

3.1 CANONICAL NEUROGENESIS

New neurons are added throughout life, but their generation is restricted to two small regions of the mammalian brain, namely the subventricular zone, where neural stem cells are found lining the walls of the lateral ventricles, and the dentate gyrus of the hippocampus (Gage, 2019; Gould, 2007). Neurogenesis occurs through activation of neural stem cells, which proliferate and give rise to neural progenitors. These then migrate toward their target neural circuit, where they differentiate into mature neurons. The process of activation of neural stem cells within the two neurogenic niches is similar (Llorens-Bobadilla et al., 2015; Shin et al., 2015), although their post-mitotic fates diverge as the neurogenic progeny activates transcription factors implicated in the specification of the region-appropriate neural subtypes. Adult neural stem cells originate from a subset of developmental radial glia that ceases to proliferate mid-gestation, only to exit quiescence in adulthood to initiate gliogenic or neurogenic programs throughout life (Fuentealba et al., 2015; Furutachi et al., 2015). Albeit seemingly homogeneous, the adult neural stem cell population encompasses a collection of cells primed for distinct lineage fates, which are instructed based on embryonically-determined cell-autonomous cues (Fuentealba et al., 2015), as well as environmental exposure to morphogen gradients, such as Wnt, which primes dorsal subventricular zone neural stem cells towards the oligodendrocyte lineage (Azim et al., 2014; Ortega et al., 2013), or sonic hedgehog (Shh), which instructs specification of deep olfactory bulb granule cell in ventral neural stem cells (Ihrie et al., 2011). Cell states along the entire neurogenic lineage can be found at any time in the germinal niche, which has enabled computational reconstruction of the complex cascade of molecular events leading to the generation of new neurons (Hochgerner et al., 2018; Llorens-Bobadilla et al., 2015; Shin et al., 2015; Zywitza et al., 2018). The quiescent neural stem cell state is characterized by expression of prominin-1 (CD133) and of astroglial markers, such as Gfap and aquaporin 4 (Aqp4) (Llorens-Bobadilla et al., 2015), and presents high glycolytic metabolism and low translational activity (Llorens-Bobadilla et al., 2015; Shin et al., 2015). Quiescent neural stem cells also express higher levels of membrane receptors, reflecting their reliance on environmental cues to maintain their dormant state (Shin et al., 2015). By contrast,

activated neural stem cells, which downregulate expression of these receptors, become progressively less responsive to the influence of the niche, so that they can even complete differentiation *ex vivo* (Costa et al., 2011). Activated neural stem cells induce expression of epidermal growth factor (Egf) receptor (Codega et al., 2014; Marques-Torrejon et al., 2021), along with genes implicated in ribosomal biogenesis (Baser et al., 2019), while gene sets related to glial functions (i.e., ion transport and glycolytic metabolism) are progressively silenced (Dulken et al., 2017; Llorens-Bobadilla et al., 2015). Actively dividing cells, expressing classical cell cycle genes, such as proliferating cell nuclear antigen (Pcna) and marker of proliferation Ki-67 (Mki67), are characterized by high levels of genes involved in the protein synthesis machinery (Baser et al., 2019), and concomitant upregulation of neuronal lineage-specific transcription factors (Llorens-Bobadilla et al., 2015). Interestingly, clonal analyses have suggested that activated neural stem cells are rapidly depleted after generating their progeny, while only a subset of cells contribute to the long-term self-renewal capacity of the population (Calzolari et al., 2015; Obernier et al., 2018). Furthermore, there is an increased tendency to quiescence in the aging subventricular zone to avoid depletion of the neural stem cell pool (Kalamakis et al., 2019). However, once activated older neural stem cells proliferate and differentiate at similar rates compared to the younger counterparts and display minimal transcriptional differences (Kalamakis et al., 2019). Similarly, Harris and colleagues (2021) have shown that albeit neurogenesis rapidly decreases in the dentate gyrus with age, the neural stem cell pool is maintained throughout life through an increased propensity to returning to quiescence, a process regulated by degradation of the pro-neuronal transcription factor Ascl1 (Urban et al., 2016).

The germinal niche is characterized by a privileged microenvironment, directly contacting cerebrospinal fluid and vasculature, and interacting with several types of neuronal and glial cells that participate in the neurogenic process (Ihrie and Alvarez-Buylla, 2011). Contrary to other parenchymal areas of the brain, characterized by a tightly regulated blood-brain barrier, the subventricular zone gains direct access to secreted molecules through an increased leakage of blood serum into the area (Tavazoie et al., 2008). Additionally, the vascular niche influences quiescence via direct contact of endothelial cells and quiescent neural stem cells, expressing Jagged-1 ligand and Notch receptor, respectively (Ottone et al., 2014). Similarly, contact with the cerebrospinal fluid regulates neural stem cells activity (Silva-Vargas et al., 2016) and the flow of the fluid through the ventricular system directs migration of neuroblasts along the rostral migratory stream and into the olfactory bulbs (Sawamoto et al., 2006). Active proliferation of neural stem cells progressively decreases as a function of age, likely due to lower availability of growth and neurotrophic factors, including bone morphogenic protein 5 (BMP5) and insulin-like growth factor 1 (IGF1) (Silva-Vargas et al., 2016). Interestingly, heterochronic infusion of cerebrospinal fluid from juvenile mice is sufficient to restore youthful rates of neural stem cell expansion in aged brains (Silva-Vargas et al., 2016). The niche encompasses also a collection of glia cells and neural innervations that regulate stem cell behavior. Importantly, neural stem cells themselves have an active feedback system through which activated cells promote quiescence of the neighboring cells through Notch and Nogo

signaling, to avoid depletion of the neural stem cell pool (Kawaguchi et al., 2013; Rolando et al., 2012). Ependymal cells modulate BMP signaling, known for its role in promoting astrogliogenesis at the expense of neuronal lineage commitment (Lim et al., 2000). Interestingly, ependymal cells are another example of latent neural stem cells, which exit quiescence and acquire neurogenic properties after stroke, through a process that requires canonical Notch signaling inhibition (Carlen et al., 2009). Non-neurogenic niche astrocytes play roles in maintaining neural stem cell quiescence through secretion of Notch ligands Jagged-1 and Delta like non-Canonical notch ligand 1 (Dlk-1) (Ferron et al., 2011), while they also regulate proliferation and differentiation through production of Wnt ligands (Adachi et al., 2007). Similarly, microglia regulate neurogenesis in both the developmental (Zhu et al., 2008) and the adult brain via secretion of factors, such as Transforming growth factor beta (TGFb) and IGF1 (Battista et al., 2006; Ueno et al., 2013). Finally, several neuronal populations innervate the niche and promote neural stem cell activity via neurotransmitter release (Tong et al., 2014).

3.2 FUNCTIONAL IMPACT OF ADULT-BORN NEURONS

In hippocampal neurogenesis, radial glial cells are found in the subgranular zone of the dentate gyrus (Seri et al., 2001) where they give rise to highly proliferative intermediate progenitors and subsequently neural lineage-committed progenitors (Bonaguidi et al., 2011). The neurogenic progeny, the neuroblasts, then migrates short distances and populate the granule cell layer, where they complete maturation (Goncalves et al., 2016b) extending their dendrites towards the molecular layer and their axon through the hilus and cornu ammonis (CA)3 and CA2 subregions, where they make synaptic contact with pyramidal neurons and mossy cells by 2-3 weeks post-mitosis (Llorens-Martin et al., 2015; Toni et al., 2008; Zhao et al., 2006). It has been estimated that as many as 9000 new neurons are generated every day in the rodent hippocampus (Cameron and McKay, 2001). Likewise, hippocampal neurogenesis remains a robust process also in the human brain (Boldrini et al., 2018; Knoth et al., 2010; Moreno-Jimenez et al., 2019; Spalding et al., 2013). Immature neurons are electrophysiologically active already one week after mitosis, but unlike mature granule cells, they respond with excitation to GABAergic inputs (Chancey et al., 2013). Furthermore, they present highly plastic synaptic activity and have a lower threshold for long-term potentiation (Ge et al., 2007), making them hyper-responsive to incoming signals. Although hippocampal radial glia only generate one type of neuron, namely granule cells, different regions of the hippocampus are implicated in distinct cognitive and affective behaviors, suggesting that new neurons originated across the dorsoventral axis of the hippocampus may be implicated in different functions (Ayhan et al., 2021; Cope and Gould, 2019).

The hippocampal formation is known as a gateway to memory, which guides formation of new memories through the establishment of connections between pre-existing neurons. In this context, synaptic plasticity is crucial to sustain life-long learning abilities and hippocampal neurogenesis also aids this process of memory formation. Furthermore, neurogenesis has been

linked to learning of pattern separation (Clelland et al., 2009), as well as forgetting of old memories (Akers et al., 2014). The unique electrophysiological properties of young neurons allow them to be highly receptive to new information that may be carried with weaker signal due to their connections not yet being fully established. Interestingly, the rate at which new neurons are added to the dentate gyrus is influenced by genetic and environmental factors. Major depressive disorder is associated with lower levels of neurogenesis and adult-born neurons are virtually depleted in the brains of individuals suffering from Alzheimer's disease (Berger et al., 2020; Moreno-Jimenez et al., 2019). The impaired neurogenic program may be at the basis of the memory problems encountered in the neurodegenerative condition, but it may also be associated with affective dysregulation since the hippocampus is part of the limbic system, a collection of brain structures that participate in emotional regulation (Anacker and Hen, 2017; Anacker et al., 2018). Conversely, hippocampal neurogenesis has been shown to benefit from environmental experiences, such as learning, physical exercise and exposure to an enriched environment (van Praag et al., 2000), all of which increases proliferation and survival of the progenitors, as well as enhances complexity and maturation of afferent and efferent connections, and in turn, benefits cognitive performance (Llorens-Martin et al., 2015).

The functional role of adult-born neurons from the subventricular zone is less well understood. In the subventricular zone, neural stem cells are found at the basal side of the ependymal cell layer lining the lateral ventricles (Kriegstein and Alvarez-Buylla, 2009). They extend an apical process passed the ependymal cell layer to reach the cerebrospinal fluid and another that makes contact with the vasculature (Doetsch et al., 1999), emphasizing the importance of these two compartments in regulating neurogenesis. Activated neural stem cells give rise to transit amplifying cells, which expand rapidly before generating neuroblasts that migrate toward their target neural circuit. In rodents, neuroblasts move along the rostral migratory stream to reach and innervate the olfactory bulb (Belluzzi et al., 2003; Doetsch et al., 1999). Once there, they mature into interneurons that may participate in olfactory discrimination tasks (Bragado Alonso et al., 2019). It has been shown that neural stem cells along the walls of the subventricular zone are regionally specialized since embryogenesis (Fuentealba et al., 2015) and generate neuroblasts that mature into distinct neuronal subtypes in the olfactory bulb (Delgado and Lim, 2015; Mizrak et al., 2019). The dorsal domain of the subventricular zone gives rise to superficial olfactory bulb granule cells and dopaminergic periglomerular cells, whereas the ventral domain specifies maturation of deep olfactory bulb granule neurons and calbindin-expressing periglomerular cells (Merkle et al., 2007). Interestingly, no neurogenesis occurs in the human olfactory bulb (Bergmann et al., 2012), which may reflect the decreased reliance of our species on the sense of smell, compared to mice. Instead, neuroblasts in humans seem to migrate to the striatum, where they mature into calretinin-expressing interneurons, which are a rare population in rodents but make up about 10% of interneurons in the human striatum (Ernst et al., 2014). The functional relevance of striatal neurogenesis is still enigmatic, although it is striking that new neurons are predominantly added to the medial striatum, which is part of the limbic system, receiving afferents from medial prefrontal and anterior cingulate cortex. The human brain is not unique in displaying striatal neurogenesis in homeostatic conditions, which

has also been recorded in rats (Dayer et al., 2005) and rabbits (Luzzati et al., 2006). These model organisms could, thus, be exploited in the future to investigate physiological properties and roles of the adult-born neurons and help us clarify their functional relevance in humans.

3.3 REACTIVE NEUROGENESIS

While at the population level, neural stem cells have multilineage and self-renewal potential, the individual cell appears to be fate-biased since embryonic development (Fuentealba et al., 2015; Merkle et al., 2007) and can quickly be depleted after one or two rounds of expansion (Obernier et al., 2018), which could hinder efficient brain repair after injury. However, several studies have demonstrated that injury elicits a process of reactive neurogenesis that promotes recruitment of quiescent stem cells (Llorens-Bobadilla et al., 2015), increases their lineage plasticity (Faiz et al., 2015), and reroutes their differentiated progeny towards sites of damage (Arvidsson et al., 2002; Faiz et al., 2015). After an ischemic event, quiescent neural stem cells have shown to selectively respond to injury-induced interferon gamma activity, which primes them for activation (Llorens-Bobadilla et al., 2015). Furthermore, in response to stroke, neural stem cells of the subventricular zone display trilineage potential and generate neurons, oligodendrocytes, as well as astrocytes to attempt to repopulate the damaged area (Li et al., 2010). Some reports have suggested that neurogenic subventricular zone cells are also able to generate region-appropriate neurons in the target area, such as medium spiny neurons that have been recorded in the striatum after stroke (Arvidsson et al., 2002), albeit these findings remain controversial (Liu et al., 2009). Astrocytes generated by subventricular zone cells in response to injury may participate with resident reactive glia in restricting tissue damage. They also display distinctive features and may exhibit increased capacity for repair (Faiz et al., 2015). Demyelinating lesions elicit lineage-fate switch of neuron-committed progenitors to favor oligodendrogenesis (Jablonska et al., 2010). After stroke, subventricular zone neuroblast migrate towards striatal sites of damage. The partial rerouting of the progenitors occurs along newly generated blood vessels (Thored et al., 2007) and is sustained for several months after the ischemic event has occurred (Thored et al., 2006). Nevertheless, a minority of injury-induced neurogenic cells survive long-term in the striatum and only a small percentage (about 0.2%) of the neurons lost to injury is eventually replaced (Arvidsson et al., 2002).

Interestingly, the brain encompasses cells that can be viewed as latent neural stem cells, whose regenerative potential is revealed in response to injury. Injury prompts a bidirectional fate switch between the two main cellular components of the subventricular zone, namely neural stem cells and ependymal cells, a process regulated by Ephrin B2, downstream of Notch signaling (Nomura et al., 2010). In fact, ependymal cells, which are generally quiescent in homeostatic conditions, have shown trilineage potential when cultured *in vitro* (Meletis et al., 2008), and have demonstrated the ability to proliferate robustly after brain and spinal cord injury, and to generate neuroblasts, astrocytes and oligodendrocytes (Barnabe-Heider et al., 2010; Carlen et al., 2009; Llorens-Bobadilla et al., 2020; Sabelstrom et al., 2013). Interestingly, the latter lineage is not favored after spinal cord injury, although multiomic analysis has

demonstrated a permissive chromatin state for oligodendrogenic gene expression programs (Llorens-Bobadilla et al., 2020). After injury, ectopic expression of the transcription factor Olig2 instructs ependymal cells towards generation of oligodendrocytes that myelinate and appear transcriptionally and functionally comparable to local glia (Llorens-Bobadilla et al., 2020). This suggests that learning the transcriptional and epigenetic landscape of canonical and latent neural stem cells may guide design of regenerative therapies to replace specific cell types lost to injury.

Reactive neurogenesis can also occur ectopically, at the lesion site, from resident cells that can reveal a latent neurogenic program. This has been demonstrated for parenchymal astrocytes, which generate new neurons in the striatum following ischemic damage (Magnusson et al., 2014), as well as an experimental model of Huntington's disease (Nato et al., 2015). The recruitment of astrocytes into neurogenesis is controlled by Notch signaling inhibition, which is by itself sufficient to trigger a similar response in the striatum in the absence of injury (Magnusson et al., 2014). Once engaged, resident glia enter a neurogenic program that is comparable to canonical neural stem cell behavior (Magnusson et al., 2020), generating transit amplifying progenitors and neuroblasts, before maturing into striatal interneurons (Magnusson et al., 2014). Interestingly, silencing of Notch signaling in the context of a cortical injury enables astrocytes from the somatosensory and the occipital cortices to mount a similar neurogenic response, a process that unfolds independent of reactive gliosis (Zamboni et al., 2020). Several laboratories have also proposed NG2 cells as a possible source of new neurons based on observations that suggest trilineage potential of these cells when cultured *in vitro* (Kondo and Raff, 2000). The evidence for NG2 cell-mediated neurogenesis is, however, scarce and controversial both in physiological conditions, as well as in the context of injury (Kirdajova and Anderova, 2020). NG2 cells, however, have been shown to be a valuable target for *in vivo* reprogramming strategies, where they can generate multiple subtypes of neurons using ectopic expression of lineage-specific transcription factors (Heinrich et al., 2014; Pereira et al., 2017; Torper et al., 2015).

The role of immune signals in neurogenesis is relevant but complex. Interferon gamma activity has been shown to prime quiescent stem cells for activation shortly after stroke (Llorens-Bobadilla et al., 2015). Furthermore, injury-induced changes in the immune signals are sufficient to trigger regenerative responses in the zebrafish brain (Kyritsis et al., 2012), through gene regulatory mechanisms that are not active in canonical neurogenesis, but unique to the regenerating tissue (Kang et al., 2016). In mammals, studies have also suggested that inflammation is sufficient to trigger neurogenesis (Chapman et al., 2015). Microglia have been shown to regulate neurogenesis and plasticity in homeostatic conditions, both in the developing brain, as well as in the adult germinal niche. However, several investigations have shown that activation of the immune cells may also hinder neurogenesis. For instance, Liu and colleagues (2007) have shown that depletion of activated microglia in the chronic phase of stroke supported survival and maturation of new neurons after stroke. Similarly, Ekdahl and colleagues (2003) suggest that survival of newly generated hippocampal neurons is negatively impacted by the extent of microglia activation. Furthermore, mimicking bacterial infections

with lipopolysaccharide administration is associated with a shift to a pro-inflammatory phenotype of microglia, which is linked to impaired neurogenesis (Ekdahl et al., 2003). Finally, in aging and neurodegeneration, microglia adopt a pro-inflammatory phenotype that may impair neurogenesis (Biscaro et al., 2012; Sierra et al., 2007). Altogether, reports studying the involvement of immune cells in the regulation of reactive neurogenic responses suggest that their implication can be either favorable or detrimental at different stages of disease progression, as well as depending on disease severity.

The functional relevance of the injury-induced neurons is unclear, but it may differ based on the type of injury and the site at which neurogenesis is occurring. Interestingly, epilepsy is associated with increased proliferation of dentate gyrus radial glia and faster maturation of granule cells. Yet, long-lasting epilepsy is linked to cognitive decline and inhibiting seizure-induced neurogenesis has been shown to directly prevent impairment, as well as lower the risk for chronic seizures (Cho et al., 2015). Similarly, a recent study has shown that aberrant hippocampal neurogenesis recorded after stroke exacerbates injury-related symptoms of cognitive impairment (Cuartero et al., 2019). It is possible that abnormal rates of generation and maturation of neurons lead to the establishment of aberrant connections in the neural circuit, which in turn can alter excitability of the dentate gyrus (Overstreet-Wadiche et al., 2006). Arguably, even the process of reactive neurogenesis recorded at the injury sites, which leads to the replacement of just a small proportion of lost neurons, is unlikely to contribute to efficient repair, as highlighted by the fact that patients who suffer a stroke may never regain full functionality. However, reports have shown that specifically ablating neural progenitor populations shortly after ischemic injury, leads to worsened outcomes in terms of lesion volumes and mortality (Butti et al., 2012). The reactive response of subventricular zone cells appears to be beneficial, in that it provides protection against excitotoxic damage induced by glutamatergic neurons after stroke (Butti et al., 2012) Furthermore, injury-induced neuronal cells that survive long-term, successfully integrate in the local circuit (Hou et al., 2008), suggesting that their existence may have some beneficial contribution, and that further promoting their survival may prove valuable to enhance their positive influence on recovery (Wang et al., 2012).

Even with injury-mediated changes in the rate of neurogenesis and lineage plasticity of neural stem cells, the brain remains inefficient at repairing itself. The restricted number of neural stem cells found in the adult brain is unlikely to be sufficient to repopulate brain regions afflicted by widespread neuronal death. Furthermore, most neural progenitors and neurons induced after injury die shortly after they are generated. Thus, novel strategies for regenerative repair could be designed in order to promote survival of the neural stem cell progeny, as well as to stimulate differentiation into region-appropriate neurons that are functionally comparable to the original cells. Furthermore, the discovery that resident parenchymal cells may activate a neurogenic program in response to injury opens the doors to regenerative therapies that target these abundant cell types to achieve more widespread neuronal replacement. These topics are discussed further in the next chapter of this thesis.

4 CELL REPLACEMENT AND REGENERATION

> *"Awakening, basically, is a reversal of this: the patient ceases to feel the presence of illness and the absence of the world, and comes to feel the absence of his illness and the full presence of the world."*
>
> — Oliver Sacks, 1973

Despite lifelong generation of new neurons in the specialized niches of the brain, the central nervous system of mammals is largely inefficient at repairing itself after widespread damage and neuronal death. Ultimately, cell replacement therapies for neurologic conditions aim at repopulating the injured brain with the appropriate cell types that were lost to injury, allowing them to integrate correctly into the neuronal network, thus, providing functional recovery. Two main categories of cell replacement therapies are currently being explored, namely cell transplantation and recruitment of endogenous cells. Aside from the specific strengths and limitations of the therapeutic approaches, each strategy will have to overcome significant hurdles imposed by the target tissue in order to become successful. Indeed, the adult brain offers an unsupportive environment for the young neurons to achieve maturation and functional integration, whatever source these cells are originating from. Much unlike in the embryonic brain, characterized by a supportive microenvironment where neurogenesis is prevalent at the expense of the gliogenic fate, starting at birth and throughout life, gliogenesis becomes the default fate (Gotz et al., 2016). Indeed, early transplantation studies have shown that neural progenitors, with recognized neurogenic potential *in vitro*, are unable to generate neurons when grafted to the adult brain, unless they are placed at the germinal niche (Herrera et al., 1999). The strong influence of a non-permissive environment is also evident in the context of reprogramming studies, where ectopic expression of neuronal fate determinants efficiently drives lineage conversion *in vitro* but proves considerably more challenging when attempted *in vivo* (Buffo et al., 2005; Grande et al., 2013). This may lead to the therapeutic cells differentiating into glial cells, or it may hinder their survival altogether. The developing brain is also characterized by the presence of a well-defined network of cells and chemoattractant signals that encourage progenitors' migration and establishment of synaptic connections at their target sites (Gotz et al., 2016). In the adult brain, radial glia providing structural support to migrating cells are absent and sprouting axons are no longer guided by an abundance of signals that ensure path finding and connectivity with the appropriate cells. In fact, it was observed that, after transplantation of neural progenitors, the cells tended to form clusters around the injection site and did not display considerable migration (Ladewig et al., 2014). Interestingly, neural progenitors at different stages of maturation are differentially capable of migrating once transplanted, with cells at early or intermediate stages of differentiation covering the longest distances (Ganat et al., 2012). Similarly, in a mouse model of stroke, overexpression of Slit1, which is associated with neuronal migration, promoted movement of subventricular zone neuroblasts passed the reactive astrocyte layer limiting the injury site, and

led to greater repopulation of new neurons at the infarcted area (Kaneko et al., 2018). In another study, polysialic acid (PSA), a carbohydrate implicated in migration and synapse formation, was overexpressed in embryonic cells transplanted into a rodent model of Parkinson's disease and shown to make cells more responsive to the scarce axon-guiding cue found in the adult brain and more efficient at establishing connections (Battista et al., 2014). Finally, the aging brain becomes progressively less plastic and is characterized by a generalized state of neuroinflammation (Biscaro et al., 2012; Sierra et al., 2007), which may be detrimental for survival, proliferation, and integration of newly generated cells. The situation becomes exacerbated after injury or in a brain afflicted by neurodegenerative diseases (Biscaro et al., 2012), which are generally the targets of regenerative medicine efforts. Monje and colleagues (2003) have demonstrated that inflammation challenges the proliferative behavior of hippocampal neural stem cells, and that their activity can be restored through administration of non-steroidal anti-inflammatory drugs. Thus, combining replacement therapies with strategies that support survival and maturation of the newly generated cells, or that consider comorbidities of the disease, such as neuroinflammation, will likely prove greater potential in promoting regenerative repair. Additionally, considering that cell therapies are generally aimed at treating age-related neurological conditions, extensive investigation of their potential in aged animal models may support their translation into clinical application.

4.1 REPLACEMENT THERAPY USING CELL TRANSPLANTATION

The earliest attempts at promoting regeneration of injured brains date back to the 1970s when researchers succeeded in replacing degenerated dopaminergic neurons in a mouse model of Parkinson's disease, effectively rescuing motor function (Perlow et al., 1979). Since then, replacement therapies utilizing exogenous sources of cells have been refined and, even, tested in clinical trials (Barker et al., 2015; Parmar et al., 2020). Multiple sources of cells for transplantation have been explored so far. Initial experiments utilized allografts of human fetal origin (Lindvall et al., 1990), which were shown to differentiate *in situ*, establish meaningful connections, and release neurotransmitters at desired sites. The outcomes for treating Parkinson's disease with fetal ventro-mesencephalic tissue have been promising (Parmar et al., 2020) and attempts have been made for restoring basal ganglia functionality in Huntington's disease (Hauser et al., 2002), albeit with limited success owing to the poor survival rate of grafted cells (Cisbani and Cicchetti, 2014). Nevertheless, the use of human fetal tissue poses great limitations that hinders this strategy from becoming a therapy. Firstly, the scarce availability of the source and the large amounts required to repopulate an adult brain have led to a large number of surgeries being cancelled owing to the lack of tissue (Barker and consortium, 2019). Furthermore, there is poor control over the genetic background of the donors and the compositions of cells collected, because multiple fetal brains are used to treat each patients, and they may differ in age and the way they are being dissected, leading to high variability of the therapeutic material being delivered (Parmar et al., 2020). More recently, cell grafts from human embryos have also shown some therapeutic promise through successful

structural and functional integration in compromised neural networks (Barker, 2014; Falkner et al., 2016; Steinbeck et al., 2015). Interestingly, a study by Li and colleagues (Li et al., 2016) showed that a patient with Parkinson's disease who received embryonic stem cell transplantation, retained some of the grafted cells for as long as 24 years, and these cells connected with the putamen through dopaminergic innervation. This suggests that the beneficial effect of cell transplantation derives from cell replacement, which is, in fact, the ultimate goal of the therapy. However, other studies have also observed bystander beneficial effects, such as increased survival of host neurons thanks to trophic support offered by the transplanted cells (Mendonca et al., 2015; Wang et al., 2013). Compared to fetal cells, pluripotent human embryonic stem cells (hESCs) are more easily accessible because cultures can be expanded virtually indefinitely. In addition, there is better control over the production and differentiation processes, which results in greater purity and decreased inter-batch variability (Parmar et al., 2020). However, embryonic stem cells still carry the risk of immune rejection and, therefore, need to be combined with immunosuppression. Furthermore, their pluripotent and self-renewal potential raises concerns regarding possible tumorigenicity. Notably, many studies in model organisms have been carried out and, so far, none has recorded problems of uncontrolled proliferation or tumor formation. However, the longer lifespan of the patients combined with the greater numbers of cells being injected may carry greater risks, which will be carefully evaluated in clinical trials (Parmar et al., 2020). These hurdles, as well as risks associated with immunosuppressive treatments, which still need to be imposed to avoid rejection of cell grafts, may be overcome with the use of induced pluripotent stem cells (iPSCs), which can be harvested in large numbers from the patient's own organs (Takahashi and Yamanaka, 2006). Fibroblasts from the skin can be reprogrammed *in vitro* using refined differentiation protocols that allow correct patterning and maturation into midbrain dopaminergic neurons (Soldner et al., 2009). Specific neuronal and glial subtypes can now be reliably and efficiently generated from iPSCs through activation of key transcription factors (Canals et al., 2021). Nerve cells originated from iPSCs and transplanted into the brain have remarkable capacity for neurite outgrowth, and synthesis and release of neurotransmitters with potency and specificity that parallels that of endogenous cells (Kirkeby et al., 2012; Kriks et al., 2011). Stem cell transplants have demonstrated successful integration of grafted cells even in highly complex neocortical circuits in mouse models of Alzheimer's disease, cortical injury, and stroke (Espuny-Camacho et al., 2017; Espuny-Camacho et al., 2013; Falkner et al., 2016). Importantly, as studies have shown that grafted cells may begin to bear pathological hallmarks of host neurons after transplantation (Li et al., 2008a), stem cell therapy has the potential advantage to be genetically engineered to withstand disease pathology, which is particularly relevant in case the cells to be injected directly derive from the patient (Kikuchi et al., 2017). Based on the promising outcomes of preclinical studies utilizing iPSCs-originated neurons (Kikuchi et al., 2017; Sharma et al., 2019; Tsuji et al., 2019), trials in humans are currently being designed for patients afflicted by Parkinson's disease, as well as other conditions, such as macular degeneration and spinal cord injury (Cyranoski, 2018a, b; Mandai et al., 2017).

4.2 ENDOGENOUS SOURCES FOR REGENERATIVE REPAIR

The risks associated with cell transplantation and the limited contribution of adult neural stem cells to widespread regeneration have encouraged research towards the discovery of alternative sources for safer and more efficient repair. Induction of ectopic neurogenesis *in situ* has been proposed as a novel therapeutic strategy since the realization that several differentiated cell types can be instructed to undertake a neuronal identity, when stimulating expression of key transcription factors (Chanda et al., 2014). There are fundamental advantages associated with strategies that aim at repurposing local cells to repopulate neural circuitries, including the vast availability of these cells *in situ* and the lack of risks associated with graft rejection. However, more research is needed in this field to achieve efficient lineage conversion and to demonstrate that newly generated neurons are molecularly and functionally comparable to the cells they aim to replace. Several studies have provided evidence that direct reprogramming through ectopic expression of transcription factors implicated in development as master regulators of neurogenesis, can drive lineage fate conversion of brain cells into neurons. The earliest example of such approach was a study published by Heins and colleagues (2002), who showed that neocortical glia from postnatal brains can convert to neurons under Paired box 6 (Pax6) overexpression when cultured *in vitro*. Since then, numerous other studies have shown the possibility to generate different types of neurons using lineage-specific fate determinants, individually or in combination. For instance, Distal-less homeobox 2 (Dlx2) enables induction of GABAergic neurons from astrocytes isolated from neonatal cortex (Heinrich et al., 2011), whereas, ectopic activation of Neurogenin 2 (Neurog2) or Neuronal differentiation 1 (NeuroD1) drives conversion into functional glutamatergic neurons from postnatal astrocytes, as well as NG2 cells, which can give rise to both deep layer pyramidal cells and GABAergic interneurons (Guo et al., 2014). Earlier investigations were successful at converting cells *in vitro* or in the postnatal brain, but it has been challenging to achieve the same level of efficiency in the adult brain, given the less permissive parenchymal environment (Gotz et al., 2016). Nevertheless, new efforts are demonstrating promising results and are even showing that reprogrammed cells can restore tissue functionality. For instance, expression of Ascl1/Lmx1a/NeuroD1 together with miRNA128 has proven able to convert striatal astrocytes into dopaminergic neurons and rescue injury-induced motor impairment in a mouse model of Parkinson's disease (Rivetti di Val Cervo et al., 2017). Similarly, transcription factors implicated in rod cell fate specification have been co-expressed in the retina to reveal the neurogenic potential of Müller glia, and restore vision in a mouse model of congenital blindness (Yao et al., 2018). Better efficiency of direct reprogramming can also be achieved with adjunct strategies that aid survival of the new neurons, rather than progressively increasing the number of transcription factors that are being modulated. For instance, conversion rates and survival can be promoted in reprogrammed cells through co-expression of Bcl2 and administration of anti-oxidative treatment (Gascon et al., 2016), which has increased efficiency of glia-to-neuron conversion up to 90%.

Research from our lab has shown that activation of regenerative programs in local glia with neural stem cell properties could be a valuable alternative for promoting repair (Llorens-

Bobadilla et al., 2020; Magnusson et al., 2014; Magnusson et al., 2020; Zamboni et al., 2020). Notably, direct reprogramming studies have shown that alternative fates, unrelated to the neuronal lineage, could arise under ectopic manipulation of transcription factors (Karow et al., 2018; Treutlein et al., 2016). For instance, Treutlein and colleagues (2016) have observed generation of cells from the myogenic lineage following Ascl1 overexpression in fibroblasts, suggesting that transcription factor modulation alone may not be sufficient to reliably induce the neuronal identity, but that other factors, such as epigenetic regulation, may be at play. Our investigations, instead, show that Rbpj-depleted astrocytes unfold a neurogenic program that is virtually indistinguishable from canonical neural stem cell behavior, and does not lead to the generation of aberrant lineage fates (Magnusson et al., 2020; Zamboni et al., 2020). Additionally, contrary to direct reprogramming, activation of the neurogenic program allows astrocytes to enter a phase of amplification, which enables generation of up to 40 neuroblasts from each cell (Zamboni et al., 2020). This could greatly increase the extent of neuronal replacement without the risk of depleting the astroglial population, making local recruitment of latent neural stem cells an attractive strategy for widespread regeneration. More research is, however, needed in this field in order to improve efficiency of recruitment, promote survival of the progenitors, and verify their functional integration. A recent attempt has been made in this context, where researchers showed that new striatal neurons derived from Rbpj-depleted astrocytes are electrophysiologically active and receive synaptic input from pre-existing neurons, effectively demonstrating functional integration (Dorst et al., 2021). The study, however, suggests that these cells would provide excitatory signals to the striatum (Dorst et al., 2021), which is inconsistent with our findings proposing that new striatal neurons have a GABAergic interneuronal identity (Magnusson et al., 2014; Magnusson et al., 2020).

A limiting factor for achieving effective cell replacement in the adult brain is the mode of administration of the therapy. Studies have demonstrated the feasibility of using small molecules to attain direct reprogramming of glia into neurons (Zhang et al., 2015). However, the lack of target specificity of pharmaceutical options will likely lead to side effects, which may outweigh the benefits. Furthermore, compounds need to be able to cross the blood-brain barrier in order to reach their target, or they must be administered directly into the brain through surgical intervention. Viral gene therapy is an alternative strategy that is being explored and has proven promising in pre-clinical and clinical settings. In particular, the use of adenoassociated viruses (AAVs) has been explored in humans owing to their ability to deliver constructs with high efficiency and without triggering extensive inflammatory reactions (Peel and Klein, 2000). Surprisingly, the tropism of AAVs can change in injury conditions and depending on the vector being delivered, which emphasizes the importance of carefully designed studies that verify the identity of infected cells (Wang et al., 2021). Indeed, this was recently brought up in a study by Wang and colleagues (2021), who discovered that local neurons may have been misidentified as reprogrammed cells in several published astrocyte-to-neuron conversion studies. Current research is aimed at improving transduction specificity, so that only the target cells would reliably be expressing the therapeutic genes. This can be enabled through capsid evolution, which has improved efficiency and specificity in other tissues

(Tabebordbar et al., 2021), as well as the use of cell-specific genomic elements, which regulate expression of the transgene in the desired cell population. In this context, innovative viral vector design is leveraging the great diversity of enhancer sequences in neural cells (Li et al., 2021b) to achieve expression of the transgene exclusively in specific subtypes of brain cells (Graybuck et al., 2021; Mich et al., 2021). Additionally, combined with recent development in viral vector design, advances in the CRISPR/Cas9 technology can offer versatile and effective strategies for gene-editing, as well as transcriptional inhibition or activation (Dahlman et al., 2015). For instance, CRISPR/Cas9 has been implemented to force concomitant activation of ten endogenous neurogenic genes and one long noncoding RNA, which enabled conversion of dorsal midbrain astrocytes into functional neurons (Zhou et al., 2018). Furthermore, a CRISPR activation screen has been implemented to identify transcription factors that would promote neuronal fate commitment in embryonic stem cell cultures (Liu et al., 2018). This study has demonstrated that combined expression of specific genes facilitates conversion and reported a novel role for Ezh2 in inducing neuronal identity.

Single-cell RNA-sequencing (scRNA-seq) investigations have provided us with the opportunity to interrogate cellular composition and developmental dynamics of complex tissues with unprecedented resolution (Briggs et al., 2018; La Manno et al., 2021; Zeisel et al., 2018) and it has made it possible to portray a complete transcriptional framework of the neurogenic process in homeostatic conditions (Hochgerner et al., 2018) and under perturbations. For instance, it revealed how ischemic injury promotes recruitment of dormant neural stem cells through interferon-gamma signaling (Llorens-Bobadilla et al., 2015), or that conditional deletion of Lrp2, an important factor implicated in neural stem cell maintenance, dampens proliferation, globally decreases ribosomal activity, and interferes with Wnt and BMP signaling (Zywitza et al., 2018). Transcriptomics analysis has also been employed to fully characterize the reprogramming process of human pericytes overexpressing Ascl1 and Sox2, and to compare the molecular profile of induced neurons to that of endogenous cells (Karow et al., 2018). In line with the canonical neurogenic program, pericytes transit through a neural stem cell-like state, albeit in the absence of cell division, prior to developing a neuron-specific molecular phenotype. Furthermore, the researchers were able to demonstrate that reprogramming success depends on the source of cells targeted for neuron production, as distinct pericyte clusters display heterogeneous reprogramming responses. Overall, single-cell resolution and access to whole-transcriptome dynamics will allow us to refine our treatment protocols considering cell heterogeneity and the most promising temporal window for delivering therapeutic agents. Furthermore, since treatment strategies for potentiating neurogenesis are predominantly tested in pre-clinical settings, scRNA-seq analysis may help generating predictions on the effectiveness of treatments in humans, through direct comparison of shared modes of neuronal development, patterning, and integration across species (Zhu et al., 2018).

5 RESEARCH AIMS

Paper I – To learn the transcriptional mechanisms underlying neurogenesis by striatal astrocytes and to explore manipulations to recruit a greater number of astrocytes into the neuronal lineage fate.

Paper II – To assess whether neurogenesis by astrocytes is retained in different regions of the cortex in the presence of an injury, when Notch signaling is inhibited and to compare the transcriptional program of neurogenic astrocytes with that of canonical neurogenic cells, to support the notion that parenchymal astrocytes can be considered latent neural stem cells.

Paper III – To provide a reliable and unbiased framework to study human hippocampal neurogenesis with unprecedented resolution, through transcriptional profiling of neural stem and progenitor cells in the dentate gyrus across ages.

6 METHODS

> *"What functional significance can be attributed to the neuroglia? Unfortunately, the present state of science does not allow to answer this important question but through more or less rational conjectures. When facing this problem, the physiologist is totally disarmed for lack of methods."*
>
> — Ramón y Cajal, 1899

6.1 MODELS AND TOOLS TO STUDY ASTROCYTE BIOLOGY

It has been challenging to investigate the plethora of physiological functions astrocytes are responsible for in the healthy and the injured brain. Experiments that study cells *in vitro* have the advantage of being able to tease biological functions apart, as well as study processes that are specific to particular cell types, which can be cultured as single populations or co-cultured to learn about interaction between cellular communities. The original protocol for isolation and culture of glia, by McCarthy and deVellis (1980), leverages the fact that astrocytes bind more firmly than other neural cells in a dish, so that purity is achieved by washing loose cells away. More recently, protocols have used antibody-conjugated magnetic beads or immunopanning for achieving high purity cultures and developed serum-free conditions to give rise to culture settings that more closely resemble physiological environments (Guttenplan and Liddelow, 2019; Li et al., 2021a). Initial discoveries of active participation of astrocytes in neuronal communication have been possible thanks to *in vitro* modelling, although recent technological developments have also enabled *in vivo* recordings of, for instance, calcium signaling which can now be performed even in awake, freely moving animals (Qin et al., 2020). Cultures of astrocytes have also permitted manipulation of human cells and recording their response to stressors, such as hypoxia and oxidative damage, as well as identified species-specific behaviors (Li et al., 2021a). Nevertheless, the importance of astrocytes is to be fully appreciated when the cells are immersed in their original microenvironment, in contact with neurons whose activity they support, as well as blood vessels and other glial cell types they interact with. In this sense, refinement of *in vivo* experimental models has advanced our understanding of the specific contribution of astrocytes in health and disease. For instance, transgenic models have provided tools to specifically label and genetically manipulate glia to investigate their roles through gain- and loss-of-function designs (Srinivasan et al., 2016). Cell type-specific expression of fluorescent reporter tags, has facilitated isolation of astrocyte populations and the investigation of their molecular heterogeneity across and within regions (Batiuk et al., 2020; Lanjakornsiripan et al., 2018), as well as in response to injury (Hasel et al., 2021). They have, furthermore, assisted lineage-tracing of the glial progeny (Slezak et al., 2007), which enabled us to demonstrate an astroglial origin of the newly generated neurons after striatal (Magnusson et al., 2014) and cortical injuries (Zamboni et al., 2020).

6.2 MODELS AND TOOLS TO STUDY ADULT NEUROGENESIS

Adult neurogenesis has been extensively studied in mice and other model organisms, but it has been challenging to investigate the same processes in the human brain. Concerns around the limitations and technical challenges of the current detection methods are, in fact, continuing to be raised, while the scientific community demands rigorous studies to validate and extend the methods at our disposal for identifying cellular and molecular mechanisms of neurogenesis (Kempermann et al., 2018; Lucassen et al., 2020). Incorporation of thymidine analogs in cells that had divided in adulthood has provided the first line of evidence supporting a lifelong addition of cells in the hippocampus (Eriksson et al., 1998). Subsequent detection of BrdU with immunostaning and its colocalization with neuron-specific markers has become the gold-standard to prove the existence of adult-born neurons in the human hippocampus and striatum (Ernst et al., 2014; Kuhn and Cooper-Kuhn, 2007), and has been validated with independent measurements, such as C^{14}-based birth dating (Spalding et al., 2013). Neurogenesis, however, comprises a complex cascade of cellular and molecular events, which are continuously being unveiled, thanks to the incessant development of technologies to track and influence generation and maturation of neurons. Immunostaining studies have provided information regarding the expression proteins at specific stages along the neurogenic trajectory (Knoth et al., 2010), which have also facilitated creation of transgenic lines and viral vectors that can be used for visualizing neural stem and progenitor cells and manipulating their function (Kuhn et al., 2018). The use of postmortem tissue for immunostaining has, however, constrained appreciation of the dynamic processes that lead to the generation of new neurons. Consequently, the development of novel technologies that allow *in vivo*, multiphoton imaging of hippocampal neurogenesis, has enabled researchers to uncover details on neural plasticity, progenitors' survival, and fate choices, that would have been difficult to estimate in fixed postmortem samples (Danielson et al., 2017; Goncalves et al., 2016a; Pilz et al., 2018). Furthermore, the establishment of lineage tracing techniques has revolutionized our understanding of fate commitment and development, particularly when these techniques are combined with multiomics analysis (Ratz et al., 2021; Weinreb et al., 2020). In model organisms, lineage-tracing relies on engineered genetic tags that label cells and their progeny, such as constitutive expression of fluorescent reporters, integration of barcode libraries, and performing heritable genetic scars (Kester and van Oudenaarden, 2018; Wagner and Klein, 2020), all of which have helped us reconstruct lineage trees for developing organisms or stem cell niches (Weinreb et al., 2020). Lineage tracing in humans, on the other hand, exploits naturally occurring somatic mutations, which are propagated in the progeny of dividing cells, but absent in distantly related populations (Evrony et al., 2015; Hard et al., 2019). This can be achieved via whole-genome or targeted sequencing of specific mobile genetic elements, as well as by leveraging the high mutation rate of mitochondrial DNA (Ludwig et al., 2019). Single-cell transcriptomics analysis has, also, been performed by multiple laboratories to fully appreciate the complex molecular dynamics underlying neurogenesis in the subventricular zone (Llorens-Bobadilla et al., 2015; Zywitza et al., 2018) and dentate gyrus of mice (Hochgerner et al., 2018; Shin et al., 2015), and has provided a wealth of information regarding the transcriptional mechanisms implicated in the activation of neural stem cells, as well as their differentiation into mature neurons. These

investigations will be crucial to help us answer fundamental questions related to the heterogeneity of neural stem and progenitor cells, the molecular identifiers and functions of progenitors along the course of differentiation, as well as the differential vulnerability of cells and processes in the presence of neurological conditions.

6.3 SINGLE-CELL TRANSCRIPTOMICS

In the past decade, scRNA-seq has established itself as a groundbreaking tool that allows to investigate the transcriptional profile of cells at an unprecedented resolution, with the potential to completely revolutionize biomedical research. Since the pioneering study of Tang and colleagues (2009) that recorded mRNA expression in a single mouse blastomere, the field has progressed at an astonishing pace, and laboratories are now sampling hundreds of thousands of cells to characterize their behavior and composition in health and disease (Eraslan et al., 2021), as well as throughout development (Cao et al., 2019; La Manno et al., 2021) and across species (Bakken et al., 2021). Novel technological advances in the omics field are also enabling epigenomics profiling at the single-cell level (Buenrostro et al., 2015), as well as to determine transcriptional state along with its spatial organization (Stahl et al., 2016). Multiomic analysis is now even accessible in the same cell, for which we can record transcriptional information, along with genome-wide chromatin accessibility, and protein expression (Mimitou et al., 2021). Ultimately, multidimensional genomic measurements will bring us closer to better understanding gene regulation and will improve our abilities to predict cellular responses to genetic perturbations (Dixit et al., 2016; Kamimoto et al., 2020).

The scRNA-seq protocols rely on dissociation of tissue into single-cell suspensions and capture of RNA from each lysed cell, which is converted into complementary DNA (cDNA) by reverse transcription and PCR-amplified to obtain enough biological material for deep sequencing. Different chemistries have been developed for library preparation, and their use depends on the scientific question they are addressing. Full-length approaches, such as Smart-seq2 (Picelli et al., 2013; Picelli et al., 2014), can quantify specific transcript isoforms and be used to monitor allele-specific expression (Deng et al., 2014; Reinius et al., 2016). The scRNA-seq reactions in these protocols are generally performed in well-plates, are laborious and require relatively large volumes of reagents, which preclude carrying out affordable, large-scale experiments. Droplet-based methods, such as Drop-seq and inDrop, produce reads that are biased towards the 5’ end, thus sacrificing full-length coverage, but rely on microfluidic platforms to perform the reactions, which grants cost-effective scaling of the experiment (Klein et al., 2015; Macosko et al., 2015). These approaches embed each cell in droplets before lysing, capturing mRNA molecules, and introducing cell-specific barcodes so that cells can be pooled and processed together. Furthermore, they incorporate the unique molecular identifier (UMI) technology (Kivioja et al., 2011), which enables estimation of absolute numbers of molecules to control for amplification bias during downstream analysis. Commercialization of the droplet-based technology by 10x Genomics has allowed popularization of high-throughput transcriptomics without the need of extensive technical expertise in microfluidics. Notably, the Smart-seq3

protocol was recently released, which combines the major advantages of each library preparation method, to obtain full-length coverage combined with UMI counting strategy (Hagemann-Jensen et al., 2020).

Paralleling the rapid technological advances, many computational tools have been developed to extract relevant biological information from scRNA-seq datasets. The statistical analysis of single-cell experiments is fundamentally different from classic gene expression queries owing to the unique structure of the data. The experimental procedure does not capture all mRNA molecules in each cell and genes are expressed in discrete bursts (Larsson et al., 2019). This leads to generating sparse matrices that require pre-processing and transformation to improve their interpretation. These initial steps generally include filtering of low-quality cells, followed by normalization of sequencing depth and logarithmic transformation of the data to equalize variance across genes that vary widely in expression levels. Subsequently, feature selection allows to identify genes that fluctuate more prominently across the dataset and that are, thus, more likely to reveal relevant biological variation. Finally, data are scaled and centered around the mean to allow easier comparison of transcriptional differences across cells. Several approaches have also been developed to deal with technical sources of variation, such as batch effects, which can be regressed out using linear models or accounted for in more sophisticated approaches (Butler et al., 2018). Doublets are two (or more) cells that share the same cell-specific barcode, which need to be handled to avoid misleading biological inferences. Multiple approaches have been designed to identify these outliers through generation of simulated doublets and training of machine learning algorithms to label cells in the sample based on similarity to the simulated data (Bernstein et al., 2020; McGinnis et al., 2019).

Following pre-processing, data can be visualized and grouped into populations to learn about sample composition. Visualization approaches aim at reducing the high dimensional structure of transcriptomics data to fewer (generally two) dimensions that retain at best the variation in the data but are easier to interpret. Uniform manifold approximation and projection (UMAP) is one such example that has gained popularity because it scales well with increasing size of datasets and because it is able to better retain the global structure of transcriptional data, compared to its predecessors (Becht et al., 2018). Cells can then be grouped in communities using different clustering approaches, such as k-means algorithms, which groups transcriptionally similar cells under a predetermined number of equally sized clusters, or graph-based methods, which can accurately handle clusters that vary in size, but whose performance heavily relies on how well transcriptional data are converted into a graph representation (Andrews and Hemberg, 2018). Clustering algorithms are employed to identify cell types and states that share transcriptional signatures, but there exists a trade-off between resolution limit and robustness of clustering, which may preclude identification of rare cell populations in one case or introduce irrelevant subdivisions of the dataset in the other. After identifying putative cell types, differential expression analysis is usually employed to determine cluster-specific transcriptional signatures.

In a developing system, cells may not be synchronized but can be found in a continuum of states. This allows scRNA-seq experiments that sample tissues at one timepoint to reconstruct a continuous dynamic process and order cells along the resulting trajectory. Multiple algorithms have been developed to perform this 'pseudotemporal' ordering, which can be used to infer developmental transitions in simple trajectories (Bendall et al., 2014; Qiu et al., 2017; Trapnell et al., 2014), as well as model branching lineage trees in datasets encompassing common progenitors along with progeny that differentiates into distinct cell types (Cao et al., 2019; Setty et al., 2016). This type of analysis can, thus, reveal gene expression dynamics that drive lineage fate specification and identify transcriptional signatures that may be implicated in fate decisions. Interestingly, an algorithm developed by La Manno and colleagues (2018) leverages knowledge of transcription kinetics to infer future gene expression based on proportions of unspliced and spliced mRNA molecules in each cell. This RNA velocity analysis allows to predict future cell states on a timescale of hours. Finally, algorithms have been developed to infer gene regulatory network activity based on co-expression of transcription factors and putative target genes with enriched transcription factor-binding motifs (Aibar et al., 2017; Van de Sande et al., 2020). Investigation of gene regulation with this type of analysis can be used to better understand the mechanisms that drive cell identity, as well as make predictions of cell fate conversions under transcription factor perturbation (Kamimoto et al., 2020).

Transcriptomics analysis has proven to be a powerful tool for dissecting tissue composition and cellular heterogeneity, and to study gene expression dynamics in development and disease conditions. Technological and computational advances in the field have enabled the scientific community to reveal and characterize novel cell types, as well as explore their functional relevance. These efforts are culminating in the ambitious production and dissemination of informative atlases of human tissues and model organisms (Bakken et al., 2021; Network, 2021; Osumi-Sutherland et al., 2021; Zeisel et al., 2018) that can ultimately aid better understanding of cellular behavior and promote the establishment of systematic, data-driven approaches for therapeutics development.

7 RESULTS AND DISCUSSION

7.1 PAPER I

7.1.1 Summary of the results

This study builds on previous findings of the lab demonstrating that stroke pushes striatal astrocytes into neurogenesis, a process regulated by Notch signaling inhibition. Here, we use a conditional knock-out of Rbpj-K to silence Notch signaling and characterize the neurogenic program of striatal astrocytes using scRNA-seq technologies.

In pseudotemporal ordering, we see that parenchymal astrocytes are located upstream of neural stem cells. However, once activated, Notch-depleted astrocytes closely resemble subventricular zone neural stem cells in the cell states and molecular dynamics they originate. Additionally, striatal neurogenesis resembles hippocampal neurogenesis at the earliest stages, but it diverges through their differentiation, likely reflecting the different neuronal cell types each region gives rise to.

We analyzed astrocytes outside the striatum and show that, albeit they fail to undertake the full neurogenic program, they too upregulate expression of neural stem cell genes.

Finally, we demonstrate that striatal astrocytes that are halted at the pre-division stage can be recruited to produce greater numbers of transit-amplifying cells and neuroblasts via administration of EGF.

7.1.2 Discussion for Paper I

The brain has poor regenerative capacity and efforts towards achieving functional recovery recognize the importance of replacing neurons that are lost to injury. Recruitment of endogenous cells with neural stem cell capabilities is an attractive strategy for replacing neurons and astrocytes represent a valuable source of resident cells for this purpose.

The present study aimed at characterizing the neurogenic program of striatal astrocytes following inhibition of Notch signaling and demonstrated that the molecular dynamics delineating the astrocyte-to-neuron transition are near-identical to those seen in canonical neurogenesis. We further demonstrate that even astrocytes that fail to be recruited into transit-amplifying division show activation of a neural stem cell program.

Our data, thus, suggests that all astrocytes may have potential to generate new neurons given the right molecular cues. Additionally, once recruited into neurogenesis, striatal astrocytes give rise to the same cell types and activate largely similar molecular programs as seen in subventricular zone neurogenesis, further supporting our view of parenchymal astrocytes as a form of latent neural stem cells.

7.2 PAPER II

7.2.1 Summary of the results

In this study we showed that cortical astrocytes retain the potential to generate neurons. Their neurogenic program is activated following Notch depletion, via conditional deletion of Rbpj-K, and when animals are subjected to a stab wound injury.

We further demonstrate that the neurogenic potential of astrocytes is not restricted to specific regions of the mouse neocortex but could be revealed across distal structures, as well as among all cortical layers.

We show that silencing of Notch signaling elicits a neural stem cell program in the astrocytes, regardless of injury. However, it is only after injury that glia undergo cell division and generate neuroblasts committed to mature into cortical interneurons.

Furthermore, we suggest that the neurogenic program can unfold irrespective of reactive gliosis, and that it recapitulates molecular dynamics characteristics of canonical neurogenesis.

7.2.2 Discussion for Paper II

Our previous investigations suggested that astrocytes may be considered as a source of neurogenic cells to replace neurons lost to injury. Notch signaling inhibition forces striatal astrocytes into the neurogenic program, but the same fate is not shared by glia in other regions of the brain. In order to demonstrate the widespread potential of astrocytes for cell replacement therapies, we turned to the cortex to show that Rbpj-K depleted astrocytes can, in fact, undergo neurogenesis in response to injury.

Similar to our previous findings, we confirm that Rbpj-K deletion alone results in the activation of a neural stem cell program in astrocytes throughout the cortical layers, whereas it is only after injury that cortical cells engage in transit-amplifying division.

We demonstrate high transcriptional similarity between neurogenesis by cortical astrocytes and by subventricular zone stem cells and show that cortical cells are committed to generate GABAergic interneurons.

The data produced in this study may represent a valuable resource to learn the mechanisms that drive the commitment of astrocytes to the neurogenic fate and may support identification of molecular cues that enhance their recruitment. This study, further, supports the idea that astrocytes are a precious source of latent neural stem cells. It, also, suggests that the same regenerative behavior is shared among glia from different cortical layers and regions, indicating that astrocytes may be recruited for repair following types of damage and diseases that interest different cortical structures.

7.3 PAPER III

7.3.1 Summary of the results

This manuscript aims at characterizing the molecular profile of cells across the neurogenic program in the human hippocampus. We profiled about 500,000 cells from childhood and adult dentate gyri and identified all major cell types, including neural stem and progenitor cells.

With the childhood dataset we performed an in-depth characterization of the transition from neural stem cells to neurons and identified markers describing cells at each state.

We compared juvenile human and mouse neurogenic programs and demonstrated similar transcriptional dynamics occurring between the two species.

The childhood dataset served as reference for integration with the adult samples and allowed us to identify adult cells with a transcriptional profile resembling that of the childhood neurogenic cells. Following identification of adult hippocampal neurogenic cells, we characterized them molecularly to detect their active gene regulatory network and specific cell type markers.

7.3.2 Discussion for Paper III

Hippocampal neurogenesis is important for memory formation and mood regulation and alterations in the neurogenic process has been linked to psychiatric and neurodegenerative conditions. Studies of adult hippocampal neurogenesis has relied on model animals for characterizing neural stem and progenitor cells contributing to the addition of young neurons to the hippocampal neural circuit. The molecular characteristics of human neurogenic cells have instead remained elusive due to the challenges of working with human specimens.

In this study, we leveraged the high-throughput and transcriptome-wide resolution of snRNA-seq experiments to provide a comprehensive characterization of the molecular profile of human neurogenesis across cell states, as well as throughout ages. We were able to identify neural stem cells, dividing and postmitotic progenitors, as well as immature neurons and show combinations of markers that distinguish each cell state. We, further, provided a characterization of active gene regulatory networks across the neurogenic trajectory and compared childhood and adult programs in terms of numbers of cells captured and their molecular features.

We also performed a cross-species comparison to assess how closely related are mouse and human neurogenic programs. We found good overlap of cell types between species and obtained collections of conserved cell type markers that reflect the overall similarity of the transcriptional programs undertaken by the neural stem cells in the two species. This investigation, thus, supports the use of mice as a valuable model organism to study adult neurogenesis, but also provides a resource for identifying molecular mechanisms that are unique to the neurogenic program in each species.

Ultimately, we hope that our molecular characterization of human neurogenesis will facilitate further investigations into its (dys-)regulation in health and disease.

8 CONCLUSIONS

The work presented in this thesis aims at proposing parenchymal astrocytes as a valuable source of latent neural stem cells that can be recruited, given the right molecular cues, to mount a regenerative response after injury in the adult brain. We have shown that astrocytes from different brain regions retain the potential to generate new neurons in response to Notch signaling inhibition and traumatic injury. Once recruited, the neurogenic program of parenchymal glia follows remarkably similar molecular dynamics as seen in canonical neurogenesis. This suggests that a better understanding of the adult neural stem cell behavior and the mechanisms that drive their activation and neuronal specification in the germinal niches may help us develop therapies that promote efficient conversion of astrocytes into neurons. We have worked towards this end to characterize the neurogenic trajectory of human hippocampal neurogenesis and produced a rich compendium of transcriptional data that can be exploited to learn about key transcriptional signatures of the neurogenic cells. Because the brain is incapable of fully repairing itself after injury, treatments that aim at replacing neurons lost to injury have the potential to rebuild neural circuits and achieve recovery of tissue functionality that are otherwise difficult to attain. Future studies in this field will have to determine if the same molecular mechanisms implicated in canonical neurogenesis to activate quiescent stem cells can be utilized to unfold an astroglial neurogenic program. Furthermore, future research may help us determine how to effectively integrate the new neurons in pre-existing neural circuits to provide functional recovery. Finally, it remains to be established whether astrocytes have the potential to replace neurons in different disease conditions, such as in neurodegenerative diseases.

9 ACKNOWLEDGEMENTS

First and foremost, I would like to thank my main supervisor, **Jonas Frisén**, for giving me the wonderful opportunity to work in the lab. You have always been supportive and available, while giving me the freedom and flexibility to pursue the research questions I was passionate about. These years have been the most fun and stimulating of my life. Thank you also to my co-supervisor **András Simon** for your support.

I would also like to thank all my colleagues, past and present. **Jens Magnusson** for introducing me to the stars of the brain. **Giuseppe Santopolo** for being an amazing friend and supportive colleague: I've been missing having you around the past year and I'm happy you didn't end up moving too far! **Enric Llorens-Bobadilla**, for teaching me a ton and always being excited to talk about new findings. I feel so lucky to have had the chance to work with you during the PhD and I'm thrilled to see what awaits me in the postdoctoral adventure. **Ionut Dumitru**, for always making sure I was well fed with the constant provision of cookies and for involving me in the adult neurogenesis quest. **Camilla Engblom**, for sharing the maternity adventure and making it a whole lot of fun. **Ilke Demirci**, for always cheering me up with the latest gossip and the crazy nights out (although it's been a while since I last managed to join). **Martyna Lukoseviciute**, for the inspiring science and career talks after our dance lessons. **Carl-Johan Eriksson**, for making the last experiments in the animal house much more enjoyable. **Johanna Classon**, for including me on cool new projects. **Helena Lönnqvist** for an endless supply of the hottest chili peppers and your help in the lab throughout the years. Thank you also to **Kanar Alkass**, **Sarantis Giatrellis**, **Joanna Hård**, **Qirong Lin**, **Jeff Mold**, **Marta Paterlini**, **Michael Ratz**, **Moa Stenudd**, **Embla Steiner**, **Mathew Tata**, **Leonie von Berlin**, you have made Frisén lab a great place to work. I am incredibly fortunate to have been a part of such an amazing team of inspiring scientists and great friends.

Thank you also to **Marion**, **Enikő** and **Áron**, **Elin** and **Leland**, **Mehdi**, **Michel** and **Mod**, **Aga**, **Sinan**, **Isak**, **Alexandra**, **Maëlle**, **Ernesto**, **Alrik**, **Alvaro**, and all the other friends in Sweden who have made me feel home during the past five years.

A big thank you to my wonderful parents **Raffaella** and **Sandro**, my brother **Jacopo**, my sisters **Martina** and **Xiaolu**, and my nephew (and newest addition to the family) **Enrico**, for all the love and support throughout the years. Thank you also to my American family, **Emily** and **Dan** for welcoming me so warmly.

Finally, I would like to thank my amazing husband **John Paul**, for your love and for incessantly trying to instill some confidence in me. You have given me the strength to pursue my life's passions no matter how challenging. And forever thank you for giving me the most wonderful and loving family, **Melvin**, **Ray**, **Pinguin**, and our beautiful and perfect son **Oliver Gene**.

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