Technische Universität München
Fakultät für Medizin
Neurologische Klinik und Poliklinik des Klinikums rechts der Isar
(Direktor: Univ.-Prof. Dr. Bernhard Hemmer)

Die Rolle des EphB3-ephrinB3 Signalwegs bei der hypoxischen Zellantwort adulter neuronaler Stamm-/Vorläuferzellen

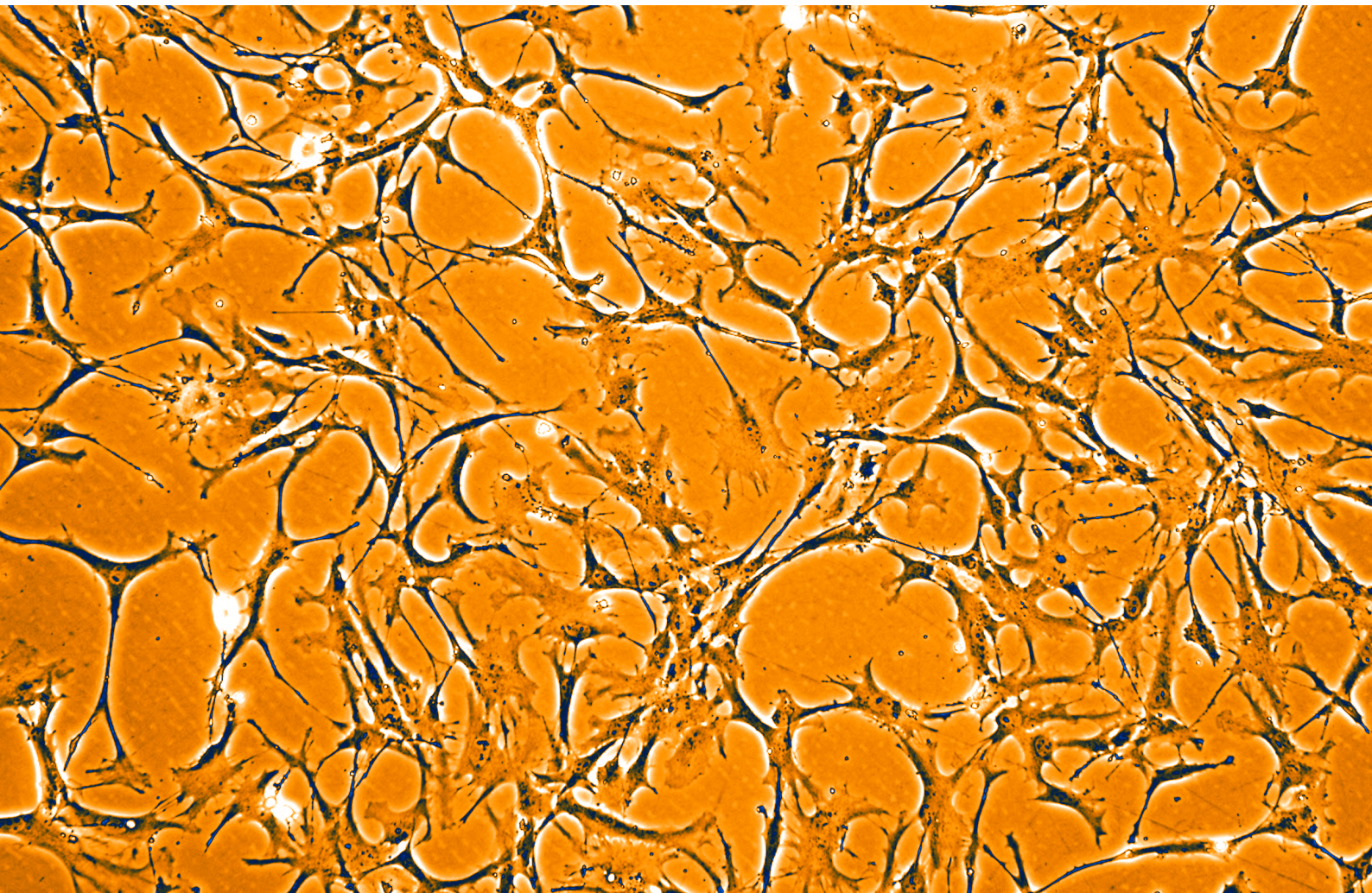
Gisela Katharina Baumann

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Medizin genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. Ernst J. Rummeny

Prüfer der Disseration: 1. apl. Prof. Dr. Achim Berthele
2. Univ.-Prof. Dr. Bernhard Hemmer

Die Disseration wurde am 17.08.2015 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 06.04.2016 angenommen.



in cooperation with

University of Miami
The Miami Project to Cure Paralysis

English title

The role of EphB3-ephrinB3 signaling during the adult neural stem/progenitor cell response to hypoxia

Hainfeld, 2014

For Elisabeth and Wolfgang,

our parents,

for nurturing our curiosity.

Variability is the law of life, and as no two faces are the same, so no two bodies are alike, and no two individuals react alike and behave alike under the abnormal conditions which we know as disease.

William Osler

Preface and Acknowledgements

Research - the quest for acquiring new insights into natural phenomena and development of therapeutic approaches. It is an interesting, inspiring, fascinating and intense field of work, which requires a fair degree of observation, thought, skill, discipline, questioning, patience, flexibility and criticism from the scientists conducting it. In the beginning stand the questions raised: *How is adult neurogenesis regulated and what are the changes post - brain injury? Why does recovery in the Central Nervous System only have limited capacity? What are the changes in low oxygen tension which provide better growth conditions for neural progenitors? How can we utilize and guide our innate resources for improved brain repair?* This thesis focuses on answers to these questions, though minimized to finding proof to more detailed hypotheses, as applicable to methods of modern science.

For the course of one year, I had the privilege to learn some of 'the essentials' in science in the laboratory of Dr Liebl at the University of Miami, emphasizing on the processes 'around' experiments rather than only following existent experimental protocols. The investigation of a project's background in literature, proposing hypotheses and planning of experimental designs, modifying protocols, critical interpretation of own and foreign data, staying up-to-date with recent findings in the field as well as clear presentation of results for fruitful discussion; these are the cornerstones of a scientific project and the treasures of science one should aim to learn and profit from, to utilize them in any kind of future career.

All my thanks go to the team of Dr Liebl's laboratory for sharing and discussing ideas and thoughts, providing profound education and experience, and simultaneously supporting my independence in scientific work. Special thanks I owe to Dr Daniel Liebl for giving me the great opportunity to work a year in his lab and to Dr Michelle Theus for being a brilliant tutor and co-worker throughout my project.

Results from this thesis project have been previously published in a paper journal in July 2013 (*Baumann, G. et al., 2013*). The paper includes relevant experiments conducted in our laboratory but not by myself, which I have presented and discussed in this thesis as "Background Results" for the purpose of better understanding through exposure to the whole research project.

CONTENTS

ABBREVIATIONS

in alphabetical order

#	number
%	percent
approx.	approximately
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblastic growth factor
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
CCI	controlled cortical impact
CDK	cyclin-dependent kinases
CNS	central nervous system
ddH2O	deionized, distilled water
DVR	Division of Veterinary Resources
E2F1	transcription factor E2F1
eB3	ephrinB3-Fc
e.g.	for example (*lat.* exempli gratia)
EGF	epidermal growth factor
Eph	erythropoietin-producing hepatocellular
$EphB3^{-/-}$	EphB3 receptor knock-out
$ephrinB3^{-/-}$	ephrinB3 ligand knock-out
EPO	erythropoietin
et al.	*lat.* et altera (*engl.* and others)
Fig.	figure
GDNF	glial-derived neurotrophic factor
germ.	in German language
GFAP	glial fibrillary acidic protein
GPI	anchor glycosylphosphatidylinositol anchor
HIFs	hypoxia-inducible factors
HIF-1α	hypoxia-inducible factor 1-alpha

HPb	pimonidazole hydrochloride
HRPO	horseradish peroxidase
hr(s)	hour(s)
IGF-1	insulin-like growth factor-1
JM	juxtamembrane
kg	kilogram
min	minute(s)
mM/μM	millimolar/micromolar
mmHg	millimeter of mercury
MW	molecular weight
NSPC(s)	neural stem/progenitor cell(s)
NSVZ	germ. neuronale Stamm-/Vorläuferzellen
OB	olfactory bulb
p-AKT	phosphorylated/phospho-AKT
PCR	polymerase chain reaction
PDZ-domain	acronym: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
pO	partial pressure of oxygen
Rb	retinoblastoma protein
RMS	rostral migratory stream
RT	room temperature
RTK	receptor tyrosine kinase
SAM	sterile a-domain
SCF	stem cell factor
sham	non-injured/ negative control
SHT	*germ.* Schädel-Hirn-Trauma
SVZ	subventricular zone; *germ.* subventrikuläre Zone
TBI	traumatic brain injury
VEGF	vascular endothelial growth factor
WT	wild type

I. 1. SUMMARY

Many common neurological disorders, including traumatic brain injury, stroke and neurodegenerative diseases, are characterized by the loss of neurons and glial cells. Research strategies to reduce cell loss after injury or disease have focused on two approaches; acute protection and chronic recovery. Efforts to develop restorative therapies principally involve either exogenous transplantation of stem/progenitor cells or the stimulation of residential stem cells within the adult central nervous system. It is now widely accepted that the adult brain retains the capacity to develop new neurons, and the subventricular zone (SVZ) and hippocampus represent two regions where neurogenesis remains highly active. In the past decade, significant advancements have been made in our understanding of the molecular and cellular mechanism that regulate adult neurogenesis. Ephrins and Eph receptors are membrane-bound molecules that play important roles in regulating adult neurogenesis in the SVZ (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). Following traumatic brain injury (TBI), deficiency or activation of EphB3 signaling leads to alterations in proliferation and survival of SVZ-derived neural stem/progenitor cells (NSPCs) (*Theus, M.H. et al., 2010*). In the present study, we build upon these findings by examining the role of hypoxia, commonly associated with TBI, on EphB3 signaling, expression and function as it relates to adult neurogenesis in the SVZ. Ultimately, these findings will improve our understanding of adult neurogenesis, and may lead to the development of therapeutic strategies to enhance recovery from brain injury and disease.

To examine NSPC function in hypoxic environment associated with TBI, we isolated and cultured NSPCs from the SVZ of adult CD1 mice from wild type and gene-targeted knockout mice, and examined the role of ephrinB3-EphB3 signaling in mediating NSPC proliferation and survival under normoxic and hypoxic conditions. Low oxygen culture resulted in down-regulation of EphB3 expression, which coincided with enhanced survival and proliferation of wild type NSPCs compared to normoxic controls. In the absence of EphB3 (i.e. EphB3$^{-/-}$) and under normoxic conditions, we observed an increase in NSPC proliferation and survival similar to hypoxia-induced conditions, supporting the hypothesis that hypoxia-induced reduction in EphB3 leads to the observed improvements in NSPC proliferation and survival. Interestingly, activation of EphB3 receptors with soluble pre-clustered ephrinB3-Fc molecules leads to reduced proliferation but enhanced survival of wild type NSPC in both normoxic and hypoxic conditions. This demonstrates that stimula-

tion with ephrinB3 functions to limit both NSPC proliferation and cell death of cultured NSPCs. Consistently, reduced EphB3 expression was observed *in vivo* post-TBI in the SVZ, and both increased survival and attenuated proliferation were found following ephrinB3-Fc infusion in the SVZ of wild type mice (*Theus, M.H. et al., 2010*). Tissue hypoxia is part of the pathophysiologic response after TBI (*Baumann, G. et al., 2013*) and down-regulation of EphB3 under these hypoxic conditions may be an important regulatory component of NSPC numbers and therefore, endogenous repair.

We also found that cell cycle regulator phosphorylated (p)-AKT and hypoxia inducible factor-1 alpha (Hif-1α) are up-regulated in hypoxia, inversely to EphB3 down-regulation. p-AKT is known to promote p53 degradation, where p53 is a known anti-proliferative factor (*Mayo, L.D. and Donner, D.B., 2002*). Hif-1α regulates factors that stimulate stem cell proliferation, survival and differentiation under low oxygen conditions (*Panchision, D.M., 2009; Zhao, T. et al., 2008*). Previously, we have shown that p53 is downstream of the EphB3 signaling cascade and plays an important role in the ability of EphB3 to regulate anti-proliferative functions (*Theus, M.H. et al., 2010*). We hypothesize that hypoxia leads to increased p-AKT and Hif-1α levels, but only p-AKT is downstream of EphB3 signaling (*Blits-Huizinga, C.T. et al., 2004*). EphrinB3-Fc stimulation of EphB3 leads to reduced p-AKT levels but not Hif-1α, suggesting that hypoxia results in increased p53 degradation as a result of reduced EphB3 expression that ultimately mediates hypoxia-induced proliferation and survival of NSPCs.

These data demonstrate that EphB3 plays an important role in controlling the numbers of adult NSPCs in the SVZ under normoxic and hypoxic conditions and that it might be a key player regulating a reparative response in the SVZ after CNS injury.

I. 2. ZUSAMMENFASSUNG

Im Zentrum der Pathogenese vielerlei neurologischer Erkrankungen steht der Verlust von Neuronen und Glia, wie beispielsweise beim Schädel-Hirn-Trauma, Schlaganfall und neurodegenerativen Erkrankungen. Zweierlei Strategien stellen den Kern der derzeitigen Forschung dar, um genannten Zellverlust zu reduzieren; einerseits akute Zellprotektion und zum Anderen Zellregeneration im chronischen Verlauf. Die Erforschung regenerativer

Therapien beinhaltet sowohl Transplantation exogener Stamm-/Vorläuferzellen als auch Stimulierung und Rekrutierung lokaler neuronaler Stammzellen. Seit wenigen Jahrzehnten ist es nun die allgemein gültige Forschungsmeinung, dass das adulte zentrale Nervensystem die Möglichkeit zur Neubildung von Neuronen - die sogenannte Neurogenese - beibehält. Sowohl die subventrikuläre Zone (SVZ) als auch der Hippocampus sind die Regionen, innerhalb derer die Neurogenese bis ins Erwachsenenalter hochaktiv bleibt. Zusätzlich wurden während der letzten zehn Jahre signifikante Fortschritte in der molekularen und zellulären Forschung erzielt, um die Mechanismen zur Regulierung adulter Neurogenese aufzuklären. Ephrine und Eph Rezeptoren sind membranständige Moleküle, die eine wichtige Rolle bei der Regulierung der Neurogenese in der adulten SVZ übernehmen (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). Nach Schädel-Hirn-Trauma (SHT) führen sowohl Verlust als auch Aktivierung des EphB3-Rezeptorsignals zu Veränderungen von Zellproliferation und -überleben der neuronalen Stamm-/Vorläuferzellen (NSVZ) der SVZ (*Theus, M.H. et al., 2010*). Daraufhin haben wir in der vorliegenden Studie untersucht, wie hypoxische Bedingungen - die nachweislich nach SHT vorliegen - Einfluss nehmen auf das EphB3-Rezeptorsignal, die Expression und Funktion des Rezeptors, da dies letztendlich die adulte Neurogenese in der SVZ modifizieren könnte. Es ist das Ziel dieser Studie, unser Wissen und Verständnis der adulten Neurogenese zu erweitern, um zur Entwicklung neuer therapeutischer Strategien beizutragen, die auf die Wiederherstellung zentraler Funktionen nach Krankheit oder Trauma abzielen.

Zur Untersuchung der NSVZ in einem hypoxischen Umfeld post SHT isolierten wir zunächst adulte NSVZ von der SVZ adulter CD1 Mäuse, sowohl vom Wildtyp als auch von genveränderten „knock-out“ Mäusen, um die Rolle des EphB3-ephrinB3 Signalwegs auf Wachstum und Zelltod adulter NSVZ unter normoxischen und hypoxischen Bedingungen zu ermitteln. Zellkultur von NSVZ des Wildtyps bei niedrigem Sauerstoffgehalt führte zu einer Herabregulierung des EphB3-Rezeptors, was wiederum ein gesteigertes Zellüberleben und -wachstum begünstigte im Vergleich zu normoxischen Kontrollen. In Abwesenheit des EphB3-Rezeptors (EphB3$^{-/-}$ Genotyp) und unter normoxischen Bedingungen konnten wir eine Zunahme des NSVZ-Überlebens und der Proliferation beobachten, ähnlich wie zuvor unter hypoxischen Bedingungen. Dies unterstreicht unsere These, dass die Hypoxie-bedingte Reduzierung des EphB3-Rezeptors zu der genannten Steigerung von Zellwachstum und -überleben führen könnte. Interessanterweise resultiert die Aktivierung des EphB3-Rezeptors mittels löslicher ephrinB3-Fc Moleküle in einer erhöhten Zellüberlebensrate nebst

reduzierter Proliferation in normoxischer sowie hypoxischer Zellkultur. Dies unterstreicht, dass die ephrinB3-vermittelte Aktivierung von EphB3 sowohl Proliferation als auch Zelltod der kultivierten NSVZ einschränkt. Gleichsam war die Exprimierung des EphB3 Rezeptors in der SVZ nach SHT *in vivo* signifikant reduziert, und infolge der Infusion von ephrinB3-Fc in die SVZ von Mäusen des Wildtyps wurde das Zellüberleben gesteigert, während Zellwachstum verringert war (*Theus, M.H. et al., 2010*). Hypoxie ist Teil der Gewebs-Pathophysiologie nach SHT (*Baumann, G. et al., 2013*) und Herabregulierung des EphB3-Rezeptors unter hypoxischen Bedingungen könnte ein wichtiges Komponent sein um die Anzahl der NSVZ zu regulieren, und damit nicht zuletzt auch ein Einflussfaktor der endogenen Regeneration.

Schließlich konnten wir zeigen, dass phosphoryliertes (p)-AKT, ein Regulatorprotein des Zellzykluses, und Hypoxia inducible factor-1 alpha (Hif-1α) umgekehrt proportional zur Herabregulierung des EphB3 Rezeptors unter hypoxischen Bedingungen heraufreguliert werden. p-AKT ist ein bekannter Promotor der p53-Degradierung, während p53 ein zentraler anti-proliferativer Faktor ist (*Mayo, L.D. und Donner, D.B., 2002*). Hif-1α reguliert Signalmoleküle, die unter anderem die Stammzellproliferation, -überleben und -differenzierung unter niedrigem Sauerstoff begünstigen (*Panchision, D.M., 2009; Zhao, T. et al., 2008*). Wie in früheren Studien beschrieben, ist p53 ein Glied der EphB3-Signalkaskade und spielt eine wichtige Rolle in der Vermittlung der anti-proliferativen Funktion des EphB3-Rezeptors (*Theus, M.H. et al., 2010*). Wir vermuten also, dass Hypoxie zu erhöhten p-AKT und Hif-1α Werten führt, allerdings ist lediglich p-AKT ein Faktor der EphB3-Signalkaskade. Stimulierung des EphB3-Rezeptors mittels ephrinB3-Fc führt zur Reduzierung von p-AKT aber nicht von Hif-1α; dies deutet darauf hin, dass p53 vermehrt degradiert wird als Folge einer verminderten Exprimierung des EphB3-Rezeptors unter hypoxischen Bedingungen, und dies führt schließlich zu einer Hypoxie-bedingten erhöhten Proliferations- und Zellüberlebensrate der NSVZ.

Diese Daten demonstrieren, dass EphB3 eine maßgebliche Rolle spielt bei der Kontrolle adulter NSVZ in der SVZ, sowohl unter normalen Sauerstoffbedingungen als auch unter Hypoxie. Der EphB3-Rezeptor könnte somit ein Schlüsselprotein bei der Regulierung einer posttraumatischen endogenen, regenerativen Zellantwort der SVZ sein.

II. INTRODUCTION

1. Neurogenesis in adult vertebrates

For a long time in the neuroscience field, it was thought that 'in the adult centers, the nerve paths are something fixed, ended and immutable', as Ramón y Cajal was widely quoted. The brain was believed to be incapable of generating new neurons or including neurons to its complex circuitry. However, the development of new techniques during the past twenty years has lead to a new generation of research demonstrating that neurogenesis constitutively occurs in adult mammals, primarily in two specific brain regions. These are the subventricular zone (SVZ) of the olfactory system and the dentate gyrus of the hippocampus functioning in learning and memory (*Altman, J. and Das, G.D., 1966; Reynolds, B.A. and Weiss, S., 1992; Lois, C. and Alvarez-Buylla, A., 1993; Eriksson, P.S. et al., 1998; Gage, F.H., 2000; Alvarez-Buylla, A. and Garcia-Verdugo, J.M., 2002; Sohur, U.S. et al., 2006; Curtis, M.A. et al., 2011*). Other neurogenic regions with similar properties but smaller size have been found in the central nervous system (CNS) as well, e.g. the cortex, retina, spinal cord and substantia nigra (*Palmer, T.D. et al., 1999; Weiss, S. et al., 1996; Tropepe, V. et al., 2000; Lie, D.C. et al., 2002*). By definition, neurogenesis, the birth of new neurons, comprises the entire set of events from precursor cell division to the development of mature, integrated and functioning new neurons. A number of studies have demonstrated that in the human brain, there are more proliferating cells in the SVZ compared with the hippocampus (*Curtis, M.A. et al., 2011; Lucassen, P.J. et al., 2010*). In the adult CNS, 'stem cells' are defined and characterized by three main features: (1) they have some capacity for self-renewal, (2) they are proliferative, continue to undergo mitosis and can give rise to cells other than themselves through asymmetric cell division and (3) they are multi-potent for the different neuroectodermal CNS lineages and can therefore generate neural and glial tissue (*Gage, F.H., 2000*).

The discovery of resident adult neural stem/progenitor cells (NSPCs) has lead towards subsequent investigations, researching how the brain's germinal regions respond to CNS injuries. Generally spoken, the idea of cellular replacement strategies is appealing for the treatment of ischemic or traumatic CNS injuries as well as neurodegenerative diseases and several others, as they are all associated with the loss of functional neurons and/or glia. It has been observed that adult NSPCs of the SVZ show increased survival and proliferation, migrate and differentiate following CNS damage. This indicates that endogenous neural

progenitor cells could be recruited to replace or support the recovery of damaged tissue as a potential endogenous repair mechanism in the adult brain, although it only occurs in a very limited manner and with controversial functional outcome (*Salman, H. et al., 2004; Arvidsson, A. et al., 2002; Yamashita, T. et al., 2006; Sundholm-Peters, N.L. et al., 2005; Nakatomi, H. et al., 2002*). Interestingly, functional neurogenesis occurs successfully in non-mammalian vertebrates, for example in goldfish undergoing permanent retinal neurogenesis or in songbirds, where new neurons are continuously added to the high vocal centers (*Johns, P.R. and Easter, S.S. Jr., 1977; Goldman, S.A. and Nottebohm, F., 1983*).

Therefore, understanding the organization in the SVZ in regards of cellular proliferation and apoptosis, precursor migration and differentiation in the microenvironment of the neurogenic niche and its changes post-injury might be crucial for developing future therapeutic strategies to promote recovery.

1. 1. The subventricular zone

The mechanisms that govern neurogenesis in the SVZ from stem cell to neuron are tightly regulated. Under normal physiological conditions, SVZ neuroblasts (type A cells) migrate in homotypic chains through pathways distributed throughout the wall of the lateral ventricle (*Doetsch, F. and Alvarez-Buylla, A., 1996; Alvarez- Buylla, A. and García-Verdugo, J.M., 2002*). These chains are ensheathed by the processes of slowly proliferating neural stem cells (type B cells), sharing astrocytelike (glial fibrillary acidic protein (GFAP)-positive) features and giving rise to rapidly dividing progenitor cells (type C cells) (*Lois, C. et al., 1996; Doetsch, F. et al., 1999*). Type C cells are scattered in clusters along the chains of type A neuroblasts, represent transient amplifying progenitors and differentiate into neuroblasts (*Doetsch, F. et al., 1997*). The chains of neuroblasts merge into a structure called the rostral migratory stream (RMS) leading towards the olfactory bulb (OB). Lateral to the ventricle a layer of multi-ciliated ependymal cells (type E cells) lies adjacent to the SVZ; Type B cells interact closely with type E cells and occasionally contact the ventricle lumen as well by reaching out a ciliated process (*Alvarez-Buylla, A. and García-Verdugo, J.M., 2002*). The RMS carries more than 30,000 neuroblasts per day into the OB, where a fraction of these differentiate into interneurons specific to the OB, olfactory granule neurons and a few peri-glomerular interneurons (*Lois, C. and Alvarez-Buylla, A., 1994; Alvarez-Buylla, A. et al., 2001*). Evidence exists that not only lost neurons are replaced but that there is also a net increase in neurons

in the OB over time, in contrast to the hippocampus (*Winner, B. et al., 2002*). The adult SVZ is highly vascularized, showing a prominent network of blood vessels and GFAP-expressing precursors lying intimately close to the vascular surface (*Shen, Q. et al., 2008*). It is suggested that blood vessels serve as a physical substrate for migrating neuroblasts, possibly triggered by brain-derived neurotrophic factor (BDNF) as a molecular signal released from endothelial cells (*Snapyan, M. et al., 2009*). To ensure that there is an adequate pool of stem cells, progenitors, precursors, neuroblasts and neurons at the respective state of differentiation, proliferation and cell death must occur both at the same time in a controlled manner.

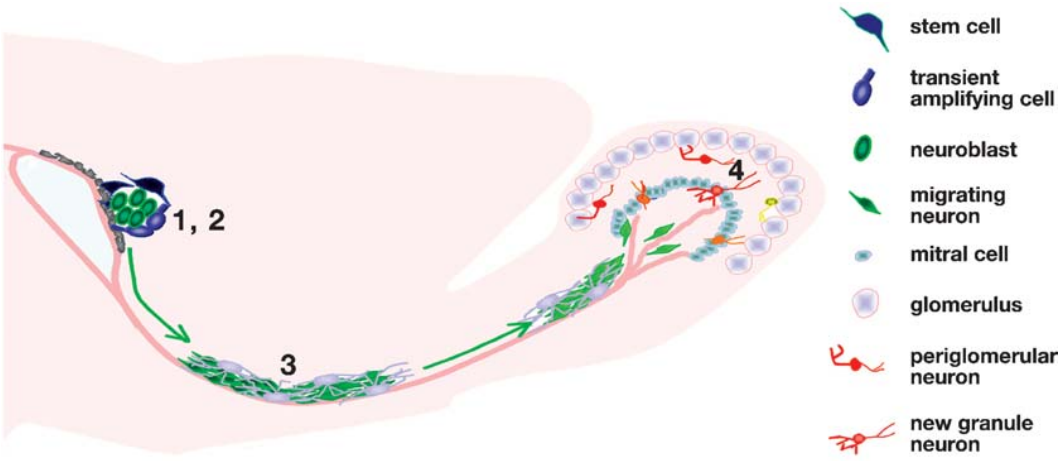


Figure 1. The course of neurogenesis in the SVZ.
Adult neural stem cells proliferate (1), give rise to transient amplifying progenitor cells and start to differentiate (2) into neuroblasts in the lateral side of the ventricle. The neuroblasts migrate in chains (3) towards the olfactory bulb where they integrate synaptically (4) and function as new interneurons, peri-glomerular and granule neurons. (*Figure: Ming, G.L. and Song, H., 2005; with permission from Copyright Clearance Center*)

1. 2. Adult neurogenesis in the SVZ for cellular brain repair

Recent studies have begun to evaluate the potential of adult NSPCs from the SVZ for cellular replacement following CNS injury. Adult neural precursor cells are extremely promising for the goals of CNS cellular repair, as they are undifferentiated, often highly mobile cells, relatively resistant to hypoxia and injury, actively proliferate, and are able to produce mature neurons and glia (*Sohur, U.S. et al., 2006*). It has been demonstrated that proliferating cells migrate from the SVZ to cortical and subcortical structures in mice affected by TBI, and that some of these cells are migrating neuroblasts (*Ramaswamy, S. et al., 2005*).

Experimental ischemic injury, caused by transient middle cerebral artery occlusion in adult rats, leads to increased cell proliferation in the SVZ, neuroblast migration, differentiation into neurons, and integration in the damaged striatum (*Arvidssen, A. et al., 2002*). Apoptotic degeneration of corticothalamic projection neurons has also been shown to increase NSPC migration, neural differentiation and induce formation of appropriate longdistance connections with long-term survival in the adult CNS (*Magavi, S.S. et al., 2000*). Interestingly, neurogenic regions also respond to neurodegenerative diseases, e.g. Alzheimer‘s disease, where neuronal replacement in the hippocampus dentate gyrus is increased (*Jin, K. et al., 2004*). In Huntington‘s disease, the SVZ is significantly thicker (2.8-fold), has an altered cellular composition and contains an increased number of proliferating cells compared with the normal SVZ (*Curtis, M.A. et al., 2003; Curtis, M.A. et al., 2005*). Conversely, dopamine depletion in Parkinson‘s disease leads to a decrease in precursor cell proliferation of both neurogenic regions via reduction of D2-like receptor signaling, which is evident in postmortem human brains of affected individuals (*Hoglinger, G.U. et al., 2004*).

After focal cortical ischemic injury in rats Leker and colleagues describe that SVZ-derived progenitors migrate through white matter and form a regenerative zone adjacent to the injured tissue (*Leker, R.R., 2007*). Furthermore it is mentioned that this is an ongoing process that occurs over long periods of time. The functional relevance of this SVZ proliferation, altered migration and neuronal differentiation following CNS injury and disease remains poorly defined, but it may in part explain some of the spontaneous recovery that can occur after CNS injury. Furthermore, it has been shown that manipulations through stimulating factors which are directed to increase neurogenesis have not only lead towards a higher number of proliferating and migrating precursors from the SVZ but have also improved functional outcome (*Leker, R.R. et al., 2007; Ohab, J.J. et al., 2006*). Most importantly, the major challenges for endogenous repair in the adult brain seem to be inducing sufficient proliferation and migration of progenitor cells to repair the damaged tissue as well as encouraging cellular survival and functional integration in the ischemic environment to which they migrate.

1. 3. *In vitro* culture of adult NSPCs

After isolation of adult NSPCs from neurogenic regions of the brain, there are two main forms how to maintain and culture them. Traditional approaches for the isolation

and maintenance of NSPCs have relied on the *in vitro* culture as floating neurospheres, but recent development of cultural techniques has provided an alternative for maintaining immature marker nestin-positive progenitors derived from the SVZ in adherent, monolayer conditions (*Scheffler, B. et al., 2005*). An advantage of culturing NSPCs in monolayers is that it permits each cell to receive similar amounts of nutrients and oxygenation, which does not occur in neurospheres, and also permits investigators more accurate assessment to quantify cellular changes in proliferation, differentiation and survival rates. Through the substitution of growth factors like epidermal and basic fibroblastic growth factors (EGF and bFGF) in culture, monolayer adult NSPCs maintain their immature and mitotic state (*Reynolds, B.A. and Weiss, S., 1992*). Although the regional *in vivo* microenvironment as a whole cannot be mimicked *in vitro*, cultural approaches are indispensable to investigate cellular events such as self-renewal and differentiation as well as the local influence of drug actions.

2. The effect of hypoxia on adult NSPCs in culture and in situ

Low oxygen culture has been shown previously to maintain undifferentiated states of embryonic, hematopoietic, mesenchymal, and neural stem cell phenotypes in culture and influences a number of various stem cell types via changes of proliferation and cell-fate (*Mohyeldin, A. et al., 2010*). Investigations of embryonic neural precursors grown in hypoxic culture showed that in contrast to the response of adult neurons/glia, proliferation and survival were enhanced, and a higher percentage of neurons was generated (*Studer, L. et al., 2000; Morrison, S.J. et al., 2000; Zhao, H.Q. et al., 2004*). Human fetal mesencephalic precursor cells from 9- to 12-week-old fetal brains showed long-term proliferation when cultured in low oxygen (3%) and could give rise to dopaminergic neurons (*Storch, A. et al., 2001; Storch, A. et al., 2003*). *In vivo* studies in adult male Wistar rats showed that the numbers of proliferating (BrdU-positive) cells in the SVZ and dentate gyrus increased greater than 62% and 35%, respectively, following intermittent hypoxia mimicking high altitude (*Zhu, L.L. et al., 2005*). Interestingly, physiologic oxygen concentrations in situ in the brain, although consuming a large portion of bodily oxygen, are substantially lower than the 21% found in the atmosphere with values ranging from 8% (19–40 mmHg) in the pia down to 0.55% (4.1 mmHg) in the midbrain (*De Filippis, L. and Delia, D., 2011; Erecinska, M. and Silver, I.A., 2001*). Severe hypoxia refers to tissue oxygen levels <1% and can in turn lead to quiescence and apoptosis of fetal human neural stem cells in culture (*Santilli, G. et al., 2010*). Hypoxia further occurs under pathological conditions such as stroke and TBI and the changes of

tissue oxygen tension and their consequences are a large subject for research (*Maas, A.I. et al., 1993; Zauner, A. et al., 1996; van den Brink, W. et al., 2000; Bader, M.K., 2006*). After middle cerebral artery occlusion in rats, which is commonly used as a model for stroke, pO_2 levels dropped from 33.4 mmHg to 10.7 mmHg in the penumbra and to 1.2 mmHg in the ischemic core 1 hour post-occlusion (*Liu, S. et al., 2004*). These studies demonstrate that hypoxic exposure takes part in the pathophysiology of CNS damage and indicate that tissue oxygen levels will contribute to the outcome after CNS injury.

The molecular mechanisms underlying these hypoxia-mediated changes in neural precursor cell biology are not well understood. Various pathways including Notch1, Bone morphogenetic protein (BMP) and Wnt signaling have been implied in the regulation of cellular behaviors of neural stem cells in the hypoxic environment (*Zhang, K. et al., 2011*). One central role of the oxygen-sensitive pathway is played by hypoxia-inducible factors (HIFs), transcription factors which are stabilized under hypoxia. Their activation leads to the induction of an hypoxia-adaptive phenotype through up-regulation of more than 50 downstream genes, e.g. vascular endothelial growth factor (VEGF) and erythropoietin (EPO), functioning in erythropoiesis, glycolysis and angiogenesis amongst others (*Ke, Q. and Costa, M., 2006; Keith, B. and Simon, M.C., 2007; Panchision, D.M., 2009*). In neurogenesis, HIF-1α has been demonstrated to be crucial in the process mediating the positive effect of moderate hypoxia on the proliferation of embryonic NSPC (*Zhao, T. et al., 2008*).

3. Further factors possibly involved in the promotion or inhibition of SVZ neurogenesis post-injury

The exact mechanisms that initiate and regulate the SVZ response to CNS injury are not well understood. Studies investigating the changes in gene expression in the SVZ post-TBI revealed that of 9,596 genes screened, 97 were up-regulated and 204 were down-regulated (*Yoshiya, K. et al., 2003*), which implies a certain level of complexity in the SVZ response. Among those are genes encoding transcription factors, extracellular messengers, membrane proteins and many others, suggesting that brain injury influences a broad range of cellular functions in the SVZ. After ischemic injury to the brain, several diffusible factors have been shown to influence the response of endogenous repair, including EGF and bFGF, BDNF, glial-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF-1), VEGF, stem cell factor (SCF), erythropoietin (EPO)/EPO-receptor signaling, nitric oxide

and enriched environment (*Dempsey, R.J. et al., 2003; Jin, K. et al., 2002-1/2; Tsai, P.T. et al., 2006; Wang, L. et al., 2004; Wang, Y. et al., 2007; Yan, Y.P. et al., 2006; Yoshimura, S. et al., 2001; Zhu, D.Y. et al., 2003; Wiltrout, C. et al., 2007; Kuhn, H.G. et al., 1997; Zhang, R. et al., 2001; Leker, R.R. et al., 2007*). Conversely, decreased expression of growth factors impairs SVZ neurogenesis after stroke (*Chen, J. et al., 2005; Tsai, P.T. et al., 2006; Yan, Y.P. et al., 2006*). In addition to diffusible factors, local factors present in the extracellular regions and the surrounding tissues may also influence endogenous adult NSPCs.

It is important to note that successful neurogenesis also depends on the neurogenic permissiveness of the local environment, suggesting neurogenesis depends on both intrinsic and extrinsic responses. Precursors have been found in various CNS regions, for example in the spinal cord, and transplantation of these cells into the 'neurogenic region' of the hippocampus resulted in the generation of region-specific neurons, whereas when transplanted back into the spinal cord, only glial cells are produced (*Shihabuddin, L.S. et al., 2000*). Similarly, when SVZ precursors were transplanted into the hippocampus they generated hippocampal neurons, as well as hippocampal precursors generated olfactory interneurons when transplanted into the RMS (*Suhonen, J.O. et al., 1996*). Interestingly, adult neural precursors have been shown to have a broad potential for differentiation which is not limited to the neuroectodermal cell lineage, as they integrate into the hematopoietic system following intravenous application into irradiated mice (*Bjornson, C.R. et al., 1999*) and can contribute to the formation of chimeric chick and mouse embryos, giving rise to cells of all germ layers (*Clarke, D.L. et al., 2000*). Therefore, adult NSPCs are able to respond to and rely on various environmental cues in order to proliferate, migrate, differentiate and integrate functionally.

Similar or connected to the importance of local environment, vascularization seems to highly influence neurogenesis and the migration of neuroblasts to regions of ischemic damage. As mentioned above, vascularization is highly present in the SVZ and guides physiologic neurogenesis. In the ischemic striatum, many neuroblasts are found in close proximity to blood vessels (*Ohab, J.J. et al., 2006; Thored, P. et al., 2007; Yamashita, T. et al., 2006*), and infusion of pro- or antiangiogenic factors stimulates or inhibits neuroblast migration to peri-infarct regions respectively, indicating a tight link between angiogenesis and neurogenesis (*Ohab, J.J. et al., 2006*). VEGF, which is a downstream factor of HIF-1α in hypoxia, stimulates proliferation of neural precursors *in vitro* and *in vivo* (*Jin, K. et al., 2002-1*), and VEGF over-expression leads to an increase in the number of migrating precursors

and newly generated cortical neurons, resulting in reduced infarct volume and improved post-ischemic motor function (*Wang, Y. et al., 2007*). As angiogenesis and VEGF are tightly linked to and augmented under hypoxia, they might represent strong factors contributing to hypoxia-induced proliferation and survival in the neurogenic niche.

In summary, proliferation, survival and migration of SVZ-derived NSPCs and neuroblasts following CNS injury might be regulated by a number of molecular mechanisms that synergize to orchestrate a neurogenic response to endogenous repair.

4. Ephrins and Eph receptors

As the adult SVZ maintains developmental features such as proliferation and migration of NSPCs, it has been proposed that the regulation of such events requires factors important during embryonic and fetal processes. Erythropoietin-producing hepatocellular (Eph) receptors/ephrin ligands are a group of membrane-bound growth and guidance molecules highly expressed in the CNS throughout development which are down-regulated in most regions following birth (*Liebl, D.J. et al., 2003*). Exceptions include neurogenic regions and high plasticity centers in the brain, which is why they have been recently implicated in proliferation and survival of adult NSPC cells in the SVZ (*Ricard, J. et al., 2006*). Eph receptors are the largest known subgroup of transmembrane receptor tyrosine kinases and currently number 16 members in warm-blooded vertebrates (*Gale, N.W. et al., 1996; Friedman, G.C. and O'Leary, D.D., 1996*). They have been implicated in developmental patterning, axonal guidance and fasciculation, neural crest migration, midline development, and synaptic plasticity (*Gale, N.W. et al., 1996; Henkemeyer, M. et al., 1996; Orioli, D. et al., 1996; Torres, R. et al., 1998; Gao, W.Q. et al., 1998; Gao, P.P. et al., 1999; Feng, G. et al., 2000*). The Eph family is divided into two distinct receptor subfamilies, EphA (1-10) and EphB (1-6), both of which bind to membrane-anchored ephrin ligands. The EphA receptors can each bind a group of six A-class ephrin ligands that are anchored to the membrane via a glycosylphosphatidylinositol (GPI) linkage, while the EphB receptors can interact with three related transmembrane spanning B-class ephrins (*Blits-Huizinga, C.T. et al., 2004*). Upon cell contact, two Eph receptors and two ephrins can form heterotetramers, which are further arranged into higher-order multimeric signaling complexes (Fig. 2). Of all the receptors, only EphA4 and EphB2 have been shown to bind with high affinity to both the A-class and B-class ephrins. A broad overlap of binding specificities within the subclasses suggests shared activities and

possible redundancy among family members; EphB3 receptor for example binds to all ephrinB ligands but not to ephrinA ligands, whereas ephrinB3 binds strongest to EphB3 and EphA4, and more weakly to EphB1 and EphB2 (*Orioli, D. and Klein, R., 1997*). B-class ephrins and all Eph receptors have intracellular signaling capacities that are elicited by ligand-receptor interactions (*Henkemeyer, M. et al., 1996; Holland, S.J. et al., 1996; Brückner, K. et al., 1997*). Eph receptors share common features of receptor tyrosine kinases (RTK) (*Pawson, T., 1995; Pawson, T. and Scott, J.D., 1997; Kalo, M.S. and Pasquale, E.B., 1999*). Unlike many other RTK, Eph receptors are membrane-tethered molecules that function through cell-cell interaction requiring close cellular contact and not through diffusible gradients. This provides a mechanism by which Eph receptors and ephrins can function as cell autonomous receptors and ligands. The ensuing signals can propagate bidirectionally into both the Eph (A or B) -receptor-expressing cells (in a process known as forward signaling) and the ephrinB-expressing cells (reverse signaling) (*Holland, S.J. et al., 1996; Brückner, K. and Klein, R., 1998*). Due to the complexity of downstream signaling and broad binding possibilities the effects produced by Eph/ephrin signaling vary widely, e.g. both repulsive and adhesive functions in cells are mediated (*Orioli, D. and Klein, R., 1997; Zimmer, M. et al., 2003*).

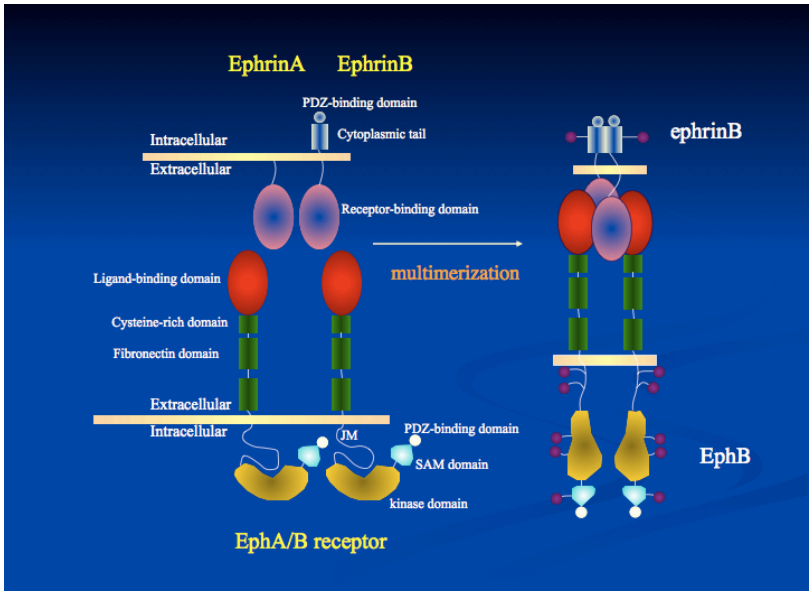


Figure 2. Structure and multimerization of Eph receptors and ephrin ligands.
A-class ephrins consist of an extracellular receptor-binding domain linked to the membrane through a GPI anchor, while B-class ephrins have transmembrane and cytoplasmic domains and a C-terminal PDZ-binding motif. Eph receptors have an N-terminal extracellular ligand-binding domain, followed by a cyteine-rich domain and two fibronectin type III repeats, a transmembrane region and a cytoplasmic region containing a juxtamembrane (JM) domain, tyrosine kinase domain, C-terminal sterile a-domain (SAM), and PDZ-binding motif. Cis-dimerized Eph receptors will bind with cis-dimerized ephrin ligands forming a trans-heterotetramer. Tetramerization leads to repositioning of several domains, and allows the formation of multimeric complexes. Upon binding, the intracellular domains of both ephrins and Eph receptors get phosphorylated at specific residues initiating bidirectional signaling (*Legend: Blits-Huizinga, C.T. et al., 2004; Figure: Dr. Jerome Ricard, laboratory Dr. Daniel J. Liebl*)

4. 1. The role of Eph/ Ephrin signaling in regulation of SVZ neurogenesis

Previously, Ephrins and Eph receptors have been investigated extensively for their role in developmental control of axon growth and guidance, midline development etc., but recently these molecules have also been implicated in regulate proliferation, survival and migration in the adult SVZ (*Conover, J.C. et al., 2000; Holmberg, J. et al., 2005; Depaepe, V. et al., 2005; Katakowski, M. et al., 2005; Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). The role of ephrins and Eph receptors in neurogenesis is just beginning to be elucidated. Conover and colleagues have shown that *in vivo* disruption of ephrins and Eph receptors can result in altered proliferation and migration of neuroblasts in the adult mouse SVZ (*Conover, J.C. et al., 2000*). These early infusion studies, however, were unable to elucidate the mechanisms of specific ephrins/Eph receptors involved in regulating SVZ functions. Addition of clustered EphB2 to neurosphere cultures directly activated the proliferation of SVZ precursors and promoted a neuronal fate (*Katakowski, M. et al., 2005*). Moreover, the A-class was implicated in the control of neurogenesis: EphA7- induced ephrinA2 reverse signaling was identified as negative regulator of neural precursor proliferation (*Holmberg, J. et al., 2005*). Previous investigations in our laboratory have identified ephrinB3 as negative regulator of proliferation in the SVZ, using genetic approaches through specific gene targeted knockout mice (*Ricard, J. et al., 2006*). In absence of ephrinB3, both the rate of proliferation and cell death are increased in SVZ-progenitors (*Ricard, J. et al., 2006*), whereas in absence of its receptor EphB3, proliferation is increased but survival improved (*Theus, M.H. et al., 2010*). Morphological and RT-PCR analysis showed that ephrinB3 ligand is only expressed outside the SVZ, in the striatum lining the area next to the lateral ventricle, while its major binding partners, EphB3 and EphA4, are expressed on NSPCs within the SVZ (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). A new role was found for EphA4 as "dependence receptor" in NSPCs, as pro- or anti-apoptotic signaling will be initiated, depending on the presence of ligand ephrinB3 (*Furne, C. et al., 2008*). Dependence receptors are involved in tumorigenesis and can play supportive roles during homeostasis, such as anti-apoptotic signaling when their respective ligand is present and binds to the receptor, but in the absence of ligand stimulation the receptor reverts to pro-apoptotic functions (*Mehlen, P. and Bredesen, D.E., 2004; Mehlen, P. and Thibert, C., 2004; Tauszig-Delamasure, S. et al., 2007*). We further examined the role of ephrinB3-EphB3 interactions in regulation of proliferation and survival in the SVZ following unilateral CCI injury (*Theus, M.H. et al., 2010*): proliferation in the SVZ was augmented in mice deficient in either ephrinB3 or EphB3 compared to wild

type, which could be restored via infusion of ephrinB3-Fc only in ephrinB3-deficient and wild type mice (but not EphB3-deficient), showing that loss of EphB3 forward signaling triggers increased SVZ-proliferation. EphB3-induced suppression of NSPC proliferation was further shown to be mediated by downregulation of p-AKT and subsequent p53 activation. Furthermore, cell death in the SVZ was decreased in EphB3-deficient but increased in ephrinB3-deficient mice compared to wild type mice, being reduced back to normal levels via infusion of ephrinB3-Fc in ephrinB3-deficient mice only; this suggests that EphB3 receptor may function as pro-apoptotic dependence receptor in the SVZ *in vivo*, similarly as shown for EphA4 *in vitro*. EphB3 expression is down-regulated in the adult SVZ after brain injury, which might represent a mechanism to induce proliferation and survival in the SVZ post-injury (*Theus, M.H. et al., 2010*).

4. 2. p53 and p-AKT are involved in neurogenesis and Eph downstream pathways

The protein p53, synonymously known as „the guardian of the genome", plays a pivotal role in regulating anti-proliferative and pro-apoptotic functions (*Roger, L. et al., 2006; Bose, I. and Ghosh, B., 2007; Vogelstein, B. et al., 2000*). Recent studies have shown that p53 is an important regular of adult neurogenesis, i.e. p53 activation suppresses proliferation in the adult SVZ (*Medrano, S. and Scrable, H., 2005; Meletis, K. et al., 2006; Medrano, S. et al., 2009*). p53 stabilization is known to be regulated by various pathways, including the interaction with phosphorylated AKT (p-AKT). p-AKT signaling suppresses p53 function by phosphorylation and activation of Mdm2, which translocates to the nucleus, ligates ubiquitin to p53 to target it for degradation by the proteasome (*Mayo, L.D. and Donner, D.B., 2002*). Our previous studies show that basal p53 expression is reduced *in vivo* in the SVZ of ephrinB3-deficient and EphB3-deficient mice compared to wild type, and it has been demonstrated that EphB3 forward signaling suppresses NSPC proliferation through up-regulation of p53 (*Theus, M.H. et al., 2010*). Since p-AKT levels are reduced when ephrinB3-Fc is infused in wild type mice but remain unchanged after infusion in EphB3-deficient mice, p53 stability is directly regulated by EphB3 via down-regulation of p-AKT activity (*Theus, M.H. et al., 2010*). It has been previously shown that p-AKT is suppressed downstream of Eph receptor activation (*Ricard, J. et al., 2006, Pasquale, E.B., 2008; Menges, C.W. and McCance, D.J., 2008*).

5. Specific aims of this work

The long-term objectives of these studies are to provide a better understanding of stem cell biology and the cellular and molecular changes post-CNS injury, as well as to contribute to the development of therapeutic strategies for recovery following CNS injury, e.g. through the promotion of endogenous repair by SVZ-derived progenitors. As an initial step we aimed to further elucidate the mechanisms that regulate proliferation and survival of adult NSPCs in the SVZ. In the present studies, we investigated the role of EphB3-ephrinB3 signaling in mediating proliferation and survival of SVZ-derived adult NSPCs in monolayer culture under normoxic and hypoxic conditions. The following issues were addressed:

A. Role of ephrinB3-EphB3 signaling in mediating proliferation and survival of SVZ-derived progenitors under ambient oxygen conditions.

Based on previous findings, we hypothesized that EphB3 forward signaling negatively regulates proliferation and survival of adult NSPCs derived from the SVZ. We employed loss-of-function (EphB3 knockout) and gain-of-function (addition of clustered ephrinB3) approaches to assess this matter.

B. Expression of different Eph/ephrins in adult NSPC monolayer culture.

Concurrent with previous results, we strongly hypothesized that ligand ephrinB3 is not expressed in monolayer culture of adult SVZ-derived progenitors. To prove this, we examined the expression of different Eph/ephrins on a transcriptional (RT-PCR) and on a translational level (immunohistochemistry). Further studies in our laboratory included morphological studies of ephrinB3 expression.

C. Development of the hypoxia model.

Studies in our laboratory aimed to examine oxygen changes in the brain tissue post-TBI. We employed a specific stain (hypoxyprobe) to visualize tissue hypoxia on brain slides following CCI to then use a similar oxygen tension for *in vitro* culture of NSPCs.

D. Expression of EphB3 receptor in hypoxic wild type adult NSPCs

We aimed to investigate whether EphB3 expression is altered under conditions of lowered oxygen. We hypothesized that a change of EphB3 expression in adult NSPCs could result in significant changes of survival and proliferation under hypoxic conditions. To examine levels of EphB3 expression, we performed Western Blot analysis.

E. Role of ephrinB3 in mediating proliferation and survival of adult NSPCs under hypoxic conditions

We hypothesized that ephrinB3-EphB3 signaling is involved in regulating hypoxia-mediated changes of adult NSPC survival and proliferation. To address this issue, we employed a gain-of-function (addition of clustered ephrinB3) approach under hypoxic culture conditions.

F. Levels of p-AKT and HIF-1α during hypoxic culture conditions of wild type adult NSPCs

In order to investigate whether expression of p-AKT and HIF-1α are changed in response to hypoxic culture and whether these changes depend on ephrinB3/EphB3 signaling, we performed Western Blot analysis of wild type adult NSPCs, with and without addition of ephrin ligands. We hypothesize that levels of p-AKT and HIF-1α might be part of a mechanism regulating proliferation and/or survival in adult NSPCs under hypoxic conditions.

III. MATERIALS AND METHODS

1. Animals and tissue preparation

1. 1. Dissection of adult mouse neural stem/progenitor cells

Both wild type and mutant CD1 mice were housed in a 12h light/dark cycle with *ad libitum* (*lat.* freely, as desired) access to food and water. Surgery and animal care met or exceeded institutional guidelines. The animals were treated in accordance with the guidelines of the NIH and the University of Miami Miller School of Medicine. The generation of the mutant CD1 mice has been described (*Henkemeyer, M. et al., 1996; Orioli, D. et al., 1996; Cowan, C.A. et al., 2000; Yokoyama, N. et al., 2001*). Genotyping was performed by PCR analysis (Promega). Animals were sacrificed by decapitation under anesthesia.

DVR cocktail for anesthesia:

Ketamin..... 43 mg/ml

Xylaxine..... 8.6 mg/ml

Acepromazine..... 1.4 mg/ml

Dose: 0.5 – 0.7 ml/kg for mice (intramuscular or subcutaneous administration)

Adult neural stem/progenitor cells (NSPCs) were isolated from the lateral ventricle wall of 2-4 months old CD1 wild type and EphB3$^{-/-}$ mutant mice. Under anesthesia the skullcap was opened and the brain was removed completely and placed into Leipovitz-15 medium (Gibco) with penicillin/streptomycin (Invitrogen). One mm coronal slices corresponding to anteroposterior coordinates from +1.0 mm to 0 relative to bregma were cut. The ventricle wall on the striatal side was dissected out and the tissue was dissociated in 1.33 mg/ml trypsin, 0.7 mg/ml hyaluronic acid and 0.2 mg/ml kynurenic acid (all Sigma) in Hank's balanced salt solution (HBSS; [Invitrogen]).

1. 2. Transfer of adult NSPCs to tissue culture as monolayers

Monolayer media:

DMEM/F12 (Gibco)

N-2 supplement (Invitrogen)

Fetal Bovine Serum(FBS; HyClone).....5 %

Bovine Pituitary Extract (BPE; Invitrogen)..... 35 µg/ml

Penicillin (Invitrogen) ..... 100 units/ml

Streptomycin (Invitrogen)..... 100 µg/ml

Epidermal Growth Factor (EGF; Sigma) ..... 20 ng/ml

Basic Fibroblast Growth Factor (bFGF; Sigma) ..... 20 ng/ml

Isolated adult neural stem/progenitor cells were cultured as monolayers according to Scheffler and colleagues (*Scheffler, B. et al., 2005*). Briefly, dissociated tissue was placed overnight in monolayer media. Following overnight incubation, unattached cells were collected, gently triturated and replated onto a fresh uncoated 100mm bacterial petri dish. EGF and bFGF were freshly added to the medium every other day. Once the cells reached approximately 70-80% confluency in the dish, they were passed by using sterile 0.05% Trypsin/ EDTA (Invitrogen) for 5 min at 37 ºC. One volume monolayer media was added to stop the trypsin reaction and the cells were spun down for 5 min at 1500 rpm. Cells were resuspended in monolayer media and counted with the hemocytometer (Hausser) to plate 0.5 x 106 cells in one 100mm dish. For long-term storage, cells were placed in cryogenic vials (Corning) in 85 % DMEM/F12, 5 % FBS and 10 % Dimethylsulphoxide (DMSO; [Sigma]) at - 80°C. For all experiments used in this study, the passage number did not exceed seven passages.

2. Cell culture experiments

2. 1. Placement of adult NSPCs

Cells were plated at 2,000 cells/ well (96-well plates) containing 100µl monolayer media. Cells were grown at 37ºC, 21% O_2, 5% CO_2, for 24, 48 or 72 hrs to assess proliferation (Quick cell proliferation assay/ percentage of BrdU-positive cells) and cell death (LIVE/

DEAD assay). For experiments investigating the effect of EphB3 stimulation, pre-clustered ephrinB3 protein aggregates were added to the media 24 hrs after plating to reach a final concentration of 5µg/ml per well (cells harvested 24 hrs after) or 10µg/ml per well (cells harvested after up to 72 hrs in culture). Equal amounts of elution buffer (vehicle) were added as negative control.

EphrinB3 protein aggregate: Fusion protein of mouse extracellular domain of ephrinB3, aminoacids 28-227 (345 total + His Tag for purification, MW 22 kD+17.7 kD TRX Tag

Elution buffer (vehicle):
- 50 mM Na-phosphate pH 7.0
- 150 mM Imidazole (covers His Tag)
- 300 mM NaCl

2. 2. Hypoxic Treatment

In order to determine the effects of hypoxia on aNSPC growth, cells were passed and 2,000 cells were plated into each well of a 96-well plate containing 100µl monolayer media. Following overnight incubation at 37°C, 21% O_2, 5% CO_2, plates either remained in same control environment (referred to as normoxia) or were placed in an airtight oxygen controlled ProOx-C-chamber housed in a 37 °C incubator to maintain constant and appropriate temperature and humidity. The hypoxia chamber was maintained at 1% O_2, 5% CO_2 by infusing a balanced Nitrogen gas mixture in order to reach and maintain the oxygen level at 1%, which was measured and controlled by a ProOx-C oxygen sensor (Model No. 110). Normoxic control was provided according to each time point.

2. 3. Quick cell proliferation assay

Proliferation was examined at different times in normoxia and hypoxia using Quick cell proliferation assay (BioVision). The Quick cell proliferation assay is based on the cleavage of the tetrazolium salt (WST-1) to formazan by cellular mitochondrial dehydrogenases. Expansion in the number of viable cells results in an increase in the activity of the mitochondrial dehydrogenases, which leads to the increase in the amount of formazan dye formed. Therefore, both the generation of new cells and cell loss are included in this assay. For assess-

ment, 10 µl of WST-1/ECS solution were added to each well containing 100µl media. After three hours of incubation, plates were shaken for 1 min and formazan dye was quantified by measuring absorbance at 450nm for 0.1sec, using a multi-well spectrophotometer.

2. 4. BrdU preparation and analysis

Bromodesoxyuridine (BrdU; [Sigma]) powder was dissolved in 1N NaOH, neutralized in equal amounts of 1N HCl and diluted to a concentration of 10 mg/ml in 1x PBS. For sterile use *in vivo* and *in vitro*, it is filter sterilized and stored at -20°C. We pulsed cells with 10µM bromodesoxyuridine (BrdU) for one hour before fixation with 10% buffered formalin and staining with anti-BrdU antibody (1:100; [Roche]). BrdU as nucleotide substitute replaces thymidine with uridine in the DNA structure of dividing cells, therefore identifying BrdU-positive cells as mitotic, during the S-phase of mitosis. Hoechst stain 33342 (Molecular Probes) was used for nuclei staining. Employing Cellomics cell count (TRITC/DAPI filters) we assessed proliferation as the percentage of Hoechst-positive cells. Cells were analyzed under TRITC/DAPI filters on the fluorescent Axiophot microscope (Zeiss) equipped with a CCD camera. Pictures were processed in Axiovision software. For quantification of the BrdU analysis, six images per well were acquired by using the Cellomics kinetic scan HCS reader (Thermo Fisher) and quantified as percentage of proliferation by VHCS scan software (TRITC average intensity greater than 100).

1x Phophate buffered saline (PBS):

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM

2. 5. LIVE/DEAD assay

Cell death of adult NSPCs in culture was assessed by the addition of SYTOX orange (Molecular Probes) and Hoechst stain (LIVE/DEAD assay) for staining dead and total nuclei. 1-2 µl SYTOX and Hoechst dyes were added per well, mixed with the media and after 10 min incubation we analyzed the percentage of cell death, employing Cellomics cell count (see BrdU analysis).

3. Molecular methods

3. 1. RT-PCR

Total RNA of monolayers was isolated by applying Trizol reagent (Invitrogen), following the manufacturer's protocol. RNA quantification was carried out by measuring absorbance with spectrophotometer ND-1000 (NanoDrop). For DNA contamination, RNA was DNase-treated (Deoxyribonuclease [DNase] I Amplification Grade [Invitrogen]). RNA was reverse transcribed into cDNA with Im-Prom II Reverse Transcription System (Promega). The PCR products were loaded on 1% agarose gel and were visualized with ethidium bromide. PCR analysis was performed for the genes of interest EphB1, B2, B3, A4 and ephrin B1, B2, B3 (Nova Taq Polymerase [Novagen] or GoTaq Flexi DNA Polymerase [Promega]). The reverse transcriptase was omitted in the negative control reaction and cDNA generated from adult mouse brain RNA were used in the positive control reaction. Both controls were applied for each PCR reaction.

Primers:

EphB1 _forward.....5' ATC CGG AAC CCA GCT AGT CTC AAG 3'
EphB1 _reverse.....5' GGT GGT AAA GGC CGT GAA GTC TG 3'
EphB2 _forward.....5' ATC CGG AAC CCA GCT AGT CTC AAG 3'
EphB2 _reverse.....5' GGT GTG TAA TGT GCG TGA GT 3'
EphB3 _forward.....5' GGT GTG TAA TGT GCG TGA GT 3'
EphB3 _reverse.....5' TGG TGT CCA CTT TCA CGT AG 3'
EphA4 _forward.....5' AGG AAG TGA GCA TTA TGG ATG A 3'
EphA4 _reverse .....5' TGC TCC TCG TGC CCA GCG TT 3'
EphrinB1 _forward.....5' CAC CAT CAA GTT CCA AGA T 3'
EphrinB1 _reverse.....5' CTT CTC TCC TGG TTC ACA G 3'
EphrinB2 _forward.....5' TGG AAG TAC TGT TGG GGA CT 3'
EphrinB2 _reverse .....5' TCA CAT CTT GGT CTG GTC TG 3'
EphrinB3 _forward.....5' GGG ACC GGC TAG ATC TAC TT 3'
EphrinB3 _reverse .....5' GGC TGT ATT CCT GGA ACT TG 3'

3. 2. Western Blot analysis

Protein of monolayer cultures was extracted by lysing cells in RIPA buffer in the presence of complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail II (Sigma). Cells were mechanically scraped off the plate using a cell lifter and transferred to a 1.7 ml tube. Supernatant was collected by centrifuging at 13.000g for 30min at 4ºC and Lowry assay (high) kit (BioRad) was used for determination of protein concentration (reading absorbance at 650 nm with a spectrophotometer). 6x sample buffer was added to protein (1:5) and the protein samples were separated on 8% SDS-PAGE gels in 1x Running buffer. We blotted onto PVDF membranes in 1x Transfer buffer and blocked with 5% BSA (Bovine serum albumin; [Calbiochem]) or 7% milk in TBST buffer. Primary and HRPconjugated secondary antibodies in block were applied to the membrane using different concentrations and incubation times (see table below). After each antibody incubation membranes were washed in TBST at least 4 x 10 min. Secondary antibodies bound to horse-raddish peroxidase (HRP) and chemiluminescent HRP substrate (Pierce) were added before the film was developed. Blots were quantified by densitometry using acquisition into Adobe Photoshop and analyzing by the NIH Image software (National Institutes of Health, Bethesda, MD, USA). For removing antibody binding, membranes were placed in stripping buffer at 50ºC for 30 min while rotating. The level of protein expression was normalized according to β-actin controls or total AKT levels. Samples were run in triplicate.

RIPA buffer:

Nonidet P-40	1 %
Sodium-deoxycholate	1 %
SDS	1 %
NaCl	0.15 M
EDTA	2 mM
Sodium phosphate pH 7.2	0.01 M

8 % SDS-PAGE:

Tris HCl	1.5 M
Acrylamide	30 %
SDS	10 %

APS ..... 10 %

TEMED ..... 5 µl

dH_2O ..... to total 10 ml

6x sample buffer:

4x Tris HCl/SDS (pH 6.8)

Glycerol ..... 30 %

SDS ..... 10 %

Dithiothreitol (DTT) ..... 0.6 M

Bromphenol blue ..... 0.012 %

1x Running buffer: (pH 8.3-8.6)

Tris base ..... 24.8 mM

Glycine ..... 250.5 mM

SDS ..... 0.1 %

1x Transfer buffer:

Tris base ..... 25 mM

Glycine ..... 190 mM

SDS ..... 0.05 %

Methanol ..... 20 %

TBST buffer: (pH 7.5)

Tris HCl ..... 100 mM

NaCl ..... 150 mM

Tween 20 ..... 0.1 %

Stripping buffer:

Dithiothreitol (DTT) ..... 50 mM

SDS ..... 2 %

Tris HCl (pH 6.7) ..... 62.5 mM

Primary and secondary antibodies (purchased from)	Dilution / Incubation
Mouse anti-EphB3 (ABCAM)	1:1000 / overnight at 4ºC
Mouse anti-HIF-1α (Novus)	1:2000 / overnight at 4ºC
Rabbit anti-p-AKT (Cell Signaling)	1:4000 / overnight at 4ºC
Rabbit anti-AKT (Cell Signaling)	1:4000 / overnight at 4ºC
Mouse anti-β-actin (Sigma)	1:5000 / overnight at 4ºC
Goat anti-mouse IgG (Jackson laboratory)	1:5000 / 3 hrs. at RT
Donkey anti-rabbit IgG (Jackson laboratory)	1:5000 / 3 hrs. at RT

4. Immunohistochemistry

For staining, monolayer cultures were fixated in 10% buffered formalin and washed in 1x PBS. For antigen retrieval, wells were placed in a 2:1 ethanol:acetic acid solution. Cells were permeabilized in 0.4% Triton-100X and blocked in 5% BSA/0.2% Triton-100X before the application of primary and secondary antibodies (for dilution of antibodies and incubation time see table below).

Primary and secondary antibodies (purchased from)	Dilution/ Incubation
Mouse anti-nestin (Millipore)	1:1000 / overnight at 4ºC
Goat anti-EphB3 (Abcam)	1:400 / overnight at 4ºC
Rabbit anti-EphA4 (Abcam)	1:400 / overnight at 4ºC
Rabbit anti-ephrinB3 (Zymed)	1:500 / overnight at 4ºC
Donkey anti-mouse (Abcam)	1:5000 / 3 hrs at RT
Goat anti-rabbit IgG (Jackson laboratory)	1:5000 / 3 hrs at RT
Donkey anti-goat (Abcam)	1:5000 / overnight at 4ºC

5. Statistical analysis

Data were graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). Student‘s two-tailed t-test was used for comparison of two experimental groups. Changes were identified as significant if p was less than 0.05. Data was expressed as mean values ± standard error of mean (SEM).

6. Materials

Equipment in alphabetical order	**purchased from**
Adobe Photoshop	Apple
Axiophot fluorescent microscope	Zeiss
Cell culture dish (100mm)	Falcon
Cell lifter	Corning
Cellomics kinetic scan HCS reader	ThermoFisher
Centricon membrane collum	Millipore
Centrifuges	Eppendorff, Fisher, Beckman Coulter
Cryogenic vials	Corning
Film	Midsci
Freezer (-20°C)	Fisher
Freezer (-80°C)	VWR
Hemocytometer	Hausser
Hot plate	Corning
Incubator (Cell culture)	ThermoForma
Nitrogen tank (5 % CO_2-balanced)	Airgas
PCR machine	Eppendorff
Pipettes	Gilson, Eppendorff
Pipet tips	Midwest, Transax, Corning
Plates (96-well)	Falcon
ProOx-C oxygen controlled chamber	BioSpherix
ProOx-C oxygen sensor; Model No. 110	BioSpherix
PVDF membrane	Millipore
Refrigerator	VWR
Scales	Mettler Toledo, Ohaus
Shaker	Midwest
Spectrophotometer	Beckman

Equipment in alphabetical order	**purchased from**
Spectrophotometer (multi-well)	Perkin Elmer
Spectrophotometer ND-1000	Nanodrop
Stirrer	Corning
Tube, 1.7 ml	Midwest
Tube, 15 ml conical	Sarstedt
Tube, 50 ml conical	Corning
VHCS scan software	ThermoFisher

Chemicals

All chemicals are of highest purity and purchased either from Sigma, Amresco, BioRad.

IV. RESULTS

1. EphB3 influences proliferation and survival of adult NSPCs *in vitro* under normoxic conditions

Ephrins and Eph receptors have been implicated previously in playing a role in the proliferation and survival of NSPCs in the SVZ of the adult forebrain (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). To evaluate cell autonomous effects of EphB3 on the growth and survival of NSPCs, we isolated SVZ-derived progenitors from wild type and EphB3$^{-/-}$ mice. When grown *in vitro* as monolayer cultures, both wild type and EphB3$^{-/-}$ NSPCs retain an undifferentiated phenotype after 96 hrs in culture as defined by positive staining with neural progenitor marker anti-nestin (Fig. 1a).

Previously, deletion of ephrinB3 resulted in an increase of proliferation and cell death in the SVZ (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). When only the extracellular binding domain of ephrinB3 was preserved by addition of ephrinB3-Fc, the above effects of increased proliferation and cell death were reversed, suggesting that activation/binding of Eph receptors is essential for restrictive control of cell proliferation and cell death in the SVZ (*Ricard, J. et al, 2006; Furne, C. et al., 2009*). To investigate whether proliferation and survival of NSPCs is dependent on EphB3, we cultured wild type and EphB3$^{-/-}$ NSPCs derived from the SVZ.

1. 1. Loss of EphB3 promotes proliferation of adult NSPCs

First we examined the cellular growth and proliferation of wild type and EphB3$^{-/-}$ NSPCs by means of overall metabolic rate (Quick assay), number of total nuclei in culture (Hoechst) and percentage of mitotic cells (BrdU). The Quick cell proliferation assay can be used to quantify the expansion of the number of viable cells by testing the activity of the mitochondrial dehydrogenases, which leads to an increase in the amount of formazan dye formed, measured as absorbance and representing metabolic activity of total cells. Therefore, both the generation of new cells and cell loss are assessed through this assay. Employing Quick assay, we analyzed the growth curves of both genotypes in culture up to 72 hrs (Fig. 1b). 24 hrs after plating equal amounts of wild type and EphB3$^{-/-}$ NSPCs, both

cultures show similar amounts of absorbance (wild type: 0.24 ± 0.008 vs. EphB3$^{-/-}$: 0.26 ± 0.011). At 48 hrs, metabolic activity is showing a slightly a higher rate in EphB3$^{-/-}$ NSPCs compared to wild type cultures (wild type: 0.29 ± 0.016 vs. EphB3$^{-/-}$: 0.34 ± 0.020). This continues to increase up to 72 hrs in culture (wild type: 0.46 ± 0.015 vs. EphB3$^{-/-}$: 0.61 ± 0.040). It demonstrates that EphB3$^{-/-}$ progenitors have an increased growth rate compared to wild type cultures, which could be represented by an increase of the proliferative rate and/or a decrease of the cell death rate.

Likewise, we cultured wild type and EphB3$^{-/-}$ NSPCs up to 96 hrs and counted Hoechst-positive nuclei per field employing Cellomics scan, viewing at 6 fields per well using light microscopy and 10x magnification. Here we verified the results above showing that the increase in metabolic activity represents an increase in the total number of Hoechst-positive cells per field at 48 and 72 hrs after plating (Fig. 1c). At 72 hrs we counted 425.9 ± 38.48 nuclei per field in EphB3$^{-/-}$ as opposed to 270.8 ± 32.94 in wild type cultures. The increase also extended to 96 hrs in culture, when 614.7 ± 72.54 EphB3$^{-/-}$ compared to 317.4 ± 41.22 wild type cells were counted. Over 48 hours in culture, this represents a cellular expansion of 113% in EphB3$^{-/-}$ compared to 42% in wild type cells.

To exclusively analyze the rate of proliferation in wild type and EphB3$^{-/-}$ adult NSPCs, we added bromodesoxyuridine (BrdU) to 24-hr-cultures 1 hour prior to harvesting and staining. BrdU as nucleotide substitute replaces thymidine with uridine in the DNA structure of dividing cells, therefore identifying BrdU-positive cells as mitotic, during the S-phase of mitosis. Employing Cellomics cell count we assessed proliferation as the percentage of mitotic BrdU-positive divided by total Hoechst-positive cells. At 24 hrs in culture, the percentage of BrdU-positive cells was significantly higher in EphB3$^{-/-}$ (18.3% ± 2.77) compared to wild type cultures (9.77% ± 0.97) (Fig. 1d-f). This data demonstrates that proliferation of NSPCs increases by almost 50% when EphB3 receptor is absent.

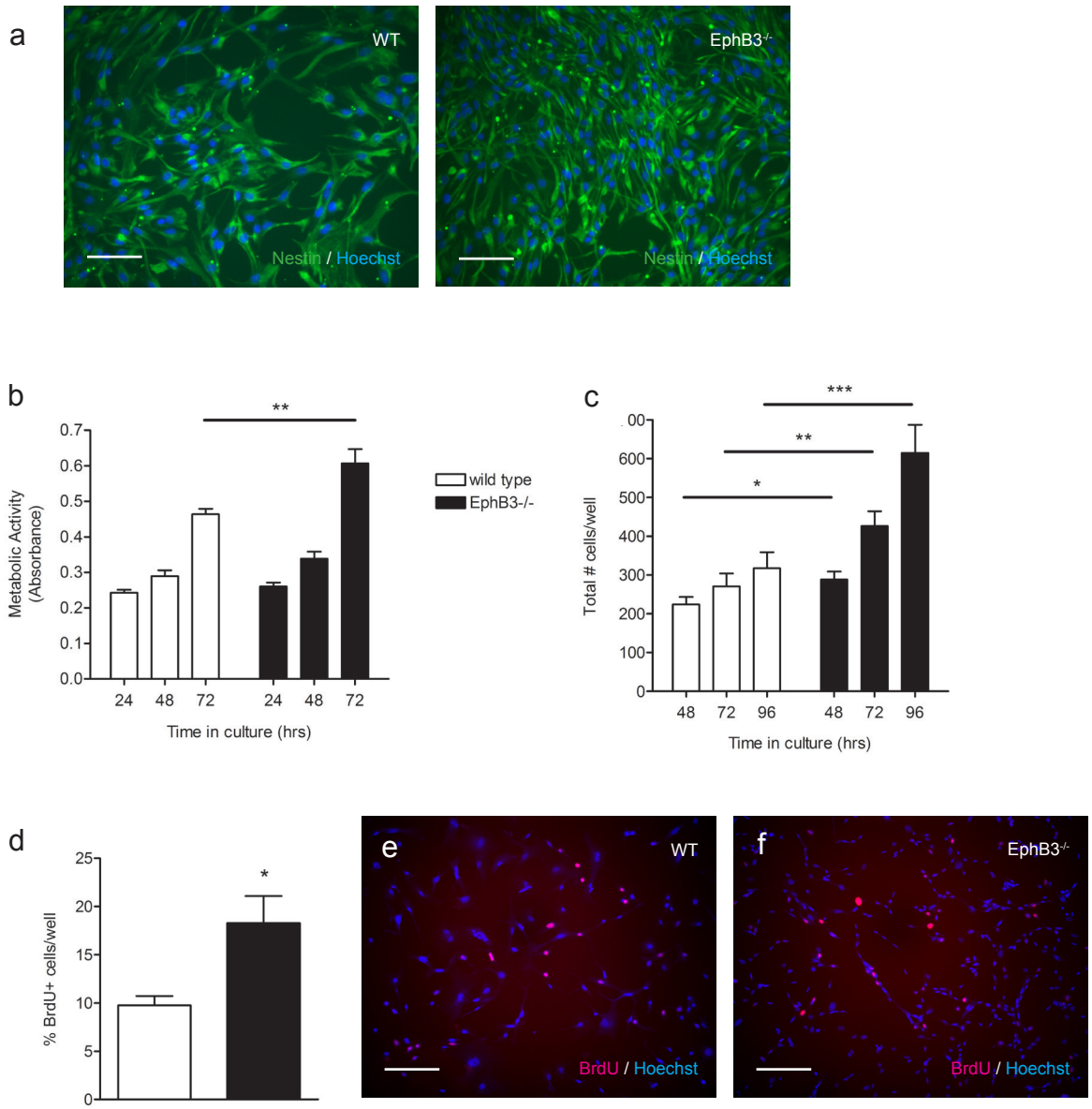


Figure 1. Loss of EphB3 promotes proliferation of NSPCs.

(a) Adult NSPCs derived from wild type (WT) and from EphB3$^{-/-}$ mice retain an undifferentiated phenotype after 96 hrs in monolayer culture as defined by staining with neural progenitor marker anti-nestin (green); Hoechst as nuclei stain (blue). (b,c) Growth curves of wild type and EphB3$^{-/-}$ NSPCs in monolayer culture. (b) Absorbance as means of metabolic activity, measured by Quick cell proliferation assay from 24 hrs to 72 hrs in culture, showing increased absorbance of EphB3$^{-/-}$ NSPCs compared to wild type at 72 hrs. (c) Hoechst nuclei stain: after culture from 48 hrs to 96 hrs, an increased number of Hoechst-positive (+) cells per field were counted in EphB3$^{-/-}$ NSPCs compared to wild type. (d-f) Loss of EphB3 promotes proliferation of adult NSPCs. (d) Cell proliferation rate was quantified as the percentage of BrdU-positive (+) of total Hoechst-positive (+) cells per field. The proliferation rate at 24 hrs in culture was nearly doubled in EphB3$^{-/-}$ cells. (e,f) Immunofluorescence labeling of cultured NSPCs showed a significant increase in BrdU (red) incorporation of EphB3$^{-/-}$ (f) compared to WT (e). Hoechst nuclei stain (blue) for visualization of all nuclei. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, EphB3$^{-/-}$ compared to wild type cultures. Scale bar (a, e, f) = 200 µm

1. 2. Loss of EphB3 as well as EphB3 binding improve survival of NSPCs

Secondly, we investigated whether EphB3 receptor is involved in alterations of NSPC survival as well. Thus, we employed loss-of-function (i.e. EphB3$^{-/-}$) and gain-of-function approaches (i.e. addition of ligand ephrinB3). We compared the rates of cell death quantitatively using the SYTOX assay kit. SYTOX is a nucleic acid stain which easily penetrates cells with compromised plasma membranes due to its high affinity, yet intact membranes of live cells will not be crossed. SYTOX-positive cells are identified as damaged and/or dying cells, which are then divided by total Hoechst-positive nuclei, resulting in the percentage of cell death. We again grew wild type and EphB3$^{-/-}$ NSPCs up to 96 hrs and counted SYTOX-positive cells using Cellomics scan. At all time points from 48 to 96 hrs, we see a marked decrease of cell death if EphB3 receptor was absent compared to wild type NSPCs. At 48 hrs in culture, cell death in wild type cultures is almost doubled compared to EphB3$^{-/-}$ cultures (wild type: 17.45% ± 1.42 vs. EphB3$^{-/-}$: 9.53% ± 1.89). Moreover, at 72 and 96 hrs in culture, cell death is reduced to one forth in EphB3$^{-/-}$ compared to wild type cultures (Fig. 2a-c). These data demonstrate that the loss of EphB3 expression results in enhanced survival of adult NSPCs *in vitro*. It also supports previous data, showing that the number of cell death marker TUNEL-positive cells is reduced in the SVZ of EphB3$^{-/-}$ animals *in vivo* (*Theus, M.H. et al., 2010*)

We now aimed to analyze the effect of EphB3 stimulation on cell death by adding back ligand ephrinB3. Pre-clustered ephrinB3-Fc (final concentration 5µg/ml, dissolved in elution buffer) was added to culture and survival was assessed 24 hrs after. Elution buffer only was added for negative control. Likewise, we examined the rate of cell death using SYTOX assay kit and Cellomics cell count with Hoechst as total nuclei stain. When ligand ephrinB3 was present in culture, percentage of cell death could be reduced to 5.66% ± 2.25 compared to 18.37% ± 2.02 when only vehicle had been added (Fig. 2d). This shows a 69% reduction of cell death through addition of ligand ephrinB3, which binds and activates mainly its cognate receptors EphB3 and/or EphA4 (*Orioli, D. and Klein, R., 1997*). It supports our previous data *in vivo* demonstrating that the number of cell death marker TUNEL-positive cells in the SVZ decreases by infusion of pre-clustered ephrinB3 into the lateral ventricle of sham wild type mice (*Theus, M.H. et al., 2010*). This data summarizes that cell death of NSPCs is reduced in two cases: i) in the absence of receptor EphB3 and ii) in the presence of ephrinB3. It suggests that EphB3 may function as pro-apoptotic dependence receptor in NSPCs, a role that has been previously accredited to EphA4 as well (*Furne, C. et al., 2009*).

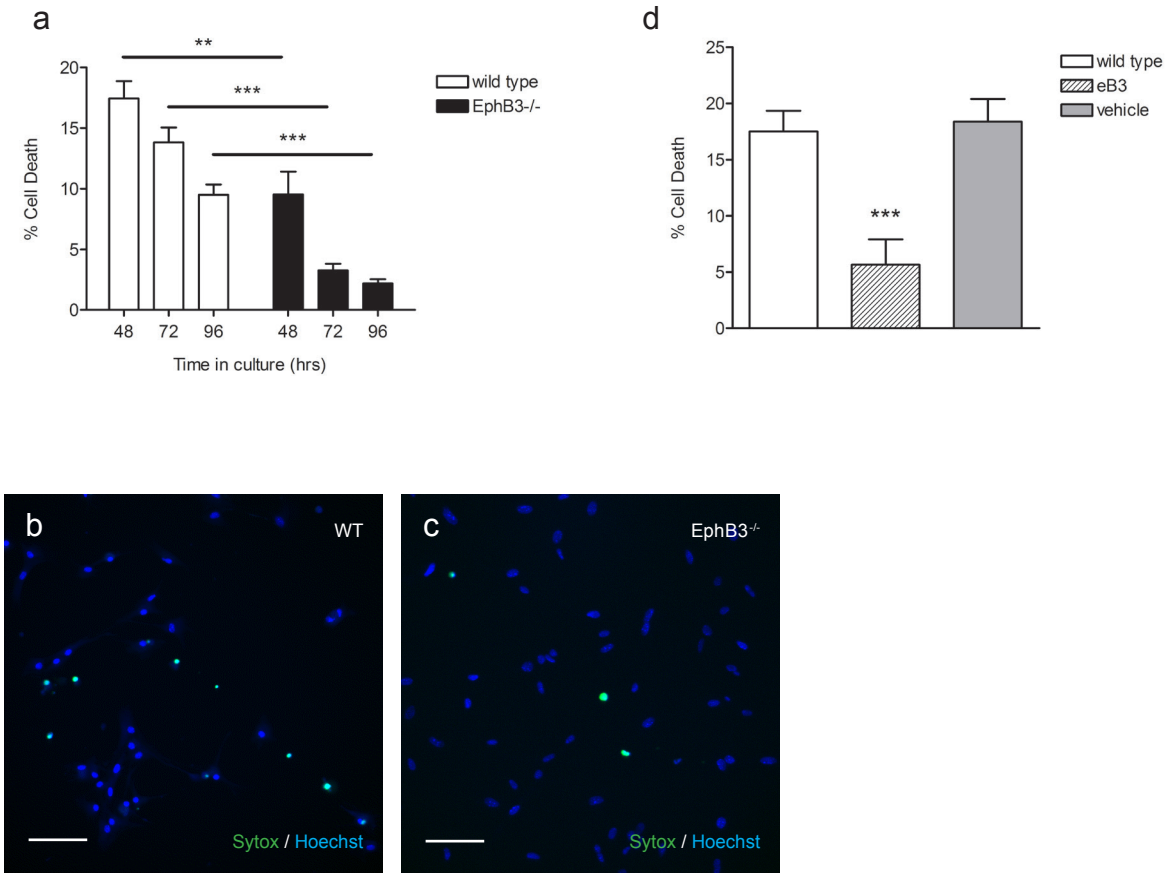


Figure 2. EphB3-ephrinB3 signaling influences the survival of NSPCs.

(a-c) The loss of EphB3 promotes the survival of adult NSPCs. (a) Cell death studies counting SYTOX-positive (+) (green) cells and total Hoechst-positive (+) (blue) nuclei in wild type and EphB3$^{-/-}$ cultures. Over all time points from 48 hrs to 96 hrs., cell death is reduced in EphB3$^{-/-}$ compared to wild type cultures. (b,c) Immunofluorenscence staining of wild type (WT) and EphB3$^{-/-}$ cultures showing SYTOX-positive (+) cells (green) are increased in wild type cultures; Hoechst (blue) stain for all nuclei. (d) Effect of pre-clustered ligand ephrin B3-Fc ('+EB3') on the survival of wild type (WT) adult NSPCs in culture. Quantification as the percentage of SYTOX-positive (+) of total Hoechst-positive (+) cells per field. With addition of ephrinB3, cell death of wild type NSPCs could be reduced from 18% to 6%. Addition of vehicle only ('+vehicle') as negative control. **$p < 0.01$ and ***$p < 0.001$, EphB3$^{-/-}$ compared to wild type cultures/ ephrinB3-Fc compared to vehicle control. Scale bar (b-c) = 200 µm

2. The hypoxia model: an *in vitro* model for changes post-injury

Previous studies in our laboratory have focused on Eph/ephrin involvement in the SVZ response after TBI. Shortly, ephrinB3-EphB3 interactions have been implicated in regulating proliferation and survival in the SVZ, as TBI resulted in a significant reduction of EphB3 expression, which coincided with enhanced NSPC survival and proliferation at 3 and 7 days post-injury (*Theus, M.H. et al., 2010*). Cerebral hypoxia develops as part of the secondary injury following primary mechanical and/or ischaemic trauma to the brain (*Maas, A.I. and Fleckenstein, W., 1993; Zauner, A. et al., 1996; van den Brink, W.A. et al., 2000; Bader, M.K., 2006; Liu, S. et al., 2004*). To confirm the presence of hypoxia post-TBI, we visualized the changes of oxygen levels in the adult mouse brain after CCI, a model for TBI, injecting HPb (pimonidazole hydrochloride, hypoxyprobe staining) at 0 hrs, 1.5 hrs and 24 hrs post-injury (*Baumann, G. et al., 2013*). HPb detects hypoxic gradients, as tissue requires a hypoxia level of $pO_2 < 10$ mmHg for successful binding (i.e. < 10 mmHg, equivalent to < 1.32 % oxygen). As expected, the presence of HPb was observed at the site of injury in the cortex (CTX) and underlying corpus callosum (CC) of CCI-injured mice (*Baumann, G. et al., 2013*; see also Background Results, Fig. B-1c to f; brown staining). Importantly, the area lining the left ventricle, both dorsal and ventral subregions of the SVZ, was positive for HPb staining at all time points but most notable at 24 hrs suggesting that the NSPCs residing in the lateral wall of the ventricle may be influenced by the low oxygen environment (*Baumann, G. et al., 2013*; see also Background Results, Fig. B-1f; black arrows). Using confocal image analysis, HPb colabeled with neuroblasts marker doublecortin (DCX) and NSPC markers MASH1 and glial fibrillary acidic protein (GFAP) (*Baumann, G. et al., 2013*; see also Background Results, Fig. B-2k-m). These data indicate that HPb expression is evident on stem/progenitor and neuroblast cell types in the SVZ following TBI, which suggests that neural progenitors are likely to be influenced by low oxygen tension after brain injury.

Transient low oxygen levels have been shown to promote the growth of neural stem cells and maintain their survival both *in vitro* and *in vivo* (*Zhu, L.L. et al., 2005*). Furthermore, Studer and colleagues grew embryonic day 12 rat mesencephalic precursor cells in traditional cultures with 20% O_2 and in lowered O_2 (3 ± 2%), demonstrating that proliferation was promoted and apoptosis was reduced when cells were grown in lowered O_2, yielding greater numbers of precursors (*Studer, L. et al., 2000*). Therefore, reduced oxygen levels could be a trigger for endogenous progenitor/stem cells to propagate and enable endogenous repair.

We aimed to confirm these previous findings and analyzed growth and survival rates of wild type NSPCs derived from the adult SVZ under conditions of 21% (normoxia) and 1% (hypoxia) oxygen in culture, grown from 24 to 72 hrs. Employing Hoechst and SYTOX staining as well as subsequent Cellomics cell count we assessed the number of total cells and the percentage of cell death. As expected, total cell numbers were increased at 96 (72) hrs, when cultured under low oxygen conditions compared to normoxia (hypoxia: 748.0 ± 79.13 vs. normoxia: 525.6 ± 66.62; Fig. 3a). Conversely, hypoxia minimized the percentage of cell death (hypoxia: 5.97% ± 0.77 vs. normoxia: 9.50% ± 0.85; Fig 3b). This confirms previous findings identifying decreased levels of oxygen as a trigger for growth and survival of NSPCs, extending the focus from embryonic to adult NSPCs and to relatively low oxygen levels of 1% (*Studer, L. et al., 2000; Morrison, S.J. et al., 2000; Zhao, H.Q. et al., 2004; Zhu, L.L. et al., 2005*).

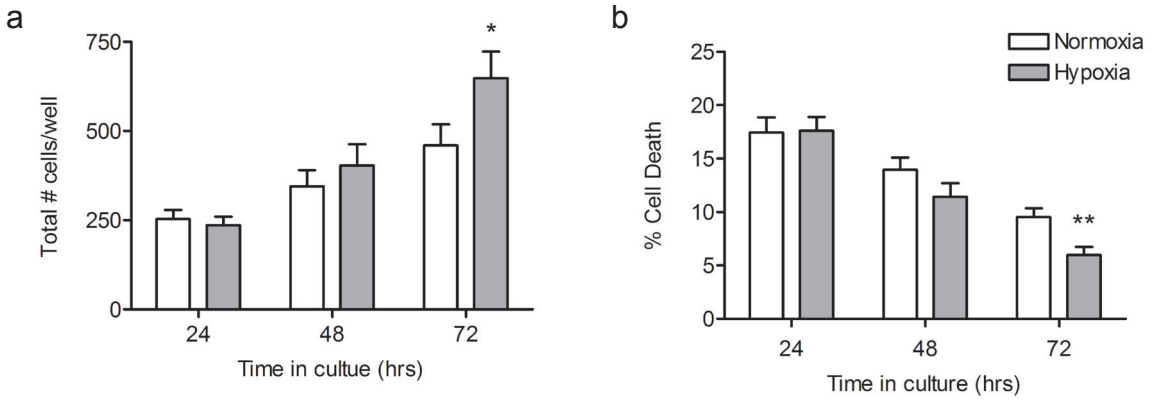


Figure 3. Growth and survival of wild type NSPCs are improved under hypoxic conditions.

(a) Growth curve of wild type NSPCs in hypoxia and normoxia, counting total Hoechst-positive (+) cells under conditions of 1% and 21% oxygen. Cells were cultured from 24 to 72 hrs. (b) Cell death studies counting the percentage of SYTOX-positive (+) cells to total Hoechstpositive (+) nuclei in normoxic compared to hypoxic NSPC cultures. **p < 0.01 hypoxia compared to normoxia conditions.

3. Expression of EphB3 receptor but not ligand ephrinB3 in NSPCs

Our previous findings indicate that EphB3 receptor is expressed on SVZ-derived NSPCs and negatively regulates NSPC proliferation when ephrinB3 ligand is present (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). Immunohistochemical studies have demonstrated that the ligand ephrinB3 is expressed in the tissues surrounding the SVZ and the rostral migratory stream (RMS) but not within the SVZ (*Ricard, J. et al., 2006*). The following results have been previously published (*Baumann, G. et al., 2013*): We isolated RNA from wild type NSPCs monolayer cultures to examine the expression of ephrinB ligands and/or their cognate receptors on a transcriptional level. Using RT-PCR, we confirmed that EphB3 is expressed on NSPCs, while ephrinB3 is absent (Fig. 4). As expected, we verified the expression of ephrins B1 and B2 as well as their receptors EphB1 and EphB2 (Fig. 4d). EphA4, which has been shown to bind ephrinB3 with high affinity is also expressed in monolayer culture. In addition to the presence of EphB3 and EphA4 transcripts, we show their cellular localization on NSPCs by immunofluorescence (Fig. 4a,b) but not ephrinB3 ligand (Fig. 4c).

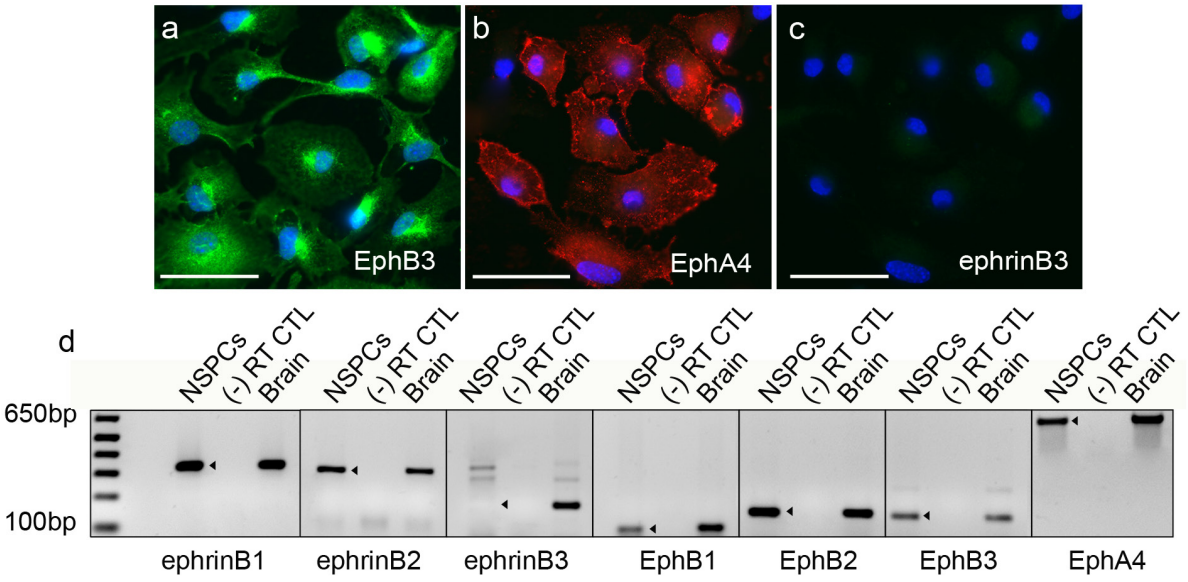


Figure 4. Expression of Eph receptors and ephrins in wild type NSPCs derived from the SVZ, cultured as monolayers.

Immunofluorescence labeling shows expression of EphB3 (a) and EphA4 (b) receptors but not ephrinB3 ligand (c) on NSPCs. Hoechst used as nuclei staining. (d) Using reverse transcriptase (RT)-PCR, we confirmed the expression of EphB3, EphA4 and the absence of ephrinB3 transcripts. NSPCs also express transcripts for EphB1, EphB2, ephrinB1 and ephrinB2. Lane 1: NSPCs; lane 2: RT(-) control; lane 3: total adult brain RNA extract. Scale bar (a-c) = 50 µm. (*Baumann, G., et al., 2013, figure pre-published*)

We also confirmed our previous findings by taking advantage of a transgenic knock-in mouse where β-galactosidase (ephrinB3$^{\beta gal}$) replaces the cytoplasmic domain of ephrinB3. β-gal staining visualized ephrinB3 expression throughout the brain, including the striatum (St), septum, and corpus callosum (CC) (*Baumann, G. et al., 2013*; Background Results, Fig. B-3); however, no ephrinB3 expression was observed within the SVZ (Background Results, Fig. B-3c1). Co-labeling GFAP-positive NSPCs (green) and PSA-NCAM-expressing neuroblasts (red) on ephrinB3$^{\beta gal}$ tissue sections show NSPCs residing in the SVZ exist along a border, yet separate of ephrinB3 expression in the adjacent tissue (*Baumann, G. et al., 2013*; Background Results, Fig. B-3a1 to c1). EphB3, on the other hand, is expressed in the SVZ and is absent in the neighboring striatum, as well as co-labels with hypoxia marker HPb at 24 hrs after TBI (*Baumann, G. et al., 2013*; Background Results, Fig. B-3d to g). This shows that neural progenitors in the SVZ are expressing EphB3 receptor and are meanwhile exposed to neighboring areas expressing ephrinB3, which possibly serves to regulate NSPC functions.

4. EphB3 expression and p-AKT activation in hypoxic NSPCs

In reference to our previous results, we demonstrated that ephrinB3 binding of EphB3 acts as negative control of proliferation in SVZ-derived NSPCs (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). This effect is non-existent in the absence of EphB3 receptor, which accounts for the specificity of EphB3-ephrinB3 signaling (*Theus, M.H. et al., 2010*). Following TBI, EphB3 expression is down-regulated, which diminishes the inhibitory signal of EphB3-ephrinB3 interaction, leading to increased NSPC proliferation (*Theus, M.H. et al., 2010*). The following results have been previously published (*Baumann, G. et al., 2013*): In order to assess whether hypoxia could be an early initiating factor that modulates EphB3 signaling, we employed our *in vitro* hypoxia model. Using Western Blot analysis, we first observed that EphB3 expression on NSPCs was significantly reduced when exposed to hypoxia at 24 hrs (0.23 ± 0.04 compared to 0.38 ± 0.05 normoxia), 48 hrs (0.28 ± 0.04 compared to 0.41 ± 0.03 normoxia), and 72 hrs (0.27 ± 0.04 compared to 0.51 ± 0.08 normoxia), (Fig. 5a,b). As EphB3 acts as negative regulator of NSPC proliferation and survival (in unbound form) (*Ricard, J. et al., 2006; Theus, M.H. et al., 2010*), this down-regulation of EphB3 might be part of a signaling pathway to promote proliferation and/or survival in hypoxic NSPCs or post-TBI. EphB3 forward signaling has previously shown to suppress intracellular levels of phosphorylated AKT (p-AKT) (*Pasquale, E.B., 2008, Ricard, J. et al., 2006*). Here we show,

that conversely to EphB3 down-regulation, p-AKT is increased under hypoxia at 24 hrs (0.51 ± 0.09 compared to 0.25 ± 0.05 normoxia) and 72 hrs (1.03 ± 0.22 compared to 0.65 ± 0.11 normoxia) (Fig. 5a,c).

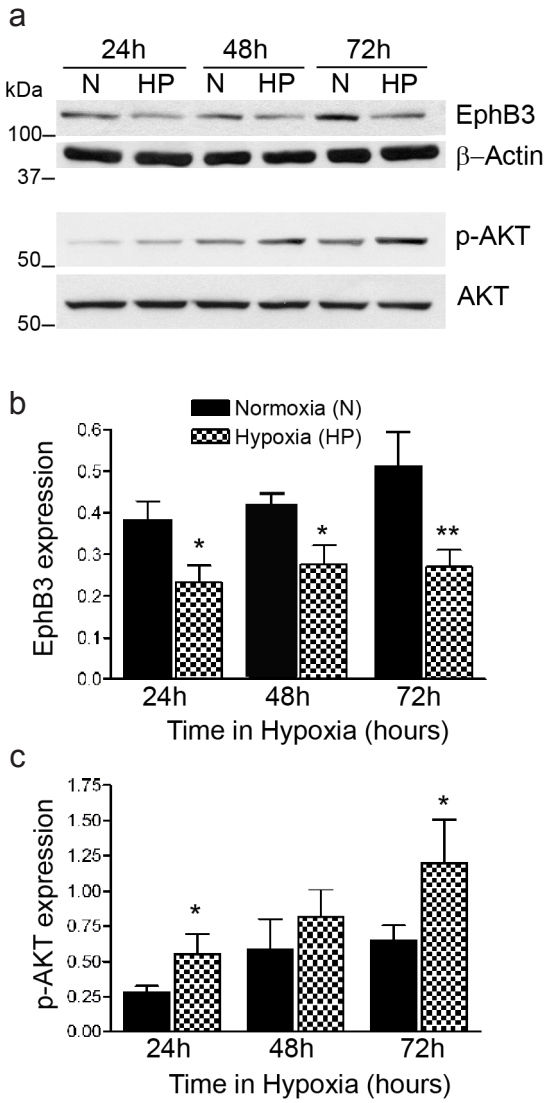


Figure 5. EphB3 expression is reduced under hypoxia cell culture conditions.

NSPCs were grown under hypoxic (HP) or normoxic (N) cell culture conditions for 24–72 h. (a) Using Western Blot analysis, we found EphB3 expression was reduced; conversely, p-AKT was increased under hypoxia compared to normoxia. (b) Bar graph representing quantified relative expression of EphB3 at 24– 72 h in hypoxic or normoxic cultures. Grey intensity values were normalized against b-actin control levels. (c) Bar graph representing quantified data for p-AKT normalized to total AKT levels at 24–72 h in culture. *$p < 0.05$ and **$p < 0.01$ compared to normoxia control NSPC cultures. (*Baumann, G. et al., 2013, figure pre-published*)

5. EphB3 stimulation limits hypoxia-induced proliferation in cultured NSPCs

The following results have been previously published (*Baumann, G. et al., 2013*): We next aimed to assess whether hypoxia-induced reduction of EphB3 expression was concomitant with increased proliferation and whether the presence of soluble aggregated ephrinB3-Fc, which mimics the *in vivo* environment within the neurogenic compartment, would limit this effect. To test this, we treated SVZ-derived NSPCs with either 10 μg/mL ephrinB3 or vehicle control and placed them under hypoxia or normoxia for 24, 48 and 72 hrs followed by 1 hr incubation in 10 μM BrdU (Fig. 6). As expected, we observed a significant increase in the number of BrdUpositive NSPCs when exposed to hypoxia at 24 hrs (21.34 ± 0.8% compared to 16.9 ± 0.9% normoxia), 48 hrs (29.5 ± 0.8% compared to 24.4 ± 1.8% normoxia) and 72 hrs (28.7 ± 0.6% compared to 23.5 ± 0.6% normoxia) (Fig. 6e). The total number of NSPCs per well was also significantly increased under hypoxia at 48 hrs (1312 ± 83.7 compared to 723.4 ± 86.9 normoxia) and 72 hrs (3248 ± 124.7 compared to 2158 ± 87.69 normoxia (Fig. 6f). We next assessed the effect of adding ligand ephrinB3 on hypoxia-induced proliferation. At 24 hrs after addition of ephrinB3, hypoxia-mediated proliferation was significantly reduced compared to vehicle control (21.34 ± 0.8% vehicle compared to 16.3 ± 1.7% ephrinB3). This effect was similar but less pronounced at 48 hrs (29.5 ± 0.8% vehicle compared 26.6 ± 0.6% ephrinB3) (Fig. 6c-e) which subsequently lead to an overall reduction in the total number of cells per well at 72 hrs (3248 ± 124.7 vehicle compared to 2577 ± 132.5 ephrinB3) compared to vehicle control (Figure 6f). While the presence of ephrinB3 attenuated hypoxia-induced proliferation, the numbers were not restored completely back to normoxic levels. Additionally, ephrinB3 failed to attenuate hypoxia-induced proliferation in NSPCs derived from EphB3$^{-/-}$ mice (*Baumann, G. et al., 2013; data not shown*). These results indicate that ephrinB3 limits the NSPC response to hypoxic stimuli via EphB3 forward signaling and suggests that the NSPC response *in vivo* post-injury could be enhanced on those NSPCs not directly in contact with ephrinB3 ligand.

We also assessed whether ephrinB3 could reduce proliferation by elevating cell death. Although we previously demonstrated reduced cell death in the presence of ephrinB3 (*Theus, M.H. et al., 2010*), we show that ephrinB3 also reduced cell death under hypoxic conditions (Fig. 6g) at 24 hrs (16.7 ± 1.2% vehicle compared to 5.6 ± 1.4%), 48 hrs (11.3 ± 1.6% vehicle compared to 3.5 ± 1.0%), and 72 hrs (7.8 ± 1.3% vehicle compared to 2.1 ± 0.9%).

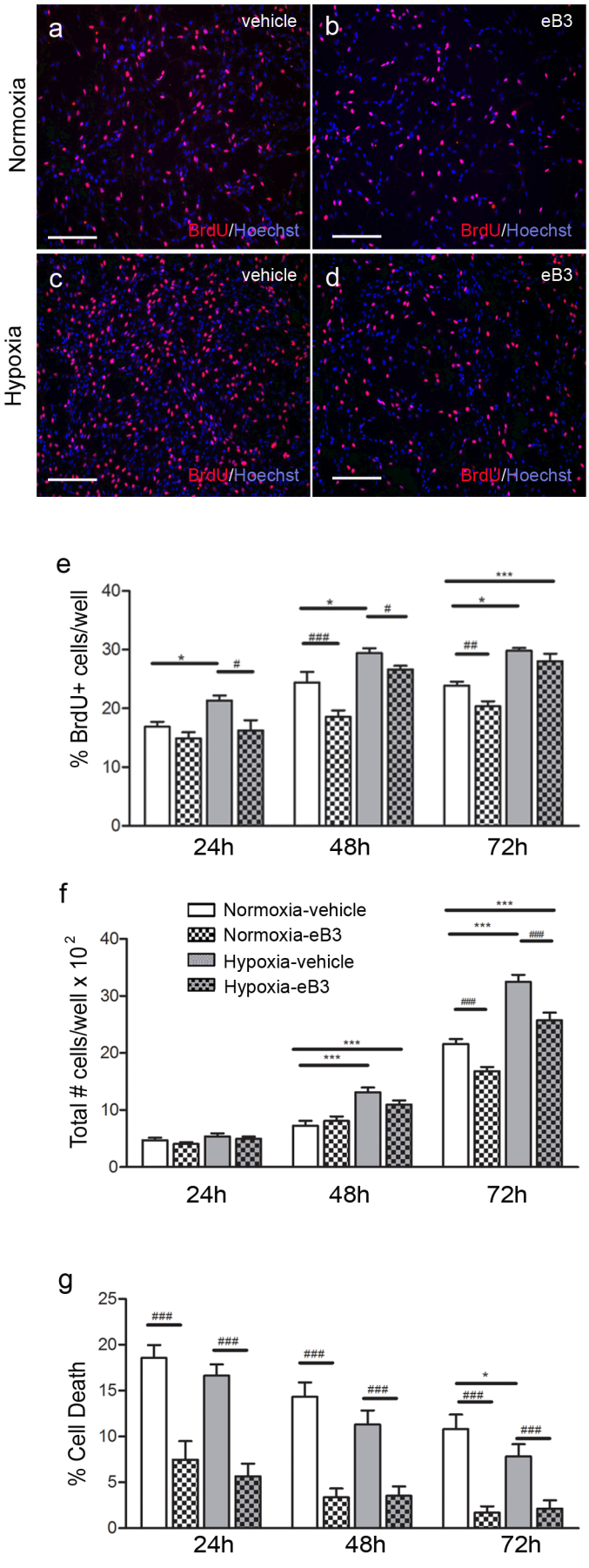


Figure 6. Stimulation with ephrinB3 attenuates hypoxia-induced proliferation on SVZ-derived NSPCs.
The proliferation of SVZ-derived NSPCs was assessed using bromodeoxyuridine (BrdU) incorporation in the presence and absence of soluble aggregated ephirnB3 ligand (eB3) at 24–72 hrs under low oxygen conditions. (a–d) Immunofluorescence labeling of cultured NSPCs under hypoxia showed a significant increase in BrdU incorporation at 72 hrs hypoxia exposure (c) compared to normoxia (a). However, cell numbers are reduced when normoxic or hypoxic NSPCs were grown in the presence of eB3 (b and d). (e) Bar graph representing the percentage (%) of BrdU-positive NSPCs shows that hypoxia-induced proliferation was significantly attenuated following 24 and 48 hr exposure to eB3 compared to vehicle control. (f) Bar graph representing the total number of NSPCs per well demonstrates a significant overall reduction in the amount of NSPCs present after 72 hr stimulation with eB3 under hypoxia compared to vehicle control. (g) Bar graph showing reduced cell death in the presence of eB3 and hypoxia. *p < 0.05 and ***p < 0.001 compared to normoxia-vehicle; #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to hypoxia-vehicle. Scale bar (a-d) = 200 µm (*Baumann, G., et al., 2013, figure pre-published*)

6. p-AKT but not Hif-1α is regulated by EphB3-ephrinB3 signaling under hypoxia

The following results have been previously published (*Baumann, G. et al., 2013*) and are included here for completeness of contents: Hypoxia inducible factor-1 alpha (Hif-1α) elicits the expression of a plethora of growth-promoting genes responsible for stimulating proliferation, survival and differentiation of stem cells under low oxygen conditions (*Milosevic, J. et al., 2007; Keith, B. and Simon, M.C., 2007*). To further elucidate the mechanism(s) by which ephrinB3-EphB3 signaling limits hypoxia-induced proliferation of NSPCs, subsequent studies in our laboratory examined whether EphB3 stimulation could prevent early activation of Hif-1α. Soluble ephrinB3 was added at the time NSPCs were placed in hypoxia and the effects of EphB3 stimulation on early Hif-1α activation was assessed using Western Blot analysis. We found that Hif-1α expression increased at 12 hrs hypoxia exposure (0.44 ± 0.05 compared to 0.13 ± 0.03 normoxia), which was unaffected by the addition of either 10 μg/mL soluble aggregated ephrinB3 (0.48 ± 0.07) or 10 μg/mL ephrinB1 (0.33 ± 0.08) compared to vehicle control (0.4370 ± 0.05) (*Baumann, G. et al., 2013*; see also Background Results, Fig. B-4a,b). As expected, Hif-1α accumulation could be prevented following treatment with 50 μM AKT inhibitor LY294002 (0.14 ± 0.01 compared to vehicle 0.44 ± 0.05) (*Baumann, G. et al., 2013*; see also Background Results, Fig. B-4a,b). As evaluated before, the AKT pathway is blocked downstream of EphB3 forward signaling (*Pasquale, E.B., 2008, Ricard, J. et al., 2006*), whereas it is also known to act as inducer of Hif-1α under hypoxic conditions (*Mottet, D. et al., 2003; Pore, N. et al., 2006*). Although early hypoxic p-AKT levels were significantly attenuated in the presence of ephrinB3 (0.42 ± 0.06 compared to vehicle control 0.76 ± 0.09) (*Baumann, G. et al., 2013*; see also Background Results, Fig. B-4a,c), this had no measurable effect on the expression of Hif-1α, suggesting a threshold of p-AKT inactivation must be reached to affect Hif-1α induction. These data therefore indicate that EphB3 limits hypoxia-induced proliferation in the presence of ephrinB3 through Hif-1α independent pathway(s).

V. DISCUSSION

1. EphB3 signaling regulates proliferation of adult NSPCs

Adult NSPCs residing in the SVZ represent a potential cell source for restorative therapy after CNS injury (*Salman, H. et al., 2004; Arvidsson, A. et al., 2002; Yamashita, T. et al., 2006; Sundholm-Peters, N.L. et al., 2005; Nakatomi, H. et al., 2002*). Whereas various soluble factors have been implicated previously in altering SVZ-derived NSPC turnover, migration and differentiation (*Dempsey, R.J. et al, 2003; Jin, K. et al., 2002-1; Jin, K. et al., 2002-2*), our study focused on evaluating the role of membrane-bound ephrins and Eph receptor signaling as local cues maintaining homeostasis of adult NSPCs. Specifically, we investigated the role of EphB3 receptor in the proliferation and survival of NSPCs, based on the particular expressional pattern of its primary ligand ephrinB3 bordering the SVZ (*Ricard, J. et al., 2006; Baumann, G. et al., 2013*). As expected, we confirmed that ephrinB3 was not expressed in monolayer culture of adult progenitor cells in contrast to its binding receptors EphB1-3 and EphA4 and fellow ephrinB ligands ephrinB1 and B2. Employing a loss-of-function approach, NSPCs deficient of EphB3 receptor showed an increased growth rate (i.e. absorbance representing metabolic activity and total cell counts) as well as an increase of BrdU-positive proliferative cells compared to wild type. This effect was reversed in wild type NSPCs grown with ephrinB3 in culture, equivalent to a gain-of-function approach activating EphB3 signaling, which resulted in decreased numbers of proliferating cells and total cells in culture. A similar effect had been shown *in vivo*, as the SVZ of EphB3-deficient as well as ephrinB3-deficient mice contain a higher number of BrdU-positive proliferative cells compared to their wild type littermates, and infusion of ephrinB3 attenuated proliferation in the SVZ of ephrinB3-deficient and wild type mice (*Theus, M.H. et al., 2010*). Together, these results verify that activated EphB3 forward signaling suppresses proliferation in cultured adult neural progenitors derived from the SVZ and *in vivo*. Once either part of ephrinB3-EphB3 interaction is lost, i.e. receptor or ligand, the anterograde cell signal will lose an inhibitory influence on cell cycle control, leading to augmented proliferation.

Moreover, ephrinB3-EphB3 boundaries have been shown to regulate NSPC proliferation through downstream control of tumor suppressor protein p53. The degradation of p53 is promoted by p-AKT (*Mayo, L.D. and Donner, D.B., 2001; Mayo, L.D. and Donner, D.B., 2002*), a cell cycle regulating protein whose intracellular level has previously been shown to be

suppressed secondary to EphB3 signaling (*Pasquale, E.B., 2008; Ricard, J. et al., 2006*). Consequently, diminution of EphB3 signaling leads to increased p-AKT, which in turn contributes to p53 degradation, resulting in inhibition of cell cycle control and increased proliferation. It is well established that p53 initiates various cellular responses leading to cell-cycle arrest, cellular senescence, differentiation and apoptosis (*Bose, I. and Ghosh, B., 2007; Zhou, J. et al., 2004*). Recent reports have demonstrated that adult NSPCs in the SVZ highly express p53, which was discussed to protect adult NSPCs from uncontrolled cellular division (*Medrano, S. and Scrable, H., 2005; Medrano, S. et al., 2009; Meletis, K. et al., 2006; Theus, M.H. et al., 2010*). *In vivo* p53 levels were markedly reduced in the SVZ of EphB3-deficient and ephrinB3-deficient compared to wild type mice, as they were in EphB3 knock-out cultures; higher levels of total protein, phosphorylation and nuclear translocation of p53 were found subsequent to ligand ephrinB3 stimulation and as such, p53 proves the responsible factor for suppression of NSPC proliferation (*Theus, M.H. et al., 2010*).

Consequently, the anatomical proximity of ephrinB3-bearing striatal cells adjacent to the SVZ and RMS could potentially function as proliferative control in itself; if an increased number of EphB3-bearing SVZ progenitors/neuroblasts was generated, closer cellular contact to neighboring tissues would occur, leading to increased ephrinB3-EphB3 interaction and EphB3 forward signaling, activating the intracellular cascade involving p-AKT suppression and p53 activation, which ultimately compromises cellular turnover and contributes to cellular senescence in the lateral layers of the SVZ. This could represent a mechanism maintaining cellular control and homeostasis of SVZ and/or RMS, preventing over-production of neural progenitors and/or neuroblasts. Migration from the SVZ via the RMS route to OB has not shown to be affected by ephrinB3 deficiency, although fewer cells reach the OB (*Ricard, J. et al., 2006*).

2. EphB3 - a dependence receptor in adult NSPCs

Previous results have shown that in contrast to its influence on proliferation, downstream control of p-AKT and p53 was not responsible for changes regarding cell survival following EphB3 signaling (*Theus, M.H. et al., 2010*). Here, we demonstrate that EphB3 forward signaling in NSPC culture is pro-apoptotic in the absence of ephrinB3 ligand, whereas addition of ephrinB3 to EphB3-expressing wild type cultures as well as lack of EphB3 expression resulted in a pronounced improvement of NSPC survival. *In vivo* stud-

ies have given similar results, showing less apoptotic TUNEL-positive cells in the SVZ of EphB3-deficient mice compared to wild type, whereas infusion of ligand ephrinB3 into the lateral ventricle lowers cell death in the wild type SVZ (*Theus, M.H. et al., 2010*). To summarize, both loss and activation of EphB3 receptor lead to lower rates of cell death. Under both circumstances, the amount of unbound EphB3 receptor is diminished, which provides strong support for the role of EphB3 as pro-apoptotic dependence receptor during adult neurogenesis in the SVZ. Dependence receptors depend on their ligands to play supportive roles during development and homeostasis, but revert to pro-apoptotic functions in the absence of ligand stimulation (*Mehlen, P. and Bredesen, D.E., 2004; Mehlen, P. et al., 1998; Mehlen, P. and Thibert, C., 2004; Tulasne, D. et al., 2004; Tauszig-Delamasure, S. et al., 2007*). This hypothesis could explain the discrepancy between mice deficient in EphB3 (greater cell survival in the SVZ) or deficient in ephrinB3 (greater cell death in the SVZ) (*Theus, M.H. et al., 2010*). Just recently, EphA4 had been identified as a pro-apoptotic dependence receptor in the absence of ligand ephrinB3, whereas ephrinA ligands, which bind EphA4 with great affinity *in vitro* were not able to inhibit cell death (*Furne, C. et al., 2008*). EphA4 and especially EphB3 are both primary receptors for ligand ephrinB3, whereas EphB1 and EphB2 receptors share very little binding capacity with ephrinB3 (*Blits-Huizinga, C.T. et al., 2004*). Like EphA4, EphB3 also contains the dependence-associated receptor transmembrane (DART) motif, which has been found as a unique sequence common to many described dependence receptors (*Del Rio, G. et al., 2007*). Both EphB3 and EphA4 might therefore function as dependence receptors in adult NSPCs, with possible compensatory mechanism between the two. In mice lacking EphB3, the expression and function of subsequent Eph receptors might be altered in order to neutralize loss of EphB3 function. Future studies are planned to involve EphB3/EphA4 double knock-out mice to study survival in the SVZ (*Theus, M.H. et al., 2010*).

The expression of ligand ephrinB3 and its interesting distribution mentioned above may support the importance of ephrinB3-EphB3 (and EphA4) interactions in adult neurogenesis in the SVZ. EphrinB3 lining the neurogenic region (*Ricard, J. et al., 2006; Baumann, G. et al., 2013*) would have to have close contact to EphB3/EphA4 expressing neural progenitors in order to bind these ephrinB3-dependent receptors to contribute to cell survival and maintain cell numbers. Similar to control of SVZ proliferation, the ephrinB3 lining may represent a mechanism to regulate numbers of neural progenitors by inducing cell death in cells with unbound EphB3/EphA4, avoiding excessive growth of neural progenitors towards the

medial side of the SVZ. The long, slim shape of the SVZ in the lateral lining of the ventricle might be the consequence of EphB3-/EphA4-expressing progenitors requiring this ephrinB3 contact for survival, supporting the hypothesis of their role as dependence receptors functioning in maintaining homeostatic numbers in the SVZ.

In contrast, progenitors of the adult SVZ have been previously implicated in tumorigenesis (*Oliver, T.G. and Wechsler-Reya, R.J., 2004*). In the cancer research field, the absence of pro-apoptotic dependence receptors is associated with thriving of malignant metastatic tumors (*Mehlen, P. and Puisieux, A., 2006*). Down-regulation of various Eph receptors, like EphA4 in breast cancer or EphB3 in colorectal cancer correlate with tumor progression, invasiveness, and malignancy of tumors (*Fox, B.P. and Kandpal, R.P., 2004; Hafner, C. et al., 2004; Batlle, E. et al., 2005; Guo, D.L. et al., 2006*). In contrast to that, Eph receptor overexpression reduces cell growth in certain cancer cell lines (*Jubb, A.M. et al., 2005; Davalos, V. et al., 2006; Huusko, P. et al., 2004*). The involvement of ephrins and Eph receptors in both neurogenesis and tumorigenesis accounts for their important role in cell cycle control of various cell lineages. Future studies in both cancer and neurogenesis research should further investigate the significance of Eph-ephrin signaling and the implication of expressional changes on cellular fate of adult NSPCs and other cell lines, before therapeutic approaches to induce neurogenesis, e.g. through EphB3 down-regulation are attempted.

3. The hypoxia model: an *in vitro* model for changes post-injury - EphB3 down-regulation affects NSPC proliferation and survival under hypoxia and post-injury

Following TBI, the SVZ of wild type mice demonstrate signs of regeneration for repair, including increased proliferation, cell survival and migration of proliferating cells from the SVZ to cortical and subcortical structures in mice affected by TBI (*Theus, M.H. et al., 2010; Ramaswamy, S. et al., 2005*). Similarly, hypoxia, which takes part in the secondary injury post-TBI, has been shown to promote the growth of neural stem cells and maintain their survival both *in vitro* and *in vivo* (*van den Brink, W.A. et al., 2000; Zhu, L.L. et al., 2005; Studer, L. et al., 2000; Morrison, S.J. et al., 2000; Zhao, H.Q. et al., 2004*). Here, using the HPb biochemical marker of hypoxia, we show HPb localization in the SVZ on stem cells, intermediate progenitor cells and neuroblasts in the first 24 hrs after TBI, which indicates that the local milieu within the SVZ niche has a pO_2 less than 10mmHg (≤ 1%) (*Baumann, G. et al., 2013*). Based on results above, we conclude that the hypoxic changes post-injury represent an ad-

vantageous environment for NSPC growth. When we cultured NSPCs as monolayers under hypoxic (1% O_2) conditions similar to tissue hypoxia post-TBI, growth and survival were increased. Searching for the mechanisms responsible for these hypoxia-triggered growth/survival changes of NSPCs, we investigated the influence of ephrinB3-EphB3 interaction, based on our previous observations that loss of EphB3 signaling favors proliferation and cell survival, as well as down-regulation of EphB3 in the SVZ post-TBI (*Theus, M.H. et al., 2010*). Here, we show that expression of EphB3 receptor is down-regulated in NSPCs cultured in lowered oxygen (1% O_2) over a sustained period of 72 hrs, which is consistent to results post-TBI. This leads to the hypothesis that down-regulation of EphB3 receptor is part of the endogenous cellular response to hypoxia and post-injury creating conditions facilitating the growth response of neural progenitors in the SVZ for tissue repair. We then sought to understand how hypoxic NSPCs would behave in the presence and absence of EphB3 stimulator ephrinB3. The number of proliferative BrdU-positive NSPCs was generally increased in hypoxic culture, but significantly reduced in presence of ephrinB3. This suggests that NSPCs in the SVZ may have a greater growth response to lowered oxygen when not directly in contact with ephrinB3 (*Baumann, G. et al., 2013*). The reducing effect of ephrinB3 on proliferation seems to alleviate over time in hypoxic culture until absent at 72 hrs while addition of ephrinB3 still significantly suppresses proliferation in normoxic cultures after 72 hrs. This could possibly be explained by down-regulation of EphB3 in hypoxia, which decreases the susceptibility to binding of anti-proliferative ephrinB3 after 48 to 72 hrs in culture. Alternatively, one has to take into account that ephrinB3-Fc would be excessively used, degraded or internalized over time in culture, so its concentration is possibly much reduced at later time points. Also, the proliferative rate in terms of BrdU incorporation could generally reach its saturation level around 30% in our experiment. Early hypoxia-induced proliferation might be predominantly regulated through Hif-1α, whose stabilization at 12 hrs in hypoxia we show to be unaltered by ephrinB3 stimulation (*Baumann, G. et al., 2013*). Cell death was consistently reduced when ephrinB3 was present in culture, irrelevant of and to a similar degree in normoxic or hypoxic conditions. This reduction occurs continuously over 72 hrs, which counterposes excessive usage of ephrinB3-Fc.

Mild hypoxia (2.5–5% O_2), which represents the *in vivo* situation of the neural stem cell niche, is optimal for proliferation and neuronal differentiation (*De Filippis, L. and Delia, D., 2011*), while severe hypoxia (< 1% O_2) can contrarily lead to quiescence and apoptosis of fetal human neural stem cells in culture (*Santilli, G. et al., 2010*). The functional role of hy-

poxia occurring in the CNS post-injury for reparative neurogenesis is equally controversial. On one hand, acute hypoxia positively promotes self-renewal of neural stem cells and neurogenesis in the neurovascular niche employing a mechanism including nitric oxide, VEGF and BDNF (*Madri, J.A., 2009*). Also, global ischemia was necessary for transplanted human neural stem cells to migrate towards lesioned areas and not to remain near the injection site as when transplanted into a healthy rat brain (*Rota Nordari, L. et al., 2010*). On the other hand, there is a tight link between angiogenesis and neurogenesis showing that following injury, neural stem cells are supported by their local oxygen-carrying vasculature to proliferate, migrate to and differentiate at injury sites (*Madri, J.A., 2009*). Studies in mice and rats with transient or permanent occlusion of the middle cerebral artery demonstrated that the endothelial cells surrounding the infarcted area started to proliferate as early as 12 to 24 hrs after injury (*Yang, X.T. et al., 2011*). In summary, the response of neural progenitors to hypoxia seems to have two sides: promotion of growth and survival as well as quiescence and apoptosis. This ambiguity of reaction within a rather narrow spectrum of oxygen tension in the brain accounts for the complexity and susceptibility of the neuronal environment to the oxygen supply during health and different phases of disease and recovery. Therefore a fine tuning of oxygen levels seems to play an essential role in regulating the balance between regeneration and degeneration (*De Filippis, L. and Delia, D., 2011*). In addition, several studies indicate that redox metabolism in stem cells is differentially and uniquely regulated to protect them from oxidative damage (*Ogasawara, M.A. and Zhang, H., 2009*). The ability of stem cells to properly control redox balance is an important property of adult progenitor cells and could help explain why transient even severe hypoxia may create a favorable environment in the neural stem niche following brain injury (*Keith, B. and Simon, M.C., 2007; Ostrakhovitch, E.A., Semenikhin, O.A., 2013; Vieira, H.L. et al., 2011; Panchision, D.M., 2009*).

Hypoxia was shown here to be a factor causing down-regulation of EphB3. Hypoxia-inducible factors (HIFs) represent the key regulators of cellular adaption to hypoxia, including proliferation, neurogenesis and angiogenesis (*Ke, Q. and Costa, M., 2006; Keith, B. and Simon, M.C., 2007; Panchision, D.M., 2009; Zhao, T. et al., 2008*). Enhancement of self-renewal and maintenance of human embryonic stem cells was associated with changes in the activity of HIFs, namely long-term stabilization of HIF-2α and HIF-3α ensured expression of pluripotency markers whereas HIF-1α is only involved in the transient initial adaptation to the hypoxic environment (*Forristal, C.E. et al., 2010*). Alternatively, HIF-1α downstream signals have also been shown to promote hypoxia-induced cell death of embryonic stem cells and

lack of HIF-1α lead to decreased hypoxia-induced apoptosis and increased stress-induced proliferation, suggesting at least two different adaptive response mechanisms to hypoxia (*Carmeliet, P. et al., 1998*). This reported ambivalent response to HIF-1α up-regulation could represent the above mentioned two-sided cellular response to hypoxia, ranging from cell growth to hypoxia-induced cell death. We show that hypoxia-induced early HIF-1α stabilization in cultured NSPCs, but this effect is blocked in the presence of the AKT inhibitor LY294002 (*Baumann, G. et al., 2013*). Although stimulation of EphB3 limited hypoxia-mediated p-AKT expression, early activation of HIF-1α was unchanged in the presence of ephrinB1 or B3 suggesting that a threshold of p-AKT suppression may be necessary for blocking HIF-1α induction (*Baumann, G. et al., 2013*). Alternatively, growth suppression in presence of ephrinB3 may be regulated downstream of HIF-1α by other signaling pathways known to be suppressed by Eph signaling such as AKT. It is also possible that EphB3/ephrinB3 signaling may affect HIF-1α expression at later time points or influence HIF-2α and/or HIF-3α expression.

In summary, EphB3 down-regulation may be a factor in transient tissue hypoxia after CNS injury required to activate NSPC cycling, enabling recruitment of endogenous progenitors for repair. Future investigations might also focus on the expression of ligand ephrinB3 in cell layers surrounding the neurogenic region in response to hypoxia/CNS-injury, as changes of ephrinB3 could influence survival and proliferation of neural progenitors in the SVZ.

4. EphB3 induced control of proliferation acts through cell cycle regulators p-AKT and p53

Activation of cell cycle regulator p-AKT is suppressed downstream of EphB3 signaling (*Ricard, J. et al., 2006; Pasquale, E.B., 2008; Theus, M.H. et al., 2010*) and acts as a promotor of p53 degradation (*Mayo, L.D. and Donner, D.B., 2001; Mayo, L.D. and Donner, D.B., 2002*). Accordingly, we show increasing p-AKT levels in hypoxic culture coinciding with down-regulation of EphB3 expression. Tumor suppressor protein p53 is involved in cell-cycle control of both the developing and adult brain. Mice deficient of p53 exhibit an increase in BrdU incorporation compared to p53 expressing littermates, whereas an increase of p53 activity leads to premature loss of neurogenic capacity (*Medrano, S. and Scrable, H., 2005; Medrano, S. et al., 2009; Meletis, K. et al., 2006; Gil-Perotin, S. et al., 2006*). Our previous studies

in vivo confirm that EphB3 stimulation suppresses NSPC proliferation in a p53-dependent manner. Being markedly reduced in the SVZ of uninjured EphB3-deficient and ephrinB3-deficient compared to wild type mice, p53 levels in the SVZ were unchanged in response to TBI in EphB3-deficient mice, whereas in wild type they decrease post-injury concurrent with the burst of proliferation in the SVZ (*Theus, M.H. et al., 2010*). Future studies will investigate p53 levels in normoxic and hypoxic NSPCs. Expectably, increased levels of p-AKT in hypoxic culture will lead to a decrease of p53, resulting in disinhibition of strict cell cycle control and thus promotion of cellular turnover. We have also shown that basal levels of p53 expression were reduced in cultures of EphB3-deficient compared to wild type NSPCs (*Theus, M.H. et al., 2010*). The hypothesis of Eph forward signaling influencing levels of cell cycle proteins such as p-AKT and p53 represents a molecular background for the involvement of Eph-ephrin signaling in guiding events of cellular maintenance, tumorigenesis and neurogenesis. The pathway governing proliferation during adult neurogenesis has previously also been shown to require activity of the transcription factor E2F1 (*Cooper-Kuhn, C.M. et al., 2002; Medrano, S. and Scrable, H., 2005*). In mice lacking E2F1 expression, a significantly decreased amount of stem cell and progenitor division was found in the proliferative zones of the lateral ventricle wall and the hippocampus resulting in a decreased production of newborn neurons in the adult olfactory bulb and dentate gyrus (*Cooper-Kuhn, C.M. et al., 2002*). E2F1 activity is blocked by interaction with the unphosphorylated retinoblastoma protein (Rb); p53 exerts control over this pathway by regulating the expression of the cyclin-dependent kinase (CDK) inhibitors, p21Cip1 and p27Kip1, which inhibit the phosphorylation of Rb by the cyclin-dependent kinases CDK4/6 and CDK2, allowing Rb to bind E2F1 and therefore preventing E2F1 to interact with its co-factor (*Medrano, S. and Scrable, H., 2005*). CDK inhibitor p27Kip1 is decreased in the SVZ of ephrinB3-deficient mice compared to wild type, in agreement with an antiproliferative pathway downstream of EphB3 signaling, involving p-AKT activation, p53 down-regulation and E2F1 activation (*Ricard, J. et al., 2006*).

In regard of these results demonstrating the impact of EphB3/p-AKT/p53/E2F1 on adult NSPC turnover, a careful regulation of their activation and degradation might be required to control events ranging from cellular quiescence to excessive growth. Future studies examining p-AKT/p53/E2F1 levels in wild type and various Eph/ephrin-deficient adult NSPC cultures both in normoxia and hypoxia will aid to understand their functions and interplay.

5. Eph-ephrin signaling is ubiquitously involved in cellular processes - a result of great expressional variability and promiscuity of Eph-ephrin interaction?

The function of Eph-ephrin signaling in adult neurogenesis has just recently begun to be further investigated. Ephrins and their receptors have been shown to play an important role maintaining and regulating the adult SVZ (*Conover, J.C. et al., 2000; Holmberg, J. et al., 2005; Depaepe, V. et al., 2005; Katakowski, M. et al., 2005; Ricard, J. et al., 2006; Theus, M.H. et al., 2010*). The fact that Eph/ephrins are highly expressed and seen as important guidance cues during development, but nearly not present in most cells after birth (*Liebl, D.J. et al., 2003*) except for centers retaining high plasticity such as the neurogenic regions implicates their important role in the regulation of adult neurogenesis. Previously, Eph receptors and ephrins have also been known to orchestrate sophisticated cell movements during multiple early embryonic and adult morphogenetic processes, including gastrulation, segmentation, angiogenesis, neuron axonal path finding and neural crest cell migration (*Zhang, J. and Hughes, S.E., 2006*). Several processes of adult cellular repair involve Eph/ephrin signaling: Stark and colleagues demonstrate that ephrins and Eph receptors guide the processes of muscle regeneration, including the escape from the regenerative niche, directed migration to sites of injury, and patterning of regenerated muscle (*Stark, D.A. et al., 2011*). EphB-ephrinB interactions may also mediate the recruitment, migration and differentiation of mesenchymal stem/stromal cells during bone repair (*Arthur, A. et al., 2011*). Previous to our studies involving ephrinB3 and EphB3 signaling during neuro-regenerative changes post-TBI, neuroblast chain migration in the SVZ has also been shown to be disrupted by infusion of ephrinB2-Fc or EphB2-Fc in the lateral vehicle, whereas infusion of ephrinB1-Fc or Fc alone had no effect (*Conover, J.C. et al., 2000*). EphA4 also regulates CNS vascular formation and guidance during development and has an additional role in the repair of the blood-brain barrier (*Goldshmit, Y. et al., 2006*). These studies demonstrate the plenitude of Eph/ephrin involvement in (inter)cellular signaling, while erroneous appearances occur following disruption of natural Eph/ephrin expression.

Interestingly, Eph/ephrin signaling is defined by high complexity, accounting to the high number of different Eph receptors (16) and ligands (9) expressed in warmblooded vertebrates as well as their numerous possible interactions depending on co-expression and promiscuous binding amongst their members, which creates a great variety of possible Eph-ephrin interactions and bi-directional signaling (*Blits-Huizinga, C.T. et al, 2004*). Depend-

ing on cell type and context, a limited and conserved set of receptor–ligand interactions is translated into a large variety of downstream signaling processes (*Pitulescu, M.E. and Adams, R.H., 2010*). In NSPC monolayer culture, we confirmed expression of EphB1-3 and EphA4 receptors as well as ligands ephrinB1 and 2, whereas ephrinB3 was not present within the SVZ, but outside in the striatum bordering the SVZ. The observation of expressional patterns includes differences in quantity, e.g. showing high expression of ephrinB1 but less ephrinB2 stands for a careful balance of Eph/ephrin protein levels. As a result, multiple probabilities of Eph-ephrin interactions occur, creating a rich variability of forward and reverse intracellular signals sent, which guide both cells towards their respective response in cell behavior.

In conclusion, Ephrins and Eph receptors function to guide numerous and ubiquitous cellular events, by the help of carefully established cellular expressional patterns, through mediating processes like adhesion and repulsion, survival and cell death, proliferation and quiescence, in a cell-interacting manner involving close intercellular contact and a proximate cellular environment. Studies investigating their expressional pattern in different cell types and the development of improved Eph/ephrin-specific imaging will help to elucidate the function of their various interactions.

6. HIF-1α as potential upstream factor of Eph/ephrin expression

Although ephrins and their receptors have been implicated in various processes guiding cellular function, mechanisms controlling their expression are not well understood. In the present studies, we found that EphB3 receptor is downregulated in adult NSPCs maintained in low oxygen culture. Examination of the temporal changes of EphB3 expression *in vitro* under hypoxic exposure shows an early decrease after 24 hours, which is sustained until 72 hours in culture, correlating with increased growth and survival of wild type NSPCs. EphB3 down-regulation was also observed in the SVZ 3 and 7 days after TBI, which correlated with increased proliferation and survival of SVZ-derived adult NSPCs (*Theus, M.H. et al., 2010*). Generally, the mechanisms and prior events which elicit changes of Eph/ephrin expression are not well elaborated, neither after injury or in hypoxia. Vihanto and colleagues have related an up-regulation of ephrins or Eph receptors of both A and B subclasses in hypoxic mouse skin to HIF-1α induction, as RNA interference of HIF-1α in cell lines resulted in prominent reduction of relative Eph/ephrin mRNA levels (*Vihanto, M.M. et*

al., 2005). EphrinB2 has also been found to be up-regulated on endothelial cells in hypoxia (*Suenobu, S. et al., 2002*). As shown here, HIF-1α was stabilized early after 12 hours in hypoxic culture, which could potentially alter transcription of EphB3 expression, shown reduced at 24 hours. This would support the general hypothesis of HIF-influence as main adaptive process under hypoxia, as EphB3 down-regulation enables proliferation and improves survival of NSPCs. We suggest further studies employing suppression of HIF-1α stabilization e.g. via siRNA interference, to assess its effect on Eph/ephrin levels in hypoxic NSPCs.

7. Conclusion

We have demonstrated that Eph-ephrin signaling plays an important role regulating adult NSPCs homeostasis. Ephrins and their receptors are local guiding cues during development and continue to be highly expressed in and outside of the neurogenic region of adult mice. They mediate events affecting proliferation and cell survival of adult neural progenitors in the SVZ. We identify EphB3 as a regulator of adult NSPC turnover, showing that EphB3 forward signaling activates anti-proliferative and anti-apoptotic pathways in adult neural progenitors. EphB3 also acts as dependence receptor, promoting cell survival when bound to ephrinB3, but leading to induction of cell death in absence of its ligand. Concomitantly, ephrinB3 is not expressed within the population of SVZ-derived progenitors, but in cell layers lining the neurogenic region, suggesting to play a bordering, regulatory role maintaining SVZ numbers. Secondly, we confirm secondary hypoxic exposure as low oxygen levels of less than one percent are present during 24 hours after TBI. In low oxygen culture representing conditions post-injury, EphB3 receptor is down-regulated similar to events post-TBI, both leading to increased proliferation and survival of NSPCs. Cell cycle regulator p-AKT is up-regulated both in hypoxia and post-injury, then having shown to lower p53 levels. Addition of ephrinB3 negatively regulates hypoxia-mediated NSPC proliferation. Together, we show that EphB3/ephrinB3 negatively regulates the proliferation of NSPCs under homeostatic and hypoxic conditions and that down-regulating this pathway is critical for NSPC expansion (*Baumann, G. et al., 2013*). The results of these studies provide valuable insights into stem/progenitor cell biology, specifically the influence of Eph-ephrin signaling on adult NSPCs derived from the SVZ, and aim to contribute to the development of restorative therapies to improve neurological outcome after CNS injuries.

VI. REFERENCES

Altman, J., Das, G.D.
Autoradiographic and histological studies of postnatal neurogenesis.
J. Comp. Neurol. 126 (1966) 337-89

Alvarez-Buylla, A., García-Verdugo, J.M.
Neurogenesis in adult subventricular zone.
J. Neurosci. 22 (2002) 629-34

Alvarez-Buylla, A., García-Verdugo, J.M., Tramontin, A.D.
A unified hypothesis on the lineage of neural stem cells.
Nat. Rev. Neurosci. 2 (2001) 287-93

Arthur, A., Zannettino, A., Panagopoulos, R., Koblar, S.A., Sims, N.A., Stylianou, C.,
Matsuo, K., Gronthos, S.
EphB/ephrin-B interactions mediate human MSC attachment, migration and osteochondral differentiation.
Bone 48 (2011) 533-42

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., Lindvall, O.
Neuronal replacement from endogenous precursors in the adult brain after stroke.
Nat. Med. 8 (2002) 963-70

Bader, M.K.
Recognizing and treating ischemic insults to the brain: the role of brain tissue oxygen monitoring.
Crit. Care Nurs. Clin. North Am. 18 (2006) 243-56, xi.

Batlle, E., Bacani, J., Begthel, H., Jonkeer, S., Gregorieff, A., van de Born, M., Malats, N., Sancho, E., Boon, E.,
Pawson, T., Gallinger, S., Pals, S., Clevers, H.
EphB receptor activity suppresses colorectal cancer progression.
Nature Letters 435 (2005) 1126-30

Baumann, G., Travieso, L., Liebl, D.J., Theus, M.H.
Pronounced hypoxia in the subventricular zone following traumatic brain injury and the neural stem/progenitor cell response.
Exp. Biol. Med. (Maywood) 238 (2013) 830-41

Bjornson, C.R., Rietze, R.L., Reynolds, B.A., Magli, M.C., Vescovi, A.L.
Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*.
Science 283 (1999) 534-7

Blits-Huizinga, C.T., Nelersa, C.M., Malhotra, A., Liebl, D.J.
Ephrins and their receptors: binding versus biology.
I.U.B.M.B. Life 56 (2004) 257-65

Bose, I. and Ghosh, B.
The p53-MDM2 network: from oscillations to apoptosis.
J. Biosci. 32 (2007) 991-7

Brückner, K., Pasquale, E.B., Klein, R.
Tyrosine phosphorylation of transmembrane ligands for Eph receptors.
Science 275 (1997) 1640-3

Brückner, K. and Klein, R.
Signaling by Eph receptors and their ephrin ligands.
Curr. Opin. Neurobiol. 8 (1998) 375-82

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumara, D., Brusselmans, K, Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D., Keshert, E.
Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis.
Nature 394 (1998) 485-90

Chen, J., Zacharek, A., Zhang, C., Jiang, H., Li, Y., Roberts, C., Lu, M., Kapke, A., Chopp, M.
Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice.
J. Neurosci. 25 (2005) 2366-75

Clarke, D.L., Johansson, C.B., Wilbertz, J., Veress, B., Nilsson, E., Karlström, H., Lendahl, U., Frisén, J.
Generalized potential of adult neural stem cells.
Science 288 (2000) 1660-3

Conover, J.C., Doetsch, F., Garcia-Verdugo, J.M., Gale, N.W., Yancopoulos, G.D., Alvarez-Buylla, A.
Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone.
Nat. Neurosci. 3 (2000) 1091-7

Cooper-Kuhn, C.M., Vroemen, M., Brown, J., Ye, H., Thompson, M.A., Winkler, J.,Kuhn, H.G.
Impaired adult neurogenesis in mice lacking the transcription factor E2F1.
Mol. Cell. Neurosci. 21 (2002) 312–323

Cowan, C.A., Yokoyama, N., Bianchi, L.M., Henkemeyer, M., Fritzsch, B.
EphB2 guides axons at the midline and is necessary for normal vestibular function.
Neuron 26 (2000) 417–430

Curtis, M.A., Penney, E.B., Pearson, A.G., Van Roon-Mom, W.M., Butterworth, N.J., Dragunow, M., Connor, B., Faull, R.L.
Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain.
Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 9023-7

Curtis, M.A., Penney, E.B., Pearson, J., Dragunow, M., Connor, B., Faull, R.L.
The distribution of progenitor cells in the subependymal layer of the lateral ventricle in the normal and Huntington's disease human brain.
Neuroscience 132 (2005) 777-88

Curtis, M. A., Kam, M., Nannmark, U., Anderson, M.F., Axell, M.Z., Wikkelso, C., Holtås, S., van Roon-Mom, W.M., Björk-Eriksson, T., Nordborg, C., Frisén, J., Dragunow, M., Faull, R.L., Eriksson, P.S.
Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension.
Science 315 (2007) 1243-9

Curtis, M.A., Kam, M., Faull, R.L.M.
Neurogenesis in humans.
Eur. J. Neurosci. 33 (2011) 1170-4

Davalos, V., Dopeso, H., Castaño, H., Wilson, A.J., Vilardell, F., Romero-Gimenez, J., Espín, E., Armengol, M., Capella, G., Mariadason, J.M., Aaltonen, L.A., Schwartz, S. Jr., Arango, D.
EPHB4 and Survival of Colorectal Cancer Patients.
Cancer Res. 66 (2006) 8943-9

De Filippis, L. and Delia, D.
Hypoxia in the regulation of neural stem cells.
Cell. Mol. Life Sci. 68 (2011) 2831-44

Del Rio, G., Kane, D.J., Ball, K.D., Bredesen, D.E.
A novel motif identified in dependence receptors.
PLoS ONE 2 (2007); e463

Dempsey, R.J., Sailor, K.A., Bowen, K.K., Türeyen, K., Vemuganti, R.
Stroke-induced progenitor cell proliferation in adult spontaneously hypertensive rat brain: effect of exogenous IGF-1 and GDNF.
J. Neurochem. 87 (2003) 586-97

Depaepe, V., Suarez-Gonzalez, N., Dufour, A., Passante, L., Gorski, J.A., Jones K.R., Ledent, C., Vanderhaeghen, P.
Ephrin signalling controls brain size by regulating apoptosis of neural progenitors.
Nature 435 (2005) 1244-50

Doetsch, F. and Alvarez-Buylla, A.
Network of tangential pathways for neuronal migration in adult mammalian brain.
Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 14895-900

Doetsch, F., García-Verdugo, J.M., Alvarez-Buylla, A.
Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain.
J. Neurosci. 17 (1997) 5046-61

Doetsch, F., Caille, I., Lim, D.A., García-Verdugo, J.M., Alvarez-Buylla, A.
Subventricular zone astrocytes are neural stem cells in the adult mammalian brain.
Cell 97 (1999) 703-16

Erecinska, M. and Silver, I. A.
Tissue oxygen tension and brain sensitivity to hypoxia.
Respir. Physiol. 128 (2001) 263-76

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., Gage, F.H.
Neurogenesis in the adult human hippocampus.
Nat. Med. 4 (1998) 1313-7

Feng, G., Laskowski, M.B., Feldheim, D.A., Wang, H., Lewis, R., Frisen, J., Flanagan, J.G., Sanes, J.R.
Roles for ephrins in positionally selective synaptogenesis between motor neurons and muscle fibers.
Neuron 25 (2000) 295-306

Fox, B.P. and Kandpal, R.P.
Invasiveness of breast carcinoma cells and transcript profile: Eph receptors and ephrin ligands as molecular markers of potential diagnostic and prognostic application.
Biochem. Biophys. Res. Commun. 318 (2004) 882-92

Forristal, C.E., Wright, K.L., Hanley, N.A., Oreffo, R.O., Houghton, F.D.
Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions.
Reproduction 139 (2010) 85–97

Friedman, G.C. and O'Leary, D.D.
Eph receptor tyrosine kinases and their ligands in neural development.
Curr. Opin. Neurobiol. 6 (1996) 127-33

Furne, C., Ricard, J., Cabrera, J.R., Pays, L., Bethea, J.R., Mehlen, P., Liebl, D.J.
EphrinB3 is an anti-apoptotic ligand that inhibits the dependence receptor functions of EphA4 receptors during adult neurogenesis.
Biochim. Biophys. Acta 1793 (2009) 231-8

Gage, F.H.
Mammalian neural stem cells.
Science 287 (2000) 1433-8

Gale, N.W., Holland, S.J., Valenzuela, D.M., Flenniken, A., Pan, L., Ryan, T.E., Henkemeyer, M., Strebhardt, K., Hirau, H., Wilkinson, D.G., Pawson, T., Davis, S., Yancopoulos, G.D.
Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis.
Neuron 17 (1996) 9-19

Gao, W.Q., Shinsky, N., Armanini, M.P., Moran, P., Zheng, J.L., Mendoza-Ramirez, J.L., Phillips, H.S., Winslow, J.W., Caras, I.W.
Regulation of hippocampal synaptic plasticity by the tyrosine kinase receptor, REK7/EphA5, and its ligand, AL-1/Ephrin-A5.
Mol. Cell Neurosci. 11 (1998) 247-59

Gao, P.P., Yue, Y., Cerretti, D.P., Dreyfus, C., Zhou, R.
Ephrin-dependent growth and pruning of hippocampal axons.
Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 4073-7

Gil-Perotin, S., Marin-Husstege, M., Soriano-Navarro, M., Zindy, F., Roussel, M.F., Garcia-Verdugo, J.M., Casaccia-Bonnefil, P.
Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumors.
J. Neurosci. 26 (2006) 1107–16

Goldman, S.A. and Nottebohm, F.
Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain.
Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 2390-4

Goldshmit, Y., Galea, M.P., Bartlett, P.F., Turnley, A.M.
EphA4 Regulates Central Nervous System Vascular Formation
J. Comp. Neurol. 497 (2006) 864–875

Guo, D.L., Zhang, J., Yuen, S.T., Tsui, W.Y., Chan, A.S., Ho, C., Ji, J., Leung, S.Y., Chen, X.
Reduced expression of EphB2 that parallels invasion and metastasis in colorectal tumours.
Carcinogenesis 27 (2006) 454-64

Hafner, C., Schmitz, G., Meyer, S., Bataille, F., Hau, P., Langmann, T., Dietmaier, W., Landthaler, M., Vogt, T.
Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers
Clin Chem. 50 (2004) 490-9

Henkemeyer, M., Orioli, D., Henderson, J.T., Saxton, T.M., Roder, J., Pawson, T., Klein, R.
Nuk controls pathfinding of commissural axons in the mammalian central nervous system.
Cell 86 (1996) 35-46

Hoglinger, G.U., Rizk, P., Muriel, M.P., Duyckaerts, C., Oertel, W.H., Caille, I., Hirsch, E.C.
Dopamine depletion impairs precursor cell proliferation in Parkinson disease.
Nat. Neurosci. 7 (2004) 726-35

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M., Pawson, T.
Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands.
Nature 383 (1996) 722-5

Holmberg, J., Armulik, A., Senti, K.A., Edoff, K., Spalding, K., Momma, S., Cassidy, R., Flanagan, J.G., Frisén, J.
Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis.
Genes Dev. 19 (2005) 462-71

Huusko, P., Ponciano-Jackson, D., Wolf, M., Kiefer, J.A., Azorsa, D.O., Tuzmen, S., Weaver, D., Robbins, C., Moses, T., Allinen, M., Hautaniemi, S., Chen, Y., Elkahloun, A., Basik, M., Bova, G.S., Bubendorf, L., Lugli, A., Sauter, G., Schleutker, J., Ozcelik, H., Elowe, S., Pawson, T., Trent, J.M., Carpten, J.D., Kallioniemi, O.P., Mousses, S.
Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer.
Nat. Genet. 36 (2004) 979-83

Jin, K., Zhu, Y., Sun, Y., Mao, X.O., Xie, L., Greenberg, D.A.
Vascular endothelial growth factor (VEGF) stimulates neurogenesis *in vitro* and *in vivo*.
Proc. Natl. Acad. Sci. U.S.A. 99 (2002-1) 11946-50

Jin, K., Mao, X.O., Sun, Y., Xie, L., Greenberg, D.A.
Stem cell factor stimulates neurogenesis *in vitro* and *in vivo*.
J. Clin. Invest. 110 (2002-2) 311-9

Jin, K., Peel, A.L., Mao, X.O., Xie, L., Cottrell, B.A., Henshall, D.C., Greenberg, D.A.
Increased hippocampal neurogenesis in Alzheimer's disease.
Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 343-7

Johns, P.R. and Easter, S.S. Jr.
Growth of the adult goldfish eye.
J. Comp. Neurol. 176 (1977) 331-41

Jubb, A.M., Zhong, F., Bheddah, S., Grabsch, H.I, Frantz, G.D., Mueller, W., Kavi, V., Quirke, P, Polakis, P., Koeppen, H.
EphB2 is a prognostic factor in colorectal cancer.
Clin. Cancer Res. 11 (2005) 5181–7.

Kalo, M.S. and Pasquale, E.B.
Multiple *in vivo* tyrosine phosphorylation sites in EphB receptors.
Biochemistry 38 (1999) 14396-408

Katakowski, M., Zhang, Z., deCarvalho, A.C., Chopp, M.
EphB2 induces proliferation and promotes a neuronal fate in adult subventricular neural precursor cells.
Neurosci. Lett. 385 (2005) 204-9

Ke, Q. and Costa, M.
Hypoxia-inducible factor-1 (HIF-1).
Mol. Pharmacol. 70 (2006) 1469-80

Keith, B. and Simon, M. C.
Hypoxia-inducible factors, stem cells, and cancer.
Cell 129 (2007) 465-72

Kuhn, H.G., Winkler, J., Kempermann, G., Thal, L.J., Gage, F.H.
Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain.
J. Neurosci. 17 (1997) 5820-9

Leker, R.R., Soldner, F., Velasco, I., Gavin, D.K., Androutsellis-Theotokis, A., McKay, R.D.
Long-lasting regeneration after ischemia in the cerebral cortex.
Stroke 38 (2007) 153-61

Lie, D.C., Dziewczapolski, G., Willhoite, A.R., Kaspar, B.K., Shults, C.W., Gage, F.H.
The adult substantia nigra contains progenitor cells with neurogenic potential.
J. Neurosci. 22 (2002) 6639-49

Liebl, D.J., Morris, C.J., Henkemeyer, M., Parada, L.F.
mRNA expression of ephrins and Eph receptor tyrosine kinases in the neonatal and adult mouse central nervous
system.
J. Neurosci. Res. 71 (2003) 7-22

Liu, S., Shi, H., Liu, W., Furuichi, T. Timmins, G.S., Liu, K.J.
Interstitial pO_2 in ischemic penumbra and core are differentially affected following transient focal cerebral ischemia in rats.
J. Cereb. Blood Flow Metab. 24 (2004) 343-9

Lois, C. and Alvarez-Buylla, A.
Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia.
Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2074-7

Lois, C. and Alvarez-Buylla, A.
Long-distance neuronal migration in the adult mammalian brain.
Science 264 (1994) 1145-8

Lois, C., García-Verdugo, J.M., Alvarez-Buylla, A.
Chain migration of neuronal precursors.
Science 271 (1996) 978-81

Lucassen, P.J., Stumpel, M.W., Wang, Q., Aronica, E.
Decreased numbers of progenitor cells but no response to antidepressant drugs in the hippocampus of elderly depressed patients.
Neuropharmacology 58 (2010) 940-9

Maas, A. I., Fleckenstein, W., de Jong, D.A., van Santbrink, H.
Monitoring cerebral oxygenation: experimental studies and preliminary clinical results of continuous monitoring of cerebrospinal fluid and brain tissue oxygen tension.
Acta Neurochir. Suppl. (Wien) 59 (1993) 50-7

Madri, J. A.
Modeling the neurovascular niche: implications for recovery from CNS injury.
J. Physiol. Pharmacol. 60 (2009) 95-104

Magavi, S.S., Leavitt, B.R., Macklis, J.D.
Induction of neurogenesis in the neocortex of adult mice.
Nature 405 (2000) 951-5

Mayo, L.D. and Donner, D.B.
A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus.
Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 11598-603

Mayo, L.D. and Donner, D.B.
The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network.
Trends Biochem. Sci. 27 (2002) 462-7

Medrano, S. and Scrable, H.
Maintaining appearances - the role of p53 in adult neurogenesis.
Biochem. Biophys. Res. Commun. 331 (2005) 828-33

Medrano, S., Burns-Cusato, M., Atienza, M.B., Rahimi, D., Scrable, H.
Regenerative capacity of neural precursors in the adult mammalian brain is under the control of p53.
Neurobiol. Aging 30 (2009) 483-97

Mehlen, P., Rabizadeh, S., Snipas, S.J., Assa-Munt, N., Salvesen, G.S., Bredesen, D.E.
The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis.
Nature 395 (1998) 801-4

Mehlen, P. and Bredesen, D.E.
The dependence receptor hypothesis.
Apoptosis 9 (2004) 37-49

Mehlen, P. and Thibert, C.
Dependence receptors: between life and death.
Cell. Mol. Life Sci. 61 (2004) 1854-66

Mehlen, P. and Puisieux, A.
Metastasis: a question of life or death.
Nat. Rev. Cancer 6 (2006) 449-58.

Meletis, K., Wirta, V., Hede, S.M., Nistér, M., Lundeberg, J., Frisén, J.
p53 suppresses the self-renewal of adult neural stem cells.
Development 133 (2006) 363-9

Menges, C.W. and McCance, D.J.
Constitutive activation of the Raf-MAPK pathway causes negative feedback inhibition of Ras-PI3K-AKT and cellular arrest through the EphA2 receptor.
Oncogene 27 (2008) 2934-40

Milosevic, J., Maisel, M., Wegner, F., Leuchtenberger, J., Wenger, R.H., Gerlach, M., Storch, A., Schwarz, J.
Lack of Hypoxia-Inducible Factor-1α impairs midbrain neural precursor cell involving vascular endothelial growth factor signaling.
J. Neurosci. 27 (2007) 412– 21

Ming, G.L. and Song, H.
Adult neurogenesis in the mammalian central nervous system.
Annu. Rev. Neurosci. 28 (2005) 223-50

Mohyeldin, A., Garzón-Muvdi, T., Quiñones-Hinojosa, A.
Oxygen in stem cell biology: a critical component of the stem cell niche.
Cell Stem Cell 7 (2010) 150-61

Morrison, S.J., Csete, M., Groves, A.K., Melega, W., Wold, B., Anderson, D.J.
Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells.
J. Neurosci. 20 (2000) 7370-6

Mottet, D., Dumont, V., Deccache, Y., Demazy, C., Ninane, N., Raes, M., Michiels, C.
Regulation of hypoxia-inducible factor-1 alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/ glycogen synthase kinase 3 beta pathway in HepG2 cells.
J. Biol. Chem. 278 (2003) 1277–85

Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., Tamura, A., Kirino, T., Nakafuku, M.
Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors.
Cell 110 (2002) 429-41

Ogasawara, M.A. and Zhang, H.
Redox regulation and its emerging roles in stem cells and stem-like cancer cells.
Antioxid. Redox. Signal 11 (2009) 1107–22

Ohab, J.J., Fleming, S., Blesch, A., Carmichael, S.T.
A neurovascular niche for neurogenesis after stroke.
J. Neurosci. 26 (2006) 13007-16

Oliver, T.G. and Wechsler-Reya, R.J.
Getting at the root and stem of brain tumors.
Neuron 42 (2004) 885-8.

Orioli, D., Henkemeyer, M., Lemke, G., Klein, R., Pawson, T.
Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation.
Embo J. 15 (1996) 6035-49

Orioli, D. and Klein, R.
The Eph receptor family: axonal guidance by contact repulsion.
Trends Genet. 13 (1997) 354-9

Ostrakhovitch, E.A., Semenikhin, O.A.
The role of redox environment in neurogenic development.
Arch. Biochem. Biophys. 534 (2013) 44-54

Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F., Gage, F.H.
Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS.
J. Neurosci. 19 (1999) 8487-97

Panchision, D.M.
The role of oxygen in regulating neural stem cells in development and disease.
J. Cell. Physiol. 220 (2009) 562-8

Pasquale, E.B.
Eph-ephrin bidirectional signaling in physiology and disease.
Cell 133 (2008) 38-52

Pawson, T.
Protein-tyrosine kinases. Getting down to specifics.
Nature 373 (1995) 477-8

Pawson, T. and Scott, J.D.
Signaling through scaffold, anchoring, and adaptor proteins.
Science 278 (1997) 2075-80

Pitulescu, M.E. and Adams, R.H.
Eph/ephrin molecules - a hub for signaling and endocytosis.
Genes & Development 24 (2010) 2480-2492

Pore, N., Jiang, Z., Shu, H. K., Bernhard, E., Kao, G. D., Maity, A.
Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycinindependent pathway.
Mol. Cancer Res. 4 (2006) 471-9

Ramaswamy, S., Goings, G.E., Sonderstrom, K.E., Szele, F.G., Kozlowski, D.A.
Cellular proliferation and migration following a controlled cortical impact in the mouse.
Brain. Res. 1053 (2005) 38-53.

Reynolds, B.A. and Weiss, S.
Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system.
Science 255 (1992) 1707-10

Ricard, J., Salinas, J. Garcia, L., Liebl, D.J.
EphrinB3 regulates cell proliferation and survival in adult neurogenesis.
Mol. Cell. Neurosci. 31 (2006) 713-22

Roger, L., Gadea, G., Roux, P.
Control of cell migration: a tumour suppressor function for p53?
Biol Cell 98 (2006) 141-52

Rota Nodari, L., Ferrari, D., GIANI, F., BOSSI, M., Rodriguez-Menendez, V., Tredici, G., Delia, D., Vescov, A.L.. De Filippis, L.
Long-term survival of human neural stem cells in the ischemic rat brain upon transient immunosuppression.
PLoS One 5 (2010): e14035.

Salman, H., Ghosh, P., Kernie, S.G.
Subventricular zone neural stem cells remodel the brain following traumatic injury in adult mice.
J. Neurotrauma 21 (2004) 283-92

Santilli, G., Lamorte, G., Carlessi, L, Ferrari, D., Rota Nodari, L, Binda, E., Delia, D., Vescovi, A.L., De Filippis, L.
Mild hypoxia enhances proliferation and multipotency of human neural stem cells.
PLoS One 5 (2010) e8575.

Scheffler, B., Walton, N.M., Lin, D.D., Goetz, A.K., Enikolopov, G., Roper, S.N., Steindler, D.A.
Phenotypic and functional characterization of adult brain neuropoiesis.
Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 9353-8

Shen, Q., Wang, Y., Kokovay, E., Gang, L., Chuang, S.-M., Goderie, S.K., Roysam, B., Temple, S.
Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions.
Cell Stem Cell 3 (2008) 289-300

Shihabuddin, L.S., Horner, P.J., Ray, J., Gage, F.H.
Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus.
J. Neurosci. 20 (2000) 8727-35

Snapyan, M., Lemasson, M., Brill, M.S., Blais, M., Massouh, M., Ninkovic, J., Gravel, C., Berthod, F., Götz, M., Barker, P.A., Parent, A., Saghatelyan, A.
Vasculature guides migrating neuronal precursors in the adult mammalian forebrain via brain-derived neurotrophic factor signaling.
J Neurosci 29 (2009) 4172-88

Sohur, U.S., Emsley, J.G., Mitchell, B.D., Macklis, J.D.
Adult neurogenesis and cellular brain repair with neural progenitors, precursors and stem cells.
Philos. Trans. R. Soc. Lond. B. Biol. Sci. 361 (2006) 1477-97

Stark, D.A., Karvas, R.M., Siegel, A.L., Cornelison, D.D.
Eph/ephrin interactions modulate muscle satellite cell motility and patterning.
Development. 138 (2011) 5279-89

Storch, A., Lester, H.A., Boehm, B.A., Schwarz, J.
Functional characterization of dopaminergic neurons derived from rodent mesencephalic progenitor cells.
J. Chem. Neuroanat. 26 (2003) 133-42

Storch, A., Paul, G., Csete, M., Boehm, B.O., Carvey, P.M., Kupsch, A., Schwarz, J.
Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells.
Exp. Neurol. 170 (2001) 317-25

Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B., McKay, R.
Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen.
J Neurosci 20 (2000) 7377-83

Suenobu, S., Takakura, N., Inada, T., Yamada, Y., Yuasa, H., Zhang, X.-Q., Sakano, S., Oike, S., and Suda, T.
A role of EphB4 receptor and its ligand, ephrin-B2, in erythropoiesis.
Biochem. Biophys. Res. Commun. 293 (2002) 1124-1131

Suhonen, J.O., Peterson, D.A., Ray J., Gage, F.H.
Differentiation of adult hippocampus-derived progenitors into olfactory neurons *in vivo*.
Nature 383 (1996) 624-7

Sundholm-Peters, N.L., Yang, H.K., Goings, G.E., Walker, A.S., Szele, F.G.
Subventricular zone neuroblasts emigrate toward cortical lesions.
J. Neuropathol. Exp. Neurol. 64 (2005) 1089-100

Tauszig-Delamasure, S., Yu, L.Y., Cabrera, J.R., Bouzas-Rodriguez, J., Mermet-Bouvier, C., Guix, C., Bordeaux, M.C., Arumäe, U., Mehlen, P.
The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm.
Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 13361-6

Theus, M.H., Ricard, J., Bethea, J.R., Liebl, D.J.
EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury.
Stem Cells 28 (2010) 1231-42

Thored, P., Wood, J., Arvidsson, A., Cammenga, J., Kokaia, Z., Lindvall, O.
Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke.
Stroke 38 (2007) 3032-9

Torres, R., Firestein, B.L., Don, H., Staudinger, J., Olson, E.N., Huganir, R.L., Bredt, D.S., Gale, N.W., Yancopoulos, G.D.
PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands.
Neuron 21 (1998) 1453-63

Tropepe, V., Coles, B.L., Chiasson, B.J., Horsford, D.J., Elia, A.J., McInnes, R.R., van der Kooy, D.
Retinal stem cells in the adult mammalian eye.
Science 287 (2000) 2032-6

Tsai, P.T., Ohab, J.J., Kertesz, N., Groszer, M., Matter, C., Gao, J., Liu, X., Wu, H., Carmichael, S.T.
A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery.
J. Neurosci. 26 (2006) 1269-74

Tulasne, D., Deheuninck, J., Lourenco, F.C., Lamballe, F., Ji, Z., Leroy, C., Puchois, E., Moumen, A., Maina, F., Mehlen, P., Fafeur, V.
Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage.
Mol. Cell. Biol. 24 (2004) 10328–39

Van den Brink, W.A., van Santbrink, H., Steyerberg, E.W., Avezaat, C.J., Suazo, J.A., Hogesteeger, C., Jansen, W.J., Kloos, L.M., Vermeulen, J., Maas, A.I.
Brain oxygen tension in severe head injury.
Neurosurgery 46 (2000) 868-78

Vieira, H.L., Alves, P.M., Vercelli, A.
Modulation of neuronal stem cell differentiation by hypoxia and reactive oxygen species.
Prog. Neurobiol. 93 (2011) 444–55

Vihanto, M.M., Plock, J., Erni, D., Frey, B.M., Frey, F.J., Huynh-Do, U.
Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin.
FASEB J. 19 (2005) 1689-91

Vogelstein, B., Lane, D., Levine, A.J.
Surfing the p53 network.
Nature 408 (2000) 307-10

Wang, L., Zhang, Z., Wang, Y., Zhang, R., Chopp, M.
Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats.
Stroke 35 (2004) 1732-7

Wang, Y., Jin, K., Mao, X.O., Xie, L., Banwait, S., Marti, H.H., Greenberg, D.A.
VEGF-overexpressing transgenic mice show enhanced post-ischemic neurogenesis and neuromigration.
J. Neurosci. Res. 85 (2007) 740-7

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., Reynolds, B.A.
Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis.
J. Neurosci. 16 (1996) 7599-609

Wiltrout, C., Lang, B., Yan, Y., Dempsey, R.J., Vemuganti, R.
Repairing brain after stroke: a review on post-ischemic neurogenesis.
Neurochem. Int. 50 (2007) 1028-41

Winner, B., Cooper-Kuhn, C.M., Aigner, R., Winkler, J., Kuhn, H.G.
Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb.
Eur. J. Neurosci. 16 (2002) 1681-9

Yamashita, T., Ninomiya, M., Hernández Acosta, P., García-Verdugo, J.M., Sunabori, M., Sakaguchi, M., Adachi, K., Kojima, T., Hirota, Y., Kawase, T., Araki, N., Abe, K., Okano, H., Sawamoto, K.
Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum.
J. Neurosci. 26 (2006) 6627-36

Yan, Y.P., Sailor, K.A., Vemuganti, R., Dempsey, R.J.
Insulin-like growth factor-1 is an endogenous mediator of focal ischemiainduced neural progenitor proliferation.
Eur. J. Neurosci. 24 (2006) 45-54

Yang, X.T., Bi, Y.Y. and Feng D.F.
From the vascular microenvironment to neurogenesis.
Brain Res. Bull. 84 (2011) 1-7

Yokoyama, N., Romero, M.I., Cowan, C.A., Galvan, P., Helmbacher, F., Charnay, P., Parada, L.F., Henkemeyer, M.
Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline.
Neuron 29 (2001) 85–97

Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S.S., Waeber, C., Bakowska, J.C., Breakefield, X.O., Moskowitz, M.A.
FGF-2 regulation of neurogenesis in adult hippocampus after brain injury.
Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 5874-9

Yoshiya, K., Tanaka, H., Kasai, K., Irisawa, T., Shiozaki, T., Sugimoto, H.
Profile of gene expression in the subventricular zone after traumatic brain injury.
J. Neurotrauma. 20 (2003) 1147-62

Zauner, A., Bullock, R., Kuta, A.J., Woodward, J., Young, H.F.
Glutamate release and cerebral blood flow after severe human head injury.
Acta Neurochir. Suppl. 67 (1996) 40-4

Zhang, J. and Hughes, S.
Role of the ephrin and Eph receptor tyrosine kinase families in angiogenesis and development of the cardiovascular system.
J Pathol 208 (2006) 453-61

Zhang, K., Zhu, L., Fan, M.
Oxygen, a key factor regulating cell behavior during neurogenesis and cerebral diseases.
Front. Mol. Neurosci. 4 (2011) 1-11

Zhang, R., Zhang, L., Zhang, Z., Wang, Y., Lu, M., Lapointe, M., Chopp, M.
A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats.
Ann Neurol 50 (2001) 602-11

Zhao, H.Q., Zhu, L.L., Zaho, T., Wu, L.Y., Ding, A.S., Fan, M.
Effects of hypoxia on the proliferation of embryonic stem cells
Zhongguo Ying Yong Sheng Li Xue Za Zhi 20 (2004) 209-13

Zhao, T., Zhang, C.P., Lie, Z.H., Wu, L.Y., Huang, X. Wu, H.T., Xiong, L., Wang, X., Wang, X.M., Zhu, L.L., Fan, M.
Hypoxia-driven proliferation of embryonic neural stem/progenitor cells-role of hypoxia-inducible transcription factor-1 alpha.
FEBS J. 275 (2008) 1824-34

Zhou, J., Schmid, T., Brune, B.
HIF-1α and p53 as targets of NO in affecting cell proliferation, death and adaptation.
Curr. Mol. Med. 4 (2004) 741–51

Zhu, D.Y., Liu, S.H., Sun, H.S., Lu, Y.M.
Expression of inducible nitric oxide synthase after focal cerebral ischemia stimulates neurogenesis in the adult rodent dentate gyrus.
J. Neurosci. 23 (2003) 223-9

Zhu, L.L., Wu, L.Y., Yew, D.T., Fan, M.
Effects of hypoxia on the proliferation and differentiation of NSCs.
Mol Neurobiol 31 (2005) 231-42

Zimmer, M., Palmer, A. Köhler, J., Klein, R.
EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion.
Nat. Cell. Biol. 5 (2003) 869-78

VII. APPENDIX

1. Previous publication

Baumann, G., Travieso, L., Liebl, D.J., Theus, M.H.

Pronounced hypoxia in the subventricular zone following traumatic brain injury and the neural stem/progenitor cell response.

Exp. Biol. Med. (Maywood) 238 (2013) 830-41

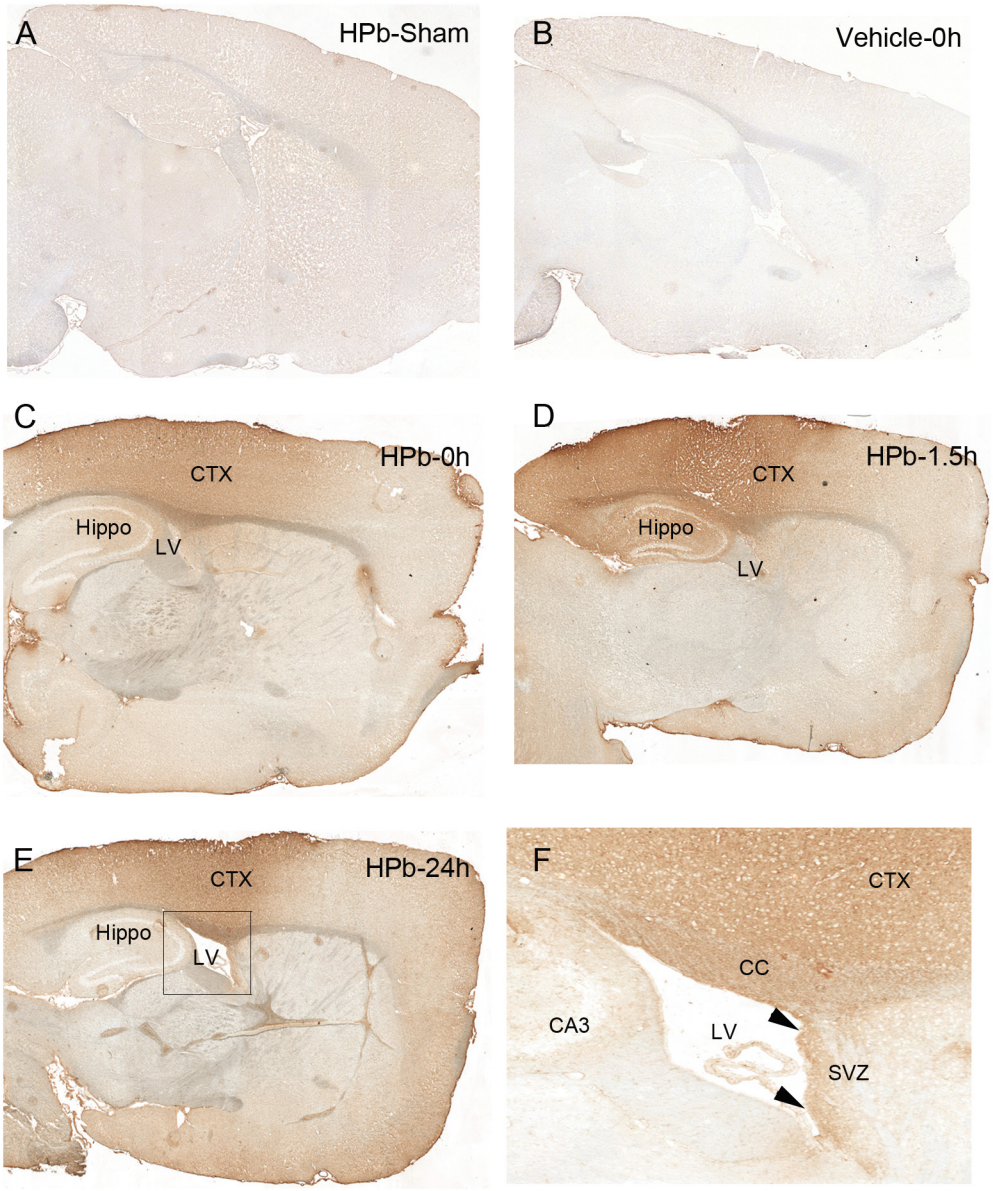


Figure B-1. HPb expression in the adult murine brain after CCI injury.

Uptake of hypoxyprobe (HPb) by low oxygen-bearing cells was visualized using immunohistochemistry and diaminobenzidine (DAB) staining on sagittal brain sections. Compared to sham-injured (a) or CCI-injured vehicle injected control (b), induction of HPb expression was seen at 0 h (c), 1.5 h (d), and 24 h (e) after CCI injury. DAB staining (brown) shows HPb expression is localized to the impacted cortex, corpus callosum, and the lateral wall of the lateral ventricle. HPb expression was also seen in the hippocampus but only at 1.5 h post-injury. At higher magnification, we observed that HPb remained expressed in the SVZ at 24 h (f; inset from e; black arrows) post-CCI injury CTX: cortex; CC: corpus callosum; LV: lateral ventricle; Hippo: hippocampus. (*Baumann, G. et al., 2013, figure pre-published*)

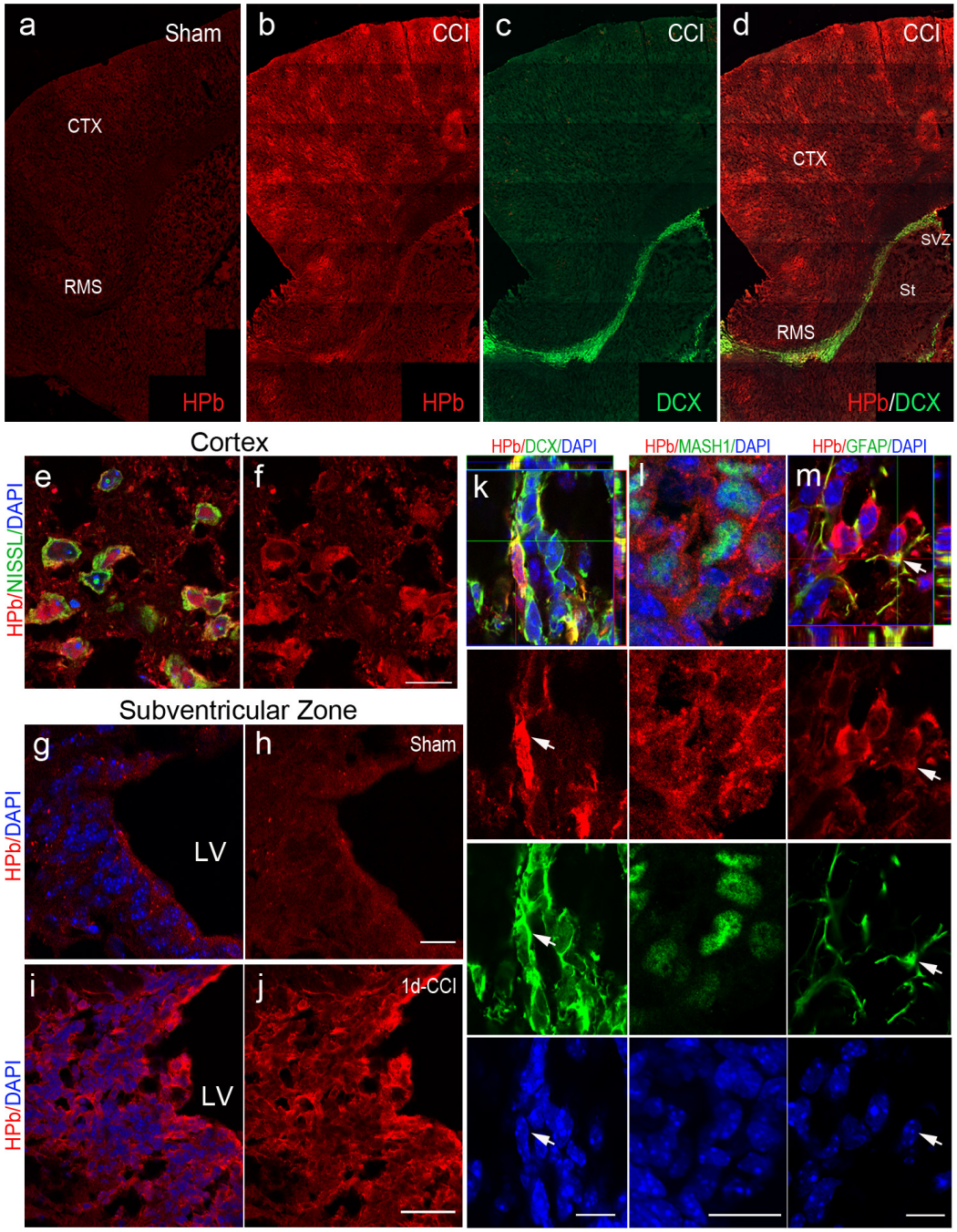


Figure B-2. HPb expression in the adult murine forebrain after CCI injury.

(a–d) HPb (red) expression by immunofluorescence on sagittal sections following sham or CCI injury with HPb injection. Representative low-magnification images demonstrate that the SVZ and RMS reside within hypoxic regions of the brain after 1.5 h CCI injury (b–d) compared to sham-injured (a) controls. HPb is expressed on injured NISSL-positive neurons in the cortex (e and f; scale bar 1/4 20 mm), weakly expressed in the sham-injured SVZ (g and h; scale bar 1/4 20 mm) and significantly present at 1 day post-TBI in the SVZ (i and j; scale bar 1/4 50 mm). HPb co-localized with DCX-positive neuroblasts (k; scale bar 1/4 10 mm), MASH1-positive intermediate progenitors (l; scale bar 1/4 20 mm) and GFAP-expressing NSPCs (m; scale bar 1/4 10 mm), in the SVZ neurogenic compartment. (*Baumann, G. et al., 2013, figure pre-published*)

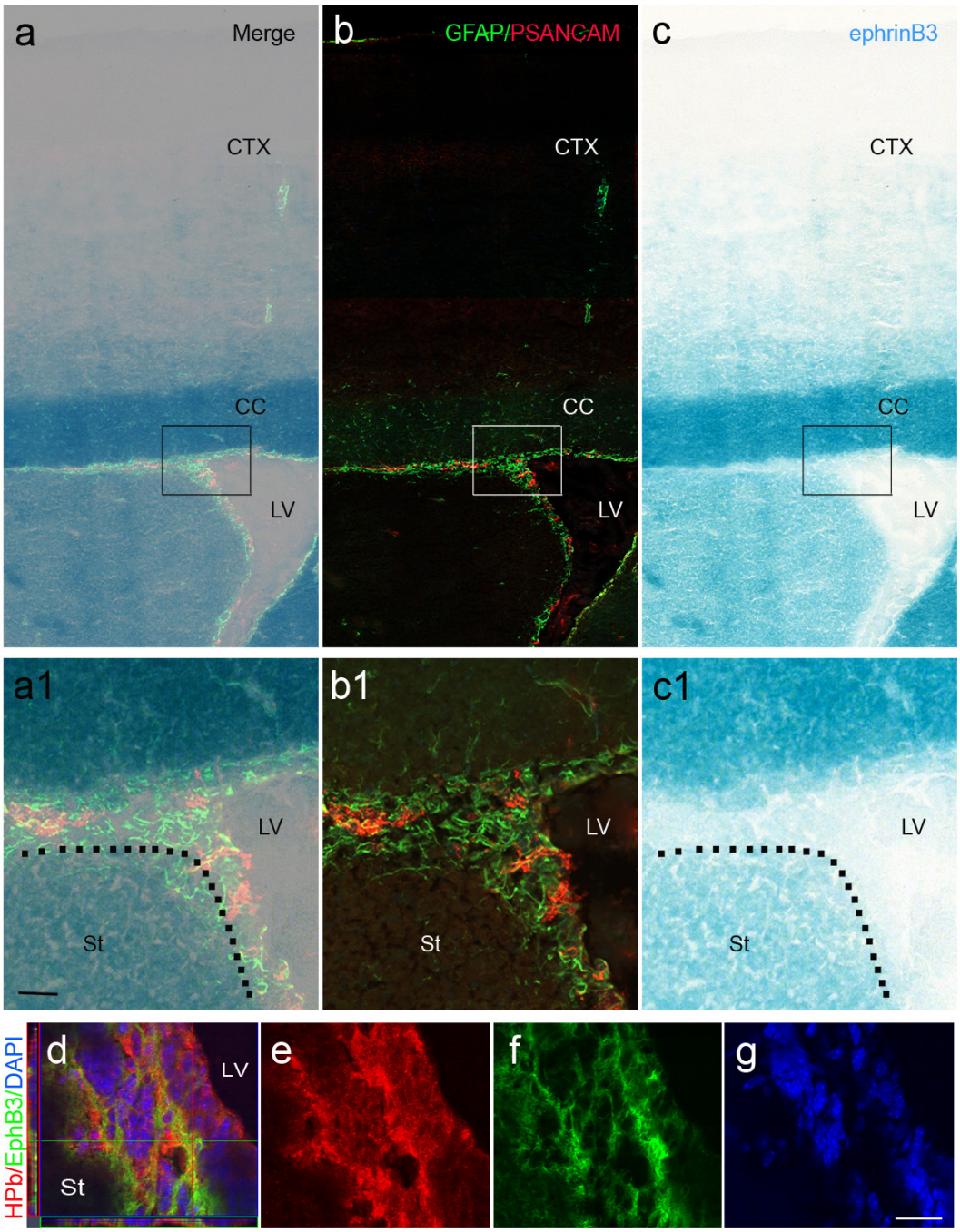


Figure B-3. EphB3 and ephrinB3 expression in the adult murine forebrain.

(a–c) Immunofluorescence labeling and β-gal staining on sagittal sections from naïve ephrinB3$^{\beta\text{-gal}}$ mice. (a) Low magnification overlay image of immunofluorescence labeling of anti-GFAP and anti-PSA-NCAM (b) with β-gal staining of ephrinB3 (c). EphrinB3 is robustly expressed in the corpus callosum, striatum, and weakly expressed in the ventral cortex. (a1–c1) Conversely, high-magnification images show the absence of ephrinB3 in the SVZ. GFAP-expressing NSPCs (green) and PSA-NAM-positive neuroblasts (red) reside in the lining of the lateral ventricular wall adjacent to the β-gal staining (insets from a–c; black dotted lines). (d–g) EphB3 expression co-labels with HPb hypoxia marker in the SVZ and is absent in the striatum. CTX: cortex; CC: corpus callosum; LV: lateral ventricle; St: striatum. Scale bar (a1–c1) 1⁄4 100 mm; (d–g) 1⁄4 20 mm. (*Baumann, G. et al., 2013, figure pre-published*)

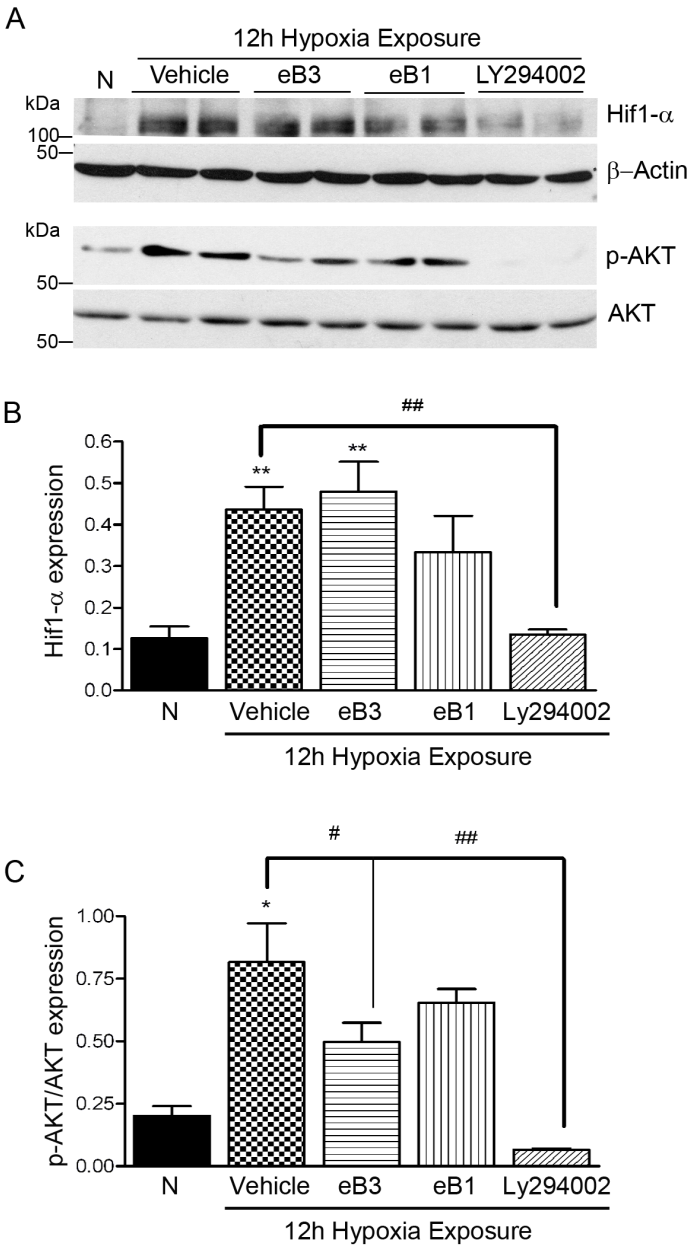


Figure B-4. Hif-1α induction is not regulated by ephrinB3.

(a) The expression of Hif-1α and p-AKT in cultured NSPCs was assessed using Western Blot analysis following 12 hr hypoxia exposure. Both Hif-1α and p-AKT expression are increased with hypoxia compared to normoxia (N). Inhibition with 50 mm AKT pharmacological inhibitor LY294002, prevented AKT phosphorylation and blocked Hif-1α induction compared to vehicle treatment. Although the application of 10 mg/mL eB3 and 10 mg/mL eB1 also significantly reduced p-AKT levels under hypoxia, the induction of Hif-1α was unaffected. (b) Bar graph representing quantified relative expression of Hif-1α at 12 hr hypoxia or normoxia exposure. Grey intensity values were normalized against β-actin control levels. (c) Bar graph representing quantified data for p-AKT normalized to total AKT levels. *$p < 0.05$ and **$p < 0.01$ compared to normoxia control NSPC cultures. #$p > 0.05$ and ##$p < 0.01$ compared to hypoxia treated vehicle control. (*Baumann, G. et al., 2013, figure pre-published*)

3. Curriculum Vitae

Personal Data

Gisela Katharina Baumann

born 5 January, 1984 in Bad Bergzabern, Germany

Address: Weyherer Straße 5, 76835 Hainfeld

Mobile: + 49 (0)176 / 2345 3219

E-Mail: gisela.k.baumann@googlemail.com

Education

1990 - 1994	Primary School, Edesheim/ Pfalz
1994 - 2003	Eduard-Spranger-Gymnasium, Landau/Pfalz
	General qualification for university entrance ("Abitur") : 1,7
2003 - 2005	Ludwig-Maximilians-University and Technical University, Munich
	Preclinical studies of human medicine
	First part of doctor's degree : 2,5
	Elective: "PINK1 in Parkinson's syndrome" (Adolf-Butenandt-Institute) : 1,0
2005 - 2010	Technical University, Munich
	Clinical studies of human medicine
	Second part of doctor's degree : 1,5
	Elective: Diagnostic Radiology

Practical Experience

2006 - 2007	The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami (FL), USA
	Research Scholar in Neuroscience, laboratory of Dr Daniel J Liebl
	Thesis project: „Eph B3 and its role in Adult Neural Stem Cells"
	• MD scholarship (Boehringer-Ingelheim-Fonds)
2011 - 2012	Royal Blackburn Hospital, Blackburn, UK
	Foundation Year One Doctor
	• Rotations: Paediatrics, Repiratory Medicine, Colorectal Surgery
2012 - 2013	James Cook University Hospital, Middlesbrough, UK
	Foundation Year Two Doctor
	• Rotations: Intensive Care, Geriatrics, Gastroenterology