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ADULT NEURAL STEM CELLS AND THEIR DIFFERENTIATION CHOICES

-examples from experimental inflammation and transplantation

Cynthia Pérez Estrada

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Cover image:
Far left panel (blue and red), SVZ NPCs neurosphere stained with DAPI and Laminin B1.
The different colored variations in the following panels, represent the different NPC specialization states, which they aquaire through interactions with their envrionment.

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TO MY ABUELITO, MOTHER, FATHER, AND MY FAMILY

ABSTRACT

The aim of this thesis is to study how inflammation influences the fate of NPCs, which can impact the regenerative capacities of the CNS.

In paper I, we used a hypoglossal nerve avulsion model, to study if survival, differentiation, and integration of transplanted NPCs could be achieved. Transplanted NPCs were present after 3 months post grafting, and expressed differentiation markers specific for neurons, astrocytes, and oligodendrocytes. Motor neuronal cell survival was increased in injured animals which received the NPC graft. This results show that transplanted NPCs can modulate the microenvironment increasing motor neuronal survival. Further, in Paper II, we explored the same transplantation setting in a transection injury of the hypoglossal nerve, i.e. an injury that yields significantly less neuronal cell death in contrast to the more severe avulsion injury. Comparison of transplanted NPCs into both injury models was then investigated. In the transection injury model we found that NPC transplanted to the hypoglossal nucleus, maintained their Sox2 expression after three months transplantation. Also, no differentiated progeny derived from the graft was observed. Motor neuronal survival was not significantly different from non-transplanted animals, indicating that the cues for NPCs differentiation provided from the environment were different comparing to the avulsion model.

Reactive oxygen species (ROS), are an integral part of inflammation, so we next questioned whether H_2O_2 a member of the ROS family would have an effect on the differentiation outcome of NPC from the brain lateral ventricles. In paper III I found that NPCs exposed to H_2O_2 resulted in more neurons and oligodendrocytes. H_2O_2 exposure influenced proliferation and the expression of genes involved in chromatin remodeling and antioxidant defense. These results indicate that H_2O_2 has a specific modulatory effect on the differentiation potential of NPCs.

In paper IV the global gene expression and differentiation outcome from NPCs from the brain and the spinal cord were analyzed under normal conditions and after Experimental Allergic Encephalomyelitis (EAE), a model for multiple sclerosis (MS). We found that NPC from naïve rat brains were more neurogenic compared to spinal cord (SC) naïve NPCs. In contrast, NPCs from the SC of EAE rats became more neurogenic comparing to healthy SC NPCs. Thus, the inflammatory environment produced during EAE proves to be able to change NPC fate.

Overall the findings presented in this thesis suggest the inflammatory milieu as well as its individual components, like H_2O_2, act as mediators of the regenerative properties of NPCs.

POPULAR SCIENCE ABSTRACT

In recent years the number of companies offering “the promise” that stem cells will regenerate new organs, or even help us to live forever has become increasing, But, how much do we really know about stem cells, and what can they really do for our health? In my thesis I concentrate on a special type of stem cell, the adult neural stem cell. These cells are found in our brain and spinal cord, and maintain limited potential to regenerate new brain cells throughout adulthood. I investigate on how the environment instructs these stem cells to choose different fates, through a process called differentiation. Differentiation allows these cells to become the brains three most important cell types, neurons, astrocytes and oligodendrocytes. After injury an inflammatory response arises, where several types of free radicals are present, causing various changes to the microenvironment and to the neural stem cells. These changes can range from altered cellular behavior to cell death. Understanding the role that inflammation and free radicals play in the functionality of adult neural stem cells could help us to design better therapies to regenerate the damaged adult central nervous system. My results show that free radicals can direct the NPCs to become neurons; crucial for the communication of information in the brain, and oligodendrocytes; necessary to ensure neuronal communication. Further I also show that after injury there is an initial attempt for regeneration by the nervous system, instructing the stem cells to become neurons, however inherent changes caused by inflammation, also provide cues towards malfunctioning of these newly differentiated cells. Thus, to answer the initial questions, we have to remain cautious on the use of stem cells for clinical therapies, as we still need to learn further on applying and controlling stem cell potential in inflammatory conditions, to help us optimize regenerative therapies. My thesis work, contributes to the path towards understanding these possibilities envisioning a future where we can unleash the regenerative potential of the CNS.

POPULAR SCIENCE ABSTRACT (SPANISH)

En los últimos años nuevas compañías que prometen regenerar nuevos órganos y hasta vida eterna usando células madre ha incrementado, Pero, ¿cuánto sabemos realmente sobre las células madre, y que pueden hacer estas células por nuestra salud? En mi tesis me concentro en una clase especial de célula madre, las células madre neuronales adultas. Estas células se encuentran en el cerebro y la médula espinal, y se mantienen con el potencial para regenerar nuevas células cerebrales en la edad adulta. Así pues, he investigado los efectos que el microambiente tiene en el comportamiento de estas células, influyendo la decisión que toman al elegir diferentes destinos celulares, a través de un proceso llamado diferenciación. La diferenciación permite a estas células transformarse en tres importantes tipos de células cerebrales; neuronas, atrocitas y oligodendrocitos. Después de que una lesión ocurre en el sistema nervioso central (SNC), una reacción inflamatoria ocurre, donde varios tipos de radicales libres están presentes, causando diversos cambios en el microambiente y a las células progenitoras existentes. Estos cambios pueden modificar el comportamiento celular o hasta causar la muerte de dichas células.

El entendimiento del rol que la inflamación y los radicales libres desempeñan en el funcionamiento de las NPCs podría ayudarnos a diseñar mejores terapias para regenerar el SNC adulto después de haber sido lesionado. En esta tesis investigue los efectos que los radicales libres y la inflamación en el SNC tienen sobre la capacidad de diferenciación de las NPCs.

Mis resultados muestran que los radicales libres pueden instruir a las NPCs para convertirse en neuronas; cruciales para la transferencia de información en el cerebro, y oligodendrocitos; necesarios para garantizar la comunicación neuronal. Además, también muestro que después de que ocurren daños en el SNC, el SNC hace in intento inicial para repararse, instruyendo a las células madre para convertirse en neuronas, sin embargo cambios inherentes dentro de las células en estas circunstancias, conllevan al inicio de mecanismos que pueden significar el mal funcionamiento de estas células al madurar. Por lo tanto, respondiendo a las preguntas iniciales, se tiene que mantener cautela en cuanto al uso de las células madre para terapias clínicas, ya que todavía tenemos que aprender más sobre el funcionamiento y el control del potencial de éstas en las enfermedades inflamatorias, para ayudar a optimizar terapias regenerativas. Mi trabajo de tesis, contribuye al entendimiento de estas posibilidades imaginando un futuro en el que podamos liberar el potencial de regeneración del Sistema Nervioso Central.

LIST OF PUBLICATIONS

I. Neural stem/progenitor cells transplanted to the hypoglossal nucleus integrates with the host CNS in adult rats and promotes motor neuron survival.

Michael Fagerlund*, Cynthia Pérez Estrada*, Nasren Jaff, Mikael Svensson and Lou Brundin

Cell Transplantation 2012, 21 (4):739-747

II. Integration differences of transplanted adult neural progenitors in two models of axotomy.

Cynthia Pérez Estrada*, Michael Fagerlund*, Nasren Jaff, Lou Brundin and Mikael Svensson

Manuscript

III. Oxidative stress increases neurogenesis and oligodendrogenesis in adult neural progenitor cells.

Cynthia Pérez Estrada, Ruxandra Covacu, Mikael Svensson, Lou Brundin

Stem Cell and Development 2014, (Epub ahead of print).

IV. Change of fate commitment in adult neural progenitor cells subjected to chronic inflammation.

Ruxandra Covacu*, Cynthia Pérez Estrada*, Lisa Arvidsson*, Mikael Svensson & Lou Brundin

The Journal of Neuroscience, 2014, 34 (35): 11571-11582

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The author has also contributed to the following publications/manuscripts.

I. Nitric oxide-induced neuronal to glial lineage fate-change depends on NRSF/REST function in neural progenitor cells

Bergsland M, Covacu R, Perez Estrada C, Svensson M, Brundin L.

Stem Cells, 2014, 32(9):2539-49

II. Pericytes generate scar tissue across multiple central nervous system disorders

*David O. Dias *, Yildiz Kelahmetoglu *, Cynthia Pérez-Estrada **, Jemal Tatarishvili **, Aurélie Ernst 1, Zaal Kokaia , Olle Lindvall , Lou Brundin , Christian Göritz , Jonas Frisén*

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Manuscript

III. Altered gene expression and differentiation in spinal cord neural progenitor cells, after exposure to low level inflammation.

Lisa Arvidsson, Ruxandra Covacu, Cynthia Peréz Estrada, Sreenivasa Raghavan Sankavaram, Mikael Svensson, Lou Brundin

Manuscript

IV. Characterization of the response of endogenous spinal cord neural stem cells to EAE.

Ongoing collaboration with Jonas Frisen´s group.

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LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
Ascl1	Achaete-scute homolog 1
CAM	Cell Adhesion Molecule
CAT	Catalase
CC	Central canal
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DA	Dark Agouti
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCX	Doublecortin
DG	Dentate Gyrus
EAE	Experimental Allergic Encephalomyelitis
eGFP	Enhanced –GFP
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
HDACs	Histone Deacetylases
IHC	Immunohistochemistry
IPA	Ingenuity Systems Pathway Analysis
MeV	Multiexperiment Viewer
MOG	Myelin Oligodendrocyte Glycoprotein
NAD	Nicotinamide Adenine Dinucleotide
NO	Nitric Oxide
NPC	Neural progenitor cell
Prxs	Peroxiredoxins
ROS	Reactive Oxygen Species
SC	Spinal Cord
SOD	Super Oxide Dismutase
SOX	Sry-containing HMG box
SVZ	Sub-Ventricular Zone
VEGF	Vascular Endothelial Growth Factor
WebGestalt	WEB-based Gene Set Analysis Toolkit

INTRODUCTION

In the adult brain there is a cavity that persists through development, the ventricular system. A lateral ventricle occupies each cerebral hemisphere. The lateral ventricles cell architecture, as well as the spinal cord organization contain a special type of CNS cell, the neural progenitor cell (NPC). NPCs were not discovered but until 1996 by Weiss et al. (Weiss et al., 1996). NPC have the capacity to self-renew and to differentiate into specialized Central Nervous System (CNS) cells (Doetsch et al., 1999, Weissman et al., 2001, Gage, 2000). CNS pathologies ranging from neurodegenerative disorders to neuro trauma challenge the regeneration and plasticity capacities of the adult mammalian CNS. In other organisms like planarians and salamanders, the capacities for regenerating entire damaged nerve networks are immense, however in mammals like humans and rodents, this capacity is limited or non existant. Through evolution the mechanisms for loss of regeneration in humans could have compensated a more intricate and complex nerve wiring for a lower regenerative capacity, as rewiring existing circuits could represent a major challenge to guide, differentiate and reconnect to the right terminals. There is increasing evidence that the adult CNS in mammals retains poor plasticity, and neurogenesis takes place at a limited occurrence. The environment surrounding the adult NPC is a strong regulator of NPC behavior. The focus of my thesis centers on the interactions that the environment and the adult NPCs play together, interactions which challenge the differentiation and integration of the NPC.

1 ADULT NEUROGENESIS

Exactly 101 years ago, in 1913 Ramón y Cajal introduced the prevalent notion that neuronal birth exists only at embryonic stages, “In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.” (extract from Ramón y Cajal “ Estudios sobre la degeneración y regeneración del sistema nervioso”). Ramón y Cajal died in 1934, only 28 years later in 1962 Joseph Altman, using H^{3-}thymidine labeling, was able to follow cells that had incorporated it into new chromosomal DNA, during mitotic cell division. This lead to the first report on new neurons in the adult rat brain. More evidence reporting adult neurogenesis in birds, rodents and in non-human primates in both the sub ventricular zone (SVZ) and the dentate gyrus of the hippocampus would follow (Morshead et al., 1994, Palmer et al., 1997, Nottebohm, 1989). Further Bernier et al, showed that the SVZ NPCs contribute to neurogenesis in the amygdala of adult monkeys (Bernier et al., 2002), but a central question remained, is there neurogenesis in the adult human CNS?

The first report on human adult neurogenesis came in 1998 by Eriksson et al, who by using BrdU labeling found ongoing neurogenesis in the adult human dentate gyrus (Eriksson et al., 1998). These results were confirmed by Jonas Frisen´s lab last year (Spalding et al., 2013) and this year (Ernst et al., 2014). Further, in 2012 using C^{14} cell dating, Bergman et al showed none or limited neurogenesis in the human olfactory bulb (OB) (Bergmann et al., 2012). Given the presence of NPCs in the adult human SVZ (Quinones-Hinojosa et al., 2006) this result re-opened the question, if there really is neurogenesis in the adult human brain. In rodents the OB is vital for survival, as it plays a crucial role in their interaction with the environment. Humans on the contrary, rely more on other brain functions which may receive constant environmental stimuli, like learning and memory in the cortex. However, Frisen et al using the C^{14} cell dating, found that there was no neurogenesis in the adult human cortex (Spalding et al., 2005, Bhardwaj et al., 2006).

All together, these studies suggest adult neurogenesis in the adult human brain occurs in specific locations of the adult brain, highlighting the SVZ and the Dentate gyrus (DG) as neurogenic areas. In conclusion the adult human brain has some extent of plasticity, further investigation on new born and integrated neurons in the adult human brain will have to be addressed in the future.

I will next shortly describe the three areas in the CNS which harbor NPC niches in the adult human and rat CNS; the SVZ of the brain, the sub granular area of the hippocampus and the spinal cord and the filum terminale at the caudal end of the SC.

Brain lateral sub-ventricular wall structure

In the adult brain, the major germinal niche is in the sub-ventricular zone of the lateral ventricles. Through development ependymal cells displace progenitor cells from the lateral ventricle, into the sub-ventricular zone. The architecture of this area is populated by ependymal cells which constitute the E1 and E2 cell types. They have cilia which contacts the cerebrospinal fluid (CSF) and are found surrounding the radial glia (Mirzadeh et al., 2008). Radial glia (RG) are the NPC during development (Malatesta and Gotz, 2013) and in adulthood the neural progenitors cells of the SVZ, RG are known as the B1 cell type which expresses $GFAP^{+}$ (Doetsch et al., 1999) and similarly to E cells, the B1 cells also protrude cilia contacting the CSF on one side and protruding with long specialized processes to directly contact blood vessels (Fuentealba et al., 2012). B1 cells give rise to oligodendrocyte progenitors (Olig2+) and transit amplifying type C cells (Ascl1+) (Parras et al., 2004), C cells then further commit to become A type cells, expressing doublecourtin (DCX) (Nacher et al., 2001), which are also known as the transit amplifying neural progenitor lying underneath the B1 cells. Surrounding this germinal zone are microglial cells, blood vessels and CSF. It becomes apparent the role that the environment may play in the behavior of the NPCs, signaling through blood vessels and CSF.

Spinal cord central canal cyto-architechture

NPC in the spinal cord are located at the central canal (CC) region of the SC. This area is divided on to two compartments an ependymal compartment and a sub-ependymal compartment (Hamilton et al., 2009, Johansson et al., 1999). At the ependymal compartment, ependymal cells in the CC are in closed contact to the vasculature, and have from one to three cilia contacting the CSF, also some ependymal cells in this area are bi-nucleated and have four cilia contacting the CSF (Alfaro-Cervello et al., 2012a). Ependymal cells of the SC CC have been described to express Nestin (Meletis et al., 2008) but also Afaro-Cervello et al have identified an ependymal cell population in the SC CC which is Nestin negative (Alfaro-Cervello et al., 2012b), both of this described ependymal cell populations possess neural progenitor properties. Ependymal cells have been described to activate their stem cell potential after injury, where they were found to proliferate, migrate and differentiate, in response to SC injury (Johansson et al., 1999, Brundin et al., 2003, Meletis et al., 2008) . Dromard et al, (Dromard et al., 2008) describe a subset of ependymal

cells, the SC subependymal cells which express markers common to NPC from SVZ of the adult brain and are able to differentiate into neurons, astrocytes and oligodendrocytes.

The cellular microenvironment organization of the SC CC consists of neurons contacting the CSF (Alfaro-Cervello et al., 2012b), GFAP+ positive cells, and rare proliferating ependymal cells which generate ependymal cell doublets. The sub-ependymal layer's ependymal cells, express GFAP, and are surrounded by neurons, both in contact with the CSF and the microenvironment vasculature. Also, oligodendrocyte precursors are an integral part of this niche (Hamilton et al., 2009).

It's important to mention that NPC from the different regions of the SC present distinct differentiation properties (Kulbatski and Tator, 2009, Shihabuddin et al., 1997) (Paper IV), these differences could also indicate variations in the molecular and physical constitution of the NPC niche at the different levels of the SC; cervical, thoracic, and caudal which could define their characteristics.

Adult spinal cord radial glia has NPC properties

Within the adult rodent SC there is another cell type with NPC properties, these cells are a heterogeneous population, sharing diverse genes with SVZ NPCs, and CC NPCs, the spinal cord radial glia (RG) (Petit et al., 2011) (Table 1). The RG cells are found in the white matter of the adult SC, these cells may proliferate in response to injury and differentiate in to astrocytes (Bannerman et al., 2007), but they can also become oligodendrocytes and neurons (Ohori et al., 2006). The identity of radial glia has been elusive, but they are known to express several markers, PDGFβ, vimentin, NG2 and GFAP (Sabourin et al., 2009, Petit et al., 2011). Some of these markers like PDGFb and vimentin are shared by other cell types like pericytes, the scar building cells after SC injury (Goritz et al., 2011). It would definitely be of help to find specific markers to define this cell population, to elucidate contributions to neurogenesis and possible uses in regenerative therapies.

Filum terminale

At the most caudal part of the adult mammalian spinal cord, another NPC population resides, as the CC narrows down to disappearing caudally, ependymal cells are found surrounded by fibroblast, adipocytes, non-ciliated ependymal cell conglomerates, neuroblasts, neurons and glial cells (Fontes et al., 2006, Jha et al., 2013). Culturing of the human and rodent filum terminale area yields free floating neurospheres which differentiate into neurons, astrocytes and oligonderocyes (Varghese et al., 2009, Arvidsson et al., 2011, Jha et al., 2013), however

the true identity of the NPC in this region has not yet been as well characterized as in the SVZ and the CC of the SC.

All together the NPCs from the spinal cord show heterogenic qualities, further characterization of these NPC populations would shed light into the roles of each of these NPCs during inflammation and their impact in neuro repair.

Table 1, shows the most commonly described NPC markers expressed in the heterogeneous populations of the adult NPC in the CNS. Two markers are present among all the different NPC populations, Sox2 and Nestin, being Sox2 the most widely used pluripotency marker.

Sox2 belongs to the group of genes SoxB1, crucially expressed during neurogenesis in the embryo (Avilion et al., 2003, Masui et al., 2007) (For review see (Uchikawa et al., 2011)). In the adult CNS Sox2 expression has been shown in NPCs (Brazel et al., 2005, Ellis et al., 2004, Suh et al., 2007), and proven to be necessary for maintaining the undifferentiated state (Taranova et al., 2006) as well as for neurogenesis in the adult CNS (Ferri et al., 2004). The validation of Sox2 in these studies shows its importance in stablishing and maintaining NPCs, and makes Sox2 a valuable marker for pluripotency.

The adult NPC niche

The adult NPC niche plays a crucial role in regulating stemness, cell proliferation, and differentiation. The components of the NPC niche orchestrate a dynamic architecture capable of providing the NPCs with the necessary environmental conditions to maintain their properties. The study of the stem cell niche is an emerging field, next I provide a short summary of some of the most important components of the adult NPC niche. The Basal laminae extra cellular matrix, contains fractones (Mercier et al., 2002) which can compartmentalize growth factors and cytokines (Bernfield et al., 1984) and play a special role in the niche, where fractones sequester and hold mitogens like FGF, which bind to specific cell types. Fractones contribute to an environment rich in stemness promoting factors (Kerever et al., 2007), playing a crucial role in the maintenance of the NPCs pluripotent state.

Mechanical force. External forces on the stem cells are also part of the stem cell niche, some groups have demonstrated that tension forces can regulate pluripotency in the presence of the appropriate growth factors, suggesting the fine relationship between the chemical and the physical environment that regulate stem cell behavior. (Nava et al., 2012) An example of this quality are Cell Adhesion Molecules (CAMs) which are anchoring factors providing physical tension, influencing cell geometry and cell differentiation, or loosing this physical tension and allowing differentiation. Among all the CAMs, E-cadherin is considered a key molecule for stem cell pluripotency (Li et al., 2012). Other important components of the niche, include other

cell types which participate of the architectural components of the niche; at the supraependymal area of the SVZ there is a network of 5HT positive neurons, which innervates the SVZ and may trigger proliferation of B1 and E1 cells upon release of 5HT (Tong et al., 2014). B1 and E1 cells are also in close proximity to blood vessels, pericytes, endothelial cells, and also microphages and fibroblasts (Mercier et al., 2002) which in turn could further provide different molecular signals regulating stem cell behavior.

Table 1. NPC markers and their expression in the different NPC populations in the CNS.

Markers present in all adult NPCs types, are highlighted in red.

	1	2	3	4	5	6
Marker	**Brain SVZ NPC**	**Brain SGZ NPC**	**Spinal cord NPC**	**Ependymal cells of the Spinal cord**	**Spinal cord Radial glia**	**Filum terminale**
BLBP					+	
CD15	+		+			
CD133	+	+		+		
CD24				+		
Crocc				+		
Fox J1				+		
GFAP	+	+	+		+	+
Lex	+					
Musashi-1	+	+		+		+
Nestin	+	+	+	+	+	+
Pax6	+					
PDGFR-α				+		
Sox2	+	+	+	+	+	+
Sox3				+		
Sox9				+		
Vimentin			+	+	+	+

1. *(Doetsch et al., 1999, Merkle et al., 2004)*
2. *(Seri et al., 2001, Suh et al., 2007, Walker et al., 2013)*
3. *(Johansson et al., 1999, Dromard et al., 2008)*
4. *(Pfenninger et al., 2011, Alfaro-Cervello et al., 2012b, Meletis et al., 2008)*
5. *(Petit et al., 2011, Kulbatski et al., 2007, Kulbatski and Tator, 2009)*
6. *(Jha et al., 2013, Arvidsson et al., 2011, Varghese et al., 2010)*

2. ADULT NEUROREGENERATION

It's of importance to model CNS regeneration and find new candidate genes in organisms with better regenerative capacities than humans. Evolutionary conserved genes have been identified across species, like *Drosophila M*. genes present in human development and higher mammals, allowing for the modeling and discovery of possible candidate genes in humans. Salamander and planarians, possess copious regenerative capacities, and have therefore been widely studied. Their regenerative capacity is very successful and could help us to model new approaches to regenerate the mammalian CNS; Here I attempt to highlight some of the most significant regenerative features from these organisms, to give a broader perspective to the human regenerative features and possibilities.

Regenerative potential in planarians (plathelmynthus)

Planarians, members of the phylum Platyhelminthes, are widely studied due to their outstanding regenerative capacities. Planarians can regenerate an entire new planarian from a very small piece of tissue, and any cell type and organ of their adult body can be regenerated from a single pluripotent adult stem cell type, known as the neoblast (Elliott and Sanchez Alvarado, 2013, Reddien, 2013). There is crescent interest for finding the master genes for pluripotency in the neoblast, since these genes could have analogues in mammals which could allow for the discovery and implementation of regenerative therapies (Lobo et al., 2012). Important gene candidates have already been identified, among which are genes homologues to human genes like peroxiredoxin genes (Galloni, 2012), and possibly *Oct4* and *Sox2* (Onal et al., 2012). Understanding the role of these factors in the topology of gene networks would give us the opportunity to elucidate their possible role in mammalian regeneration.

Salamander's regenerative capacities

Salamanders belong to the phylum cordata and have a remarkable potential for regeneration. Salamanders are capable of regenerating the tail and limbs, (Simon and Tanaka, 2013) brain (Parish et al., 2007) and spinal cord (Davis et al., 1990), but also the retina (Stone, 1950), lens (Stone, 1967)and jaws (Morrison et al., 2006). The best studied regenerative area in salamander is the limb blastema, which refers to the area from which a new limb grows back after amputation. One of the mechanisms for limb regeneration, occurs after adult stem cells in this area reverse to a more immature state (Sandoval-Guzman et al., 2014). Inflammation could possibly initiate epigenetic mechanisms which unlock the cells dedifferentiation towards

rebuilding a new limb. There is a continuous effort to understand limb regeneration in salamander and to develop new molecular markers to trace cell commitment in either dedifferentiated cells or into the inherent stem cell pool which contribute to the limb formation. Injured neurons and muscle cells may also exert specific secretomes orchestrating the patterning and activation of the regenerative machinery (King and Newmark, 2012). Although gene homologues to human genes have been identified like retinoic acid, Hox A genes (Nacu and Tanaka, 2011) and *Hdac* genes (Taylor and Beck, 2012), many more remain to be unveiled in this area.

Human regenerative potential

In adult humans some organs retain their ability for regeneration, like liver, bone and blood, however CNS regeneration after injury is very limited. As mentioned earlier Eriksson et al (Eriksson et al., 1998), and Frisen et al (Ernst et al., 2014) have reported neurogenesis in the striatum and hippocampus, this findings imply a possible difference in between rodent neurogenesis and human neurogenesis. Also Frisen´s lab, reported increased neurogenesis in the striatum after stroke, however whether this young neuroblasts mature and integrate in the environment is not known (Ernst et al., 2014). Another brain pathology which stimulates neurogenesis is epilepsy, where neurogenesis occurs in the subgranular zone of the DG (Ekdahl, 2012). Additionally, In the environment surrounding CNS injury, factors which prevent regeneration have been found, like inhibitory molecules secreted by the CNS (Pernet and Schwab, 2012), the immune system (Fukazawa et al., 2009) and in the scarring which occurs after injury (Fawcett et al., 2012) among others. It is still debatable to at what extent the immune system activation is beneficial or detrimental to regeneration (Monje et al., 2003) (Paper III, Paper IV) but for example in amphibians (Fukazawa et al., 2009) and rats (Monje et al., 2003) inhibiting immune cells activation after injury rescues regeneration. Also the development of a thick glial scar after injury is now known to inhibit regeneration (Fawcett et al., 2012), studies in axolotl salamanders, show wound healing occurs scar free (Seifert et al., 2012).

Studying CNS regeneration in other organisms could help us find mechanisms and pharmacological targets for improved regeneration in the adult mammalian CNS, and for the possible discovery of silent genes present in humans with the potential to regenerate the CNS after injury (Fig. 1).

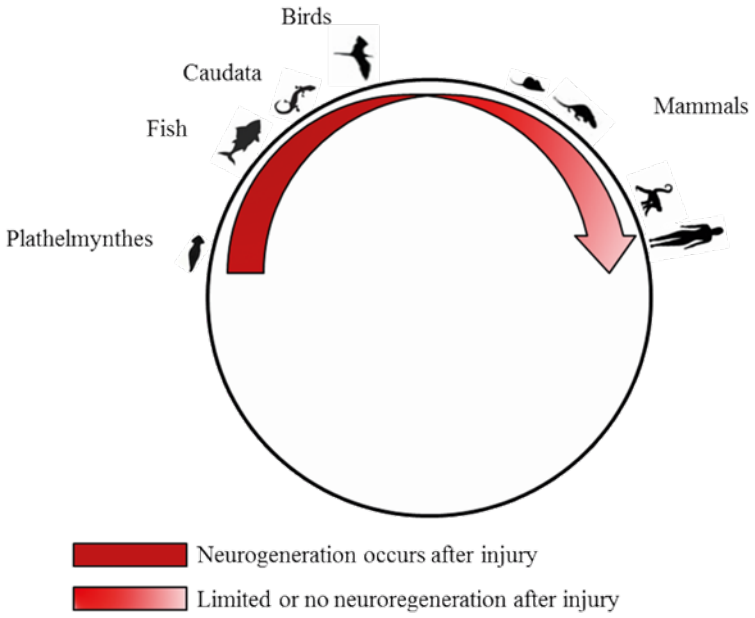

Figure 1. The translational value of the study of neurogenesis in other species. *The species shown are the most widely studied in the regenerative field, the different species are positioned in evolutionary order, the faded red color indicates the progressive loss of neuroregeneration over evolution (Ferretti, 2011, Tanaka and Ferretti, 2009). Studying neurogenesis in other species could lead to the discovery of evolutionary conserved genes relevant to neuro repair, allowing for the development of cross species integrative gene analysis to develop new regenerative concepts for the regeneration of the human CNS. The illustration scale is arbitrary.*

3. Nerve Avulsion and Transection Injuries

Nerve avulsion injuries

Nerve avulsion injuries occur when a nerve is pulled and detached from its roots, due to trauma or surgery. Avulsion injury often leads to proximal breaking of axons near the CNS- peripheral nervous system (PNS) border or/and also on the cell bodies in the CNS. Avulsion injury is commonly categorized as a CNS injury, inducing retrograde degeneration and considerable neuronal death (Martin et al., 1999). The conformational changes that then occur to the nerve itself and to the surrounding microenvironment are various.

The environmental changes surrounding the area of injury are characterized by activation of immune cells, glial cells and Wallerian degeneration. Wallerian degeneration is characterized by anterograde degeneration (Society, 1851, Beirowski et al., 2005), where the distal portions of axons fragment. Mitochondrial dysfunction and ROS among others have been suggested to play an important role in the occurring axonal fragmentation (For review see (Conforti et al., 2014). Wallerian degeneration can also cause the cell death of neurons which are extensively

connected with the avulsed neuron, causing proximal cell death, or permanent atrophy (Koliatsos et al., 1994, Jiang et al., 2000). Regenerative mechanisms are required to occur across the verge of the CNS and the PNS.

Transection injuries

A transection injury leads to disruption of all of the components of the nerve, however in contrast to the avulsion injury the number of motor neuronal death is lower. Importantly the more distal the injury is to the site of the motor neuron, the lesser the motor neuronal death, which in turn influences the load of inflammatory components into the environment (Yu, 1997, Svensson and Aldskogius, 1993a, Mattsson et al., 1999). Transection injuries also lead to Wallarian degeneration in the CNS, which as mentioned earlier is retrograde degeneration occurs as described for the avulsion model (Conforti et al., 2014). Because of being in the CNS-PNS border, both injury types generate reactions in the CNS and PNS. In the CNS activation of glial cells occurs (Svensson and Aldskogius, 1993b), leading to ROS production and conformational changes to the synapses, initiating synaptic stripping. The PNS reaction involves, Schwann cell activation and proliferation, providing trophic support and clearing the area from debris. The axon starts a regrowth mode, and in the best of cases may even reestablish connectivity with other axons returning to a normal state (Brosius Lutz and Barres, 2014).

Hypoglossal nerve injury model

In order to be able to study if nerve regeneration and survival after nerve injury could be improved using NPC transplantation, we used a model of nerve avulsion/transection of the hypoglossal nerve. The hypoglossal nerve is the XII cranial nerve of the hypoglossal nucleus in the brain stem. The hypoglossal nerve innervates the tongue muscle intrinsically and extrinsically. This nerve is essential for swallowing, eating and in humans also for talking. The anatomy of the area takes us to the upper part of the nucleus, protruding slightly in to the fourth ventricle, adjacent to where the NPC niche is found. The lower part of the nucleus lies in close proximity to the central canal, again near to a NPC niche. After avulsion injury limited proliferation of ependymal cells is detected, and migration of NPCs is scarce (Fagerlund et al., 2011).

There is a hypoglossal analogous nerve nucleus at each side of the CC. The anatomical characteristics of this nerve allowed us to injure the hypoglossal nerve on one side of the CC, keeping intact the analogous area, which served as a control for the injury. Thus the analysis could be carried out in parallel, in the same section from the brain stem. The nuclei are also ideal for microinjections, since the floor of the fourth ventricle adjacent to the nuclei is

surgically accessible, leading to minor additional trauma. Based on this, the hypoglossal injury model became ideal for NPC transplantation delivered through microinjections. Furthermore, the model can be extrapolated to a clinical setting, since it can be considered equivalent to ventral nerve or root injury.

4. Stem Cell Transplantations

As discussed in section 2, mammalian regeneration is not successful (Fig. 1), therefore not only the search for the application of evolutionary conserved genes to help CNS regeneration is necessary, but also, to investigate how exogenous cell transplantation can contribute to repair. There has been a plethora of attempts to achieve this primary goal, transplantation of embryonic tissue, nerve grafts, mesenchymal stem cells and bone marrow stem cells. In my thesis I will only discuss NPCs transplantation.

NPC transplantation

In order to alleviate incurable injuries and diseases NPC transplantation has been extensively investigated, and have been shown to exert beneficial effects onto different neurodegenerative conditions, like Parkinson's disease (Gaillard and Jaber, 2011, Ziavra et al., 2012), Amyotrophic Lateral Sclerosis (Xu et al., 2006, Lepore, 2011), MS or EAE (Pluchino et al., 2003, Pluchino et al., 2009, Ben-Hur, 2008) and SC injury (Zhao et al., 2013). The beneficial effects observed, have been considered to be achieved by direct cell replacement, neuroprotection, or by the production of factors which can modulate the microenvironment, like growth factors or immunomodulatory molecules like bone morphogenic protein-4, VEGF and guidance molecules among others (De Feo et al., 2012, Lepore and Maragakis, 2007, Fagerlund et al., 2012). Cell differentiation state for transplantation therapies is vital. Adult NPCs grafting have shown no evidence of theratoma formation, comparing to cells from an embryonic state (Brederlau et al., 2006). Hence, adult NPCs may be a better choice for regenerative therapies. However the gathering of adult NPC tissue is scarce and challenging. To solve this issue, the use of iPSC technology (Takahashi and Yamanaka, 2006) becomes a viable option, which allows for the development of personalized regenerative therapies.

It is important to mention that even when cell transplantation is a promising therapy and should continue to be investigated, reports on side effects exist (Bjorklund, 2004, Bjorklund and Kordower, 2013), indicating further studies on the behavior of NPC transplantation in vivo are required. Animal models where the study of transplantation cell state, type and numbers as well as site and timing for transplantation are crucial to provide solid evidence to continue with clinical trials.

5. Epigenetic Regulation of Adult Progenitor Cells Differentiation

The word epigenetic means on top of the DNA. Eukaryotic DNA is packaged in chromatin; chromatin packaging is highly dynamic and its controlled by epigenetic changes which lead to gene expression modulation (Berger et al., 2009). The fundamental unit of chromatin, is the nucleosome, which is an octamere histone core, around which DNA is wrapped (Kouzarides, 2007). Epigenetic changes are regarded as changes to chromatin structure and not genomic sequence alterations. Epigenetic changes can be inherited through generations (Berger et al., 2009). In order to elucidate a mechanism by which H_2O_2 exposure in Paper III, resulted in higher number of neurons and oligodendrocytes from NPCs, we were interested in studying the gene expression of chromatin architecture enzymes. Chromatin architecture enzymes contribute to variations in chromatin structure by influencing histone modifications, leading to gene expression changes. Epigenetic regulation of NPC differentiation among others involves; chromatin modification enzymes, like DNA methylation and demethylation, acetylation and deacetylation, but also activation of micro RNAs, and complexes that modify chromatin structure (Juliandi et al., 2010).

Intrinsic and environmental cues trigger epigenetic mechanisms. This environmental influence has long lasting effects on chromatin structure. Thus, mircroenvironmental changes like disease or medical treatment, may control transitions from one cellular state to another (Mirbahai and Chipman, 2014). Here I will solely introduce methylation, demethylation and acetylation, as these modifications are relevant to the chromatin architecture enzymes expression we found affected by H_2O_2.

Histone methylation and demethylation

Methylation is processed by enzymes which add methyl groups to the histone tails of specific nucleosomes, causing chromatin structure modifications (Chen and Riggs, 2011). Methyl groups bound to DNA's cytosine residues (CpG islands) can also mediate the recruitment of other complexes, like histone deacetylases. Methyl groups are the bases of epigenetic gene silencing. Methylation is reversible and methyl groups can be removed by demethylating enzymes, although methyl transferases themselves may also initiate the demethylation process. Specific mechanisms of active demethylation are yet not well understood (Ooi and Bestor, 2008). Methylation changes the structure of chromatin, modifying the interaction that DNA has with different proteins, blocking the binding of such proteins to the DNA, and regulating transcription activation, if this site incurs a promoter binding site (Jones and Takai, 2001).

Specific demethylating enzymes which take away methyl groups from specific histone sites, play an important role between the self-renewal state transition of the NPC into the neurogenic sate, it is also known that methylation of specific histone tails is necessary for neuronal differentiation, allowing a dynamic interaction between specific transcription binding factors which can transcribe neurogenic genes, for review see (Ma et al., 2010).

Histone Acetylation and Deacetylation

Acetylation and deacetylation of lysine residues in histone tails is mediated through the enzymes histone acetyl transfrases (HATs) and histone deacetylases (HDACs) respectively. Histone acetylation of specific sites of the core histones, occurs at lysine residues located at the N-termini. *In vivo,* all core histones are acetylated and increased levels of acetylation, are often correlated to increase transcriptional activity. In contrast to methylation, recruitment of HDAC enzymes, and the consecutive deacetylation of N-Termini, results in repression of gene expression (Cress and Seto, 2000) since DNA becomes more tightly compacted, preventing transcription binding to promoter regions, for review see (Bose et al., 2014). During neurogenesis acetylation has been recognized to play an important role (Sun et al., 2011), leading to a more open chromatin state, allowing the binding of transcription factors to specific sites which can initiate the gene transcription of neuronal genes. Differentiation of both oligodendrocyte and neuronal lineages are dependent on HDACs activity (Juliandi et al., 2010). Inhibition of HDACs at the right time window has been shown to decrease neurogenesis by the silencing of specific neuronal genes (Shaked et al., 2008), whereas up-regulation of HDACs could lead to oligodendrocyte differentiation (Ji et al., 2011).

HDACs family of enzymes, are composed of Class I, II and Class III HDACs. Here I will only refer to Class III HDACs as *Sirt2* the HDAC found relevant in Paper III, is part of this family.

Sirtuins are class III HDACs and are NAD+ dependent enzymes. Among the sirtuins, is Sirt2 which is found in the nucleus and in the cytoplasm of cells, Sirt2 has a strong expression in the brain, and is highly expressed by oligodedrocytes. Sirt2 can be activated by cellular stress and its upregulation its known to cause cell cycle exit (North and Verdin, 2007), and also to influence differentiation towards the oligodercyte lineage (Ji et al., 2011). Specific chromatin states can be correlated with specific differentiation status (Larson and Yuan, 2012). The importance for the understanding of the orchestration of chromatin architecture could lead us to develop better therapies by guiding the differentiation of NPC to the desired cell type.

6. Experimental Allergic Encephalomyelitis (EAE)

The lamentable accidental induction of symptoms which resembled multiple sclerosis (MS) was observed after an anti-rabies vaccine in humans containing myelin-like components was administered (Remlinger, 1905). This unfortunate event initiated the beginning of the development of MS models like EAE. This condition may be induced by immunization of myelin antigens in Freund adjuvant which enhances the immune response initiating demyelination

(Wekerle and Lassmann, 1994). Widely, the EAE model is regarded as a helpful MS model, (t Hart et al., 2011, Mix et al., 2010) and in the context of my thesis, to study NPCs in an inflammatory environment, involving both CNS inflammation and demyelination.

Rat clinical symptoms

In our MOG induced EAE model in the Dark Agouti rat, clinical signs often begin at the 9th day post immunization. An initial weight loss precedes the beginning of the symptoms which are rated as follows; 0-no clinical symptoms; 1-tail weakness or tail paralysis; 2- hind-limb paraparesis; 3- hind-limb paralysis; 4-tetraplegia; and 5-death. EAE data suggests that the inflammatory cascade produced during disease may contain both, beneficial and detrimental effector molecules, influencing disease progression (Olsson, 1995). Further, proteomic EAE CSF analysis showed the up-regulation of members of the complement system and vitamin D binding protein among others (Rosenling et al., 2012), inflammatory mediators have also been detected, among which are TNF-α and IL-2 (Diab et al., 1997), all of which correlate with disease severity. In the majority of rat EAE models, paralysis is accompanied by inflammation and CNS infiltration of immune cells (Friese et al., 2014), the formation of reactive oxygen species (ROS) (Gilgun-Sherki et al., 2004), demyelination, glial scar formation and remyelination (Voskuhl et al., 2009). Clinical outcome may vary, as different rat strains carry different genetic immune susceptibilities (Storch et al., 1998) (Weissert et al., 1998, Stefferl et al., 1999, Sakuma et al., 2004) producing a broad spectrum of EAE clinical symptoms relating to the clinical outcomes observed during human MS, similarities which I will review next.

Clinical similarities between EAE and MS

MS is a chronic inflammatory disease, characterized by demyelinating plaques in the CNS. MS is most prevalent in females (Compston and Coles, 2002, Duquette et al., 1992) , a difference which has also been stablished in mice EAE (Cruz-Orengo et al., 2014), Cruz-Orengo et al, validated their data in humans showing increased gender susceptibility to MS and EAE is in part attributed to higher expression of S1PR2 which increases BBB permeability in females.

MS clinical symptoms often start as a relapsing-remitting form of the disease. Already early in the disease course neurons are lost (De Stefano et al., 2002) and CNS atrophy can be measured. Inflammation is accompanied by neurodegeneration accumulating irreversible neurological disabilities and worsening CNS deficits (Natowicz and Bejjani, 1994). DA rats with MOG induced EAE, also developed relapsing-remitting disease with focal demyelination in the spinal cord (Lorentzen et al., 1995).

High levels of inflammatory agents have been detected in MS CSF, comparable to EAE, among which are NO (Svenningsson et al., 1999, Danilov et al., 2003b), TNF-α, IFNγ, IL-2, and IL-17A (Duan et al., 2013) , but also other molecules important for immune system regulation have been identified, like , vitamin D binding protein and the calcium regulating protein Fetuin A (Olsson, 1995, Ottervald et al., 2010).

In this thesis work, I used the female DA rat strain which has shown EAE susceptibility (Stepaniak et al., 1995). This MOG EAE model leads to a variety of clinical symptoms representing a complex MS mimicking model (Storch et al., 1998).The inflammatory milieu initiated by MOG- EAE, could have a potential effect on the inner NPC pools, possibly affecting NPC behavior, inflicting regeneration (Nait-Oumesmar et al., 2007). Importantly MS lesions are often found lining the lateral ventricles, suggesting possible changes on the NPC nature may occur.

NPC transplantation on the EAE model

Several studies have reported the benefits of NPC transplantation on EAE animals, exerted via modifying the inflammatory environment (Deboux et al., 2013, Pluchino et al., 2009). Also some reports showed lower proliferation of NPC after EAE (Pluchino et al., 2008) and differentiation changes from NPC after EAE (Picard-Riera et al., 2002, Kuhlmann et al., 2008). Based on all the above, in this thesis the model of MOG-EAE on the female DA rat was ideal to study NPC characteristics in an inflammatory environment, allowing us to produce data which could be of possible clinical relevance.

7. Reactive oxygen species and their role in NPC signaling

Except for some anaerobic microorganisms, oxygen is necessary for the life of almost every organism on earth. However, even though oxygen gives life to most organisms it also represents a risk every time we use it, due to its highly reactive and mutagenic nature. We and aerobic organisms only survive due to the antioxidant defense mechanisms we have evolved. As oxygen levels fluctuated the atmosphere to the current value of 21% (Berner, 1999) organisms became more complex, and adapted better mechanisms to produce oxygen energy

based products (Powell, 2010, Taylor and McElwain, 2010). In humans, tissue oxygen levels are critical for normal embryonic development, cellular differentiation, and stem-cell maintenance (Stamati et al., 2011). Also, the immune system has to be able to react and adapt to environmental oxygen changes in order to generate the bioenergetics resources that allows it to initiate an immune response/inflammation. Immune cells like neutrophils and macrophages, have adapted mechanisms to combat infections which use oxygen consumption to produce toxic products which can kill bacteria (Bedard and Krause, 2007). One of the most classical immune mechanisms in which oxygen is used to produce toxic oxygen derivatives is the oxidative burst. The membrane bound NADPH oxidase generates the super oxide anion (O_2^-) (Bedard and Krause, 2007), which is converted to H_2O_2 by the enzyme super oxide dismutase (SOD) , H_2O_2 is then enzymatically converted to more reactive chemical forms, like the hydroxyl radical (HO^-), hypochlorite (OCL^-) and hypobromite (OBr^-) (Yang et al., 2013). These toxic oxygen derivatives are known as free radicals, and form part of a family of molecules known as Reactive Oxygen Species (ROS).

Free Radicals

Free radicals are formed when any chemical species loses a single electron, which results in one or more unpaired electrons. This condition of one or more unpaired electrons can produce high reactivity, as the unpaired electron would make the chemical species more attracted to a magnetic field. The oxygen molecule (O_2) has two unpaired electrons, making it qualify as a free radical, however this unpaired electrons have parallel spins, which makes the molecule highly stable, being the form in which O_2 exist in the air we breathe. Free radicals can react differentially upon contact with different molecules, like hydrogen peroxide for example. H_2O_2 is a free radical which selectively reacts with other molecules, allowing it to also be used as a signaling molecule by the cell. H_2O_2 can easily cross cell membranes, after which it reacts with Fe and Cu, possibly by donating an electron to this elements (Prabhakar et al., 2004) to form more reactive species, like HO^-, which accounts for much of the DNA damage caused by H_2O_2 to cells.

$Fe^{2+} + H_2O_2 \rightarrow HO\bullet + OH^- + Fe^{3+}$ (Gutteridge and Halliwell, 2007, Fourth Edition)

$Cu^{2+} + H_2O_2 \rightarrow HO\bullet + OH^- + Cu^{+}$ (Simpson et al., 1988)

Simplified overview of H_2O_2 reacting with iron and Cu. The iron reaction its known as the Fenton reaction after its discovery by H.J.H Fenton in 1894, where the oxidation of Fe^{2+} to Fe^{3+} produces a hydroxyl radical and hydroxyl ion.

Inflammation and H_2O_2

Inflammation is characterized by the production of ROS among which we find nitric oxide ($NO^{\cdot}$), super oxide $O^{-\cdot}$, hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2). Interestingly, $NO^{\cdot}$ and H_2O_2 can also play a signaling role in the cell as they can mediate cell to cell communication and are classified as first type cell messengers (Holmquist et al., 2007).

In this thesis I will focus on H_2O_2 in relation to NPC differentiation. H_2O_2 may be produced by neutrophils and macrophages during inflammation, where it can be found compartmentalized in the cells mitochondria and the endoplasmic reticulum (Gough and Cotter, 2011). During oxygen metabolism H_2O_2 contributes to the generation of energy in the cell mitochondria. Low physiological levels of H_2O_2 are known to allow H_2O_2 signaling properties. As mentioned earlier, comparing to other ROS, H_2O_2 is poorly reactive for example, depending on cell type, oxidation of DNA, lipids and proteins does not occur at mM concentrations of H_2O_2 (Gutteridge and Halliwell, 2007, Fourth Edition).

H_2O_2 and Neuroinflammation

In the brain Fe contents are low, however after brain injury and MS iron concentrations rise rapidly (Mehta et al., 2013), Fe increase could then react with H_2O_2 and contribute to higher levels of $HO^{\cdot}$ and $O^{-\cdot}$ in the cell increasing oxidative stress in the environment and contributing further to cell toxicity. Further damage can continue by the formation of more H_2O_2 from neurotransmitters like noradrenalin among others (Swaroop et al., 1983). The low oxygen concentrations found in the brain, which are around 10 times lower than atmospheric levels (Silver and Erecinska, 1998), are so perhaps to prevent formation of oxygen free radicals due to the already high oxygen consumption by the brain. However a recent publication evaluates ROS production from proliferating astrocytes after stroke, establishing an important contribution to ROS levels in the brain (Walton et al., 2012), which in an inflammatory context could further contribute to cell damage given the already hostile environment (Nikic et al., 2011, Marti-Fabregas et al., 2010) and for review see (Rafalski and Brunet, 2011).

ROS are necessary for the adult SVZ stem cell niche and NPCs function

The NPCs population from the brain SVZ, are influenced by basal levels of ROS. ROS in NPCs act as signaling mediators of self-renewal and normal neurogenesis, and are proven to be necessary for NPCs normal functionality (Le Belle et al., 2011)(Paper III). Importantly ROS are an important regulator of NPC proliferation and cell cycle entry (Chaudhari et al., 2012), which leads to fate commitment and loss of stemness. Concomitantly the regulation of

chromatin architecture genes like the HDAC *Sirt2*, which can influence NPC fate decisions, are also regulated by ROS (Nakagawa and Guarente, 2011, Perez Estrada et al., 2014) .

ROS are produced during energy production in the NPC by mitochondria (Zorov et al., 2014). Mitochondrial activity is known to be higher in adult NPCs than in their differentiated progeny yet, their ROS content in the undifferentiated state is low, the reason for this may be the high concentration of antioxidants like glutathione peroxidase among others in this cells (Madhavan et al., 2006), thus the adult NPCs show to have a higher metabolism with which they cope efficiently by up-regulating antioxidant enzymes.

The NPC niche and ROS

Also the SVZ NPC niche has shown to be tightly regulated by ROS, since ROS is able to control the anchoring molecules which give the NPC niche its cell architectural structure. The B cells *in-vivo* structure depends on the dynamics of Cell Adhesion Molecules (CAMs) (Kokovay et al., 2012). The close association of NPC to the neurovascular system (Shen et al., 2008), may not only provide physical support but also, contribute to oxygen supply and or signaling from the bloodstream. Disruption of the ROS levels could cause the malfuctionality of molecules like V-CAM1 (Kokovay et al., 2012), and allow the proliferation of the NPC which could in turn enfeeble the NPC population. Also, during ischemic conditions, oxygen levels drop, low oxygen levels stimulate mitochondria to produce more ROS (Klimova and Chandel, 2008). This oxygen increase can lead to the activation of the NPC pool, initiating proliferation and cell cycle entry leading to NPC differentiation and as mentioned above, the consequences of such activation could possibly result in the exhaustion of the NPC pool. Moreover the hostile environment could make it difficult for the newly differentiated cells to exert any beneficial properties onto the injury area.

NPC Antioxidant Mechanisms

NPCs cultures show lower profile of ROS than their or differentiated counterparts, possibly through a more active antioxidant repertoire, where Glutathione peroxidase (GPx), super oxide dismutase (SOD), and catalase (CAT) regulate the redox content of these cells (Madhavan et al., 2006). Disruption of these defense mechanisms in the NPC pool could lead to mitochondrial dysfunction, energy production deficiencies, and ultimately cell death affecting the regenerative potential of the organism. Next I will shortly describe these three important antioxidant enzymes.

Super oxide dismutase (SOD)

SODs are located in the cytosol and in the mitochondria of eukaryotic cells. The SODs family of enzymes comprises SOD1 and SOD2. SOD1 which at its active site uses Cu and Zn, and is localized in the cytosol. SOD2 uses Mn in its catalytic center (Fridovich, 1995) and is localized in the cell mitochondrion, near by the mitochondrial respiratory chain, which leads to the production of O_2^- as the end product . Further, SOD can inhibit the levels of the free radical $HO^{\cdot}$ produced by the Fenton reaction, by reducing O_2^- levels, however the dismutation of O_2^- results in H_2O_2 production (Luk et al., 2003). H_2O_2 then needs to be reduced, converting H_2O_2 into water by another antioxidant molecular set, the glutathione peroxidase family of enzymes.

Glutathione peroxidase (GPx)

A simplified way of describing GPx mode of action is as follows, GPx reduces lipid peroxides to their alcohol more stable forms, and reduces H_2O_2 to water. GPx does that by using reduced glutathione (GSH) as an electron donor, producing two H_2O molecules as the end product;

$H_2O_2 + 2GSH = GS\text{-}SH + 2H_2O$

GPxs are a family of enzymes, composed of four different GPxs, classically described by their common location in the cell or in a specific organ. Gpx1is found in the cytosol, nucleus, and mitochondria. GPx2 is found in the cytosol and nucleus, and is classically localized on the cells lining the gastrointestinal tract. GPx3 is found in the cytosol. All of these enzymes use selenium in their active site to perform their antioxidant function. The GPx4 which not only takes care of H_2O_2 but also peroxidized fatty acid residues in the cell membrane, GPx4 is found abundantly in testis, playing a role in sperm maturation, and in the cell it can be found in the cytosol, nucleus, mitochondria and cell membrane (Gutteridge and Halliwell, 2007, Fourth Edition). More recently there have been additional forms of GPx described, GPx5,GPx6 and GPx7 (Margis et al., 2008).

Catalase (CAT)

Another cell defense mechanism against H_2O_2 is the enzyme CAT, which catalyzes the direct decomposition of H_2O_2 into O_2. CAT is localized in the cell peroxisomes, CAT uses Fe at its active site and its mode of action is similar to that of SOD, a dismutation reaction, reducing H_2O_2 to H_2O and O_2. CAT function is optimal during high H_2O_2 levels, as the reaction velocity and complete H_2O_2 clearance efficiency need two H_2O_2 molecules, when H_2O_2 concentrations drop this efficiency becomes less likely, and CAT less efficient (Gutteridge and Halliwell, 2007, Fourth Edition).

Peroxiredoxins (Prxs)

Are a family of peroxidases which reduce H_2O_2, peroxiredoxins redox reactions depend on cysteine at their active site. There are known six different types of peroxiredoxins. Prxs are very abundant in the cell, and can be found in different cellular compartments; Prx 1,2 and 6 found in the cytosol. There is the mitochondrial Prx3, the endoplasmic reticulum found Prx4 , and Prx5 found both in mitochondria and peroxisomes (Gutteridge and Halliwell, 2007, Fourth Edition).

AIMS

The aim of this thesis is to examine the NPCs survival, function and fate changes on a transcriptional and functional level after exposure to three models of experimental inflammation; A brain stem injury, *in vitro* exposure to free radicals, and EAE.

RESULTS AND DISCUSSION

PAPER I

NPCs Transplanted to the Hypoglossal Nucleus show signs of integration and promote motor neuron survival

In this study we culture primary NPCs from the SVZ of the adult rat brain, isolated accordingly to a modified protocol by Johansson et al. (Johansson et al., 1999). For NPC culture we used the homozygote offspring from a backcrossed Lew rat which carries an eGFP insertion under the ubiquitin promoter (Lois et al., 2002). These cells were transplanted into a hypoglossal nerve avulsion injured wild type Lew rat sibling. We determined the survival, differentiation and possible integration and or beneficial effect from the graft into the microenvironment of the hypoglossal nerve. The advantage of the hypoglossal nerve avulsion model is that the injured side is also in close vicinity to the corresponding uninjured side which serves as a control and is present at each section. These characteristics allowed us to assess the potential effects of NPCs transplantation onto the hypoglossal nucleus injured side, in contrast to its un-injured site. After the second passage SVZ NPCs were prepared for transplantation in a single cell suspension of 100,000 cells in 5ul. For the NPC transplantation the brain stem was exposed and the obex identified. NPCs were injected into the right hypoglossal nucleus through a glass micropipette sealed onto a Hamilton syringe. The experimental groups are described in Fig. 2. NPCs were transplanted at the 9^{th} day post injury in order to avoid the peak of microglial reaction known to occur the first week after injury (Aldskogius and Svensson, 1993), and which could influence the transplantation result.

Transplanted NPCs survive, differentiate and express neuronal markers

To prevent transplantation rejection, all transplanted animals, including controls received cyclosporine injections. 10mg/kg cyclosporine were injected daily, from 1d prior to transplantation, and diminished to 3x/week after time point 28d. Animals were perfused after 2, 7, and 28 days and 3 months (n = 8 in each group and survival time). The brain stems were removed and postfixed. The survival, differentiation, integration and possible beneficial

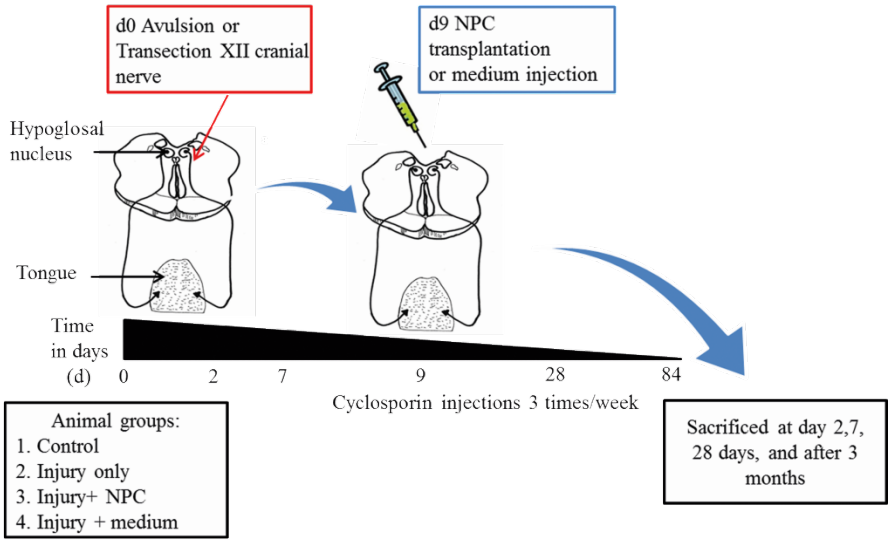

Figure 2. Illustration showing the experimental design in paper I and II. *Wild type animals were injured and transplanted groups received NPCs from eGFP+ siblings. In paper I animals were subjected to avulsion of the XII cranial nerve and in paper II, two groups were studied, nerve transection and nerve avulsion injury of the XII cranial nerve.*

effects of the graft onto the microenvironment were assessed. Morphologically rounded eGFP NPC were found after 7 days transplantation, this morphology could indicate that these cells were in an undifferentiated state. Interestingly after three months, the transplanted eGFP NPC displayed different morphological features. Immunohistochemical analysis of these areas, showed eGFP transplanted cells, were co-stained with GFAP (marker for astrocytes), O4 (marker for oligodendrocytes) and Tuj-1 (early neuronal marker). The presence of the neuronal marker Neu-N allowed us to asses if the graft had differentiated in to more mature neurons. These results indicate that the environmental cues of the injured micro environment could have influenced the fate decision of the graft and that the transplanted graft can differentiate *in vivo*, producing the major CNS cell types, neurons, astrocytes and oligodendrocytes.

The differentiated graft showed the expression of early synaptic markers

Additionally, we were interested in studying if the differentiated neurons could have been functionally integrated in the microenvironment, for which we tested if neural structures in the graft were co-stained with bassoon, and synaptophysin. Our findings revealed positive immunostaining of the transplanted NPCs with these two markers. Bassoon, is found at the presynaptic active zone, and is commonly one of the earliest proteins appearing in newly formed synapses, (Friedman et al., 2000), playing an important role, in appropriately anchoring synaptic proteins to the newly formed active site. (Dick et al., 2003). Synaptophysin which is a

synaptic vesicle membrane protein, is widely used as a marker for presynaptic terminals, and play several roles in the presynaptic terminals; endocytosis of vesicles, synapse formation and exocytosis, highly relevant during neuronal activity (Becher et al., 1999). Together these findings indicate that the transplanted NPC graft was capable of differentiating into astrocytes, early oligodendrocytes and neurons, and that the newly differentiated neurons showed signs of possible integration in to the new microenvironment, forming early synaptic signatures.

The number of surviving motor neurons was higher in transplanted animals

After nerve avulsion motor neuronal death is significant (Martin et al., 1999)(Paper I), with numbers ranging from 80% after 3 months injury. Concomitantly we were interested to study if the NPC graft would have any effect on motor neuronal viability. Quantification of motor neurons showed that in animals which were injured but did not received NPC transplantation, the number of surviving neurons kept decreasing with time and animals which were injured and received the NPCs graft, showed a significantly higher number of surviving neurons after nerve avulsion. Subsequent to nerve avulsion, the degeneration that follows is known as Wallarian degeneration (described in Nerve avulsion injuries section page 10), our results then indicate that the NPCs mediated a nursing effect on the motor neurons surrounding the lesion, providing trophic support needed during this inflammatory condition. Also, this support was able to be sustained for up to three months, as in the non NPC transplanted animals, cells continued to degenerate whereas in the transplanted animals survival was consistent at this time point.

Long lasting VEGF expression from the transplanted NPCs

The beneficial effects on motor neuron cell survival observed in the transplanted animals, lead to another question; by which mechanism could the NPCs provide trophic support to the injured microenvironment? We decided to study if the transplanted NPCs, could express vascular endothelial growth factor (VEGF) on to the site of injury. VEGF is known to have beneficial effects on different neurodegenerative landscapes, like during the devastating disorder of amyotrophic lateral sclerosis (ALS), where motor neurons die irremediably. In this context VEGF has been observed to reduce degeneration of motor neurons when infused onto an animal model of ALS (Storkebaum et al., 2005). Also it has been shown that VEGF treatment enhances neuronal survival and neurite outgrowth (Rosenstein et al., 2003) (Sondell et al., 1999), and it can also protect neurons during hypoxic conditions (Jin et al., 2000). Our results showed that NPCs transplanted to the avulsed injured animals, co-expressed VEGF three months post-injury, suggesting a possible mechanism by which the NPCs could exert their beneficial effect onto the injured area, supporting motor neuronal survival.

In Paper I, I present that transplanted adult NPCs can survive up to three months after transplantation and are able to differentiate into astrocytes, oligodendrocytes, and neurons on to the site of injury. Differentiated neurons expressed signs of newly formed synapses indicating possible integration of the graft into the microenvironment. Besides differentiation, the graft proved to be beneficial to the injured area, by decreasing the numbers of motor neuronal cell death, a mechanism possibly influenced by VEGF. This study contributes to the regenerative field by demonstrating that transplanted NPCs in to the injured CNS increased motor neuronal survival, showing that external sources of NPCs can potentially contribute to the repair of the CNS.

PAPER II

Transplanted NPCs showed activation differences depending on the injury severity

We were interested in studying whether the NPC activity was affected by the extent of inflammation, which was modelled by two injury models: nerve transection and nerve avulsion injury of the hypoglossal nerve.

A transection nerve injury differs from the avulsion model by causing less damage to the site of injury (see page 11 of the introduction) and less neuronal death to the neurons in close contact to the transected nerve. In this model the right side of the hypoglossal nerve was transected at the level where it passes the carotid artery (groups II-IV). The experimental design was the same as for Paper I (Figure 2). We speculated that the microenvironment of inflammation could differ in the two different injury models and could also differentially activate the transplanted NPCs and therefore affect the possible regeneration. After 9 days post transection and avulsion injury, 100,000 primary eGFP NPCs were transplanted in injured animals into the right hypoglossal nucleus as described in paper I. Our findings revealed differences between the two injury models, with respect to transplanted NPCs distribution, differentiation, and expression of the antioxidant defense molecule glutathione peroxidase (Gpx).

Higher motor neuronal survival in nerve transected injury model

When quantifying the motor neuronal survival, we found that at 28 days post injury 89% of motor neurons were persistent. In contrast as discussed in Paper I, motor neuronal survival in the avulsion model is 25% at this time point.

Transplanted NPC distribution at the injury site

After three months we found that the transplanted NPCs density and morphology was different between the transection model and the avulsion model. We observed 10% less $eGFP^+$ NPCs present in the transection injury model and scattered distant to the injury site, whereas in the avulsion injury, higher numbers of $eGFP^+$ cells were found and their distribution was observed to remain at the site of injury, suggesting differences in instructive signals in each inflammatory milieu directing the cells towards different distribution and survival patterns.

Transplanted NPC showed differentiation differences depending on injury type

Differentiation of the graft was observed three months post transplantation in paper I. Immunohistochemistry for GFAP and Tuj-1 showed co-staining of $eGFP^+$ NPCs to these markers only in the avulsion model. The $eGFP^+$ NPCs in the transection model, displayed more rounded morphologies but also protrusions, however these cells did not show co-staining with any of these differentiation markers. These differences in fate choice could be attributed to components in the inflammatory milieu and differential regenerative needs in each injury model. The nerve avulsion injury model is a more severe injury, which may involve different instructive signals for the graft to express distinctive molecular components, like VEGF among others. This could provide different cues to the NPCs to choose different lineage engagement, however this will have to be further studied.

Interestingly at the later time point (3 months) we found several $eGFP^+$ NPCs which were Sox2 positive in the transection injury model (25-30%), whereas in the avulsion model, we could not observe Sox2 expression from the grafted cells at this time point. Sox2 expression, indicates that a portion of the transplanted cells, remained in an undifferentiated state, possibly by the lack of environmental cues which could initiate the process for fate specification, or exerting other beneficial effects to the microenvironment rather than integration, as has been previously reported in other injury model (Bacigaluppi et al., 2009).

Transplanted NPCs expressed antioxidant markers

Despite the undifferentiated state of the NPC into the transected nerve injury, we wanted to study the possible expression of other molecules which could benefit the inflamed microenvironment, for which we tested the expression of glutathione peroxidase (Gpx1).

Gpx1 is an antioxidant enzyme which protects the cell from ROS, in particular from the inflammation produced by H_2O_2, for review see (Brigelius-Flohe and Maiorino, 2013). Immunohistochemical staining for this marker showed a higher expression of Gpx1 in the avulsion model, than in the transection injury model. The distribution of the Gpx1 staining

showed that in the avulsion injury, the expression of Gpx1, was not solely confined to the eGFP NPCs, but also there was Gpx expression in the parenchyma of the hypoglossal nucleus. Expression of Gpx1 in the transection injury model, showed different distribution, as it was confined mostly to the transplanted graft, and the intensity of the expression was lower than in the avulsion model, also, the hypoglossal nerve parenchyma did not show expression of Gpx1. The presence of Gpx1 in the site of injury in the avulsion model, could be one of the beneficial effects from the transplanted NPCs on to the injury site, providing stronger antioxidant protection against the presence of free radicals produced during the inflammatory response after nerve avulsion, leading to a better outcome of motor neuronal survival as observed in paper I. The differential expression of Gpx1 in the transection injury, could again, be the result of the less adverse inflammatory milieu, which do not instruct the cells to express higher antioxidant components.

Comparison of VEGF protein expression from the transplanted NPCs in both injury models

As reported in paper I, VEGF was expressed from transplanted NPCs in the avulsion injury model. We aimed to investigate if this expression was similar in the transection model. The analysis showed that expression of VEGF by NPCs in the avulsion model, was more intense and abundant than in the transection model, were VEGF expression was weaker. As discussed earlier in this thesis, VEGF is a neurotropic factor, and our group and others have previously shown that VEGF can be expressed by transplanted adult NPCs after injury, this finding could also indicate the different needs of the specific injury, a more severe injury would be in more need of neurotrophic support and nursing than a milder injury.

Thus here we present two models which show differential activation of NPCs depending of injury severity. Two important points became clear when comparing both models, the first one;

The differentiation of the graft at three months post transplantation in the avulsion injury model, showed astrocytic, neuronal, and oligodendroglial differentiation, whereas in the transection model, we could not find transplanted NPCs which had differentiated into these cell types but cells that continue to express Sox2.

The second one, in the avulsion model, transplanted NPCs expressed the neurotrophic factor VEGF and the antioxidant enzyme Gpx1, which could modulate the inflammatory microenvironment, into a less hostile environment for the motor neurons to achieve a better survival rate. The transection model in contrast showed little expression of these markers, suggesting the microenvironment may instruct the cells differently leading to differential

expression of these proteins. A comparison of study I and study II clearly show that a more severe injury benefits more from NPC transplantation than a milder injury. Moreover, the injury microenvironment plays an important role in dictating the transplanted NPCs to exert different effects depending on injury type, this study could help us to elucidate better directed therapies depending on injury type and to consider the environment as a major regulator of NPC fate.

PAPER III

Oxidative stress influences the NPCs differentiation result by increasing numbers of neurons and oligodendrocytes

In this study, we investigated the NPCs differentiation result after being exposed to the free radical H_2O_2 a constituent part of the inflammatory cascade and immunity. We cultured primary adult NPCs from the SVZ of adult DA rat brains as described in the above studies (Johansson et al., 1999), and exposed them to different concentrations of H_2O_2. 100µM H_2O_2 was chosen since it did not have considerable effects in cell death, as was observed at 330mM H_2O_2 a concentration level that has been measured during severe neuroinflammation (Nikic et al., 2011). After 24h 100uM H_2O_2 exposure, NPCs were still viable. After exposure we assessed, proliferation, cell viability (Lecoeur, 2002), expression of genes relevant during differentiation, and immunohistochemistry and western blot analysis of the differentiation result (Fig. 3).

Cell death and proliferation after H_2O_2 exposure

Ki67 labeling showed that proliferation was increased in NPC cultures exposed to H_2O_2. These findings prompt us to investigate if there was a particular cell population proliferating more than another in the exposed cultures. Cells death was increased in exposed samples however this effect was not significant.

H_2O_2 exposure resulted in more neurons and oligodendrocytes

In differentiated NPCs derived from exposed cultures, we observed a significant increase in the number of neurons and oligodendrocytes. This finding prompted us to characterize our undifferentiated NPC culture in order to observe possible changes occurring immediately after exposure and which could have led to this differentiation result.

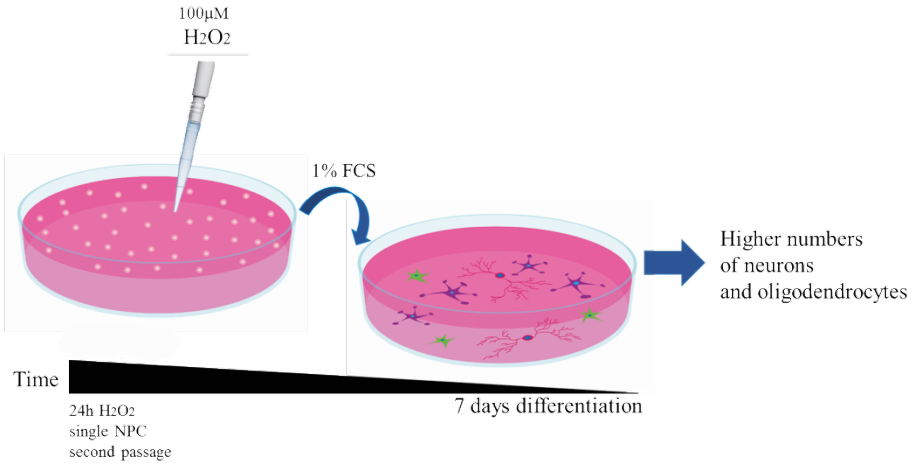

Figure 3. Experimental approach followed for NPC exposure. *After second passage, undifferentiated NPCs were exposed for 24h to the free radical H_2O_2 and then washed, and differentiated. Differentiation of exposed NPCs resulted in more neurons and oligodendrocytes.*

Olig2 was the most abundant population in NPCs exposed to H_2O_2.

When characterizing the NPC culture for the different subpopulation types; stem cells (B1 cells, $GFAP^+$)(Doetsch et al., 1999), intermediate progenitors (C cells, $Ascl1^+$) (Sommer et al., 1996), oligodendrocyte progenitors ($Olig2^+$) (Takebayashi et al., 2000), and neuroblasts (A cells, DCX^+). We found that B1 cells and neuroblast populations were diminished in H_2O_2 exposed cultures, see Fig. 4. This finding was congruent with the knowledge of young neurons being susceptible to free radical exposure (Manda et al., 2009). Olig2 showed to be the most abundant population in exposed NPC cultures, followed by C cells ($Ascl1^+$) cells (Fig. 4). Assessing proliferation by ki67 IHC revealed that the most proliferative population in the exposed NPCs cultures was $Ascl1^+$ cells, followed by Olig2+ cells. This result reflects our finding of more neurons and oligodendrocytes, as $Ascl1^+$ cells are known to be cells with long term neurogenic potential (Yu et al., 2013) and Olig2 presence promotes oligodendrocyte differentiation (Kim et al., 2011).

Gene expression dynamics correlate with the finding of more neurons and more oligodendrocytes

In order to further understand changes occurring during the differentiation process after H_2O_2 exposure, we assess the gene expression changes with real time RT-PCR at 0,2,4,8, 24 hours and after 7 days of differentiation.

Regarding neuronal differentiation, we found the up-regulation of the proneural gene *Ngn2* at 8 hours which is known to be highly express during the early neurogenic periods (Ma et al., 1996) and concomitantly with this finding an increase in the neuronal marker *βIII-tubulin* at 7 days which was in agreement with the increased number of neurons we observed after 100 uM H_2O_2.

The *Notch1* gene expression known to be the brake from the immature state to the differentiated state, but also necessary for oligodendrocyte differentiation (Park and Appel, 2003), was up-regulated at 24hours after H_2O_2 exposure. This up-regulation contrasted with the down-regulation of *Ngn-2*, this expression dynamics are necessary for the orchestration of oligodendrocyte differentiation. Sustained *Notch1* expression is necessary for oligodendrocyte production, which in turns leads to a down regulation of the pro neural gene *Ngn2* (Zhou et al., 2001). These results might suggest that, as during development, neurogenic events precede oligodendrogenesis in H_2O_2 exposed NPCs, leading to increased numbers of neurons and oligodendrocytes.

H_2O_2 exposures negatively impacts the antioxidant protection network of NPC

We were interested in studying possible effects of H_2O_2 on the antioxidant repertoire of the NPCs. We used a real time RT-PCR gene arrays composed of a set of 84 genes related to antioxidant defense components. Our findings demonstrated that H_2O_2 down regulated the expression of the glutathione peroxidase pathway which reduces H_2O_2 to H_2O. A less efficient H_2O_2 detoxification could lead to a more effective H_2O_2 load in the cells.

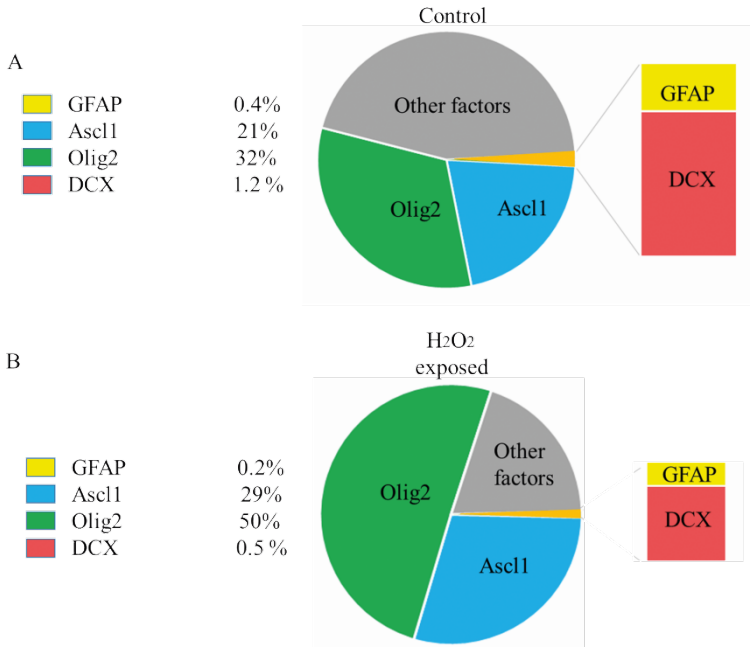

Figure 4. Population shift in undifferentiated NPCs exposed to H_2O_2. *Average percentage of the IHC quantification of NPC cultures labeled for Olig2, Ascl1, Sox2 (not shown), DCX and GFAP, in A) control cultures and B) H_2O_2 exposed cultures. Exposed cultures had increased numbers of Ascl1$^+$ cells and Olig2$^+$ cells. A fraction of the cells named "other factors" didn't express Ascl1 or Olig2. Cells were cultured for two passages and 91% of the cells expressed Sox2 at the time of analysis.*

H_2O_2 exposure changed the expression of genes identified for enzymes which modify chromatin architecture

To be able to further explain our differentiation results, we investigated if H_2O_2 could have changed the expression of chromatin structure modifying enzymes. We used a gene array containing 84 different genes predicted to have an effect on chromatin structure, our results showed that H_2O_2 down regulated the gene expression of 54 genes among which were histone methyltransferases and demethylases, and histone acetyltransferases and deacetylases. H_2O_2 significantly upregulated 6 genes, 4 of them were histone deacetylases and 2 histone methyltranspherases. These results indicated that H_2O_2 has the potential to exert chromatin architectural changes on the NPCs genome.

Using the open source DAVID plataform to find biological relevance allowed us to identify three genes from the histone deacetylase (HDAC) family of genes, *Hdac 7, 9,* and *3* to be part of the down-regulated gene group. Down regulation of HDACs is known to be involved in neuronal differentiation (Hsieh et al., 2004). In contrast we found the HDAC Sirt2 to be upregulated in cultures exposed to H_2O_2. *Sirt2* it's known to be involved in cell cycle exit, and

oligodendrocyte differentiation (Li et al., 2007). Consequently, it became of interest to investigate if H_2O_2 could affect *Sirt2* expression and in turn inflict oligodendrocyte numbers.

To inhibit *Sirt2* and to assess its requirement to oligodendrocyte differentiation, Sirtinol a *Sirt2* inhibitor, was used in NPCs previously exposed to H_2O_2 and in unexposed NPCs. Sirtinol was able to reduce the expression of *Sirt2* in both conditions, and this effect was able to be counteracted by the addition of H_2O_2 for 24h. The finding that *Sirt2* was upregulated after H_2O_2 exposure correlates with other studies reporting the *Sirt2* expression triggered after cell stressors are present in the cells microenvironment, (Zhang et al., 2013). When we assessed oligodendrocyte numbers using IHC, in cultures exposed to sirtinol, we found that sirtinol was able to decrease oligodendrocyte numbers and H_2O_2 exposure was able to restore them, these results suggested a relationship between Sirt2 and oligodendrocyte differentiation. Further our results confirm previous findings, demonstrating a role for HDACs in OPC maturation (Nave, 2008).

In paper III I describe the effect that H_2O_2 has on the NPCs differentiation potential, initiating different fate dynamics in the NPC population, where the numbers of Olig2 and Ascl1 expressing cells increased. This increase lead to more oligodendrocytes and neuronal numbers. Gene expression of chromatin modification enzymes was changed indicating DNA architecture changes may allow the expression of genes favoring oligodendrocyte and neuronal differentiation. In Fig. 5 I illustrate the network of molecules found relevant in this study for NPC differentiation after H_2O_2 exposure.

In conclusion; Specific inflammatory components could offer specialized effects on the regeneration of the CNS. Fine tuning of the inflammatory components could lead to the design of new therapeutical approaches for regenerating the injured CNS.

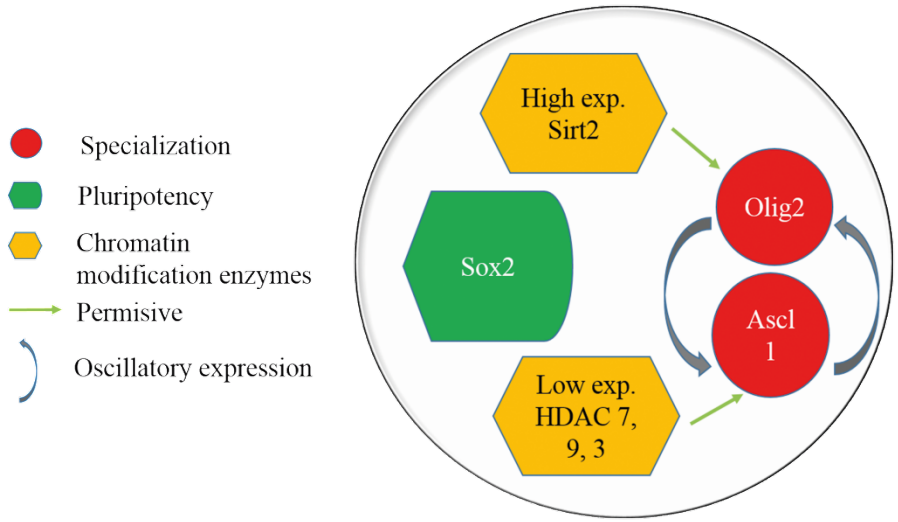

Figure 5. Simplified illustration, modeling specification of NPC after 24h H_2O_2 100uM, showing the contribution of the factors found relevant in paper III. *Differentiation is a complex mechanism where many genes participate of the journey the cell takes when specializing into a differentiated cell. Representation of a cell nuclei containing Sox2 (in green), the pluripotency master regulator, expressed during the H_2O_2 exposure and before differentiation. Chromatin modification enzymes (in yellow), like Sirt2 (high expression) and Hdac 7, 9, and 3 (lower expression) regulate the kinetics of transcription factors binding to DNA permitting cell specification (Bose et al., 2014), by factors like Olig2 or Ascl1 (in red). The sustained expression of these factors will then result in the differentiation of the NPC towards an oligodendrocyte or a neuron (Imayoshi et al., 2013).*

Paper IV

Chronic Inflammation prime SC NPCs to undergo fate conversion

Our interest to continue the investigation of the inflammatory effects on NPC differentiation, lead to further studying the overall effects of EAE on the differentiation potential of SVZ and SC NPCs of female DA rats. In the CNS different NPC populations reside, these populations differ from each other in molecular components (See Table 1), fate commitment, and microenvironmental locations as mentioned in the introduction.

For paper IV, we asked the question if SC NPCs are affected at the transcriptional and functional level by chronic inflammation. To be able to analyze the impact of neuroinflammation on the NPCs, we first addressed the differences between NPC from the SVZ and SC under healthy conditions, this data provided a baseline for comparison to when

these cells are in an inflamed environment. We studied the global gene expression differences using an Affymetrix chip array, which allowed us to measure the global expression of the genome in healthy NPCs and EAE derived NPCs, from both the SVZ and the SC. For the Affymetrix experiment the experimental groups consisted of healthy derived samples, and EAE derived samples, both groups with matched clinical scores of 3. We analysed the cells in undifferentiated and differentiated conditions. The cells were extracted from the SVZ and the cervical, thoracic and caudal parts of the spinal cord and from the SVZ biopsy prior to NPC culturing. In parallel, NPC were cultured from the same CNS regional areas and gene expression, immunohistochemistry, and western blot analysis were carried out in undifferentiated and differentiated cells.

In healthy conditions the SVZ NPCs prove to be more neurogenic than the SC NPCs which show higher gliogenic potential

In the undifferentiated NPC cultures, 187 of the genes were found to be differentially expressed between the SVZ and the SC of healthy rats NPCs derived cultures, this expression was higher in the SVZ comparing to the SC. The genes expressed in this fashion were neurogenic, and some were associated with central nervous system development, generation of neurons and neuronal differentiation. Gene expression analysis of the differentiated progeny of the healthy SVZ revealed mature neuron functional gene expression validating our finding of a neurogenic profile from the SVZ undifferentiated NPCs. The SC gene analysis profiling presented a less neurogenic signature in both undifferentiated and differentiated NPCs.
Functional studies from both NPC regions, showed a significantly higher number of neurons from the SVZ versus the SC, demonstrating the neurogenic nature of SVZ NPCs. The SC on the other hand produced more oligodedrocytes than the SVZ, showing innate regional differences in these two areas of the CNS.

EAE changed the genotypic characteristics of SC NPCs

We used 2 methods, Ingenuity System Pathway Analysis (IPA) and WebGestalt analysis (Wang et al., 2013) to better understand the biological relevance of the global gene expression data from NPCs derived from healthy and EAE SC comparison. The results revealed down regulation of crucial genes participating of astrogliogenesis and oligodendrogenesis in the SC. Functional studies of the spinal cord NPCs derived from EAE animals displayed lower numbers of oligodendrocytes and higher numbers of neurons, in contrast to the healthy control counterparts.

EAE increases the neurogenic potential of the SC NPCs as indicated by increased numbers of Ascl1+ cells

As described in paper III Ascl1 and Olig 2 positive cells are known to generate neurons and oligodendrocytes respectively, we investigated if there were differences in the numbers of these cells in the SVZ and SC NPCs from healthy and from EAE animals. Quantification of the SVZ NPCs showed a decreased in the numbers of $Olig2^+$ cells in EAE derived NPCs, also a trend towards increasing numbers of $Ascl1^+$ cells was observed (Fig. 6). The analysis of the SC-NPCs revealed an increase in the numbers of Ascl1 positive cells in EAE derived NPCs. $Olig2^+$ cell numbers were not different from the control cultures, however double labeling of Olig2/Ascl1, revealed a significant higher number of $Olig2^+$ cells which were also $Ascl1^+$ confirming a newly acquired neurogenic signature by the Olig2 cells (Fig. 7). A dominant expression of either Ascl1 or Olig2 will determine the final phenotype of these cells (Imayoshi et al., 2013), which the functional analysis confirmed to be neuronal since we found an increase in the numbers of these cells in EAE SC derived NPCs.

EAE SC derived NPCs develop a neurodegenerative signature

The transcriptome analysis revealed an increase in neurodegeneration related genes. Importantly we observed a strong decrease in the expression of several functional groups which control neurite developmental mechanisms, which could in turn affect the maturation and integration of newly differentiated neurons. Another finding which can potentially contribute to neuronal degeneration, is the observation of three functional groups predicted to decreased functionality in lipid metabolism since lipid turnover and degradation are vital for normal cellular mechanics, disruption of this system could have two outcomes, the instability

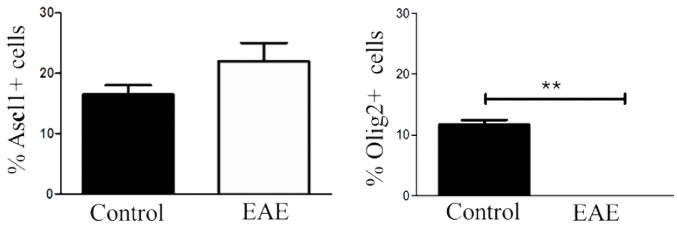

Figure 6. Quantification of $Ascl1^+$ and $Olig2^+$ cells in SVZ-NPCs from control and EAE. *Even though nitrite and nitrate levels were not detected in the SVZ-NPCs cultures, quantification of $Ascl1^+$ and $Olig2^+$ cells revealed a significant difference in the Olig2*

population, were EAE decreased the numbers of these cells. Further Ascl1^{+} cells showed a tendency towards higher numbers in EAE derived NPCs. n=3.

of neuronal homeostasis, or it could also contribute to the fate switch we observe. Since changes in stem cell metabolism have been show to contribute to fate conversions (Folmes et al., 2011). Finally, the NPCs fate change we observed may be an initial attempt to regenerate the system after EAE, however, intrinsic mechanisms within this instruction, caused by the inflammatory environment, may inhibit appropriate neuroregeneration and induce the failure of the maturation and integration of the newly differentiated neurons.

The NPC derived from EAE animals expressed a high inflammatory gene profile

Further, to identify patterns in our gene expression data, we used the open source MeV (Saeed et al., 2003) platform, and WEBGESTALT to functionally classify these genes. We found a strong immune signature from the caudal part of the SC, in NPCs derived from EAE animals. To exclude possible contributions from glial cells in this result, we assessed the presence of Cd11b positive cells in our cultures. Cd11b labels both, microglia, and macrophages. We didn't find a significant difference in Cd11b expressing cells in NPC cultures from the SVZ, and from the SC of EAE derived cultures comparing to the healthy derived NPCs, a finding correlated with the selectivity of NPCs cell culture conditions.

Additionally since $NO^{\cdot}$ has been correlated to inflammatory levels in MS (Johnson et al., 1995, Danilov et al., 2003a), measuring levels of $NO^{\cdot}$ by the nitrite and nitrate NO products with the Griess method (Griess, 1864), could provide us with information on the inflammatory state of our cultures, both in cell culture supernatants from NPC derived from healthy CNS, and from EAE animals. In the SVZ we could not detect nitrite and nitrate levels with the Griess method, however this result, changes in SVZ-NPCs populations were observed (Fig.6). This indicates EAE inflammation is present in the brain, perhaps at lower levels or with a different molecular signature, involving different inflammatory components. In contrast, the SC showed a significant increase of nitrite and nitrate, compared to the SVZ.

In paper IV the use of Affymetrix chip microarray platform allowed us to analyze the global gene expression of primary NPCs, guiding us towards possible biological phenotypes which combining other technologies allowed us to identify an inherent heterogeneity of the CNS NPC, and the fate conversion effect that inflammation produced by EAE has on the SC NPCs population. In healthy derived NPCs, we characterized the SVZ

NPC as a neurogenic population and the SC as a more gliogenic population. Interestingly, EAE converted the SC NPCs to a neurogenic population (Fig. 7 and Fig. 8), which was characterized by a neurodegenerative signature. In summary, beyond fueling regenerative needs by increasing neuronal numbers, inflammation also impacts regulators of neuronal survival and integration, which may impair initial attempts by the organism to regenerate the CNS cells.

Conclusively, the CNS NPCs have heterogenic differences depending on their region nativity; this heterogeneity is permuted when NPCs are exposed to an inflammatory environment.

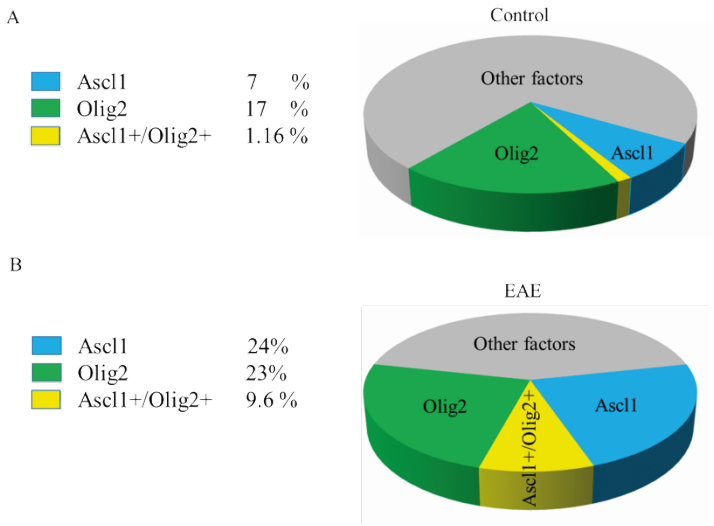

Figure 7. NPCs derived from EAE SCs showed increased numbers of cells with neurogenic potential labeled with Ascl1. *Pie chart showing the proportion of NPC populations in average percentage of the IHC quantification of NPC cultures labeled for Olig2, Ascl1, Sox2 (not shown) and Olig2 and Ascl1 together, in A) healthy SC derived cultures and B) EAE SC derived cultures. Cells were cultured for two passages and 90% of the cells expressed Sox2 at the time of analysis. EAE derived cultures had higher numbers of Ascl1$^+$ cells and Olig$^+$/Ascl1$^+$ double positive cells indicating the neurogenic population shift occurred in this culture.*

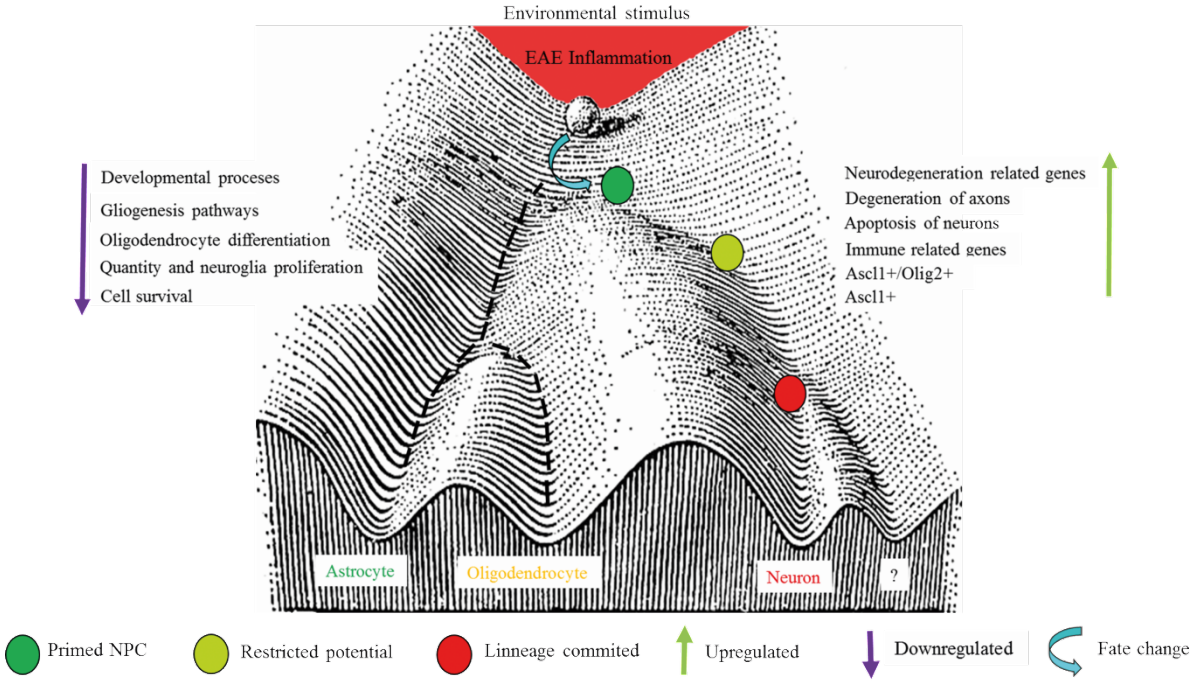

Figure 8. Modified 1957 Waddington's model (Waddington, 2014) depicting molecular changes in the NPCs triggered after inflammation modifies the NPC environment*. Colored marbles correspond to NPCs commitment states. The dashed lines reflect the fate of the NPCs under normal conditions. Arrows indicate upregulation or downregulation of the indicated functions or proteins.*

CONCLUDING REMARKS

My thesis work shows that NPCs derived from the adult brain and SC have neuroregenerative capacities in animal models of severe inflammation; a motor nerve injury model, and EAE. Also the NPCs neurogenic potential is enhanced when cells are exposed to the inflammatory component, the free radical H_2O_2. Three major points can be addressed from the present work;

a) CNS NPCs are heterogeneous, and present inherent genomic and functional differences which are regionally dependent, where, under basal conditions the SVZ NPCs are more neurogenic, and the SC more gliogenic. This NPC nature, may be changed during neuroinflammation, proving the NPC plasticity in response to the surrounding microenvironment.

b) Specific exposure of H_2O_2, a molecular component in the inflammatory milieu, activates the neurogenic capacities of NPCs leading to more neurons as the differentiation result.

c) Different injury/inflammation degrees generate different regenerative responses from the NPCs (Fig. 9). My work identifies severe inflammation as a permissive factor for NPC neuronal differentiation, which at the same time also activates detrimental mechanisms within the NPCs.

We are currently unable to ameliorate the degenerative processes occurring after CNS injury. The CNS does activates a regenerative response after injury, where the proliferation, migration and differentiation of NPCs occurs (Fagerlund et al., 2011, Danilov et al., 2006), however this response is insufficient and defective. The more phylogenetically sophisticated mammals are, and to this matter, humans, the more archaic the regenerative potential is. Then, the need to empower our CNS regenerative potential becomes obvious. A viable approach is the transplantation of exogenous NPCs which we and others demonstrate to be beneficial to the injury site. Also inflammation, the first detectable reaction after injury, has demonstrated to play a double role; Beneficial, as its not only capable of activating NPCs (Danilov et al., 2006, Fagerlund et al., 2011), and diverting the NPC nature, allowing the otherwise gliogenic SC NPCs to become neurogenic (Paper IV). But also an adverse role; as inflammation may initiate intrinsic detrimental neuronal responses in the NPCs progeny, which may result in cell death or poor cell integration into their new environment.

It becomes tempting to speculate that the latest is one of the mechanisms responsible for a poor regenerative outcome in the CNS. Further, together with this micro environmental determinants, we have the process of NPC differentiation. As described earlier

in this thesis, differentiation it's a complex machinery, which is in strict communication with the cellular environment, involving the interactions of large numbers of molecules. The expression of these molecules is dynamic, it can rise and fall, bind and unbind DNA, and remain in the microenvironment or degrade, a process which is not a step by step course but it's neither random (Imayoshi et al., 2013, Zhang and Wolynes, 2014). Mechanistic insights into this elementary process in relation to the injured microenvironment will translate into better therapeutical design, modulating NPCs function.

All together my scientific findings brings us one step closer towards understanding NPCs in an inflammatory context which hopefully will contribute to the development of better therapies and insights into how the CNS regenerative potential can be improved in the adult injured human CNS.

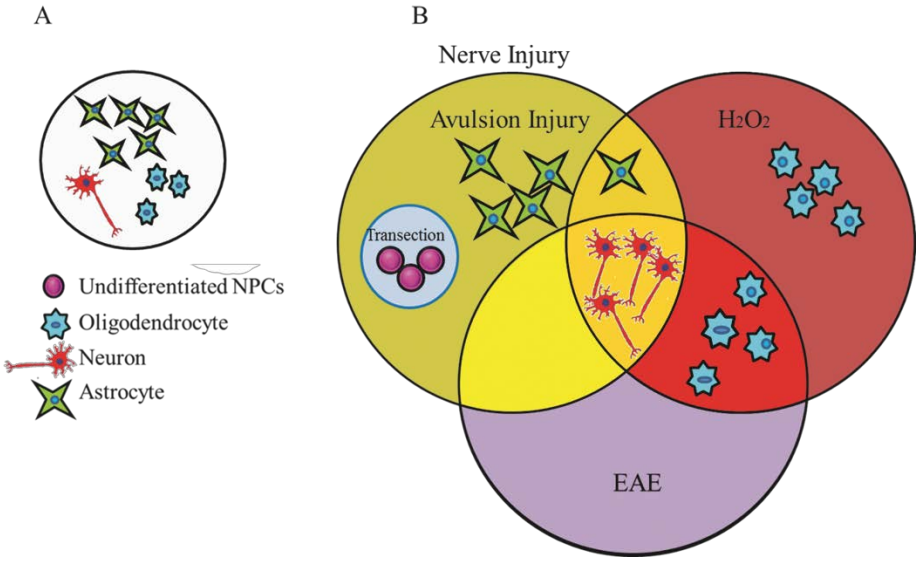

Figure 9. The micro environment dictates NPCs behavior.

In my thesis, I have found that severe inflammation like nerve avulsion or EAE, as well as components from the inflammatory cascade like the free radical H_2O_2 convert the fate of NPCs to produce more neurons from NPCs from the SVZ, and from the SC. Diagram (A) shows differentiated NPCs in a normal environment. The Venn diagram (B) illustrates the differentiation result after NPCs are exposed to different inflammatory conditions. In the case of EAE, the SC NPCs whose nature is more gliogenic, inflammation converts this gliogenic quality to a neurogenic one. In nerve avulsion injury but not transection injury, transplanted NPCs differentiated into neurons as well as astrocytes and oligodendrocytes. The majority of NPCs cells transplanted onto the transection injury model remained undifferentiated indicating the role of different inflammatory milieus onto NPCs nature.

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