DOPAMINE

A Versatile Player in Development, Regeneration and Disease

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Cover photo: fibres and cells in the brain of the salamander *Pleurodeles waltl*. Glial fibrillary acid protein fibres (yellow), dopaminergic fibres (green) and cell nuclei (blue). First prize in the Neuro Art image contest February 2017.

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By

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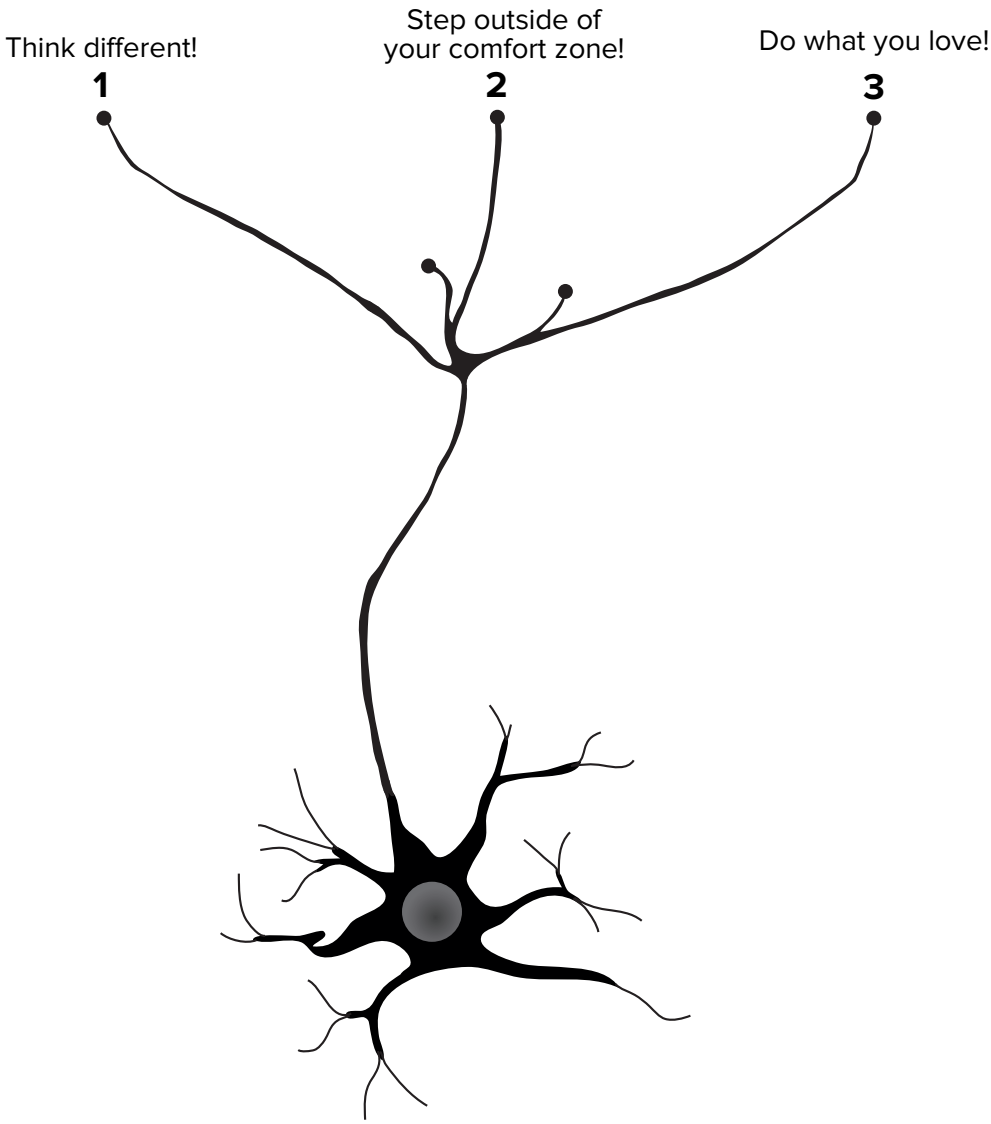

“Any man could, if he were so inclined, be the sculptor of his own brain.”

— Santiago Ramón y Cajal, Advice for a Young Investigator

with love to my family

Abstract

The dopamine neurotransmitter is present in all multicellular organisms. In the brain, the dopaminergic system orchestrates reward-motivation pathways and is involved in the control of voluntary movements and endocrine hormone secretion. Dysfunction of dopamine signalling may lead to pathological conditions such as Parkinson's disease, where dopaminergic neurons of the midbrain degenerate. Moreover, modulation of dopamine receptor signalling influences tumour growth. The aim of this work was to explore the regeneration capacity of the dopaminergic system in the vertebrate brain and to test whether dopamine may control the growth of brain tumours. To this end we performed two sets of studies. Initially, we investigated the development and regeneration of the dopaminergic system in newts, which are aquatic salamanders capable of complete regeneration of the dopaminergic system in the brain. Thereafter, we investigated how dopaminergic ligands impinge specifically on brain tumour cells.

In **paper I**, we screened a library of dopaminergic ligands for their ability to stimulate or to inhibit glioblastoma cell growth and survival. We identified the dopamine receptor 2 antagonist, trifluoperazine, as an inhibitor of glioblastoma growth. We also showed that susceptibility to trifluoperazine correlates with the dopamine receptor expression profile of the investigated glioblastoma cell lines. We concluded that dopamine receptor signalling pathways are promising targets for pharmacological interventions to inhibit glioblastoma growth.

In **paper II**, we characterized the cellular basis of brain development and stereotyped behaviour in two regeneration model salamander species. These data provide insight into the maturation of neural stem cells that are found in the adult salamander brain. Furthermore, we showed how lesioning of the dopaminergic innervation affects neurogenesis in the forebrain and behavioural performance. This study provides a new evolutionary perspective on the genesis and dynamics of brain cells in the salamander brain, including dopaminergic cells.

In **paper III**, we developed a tissue clearing method, CUBICe, to extend our study of dopaminergic neurite outgrowth during development as well as regeneration. We demonstrated that CUBICe is compatible for high resolution imaging of whole salamander brains. It is also a faster and more robust method, which allows to maintain a better sample integrity of embryonic brains in general, compared to Advanced CUBIC and Advanced CLARITY. In addition, using CUBICe we achieved tracing of genetically marked cells with neurite outgrowth of over 3600 µm. Ultimately, we showed that our method is ideal for tracing genetically marked dopaminergic cells in the salamander brain and for quantifying dopaminergic neurite density and regeneration in whole brain regions. In summary, this thesis provides insight into the versatile role of dopamine in both normal and pathological conditions of the vertebrate brain, as well as offers innovative tools for studying the regeneration of the dopaminergic system.

List of Scientific Papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

I. A chemical screen identifies trifluoperazine as an inhibitor of glioblastoma growth

Tiago Pinheiro, Magdalena Otrocka, Brinton Seashore-Ludlow, Vilma Rraklli, Johan Holmberg, Karin Forsberg-Nilsson, András Simon and Matthew Kirkham

Biochemical and Biophysical Research Communications, 2017 Dec 16; 494(3-4):477-483

II. Cellular basis of brain maturation and acquisition of complex behaviors in salamanders

Alberto Joven, Heng Wang, **Tiago Pinheiro**, L. Shahul Hameed, Laure Belnoue and András Simon

Development, 2018 Jan 8; 145:1

III. CUBICe: an optimized clearing method for cell tracing and evaluation of neurite density in the salamander brain

Tiago Pinheiro, Steven Edwards, Ivanna Mayorenko, Alberto Joven, Christina Kantzer, András Simon and Matthew Kirkham,

Manuscript

Contents

List of Abbreviations

2D	two-dimensional
3D	three-dimensional
3DISCO	3D imaging of solvent-cleared organs
5-HT1-7	5-hydroxytryptamine1-7
6-OHDA	6-hydroxydopamine
ACT-PRESTO	active clarity technique-pressure related efficient and stable transfer of macromolecules into organs
ALDH	aldehyde dehydrogenase
AML	acute myeloid leukaemia
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASC	adult stem cell
Ascl1	achaete-Scute Family BHLH Transcription Factor 1
BABB	benzyl benzoate
BLBP	brain lipid-binding protein
Bmi-1	B cell-specific Moloney murine leukemia virus integration site 1
BrdU	bromodeoxyuridine
CAST	clearing assisted scattering tomography
Ce3D	clearing-enhanced 3D microscopy
CNS	central nervous system
CUBIC	clear, unobstructed brain imaging cocktails and computational analysis
CUBICe	CUBICembryonic
DARP23	dopamine-releasing protein 23
DBE	dibenzyl ether
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
DPE	diphenyl ether
DRD1-5	dopamine receptor subtype 1-5

EC50	half maximal effective concentration
EdU	5-Ethynyl-2'-deoxyuridine
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGR-1	early growth response protein 1
En	homeobox protein engrailed-1
eNSC	embryonic neural stem cell
ERK1/2	extracellular signal–regulated kinases 1/2
ESC	embryonic stem cell
eYFP	enchanced yellow fluorescent protein
FAST-Clear	free-of-Acrylamide SDS-based Tissue Clearing
FGF8	fibroblast growth factor 8
FoxA2	forkhead box protein A2
GABA	gamma-aminobutyric acid
GAD6	glutamate decarboxylase 6
GBM	glioblastoma
Gbx2	gastrulation Brain Homeobox 2
GFAP	glial fibrillary acid protein
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
Gli1/2/3	glioma-associated oncogene homolog 1/2/3
GNS	glioblastoma neural stem cell
GS	glutamine synthetase
Gsx2	GS homeobox 2
HCl	hydrochloric acid
HER2	human epidermal growth factor receptor 2
HGCC	human glioblastoma cell culture
IDH	isocitrate dehydrogenase
iDISCO	immunolabeling-enabled three-dimensional imaging of solvent-cleared organs

iExM	iterative expansion microscopy
iPSC	induced pluripotent stem cell
L-DOPA	levodopa/L-3,4-dihydroxyphenylalanine
Lmx1a/b	LIM homeobox transcription factor 1 alpha/beta
m-AchR	muscarinic acetylcholine receptor
MAGIC	multi-addressable genome-integration colour
MAP	microtubule-associated protein
MCM2	minichromosome Maintenance protein complex 2
MGMT	O6-methylguanine–DNA methyltransferase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
Msx1	mutS protein homolog homeobox 1
mTOR	mechanistic target of rapamycin
MTPT	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
n-AchR	nicotinic acetylcholine receptor
NA	numerical aperture
NeuN	neuronal nuclei protein
NeuroD1	neurogenic differentiation 1
NGN2	neurogenin 2
NMDA	N-methyl-D-aspartate receptor
NPY	neuropeptide Y
NSC	neural stem cell
NSPC	neural stem/progenitor cell
Nurr1	nuclear receptor related-1 protein
Otx2	orthodenticle homeobox 2
PACT	passive clarity technique
PARS	perfusion-assisted agent release in situ
Pax	paired box gene
PCNA	proliferating cell nuclear antigen
PD	Parkinson’s disease

PD-L1	programmed death-ligand 1
PDGFRb	beta-type platelet-derived growth factor receptor
Pitx3	pituitary homeobox 3
POMC	pro-opiomelanocortin
Prox1	prospero homeobox protein 1
PSA-NCAM	polysialylated neuronal cell adhesion molecule
PSC	pluripotent stem cell
RI	refractive index
RMS	rostral migratory stream
ROS	reactive oxygen species
SCM	simplified CLARITY method
seeDB	see deep brain
Shh	sonic hedgehog
SNc	substantia nigra pars compacta
Sox2	sex determining region Y-box 2
Sp8	specificity protein 8
STP	serial two-photon
SVZ	subventricular zone
SWITCH	system-wide control of interaction time and kinetics of chemicals
TCGA	the cancer genome atlas
TDE	2,2′-thiodiethanol
TH	tyrosine hydroxylase
Thy1	thymocyte antigen 1
TLX	orphan nuclear receptor tailless
uDISCO	ultimate DISCO
VTA	ventral tegmental area
VZ	ventricular zone
WHO	world health organization
Wnt	wingless-type mouse mammary tumor virus integration site

1. Introduction

"We all woke up this morning and we had with it the amazing return of our conscious mind. We recovered minds with a complete sense of self and a complete sense of our own existence — yet we hardly ever pause to consider this wonder."

António Damásio[1]

The brain… comprised of 86 billions of neurons and another 85 billions of other brain cells (Farfel et al., 2009), interconnected and working together to bring about our conscious mind. What a marvellous structure! Between 400,000-600,000 of these cells are dopaminergic neurons in humans (Björklund & Dunnett, 2007), a tiny fraction of the overall picture and yet, so critical for maintaining correct brain function. More than 70% of these neurons are located in the substantia nigra, a small brain region that suffers severe neurodegeneration in Parkinson's disease (Björklund & Dunnett, 2007; Höglinger et al., 2004; Winner & Winkler, 2015). This level of degeneration of these dopaminergic neurons is correlated with the degree of motor movement deficiencies observed in Parkinson patients. The human brain has limited capacity for regeneration, illustrated by its incapacity to recover from dopaminergic degeneration. The reasons behind this are unclear. Perhaps it has something to do with brain complexity, brain size or a higher degree of specialization of its constituent cells during embryonic development. The historical case of Phineas Gage, a man who suffered severe brain injury by a metal rod which crossed through his skull and brain (Ratiu, Talos, Haker, Lieberman, & Everett, 2004), has shown us the human brain's remarkable focus on damage control. By this I refer to brain plasticity, the capacity of some brain regions to replace the functions of other injured areas. From my perspective, this is a rather limited mechanism and is far from a full regeneration response where the lost cells would actually be replaced. Some organisms, such as salamanders, possess a much greater repertoire of regenerative capacities than humans. Only through the mechanistic understanding of the cellular and molecular regenerative processes in these animals can we try to elicit a similar regeneration potential in humans. Perhaps the regeneration potential of the human brain too lies quiescent, with its cellular mechanisms waiting to be unlocked.

The neurotransmitter, dopamine, is one of the regulating components of regeneration in the salamander brain. Indeed, its absence leads to activation of progenitor cells in the midbrain and subsequent regeneration of an injured dopaminergic population. Dopamine is also present in all known multicellular

[1] "Self Comes to Mind: Constructing the Conscious Brain" (2012), ISBN: 978-0099498025.

organisms (Barron, Søvik, & Cornish, 2010; Berg, Belnoue, Song, & Simon, 2013), providing further support for its biological importance. Moreover, its functions go far beyond neurotransmission and the regulation of regeneration. Dopamine has been shown to be involved in development, by controlling the proliferation of cells which later give rise to different brain functions and behaviours (Freundlieb et al., 2006). Dopamine agonists and antagonists have been shown to regulate cancer progression (Rubí & Maechler, 2010) and dopamine has been found to be involved in the progression of Parkinson's disease, as previously mentioned in the beginning of this chapter. In the case of cancer, dopamine signalling has been shown to be involved in the regulation of cancer growth (Dolma et al., 2016).

The present work provides insight into the versatile role of dopamine in development, regeneration and disease. Furthermore, it provides tools for studying the regeneration of the dopaminergic system.

In the words of Emerson Pugh[2], *"if the human brain were so simple that we could understand it, we would be so simple that we couldn't"*. Studying the brain is an immeasurable challenge and it can be argued that progress in this research field occurs at a somewhat slower pace compared to others. However, we can only try (and hopefully succeed).

1.1. Aims

In order to explore how modulation of dopamine signalling could be used in future clinical therapies, we need to understand its action, the extent of its effects and to have the right tools for its study.

The overall aim of this thesis was to progress the understanding of dopamine's functions in the contexts of development, regeneration and disease, departing from the paradigm of dopamine's control of regenerative processes in the salamander brain (Fig. 1; Berg, Kirkham, Wang, Frisén, & Simon, 2011).

Specific aims for the scientific **papers I-III** were:

I. To evaluate the capacity of dopamine signalling to inhibit glioblastoma proliferation and survival.

II. To provide an evolutionary perspective on the development of the vertebrate brain and associated behaviours, with a focus on the dynamics of dopaminergic neurogenesis.

III. To develop methods for the study of both the neurite outgrowth of dopaminergic neurons and also the fibre projections of ependymoglial

[2] "Biological Origin of Human Values" (1978), ISBN: 978-0710089250.

cells (the progenitors of dopaminergic cells). To undertake this in a regeneration and development setting in the newt brain.

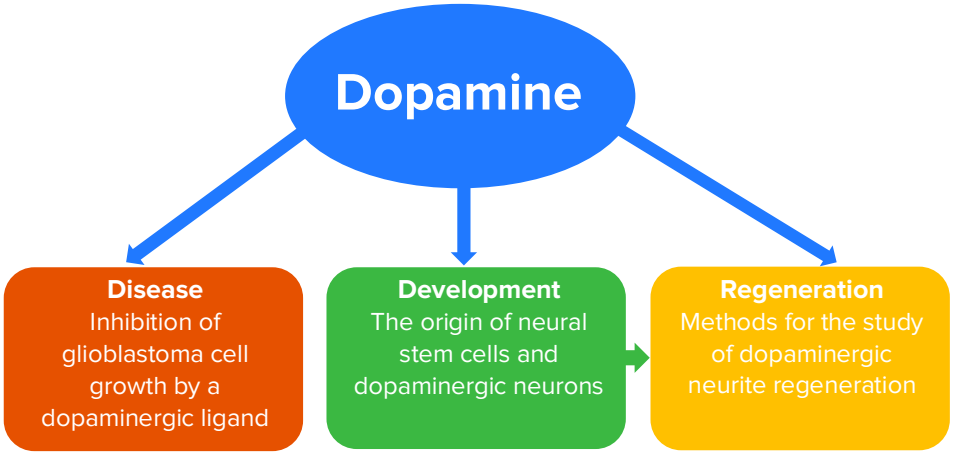

Figure 1 | Dopamine's involvement in the three papers of this thesis as well as in their respective fields: disease, development and regeneration.

1.2. Thesis content

Both **papers II** and **III** included in this thesis utilize the newt, a subfamily of subaquatic salamanders, as an animal model. To understand the importance of the newt as a regeneration model organism, I discuss its advantages in Chapter 2. In Chapter 3, I examine the differences between stem and progenitor cells as well as how to distinguish these cell types from each other. **All the papers** in this thesis study the effects of dopamine or the dopaminergic system, and all of these studies stemmed from observations of the unique neurogenic capacity of the adult newt after dopaminergic lesion. In Chapter 4 I allude to the neurogenic differences between humans, rodents and newts. In **paper II** I attained the need for better methods to study the development and regeneration of dopaminergic neurites in the newt brain, which gave rise to **paper III**. In Chapter 5 I discuss the methodological background that was the genesis for the CUBICe method development (**paper III**). In Chapter 6 I discuss fundamentals of cancer biology, which gave rise to our hypothesis that dopamine is a player in a type of brain cancer, glioblastoma (**paper I**). In Chapter 7, I discuss the background studies of dopamine that bring **papers I-III** together: dopamine's involvement in development, regeneration and disease. In Chapter 8, I summarize and discuss the results of **papers I-III,** as well as presenting some of their limitations**.**

2. The newt: a versatile animal model

The species of salamanders studied in **papers II** and **III**, *Notophthalmus viridescens* (North American) and *Pleurodeles waltl* (Iberian), are newts (Fig. 2; J. Brockes & Kumar, 2005). *Cynops pyrroghaster* (Japanese) is another species of newts which is commonly used in research laboratories (Fig. 2). Newts belong to the subfamily Pleurodelinae of aquatic salamanders and are part of the class Amphibia as well as the superclass of tetrapods. Urodeles, also known as Caudata, is the order containing all salamanders and is characterized by the presence of tail in the adult (Laudet, 2011). Newts are a subfamily of urodeles/salamanders, which can be distinguished from other salamanders by their aquatic life in adulthood (J. Brockes & Kumar, 2005), while other salamanders are mostly terrestrial.

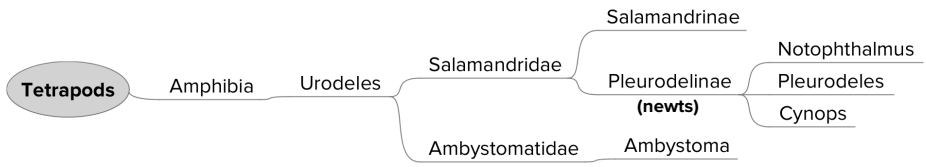

Figure 2 | Phylogenetic positions of Notophthalmus, Pleurodeles, Cynops and Ambystoma among the tetrapod superclass.

To reach adulthood newts go through three developmental phases: larval, terrestrial juvenile and aquatic adult (J. Brockes & Kumar, 2005; Joven, Kirkham, & Simon, 2015). These developmental phases were used as research models in **papers II** and **III**. During the larval phase, newts undergo metamorphosis, which is considered to be a rather dramatic postembryonic developmental process, where abrupt morphological changes occur (Reiss, 2002). Among non-extinct amphibians, frogs, salamanders and caecilians demonstrate distinct metamorphic capacities. These morphological changes have three bases aimed at functional adaptation: (i) environmental – a switch from water to land/air (ii) locomotional – a switch from swimming to hopping (iii) feeding – a switch from feeding on water-suspended food to predation. However, in newts, metamorphosis is a rather mild, less dramatic process, when compared to frogs. This is especially so when considering newts do not undergo environmental functional adaptation through metamorphosis: they remain living an aquatic life after adulthood instead of

switching to a terrestrial habitat. Generally, metamorphosis in the newt involves the reabsorption of the gills and the development of the limbs (Cameron, Beug, & Tsilfidis, 2004). During juvenile to adult phases, newts become sexually mature.

Another species of salamanders commonly used in research laboratories is the axolotl, *Ambystoma mexicanum* (Fig. 2). This species is often incorrectly identified as a newt due to its aquatic life. Instead, the aquatic characteristics of the axolotls are due to paedomorphosis, where it retains the larval characteristics in the adult form (Laudet, 2011). The axolotl is extensively used in limb regeneration studies. Interestingly, rather different cellular and molecular mechanisms are involved in limb regeneration in the axolotl, when compared to the newt (Sandoval-Guzmán et al., 2014).

Among vertebrates, newts display the largest repertoire of regenerative capacities in adulthood (Tsonis & Fox, 2009). Additionally, their evolutionary proximity to mammals make them ideally suited for the cross-species study of the basal foundations of their regenerative capacities (Laudet, 2011). Newts are able to regenerate the heart (Oberpriller & Oberpriller, 1974; Witman, Murtuza, Davis, Arner, & Ian, 2011), limbs (J. P. Brockes, 1997), spinal cord (Davis, Ayers, Koran, Anderson, & Simpson, 1990), lens (Eguchi, 1988), brain (Parish, Beljajeva, Arenas, & Simon, 2007), male but not female gonads (Scadding, 1977), tail (Iten & Bryant, 1976), intestine (O'Steen, 1958) and jaw (Goss & Stagg, 1958). Interestingly, the kidney has been reported as a structure not capable of regeneration in the newt (Scadding & Liversage, 1974). The capacity for brain regeneration in newts is discussed in detail in Chapter 7.

A striking feature of newt regeneration is its independence of age and successive number of injuries. Such findings were demonstrated in a series of experiments spanning 30 years, where some animals underwent 17 consecutive rounds of lens removal and subsequent regeneration episodes, without any sign of regenerative decline in terms of pace, morphology, or gene expression pattern (Eguchi et al., 2011).

The dopaminergic innervation pathways of the basal ganglia, such as the midbrain-to-striatum pathway studied in **papers II** and **III**, are highly conserved in tetrapods (O Marín, Smeets, & González, 1998). Taken together, the evolutionary proximity to mammals, the capacity for regeneration of dopaminergic cell subpopulations and the high degree of conservation of the dopaminergic midbrain-to-striatum pathway make newts an ideal animal model for the mechanistic understanding of the dopaminergic system's regeneration (Laudet, 2011; O Marín et al., 1998; Parish et al., 2007).

3.Of stem and progenitor cells

The concepts of stem and progenitor cells are sometimes used ambiguously, which can lead to confusion (Seaberg & Van Der Kooy, 2003).

The concept of stem cell properties derives from studies by McCulloch and Till, who first demonstrated transplanted mouse marrow cells could proliferate and form colonies in the spleen of irradiated mice (Becker, McCulloch, & Till, 1963). This finding added to the then existing model of haematopoiesis, which assumed blood cells would arise from progenitor cells with proliferation capacity. Based on these studies, it was then suggested hematopoietic tissue would have a hierarchical structure, composed of progenitor cells, cells with limited capacity for division and fully differentiated cells. At the same time, the concept of stemness, the unique characteristics of what defines a stem cell, was proposed (Becker et al., 1963; McCulloch, Minden, Miyauchi, Kelleher, & Wang, 1988; Seaberg & Van Der Kooy, 2003). Currently, these characteristics are: (i) long-term capacity for division and self-renewal; (ii) an undifferentiated state; (iii) capacity to give rise to differentiated cell types (Bethesda, 2016).

Stem cells can be classified according to different degrees of potency (Banerjee, 2011): (i) totipotent – capable of giving rise to complete embryos and the extra embryonic tissue; (ii) pluripotent – capable of giving rise to every cell type in the body; (iii) multipotent – capable of giving rise to multiple cell types in a given tissue; (iv) unipotent – capable of giving rise to only one cell type in a given tissue.

Stem cells can also be classified according to their origin (Banerjee, 2011): (i) pluripotent embryonic stem cells (ESCs); (ii) multipotent or unipotent adult stem cells (ASCs); (iii) cancer stem cells (still a hypothesis, as explained in Chapter 6); (iv) induced pluripotent stem cells (iPSCs), where the term “induced” refers to the fact that these cells are genetically or chemically reprogrammed to become stem cells from adult somatic cells. Pluripotent ESCs can give rise to any of the three embryonic germ layers: ectoderm, mesoderm and endoderm. From each of these layers, different lineages of ASCs are derived, which in its turn give rise to cell types belonging to each of the specific tissues in the body (e.g. pancreatic or neural tissue).The concept of stem cell has been blurred by the discovery of stem cell plasticity, a concept that implicates different types of stem cells in the crossing of

lineage boundaries to give rise to tissue belonging to different germ layers than the one they originate from (Blau, Brazelton, & Weimann, 2001).

Progenitor cells are an intermediate state in the differentiation path from stem cell to differentiated cell, and can be in an undifferentiated or partly differentiated state (Bethesda, 2016). Stem cells can be distinguished from progenitor cells by functional assays (Seaberg & Van Der Kooy, 2003). However, the assessment of these functional characteristics can be challenging, either in vitro or in vivo. Stem cells can divide symmetrically to give rise to either two other stem cells with capacity for self-renewal or two progenitor cells with no capacity for self-renewal but with proliferative capacity. Alternatively, stem cells can divide asymmetrically and generate one stem cell and one progenitor cell.

In vitro, it is possible to assess if a cell has stem properties. However, assessing proliferation of cells alone is not evidence of being a stem cell, as different kinds of progenitor cells are also capable of proliferation. In fact, being proliferative does not even imply capacity for self-renewal (Clarke et al., 2006). To assess the functional characteristics of stem cells in vitro, one can evaluate if the cell proliferates and track its progeny to check whether specialized cell types are formed. Thus, indicating multipotentiality or unipotentiality.

4. Neurogenesis

Neurogenesis is defined as the production and functional integration of new neurons. In the adult brain, neurogenesis is fuelled by neural stem/progenitor cells (NSPCs), which are located in discrete neurogenic niches. The mammalian adult brain displays a limited neurogenic potential and this could explain the inability of mammals to regenerate neurons lost after stroke-injuries or during the development of neurodegenerative diseases. A wide variation in the extent of adult neurogenesis is seen among species, and this variation correlates with habitat and absolute age (Amrein, 2015). Specifically, rodents living in environmentally challenging habitats were shown to have higher adult hippocampal neurogenesis than rodents living in protected environments (Amrein et al., 2014). Furthermore, cell proliferation decreases with age in the hippocampus of rodents in both environmentally challenging and protected habitats (Amrein et al., 2014; Amrein, Isler, & Lipp, 2011a; Cavegn et al., 2013). While such variation represents a difficulty for the development of a common mechanistic understanding of neurogenesis, it also represents an opportunity to extend the limited regenerative capacity of the mammalian brain. This might be achieved through the understanding of neurogenesis in various species followed by the developing of methodological approaches to elicit neuron regeneration in the species where this capacity is lacking.

4.1. In humans

The cellular composition of the adult human brain has long been viewed as static. This is partly because research on the birth of new neurons has been undertaken more intensively in rodent models than in humans due to methodological limitations. However, we know today that neurogenesis also occurs in the human brain (Eriksson et al., 1998; Spalding, Bhardwaj, Buchholz, Druid, & Frisén, 2005). NSPCs have been found in two neurogenic niches: in the subventricular zone (SVZ) of the lateral ventricles and in the dentate gyrus (DG) of the hippocampus (Fig. 3; Braun & Jessberger, 2014). These findings were based on the expression of neuroblast markers and cell morphology (Frisén, 2016). Moreover, neuroblasts were deemed to decrease in numbers after the initial post-natal weeks, potentially explaining the limited regenerative capacity observed in the adult human brain (Göritz & Frisén, 2012). Neurogenesis has also been reported to occur in a third

neurogenic niche, the human striatum (Fig. 3). However, the source of these new neurons is still debated and is discussed later in this chapter (Ernst et al., 2014).

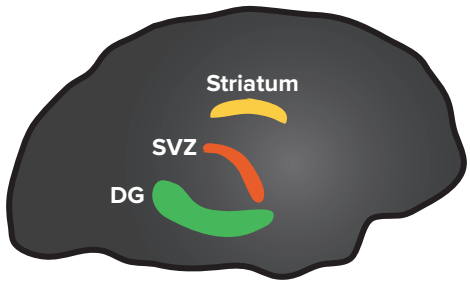

Figure 3 | Neurogenic niches in the human brain: DG, SVZ and striatum. ***Adapted from Braun & Jessberger, 2014.***

The first evidence for neurogenesis in the adult human brain was the finding of the incorporation of a nucleotide analogue, Bromodeoxyuridine (BrdU), in the cells in the DG of post mortem brains from patients with head and neck cancer (Eriksson et al., 1998). Here, BrdU was administered for diagnostic purposes. Since then, the development of a method to date cell birth based on the levels of carbon-14 in genomic DNA has paved the way to new insights into adult human neurogenesis (Spalding et al., 2005).

* Dentate gyrus of the hippocampus

In the DG of the hippocampus, two populations of neurons are known to exist. In one population, no cell turnover has been detected, while the other population has a median annual turnover of 1.75% (Spalding et al., 2013). This rate modestly declines with age in humans, while in rodents it declines more emphatically from young to old age (Ben Abdallah, Slomianka, Vyssotski, & Lipp, 2010). In mice, during the hippocampal neurogenic process, a subpopulation of about 10% of the dentate granule neurons are replaced throughout adulthood (Fig. 4; Imayoshi et al., 2008; Ninkovic, Mori, & Götz, 2007). In contrast, all of the dentate granule neurons are thought to be replaced during adulthood in humans (Fig. 4; Bergmann, Spalding, & Frisén, 2015; Spalding et al., 2013). While the functional relevance of hippocampal neurogenesis in mice has been correlated with pattern separation in memory formation and cognition, evidence for the same functions in humans are still lacking (Bergmann et al., 2015).

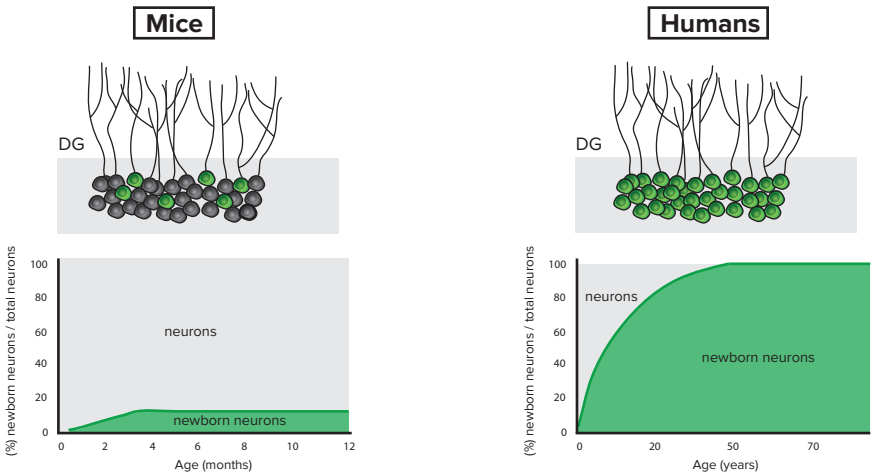

Figure 4 | Proportion of dentate granule neurons replaced throughout the lifetime of mice and humans. While in humans virtually all of these neurons are replaced throughout life, in mice this proportion accounts for only 10%. *Adapted from Ernst et al., 2014*.

* Subventricular zone of the lateral ventricle walls

Another neurogenic niche in the adult human brain is the SVZ in the walls of the lateral ventricles. Whether NSPCs give rise to migratory neuroblasts that travel to the human olfactory bulb is controversial. Upon studying of the identity of the NSPCs in the human SVZ, Sanai and colleagues have shown a ribbon of astrocytes lining the lateral ventricles which express glial fibrillary acid protein (GFAP) and vimentin (Sanai et al., 2004). Contrary to the situation in mice, the same study has found no evidence for neuroblast migration. However, in the forebrain of human foetal samples, another study reported substantial proliferation and migrating neuroblasts expressing doublecortin (DCX) in the human SVZ (C. Wang et al., 2011). The DCX expression in the SVZ was confirmed by another study performed with infant human brain samples (Sanai et al., 2011). Curtis *et al.* argued that a rostral migratory stream (RMS) exists in humans, through which neuroblasts migrate to the olfactory bulb (Curtis et al., 2007). However, the measurement of the proliferating cell nuclear antigen (PCNA) in this study, as a proliferation marker of neuroblasts, did not correlate with the expression of ki-67 and MCM2 markers used in similar studies (Sanai et al., 2004; C. Wang et al., 2011). These findings indicate PCNA might not be specific to proliferating cells in the human brain (as reviewed in Bergmann 2015). An eventual neuronal turnover in the olfactory bulb of adult humans would indirectly argue for the existence of the RMS. Yet, the turnover rate of these neurons has been reported to have an exchange rate of less than 1% in 100 years, a striking contrast to the 50% annual exchange rate seen in rodents (Bergmann et al., 2012; Imayoshi et al., 2008). Taken together, the existence of

migrating neuroblasts from the human SVZ and of a human RMS remain elusive. It is therefore unclear what the fate of the neuroblasts found in SVZ may be, especially considering the absence of apoptosis (Ernst et al., 2014).

* Striatum

As opposed to mice, neurogenesis has been reported to also occur in the human striatum (Ernst et al., 2014). Both protein and transcriptomic interrogations demonstrated that the neuroblast marker DCX is present in the striatum with expression levels comparable to the human hippocampus. Additionally, striatal interneurons were shown to be born in the adult brain of cancer patients who had been administered with a thymidine analogue for diagnostic purposes (Ernst et al., 2014). Furthermore, a turnover rate of 2.7%/year was found in the DARP23^{-} interneuron subpopulation in the striatum, indicating that new interneurons are formed through neurogenesis in the striatum (Bergmann et al., 2015; Ernst et al., 2014). Whether these newly born interneurons originate from the SVZ is unclear. Contrary to this theory, in another study performed in mice, parenchymal astrocytes have been found to give rise to striatal neurons post stroke, opening new possibilities for the role of astrocytes in striatal neurogenesis (Magnusson et al., 2014). Indeed, the proposed roles of the striatum in motor function and cognitive flexibility would argue for the occurrence of striatal neurogenesis throughout adulthood (Ernst & Frisén, 2015).

* Neurogenesis after acute brain injury - ischemic stroke

Despite the presence of NSPCs in humans, brain regeneration is not known to occur after injury, and our capacity to halt neurodegeneration is modest. However, an increase in cell proliferation was found after ischemic stroke in the ipsilateral SVZ, raising questions on the existence of a neurogenic response after injury (Marti-Fabregas et al., 2010). Additionally, preliminary data on the age of striatal neurons in human samples with ischemic stroke has shown likelihood for two possibilities: either there is increased neurogenesis in the striatum after striatal stroke or there is selective death of old neurons compared to young neurons (Magnusson, 2017). The answer could also be explained by a combination of the two possibilities. Although stroke patients can show some signs of recovery, months or even years after stroke, the mechanism currently believed to be responsible for this is brain plasticity rather than neurogenesis (Nudo, 2013).

* Neurogenesis after chronic brain damage - Parkinson's disease

In the case of chronic pathological conditions, such as Parkinson's disease (PD), where patients do not regenerate lost dopaminergic neurons in the midbrain, humans once again show limited capacity for brain regeneration. However, one

study has found expression of a marker of immature migrating neuroblasts, the polysialylated neuronal cell adhesion molecule (PSA-NCAM). This finding is indicative of activation of resident glial cells, which are known to also express PSA-NCAM (Arias-Carrión, Freundlieb, Oertel, & Höglinger, 2007; Nomura, Yabe, Rosenthal, Krzan, & Schwartz, 2000; Yoshimi et al., 2005). This process could be the first step to an incomplete neurogenic response. The involvement of dopamine and neurogenesis in PD is further discussed in Chapter 7.

* Cell replacement therapies

Cell replacement therapies hold great promise towards the replacement of the lost cells after brain injury, though success in human trials has been limited (Lindvall, Kokaia, & Martinez-Serrano, 2004). In principle, there are two main routes for cell replacement therapy, involving the use of endogenous or exogenous cell sources (Grade & Götz, 2017). In an endogenous approach, the NSPCs in the DG and SVZ neurogenic niches, or the latent local progenitors can be induced to expand and differentiate, and possibly to migrate, to replace a lost cell type at an injury site. For instance, astrocytes have been successfully induced to enter a neurogenic program by blocking of notch signalling in the striatum of mice (Magnusson et al., 2014). Another way to pursue an endogenous approach is to reprogram brain cells close to the injury site to the lost cell type, using either transcription factors or small molecules (Pfisterer et al., 2016; Takahashi et al., 2007). Exogenous approaches involve the transplantation of committed or differentiated donor cells to the injury site, with the aim of replacing lost cells. In this approach donor cells can be foetal neurons, embryonic neural stem cells (eNSCs), embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The use of foetal neurons for cell replacement therapies has already been applied in Parkinson's disease patients with variable results. The start of the first pluripotent stem cells (PSC) human trials in Parkinson patients is imminent. These findings are further discussed in Chapter 7.

4.2.In rodents

In rodents, the two most extensively studied neurogenic niches are the SVZ of the lateral ventricles and the DG of the hippocampus (Fig. 5; Braun & Jessberger, 2014). NSPCs are mostly in a quiescent state in the SVZ and DG, but upon activation by internal or external signals, they undergo proliferation and differentiation into new neurons, followed by maturation and integration into axonal networks in their surroundings (Braun & Jessberger, 2014; Chunmei Zhao, Deng, & Gage, 2008).

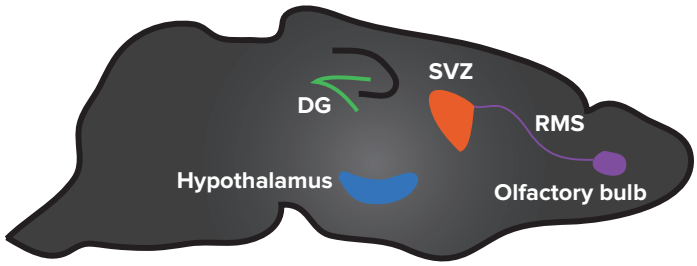

Figure 5 | Neurogenic niches in the mouse brain: DG, SVZ and Hypothalamus. ***Adapted from Braun & Jessberger, 2014.***

* Dentate gyrus of the hippocampus

In the DG of the hippocampus, new neurons are generated by radial NSPCs, also called radial-glia-like cells. These are activated to produce numerous non-radial NSPCs, thereby increasing the number of cells with neurogenic capacity. These cells become immature neurons and migrate to the granule cell layer where they integrate their projections in the hilus and area 3 of cornu ammonis, thereby becoming granule neurons (Toni et al., 2008; C. Zhao, 2006). One of the functions of the neurogenic process in the DG is believed to be the pattern separation in learning and memory (Sahay, Wilson, & Hen, 2011). Interestingly, cell proliferation in the hippocampus declines with age. Studies in both wild and laboratory mammals indicate that this decline is similar across several species, independent of the ontogenetic rate and correlated with absolute age (Amrein, 2015; Amrein, Isler, & Lipp, 2011b). However, the levels of actual hippocampal neurogenesis have been reported to differ based on the original habitats of a pool of 11 rodent species. Here, mole-rats with subterranean habitats and relatively stable habitats show lower levels of hippocampal neurogenesis than southern African mice living in dry demanding climates (Amrein, 2015; Amrein et al., 2014; Cavegn et al., 2013). Such findings argue for a role of hippocampal neurogenesis in behavioural flexibility. In accordance with a role of the surrounding environment in the regulation of the neurogenic process, physical exercise in the form of running has been shown to increase neurogenesis in the dentate gyrus of the hippocampus (Van Praag, 2008). Furthermore, such variations in neurogenesis levels between species highlight the need for undertaking cross-species studies before generalization of the dynamics and cellular processes involved in neurogenesis.

* Subventricular zone of the lateral ventricle walls

In another neurogenic niche in mice, the SVZ of the lateral ventricle, neurogenesis occurs in two phases. First, type B NSPCs, also called radial-glia like cells, are activated to produce non-radial type C NSPCs with neurogenic capacity. These

cells become immature neurons (type A neuroblast cells) and migrate to the olfactory bulb through the rostral migratory stream (RMS). Then, upon arrival to the olfactory bulb, neuroblasts differentiate to produce interneurons (Braun & Jessberger, 2014; Kempermann, Jessberger, Steiner, & Kronenberg, 2004; Merkle & Alvarez-Buylla, 2006). During this process, about 40% of the maturated neurons integrate and survive for up to 19 months (Winner, Cooper-Kuhn, Aigner, Winkler, & Kuhn, 2002).

* Hypothalamus

Several studies have reported neurogenesis to occur in the hypothalamus of rodents (Kokoeva, 2005; Pierce & Xu, 2010). One of the progenitor cell sources in this neurogenic process is believed to be the β2-tanycyte population, a type of radial glia-like ependymal cells at the base of the third ventricle of the hypothalamic median eminence (D. A. Lee et al., 2012). These cells have been shown to give rise to new born neurons which express mature hypothalamic neuron markers such as GAD6, MAP2, NPY, POMC and c-fos (D. A. Lee et al., 2012). The functions of hypothalamic neurogenesis are still under interrogation. Interestingly, mice fed with a high-fat diet had 4-fold higher neurogenesis than the controls, demonstrating the effect of dietary changes in adult hypothalamic neurogenesis (D. A. Lee et al., 2012).

* Molecular regulation of adult neurogenesis

Transcriptional regulators are involved in the regulation of the neurogenic process in the SVZ and the DG. These are Prox1, Sox2, NeuroD1, Ascl1, Pax6, Gsx2, Sp8, Prox1 and TLX (Braun & Jessberger, 2014; Chunmei Zhao et al., 2008). Interestingly, neurotransmitters have also been shown to control adult neurogenesis in the SVZ and DG, implicating neurotransmitter signalling into broader brain functions than the signal transmission between neurons (Berg et al., 2013). These neurotransmitters are acetylcholine, dopamine, GABA, glutamate, nitric oxide, neuropeptide Y, noradrenaline and serotonin. In Chapter 7 the role of the neurotransmitter dopamine in brain regeneration in connection with neurogenesis is further discussed. The literature on the molecular regulation of adult neurogenesis is vast. Besides the regulation exerted by neurotransmitters and transcription factors, several proteins and hormones also play a role (Egeland, Zunszain, & Pariante, 2015; Guillemot, 2005; Hagg, 2005; H. Liu & Song, 2016).

* Neurogenesis after ischemic stroke

Rodents show some capacity for adult neurogenesis after ischemic stroke. In rats, a study shows that ischemic stroke in the striatum induces neurogenesis of cholinergic and GABAergic neurons, which become functionally integrated (S. W.

Hou et al., 2008). In gerbils, an increased neurogenesis was detected in the dentate gyrus post ischemic stroke, which is shown by newly generated cells expressing neuronal markers such as calbindin-D28K, microtubule-associated protein-2 and neuronal nuclear antigen (J. Liu, Solway, Messing, & Sharp, 1998). Although rodents seem to show a higher capacity for neurogenic-based regeneration after ischemic stroke than humans this is still modest and does not lead to full recovery. The lack of studies in humans maybe misleading, thus there might also be an eventual neurogenic response after stroke, similar to the one observed in rodents.

4.3.In newts

Newts exhibit the most extensive regenerative capacity among vertebrates, as previously discussed in Chapter 2. Parish and colleagues examined the regenerative/neurogenic ability of the adult newt brain, *Notophthalmus viridescens*, by using a cell type-specific chemical ablation injury model, which resembles a Parkinson-like animal model (Parish et al., 2007). This ablation was performed with 6-hydroxydopamine (6-OHDA), which is a neurotoxin that selectively ablates catecholaminergic neurons and is used in the creation of other parkinsonian animal models. Here the authors have shown that, after selective ablation of dopaminergic neurons in the ventral midbrain, a neurogenic program was natively initiated. This lead to full regeneration of the lost neuronal population as well as reacquisition of normal motor performance. These findings demonstrate that that the adult vertebrate brain is capable of regulating its neurogenic regenerative potential by activation and deactivation of quiescent regions, such as the ventral midbrain in the newt. A more detailed discussion on the role of dopamine in neurogenesis is provided in Chapter 7.

The regeneration of dopaminergic neurons in the ventral midbrain of the newt is fuelled by ependymoglial cells, which line the lateral ventricle and express GFAP (Berg et al., 2010). Due to the lack of astrocytes in the newt brain this is the only cell type expressing GFAP (Benraiss, Arsanto, Coulon, & Thouveny, 1999; Lazzari, Franceschini, & Ciani, 1997). In the newt telencephalon, ependymoglial cells have been found to be of two types (Kirkham, Hameed, Berg, Wang, & Simon, 2014): Type-1 are stem-cell-like, $GFAP^+$ and glutamine synthetase $(GS)^+$, and are the main quiescent; Type-2 are transit-amplifying-progenitor-like, $GFAP^+$ and GS^-, and correspond to the majority of the proliferating $GFAP^+$ cells in activate neurogenic regions of the brain or the so called “hotspot regions”. Interestingly, after cholinergic ablation, mainly quiescent regions of the brain, “non-hotspots”, became more active. This was shown by the proliferation of type-2 cells and PSA-$NCAM^+$ cells which could now be found in these previously quiescent regions. PSA-NCAM is a marker of immature migrating neuroblasts (Arias-Carrión et al., 2007; Nomura et al., 2000; Yoshimi et al., 2005). Taken together, the dynamics of type-

1, type-2 and PSA-NCAM$^+$ cells upon cholinergic lesion suggest an orchestrated neurogenic process in both regions of active neurogenesis and regions that were previously quiescent in the newt brain.

Another study has shown that accumulation of reactive oxygen species (ROS) is required for ependymoglial cell activation as well as subsequent neurogenesis (Hameed, Berg, Belnoue, & Jensen, 2015). Furthermore, ROS was specifically shown to be necessary for the regeneration of the ventral midbrain dopaminergic neurons after lesion.

In **paper II**, we aimed to further characterize the mechanisms behind the brain regenerative capacity in the newt. To this end, we studied the basis of cellular brain development in two newt species, *Pleurodeles waltl* and *Notophthalmus viridescens*. We described the regional peaks of neurogenesis throughout development and correlate it with the acquisition of stereotypical behaviours. We also found that injury-induced lack of dopaminergic innervation in the striatum decreased proliferation in the brain of *Pleurodeles waltl*. Such findings gave rise to the hypothesis that a mechanism of striatal neurogenesis is controlled by dopaminergic midbrain-to-striatum fibres.

In summary, newts possess a native brain regeneration capacity and an ability to activate their quiescent cells to an extent that has not been observed in mammalian brains. The understanding of such processes could represent an important contribution towards developing native regeneration therapies for brain injuries.

5. Tissue clearing methods for the brain

Tissue clearing methods facilitate the imaging of whole brain volumes without the need for mechanical sectioning and preserve structural relationships between the components of a sample. These advantageous features are especially useful for neurite or fibre tracing studies. Generally, whole-structure imaging methods, such as magnetic resonance imaging (MRI), stand as an alternative to the mechanical sectioning of samples. In fluorescent or light microscopy, however, imaging deep into the tissue is largely limited by light scattering, caused by the different phases of water and organic compounds within the sample, which result in a variable refractive index (RI) throughout the tissue. These variations bend the light and make the tissue opaque. In order to achieve transparency and, in consequence, deep tissue imaging, one needs to homogenize these RI variations. In biological samples, lipids are one of the main sources of light scattering. Hence, the removal of lipids is at the basis of achieving tissue transparency. In essence, many tissue clearing techniques do exactly that and make it feasible to image large whole-samples with fluorescence or light microscopy, without mechanical sectioning.

5.1. Advantages

+ Retaining of the structural information of a sample

Biological samples are inherently three-dimensional (3D). In most classical methods, aimed at the analysis of both structural and molecular information, samples are divided into smaller sections. These sections of the, initially, 3D sample often resemble a two-dimensional (2D) structure. A common process employed to transform a sample into a 2D structure is mechanical sectioning with cryostats or microtomes. This process destroys the structural relationship between the different components within a sample, such as cells or neurites, and therefore prevents the tracking of features between serial sections, such as fibre tracing. It is possible to reconstruct the 3D structure of a sample from sections but this process is usually laborious, and may introduce structural innacuracies due to tissue deformation introduced during the mechanical cutting process or during

histological processing. Furthermore, mechanical sectioning can be generally time consuming if one aims to manually image every section of a sample.

Automated high-throughput methods for mechanical sectioning have tackled some of its drawbacks, such as its laboriousness, expensiveness and the user-introduced damage to the tissue. These include serial block-face mechanical methods (A. Li, 2010), and can be combined with both two-photon tomography (Ragan et al., 2012), electron microscopy (Winfried Denk & Horstmann, 2004) or array tomography (Micheva & Smith, 2007). The major limitations of these techniques include the non-compatibility with molecular labelling, the speed of acquisition, the difficulty of set up and, ultimately, the destruction of the sample. Economo and colleagues tackled the lack of molecular labelling and, in part, the speed of acquisition with the development of a high-speed volumetric serial two-photon (STP) tomography (Economo et al., 2016). While this technology is capable of imaging over 50 mm^3/day and 16 megavoxels/s, representing a 16-48x increase over conventional laser-scanning microscopes, it still takes approximately one week of imaging time for a whole mouse brain. As a comparison, a light sheet Z.1 microscope (ZEISS) can acquire 127 megavoxels/s[3]. Therefore, limitations remain for high-speed volumetric STP tomography in terms of speed of acquisition for large samples and lack of commercial availability.

Tissue clearing methods have the advantage of allowing for the imaging of intact biological samples without the need for mechanical sectioning, thanks to rendering the sample transparent. The imaging of a whole transparent sample can be achieved with conventional microscopy methods, which are widely available, such as light sheet, confocal or two-photon microscopy. In this manner, the structural information of a sample is retained.

+ Compatibility with molecular phenotyping

Molecular phenotyping of a biological sample is achieved either by the expression of a genetic reporter coupled to the molecular component of interest, such as a fluorescent protein, or by the detection of a molecular component with reporter-tagged affinity molecules, such as whole-tissue immunostaining with antibodies. Different tissue clearing methods are compatible with one or both of these approaches of molecular phenotyping, thus providing a substantial advantage for the molecular interrogation of research samples, as opposed to whole-structure imaging methods such as MRI. In addition, fluorescent molecular phenotyping allows for the multiplexed interrogation of molecular components in the same sample, by the application of fluorescent reporters with differing excitation and emission spectra. Specifically, the CLARITY method allows for the molecular

[3] Own calculations assuming a frame of 1920x1920 pixels and a minimum camera exposure time of 29 ms.

phenotyping of not only proteins but also microRNAs and mRNAs through multiplexed *in situ* hybridization (Sylwestrak, Rajasethupathy, Wright, Jaffe, & Deisseroth, 2016).

+ Multiple rounds of molecular phenotyping in the same sample

Even with the use of optically separable fluorescent reporters for multiplexing of molecular phenotyping, one is still limited by the often broad excitation and emission spectra. Such phenomena place the cap for multiplexing in cleared samples at around 3-5 fluorescent reporters per sample, depending on the imaging system. Conveniently, some tissue clearing methods allow for the detergent-mediated removal of affinity-based reporters which were applied during a previous round of molecular phenotyping (Chung et al., 2013; Murray et al., 2015). This process facilitates the re-interrogation of the same sample with another set of affinity based-reporters and, in this manner, dispenses the need for more biological replicates and prevents the difficulties associated with biological variability between samples.

+ Speed of acquisition

When tissue clearing and imaging by light sheet microscopy are combined, an unprecedented speed of acquisition is achieved. The merge between these technologies make it feasible to image large biological samples in high-resolution and in enough replicates to answer a biological question. For example, Tomer and colleagues achieved the imaging of a Thy1-eYFP whole mouse brain at 10x magnification in ~4 h (Tomer, Ye, Hsueh, & Deisseroth, 2014).

+ Easy digital reconstruction and 3D visualization

The numerical aperture (NA) of a microscope objective refers to its ability to capture light and resolve details of a sample. Typically, NA increases with an objective's magnification, which in turn is inversely correlated with the field of view. Upon imaging, if a sample does not fit in the field of view and one does not wish to decrease the resolution, images need to be separately acquired at multiple positions and encompassing the whole volume of interest in a process named tiling. Post acquisition, these tiles need to be reconstructed into a single 3D volume, in a process named stitching. In other words, while in mechanical sectioning methods the sample is physically cut, in tissue clearing imaging the sample is instead optically cut, with accurate preservation of the positional coordinates of each tile in order to ensure faithfull reconstruction of its 3D structure. A digitally reconstructed 3D sample allows for the visualisation of its original morphology and provides an accurate representation of the location of its components relative to one another. Substantial advances in bioinformatic tools

for tile stitching have progressively made this reconstruction process straight forward and time. Some examples of these tools are Terasticher, Arivis, Imaris Stitcher and Big Stitcher (Bria & Iannello, 2012).

+ Fibre tracing

The understanding of the brain can greatly benefit from the study of the interconnections between structure and function at a whole organ level, as exemplified by the brain's long ranging connections. The connectome is defined by Defelipe as the map of brain connections at the macroscopic and intermediate levels, while the synaptome is regarded as the same study at an ultrastructural level (DeFelipe, 2010). Methods for the study of the connectome and synaptome traditionally require large amounts of time for imaging and data processing (Kasthuri & Lichtman, 2007). The tissue clearing method CLARITY has been shown to achieve high-speed imaging while allowing for brain-wide projection mapping, by direct observation of cell fibres (Ye et al., 2016) or, indirectly, by observation of their input/output cell bodies (Menegas et al., 2015). Individual fibre tracing has also been demonstrated in other tissue clearing methods, as in 3DISCO (Belle et al., 2014), and synaptic-scale resolution can be achieved in some cases, as in SeeDB2 (M. Ke et al., 2016).

In studies addressing the function of a specific brain region, or in cases where fibre density is too high to trace an individual neurite, it can be of use to fluorescently mark the neurites belonging to a subpopulation of neurons. Lipophilic neuronal tracers, such as DiI, can achieve this and have the advantage of binding to cellular membranes, which prevent them from diffusing before sample fixation. However, many tissue clearing methods work precisely by removing lipids to reduce light scattering and, thereby, turn the sample transparent. Therefore, during the clearing process the lipophilic tracers are also washed away. However, the lipophilic dye DiD can be used by direct dissolution in the clearing reagent, as shown by the labelling of myelinated fibres in the SWITCH protocol (Murray et al., 2015). Yet, this approach labels all myelinated populations of neurons within the samples, rather than specific populations of interest. Certain aqueous-based detergent free clearing protocols, such as Sca*l*eS, seeDB, Clear^{T} and FRUIT, are able to preserve lipids, and, in consequence, the lipophilic neuronal tracers (Hama et al., 2015). Yet, seeDB, clear^{T} and FRUIT are limited in the depth of imaging they facilitate. Furthermore, Sca*l*eS' clearing capability is slightly lower than alternative lipid clearing methods, such as CLARITY and CUBIC. Jensen and Berg have addressed this limitation by testing DiI-analogue dyes with molecular modifications that make them aldehyde-fixable and, thus, less clearable during the CLARITY protocol (Kristian H R Jensen & Berg, 2016). Indeed, these DiI-analogue dyes are compatible with CLARITY, with CM-DiI displaying the sharpest and FM1-43FX the strongest fluorescent signal. These methodological advances open new avenues to neuronal tracing in conjunction with tissue clearing.

In **paper III**, we aimed to apply the fibre tracing capacities of tissue clearing to the study of dopaminergic fibres in the newt brain, both in development and in regeneration. Although we used a different approach to fibre tracing, neuronal tracing dyes have previously been used in the newt, *Pleurodeles waltl* newts to inform on afferent and efferent connections to the striatum, as well as catecholaminergic innervation of the striatum and nucleus accumbens (Oscar Marín, González, & Smeets, 1997a, 1997b; Oscar Marín, Smeets, & González, 1997). The joining of tissue clearing with neuronal tracing dyes could further enlighten the suggestive regeneration phenomenon in the midbrain-to-striatum pathway of newts.

+ Extraction of 3D measurements

The study of samples in 3D allows for the measurement of unique properties of brain-wide projecting fibres, that would not be possible in 2D representations without accurate reconstruction. Some examples of these parameters are the measurement of the projection angle of a fibre, the number of dendrite terminal points, the length of all the fibres belonging to a specific cell body and the number of branching points (**paper III**). If aiming to find out the density of arborisation in a 3D region, one can also perform Sholl analysis, which measures the number of intersecting fibres in a number of concentric circles (Langhammer et al., 2010; Sholl, 1956). Such measurements can inform on differences between cell types, through characterization, and cell function, through correlation with *in vivo or ex vivo* studies.

5.2. Limitations

– Alterations in tissue morphology

Many tissue clearing methods induce the expansion or shrinking of a sample, throughout the clearing protocol (Kolesová, Čapek, Radochová, Janáček, & Sedmera, 2016). In some, the alteration in morphology is transient while in others it is permanent. These changes in tissue size can potentially result in non-homogeneous deformations of tissue morphology. While clearing techniques such as SeeDB and Sca*l*e U2 are able to clear tissue without morphology alterations, the extent of clearing in large samples and the length of the protocol are certainly issues (Richardson & Lichtman, 2015). To address the alteration of morphology limitation in tissue clearing techniques, cellular and subcellular structures can be compared by hematoxylin and eosin staining in cleared and uncleared samples (Gómez-Gaviro et al., 2017). For nanoscale structure one can only monitor changes with transmission electron microscopy (Gómez-Gaviro et al., 2017). It has been shown that different clearing methods have a different degree of preservation of

the subcellular structure (Hama et al., 2015). This should be taken into account upon the choice of a method, if the preservation of the subcellular structure is important. Morphological alteration may also be less of a problem if quantifications are compared within the same clearing protocol, rather than between different ones.

– Protein loss

Tissue clearing techniques can result in protein loss throughout the clearing process (Fig. 6). If the sample being cleared has a weak expression of a reporter or if the protein to be detected is present already in very low quantities, then molecular phenotyping may be limited after clearing. In the cases where protein loss represents a problem this can be minimized by a process called hydrogel-tissue hybridization, where hydrogel monomers enter the tissue and are subsequently polymerized into a hydrogel mesh which covalently cross-links proteins in the tissue (Fig. 7; Chung et al., 2013).

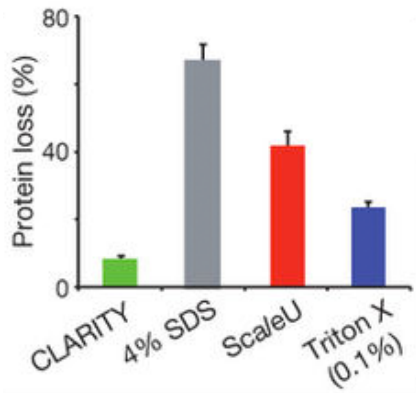

Figure 6 | Amount of protein loss by the end of in four tissue clearing processes: CLARITY, 4% SDS, Sca*l*eU, Triton X (0.1%). ***Reproduced with permission from Chung et al. (2013).***

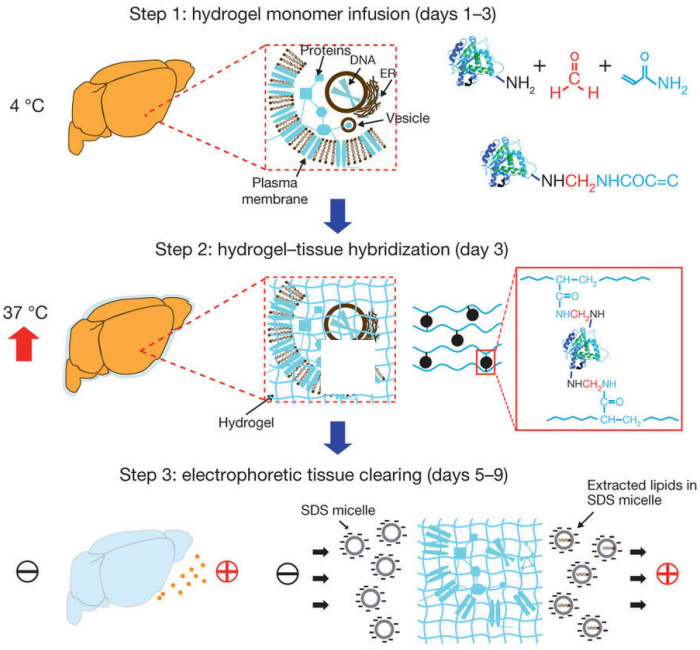

Figure 7 | Hydrogel-tissue infusion, hybridization and tissue clearing process in the CLARITY protocol. First, the tissue is infused with hydrogel monomers and cross-linked with formaldehyde. Second, the hydrogel monomers are induced to polymerize into a mesh by a temperature increase. This is followed by SDS-based lipid clearing. A major proportion of the protein content is kept due to the hydrogel mesh which is in place during the clearing process. *Reproduced with permission from Chung et al. (2013).*

– Duration of the protocol

From start to end, tissue clearing protocols can take days, weeks or even months, depending on the technique and thickness of the sample (Richardson & Lichtman, 2015). While this is a limitation, depending on the technique, actual hands-on time can be very limited and comparable to traditional sectioning and immunostaining. Furthermore, we rely more and more on the development and commercialization of technological solutions capable of performing most of the protocol steps automatically and with high reproducibility (e.g. solutions from LifeCanvas Technologies and the X-CLARITY system from Logo Biosystems).

In **paper III**, we optimized the Advanced CUBIC method, not only in terms of minimizing sample fragility but also in terms of reducing the protocol time from 16 to 9 days.

– High start-up costs

Some tissue clearing techniques represent high costs in terms of reagents and RI matching solutions, such as FocusClear currently selling at 36$/ml. Furthermore, specialized equipment may also be required, for example the need for an electrophoretic tissue clearing chamber in the initial CLARITY publication (Chung et al., 2013). In this case, the development of passive clarity has dispensed the need for the chamber but, in exchange, increased protocol duration (Tomer et al., 2014). Tissue clearing techniques with cheaper reagents and lower equipment requirements exist, such as CUBIC (E. a. Susaki et al., 2014). However, the choice of technique depends on many factors, such as the size of the sample, the need to remain free of toxic reagents, the retaining of functional fluorescent proteins and the compatibility with immunostaining (Richardson & Lichtman, 2015). If the purchase of a light sheet microscopy is required, this is another substantial cost to consider. The establishment of collaborations with facilities which can provide access to this technology is a way to circumvent such investment. Light sheet microscopy is becoming more widespread across academic institutions and, perhaps in the future, we will see a light sheet microscope in every laboratory/department just as with confocal microscopy.

– Big data

The imaging of large samples in high-resolution results in large amounts of data being created, often in the terabyte range. Data processing, such as stitching, creates further copies of the original datasets, adding to the data storage requirements. Handling such big datasets requires also large amounts of computational memory so that the computational algorithms can run effectively. Besides the need for high computing power and large storage space, high network transfer speeds are also required to move the data or share it. Despite the existence of cloud storage services like Amazon S3, pushing terabytes of data routinely over the network is not practical. The solution is to keep the data locally and to invest in high-speed local networks, computer power and storage space. Such solutions are more easily implemented at the level of core facilities rather than individual laboratories. Apart from data volume, there is no file format or framework standard for sharing light sheet microscopy data or comparing it with data from other systems. Partly addressing this gap, Fürth and colleagues proposed an interactive framework for sharing connectivity and mapping data across laboratories and imaging technologies, including wide field, confocal and light sheet microscopy (Fürth et al., 2017).

5.3. A historical perspective

Both the use and development of tissue clearing techniques have proliferated in the last decade, but its first description is attributed to Spalteholz a century ago (Spalteholz, 1911, 1914; Vigouroux, Belle, & Chédotal, 2017). Spalteholz treated tissues with a mixture of benzyl alcohol and methyl salicylate, thus rendering them transparent by replacing its water with organic compounds having higher RI. At the time, there were no microscopes capable of imaging deep enough into cleared tissue to take advantage of is transparency.

It was not until 1990 that the first two-photon laser scanning fluorescent microscope was made available (W Denk, Strickler, & Webb, 1990), soon followed by the first light sheet microscope (Voie, Burns, & Spelman, 1993). Since then and throughout its development, these technologies increasingly pushed the depth boundary of tissue imaging. One more decade passed before the introduction of the first commercially available clearing solution – FocusClear (Y. C. Liu & Chiang, 2003). While this allowed for high resolution deep imaging by making use of a confocal microscope, its contents remained proprietary and highly expensive for use with large samples/experiments (Y. C. Liu & Chiang, 2003). Helmchen further demonstrated the potential of deep tissue imaging by successfully using a two-photon microscope to acquire images 800 µm deep into the mouse neocortex (Helmchen & Denk, 2005). Following this depth breakthrough, a whole mouse brain was successfully imaged with a modified ultramicroscope, by making use of a clearing solution made of a mixture of benzyl-alcohol and benzyl-benzoate (BABB) (Dodt et al., 2007). Although BABB does render tissue highly transparent, it has toxic properties and molecular phenotyping is hindered by the fast quenching of fluorescent protein emission (~half a day) (Dodt et al., 2007; Richardson & Lichtman, 2015). This quenching issue has later been addressed in a modification of the BABB protocol, entitled FluoClearBABB (Schwarz, Scherbarth, Sprengel, & Engelhardt, 2015).

It was, however, in 2011 that three essential factors for the success of tissue clearing methods come together: (i) a new clearing agent with an open source formula and capable of preserving fluorescent signals in labelled samples – Sca*l*e (Hama et al., 2011); (ii) the launch of the first commercial light sheet microscope by LAVision, providing rapid sample imaging in high resolution; (iii) developments within computer processing power, network infrastructure and data storage, making practical analysis of light sheet data feasible. Thanks to this combined effort the tissue-clearing field is currently branching out towards numerous applications, uncovering brain features previously deemed inaccessible by depth, reconstruction or resolution limitations. Furthermore, constant bioinformatic developments are making the data stitching and neuronal tracing faster, more accurate and more user-friendly (Bria & Iannello, 2012; Hörl, Rusak, Harz, Treier, & Preibisch, 2017; Usher et al., 2017).

5.4.The different methods

Since the introduction of Sca*l*e we have seen the development of a vast number of clearing techniques, tackling issues such as fluorescent protein quenching, speed of clearing, sample deformation and toxicity (Feuchtinger, Walch, & Dobosz, 2016; Richardson & Lichtman, 2015, 2017), or focusing on protocol optimizations for specific applications (Kubota et al., 2017). The best taxonomy to categorize modern clearing methods is still debatable. Richardson and Lichtman proposed clustering the techniques based on the physical mechanism underlying the clearing process: (i) Solvent-based; (ii) Simple immersion; (iii) Hyperhydration; (iv) Hydrogel embedding; (v) Hydrogel embedding and hyperhydration (Richardson & Lichtman, 2015, 2017). Clusters ii-v belong, at a higher-level category than aqueous-based clearing. Instead, Silvestri and colleagues proposed clustering clearing techniques based on their intended application: (i) whole organ light sheet microscopy-based imaging; (ii) large area confocal or two-photon microscopy-based imaging (Silvestri, Costantini, Sacconi, & Pavone, 2016). Each of these two categories is then split into endogenous fluorescence or *ex-vivo* staining. While both taxonomies have logic, the constant specialization and application-based variations of clearing protocols might create too much of an overlap between techniques, thereby making it difficult to attribute them to a specific classification. In this section, we will use the taxonomy based on the physical mechanism of clearing to discuss the differences between methods.

Most tissue clearing methods have 2-4 protocol steps in common (Richardson & Lichtman, 2017; Tainaka, Kuno, Kubota, Murakami, & Ueda, 2016): (i) pre-treatment; (ii) permeabilization and delipidation; (iii) Immunolabelling; iv) RI matching. First, during the pre-treatment step, samples are treated with decolourization/bleaching reagents to remove light-absorbing or auto fluorescent pigments. During this step, certain techniques opt instead to embed the sample in a hydrogel to prevent protein loss or keep the components together during sample expansion. Secondly, during the permeabilization and delipidation step, the sample is exposed to detergents or organic solvents in order to remove lipids and facilitate the penetration of reagents in later steps. Thirdly, during the immunolabelling step, the sample is exposed to affinity molecules that will bind to the targets to be detected. These molecules can penetrate the sample passively, through simple diffusion, or actively, through the exertion of pressure or electric current. If immunolabelling is not required or if the sample already contains natively expressed fluorescent proteins, then this step can be omitted. Lastly, during the RI matching step, the RI of the sample is equilibrated by impregnation with a variety of solutions which homogenize the RI.

* Solvent-based clearing methods

These methods work by a dehydration and lipid solvation step, followed by a RI adjustment step with an organic solvent with a RI close to the dehydrated tissue (Richardson & Lichtman, 2015).

Since solvent-based methods, such as 3DISCO, remove water from the sample and water is needed to maintain emission of most fluorescent proteins, these end up quenched, thereby preventing the analysis of these endogenous reporters (Ertürk, Lafkas, & Chalouni, 2014). Later developments of these techniques (uDISCO) have minimized this issue by the substitution of dibenzyl ether (DBE) with diphenyl ether (DPE) (C. Pan et al., 2016). In this case, fluorescent emission is preserved for at least a few weeks at imageable levels, although still decreasing with time. However, this progressive drop in fluorescence limits the comparison of expression levels based on fluorescent signal intensity, unless the samples are compared at the exact same time points post clearing, which is a rather challenging. Furthermore, the solvents used in these methods are corrosive and can potentially damage imaging objectives in some microscopes. This can, however, be circumvented by isolating cleared samples in tubing with a RI which matches the imaging media and objective. Lastly, solvent-based methods typically induce sample shrinking, which can be an advantage in terms of the amount of data acquired or a disadvantage, if the features to be observed are at the limit of the microscopes resolution.

In **paper III**, we chose not to use solvent-base methods due to the corrosive characteristics of the solvents involved and their incompatibility with the objectives used in the light sheet Z.1 microscope. Another reason for our choice, is the fluorescence quenching which is observed over time with these methods.

Solvent-based clearing methods include Spalteholz method, BABB/Murray's clear, 3DISCO, iDISCO, iDISCO+ and uDISCO (Dodt et al., 2007; Ertürk et al., 2012; C. Pan et al., 2016; Renier et al., 2014, 2016, Richardson & Lichtman, 2015, 2017).

* Simple immersion

These methods work by replacing the liquid inside the tissue with a high RI solution, in order to average the RI of the sample and thereby achieve a similar sample RI to the liquid it is immersed in (Richardson & Lichtman, 2015). Most of these techniques are easy to implement and preserve lipids, which can be an advantage if using lipophilic neuronal tracing dyes, as they will be not be removed during clearing. It can however be a disadvantage, as clearing effectiveness can be lower, as well as antibody penetration during immunostaining.

Simple immersion clearing methods include sucrose, FocusClear, Clear^{T}, Clear^{T2}, SeeDB, SeeDB2G, SeeDB2S, FRUIT, TDE, CUBIC-Cancer, FAST-Clear and C_e3D (M. Ke et al., 2016; Kubota et al., 2017; W. Li, Germain, & Gerner, 2017; A. K.L. Liu, Lai, Chang, & Gentleman, 2017; Richardson & Lichtman, 2015, 2017).

* Hyperhydration

These methods work by removing lipids and thereby reducing the RI of the sample (Richardson & Lichtman, 2015). For lipid removal, they make use of detergents, which is a slow process and requires multiple solution changes. Hyperhydration methods can result in high protein losses (24-41%) and, in consequence, a weak immunostaining signal due to the reduced amount of epitopes (Chung et al., 2013; Richardson & Lichtman, 2015). These methods use urea to facilitate penetration of the clearing detergents, by partially denaturing proteins in hydrophobic regions of the tissue.

The clearing protocol presented in **paper III** is an optimization of a hyperhydration method, CUBIC (E. a Susaki et al., 2015). The first reagent in this protocol uses urea for hyperhydration, quadrol for decolourization and Triton-X 100 for delipidation (Tainaka et al., 2016). The second reagent expedites the clearing process by using sucrose and again urea for hyperhydration and triethanolamine for delipidation (Richardson & Lichtman, 2015; Tainaka et al., 2016).

Hyperhydration clearing methods include Sca*l*e A2, Sca*l*e U2, Sca*l*e S CUBIC and whole-body CUBIC (Hama et al., 2011, 2015; E. a. Susaki et al., 2014; E. A. Susaki & Ueda, 2016; E. a Susaki et al., 2015).

* Hydrogel embedding

These methods address the protein loss that occurs during hyperhydrating alternatives. They do so by embedding the tissue in a hydrogel mesh prior to clearing (Chung et al., 2013). Then, lipids are removed with detergent solutions and electrophoresis, followed by RI matching. By doing so, high transparency and antibody penetration can be achieved in large samples, with only about 8% protein loss (for CLARITY cleared samples). Some hydrogel based methods have additional advantages, such as improving antibody distribution by activating antibody binding post-antibody penetration (SWITCH), or enhancing antibody penetration by the use of pressure (ACT-PRESTO) or an electric field (stochastic electrotransport) (Kim et al., 2015; E. Lee et al., 2016; Murray et al., 2015).

Hydrogel embedding methods include CLARITY, PACT, PARS, Bone CLARITY, ACT-PRESTO, SWITCH, SCM and stochastic electrotransport (Chung et al., 2013; Greenbaum et al., 2017; Kim et al., 2015; E. Lee et al., 2016; Murray et al., 2015; Sung et al., 2016; B. Yang et al., 2014).

* Hydrogel embedding and hyperhydration

These methods take advantage of the sample expansion observed in hyperhydration methods and the capacity of the hydrogel-based methods to keep the sample components together to perform controlled expansion of the sample

with minimal morphological distortion. This process gave rise to expansion microscopy, in which diffraction-limited microscopes can be used to image samples at biological scales only before deemed possible by super resolution microscopy.

At the time of writing of this thesis two expansion tissue clearing protocols exist: (i) MAP, which allows sample expansion up to 4-5x the original size (Ku et al., 2016); (ii) iExM, which allows for sample expansion up to 20x the original volume by using more than one round of expansion (Chang et al., 2017).

The direct and unbiased comparison of clearing methods is necessary for users to perform an informed choice of the best alternative to their application. Orlich and Kiefer have partially filled this gap by directly comparing ten clearing techniques in the mouse brain, muscle, heart, kidney and lung, and also in embryos and foetuses at different developmental stages (Orlich & Kiefer, 2017). The clearing methods compared in this study were: Murray's clear, 3DISCO, Sca*l*e, SeeDB, FRUIT, CUBIC, CUBIC-perfusion, Clear^{T2}, CLARITY, PACT,

For CLARITY based protocols, Jensen and Berg provide a comprehensive review of the different alternatives and optimizations, as well as providing advice on which factors to consider when choosing a protocol, such as the type of tissue, the speed of clearing, the type of staining required, the price of the optical clearing solution and the imaging options available (Kristian H.Reveles Jensen & Berg, 2017).

5.5. Applications

Imaging deep into tissue and the visualization of large samples in 3D by tissue clearing methods have allowed for novel research studies and applications. Here we discuss some of these in respect to brain studies.

* Cancer metastasis profiling at a whole-brain level

Kubota and colleagues optimized the CUBIC tissue clearing protocol for the imaging of cancer metastasis at a whole body/organ level (Kubota et al., 2017). Given this method's capacity for single-cell resolution, even micro metastasis can be detected. In this study, mice were intracardiacally injected with two metastatic cancer cell lines and the newly developed metastasis in their brains were classified in terms of their histological patterns. The results suggest that different cell lines spread differently through the blood vasculature. While some may promote angiogenesis of new vasculature to support their growth, others migrate to pre-existing vasculature to take advantage of their nutritional maintenance potential. The same study demonstrates the use of tissue clearing to study the spatiotemporal dynamics of metastatic progression and the therapeutic effect of anti-tumour drugs, by screening for volume and number of foci. Other studies have merged tissue clearing, automatic segmentation and quantification methods and

the study of anti-tumour drugs. By doing so it is possible to inform on tumour morphology, vasculature and drug penetration, providing a more representative overview than with mechanical sectioning (Dobosz, Ntziachristos, Scheuer, & Strobel, 2014; Feuchtinger et al., 2016).

These approaches could be of value when performing in vivo studies on the anti-tumour effect of trifluoperazine, the anti-glioblastoma drug reported in **paper I**.

* Integration of brain grafts in a hosts brain

One study has examined the integration of neuroepithelial-like stem cells in mice brains by marking cells with lentiviral vectors, followed by transplantation of the grafts into the brain and infection with rabies virus (Doerr et al., 2017). Such procedure allows for rabies-mediated trans-synaptic tracing and shows that, not only can the grafted cells integrate with the hosts neurons, but they can also project in different amounts and to different areas depending on their input transplanted region. Another study has used the tissue clearing technique SeeDB to research the interaction of implanted glioblastoma cells and mesenchymal stem cells in the zebrafish brain (Vittori et al., 2016).

* Wiring and molecular features of brain regions

CLARITY tissue clearing has been used in the study of the projections and molecular phenotype of cell subpopulations in the prefrontal cortex of mice (Ye et al., 2016). This study shows that different cell populations in the prefrontal cortex represent positive or negative emotion judgments and that this affects their wiring pattern, behaviour and gene expression.

The same clearing method, CLARITY, has been applied in the study of the substantia nigra pars compact (SNc) dopaminergic subcircuits, facilitating the identification of two different nigrostriatal dopaminergic circuits, based on their inputs, outputs, biophysical parameters and how their environmental information was represented (Lerner et al., 2015).

* Spatiotemporal study of nerve regeneration

The optic nerve is not part of the brain but we comment on this study in the context of nerve regeneration within the central nervous system (CNS) in **paper III**.

Diekmann *et al.* used FocusClear to perform whole mount tissue clearing of retinal cell cultures of transgenic zebrafish (Diekmann, Kalbhen, & Fischer, 2015). They show the spatiotemporal regeneration of individual axons in the zefrafish retina, setting up a platform for the mechanistic understanding of CNS regeneration in zebrafish.

* Mapping of brain activity

Renier and colleagues have combined the tissue clearing method iDISCO+ with automated bioinformatics pipelines for cell detection and registration in order to observe brain activity at cellular resolution (Renier et al., 2016). Brain activity is measured by expression of the immediate early gene, c-fos, whose expression reflects recent neuronal activity. The bioinformatics part of this method is entitled ClearMap. By applying iDISCO+ and ClearMap, this study was able to detect brain regions activated in response to haloperidol, a dopamine receptor (DR) antagonist and anti-psychotic drug. The same study could also link brain activity with specific behaviours, such as the exploration of new environments with or without whiskers in mice.

* Forensic medicine investigations

To investigate infant head injury post-mortem in suspected cases of abusive head trauma (Cheshire et al., 2015). This method allows for the detection of small amounts of bleeding in the dura mater.

* Label-free brain imaging

The tissue clearing method SWITCH was reported to extend the imaging depth of optical frequency domain imaging, a technology based on detecting photons scattered from tissues, from 1-2 mm to the whole mouse brain depth (Ren, Choi, Chung, & Bouma, 2017). This methodological combination is entitled Clearing Assisted Scattering Tomography (CAST) and makes it possible to perform connectome and volumetric studies completely label-free.

* Neuroanatomical studies

One study has applied BABB tissue clearing to samples of mice cerebellum in order to study the spatial distribution of Purkinje cells in 3D (Silvestri et al., 2015). They developed automated algorithms for cell identification, which then facilitated the identification of gaps in the lamellar organization of Purkinje cells, which are suggested to play a role in autism disorders. This study also opens new possibilities for new ways of anatomical division, based on the clustering analysis of Purkinje cells.

Another study has used CLARITY and light sheet microscopy to answer if midbrain dopaminergic neurons projecting to different brain regions would form distinct subclasses based on their projection inputs (Menegas et al., 2015). By applying rabies retrograde trans-synaptic tracing systems, this study demonstrates that midbrain dopaminergic neurons with projections to the posterior striatum receive

major inputs from the zona incerta, the globus pallidus and the subthalamic nucleus.

* Neurodegeneration studies

Liebmann and colleagues have used iDISCO and the ClearMap bioinformatic software to detect amyloid plaques in the brains of mice and in human archival samples (Liebmann et al., 2016). They demonstrate this to be a rapid and accurate method to quantify amyloid plaques in terms of number, density, volume, relative distance and sphericity.

Other studies have also shown tissue clearing to be able to inform on neurodegeneration, such as in the imaging of Lewy bodies associated with PD and of mitochondrial proteins associated with respiratory chain deficiency in mitochondrial disease (Alan King Lun Liu et al., 2015; Phillips et al., 2016).

* Development of brain organoids

Renner et al. demonstrated the use of SWITCH tissue clearing in the study of the development and differentiation of human cerebral organoids (Renner et al., 2017). They show the existence of distinct ventral and dorsal regions in these organoids, many of which being linked. They further identify a forebrain organizing centre which secrete growth factors and provide spatiotemporal insights into the generation of different types of brain cells, such as neurons, astrocytes and oligodendrocytes.

* Ischemic brain studies

To quantify micro-vessels in a model of brain ischemic injury, one study applied 3DISCO tissue clearing in combination with a hydrogel-fluorescent dye solution that marks micro-vessels (Lugo-Hernandez et al., 2017). This method allows for the quantification of vessel length and vessel density, as well as the distinction between vessels with different diameter, which differ in areas affected by focal cerebral ischemia.

* Paper III – study of dopaminergic neurites and cell projections

In **paper III** we have evaluated the performance of Advanced CUBIC and Advanced CLARITY in the study of dopaminergic neurites and ependymoglia radial projections (E. a Susaki et al., 2015; Tomer et al., 2014). The choice of these particular methods was based on the following factors:

- Reducing requirements for specialized equipment.

- Compatibility with immunostaining.
- Easy-to-apply protocol.
- Prevention of protein loss (only in Advanced CLARITY).
- Specifically compatible with the light sheet microscope ZEISS Z.1.
    - ◊ In this microscope the sample is placed within a chamber which is open at the top and forced ventilation is not available. For this reason, toxic RI matching solutions, as the ones often used in solvent-based clearing methods, should be avoided.
    - ◊ The objective of this microscope does not support RI's over 1.48, thereby limiting the choice of tissue clearing methods. For example, the solvent-based clearing method 3DISCO uses DBE (RI=1.56) as final clearing solution and is therefore incompatible with this objective (Ertürk et al., 2012).

6. Cancer biology

In order to develop new therapies that can halt or reduce cancer growth we need to understand how cancer cells within a tumour differ from each other. There are two main models to explain cancer cell heterogeneity: the classical clonal evolution model and the more recent cancer stem cell model (Shackleton, Quintana, Fearon, & Morrison, 2009). The first, the classical clonal evolution model, postulates that the different cell types within a tumour arise from genetic and epigenetic changes occurring in each individual cancer cell. The second, more recent model, argues for the existence of cancer stem cells, a discrete subpopulation of cells with tumorigenic potential, which exhibit self-renewal and differentiation capacities and are able to give rise to all the cancer cell types that are found within a tumour. Evidence for the cancer stem cell hypothesis come from several sources. For instance, discrete cell subpopulations in human cancers have been found to be the source of new cancer cells when transferred into immunocompromised mice. This has been shown in several cancers models, (Shackleton et al., 2009) including: breast (M. Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003), germ cell (Illmensee & Mintz, 1976; Kleinsmith & Pierce, 1964), leukaemia (Bonnet & Dick, 1997; Lapidot et al., 1994), brain (Singh et al., 2004) and colon (P. Dalerba et al., 2007; O'Brien, Pollett, Gallinger, & Dick, 2007; Ricci-Vitiani et al., 2007). Furthermore, these putative cancer stem cells all expressed particular marker combinations, such as $CD44^{+}/CD24^{-}$ in breast cancer (M. Al-Hajj et al., 2003). The existence of cancer stem cells, with the ability to extensively proliferate, and other tumour cells, with limited ability for proliferation, implies a heterogeneous and hierarchical organization of cell types during cancer development (M. Al-Hajj et al., 2003). Hierarchical organization in cancer refers to the existence of a usually unidirectional process of epigenetic changes from a tumorigenic to a non-tumorigenic cancer cell phenotype (Shackleton et al., 2009). In this paradigm, non-tumorigenic cells compose the bulk of the tumour. This hierarchical organisation has major implications for cancer biology since drugs targeting the cells which fuel cancer growth - i.e. the cancer stem cells - could prove more effective than targeting the already differentiated non-dividing cells.

However, the cancer stem cell hypothesis cannot account for why lymphoblastic leukaemia with high-grade B cells do not naturally exhibit a hierarchical organization (Williams, Den Besten, & Sherr, 2007). However, a hierarchy can be artificially induced upon imatinib therapeutic treatment. Interestingly, the cancer stem cell model does not completely exclude the clonal evolution model. Indeed,

cancer stem cells could evolve through clonal evolution while simultaneously giving rise to their progeny, less stem-like, cancer cells (Shackleton et al., 2009).

In this chapter we further explore the concepts of the cancer stem cell theory and its links to the differentiation of normal stem cells in homeostatic conditions. We also discuss the resistance of newts to cancer formation and the role of neurotransmitters in cancer. These two paradigms influenced our hypothesis that the neurotransmitter dopamine has a key role in glioblastoma (**paper I**).

6.1. A stem cell disease

Just as a normal tissue is heterogeneous and can contain self-renewing stem cells, progenitor and differentiated cells, tumour tissues are also believed to be heterogeneous by containing different cell types as well as homogeneous within each specific cell population. The cancer stem cell hypothesis explains tumour heterogeneity by the existence of cancer stem cells with tumorigenic potential, which can give rise to diverse types of non-tumorigenic cells within a tumour. Therefore, a cancer stem cell is defined as, a cell belonging to a tumour which has the capacity for self-renewal and for multipotentiality towards the different lineages of cancer cells that make up a tumour (Clarke et al., 2006). This model could partly explain both the resistance to anti-cancer drugs, as these could be targeting tumour cells that are not responsible for tumour growth, and also recurrence, as cancer stem cells can be left in the body after drug or surgical treatment and thereby serve as the genesis of new tumours (Shackleton et al., 2009).

Cancer stem cells were first defined in 1997 by Bonnet and Dick in human acute myeloid leukaemia (AML) (Bonnet & Dick, 1997). They observed that a subpopulation of AML cells, with a marker profile of $CD34^{++}$ $CD38^{-}$, were the ones responsible for initiating human AML in mice. As mentioned at the beginning of the chapter, the identification of cancer stem cells has been replicated in many other cancers (Shackleton et al., 2009).

The terms tumour-initiating cell and cancer stem cell are sometimes used interchangeably. This is because, to define a cell as a cancer stem cell, its properties, self-renewal and multipotentiality, need to be determined experimentally, just as with normal stem cells (Clarke et al., 2006). When this is not possible, the term tumour-initiating cell is more frequently used. The need for further clarification of these concepts has been identified in a review by Visvader (Visvader, 2011). Here, it is suggested that naming of “tumour-initiating cells” is a better term to denote the cell of origin in a given tumour, while “cancer stem cells” is a better term for the cells fuelling cancer propagation.

Cancer stem cells were found to give rise to multilineage differentiated tumour cells with restricted proliferation potential, demonstrating a tumour hierarchical organization that, in turn, argues in support of the cancer stem cell hypothesis

(Piero Dalerba et al., 2011). Because of this hierarchical organization, the route for cancer stem cell differentiation from tumorigenic to non-tumorigenic cell, as well as the maintenance of cancer stem cells, is believed to be based on epigenetics. If cancer would evolve by stochastic genetic mutations then it would be hard for cancer stem cells to remain as a discrete cell population expressing particular markers. Several mechanisms behind the epigenetic regulation of cancer stem cells and cancer progression have been elucidated and are believed to act through pathways such as Wnt, Notch and sonic hedgehog (Shh) signalling (Toh, Lim, & Chow, 2017).

While differentiated tumour cells are usually sensitive to conventional therapeutic agents such as radiation therapy and chemotherapy, it is believed that cancer stem cells display marked resistance to these therapeutic approaches (Muhammad Al-Hajj, Becker, Wicha, Weissman, & Clarke, 2004). To tackle this, combination therapies could target both differentiated tumour cells and cancer stem cells, in this manner potentially preventing tumour relapses. Many clinical trials on epigenetic modulating drugs which are capable of targeting cancer stem cells therefore hold great promise towards the improvement of cancer therapies. Another possible avenue to the specific targeting of cancer stem cells is using immunotherapeutic approaches directed to the cancer stem cell phenotype (Q. Pan et al., 2015). Immunotherapy makes use of the immune system's capacity to counteract diseases such as cancer. Break-through milestones in this field have already been achieved, such as the identification of the programmed death-ligand 1 (PD-L1) immune checkpoint modulator which is expressed in some human cancers (Chen & Mellman, 2013). By blocking the interaction of PD-L1 with the PD-1 receptor, current therapies are able to restore the capacity for a patient's T-cells to fight a tumour. This therapy has already shown a clinical benefit in lung cancer, melanoma and Hodgkin's disease (Ansell et al., 2015; Q. Pan et al., 2015; Sharma, Wagner, Wolchok, & Allison, 2011; Topalian et al., 2012). PD-L1 expression in cancer stem cells is variable. While cancer stem cells in the head and neck carcinoma do express this immune checkpoint (Y. Lee & Sunwoo, 2014), malignant melanoma-initiating cells show instead decreased expression levels (Schatton et al., 2010). Cancer stem cell antigens such as CD133, CD44, ALDH and HER2 are possible targets for further immunologically-based targeting (Q. Pan et al., 2015). Certain tumour microenvironment factors, such as IL-6, also have been shown to enhance cancer stem cells and could therefore also be potential targets for immunotherapy (L. Wang et al., 2009).

6.2. The link to regenerative biology

As discussed in the previous section, cancer can be considered a stem cell disease. Furthermore, it exhibits parallels in stem cell regulation to normal tissue homeostasis, regenerative responses and tissue repair. One example of this is the regulation of the Wnt and Shh pathways. In cancer stem cells, these are believed to be epigenetically regulated to achieve maintenance and progression of the cancer stem cell identity (Beachy, Karhadkar, & Berman, 2004; Toh et al., 2017). Organ or appendage regeneration responses can resemble in part the embryonic signalling and pattern formation for a given tissue. In salamanders, Shh is expressed in both bud formation during limb development as well as in blastemas of regenerating limbs (Imokawa & Yoshizato, 1997). In accordance, inhibiting Shh signalling results in limb regeneration with loss of digits (Roy & Gardiner, 2002). A similar process is observed also in salamander lens, with Shh being expressed in both the developing and regenerating lens and inhibition of this pathway resulting in proliferation and differentiation defects (Tsonis et al., 2004). In single tissue regeneration responses, the Shh or Wnt pathways are also activated, as during the regeneration of prostate epithelium (Karhadkar et al., 2004) and during muscle regeneration (Polesskaya, Seale, & Rudnicki, 2003), respectively. Additionally, Wnt and Shh pathways are involved in the regulation of some cancers (Scales & de Sauvage, 2009; Zhan, Rindtorff, & Boutros, 2017). In fact, cancer could be seen as the prevention of return to normal conditions of the Wnt and Shh pathway activation. Adding to this line of thinking, Bmi-1, a gene dependent on the Shh pathway activity, is required in both hematopoietic stem cell renewal and in leukaemia propagation (Lessard & Sauvageau, 2003; Park et al., 2003). Taken together, the conservation of Shh and Wnt in cancer and regenerative responses suggests these processes to use similar signalling pathways and similar mechanisms for stem cell activation. However, it is important to note that only a limited number of pathways have been identified in the discussed scenarios and that Wnt and Shh are involved in many processes in, possibly, all multicellular organisms (N. Barker, 2008; Choudhry et al., 2014).

When it comes to tissue repair and cancer, there are also suggestive links between these two phenomena. Conditions involving chronic tissue damage and repair, such as toxin exposure, chronic infection and inflammatory conditions, are also associated with a higher cancer risk (Coussens & Werb, 2002; Dvorak, 1986). The parallels between normal homeostatic conditions, cancer and tissue repair, suggest that cancer could be perceived as a permanent state of repair/regeneration (Fig. 8). With this model in mind, in acute injuries, stem cells are activated, give rise to differentiated progeny and then return to its quiescent state. However, in chronic injuries, stem cells are activated multiple times and in increasing numbers in response to constant injury. This increases the likelihood for an oncogenic event to occur at the stem cell level that will induce the genesis of oncogenic cells, or in other words transformation of a stem cell to a cancer stem cell. If following this theory, tissue which experiences higher levels of renewal

should also exhibit higher numbers of cancers. Indeed this is the case for the skin and lungs (Beachy et al., 2004). Hence, while regeneration is often seen as self-renewal under control, cancer might be self-renewal outside of control.

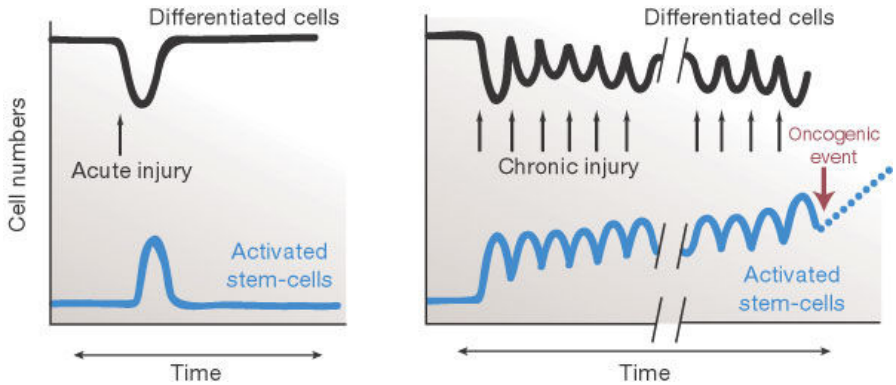

Figure 8 | The hypothesized roles of acute and chronic injury in stem cell activation. (Left) Acute injury leads to the loss of a portion of differentiated cells, which is then repaired through stem-cell activation. (Right) Chronic injury leads to the repeated expansion of the stem cell pool, preventing the number of activated cells from returning to normal values. This permanent expansion of the stem cell pool increases the probability of an oncogenic event to occur which forces the stem cell into a perpetual activated state. *Reproduced with permission from Beachy et al. (2004).*

6.3.Resistance of newt tissues to cancer formation

As highlighted earlier in Chapter 4, the newts display striking capacities for regeneration of a variety of organs and appendages. Surprisingly, these animals also have low frequencies of tumour formation upon exposure to carcinogenic compounds, which has been recorded in the regenerating lens (Okamoto, 1997) and limb tissues (Breedis, 1952; Tsonis & Eguchi, 1982).

After lens removal, the dorsal iris epithelial cells re-enter the cell cycle and transdifferentiate to form a new lens, whereas, the ventral iris does not, highlighting cellular and molecular differences between these two tissues (Oviedo & Beane, 2009). In an experimental context such as lentectomy, where the animals were exposed to carcinogenic substances, either the regeneration proceeded as normal, or the ventral iris, the tissue that is assumed not be the source of regenerative tissue, produced extra lenses with or without abnormalities (Okamoto, 1997; Oviedo & Beane, 2009). Because the exposure to carcinogens did not alter the structures formed by dorsal iris during regeneration, a causal relationship for the insensitivity of regenerative tissue to carcinogenic compounds and its resistance to cancer formation cannot be overruled.

In one study, forelimbs of newts (*Triturus viridescens*) were injured by thermal or mechanic injury. Then, these animals were injected with either one of seven

different types of carcinogens or with neoplasm grafts from other amphibians. Surprisingly, non-neoplastic supernumerary limbs were induced in most cases, and sarcoma was only seen in 2 out of 500 animals (Breedis, 1952). In another experiment, forelimb blastemas of Japanese newts (*Cynops pyrrhogaster*) were treated with potent carcinogens, 20-methylcholanthrene and benzo(α)pyrene, 7 days after limb amputation. Exposure to carcinogens resulted in altered or delayed regeneration patterns, but no tumour formation was observed (Tsonis & Eguchi, 1982). In yet another investigation, the newts (*Triturus cristatus*) were subcutaneously injected with carcinogens (benzpyrene, dibenzanthracene or a combination of both) at sites in the regenerative area of the tail or the non-regenerative area of the trunk (Seilern-Aspang & Kratochwill, 1965). This study has recorded the appearance of tumours in regenerative tissues in 9% of injected animals, but this proportion was higher (15-16%) in non-regenerative tissues. Additionally, while 90% of the tumours in non-regenerative tissue were shifted to infiltrative and metastatic growth, the tumours in the regenerative tissue showed minimal infiltrative growth of 7%, and the remaining cases grew without invasion (Seilern-Aspang & Kratochwill, 1965). Altogether, these observations suggest an intriguing possibility of resistance to tumour formation in the regenerative tissue of the newts. Moreover, the mechanisms regulating cell dedifferentiation and differentiation appear to be distinct in newts, and this aspect is of particular interest in the context of tumorigenesis. As suggested by Oviedo and Beane, perhaps the regeneration-capable newt cells might not reach a fully dedifferentiated stem cell state, and therefore, carcinogenic compounds might act differently on them (Oviedo & Beane, 2009). Another possibility is that of the regulatory machinery in newt cells, which might repair carcinogen-induced damage better than other organisms. It is possible to test this hypothesis by single-cell sequencing of newt cells which are responsible for regeneration, before and after exposure to carcinogens and the availability of salamander genomes facilitates such studies (Elewa et al., 2017; Keinath et al., 2015; Nowoshilow et al., 2018).

6.4. Neurotransmitters and cancer

As the peripheral nervous system extends to all parts of the body, so can neurotransmitters reach all these body regions. Besides its classical function in neurotransmission through chemical synapses, many of these molecules have been linked to cancer initiation, regulation and maintenance, such as: acetylcholine, serotonin, dopamine, norepinephrine, epinephrine, glutamate and gamma-Aminobutyric acid (GABA) (Mancino, Ametller, Gascón, & Almendro, 2011).

* Acetylcholine

Acetylcholine binds to two families of receptors: muscarinic (m-AchR) and nicotinic (n-AchR) (Mancino et al., 2011). Activation of the M-AchR type 2 with a specific agonist has been shown to inhibit glioblastoma cell proliferation as well as inducing apoptosis (Di Bari et al., 2015). In contrast, activation of M-AchR type 1 induced tumour invasion and metastasis in studies of prostate cancer (Magnon et al., 2013). In yet another study, m-AchR type 3 was found to have a growth-promoting effect in colon cancer cells (Ukegawa, Takeuchi, Kusayanagi, & Mitamura, 2003). Also, m-AchR activation was suggested to be implicated in the proliferation and propagation of breast cancer cells (Jiménez & Montiel, 2005). In the case of n-AchR receptors, nicotine and a derivate agonist which bind to n-AchR were found to stimulate proliferation of human small cell lung cancer cells (Schuller, 1989). Similar effects on n-AchR pathway activation have been found in mesothelioma, colon and gastric cancer cells (Mancino et al., 2011).

* Serotonin

Serotonin binds to seven families of receptors: 5-hydroxytryptamine$_{1\text{-}7}$ (5-HT$_{1\text{-}7}$) (Sarrouilhe, Clarhaut, Defamie, & Mesnil, 2015). It has been found to have a cancer stimulating effect, usually through the activation of 5-HT$_1$ and 5-HT$_2$ receptors. This cancer stimulating effect has been shown in the following cancers: prostate, bladder, small-cell lung, colorectal, bile ducts, womb, breast, hepatocellular and neuroendocrine (Sarrouilhe et al., 2015). The role of serotonin in cancer is believed to be concentration dependent, as low concentrations of this neurotransmitter lead to lower blood supply and therefore reduced tumour growth. In glioblastoma, lower levels of serotonin expression are related to longer survivals and it is believed to induce proliferation and angiogenesis (Caragher, Hall, Ahsan, & Ahmed, 2017). There are also indirect indications suggesting glioblastoma may synthesize and secrete its own serotonin molecules.

* Norepinephrine and epinephrine

Norepinephrine and epinephrine bind to two families of adrenergic receptors: α and β (Mancino et al., 2011). These neurotransmitters have been shown to regulate proliferation, survival and angiogenesis in ovarian cancer cells (Lutgendorf et al., 2003; Thaker et al., 2006) as well as to induce metastasis formation in colon, lung and breast cancer cases (Ben-Eliyahu, Yirmiya, Liebeskind, Taylor, & Gale, 1991; Masur, Niggemann, Zanker, & Entschladen, 2001; Melamed et al., 2005). Furthermore, depletion of the β2 and β3 adrenergic receptors has been found to prevent early phases of prostate cancer development (Magnon et al., 2013).

* Glutamate

Glutamate binds to two classes of receptors: metabotropic and ionotropic, which in its turn contain the AMPA, NMDA and kainite receptor families(Mancino et al., 2011). The following cancers have been found to express glutamate or parts of its receptors: colorectal, glioma, gastric, prostate, oral squamous cell carcinoma, melanoma and osteosarcoma (Mancino et al., 2011). While higher levels of glutamate receptors are often associated with poorer prognosis in oral squamous cell and colorectal cancer, in colon cancer it is linked to lower drug resistance. Conversely, in medulloblastoma it correlates with higher tumour aggressiveness and recurrence.

* Gamma-aminobutyric acid (GABA)

GABA binds to two classes of receptors: ionotropic and metabotropic (Mancino et al., 2011). The following types of cancer have shown increased levels of this neurotransmitter: breast, ovarian, gastric and colon. Likewise, GABA receptors were found to be upregulated in breast and pancreatic cancer.

The effects of the neurotransmitter dopamine in cancer are discussed in Chapter 7.

The link between cancer and regenerative biology, the resistance of newt tissues to cancer formation, the role of neurotransmitters in cancer and the capacity of the neurotransmitter dopamine to control neural stem cell fate in the newt brain, all led us to question of whether dopamine could exert a regulatory function in a brain cancer condition, glioblastoma. This reasoning gave rise to **paper I**.

6.5. Glioblastoma

* Classification

A tumour of the brain with its origin in glial cells is generally termed glioma (“Definition of glioma,” 2018). Gliomas can be classified based on the type of glial cell in its origin (“Gliomas,” 2018): (i) ependymomas – arising from the tissue lining the ventricular system, the ependyma; (ii) astrocytomas – arising from astrocytes; (iii) oligodendrogliomas – arising from oligodendrocytes; and (iv) oligoastrocytomas – a mix between astrocytomas and oligodendrogliomas. A more clinical approach to glioma classification is the World Health Organization (WHO)

four-grade malignancy scale (I-IV) (Louis et al., 2016). Grade I glioma is the least malignant and is prevalent within children and young adults. Grade II-IV are used for infiltrating gliomas and its classification is based on histopathological characteristics of poor cellular differentiation, such as nuclear atypia, mitotic activity, proliferation of micro-vessels and necrosis. The most malignant glioma with the worse prognosis is grade IV, including for example glioblastoma.

In a recent update of the WHO classification of tumours of the central nervous system, glioblastoma are classified into molecular subtypes, bases on their biomarker status for mutations in the isocitrate dehydrogenase (IDH) gene (Louis et al., 2016). The absence of IDH mutations, wildtype, has been associated with poorer prognosis, while its presence is associated with better prognosis (Combs et al., 2011). Genomic analyses have been able to identify further molecular subtypes of glioblastoma based on mutations or gene expression of EGFR, NF1, IDH1 and PDGFRA (Verhaak et al., 2010). This gave rise to a molecular subtype classification of glioblastoma: classical, mesenchymal and proneural.

* Epidemiology and treatment

Glioblastomas are the most prevalent malignant brain tumour group, with a survival median of only 12-14 months (Zhang et al., 2016). Standard treatment consists on surgical resection, radiotherapy and chemotherapy (Olson, Nayak, Ormond, Wen, & Kalkanis, 2014). Furthermore, the chemotherapeutic agent temozolomide has been found to increase survival by a few months. Whether or not a patient group benefits from this drug is correlated with the epigenetic status of the O6-methylguanine–DNA methyltransferase (MGMT) promoter (Hegi et al., 2005; Jacinto & Esteller, 2007). Patients with a methylated MGMT promoter benefit from temozolomide while the ones without methylation do not. There is an unmet need for more effective drugs towards the treatment of glioblastoma. However, this is lacking not only due to a sparse amount of drug candidates but also due to the challenge of drug delivery across the brain barrier (Huse & Holland, 2010). While increasing drug dosing is a usual approach to achieve this crossing, this is largely limited by the drug's toxicologically profile and associated maximum safely administered dose.

* Cancer stem cells

The marker CD133/Prominin 1 has been reported as specific to neural stem cells and cancer stem cells in brain tumours (Singh et al., 2003, 2004). Additionally, cell populations expressing this marker were linked to radio resistance (Bao et al., 2006). However, more recent studies have created controversy over the specificity of this marker to tumour initiating cells. First, $CD133^{-}$ glioma cells were able to form tumours in mice (J. Wang et al., 2008). Second, CD133- tumour initiating cells were found in adult human gliomas (Ogden et al., 2008). Two hypotheses have been

provided to explain these controversial findings. One is that some gliomas might not follow a cancer stem cell model (Shackleton et al., 2009). Another is the primary cell culture and cell handling differences between studies, which could introduce biological variations (Huse & Holland, 2010). In the case of glioblastoma, the high heterogeneity between tumours may also represent a major hurdle to the finding of a specific cancer stem cell marker (Patel et al., 2014).

7. A discussion on dopamine

Dopamine's role as a neurotransmitter was discovered by Arvid Carlsson in 1957 (Carlsson, 1993). We now know dopamine is a catecholamine, together with epinephrine and norepinephrine. Twenty catecholaminergic cell populations have been identified in the mouse brain (A1-A17 for dopamine and norepinephrine groups and C1-C3 for epinephrine groups) (Björklund & Dunnett, 2007). Of these, 9 are dopaminergic populations (A9-A17; Fig. 9). The ventral midbrain contains 3 dopaminergic populations: (i) retrorubral field (A8); (ii) SNc (A9); (iii) VTA (A10).

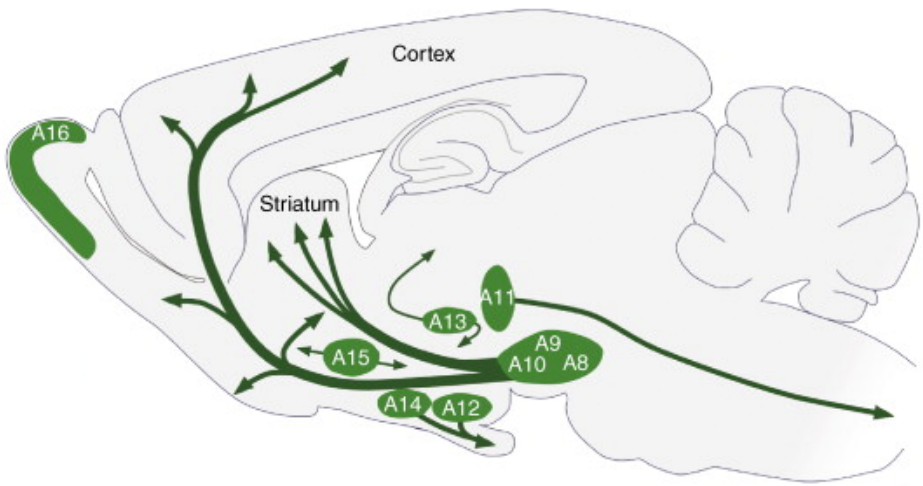

Figure 9 | Distribution of the 9 dopaminergic neuron populations in the adult mouse brain. ***Reproduced with permission Björklund & Dunnett (2007).***

Dopamine plays a biological role in most multicellular organisms (Barron et al., 2010). This is the case even for nematodes, which exhibit one of the simplest nervous systems, but still have dopaminergic neurons. The dopaminergic system is thought to be classically involved in reward-motivation pathways, control of voluntary movements and endocrine hormone secretion (Harsing, 2008). However, the functions of a molecule so widespread within the animal kingdom are more diverse (Berg et al., 2013).

The role of dopamine in disease, development and regeneration is the common denominator bringing **papers I**, **II** and **III** together, as well as this thesis. In **paper I**, we demonstrate that trifluoperazine, a dopamine receptor 2 (DRD2) antagonist, is

an inhibitor of glioblastoma growth. In **paper II**, we elucidate on the role of dopamine in development by studying the spatiotemporal developmental dynamics of the neural stem cells that give rise to dopaminergic neurons in the newt. We further show how ablation of the dopaminergic innervation affects neurogenesis in the forebrain as well as behaviour. In **paper III**, we extend our capacity to study the role of dopamine in regeneration by optimizing a tissue clearing method in order to quantify and trace dopaminergic neurites, as well as the radial extensions of newt neural stem cells.

In this chapter we discuss dopamine's role in disease, development and regeneration.

7.1. The receptors

The dopamine which acts on dopamine receptors is synthesized in the cytoplasm of cells, close to the synaptic terminals (Harsing, 2008). First, the aminoacid tyrosine is transformed into L-DOPA by the enzyme tyrosine hydroxylase (TH). Then, the enzyme DOPA decarboxylase converts L-DOPA into dopamine. Dopamine is then loaded into vesicles by vesicular monoamine transporters and released, so that it can interact with the DR.

Five subtypes of DRs have been identified and constitute two families: D1-like (DRD1 and DRD5) and D2-like (DRD2, DRD3 and DRD4) (Missale, Nash, Robinson, Jaber, & Caron, 1998). The D1-like family is known to up regulate adenyl cyclase, while the D2-like family is known to down regulate it (Sibley & Monsma, 1992). Both families exert a regulatory effect on adenyl cyclase through their coupling to G proteins. D1-like family genes are intron-less (Missale et al., 1998), whereas D2-like family receptors have introns, which results in alternative splicing. Furthermore, DRD4 has polymorphisms in its coding sequence which results in a variable number of repeats in its third intracellular protein loop (Missale et al., 1998).

The different subtypes of DRs are localized differently across the brain. The following localization list is adapted from a review from Strange and Neve (Strange, 2013):

- DRD1: caudate/putamen, nucleus accumbens, olfactory tubercle, hypothalamus, thalamus, frontal cortex.
- DRD2: caudate/putamen, nucleus accumbens, olfactory tubercle, cerebral cortex.
- DRD3: nucleus accumbens, olfactory tubercle, islands of Calleja, putamen, cerebral cortex.
- DRD4: frontal cortex, midbrain, amygdala, hippocampus, hypothalamus, medulla.

- DRD5: hippocampus, thalamus, lateral mamillary nucleus, striatum, cerebral cortex.

7.2. In regeneration

* The newt

The newt is a fascinating animal model to study regeneration, as discussed in Chapter 2. Perhaps one day we will be able to cure neurodegenerative diseases by transferring these regenerative capacities to humans. This can only be achieved by the thorough understanding of the molecular processes that are involved.

One of the most striking regenerative processes in the brain of one species of adult newts (*Notophthalmus viridescens*) is regulated by dopamine. This is the regeneration of the dopaminergic neurons in the ventral midbrain after dopaminergic lesion. The first step towards this finding was taken by Parish *et al*., who have created a Parkinson-like newt model by chemically ablating the dopaminergic system in the diencephalon and mesencephalon of the brain with 6-OHDA (Parish et al., 2007). They demonstrated this lesion was effective by loss of DAPI, neuronal and dopaminergic markers. Full regeneration of the dopaminergic cell numbers occurred 30 days after neuronal ablation and this was accompanied by the recovery of locomotor activity. Particularly, the ventral midbrain of *Notophthalmus* is quiescent and, hence, no neurogenesis or proliferation of ependymoglial cells (progenitor cells in the newt) occurs in this region without injury. Thus, this demonstrates an unprecedented capacity for brain regeneration which has not been seen in post juvenile mammals to the same extent. This regenerative process is driven by *de novo* adult neurogenesis and was supported by the facts that after dopaminergic ablation: (i) The first cells to show incorporated BrdU were ependymoglial (progenitor cells in the newt); (ii) The progeny of ependymoglial cells migrated away from the ventricular layers and were positive for dopaminergic neuronal markers; (iii) Blocking of proliferation by administering cytosine arabinoside, reduced the number of new-born TH^+ (dopaminergic) cells and the subsequent recovery in locomotion (Berg et al., 2010; Parish et al., 2007). This inherent capacity for regeneration in the adult newt brain raised questions on the distribution of the germinal niches across different brain regions, as these niches are known to fuel neurogenesis (Braun & Jessberger, 2014). Such question is especially relevant when considering the limited neurogenic capacity exhibited by mammals, as described in Chapter 4. Surprisingly, the distribution of germinal niches in homeostatic conditions is similar in the newt and in mammals (Berg et al., 2010). Particularly, the adult newt forebrain displays proliferative and neurogenic activity in normal conditions, while the midbrain shows no proliferative activity, thus being a quiescent region. However, upon dopaminergic lesion, ependymoglial cells in the midbrain exit their quiescent state, start expressing the neuronal marker

NeuN and become Tyrosine Hydroxylase (TH, the rate limiting enzyme in the biosynthesis of dopamine) positive neurons, thereby achieving regeneration of the midbrain dopaminergic population.

Msx1 is a transcriptional repressor that is needed in the development of midbrain dopaminergic neurons in mice (Andersson et al., 2006). The number of cells expressing this factor in the newt decreases in the ventral diencephalon and mesencephalon after dopaminergic lesion, followed by a progressive recover to normal values (Berg et al., 2010). This demonstrates Msx1 likely plays a role in the regeneration of TH^+ midbrain neurons in the adult newt, in addition to its role in the homeostatic development of these neurons in mice. The similarities between this regenerative process in the newt and the developmental process in mammals raises the possibility of mammals retaining latent mechanisms for brain regeneration that could possibly be unlocked by external induction. Uncovering the processes in control of the activation of regenerative mechanism is key to the understanding how such findings can be transferred to a mammalian setting. Interestingly, the ligand Shh was found to be upregulated in the midbrain after dopaminergic lesion and blocking its expression inhibited regeneration (Berg et al., 2010). This factor was previously found to be involved in the specification of neural stem cells giving rise to midbrain dopaminergic neurons (Arenas, 2008; Kittappa, Chang, Awatramani, & McKay, 2007), as well as in the regulation of proliferation of neuronal/glial precursors (Traiffort, Angot, & Ruat, 2010). Shh has also been involved in newt regeneration, particularly in the limb and the lens.

Another factor involved in the regeneration of midbrain dopaminergic neurons in the newt is dopamine. In fact, dopamine has been reported to control ependymoglia quiescence in a feedback mechanism fashion (Fig. 10; Berg et al., 2011). In homeostatic conditions, and while the dopaminergic neuron population in the midbrain is present, dopamine inhibits the proliferation of ependymoglial cells as well as lesion-induced neurogenesis into dopaminergic neurons. Conversely, in dopaminergic lesion conditions, absence of dopamine results in activation of ependymoglial cells to proliferate, which in turn give rise to regenerative dopaminergic population to substitute the one that has been lost. These findings are strengthened by the fact ependymoglial cells express the DRD1 and DRD2 receptors and are thus sensitive to dopamine signalling regulation. Furthermore, administration of L-DOPA, a dopamine precursor used as dopamine replacement therapy in PD, result in inhibition of ependymoglia proliferation and subsequent regenerative response. This finding highlights the mechanistic requirement of dopamine depletion for neural stem cell activation. In line with these findings, blocking of the dopamine signalling in normal homeostatic conditions with a dopamine antagonist, haloperidol, activates ependymoglia proliferation and leads to genesis of an above-normal number of developing and mature dopaminergic neurons.

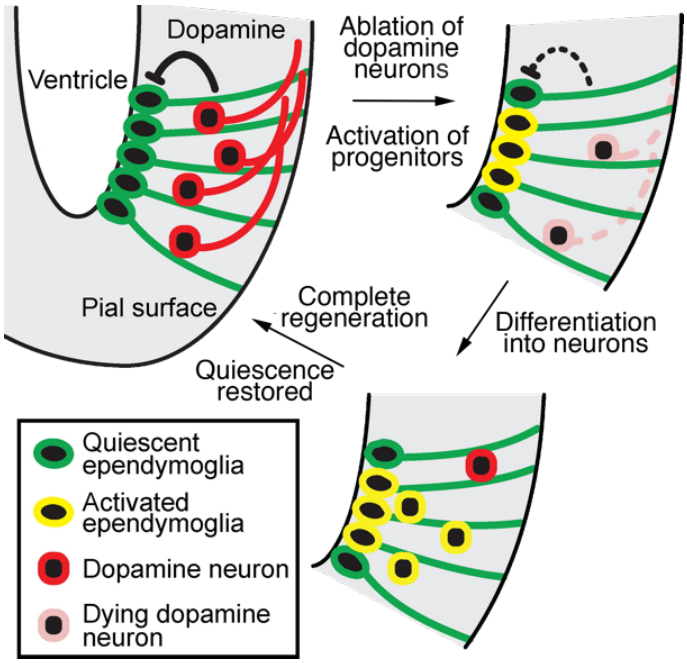

Figure 10 | Model of control of quiescent ependymoglia proliferation in the newt brain by dopamine. If dopamine is depleted due to the ablation of dopaminergic neurons, the ependymoglial cells in the ventricular zone are activated from a quiescent state and lead to a regenerative response of the lost neurons. *Adapted from Berg (2011).*

Taken together, adult newts possess a capacity for activation of brain regeneration processes out of a quiescent state, thereby achieving full regeneration of the midbrain dopaminergic neuron population. This is however not the case of PD patients, who do not natively regenerate the dopaminergic neurons which are lost in the SNc in the course of this disease. Cross-species studies are therefore paramount for the understanding of the biological differences which are key to regenerative capacities.

* Mammals

Dopamine has been implicated in proliferation regulation in the mammalian brain (Berg et al., 2013) and this control was observed mainly in the SVZ neurogenic niche of the forebrain of adult mice (Chapter 4). Cells in this niche express dopamine receptors (C-cells, D2-like receptors and A-cells, D1-like and D2-like receptors) and are innervated by dopaminergic fibres which have their cell bodies in the SNc (Freundlieb et al., 2006; Höglinger et al., 2004). If these dopaminergic cell bodies in the SNc are ablated, and with them the release of dopamine to the SVZ, then the proliferation of progenitor cells in the SVZ is decreased, as well as

neurogenesis (Baker, Baker, & Hagg, 2004; Höglinger et al., 2004; L'Episcopo et al., 2014; Winner et al., 2009). Dopamine receptor agonists have been shown to rescue proliferation as a consequence of lack of dopamine release (Höglinger et al., 2004; Winner et al., 2009; P. Yang, Arnold, Habas, Hetman, & Hagg, 2008), while another study reports a similar effect with the dopamine receptor antagonist, haloperidol (Kippin, Kapur, & van der Kooy, 2005). Berg and colleagues proposed that these contradictory findings could be explained by dopamine having an anti-proliferative effect on neural stem cells but pro-proliferative effect on transit-amplifying cells (Berg et al., 2013). As neural stem cells can divide less frequently than transit-amplifying cells then perhaps only long-term studies can uncover the anti-proliferative effect of dopamine receptor antagonists acting on them.

7.3. In development

Understanding the ontogeny of dopaminergic neurons in the brain is necessary in order to be able to artificially recreate this process for clinical purposes. How do the different dopaminergic neurons occupy their right positions? Which factors regulate their development? How do they acquire their dopaminergic phenotype? A vast amount of research on the ontogeny of dopaminergic neurons has focused on the ventral midbrain populations (A8-A10). This is due to interest in the degenerative processes of the dopaminergic populations of the ventral midbrain in Parkinson's disease, particularly in the VTA and the SNc (Alberico, Cassell, & Narayanan, 2015). The hope is that, by better understanding the ontogeny of these neurons, one will be capable of recapitulating its identity in cell replacement therapies, which are aimed at the treatment of neurodegenerative diseases (R. A. Barker, Parmar, Studer, & Takahashi, 2017; Kikuchi et al., 2017).

* Development of the ventral midbrain dopaminergic neurons

A midbrain dopaminergic neuron is born through a complex series of molecular cascades and feedback loops, some of which are still not fully understood. Three main phases can be generally recognized: (i) the early patterning of the ventral mesencephalon; (ii) acquisition of a dopaminergic phenotype by the ventral midbrain neural precursors; (iii) development of post-mitotic dopaminergic neurons in the ventral midbrain. For better understanding we present detailed diagrams for each phase of development of the ventral midbrain dopaminergic neurons, based on the review texts by Hegarty and colleagues (Hegarty, Sullivan, & O'Keeffe, 2013). These are in a time organized fashion, with events happening later being shown lower on axis of the main cascade and events happening earlier are shown higher. These diagrams are annotated for which factor induces/represses each molecular event, as well as for the mouse developmental stage correspondent to these events. These annotations are also performed for pathways that are

suspected to be involved but for which there is not yet enough scientific evidence to establish a correlation yet. We briefly discuss the highlights of the 3 phases of development of the ventral midbrain dopaminergic neurons below.

During the early patterning of the ventral mesencephalon the dorsal ectoderm gives rise to the neural plate which, upon closing of this structure, becomes the neural tube. In the neural tube two structures are formed that are later required for the genesis of dopaminergic neurons: the floor plate and the isthmus organizer (Fig. 11). The floor plate will be the source for the dopaminergic neural precursors, while the isthmus organizer is involved in the regional specification of the ventral midbrain. The isthmus organizer is placed at the right anatomical position by a positional repression of Otx2 and Gbx2 against each other. The isthmus organizer then expresses secondary transcription factors: Pax2, Lmx1b, Wnt1 and En-1. Wnt1 and En-1 induce FGF8 and, in this manner, refine the position of the isthmus. All of the secondary transcription factors here mentioned contribute to the regional specification of the ventral midbrain. En-2 and Pax5 have also been found to play a role in this process.

The second phase in the development of the dopaminergic neurons of the ventral midbrain is the induction of a dopaminergic phenotype (Fig. 12). Here, floor plate radial glia-like neuronal precursors, which are positive for Vimentin, BLBP and GLAST, start expressing Shh. Shh then participates in a molecular cascade with Gli1/2/3, ending up in the induction of FoxA2. In turn FoxA2 induces the expression of proneural protein NGN2 and Lmx1a/b. Lmx1a then induces Msx1 which further induces NGN2. Lmx1a and Msx1 are determinants of midbrain dopaminergic neurons and have been found to be required for differentiation into dopaminergic neurons (Andersson et al., 2006). Wnt signalling also plays an important role in the induction of a dopaminergic phenotype. It does so by inducing Lmx1a and FGF8, and by repressing Shh which in turn blocks dopaminergic neurogenesis for the time being. This blocking of dopaminergic neurogenesis is lifted later at developmental stage E12. Lmx1a has also been found to induce Otx2, which then regulates proliferation of dopaminergic neural precursors.

The last and third phase in the development of dopaminergic neurons of the ventral midbrain is the development of post-mitotic dopaminergic neurons (Fig. 13). After the neuronal precursors in the floor plate have acquired a neuronal phenotype, culminating in the induction of NGN2, these cells will start to express TH, gradually stop mitosis and migrate to their respective position in the SNc and VTA. First, ventral migration takes place, along tenascin-expressing radial glia. Second, lateral migration takes place, along L-1 expressing tangentially oriented fibres. Eventually migration halts once the cells are at their respective positions. The factors involved in the regulation of the end of migration are not yet known. After the stop of migration several transcription factors which contribute to differentiation and survival of the ventral midbrain dopaminergic neurons are expressed: Lmx1b, Nurr1, Pitx3 and En-1/En-2. The respective pathways induced by these transcription factors are represented in the diagrams of Fig. 13 and Fig. 14.

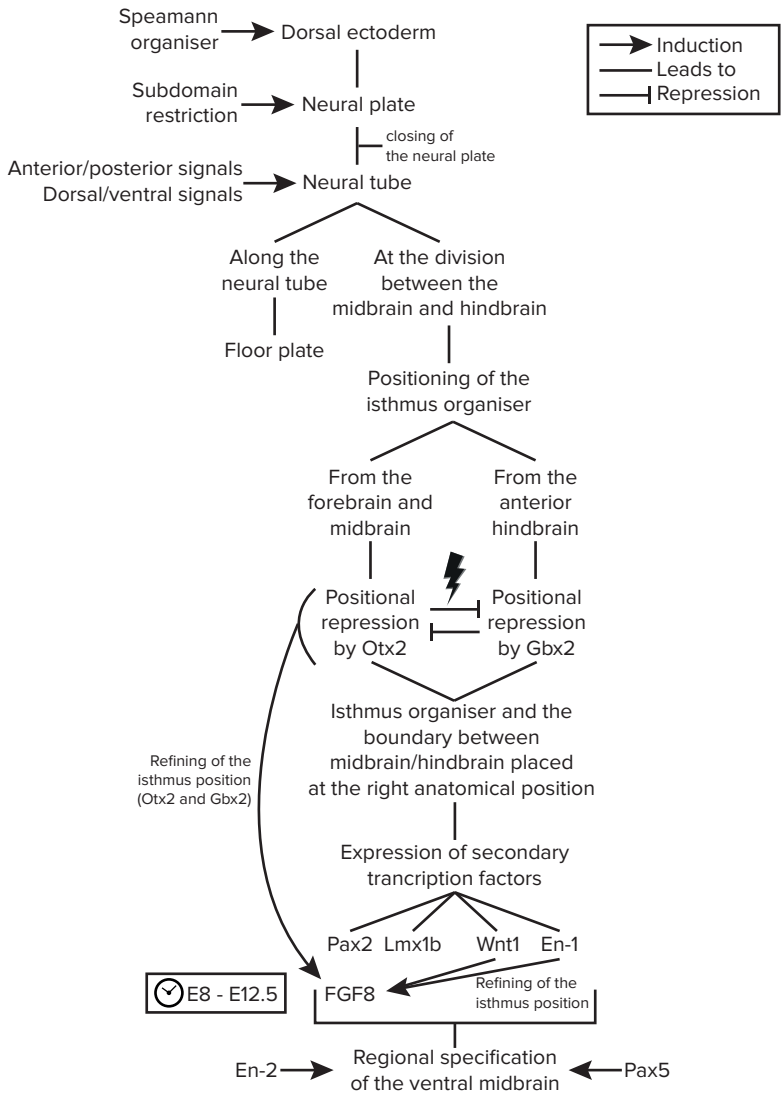

Figure 11 | Cellular and molecular events in the early patterning of the ventral mesencephalon. These processes are involved in the development of the dopaminergic neurons in the ventral midbrain.

Lmx1a^{+} cells arise during embryonic development but they remain in the midbrain of the adult even after the neurogenic process has finished (Hedlund et al., 2016). These cells, however, decrease in number and their fibre projections become shorter as the adult ages. Interestingly, lmx1a+ cells in the midbrain were found to express DRD2. Additionally, the brain region where they are located is innervated by midbrain dopaminergic neurons. If embryonic mice (E12-E17.5) are treated with

the dopamine antagonist haloperidol then proliferation of Lmx1a$^+$ cells is increased, as well as dopaminergic neurogenesis. Furthermore, a similar effect on proliferation and neurogenesis was observed in mouse embryonic midbrain cell cultures with haloperidol and another dopamine receptor antagonist, sulpiride. Taken together, dopaminergic antagonists seem to be in control of proliferation of Lmx1a$^+$ neuronal progenitors which are committed to differentiation into dopaminergic neurons, after the neurogenic period of these neurons has ended. The question remains on whether it is possible to induce a similar neurogenic process in the ventral midbrain of the adult mouse.

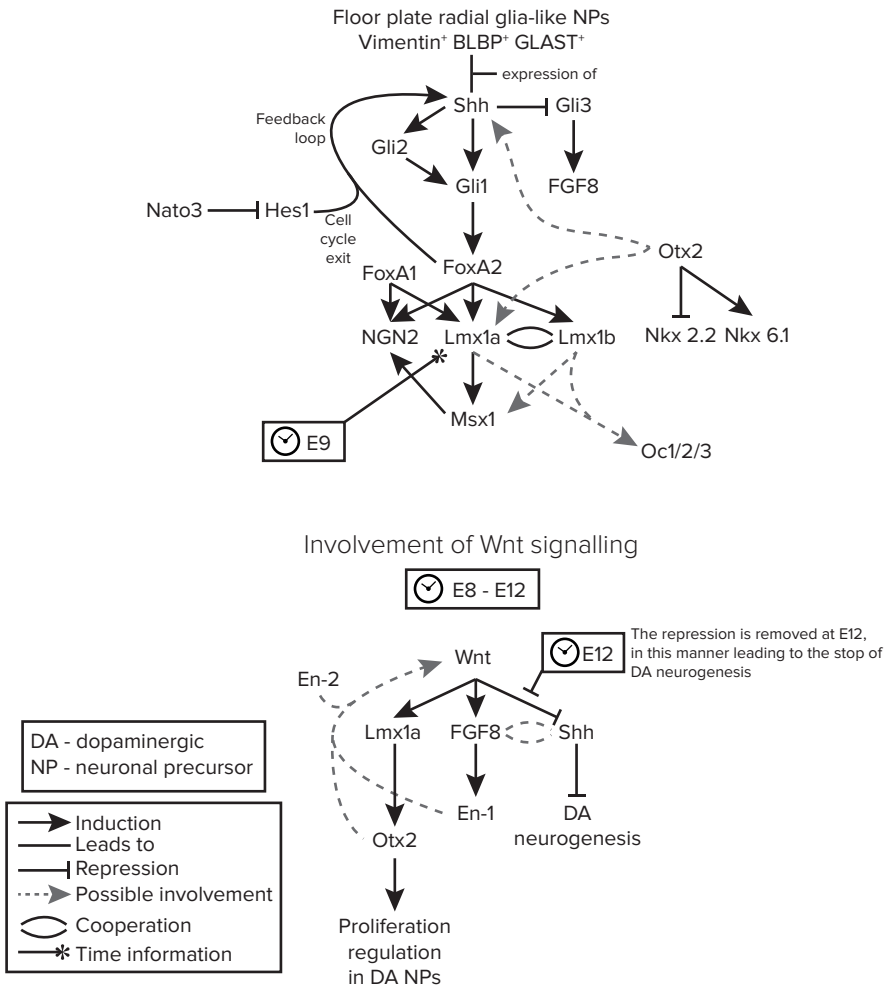

Figure 12 | Cellular and molecular events in the induction of a dopaminergic phenotype. These processes are involved in the development of the ventral midbrain dopaminergic neurons.

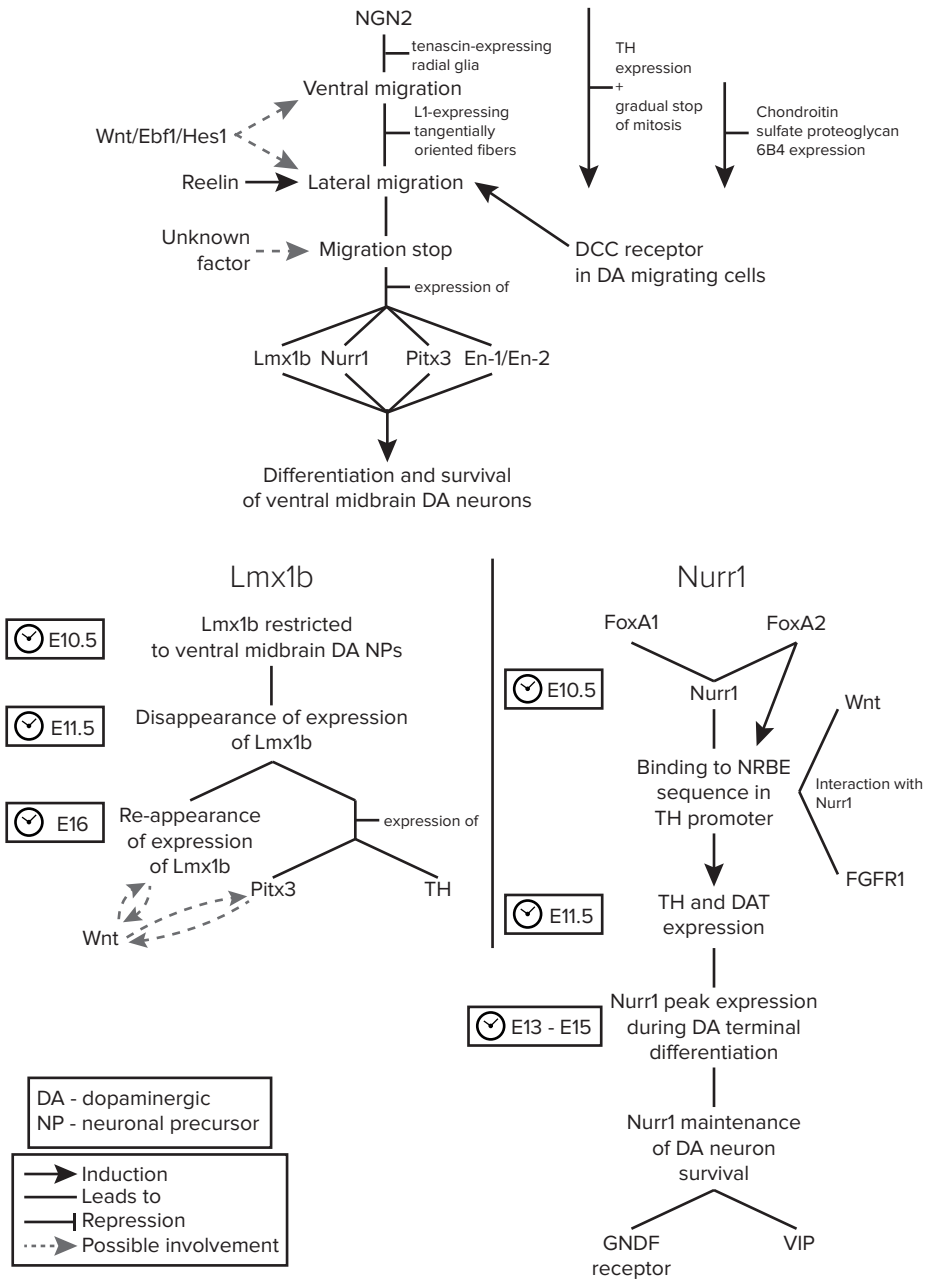

Figure 13 | Cellular and molecular events involved in the development of post-mitotic dopaminergic neurons in the ventral midbrain.

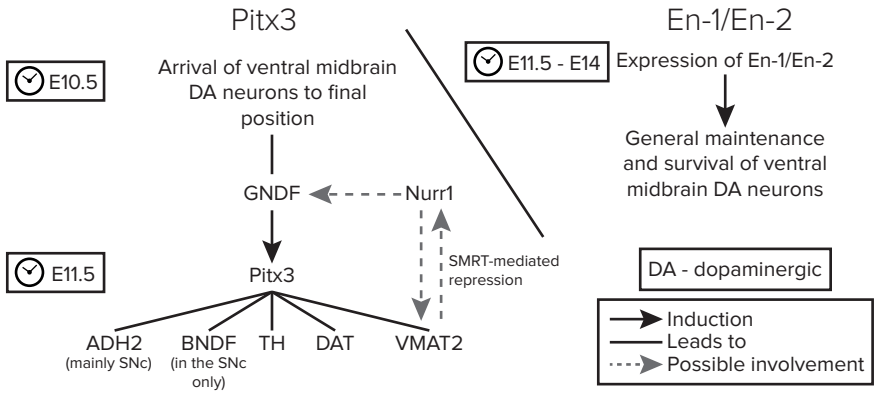

Figure 14 | Molecular events of Pitx3 and En-1/En-2 during the development of post-mitotic dopaminergic neurons in the ventral midbrain.

7.4. In disease

7.4.1. Parkinson's disease

Epidemiological studies report PD has the second most common neurodegenerative disease, after Alzheimer's disease (Ascherio & Schwarzschild, 2016). PD is characterized by slow movement (bradykinesia), postural imbalance, tremors upon resting and rigidity. Earlier, non-motor related symptoms, have also been associated with PD. These are: anxiety, depression, cognitive impairment, decrease in olfaction and dysfunction of the autonomic nervous system (Winner & Winkler, 2015).

PD symptoms are believed to be linked to the degeneration of dopaminergic neurons in the SNc of the ventral midbrain, together with the degeneration of dopaminergic forebrain innervation where VTA and SNc dopaminergic cell bodies project to (Höglinger et al., 2004; Winner & Winkler, 2015). Some degeneration of the VTA dopaminergic population has also been reported in PD patients, although this degeneration is less extensive than the one observed in the SNc (Alberico et al., 2015). A hallmark of the degeneration of dopaminergic neurons in PD is the intracellular deposition of alpha-synuclein in abnormal protein aggregates, Lewy bodies (Spillantini et al., 1998).

* Impact of dopaminergic degeneration in the ventral midbrain

As discussed in Chapter 4 the SVZ of the lateral ventricles and the DG of the hippocampus contain NSPCs with neurogenic potential. These niches are innervated by dopaminergic fibres from three dopaminergic cell populations in the ventral midbrain, two of which have been shown to degenerate with the

progression of PD (Höglinger, Arias-Carrión, Ipach, & Oertel, 2014). In mice, the SNc dopaminergic neurons of the ventral midbrain were found to project to the SVZ as well as to the RMS. Whereas, the VTA dopaminergic neurons innervate the SVZ alone. The SVZ is adjacent to the striatum and the nucleus accumbens, which both receive dopaminergic afferents from the three ventral midbrain dopaminergic groups (SNc, VTA and retrorubral fields; A8-A10). Both the SNc and the VTA dopaminergic neurons project to the DG of the hippocampus. In macaques, the SNc dopaminergic neurons, but not the ones from the retrorubral field, were also found to project to the SVZ (Freundlieb et al., 2006).

As the dopaminergic neurons in the ventral midbrain project to two of the three neurogenic niches, what is the impact of dopaminergic fibre degeneration on neurogenesis, such as it happens in PD? In the SVZ of mice, Höglinger and colleagues have shown proliferative precursors express dopamine receptors, meaning they can potentially respond to dopamine signalling (Höglinger et al., 2004). The same study demonstrates that, in PD mouse models, generated by dopaminergic lesion with MTPT, decreased proliferation levels were observed in the precursor cells of the SVZ and DG. Furthermore, in the SVZ, the cells which exhibited this lower proliferation are EGFR positive. The effect of dopaminergic lesion on proliferation is rescued by ropinirole, a D2-like agonist. Höglinger *et al.* also report similar findings in PD patient samples. In these patients, the SVZ has lower numbers of proliferating cells and in the DG and olfactory bulb the numbers of neural precursor cells are also lower than in the controls. Freundlieb and colleagues performed a similar study in PD macaque models (Freundlieb et al., 2006). They find that, after dopaminergic denervation, lower numbers of proliferating cells as well as neuroblasts were found in the SVZ. A later study elucidated on the molecular mechanisms involved in the control of proliferation by dopaminergic fibres (O'Keeffe et al., 2009). In mice, dopaminergic fibres release EGF at the SVZ, which then activates the EGFR to induce proliferation of SVZ precursor cells. Interestingly, the same study demonstrates dopamine also has the capacity to control self-renewal of stem cell populations which are positive for GFAP. Taken together, these findings implicate dopaminergic fibre degeneration and a PD phenotype in a lower proliferation capacity at the SVZ and DG neurogenic niches. This can in turn represent the occurrence of lower neurogenic levels in PD patients. In accordance to the findings above, O'Keefe *et al.* also find a decreased number of $EGFR^+$ cells in PD patients. This means that not only is the dopaminergic signalling towards stimulation of proliferation decreased in PD but also the capacity to receive these signals.

However, the impact of the dopaminergic degeneration in the ventral midbrain in proliferative changes of neurogenic niches is still controversial. Indeed, Berge et al. reported opposite results when finding no decrease in neural stem cell number or in proliferation between PD and control groups (Van Den Berge et al., 2011). Likewise, the same study found no effect on precursor cell proliferation in PD mice models. Winner and Winkler propose these contrasting findings could be due to

the use of samples with different post mortem times, differences in the region of the SVZ that was analysed or the use of distinct proliferation markers.

* Adult dopaminergic neurogenesis in the substantia nigra

On the topic of whether adult dopaminergic neurogenesis occurs in the substantia nigra, several studies have reported opposite results. First, progenitor cells have been identified in the adult substantia nigra but show no capacity to differentiate into neurons in vivo possibly due to the lack of the right environmental signals (Lie et al., 2002). Second, Zhao *et al.* brought forward evidence on the generation of very low numbers of dopaminergic neurons in the substantia nigra of rodents, with an increase in the neurogenic level after MPTP-induced dopaminergic lesion (M. Zhao et al., 2003). Shan *et al.* report similar findings with increased dopaminergic neurogenesis in the substantia nigra, with origin on neural precursor cells, after MPTP lesion (Shan et al., 2006). However, another study reports contradictory results by showing that in a mouse model with 6-OHDA-induced dopaminergic lesion, there is no sign of neurogenesis of new dopaminergic neurons or migration of neural stem cells to the substantia nigra (Frielingsdorf, Schwarz, Brundin, & Mohapel, 2004). In normal and PD human tissue, similar findings were obtained, with no evidence for newborn neurons in the substantia nigra (Höglinger et al., 2007). Taken together, the notion of no occurrence of adult dopaminergic neurogenesis in the substantia nigra remains stronger, although the topic remains highly controversial.

* Cell replacement therapies

A type of exogenous cell replacement therapy, the intrastriatal transplants of dopaminergic neuron grafts, have been performed in PD patients with the aim of alleviating the symptoms caused by dopaminergic neuron degeneration (Lindvall & Bjorklund, 2004; Lindvall et al., 2004). These clinical studies made use of foetal mesencephalic tissue for transplantation. The dopaminergic neurons in this tissue successfully integrated in the target striatum, restored dopamine release and provided some degree of functional and movement-related recovery. However, a couple of drawbacks afflicted these studies: (i) the difficulty in obtaining foetal brain tissue and ethical constraints involved make it difficult for such a clinical approach to be available for a large cohort of PD patients; (ii) the degree of achieved recovery was considerably variable among patients; (iii) dyskinesia side effects which occurred in a large proportion of patients. Due to the development of iPSC cells we can now derive dopaminergic neurons from somatic cells, thereby removing one of the difficulties involved in the undertaking of exogenous cell replacement therapies in PD. Furthermore, Kikuchi *et al.* successfully performed pre-clinical studies involving the transplantation of iPSC-derived dopaminergic progenitor cells into the midbrain of macaque PD models (Kikuchi et al., 2017). They show that these neurons maturated into dopaminergic neurons and their neurites projected to the

striatum. Additionally, movement recovery was achieved and no tumours were formed parallel to graft survival for up to 24 months. This study is the first step towards the human trials of iPSC-derived dopaminergic neurons in PD, which have already started, at the time of writing of this thesis (R. A. Barker et al., 2017). These trials, which take place concurrently in several countries, are aggregated under the umbrella organization GForce-PD (www.gforce-pd.com).

7.4.2. Cancer

7.4.2.1. Glioblastoma

Brain tumour incidence was found to be reduced in patients with neuropsychiatric disorders, such as PD and schizophrenia (Diamandis, Sacher, Tyers, & Dirks, 2009; Olsen et al., 2005). One explanation for this would be the neuropharmacological drugs which are taken by these patients, such as the dopamine precursor L-DOPA, to have an inhibitory effect towards brain tumour development. Indeed, dopamine receptor signalling was found to be involved in the proliferation and survival of glioblastoma neural stem cells (GNS) (Dolma et al., 2016). Such findings are in line with the capacity of dopamine receptor antagonists to induce proliferation and control fate of normal neural stem cells (Berg et al., 2011; Dolma et al., 2016; Hedlund et al., 2016), although in GNS dopamine receptor antagonists seem to act rather through an anti-proliferation mechanism (Dolma et al., 2016; Karpel-Massler et al., 2015). Dolma *et al.* investigated the mechanisms by which dopamine signalling is inhibitory to GNS. In this study, they screened GNS, NSC and fibroblast lines with 680 neuroactive compounds in proliferation assays. They found dopaminergic compounds to represent the largest class of hits which are selective for GNS. After a secondary screening, they showed that 2 of the 3 most selective hits for GNS are DRD4 antagonists: PNU 96145E and L-741,742. These two DRD4 antagonists reduced GNS cell viability and reduced the clonogenic capacity of primary glioblastoma (GBM) cells, with a mild reduction effect on the cell viability of normal NSC. Particularly, the DRD4 antagonist L-741,742 induced differentiation of neural stem cells, in line with previous studies with other DRD4 antagonists (Berg et al., 2011; Hedlund et al., 2016). Of particular clinical value, both DRD4 antagonists were found to act synergistically with the most common therapeutic agent for glioblastoma, temozolomide. Dolma and colleagues also showed the DRD4 receptor in GNS to be functional and that its functional loss by short hairpin RNA knockout lead to reduced proliferation. Interestingly, the inhibitory effect on GNS by DRD4 antagonists seemed to be due to an impairment of autophagic-flux mediated by inhibition of DRD4 signalling. Other effects of these DRD4 antagonists on GNS cells were the disruption of the PDGFRb-ERK1/2 pathway and mTOR signalling as well as the triggering of G_0/G_1 cell cycle arrest and apoptosis. This effect was observed both in *in vitro* cell lines and *in vivo* tumours. Lastly, DRD4 antagonists were also found to increase survival in mice. Taken together, DRD4

antagonists are strongly implicated in the inhibition of glioblastoma growth and some of the molecular mechanisms involved in this response are now understood. Another study identified Olanzapine, a DRD2 antagonist, as inhibitory of proliferation and migration in GBM cell lines (Karpel-Massler et al., 2015). In similarity to DRD4 antagonists, this DRD2 antagonist acted synergistically with the chemotherapeutic agent temozolomide. Another study showed a cell viability inhibition effect on long term passaged GBM cell lines and GNS by DRD2 antagonists: spiperone, haloperidol, risperidone and L-741,626 (J. Li et al., 2014). In accordance to the findings discussed above, dopamine itself was found to inhibit glioma cell proliferation as well as to induce apoptosis through the activation of mitochondrial pathways (Lan, Wang, Xing, Yu, & Lou, 2017).

From a prognostic perspective, data from the Cancer Genome Atlas (TCGA) correlates both higher DRD4 and also TH expression in GBM with worse survival (Dolma et al., 2016). Furthermore, the expression of DRD1, DRD2 and DRD5 was found to be reduced in GBM in the same database (Caragher et al., 2017). Contrastingly to DRD4, the expression of all the remaining DR subtypes was found associated with a survival benefit in GBM patients.

In **paper I**, we show that a DRD2 antagonist, trifluoperazine, inhibits glioblastoma cell line growth and survival differently in cell lines expressing dissimilar DR expression profiles. These findings are further discussed in Chapter 8.

7.4.2.2. Other cancer types

Dopamine signalling has been found to have a regulatory effect in other tumour types than glioblastoma. A general (DRD1-DRD5) dopamine receptor antagonist, thioridazine, was found to prevent initiation of acute myeloid leukaemia by targeting dopamine receptors in human somatic cancer stem cells (Sachlos et al., 2012). In a review by Rubi and Maechler, the effect of dopamine and its antagonists/agonists on cancerous cells is discussed. Reportedly, dopamine and dopamine agonists were shown to impair cell growth or slow tumour growth in small lung cancer, meningioma, B cells from lymphoid malignancy and gastric cancer (Rubí & Maechler, 2010). In breast and colon tumours dopamine seems to increase the efficiency of anticancer drugs. Furthermore, dopamine was shown to stabilize tumour blood vessels, which is necessary to improve drug delivery and prevent worsening of tumour hypoxia, a factor increasing resistance to cancer drugs (Chakroborty et al., 2011).

8. Present investigations

8.1. Paper I – dopamine and disease

The aim of this study was to evaluate the effect of dopamine ligands and their receptors in the inhibition of GBM proliferation and survival.

* Summary of results

We hypothesized dopamine signalling to have a regulatory effect on glioblastoma proliferation and survival based on: (i) the similarities of stem cell regulation in homeostasis, regeneration and in cancer; (ii) the capacity of dopamine signalling to control the growth of other cancer types; (iii) how dopamine influences neural stem cell quiescence in the brain, as for example in the regenerative process of dopaminergic neurons in the ventral midbrain of the newt. Please refer to Chapters 6 and 7 for further details on these topics.

To identify dopamine signalling compounds capable of modulating metabolic activity in GBM we screened three primary GBM cell lines, U3005MG, U3028MG and U3046MG, with a library of 80 dopaminergic ligands. These specific cell lines were selected for this study due to their diverse dopamine receptor expression profiles. In this primary screening, we find 45 metabolically active compounds. Of these, 42 resulted in a decrease and 3 in an increase in GBM cell viability. We also observed some of these compounds to be active in certain GBM cell lines but not in others, while other compounds showed a strong inhibitory effect in all studied GBM cell lines. This suggested a selective effect of some dopamine ligands to specific GBM cell lines.

In order to further validate these findings, 8 out of the 45 identified compounds were selected for further screenings. Concentration-response curves were then run on: (i) 2 of the 3 compounds found to promote cell viability; (ii) 6 of the 42 compounds found to inhibit cell viability. We found that the cell viability promoting compounds showed no effect in these concentration-response curves. We also considered 5 of the 6 tested inhibitory compounds to be acting through cytotoxic non-selective mechanisms, based on their EC50 values and the shape of their dose-response curves. However, a DRD2 antagonist, trifluoperazine 2HCl displayed an effective inhibition of cell viability of GBM cell lines in a dose dependent manner. We also find this dopamine ligand to have a selective inhibitory

effect on U3005MG and U3046MG, while U3028MG showed some resistance to this inhibition at lower concentrations.

The 3 GBM cell lines in this study express different profiles of DR subtypes, specific to each cell line. We asked whether trifluoperazine 2HCl, being a DRD2 antagonist, targets GBM cell lines differently based on their expression of DR subtypes. We therefore validated the expression levels of DRD1, DRD2 and DRD3 in the GBM cell lines of this study and compared this with the values reported in the Human Glioblastoma Cell Culture (HGCC) resource. We find the expression levels to be in accordance to the HGCC reported levels, demonstrating our cells have not substantially changed during the course of our experiments and are therefore representative of their dopamine receptor expression profiles. In summary, these cell lines have the following relative expression values: (i) U3005MG has high DRD2 and DRD5; (ii) U3028MG has high DRD1, DRD4 and DRD5; (iii) U3046MG has low of DRD1-DRD5 (all subtypes).

Metabolic based assays, as used in the primary and secondary screenings of this study, are not a direct indication of changes in cell proliferation. Therefore, we performed nucleotide analogue EdU based assays to assess proliferation changes upon treatment with trifluoperazine. We find the GBM cell line U3028MG to retain some base line proliferation levels 73h after the treatment with the highest concentration of trifluoperazine. However, U3005MG proliferation rate dropped sharply or could not be calculated in U3028MG due to a considerable amount of dying cells. In accordance, U3028MG total cell number is found unaltered at the highest concentration of trifluoperazine treatment, showing this drug inhibits proliferation but does not lead to cell death.

We conclude trifluoperazine 2HCl's inhibition of GBM cells is cell line dependent and correlates with variations in the DR expression profile of these cell lines.

* Discussion

From our screening of 80 dopaminergic ligands we have identified and validated a DRD2 antagonist, trifluoperazine, to have an inhibitory effect on GBM cell lines. This compound has been linked to cancer inhibition in several studies and this inhibition has been linked to several pathways, including DRD2 signalling. First, trifluoperazine inhibits GBM invasion in a calcium signalling mediated manner (Kang et al., 2017). In another GBM study, trifluoperazine is demonstrated to inhibit proliferation of U87MG GBM cell line after EGR-1 upregulation (Shin, Kim, Hong, Kim, & Lee, 2004). Furthermore, in a lung cancer setting, this compound inhibits cancer stem cell growth and overcomes drug resistance (Yeh et al., 2012). In yet another study, trifluoperazine reduces angiogenesis and prevents metastasis of human prostate cancer cell lines, with these effects being linked to DRD2 signalling which is believed to act in this context through a DRD2-AKT-ß-catenin network (Pulkoski-Gross et al., 2015). Upon screening for GBM inhibiting compounds, Dolma

and colleagues identified trifluoperazine, together with another DRD2 antagonist, thioridazine, as hits (Dolma et al., 2016). Moreover, concurrent inhibition of DRD2 and EGFR signalling leads to an anti-tumour effect in *in vitro* and *in vivo* glioblastoma settings (J. Li et al., 2014). Taken together, the findings of **paper I** and previous research suggest trifluoperazine's inhibition of GBM growth may act through DRD2 signalling but also through additional mechanisms.

Interestingly, the GBM cell line that shows the highest resistance to trifluoperazine, U3028MG, is also the one with the highest expression level of all DR subtypes together. Perhaps the D2-like receptor subtypes (DRD2, DRD3 and DRD4) act in synergy with each other as all of these are known to down regulate adenyl cyclase. We also observe U3028MG has the highest expression levels of DRD4 out of the 3 tested GBM cell lines. Dolma and colleagues have elucidated a mechanism for GBM inhibition *in vitro* and *in vivo* which acts through DRD4 signalling (Dolma et al., 2016). In this study the DRD4 antagonists L-741,742 and PNU 96415E disrupt the autophagy-lysosomal pathway, specifically in GNSs and not in NSCs, in this manner inhibiting proliferation and survival of GNS.

The results from **paper I** as well as previous studies indicate trifluoperazine as a potential candidate for treatment of GBM. Moreover, some of the compounds on the primary screening of this study were not further validated and thus could be additional candidates in the context of dopamine signalling inhibition of GBM.

* Study limitations

Some of the limitations of **paper I** are:

- No mechanism has been elucidated to explain trifluoperazine's inhibition of cell proliferation and survival through DRD2 in a GBM setting. In prostate cancer trifluoperazine has been shown to act on DRD2 through a DRD2-AKT-ß-catenin network but this has not been validated in a GBM context or in *in vivo* experiments (Pulkoski-Gross et al., 2015).
- Trifluoperazine's effect on glioblastoma has not been tested *in vivo*, meaning its inhibititory activity may or may not reproduce itself in this setting.
- Sinergestic effects between DR subtypes need further clarification in a cancer context.
- Many of the dopamine ligands which were introduced in the primary and secondary screening have shown no effect on inhibition of GBM cell lines. This may represent different acting mechanisms by similar dopamine agonists/antagonists or raise concerns about its specificity.
- It can not be excluded that trifluoperazine acts through a non-DRD2 pathway during inhibition of GBM proliferation and survival.

8.2. Paper II – dopamine and development

The aim of this study was to provide a new evolutionary perspective on the origin of adult neural stem cells, brain maturation and acquisition of associated behaviours. This work was also aimed to evaluate the dynamics of dopaminergic neurogenesis during development and the consequences of dopaminergic neuronal ablation at the cellular and behaviour level.

* Summary of results

This study made use of two newt species, *Notophthalmus viridescens* and *Pleurodeles waltl*. Due to different body sizes of these animals at the juvenile and adult stages it was unclear whether their brain size would also differ. Indeed, in absolute size the brain of *Pleurodeles* is bigger than the one of *Notophthalmus* but if this measurement is undertaken as a ratio to body size then the *Notophthalmus* brain is instead bigger.

By studying cell proliferation in the brain of these two species we found the following similarities: (i) The marker combination Sox2+/GFAP+ characterizes the main proliferative population; (ii) Quiescent regions appear in a caudal-to-rostral fashion across development.

We also find the following differences in cell proliferation between species and brain regions: (i) proliferation rates are generally higher across *Pleurodeles* brain regions during development; (ii) *Notophthalmus* has more regions becoming quiescent by adulthood than *Pleurodeles*.

Proliferating ependymoglial cells in the ventricular zone of the *Notophthalmus* brain are considered to be the source of new neurons (Kirkham et al., 2014). Ependymoglia can be of two types: (i) type-1, $GFAP^{+}/GS^{+}$ and the main proliferating type in quiescent "non-hotspot" regions; (ii) type-2, $GFAP^{+}/GS^{-}$ and the majority of the progenitors in proliferating "hotspot" regions. We find that in *Pleurodeles* and at early developmental stages, all the ependymoglial cells in the VZ and SVZ are of type-2. Type-1 cells arise later during brain maturation. We also correlated the appearance of type-2 cells with the appearance of structures responsible for the anatomical separation of brain regions, sulci. These data indicated ependymoglia distribution to differ from previous observations in the adult *Notophthalmus* brain. Specifically, in adult *Pleurodeles*, in addition to developmental *Notophthalmus/Pleurodeles*, type-2 cells correspond to the main proliferating population in all brain regions, instead of only the "hotspot" regions. Experiments with conditional reporter transgenic newts are in support of these findings. Here, type-1 cells give rise to small clone groups in the brain parenchyma, arguing for their quiescent state. Contrastingly, type-2 cells are found in large groups in the brain parenchyma, arguing for their proliferative state.

We hypothesized the acquisition of quiescence could be linked to variations in cell cycle length in different brain regions. We then measured cell cycle length at a population level by sequential injection of EdU/BrdU nucleotide analogues (Martynoga, Morrison, Price, & Mason, 2005; Nowakowski, Lewin, & Miller, 1989). For initial larvae we find that the cell cycle is longer in *Pleurodeles* than in *Notophthalmus* in all studied brain regions. For late larvae, the regions that were in the process of becoming quiescent accumulated slowly cycling cells. Particularly, the cell cycle length of the pallium is longer in *Pleurodeles* than in *Notophthalmus*. In accordance, the telencephalon of *Notophthalmus* becomes quiescent at a later stage than in *Pleurodeles*. We also measured cell cycle length at a cellular level by using transgenic Nucbow and Cytbow salamanders, created with the aid of the Multi-addressable genome-integration colour (MAGIC) method (Loulier et al., 2014). This method works by inducing a one-time recombination that leads to the expression of the same fluorescent protein by both the recombined cell and its upcoming progeny. We find that clones in quiescent areas remained local and in low absolute numbers, whereas clones in expanding regions had higher cell numbers, some of which spread into the mantle zone. Taken together, these results confirm type-1 ependymoglia are slow diving progenitor cells.

We then asked how neurogenesis is spatially and temporally regulated in the developing newt brain. We answer this by first analysing the expansion of the *Pleurodeles* telencephalon with markers for ependymoglial cells (Sox2), for mature neurons (NeuN) and EdU pulse chase experiments. We observe brain regions reach maturation in the following order: striatum and medial pallium, lateral and ventral pallia, dorsal pallium and pallidum. After performing similar experiments in the *Notophthalmus* brain, we show that, in both species, highly neurogenic areas correlate with highly proliferative areas across developmental stages and brain regions. We further demonstrate the following correlations between the appearance of specific behaviours and the development of specific neuroanatomical features: (i) a mix of locomotor and feeding behaviours is associated with the maturation of the striatum and the medial pallium region; (ii) the active hunting-foraging behaviour, first use of olfaction and motivationally mediated locomotion is associated with the thickening of the fibre zone; (iii) olfaction-driven exploratory and feeding behaviour is associated with the rise of $NeuN^{+}$ cell numbers in the dorsal pallium and the pallidum.

Next, we set to examine the dopaminergic and cholinergic neurogenesis across development. We find suggestive evidence of the following: (i) the ventral midbrain dopaminergic cells arise first from caudal-most regions; (ii) dopaminergic and cholinergic neurogenesis slows down but still takes place in juvenile stages; (iii) *Pleurodeles* have higher dopaminergic cell numbers than *Notophthalmus* during development and in adulthood; (iv) *Pleurodeles* males have more dopaminergic neurons in some brain regions, while no sex-based difference is observed in *Notophthalmus*.

We also investigated the role of dopaminergic neurogenesis during development by performing dopaminergic ablations followed by behavioural testing. This procedure ablated the dopaminergic neurons of the ventral midbrain as well as its ventral midbrain-to-striatum projections. We find evidence of the following: (i) forebrain dopaminergic innervation is involved in cognitive function related to learning, fear and decision-making; (ii) a regenerative response of the ventral midbrain dopaminergic neurons during *Pleurodeles* development; (iii) striatal proliferation is decreased in animals where the ventral midbrain-to-striatum pathway has been lesioned; (iv) shorter cell migratory distances in the striatum region support the effect of dopaminergic lesion on cell proliferation.

* Discussion

These results provide a descriptive analysis of the processes involved in brain maturation in two newt species: *Notophthalmus* and *Pleurodeles*. This analysis was performed at the different levels: cell proliferation, ependymoglial cell maturation, neurogenesis (general and of specific subpopulations) and acquisition of behaviours.

One of the findings was that proliferative regions are not conserved in two newt species. This is interesting since in the adult axolotl proliferation was found to be present in many regions (Amamoto et al., 2016; Maden, Manwell, & Ormerod, 2013; Richter & Kranz, 1981), while the adult *Notophthalmus* brain is largely quiescent (Berg et al., 2010). Our data indicates the proliferation dynamics of the *Pleurodeles* brain during development to more closely resemble the axolotl and teleost fishes. This provides one more evolutionary perspective to brain ontogeny of vertebrates. Interestingly, in our study, proliferating cells are restricted to the VZ and SVZ of the newt brain, just as in other vertebrates (D'Amico, Boujard, & Coumailleau, 2011; Garcı□a-Verdugo et al., 2002; Lam, März, & Strähle, 2009; Lin et al., 2015; Montiel & Aboitiz, 2015; Moreno & González, 2017). We also show *Notophthalmus* and *Pleurodeles* brain development to have similarities to other tetrapods. This is evident as the cell cycle length slows down at later developmental stages in both cases (Thuret, Auger, & Papalopulu, 2015; Watanabe, Kageyama, & Ohtsuka, 2015). In addition, in this study the S-phase of newts was also found to have similar length to tetrapods (Caviness, Takahashi, & Nowakowski, 1995; Thuret et al., 2015).

In terms of ependymoglia maturation this study presents evidence for type-1 ependymoglia to appear during developmental stages after type-2 cells. Furthermore, type-1 cells appear in increasingly quiescent regions and have longer cell cycles. These data provide new insights into the developmental origin of ependymoglia types in the adult newt brain. This is important also in the context these cells play an important role in the regeneration of the ventral midbrain dopaminergic neurons in the *Notophthalmus* adult brain, under the control of dopamine (Berg et al., 2011; Kirkham et al., 2014). In **paper III**, we present a

methodological development that allows for the study of the morphological characteristics of ependymoglial cells' radial extensions. Future work with this method may identify additional ependymoglial cell subtypes that play a role in the brain maturation processes studied in **paper II**.

This study also sheds new light into the neurogenesis of dopaminergic populations in the newt brain. *Pleurodeles* are not only found to have more dopaminergic cells in general but also to have a higher number of these cells in males than in females. Sexual dimorphism has also been shown in mammals but these results were rather opposite, with female mammals having more dopaminergic cells than males, except in the olfactory bulb (Abel & Rissman, 2012; Bleier, Byne, & Siggelkow, 1982; Gillies, Virdee, McArthur, & Dalley, 2014; Gómez et al., 2007; McArthur, McHale, & Gillies, 2007).

We also find the neurogenesis of specific brain regions to correlate with the appearance of specific behaviours and being in accordance to the functions of corresponding regions in other tetrapods. For example, we observed an increase in the number of dopaminergic cells in the olfactory bulb and that this occurs around the time *Pleurodeles* develop olfaction sensing. At the same time, we also detected a neurogenic wave in the ventral/lateral pallia. The lateral pallium in mammals gives rise to the olfactory cortex, among other structures. It is therefore likely that the neurogenic wave observed in this region in *Pleurodeles* is also linked to the increase in the number of dopaminergic cells in the olfactory bulb (Medina & Abellán, 2009).

In this study, we performed dopaminergic lesioning to investigate the role of dopaminergic neurogenesis during development. Our behavioural experiments indicated dopaminergic innervation in the striatum and nucleus accumbens to be involved in behaviours such as associative learning, decision making and fear. Interestingly, in humans the striatum also plays a role in learning and decision making (Hiebert et al., 2014), while the nucleus accumbens is involved in fear responses (Levita, Dalley, & Robbins, 2002; Martinez, Oliveira, Macedo, Molina, & Brandão, 2008). Taken together these observations suggest that many neuroanatomically-related behaviours are highly conserved among tetrapod evolution. We also demonstrated that lack of dopaminergic innervation in the forebrain correlated with lower proliferative activity in this region. This finding is in line with studies in primates, where dopaminergic innervation projecting from the substantia nigra to the forebrain was found responsible for the stimulation of proliferation (Freundlieb et al., 2006). Thus, we once again find brain maturation dynamics to be highly conserved in vertebrates.

* Study limitations

Some of the limitations of **paper II** are:

- Labelling by electroporation marks a random fraction of ependymoglial cells in the ventricular/subventricular layer of the electroporated brain region. These randomly labelled cells can be ependymoglia type-1 or type-2. In this paper, and to determine characteristics of these ependymoglia, we screened for labeled parenchymal cells. A parenchymal cell was assumed to have migrated radially and to have arisen from one of its nearest radial labelled ependymoglial cells, by using a transgenic line that can express a distinctive combination of fluorescent proteins. This is because ependymoglia radial projections are oriented in a ventricle-to-pial-surface fashion. Assuming such origin for parenchymal cells makes sense as neuroblasts have been shown to use radial glia (ependymoglia) projections as scaffolds for migration (Kawauchi et al., 2010). Based on this reasoning we assume isolated labelled cells in the parenchyma arise from type-1 ependymoglia (NSC) and grouped labelled cells instead arise from type-2 ependymoglia (more frequently dividing progenitors). However, as we could not label type-1 or type-2 ependymoglia alone we cannot exclude lateral migration to occur during maturation from ependymoglia and parenchymal cell stage. If lateral migration would occur then the grouped and isolated cells, which are seen in the parenchyma, could have migrated from ependymoglia in alternative locations. If this would be the case it would be hard to accurately determine the origin of the cells and the thus group size. Although unlikely, this possibility cannot be fully excluded without more specific labelling of each of the ependymoglia types.
- Regeneration of dopaminergic neurons in the ventral midbrain of *Pleurodeles* could be due to developmental compensatory mechanisms rather a regeneration response similar to the one observed in adult Notophthalmus brains.
- A regeneration response in *Pleurodeles* brains requires higher proliferation ($MCM2^+$) and higher levels of cycling cells (EdU^+), in order to replace the lost cells. This response also allows for the catch up with the developmental processes of dopaminergic cells which are seen in control animals. While higher numbers of cycling cells are shown with EdU, higher proliferation with MCM2 data is lacking.
- The molecular mechanism by which decreased dopaminergic innervation leads to lower proliferation levels in the striatum is not elucidated.

8.3.Paper III – dopamine and regeneration

As discussed in chapter 5, available methods for the study of brain cell projections are limited by the destruction of the 3D relationship between a sample's components. The aim of this study was to optimize tissue clearing methods for the study of dopaminergic neurite outgrowth, as well as of ependymoglia radial projections (the progenitors of dopaminergic cells) in the newt brain. These methods can aid with the study of the regeneration and development of the dopaminergic system. With these methods we hope to be able to answer how long-range projections between the ventral midbrain and the forebrain are restored in the newt brain after the ablation of dopaminergic neurons.

* Summary of results

We first asked whether two commonly used tissue clearing techniques, Advanced CUBIC and Advanced CLARITY, could be used to image whole developmental and adult newt brains. We found both techniques performed sub optimally in these small and fragile samples. This was either due to compromising of sample integrity in Advanced CUBIC that led to the discarding of a high number of sample replicates or due to low resolution and signal intensity challenges in Advanced CLARITY. Sample deformation which is induced in newt brains by Advanced CUBIC is probably due to the embryonic characteristics of the adult newt brain, as the embryonic mouse brain also becomes deformed. Interestingly, adult newt brains also become deformed and may retain some of these embryonic characteristics and ended up deformed as well. We further reported on the development of a flexible and optically optimized method for mounting of fragile and small newt brains in light sheet Z.1 microscopes.

To be able to study small and fragile samples we set out to optimize the Advanced CUBIC protocol in order to reduce sample deformation. We achieved this by replacing CUBIC reagent 2 with the RI matching solution Omnipaque 350 and by shortening all the clearing and staining times within the original protocol. Additionally, we reduced the reagent volumes required for achieving clearing of each sample. We demonstrated this optimized protocol to retain sample integrity of newt brains and denoted it CUBICembryonic (CUBICe). When compared with Advanced CLARITY, CUBICe excels at depth, contrast and signal intensity of imaging in stained samples. Moreover, transgenic GFP^+ expressing newt brains, as opposed to stained samples, could also be imaged with this technique.

By joining CUBICe with electroporation methods for genetic labelling we showed it capable of tracing long range projections, dopaminergic fibres and characterization of ependymoglial cells. Specifically, we found morphological differences between ependymoglial cells of the forebrain by measuring the following parameters: the degree of branching, the projections' total length, the distribution of distances from each projection origin to the respective terminal and Sholl intersections. In terms of long range projection tracing, we achieved a

maximum traced distance of 3647 µm. Lastly, the neurites of one ventral midbrain dopaminergic cell were also successfully traced with CUBICe.

Our main objective with applying tissue clearing methods to the newt brain was to study dopaminergic neurites and their cell bodies across the ventral midbrain-to-striatum region. We found that, in Advanced CLARITY cleared tissue, the ventral midbrain dopaminergic cells and their neurites were not clearly visible. However, in CUBICe cleared tissue these structures were clearly discernible. We then performed dopaminergic lesion in newt brains by injection of 6-OHDA (Parish et al., 2007). Then, these brains were cleared using CUBICe. We were able to successfully quantify the degree of dopaminergic lesion in midbrain and forebrain regions by both contrast and intensity measurements. This demonstrates that CUBICe, coupled with bioinformatics pipelines and light sheet microscopy, is an easily applicable method for the quantification of dopaminergic lesions in the newt brain. We further achieved contrast quantification of the midbrain and forebrain regions 30 days after dopaminergic lesion, in the same newt species (*Notophthalmus viridescens*) which was, in a previous study, shown to regenerate the ventral midbrain dopaminergic cell bodies. Due to lack of a sufficient number of experimental animals we relied on previous investigations which showed full dopaminergic cell body regeneration after 6-OHDA lesion is attained at this time point (Parish et al., 2007). We found no difference in dopaminergic neurite and cell body contrast between control and lesion groups in the forebrain region. However, in the midbrain we detected a slight excess of fibres than in the control group, which could represent a compensatory mechanism to the performed lesion.

* Discussion

As far as we know, no tissue clearing protocol has previously been fine tuned to the amphibian and the dissected embryonic mouse brain. However, a whole-mount method for imaging of the newt spinal cord already exists but it is limited by lack of quantification and the thickness of the tissue that can be imaged (Zukor, Kent, & Odelberg, 2010).

CUBICe is a method that makes it possible to image whole cleared newt brains in high resolution without sacrifices in sample fragility and loss. Furthermore, its protocol is faster and uses less reagents than the method it was initially based on, Advanced CUBIC. Using CUBICe one can quantify dopaminergic lesions and subsequent regeneration in regions of the newt brain. A previous study has studied axonal regrowth in the optical nerve of zebrafish by resourcing to tissue clearing methods (Diekmann et al., 2015). Future work using CUBICe will likely focus on studying the targets of regenerated dopaminergic neurites as well as the projection differences between regenerated and control dopaminergic neurites.

By applying CUBICe to newt brains, it is possible to perform neurite tracing to distances over 3600 µm. The cells traced in this study were genetically labelled by

electroporation. This is a transient method where the labelling intensity becomes diluted over repeated cell divisions and becomes, therefore, increasingly hard to detect. In future experiments we intend to make use of transgenic lines to stably and conditionally label these cells in a long term, non-intensity-dilutable manner (Joven et al., 2018). The use of neuronal tracing dyes is another method that can be employed in the labelling of brain cells and their corresponding neurites. Many neuronal tracing dyes are lipophilic and are therefore incompatible with tissue clearing methods which work by facilitating lipid removal from samples. However, recent studies have identified some of these dyes to be compatible with tissue clearing (B. Hou et al., 2015; M.-T. Ke, Fujimoto, & Imai, 2013; Launay et al., 2015) by, for example, binding to both cell membranes and proteins and being aldehyde-fixable (Kristian H.Reveles Jensen & Berg, 2017). Such a methodological approach for the direct labelling of neuronal cells is promising but the injection of these compounds in the newt brain is challenging due to the small brain size and the unavailability of stereotaxic brain coordinates. Despite these difficulties, some studies have achieved the use of these dyes in the newt brain to inform on the ventral midbrain-to-striatum dopaminergic pathways (Oscar Marín, González, et al., 1997a, 1997b; Oscar Marín, Smeets, et al., 1997).

In this study we were also able to trace several morphological parameters of ependymoglial cell radial projections, such as the degree of branching and their projection targets. Future studies should focus on the correlation of these morphological features with the gene expression patterns that are representative of these two types of ependymoglial cells, in the brain of the Notophthalmus viridescens newt (Kirkham et al., 2014). The undertaking of such work could also further the study of ependymoglia maturation (**paper II**) by possibly uncovering new ependymoglial cell types corresponding to progenitor transit amplifying populations which are pre-committed to the genesis of a specific cell type, such as dopaminergic neurons. Ependymoglial cells give rise to regenerated ventral midbrain dopaminergic neurons as well as other cell types in the newt (Parish et al., 2007). Furthermore, radial glial cells, the mammal counterpart of ependymoglial cells in the newt, were found to provide a scaffold which aids neuroblast migration and promotes neuronal regeneration in the mouse neonatal cortex (Jinnou et al., 2018). Therefore, the regeneration of ventral midbrain dopaminergic neurons in the newt brain could follow the signalling cues of an ependymoglial radial projection network and the identification of different ependymoglial cell types can help with the understanding of how these processes work.

* Study limitations

Some of the limitations of **paper III** are:

- The number of cells which develop to be positive for dopaminergic and genetical labelling markers (TH^+/GFP^+) in the ventral midbrain is very low.

Therefore, a substantial amount of animals is required to achieve labelling and subsequential tracing of these cells.

- The labelling of cells genetically marked through electroporation becomes diluted as cell divisions take place. This limits the undertaking of long-term labelling experiments.
- Due to lack of sufficient number of animals during the regeneration experiments we were not able to assess the degree of the lesion. Also for the same reason we have only three animals per control and lesion group. Given the inherent variability between samples this limits the power of our experimental interpretation.

9. Conclusions

This thesis highlights the diverse and complex roles of dopamine in development, regeneration and disease.

In **development**, dopamine controls proliferation of progenitor cells which is indirectly responsible for the later development of specific behaviours.

In **regeneration**, dopamine's role revolves around the maintenance of a quiescent state in progenitor cells, some of which are capable of regenerative responses.

In **disease**, manipulation of dopamine signalling might be a treatment option for glioblastomas. Further investigations could reveal to what extent endogenous dopamine signalling influences glioblastoma growth in the brain. Furthermore, dysfunction of dopamine signalling is one of the hallmarks of Parkinson's disease.

Specific conclusions for **papers I-III** are:

I. Trifluoperazine, a dopamine receptor 2 antagonist, inhibits growth and proliferation of glioblastoma cell lines, indicating the possibility of using dopamine receptor signaling pathways as targets for pharmacological interventions.
II. During developmental maturation, the newt brain exhibits distinct features and substantial similarities among closely related species. This developmental map constitutes the groundwork for finding molecular keys behind the salamanders' capacity for brain regeneration.
III. CUBICe is a superior method to other currently available protocols for the study of neurites and fibres in the developmental and adult newt brains. It allows for tracing of neurons and other brain cells and specifically the quantification of dopaminergic innervation after ablation and subsequent regeneration.

In the words of the great neuroscientist António Damásio[4], "*we almost never think of the present, and when we do, it is only to see what light it throws on our plans for the future*". Indeed, the discussion of the present work leads us into considering the future developments in this field. From my perspective, the holistic view of one single researcher working on his/her own and thereby bringing breakthrough

[4] "Descartes' Error: Emotion, Reason and the Human Brain" (2006), ISBN 978-0099501640. These exact words are paraphrased by António Damásio from a text whose authorship is originally attributed to Blaise Pascal.

scientific discoveries to the eyes of the scientific community is long gone. The methods utilized in answering the research questions in this field, such as the ones in this thesis, profoundly and constantly increase in complexity and cost. It is perhaps acceptable to say one laboratory is not enough. We should disengage from working in our own bubbles and, instead, establish strategic and effective collaborations so that new scientific grounds can be reached and explored more rapidly. **Papers I** and **III** in this thesis are a result of successful inter-laboratory collaborations, with the Chemical Biology Consortium Sweden and the National Microscopy Infrastructure, respectively. These provided both essential knowledge and technology, that were critical for answering the questions we set out in these projects.

As a final note, the brain is a marvellous structure filled with all kinds of secrets and riddles. Having had the opportunity to study it and in this manner unravel little pieces of its mysteries has been a true privilege. Until we meet again.

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References

Abel, J. L., & Rissman, E. F. (2012). Location, location, location: Genetic regulation of neural sex differences. *Reviews in Endocrine and Metabolic Disorders*, *13*(3), 151–161. http://doi.org/10.1007/s11154-011-9186-0

Al-Hajj, M., Becker, M. W., Wicha, M., Weissman, I., & Clarke, M. F. (2004). Therapeutic implications of cancer stem cells. *Current Opinion in Genetics & Development*, *14*(1), 43–47. http://doi.org/10.1016/J.GDE.2003.11.007

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences*, *100*(7), 3983–3988. http://doi.org/10.1073/pnas.0530291100

Alberico, S. L., Cassell, M. D., & Narayanan, N. S. (2015). The Vulnerable Ventral Tegmental Area in Parkinson's Disease. *Basal Ganglia*, *5*(2–3), 51–55. http://doi.org/10.1016/j.baga.2015.06.001

Amamoto, R., Huerta, V. G. L., Takahashi, E., Dai, G., Grant, A. K., Fu, Z., & Arlotta, P. (2016). Adult axolotls can regenerate original neuronal diversity in response to brain injury. *eLife*, *5*, e13998. http://doi.org/10.7554/eLife.13998

Amrein, I. (2015). Adult hippocampal neurogenesis in natural populations of mammals., *7*(5), a021295. http://doi.org/10.1101/cshperspect.a021295

Amrein, I., Becker, A. S., Engler, S., Huang, S.-H., Müller, J., Slomianka, L., & Oosthuizen, M. K. (2014). Adult neurogenesis and its anatomical context in the hippocampus of three mole-rat species. *Frontiers in Neuroanatomy*, *8*, 39. http://doi.org/10.3389/fnana.2014.00039

Amrein, I., Isler, K., & Lipp, H.-P. (2011a). Comparing adult hippocampal neurogenesis in mammalian species and orders: influence of chronological age and life history stage. *The European Journal of Neuroscience*, *34*(6), 978–87. http://doi.org/10.1111/j.1460-9568.2011.07804.x

Amrein, I., Isler, K., & Lipp, H. P. (2011b). Comparing adult hippocampal neurogenesis in mammalian species and orders: Influence of chronological age and life history stage. *European Journal of Neuroscience*, *34*(6), 978–987. http://doi.org/10.1111/j.1460-9568.2011.07804.x

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., … Ericson, J. (2006). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell*, *124*(2), 393–405. http://doi.org/10.1016/j.cell.2005.10.037

Ansell, S. M., Lesokhin, A. M., Borrello, I., Halwani, A., Scott, E. C., Gutierrez, M., … Armand, P. (2015). PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *New England Journal of Medicine*, *372*(4), 311–319. http://doi.org/10.1056/NEJMoa1411087

Arenas, E. (2008). Foxa2: The Rise and Fall of Dopamine Neurons. *Cell Stem Cell*, *2*(2), 110–112. http://doi.org/10.1016/J.STEM.2008.01.012

Arias-Carrión, O., Freundlieb, N., Oertel, W. H., & Höglinger, G. U. (2007). Adult Neurogenesis and Parkinson ' s Disease. *Science*, 1–10.

Ascherio, A., & Schwarzschild, M. A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. *The Lancet Neurology*, *15*(12), 1257–1272. http://doi.org/10.1016/S1474-4422(16)30230-7

Baker, S. A., Baker, K. A., & Hagg, T. (2004). Dopaminergic nigrostriatal projections regulate neural precursor proliferation in the adult mouse subventricular zone. *European Journal of Neuroscience*, *20*(2), 575–579. http://doi.org/10.1111/j.1460-9568.2004.03486.x

Banerjee, C. (2011). Stem cells therapies in basic science and translational medicine: current status and treatment monitoring strategies. *Current Pharmaceutical Biotechnology*, *12*(4), 469–87. http://doi.org/10.2174/138920111795164002

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., … Rich, J. N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*, *444*(7120), 756–760. http://doi.org/10.1038/nature05236

Barker, N. (2008). The Canonical Wnt/β-Catenin Signalling Pathway. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 468, pp. 5–15). http://doi.org/10.1007/978-1-59745-249-6_1

Barker, R. A., Parmar, M., Studer, L., & Takahashi, J. (2017). Human Trials of Stem Cell-Derived Dopamine Neurons for Parkinson ' s Disease : Dawn of a New Era. *Stem Cell*, *21*(5), 569–573. http://doi.org/10.1016/j.stem.2017.09.014

Barron, A. B., Søvik, E., & Cornish, J. L. (2010). The Roles of Dopamine and Related Compounds in Reward-Seeking Behavior Across Animal Phyla. *Frontiers in Behavioral Neuroscience*, *4*(October), 1–9. http://doi.org/10.3389/fnbeh.2010.00163

Beachy, P. a, Karhadkar, S. S., & Berman, D. M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature*, *432*(7015), 324–331. http://doi.org/10.1038/nature03100

Becker, A. J., McCulloch, E. A., & Till, J. E. (1963). Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells. *Nature*, *197*(4866), 452–454. http://doi.org/10.1038/197452a0

Belle, M., Godefroy, D., Dominici, C., Heitz-Marchaland, C., Zelina, P., Hellal, F., … Chédotal, A. (2014). A Simple Method for 3D Analysis of Immunolabeled Axonal Tracts in a Transparent Nervous System. *Cell Reports*, *9*(4), 1191–1201. http://doi.org/10.1016/j.celrep.2014.10.037

Ben-Eliyahu, S., Yirmiya, R., Liebeskind, J. C., Taylor, A. N., & Gale, R. P. (1991). Stress increases metastatic spread of a mammary tumor in rats: evidence for mediation by the immune system. *Brain, Behavior, and Immunity*, *5*(2), 193–205. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1654166

Ben Abdallah, N. M.-B., Slomianka, L., Vyssotski, A. L., & Lipp, H.-P. (2010). Early age-related changes in adult hippocampal neurogenesis in C57 mice. *Neurobiology of Aging*, *31*(1), 151–61. http://doi.org/10.1016/j.neurobiolaging.2008.03.002

Benraiss, A., Arsanto, J. P., Coulon, J., & Thouveny, Y. (1999). Neurogenesis during caudal spinal cord regeneration in adult newts. *Development Genes and Evolution*, *209*(6), 363–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10370118

Berg, D. a., Kirkham, M., Wang, H., Frisén, J., & Simon, A. (2011). Dopamine controls neurogenesis in the adult salamander midbrain in homeostasis and during regeneration of dopamine neurons. *Cell Stem Cell*, *8*(4), 426–433. http://doi.org/10.1016/j.stem.2011.02.001

Berg, D. a, Belnoue, L., Song, H., & Simon, A. (2013). Neurotransmitter-mediated control of neurogenesis in the adult vertebrate brain. *Development (Cambridge, England)*, *140*(12), 2548–61. http://doi.org/10.1242/dev.088005

Berg, D. a, Kirkham, M., Beljajeva, A., Knapp, D., Habermann, B., Ryge, J., … Simon, A. (2010). Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain. *Development (Cambridge, England)*, *137*(24), 4127–4134. http://doi.org/10.1242/dev.061754

Bergmann, O., Liebl, J., Bernard, S., Alkass, K., Yeung, M. S. Y., Steier, P., … Frisén, J. (2012). The Age of Olfactory Bulb Neurons in Humans. *Neuron*, *74*(4), 634–639. http://doi.org/10.1016/j.neuron.2012.03.030

Bergmann, O., Spalding, K. L., & Frisén, J. (2015). Adult Neurogenesis in Humans. *Cold Spring Harbor Perspectives in Biology*, *7*(7), a018994. http://doi.org/10.1101/cshperspect.a018994

Bethesda, M. (2016). NIH Stem Cell Information Home Page. Retrieved February 6, 2018, from stemcells.nih.gov/info/basics/2.htm

Björklund, A., & Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends in Neurosciences*, *30*(5), 194–202. http://doi.org/10.1016/j.tins.2007.03.006

Blau, H. M., Brazelton, T. R., & Weimann, J. M. (2001). The evolving concept of a stem cell: entity or function? *Cell*, *105*(7), 829–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11439179

Bleier, R., Byne, W., & Siggelkow, I. (1982). Cytoarchitectonic sexual dimorphisms of the medial preoptic and anterior hypothalamic areas in guinea pig, rat, hamster, and mouse. *The Journal of Comparative Neurology*, *212*(2), 118–130. http://doi.org/10.1002/cne.902120203

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, *3*(7), 730–737. http://doi.org/10.1038/nm0797-730

Braun, S. M. G., & Jessberger, S. (2014). Adult neurogenesis: mechanisms and functional significance. *Development*, *141*(10), 1983–1986. http://doi.org/10.1242/dev.104596

Breedis, C. (1952). Induction of accessory limbs and of sarcoma in the Newt (Triturus viridescens) with carcinogenic substances. *Cancer Research*, *12*(12), 861–866.

Bria, A., & Iannello, G. (2012). TeraStitcher - A tool for fast automatic 3D-stitching of teravoxel-sized microscopy images. *BMC Bioinformatics*, *13*(1), 316. http://doi.org/10.1186/1471-2105-13-316

Brockes, J., & Kumar, A. (2005). Newts. *Current Biology*, *15*(2), 42–44.

Brockes, J. P. (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science (New York, N.Y.)*, *276*(5309), 81–87. http://doi.org/10.1126/science.276.5309.81

Cameron, C., Beug, S., & Tsilfidis, C. (2004). Captive Breeding of Notophthalmus viridescens Through. *Herpetological Review*, *35*(3), 257–259.

Caragher, S. P., Hall, R. R., Ahsan, R., & Ahmed, A. U. (2017). Monoamines in glioblastoma: complex biology with therapeutic potential. *Neuro-Oncology*, (January), 1–12. http://doi.org/10.1093/neuonc/nox210

Carlsson, A. (1993). Thirty years of dopamine research. *Advances in Neurology*, *60*, 1–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8093570

Cavegn, N., van Dijk, R. M., Menges, D., Brettschneider, H., Phalanndwa, M., Chimimba, C. T., … Amrein,

I. (2013). Habitat-specific shaping of proliferation and neuronal differentiation in adult hippocampal neurogenesis of wild rodents. *Frontiers in Neuroscience*, *7*, 59. http://doi.org/10.3389/fnins.2013.00059

Caviness, V. S., Takahashi, T., & Nowakowski, R. S. (1995). Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. *Trends in Neurosciences*, *18*(9), 379–383. http://doi.org/10.1016/0166-2236(95)93933-O

Chakroborty, D., Sarkar, C., Yu, H., Wang, J., Liu, Z., Dasgupta, P. S., & Basu, S. (2011). Dopamine stabilizes tumor blood vessels by up-regulating angiopoietin 1 expression in pericytes and Kruppel-like factor-2 expression in tumor endothelial cells. *Proceedings of the National Academy of Sciences*, *108*(51), 20730–20735. http://doi.org/10.1073/pnas.1108696108

Chang, J.-B., Chan, F., Jung, E., Babcock, H., Zhuang, X., & Boyden, E. S. (2017). Iterative expansion microscopy. *In Preparation*, (September 2016), 1–11. http://doi.org/10.1038/nmeth.4261

Chen, D. S., & Mellman, I. (2013). Oncology meets immunology: The cancer-immunity cycle. *Immunity*, *39*(1), 1–10. http://doi.org/10.1016/j.immuni.2013.07.012

Cheshire, E. C., Malcomson, R. D. G., Joseph, S., Biggs, M. J. B., Adlam, D., & Rutty, G. N. (2015). Optical clearing of the dura mater using glycerol: a reversible process to aid the post-mortem investigation of infant head injury. *Forensic Science, Medicine, and Pathology*. http://doi.org/10.1007/s12024-015-9691-7

Choudhry, Z., Rikani, A. A., Choudhry, A. M., Tariq, S., Zakaria, F., Asghar, M. W., … Mobassarah, N. J. (2014). Sonic hedgehog signalling pathway: a complex network. *Annals of Neurosciences*, *21*(1), 28–31. http://doi.org/10.5214/ans.0972.7531.210109

Chung, K., Wallace, J., Kim, S.-Y., Kalyanasundaram, S., Andalman, A. S., Davidson, T. J., … Deisseroth, K. (2013). Structural and molecular interrogation of intact biological systems. *Nature*, *497*(7449), 332–7. http://doi.org/10.1038/nature12107

Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H. M., Jones, D. L., … Wahl, G. M. (2006). Cancer stem cells - Perspectives on current status and future directions: AACR workshop on cancer stem cells. *Cancer Research*, *66*(19), 9339–9344. http://doi.org/10.1158/0008-5472.CAN-06-3126

Combs, S. E., Rieken, S., Wick, W., Abdollahi, A., von Deimling, A., Debus, J., & Hartmann, C. (2011). Prognostic significance of IDH-1 and MGMT in patients with glioblastoma: one step forward, and one step back? *Radiation Oncology (London, England)*, *6*, 115. http://doi.org/10.1186/1748-717X-6-115

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature*, *420*(6917), 860–867. http://doi.org/10.1038/nature01322

Curtis, M. A., Kam, M., Nannmark, U., Anderson, M. F., Axell, M. Z., Wikkelso, C., … Eriksson, P. S. (2007). Human Neuroblasts Migrate to the Olfactory Bulb via a Lateral Ventricular Extension. *Science*, *315*(5816), 1243–1249. http://doi.org/10.1126/science.1136281

D'Amico, L. A., Boujard, D., & Coumailleau, P. (2011). Proliferation, migration and differentiation in juvenile and adult Xenopus laevis brains. *Brain Research*, *1405*, 31–48. http://doi.org/10.1016/j.brainres.2011.06.032

Dalerba, P., Dylla, S. J., Park, I.-K., Liu, R., Wang, X., Cho, R. W., … Clarke, M. F. (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences*, *104*(24), 10158–10163. http://doi.org/10.1073/pnas.0703478104

Dalerba, P., Kalisky, T., Sahoo, D., Rajendran, P. S., Rothenberg, M. E., Leyrat, A. A., … Quake, S. R. (2011). Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nature Biotechnology*, *29*(12), 1120–1127. http://doi.org/10.1038/nbt.2038

Davis, M., Ayers, J. L., Koran, L., Anderson, M. C., & Simpson, S. B. (1990). Time Course of Salamander Spinal Cord Regeneration and Recovery of Swimming : HRP Retrograde Pathway Tracing and Kinematic Analysis, *213*, 198–213.

DeFelipe, J. (2010). From the connectome to the synaptome: An epic love story. *Science*, *330*(6008), 1198–1201. http://doi.org/10.1126/science.1193378

Definition of glioma. (2018). Retrieved January 21, 2018, from https://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=45700

Denk, W., & Horstmann, H. (2004). Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. *PLoS Biology*, *2*(11). http://doi.org/10.1371/journal.pbio.0020329

Denk, W., Strickler, J. H., & Webb, W. W. (1990). Two-photon laser scanning fluorescence microscopy. *Science (New York, N.Y.)*, *248*(4951), 73–76. http://doi.org/10.1126/science.2321027

Di Bari, M., Tombolillo, V., Conte, C., Castigli, E., Sciaccaluga, M., Iorio, E., … Tata, A. M. (2015). Cytotoxic and genotoxic effects mediated by M2 muscarinic receptor activation in human glioblastoma cells. *Neurochemistry International*, *90*, 261–270. http://doi.org/10.1016/j.neuint.2015.09.008

Diamandis, P., Sacher, A. G., Tyers, M., & Dirks, P. B. (2009). New drugs for brain tumors? Insights from

chemical probing of neural stem cells. *Medical Hypotheses*, *72*(6), 683–687. http://doi.org/10.1016/j.mehy.2008.10.034

Diekmann, H., Kalbhen, P., & Fischer, D. (2015). Characterization of optic nerve regeneration using transgenic zebrafish. *Frontiers in Cellular Neuroscience*, *9*(April), 1–11. http://doi.org/10.3389/fncel.2015.00118

Dobosz, M., Ntziachristos, V., Scheuer, W., & Strobel, S. (2014). Multispectral Fluorescence Ultramicroscopy: Three-Dimensional Visualization and Automatic Quantification of Tumor Morphology, Drug Penetration, and Antiangiogenic Treatment Response. *Neoplasia*, *16*(1), 1-W7. http://doi.org/10.1593/neo.131848

Dodt, H.-U., Leischner, U., Schierloh, A., Jährling, N., Mauch, C. P., Deininger, K., … Becker, K. (2007). Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. *Nature Methods*, *4*(4), 331–336. http://doi.org/10.1038/nmeth1036

Doerr, J., Schwarz, M. K., Wiedermann, D., Leinhaas, A., Jakobs, A., Schloen, F., … Brüstle, O. (2017). Whole-brain 3D mapping of human neural transplant innervation. *Nature Communications*, *8*, 14162. http://doi.org/10.1038/ncomms14162

Dolma, S., Selvadurai, H. J., Lan, X., Lee, L., Kushida, M., Voisin, V., … Dirks, P. B. (2016). Inhibition of Dopamine Receptor D4 Impedes Autophagic Flux, Proliferation, and Survival of Glioblastoma Stem Cells. *Cancer Cell*, *29*(6), 859–873. http://doi.org/10.1016/j.ccell.2016.05.002

Dvorak, H. F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *New England Journal of Medicine*, *315*(26), 1650–1659. http://doi.org/10.1056/NEJM198612253152606

Economo, M. N., Clack, N. G., Lavis, L. D., Gerfen, C. R., Svoboda, K., Myers, E. W., & Chandrashekar, J. (2016). A platform for brain-wide imaging and reconstruction of individual neurons, 1–22. http://doi.org/10.7554/eLife.10566

Egeland, M., Zunszain, P. A., & Pariante, C. M. (2015). Molecular mechanisms in the regulation of adult neurogenesis during stress. *Nature Reviews Neuroscience*, *16*(4), 189–200. http://doi.org/10.1038/nrn3855

Eguchi, G. (1988). Cellular and molecular background of Wolffian lens regeneration. *Regulatory Mechanisms in Developmental Processes*, 147–158.

Eguchi, G., Eguchi, Y., Nakamura, K., Yadav, M. C., Millán, J. L., & Tsonis, P. a. (2011). Regenerative capacity in newts is not altered by repeated regeneration and ageing. *Nature Communications*, *2*, 384. http://doi.org/10.1038/ncomms1389

Elewa, A., Wang, H., Talavera-lópez, C., Joven, A., Brito, G., Kumar, A., … Simon, A. (2017). reveals novel features of tetrapod regeneration. *Nature Communications*, *8*(2286), 1–9. http://doi.org/10.1038/s41467-017-01964-9

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., & Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nat Med*, *4*(11), 1313–1317. http://doi.org/10.1038/3305

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., … Frisén, J. (2014). Neurogenesis in the striatum of the adult human brain. *Cell*, *156*(5), 1072–1083. http://doi.org/10.1016/j.cell.2014.01.044

Ernst, A., & Frisén, J. (2015). Adult Neurogenesis in Humans- Common and Unique Traits in Mammals. *PLoS Biology*, *13*(1), 1–12. http://doi.org/10.1371/journal.pbio.1002045

Ertürk, A., Becker, K., Jährling, N., Mauch, C. P., Hojer, C. D., Egen, J. G., … Dodt, H.-U. (2012). Three-dimensional imaging of solvent-cleared organs using 3DISCO. *Nature Protocols*, *7*(11), 1983–1995. http://doi.org/10.1038/nprot.2012.119

Ertürk, A., Lafkas, D., & Chalouni, C. (2014). Imaging Cleared Intact Biological Systems at a Cellular Level by 3DISCO. *Journal of Visualized Experiments : JoVE*, (89), 1–13. http://doi.org/10.3791/51382

Farfel, M., Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. E. A. T., Ferretti, R. E. L., Leite, R. E. P., … Lent, R. (2009). Equal Numbers of Neuronal and Nonneuronal Cells Make the Human Brain an Isometrically Scaled-Up Primate Brain, *541*(June 2008), 532–541. http://doi.org/10.1002/cne.21974

Feuchtinger, A., Walch, A., & Dobosz, M. (2016). Deep tissue imaging: a review from a preclinical cancer research perspective. *Histochemistry and Cell Biology*, *146*(6), 781–806. http://doi.org/10.1007/s00418-016-1495-7

Freundlieb, N., François, C., Tandé, D., Oertel, W. H., Hirsch, E. C., & Höglinger, G. U. (2006). Dopaminergic Substantia Nigra Neurons Project Topographically Organized to the Subventricular Zone and Stimulate Precursor Cell Proliferation in Aged Primates. *Journal of Neuroscience*, *26*(8), 2321–2325. http://doi.org/10.1523/JNEUROSCI.4859-05.2006

Frielingsdorf, H., Schwarz, K., Brundin, P., & Mohapel, P. (2004). No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. *Proceedings of the National Academy of*

Sciences of the United States of America, *101*(27), 10177–82. http://doi.org/10.1073/pnas.0401229101

Frisén, J. (2016). Neurogenesis and Gliogenesis in Nervous System Plasticity and Repair. *Annual Review of Cell and Developmental Biology*, *32*(1), 127–141. http://doi.org/10.1146/annurev-cellbio-111315-124953

Fürth, D., Vaissière, T., Tzortzi, O., Xuan, Y., Märtin, A., Lazaridis, I., … Meletis, K. (2017). An interactive framework for whole-brain maps at cellular resolution. *Nature Neuroscience*. http://doi.org/10.1038/s41593-017-0027-7

Garcı□a-Verdugo, J. M., Ferrón, S., Flames, N., Collado, L., Desfilis, E., & Font, E. (2002). The proliferative ventricular zone in adult vertebrates: a comparative study using reptiles, birds, and mammals. *Brain Research Bulletin*, *57*(6), 765–775. http://doi.org/10.1016/S0361-9230(01)00769-9

Gillies, G. E., Virdee, K., McArthur, S., & Dalley, J. W. (2014). Sex-dependent diversity in ventral tegmental dopaminergic neurons and developmental programing: A molecular, cellular and behavioral analysis. *Neuroscience*, *282*, 69–85. http://doi.org/10.1016/J.NEUROSCIENCE.2014.05.033

Gliomas. (2018). Retrieved January 21, 2018, from https://www.hopkinsmedicine.org/healthlibrary/conditions/nervous_system_disorders/gliomas_134,22

Gómez-Gaviro, M. V., Balaban, E., Bocancea, D., Lorrio, M. T., Pompeiano, M., Desco, M., … Vaquero, J. J. (2017). Optimized CUBIC protocol for three-dimensional imaging of chicken embryos at single-cell resolution. *Development*, *144*(11), 2092–2097. http://doi.org/10.1242/dev.145805

Gómez, C., Briñón, J. G., Valero, J., Recio, J. S., Murias, A. R., Curto, G. G., … Alonso, J. R. (2007). Sex differences in catechol contents in the olfactory bulb of control and unilaterally deprived rats. *European Journal of Neuroscience*, *25*(5), 1517–1528. http://doi.org/10.1111/j.1460-9568.2007.05407.x

Göritz, C., & Frisén, J. (2012). Neural Stem Cells and Neurogenesis in the Adult. *Cell Stem Cell*, *10*(6), 657–659. http://doi.org/10.1016/J.STEM.2012.04.005

Goss, R. J., & Stagg, M. W. (1958). Regeneration in lower jaws of newts after excision of the intermandibular regions. *The Journal of Experimental Zoology*, *137*(1), 1–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13563783

Grade, S., & Götz, M. (2017). Neuronal replacement therapy: previous achievements and challenges ahead. *Npj Regenerative Medicine*, *2*(1), 29. http://doi.org/10.1038/s41536-017-0033-0

Greenbaum, A., Chan, K. Y., Dobreva, T., Brown, D., Balani, D. H., Boyce, R., … Gradinaru, V. (2017). Bone CLARITY: Clearing, imaging, and computational analysis of osteoprogenitors within intact bone marrow. *Science Translational Medicine*, *9*(387). http://doi.org/10.1126/scitranslmed.aah6518

Guillemot, F. (2005). Cellular and molecular control of neurogenesis in the mammalian telencephalon. *Current Opinion in Cell Biology*, *17*(6), 639–647. http://doi.org/10.1016/j.ceb.2005.09.006

Hagg, T. (2005). Molecular regulation of adult CNS neurogenesis: An integrated view. *Trends in Neurosciences*, *28*(11), 589–595. http://doi.org/10.1016/j.tins.2005.08.009

Hama, H., Hioki, H., Namiki, K., Hoshida, T., Kurokawa, H., Ishidate, F., … Miyawaki, A. (2015). ScaleS: an optical clearing palette for biological imaging. *Nature Neuroscience*, (September). http://doi.org/10.1038/nn.4107

Hama, H., Kurokawa, H., Kawano, H., Ando, R., Shimogori, T., Noda, H., … Miyawaki, A. (2011). Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. *Nature Neuroscience*, *14*(11), 1481–1488. http://doi.org/10.1038/nn.2928

Hameed, L. S., Berg, D. A., Belnoue, L., & Jensen, L. D. (2015). Environmental changes in oxygen tension reveal ROS-dependent neurogenesis and regeneration in the adult newt brain, 1–16. http://doi.org/10.7554/eLife.08422

Harsing, L. G. (2008). Handbook of Neurochemistry and Molecular Neurobiology. In A. Lajtha (Ed.), (pp. 151–167).

Hedlund, E., Belnoue, L., Theofilopoulos, S., Salto, C., Bye, C., Parish, C., … Simon, A. (2016). Dopamine Receptor Antagonists Enhance Proliferation and Neurogenesis of Midbrain Lmx1a-expressing Progenitors. *Scientific Reports*, *6*(April), 26448. http://doi.org/10.1038/srep26448

Hegarty, S. V., Sullivan, A. M., & O'Keeffe, G. W. (2013). Midbrain dopaminergic neurons: A review of the molecular circuitry that regulates their development. *Developmental Biology*, *379*(2), 123–138. http://doi.org/10.1016/j.ydbio.2013.04.014

Hegi, M. E., Diserens, A.-C., Gorlia, T., Hamou, M.-F., de Tribolet, N., Weller, M., … Stupp, R. (2005). *MGMT* Gene Silencing and Benefit from Temozolomide in Glioblastoma. *New England Journal of Medicine*, *352*(10), 997–1003. http://doi.org/10.1056/NEJMoa043331

Helmchen, F., & Denk, W. (2005). Deep tissue two-photon microscopy. *Nature Methods*, *2*(12), 932–940. http://doi.org/10.1038/nmeth818

Hiebert, N. M., Vo, A., Hampshire, A., Owen, A. M., Seergobin, K. N., & MacDonald, P. A. (2014). Striatum in stimulus–response learning via feedback and in decision making. *NeuroImage*, *101*, 448–457.

http://doi.org/10.1016/J.NEUROIMAGE.2014.07.013

Höglinger, G. U., Arias-Carrión, O., Ipach, B., & Oertel, W. H. (2014). Origin of the dopaminergic innervation of adult neurogenic areas. *Journal of Comparative Neurology*, *522*(10), 2336–2348. http://doi.org/10.1002/cne.23537

Höglinger, G. U., Breunig, J. J., Depboylu, C., Rouaux, C., Michel, P. P., Alvarez-Fischer, D., … Hunot, S. (2007). The pRb/E2F cell-cycle pathway mediates cell death in Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(9), 3585–90. http://doi.org/10.1073/pnas.0611671104

Höglinger, G. U., Rizk, P., Muriel, M. P., Duyckaerts, C., Oertel, W. H., Caille, I., & Hirsch, E. C. (2004). Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nature Neuroscience*, *7*(7), 726–735. http://doi.org/10.1038/nn1265

Hörl, D., Rusak, F. E. R., Harz, H., Treier, M., & Preibisch, S. (2017). BIG STITCHER - EFFICIENT ALIGNMENT OF MULTI-TILE AND MULTI-VIEW LIGHTSHEET DATA OF CLEARED SAMPLES David. In *Focus on Microscopy 2017*. Retrieved from http://www.focusonmicroscopy.org/2017/PDF/1075_Preibisch.pdf

Hou, B., Zhang, D., Zhao, S., Wei, M., Yang, Z., Wang, S., … Jiang, T. (2015). Scalable and DiI-compatible optical clearance of the mammalian brain. *Frontiers in Neuroanatomy*, *9*(February), 1–11. http://doi.org/10.3389/fnana.2015.00019

Hou, S. W., Wang, Y. Q., Xu, M., Shen, D. H., Wang, J. J., Huang, F., … Sun, F. Y. (2008). Functional integration of newly generated neurons into striatum after cerebral ischemia in the adult rat brain. *Stroke*, *39*(10), 2837–2844. http://doi.org/10.1161/STROKEAHA.107.510982

Huse, J. T., & Holland, E. C. (2010). Targeting brain cancer: Advances in the molecular pathology of malignant glioma and medulloblastoma. *Nature Reviews Cancer*, *10*(5), 319–331. http://doi.org/10.1038/nrc2818

Illmensee, K., & Mintz, B. (1976). Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts. *Proceedings of the National Academy of Sciences of the United States of America*, *73*(2), 549–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1061157

Imayoshi, I., Sakamoto, M., Ohtsuka, T., Takao, K., Miyakawa, T., Yamaguchi, M., … Kageyama, R. (2008). Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nature Neuroscience*, *11*(10), 1153–61. http://doi.org/10.1038/nn.2185

Imokawa, Y., & Yoshizato, K. (1997). Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(17), 9159–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9256452

Iten, L. E., & Bryant, S. V. (1976). Stages of tail regeneration in the adult newt,Notophthalmus viridescens. *Journal of Experimental Zoology*, *196*(3), 283–292. http://doi.org/10.1002/jez.1401960303

Jacinto, F. V., & Esteller, M. (2007). MGMT hypermethylation: A prognostic foe, a predictive friend. *DNA Repair*, *6*(8), 1155–1160. http://doi.org/10.1016/j.dnarep.2007.03.013

Jensen, K. H. R., & Berg, R. W. (2016). CLARITY-compatible lipophilic dyes for electrode marking and neuronal tracing, (May), 1–13.

Jensen, K. H. R., & Berg, R. W. (2017). Advances and perspectives in tissue clearing using CLARITY. *Journal of Chemical Neuroanatomy*, *86*, 19–34. http://doi.org/10.1016/j.jchemneu.2017.07.005

Jiménez, E., & Montiel, M. (2005). Activation of MAP kinase by muscarinic cholinergic receptors induces cell proliferation and protein synthesis in human breast cancer cells. *Journal of Cellular Physiology*, *204*(2), 678–686. http://doi.org/10.1002/jcp.20326

Jinnou, H., Sawada, M., Kawase, K., Ajioka, I., Saitoh, S., & Sawamoto, K. (2018). Radial Glial Fibers Promote Neuronal Migration and Functional Recovery after Neonatal Brain Injury. *Cell Stem Cell*, (22), 1–10. http://doi.org/10.1016/j.stem.2017.11.005

Joven, A., Kirkham, M., & Simon, A. (2015). Husbandry of Spanish Ribbed Newts (Pleurodeles waltl). In A. Kumar & A. Simon (Eds.), *Salamanders in Regeneration Research: Methods and Protocols* (pp. 47–70). New York, NY: Springer New York. http://doi.org/10.1007/978-1-4939-2495-0_4

Joven, A., Wang, H., Pinheiro, T., Hameed, L. S., Belnoue, L., & Simon, A. (2018). Cellular basis of brain maturation and acquisition of complex behaviors in salamanders. *Development*, *145*(1). http://doi.org/10.1242/dev.160051

Kang, S., Hong, J., Lee, J. M., Moon, H. E., Jeon, B., Choi, J., … Kang, S. S. (2017). Trifluoperazine, a Well-Known Antipsychotic, Inhibits Glioblastoma Invasion by Binding to Calmodulin and Disinhibiting Calcium Release Channel IP $_3$ R. *Molecular Cancer Therapeutics*, *16*(1), 217–227. http://doi.org/10.1158/1535-7163.MCT-16-0169-T

Karhadkar, S. S., Steven Bova, G., Abdallah, N., Dhara, S., Gardner, D., Maitra, A., … Beachy, P. A. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature*, *431*(7009),

707–712. http://doi.org/10.1038/nature02962

Karpel-Massler, G., Kast, R. E., Westhoff, M. A., Dwucet, A., Welscher, N., Nonnenmacher, L., … Halatsch, M. E. (2015). Olanzapine inhibits proliferation, migration and anchorage-independent growth in human glioblastoma cell lines and enhances temozolomide's antiproliferative effect. *J Neurooncol*, *122*(1), 21–33. http://doi.org/10.1007/s11060-014-1688-7

Kasthuri, N., & Lichtman, J. W. (2007). The rise of the "projectome." *Nature Methods*, *4*(4).

Kawauchi, T., Sekine, K., Shikanai, M., Chihama, K., Tomita, K., Kubo, K., … Hoshino, M. (2010). Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking. *Neuron*, *67*(4), 588–602. http://doi.org/10.1016/j.neuron.2010.07.007

Ke, M.-T., Fujimoto, S., & Imai, T. (2013). SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. *Nature Neuroscience*, *16*(8), 1154–61. http://doi.org/10.1038/nn.3447

Ke, M., Nakai, Y., Ke, M., Nakai, Y., Fujimoto, S., Takayama, R., … Kitajima, T. S. (2016). Super-Resolution Mapping of Neuronal Circuitry With an Index-Optimized Clearing Agent Resource Super-Resolution Mapping of Neuronal Circuitry With an Index-Optimized Clearing Agent. *CellReports*, 1–15. http://doi.org/10.1016/j.celrep.2016.02.057

Keinath, M. C., Timoshevskiy, V. A., Timoshevskaya, N. Y., Tsonis, P. A., Voss, S. R., & Smith, J. J. (2015). Initial characterization of the large genome of the salamander Ambystoma mexicanum using shotgun and laser capture chromosome sequencing. *Scientific Reports*, *5*(1), 16413. http://doi.org/10.1038/srep16413

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends in Neurosciences*, *27*(8), 447–452. http://doi.org/10.1016/j.tins.2004.05.013

Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., … Takahashi, J. (2017). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature Publishing Group*, *548*(7669), 592–596. http://doi.org/10.1038/nature23664

Kim, S.-Y., Cho, J. H., Murray, E., Bakh, N., Choi, H., Ohn, K., … Chung, K. (2015). Stochastic electrotransport selectively enhances the transport of highly electromobile molecules. *Proceedings of the National Academy of Sciences*, *112*(46), E6274–E6283. http://doi.org/10.1073/pnas.1510133112

Kippin, T. E., Kapur, S., & van der Kooy, D. (2005). Dopamine Specifically Inhibits Forebrain Neural Stem Cell Proliferation, Suggesting a Novel Effect of Antipsychotic Drugs. *Journal of Neuroscience*, *25*(24), 5815–5823. http://doi.org/10.1523/JNEUROSCI.1120-05.2005

Kirkham, M., Hameed, L. S., Berg, D. a, Wang, H., & Simon, A. (2014). Progenitor Cell Dynamics in the Newt Telencephalon during Homeostasis and Neuronal Regeneration. *Stem Cell Reports*, *2*(4), 507–19. http://doi.org/10.1016/j.stemcr.2014.01.018

Kittappa, R., Chang, W. W., Awatramani, R. B., & McKay, R. D. G. (2007). The foxa2 Gene Controls the Birth and Spontaneous Degeneration of Dopamine Neurons in Old Age. *PLoS Biology*, *5*(12), e325. http://doi.org/10.1371/journal.pbio.0050325

Kleinsmith, L. J., & Pierce, G. B. (1964). Multipotentiality of single embryonal carcinoma cells. *Cancer Research*, *24*, 1544–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14234000

Kokoeva, M. V. (2005). Neurogenesis in the Hypothalamus of Adult Mice: Potential Role in Energy Balance. *Science*, *310*(5748), 679–683. http://doi.org/10.1126/science.1115360

Kolesová, H., Čapek, M., Radochová, B., Janáček, J., & Sedmera, D. (2016). Comparison of different tissue clearing methods and 3D imaging techniques for visualization of GFP-expressing mouse embryos and embryonic hearts. *Histochemistry and Cell Biology*, *146*(2), 141–152. http://doi.org/10.1007/s00418-016-1441-8

Ku, T., Swaney, J., Park, J. Y., Albanese, A., Murray, E., Hun Cho, J., … Chung, K. (2016). Multiplexed and scalable super-resolution imaging of three-dimensional protein localization in size-adjustable tissues. *Nature Biotechnology*, *34*(9), 973–981. http://doi.org/10.1038/nbt.3641

Kubota, S. I., Takahashi, K., Nishida, J., Morishita, Y., Ehata, S., Tainaka, K., … Ueda, H. R. (2017). Whole-Body Profiling of Cancer Metastasis with Single-Cell Resolution. *Cell Reports*, *20*(1), 236–250. http://doi.org/10.1016/j.celrep.2017.06.010

L'Episcopo, F., Tirolo, C., Testa, N., Caniglia, S., Morale, M. C., Serapide, M. F., … Marchetti, B. (2014). Wnt/β-Catenin Signaling Is Required to Rescue Midbrain Dopaminergic Progenitors and Promote Neurorepair in Ageing Mouse Model of Parkinson's Disease. *STEM CELLS*, *32*(8), 2147–2163. http://doi.org/10.1002/stem.1708

Lam, C. S., März, M., & Strähle, U. (2009). gfap and nestin reporter lines reveal characteristics of neural progenitors in the adult zebrafish brain. *Developmental Dynamics*, *238*(2), 475–486. http://doi.org/10.1002/dvdy.21853

Lan, Y., Wang, X., Xing, J., Yu, Z., & Lou, J. (2017). Anti-cancer effects of dopamine in human glioma : involvement of mitochondrial apoptotic and anti-inflammatory pathways, *8*(51), 88488–88500.

Langhammer, C. G., Previtera, M. L., Sweet, E. S., Sran, S. S., Chen, M., & Firestein, B. L. (2010). Automated Sholl analysis of digitized neuronal morphology at multiple scales: Whole cell Sholl analysis versus Sholl analysis of arbor subregions. *Cytometry Part A*, *77 A*(12), 1160–1168. http://doi.org/10.1002/cyto.a.20954

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., … Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, *367*(6464), 645–648. http://doi.org/10.1038/367645a0

Laudet, V. (2011). The Origins and Evolution of Vertebrate Metamorphosis. *Current Biology*, *21*(18), R726–R737. http://doi.org/10.1016/j.cub.2011.07.030

Launay, P.-S., Godefroy, D., Khabou, H., Rostene, W., Sahel, J.-A., Baudouin, C., … Reaux-Le Goazigo, A. (2015). Combined 3DISCO clearing method, retrograde tracer and ultramicroscopy to map corneal neurons in a whole adult mouse trigeminal ganglion. *Experimental Eye Research*, (June), 6–13. http://doi.org/10.1016/j.exer.2015.06.008

Lazzari, M., Franceschini, V., & Ciani, F. (1997). Glial fibrillary acidic protein and vimentin in radial glia of Ambystoma mexicanum and Triturus carnifex: an immunocytochemical study. *Journal Fur Hirnforschung*, *38*(2), 187–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9176731

Lee, D. A., Bedont, J. L., Pak, T., Wang, H., Song, J., Miranda-Angulo, A., … Blackshaw, S. (2012). Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. *Nature Neuroscience*, *15*(5), 700–702. http://doi.org/10.1038/nn.3079

Lee, E., Choi, J., Jo, Y., Kim, J. Y., Jang, Y. J., Lee, H. M., … Sun, W. (2016). ACT-PRESTO: Rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. *Scientific Reports*, *6*, 1–13. http://doi.org/10.1038/srep18631

Lee, Y., & Sunwoo, J. (2014). PD-L1 is preferentially expressed on CD44+ tumor-initiating cells in head and neck squamous cell carcinoma. *Journal for ImmunoTherapy of Cancer*, *2*(Suppl 3), P270. http://doi.org/10.1186/2051-1426-2-S3-P270

Lerner, T. N., Shilyansky, C., Davidson, T. J., Evans, K. E., Beier, K. T., Zalocusky, K. A., … Deisseroth, K. (2015). Intact-Brain Analyses Reveal Distinct Information Carried by SNc Dopamine Subcircuits. *Cell*, *162*(3), 635–647. http://doi.org/10.1016/j.cell.2015.07.014

Lessard, J., & Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature*, *423*(6937), 255–260. http://doi.org/10.1038/nature01572

Levita, L., Dalley, J. W., & Robbins, T. W. (2002). Nucleus accumbens dopamine and learned fear revisited: a review and some new findings. *Behavioural Brain Research*, *137*(1–2), 115–127. http://doi.org/10.1016/S0166-4328(02)00287-5

Li, A. (2010). Micro-Optical Sectioning Tomography. *Science*, *1404*(2010), 1404–1408. http://doi.org/10.1126/science.1191776

Li, J., Zhu, S., Kozono, D., Ng, K., Futalan, D., Shen, Y., … Chen, C. C. (2014). Genome-wide shRNA screen revealed integrated mitogenic signaling between dopamine receptor D2 (DRD2) and epidermal growth factor receptor (EGFR) in glioblastoma. *Oncotarget*, *2*(4), Advanced online publication. http://doi.org/10.18632/oncotarget.1801

Li, W., Germain, R. N., & Gerner, M. Y. (2017). Multiplex, quantitative cellular analysis in large tissue volumes with clearing-enhanced 3D microscopy (C $_e$ 3D). *Proceedings of the National Academy of Sciences*, 201708981. http://doi.org/10.1073/pnas.1708981114

Lie, D. C., Dziewczapolski, G., Willhoite, A. R., Kaspar, B. K., Shults, C. W., & Gage, F. H. (2002). The adult substantia nigra contains progenitor cells with neurogenic potential. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(15), 6639–6649. http://doi.org/20026700

Liebmann, T., Renier, N., Bettayeb, K., Greengard, P., Tessier-Lavigne, M., & Flajolet, M. (2016). Three-Dimensional Study of Alzheimer's Disease Hallmarks Using the iDISCO Clearing Method. *Cell Reports*, *16*(4), 1138–1152. http://doi.org/10.1016/j.celrep.2016.06.060

Lin, R., Cai, J., Nathan, C., Wei, X., Schleidt, S., Rosenwasser, R., & Iacovitti, L. (2015). Neurogenesis is enhanced by stroke in multiple new stem cell niches along the ventricular system at sites of high BBB permeability. *Neurobiology of Disease*, *74*, 229–239. http://doi.org/10.1016/J.NBD.2014.11.016

Lindvall, O., & Bjorklund, A. (2004). Cell therapy for Parkinson disease. *NeuroRx: The Journal of the Americal Society of Experimental NeuroTherapeutics*, *1*(October), 382–389. http://doi.org/10.1602/neurorx.1.4.382

Lindvall, O., Kokaia, Z., & Martinez-Serrano, A. (2004). Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nature Medicine*, *10 Suppl*(July), S42–S50. http://doi.org/10.1038/nm1064

Liu, A. K. L., Hurry, M. E., Ng, O. T.-W., DeFelice, J., Lai, H. M., Pearce, R. K., … Gentleman, S. M. (2015). Bringing CLARITY to the human brain: visualisation of Lewy pathology in three-dimensions.

Neuropathology and Applied Neurobiology, n/a-n/a. http://doi.org/10.1111/nan.12293

Liu, A. K. L., Lai, H. M., Chang, R. C. C., & Gentleman, S. M. (2017). Free of acrylamide sodium dodecyl sulphate (SDS)-based tissue clearing (FASTClear): a novel protocol of tissue clearing for three-dimensional visualization of human brain tissues. *Neuropathology and Applied Neurobiology*, *43*(4), 346–351. http://doi.org/10.1111/nan.12361

Liu, H., & Song, N. (2016). Molecular Mechanism of Adult Neurogenesis and its Association with Human Brain Diseases. *Journal of Central Nervous System Disease*, *8*, JCNSD.S32204. http://doi.org/10.4137/JCNSD.S32204

Liu, J., Solway, K., Messing, R. O., & Sharp, F. R. (1998). Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J Neurosci*, *18*(19), 7768–7778. Retrieved from http://www.jneurosci.org/content/18/19/7768.full.pdf

Liu, Y. C., & Chiang, A. S. (2003). High-resolution confocal imaging and three-dimensional rendering. *Methods*, *30*(1), 86–93. http://doi.org/10.1016/S1046-2023(03)00010-0

Louis, D. N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W. K., … Ellison, D. W. (2016). The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathologica*, *131*(6), 803–820. http://doi.org/10.1007/s00401-016-1545-1

Loulier, K., Barry, R., Mahou, P., Franc, Y. Le, Supatto, W., Matho, K. S., … Livet, J. (2014). Multiplex Cell and Lineage Tracking with Combinatorial Labels. *Neuron*, *81*(3), 505–520. http://doi.org/10.1016/j.neuron.2013.12.016

Lugo-Hernandez, E., Squire, A., Hagemann, N., Brenzel, A., Sardari, M., Schlechter, J., … Hermann, D. M. (2017). 3D visualization and quantification of microvessels in the whole ischemic mouse brain using solvent-based clearing and light sheet microscopy. *Journal of Cerebral Blood Flow and Metabolism*, *37*(10), 3355–3367. http://doi.org/10.1177/0271678X17698970

Lutgendorf, S. K., Cole, S., Costanzo, E., Bradley, S., Coffin, J., Jabbari, S., … Sood, A. K. (2003). Stress-related mediators stimulate vascular endothelial growth factor secretion by two ovarian cancer cell lines. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *9*(12), 4514–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14555525

Maden, M., Manwell, L. a, & Ormerod, B. K. (2013). Proliferation zones in the axolotl brain and regeneration of the telencephalon. *Neural Development*, *8*(1), 1. http://doi.org/10.1186/1749-8104-8-1

Magnon, C., Hall, S. J., Lin, J., Xue, X., Gerber, L., Freedland, S. J., & Frenette, P. S. (2013). Autonomic Nerve Development Cancer Progression. *Science (New York, N.Y.)*, *341*(6142), 1–10. http://doi.org/10.1038/aja.2013.113

Magnusson, J. P. (2017). *Astrocytes: A Reservoir for New Neurons*. *Karolinska Institute*. Karolinska Institute.

Magnusson, J. P., Göritz, C., Tatarishvili, J., Dias, D. O., Smith, E. M. K., Lindvall, O., … Frisén, J. (2014). A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science*, *346*(6206), 237–241. http://doi.org/10.1126/science.346.6206.237

Mancino, M., Ametller, E., Gascón, P., & Almendro, V. (2011). The neuronal influence on tumor progression. *Biochimica et Biophysica Acta - Reviews on Cancer*, *1816*(2), 105–118. http://doi.org/10.1016/j.bbcan.2011.04.005

Marín, O., González, A., & Smeets, W. J. A. J. (1997a). Basal Ganglia Organization in Amphibians : Afferent Connections to the Striatum and the Nucleus Accumbens, *49*(July 1996), 16–49.

Marín, O., González, A., & Smeets, W. J. A. J. (1997b). Basal ganglia organization in amphibians: Efferent connections of the striatum and the nucleus accumbens. *Journal of Comparative Neurology*, *380*(1), 23–50. http://doi.org/10.1002/(SICI)1096-9861(19970331)380:1<23::AID-CNE3>3.0.CO;2-V

Marín, O., Smeets, W. J. A. J., & González, A. (1997). Basal Ganglia Organization in Amphibians: Catecholaminergic Innervation of the Striatum and the Nucleus, *49*(July 1996), 16–49.

Marín, O., Smeets, W. J., & González, a. (1998). Evolution of the basal ganglia in tetrapods: a new perspective based on recent studies in amphibians. *Trends in Neurosciences*, *21*(11), 487–494. http://doi.org/10.1016/S0166-2236(98)01297-1

Marti-Fabregas, J., Romaguera-Ros, M., Gomez-Pinedo, U., Martinez-Ramirez, S., Jimenez-Xarrie, R. M. E., R, J. L. M.-V., & Garcia-Verdugo, J. M. (2010). Proliferation in the human ipsilateral subventricular zone after ischemic stroke: Neurology 2010;Vol.74:357-365. *Ann Neurosci*, *17*(3), 134–135. http://doi.org/10.5214/ans.0972-7531.1017308

Martinez, R. C. R., Oliveira, A. R., Macedo, C. E., Molina, V. A., & Brandão, M. L. (2008). Involvement of dopaminergic mechanisms in the nucleus accumbens core and shell subregions in the expression of fear conditioning. *Neuroscience Letters*, *446*(2–3), 112–116. http://doi.org/10.1016/J.NEULET.2008.09.057

Martynoga, B., Morrison, H., Price, D. J., & Mason, J. O. (2005). Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor

proliferation and apoptosis. *Developmental Biology*, *283*(1), 113–127. http://doi.org/10.1016/j.ydbio.2005.04.005

Masur, K., Niggemann, B., Zanker, K. S., & Entschladen, F. (2001). Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. *Cancer Research*, *61*(7), 2866–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11306460

McArthur, S., McHale, E., & Gillies, G. E. (2007). The Size and Distribution of Midbrain Dopaminergic Populations are Permanently Altered by Perinatal Glucocorticoid Exposure in a Sex- Region- and Time-Specific Manner. *Neuropsychopharmacology*, *32*(7), 1462–1476. http://doi.org/10.1038/sj.npp.1301277

McCulloch, E. A., Minden, M. D., Miyauchi, J., Kelleher, C. A., & Wang, C. (1988). Stem cell renewal and differentiation in acute myeloblastic leukaemia. *Journal of Cell Science. Supplement*, *10*, 267–81. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3077940

Medina, L., & Abellán, A. (2009). Development and evolution of the pallium. *Seminars in Cell & Developmental Biology*, *20*(6), 698–711. http://doi.org/10.1016/J.SEMCDB.2009.04.008

Melamed, R., Rosenne, E., Shakhar, K., Schwartz, Y., Abudarham, N., & Ben-Eliyahu, S. (2005). Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a beta-adrenergic antagonist and a prostaglandin synthesis inhibitor. *Brain, Behavior, and Immunity*, *19*(2), 114–26. http://doi.org/10.1016/j.bbi.2004.07.004

Menegas, W., Bergan, J. F., Ogawa, S. K., Isogai, Y., Umadevi Venkataraju, K., Osten, P., … Watabe-Uchida, M. (2015). Dopamine neurons projecting to the posterior striatum form an anatomically distinct subclass. *eLife*, *4*, 1–30. http://doi.org/10.7554/eLife.10032

Merkle, F. T., & Alvarez-Buylla, A. (2006). Neural stem cells in mammalian development. *Current Opinion in Cell Biology*, *18*(6), 704–709. http://doi.org/10.1016/j.ceb.2006.09.008

Micheva, K. D., & Smith, S. J. (2007). Array Tomography: A New Tool for Imaging the Molecular Architecture and Ultrastructure of Neural Circuits. *Neuron*, *55*(1), 25–36. http://doi.org/10.1016/j.neuron.2007.06.014

Missale, C., Nash, S. R. S., Robinson, S. W., Jaber, M., & Caron, M. G. (1998). Dopamine receptors: from structure to function. *Physiological …*, *78*(1), 189–225. http://doi.org/10.1186/1471-2296-12-32

Montiel, J. F., & Aboitiz, F. (2015). Pallial patterning and the origin of the isocortex. *Frontiers in Neuroscience*, *9*, 377. http://doi.org/10.3389/fnins.2015.00377

Moreno, N., & González, A. (2017). Pattern of Neurogenesis and Identification of Neuronal Progenitor Subtypes during Pallial Development in Xenopus laevis. *Frontiers in Neuroanatomy*, *11*, 24. http://doi.org/10.3389/fnana.2017.00024

Murray, E., Cho, J. H., Goodwin, D., Ku, T., Swaney, J., Kim, S.-Y., … Chung, K. (2015). Simple, Scalable Proteomic Imaging for High-Dimensional Profiling of Intact Systems. *Cell*, *163*(6), 1500–1514. http://doi.org/10.1016/j.cell.2015.11.025

Ninkovic, J., Mori, T., & Götz, M. (2007). Distinct modes of neuron addition in adult mouse neurogenesis. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(40), 10906–11. http://doi.org/10.1523/JNEUROSCI.2572-07.2007

Nomura, T., Yabe, T., Rosenthal, E. S., Krzan, M., & Schwartz, J. P. (2000). PSA-NCAM distinguishes reactive astrocytes in 6-OHDA-lesioned substantia nigra from those in the striatal terminal fields. *Journal of Neuroscience Research*, *61*(6), 588–596. http://doi.org/10.1002/1097-4547(20000915)61:6<588::AID-JNR2>3.0.CO;2-M

Nowakowski, R. S., Lewin, S. B., & Miller, M. W. (1989). Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *Journal of Neurocytology*, *18*(3), 311–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2746304

Nowoshilow, S., Schloissnig, S., Fei, J.-F., Dahl, A., Pang, A. W. C., Pippel, M., … Myers, E. W. (2018). The axolotl genome and the evolution of key tissue formation regulators. *Nature 2018*. http://doi.org/10.1038/nature25458

Nudo, R. J. (2013). Recovery after brain injury: mechanisms and principles. *Frontiers in Human Neuroscience*, *7*(December), 1–14. http://doi.org/10.3389/fnhum.2013.00887

O'Brien, C. A., Pollett, A., Gallinger, S., & Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*, *445*(7123), 106–110. http://doi.org/10.1038/nature05372

O'Keeffe, G. C., Tyers, P., Aarsland, D., Dalley, J. W., Barker, R. A., & Caldwell, M. A. (2009). Dopamine-induced proliferation of adult neural precursor cells in the mammalian subventricular zone is mediated through EGF. *Proceedings of the National Academy of Sciences*, *106*(21), 8754–8759. http://doi.org/10.1073/pnas.0803955106

O'Steen, W. K. (1958). Regeneration of the intestine in adult urodeles. *Journal of Morphology*, *103*(3),

435–477. http://doi.org/10.1002/jmor.1051030303

Oberpriller, J. O., & Oberpriller, J. C. (1974). Response of the adult newt ventricle to injury. *The Journal of Experimental Zoology*, *187*(2), 249–53. http://doi.org/10.1002/jez.1401870208

Ogden, A. T., Waziri, A. E., Lochhead, R. A., Fusco, D., Lopez, K., Ellis, J. A., … Bruce, J. N. (2008). Identification of A2B5+CD133− Tumor-Initiating Cells in Adult Human Gliomas. *Neurosurgery*, *62*(2), 505–515. http://doi.org/10.1227/01.neu.0000316019.28421.95

Okamoto, M. (1997). Simultaneous demonstration of lens regeneration from dorsal iris and tumour production from ventral iris in the same newt eye after carcinogen administration. *Differentiation*, *61*(5), 285–292. http://doi.org/10.1046/j.1432-0436.1997.6150285.x

Olsen, J. H., Friis, S., Frederiksen, K., McLaughlin, J. K., Mellemkjaer, L., & Møller, H. (2005). Atypical cancer pattern in patients with Parkinson's disease. *British Journal of Cancer*, *92*(1), 201–205. http://doi.org/10.1038/sj.bjc.6602279

Olson, J. J., Nayak, L., Ormond, D. R., Wen, P. Y., & Kalkanis, S. N. (2014). The role of cytotoxic chemotherapy in the management of progressive glioblastoma: A systematic review and evidence-based clinical practice guideline. *Journal of Neuro-Oncology*, *118*(3), 501–555. http://doi.org/10.1007/s11060-013-1338-5

Orlich, M., & Kiefer, F. (2017). A qualitative comparison of ten tissue clearing techniques, 1–35. http://doi.org/10.14670/HH-11-903

Oviedo, N. J., & Beane, W. S. (2009). Regeneration: The origin of cancer or a possible cure? *Seminars in Cell and Developmental Biology*, *20*(5), 557–564. http://doi.org/10.1016/j.semcdb.2009.04.005

Pan, C., Cai, R., Quacquarelli, F. P., Ghasemigharagoz, A., Lourbopoulos, A., Matryba, P., … Ertürk, A. (2016). Shrinkage-mediated imaging of entire organs and organisms using uDISCO. *Nature Methods*, *13*(10), 859–867. http://doi.org/10.1038/nmeth.3964

Pan, Q., Li, Q., Liu, S., Ning, N., Zhang, X., Xu, Y., … Wicha, M. S. (2015). Concise review: Targeting cancer stem cells using immunologic approaches. *Stem Cells*, *33*(7), 2085–2092. http://doi.org/10.1002/stem.2039

Parish, C. L., Beljajeva, A., Arenas, E., & Simon, A. (2007). Midbrain dopaminergic neurogenesis and behavioural recovery in a salamander lesion-induced regeneration model. *Development (Cambridge, England)*, *134*(15), 2881–2887. http://doi.org/10.1242/dev.002329

Park, I., Qian, D., Kiel, M., Becker, M. W., Pihalja, M., Weissman, I. L., … Clarke, M. F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature*, *423*(6937), 302–305. http://doi.org/10.1038/nature01587

Patel, A. P., Tirosh, I., Trombetta, J. J., Shalek, A. K., Gillespie, S. M., Wakimoto, H., … Bernstein, B. E. (2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science (New York, N.Y.)*, *344*(6190), 1396–401. http://doi.org/10.1126/science.1254257

Pfisterer, U., Ek, F., Lang, S., Soneji, S., Olsson, R., & Parmar, M. (2016). Small molecules increase direct neural conversion of human fibroblasts. *Scientific Reports*, *6*(August), 1–9. http://doi.org/10.1038/srep38290

Phillips, J., Laude, A., Lightowlers, R., Morris, C. M., Turnbull, D. M., & Lax, N. Z. (2016). Development of passive CLARITY and immunofluorescent labelling of multiple proteins in human cerebellum: understanding mechanisms of neurodegeneration in mitochondrial disease. *Scientific Reports*, *6*(April), 26013. http://doi.org/10.1038/srep26013

Pierce, A. A., & Xu, A. W. (2010). De Novo Neurogenesis in Adult Hypothalamus as a Compensatory Mechanism to Regulate Energy Balance. *Journal of Neuroscience*, *30*(2), 723–730. http://doi.org/10.1523/JNEUROSCI.2479-09.2010

Polesskaya, A., Seale, P., & Rudnicki, M. A. (2003). Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell*, *113*(7), 841–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12837243

Pulkoski-Gross, A., Li, J., Zheng, C., Li, Y., Ouyang, N., Rigas, B., … Cao, J. (2015). Repurposing the antipsychotic trifluoperazine as an antimetastasis agent. *Molecular Pharmacology*, *87*(3), 501–12. http://doi.org/10.1124/mol.114.096941

Ragan, T., Kadiri, L. R., Venkataraju, K. U., Bahlmann, K., Sutin, J., Taranda, J., … Osten, P. (2012). Serial two-photon tomography for automated ex vivo mouse brain imaging. *Nature Methods*, *9*(3), 255–258. http://doi.org/10.1038/nmeth.1854

Ratiu, P., Talos, I.-F., Haker, S., Lieberman, D., & Everett, P. (2004). The Tale of Phineas Gage, Digitally Remastered. *Journal of Neurotrauma*, *21*(5), 637–643. http://doi.org/10.1089/089771504774129964

Reiss, J. O. (2002). The phylogeny of amphibian metamorphosis. BT - Zoology. *Zoology*, *105(2)*, 85–96. Retrieved from e:%5CLUCAS FIORELLI%5CBDD152-0008.pdf

Ren, J., Choi, H., Chung, K., & Bouma, B. E. (2017). Label-free volumetric optical imaging of intact murine brains. *Scientific Reports*, *7*, 1–8. http://doi.org/10.1038/srep46306

Renier, N., Adams, E. L., Kirst, C., Wu, Z., Azevedo, R., Kohl, J., … Tessier-Lavigne, M. (2016). Mapping of Brain Activity by Automated Volume Analysis of Immediate Early Genes. *Cell*, *165*(7), 1789–1802. http://doi.org/10.1016/j.cell.2016.05.007

Renier, N., Wu, Z., Simon, D. J., Yang, J., Ariel, P., & Tessier-Lavigne, M. (2014). iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging - suppl original. *Cell*, *159*. Retrieved from http://www.sciencedirect.com/science/article/pii/S0092867414012975

Renner, M., Lancaster, M. A., Bian, S., Choi, H., Ku, T., Peer, A., … Knoblich, J. A. (2017). Self-organized developmental patterning and differentiation in cerebral organoids. *The EMBO Journal*, *36*(10), 1316–1329. http://doi.org/10.15252/embj.201694700

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., & De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature*, *445*(7123), 111–115. http://doi.org/10.1038/nature05384

Richardson, D. S., & Lichtman, J. W. (2015). Clarifying Tissue Clearing. *Cell*, *162*(2), 246–257. http://doi.org/10.1016/j.cell.2015.06.067

Richardson, D. S., & Lichtman, J. W. (2017). SnapShot: Tissue Clearing. *Cell*, *171*(2), 496–496.e1. http://doi.org/10.1016/j.cell.2017.09.025

Richter, W., & Kranz, D. (1981). [Autoradiographic investigations on postnatal proliferative activity of the telencephalic and diencephalic matrix-zones in the axolotl (Ambystoma mexicanum), with special references to the olfactory organ (author's transl)]. *Zeitschrift Fur Mikroskopisch-Anatomische Forschung*, *95*(6), 883–904. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7336815

Roy, S., & Gardiner, D. M. (2002). Cyclopamine induces digit loss in regenerating axolotl limbs. *Journal of Experimental Zoology*, *293*(2), 186–190. http://doi.org/10.1002/jez.10110

Rubí, B., & Maechler, P. (2010). Minireview: New roles for peripheral dopamine on metabolic control and tumor growth: Let's seek the balance. *Endocrinology*, *151*(12), 5570–5581. http://doi.org/10.1210/en.2010-0745

Sachlos, E., Risueño, R. M., Laronde, S., Shapovalova, Z., Lee, J. H., Russell, J., … Bhatia, M. (2012). Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell*, *149*(6), 1284–1297. http://doi.org/10.1016/j.cell.2012.03.049

Sahay, A., Wilson, D. a., & Hen, R. (2011). Pattern Separation: A Common Function for New Neurons in Hippocampus and Olfactory Bulb. *Neuron*, *70*(4), 582–588. http://doi.org/10.1016/j.neuron.2011.05.012

Sanai, N., Nguyen, T., Ihrie, R. A., Mirzadeh, Z., Tsai, H.-H., Wong, M., … Alvarez-Buylla, A. (2011). Corridors of migrating neurons in the human brain and their decline during infancy. *Nature*, *478*(7369), 382–6. http://doi.org/10.1038/nature10487

Sanai, N., Tramontin, A. D., Quiñones-Hinojosa, A., Barbaro, N. M., Gupta, N., Kunwar, S., … Alvarez-Buylla, A. (2004). Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature*, *427*(6976), 740–744. http://doi.org/10.1038/nature02301

Sandoval-Guzmán, T., Wang, H., Khattak, S., Schuez, M., Roensch, K., Nacu, E., … Simon, A. (2014). Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell*, *14*(2), 174–187. http://doi.org/10.1016/j.stem.2013.11.007

Sarrouilhe, D., Clarhaut, J., Defamie, N., & Mesnil, M. (2015). Serotonin and Cancer: What Is the Link? *Current Molecular Medicine*, *15*(1), 62–77. http://doi.org/10.2174/1566524015666150114113411

Scadding, S. R. (1977). Response of the adult newt,Notophthalmus viridescens to partial or complete gonadectomy. *The Anatomical Record*, *189*(4), 641–647. http://doi.org/10.1002/ar.1091890408

Scadding, S. R., & Liversage, R. A. (1974). Studies on the response of the adult newt kidney to partial nephrectomy. *American Journal of Anatomy*, *140*(3), 349–367. http://doi.org/10.1002/aja.1001400304

Scales, S. J., & de Sauvage, F. J. (2009). Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. *Trends in Pharmacological Sciences*, *30*(6), 303–312. http://doi.org/10.1016/j.tips.2009.03.007

Schatton, T., Schütte, U., Frank, N. Y., Zhan, Q., Hoerning, A., Robles, S. C., … Frank, M. H. (2010). Modulation of T-cell activation by malignant melanoma initiating cells. *Cancer Research*, *70*(2), 697–708. http://doi.org/10.1158/0008-5472.CAN-09-1592

Schuller, H. M. (1989). Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. *Biochemical Pharmacology*, *38*(20), 3439–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2573356

Schwarz, M. K., Scherbarth, A., Sprengel, R., & Engelhardt, J. (2015). Fluorescent-Protein Stabilization and High- Resolution Imaging of Cleared , Intact Mouse Brains, 1–26. http://doi.org/10.1371/journal.pone.0124650

Seaberg, R. M., & Van Der Kooy, D. (2003). Stem and progenitor cells: The premature desertion of

rigorous definitions. *Trends in Neurosciences*, *26*(3), 125–131. http://doi.org/10.1016/S0166-2236(03)00031-6

Seilern-Aspang, F., & Kratochwill, K. (1965). *Regeneration in animals and related problems*. (V. Kiortsis & H. Trampusch, Eds.).

Shackleton, M., Quintana, E., Fearon, E. R., & Morrison, S. J. (2009). Heterogeneity in Cancer: Cancer Stem Cells versus Clonal Evolution. *Cell*, *138*(5), 822–829. http://doi.org/10.1016/j.cell.2009.08.017

Shan, X., Chi, L., Bishop, M., Luo, C., Lien, L., Zhang, Z., & Liu, R. (2006). Enhanced de novo neurogenesis and dopaminergic neurogenesis in the substantia nigra of MPTP-induced Parkinson's disease-like mice. *Stem Cells*, *24*(5), 1280–1287. http://doi.org/10.1634/stemcells.2005-0487

Sharma, P., Wagner, K., Wolchok, J. D., & Allison, J. P. (2011). Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nature Reviews. Cancer*, *11*(11), 805–12. http://doi.org/10.1038/nrc3153

Shin, S. Y., Kim, C. G., Hong, D. D., Kim, J.-H., & Lee, Y. H. (2004). Implication of Egr-1 in trifluoperazine-induced growth inhibition in human U87M G glioma cells. *Experimental & Molecular Medicine*, *36*(4), 380–386.

Sholl, D. A. (1956). The measurable parameters of the cerebral cortex and their significance in its organization. *Progress in Neurobiology*, (2), 324–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13441807

Sibley, D. R., & Monsma, F. J. (1992). Molecular biology of dopamine receptors. *Trends in Pharmacological Sciences*, *13*(2), 61–69. http://doi.org/10.1016/0165-6147(92)90025-2

Silvestri, L., Costantini, I., Sacconi, L., & Pavone, F. S. (2016). Clearing of fixed tissue: a review from a microscopist's perspective. *Journal of Biomedical Optics*, *21*(8), 81205. http://doi.org/10.1117/1.JBO.21.8.081205

Silvestri, L., Paciscopi, M., Soda, P., Biamonte, F., Iannello, G., Frasconi, P., & Pavone, F. S. (2015). Quantitative neuroanatomy of all Purkinje cells with light sheet microscopy and high-throughput image analysis. *Frontiers in Neuroanatomy*, *9*(May), 1–11. http://doi.org/10.3389/fnana.2015.00068

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., & Dirks, P. B. (2003). Identification of a Cancer Stem Cell in Human Brain Tumors Identification of a Cancer Stem Cell in Human Brain Tumors, 5821–5828.

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., … Dirks, P. B. (2004). Identification of human brain tumour initiating cells. *Nature*, *432*(7015), 396–401. http://doi.org/10.1038/nature03128

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., … Frisén, J. (2013). Dynamics of Hippocampal Neurogenesis in Adult Humans. *Cell*, *153*(6), 1219–1227. http://doi.org/10.1016/j.cell.2013.05.002

Spalding, K. L., Bhardwaj, R. D., Buchholz, B. A., Druid, H., & Frisén, J. (2005). Retrospective Birth Dating of Cells in Humans. *Cell*, *122*(1), 133–143. http://doi.org/10.1016/j.cell.2005.04.028

Spalteholz, W. (1911). *Uber das durchsichtigmachen von menchlichen und Tierichen Praparaten und Seine Theoretischen Bedingungen*. Leipzig: S Hirzel.

Spalteholz, W. (1914). *Über das Durchsichtigmachen von menschlichen und tierischen Präparaten und seine theoretischen Bedingungen, nebst Anhang*.

Spillantini, M. G., Crowther, R. A., Jakes, R., Cairns, N. J., Lantos, P. L., & Goedert, M. (1998). Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. *Neuroscience Letters*, *251*(3), 205–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9726379

Strange, P. G. (2013). Dopamine Receptors. *Tocris Bioscience Scientific Review Series*.

Sung, K., Ding, Y., Ma, J., Chen, H., Huang, V., Cheng, M., … Kulkarni, R. P. (2016). Simplified three-dimensional tissue clearing and incorporation of colorimetric phenotyping. *Scientific Reports*, *6*(July), 1–7. http://doi.org/10.1038/srep30736

Susaki, E. a., Tainaka, K., Perrin, D., Kishino, F., Tawara, T., Watanabe, T. M., … Ueda, H. R. (2014). Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. *Cell*, *157*(3), 726–739. http://doi.org/10.1016/j.cell.2014.03.042

Susaki, E. A., & Ueda, H. R. (2016). Whole-body and Whole-Organ Clearing and Imaging Techniques with Single-Cell Resolution: Toward Organism-Level Systems Biology in Mammals. *Cell Chemical Biology*, *23*(1), 137–157. http://doi.org/10.1016/j.chembiol.2015.11.009

Susaki, E. a, Tainaka, K., Perrin, D., Yukinaga, H., Kuno, A., & Ueda, H. R. (2015). Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. *Nature Protocols*, *10*(11), 1709–1727. http://doi.org/10.1038/nprot.2015.085

Sylwestrak, E. L., Rajasethupathy, P., Wright, M. A., Jaffe, A., & Deisseroth, K. (2016). Multiplexed Intact-Tissue Transcriptional Analysis at Cellular Resolution. *Cell*, *164*(4), 792–804.

http://doi.org/10.1016/j.cell.2016.01.038

Tainaka, K., Kuno, A., Kubota, S. I., Murakami, T., & Ueda, H. R. (2016). Chemical Principles in Tissue Clearing and Staining Protocols for Whole-Body Cell Profiling. *Annual Review of Cell and Developmental Biology*, *32*(1), annurev-cellbio-111315-125001. http://doi.org/10.1146/annurev-cellbio-111315-125001

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*, *131*(5), 861–872. http://doi.org/10.1016/j.cell.2007.11.019

Thaker, P. H., Han, L. Y., Kamat, A. A., Arevalo, J. M., Takahashi, R., Lu, C., … Sood, A. K. (2006). Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nature Medicine*, *12*(8), 939–44. http://doi.org/10.1038/nm1447

Thuret, R., Auger, H., & Papalopulu, N. (2015). Analysis of neural progenitors from embryogenesis to juvenile adult in Xenopus laevis reveals biphasic neurogenesis and continuous lengthening of the cell cycle. *Biology Open*, *4*(12), 1772–81. http://doi.org/10.1242/bio.013391

Toh, T. B., Lim, J. J., & Chow, E. K.-H. (2017). Epigenetics in cancer stem cells. *Molecular Cancer*, *16*(1), 29. http://doi.org/10.1186/s12943-017-0596-9

Tomer, R., Ye, L., Hsueh, B., & Deisseroth, K. (2014). Advanced CLARITY for rapid and high-resolution imaging of intact tissues. *Nature Protocols*, *9*(7), 1682–97. http://doi.org/10.1038/nprot.2014.123

Toni, N., Laplagne, D. A., Zhao, C., Lombardi, G., Ribak, C. E., Gage, F. H., & Schinder, A. F. (2008). Neurons born in the adult dentate gyrus form functional synapses with target cells. *Nature Neuroscience*, *11*(8), 901–907. http://doi.org/10.1038/nn.2156

Topalian, S. L., Hodi, F. S., Brahmer, J. R., Gettinger, S. N., Smith, D. C., McDermott, D. F., … Sznol, M. (2012). Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *New England Journal of Medicine*, *366*(26), 2443–2454. http://doi.org/10.1056/NEJMoa1200690

Traiffort, E., Angot, E., & Ruat, M. (2010). Sonic Hedgehog signaling in the mammalian brain. *Journal of Neurochemistry*, *113*(3), 576–590. http://doi.org/10.1111/j.1471-4159.2010.06642.x

Tsonis, P. A., & Eguchi, G. (1982). Abnormal Limb Regeneration without Tumor Production in Adult Newts Directed by Carcinogens, 20-Methylcholanthrene and Benzo (α) pyrene. *Development, Growth & Differentiation*, *24*(2), 183–190. http://doi.org/10.1111/j.1440-169X.1982.00183.x

Tsonis, P. A., & Fox, T. P. (2009). Regeneration According to Spallanzani, (August), 2357–2363. http://doi.org/10.1002/dvdy.22057

Tsonis, P. A., Vergara, M. N., Spence, J. R., Madhavan, M., Kramer, E. L., Call, M. K., … Del Rio-Tsonis, K. (2004). A novel role of the hedgehog pathway in lens regeneration. *Developmental Biology*, *267*(2), 450–461. http://doi.org/10.1016/j.ydbio.2003.12.005

Ukegawa, J.-I., Takeuchi, Y., Kusayanagi, S., & Mitamura, K. (2003). Growth-promoting effect of muscarinic acetylcholine receptors in colon cancer cells. *Journal of Cancer Research and Clinical Oncology*, *129*(5), 272–278. http://doi.org/10.1007/s00432-003-0433-y

Usher, W., Klacansky, P., Federer, F., Bremer, P. T., Knoll, A., Angelucci, A., & Pascucci, V. (2017). A Virtual Reality Visualization Tool for Neuron Tracing. *IEEE Transactions on Visualization and Computer Graphics*. http://doi.org/10.1109/TVCG.2017.2744079

Van Den Berge, S. A., Van Strien, M. E., Korecka, J. A., Dijkstra, A. A., Sluijs, J. A., Kooijman, L., … Hol, E. M. (2011). The proliferative capacity of the subventricular zone is maintained in the parkinsonian brain. *Brain*, *134*(11), 3249–3263. http://doi.org/10.1093/brain/awr256

Van Praag, H. (2008). Neurogenesis and exercise: Past and future directions. *NeuroMolecular Medicine*, *10*(2), 128–140. http://doi.org/10.1007/s12017-008-8028-z

Verhaak, R. G. W., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., … Hayes, D. N. (2010). Integrated Genomic Analysis Identifies Clinically Relevant Subtypes of Glioblastoma Characterized by Abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*, *17*(1), 98–110. http://doi.org/10.1016/j.ccr.2009.12.020

Vigouroux, R. J., Belle, M., & Chédotal, A. (2017). Neuroscience in the third dimension: Shedding new light on the brain with tissue clearing. *Molecular Brain*, *10*(1), 1–10. http://doi.org/10.1186/s13041-017-0314-y

Visvader, J. E. (2011). Cells of origin in cancer. *Nature*, *469*(7330), 314–322. http://doi.org/10.1038/nature09781

Vittori, M., Breznik, B., Gredar, T., Hrovat, K., Bizjak Mali, L., & Lah, T. T. (2016). Imaging of human glioblastoma cells and their interactions with mesenchymal stem cells in the zebrafish (Danio rerio) embryonic brain. *Radiology and Oncology*, *50*(2), 159–167. http://doi.org/10.1515/raon-2016-0017

Voie, A., Burns, D., & Spelman, F. (1993). Orthogonal-plane fluorescence optical sectioning: three-dimensional imaging of macroscopic biological specimens, *170*(3), 229–36.

Wang, C., Liu, F., Liu, Y.-Y., Zhao, C.-H., You, Y., Wang, L., … Yang, Z. (2011). Identification and characterization of neuroblasts in the subventricular zone and rostral migratory stream of the adult human brain. *Cell Research*, *21*(11), 1534–50. http://doi.org/10.1038/cr.2011.83

Wang, J., Sakariassen, P. Ø., Tsinkalovsky, O., Immervoll, H., Bøe, S. O., Svendsen, A., … Enger, P. Ø. (2008). CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *International Journal of Cancer*, *122*(4), 761–768. http://doi.org/10.1002/ijc.23130

Wang, L., Yi, T., Kortylewski, M., Pardoll, D. M., Zeng, D., & Yu, H. (2009). IL-17 can promote tumor growth through an IL-6–Stat3 signaling pathway. *The Journal of Experimental Medicine*, *206*(7), 1457–1464. http://doi.org/10.1084/jem.20090207

Watanabe, N., Kageyama, R., & Ohtsuka, T. (2015). Hbp1 regulates the timing of neuronal differentiation during cortical development by controlling cell cycle progression. *Development (Cambridge, England)*, *142*(13), 2278–90. http://doi.org/10.1242/dev.120477

Williams, R. T., Den Besten, W., & Sherr, C. J. (2007). Cytokine-dependent imatinib resistance in mouse BCR-ABL+, Arf-null lymphoblastic leukemia. *Genes and Development*, *21*(18), 2283–2287. http://doi.org/10.1101/gad.1588607

Winner, B., Cooper-Kuhn, C. M., Aigner, R., Winkler, J., & Kuhn, H. G. (2002). Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. *The European Journal of Neuroscience*, *16*(9), 1681–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12431220

Winner, B., Desplats, P., Hagl, C., Klucken, J., Aigner, R., Ploetz, S., … Winkler, J. (2009). Dopamine receptor activation promotes adult neurogenesis in an acute Parkinson model. *Experimental Neurology*, *219*(2), 543–52. http://doi.org/10.1016/j.expneurol.2009.07.013

Winner, B., & Winkler, J. (2015). Adult neurogenesis in neurodegenerative diseases., *7*(4), a021287. http://doi.org/10.1101/cshperspect.a021287

Witman, N., Murtuza, B., Davis, B., Arner, A., & Ian, J. (2011). Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury. *Developmental Biology*, *354*(1), 67–76. http://doi.org/10.1016/j.ydbio.2011.03.021

Yang, B., Treweek, J. B., Kulkarni, R. P., Deverman, B. E., Chen, C. K., Lubeck, E., … Gradinaru, V. (2014). Single-Cell Phenotyping within Transparent Intact Tissue through Whole-Body Clearing. *Cell*, *158*(4), 945–958. http://doi.org/10.1016/j.cell.2014.07.017

Yang, P., Arnold, S. A., Habas, A., Hetman, M., & Hagg, T. (2008). Ciliary Neurotrophic Factor Mediates Dopamine D2 Receptor-Induced CNS Neurogenesis in Adult Mice. *Journal of Neuroscience*, *28*(9), 2231–2241. http://doi.org/10.1523/JNEUROSCI.3574-07.2008

Ye, L., Allen, W. E., Thompson, K. R., Tian, Q., Hsueh, B., Ramakrishnan, C., … Deisseroth, K. (2016). Wiring and Molecular Features of Prefrontal Ensembles Representing Distinct Experiences. *Cell*, *165*(7), 1776–1788. http://doi.org/10.1016/j.cell.2016.05.010

Yeh, C. T., Wu, A. T. H., Chang, P. M. H., Chen, K. Y., Yang, C. N., Yang, S. C., … Huang, C. Y. F. (2012). Trifluoperazine, an antipsychotic agent, inhibits cancer stem cell growth and overcomes drug resistance of lung cancer. *American Journal of Respiratory and Critical Care Medicine*, *186*(11), 1180–1188. http://doi.org/10.1164/rccm.201207-1180OC

Yoshimi, K., Ren, Y. R., Seki, T., Yamada, M., Ooizumi, H., Onodera, M., … Mochizuki, H. (2005). Possibility for neurogenesis in substantia nigra of parkinsonian brain. *Annals of Neurology*, *58*(1), 31–40. http://doi.org/10.1002/ana.20506

Zhan, T., Rindtorff, N., & Boutros, M. (2017). Wnt signaling in cancer. *Oncogene*, *36*(11), 1461–1473. http://doi.org/10.1038/onc.2016.304

Zhang, A. S., Ostrom, Q. T., Kruchko, C., Rogers, L., Peereboom, D. M., & Barnholtz-Sloan, J. S. (2016). Complete prevalence of malignant primary brain tumors registry data in the United States compared with other common cancers, 2010. *Neuro-Oncology*, *19*(December 2016), now252. http://doi.org/10.1093/neuonc/now252

Zhao, C. (2006). Distinct Morphological Stages of Dentate Granule Neuron Maturation in the Adult Mouse Hippocampus. *Journal of Neuroscience*, *26*(1), 3–11. http://doi.org/10.1523/JNEUROSCI.3648-05.2006

Zhao, C., Deng, W., & Gage, F. H. (2008). Mechanisms and Functional Implications of Adult Neurogenesis. *Cell*, *132*(4), 645–660. http://doi.org/10.1016/j.cell.2008.01.033

Zhao, M., Momma, S., Delfani, K., Carlen, M., Cassidy, R. M., Johansson, C. B., … Janson, A. M. (2003). Evidence for neurogenesis in the adult mammalian substantia nigra. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(13), 7925–30. http://doi.org/10.1073/pnas.1131955100

Zukor, K. A., Kent, D. T., & Odelberg, S. J. (2010). Fluorescent whole-mount method for visualizing three-dimensional relationships in intact and regenerating adult newt spinal cords. *Developmental Dynamics*, *239*(11), 3048–3057. http://doi.org/10.1002/dvdy.22441