THE NEUROGENIC NICHE AND INJURY-INDUCED NEUROGENESIS

by

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DEDICATION

For Mom and Dad

For Stephanie and Jared

For Joe

and For Baby and Mayz

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TABLE OF CONTENTS

List of Figures

Figure:

List of Tables

ABSTRACT

Neurogenesis persists in the forebrain subventricular zone (SVZ) where neural stem cells (NSCs) reside in a specific niche. Stroke stimulates SVZ NSCs, which offer reparative potential, but few new neurons survive long-term. Thus, the capacity for self-repair appears insufficient. This thesis focuses on NSC modulation by endothelial cells within the niche, and stroke or inflammation, with the goals of understanding how injury influences neurogenesis and whether stimulating injury-induced neurogenesis improves functional recovery.

Evaluation of the components of injury may improve our understanding of how it modulates SVZ neurogenesis. We examined the influence of *in vivo* exposure to hypoxia or inflammation, or both, on SVZ NSCs using the neurosphere (NS) assay. Mice exposed to acute hypoxia or systemic inflammation produced significantly more NS, neurons and oligodendrocytes. The combined insults, however, significantly reduced neurogenesis. This effect may be mediated by IL-1β as IL-1β deficient mice generated fewer NS and neurons.

NSCs reside in a vascular niche composed of glia and endothelial cells. Angiogenesis and neurogenesis interact in neurogenic regions stimulated by stroke. We hypothesized that factors secreted by intact or oxygen-glucose deprived (OGD) endothelial cells enhance SVZ neurogenesis. We found that

SVZ NS exposed to endothelial cell co-culture or endothelial cell conditioned media (ECCM) increased in numbers and generated neurons and glia that appeared more immature. NS exposure to OGD-ECCM, in contrast, stimulated neuronal maturation and migration. These data suggest that intact and injured endothelial cells secrete factors that differentially influence SVZ NSCs, and offer potential targets to stimulate NSC expansion and integration of new neurons after brain injury.

We also hypothesized that augmenting stroke-induced neurogenesis would enhance functional recovery. We exposed rats to retinoic acid (RA) and environmental enrichment (EE), to promote SVZ neurogenesis and post-stroke recovery. RA-treatment preserved striatal and hemispheric tissue, and combined RA/EE treatment increased SVZ cell proliferation and neurogenesis. These interventions did not improve behavioral recovery. Together, these studies underscore the complexity of how the stem cell niche and injury influence postnatal SVZ neurogenesis. Future work should target factors from endothelial cells, hypoxia and inflammation to promote neurogenesis and enhance the brain's natural repair process.

CHAPTER I:

INTRODUCTION

Adult Neurogenesis

Scientists once believed that the adult brain was not capable of regeneration; that we were born with a set number of neurons and when these neurons died, they were not replaced. In the late 1960's, however, Altman and Das labeled mitotic cells with tritiated thymidine and discovered that these cells produced new neurons in the adult rodent olfactory bulb and dentate gyrus of the hippocampus (Altman, 1969, Altman and Das, 1965, Altman and Das, 1966). The process of forming new neurons is called neurogenesis and these proliferating cells are often referred to as neural stem cells (NSCs). Advances in technology such as the advent of the thymidine analog bromodeoxyuridine (BrdU) and retroviral labeling, allowed scientists to more easily identify and study these cells. These advances led to the discovery of proliferative populations of cells in the central nervous system of every mammalian species studied to date, including humans, as well as many non-mammalian vertebrates (Alvarez-Buylla, 1992, Bedard and Parent, 2004, Eriksson, et al., 1998, Gould, et al., 1999, Kornack and Rakic, 1999, Lois and Alvarez-Buylla, 1993, Pencea, et al., 2001). The existence of these neurogenic regions offers potential for the brain to repair itself and provides a target for developing therapeutic strategies.

Important treatment strategies for the future may include enhancing proliferation, migration, differentiation and survival of this population of cells. Thus, the scope of this thesis focuses on factors that regulate neurogenesis in the postnatal and adult brain, especially after injury, and that may promote brain repair.

The rodent forebrain contains two proliferative or neurogenic regions that persist throughout life, the subventricular zone (SVZ) and the hippocampal dentate gyrus (Figure 1.1A-C). The work encompassed by this dissertation focuses on the forebrain SVZ neurogenic region. The SVZ lies along the lateral ventricle and gives rise to olfactory bulb interneurons while the dentate gyrus produces granule cell layer neurons (Figure 1.1A-C; Reviewed in (Lichtenwalner and Parent, 2006). The SVZ contains three types of cells, named type A cells, type B cells and type C cells (Doetsch, et al., 1997), all of which are mitotic (Figure 1.2). Type B cells are the putative neural stem cells which comprise approximately 23% of the SVZ and are typically identified by expression of glial-fibrillary acidic protein (GFAP), vimentin or nestin (Figure 1.2). These self-renewing cells are relatively quiescent in the normal postnatal/adult brain. Type B cells also give rise to type C cells, known as transit-amplifying cells. Type C cells typically express nestin or the transcription factor Dlx2, are the most actively dividing cells of the SVZ and make up about 11% of the SVZ cell population (Figure 1.2). Type C cells give rise to Type A cells, which are neuroblasts (Doetsch, et al., 1997, Morshead, et al., 1994) and which account for about 33% of the SVZ cells. Type A cells are migratory and typically express doublecortin (DCx), poly-sialylated neural cell adhesion molecule (PSA-NCAM), and class III

β-tubulin (TUJ1) [Figure 1.2; (Doetsch, et al., 1997, Doetsch, et al., 2002)]. These cells also express nestin and Dlx2 but are typically identified with one of the immature neuronal markers mentioned above. Type A cells are generated in the SVZ, then migrate tangentially inside astrocytic tubes through the rostral migratory stream (RMS) to the olfactory bulb, where they migrate radially and differentiate into granule and periglomerular interneurons (Figure 1.1A).

Integration of Adult-Born Neurons

Evidence exists for integration of new born neurons in the uninjured adult brain, but the exact function of these new neurons is still under investigation. In the uninjured brain, new dentate gyrus neurons appear to integrate, as they initially receive GABA-mediated synaptic input, send projections to the hippocampal CA3 area and display mature electrophysiological properties (Carlen, et al., 2002, Hastings and Gould, 1999, Markakis and Gage, 1999, Song, et al., 2002, Stanfield and Trice, 1988, van Praag, et al., 2002, Wang, et al., 2000). Given the role of the hippocampus in learning and memory, it is likely that these cells contribute to some aspect of learning and memory formation. Current studies are investigating this hypothesis and early studies suggest that ablation of these new neurons disrupts some forms of memory (Lemaire, et al., 1999, Leuner, et al., 2004, Shors, et al., 2001). Newborn olfactory bulb interneurons also appear to become functional in the uninjured adult brain. These granular and periglomerular neurons survive long-term, extend dendrites into other olfactory bulb layers, receive GABAergic synapses followed by

glutamatergic input, and display mature electrophysiological properties (Belluzzi, et al., 2003, Carlen, et al., 2002, Carleton, et al., 2003, Petreanu and Alvarez-Buylla, 2002, Winner, et al., 2002). Neurons in the olfactory bulb may function in olfactory memory and odor discrimination and evidence suggests that new olfactory bulb neurons integrate in these circuits {Rochefort, 2002 #217; Gheusi, 2000 #216; Petreanu, 2002 #215; Carlen, 2002 #213}.

Regulation of the Postnatal/Adult SVZ-Olfactory Bulb Pathway

Neurogenesis in the postnatal/adult SVZ-olfactory bulb pathway is regulated by numerous factors. These factors affect various stages of neurogenesis, including proliferation, migration, and differentiation. Some factors influence more than one step in this process. SVZ proliferation increases both in vivo and in vitro in response to epidermal growth factor (EGF) in both rats and mice (Craig, et al., 1996, Kuhn, et al., 1997, Reynolds, et al., 1992). SVZ proliferation is increased by a number of other growth factors as well, including fibroblast growth factor-2 (FGF2), brain derived neurotrophic factor (BDNF), heparin-binding epidermal growth factor (HB-EGF), and vascular endothelial growth factor (VEGF; (Jin, et al., 2002, Jin, et al., 2003, Kuhn, et al., 1997, Pencea, et al., 2001, Rossi, et al., 2006, Zigova, et al., 1998). Cytokines also regulate SVZ proliferation. Transforming growth factor- α (TGF-α) and ciliary neurotrophic factor (CNTF) promote SVZ proliferation (Craig, et al., 1996, Emsley and Hagg, 2003, Tropepe, et al., 1997). Other factors such as hormones and morphogenic factors may also influence SVZ proliferation. Noggin and prolactin

increase SVZ proliferation while bone morphogenetic proteins (BMPs) decrease it (Lim, et al., 2000, Shingo, et al., 2003).

Progenitor cell migration to the olfactory bulb occurs via a gradient of signals providing both attractive and repulsive cues. Receptors in the ephrin family, EphB1-3 and EphA4, are expressed in the SVZ and endogenous administration of EphB2 or ephrin-B2 disrupts SVZ NSC migration to the olfactory bulb (Conover, et al., 2000, Holmberg, et al., 2005). The neuregulins and selected integrins also provide guidance cues for chain migration of progenitors to the olfactory bulb (Anton, et al., 1997, Jacques, et al., 1998), whereas Slit-1 and Slit-2 provide repulsive cues to maintain these cells within the RMS during migration (Hu, 2001, Mason, et al., 2001, Nguyen-Ba-Charvet, et al., 2004, Nguyen Ba-Charvet, et al., 2001, Wu, et al., 1999). Another regulator of migration in the SVZ-olfactory bulb pathway is PTEN, which negatively regulates migration (Li, et al., 2002), while administration of erythropoietin enhances migration (Wang, et al., 2008, Wang, et al., 2004). Finally, factors such as reelin and tenascin-R regulate migration of SVZ neuroblasts once they reach the olfactory bulb (Hack, et al., 2002, Saghatelyan, et al., 2004).

The factors that regulate proliferation in the SVZ-olfactory bulb pathway are often involved in differentiation as well. For example, FGF promotes neurogenesis and increases neuron production in the olfactory bulb while EGF directs SVZ-derived cells towards an astrocytic fate (Jin, et al., 2003, Jin, et al., 2003, Kuhn, et al., 1997). Other growth factors such as BDNF and HB-EGF also increase neurogenesis in the olfactory bulb while VEGF promotes SVZ-derived

neuron survival (Ahmed, et al., 1995, Jin, et al., 2002, Jin, et al., 2004, Jin, et al., 2003, Jin, et al., 2002, Kirschenbaum and Goldman, 1995, Zigova, et al., 1998). Finally, noggin and prolactin also increase neuronal differentiation while BMPs decrease it (Lim, et al., 2000, Shingo, et al., 2003).

Retinoic Acid

Neonatal and adult neurogenesis are regulated by developmental signals that persist in the brain. One of these factors is retinoic acid (RA), a vitamin A derivative that regulates many processes in central nervous system development. It plays an essential role in anteroposterior patterning of the hindbrain and spinal cord (Liu, et al., 2001, Maden, 2002, Melton, et al., 2004), dorsoventral patterning of spinal cord neurons (Diez del Corral, et al., 2003, Novitch, et al., 2003, Wilson and Maden, 2005) and striatal neuron differentiation (Toresson, et al., 1999, Valdenaire, et al., 1998). RA expression and signaling continues in the postnatal and adult brain. Retinoid binding proteins are expressed in the olfactory bulb and SVZ (Zetterstrom, et al., 1999, Zetterstrom, et al., 1994), RA receptors persist in the olfactory bulb (Krezel, et al., 1999), and the RA synthesizing enzyme RALDH3 is present in the olfactory bulb, rostral migratory stream (RMS), and SVZ (Wagner, et al., 2002). Recently our laboratory demonstrated that retinoid signaling enhances proliferation in the neonatal mouse SVZ and RMS, directs SVZ-derived NSC migration to the olfactory bulb, and increases the numbers of SVZ-derived neurons (Wang, et al., 2005). These effects are blocked by inhibiting RA-signaling in postnatal SVZ

neurospheres and SVZ-OB slices. Further, recent studies provide evidence for RA influences on the adult brain. RA-signaling continues in the adult SVZ and olfactory bulb, where it influences subtypes of olfactory bulb neurons and SVZ neural precursors (Hagglund, et al., 2006, Haskell and LaMantia, 2005). Oral retinoid administration increases adult rat SVZ proliferation (Giardino, et al., 2000), and depletion of RA in adult mice decreases dentate granule cell differentiation (Jacobs, et al., 2006).

Environmental Enrichment

Adult neurogenesis is also influence by environmental or behavioral interventions. Environmental enrichment (EE) is a commonly used behavioral intervention for rodents and primates after brain injury that provides complex sensorimotor experiences to promote recovery. Many studies demonstrate improved motor and cognitive function after EE treatment (Dahlqvist, et al., 1999, Gobbo and O'Mara, 2004, Komitova, et al., 2002, Ohlsson and Johansson, 1995). The timing of EE or related interventions after injury is one factor that determines whether it provides beneficial or deleterious effects on recovery or secondary neural degeneration (Bland, et al., 2001, Kleim, et al., 2003, Schallert, et al., 2000, Tillerson, et al., 2001). Improvements in function after EE may relate to effects on neurogenesis as studies show that it increases dentate gyrus neurogenesis by enhancing survival of newly differentiated neurons (Kempermann, et al., 1998, Kempermann, et al., 1997, Komitova, et al., 2002,

Nilsson, et al., 1999, Wurm, et al., 2007); however, few studies have examined the effects of EE on striatal neurogenesis.

Vascular Niche: Uninjured Brain

Given that neural stem cells with in vivo neurogenic potential are housed in two distinct locations in the brain, rather than throughout the brain, the microenvironment of these cells likely plays a critical role in their maintenance. Influences may be exerted on NSCs through other cell types present in the niche, via direct cell-cell contact or secreted factors. It has long been noted that the lay-out of blood vessels and neurons follows similar branching patterns (Carmeliet and Tessier-Lavigne, 2005, Vesalius, 1543) and reciprocal interactions are seen during development. Factors produced by blood vessels provide axonal guidance for neurons while nerves produce signals such as vascular-endothelial growth factor (VEGF) that guide blood vessels (Honma, et al., 2002, Kuruvilla, et al., 2004, Mukouyama, et al., 2002). Neurons and blood vessels also respond to the same guidance cues, such as the neuropilins and ephrins (Adams, et al., 1999, Soker, et al., 1998, Wang, et al., 1998). Evidence suggests that factors made by blood vessels may influence other nearby cell types via cell contact or at a distance (Lichtenwalner and Parent, 2006, Ohab and Carmichael, 2007, Riquelme, et al., 2008, Zhang, et al., 2005). Research on the neurovascular unit, the interaction between vessels, astrocytes and neurons, suggests that these cell types interact to maintain a homeostatic microenvironment and regulate normal central nervous system functioning (Abbott, et al., 2006, Takahashi and

Macdonald, 2004). The neural stem cells of the SVZ are maintained in a stem cell niche containing glial and vascular elements, including astrocytes and endothelial cells. Thus, it is likely that astrocytes, endothelial cells, or both interact with NSCs in their microenvironment to regulate the stem cell state.

Astrocyte Influence on SVZ Neural Stem Cells

The location of astrocytes within the neurogenic niche makes this cell type a prime candidate for influencing SVZ NSCs. Research to date suggests that astrocytes may influence multiple stages of neurogenesis in the normal brain. Radial glia or radial-glia-like astrocytes in the SVZ and dentate gyrus appear to be the neural stem cells that give rise to neuroblasts (Doetsch, et al., 1999, Garcia, et al., 2004, Merkle, et al., 2004, Seri, et al., 2001). Astrocytes also promote proliferation and neuronal differentiation when cultured with SVZ or hippocampal progenitor cells (Lim and Alvarez-Buylla, 1999, Song, et al., 2002). Chains of doublecortin-positive neuroblasts migrate from the SVZ thru the RMS surrounded by astrocytic tubes (Peretto, et al., 1997, Thomas, et al., 1996). Gliosis occurs after injury and the presence of astrocytes within the injury border suggests that they may also regulate injury-induced neurogenesis. Newly formed cells localize to sites of injury that contain an increased population of astrocytes (Zhang, et al., 2004) that produce trophic factors, such as vascular endothelial growth factor (VEGF), which are known to promote SVZ cell proliferation and neuron survival (Jin, et al., 2003, Nedergaard, et al., 2003, Schanzer, et al., 2004, Sun, et al., 2003). Gene expression profiling of

astrocytes from neurogenic regions cultured with adult NSCs combined with candidate testing identified interleukin-6 (IL-6) and IL-1β as mediators of neuronal differentiation promoted by astrocytes (Barkho, et al., 2006). Thus, astrocytes play a role in regulating SVZ NSCs; however, they likely interact with other cells present in the neurogenic niche to influence NSCs in the normal and injured brain.

Endothelial Cell Influence on SVZ Neural Stem Cells: Normal Brain

Endothelial cells are another key player in the SVZ stem cell niche. Evidence is emerging to suggest that the vasculature may influence adult NSCs. Recently, Palmer et al discovered that endothelial cells are located in the proliferative clusters within the neurogenic dentate gyrus, that these cells also proliferate, and that nestin-positive NSCs were always located in close proximity to blood vessels and proliferating endothelial cells (Palmer, et al., 2000). Due to the close physical proximity of these cells, they suggested that the vasculature may play a role in regulating and maintaining dentate gyrus NSCs and coined the phrase "vascular niche" to describe this phenomenon. The presence of blood vessels in and around the SVZ and forebrain neural stem cells as well as their role in guiding new, radially-migrating olfactory bulb cells to their final location within the bulb, suggests that the vasculature may regulate SVZ NSCs as well (Bovetti, et al., 2007, Capela and Temple, 2002).

Strong evidence of an endothelial cell-NSC interaction comes from work by Sally Temple's group (Shen, et al., 2004). They showed that non-contact co-

culture of embryonic mouse NSCs with endothelial cells increased NSC expansion and markedly stimulated neuron production upon differentiation compared to control (embryonic cortical neuron) co-cultures. Another group examining the effects of embryonic NSCs on endothelial cells using the same non-contact co-culture system discovered reciprocal interactions between these two cell types. They found that NSCs enhanced the blood brain barrier properties of endothelial cells, while endothelial cells suppressed differentiation of embryonic NSCs (Weidenfeller, et al., 2007). Similarly, hippocampal NSCs co-cultured with endothelial cells resulted in an increase in the neural stem cell population during co-culture and increased neuron production upon differentiation after removal of endothelial cells (Guo, et al., 2008). Other studies observed cross-talk between endothelial cells and neural stem cells when these cells were in direct contact, noting that endothelial cells migrate into embryonic cortical-derived neurospheres when placed directly on top of the spheres (Milner, 2007) and a percentage of NSCs apparently transdifferentiated to endothelial cells after co-culture (Wurmser, et al., 2004). Earlier studies of the adult songbird neurogenic region revealed mechanisms whereby endothelial cells promote neurogenesis as they implicated endothelial cell secretion of brain-derived neurotrophic factor in this effect (Louissaint, et al., 2002). Thus, endothelial cells in the embryonic and adult vertebrate brain appear to secrete factors that modulate neural stem cell expansion or neuronal production.

Injury-Induced Neurogenesis

The presence of neural stem cells in the forebrain offers the potential for brain regeneration after injury. The neurogenic regions of the postnatal/adult rodent brain are influenced by a number of factors, including various brain injuries. Many brain insults influence neural stem cells and may have a positive or negative impact on these cells, as well as varying degrees of influence depending on the extent of the injury. One of the most widely-studied of these is ischemia-induced neurogenesis, in which the effects of transient focal or global ischemia on SVZ and dentate gyrus neurogenesis are evaluated. After transient middle cerebral artery occlusion (tMCAO) in rodents, the population of BrdU-positive proliferating cells increases in the SVZ and dentate gyrus, peaking around 7-10 days after ischemia [Figure 1.3; (Arvidsson, et al., 2002, Jin, et al., 2001, Kee, et al., 2001, Parent, et al., 2002, Takagi, et al., 1999, Tureyen, et al., 2004, Zhang, et al., 2001)]. This increase is primarily due to increased production of type A and type C cells after stroke (Chen, et al., 2004). The SVZ expands with DCx-positive neuroblasts which migrate out of the SVZ in chains alongside astrocytes, similar to their normal migration in the RMS [Figure 1.3C-D; (Lois, et al., 1996, Peretto, et al., 1997)] and into the injured striatum during the weeks following tMCAO [Figure 1.3C-D; (Arvidsson, et al., 2002, Parent, et al., 2002)]. These cells become striatal projection neurons and express DARPP-32 and calbindin, typical markers for striatal medium spiny neurons (Arvidsson, et al., 2002, Parent, et al., 2002). Few new neurons persist for 5 weeks after stroke and this percentage is low compared with the substantial increase in proliferating and migrating neuroblasts (Arvidsson, et al., 2002, Parent, et al., 2002),

suggesting that these cells do not survive long-term after stroke (Figure 1.3E-G). Further, there is little evidence to suggest that new neurons that do survive become functional and integrate into the existing circuitry. Neurogenesis also increases in the dentate gyrus after tMCAO, even though this area is not directly affected by the stroke, resulting in new dentate granule cell layer neurons (Jin, et al., 2001, Liu, et al., 1998). Similarly, cell proliferation and neurogenesis increase in the gerbil dentate gyrus and hippocampal pyramidal cell layer after global ischemia (Liu, et al., 1998, Nakatomi, et al., 2002).

SVZ cell proliferation also increases after stroke induced using other models that result in cortical but not striatal damage, as well as other forms of injury. Cortical lesions produced by thermocoagulation or distal MCAO, which does not damage the SVZ, substantially increase SVZ cell proliferation by 7 days after injury (Gotts and Chesselet, 2005, Gotts and Chesselet, 2005, Ohab, et al., 2006). SVZ and dentate gyrus cell proliferation increase after aspiration lesion, traumatic brain injury and seizure as well (Chirumamilla, et al., 2002, Parent, et al., 1998, Parent, et al., 1997, Rice, et al., 2003, Sundholm-Peters, et al., 2005, Szele and Chesselet, 1996).

The response of the SVZ after injury is not limited to production of new neurons. SVZ NSCs can differentiate into neurons, astroyctes and oligodendrocytes. Thus, the increase in SVZ progenitor cells in response to injury is not limited to neural progenitors, as glial progenitors increase as well. Radial glial cells function as glial progenitors and provide a scaffold for neurogenesis during development but may also act as neural stem cells in the

adult SVZ by dividing and producing astrocytes and neurons (Doetsch, et al., 1999, Gregg and Weiss, 2003, Merkle, et al., 2004, Weissman, et al., 2003). After embolic MCAO, there is an increase in the presence of radial glial cells in the SVZ, coinciding with SVZ expansion, and these cells appear to support neuron migration after injury (Zhang, et al., 2007). The ischemic-induced astrogliosis that occurs within the ischemic striatum may also provide cues for migration of SVZ neuroblasts into the ischemic core (Ohab and Carmichael, 2007, Zhang, et al., 2005). Glial progenitors in the SVZ may also give rise to increased oligodendrocyte production after injury, but this appears to be more specific to demyelinating injuries rather than those in which neurons are primarily affected. For example, after a demyelinating lesion in the corpus callosum, SVZ NSCs produce four times as many oligodendrocytes as controls (Menn, et al., 2006). Thus, SVZ NSCs may prove to be useful for enhancing cell replacement after injury in which glial cells are the primary target of damage.

The neonatal SVZ is susceptible to ischemic influence as well. Hypoxia-ischemia, a model of neonatal stroke that results in cortical, striatal, and hippocampal damage, increases SVZ cell proliferation and induces neuroblast migration into the injured striatum of rats and mice, similar to tMCAO in the adult rat (Felling, et al., 2006, Ong, et al., 2005, Plane, et al., 2004, Romanko, et al., 2004, Yang and Levison, 2006). Very few newly generated neurons survive for up to 3 weeks after hypoxia-ischemia, in contrast to tMCAO-induced neurogenesis in the adult, where newly generated neurons are present in the

injured striatum several weeks after injury (Arvidsson, et al., 2002, Parent, et al., 2002, Plane, et al., 2004).

Increased SVZ cell proliferation after hypoxic-ischemic injury is not due solely to an increase in neural progenitors but also to an increase in glial progenitors as well. SVZ cells in the neonatal rodent brain give rise to cortical and striatal astrocytes as well as oligodendrocytes in the corpus callosum (Levison and Goldman, 1993). After hypoxia-ischemia, many newly formed oligodendrocytes are present within the infarct core 4 weeks after injury (Zaidi, et al., 2004), suggesting that injury stimulates oligodendrogliogenesis. More severe hypoxic-ischemic insult leads to increased apopotosis of SVZ oligodendrocyte progenitors with subsequent white matter dysmyelination (Levison, et al., 2001, Romanko, et al., 2004).

Hypoxia is one component of brain damage due to stroke, and thus may also influence neurogenesis. Interestingly, a short duration of hypoxia alone is sufficient to increase SVZ cell proliferation in the neonatal mouse forebrain but not in the rat (Ong, et al., 2005, Plane, et al., 2004). Chronic perinatal hypoxia also increases SVZ cell proliferation and cortical neurogenesis (Fagel, et al., 2006). Several studies examined the effects of *in vitro* hypoxia on embryonic stem cells and found that culturing embryonic or mesencephalic stem cells in lowered oxygen enhanced proliferation, survival, and dopaminergic differentiation (Morrison, et al., 2000, Storch, et al., 2001, Studer, et al., 2000).

Endothelial Cell Influence on SVZ Neural Stem Cells: Injured Brain

Stroke increases angiogenesis in the ischemic area (endothelial cell proliferation for formation of new blood vessels) and SVZ neurogenesis, but whether endothelial cells play a role in stroke-induced neurogenesis is unknown. The increased presence of blood vessels, proliferating endothelial cells and neuroblasts in the SVZ 5 days after stroke, as well as the correlation between increased blood vessel number and neuroblast number after stroke, suggests that endothelial cells may modulate stroke-induced neurogenesis (Gotts and Chesselet, 2005, Gotts and Chesselet, 2005). Endothelial cells also appear to promote neuroblast migration to the peri-infarct cortex after cortical injury produced by distal MCAO (Ohab, et al., 2006). The vasculature may also play a role in neuron survival after stroke, as one study found that neurons close to blood vessels were more apt to survive after focal ischemia, whereas those furthest from vessels were most affected by stroke (Mabuchi, et al., 2005).

Further evidence for the regulation of stem cells by the vasculature comes from research on brain tumor cells. Malignant brain tumor cells, some of which act stem cell-like in showing multipotency and the ability to self-renew (Al-Hajj, et al., 2003, Lapidot, et al., 1994, Singh, et al., 2004), are typically located next to capillaries in brain tumors (Calabrese, et al., 2007). Co-culture of these cancer stem cells with endothelial cells maintains the cancer stem cells in a self-renewing and undifferentiated state (Calabrese, et al., 2007). Together, these studies indicate that endothelial cells exert influences on neural stem cells after injury or in other pathologic states. Thus, increasing the endothelial cell population, i.e. promoting angiogenesis, or administering endothelial-derived

factors may be a useful tool for promoting neurogenesis and brain repair after stroke or other types of brain injury.

Inflammation

Inflammation is “the response of tissue to injury or irritation that is characterized by pain, swelling, redness and heat” (Dantzer, et al., 2008). It is the immune system’s primary response to injury or infection and is characterized by changes in local blood vessels and infiltration of leukocytes (Larsen and Henson, 1983). The inflammatory cascade is initiated soon after insult. Leukocyte migration and microglial activation occurs within a few hours as these cells migrate to the affected area; upregulated cytokines such as interleuikin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNFα) trigger the progression of the inflammatory cascade (Davies, et al., 1998, Rothwell and Hopkins, 1995, Schroeter, et al., 1994, Zheng and Yenari, 2004). These cytokines induce expression of adhesion molecules such as intercellular adhesion molecule-1, selectins and integrins on the surface of endothelial cells, increasing their permeability and allowing infiltration of cells and molecules as the blood-brain barrier breaks down (del Zoppo, et al., 2000, Dimitrijevic, et al., 2006, Gong, et al., 1998, Lindsberg, et al., 1996, Ritter, et al., 2000, Stamatovic, et al., 2005, Yamasaki, et al., 1995, Zhang, et al., 1998). Inflammation accompanies most types of injury and may exert beneficial or harmful effects directly on the brain as well.

Given that brain injury both stimulates neurogenesis and induces inflammation, inflammatory mediators are prime candidates for influencing NSCs and neurogenesis. However, the effects of inflammation on neurogenesis are not clear, as evidence exists for both increased and decreased neurogenesis due to inflammation, as well as maintenance of neural progenitors in an undifferentiated state during CNS inflammation (Pluchino, et al., 2005). Neural stem cells from humans and rodents express the inflammatory cytokines IL-1β, IL-6, TNF-α and transforming growth factor-β1 (TGF-β1) or receptors for TNF-α, IL-1β and interferon-gamma (IFNγ) (Ben-Hur, et al., 2003, Cacci, et al., 2005, Klassen, et al., 2003, Wang, et al., 2007). Pro-inflammatory cytokines such as TNFα and IFNγ differentially influence various aspects of neurogenesis depending on the age of the animals, locations examined and assays used for evaluation. Ben-Hur et al cultured neural progenitors from newborn rat as striatal spheres and found that these cells express receptors for TNFα and IFNγ but not IL-1 (Ben-Hur, et al., 2003). They applied either TNFα or IFNγ to spheres for 48 hours and then evaluated proliferation, migration, cell phenotype and cell death. They observed dose-dependent decreases in proliferation after treatment with TNFα and IFNγ alone or together, as well as a dose-dependent increase in cell death after IFNγ alone or with TNFα but not with TNFα alone (Ben-Hur, et al., 2003). Further, they showed increased migration after exposure to either of these cytokines alone or in combination but no difference on cell phenotype (Ben-Hur, et al., 2003).

Studies were also performed to evaluate the effects of cytokines on the adult rat neurogenic regions. One study found that infusion of TNFα into the lateral ventricle of the adult rat increased SVZ cell proliferation for the first 24 hours after infusion. Proliferation returned to baseline by 48 hours after TNFα infusion and no corresponding alterations in cell death were seen (Wu, et al., 2000). Other in vivo studies evaluating the effects of TNFα on striatal and hippocampal neurogenesis found this cytokine to have detrimental effects on neurogenesis. After stroke induced by MCAO, infusion of TNFα into the lateral ventricle of adult rats resulted in a decrease in the number of neuroblasts in the striatum and dentate gyrus two weeks after stroke (Heldmann, et al., 2005). Monje et al cultured hippocampal precursors and differentiated them in the presence of TNFα, IFNγ, IL-6 or IL-1β and found a 50% decrease in neurogenesis with TNFα or IL-6 but no significant changes after exposure to IL-1β or IFNγ (Monje, et al., 2003). The variability in results from these studies makes it difficult to predict the net in vivo effects of these pro-inflammatory cytokines on neurogenesis during inflammatory insults.

Lipopolysaccharide (LPS) is a cell-wall component of gram-negative bacteria that is used experimentally to induce systemic inflammation. LPS binds to toll-like receptor-4 (TLR-4) and initiates a signaling cascade that activates transcription factors and results in the production of pro-inflammatory cytokines including TNF-α and IL-1β. LPS administration prior to ischemia (as preconditioning) results in a neuroprotective effect (Bordet, et al., 2000, Heemann, et al., 2000, Kariko, et al., 2004, Tasaki, et al., 1997) and establishes

tolerance to a subsequent LPS challenge (Cavaillon, et al., 2003, Fan and Cook, 2004, Fan, et al., 2004). LPS administration after hypoxia-ischemia produces the opposite effect; after neonatal hypoxia-ischemia, LPS infusion increases hemispheric damage and stunts post-ischemic SVZ cell proliferation (Barks, et al., 2008). LPS administration also reduces hippocampal neurogenesis in the adult rodent, and when combined with partial status epilepticus, it reduces the injury-induced neurogenic response in the dentate gyrus (Ekdahl, et al., 2003). These studies suggest that post-insult inflammation may be detrimental to injury-induced neurogenesis.

Integration and Recovery After Injury

As described above, there is an abundance of evidence demonstrating that injury increases neurogenesis in the forebrain SVZ and dentate gyrus of the hippocampus. Unfortunately, however, there is little evidence to support integration and function of newly generated neurons after stroke or their involvement in functional recovery. One study demonstrated that selective cell death in cortical layer IV prompted neurogenesis specific to this area; the newly formed neurons exhibited pyramidal morphology and a small percentage of these cells formed long-distance corticothalamic connections (Magavi, et al., 2000). After MCAO, some newly generated striatal neurons express markers of mature medium-spiny neurons and form somatodendritic connections with nearby neurons (Arvidsson, et al., 2002, Jin, et al., 2003, Parent, et al., 2002, Yamashita, et al., 2006, Zhang, et al., 2004). While these studies provided

evidence to support the claims that newly formed neurons become mature and begin to display characteristics of integration, no study has yet to provide clear evidence for integration and long-distance connection of newly generated striatal neurons after stroke. Previous studies in our laboratory attempted to identify long-distance connections from newly generated striatal neurons after stroke using retrograde tracer labeling of FluoroGold and BrdU (Lichtenwalner and Parent, 2006). FluoroGold injections into one target of striatal projection neurons, the substantia nigra pars reticulata, revealed the presence of striatal medium spiny neurons adjacent to BrdU-labeled striatal cells, however, no double-labeling was found (Lichtenwalner and Parent, 2006). Thus, none of the stroke-generated neurons in this study formed long-distance connections with the targeted region.

Although there is little evidence to suggest that stroke-generated neurons integrate and provide functional contributions, there is a large body of evidence suggesting an indirect link of stroke-induced neurogenesis with functional recovery. For example, stroke animals treated with growth factors such as epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF), or vascular endothelial growth factor (VEGF) showed increased stroke-induced neurogenesis as well as improvements in functional recovery (Andsberg, et al., 2002, Jin, et al., 2004, Sun, et al., 2003, Zhang, et al., 2000, Zhu, et al., 2003). Other factors, such as cytokines and neurotransmitters, also appear to influence both neurogenesis and functional recovery after stroke. Administration of erythropoietin after MCAO results in increased SVZ neurogenesis and

improvements in functional recovery (Wang, et al., 2004). Mice deficient in endothelial nitric oxide synthase (eNOS) display reduced neurogenesis and poorer function after stroke (Chen, et al., 2005).

Together, these findings suggest that the brain's ability to repair itself may be incomplete without the aid of exogenous factors to support cell survival and integration and thus promote the formation of long-distance connections. Furthermore, it is unclear whether the addition of new neurons into existing networks is beneficial and whether or not neurogenesis actually contributes to functional recovery. Determining the functional role of new neurons in both the normal and injured brain will provide important clues as to the capability of these cells and may also provide insight as to how to increase their capacities after injury. Future studies to determine if neurogenesis is necessary for functional improvement should evaluate whether augmenting neurogenesis increases recovery or blocking neurogenesis corresponds with decreased functional recovery. Studies using irradiation or directed ablation of endogenous precursors or stroke-generated neurons may provide insight as to their functions in the normal and stroke-injured brain.

Focal Ischemic Injury Models

Stroke is one of the most common causes of death in the United States as approximately 780,000 people suffer a stroke and about 150,000 people die from stroke every year (American Heart Association; www.americanheart.org/statistics). Someone in the U.S. has a stroke about

every 40 seconds and most survive their first stroke, making it one of the most debilitating diseases in the U.S. (AHA online). It is estimated that stroke will cost the U.S. about $65.5 billion this year, due to long-term care and rehabilitation needed from this disease (AHA online). There are three basic kinds of stroke, including ischemic, intracerebral hemorrhage, and subarachnoid hemorrhage. Ischemic stroke is the most common, accounting for 87% of all strokes, while only 10% are intracerebral hemorrhages, and 3% are subarachnoid hemorrhages (AHA online). A number of animal models exist to study the effects of focal or global ischemic injury on the postnatal and adult brain. Models of focal ischemia, the main focus of these studies, include temporary and permanent middle cerebral artery occlusion (MCAO), distal MCAO, embolic MCAO and photothrombosis. Each model has advantages and disadvantages in replicating human stroke and will be discussed further.

Middle Cerebral Artery Occlusion

The MCAO model was introduced in the late 1980s and modified in the 1990s to reduce hemorrhage (Belayev, et al., 1996, Longa, et al., 1989, Schmid-Elsaesser, et al., 1998). This model consists of disrupting blood flow from the branches of the external carotid artery (ECA) and the extracranial branches of the internal carotid artery (ICA) to reduce collateral blood flow, then inserting a nylon suture into the ECA and advancing it through the ICA to block blood flow into the MCA (Longa, et al., 1989). The intraluminal suture is removed after a given duration of time, usually 60-120 minutes for adult rats, to produce

reperfusion. This is called transient MCAO (tMCAO); permanent MCAO, without suture removal, is also a commonly used rodent stroke model. MCAO produces primary ischemic cell death in the ipsilateral striatum and cortex and may extend to the thalamus, hypothalamus and substantia nigra (Garcia, 1995, Kanemitsu, et al., 2002, Williams, et al., 2004). Cell death begins in the striatum, is primarily necrotic, and occurs rapidly (Carmichael, 2005, Garcia, 1995, Li, et al., 1995). Cortical infarction is delayed and consists of more apoptotic cell death (Carmichael, 2005, Garcia, 1995, Li, et al., 1995, Linnik, et al., 1995). This results in an ischemic core in the striatum and ischemic penumbra in the cortex and peri-infarct striatum. The early necrotic cell death in the core followed by progressive cell death in the penumbra that is associated with hypoxia genes, pro-inflammatory cytokines and mediators of oxidative injury (Carmichael, 2005, Durukan and Tatlisumak, 2007) make this model well-suited for studying potential therapies for human ischemia. The disadvantage of this model is that it results in a fairly large amount of damage in a number of brain areas, whereas the majority of human strokes tend to be smaller and more focal, rather than involving a number of motor and sensory systems.

Another MCAO model that results in more restricted hemisphere damage than the permanent or tMCAO models described above is distal MCAO. There are two versions of this model. The original distal MCAO involved transection of the lenticulostriate branches of the MCA at the basal surface of the brain. This requires craniotomy and results in damage to the ipsilateral striatum, subcortical white matter, and cortex. A newer version of this technique, called the 3-vessel

occlusion model, involves MCAO on the surface of the brain combined with bilateral common carotid artery occlusions. This model results in damage to the cortex, subcortical white matter, and a small portion of the dorsolateral striatum. Both of these models involve reperfusion, allowing for return of collateral blood flow. These models produce an infarct core in the cortex, with infarction extending laterally and medially over a period of several days to other cortical areas as well as the dorsolateral striatum, and are characterized by increased apoptosis, microglia infiltration and increased cytokine production. The main advantage of distal MCAO vs. MCAO is that it produces smaller infarcts; however, the need for craniotomy weighs against this model.

Embolic or Photothrombotic Stroke

At least two other types of focal stroke models are described in the literature. There are three variations of embolic stroke currently in use to study cerebral ischemia (Carmichael, 2005, Durukan and Tatlisumak, 2007). These models involve injection of microspheres, macrospheres or thrombotic clots into the ICA, which become lodged in the MCA. Microsphere injection results in multiple small, focal infarcts throughout the brain, while macrosphere injection produces damage similar to the MCAO model, but without hypothalamic damage. Injection of thrombotic clots closely resembles human clot-induced stroke but results in smaller and more variable (in size and location) infarcts than the MCAO model. Another slightly newer stroke model is the photothrombotic stroke, which combines the use of a photosensitive dye and laser activation to produce

cortical infarcts. Typically rose Bengal is injected intravenously and the region of interest is laser-irradiated minutes later. This results in focal endothelial damage, platelet activation, and simultaneous microvascular occlusion and secondary ischemia in the photoactivated area (Carmichael, 2005, Durukan and Tatlisumak, 2007). Rapid apoptotic cell death occurs in the targeted area and is characterized by microglia activation and increased cytokine production. This model is appealing due to its selectiveness of infarct; a specific cortical region, for example the barrel field, can be targeted so that the infarct only occurs in the desired area. This allows for study of an already known circuit and more targeted functional therapy. There is little ischemic penumbra in this model, however, as rapid oxidative damage occurs rather than slower expansion of infarction that occurs with reperfusion. Another disadvantage of this model is the simultaneous occurrence of intra- and extracellular edema, which differs from the intracellular edema alone of human stroke.

Oxygen-Glucose Deprivation

Oxygen-glucose deprivation (OGD) is an *in vitro* stroke model that involves removal of glucose from cell culture media combined with cell exposure to anoxia. The duration of anoxia can be altered to produce variations in the extent of injury. Short durations of anoxia are equivalent to preconditioning, in which the cells are primed but not damaged, whereas longer durations of anoxia induce apoptosis (Andjelkovic, et al., 2003, Keep, et al., 2005, Stanimirovic, et al., 1997, Xu, et al., 2000, Zhang, et al., 1999). “Reperfusion” can be

accomplished by returning the OGD-exposed cells to their normal growth conditions for a given period of time. OGD durations that induce injury result in an up-regulation of pro-inflammatory cytokines, adhesion molecules, anti-oxidative enzymes, and pro-angiogenic mediators (Andjelkovic, et al., 2003, Fischer, et al., 2002, Haqqani, et al., 2007, Haseloff, et al., 2006, Hu, et al., 2006, Murphy and Horrocks, 1993, Stanimirovic and Satoh, 2000, Stanimirovic, et al., 1997, Zhang, et al., 1999).

Research Objectives

The main goal of this dissertation research was to investigate factors that regulate postnatal/adult neurogenesis in the intact and injured brain, and that may enhance adult-born neuron survival and promote recovery after stroke. Factors specific to the local NSC environment such as growth factors and other developmental molecules, other cells in the niche, or various components of injury exert influences on the SVZ NSC population. In addition, external factors such as exercise, environmental enrichment or oral administration of molecules regulating CNS development also influence neurogenesis. This research made use of both *in vitro* and *in vivo* techniques to identify how the environment-both within the stem cell niche, as well as the external environment- affected SVZ neural stem cells in the normal and injured brain. The use of in vivo and in vitro injury models was employed to study the effects of specific components of injury, including hypoxia and inflammation, and to determine general effects of stroke or treatment after stroke on SVZ neural stem cells. We discovered that factors

secreted by normal endothelial cells maintain SVZ NSCs in their stem cell-like state by promoting proliferation and slowing differentiation, while stroke-injured endothelial cells produce factors that enhance migration and differentiation of SVZ NSCs (Chapter 3). Further, we found that hypoxia, inflammation, or treatment with retinoic acid combined with environmental enrichment stimulates SVZ neurogenesis, whereas the combined insult of hypoxia and inflammation depresses SVZ neurogenesis (Chapters 2 and 4). These data provide interesting insight into the regulation of postnatal and adult SVZ neural stem cells and may provide direction for the development of regenerative therapies for stroke or other brain insults.

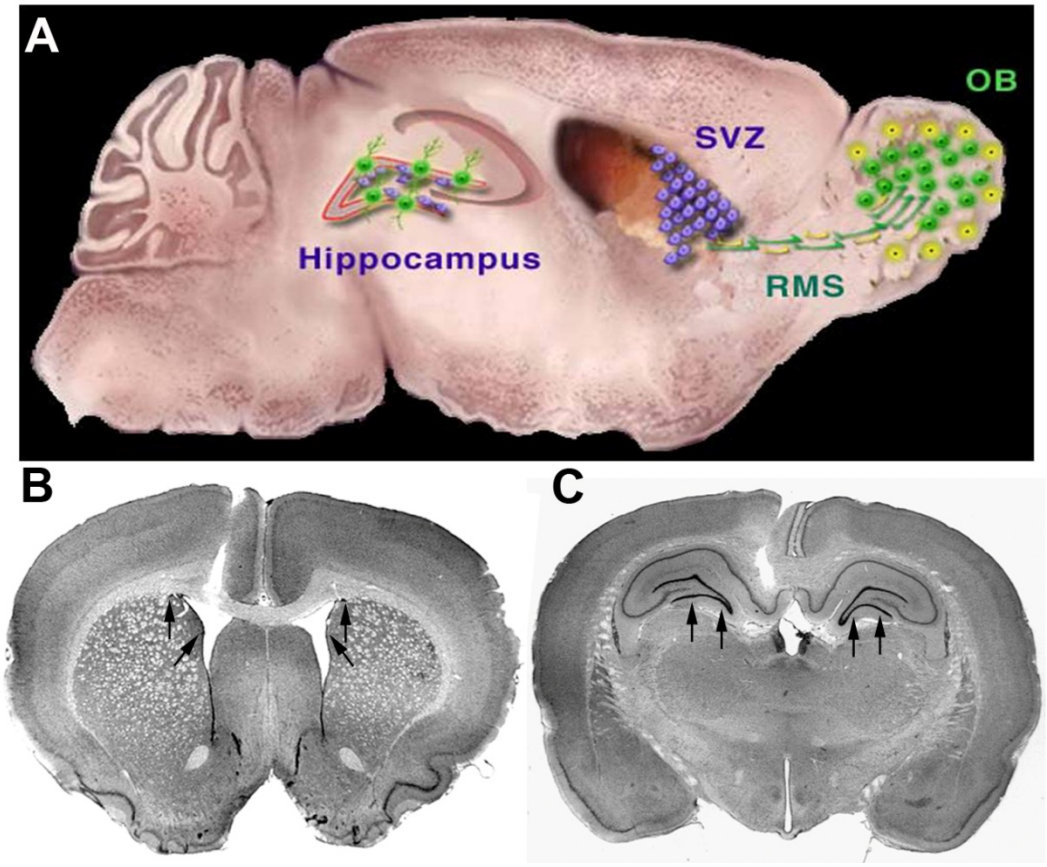

Figure 1.1: Neurogenesis in the adult rodent brain.

Neurogenesis occurs in two regions of the postnatal/adult brain, the subventricular zone (SVZ) and dentate gyrus of the hippocampus (parasagittal schematic in A). The SVZ is located along the lateral ventricle (A, arrows in the coronal section shown in B) and neural stem cells from this area migrate through the rostral migratory stream (RMS in A) to the olfactory bulb (OB in A) where they differentiate into interneurons. The neural stem cells of the dentate gyrus (A, arrows in C) migrate a short distance to become neurons in the granule cell layer of the dentate gyrus. Adapted from Lichtenwalner & Parent, JCBFM, 2006, 26:1-20.

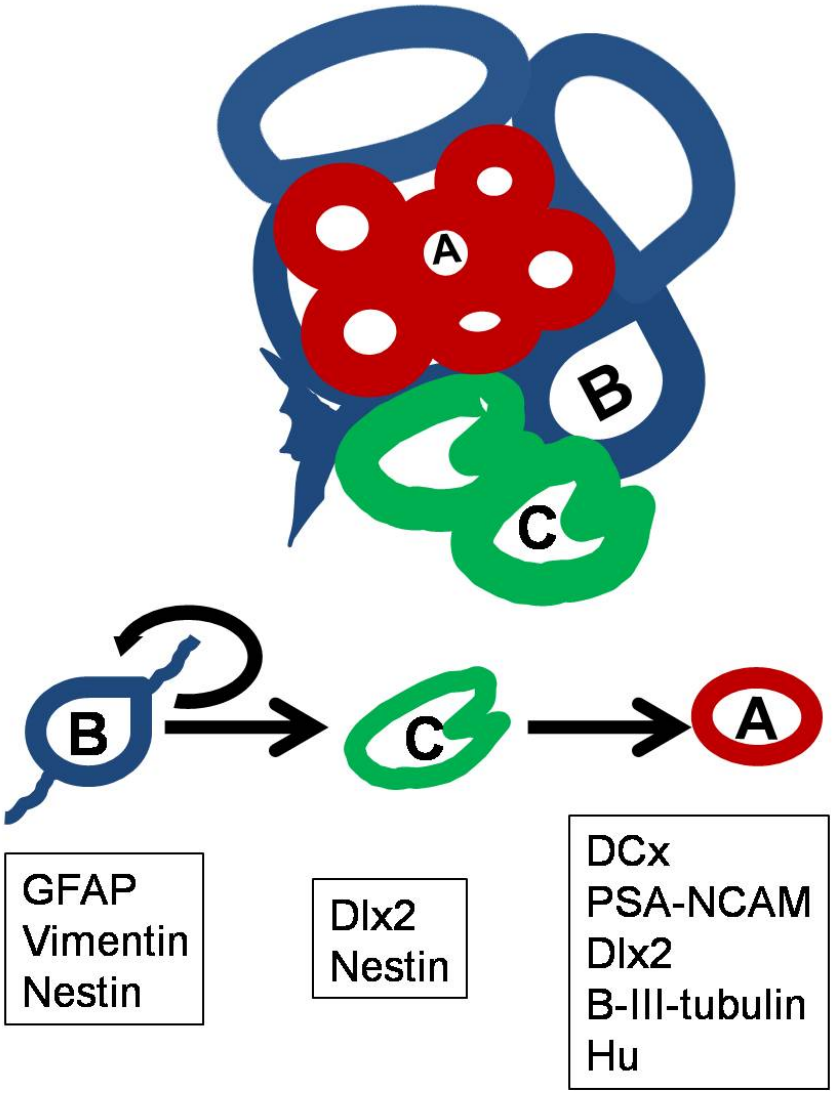

Figure 1.2: Cell types of the SVZ.

The SVZ contains Type A, Type B and Type C cells, identified by Doetsch, et al, 1997. Type B cells are the neural stem cells which are self-renewing and express GFAP, Vimentin and Nestin. Type B cells give rise to Type C cells. Type C cells are transit-amplifying cells that express Nestin and Dlx2; these cells give rise to Type A cells. Type A cells express doublecortin (DCx), PSA-NCAM, class III β-tubulin, Dlx2 and Hu. These cells are migrating neuroblasts that leave the SVZ and migrate through the RMS to the olfactory bulb.

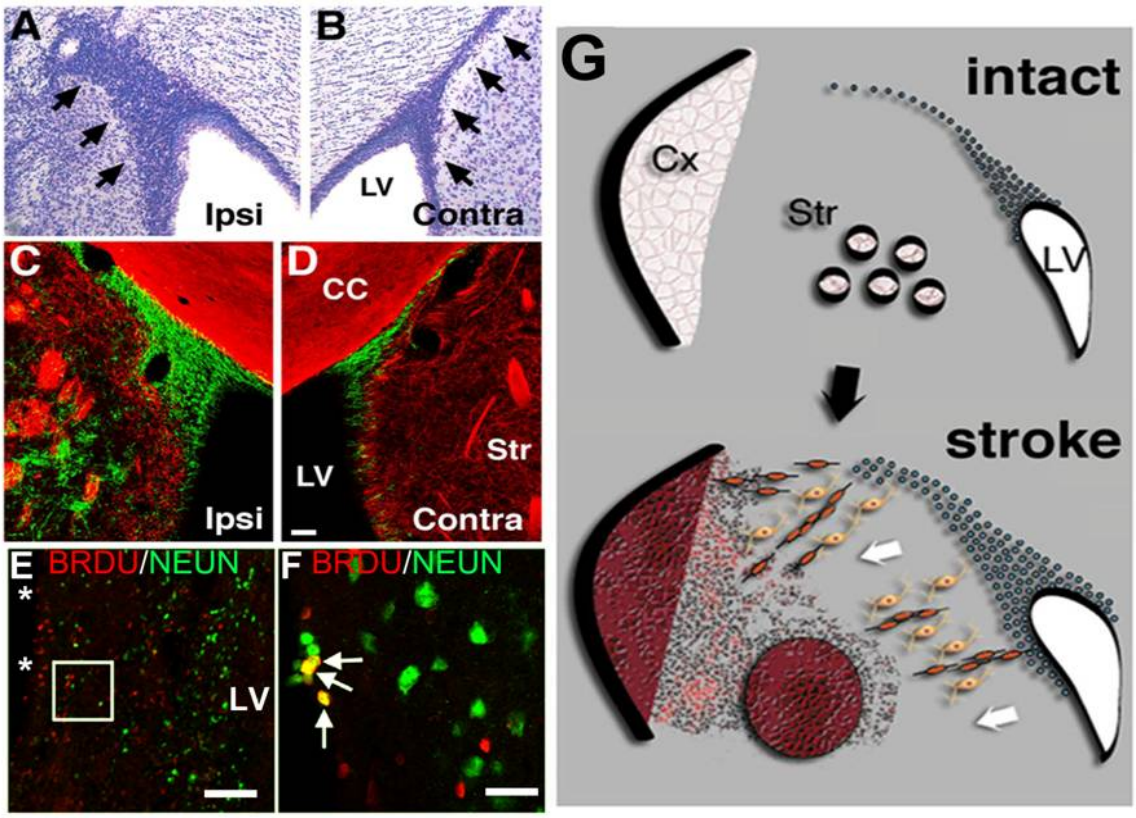

Figure 1.3: Stroke-induced neurogenesis in the adult rodent SVZ and striatum.

The ipsilateral SVZ expands after tMCAO (arrows in A) compared with the contralateral SVZ (B). The expanded SVZ contains an increased population of DCx+ cells (green in C) compared with the non-stroke side (D) and these cells migrate in to the injured striatum (C) in the weeks after tMCAO. Myelin oligodendrocyte glycoprotein immunoreactivity is shown in red. Newly formed neurons are present in the injured striatum 61 days after tMCAO (E, F). The schematic in G shows a comparison between normal and stroke-induced neurogenesis. Under normal conditions, the SVZ is relatively small and quiescent and neuroblasts migrate to the olfactory bulb via the RMS. After stroke, the SVZ expands as proliferation increases and cues from the injured tissue attract neuroblasts and proliferating cells towards the infarct and peri-infarct regions, where new neurons are formed, perhaps replacing those lost from injury. Adapted from Parent, et al, Ann Neurol 2002. Scale bars: 150 μm in E; 25 μm in F.

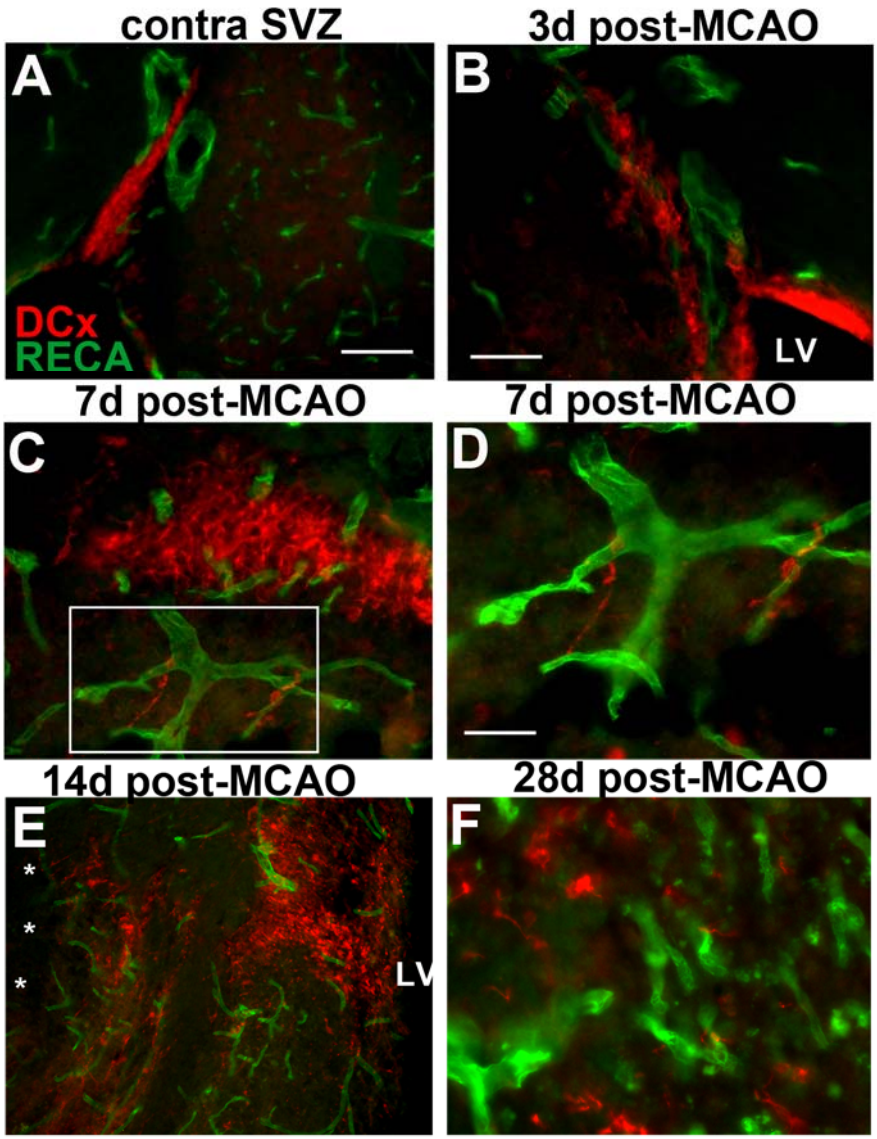

Figure 1.4: SVZ neural stem cells reside in a vascular niche in the normal and stroke-injured brain.

In the normal brain, RECA+ (green) blood vessels surround parts of the DCx+ (red) SVZ (A). By 3 days after tMCAO, there is an increase in RECA+ blood vessels surrounding the SVZ as well as coursing through the SVZ (B). By 7d, the SVZ is enlarged with DCx+ neuroblasts (C), and blood vessels appear to guide these cells out of the SVZ into the injured striatum (box in C, enlarged in D). At 14 days after tMCAO, the SVZ and striatum contain numerous DCx+ neuroblasts which appear in areas enriched with RECA+ blood vessels (E). DCx+ neuroblasts and RECA+ vessels remain in close contact in the injured striatum 28d after tMCAO (F) Scale bars: 100 μm in A (A, E); 50 μm in B (B, C); 30 μm in D (D, F).

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CHAPTER II:

POSTNATAL MOUSE SUBVENTRICULAR ZONE NEUROGENESIS IS INCREASED BY INFLAMMATION OR HYPOXIA BUT IS SUPPRESSED BY THE COMBINED INSULTS

Abstract

Neonates and infants are often exposed to a number of insults during development and birth, including infection, lack of oxygen, and stroke which can lead to a range of cognitive deficits. The persistence of neurogenesis in the postnatal brain provides a pool of cells with the potential to contribute to brain repair after injury. In the postnatal rodent brain, hypoxia-ischemia increases SVZ neural progenitor cell (NPC) proliferation and neurogenesis, but few new neurons survive long-term. Evaluation of the components of injury, including hypoxia and inflammation, may better our understanding of SVZ neurogenesis in order to promote neurogenesis after brain injury. In vitro hypoxia maintains stem cells in a proliferative state or directs neuron specification (Morrison, et al., 2000, Storch, et al., 2001, Studer, et al., 2000), but studies on inflammation and neurogenesis have shown conflicting results. Thus, we examined the influence of in vivo exposure to hypoxia or inflammation, or the combined insult, on SVZ NPCs using the neurosphere (NS) assay. Mice exposed to acute hypoxia or systemic inflammation produced significantly more primary and secondary NS.

Upon differentiation, hypoxia or inflammation alone significantly increased the generation of neurons and oligodendrocytes from SVZ NS but combined exposure led to a significant decline in neurogenesis. This effect may be mediated by IL-1β as NS production from IL-1β deficient mice decreased and IL-1β knockout mice produced fewer neurons. These results suggest that targeting the pathway involved in hypoxia or inflammation may be beneficial for enhancing postnatal SVZ neurogenesis and brain repair.

Introduction

Prematurely born babies have a high risk of developing neuronal and glial injury that often leads to developmental disabilities. Preterm infants are exposed more frequently to insults such as labor stresses, maternal health issues such as placental damage or intrauterine infection, and umbilical cord asphyxiation (Vannucci and Vannucci, 2005, Vawda, et al., 2007). Term infants also may be exposed to insults during birth such as birth asphyxia, multi-organ failure, respiratory distress or congenital cardiac anomalies (Back, et al., 2001, Inder, et al., 2003, Inder and Volpe, 2000, Inder, et al., 2003, Kinney and Back, 1998, Volpe, 2001) which lead to hypoxia or hypoxia-ischemia in the brain.

The extent of injury and subsequent recovery is influenced by the age of onset as well as the severity and repetitiveness of insult (i.e., chronic vs. acute). Survival rates for these critically ill neonates are on the rise which has lead to a substantial persistant number of children with severe motor and cognitive disabilities (Bassan, et al., 2006, Cooke, 2006, Hack, et al., 2002, Paul, et al.,

2006, Saigal, et al., 2006, Wilson-Costello, et al., 2005). The most common deficits are accompanied by loss of oligodendrocytes in the periventricular white matter in the preterm infant and loss of neurons in the cortical gray matter, which may further affect development in subcortical regions, since appropriate connections may not be formed (Back, et al., 2001, Counsell, et al., 2003, Inder, et al., 1999, Inder, et al., 2005, Kinney and Back, 1998, Peterson, 2003, Volpe, 2001). Further, neuronal and oligodendroglial progenitors may also be affected, and may contribute to prolonged developmental deficits (Back, et al., 2001, Curristin, et al., 2002, McQuillen, et al., 2003, Rothstein and Levison, 2005, Sheldon, et al., 1996, Sizonenko, et al., 2003). The persistence of a substantial number of neonates with severe cognitive and motor deficits suggests a need to determine the underlying mechanisms that cause brain injury as well as potential therapeutic options for replacing the lost neurons and oligodendrocytes.

One potential source of intervention may be to promote the endogenous neural stem cell population to proliferate, migrate to areas of injury and replace dying neurons and oligodendrocytes. The postnatal forebrain subventricular zone (SVZ) and dentate gyrus contain populations of neural stem cells that persist into adulthood. Neural stem/progenitor cells of the SVZ reside along the lateral ventricle, migrate through the rostral migratory stream and give rise to olfactory bulb interneurons, while those in the dentate gyrus of the hippocampus migrate a short distance and differentiate into dentate granule layer neurons (Altman, 1969, Cameron and Gould, 1994, Corotto, et al., 1993, Kaplan and Hinds, 1977, Kuhn, et al., 1996, Lois and Alvarez-Buylla, 1994). After

experimental stroke induced by neonatal hypoxia-ischemia or middle cerebral artery occlusion in adults, neural progenitor cells (NPCs) in the SVZ rapidly proliferate. These proliferating cells and immature neuroblasts migrate out of the SVZ into the injured striatum where they become new neurons and glia (Arvidsson, et al., 2002, Felling, et al., 2006, Jin, et al., 2001, Ong, et al., 2005, Parent, 2003, Plane, et al., 2004, Romanko, et al., 2004, Yang and Levison, 2006). More selective brain injury, such as cortical infarct induced by thermocoagulation lesion or distal middle cerebral artery occlusion, also promotes proliferation in the SVZ and migration of these neuroblasts towards the injured area (Gotts and Chesselet, 2005, Gotts and Chesselet, 2005, Gotts and Chesselet, 2005, Ohab, et al., 2006). Only a small percentage of neurons generated after stroke persist in the injured striatum for at least 5 weeks in the adult, and even fewer survive long-term after neonatal stroke, suggesting that the potential for endogenous repair is limited without further intervention (Arvidsson, et al., 2002, Ong, et al., 2005, Parent, et al., 2002, Plane, et al., 2004).

Studies on ischemic preconditioning identified hypoxia as a key player in dampening the damage incurred by subsequent cerebral ischemia. Short durations of hypoxia exposure are neuroprotective in the adult rodent brain and provide protection against stroke- and kainic acid-induced injury (Bunn and Poyton, 1996, Dimitrijevic, et al., 2006, Dimitrijevic, et al., 2007, Lin, et al., 2002). Hypoxia also alters postnatal forebrain neurogenesis. Hypoxia alone is sufficient to increase SVZ NPC expansion after chronic or acute hypoxia (Fagel, et al., 2006, Plane, et al., 2004) as well as dentate gyrus and SVZ NPC expansion after

intermittent hypoxia (Zhu, et al., 2005). After exposure to acute hypoxia on P10, which did not produce any overt brain damage, bromodeoxyuridine (BrdU) incorporation doubled in SVZ NPCs of mice 14 days after exposure to 45 minutes of 10% O_2 (Plane, et al., 2004). A similar effect was not seen, however, when P7 rats were exposed to 90 minutes of reduced O_2 (Ong, et al., 2005). After chronic hypoxia exposure on postnatal days 3-11, which elicited a transient loss in cortical neurons, SVZ NPC proliferation increased one week after cessation of hypoxia, doublecortin-positive neuroblasts were seen migrating through the corpus callosum to the nearby cortex, and significantly more BrdU+ proliferating cells were located in the cortex one month after hypoxia, many of which expressed neuronal markers (Fagel, et al., 2006). These data suggest that mild hypoxic insults or those which produce only transient neuronal damage promote SVZ neurogenesis.

Most studies examining the influence of hypoxia on stem cells do so by exposing in vitro cells to hypoxia as simplified model systems. Such studies have evaluated the influence of hypoxia on stem cell proliferation, differentiation and survival. One recent report showed that culture of neural or mesenchymal stem cells in lowered oxygen tension (3-5% O_2) maintains their proliferative potential or induces their differentiation towards a dopaminergic phenotype (Chen, et al., 2007, Milosevic, et al., 2007, Morrison, et al., 2000, Storch, et al., 2001, Studer, et al., 2000, Zhu, et al., 2005, Zhu, et al., 2005). Embryonic stem cells are maintained in an undifferentiated state at lowered oxygen (Ramirez-Bergeron, et al., 2004, Ramirez-Bergeron and Simon, 2001, Yun, et al., 2005,

Yun, et al., 2002) and rat precursors remain undifferentiated in relative hypoxia via Notch signaling (Gustafsson, et al., 2005). These studies provide evidence that hypoxia (or rather more physiological tissue oxygen concentrations) functions both to promote proliferation and stem cell maintenance as well as direct differentiation into specific neuronal sub-types. None of these studies focused on neonatal forebrain neural stem cells, however, as most focused on embryonic stem cells or those derived from regions other than the forebrain.

Activation of the inflammatory cascade is the immune system's first response to brain injury. This response is mediated in part by up-regulation of the cytokines TNF-α and IL-1β. Recent evidence indicates that these cytokines or their receptors are present in neurogenic regions of the brain (French, et al., 1999, Klassen, et al., 2003, Koo and Duman, 2008, Sairanen, et al., 1997, Wang, et al., 2007). Neurogenesis that increases after injury may be influenced by the post-injury inflammatory response. Evidence exists for the influence of pro-inflammatory cytokines on neurogenesis, but results have varied depending on age, stem cell location and assays used to evaluate neurogenesis. IL-1β is up-regulated after cerebral ischemia (Buttini, et al., 1994, Liu, et al., 1993, Liu, et al., 2005, Minami, et al., 1992, Sairanen, et al., 1997, Yabuuchi, et al., 1994) and was recently shown to enhance neuronal injury in vivo after stroke and in vitro after hypoxia exposure (Boutin, et al., 2001, Fogal, et al., 2007, Legos, et al., 2000, Minami, et al., 1992), suggesting that it may not be a beneficial player in stroke-induced neurogenesis. Indeed, administration of IL-1β decreased hippocampal neurogenesis in the adult rat brain (Koo and Duman, 2008) and

decreased proliferation of embryonic rat brain precursors in vitro (Wang, et al., 2007).

Another pro-inflammatory cytokine, TNF-α, is also expressed in neurogenic regions but conflicting data have shown both positive and negative effects on neurogenesis. In TNF-receptor-1 knockout mice, basal and seizure-induced neurogenesis increases; however, seizure-induced neurogenesis is reduced in TNF-R2 knockout mice (Iosif, et al., 2006), suggesting differential roles for this cytokine in injury-induced and basal neurogenesis, depending on which receptor is activated. After stroke, inhibition of TNF-α results in decreased striatal and hippocampal neuron survival (Heldmann, et al., 2005). In one in vitro study, TNF-α administration led to decreased proliferation but increased migration in newborn rat striatal sphere cultures (Ben-Hur, et al., 2003). Other work in vivo, however, showed that administration of TNF-α increased SVZ cell proliferation (Wu, et al., 2000). In yet another study, in vitro administration of TNF-α or IL-6, but not IL-1β, significantly reduced adult hippocampal neurogenesis (Monje, et al., 2003). Thus, the results from these studies suggest that inflammatory cytokines regulate neurogenesis, but the findings are inconsistent such that responses to specific interventions targeting these molecules are very difficult to predict.

Another technique used to study inflammation is administration of lipopolysaccharide (LPS), a bacterial cell wall component that elicits systemic inflammation via the innate immune response (Shaw, et al., 2001, Terrazzino, et al., 2002, Vallieres and Rivest, 1997). Like the effects of pro-inflammatory

cytokines on neurogenesis, the effects of LPS are incompletely understood. LPS and one of its receptors, toll-like receptor-4 (TLR-4), recently were examined for their effects on neurogenesis. Administration of LPS to adult rats caused a significant decrease in basal hippocampal neurogenesis (Ekdahl, et al., 2003, Monje, et al., 2003) and blocked status epilepticus-induced neurogenesis (Ekdahl, et al., 2003). Similarly, LPS administration after neonatal hypoxia-ischemia reduces the injury-induced neurogenic response in the SVZ (Barks, et al., 2008). The receptors for LPS, TLR-4 and TLR-2, are present on adult NPCs in the SVZ and dentate gyrus (Rolls, et al., 2007), suggesting that LPS may directly influence NPCs. Mice lacking TLR-2 have impaired hippocampal neurogenesis whereas those deficient in TLR-4 display enhanced proliferation and differentiation (Rolls, et al., 2007).

Because both hypoxia and inflammation frequently co-exist in critically ill neonates with brain injury, we investigated how hypoxia or inflammation, alone or in combination, influences neonatal SVZ neurogenesis. We exposed mice to hypoxia, LPS or a combined insult, and examined SVZ NPC self-renewal and differentiation using SVZ neurosphere cultures. We found that in vivo hypoxia or LPS exposure significantly increases in vitro NPC expansion and self-renewal as well as neurogenesis and oligodendrogliogenesis. The combined insult, however, led to decreased SVZ NS expansion and neurogenesis. These effects may be mediated in part by IL-1β, as NS cultured from IL-1β knockout mice also showed decreased NS expansion and neurogenesis.

Methods

Neurosphere (NS) Culture

NS cultures were prepared as previously described (Gritti, et al., 1996, Wang, et al., 2005) with slight modifications; all procedures were performed in accordance with the policies of the University of Michigan Committee on Use and Care of Animals. Postnatal day 13-15 (P13-15; see below and Figure 2.1) CD-1 mice (Charles River) were anesthetized with CO_2, decapitated, brains removed, and placed into ice cold Opti-mem. The forebrain containing the striatal SVZ was cut into two coronal slices and the SVZ was dissected out, minced and dissociated with trypsin. SVZ cells were plated at 4-6x10^4 in 60 mm petri dishes and cultured in serum-free media (SFM) containing growth factors [Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, Gibco), 20 ng/ml epidermal growth factor (EGF; Sigma), 10 ng/ml basic fibroblast growth factor (bFGF; Sigma), 2 μg/ml heparin, and a defined hormone and salt mixture (100 μg/ml transferrin, 25 μg/ml insulin, 60 μM putrescine, 30 nM sodium selenite and 20 nM progesterone)]. Primary NS were cultured for 6 days in vitro (DIV), then picked and re-plated for differentiation in poly-ornithine (Sigma) coated 24 well plates (Corning-Costar) or mechanically dissociated and passaged to form secondary NS. Primary NS were differentiated for 7 days (d) in DMEM/F12 plus the aforementioned hormone mixture and 1% fetal bovine serum (FBS). During differentiation, half of the medium was replaced every 3 d.

Primary NS were passaged to form secondary NS as an assay for self-renewal. Primary NS were collected and centrifuged briefly, the growth medium

aspirated, and the cells re-suspended in fresh SFM. Cells were mechanically dissociated and then plated in 60 mm petri dishes containing fresh SFM at 2-4x10^4 and allowed to expand for 5-6 d. In some experiments, secondary NS were expanded in 6 well plates to allow for treatment with LPS or vehicle. In these experiments, secondary NS were plated at 2x10^4 and were expanded for 6 d. Secondary NS were picked and re-plated for differentiation using the same protocol described above for primary NS.

Identification of Toll-Like Receptor-4 (TLR-4)

Brains were harvested from P15 mice and NS were expanded for 6 days. RNA was isolated after 6 d NS expansion using the Trizol method. NS were collected and centrifuged at 700 rpm for 2 min. NS were washed with ice-cold PBS, transferred to 1.5 ml centrifuge tubes, centrifuged at 500 rpm for 2 min at room temperature, and the supernatant was aspirated. NS were lysed with Trizol at room temperature for 4 min. Chloroform was added to the cells and mixed by vigorous shaking for 3 min. NS were then centrifuged at 1200 rpm for 16 min at 4°C. The aqueous phase was transferred to a fresh tube; isopropanol was added and incubated at -20°C for 1 h to precipitate the RNA. Tubes containing RNA were centrifuged at 1200 rpm for 15 min at 4°C and then the RNA pellet was rinsed with 75% ethanol. RNA pellet was allowed to dry at room temperature for 15 min and then was dissolved in 40 µl of water. RT-PCR was performed to identify TLR-4 and CD14 (co-factor for TLR-4). RNA from spleen and liver were used as a positive control.

LPS administration

LPS is a bacterial cell wall component that induces white matter damage and expression of the cytokines IL-1β and TNF-α when administered to neonatal animals (Cai, et al., 2000, Gilles, et al., 1977, Gilles, et al., 1976). In experiment one (Figure 2.1), LPS from E. coli (Sigma, St. Louis, MO) was dissolved (0.02 mg/ml) in sterile 0.9% (w/v) NaCl. CD-1 mice received intraperitoneal (i.p.) injections of 0.2 mg/kg LPS on days P10-P12 and 0.1 mg/kg on P13-P14 using a 25-guage needle attached to a 250 µl Hamilton syringe in a volume of 10 µl/g body weight. Control mice received an equal volume (10 µl/g body weight) of sterile 0.9% NaCl (vehicle). This administration protocol was chosen based on previous studies that reported normal weight gain, body temperature and blood glucose levels (Barks, et al., 2008) in normal and hypoxia-ischemia-exposed neonatal mice. Mice were killed on P15 for SVZ neurosphere cultures using the methods described above. Primary NS were expanded for 6 days and then counted and their diameters measured. Primary NS were picked and re-plated for differentiation for 7 days or dissociated and re-plated to form secondary NS. Secondary NS were expanded for 6 d, counted and their diameters measured, and then differentiated for 7 d. Primary and secondary NS were fixed on day 7 of differentiation and then immunostained.

In experiments four and five (Figure 2.1), LPS was applied to neurospheres during primary NS differentiation or secondary NS expansion. LPS was reconstituted to 1 mg/ml with sterile Hanks Balanced Salt Solution

(Gibco) and stored at -20°C until use. For application to primary NS during differentiation, LPS was further diluted to 1 ng/ml, 10 ng/ml, 100 ng/ml or 1 µg/ml in NS differentiation media (DMEM/F12 + hormone mix + 1% FBS). As a control, an equal volume of Hanks Balanced Salt Solution was diluted in NS differentiation media. NS were differentiated for 2 d, and then NS differentiation media was completely removed from each well and replaced with 1 ml/well of 1 ng/ml, 10 ng/ml, 100 ng/ml or 1 µg/ml LPS or vehicle. LPS or vehicle was removed 24 h later and a complete media replacement performed with NS differentiation media. Primary NS continued to differentiate until 13 DIV (total of 7 d of differentiation) and were fixed and immunostained. In some experiments, secondary NS were treated with LPS during expansion. Secondary NS were expanded for 2 d and then treated with LPS in the same doses mentioned above for differentiating primary NS. For these experiments, LPS was diluted in serum-free NS media, applied to expanding secondary NS for 24 hours, and then replaced with normal SFM for the duration of NS expansion. After 6 d of secondary NS expansion, NS were picked and re-plated for differentiation as described above.

Hypoxia

In experiment two (Figure 2.1), P10 CD-1 mice (Charles River) underwent hypoxia treatment for 45 min, which does not elicit brain injury (Jensen, 1995). Animals were placed in glass jars, partially submerged in a water bath (36.5°C), and exposed to 10% oxygen (O_2), balance nitrogen for 45 minutes; controls were

removed from the dam and placed into a warm incubator (36.5°C) for the duration of hypoxia-exposure. Brains were harvested on P13, 3 d after hypoxia, for primary NS culture, using the methods described above (See "Neurosphere Culture"). For these experiments, primary NS were expanded for 6 d and then NS were counted and their diameters measured. Primary NS were then picked and re-plated for differentiation or dissociated and re-plated for secondary NS expansion. Primary NS were differentiated for 7 d and then fixed and immunostained. Secondary NS were expanded for 6 d and then counted and their diameters measured. Some secondary NS were picked and re-plated for differentiation. Secondary NS were differentiated for 7 d and then fixed and immunostained.

Combined Hypoxia and LPS

In experiment three (Figure 2.1), P10 CD-1 mice were exposed to 45 minutes of hypoxia (10% O_2) as described above for experiment two. Control mice were removed from the dam and placed into a 36.5°C incubator with normal (normoxic) airflow for 45 minutes. All animals were subsequently returned to the dam. Two hours after hypoxia/normoxia exposure, animals received i.p. injections of 0.2 mg/kg LPS or sterile 0.9% NaCl as described above (See "LPS administration"). Mice received one injection per day thereafter, of 0.2 mg/kg LPS (or vehicle) on P11-P12 and 0.1 mg/kg LPS on P13-P14. Treatment groups were as follows: Hypoxia-exposure plus LPS-injections (Hyp+LPS), Hypoxia-exposure plus NaCl-injections (Hyp+NaCl), or normoxia-exposure plus NaCl-

injections (Norm+NaCl). We did not include an LPS-treated normoxia group because this would be a replication of the LPS-treatment group in experiment 1. Mice were killed on P15 for primary NS culture, using the aforementioned methods. Primary NS were expanded for 6 d, and then counted, their diameters measured, and then NS were picked and re-plated for differentiation or passaged for secondary NS expansion. Primary NS were differentiated for 7 d and then fixed and immunostained. Secondary NS were expanded for 6 d, and their numbers counted and diameters measured.

In experiment 4 (Figure 2.1), P10 mice underwent 45 min of hypoxia at 10% O_2 exposure using the same methods described above. Brains were harvested on P13 to start primary SVZ NS cultures. Primary NS were expanded for 6 days in SFM, and then picked and re-plated for differentiation. Primary NS were treated with 1 ng/ml, 10 ng/ml or 100 ng/ml LPS on day 2 of differentiation. LPS was diluted in NS differentiation media; media was aspirated from NS and then replaced with media containing LPS. After 24 h, LPS-treated media was removed from differentiating primary NS and replaced with normal differentiation media. Differentiation continued for 4 more days; cells were fixed and immunostained after 7 d of differentiation.

Interleukin-1β (IL-1β) experiments

For experiment 6 (Figure 2.1), primary NS were cultured from the SVZ of P15 IL-1β-knockout or wild-type mice of the same hybrid background (provided by the laboratories of Drs. David Irani and Geoff Murphy, respectively) using the

methods described above. Cells were plated at $6x10^4$ in 60 mm petri dishes and were expanded for 6 d. Primary NS were counted and their diameters measured at 6 DIV and then picked and re-plated for differentiation. Primary NS were differentiated for 7 d and then fixed and immunostained.

In additional experiments as a continuation of experiment 6 (Figure 2.1), recombinant murine IL-1β protein (PeproTech) was added to primary NS derived from IL-1β-knockout or WT mice during differentiation. Previous reports suggested that doses of 10-100 ng/ml alter hippocampal neural stem cells in vitro (Koo and Duman, 2008) and 0.8 ng/ml and higher increased proliferation of embryonic NPCs in vitro (Wang, et al., 2007). Thus, in these experiments IL-1β was diluted to a concentration of 1 ng/ml, 10 ng/ml or 100 ng/ml and added to differentiating primary NS, following the same timing used for LPS administration during differentiation (See Figure 2.1, Experiment 5).

Immunofluorescence Staining

All primary NS were fixed with 4% paraformaldehyde after 7d of differentiation and immunostained. Cells were immunostained using markers to identify neurons [1:1000 anti-rabbit β-III-tubulin, Babco; 1:800 anti-rabbit doublecortin (DCx), (Parent, et al., 2002)], astrocytes [1:500 anti-mouse Glial-Fibrillary Associated Protein (GFAP), Sigma], radial glia (1:100 anti-mouse ZRF-1, ZFIN), oligodendrocytes [1:600 anti-rat Myelin Basic Protein (MBP), Chemicon; 1:200 anti-mouse RIP, Chemicon], progenitor cells [1:100 anti-mouse Nestin, Chemicon; 1:500 anti-rabbit NG2, Chemicon; See appendix]. Cells were

washed with PBS, blocked with 10% serum (in PBS + 0.2% triton + BSA), and incubated in primary antibodies overnight at 4°C. After washes, cells were incubated in fluorescent secondary antibodies for 1.5 hour at room temperature. All secondary antibodies were used at 1:800 and included goat-anti-rabbit Alexa 594, goat-anti-mouse Alexa 488, and goat-anti-rat Alexa 488 (Molecular Probes). Cells were washed with PBS, counterstained with bisbenzamide, and stored in PBS.

Microscopy and Image Analysis

Primary and secondary NS were counted in 2x60mm petri dishes/condition for each experiment on a Leica inverted light microscope for at least 4 separate experiments. Fifty primary and secondary NS were measured at random from the center of each 60 mm petri dish using a 10X objective lens on a Leica inverted light microscope for each condition in at least 4 separate experiments. Differentiated cells were imaged using a Leica fluorescent microscope and SPOT-RT digital camera. NS were selected based on similar bisbenzamide nuclear labeling density and a minimum of 3 NS/condition were photographed for quantification using a 20X objective. Images were imported into Adobe Photoshop CS2 for analysis. β-III-tubulin+ neurons and MBP+ oligodendrocytes were counted using Adobe Photoshop, with examiner blinded to condition ($n \geq 3$ NS/condition for at least 3 experiments) and results imported into Microsoft Excel for graphic analysis.

Statistical Analysis

Analysis of variance (ANOVA) with post-hoc t-tests were used to compare differences between groups for all neuron and oligodendrocyte quantitative analyses using Statview. Quantification of neurosphere size and number was compared using ANOVA, or using paired student's t-tests for 2-group analyses. Results are presented as mean ± standard error of the mean (SEM) and considered significant when $p \leq 0.05$.

Results

LPS administration in vivo increases SVZ neurogenesis in vitro

We used the SVZ NS assay to investigate the role of *in vivo* inflammation on subsequent postnatal SVZ neurogenesis *in vitro*. Mice were injected with LPS on P10-P14 and primary NS cultures were prepared from these animals on P15 (Figure 2.1, Experiment 1). After 6 days of expansion, significantly more primary NS were produced from mice injected with LPS than from vehicle-injected mice (Figure 2.2A, $p=0.03$). No difference was seen in the size of primary NS produced from these mice (Figure 2.2B). Primary NS were dissociated and passaged to form secondary NS for assessment of self-renewal. Significantly more secondary NS were produced from LPS-injected mice than from vehicle-injected mice (Figure 2.2C, $p=0.05$). Similar to primary NS, no difference was found in the size of secondary NS produced from LPS- or NaCl-injected mice (Figure 2.2D).

We next examined the phenotype of NS-derived cells differentiated from mice that were administered LPS or vehicle (Figure 2.1, Experiment 1). Immunofluorescence staining was performed to identify neurons using antibodies to β-III-tubulin and DCx. Numerous neurons were produced from vehicle and LPS-injected mice with a significant increase in the number of neurons produced from LPS-injected mice compared with controls (Figure 2.3A-C; $p=0.04$). SVZ-derived NS also gave rise to astrocytes and oligodendrocytes after 7 days of differentiation. No obvious differences were seen in GFAP-positive astrocytes produced from LPS or vehicle-treated mice (data not shown). We then used MBP antibodies to identify myelin-forming oligodendrocytes and found an increase in MBP+ oligodendrocyte production in LPS-administered mice compared with controls (Figure 2.3D-F; arrows; $p=0.04$).

Hypoxia exposure increases SVZ NPC expansion and neurogenesis

P10 CD-1 mice underwent acute hypoxia exposure (Figure 2.1, Experiment 2) and were killed on P13 to assess hypoxia-mediated effects on SVZ NPCs in vitro. In order to determine if hypoxia increased SVZ NPC expansion, SVZ-derived NPCs were cultured in SFM containing growth factors to promote NS expansion. After 6 days of expansion, primary NS were counted and their diameters measured. Mice exposed to a brief duration of hypoxia 3 days prior to culturing NS produced significantly more primary NS than control mice (Figure 2.4A, $p<0.01$). Increased primary NS production was not accompanied by an alteration in NS size (Figure 2.4B). Hypoxia-exposed mice

also produced significantly more secondary NS than controls (Figure 2.4C, $p<0.05$), but secondary NS size was unchanged (Figure 2.4D).

We next differentiated NS from hypoxia-exposed or control mice to determine the effects of hypoxia on the production of neurons or glia. Primary NS were expanded for 6 days and then placed in differentiation media for 7 days. Immunostaining was performed with antibodies to β-III-tubulin and DCx to identify neurons, GFAP to identify astrocytes, and MBP to identify oligodendrocytes. When NS of similar density were compared, we found a significant increase in the number of neurons present in NS generated from hypoxia-exposed mice compared with controls (Figure 2.5A-C, $p=0.05$). Oligodendrocytes are typically produced in relatively small quantities in postnatal SVZ NS differentiated for 7 days. More MBP-positive oligodendrocytes were apparent in NS from hypoxia-exposed mice than were found in NS from control mice (Figure 2.5D, E). Quantification of similar density areas confirmed a significant increase in the number of oligodendrocytes produced in NS from hypoxia-exposed mice compared with controls (Figure 2.5F, $p<0.05$).

Combined exposure to hypoxia and LPS decreases neurogenesis

In experiments one and two above, we found that a single insult (hypoxia or LPS exposure) prior to culturing NS resulted in increased SVZ neurogenesis and oligodendrogliogenesis. In clinical practice, however, one of these insults often is accompanied by a second insult in the pre-term or term infant. Thus, we examined the in vitro response of SVZ NPC after in vivo exposure to hypoxia and

systemic inflammation (LPS). We first examined the influence of combined in vivo hypoxia and LPS exposure on SVZ NPC expansion and self-renewal by counting and measuring primary and secondary NS. Hypoxia alone significantly increased primary NS production (Figure 2.4A, 6A, $p \leq 0.01$) compared to normal controls (Figure 2.6A), whereas hypoxia plus LPS-exposed mice produced significantly fewer primary NS than hypoxia-exposed mice (Figure 2.6A, $p=0.01$) and trended toward decreased NS production compared to normoxia-exposed vehicle controls (Figure 2.6A, $p=0.08$). Hypoxia exposure with or without LPS exposure did not alter primary NS size (Figure 2.6B). A similar effect was seen on secondary NS production. Mice exposed to hypoxia alone produced significantly more secondary NS than controls (Figure 2.6C, $p=0.02$). Mice exposed to hypoxia and LPS produced significantly fewer secondary NS than mice exposed to hypoxia alone (Figure 2.6C, $p<0.01$) or than control mice (Figure 2.6C, $p=0.01$). No differences in secondary NS size were detected from mice exposed to hypoxia with or without LPS compared to controls (Figure 2.6D).

We next examined the effects of the combined inflammation and hypoxic insult on neurogenesis. Mice were exposed to the insult prior to the start of NS cultures, and then NS were expanded for 6 days and differentiated for 7 days (Figure 2.1, Experiment 3). Cells were fixed and immunostained on day 7 of differentiation to identify neurons and glia. Immunofluorescence labeling with antibodies to β-III-tubulin and DCx identified neurons. Very few neurons were present in NS from mice exposed to both hypoxia and LPS (Figure 2.7A) compared to controls (Figure 2.7C), whereas NS from mice exposed to hypoxia

and vehicle produced an abundance of β-III-tubulin-positive neurons (Figure 2.7B). Quantification of β-III-tubulin immunoreactive cells revealed a significant decline in neuron production in NS from hypoxia plus LPS exposed mice compared with controls (Figure 2.7D, $p<0.05$) or hypoxia alone (Figure 2.7D, $p<0.01$). NS from hypoxia and vehicle exposed mice produced substantially more neurons than controls (Figure 2.7D, $p<0.05$).

In experiments one and two, we found that systemic LPS administration or in vivo hypoxia exposure led to increased in vitro neuron and oligodendrocyte production from SVZ NPCs. As mentioned above, when these insults were combined, however, we found a decrease in neurogenesis. Therefore, we wanted to determine if a similar suppression occurred in oligodendrocyte production after a combined insult. Thus, NS were expanded and differentiated as described from mice exposed to hypoxia followed by 5 days of LPS injections (Figure 2.1, Experiment 3). Differentiated NS were immunostained with an antibody to MBP to identify oligodendrocytes. NS from mice exposed to LPS and hypoxia produced few MBP+ oligodendrocytes and their presence was typically observed only in very high density NS (Figure 2.7E). Exposure to hypoxia and vehicle prior to culture resulted in an increase in the presence of MBP-positive cells in NS differentiated from these mice (Figure 2.7F) compared to those from control mice (Figure 2.7G). NS of similar density were selected based on their bisbenzamide labeling for quantification of MBP-positive oligodendrocytes. Quantification supported the significantly increased presence of MBP-positive cells in NS from mice exposed to hypoxia and vehicle-treatment compared to NS

from normal controls (Figure 2.7H, p=0.04) or hypoxia and LPS-exposed mice (p=0.06; Figure 2.7H) with strong trends toward significant differences. Together these data indicate that the combined insult decreases SVZ neurogenesis below control levels but does not result in a similar reduction to oligodendrocyte production.

To further elucidate the effects of an in vivo insult on in vitro neurogenesis and oligodendrogliogenesis, we next examined how in vivo hypoxia exposure combined with in vitro LPS exposure affected SVZ NPCs. In these experiments, mice were exposed to hypoxia on P10 and primary NS were cultured from these animals at P13. LPS was added to differentiating NS as in previous experiments and NS were fixed and immunostained after 7 days of differentiation (Figure 2.1, Experiment 4). Immunofluorescence staining with an antibody to β-III-tubulin revealed that administration of LPS to NS from hypoxia-exposed mice resulted in production of few neurons after 7 days of differentiation (Figure 2.8A). In accordance with our results from experiment 2, we found that vehicle-administered NS from hypoxia-exposed mice resulted in increased neuron production (Figure 2.8E). Administration of LPS to NS from normal mice also resulted in increased neuron production (Figure 2.8B) compared with vehicle-treated NS (Figure 2.8F). Oligodendrocyte production was also examined in these experiments. An abundance of MBP-positive cells were present in both LPS and vehicle-treated NS from hypoxia mice (Figure 2.8C, G). Qualitatively more MBP-positive oligodendrocytes were also present in LPS-treated NS from normal mice (Figure 2.8D) compared with vehicle controls (Figure 2.8H). Thus,

combined in vivo hypoxia exposure and in vitro LPS exposure does not appear to decrease oligodendrocyte production in NS cultures.

Inflammation-induced neurogenesis occurs in a dose-dependent manner

As mentioned above, we found similar effects of decreased neurogenesis on SVZ NPCs in vitro when mice were exposed to hypoxia in vivo combined with in vivo or in vitro LPS exposure. We also found increased neurogenesis when mice were exposed to hypoxia alone or LPS alone prior to the start of NPC culture. To examine LPS effects on NPS in more detail, we exposed NPCs to different concentrations of LPS in vitro. Prior to doing this, however, we wanted to confirm that SVZ-derived NS expressed the LPS receptor TLR-4 to determine whether LPS may directly influence the cultured NPCs. RT-PCR of mRNA obtained from expanded NS showed expression of TLR-4 and its co-receptor CD14 (Figure 2.9A).

We next investigated the effects of in vitro LPS exposure on SVZ neurogenesis. Primary NS were expanded for 6 days and then passaged to form secondary NS or differentiated for 7 days. NS were exposed to incremental doses from 1 ng/ml-1 μg/ml of LPS on day 2 of primary NS differentiation or day 2 of secondary NS expansion (Figure 2.1, Experiment 5). We found no effect on the differentiation of secondary NS that were exposed to LPS during expansion (data not shown). NS that were exposed to 1 ng/ml LPS during differentiation produced substantially more β-III-tubulin-positive neurons than their vehicle counterpart (Figure 2.9B vs. G; F, $p<0.05$) or than any other dose of LPS (Figure

2.9B-J). Neuron production was similar between NS administered 10 ng/ml LPS (Figure 2.9C) and vehicle controls (Figure 2.9H). Very few β-III-tubulin-positive neurons were present in NS administered 100 ng/ml or 1 μg/ml LPS during differentiation compared to controls (Figure 2.9D-E vs. I-J; F, $p<0.05$) or lower doses of LPS (Figure 2.9, $p<0.01$).

Next we examined the influence of in vitro LPS administration on the formation of oligodendrocytes from SVZ NPCs. Because we found that in vivo LPS administration led to increased oligodendrocyte production, we examined whether this effect also occurred after in vitro LPS administration to differentiating NS. MBP immunofluorescence staining showed more oligodendrocytes present in LPS-exposed NS than vehicle-exposed NS at all doses (Figure 2.10). Quantification of MBP-immunoreactive cells revealed that the 1 μg/ml LPS treatment significantly increased oligodendrocyte number compared to vehicle-treated controls (Figure 2.10I, $p<0.05$), however, administration of lower doses of LPS trended toward an increase in MBP-positive cell number compared with their corresponding controls (Figure 2.10I, $p=0.07$-0.11).

IL-1β mediates inflammation-induced changes in SVZ neurogenesis

We sought to determine the underlying factors responsible for inflammation-induced increases in SVZ neurogenesis and oligodendrogliogenesis. LPS is a potent stimulator of the pro-inflammatory cytokine IL-1β (Cai, et al., 2000). Previous studies suggest that IL-1β decreases adult rat hippocampal NPC proliferation both in vivo and in vitro, and decreases

in vitro proliferation of embryonic rat NPCs (Koo and Duman, 2008, Wang, et al., 2007). These studies also show effects on NPC survival, in that apoptosis increases after administration of IL-1β, but it has no effect on NPC differentiation or migration (Koo and Duman, 2008, Wang, et al., 2007). We therefore investigated whether IL-1β plays a role in the neurogenic response of neonatal SVZ NPCs to in vivo or in vitro inflammatory insult as demonstrated by the results in experiments 1-5 above. We first cultured primary NS from IL-1β knockout mice and evaluated primary NS size and number, as well as differentiation of NS cells into neurons and glia (Figure 2.1, Experiment 6). We found a decrease in primary NS production from IL-1β knockout mice compared to wild-type controls (Figure 2.11A, $p=0.01$). This decrease suggests that IL-1β may be responsible, at least in part, for the inflammation-induced increase in SVZ NS expansion. To confirm that IL- 1β is involved in the inflammation-induced increase in neurogenesis, future experiments could be performed in which LPS is administered to IL-1β knockout mice and then NS production and neurogenesis evaluated. No difference was apparent in the size of NS produced from IL-1β knockout mice or controls (Figure 2.11B), also similar to our results with LPS administration (Figure 2.2).

We next examined the effects of IL-1β on NS differentiation. NS were differentiated for 7 days and then fixed and immunostained to identify neurons and glia. NS from IL-1β knockout mice produced relatively few β-III-tubulin-positive or DCx-positive neurons compared to those from wild-type controls (Figure 2.11 C-F). Similarly, NS from IL-1β knockout mice contained fewer MBP-

positive oligodendrocytes than those from wild-type controls (Figure 2.11G, H). Expression of the oligodendrocyte progenitor marker, NG2, was also examined. Fewer NG2-immunoreactive cells were present in NS from IL-1β knockout mice than from wild-type NS (Figure 2.11I, J). Together, these data implicate that IL-1β may mediate inflammation-induced increases in neurogenesis and oligodendrogliogenesis.

Further evidence supporting the role of IL-1β in mediating inflammation-induced increases in postnatal SVZ neurogenesis and oligodendrogliogenesis will be examined in future experiments. To strengthen the link between IL-1β deficiency-induced decreases in SVZ neurogenesis and inflammation-induced increases in neurogenesis, recombinant IL-1β will be added to primary NS from wild-type or IL-1β knockout mice. We hypothesize that IL-1β is responsible for mediating inflammation-induced neurogenesis in the postnatal SVZ. If our hypothesis is correct, the addition of IL-1β to differentiating wild-type NS cultures should result in increased production of neurons and oligodendrocytes. Further, addition of IL-1β protein to differentiating NS from IL-1β knockout mice should rescue the effect of decreased neuron and oligodendrocyte production. Additionally, these effects could further be confirmed by LPS administration to IL-1β knockout mice.

Discussion

Cerebral ischemia increases neurogenesis in the forebrain SVZ of the postnatal and adult rodent but the underlying mechanisms are not well

understood. We hypothesized that inflammation or hypoxia, factors associated with ischemia, alters postnatal SVZ neurogenesis. We tested this by exposing postnatal mice to hypoxia or LPS, and then culturing SVZ NPCs from the mice, or administering LPS directly to NS cultured from normal or hypoxia-exposed mice. We found that in vivo exposure to LPS or hypoxia increases primary and secondary NS production and stimulates the generation of neurons and oligodendrocytes (Table 2.1). In vitro LPS administration also increased neurogenesis and oligodendrogliogenesis (Table 2.1). When these insults were combined, however, NS formation, neurogenesis and oligodendrogliogenesis decreased significantly (Table 2.1). We also demonstrated that inflammation-induced changes in postnatal SVZ neurogenesis and oligodendrogliogenesis are dose-dependent and may be mediated via the pro-inflammatory cytokine IL-1β.

Our findings present novel insight into injury-associated alterations in postnatal SVZ NPCs that may be beneficial for developing treatment strategies to enhance the endogenous repair mechanisms after neonatal brain injury. Premature babies are often exposed to infection, hypoxia and other insults that result in brain injury or stroke. Due to improvements in pediatric care, more premature babies survive today than in the past but often suffer cognitive and motor dysfunction ranging from mild learning disabilities to severe cognitive and motor impairment. Thus, more advanced therapeutic options are needed to promote the survival of neurons and glia after these insults and to reduce long-term cognitive disability. Our findings indicate that exposure to an isolated insult may stimulate the brain's ability to repair itself, given that individual stressors

increased SVZ neurogenesis. Exposure to multiple insults, however, may be detrimental to the neonatal brain, as we found decreased neurogenesis after exposure to a combination of insults. One recent study examined the influence of an additional inflammatory challenge after hypoxia-ischemia in the neonatal rodent brain by administering LPS following hypoxia-ischemia (Barks, et al., 2008). In this study, the injury-induced response of SVZ NPCs was blunted after exposure to both insults (Barks, et al., 2008), whereas hypoxia-ischemia alone increased SVZ cell proliferation (Barks, et al., 2008, Ong, et al., 2005, Plane, et al., 2004). Our study also supports a detrimental synergistic effect of combined brain insults that may decrease the ability of SVZ NPCs to repair the damaged brain. Finding the downstream pathway(s) through which hypoxia and inflammation interact therefore may offer a potential therapeutic strategy. In addition, we identified IL-1β as a candidate mediator of inflammation-induced SVZ neurogenesis and oligodendrogliogenesis. Treatment strategies specifically targeting this cytokine to increase injury-induced neurogenesis in the postnatal brain may be useful.

Few studies have examined the influences of hypoxia and inflammation on postnatal SVZ NPCs as the majority focus on adult hippocampal NPCs. Results from these studies suggest that inflammation inhibits adult hippocampal neurogenesis. For example, administration of LPS to adult rats resulted in decreased hippocampal neurogenesis (Ekdahl, et al., 2003, Monje, et al., 2003), although administration of LPS to hippocampal precursors in culture produced no effect on neurogenesis (Monje, et al., 2003). Activation of TLR-4, the receptor for

LPS, resulted in decreased NPC proliferation in cultures from adult mice and suppression of TLR-4 resulted in increased proliferation and enhanced neuronal differentiation (Rolls, et al., 2007). Similarly, LPS administration inhibited differentiation of adult mouse NPCs into neurons. TLR-4 deficient mice also show increased proliferation and neuronal differentiation in the adult dentate gyrus (Rolls, et al., 2007). Together, these data suggest that inflammation suppresses hippocampal neurogenesis in adult mice.

In terms of the adult SVZ, the response to inflammation appears more complex. Several studies found that TNF-α administration increased SVZ proliferation in the adult rat (Wu, et al., 2000) but decreased proliferation of newborn rat SVZ NPCs and adult rat hippocampal progenitors in vitro (Ben-Hur, et al., 2003, Monje, et al., 2003). The effects of this pro-inflammatory cytokine differ after injury as well. Blockade of TNF-α after stroke results in decreased survival of new neurons in the striatum, whereas TNF-R1 deficient mice produced more hippocampal neurons after status epilepticus, but TNF-R2 deficient mice displayed decreased hippocampal neurogenesis after status epilepticus (Heldmann, et al., 2005, Iosif, et al., 2006).

Effects of other pro- and anti-inflammatory cytokines on neurogenesis appear to be complex as well. Two recent studies suggest that IL-1β decreases proliferation in embryonic forebrain NPCs and adult hippocampal NPCs (Koo and Duman, 2008, Wang, et al., 2007). Our data, however, show decreased NPC expansion (reduced number of NS) as well as decreased neurogenesis and oligodendrogliogenesis in SVZ NPCs derived from IL-1β deficient mice. Our

methods and those from the other groups all differ with regard to the age of animals used, source of isolated NPCs and assays used; thus, IL-1β mediated effects on neurogenesis may be age and location-specific. Our findings of increased SVZ NPC expansion, neurogenesis and oligodendrogliogenesis are opposite from those of previous reports of LPS administration on neural progenitors; however, ages, species, source of NPCs and methods used to evaluate these effects differed as well (Ekdahl, et al., 2003, Monje, et al., 2003, Rolls, et al., 2007).

Hypoxia may be a critical contributor to brain injury in premature infants and postmortem data show that the SVZ may be damaged after severe hypoxia (Calame, 1985; Barkovich, 1990; Barkovich, 1995; Behcer, 2004; Bell, 2005; Chao, 2006; Takizawa, 2006). In the pre-term fetal sheep brain, severe hypoxia results in SVZ cell death while severe hypoxia-ischemia in the neonatal mouse brain also results in SVZ damage, blunting the neurogenic response (Levison, et al., 2001, Plane, et al., 2004). Neonatal mice exposed to mild hypoxia or hypoxia-ischemia have an increased neurogenic response, with increased SVZ proliferation and neuroblast production (Levison, et al., 2001, Plane, et al., 2004). Similarly, SVZ proliferation is increased in mice exposed to chronic perinatal hypoxia (Fagel, et al., 2006), while both SVZ and dentate gyrus proliferation increase after intermittent hypoxia exposure (Zhu, et al., 2005, Zhu, et al., 2005). Together these studies suggest that hypoxia with or without ischemia can enhance the brain's endogenous repair mechanism or, when severe, render it incapable of contributing to brain repair.

The influence of hypoxia on NPCs may be more easily identified in vitro. As a consequence, many studies examine NPCs cultured in lowered oxygen to determine what role, if any, hypoxia plays in NPC maintenance and neurogenesis. Most studies report that in vitro hypoxia maintains stem cells in a more proliferative or undifferentiated state, regardless of age or location from which cells were isolated (Chen, et al., 2007, Gustafsson, et al., 2005, Milosevic, et al., 2007, Morrison, et al., 2000, Storch, et al., 2001, Studer, et al., 2000, Yun, et al., 2005, Zhu, et al., 2005). In vitro hypoxia also induces differentiation of neuronal subtypes, specifically dopaminergic neurons (Milosevic, et al., 2005, Morrison, et al., 2000, Storch, et al., 2001, Studer, et al., 2000). Together these studies provide support for the role of hypoxia in regulating NPCs, although none of these studies specifically examined the influence of hypoxia on postnatal SVZ NPCs. Our findings of hypoxia-induced increases in postnatal SVZ NPC self-renewal, neurogenesis and oligodendrogliogenesis are novel. The demonstration of increased NS production after hypoxia exposure in this study provides further support for the role of hypoxia in promoting NPC proliferation. Previous studies reported increased dopaminergic differentiation but not increased neurogenesis or oligodendrogliogenesis. These studies also focused on midbrain progenitors or embryonic neural stem cells and did not investigate oligodendrocyte production. Thus, the differences reported in our study both provide support to previous studies and differ from these studies as a result of age of hypoxia exposure, region of interest, and timing of NPC evaluation.

Our findings suggest that NPCs derived from the SVZ can promote neurogenesis and oligodendrogliogenesis in response to inflammation or hypoxia, while the response is dampened when both insults are combined. Inflammation and hypoxia are involved in cerebral ischemia and may be key players in the post-injury response. Targeting one or both of these pathways for treatment of perinatal brain injury therefore may enhance brain repair and decrease cognitive or motor dysfunction later in life.

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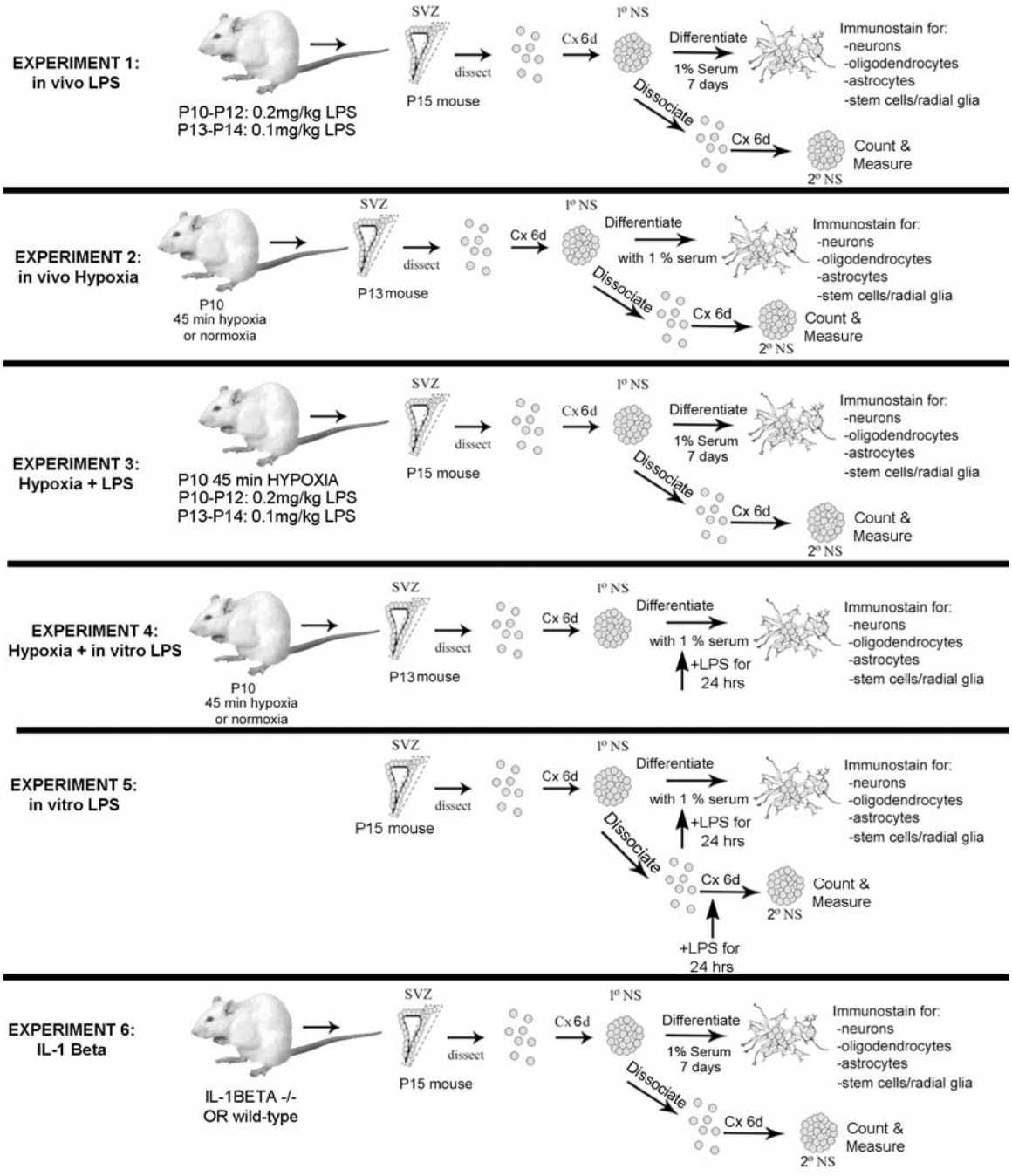
Figure 2.1: Schematic of experiments.

In experiment 1, mice were injected with 0.2 mg/kg LPS or NaCl on P10-P12 and then 0.1 mg/kg LPS or NaCl on P13-14. Mice were killed on P15 for primary NS cultures. Primary NS were expanded for 6 d and then passaged to form secondary NS, or differentiated, fixed and immunostained to identify neurons, astrocytes, oligodendrocytes and NPCs/radial glia. In experiment 2, P10 mice were exposed to 45 min of hypoxia at 10% O_2 or normal air and then were killed on P13 for primary NS cultures. Primary NS were expanded for 6 d and then differentiated for 7d or passaged to form secondary NS. In experiment 3, mice were subjected to 45 min of hypoxia at 10% O_2 (or normal air) on P10, followed by injections of 0.2 mg/kg LPS (or NaCl) on P10-P12 and 0.1 mg/kg LPS (or NaCl) on P13-P14. Brains were harvested on P15 for primary NS culture, expanded for 6 d and then differentiated or passaged to form secondary NS. In experiment 4, P10 mice underwent 45 minutes of hypoxia, as in experiment 2, and were killed on P13 for primary NS culture. Primary NS were expanded for 6 days and then differentiated. LPS was added to differentiating primary NS cultures on day 2 of differentiation and removed after 24 h. Primary NS were differentiated for a total of 7 d and then fixed and immunostained to identify neurons, astrocytes and oligodendrocytes. In experiment 5, primary NS were cultured from P15 CD-1 mice, expanded for 6 d and then differentiated. LPS was added to primary NS on day 2 of differentiation for 24 h. Primary NS were differentiated for a total of 7 d and then fixed and immunostained. Some primary NS were passaged after 6d of expansion to form secondary NS that were exposed to LPS on day 2 of expansion for 24 h. Secondary NS were expanded for 6 days total and then differentiated for 7 d, fixed and immunostained. In experiment 6, primary NS cultured from IL-1$\beta^{-/-}$ or wild-type mice were expanded for 6 d and then differentiated or passaged to form secondary NS.


EXPERIMENT 1:
in vivo LPS
P10-P12: 0.2mg/kg LPS
P13-P14: 0.1mg/kg LPS
SVZ
dissect
P15 mouse
Cx 6d
Iº NS
Differentiate
1% Serum
7 days
Immunostain for:
-neurons
-oligodendrocytes
-astrocytes
-stem cells/radial glia
Dissociate
Cx 6d
Count &
Measure
2º NS
EXPERIMENT 2:
in vivo Hypoxia
P10
45 min hypoxia
or normoxia
P13 mouse
with 1 % serum
EXPERIMENT 3:
Hypoxia + LPS
P10 45 min HYPOXIA
P10-P12: 0.2mg/kg LPS
P13-P14: 0.1mg/kg LPS
EXPERIMENT 4:
Hypoxia + in vitro LPS
+LPS for
24 hrs
EXPERIMENT 5:
in vitro LPS
EXPERIMENT 6:
IL-1 Beta
IL-1BETA -/-
OR wild-type

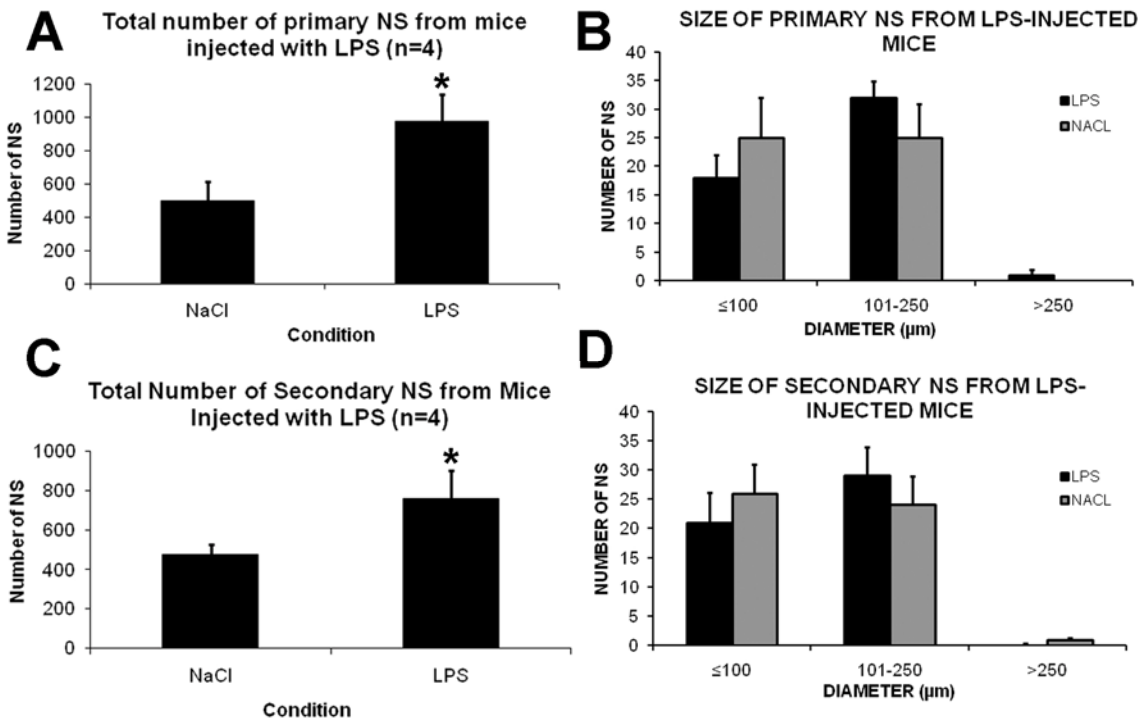
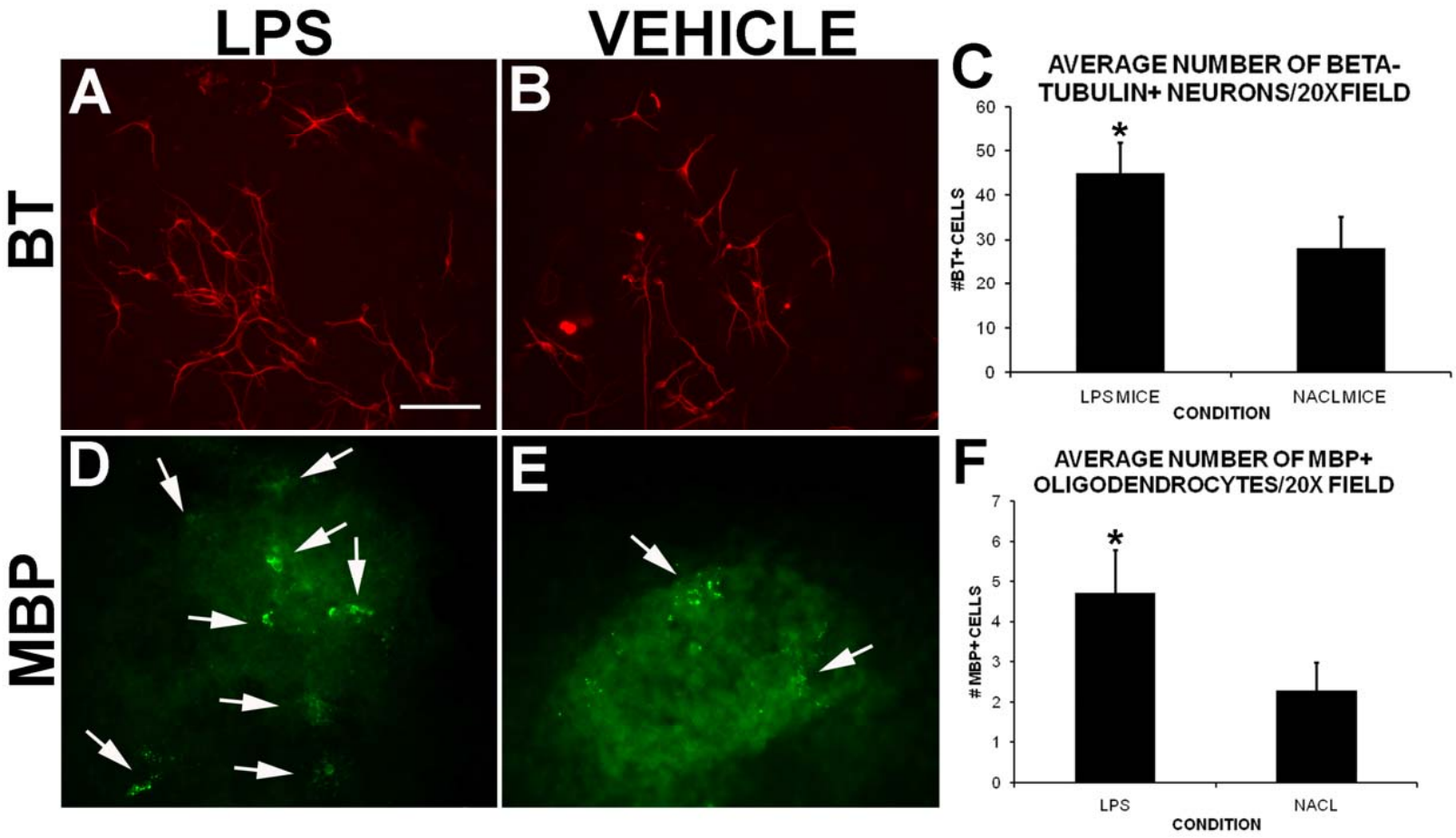


Figure 2.2: LPS-treated mice produce more NS.

Mice were injected with 0.2 mg/kg LPS (or NaCl) on P10-P12, 0.1 mg/kg LPS (or NaCl) on P13-14 and brains were harvested on P15 for primary NS culture. Mice injected with LPS produced twice as many NS as vehicle-injected controls (A, *p=0.03), with no difference in primary NS size (B; p=0.3). After passage of primary NS, LPS-injected mice produced nearly twice as many secondary NS as vehicle-injected controls (C, *p=0.05), and the NS were of similar size (D; p=0.4).

Figure 2.3: LPS-treated mice produce more SVZ-derived neurons and oligodendrocytes *in vitro*.

Mice received i.p. injections of 0.2 mg/kg LPS on P10-P12 and 0.1 mg/kg LPS on P13-P14; controls received NaCl injections. Primary NS cultured from P15 mice were expanded for 6 d and then differentiated for 7 d, fixed and immunostained with antibodies to β-III-tubulin (A, B) or MBP (D, E). LPS-injected mice produced NS that generated more β-III-tubulin+ neurons than controls (A-C; *p=0.04), and more MBP+ oligodendrocytes than vehicle-injected mice (arrows in D-E, F; *p=0.04). Scale bar: 100μm.


LPS
VEHICLE
A
B
BT
C
AVERAGE NUMBER OF BETA-TUBULIN+ NEURONS/20XFIELD
#BT+ CELLS
60
50
40
30
20
10
0
*
LPSMICE
NACLMICE
CONDITION
D
E
MBP
F
AVERAGE NUMBER OF MBP+ OLIGODENDROCYTES/20X FIELD
MBP+ CELLS
7
6
5
4
3
2
1
0
*
LPS
NACL
CONDITION

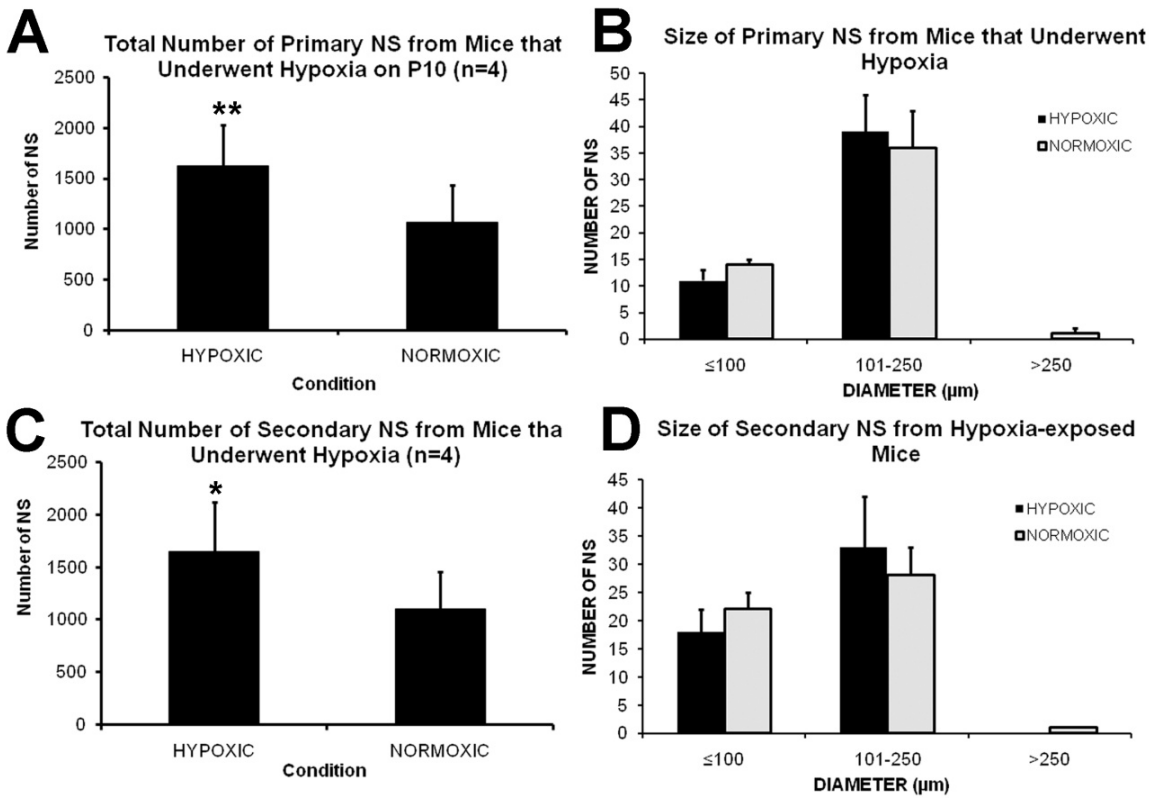


Figure 2.4: Neurosphere production increases in hypoxia-exposed mice.

P10 CD-1 mice were exposed to 45 minutes of hypoxia (10% O_2) and brains harvested on P13 for primary NS cultures. Mice exposed to hypoxia produced significantly more primary NS than controls (A, **$p<0.01$). Primary NS diameters measured after 6 days of NS expansion showed no difference in the size of primary NS produced from hypoxia versus control mice (B). Primary NS then were passaged to form secondary NS. After 6 days of secondary NS expansion, hypoxia-exposed mice produced significantly more secondary NS than controls (C, *$p<0.05$), with no difference in NS size (D).

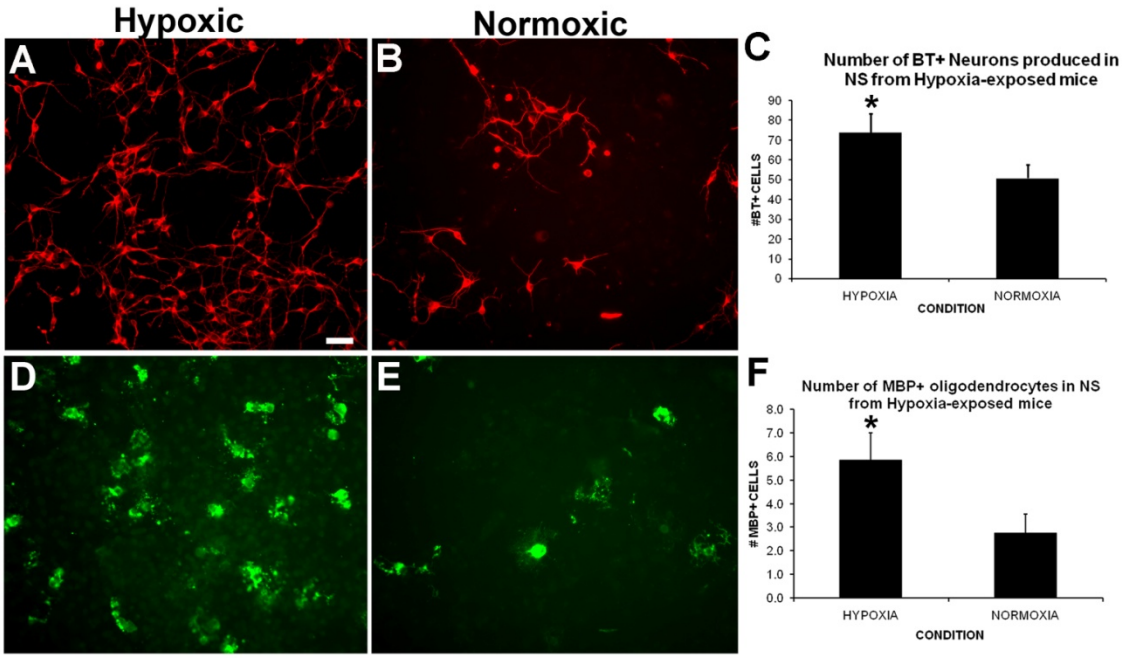


Figure 2.5: Neurogenesis and oligodendrogliogenesis increase *in vitro* after *in vivo* hypoxia-exposure.

P10 mice underwent 45 minutes of hypoxia and brains were harvested on P13 for NS culture. Primary NS were expanded for 6 days, differentiated for 7 days, and immunostained to identify neurons using an antibody to β-III-tubulin. β-III-tubulin+ neurons were present in differentiated NS from both groups, but NS from hypoxia-exposed mice contained more neurons than normoxic controls (A-C; *p=0.05). NS from mice exposed to hypoxia also produced more MBP+ oligodendrocytes than normoxic mice (F; *p=0.03). Scale bar: 50μm.

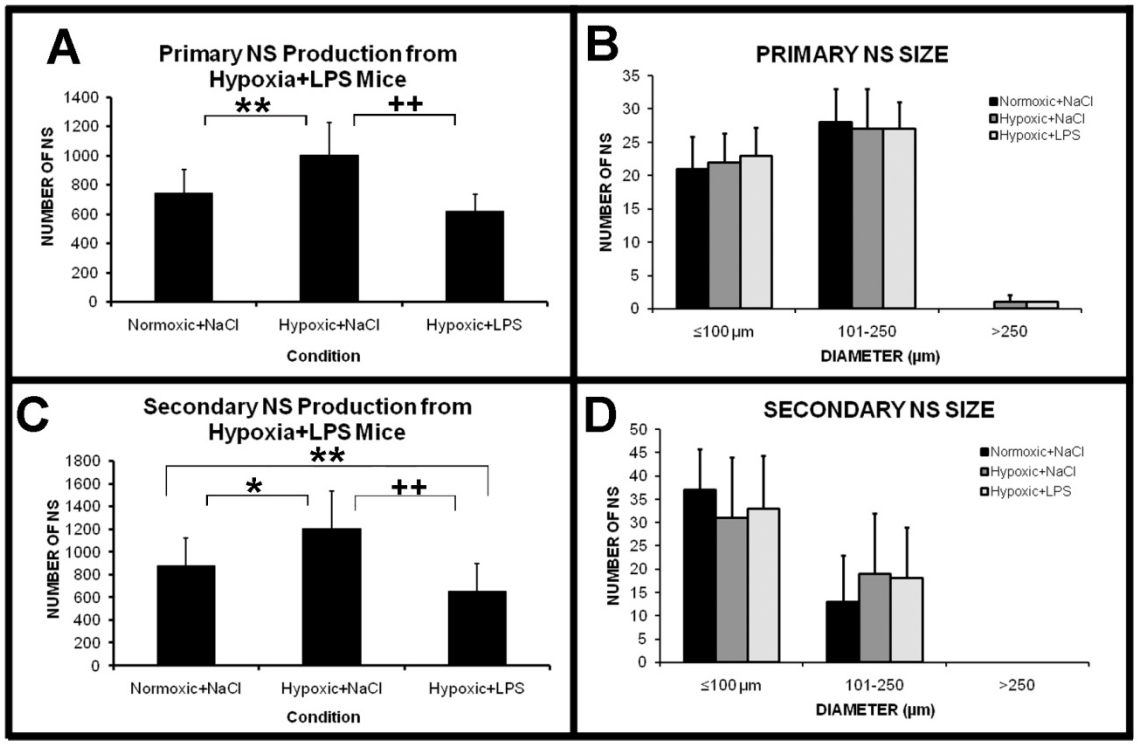


Figure 2.6: SVZ neurosphere production decreases after exposure to hypoxia and LPS.

P10 mice underwent 45 min of hypoxia and were injected with 0.2 mg/kg LPS (or NaCl) 2 hours later; mice received i.p. injections of the same dose on P11-12, followed by 0.1 mg/kg LPS on P13-14. Mice were killed on P15 for primary NS culture. Animals exposed to hypoxia and vehicle injections produced significantly more primary NS than normoxic mice that received vehicle injections (A, **p=0.01). Mice exposed to hypoxia and LPS produced significantly fewer primary NS than those exposed to hypoxia alone (A, ++p=0.01) and trended toward decreased NS production compared to normoxia-exposed vehicle controls (A, p=0.08). No difference was seen between groups in the size of primary NS produced (B). Significantly fewer secondary NS were produced from hypoxia- and LPS-exposed mice than normoxia plus vehicle (C, **p=0.01) or hypoxia plus vehicle (C,++p<0.01). Hypoxia-exposed mice produced significantly more secondary NS than normoxia mice (C,*p=0.02). We found no difference in secondary NS size between groups (D).

Figure 2.7: Combined hypoxia and LPS exposure decreases SVZ neurogenesis but not oligodendrogliogenesis *in vitro*.

Mice were exposed to hypoxia on P10 and received i.p. injections of LPS on P10-P14. Primary SVZ NS cultures were prepared from P15 mice. Mice exposed to hypoxia plus LPS produce few SVZ NS-derived β-III-tubulin+ neurons (A), whereas mice exposed to hypoxia plus vehicle treatment produce substantially more β-III-tubulin+ neurons than controls (B, C). Quantification of β-III-tubulin+ neurons revealed a significant increase in neuron production in hypoxia-exposed mice compared with normoxia-exposed controls (D, *$p<0.05$), and significantly fewer β-III-tubulin+ neurons from mice exposed to hypoxia plus LPS compared with hypoxia plus vehicle (D, ++$p<0.01$) or normoxia plus vehicle (D, *$p<0.05$). Mice exposed to hypoxia plus LPS produced NS that gave rise to a small number of MBP+ oligodendrocytes after 7 days of differentiation (E). Those exposed to hypoxia plus vehicle treatment produce numerous MBP+ oligodendrocytes, almost twice as many as NS from mice exposed to both insults or neither insult (E-G). Quantification of MBP+ oligodendrocytes revealed that NS derived from hypoxia-exposed mice generate significantly more MBP+ cells compared to controls (H, *$p=0.04$) and trended toward an increase compared to hypoxia plus LPS-exposed mice (H, $p=0.06$) and. Scale bar: 100μm.

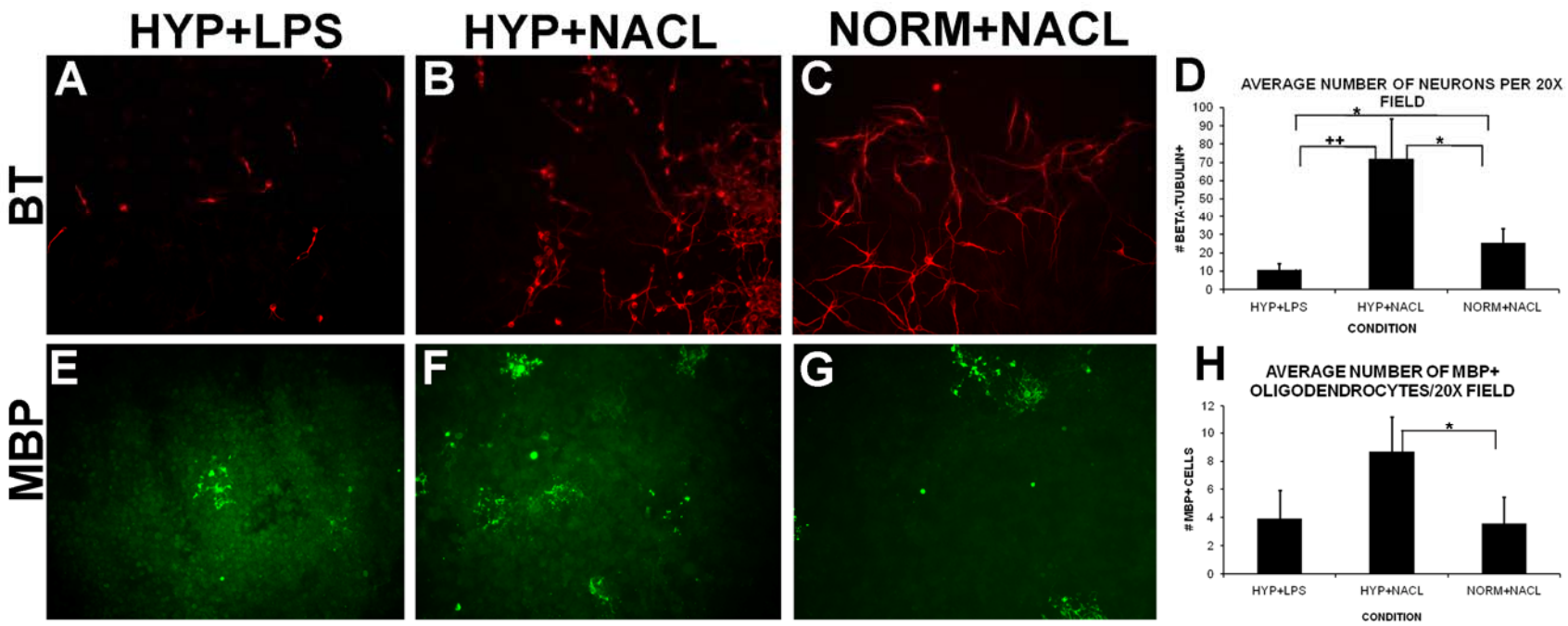

HYP+LPS
HYP+NACL
NORM+NACL
A
B
C
BT
E
F
G
MBP
D
AVERAGE NUMBER OF NEURONS PER 20X FIELD
BETA-TUBULIN+
100
90
80
70
60
50
40
30
20
10
0
*
++
*
HYP+LPS
HYP+NACL
NORM+NACL
CONDITION
H
AVERAGE NUMBER OF MBP+ OLIGODENDROCYTES/20X FIELD
MBP+ CELLS
12
10
8
6
4
2
0
*
HYP+LPS
HYP+NACL
NORM+NACL
CONDITION

Figure 2.8: In vivo hypoxia exposure combined with *in vitro* LPS also decreases SVZ neurogenesis but not oligodendrogliogenesis.

Primary NS were expanded from P13 mice that were exposed to hypoxia on P10. LPS or vehicle was added to primary NS on the second day of differentiation and removed 24 h later. NS from hypoxia-exposed mice that were treated with LPS during differentiation produced very few neurons compared to all other conditions (A, B, E, F). NS from normoxic mice treated with LPS in vitro (B) or NS from hypoxia-exposed mice that were exposed to vehicle during differentiation (E) produced considerably more neurons than controls (F). More MBP+ cells were present in NS from hypoxia-exposed mice treated with LPS (C) or vehicle (G) during differentiation than in NS from controls (H). LPS-exposed NS from normoxic mice also appeared to contain more MBP+ cells (D) than controls (H).

Hypoxia
Normoxia
Hypoxia
Normoxia
LPS
VEH
A
B
C
D
E
F
G
H

Figure 2.9: Cultured NS express TLR-4 and CD14, and increase neurogenesis in response to in vitro LPS treatment.

NS derived from P15 mice were expanded for 6 days, mRNA extracted and RT-PCR performed for TLR-4 and CD14. NS show strong expression of TLR-4 and CD14 and were present in spleen (S) and Liver (L), which were used as a positive control (A). No reverse transcriptase was present in the negative control (- in A). In experiment 5, SVZ-derived primary NS were cultured from P15 mice. NS were expanded for 6 days and then differentiated. 1 ng/ml-1 μg/ml LPS was added to differentiating NS on day 2 of differentiation and removed 24 h later. NS were differentiated for a total of 7 d and then were fixed and immunostained to identify neurons. NS treated with 1 ng/ml LPS produced numerous β-III-tubulin+ neurons (B), more than the vehicle-treated controls (G-J) or any other LPS doses (C-E). Administration of 10 ng/ml LPS also produced numerous β-III-tubulin+ neurons (C), however, these appeared similar in number to controls (H). Administration of 100 ng/ml or 1 μg/ml LPS resulted in production of fewer β-III-tubulin+ neurons (D, E) than their corresponding controls (I, J). Quantification of β-III-tubulin+ neurons revealed a significant increase in the number of neurons produced from NS administered 1 ng/ml LPS compared to the corresponding control (F, *$p<0.05$). Administration of the 2 highest doses of LPS resulted in a significant decline in neuron production versus controls (F, *$p<0.05$) or 1 ng/ml LPS (F, ++$p<0.01$). Scale bar: 100μm.

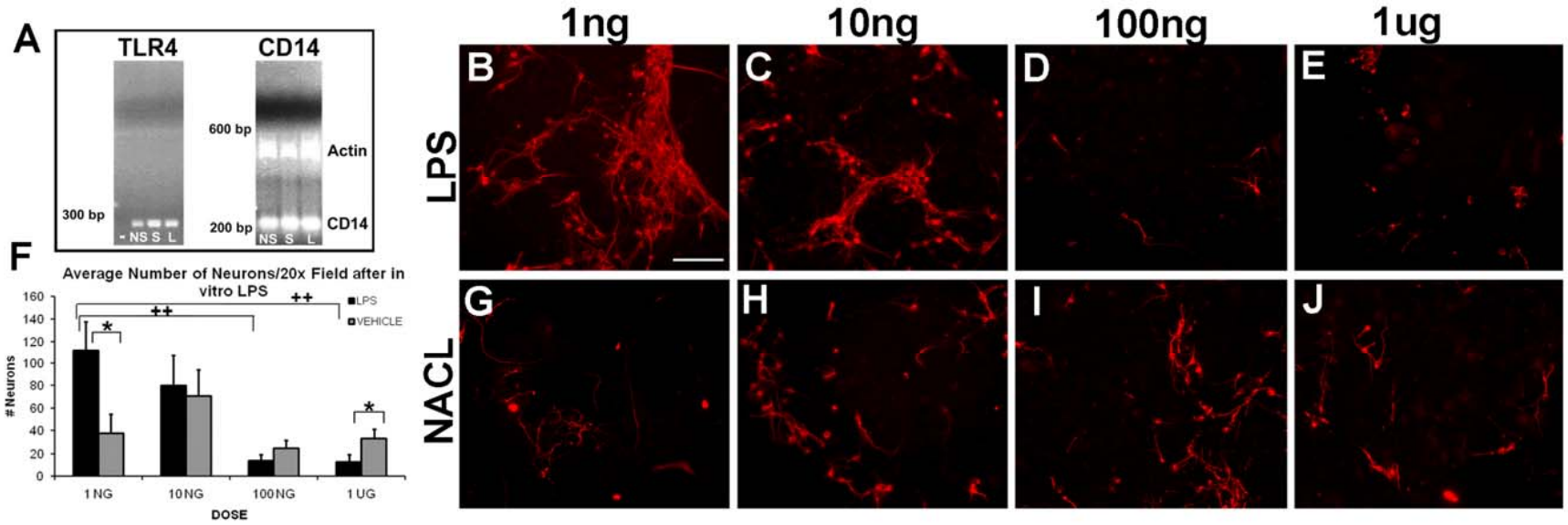
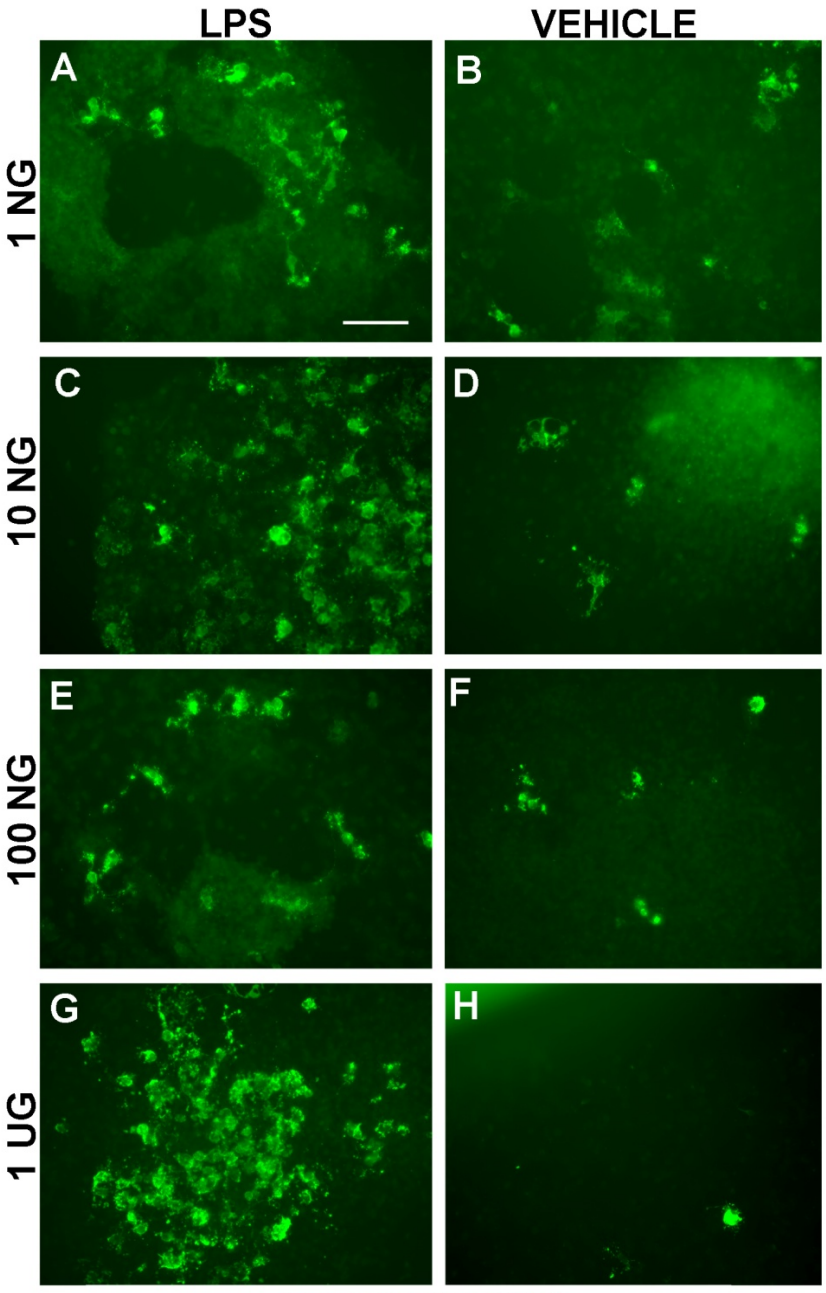

A
TLR4
CD14
600 bp
Actin
300 bp
200 bp
CD14
- NS S L
NS S L
F
Average Number of Neurons/20x Field after in vitro LPS
LPS
VEHICLE
Neurons
1 NG
10 NG
100 NG
1 UG
DOSE
1ng
10ng
100ng
1ug
LPS
NACL
B
C
D
E
G
H
I
J

Figure 2.10: LPS-treatment *in vitro* increases oligodendrogliogenesis.

In experiment 5, SVZ-derived primary NS were cultured from P15 mice, expanded for 6 d and differentiated. 1 ng/ml-1 µg/ml LPS was added to differentiating NS on day 2 for 24 h. NS were differentiated for a total of 7 d and then fixed and immunostained to identify oligodendrocytes. NS treated with 1 ng/ml, 10 ng/ml or 100 ng/ml LPS (A, C, E) during primary NS differentiation produced more MBP+ oligodendrocytes than vehicle treated NS (B, D, F). NS treated with 1 µg/ml LPS produced approximately 3-times as many MBP+ cells (G) as vehicle-treated NS (H). Quantification of MBP+ cells revealed a trend toward increased oligodendrocyte production after administration of 1ng/ml LPS (I, $p=0.07$) but no significant differences between oligodendrocyte numbers with 10 ng/ml or 100 ng/ml LPS-administration compared with corresponding vehicle-treated NS. Administration of 1µg/ml LPS significantly increased the number of MBP+ cells at 7 d of differentiation versus vehicle control (I,*$p<0.05$). Scale bar: 100 µm.


LPS
VEHICLE
A
B
1 NG
C
D
10 NG
E
F
100 NG
G
H
1 UG

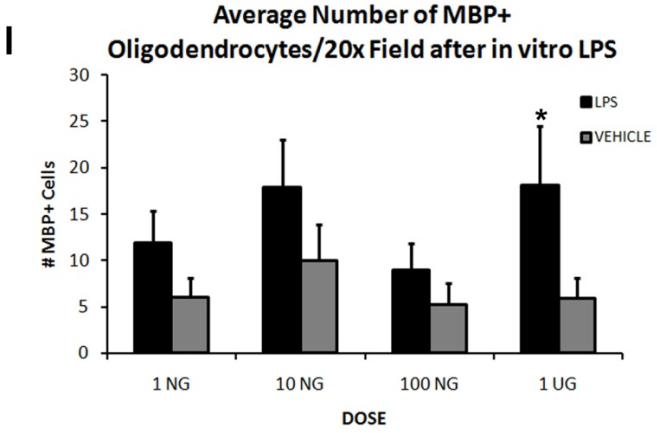

I
Average Number of MBP+
Oligodendrocytes/20x Field after in vitro LPS
MBP+ Cells
30
25
20
15
10
5
0
LPS
VEHICLE
*
1 NG
10 NG
100 NG
1 UG
DOSE

Figure 2.11: IL-1β deficient mice show reduced NS self-renewal and *in vitro* neurogenesis.

Primary NS were cultured from the SVZ of P15 IL-1β knock-out or wild-type mice. NS were expanded for 6 d, and then counted and their diameters measured. Significantly fewer primary NS were produced from IL-1β knock-out mice than from wild-type controls (A, **p=0.01), but no difference was seen in the size of primary NS (B). Primary NS were differentiated and then fixed and immunostained after 7d. Primary NS from IL-1β knock-out mice contained fewer β-III-tubulin+ (C) or DCx+ (E) neurons than NS from wild-type controls (D, F). Primary NS from IL-1β knock-out mice also gave rise to fewer MBP+ cells than wild-type controls (G, H), as well as fewer NG2+ glial progenitors (I, J). Scale bars: 100 μm in C (for C-H); 50 μm in I (for I-J).

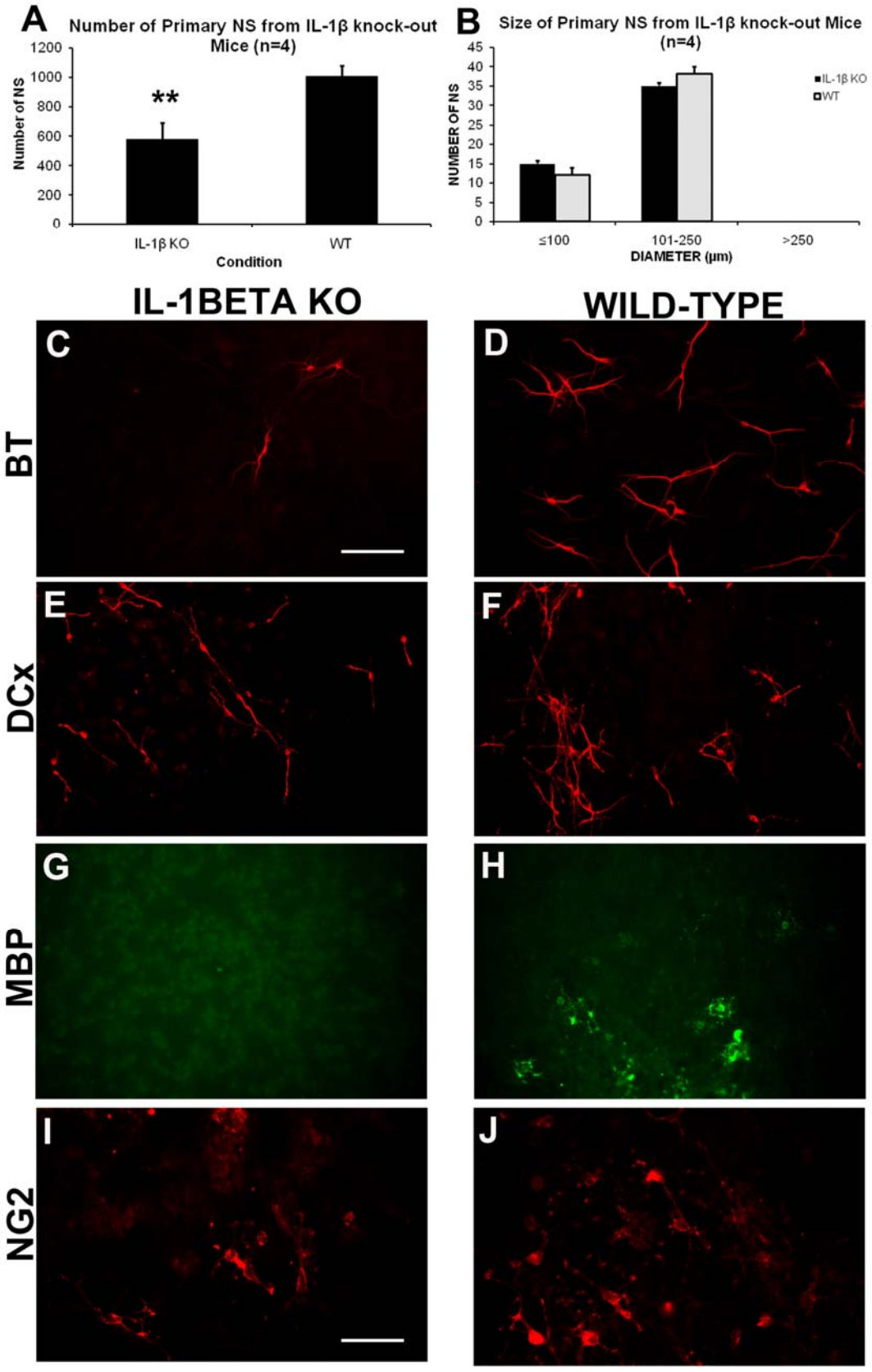

A
Number of Primary NS from IL-1β knock-out Mice (n=4)
Number of NS
1200
1000
800
600
400
200
0
**
IL-1β KO
WT
Condition
B
Size of Primary NS from IL-1β knock-out Mice (n=4)
NUMBER OF NS
45
40
35
30
25
20
15
10
5
0
IL-1β KO
WT
≤100
101-250
>250
DIAMETER (μm)
IL-1BETA KO
WILD-TYPE
BT
DCx
MBP
NG2
C
D
E
F
G
H
I
J

TABLE 2.1: Summary of effects of inflammation or hypoxia on SVZ NPCs.

	TREATMENT	SELF-RENEWAL	NEUROGENESIS	OLIGO-GENESIS
IN VITRO	LOW DOSE LPS	----------	↑ ↑ ↑	↑ ↑
IN VITRO	HIGH DOSE LPS	----------	↓ ↓ ↓	↑ ↑ ↑
IN VIVO	LPS	↑ ↑ ↑	↑ ↑ ↑	↑ ↑ ↑
IN VIVO	HYPOXIA	↑ ↑ ↑	↑ ↑ ↑	↑ ↑ ↑
IN VIVO + IN VITRO	HYPOXIA + LPS	----------	↓ ↓ ↓	↓
IN VIVO	HYPOXIA + LPS	↓ ↓	↓ ↓ ↓	↓

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CHAPTER III:

INTACT AND INJURED ENDOTHELIAL CELLS DIFFERENTIALLY MODULATE POSTNATAL MURINE FOREBRAIN NEURAL STEM CELLS

Abstract

Neural stem cells (NSCs) persist in the mammalian subventricular zone (SVZ) within a stem cell niche containing glial and vascular elements, such as endothelial cells. Recent work suggests that endothelial cells stimulate embryonic mouse NSC expansion and neuron production (Shen, et al., 2004). Stroke increases angiogenesis and SVZ neurogenesis, but the role of endothelial cells in the latter process is unknown. We hypothesized that factors secreted by intact or oxygen-glucose deprived (OGD; an *in vitro* stroke model) endothelial cells enhance postnatal SVZ neurogenesis. To test this, P15 mouse SVZ-derived neurospheres (NS) were co-cultured (non-contact) with primary mouse endothelial cells, or exposed to endothelial cell-conditioned media (ECCM). To determine the influence of injured endothelial cells on SVZ NSCs, ECCM from intact or OGD-exposed (OGD-ECCM) endothelial cells was applied to primary NS during expansion. ECCM significantly increased primary NS production and cell proliferation. Differentiated NS yielded more immature neurons after exposure to endothelial cells or ECCM. NS expanded in OGD-ECCM gave rise to neurons with longer processes compared with controls or intact ECCM.

Co-culture or ECCM altered astrocyte morphology to radial glial- or reactive astrocyte-like cells with long processes and increased nestin expression. Myelin basic protein+ oligodendrocytes also appeared more immature. OGD-ECCM also stimulated neuroblast migration compared to controls. These data suggest that intact endothelial cells secrete a factor(s) that stimulates SVZ NSC expansion and maintains NSC immaturity, while OGD-exposed endothelial cells stimulate NSC migration and maturation.

Introduction

Neural stem cells persist into adulthood in the mammalian forebrain SVZ and hippocampal dentate gyrus, where they give rise to olfactory bulb interneurons and dentate granule neurons, respectively (Altman, 1969, Cameron and Gould, 1994, Corotto, et al., 1993, Kaplan and Hinds, 1977, Kuhn, et al., 1996, Lois and Alvarez-Buylla, 1994). Many endogenous molecules and exogenous influences, such as exercise, injury and growth factors, modulate the proliferation, migration and differentiation of these cells. Little is known, however, about the factors that maintain these cells in their quiescent state or that stimulate them after injury. These factors may be produced or secreted by other cells in the local environment. SVZ NSCs reside along the lateral wall of the lateral ventricle within a stem cell niche containing vascular and glial elements which likely interact with NSCs via direct cell-cell contact or through secreted factors to maintain their stem cell-like state.

One of the first studies to investigate the stem cell microenvironment in the rodent brain identified endothelial cells as a prominent cell type within the

stem cell niche of the dentate gyrus (Palmer, et al., 2000). In that study the authors demonstrated that clusters of proliferating cells in the rodent dentate gyrus contained both endothelial cells and NSCs, suggesting that endothelial cells play an important role in dentate gyrus neural stem cell proliferation or maintenance (Palmer, et al., 2000). Two other studies revealed the important influence of the vascular niche on regulating aspects of neurogenesis. When angiogenesis was induced in the neurogenic region of the songbird brain, known as the higher vocal center, neurogenesis significantly increased in this area; when angiogenic factors were inhibited, neurogenesis decreased in the higher vocal center (Louissaint, et al., 2002). A more recent study examined the role of the vasculature in migration of new cells in the olfactory bulb and found these cells migrate radially alongside blood vessels to their final locations within the olfactory bulb (Bovetti, et al., 2007). Together, these studies suggest that endothelial cells are an important part of the stem cell niche, but their precise role in regulating NSCs may differ depending on species and location.

Evidence for the influence of endothelial cells on NSCs may be easier to obtain by evaluating their interactions *in vitro*. Three studies published to date have examined the interactions of endothelial cells and embryonic NSCs. One of these studies involved co-culture of endothelial cells with embryonic NSCs in a non-contact manner using transwell inserts, and found increased self-renewal of NSCs and increased neuron production after co-culture (Shen, et al., 2004). Another group used the same non-contact co-culture system with endothelial cells and embryonic neural progenitors to investigate the role of progenitors on

the blood-brain barrier (Weidenfeller, et al., 2007). This group focused on the influence of neural progenitors on endothelial cells and found enhanced barrier properties that required the presence of the neural progenitors (Weidenfeller, et al., 2007). As a side note, they noticed suppressed differentiation of neural progenitors after co-culture, suggesting a two-way interaction between neural and endothelial elements of the niche (Weidenfeller, et al., 2007). The importance of potential interactions between endothelial cells and NSCs during development was noted when a novel 3-D co-culture system was developed, in which endothelial cells seeded directly onto NS from embryonic NSCs attached to the NSCs and migrated into the NS (Milner, 2007). This suggests a two-way interaction between neural and vascular elements of the niche. Together, these studies suggest that endothelial cells and NSCs interact during embryonic development to influence the development of both NSCs and the blood-brain barrier.

Experimental stroke in postnatal and adult rodents increases neurogenesis in the forebrain SVZ. After neonatal hypoxia-ischemia, adult middle cerebral artery occlusion or adult thermocoagulation lesion, the SVZ expands and contains increased numbers of bromodeoxyuridine+ (BrdU) proliferating cells and doublecortin+ (DCx) neuroblasts (Arvidsson, et al., 2002, Gotts and Chesselet, 2005, Ong, et al., 2005, Parent, et al., 2002, Plane, et al., 2004). The SVZ NSCs migrate towards injured areas and differentiate into neurons and glia, potentially to replace those lost to injury (Arvidsson, et al., 2002, Parent, et al., 2002, Plane, et al., 2004). In the normal brain, SVZ NSCs

are relatively quiescent; those cells that do proliferate give rise to neuroblasts that migrate to the olfactory bulb and differentiate into olfactory bulb neurons. Thus, stroke-induced neurogenesis likely results from alterations within the neurogenic niche of the SVZ that promote proliferation combined with cues from the injured area that redirect SVZ-derived neuroblasts to migrate and differentiate in the injured regions.

In addition to stimulating neurogenesis, stroke and other brain lesions increase angiogenesis. Endothelial cell proliferation, blood vessel formation and neuroblast production increases in the SVZ after cortical thermocoagulation lesion (Gotts and Chesselet, 2005, Gotts and Chesselet, 2005), and after cortical stroke produced by distal middle cerebral artery occlusion, SVZ neuroblasts migrate to the peri-infarct cortex and locate alongside blood vessels (Ohab, et al., 2006). Additional evidence of an interaction between endothelial cells and SVZ-derived neuroblasts comes from a study in which endostatin, an angiogenesis inhibitor, was administered for one week after stroke. This treatment resulted in a significant decrease in newly formed blood vessels and neuroblasts, suggesting that their interaction may be responsible for stroke-induced increases in neurogenesis. Most recently, Teng et al co-cultured normal endothelial cells or those from the ischemic boundary with SVZ NSCs and found that normal rat brain endothelial cells increased proliferation while those from the ischemic border increased neuronal differentiation (Teng, et al., 2008). This study provides strong evidence for an interaction between endothelial cells and SVZ NSCs in the normal and stroke-injured brain (Wang, et al., 2008).

Other pathological conditions also appear to involve NSC-vascular niche interactions. One such condition is the growth of malignant brain tumors, some of which have cells with stem-like properties of multipotency and self-renewal (Al-Hajj, et al., 2003, Lapidot, et al., 1994, Singh, et al., 2004). Cancer stem cells identified by double-label expression of nestin and CD133 are often found located next to capillaries in brain tumors. These cells maintain a self-renewing and undifferentiated state when cultured with endothelial cells (Calabrese, et al., 2007). Together, these studies suggest that endothelial cells regulate SVZ NSCs after stroke or other brain insults.

One final line of evidence suggesting an interaction between endothelial cells and NSCs comes from studies demonstrating that angiogenic factors alter stroke-induced neurogenesis. Endothelial nitric oxide synthase deficient mice have decreased SVZ proliferation, decreased neuroblast production and decreased angiogenesis after focal cerebral ischemia (Chen, et al., 2005). Erythropoietin (EPO), the growth factor that regulates red blood cell production, also influences neurogenesis both in development and after stroke. EPO and EPO-receptor deficient mice show severe defects in embryonic neural development and decreased SVZ cell proliferation into the postnatal period (Tsai, et al., 2006). EPO administration after stroke increases vessel density at the infarct border, stimulates proliferation and neuroblast production in the SVZ, improves functional recovery, and increases VEGF levels (Wang, et al., 2004). Thus, SVZ NSCs reside in a microenvironment in which they likely respond to

factors produced by endothelial cells; after stroke, endothelial factors from the infarct region may also serve as modulators of SVZ-derived NSCs.

To investigate how normal or injured endothelial cell-secreted factors modulate postnatal SVZ NSCs, we examined the influence of normal and oxygen-glucose deprived (OGD, an *in vitro* stroke model) endothelial cells on postnatal SVZ NSCs cultured as NS. We hypothesized that endothelial cells secrete factors that augment neurogenesis in the intact and injured postnatal mammalian SVZ. SVZ-derived NS were co-cultured with endothelial cells in a non-contact system or differentiated in endothelial cell-conditioned media (ECCM). We also expanded NS in conditioned media collected from intact or OGD-treated endothelial cells. We found that expansion in intact ECCM increased primary NS production and cell proliferation. Intact endothelial cells delayed SVZ neural stem cell differentiation to yield neurons with shorter processes, whereas OGD-treated endothelial cells produced the opposite effect. Intact endothelial cells or ECCM also altered the morphology of glia to more immature-appearing forms and under some conditions significantly increased oligodendrocyte production. NS expansion in OGD-ECCM also promoted neuroblast chain migration. Together these results suggest that intact endothelial cells secrete factors that maintain postnatal SVZ NSCs in an immature state, but after stroke-like injury, the endothelial cell influence on NSCs changes to promote neuroblast migration and more rapid neuronal differentiation.

Methods:

Primary Neurosphere (NS) Culture

NS cultures were prepared as previously described (Gritti, et al., 1996, Wang, et al., 2005) with slight modifications; all procedures were performed in accordance with the policies of the University of Michigan Committee on Use and Care of Animals. Postnatal day 15 (P15) CD-1 mice (Charles River) were anesthetized with CO_2, decapitated, brains removed, and placed into ice cold Opti-mem. The forebrain containing the striatal SVZ was cut into two coronal slices and the SVZ was dissected out, minced and dissociated with trypsin. SVZ cells were plated at $6x10^3$ to $6x10^4$ depending on experiment and size of wells (see below) and cultured in serum-free media (SFM) containing growth factors [Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, Gibco), 20 ng/ml epidermal growth factor (EGF; Sigma), 10 ng/ml basic fibroblast growth factor (bFGF; Sigma), 2 μg/ml heparin, and a defined hormone and salt mixture (100 μg/ml transferrin, 25 μg/ml insulin, 60 μM putrescine, 30 nM sodium selenite and 20 nM progesterone)]. Primary NS were cultured for 6 days in vitro (DIV), then picked and re-plated for differentiation in poly-ornithine (Sigma) coated 24 well plates (Corning-Costar) or mechanically dissociated and passaged to form secondary NS. Unless otherwise described, primary NS were differentiated for 7 d in DMEM/F12 plus the aforementioned hormone mixture and 1% fetal bovine serum (FBS). For all experiments, half of the medium was replaced every 3 d.

Endothelial Cell Culture

Primary mouse brain endothelial cells (EC) or a brain endothelial cell line (bEnd.3) were used for all experiments and cultured as previously described (Andjelkovic, et al., 2003). Primary endothelial cells were cultured from 4- to 6-week-old CD-1 mouse brain microvessels in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% inactivated fetal calf serum (Invitrogen), 2.5 g/mL heparin (Sigma, St. Louis, MO, U.S.A.), 20 mM HEPES, 2-mM glutamine, antibiotic/antimycotic (Invitrogen), and endothelial cell growth supplement (BD Bioscience, NJ, U.S.A.) using six-well plates coated with collagen IV (BD Bioscience, KY, U.S.A.). The brain endothelial cell line (bEnd.3) was purchased from ATCC (American Type Culture Collection, VA, U.S.A.). Cells were plated and grown in the recommended cell media (DMEM, 10% FBS, 1 × AA, 2 mM glutamine) in the presence of 10% CO_2.

Co-culture Experiments

In experiment one, primary NS were cultured with primary mouse brain endothelial cells in a non-contact manner, similar to the system described by Shen et al (Shen, et al., 2004). Endothelial cells were plated in 12 well plates and weaned from 10% serum to SFM over 3 d in order to culture them in the same media as NS. SVZ cells were plated on transwell inserts (40μm pore size, Corning) at $5x10^4$ cells/insert and expanded alone for 3 d (Figure 3.1). NS-containing transwell inserts were then placed into wells containing endothelial cells in SFM. Controls included NS grown in SFM on membranous inserts with NIH3T3 cells (fibroblast cell line) or no cells in the well below. NS expansion

continued for 3 more days. At 6 DIV, similarly sized NS were picked and re-plated on poly-ornithine coated 24 well plates in differentiation media.

Conditioned Media Differentiation Experiments

Conditioned media (CM) was collected from primary mouse brain endothelial or NIH3T3 cells grown in DMEM with 10% normal calf serum (NCS). CM was collected approximately 24 h after application to cells, filtered, and stored at -20°C until use. For experiment two, NS were plated at $6x10^4$ cells/60mm dish and expanded in SFM for 6 d, then were picked and re-plated on poly-ornithine coated 24 well plates for differentiation in 100% or 50% (diluted 1:1 with DMEM) CM from endothelial or 3T3 cells, or in unconditioned (control) media containing matching 10% or 5% NCS (Figure 3.1). NS were differentiated in CM for 7 d, with half of the medium replaced every 3 d.

Oxygen-Glucose Deprivation (OGD)

All OGD experiments were performed using the mouse bEnd.3 cell line. OGD was performed as previously described (Andjelkovic, et al., 2003). Confluent bEnd.3 cells were transferred into a temperature-controlled (37° ± 1°C) anaerobic chamber (Coy Laboratory, MI) containing a gas mixture composed of 5% CO_2, 10% H_2, 85% N_2. The culture medium was replaced with deoxygenated glucose-free, serum-free DMEM and cells were maintained in the anaerobic chamber for 1 or 5 hours. OGD-exposed cells were removed from the anaerobic chamber, the medium replaced with fresh serum-free DMEM and returned to

normoxic conditions (5% CO_2/95% air) for up to 24 hours (re-oxygenation; Figure 3.6). Control cell cultures were not exposed to OGD. Serum-free conditioned media was collected from injured endothelial cells at 6, 12 or 24 hours post-OGD (OGD-ECCM), or from intact endothelial (ECCM) or NIH3T3 cells (3T3CCM). Unconditioned media was used as an additional control. All media was filtered and stored at -20°C until use.

Normal vs. OGD-Endothelial Cell CM Experiments

For experiment three, SVZ cells were plated at $1.0x10^4$ cells/well in 12 well plates and expanded alone in SFM containing doubly concentrated growth factors for approximately 24 hours (Initial experiments were performed without doubling the concentration of growth factors, which resulted in dilution of growth factors after the addition of CM. Fewer NS were produced in these experiments and differentiated cells appeared unhealthy). An equal volume of conditioned (or unconditioned) media was then added to each well and NS expansion continued until 6 DIV. Primary NS were picked and re-plated for 4 or 7 d of differentiation in 1% FBS or were passaged and re-plated in SFM (no conditioned media) to form secondary NS (Figure 3.1).

To assess migration, primary NS were grown alone for 5d, then picked and re-plated in matrigel (Figure 3.1, Experiment 4) following similar methods to those published previously (Katakowski, et al., 2005, Nguyen-Ba-Charvet, et al., 2004, Wichterle, et al., 1997). NS were incubated at 37°C for 30 minutes to allow the matrigel to solidify. CM or control media was added to each well and NS were

placed in 37°C/5% CO_2 incubator. Phase-contrast images of NS were captured at 24 hours and 3 days post-plating. NS were fixed and immunostained at 3d post-plating for astrocytes and immature neurons.

Immunofluorescence Staining

All primary NS were fixed with 4% paraformaldehyde at 4 or 7 d of differentiation and immunostained. Cells were immunostained using markers to identify neurons [(See Appendix); 1:400 anti-mouse MAP2abc, Sigma; 1:1000 anti-rabbit β-III-tubulin, Babco; 1:800 anti-rabbit doublecortin (DCx), Parent et al(Parent, et al., 2002)], astrocytes [1:500 anti-mouse Glial-Fibrillary Associated Protein (GFAP), Sigma], radial glia (1:100 anti-mouse ZRF-1, ZFIN; 1:50 anti-mouse RC2, Hybridoma Bank), oligodendrocytes [1:600 anti-rat Myelin Basic Protein (MBP), Chemicon], neural progenitors (1:100 anti-mouse Nestin, Chemicon; 1:500 anti-rabbit NG2, Chemicon), proliferating cells (1:1000 anti-rabbit Ki67, Vector Labs), and apoptotic cells (1:1000 anti-rabbit cleaved Caspase-3, BD). Cells were washed with PBS, blocked with 10% serum (in PBS+0.2% triton+BSA), and incubated in primary antibodies overnight at 4°C. After washes, cells were incubated in fluorescent secondary antibodies for 1.5 h at room temperature. All secondary antibodies were used at 1:800 and included goat-anti-rabbit Alexa 594, goat-anti-mouse Alexa 488, and goat-anti-rat Alexa 488 (Molecular Probes). Cells were washed with PBS, counterstained with bisbenzamide, and stored in PBS.

Proteomics

CM was collected from intact NIH3T3 or endothelial cells after 6 or 12 h of cell exposure, or from OGD-treated endothelial cells 6 or 12 hours after termination of OGD. Media was separated into fractions using 5kD molecular weight cut-off filters, thus separating the media into fractions containing proteins with a molecular weight of less than or greater than 5kD. These fractions were re-constituted in DMEM and applied to primary NS using the same methods as in Experiment 3 (Figure 3.1). To assess which fraction contained the proteins potentially responsible for the results found in experiment 3, we counted the number of primary NS at 6DIV, and then differentiated NS for 4d and immunostained for MAP2abc, Ki67, β-III-tubulin, and p27. One sample/condition was sent to the Michigan Proteome Consortium for further analysis. The samples were run on a 1D gel stained with Sypro Ruby to identify the molecular weights of the proteins present in CM. Then trypsin digestion was performed using the MassPrep robot. The peptides were extracted from the gel plugs with 30µl of 2% acetonitrile 0.1% TFA. Five microliters of Maldi matrix was added to each extract and the extracts were dried using a Speed Vac. The peptides were dissolved in 5µl of 60% ACN 0.1% TFA and an aliquot of each sample was spotted onto a Maldi target. MS/MS analysis was performed and the data acquired was put into an automated database search with GPS software using the MASCOT search engine (AppliedBiosystems).

Microscopy and Image Analysis

Primary and secondary NS were counted and diameter measured in 2 wells/condition/experiment on a Leica inverted light microscope for at least 4 separate experiments. Differentiated cells were imaged using a Leica fluorescent microscope and SPOT-RT digital camera. NS were selected based on similar bisbenzamide labeling density and a minimum of 3 NS/condition were photographed for quantification using a 20X objective. Images were imported into Adobe Photoshop v. 7.0 or ImageJ for analysis. β-tubulin+ neuronal processes were measured in ImageJ with examiner blinded to condition (n≥3 NS/condition for at least 3 experiments) and results imported into Microscoft Excel for graphic analysis. Oligodendrocytes were quantified in 3 or more separate experiments by counting MBP+ cells in ≥3 20x fields/condition in a blinded fashion. Cell proliferation and cell death were quantified in a similar manner for Ki67+ and Caspase3+ cells, respectively. Migration experiments were quantified in ImageJ by measuring the distance from the center of the NS to the end of each of the 3 longest chains/NS for 3 or more NS/condition/experiment.

Statistical Analysis

Analysis of variance (ANOVA) with post-hoc t-tests were used to compare differences between groups for all quantitative analyses using Statview. Results are presented as mean ± standard error of the mean (SEM) and considered significant when $p \leq 0.05$.

Results

Endothelial cell factors influence neuronal differentiation

The interaction of components in the stem cell microenvironment, such as endothelial cells in close contact with NSCs, likely serves to regulate the persistence of neurogenesis after birth. To examine the influence of endothelial cells on postnatal SVZ NSCs, we first cultured primary NS with endothelial cells (or NIH3T3 cells or alone in cell-free media) in a non-contact co-culture system. We evaluated the effects of non-contact co-culture on primary NS formation and primary NS differentiation (Figure 3.1, Experiment 1). We found no difference in primary NS number (Figure 3.2A, $p=0.3$) or size (Figure 3.2B, $p=0.3$) after co-culture with endothelial cells compared to co-culture with NIH3T3 cells or NS alone.

Recent studies of embryonic NSCs suggested that endothelial cells stimulate neurogenesis (Shen, et al., 2004). We, therefore, examined whether endothelial cells increase neuron production from postnatal SVZ-derived NSCs. We evaluated neurogenesis in NS after co-culture with endothelial cells (Figure 3.1, Experiment 1) or in NS differentiated in ECCM (Figure 3.1, Experiment 2). In experiment one, we found no increase in neuron production, however, there was a difference in the morphology of β-tubulin+ or DCx+ neurons produced from NS co-cultured with endothelial cells compared to those from controls. β-III-tubulin-positive neurons derived from endothelial co-cultured NS displayed significantly shorter processes than controls (Figure 3.3A, B, $p<0.01$). Similar results were seen for DCx-labeled immature neurons (data not shown). The length of β-

tubulin+ processes was measured and on average, β-tubulin+ processes from co-cultured NS were less than half the length of those found in control NS (Figure 3.3C). Similarly, in experiment two, NS differentiated in ECCM gave rise to clusters of β-tubulin+ neurons with very short processes (Figure 3.3D), compared with those from controls which typically contained longer processes that contact other cells (Figure 3.3E). Process length measurements revealed that NS differentiated in ECCM (50 or 100%) contained significantly shorter neuronal processes than controls at approximately one-third of their length (Figure 3.3F, $p<0.01$).

Endothelial cell-secreted factors alter glial morphology

Next we sought to determine if endothelial cells influence the formation of glial cells from SVZ-derived NS. We first examined nestin immunoreactivity to assess effects on the formation of stem cells/radial glia. In experiment one, we found that NS grown alone and differentiated in 1% FBS contained few nestin-positive cells after 7d of differentiation (Figure 3.4C). NS co-cultured with endothelial cells contained many nestin-positive cells that displayed radial processes and expressed higher levels of nestin (Figure 3.4A) than in control cultures (Figure 3.4B-C). We found similar results using the ZRF-1 antibody that labels radial glia in zebrafish (Raymond, et al., 2006, Strausberg, et al., 2002, Thisse, 2004), with minimal labeling in control cultures and enhanced expression in radial processes of endothelial co-cultured NS (data not shown). We next performed immunofluorescence staining with a GFAP antibody to identify

astrocytes. This staining revealed GFAP-positive cells with altered morphology in NS co-cultured with endothelial cells compared to controls (Figure 3.4D-F). GFAP-positive cells derived from endothelial co-cultured NS contained numerous processes extending off of each cell, resembling reactive astrocytes (Figure 3.4D) compared with those from both controls, which were typically large, flat GFAP+ astrocytes (Figure 3.4E-F).

We also performed nestin and GFAP immunostaining in experiment two to label stem cells/radial glia and astrocytes, respectively, in NS differentiated in conditioned medium. Similar to the pre-differentiation co-culture experiments, we found strong nestin-reactivity in radial processes of 100% and 50% ECCM-differentiated NS (Figure 3.4G, M). Fewer cells from 3T3CCM-differentiated NS strongly expressed nestin, and these cells resembled astrocytes without radial morphology (Figure 3.4H, N). Very little nestin expression was detected in serum controls and the rare labeled cells resembled normal astrocytes when present (Figure 3.4I, O). GFAP-positive cells from ECCM-differentiated NS resembled the nestin-positive cells in these NS, with positive staining present in radial processes (Figure 3.4J, P). GFAP-positive cells from both 3T3CCM and serum controls displayed more typical astrocyte morphology (Figure 3.4K-L, Q-R).

SVZ NSCs cultured as NS give rise to neurons, astrocytes and oligodendrocytes. Postnatal SVZ neural progenitors also give rise to oligodendrocytes in vivo (Ganat, et al., 2006, Goldman, 1995, Levison and Goldman, 1993). We therefore examined the influence of endothelial cell-secreted factors on oligodendrocytes generated by SVZ-derived NS. In

experiment one, we found that MBP-positive oligodendrocytes from endothelial co-cultured NS appeared smaller in size and displayed little branching compared with those from controls, which appeared larger with more elaborate branching patterns (Figure 3.5 A-C). We wanted to determine if there was also a difference in the number of oligodendrocytes produced from endothelial co-cultured NS. Quantification of MBP-positive cells showed no difference in the number of oligodendrocytes produced from endothelial co-cultured NS compared with NS grown alone (Figure 3.5D). NS co-cultured with 3T3 cells generated significantly fewer MBP+ cells than NS grown alone (Figure 3.5D, $p<0.01$). The morphology of MBP-positive oligodendrocytes was also examined in experiment two, in NS differentiated in conditioned medium. NS differentiated in 50 or 100% ECCM produced small MBP-positive oligodendrocytes with little branching (Figure 3.5E, G), similar to those from NS co-cultured with endothelial cells. MBP-positive oligodendrocytes from control CM differentiation were generally larger than those from ECCM-differentiated NS and contained highly branched MBP-positive cells (Figure 3.5F, H). The number of MBP-positive oligodendrocytes was significantly increased in 50% ECCM-differentiated NS compared with all other conditions (Figure 3.5I, $p<0.01$). These results suggest that factors secreted by endothelial cells alter oligodendrocyte production, but these findings should be confirmed with use of additional oligodendrocyte markers.

One recent study suggested that NSCs could trans-differentiate into endothelial cells after exposure to endothelial cells in co-culture (Wurmser, et al., 2004). We therefore performed immunostaining to assay for endothelial cells

using an anti-Glut-1 antibody. No immunoreactivity was seen in NS expanded with primary endothelial cells or in those differentiated in ECCM (data not shown). Endothelial cells from co-culture were included as a control and all of these cells expressed Glut-1 (data not shown). Previous studies examining the influence of endothelial cells on embryonic NSCs found increased self-renewal after co-culture of these two cell types (Shen, et al., 2004). The effects of endothelial cells on NS expansion or self-renewal could not be tested in the current experiments because the endothelial cells were cultured in serum. We describe additional experiments below evaluating the effects of endothelial cells on NSC self-renewal using serum-free CM.

Endothelial factors increase neurosphere production and proliferation

Stroke-induced neurogenesis is evident within a week of insult as the SVZ expands with proliferating cells (Arvidsson, et al., 2002, Jin, et al., 2001, Parent, et al., 2002, Tureyen, et al., 2005, Zhang, et al., 2001). The vascularity of the brain is also altered after stroke. Angiogenesis, the formation of new blood vessels, increases after experimental stroke in the peri-infarct regions as a result of endothelial cell proliferation (Chen, et al., 2003, Chen, et al., 2004, Chen, et al., 2003, Ohab, et al., 2006, Wang, et al., 2008, Wang, et al., 2004). Endothelial cell proliferation increases soon after injury and peaks 5 d after stroke (Gotts and Chesselet, 2005). The correlation of stroke-induced angiogenesis and neurogenesis led us to question whether stroke-injured endothelial cells may regulate SVZ NSCs. We used oxygen-glucose deprivation, a widely used *in vitro*

stroke model that results in a preconditioning effect after short durations and cell damage and eventual cell death after longer durations, to induce ischemic preconditioning or injury to endothelial cells. Conditioned media was collected for application to SVZ NS during expansion [Figure 3.6; (Andjelkovic, et al., 2003, Hu, et al., 2006, Kapinya, et al., 2002, Keep, et al., 2005, Perez-Pinzon, et al., 1995)]. In experiment three, media conditioned for 6 or 12 h by intact NIH3T3 (control) or endothelial cells, or OGD-exposed endothelial cells (labeled 6 h ECCM, 12 h ECCM, etc) was tested for its effects on NS cultures. For these experiments, the bEnd.3 cell line was used rather than primary endothelial cells because of the availability of the cell line. Data shown in the following sections is from 5 h OGD, unless otherwise noted. Results from 1 h OGD were similar but slightly less marked than those from 5 h OGD when the same assays were evaluated.

Primary NS were expanded for 24 h in serum-free media and then for 5 d in intact ECCM or 3T3CCM (or unconditioned media as an additional control), or OGD-ECCM (Figure 3.1, Experiment 3). On day 6 of NS expansion, primary NS were counted and their diameters measured to determine effects on NS formation. We found a significant increase in the number of NS produced when expanded in intact 12 h ECCM compared with control conditions (Figure 3.7A). We found similar results after 1h OGD (Figure 3.7C, $p=0.01$ for 12h ECCM vs. 12h 3T3CCM or UNCOND). Some NS were re-plated for differentiation while others were passaged to examine self-renewal as secondary NS. No significant difference was found in the number of secondary NS produced between

conditions, but a trend toward an increase in secondary NS production was seen when primary NS were expanded in 12 h ECCM ($p=0.08$; Figure 3.7D). This trend was also evident after 1h OGD followed by 12 h media collection (Figure 3.7F; $p=0.06$-0.07 for 12h ECCM vs. 12 h 3T3CCM or UNCOND and for 12 h OGD-ECCM vs. 12 h 3T3CCM or UNCOND). No significant difference from controls emerged in the size of primary or secondary NS after expansion in intact ECCM or OGD-ECCM (Figure 3.7B, E). Next we evaluated the effects of intact- or OGD-ECCM on cell proliferation. We examined Ki67 immunostaining after 4 d of differentiation and found a significant increase in the number of Ki67-positive cells after NS expansion in ECCM (Figure 3.7H, K, M) compared with controls (Figure 3.7G, J, M, $p<0.01$) and a significant decrease in Ki67 cell number in ECCM vs. OGD-ECCM (Figure 3.7I, L, M, $p<0.05$). We also assayed for apoptosis after 4 d of differentiation using activated Caspase-3 immunostaining. We found no difference in activated caspase-3 immunoreactivity between ECCM, OGD-ECCM or controls (data not shown). Together, these data indicate that factors secreted from normal endothelial cells increase SVZ NS formation and proliferation without altering apoptosis.

Endothelial-secreted factors alter NSC maturation

In our first few studies described above, NS expansion with primary endothelial cells before differentiation, or NS differentiated in ECCM, led to altered neuron and glial morphology. In those experiments, β-tubulin-positive neurons from endothelial co-culture or ECCM differentiation contained shorter

processes and typically appeared as clusters of cells, whereas controls had significantly longer processes that contacted other cells and cell bodies were more dispersed (Figure 3.3). We asked whether similar effects would be seen with NS expanded in serum-free ECCM, and if these effects would be similar if the endothelial cells were exposed to OGD before collecting ECCM. NS were expanded for 5d in serum-free conditioned media from intact or OGD-treated endothelial cells, or control 3T3 cells, and then differentiated in normal medium for 4 or 7 d (Figure 3.1, Experiment 3; Figure 3.6). After 4 d of differentiation, NS expanded in ECCM contained MAP2abc-positive cells with short or no processes (Figure 3.8C) while MAP2abc-positive cells in controls had a mix of short and medium processes (Figure 3.8A, B). NS expanded in OGD-ECCM gave rise to MAP2abc-positive neurons with much longer processes than controls (Figure 3.8D). Results were similar with 6 h and 12 h media; data shown are from 12 h media for all conditions.

Next we evaluated whether these differences persisted, or instead if process outgrowth in NS treated with intact ECCM “caught up” with controls or OGD-ECCM process outgrowth slowed to match controls. To evaluate this, we immunostained for neuron-specific β-III-tubulin after 7 d of differentiation. We found that β-tubulin-positive neurons from intact ECCM-expanded NS maintained shorter process length than controls, and β-tubulin-immunoreactive neurons from OGD-ECCM-expanded NS continued to have longer processes than controls (Figure 3.8F-I). Results were similar with 6 h and 12 h media; data shown are from 12 h media for all conditions. The number of processes per cell and length

of each β-tubulin-positive process was counted and measured in fields with similar cell densities. Quantification of β-tubulin-positive process length confirmed that neurons from intact 6h ECCM contained significantly shorter processes than controls, as did 12 h ECCM (Figure 3.8E, $p<0.05$). This analysis also confirmed that β-tubulin-positive processes from 6 h OGD-ECCM and 12 h OGD-ECCM were significantly longer than those from controls (Figure 3.8E, $p<0.01$). We found no difference between conditions in the number of processes/cell or in the number of neurons/field (Figure 3.8J, data not shown).

Endothelial factors alter glial morphology

Due to the altered morphology of Nestin-, GFAP- and MBP-positive cells seen after co-culture with endothelial cells or differentiation in serum-containing ECCM (Figures 4 & 5), we examined whether similar changes occurred after NS expansion in serum-free intact- or OGD-ECCM. After primary NS were expanded in CM as shown in Figure 3.1, Experiment 3, NS were differentiated for 4 d and then immunostained for nestin. We found that the nestin-positive cells from ECCM-expanded NS resembled those from experiments one and two in that they displayed long radial processes (Figure 3.9C, arrowheads), whereas little nestin was expressed in the unconditioned control (Figure 3.9A); although nestin morphology varied in 3T3CCM controls, it was not present in radial processes (Figure 3.9B). NS expanded in OGD-ECCM gave rise to a mixed population of nestin positive cells, some with numerous short processes (Figure 3.9D, arrows) and others with lighter expression and very long processes (Figure

3.9D, arrowheads). After 7 d of differentiation, these differences in morphology persisted. ECCM-expanded NS contained numerous GFAP-positive radial glial-like processes (Figure 3.9G). These GFAP-positive cells from ECCM-expanded NS resembled both the nestin-positive cells seen in these conditions after 4d of differentiation and the GFAP-positive cells generated after differentiation in ECCM or following NS/endothelial cell co-culture in experiments one and two. The GFAP-positive cells found in controls were typically flat astrocytes with few/no processes (Figure 3.9E-F) while those from OGD-ECCM displayed numerous short processes (Figure 3.9H, arrow) or were long radial fibers (Figure 3.9H, arrowhead), similar to nestin staining at 4 d (Figure 3.9D). We found similar results for nestin and GFAP expression with 6 h and 12 h media; data shown in Fig. 9 are from 12 h media for all conditions.

The influence of intact- or OGD-ECCM on SVZ NS-derived oligodendrocyte formation was evaluated by MBP immunostaining. Few oligodendrocytes were produced and there was no obvious difference in the number of MBP-positive cells produced by intact- or OGD-ECCM expanded NS or in their morphology (data not shown). NS in some experiments were also stained with various antibodies to identify endothelial cells (Glut-1 or CD31). We found no evidence of transformation of SVZ-derived NSCs into endothelial cells (data not shown).

Factors from OGD-ECCM increase chain migration

Given our findings of increased process outgrowth or more rapid differentiation of NSCs induced by OGD-treated endothelial cells, we next examined whether factors from intact or OGD-treated endothelial cells would increase neuronal migration from SVZ-derived NS. In these experiments, NS were expanded alone for 5d, and then were plated in 3-dimensional matrigel culture to promote chain migration. Conditioned medium collected after either 6 or 12 hours was added to the cultures after plating in matrigel, and NS were cultured for an additional three days (Figure 3.1, Experiment 4). NS incubated with intact ECCM showed some chain migration at 24 h, but appeared similar to controls at this time (Figure 3.10A, B, E, F). NS in OGD-ECCM exhibited significantly more migration at 24 h post-plating in matrigel than controls (Figure 3.10 C, G). At 3 d post-plating in matrigel, NS in OGD-ECCM contained significantly longer chains than those in controls, while NS in intact ECCM remained similar to controls or trended toward less migration (Figure 3.10H). Quantification of chain length at 3 d probably underestimated the migratory effect, as differences were more apparent at 24 h (Figure 3.10A-C, E-G, $p<0.05$) but control and ECCM cultures started to "catch-up" to OGD-ECCM cultures. Immunofluorescence double-labeling confirmed that the long chains extending from NS cultured in OGD-ECCM were MAP2abc-positive immature neurons (green) with very few GFAP-positive astrocytes (red) present (Figure 3.10D).

Preparation of CM for Proteomic Analysis

We found clear differences in the way intact endothelial cells influence SVZ NSCs compared with OGD-treated endothelial cells. In order to determine the factors secreted by intact or OGD-treated endothelial cells that may be involved in these effects, we first separated the media into two fractions. Conditioned media from intact 3T3s, intact ECs and OGD-treated ECs was separated into fractions less than or greater than 5kD with molecular weight cut-off filters. These fractions were applied to primary NS after 24hr of expansion, and then evaluated for effects on primary NS production at 6DIV and on differentiation after 6DIV expansion plus 4d differentiation. We found that the >5kD fraction of intact ECCM produced the most primary NS (Figure 3.11A) compared with the <5kD fraction of intact ECCM or fractions from any other condition. After 4d differentiation, more Ki67-immunoreactive cells were present in the >5kD ECCM condition than in any other fraction or condition (data not shown). We also observed shorter MAP2abc-immunoreactive neuronal processes in NS expanded in >5kD ECCM (Figure 3.11F), compared to controls (Figure 3.11B, E) or the <5kD fraction of ECCM (Figure 3.11C) similar to results seen with the whole ECCM. MAP2abc-positive neurons had much longer processes after NS expansion in the >5kD fraction of OGD-ECCM (Figure 3.11G) compared with the smaller fraction (Figure 3.11D) or other conditions (Figure 3.11B-C, E-F). Together these results suggest that the CM fraction containing proteins of molecular weight greater than 5kD is responsible for the increase in primary NS production and proliferation seen with intact ECCM, as well as the increased process outgrowth after NS expansion in OGD-ECCM.

Discussion

The results from these experiments suggest that both intact and OGD-treated endothelial cells influence the proliferation, migration, and differentiation of postnatal striatal SVZ-derived NSCs *in vitro* (Table 3.1). We found that NS expansion in intact ECCM promotes NS production and cell proliferation. Regardless of whether primary NS were co-cultured with endothelial cells, expanded in ECCM or differentiated in ECCM, β-tubulin- and DCx-positive neurons derived from these NS were more clustered and had significantly shorter processes than controls. Nestin- and GFAP-positive glial cells derived from these NS also displayed altered morphology, similar to radial glia or reactive astrocytes. We also found increased oligodendrocyte production during exposure to ECCM under certain conditions, and more immature-appearing oligodendrocytes were generated after neurosphere co-culture with endothelial cells or differentiation in ECCM. Finally, NS expanded in OGD-ECCM displayed faster chain migration and neuron process outgrowth, opposite of the effects of intact ECs or ECCM on SVZ neural progenitors.

NSCs may prove to be a useful therapeutic tool for many diseases and conditions; however, a better understanding of what regulates these cells is needed to optimize their use as an effective treatment. The local environment or stem cell niche likely contributes to the maintenance of these cells in the normal brain, as well as their alterations after injury. Blood vessels are in close contact with SVZ NSCs in vivo, thus allowing for the influence of endothelial cell-secreted

factors within the stem cell niche, which may play an important role in regulating NSCs. The goal of this study was to determine how normal and stroke-injured endothelial cells regulate postnatal SVZ neurogenesis. We found that intact endothelial cells secrete factors that increase primary neurosphere production and cell proliferation, and exposure to these factors before or during differentiation leads to more immature-appearing neurons and glia. Our findings suggest that endothelial cells within the stem cell niche serve as important modulators of postnatal SVZ neural progenitors by maintaining these cells in a more undifferentiated or stem-cell like state. The findings of increased NS production and cell proliferation support the notion that endothelial cell-secreted factors function to maintain postnatal SVZ NSCs in a stem cell-like state. The presence of more immature-appearing neurons and glia after exposure to endothelial cell-secreted factors provides further support for the function of endothelial cells within the SVZ niche in maintaining these NSCs in a more undifferentiated state.

Only a few recent studies have examined the interaction of intact endothelial cells and NSCs using a similar system *in vitro*. Two of these studies focused on embryonic NSCs isolated from the developing cortex and another used postnatal rat hippocampal NSCs, whereas we examined the effects of endothelial cells on postnatal mouse NSCs isolated from the striatal SVZ (Guo, et al., 2008, Shen, et al., 2004, Weidenfeller, et al., 2007). Previous reports describing the interaction of embryonic or hippocampal NSCs and endothelial cells used co-culture with transwell inserts to avoid direct cell contact (Guo, et al.,

2008, Shen, et al., 2004, Weidenfeller, et al., 2007). We followed similar methods to those published previously, although we were specifically interested in the effects of endothelial cell factors on postnatal SVZ NSCs. Besides the differences in age and location of NSCs, the timing of the previous studies differed from our own. The embryonic NSCs were cultured as NS for 4d, dissociated and then co-cultured with endothelial cells for only 1 day (Weidenfeller, et al., 2007) or cultured as NS alone for 7d, then co-cultured with endothelial cells for 3d or as NSCs at clonal density (not as spheres) with endothelial cells for 7d (Shen, et al., 2004). The study that used rat hippocampal cells dissociated the spheres and co-cultured these dissociated NSCs with endothelial cells for 7 days (Guo, et al., 2008). They evaluated NSCs after 7d of co-culture or 7d of co-culture plus 4d of differentiation (Guo, et al., 2008). All of the aforementioned studies placed the NSCs in the bottom of the well with the endothelial cells in the insert above, the opposite of our assay.

Despite the variations in methodology, some of our results were similar to those published previously, while many of our findings on the interaction of normal endothelial cells and NSCs were novel. Shen found that during NSC and endothelial cell co-culture, NSCs produced larger clones with more nestin+ and Lex+ progenitors than the control co-culture of cortical cells and NSCs (Shen, et al., 2004). This group also primarily used an endothelial cell line not of brain origin. Guo (2007) and Weidenfeller (2007) found increased nestin expression immediately after co-culture of NSCs and endothelial cells but neither group commented on clone size (Guo, et al., 2008, Weidenfeller, et al., 2007). Cancer

stem cells, which give rise to malignant tumors, express nestin and directly contact tumor capillaries in glioblastomas (Calabrese, et al., 2007). When these cells were cultured as tumor spheres with endothelial cells, the resulting spheres were 5 times larger than controls, suggesting that endothelial cells also maintain cancer stem cells in their stem cell-like state.

The previous studies, with the exception of Calabrese, et al, (2007), used dissociated cells in their co-culture systems, allowing them to use immunocytochemistry to evaluate self-renewal (Calabrese, et al., 2007, Guo, et al., 2008, Shen, et al., 2004, Weidenfeller, et al., 2007). We did not perform antibody labeling at the end of co-culture to evaluate the number of NSCs expressing nestin or Lex due to the nature of the neurosphere assay. These proliferative clusters of cells contain hundreds to thousands of cells and it is not possible to see every cell within the sphere. To evaluate self-renewal, we counted the number of primary and secondary NS produced in each condition and measured the diameter of the NS. Although we found no significant increase in the number of primary NS produced after co-culture with endothelial cells, we found a significant increase in primary NS production after growth in ECCM, further supporting the hypothesis that factors from normal endothelial cells maintain NSC expansion. The increased nestin expression seen by the other groups as well as increased clone size corroborates our findings, which suggest that factors from endothelial cells maintain postnatal SVZ neural progenitors in a stem cell-like state.

We found altered morphology of GFAP+ and Nestin+ cells, as well as the presence of radial glial markers RC2 and ZRF1 after NS co-culture with endothelial cells, expansion in ECCM or differentiation in ECCM. The altered morphology of GFAP+ and nestin+ cells, combined with increased expression of nestin, RC2 and ZRF1 suggests that endothelial cell-secreted factors also maintain postnatal SVZ-derived NSCs in a more undifferentiated or progenitor-like state, consistent with a radial glia-like phenotype. Similarly, Weidenfeller found alterations in astrocyte and progenitor cell morphology after co-culture with endothelial cells, and suggested that these cells also displayed more precursor-like morphology (Weidenfeller, et al., 2007). We evaluated cell proliferation by counting Ki67+ cells to determine if there was an increase in the number of actively proliferating stem cells. We found an increase in the number of Ki67+ cells after 4d of differentiation from NS expanded in normal ECCM compared with controls, suggesting that proliferation is increased. This increase was not due to changes in cell death, as we found no difference in caspase-3 immunoreactivity between groups. Our results concur with those from Teng et al (2008), in which proliferation was increased after co-culture of adult SVZ NSCs with endothelial cells (Teng, et al., 2008). Although Shen did not comment on the morphology of glial cells from endothelial cell/NSC co-culture, they found an increase in BrdU+ cells after co-culture, also suggesting increased proliferation of NSCs (Shen, et al., 2004).

We found no difference in the number of neurons from those co-cultured with endothelial cells compared to control cells; however, we found a significant

difference in the length of β-tubulin+ processes. Neuronal processes were significantly shorter than controls regardless of whether we co-cultured NSCs and endothelial cells during neurosphere expansion, expanded NS in ECCM, or differentiated them in ECCM. The first two conditions suggest a priming effect because NS were not exposed to endothelial cell-secreted factors during differentiation, but exposure prior to differentiation still influenced their subsequent development. Weidenfeller allowed NSCs to differentiate after removing them from co-culture with endothelial cells and also found β-tubulin+ neurons with reduced number and length of processes compared with progenitors cultured alone (Weidenfeller, et al., 2007). When hippocampal NSCs were allowed to differentiate alone for 4d after 7d of co-culture with endothelial cells, neurofilament expression increased, suggesting an increase in the number of neurons produced from co-cultured NSCs (Guo, et al., 2008). Shen found increased neuron production after 4 d of differentiation when embryonic NSCs were cultured as NS with endothelial cells, but did not report any differences in neuronal morphology (Shen, et al., 2004). In a study recently published by Teng et al, (2008) co-culture of normal adult SVZ stem cells with endothelial cells from the ischemic border led to increased neuron production whereas co-culture of normal endothelial cells and adult SVZ NSCs increased proliferation but not neuronal production (Teng, et al., 2008). In contrast, to the above studies, we found no increase in neuron production but altered neuronal morphology. This difference may reflect the different locations from which the NSCs were isolated

(e.g., VZ vs. hippocampus vs. SVZ) or differences in the ages of animals from which the cells were derived.

We noted that MBP+ oligodendrocytes generated after NS/endothelial cell co-culture or during ECCM differentiation were smaller and displayed less branching, suggesting that these oligodendrocytes may be more immature than those from control cultures. The altered oligodendrocyte morphology and increased production after differentiation in diluted ECCM are novel findings, as Shen found no difference in oligodendrocyte production or morphology and Weidenfeller did not detect oligodendrocytes in their cultures (Shen, et al., 2004, Weidenfeller, et al., 2007). The specific conditions required for this effect in our experiments, 50% diluted ECCM, may relate in part to the amount of serum (5%) in the media.

No studies to date have evaluated the influence of injured endothelial cells on postnatal SVZ NSCs. Endothelial cell proliferation increases in the SVZ after stroke and peaks around 5 days after stroke, whereas SVZ expansion is increased by 3 d after stroke and SVZ cell proliferation peaks at 7d after stroke (Gotts and Chesselet, 2005, Gotts and Chesselet, 2005, Zhang, et al., 2001). Numerous studies have shown correlation between stroke-induced angiogenesis and stroke-induced neurogenesis, however the direct effects of injured endothelial cells on NSCs have not been evaluated (Chen, et al., 2004, Gotts and Chesselet, 2005, Ohab, et al., 2006, Sun, et al., 2003, Taguchi, et al., 2004, Thored, et al., 2007). Therefore, we examined the influence of stroke-injured endothelial cells, using OGD as an *in vitro* stroke model, on SVZ NSC

proliferation, self-renewal, migration, and differentiation. We found no significant difference in self-renewal (secondary NS size or number) after neurosphere expansion in OGD-ECCM. In contrast, OGD-ECCM significantly promoted chain migration and increased neuronal process outgrowth compared with normal ECCM or controls. Thus, factors secreted by stroke-injured endothelial cells induce SVZ NSCs to migrate faster and stimulate their differentiation.

Using a stroke model that results in a cortical lesion, a recent study reported clusters of neuroblasts adjacent to newly formed endothelial cells in the peri-infarct region, as well as the presence of migrating neuroblasts alongside blood vessels towards and into the peri-infarct cortex (Ohab, et al., 2006). To provide further support for the interaction of endothelial cells and neuroblasts, the authors blocked post-stroke angiogenesis using endostatin, which resulted in a marked decrease in the number of new endothelial cells and neuroblasts in the peri-infarct cortex. However, these effects may result from either direct cell-cell relationships or secreted factors (Ohab, et al., 2006). After administration of the angiogenic factor angiopoietin-1, significantly more doublecortin+ neuroblasts were found in the peri-infarct cortex (compared with vehicle), again located in close proximity to endothelial cells (Ohab, et al., 2006). These data suggest that endothelial cells near the infarct region may provide cues that attract neuroblasts to migrate to this area. This idea fits well with our in vitro results, where OGD-injured ECCM promoted faster neuroblast chain migration and differentiation.

Our finding that factors from OGD-endothelial cells enhance neuron process outgrowth is novel and provides interesting insight into the function of

normal vs. injured endothelial cells in the neurogenic niche. Under normal circumstances, endothelial cells appear to provide factors that maintain the niche in a more proliferative and undifferentiated state. After injury, however, these factors may be down-regulated, and novel endothelial cell-secreted molecules induce NSCs to migrate out of the SVZ and differentiate more quickly. Our hypothesis is that endothelial cells in the SVZ, which is not damaged in the aforementioned stroke models, are activated to increase proliferation and self-renewal of the NSCs, while endothelial cells in the infarct and peri-infarct regions down-regulate these factors and instead provide cues to attract NSCs to migrate to this region and differentiate quickly to replace the dying cells. Thus, different combinations of endothelial cell-secreted factors likely contribute to maintain NSCs in their normal state and promote their migration and differentiation after injury. If this model is correct, identifying these factors may provide significant therapeutic benefits for a number of diseases and conditions in which neurons are lost. One set of factors could be administered to increase NSC proliferation while another could potentially be used to direct these cells towards an injured brain region and promote their differentiation.

The specific endothelial cell-secreted factors that influence neurogenesis have not yet been identified. Others have tested retinoic acid, forskolin, fetal bovine serum, or LIF+VEGF, and found that none of these factors stimulate *in vitro* neurogenesis in embryonic NSCs (Shen, et al., 2004). Several studies suggest that VEGF, a pro-angiogenic factor expressed by endothelial cells (Palmer, et al., 2000), may be a prime candidate as it promotes normal and

stroke-induced neurogenesis (Meng, et al., 2006, Schanzer, et al., 2004, Sun, et al., 2006, Sun, et al., 2003), and stimulates adult NSC proliferation and neuronal differentiation *in vitro* via upregulation in endothelial cells isolated from the ischemic border (Teng, et al., 2008), and enhances NSC survival (Wada, et al., 2006). Other pro-angiogenic factors such as stem cell factor (Jin, et al., 2002, Sun, et al., 2006), matrix metalloproteinase-2 & 9 (Wang, et al., 2006) and pigment-epithelial-derived factor (Pumiglia and Temple, 2006, Ramirez-Castillejo, et al., 2006) also regulate NSCs in a variety of ways and are potential candidates.

Our findings suggest that endothelial cells are an important component of the neurogenic niche and function to maintain postnatal SVZ neural progenitors in their stem cell-like state in the intact brain, whereas stroke-injured endothelial cells prompt SVZ neural progenitors to migrate and differentiate, potentially serving as an endogenous repair mechanism for neuronal replacement after injury. These data provide important implications for treatment of stroke and other diseases where neuron replacement could be stimulated by administration of endothelial cell-secreted factors. Thus, an important future direction is to identify these factors and determine their precise role(s) in modulating neural progenitor cells so that these factors may be manipulated after stroke or other brain diseases for improved endogenous brain repair.

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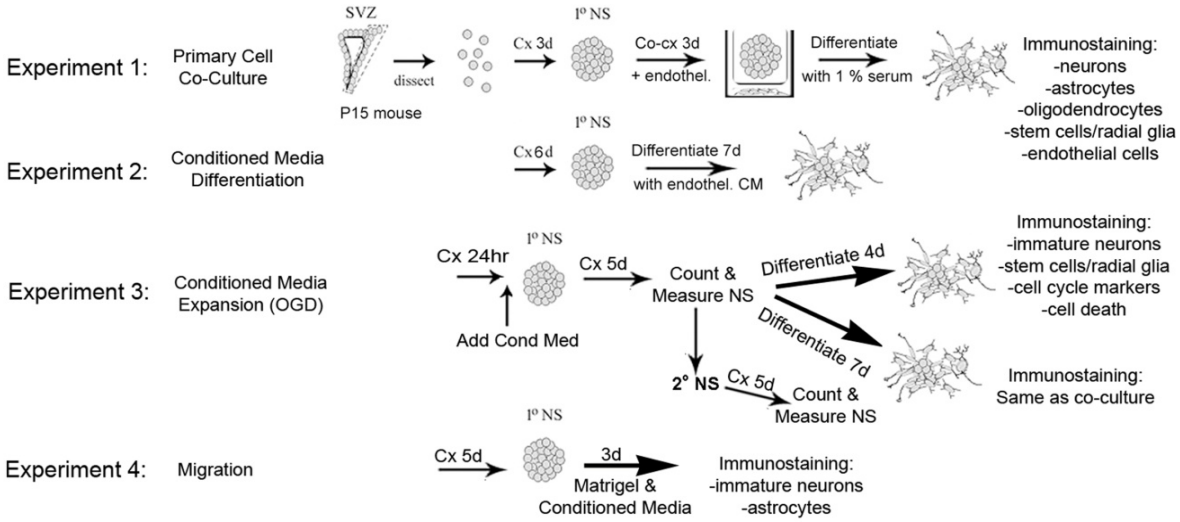


Figure 3.1: Schematic showing neurosphere (NS) culture methods for each experiment.

For all experiments, the subventricular zone (SVZ) was dissected from P15 mice, trypsinized, and plated as single cells in serum-free media containing EGF and bFGF. For experiment one, primary NS were expanded alone for 3d in transwell inserts, and then the inserts were transferred to wells containing primary endothelial cells in the serum-free media described above. NS were co-cultured with endothelial cells in this non-contact system for 3d, and then were removed and differentiated alone for 7d in media containing 1% serum. NS were then fixed and immunostained to identify neurons, astrocytes, oligodendrocytes, stem cells/radial glia and endothelial cells. For experiment two, NS were expanded alone for 6d and then were differentiated in conditioned media (CM) containing 5 or 10% serum for 7d. NS were fixed and stained as in experiment one. In experiment three, primary NS were expanded alone for 24 h, and then CM from normal or oxygen-glucose deprived (OGD) endothelial cells was added. After another 5 days cultured in (serum free) OGD- or normal CM, primary NS were counted and measured, and then 1) passaged as secondary NS; 2) differentiated for 4d (and examined for immature neurons, stem cells/radial glia, cell cycle markers and cell death); or 3) differentiated for 7d and immunostained as in experiments one and two. For experiment four, primary NS were expanded alone for 5d, and then were picked and re-plated in matrigel plus serum-free conditioned media for 3d to assess chain migration. Primary NS were then fixed and immunostained for astrocytes and immature neurons.

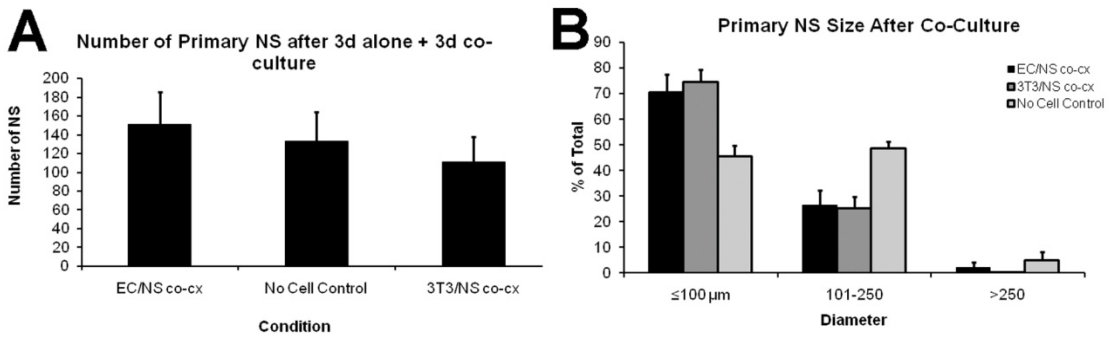


Figure 3.2: Endothelial cell co-culture with primary NS does not alter SVZ NS production.

Primary NS were expanded alone for 3d and then co-cultured with endothelial cells, 3T3 cells or alone for 3d. Primary NS numbers were counted and diameters measured at 6DIV. There was a slight but insignificant increase in primary NS production after co-culture with endothelial cells compared with controls (A). The increase in NS production was mainly due to an increase in the number of small NS produced, as NS co-cultured with endothelial cells or control cells produced a greater percentage of small NS than NS cultured alone (B).

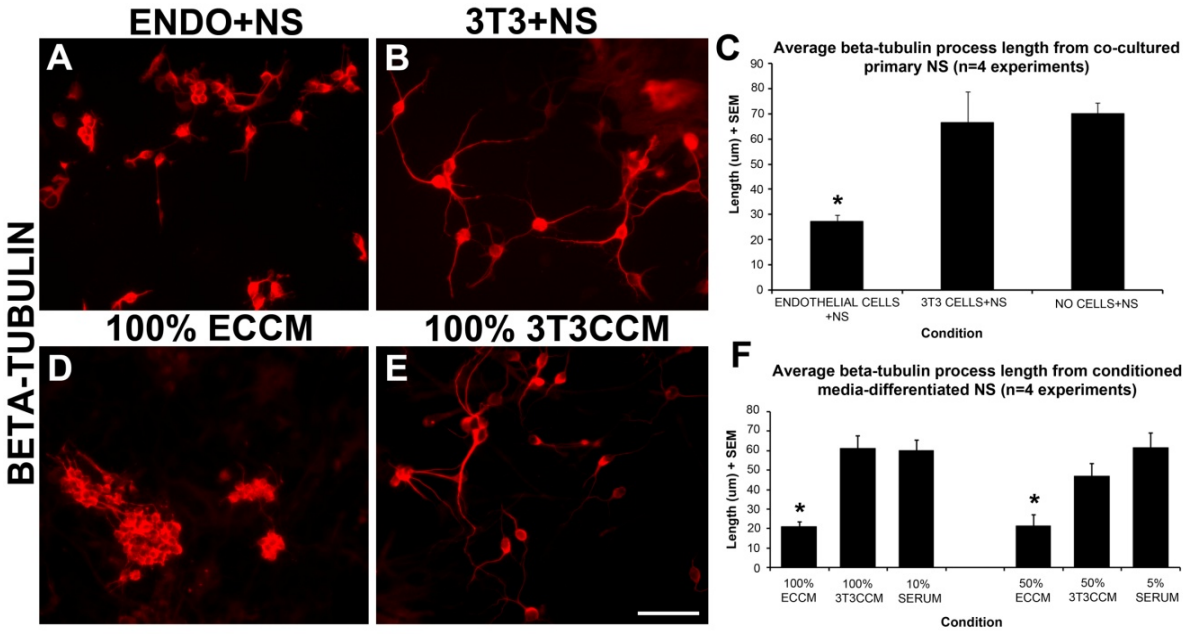


Figure 3.3: Endothelial cell-secreted factors keep neurons in a more immature state.

A-C: Primary NS were expanded in non-contact co-culture with primary endothelial cells, NIH3T3 cells or alone, and then were differentiated alone for 7d. β-tubulin+ neurons from NS expanded with endothelial cells displayed short processes and cells tend to be clustered (A), whereas those expanded with 3T3 cells or alone extended longer processes that tended to contact other neurons (B). Quantification of neuron process length revealed that neurons derived from NS co-cultured with endothelial cells had significantly shorter processes (C, *p<0.01) than either control group. D-F: Similarly, primary NS differentiated in (10% serum-containing) ECCM from primary mouse endothelial cells contained clusters of β-tubulin+ neurons with shorter processes (D) than controls, which display long processes that contacted other cells and were less clustered (E). Measurement of process length revealed that β-tubulin+ neurons from 100% or 1:1 diluted (50%) ECCM were typically one-third of the length of controls (F; *p<0.01) Scale bar: 50 μm.

Figure 3.4: Endothelial cell-secreted factors alter neural progenitor/glial morphology.

A-F: Primary NS expanded with primary endothelial cells and then differentiated for 7d showed nestin+ cells with long radial processes (A), while those expanded with 3T3 cells gave rise to fewer nestin+ cells that had a more typical astrocyte morphology (B), NS expanded alone had minimal or undetectable nestin expression (C). NS differentiated after expansion with endothelial cells showed GFAP+ cells that displayed a more reactive astrocyte-like morphology (D) compared to the more typical flat GFAP+ astrocytes from control co-cultures (E, F). G-L: After differentiation in ECCM (experiment 2), nestin is expressed in long processes (G) whereas its expression level is low and present in cells of typical astrocyte morphology in 3T3CCM-differentiated NS (H) or minimal in serum controls (I). GFAP+ cells from ECCM-differentiated NS have numerous long processes, more typical of radial glia (J) and resemble those that express nestin (G). GFAP+ cells from 3T3CCM-differentiated NS display a variety of morphologies (K), but do not typically contain radial glial-like cells, while those from serum controls have a normal astrocyte morphology (L). M-R: NS differentiated in diluted ECCM (50%) contain nestin+ cells similar to those seen after differentiation in 100% ECCM, with strong nestin-immunoreactivity in long radial processes (M). Nestin+ cells from diluted controls resemble those from 100% CM (Figure 4N-O, Q-R) with more typical astrocyte morphology. GFAP+ cells from diluted ECCM contain numerous long processes (P) similar to those seen in 100% ECCM while diluted 3T3CCM (Q) and serum controls (R) have more typical astrocyte morphology and expression. Scale bars: 100 μm in A (for A-C, M-R), 50 μm in D (for D-L).

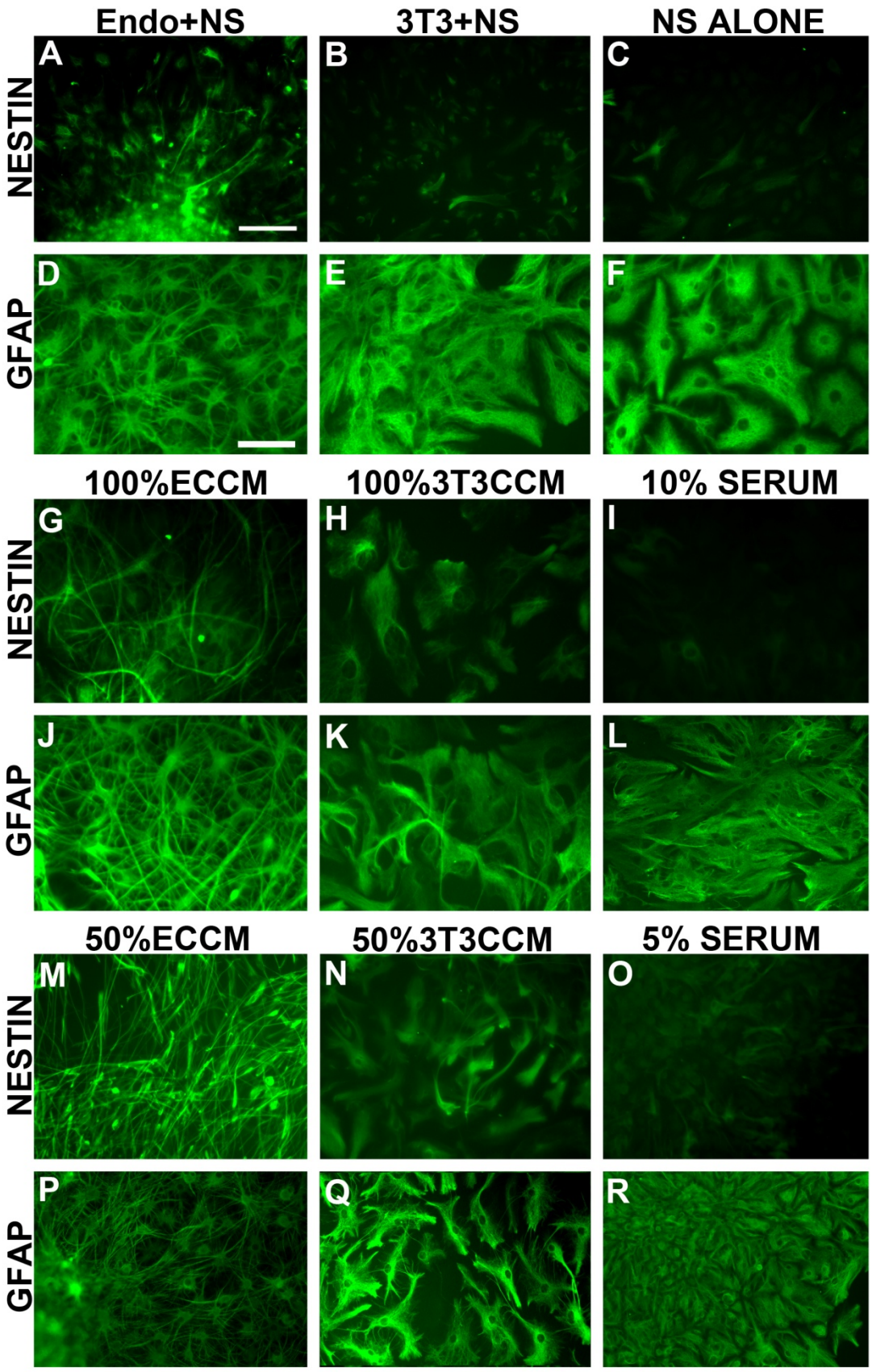

Endo+NS
3T3+NS
NS ALONE
NESTIN
GFAP
A
B
C
D
E
F
100%ECCM
100%3T3CCM
10% SERUM
G
H
I
J
K
L
50%ECCM
50%3T3CCM
5% SERUM
M
N
O
P
Q
R

Figure 3.5: Endothelial cell-secreted factors influence oligodendrocyte formation.

MBP+ oligodendrocytes derived from NS co-cultured with primary endothelial cells are typically small and display little branching (A). Significantly fewer MBP+ oligodendrocytes are formed from NS co-cultured with 3T3 cells (B, D, *$p<0.01$) compared with the no cell control (C). Those MBP+ cells from 3T3/NS co-culture that are present tend to have a larger immunoreactive center than those from other conditions and larger but more simple (one "ring") branching patterns (B). Quantification of MBP+ cells from fields of similar density revealed no difference in oligodendrocyte number between endothelial co-cultured NS and those cultured alone (D). NS also were differentiated in undiluted (100%) or half-diluted (50%) ECCM (E, G) or 3T3 cell CM (F, H), and oligodendrocyte numbers identified by MBP immunostaining were quantified. ECCM-differentiated NS contained mostly small MBP+ oligodendrocytes with little branching (E), similar to those seen in NS from co-culture with endothelial cells (A). MBP+ cells incubated with 3T3CCM displayed a variety of morphologies (F, H) and were typically similar in number to those from NS treated with 100% ECCM (I). NS differentiated in 50% ECCM produced more MBP+ oligodendrocytes (G, I, *$p<0.01$) and appeared smaller (G) than controls (H). Scale bar: 100 μm.

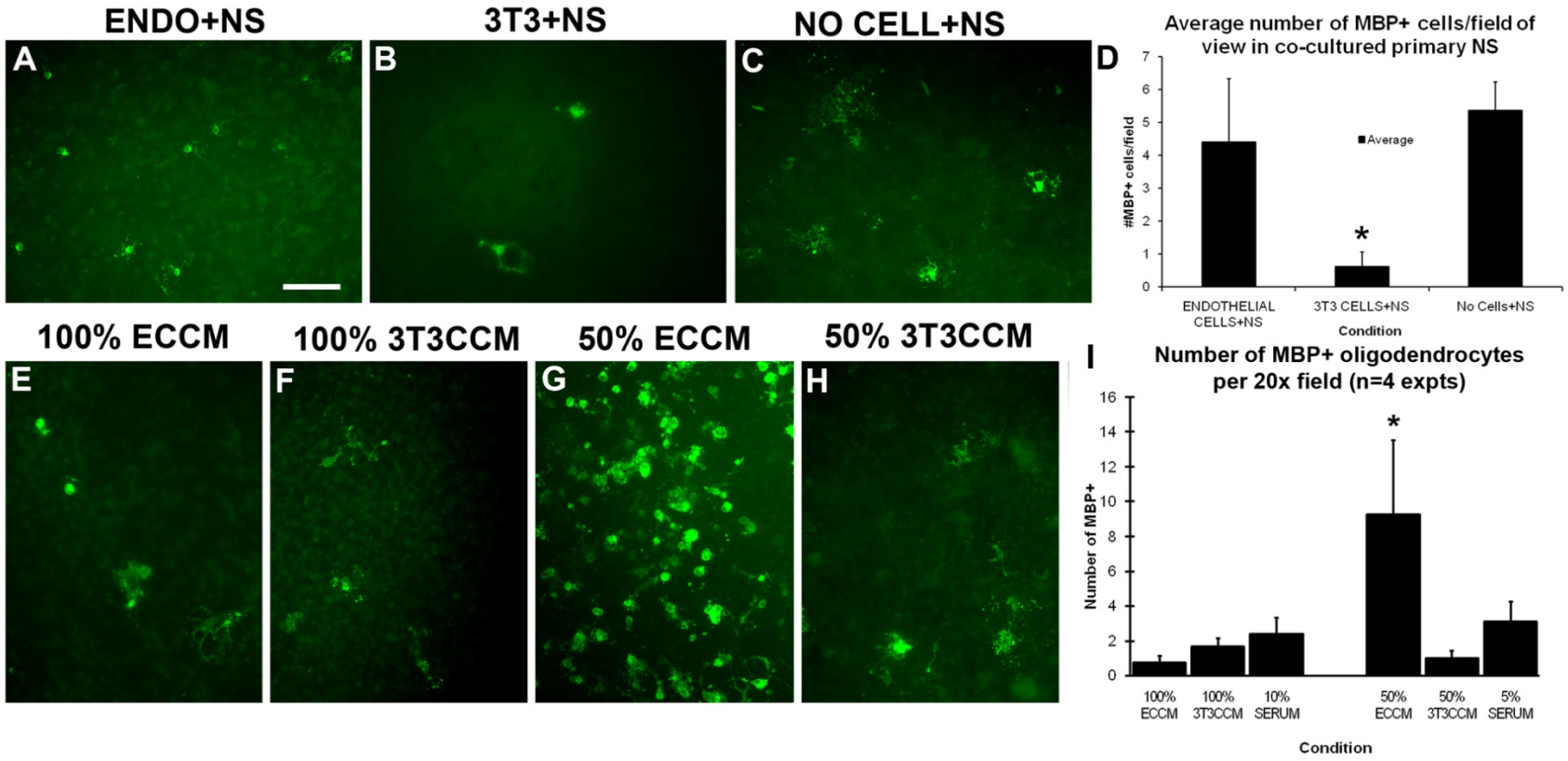
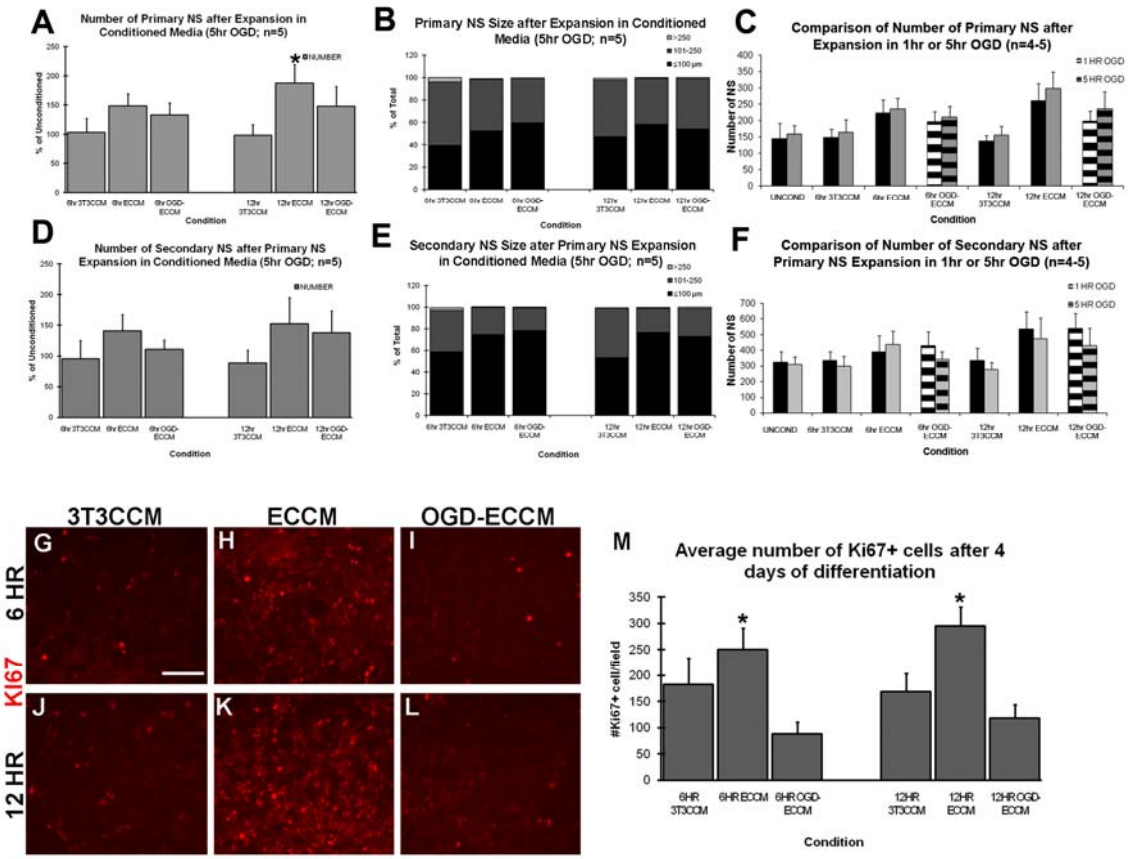

ENDO+NS
3T3+NS
NO CELL+NS
A
B
C
D
Average number of MBP+ cells/field of view in co-cultured primary NS
#MBP+ cells/field
Average
*
ENDOTHELIAL CELLS+NS
3T3 CELLS+NS
No Cells+NS
Condition
100% ECCM
100% 3T3CCM
50% ECCM
50% 3T3CCM
E
F
G
H
I
Number of MBP+ oligodendrocytes per 20x field (n=4 expts)
Number of MBP+
*
100% ECCM
100% 3T3CCM
10% SERUM
50% ECCM
50% 3T3CCM
5% SERUM
Condition

Figure 3.6: Methods for Oxygen-Glucose Deprivation (OGD) of endothelial cells.

The bEnd.3 cell line was used for all OGD experiments. Growth media was removed from bEnd.3 cells, cells were washed briefly and then put into serum-free, glucose-free media. Cells were put into an anoxic chamber and oxygen was removed from the media. Cells underwent 1 or 5hr OGD, then were removed from the chamber and placed into fresh SFM. Cells were maintained at 37°C in 10% CO_2 during the reperfusion time. SFM was collected from intact or OGD-exposed endothelial cells (or intact 3T3 cells) after 6, 12, or 24 hours for use in CM NS expansion experiments.

Figure 3.7: Endothelial cell-secreted factors increase NS production and SVZ NSC proliferation.

A-F: SVZ-derived NS were expanded in CM from intact (ECCM) or OGD-exposed endothelial cells (OGD-ECCM) or control media (3T3CCM or unconditioned). The number and size of NS was measured after 6d of expansion and data graphed as percent of unconditioned control (A-B, D-E; CM/unconditioned X 100%). Expansion in intact 12h ECCM led to significantly more primary NS produced than in other conditions (A; *$p \leq 0.01$). There was no difference in primary NS size after expansion in CM (B). Similar results were obtained for primary NS number in 1hr OGD experiments compared with 5h OGD experiments, and only the intact 12h ECCM condition showed more primary NS (C, $p<0.05$). After NS expansion in CM, cells were dissociated, re-plated in SFM and then expanded to form secondary NS. Secondary NS number was increased, though not significantly ($p=0.08$) in 12hr ECCM compared with controls (D) and there was no difference in secondary NS size (E). Secondary NS production was similar between 1hr and 5hr OGD experiments (F). G-M: Primary NS were expanded in CM for 6d, differentiated in 1% serum for 4d, and then were immunostained for Ki67. NS expanded in 3T3CCM (G, J) or OGD-ECCM (I, L) contained very few Ki67+ cells. NS expanded in intact ECCM contained significantly more Ki67+ cells compared to all other conditions (G-M). Quantification of similar density NS revealed that those exposed to intact ECCM contained 2.5-3 times as many Ki67+ cells as in the OGD-ECCM groups, and approximately twice as many as in controls (M, *$p<0.01$). Scale bar: 100 μm.


A
Number of Primary NS after Expansion in Conditioned Media (5hr OGD; n=5)
% of Unconditioned
NUMBER
Condition
B
Primary NS Size after Expansion in Conditioned Media (5hr OGD; n=5)
% of Total
Condition
C
Comparison of Number of Primary NS after Expansion in 1hr or 5hr OGD (n=4-5)
Number of NS
1 HR OGD
5 HR OGD
Condition
D
Number of Secondary NS after Primary NS Expansion in Conditioned Media (5hr OGD; n=5)
% of Unconditioned
NUMBER
Condition
E
Secondary NS Size ater Primary NS Expansion in Conditioned Media (5hr OGD; n=5)
% of Total
Condition
F
Comparison of Number of Secondary NS after Primary NS Expansion in 1hr or 5hr OGD (n=4-5)
Number of NS
1 HR OGD
5 HR OGD
Condition

3T3CCM
ECCM
OGD-ECCM
6 HR
KI67
12 HR
G
H
I
J
K
L

M
Average number of Ki67+ cells after 4 days of differentiation
#Ki67+ cell/field
6HR 3T3CCM
6HR ECCM
6HR OGD-ECM
12HR 3T3CCM
12HR ECCM
12HR OGD-ECM
Condition

Figure 3.8: Endothelial cell-secreted factors alter NSC maturation.

A-D: After 4d of differentiation, NS expanded in intact ECCM contain MAP2abc+ neurons with very short/no processes (C, arrows) compared to those from controls which contain neurons with a mixture of process lengths (A, B). MAP2abc+ neurons derived from NS expanded in OGD-ECCM display long processes, characteristic of more mature neurons (D, arrows). F-I: Similar effects were seen with β-tubulin staining. After 7d of differentiation, NS expanded in intact ECCM contained β-tubulin+ neurons with very short processes (H, arrows) compared with those from controls which contained neurons with a mixture of short and long processes (F-G). β-tubulin+ neurons derived from NS expanded in OGD-ECCM displayed long processes, characteristic of more mature neurons (I, arrowheads). E, J: Quantification of β-tubulin+ process length as a percent of the unconditioned media control (CM/unconditioned X 100%; dashed line) revealed a significant decrease in neuronal process length after NS expansion in intact ECCM and a significant increase in length after expansion in OGD-ECCM compared to controls (E, *$p \leq 0.01$, +$p \leq 0.05$ vs. controls). No difference was seen in the number of processes/cell (J). Scale bars: 50 μm in A (for A-D), 100 μm in F (for F-I).

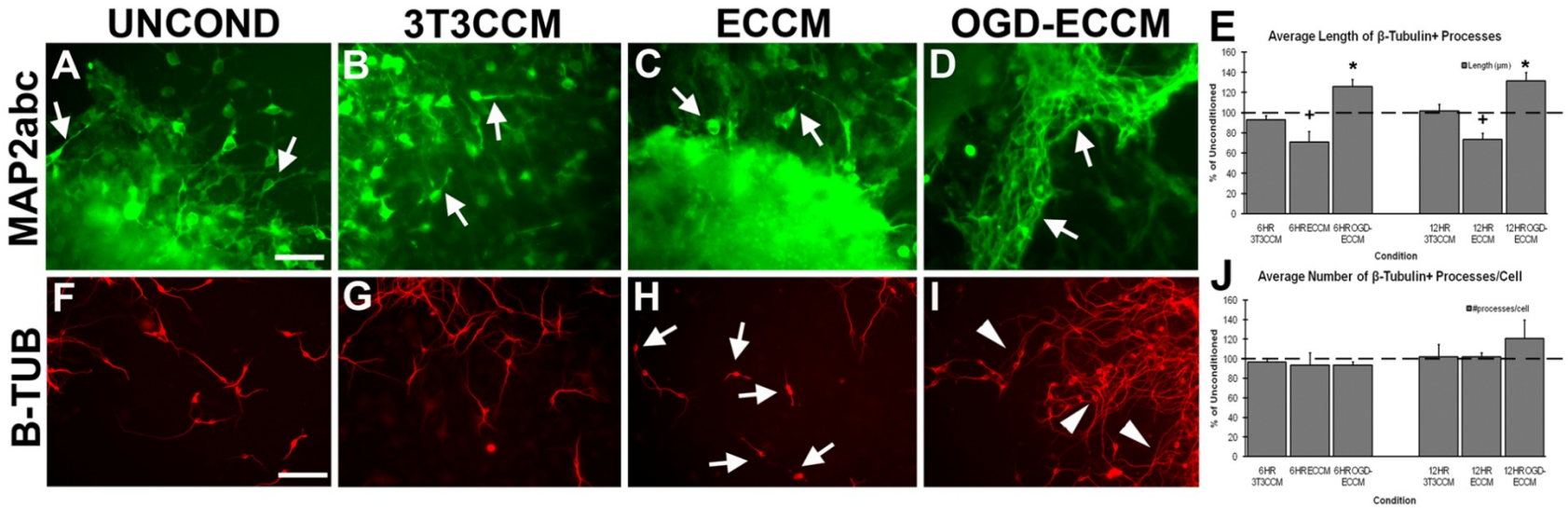

UNCOND
3T3CCM
ECCM
OGD-ECCM
MAP2abc
B-TUB
A
B
C
D
F
G
H
I
E
Average Length of β-Tubulin+ Processes
Length (μm)
% of Unconditioned
6HR 3T3CCM
6HR ECCM
6HR OGD-ECCM
12HR 3T3CCM
12HR ECCM
12HR OGD-ECCM
Condition
J
Average Number of β-Tubulin+ Processes/Cell
#processes/cell

Figure 3.9: Endothelial cell-secreted factors alter glial morphology.

A-D: Nestin expression after 4d of differentiation was low in unconditioned media (UNCOND) controls and present in astrocyte-like cells (A) while controls cultured with 3T3CCM displayed varied morphologies (B). NS expanded in intact ECCM and then differentiated for 4d contained strong nestin expression in radial glial-like cells with long processes (C, arrowheads). NS that were expanded in OGD-ECCM produced some cells that maintained strong nestin-immunoreactivity with several short processes, characteristic of type II astrocytes (D, arrows), while other cells with lower nestin expression resembled radial glia (D, arrowheads). After 7d of differentiation, NS expanded in control media contain mostly large, flat GFAP+ astrocytes (arrows in E, F), while GFAP+ cells from ECCM-expanded NS (G, arrowheads) maintain the radial glial-like morphology displayed in nestin+ cells after 4d of differentiation (C, arrowheads). GFAP+ cells from OGD-ECCM expanded NS display the morphology of type II astrocytes (H, arrows) and radial glial-like cells (H, arrowheads). Scale bars: 50 μm in A (for A-D); 100 μm in E (for E-H).

UNCOND
3T3CCM
ECCM
OGD-ECCM
NESTIN
GFAP
A
B
C
D
E
F
G
H

Figure 3.10: Endothelial cell-secreted factors alter neuroblast chain migration.

Primary NS were expanded for 5d and re-plated in matrigel for chain migration assays. CM was added to NS upon re-plating in matrigel and NS were photographed at 24 h and fixed at 3d after plating in matrigel. NS incubated with intact ECCM collected over 6 or 12hr in serum-free medium (B, F) appeared similar to controls at 24h (A, E). NS plated in OGD-ECCM collected 6 or 12h post-OGD displayed increased migration at 24h (C, G), with numerous chains extending out from the NS. After 3d in matrigel, NS immunostained for MAP2abc+ (green) and GFAP (red) typically contained numerous immature neurons and few astrocytic processes (D). The length of the longest chain was measured from the center of each neurosphere and graphed as a percent of the unconditioned control group (CM/unconditioned X 100%). OGD-ECCM-treated NS contained significantly longer chains compared with all other conditions (H, *$p \leq 0.05$ for 6h OGD-ECCM, $p \leq 0.01$ for 12h OGD-ECCM). Scale bars: 200 μm in A (for A-C, E-G), 25 μm in D.

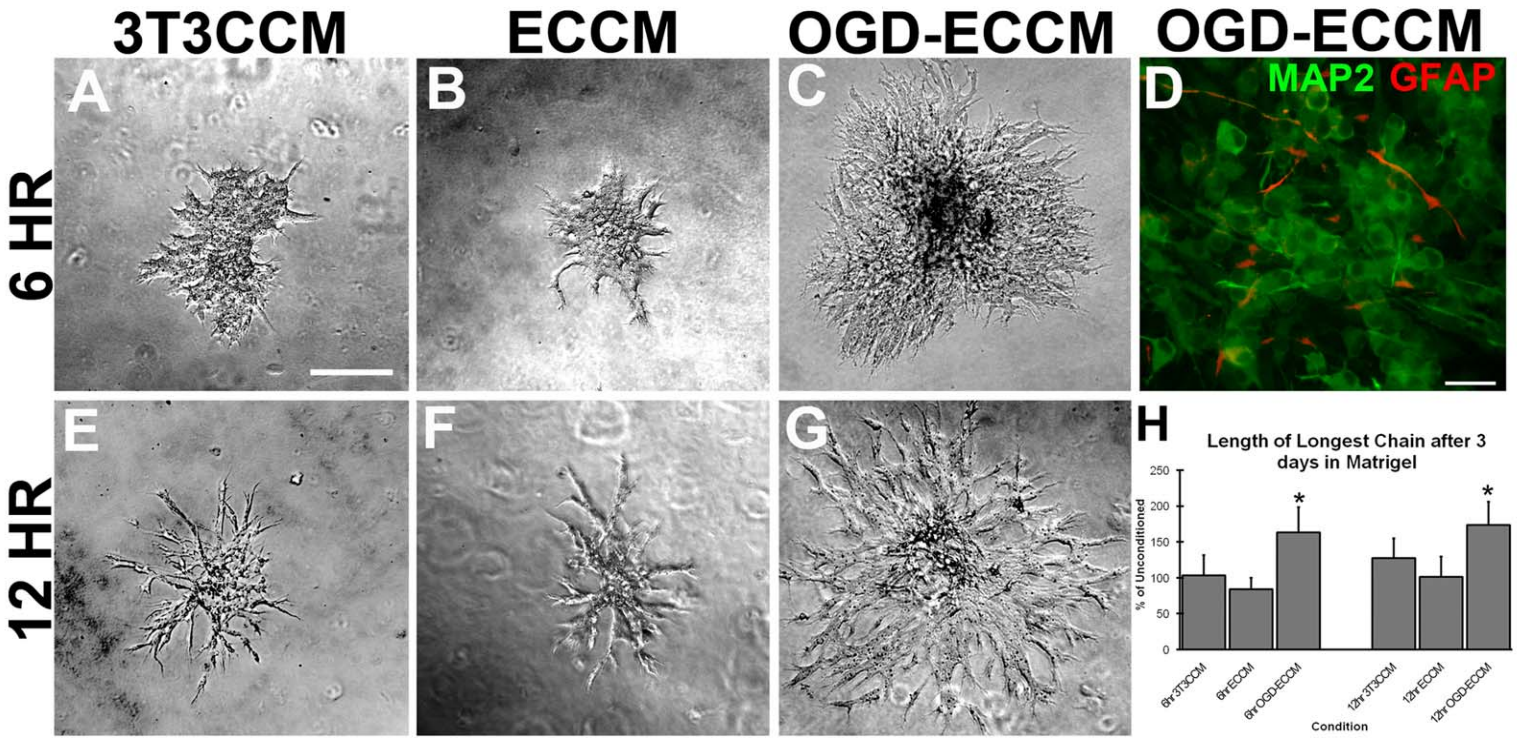

3T3CCM
ECCM
OGD-ECCM
OGD-ECCM
6 HR
12 HR
A
B
C
D
MAP2 GFAP
E
F
G
H
Length of Longest Chain after 3 days in Matrigel
% of Unconditioned
250
200
150
100
50
0
*
*
6hr-3T3CCM
6hr-ECCM
6hr-OGD-ECCM
12hr-3T3CCM
12hr-ECCM
12hr-OGD-ECCM
Condition

Figure 3.11: Effects of different CM fractions on primary NS.

CM was collected from intact 3T3 cells, intact endothelial cells or OGD-exposed endothelial cells and separated into fractions less than or greater than 5kD. These fractions were applied to primary NS 24h post-plating (same as in experiment 3) and NS allowed to expand for 5 more days. Primary NS were counted and then differentiated following the same methods as in experiment 3. The most NS were produced from the >5kD fraction of 12h ECCM (A), similar to the results seen in experiment 3. Both fractions of 3T3CCM contained MAP2abc+ cells with a mix of short, medium and long processes (B, E). MAP2abc+ neurons in the <5kD fraction of intact ECCM also displayed a mix of process lengths (C) while those from the >5kD fraction of ECCM have very short or no processes (F), similar to those from experiment 3. Both fractions of OGD-ECCM contained Map2abc+ neurons with moderate and long processes (D, G). Scale bar: 100 μm.

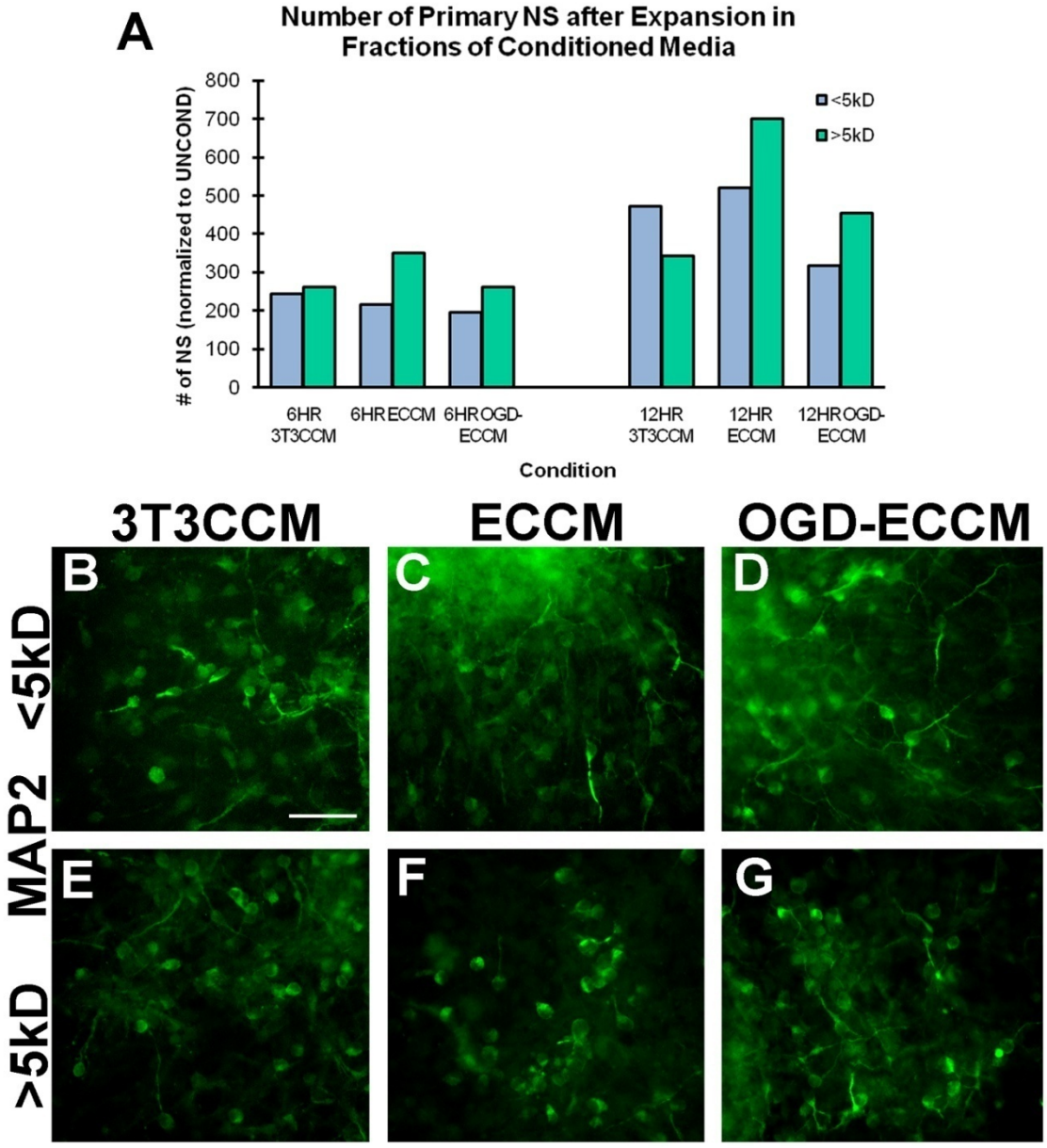

A
Number of Primary NS after Expansion in Fractions of Conditioned Media
# of NS (normalized to UNCOND)
800
700
600
500
400
300
200
100
0
<5kD
>5kD
6HR 3T3CCM
6HR ECCM
6HR OGD-ECCM
12HR 3T3CCM
12HR ECCM
12HR OGD-ECCM
Condition
3T3CCM
ECCM
OGD-ECCM
MAP2
<5kD
>5kD
B
C
D
E
F
G

Table 3.1: Summary of the effects of endothelial cells on SVZ neural stem cells. *Oligodendrocytes (oligos) increased dramatically in number only in 50% diluted ECCM. Other glial effects (+) involved alteration of Nestin- and GFAP-expressing cells toward a radial glia-like morphology.

Condition	Timing	Self-Renewal	Migration	Process Outgrowth	Glial Effects
EC +NS (primary ECs)	co-culture	↑	----------	↓↓↓	+ +
ECCM (primary ECs)	differentiation	----------	----------	↓↓↓	(*↑↑ oligos) + +
ECCM (bEND3)	during expansion	↑↑↑	NO CHANGE	↓↓↓	+ +
OGD-ECCM (bEND3)	during expansion	NO CHANGE	↑↑↑	↑↑↑	+/-

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CHAPTER IV:

RETINOIC ACID AND ENVIRONMENTAL ENRICHMENT ALTER SUBVENTRICULAR ZONE AND STRIATAL NEUROGENESIS AFTER STROKE

Abstract

Neurogenesis increases in the adult rodent forebrain subventricular zone (SVZ) after experimental stroke. Newborn neurons migrate to the injured striatum, but few survive long-term and little evidence exists to suggest that they integrate or contribute to functional recovery. One potential strategy to improve stroke recovery is to stimulate neurogenesis and integration of adult-born neurons by using treatments that enhance neurogenesis. We examined the influence of retinoic acid (RA), which stimulates neonatal SVZ and adult hippocampal neurogenesis, and environmental enrichment (EE), which enhances survival of adult-born hippocampal neurons. We hypothesized that the combination of RA and EE would promote survival of adult-generated SVZ-derived neurons and improve functional recovery after stroke. Adult rats underwent middle cerebral artery occlusion, received BrdU on days 5-11 after stroke and were treated with RA/EE, RA alone, EE/vehicle or vehicle alone and were killed 61 days after stroke. Rats underwent repeated MRI and behavioral testing. We found that

RA/EE treatment preserved striatal and hemisphere tissue and increased SVZ neurogenesis as demonstrated by Ki67 and doublecortin (DCx) immunolabeling. All treatments influenced the location of BrdU- and DCx-positive cells in the post-stroke striatum. RA/EE increased the number of BrdU/NeuN-positive cells in the injured striatum but did not lead to improvements in behavioral function. These results demonstrate that combined pharmacotherapy and behavioral manipulation enhances post-stroke striatal neurogenesis and decreases infarct volume without promoting detectable functional recovery. Further study of the integration of adult-born neurons in the ischemic striatum is necessary to determine their restorative potential.

Introduction

The capacity of the adult mammalian brain to generate new neurons has been well established over recent years. Neural stem cells from the adult forebrain subventricular zone (SVZ) give rise to olfactory bulb (OB) interneurons while those in the hippocampal dentate gyrus generate new neurons in the granule cell layer (Altman, 1969, Cameron and Gould, 1994, Corotto, et al., 1993, Kaplan and Hinds, 1977, Kuhn, et al., 1996, Lois and Alvarez-Buylla, 1994). Brain insults such as seizure, stroke, and traumatic brain injury activate these neural stem cells, causing them to proliferate more rapidly, migrate away from these areas into injured regions, and form new neurons and glia (Arvidsson, et al., 2002, Goings, et al., 2004, Parent, et al., 2002, Parent, et al., 2002, Zhang, et al., 2001). Stroke increases the production of doublecortin (DCx)- and

polysialylated neural cell adhesion molecule (PSA-NCAM)-positive neuroblasts in the SVZ and striatum; however, few cells survive long-term to become functional striatal neurons (Arvidsson, et al., 2002, Ohab, et al., 2006, Parent, et al., 2002). This latter finding suggests that interventions are necessary to enhance the long-term survival of new neurons after stroke. Moreover, structural and behavioral examination is critical to determine whether the newborn neurons integrate appropriately and contribute to functional recovery after focal ischemic insults.

Many factors are involved in regulating neurogenesis during development. The plasticity exhibited by the brain after injury suggests that these factors also may be good candidates for enhancing post-stroke neurogenesis, and one developmental molecule of particular interest in this regard is retinoic acid (RA). During development, RA plays an essential role in anteroposterior patterning of the hindbrain and spinal cord (Liu, et al., 2001, Maden, 2002, Melton, et al., 2004), dorsoventral patterning of spinal cord neurons (Diez del Corral, et al., 2003, Novitch, et al., 2003, Wilson and Maden, 2005) and striatal neuron differentiation (Toresson, et al., 1999, Valdenaire, et al., 1998). RA expression and signaling continues in the postnatal and adult brain. Retinoid binding proteins are expressed in the SVZ-olfactory bulb pathway (Zetterstrom, et al., 1999, Zetterstrom, et al., 1994), RA receptors persist in the olfactory bulb (Krezel, et al., 1999), and the RA synthesizing enzyme RALDH3 is present in the olfactory bulb, rostral migratory stream (RMS), and SVZ (Wagner, et al., 2002). We and others have found that retinoid signaling is a key regulator of postnatal and adult SVZ-olfactory bulb neurogenesis, and that exogenous retinoid

administration stimulates this process (Calza, et al., 2003, Haskell and LaMantia, 2005, Wang, et al., 2005). RA signaling also appears necessary for adult hippocampal neurogenesis as depletion of RA in adult mice decreases dentate granule cell differentiation (Jacobs, et al., 2006). These studies led us to investigate whether RA enhances post-stroke striatal neurogenesis.

Environmental enrichment is a commonly used behavioral intervention for rodents and primates after brain injury. Many studies demonstrate the positive effects of environmental enrichment (EE) on functional recovery after injury, although the timing of EE or related interventions after injury is one factor that determines whether it provides beneficial or deleterious effects on recovery or secondary neural degeneration (Bland, et al., 2001, Kleim, et al., 2003, Schallert, et al., 2000, Tillerson, et al., 2001). Other work also shows positive effects on dentate gyrus neurogenesis (Kempermann, et al., 1998, Kempermann, et al., 1997, Komitova, et al., 2002, Nilsson, et al., 1999, Wurm, et al., 2007), in that EE enhances neuron survival; however, few studies have examined the effects of EE on striatal neurogenesis.

Given that RA stimulates forebrain SVZ neurogenesis and EE enhances the survival of some adult-born neurons and improves functional recovery, we hypothesized that combining oral RA-treatment with EE would enhance the generation and long-term survival of SVZ-derived striatal neurons as well as increase functional recovery after stroke. We treated adult rats with RA/EE, RA alone, EE/vehicle or vehicle alone after experiencing a focal ischemic insult, and examined the effects of treatment on SVZ and striatal neurogenesis, stroke

volume and sensorimotor function. We found that RA/EE treatment preserved striatal and hemisphere tissue, increased SVZ neurogenesis, influenced the location of newborn cells in the post-stroke striatum, and increased the number of new striatal neurons but did not improve behavioral recovery detectably.

Methods

Transient Middle Cerebral Artery Occlusion

Adult (210-250 gram) male Sprague-Dawley rats (Charles River**)** were used for all experiments and all procedures were approved by the University of Michigan Committee on Use and Care of Animals. Animals were group housed on a 12-hour reverse light-dark cycle and provided with food and water ad libitum. Rats were anesthetized with a ketamine/xylazine mixture (100mg/kg, intraperitoneal). Body temperature was maintained using a 37°C re-circulating water pad. Transient middle cerebral artery occlusion (tMCAO) was produced for 90 minutes using the external carotid artery insertion method as described previously (Longa, et al., 1989). Briefly, the left common carotid artery was exposed through a midline incision, and the internal and external carotid arteries separated. A 3.0 nylon monofilament with tip rounded by heat was placed in the external carotid artery and advanced through the internal carotid artery until resistance was felt. The monofilament was left in place for 90 minutes and then removed under anesthesia. Animals were observed until they recovered from anesthesia. This protocol produced infarcts involving the striatum and cortex with a mortality rate of approximately 30%.

BrdU labeling

The thymidine analog, bromodeoxyuridine (BrdU, Roche, Indianapolis, IN) was used to label cells in S-phase (Miller and Nowakowski, 1988). Rats received twice daily intraperitoneal (i.p.) injections of BrdU (50mg/kg in sterile PBS, 6 hours apart) on days 5-11 after tMCAO. This timing was chosen based on previous studies showing that SVZ proliferation peaks approximately one week after stroke (Parent, et al., 2002, Zhang, et al., 2001) and to limit labeling of the earlier post-injury glial response.

MRI

All animals received T2-weighted MRIs on days 7 and 59 post-tMCAO. T2-weighted MRIs were performed using a 7.0 tesla, 18.3 cm horizontal bore magnet as previously described (Chenevert, et al., 1997, Kim, et al., 1995, Moffat, et al., 2006). Serial coronal slices were acquired at 1.5 mm intervals through the rostro-caudal extent of the brain, resulting in 13 slices/animal. Animals were randomly assigned to treatment groups using day 7 MRI. To keep infarct size consistent between groups, we excluded animals with greater than 88% or less than 65% residual hemispheric volume at the level of the striatum on day 7 (ipsilateral/contralateral hemisphere volume x 100).

Treatment

On day 7 post tMCAO, animals were randomly assigned to one of four treatment groups: combined RA-enriched diet and environmental enrichment (RA/EE), RA-enriched diet and standard environment (RA/SE), normal diet and environmental enrichment (NML/EE) or control with normal diet and SE (NML/SE). Animals in the RA groups received the RA-enriched diet on days 7-41 post tMCAO to prolong proliferation and enhance survival of newly generated neuroblasts. Animals in the EE groups were placed into monkey cages containing an assortment of tunnels, ropes and wire mesh for climbing, chew toys, hidden snacks, and a number of other rodent toys during their dark cycle on days 8-27 post tMCAO to promote functional recovery and enhance survival of newly generated neurons. Animals were rotated through 4 different monkey cages, each with a unique set-up. Unlike previous studies, EE rats were not housed continuously in EE cages, but instead were introduced to the EE cages on a gradual basis (1 hr/day for 2 days, 2 hr/day for 2 days, 3 hr/day for remainder) and housed there for a maximum of 3 hours/day. This protocol was chosen to more closely mimic the intermittent nature of human rehabilitative therapy.

Behavioral Testing

Animals underwent a set of sensorimotor tests on day 6 post-tMCAO to determine initial deficits, and the battery was repeated on days 28 and 60 post-tMCAO to measure recovery over time. All behavioral testing was performed during the dark cycle. Hind-limb deficits were measured on the tapered, ledged

beam test as previously described (Zhao, et al., 2005). The number of ipsilateral and contralateral hindlimb "foot faults" was counted on 4 video-recorded trials/animal/time point. Forelimb asymmetry was assessed using the cylinder test as well as vibrissae-evoked placing (Schallert, et al., 2000, Schallert, et al., 2002). For the cylinder test, animals were placed into a plexiglass cylinder and all trials were video-recorded via an angled mirror for blinded analysis. The number of ipsilateral, contralateral, and bilateral forepaw wall contacts for weight support or shifting was counted and the percent use of the affected limb calculated using the formula: [(ipsilateral/total)-(contralateral/total)] x 100% (Woodlee, et al., 2005). Animals underwent 10 trials/side/time point of vibrissae-evoked placing and the number of placings was recorded.

Histopathology

On day 61 post-tMCAO, animals were deeply anesthetized with sodium pentobarbital and perfusion-fixed with saline followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA overnight, cryoprotected in 30% sucrose, then frozen in powdered dry ice. Coronal serial sections were cut at 40 μm on a cryostat, collected into tris, then transferred into cryoprotectant and stored at -20°C. To confirm lesion severity from MRI analyses, 4 striatal level sections/animal were mounted onto slides, Nissl-stained using cresyl violet, and bilateral hemisphere, striatal and SVZ areas measured using Image J (see below).

Immunohistochemistry

Diaminobenzidine immunohistochemistry was performed with antibodies to doublecortin (DCx) and BrdU using previously published protocols [See Appendix; (Parent, et al., 2002, Plane, et al., 2004)]. For BrdU staining, sections were rinsed in Tris, denatured in 2N HCl at 37ºC for 30 min, neutralized in 0.1M boric acid for 10 min, rinsed, blocked with 10% normal horse serum (Invitrogen, Carlsbad, CA), and incubated overnight at 4ºC with anti-BrdU antibody (1:1000 dilution; mouse monoclonal, Roche Applied Science, Indianapolis, IN). Sections were rinsed, incubated in secondary antibody (1:200 biotinylated horse anti-mouse IgG, Vector Labs) for 1 hour, rinsed, signal amplified with Vector ABC kit, developed with stable diaminobenzidine (Invitrogen), mounted onto slides, dehydrated and cleared in ethanol and xylenes, and coverslipped using Permount (Sigma). DCx immunostaining followed a similar protocol except that sections were put into 1% H_2O_2 for 30 min instead of 2N HCl and boric acid. Sections were blocked with normal horse or goat serum and incubated either in goat anti-DCx (1:2000, overnight, Santa Cruz) or rabbit anti-DCx antibody (1:1000 for 2 nights, (Parent, et al., 2002); biotinylated horse anti-goat or goat anti-rabbit IgG secondary antibodies were used. For Ki67 immunofluorescence (See Appendix), sections were washed with Tris-Buffered Saline (TBS), blocked in goat serum, then incubated in rabbit anti-Ki67 (1:1000, Vector Labs) overnight at 4°C. Sections were washed in TBS, incubated in goat-anti-rabbit Alexa 488 (1:800, Invitrogen) at room temp for 1.5 hours. Sections were washed, mounted onto slides and allowed to dry, then coverslipped with an anti-fade medium (Pro-

Long, Invitrogen). Double-label immunofluorescence staining was performed as previously described to identify the phenotype of newly formed cells (Parent, et al., 2002, Plane, et al., 2004). Sections were stained with antibodies to (See Appendix) BrdU (1:250 rat, Accurate Chemical) and NeuN (1:1000, mouse monoclonal, Chemicon), Map2ab (1:400, mouse monoclonal, Sigma), GFAP (1:500, mouse monoclonal, Sigma), Rip (1:200, mouse monoclonal, Chemicon), ED-1 (1:200, mouse-anti-rat, Serotec) or Glut-1 (1:400, rabbit polyclonal, Chemicon).

Quantitative Analyses

Intact hemispheric and striatal areas were measured on MRIs at striatal levels (3 images/animal/timepoint) and stroke volume calculated (Area X Slice Thickness X #Slices). Area measurements were also obtained from Nissl stained striatal sections (n=4/animal) to confirm MRI findings. Unbiased stereology was performed on every 12th section (n=5 sections) to count striatal BrdU+ cells. Striatal areas were outlined at low magnification and BrdU-positive cells were counted at 100X using the optical fractionator technique (StereoInvestigator; (Plane, et al., 2004, West, et al., 1996). Peri-infarct BrdU labeling was quantified at the level of the anterior striatum in 3 sections/animal. Images of the dorsal and ventral striatum adjacent to the infarct, nearest the level of the dorsal and ventral lateral ventricle, respectively, were captured without knowledge of experimental group using a SPOT-RT digital camera at 40X magnification and all BrdU-positive cells within these fixed areas were counted. DCx immunoreactivity

was quantified in the SVZ in 4 equidistant anterior striatal sections/animal. DCx-positive SVZ area and density measurements were obtained using Image J from 10X images of the dorsolateral SVZ. Ki67-positive cells were counted in the lateral SVZ of 3 equidistant striatal sections/animal in 20X images of the lateral ventricle. Immunofluorescence double labeling and confocal microscopy (Zeiss LSM 510) were used to assess co-localization of immunofluorescence for double labeling and to quantify BrdU+ or BrdU/NeuN+ cells (3-6 63X fields/animal). All analyses were performed in a blinded manner.

Statistics

Analysis of variance (ANOVA) with post-hoc t-tests were used to compare differences between treatments for all quantitative analyses. Results were presented as means ± standard error of the mean (SEM) and considered significant when $p \leq 0.05$.

Results

RA treatment preserves hemisphere volume after stroke

RA is a key regulator of neurogenesis in the postnatal and adult SVZ-OB pathway (Haskell and LaMantia, 2005, Wang, et al., 2005). Because RA stimulates SVZ neurogenesis and EE increases the survival of adult-born neurons in the dentate gyrus, we sought to determine if RA-treatment combined with EE would promote adult SVZ neurogenesis and functional recovery after stroke. To evaluate lesion severity, assign animals to treatment groups, and

assess long-term effects of treatment on stroke volume, rats received serial MRIs on days 7 and 59 after tMCAO. Animals were randomly assigned to treatment groups or excluded from the study based on day 7 MRI results. All animals had similar lesion sizes on day 7, prior to treatment (Figure 4.1A-D, E). They underwent repeat MRI on day 59 post-tMCAO to compare treatment effects on stroke volume. Animals that received an RA-enriched diet had more residual hemisphere tissue than those on a standard diet (Figure 4.1A'-D', E, $p<0.05$ RA/EE vs. NML/EE, RA/SE vs. NML/EE or NML/SE). RA-treated animals also had less decline in hemispheric volume over time [(d59-d7)/d7] compared with controls (Figure 4.1F, $p<0.05$ RA/EE vs. NML/EE, RA/SE vs. NML/EE or NML/SE). Results were similar when the entire rostral-caudal extent of the brain was analyzed on MRI (Figure 4.2A, $p<0.05$). Analysis of cresyl violet stained sections (n=4/animal) revealed a similar trend, as smaller differences between ipsilateral and contralateral residual hemisphere were observed 61 days after MCAO in animals fed the RA-enriched diet (Figure 4.2B). Animals that received both RA and EE also lost significantly less striatal volume over time compared with untreated animals (Figure 4.1G, $p=0.03$). Finally, the ipsilateral SVZ of animals treated with RA-alone or RA/EE was substantially larger than that of vehicle-treated animals (Figure 4.2C). These differences could potentially be confounded by differences in nutrition or food consumption due to the RA-enriched diet. However, animals were weighed periodically and there was no difference in end weight between animals on normal diet vs. RA-enriched diet (Figure 4.2D, $p=0.3$). Thus, retinoic acid decreases post-stroke tissue loss

assessed by MRI in an area of the rodent brain, the striatum, where stroke-induced neurogenesis is known to occur.

RA treatment increases SVZ neurogenesis and alters neuroblast migration 61 days after stroke

To determine whether our interventions stimulated SVZ neurogenesis as a potential explanation for the preservation of tissue after stroke, we first used Ki67, an endogenous proliferation marker, to assess SVZ cell proliferation 61 days after stroke. As expected, Ki67-positive cells were present in the dorsolateral tail of the SVZ and along the lateral wall of the lateral ventricle 61 days after stroke in the control group; however, rats that received both RA and EE showed a strong trend toward greater numbers of Ki67-positive cells in the SVZ (Figure 4.3A-D, Figure 4.4A-B; 78.8 ± 8 in RA/EE vs. 53 ± 13 cells in NML/SE), although the differences were not statistically significant. We next examined whether the increased cell proliferation was associated with an increase in SVZ neurogenesis. Using DCx immunohistochemistry to identify SVZ neuroblasts, we found that the RA/EE-treated group displayed increased SVZ DCx expression in an expanded SVZ (Figure 4.3E-H, Figure 4.4C). The enlarged DCx-immunoreactive SVZ area led us to question whether the increase was simply a result of the cells being more spread out. We therefore measured the density of DCx immunostaining in the SVZ and found a significant increase in the density of DCx-positive cells in the SVZ of RA/EE-treated rats compared with controls (Figure 4.4D, $p<0.05$). Together, these findings indicate that combined

RA and EE treatment increases SVZ neurogenesis in the chronic phase after stroke.

Previous studies showed that SVZ neuroblasts migrate to the injured striatum within the first few weeks after stroke (Arvidsson, et al., 2002, Parent, et al., 2002) and continue to do so chronically (Thored, et al., 2006). To examine whether the prolonged increase in SVZ neurogenesis after RA/EE treatment corresponded with increased striatal neurogenesis after stroke, we injected adult rats with BrdU on days 5-11 after tMCAO and assessed BrdU labeling on day 61 after tMCAO. Unbiased stereology was performed on BrdU-immunostained sections to determine the number of cells generated after stroke that persisted in the ipsilateral striatum 61 days after tMCAO. There was no difference in the total number of striatal BrdU-positive cells between groups (Table 1); however, more BrdU-labeled cells appeared close to the infarct border in the three treatment groups (Figure 4.5A-E). This observation led us to quantify BrdU-positive cell numbers in the peri-infarct area of the striatum. Images of the dorsal and ventral peri-infarct striatal areas were captured and BrdU-immunoreactive cells counted for each area (Figure 4.4A). We found, on average, more BrdU-positive cells in the peri-infarct regions in all 3 treatment groups (Figure 4.5B-E; Table 4.1: 1272±350 cells for RA/EE, 1146±239 for RA/SE, 1276±253 for NML/EE) compared to controls (999±101 cells); however, the differences were not statistically significant due to the large variability between animals (Table 4.1).

We next examined the location of migrating neuroblasts at 2 months after stroke. DCx immunoreactivity was assessed in the peri-infarct region at the level

of the dorsal striatum (Figure 4.5F). We found that, compared to controls, more DCx-positive cells appeared in the injured striatum of all three treatment groups (Figure 4.5G-J). DCx-positive neuroblasts were dispersed throughout the injured striatum, but more neuroblasts were found closer to the infarct in the three treatment groups, particularly in the RA/EE group versus controls (Figure 4.5G-J). Together, these results suggest that RA and EE treatment promote migration of new cells to the peri-infarct striatum.

Combined treatment with RA and EE increases striatal neurogenesis

To determine whether RA and EE treatment yielded more surviving striatal neurons generated after stroke, double-labeling was performed for BrdU and NeuN or Map2 and confocal thin optical z-slices collected from several locations in the ipsilateral striatum. Animals received i.p. injections of BrdU on days 5-11 after MCAO to label proliferating cells and were killed on day 61 post-tMCAO to assess the long-term survival of labeled cells. Rats that received both RA and EE showed a 35% increase in the proportion of BrdU-labeled cells that co-expressed NeuN in the striatum (21±5.7%) 61 days after tMCAO (Table 4.1, Figure 4.6A-D) compared with untreated animals (15.5±3.3% of BrdU+ cells were BrdU/NeuN double-labeled; Table 4.1, Figure 4.6E-H). RA- or EE-alone did not increase the proportion of BrdU/NeuN double-labeled cells in the injured striatum (15.7±2.5% and 15.6±2.9%, respectively).

Enriched environment may transiently improve functional recovery

Behavioral testing was performed on day 6 after tMCAO to determine baseline deficits and was repeated on days 28 and 60 to assess functional improvements over time. All groups had similar and severe behavioral deficits on day 6 after injury on all 3 sensorimotor tests and showed some improvement in the cylinder and beam tests by day 28 (Figure 4.7A-B) but not in the vibrissae-evoked placing test (Figure 4.7C). The EE-alone group appeared to show the most improvement, however, and this trended toward significance on the cylinder test (Figure 4.7A, repeated measures ANOVA, $p=0.06$). The enrichment was stopped on day 27 and the probable beneficial effect was no longer apparent by day 60 (Figure 4.7A). This transient improvement and subsequent loss is likely due to the halting of EE-treatment on day 27 but suggests that short durations of EE transiently promote functional recovery. Of note, when higher doses of RA were given to animals in pilot studies they often caused weakness of hindlimbs and, to a lesser extent, forelimbs, suggesting the possibility of adverse RA effects that may have obscured the detection of functional benefit.

Discussion

Retinoic acid influences patterning and neuronal differentiation in the developing brain (Maden, 2007) and is an essential component of early neuronal differentiation and cell survival in the adult mouse dentate gyrus (Jacobs, et al., 2006). Few studies have examined the effects of retinoic acid in stroke-induced neurogenesis and no studies have evaluated the combined effects of a pro-neurogenic factor and environmental enrichment on stroke-induced neurogenesis

and functional recovery. The goal of this study was to determine whether the combined treatments improve both functional recovery and promote neurogenesis in the striatum after tMCAO. We found that RA/EE treatment decreased the loss of brain tissue months after stroke, increased neuroblast production in the SVZ and striatum, and likely influenced neuroblast migration to injury. This treatment, however, did not lead to significant improvements in functional recovery.

Very few studies have examined changes in stroke size after tMCAO within animals. Using MRI, we were able to compare initial stroke volume, prior to treatment, with stroke volume after treatment in the same animal to more accurately determine beneficial or deleterious effects of treatment. This led us to the novel finding that RA-treatment preserves brain tissue. Only one previous study found that pre-treatment with 9-cis RA, but not all-trans RA, decreased infarct size after MCAO (Harvey, et al., 2004); unlike our study, however, this work only examined the acute phase of cerebral ischemia and not regeneration. In our study the brain tissue preservation that resulted from RA-treatment was very unlikely to be a neuroprotective effect given that the treatment was not started until 8 days after stroke, although we cannot exclude an influence on very delayed cell death.

A number of potential explanations exist for the lack of a functional correlate to the tissue preservation. We found that total brain volume was preserved, as was hemisphere volume at the level of the striatum. Striatal volume was also preserved, although to a lesser extent than hemisphere volume,

leading to the possibility that RA-treatment preserved tissue in brain regions not involved in the sensorimotor tasks used to measure functional recovery. Many animals had already sustained 25-30% damage by day 7 after tMCAO so perhaps the strokes were too large and functional deficits too severe to see any modest effects of RA on functional recovery. Timing and dosage of RA is also important, as too much retinoic acid reduces cell proliferation in the mouse SVZ and dentate gyrus, reduces hippocampal neurogenesis (Crandall, et al., 2004) and causes changes in bone density (Rohde and DeLuca, 2003). In pilot studies, we observed a number of uninjured adult rats with impaired hindlimb use when given higher doses of RA, which recovered after removal of the RA-enriched diet. These observations led us to cut the dosage in half for the current study, although 3 rats (out of 18) displayed temporary impaired ipsilesional limb use with the current dose.

Another possibility is that the adult-born neurons did not effectively integrate after stroke. Such integration is likely necessary to restore function and has not yet been unequivocally demonstrated for stroke-induced striatal neurogenesis. However, following small focal cortical infarcts a combination of EGF and EPO delivered intraventricularly one week after injury enhanced SVZ cell proliferation neuronal migration, cortical tissue generation, and functional recovery (Kolb et al., 2007). Removal of the new tissue reversed, though not immediately, the functional benefit. Another possibility is that starting RA-treatment or EE at a later timepoint or for longer durations may be more

beneficial for the survival and integration of striatal neurons generated after stroke.

We found increased SVZ neurogenesis 61 days after tMCAO in RA/EE-treated rats, as shown by Ki67 and DCx labeling. Most studies assessed cell proliferation more acutely after injury and found increased neurogenesis in the SVZ in the first few weeks after MCAO (Arvidsson, et al., 2002, Jin, et al., 2001, Parent, et al., 2002, Zhang, et al., 2001), although a persistent increase in basal stroke-induced neurogenesis has been reported by one group (Thored, et al., 2006). Our data suggest that RA further increases SVZ neurogenesis in the chronic stages after stroke. Contrary to our findings, a recent study found that RA-treatment after photothrombotic stroke decreased BrdU and DCx in the SVZ (Jung, et al., 2007). This study assessed only acute (7 days after injury) proliferation effects, which may be one reason for the disparities with our findings. Further, they used photothrombosis to induce cerebral infarction, resulting in an isolated cortical infarct which differs from the tMCAO model in which rats have both striatal and cortical injury, and this larger injury may influence SVZ neurogenesis in different ways (Kolb et al., 2007). Additionally, i.p. injections of RA were administered for the first week after stroke, which differs substantially from our 5 week treatment of diet-enriched RA which began one week after tMCAO.

Another finding was that RA/EE treatment increased striatal neuroblast numbers and promoted new cells to migrate farther from the SVZ. Numerous DCx-positive neuroblasts and BrdU-positive cells were located near the infarct

border in RA/EE treated rats, whereas these cells tended to be located closer to the dorsolateral tail of the SVZ in untreated rats. Previously our lab found that RA influenced the migration of SVZ neuroblasts in the SVZ-olfactory bulb pathway of the early postnatal brain (Wang, et al., 2005), and the results from this study suggest that RA and EE may promote the migration of newly formed SVZ cells towards the injured striatum or promote their survival near the infarct border. RA/EE-treatment also appeared to increase striatal neurogenesis after tMCAO, as shown by an increase in the number of BrdU/NeuN double-labeled cells. This slight increase in new neuron survival may be a future therapeutic target to enhance neurogenesis. As shown in this study, increased neurogenesis does not necessarily mean improved function, as we saw no significant behavioral recovery in RA/EE-treated animals compared with untreated rats. In order to determine if neurogenesis is in fact necessary for functional recovery, further studies in which neurogenesis is eliminated or reversibly inhibited are needed. One would expect to see behavioral outcomes worsened by blocking neurogenesis if it is in fact important for functional recovery.

Most studies that evaluate the effects of EE on neurogenesis continuously house the animals in EE and have focused on the dentate gyrus, finding that EE enhances DG cell survival. We found that the short duration of EE used in this study seems to provide a transient improvement in sensorimotor function. Using only up to a maximum of 3 hours of EE per day, we saw a trend toward improvement in forelimb placing at the end of the treatment period. This improvement was lost, however, when the animals were tested again one month

after “therapy” was halted, suggesting that perhaps short durations of EE over a longer period of time may result in prolonged functional recovery. Similar results were found in a recent study with mice evaluating the effects of environmental enrichment for 3 hr/day for 2 weeks after MCAO (Nygren and Wieloch, 2005).

Interestingly, mice that were housed in continuous EE showed improved motor function but also had increased mortality; mice housed in EE for 3 h/d for 2 weeks, and then in standard cages for 2 weeks showed a loss of the motor function that they had recovered. These investigators also found that 4 weeks of 3 hr/day EE produced more long-lasting functional recovery. These results support our findings and the notion that a longer treatment period of short duration EE may provide substantial functional recovery. Despite this potential recovery, EE alone did not yield an increase in post-stroke neurogenesis, suggesting a dissociation between these two processes similar to that found for hippocampal-dependent memory effects of EE (Saxe, et al., 2006).

Other studies that evaluated the effects of EE on post-stroke neurogenesis found conflicting effects. After cortical stroke and EE, BrdU and Ki67 labeling increased bilaterally in the rat SVZ (Komitova, et al., 2005) and BrdU incorporation increased in the peri-infarct cortex although double-labeling revealed that most of these cells were glia (Komitova, et al., 2006). Another study from the same group found that post-stroke EE increased BrdU and DCx labeling in the SVZ 5 weeks after stroke (Komitova, et al., 2005), while others found that striatal lesioning induced by quinolinic acid or 6-hydroxydopamine plus EE increased striatal DCx expression and enhanced neuroblast migration into

the striatum (Urakawa, et al., 2007). In mice, EE increased SVZ progenitor numbers after stroke (Hicks, et al., 2007, Nygren, et al., 2006) but decreased the migration of DCx-positive cells into the striatum (Nygren, et al., 2006). Together with our results, these studies suggest that differences in species, type of injury, and duration of EE lead to varied influences on SVZ and striatal neurogenesis.

Few neurons typically survive long-term after stroke (Arvidsson, et al., 2002, Parent, et al., 2002) and our data suggest that the combination of RA and EE may provide a first step in promoting their survival. Future studies are needed to determine the most beneficial timing and dose of RA-treatment, such as whether a lower dose or longer delay prior to RA treatment may provide similar increases in neurogenesis without potential deleterious effects. Similarly, more work is required to determine if a longer timeframe of short-duration EE treatment, possibly including a tapering-off of the treatment, would be more beneficial to cell survival and functional recovery after stroke.

Acknowledgments

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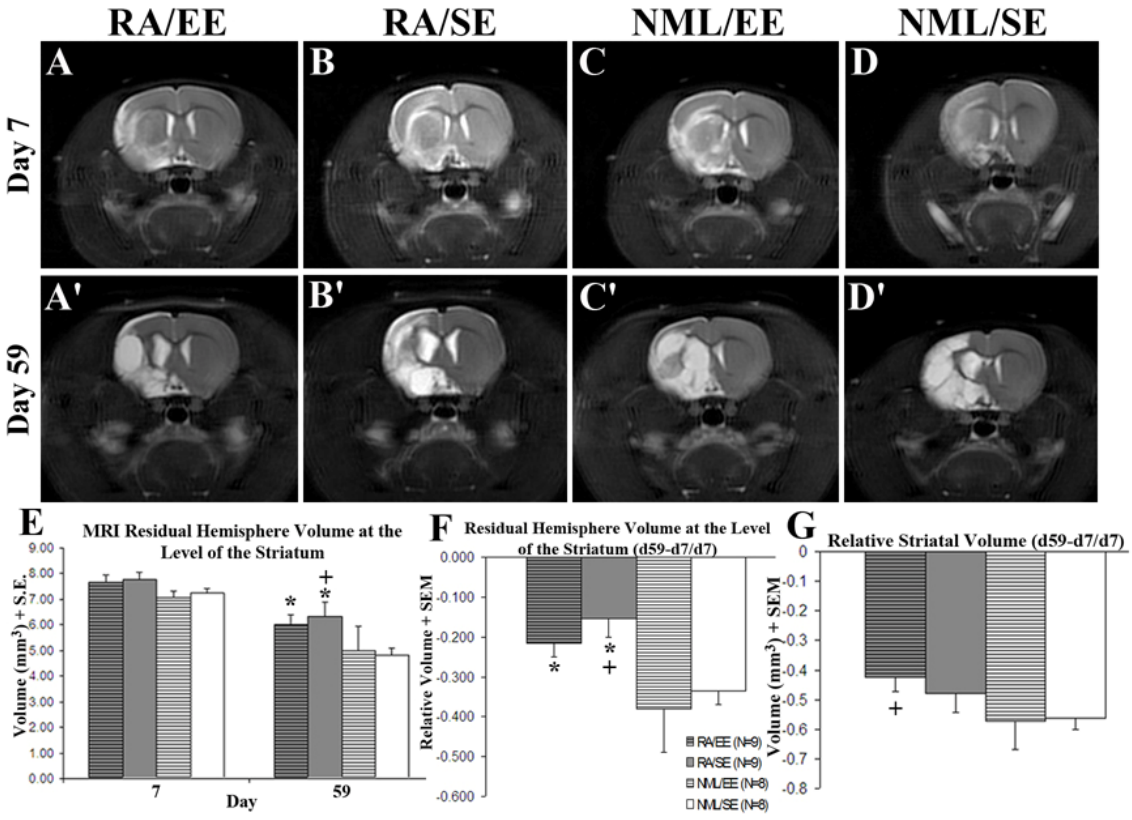

Figure 4.1: RA preserved hemisphere volume after stroke.

All animals had T2-weighted MRIs on day 7 (A-D) and day 59 (A'-D') after tMCAO and were assigned to treatment groups based on day 7 stroke volume (≥65% normal signal volume vs. intact hemisphere). All groups had similar stroke volumes prior to treatment (E). Rats that received an RA-enriched diet had more residual hemisphere volume at day 59 after MCAO compared with those on a normal diet (E, *p<0.05 vs NML/EE, +p<0.05 vs. NML/SE) as well as a smaller change in hemisphere volume over time (F, *p<0.05 vs NML/EE, +p<0.05 vs. NML/SE). Rats that received an RA-enriched diet and environmental enrichment maintained more striatal volume over time compared with normal controls (G, +p<0.05). All values are expressed as volume (mm^3) ± SEM.

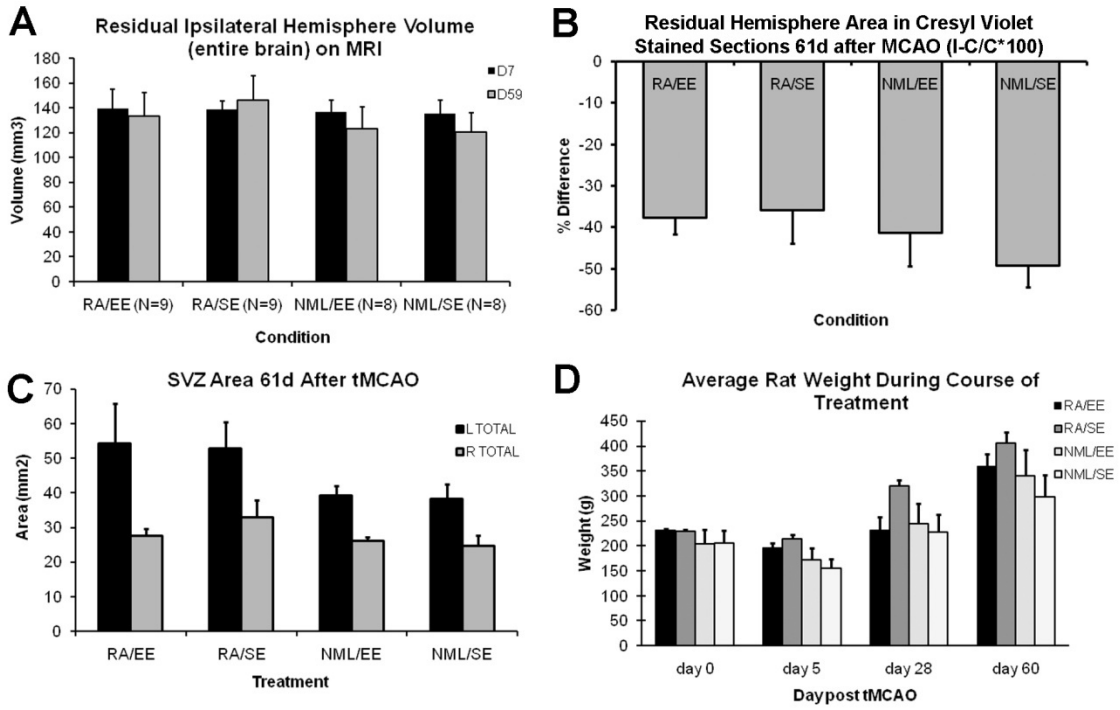

Figure 4.2: RA preserved brain volume and increased SVZ area.

Residual hemisphere tissue was measured on 13 MRI slices on days 7 and 59 after stroke. RA/EE and RA/SE animals maintained larger hemisphere volume (mm^3) over time compared with normal diet rats (A). Cresyl violet staining and residual hemisphere measurements at the level of the striatum confirmed MRI findings in Figure 1 and Figure 2A (B). The SVZ remained greatly expanded at 61d after tMCAO in RA/EE and RA/SE rats compared with NML/EE and NML/SE rats (C, cresyl violet). There was no significant difference in body weight on day 61 in RA-treated vs. vehicle-treated rats (D, $p=0.3$). All values are expressed as ± SEM.

Figure 4.3: RA/EE increased SVZ cell proliferation at 61d post-stroke.

Ki67 labeling was performed to identify proliferating cells at the time of sacrifice. All animals had Ki67+ cells present in the dorsolateral tail of the SVZ as well as along the lateral wall of the lateral ventricle. Animals that received the RA-enriched diet combined with EE had the most Ki67+ cells present in the lateral SVZ (A) and animals that received only the RA-enriched diet also displayed increased Ki67-immunoreactivity (B) compared with EE-only (C) and untreated rats (D). The enlarged SVZ contains numerous DCx+ neuroblasts and increased DCx immunoreactivity in RA/EE (E), RA alone (F) and EE alone (G), groups compared to controls (H) 61 days after MCAO. LV=lateral ventricle, shown at right-hand side in all panels. Scale bars: 50μm in A, 100 μm in E.

RA/EE
RA/SE
NML/EE
NML/SE
KI67
DCX
A
B
C
D
E
F
G
H
LV
LV
LV
LV

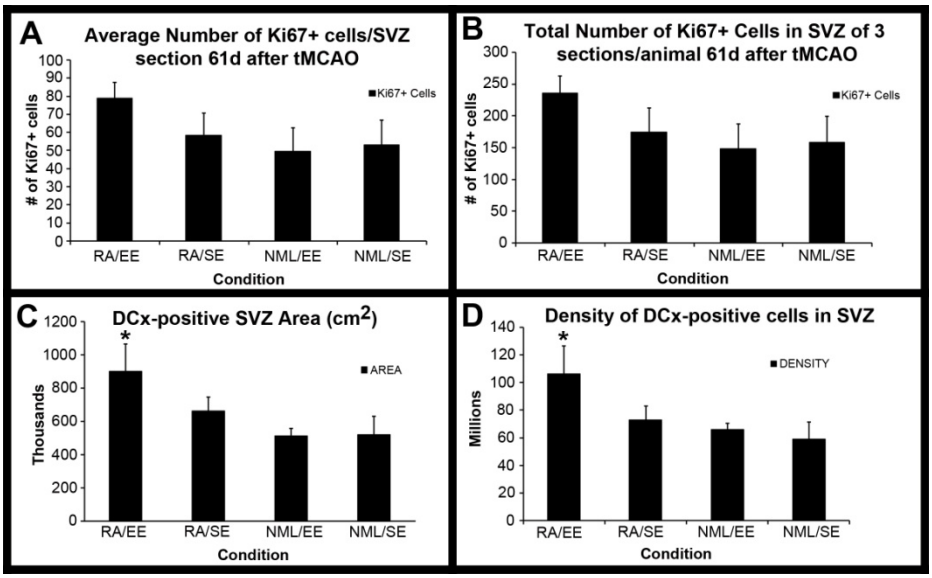

Figure 4.4: Quantification of increased SVZ neurogenesis at 61d post-stroke.

RA/EE-treatment increased the average number of Ki67+ cells/section in the lateral portion of the SVZ (A) and the total number of Ki67+ cells along the lateral wall of the lateral ventricle (B) compared with controls. RA/EE rats have a significantly larger DCx+ dorsolateral SVZ compared to EE-alone or untreated rats (C, *p=0.02). The enlarged DCx+ SVZ of RA/EE rats also contains significantly more densely packed cells (D) than that of EE alone or untreated rats (D, *p<0.05).

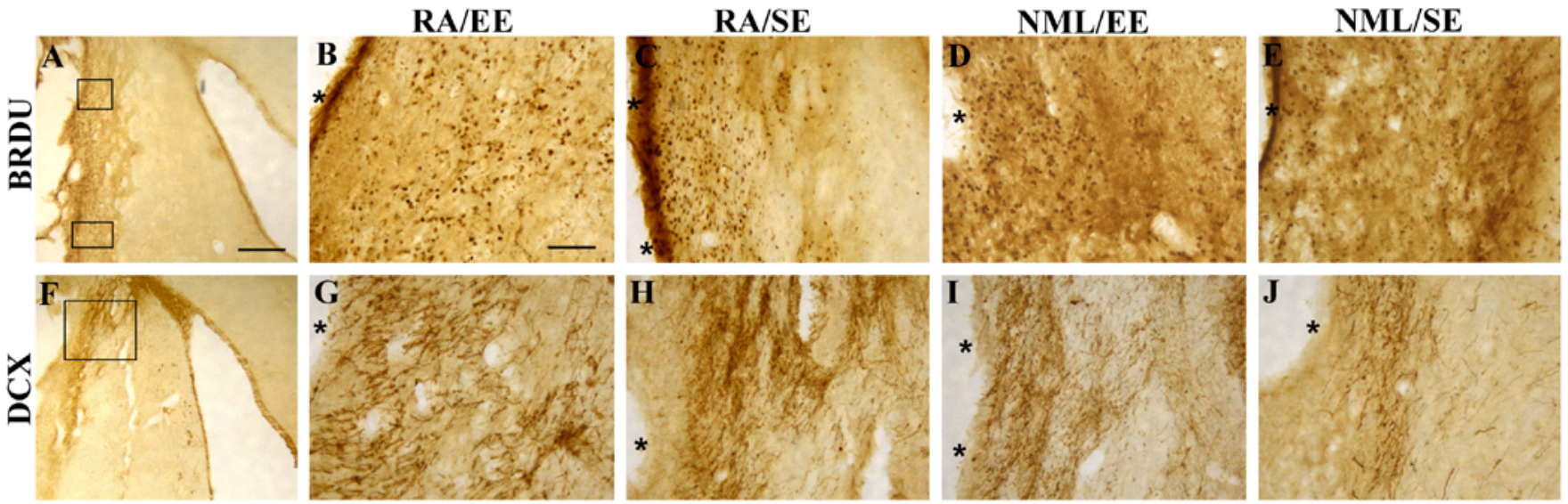

Figure 4.5: RA/EE increased striatal peri-infarct BrdU and DCx.

Rats were injected with BrdU on days 5-11 after tMCAO. Analysis of peri-infarct BrdU was performed as described in the methods section. BrdU+ cells in the peri-infarct striatum were counted in dorsal and ventral areas as shown (A). BrdU+ cells appeared more numerous within the peri-infarct region of RA/EE (B), RA/SE (C) and NML/EE (D) rats compared with untreated rats (E). The distribution of DCx expression in the injured striatum differs as well. The image in F is from a representative RA/EE rat, showing DCx expression extending from the dorsolateral tail of the SVZ, laterally to the infarct border and deep into the ventral striatum. DCx+ neuroblasts appear more dense and farther from the ventricle in the lesioned striatum in RA/EE (G) and RA/SE (H) than in NML/EE (I) or untreated rats (J). *denotes infarct border. Scale bar in A = 400 μm, in B = 100μm.

Figure 4.6: RA/EE enhanced the formation of new neurons in the injured striatum.

Rats were injected with BrdU on days 5-11 and killed 61 days after tMCAO. Double-label immunofluorescence staining for BrdU and NeuN identified new neurons born during this time of peak proliferation after tMCAO. There was a distinct border between BrdU+ cells and healthy neurons in the injured striatum of most rats, regardless of treatment, making it difficult to locate double-labeled cells (A, E). The injured striatum of RA/EE rats contained the most BrdU/NeuN double-labeled cells (arrows, B-D). Of note, very few BrdU/NeuN double-labeled cells were strongly BrdU+, rather most contained strong NeuN (C) and lighter BrdU immunoreactivity (B,D), suggesting that these cells divided numerous times. Similarly, untreated rats typically displayed strong NeuN staining (G) with more diluted BrdU (F) in the same cell (H, arrow), however, fewer double-labeled cells were found in these animals. Scale bar in A = 150μm, in I = 25μm.

BRDU NEUN
BRDU
NEUN
MERGED
A
B
C
D
E
F
G
H
RA/EE
NML/SE

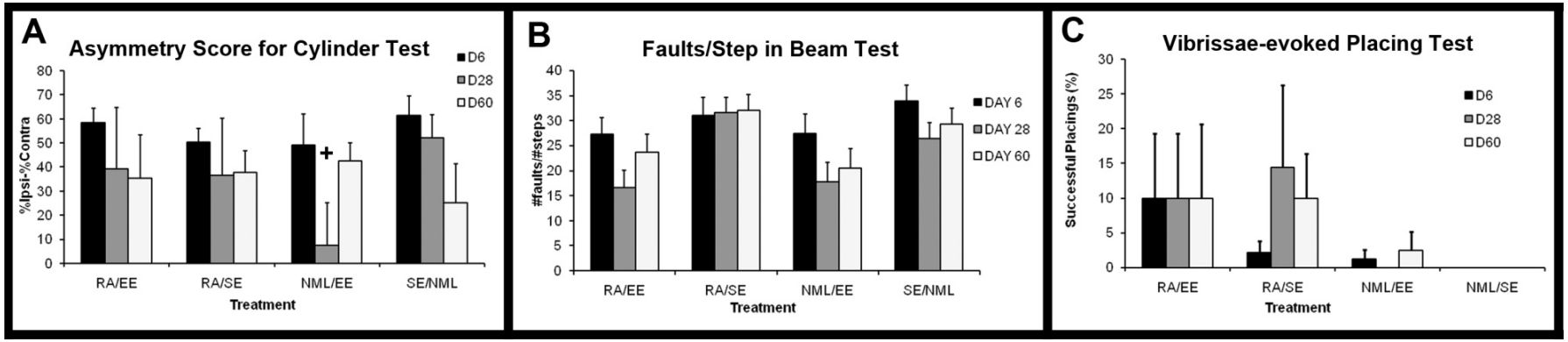

Figure 4.7: EE transiently improves functional recovery after tMCAO.

Rats were behavior tested 6 days after tMCAO to establish baseline deficits and again on days 28 and 60 after tMCAO to assess recovery. Forelimb asymmetry was assessed using the cylinder test and vibrissae-evoked placing while hindlimb asymmetry was assessed on the ledged, tapered beam. All rats displayed similar deficits on day 6 in the cylinder test (A, black bars) and beam test (B, black bars). Normal behavior in uninjured rats is 0. However, only a few animals had successful placing and in only about 10% of all trials in the vibrissae-evoked placing test (C, black bars). By day 28, all groups showed some improvement on the cylinder test (A, gray bars) but only the NML/EE group trended toward improvement (+p=0.06 vs. NML/SE). All groups except RA/SE showed some improvement on the beam test but none were significantly different (B, gray bars). RA/SE showed no improvement on the beam test (B, gray bars) but showed a slight but insignificant improvement on the vibrissae-evoked placing test (C, gray bars) while other groups showed no improvement on this test. By day 60, all groups except NML/EE displayed similar deficits on the cylinder test as were seen on day 28 (A, white bars). Interestingly, the NML/EE group had more severe deficits on the cylinder test at day 60 than at day 28 (A, white bars), suggesting that the improvements seen during the course of treatment were lost one month after treatment halted. All groups remained the same on day 60 as they were on day 28 in the beam test (B, white bars) and vibrissae-evoked placing (C, white bars).

Table 4.1. Striatal BrdU-positive cell analysis. All values are ± SEM. % BrdU/NeuN= (#BrdU/NeuN+/total BrdU+) x 100.

	BrdU Stereology	Total Peri-infarct BrdU	% BrdU/ NeuN
RA/EE	711648 ± 114529	1272 ± 350	20.97 ± 3.57
RA/SE	669720 ± 132478	1146 ± 239	15.72 ± 2.47
NML/EE	651623 ± 57937	1276 ± 253	15.63 ± 2.87
NML/SE	632630 ± 97387	999 ± 101	15.10 ± 2.90

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CHAPTER V:

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of thesis work

The data presented in this thesis provide evidence for the regulation of postnatal and adult neural stem cells at three different levels: by factors present within the local stem cell microenvironment, by factors outside of the microenvironment but present within the brain, and by exogenous factors or conditions. We found that brain insults such as hypoxia or inflammation increase SVZ neural stem cell production, neurogenesis and oligodendrogliogenesis but have detrimental effects on SVZ neural stem cell expansion and neurogenesis when these insults are combined (Figure 5.1). Our work also shows that factors secreted from normal endothelial cells, present within the stem cell niche, function to maintain SVZ neural stem cells in a more proliferative and undifferentiated state, while factors from stroke-injured endothelial cells promote SVZ neural stem cell migration and neuronal maturation (Figure 5.2). Finally, we found that treatment after stroke with retinoic acid, a vitamin-A derivative, and environmental enrichment preserves brain tissue in the injured hemisphere, increases SVZ neural stem cell proliferation and neuroblast production, but yields minimal increases in the long-term survival of neurons generated after stroke and

provides no benefit to functional recovery (Figure 5.3). In the following pages, the results and interpretations of these studies will be summarized and presented with a discussion of the clinical implications of these findings. Future studies will also be proposed to further delineate the various roles of hypoxia, inflammation and stroke on the postnatal and adult SVZ neural stem cell populations.

Regulation of the Niche

Neurogenesis occurs in very limited areas of the normal postnatal and adult brain in most mammals, whereas it is more prevalent in lower vertebrates such as lizards, which can regenerate entire brain regions (Harzsch and Dawirs, 1996, Lledo, et al., 2006). The diminished capacity for neurogenesis in the mammalian brain is likely due to factors within the brain that limit the persistence of neural stem cells to certain areas, and they likely serve more specific functions (e.g., limited neuronal turnover in plastic regions rather than brain regeneration). Currently, the main regions identified in the postnatal/adult rodent, primate or human brain that contain neural stem-like cells are the SVZ and the dentate gyrus of the hippocampus (Altman, 1969, Altman and Das, 1965, Eriksson, et al., 1998, Gould, et al., 1999, Kornack and Rakic, 1999, Lois and Alvarez-Buylla, 1993). The presence of only 2 neurogenic regions in the adult brains of these species suggests that certain environmental cues must be present to support and maintain these cells, as such, they reside in a neurogenic niche. The neural stem cells interact with elements in the niche, including each other, astrocytes and endothelial cells to maintain their stem cell-like state and actively proliferate,

adopt a precursor cell fate and migrate out of the niche (Conover and Notti, 2008, Doetsch, 2003, Lim, et al., 2007, Ohab, et al., 2006, Palmer, et al., 2000, Pierret, et al., 2007, Riquelme, et al., 2008).

The factors that regulate maintenance, migration, and differentiation are likely derived from intrinsic programs within the cells, factors external to the cell but present within the neurogenic niche, and environmental factors. Each component of neurogenesis, i.e., proliferation, migration and differentiation, may be regulated by common factors operating on a continuum or by completely separate factors/events that do not influence the previous or next step in the process of neurogenesis. The data presented in this thesis suggest that other cells within the neurogenic niche, specifically endothelial cells, influence proliferation and migration while extrinsic and environmental factors (inflammation, hypoxia, stroke-injured endothelial cells, and retinoic acid and environmental enrichment) primarily influence neuroblast production, migration and differentiation. These influences are not completely specific entities and may interact with each other or other factors to influence additional components of neurogenesis.

The postnatal SVZ is comprised of three types of neural stem cells, defined as type A, type B and type C cells (Alvarez-Buylla and Garcia-Verdugo, 2002, Doetsch, et al., 1997, Garcia-Verdugo, et al., 1998). The factors that maintain these cells may be the same or different for each cell type, as the role of each cell within the neurogenic niche differs. Type B cells are the putative neural stem cells, which have the ability to self-renew and give rise to type C cells.

Type B cells express GFAP, vimentin and nestin (See Figure 1.2) and have the appearance of radial glia or astrocytes (Doetsch, et al., 1999, Doetsch, et al., 1997, Garcia-Verdugo, et al., 1998, Lim and Alvarez-Buylla, 1999, Merkle, et al., 2004). Type C cells are the transit-amplifying cells which give rise to type A cells, known as neuroblasts [Figure 1.2; (Doetsch, et al., 1997)]. Type B cells are the only true neural stem cells in the SVZ, as type C cells and type A cells are more differentiated progenitor or precursor cells that have limited choices in cell fate (Alvarez-Buylla and Garcia-Verdugo, 2002). Each step in this progression, from type B to type C to type A cell, further commits the cell to a more specific fate. Thus, the uniqueness and multipotency of type B cells may require regulation by factors different than those that regulate type C and type A cells. In addition, type A cells are the only cell type of the three known to migrate, suggesting that factors that regulate this cell and prompt its migration may not influence type B or type C cells (Lois, et al., 1996). The potential factors that govern these cells will be discussed below in the context of their functions within the neurogenic niche and their role(s) in post-stroke neurogenesis as it pertains to the data presented in chapters 2-4.

Regulation of SVZ NSC Proliferation in the Uninjured Brain

The experiments in this thesis provide evidence that endothelial cells present within the vascular niche function to maintain postnatal SVZ neural stem cells in the normal brain. Previous studies report that endothelial cells regulate embryonic NSCs and adult hippocampal NSCs by increasing their self-renewal

(Guo, et al., 2008, Shen, et al., 2004). No studies published to date have examined the influence of normal and stroke-injured endothelial cells on the postnatal/adult SVZ. While it is tempting to make generalizations in regard to the factors that control and regulate NSCs, these populations are not identical as important differences exist between embryonic and postnatal NSCs as well as SVZ-derived vs. hippocampal NSCs. Embryonic NSCs are more plastic in terms of final cell fate, and can be directed towards more cell types or neuronal subtypes than postnatal NSCs (Delaunay, et al., 2008, Gates, et al., 1995, Gritti, et al., 2002, Imura, et al., 2003, Tramontin, et al., 2003, Watanabe, et al., 2005). The final destination of cells produced by embryonic NSCs and hippocampal NSCs also differs from that of postnatal SVZ NSCs. While factors regulating embryonic or hippocampal NSCs may provide important clues in terms of directing attention toward a given factor or stimuli, these important differences require investigations into the factors that specifically regulate postnatal SVZ NSCs.

In chapter 3, we report that SVZ NSC expansion in media conditioned by endothelial cells results in increased production of NS without a corresponding increase in NS size. SVZ-derived NS consist of type B and C cells (Alvarez-Buylla and Garcia-Verdugo, 2002, Alvarez-Buylla, et al., 2002, Doetsch, et al., 1999, Doetsch, et al., 1999, Garcia-Verdugo, et al., 1998), however, ablation of the GFAP-expressing NSC (i.e., type B cell) results in an almost complete loss of NS formation (Imura, et al., 2003, Morshead, et al., 2003). These data, together with our results, suggest that endothelial cell-secreted factors promote self-

renewal of the type B cell, and therefore promote the initial formation of neurospheres, but do not necessarily influence proliferation of the type C cells, which may result in larger spheres rather than more of them.

In addition to increasing self-renewal via NS formation, ECCM also appears to prolong proliferation of SVZ NSCs, as significantly more ECCM-expanded cells continued to express the endogenous proliferation marker, Ki67, after 4 days of differentiation. These cells may be lineage restricted neuroblasts or glioblasts rather than type B cells, but their precise nature is uncertain without additional labeling. These data further support the idea that endothelial cell-secreted factors function in the niche to maintain the NSC population by promoting and maintaining self-renewal.

Regulation of SVZ NSC Proliferation after Injury/Insult

The factors that regulate proliferation in the uninjured postnatal and adult brain may or may not contribute to increased proliferation after injury. In the normal postnatal and adult brain, SVZ NSCs are relatively quiescent (Doetsch, et al., 1999, Morshead, et al., 1994). The cell cycle is longer for adult NSCs than for embryonic NSCs, leading to fewer cell divisions and subsequent decreased neurogenesis in the adult compared to the embryonic brain (Lu, et al., 2008, Lu, et al., 2007, Maslov, et al., 2004, Zhang, et al., 2006, Zhang, et al., 2008). This makes sense, as the environment of the embryonic brain is very dynamic in order to complete development in a timely manner. In the postnatal and adult brain, however, development is complete and new cells are not in high demand. After

injury, however, the necessity and capacity for production of new cells in the postnatal and adult brain dramatically increases, rendering it more similar to the developing brain in terms of plasticity. Thus, factors that influence NSCs during development may be more likely to contribute to injury-induced neurogenesis than factors regulating NSCs in the uninjured adult brain.

Retinoic acid is involved in numerous developmental processes and was recently found to increase proliferation in the early postnatal SVZ-olfactory bulb pathway (Hagglund, et al., 2006, Haskell and LaMantia, 2005, Krezel, et al., 1999, Luo, et al., 2004, Novitch, et al., 2003, Toresson, et al., 1999, Wagner, et al., 2002, Wang, et al., 2005, Wilson and Maden, 2005, Zetterstrom, et al., 1999, Zetterstrom, et al., 1994). The results presented in chapter 4 suggest that RA also increases SVZ proliferation after stroke in the adult brain. RA alone or in combination with environmental enrichment increased DCx- and Ki67-immunoreactivity in the SVZ at 61 days after stroke. It is unclear as to whether this treatment influenced all three types of SVZ NSC, as Ki67 labels actively dividing cells, which could be type A, B or C cells. DCx-labeling is specific to type A cells and the RA-treatment was terminated 41 d after stroke. Therefore, the increased DCx population present at 61 days after stroke may be a result of initially increased type B or C cells, which have further differentiated into type A cells at this time rather than an increase in only type A cells. Regardless of whether RA acts on all 3 types of SVZ NSCs, it is clear that RA functions to promote post-stroke SVZ NSC proliferation.

While it is difficult to discern the exact mechanism(s) underlying inflammation- or hypoxia-induced increases in SVZ NSC expansion (chapter 2), these processes clearly promote postnatal SVZ NS formation. Postnatal mice exposed to hypoxia or systemic inflammation, without direct brain injury, produced more SVZ NS than control mice without altering NS size. Potential mediators of the hypoxia-induced changes in SVZ NS production include the hypoxia-inducible-factors (HIFs), which are members of the basic helix-loop-helix PAS family of transcription factors (Semenza, 2000). HIFs respond to changing oxygen levels in the brain, and are present only in low levels when oxygen is present. HIF-1α expression increases exponentially as brain oxygen levels decrease, activating target genes which include those involved in angiogenesis, energy metabolism, pH regulation, cell survival and cell proliferation (Buchler, et al., 2003, Fukuda, et al., 2003, Iyer, et al., 1998, Kasuno, et al., 2004, Kelly, et al., 2003, Semenza, 2000, Semenza, 2000, Semenza, 2000). Studies examining the effects of lowered oxygen and HIFs on stem cells reported increased dopaminergic differentiation after HIF-1α overexpression in mesencephalic NSCs while knockout of HIF-1α led to impaired survival and proliferation of midbrain precursors in addition to decreased dopaminergic differentiation (Milosevic, et al., 2007, Zhang, et al., 2006). Thus, HIF-1α activation influences proliferation and neuronal differentiation in midbrain and mesencephalic neural stem cells and may be responsible for the hypoxia-induced changes in postnatal SVZ NSCs reported here.

The inflammation induced increases in SVZ NS production are likely a result of pro-inflammatory cytokines. LPS activates its receptor, TLR-4, which initiates the inflammatory cascade, including up-regulation of the pro-inflammatory cytokines IL-1β and TNF-α (Davies, et al., 1998, Fan, et al., 2004, Larsen and Henson, 1983, Rothwell and Hopkins, 1995, Stanimirovic and Satoh, 2000, Stanimirovic, et al., 1997, Stanimirovic, et al., 1997, Zheng and Yenari, 2004). We tested whether IL-1β mediates this effect and found that IL-1β knockout resulted in decreased NS production. This suggests that IL-1β, at least in part, is involved in the post-inflammation proliferative response in the postnatal mouse SVZ. This contrasts with previous reports which suggested that IL-1β either had no effect on adult hippocampal NSCs or decreased proliferation in embryonic and adult NSCs. None of these studies, however, examined the role of IL-1β in the postnatal SVZ (Koo and Duman, 2008, Monje, et al., 2003, Wang, et al., 2007). However, more work needs to be done to confirm the involvement of IL-1β.

Regulation of Postnatal SVZ NSC Migration

The type A cells in the postnatal SVZ are neuroblasts that normally migrate in chains, ensheathed in astrocytes, through the rostral migratory stream to the olfactory bulb (Doetsch and Alvarez-Buylla, 1996, Yang, et al., 2005). Migration through the rostral migratory stream is regulated by integrins, interactions of slit with its receptor robo, ephrin signaling, and matrix metalloproteinases (Belvindrah, et al., 2007, Bovetti, et al., 2007, Conover, et al.,

2000, Murase and Horwitz, 2002, Riquelme, et al., 2008, Yang, et al., 2005). Once these cells enter the olfactory bulb, they migrate tangentially via astrocyte/endothelial interactions, to the granular and peri-glomerular layers (Bovetti, et al., 2007, Doetsch and Alvarez-Buylla, 1996). After injury, however, many of these cells migrate to the stroke-injured striatum or cortex rather than the olfactory bulb (Arvidsson, et al., 2002, Jin, et al., 2001, Parent, et al., 2002, Plane, et al., 2004, Zhang, et al., 2001). It is unclear what cues direct SVZ neuroblasts to migrate into these injured areas. Some studies suggest that astrocytes and blood vessels may direct SVZ neuroblast migration into the injured regions via EGF, stromal-derived factor-1 (SDF-1) interacting with its chemokine receptor CXCR4, or MMP-9 (Ohab, et al., 2006, Teramoto, et al., 2003, Thored, et al., 2007, Wang, et al., 2006). Few studies have directly examined the role of endothelial cells or endothelial-secreted factors on SVZ NSCs. We found SVZ NSC migration to be enhanced by conditioned medium from OGD-injured endothelial cells and observed SVZ neuroblast migration within the ischemic striatum to closely coincide with blood vessels/endothelial cells. At this time, however, we are unable to identify the specific endothelial-derived cues that likely direct SVZ neuroblast migration into the injured striatum. Some potential candidates include brain-derived neurotrophic factor (BDNF), angiopoietins, matrix metalloproteinases (MMPs), ephrins and SDF-1 (Belvindrah, et al., 2007, Conover, et al., 2000, Murase and Horwitz, 2002, Ohab, et al., 2006, Riquelme, et al., 2008, Stumm, et al., 2002, Tsai, et al., 2006, Wang, et al., 2006). Many of these molecules have already been implicated in post-

stroke migration to the peri-infarct cortex via production by glia or blood vessels in the post-stroke angiogenic environment. For example, evidence supports a role for SDF-1 in post-stroke neuroblast migration into the striatum, and the source of this factor is likely to be astrocytes and microglia within the ischemic striatum (Ohab, et al., 2006) rather than endothelial cells. It is unclear, however, what other cues mediate post-stroke neuroblast migration into the ischemic striatum and which are specifically provided by endothelial cells.

Interestingly, we found that treatment after stroke with RA in combination with EE promoted neuroblast migration into the ischemic striatum. DCx-positive neuroblasts appeared to localize to the peri-infarct striatum with greater propensity than with either treatment alone or no treatment. However, little evidence exists to suggest that RA may regulate adult SVZ NSC migration. RA-signaling was recently implicated in regulating migration of SVZ-derived NSCs to the olfactory bulb during the early postnatal period but its role in regulating post-stroke neuroblast migration has not been tested (Wang, et al., 2005). It is difficult to determine if the migratory effect demonstrated in our experiments is due to RA alone or works in combination with EE. Neuroblast migration into the peri-infarct region appeared to be increased with each treatment alone but the greatest effect was seen with the combination of RA and EE treatment. The potential interactions of RA and EE within the ischemic striatum may be mediated via BDNF, which increases with enrichment, and its receptor, trkB, which can be induced by RA in vitro (Kaplan, et al., 1993, Lucarelli, et al., 1994, Lucarelli, et al., 1995, Rossi, et al., 2006). These interactions would more likely result in an

enhanced differentiation or survival effect rather than migration, as each has been shown to promote differentiation/survival (Kempermann, et al., 1998, Kempermann, et al., 2003, Kempermann, et al., 1997, Komitova, et al., 2002, Komitova, et al., 2005, Nilsson, et al., 1999, Toresson, et al., 1999, Wurm, et al., 2007). It is difficult to interpret these results, however, as lesions sizes varied, resulting in overall differences in proliferation and migration regardless of treatment. To determine if RA plays a role in post-stroke neuroblast migration, an in vitro assay may be more suitable, for example, SVZ explants from MCAO-treated rats or mice could be cultured in matrigel in media with or without RA and effects on migration evaluated.

Regulation of Postnatal SVZ NSC Differentiation and Survival

As reported in chapter 3, SVZ NSC differentiation was influenced by factors from normal and stroke-injured endothelial cells. Intact ECCM altered differentiation by maintaining SVZ NSC-derived neurons and glia in a more immature state while OGD-ECCM promoted maturation of SVZ NSC-derived neurons. This suggests that a) normal endothelial cells secrete different factors than injured endothelial cells or b) endothelial-secreted factors that maintain SVZ NSCs in a more immature state are down-regulated after stroke. Unfortunately, we were unable to identify factors secreted by normal and injured endothelial cells in these experiments, and therefore unable to examine whether these factors are the same or different from normal versus injured endothelial cells. However, the 1-D gel used to identify protein sizes produced bands in the same

location for intact- and OGD-ECCM, but fewer bands were present in OGD-ECCM than intact ECCM. This suggests that similar factors (or at least proteins of similar size) were produced by normal and OGD-exposed endothelial cells, and that some of these were down-regulated after OGD-exposure. In these studies, factors from normal and OGD-exposed endothelial cells did not appear to alter SVZ NSC fate, only influenced their rate of differentiation/maturation. Further, they had no effect on cell survival/death.

What endothelial-secreted factor or factors manipulate the rate of differentiation/maturation in postnatal SVZ NSCs is uncertain. Endothelial cells secrete leukemia inhibitory fact (LIF) and BDNF, both of which can influence differentiation, and may play a role in promoting SVZ-derived neuronal maturation (Leventhal, et al., 1999, Louissaint, et al., 2002, Mi, et al., 2001, Nottebohm, et al., 1990). More specifically, endothelial cell-derived BDNF promotes neuronal recruitment, outgrowth and maturation in the adult songbird brain and in cultures of adult NSCs (Leventhal, et al., 1999, Louissaint, et al., 2002, Nottebohm, et al., 1990). Most studies identify factors that regulate cell fate but the temporal relationship of these factors to differentiation remains to be determined. Some of the known SVZ cell fate determinants include the bone morphogenic proteins (BMPs), which instruct SVZ NSCs toward a glial fate, and noggin, which directs SVZ NSCs toward a neuronal fate via antagonistic actions on BMP signaling (Bonaguidi, et al., 2005, Coskun, et al., 2001, Gross, et al., 1996, Lim, et al., 2000). The notch signaling pathway is also involved in directing embryonic NSC differentiation and may also regulate postnatal/adult SVZ NSC

differentiation. Notch 1 and Jagged 1 are expressed by astrocytes in the SVZ, and promote neural lineage commitment, differentiation and survival of embryonic NSCs in vitro (Androutsellis-Theotokis, et al., 2006, Guentchev and McKay, 2006, Irvin, et al., 2004, Irvin, et al., 2001, Lowell, et al., 2006, Nyfeler, et al., 2005). Further, administration of delta, one of the notch ligands, to adult rats results in increased BrdU labeling in the SVZ and increased expression of the neuronal marker Hu in BrdU-positive cells in the cortex 45 days later (Androutsellis-Theotokis, et al., 2006). Notch signaling increases after stroke and is also involved in the response of embryonic NSCs to lowered oxygen, and together these data implicate notch signaling as a potential regulator of postnatal/adult injury-induced SVZ neurogenesis (Androutsellis-Theotokis, et al., 2006, Arumugam, et al., 2006, Chen, et al., 2007, Guentchev and McKay, 2006, Gustafsson, et al., 2005). These factors, however, are produced by astrocytes and ependymal cells that reside along the SVZ.

We found that differentiation of SVZ NSC into neurons was enhanced by hypoxia or inflammation alone, but that the combined insults decreased neurogenesis. Neurogenesis may be enhanced in the inflammation experiments via the pro-inflammatory cytokine IL-1β. To test whether inflammation-induced increases in neurogenesis may be mediated by IL-1β, we cultured NS from IL-1β knockout mice and examined the effects on neurogenesis after 7 days of differentiation. We found decreased neurogenesis in the IL-1β knockout NS compared to wild-type NS. Preliminary experiments were performed in which IL-1β was added to primary NS from IL-1β knockout mice during differentiation (at

the same time that LPS was administered in previous inflammation experiments) to further implicate IL-1β in the inflammation-induced changes in postnatal SVZ neurogenesis. In these experiments, IL-1β appeared to restore SVZ neurogenesis in NS from IL-1β deficient mice.

Most studies evaluating neurogenesis in regard to IL-1β have reported its effects on modulating NSC proliferation, but not differentiation (Koo and Duman, 2008, Wang, et al., 2007). One recent study reported that blocking IL-1β decreased GFAP expression in embryonic NPCs in vitro, with no effect on neuron or oligodendrocyte formation (Wang, et al., 2007). Previous in vitro studies have shown beneficial effects of IL-1 on primary neuron survival, mediated via induction of nerve growth factor on astrocytes (Bandtlow, et al., 1990, Carman-Krzan, et al., 1991, Spranger, et al., 1990, Vige, et al., 1991, Vige, et al., 1992). Further, IL-1 increases dopaminergic neuron differentiation in mesencephalic progenitor cells and can induce FGF-2, a well-known regulator of SVZ NSCs (Albrecht, et al., 2002, Ho and Blum, 1997, Ling, et al., 1998).

The inflammation-induced increase in SVZ neurogenesis and oligodendrogliogenesis in vitro may be mediated by other cytokines as well, such as TNF-α, IL-6, and LIF, which may act in conjunction with IL-1β or downstream of it. LIF may also be responsible for decreased neurogenesis after the combined hypoxia and inflammation insult, as LIF increases after ischemia and has been shown to induce NSC differentiation into astrocytes (Carmichael, 2003, Wright, et al., 2003). The effects of inflammation on SVZ neurogenesis may also be mediated by activation of the pro-inflammatory transcription factor nuclear

factor-κ-B, which is involved in many post-insult pathways (Chang and Huang, 2006, Clemens, et al., 1997, Clemens, et al., 1997, Hu, et al., 2005, Schneider, et al., 1999, Seegers, et al., 2000, Stephenson, et al., 2000, Widera, et al., 2006). The suppressor of cytokine signaling-3 (SOCS-3) is up-regulated in ischemic tissue, negatively regulates the janus kinase/signal transducers and activators of transcription pathways (STAT), and promotes neuronal differentiation of CNS stem cells, making it another potential molecule of interest (Bates, et al., 2001, Larsen and Ropke, 2002, Lu, et al., 2003, Raghavendra Rao, et al., 2002, Turnley, et al., 2002).

After stroke, treatment with RA and EE, but not either alone, modestly increased striatal neurogenesis. Given the previous reports that EE enhances dentate gyrus neurogenesis after stroke, these findings were somewhat surprising (Kempermann, et al., 1998, Kempermann, et al., 1997, Kempermann, et al., 1998, Komitova, et al., 2005, Komitova, et al., 2002, Komitova, et al., 2005, Nilsson, et al., 1999). We hypothesized that RA combined with EE would promote survival of stroke-generated neurons, however, this was not the case. RA-treatment preserved tissue after stroke, and in combination with EE, increased SVZ cell proliferation and enhanced neuroblast migration, although this was not enough to support the generation and survival of new neurons for 2 months after stroke. This suggests that tissue sparing is not sufficient to promote neuron survival (or functional recovery), and it is likely that although this tissue was preserved, it did not provide a suitable environment for the propagation of new neurons. Further, differences in EE-induced dentate gyrus neurogenesis

have been reported for different strains of mice, suggesting that EE-induced neurogenesis is not a universal phenomenon (Kempermann, et al., 1998). Enhancing the survival of adult-generated striatal neurons after stroke may lead to significantly improved cognitive and motor function, but exogenous or in situ cues that promote this process remain unidentified.

Clinical Implications

Elucidating the factors that regulate postnatal and adult SVZ NSCs is an important step in advancing the potential of NSCs for treatment of brain injury and disease. The presence of NSCs in the postnatal and adult brain and their increased proliferation in response to injury or insult suggests that the brain has the capacity to repair itself. However, this response is not sufficient if it does not provide long-term benefits to function. By identifying the factors that regulate each step in the process of neurogenesis, including the region-specific cues, we may be able to direct postnatal and adult NSCs towards a necessary cell type to replace those lost after injury. There are multiple steps in the process of neurogenesis and it appears that each is regulated by a delicate balance of factors, both internal and external, which work together to maintain the balance between stem cell maintenance/self-renewal and differentiation into specific cell phenotypes. Each step appears to be separately regulated by multiple factors or processes but must converge in the end to maintain this balance. While the results reported in this thesis do not necessarily identify specific factors, they provide insight into pathways or cell types that could be targeted for development of future treatments.

We reported in chapter 2 that hypoxia or inflammation significantly increases postnatal SVZ NSC expansion and neurogenesis, but in combination is detrimental to postnatal SVZ neurogenesis. Hypoxia and inflammation are commonly encountered clinical problems in critically ill neonates with a broad range of cardiorespiratory and other systemic disorders and can lead to cognitive deficits later in life. The data from this thesis suggest that it may be important to target the pathways involved in these insults, such as the HIF pathway or the cascade of pro-inflammatory cytokines after a second exposure to insult. For example, administration of IL-1β or TNF-α after insult may prompt the postnatal NSCs to proliferate and subsequently form new neurons. Receptors for IL-1β are present in embryonic NSCs and IL-1β is present in human neural progenitors (Klassen, et al., 2003, Wang, et al., 2007), suggesting that exogenous administration could act on these populations. This treatment, however, would only be effective if these neurons integrated into the necessary region(s) without causing adverse side-effects, which may not be possible, given that IL-1 is present within the postnatal brain and amplifies stroke volume in the adult rodent by increasing cell death (Dziegielewska, et al., 2000, Loddick, et al., 1996, Loddick and Rothwell, 1996, Yamasaki, et al., 1995).

The data presented in this thesis also suggest that identification and administration of endothelial cell-secreted factors may be useful tools for pharmacological intervention after stroke. For example, factors secreted by normal endothelial cells could be administered to increase SVZ proliferation and then combined with factors from stroke-injured endothelial cells to promote

migration and differentiation of SVZ-derived NSCs in the injured area. Promoting angiogenesis, the formation of new blood vessels, via administration of angiogenic factors into the injured area may also serve as a useful tool to promote brain recovery, as angiogenesis and neurogenesis appear to be coupled. Potential candidate molecules include vascular endothelial growth factor, angiopoeitin, and erythropoietin, all of which are involved in angiogenesis and neurogenesis (Chang, et al., 2005, Sun, et al., 2003, Tsai, et al., 2006, Wang, et al., 2008, Wang, et al., 2004, Zhang and Chopp, 2002). However, as with the potential use of cytokines to treat neonatal brain injury, these factors may have effects on other processes as well and may produce adverse effects that outweigh their benefits, hence further investigation is needed. Although it seems ideal to promote recovery via administration of one factor, combining treatment by way of initial administration of proliferation-enhancing factors, like those from intact endothelial cells, with subsequent treatment using migration and differentiation-promoting factors from stroke-injured endothelial cells may prove to be more advantageous. Another potential caveat, however, is that promoting differentiation to replace neurons may not be useful if the injured area can not sustain these cells or if they do not integrate properly.

Although RA/EE treatment did not significantly promote long-term survival of stroke-generated neurons or provide substantial functional benefits in this study, they may still be useful treatments for stroke. One of the side effects of excessive retinoids or vitamin A is decreased bone density (Hotchkiss, et al., 2006, Kneissel, et al., 2005, Rohde and DeLuca, 2003, Rohde, et al., 1999). In

preliminary studies in which sham and tMCAO rats were treated with RA, we found that our initial dose of RA was too high, as sham-treated rats displayed decreased mobility due to limb impairment. After return to a normal diet these rats recovered, but this finding suggested that the benefit of increased proliferation came at a price. Therefore, in the studies presented within this thesis, we used a smaller dose of RA, hoping to avoid any potential negative effects. However, a few animals in our study displayed forelimb use abnormalities on the unaffected side while on the RA-enriched diet. Other animals may have been affected adversely as well, although to a lesser extent, which may have masked any benefits of neurogenesis on functional recovery. The increases in proliferation and neuroblast migration suggest that RA treatment is beneficial, however, and further studies should be performed to determine if there is a dose that substantially promotes neurogenesis without adverse consequences on motor function. In addition, perhaps the combination of RA with other physical interventions would be more beneficial in promoting neurogenesis and functional recovery. For example, exercise such as running, promotes neurogenesis (Rhodes, et al., 2003, van Praag, 2008, van Praag, et al., 1999, van Praag, et al., 1999) and when combined with RA-treatment, may provide better results than EE. Forced use of the impaired limbs may also be more beneficial in combination with RA, as forced use, when applied at the correct timeframe after stroke, promotes functional recovery (Bland, et al., 2001, Grotta, et al., 2004, Humm, et al., 1998, Kleim, et al., 2003, Schallert, 2006, Schallert, et al., 2002). Thus, RA could be administered at a low dose to

promote proliferation and migration and then combined with exercise or forced use to promote striatal neurogenesis and functional recovery.

All of these proposed treatment ideas focus on enhancing the endogenous neural stem cell population by promoting their proliferation, migration, differentiation or survival. Neural stem cells may also be used for transplantation and are often primed or directed towards a given fate prior to transplantation using techniques such as hypoxia, which specify neurons towards a dopaminergic subtype. This may not be ideal, as the internal environment into which the cells are transplanted may be subsequently altered by another insult. Our results suggest that this may lead to detrimental effects on the NSC population, rather than providing a mechanism for repair. Although the use of neural stem cells for transplantation should be pursued, we suggest that manipulating the endogenous population may be very fruitful, as this approach may be applicable to many types of brain injury and disease. This is especially true in brain diseases where proliferation is already enhanced, such as in Alzheimer's disease and Huntington's disease (Curtis, et al., 2003, Jin, et al., 2004). In this case, enhancing differentiation of endogenous NSCs into specific neuronal subtypes to replace neurons lost in these diseases may be a useful treatment strategy that may be complemented by implanting neural stem cells.

Future Directions

Further investigation into role of IL-1β in postnatal SVZ NSC regulation is needed and will be carried out in our laboratory or with our collaborators.

Preliminary experiments were performed to determine if IL-1β could rescue neurogenesis in SVZ NSCs from IL-1β knockout mice. In these initial experiments, several doses of IL-1β were applied to primary NS during differentiation. Neurogenesis appeared to be restored in NS from IL-1β knockout mice treated with IL-1β protein, compared with those treated with vehicle. These results need to be replicated and the effects of IL-1β on NSCs from wild-type also need to be determined. These experiments will be performed in the same manner as those applying exogenous IL-1β to NS from IL-1β knockout mice, and following with our previous experiments using LPS.

Similar experiments could be performed with other cytokines to determine if they play a role in inflammation-induced neurogenesis in SVZ neural stem cells. A few other interesting experiments could be performed to determine if inflammation-induced changes in SVZ neurogenesis are mediated via IL-1β. IL-1β knockout mice could be injected with LPS (or vehicle) using the same protocol as detailed in chapter 2, and then NS cultured and evaluated for NS production and differentiation effects. Similarly, LPS could be administered to NS from IL-1β knockout mice, rather than to the mice directly, and effects on neurogenesis and oligodendrogliogenesis evaluated. In this experiment, however, NS production can not be evaluated since LPS treatment occurs during differentiation. Since LPS administration increased NS production and neurogenesis and IL-1β knockout caused the opposite effect, it is hypothesized that these two may cancel each other out. However, if the LPS-induced changes were mediated through IL-1β and IL-1β is knocked-out, then administration of LPS may not

result in any changes. Another interesting experiment might be to expose IL-1β knockout mice to hypoxia and then culture NS from these animals. The mediators of hypoxia were not tested in these experiments and could also be evaluated via exposure of mice or NS to factors such as HIF-1α.

An important next step to finalize the effects of endothelial cell-secreted factors on SVZ neurogenesis is to identify what these factors are and determine their specific role in regulating neurogenesis in the normal and stroke-injured brain. Proteomic analysis of the conditioned media is an obvious choice for identifying the proteins present in intact and OGD-exposed endothelial conditioned medium. One attempt at proteomic analysis was performed, however, few candidate molecules were identified and technical issues suggest that these results may not be accurate.

Another approach that could be used is to perform candidate screening with western blot or protein microarray analyses or to perform a screen of endothelial cells using RNA interference (RNAi). Proteins that are suggested in the literature to be produced by endothelial cells could be probed for on western blots or microarrays or knocked-down using RNAi. Endothelial cells exposed to RNAi could be cultured with NS and then neurogenesis evaluated, as one would expect to see opposite effects on neurogenesis from those reported in chapter 3. Potential candidate factors could also be directly applied to primary NS and evaluated for NS production, proliferation, migration or differentiation effects. Another possible approach makes use of multiple levels of conditioned media fractioning. ECCM could be filtered with molecular weight cut-off filters in

different ranges, for example, <5kd, 5-10kD, 10-15kD and so on, and then applied to NS to determine which of the fractions are involved in expansion, proliferation, migration and differentiation. Further, these fractions could be separated by weight of specific subsets of factors (ie-proteins of size X-XX, cytokines, chemokines, etc) to further elucidate their influences on SVZ neurogenesis. Once these factors are identified, they can be tested both in vitro and in vivo. In vitro testing would allow for the specific effects presented in chapter 3 to be replicated. Most importantly, these factors need to be applied and tested *in vivo.* Comparison of *in vivo* and *in vitro* influences is vital and an important next step is to determine if these factors influence endogenous postnatal NSCs in the same way that they influence NSCs *in vitro*. Finally, these factors could also be administered to stroke animals to determine their potential in enhancing endogenous repair after injury.

In the studies described in chapters 2 and 3, the main limitation of these experiments is that they focus solely on in vitro analysis. This was necessary in order to compare various conditions in a more controlled environment. These results provide limited advancement to the field if they do not provide translational information for endogenous brain repair. Thus, the influence of hypoxia, inflammation, or the combined insult, as well as endothelial cell-secreted factors needs to be further examined in the postnatal mammalian brain. Future experiments could focus on SVZ neurogenesis in IL-1β knockout mice to determine if there are alterations in neurogenesis in these animals. These animals could be exposed to hypoxia and SVZ NSC proliferation and

differentiation examined as well. Finally, normal and stroke-injured postnatal/adult animals could be examined for changes in SVZ neurogenesis after treatment with IL-1β or endothelial cell-secreted factors.

The promise of RA as a treatment for stroke remains, although more work is needed to determine the optimal dose and timing of this treatment. As mentioned previously, RA-treatment combined with other types of therapy may be more beneficial in enhancing both post-stroke neurogenesis and functional recovery. Exercise enhances neurogenesis in the dentate gyrus and may be more beneficial than EE treatment, or EE treatment itself may be more optimal combined with RA-treatment if the duration of EE is longer. In these studies, animals received EE for only 21 days. Perhaps extending the treatment to 41 days (the timing that was used for RA-treatment) or for the duration of the study would be more beneficial than terminating EE treatment at day 28 after MCAO. It has been suggested that the simplicity of housing for laboratory animals may actually decrease neurogenesis and that EE actually restores it to baseline levels. In our study, functional improvements were seen with EE-treatment at the end of the treatment period but were lost by the time of evaluation on neurogenesis, making it difficult to speculate if these animals had EE-treatment associated enhancement of neurogenesis that was then diminished by their return to the "standard" environment. Additional studies that evaluate neurogenesis and functional recovery at the end of the treatment period and after return to standard environment may address this issue, however, comparison of

the same animal over time is not possible, again making it difficult to discern the effects of treatment versus return to the standard environment.

Yet another issue regarding enhanced neurogenesis and functional recovery after stroke is whether or not neurogenesis is necessary for enhance functional outcome. To test this, neurogenesis could be enhanced with a given treatment (RA and EE, for example) and subsequent behavior testing performed to correlate with the time frame in which new neurons have matured and contributed processes to existing circuits. Additionally, experiments in which neurogenesis is conditionally ablated after stroke and behavioral and electrophysiolocal assessments performed may also provide evidence to determine whether neurogenesis is necessary for functional recovery after stroke.

Another potential treatment strategy that could enhance striatal neuron survival and functional recovery is to combine RA treatment with forced-limb use. Studies from Tim Schallert's group suggest that forced limb-use, when applied at the proper time (too early after injury exacerbates functional deficits and too late provides no benefit) results in significant recovery of function after stroke (Grotta, et al., 2004, Kleim, et al., 2003, Schallert, et al., 2003). Although this treatment may not enhance neurogenesis alone, when combined with the proper dose and timing of RA the two may work coordinately to enhance striatal neurogenesis and promote functional recovery. Finally, once identified, factors secreted from OGD-exposed endothelial cells may also be beneficial in combination with RA to promote striatal neuron survival or integration. RA-treatment appeared to

influence neuroblast migration after stroke and in combination with stroke-injured endothelial cells could promote neuroblast migration and differentiation in the injured striatum as well.

The process of neurogenesis consists of multiple steps, including proliferation, migration, differentiation and cell survival. The factors that govern this process and their relationships to each other may be complex. Each of these processes occurs with specific spatial and temporal regulation and although each process is separate and may be regulated by distinct factors, they may also influence each other as well. In many cases, a given molecule may regulate more than one step in this process and changes to one step may influence the next. Thus, future research should continue identifying these factors and testing their roles in neurogenesis and their relationships to each other through the use of both in vivo and in vitro techniques, as demonstrated within this thesis. Such experiments should include genetic manipulations, retroviral vector labeling, neurogenesis ablation (e.g. irradiation) studies, etc to discern these relationships.

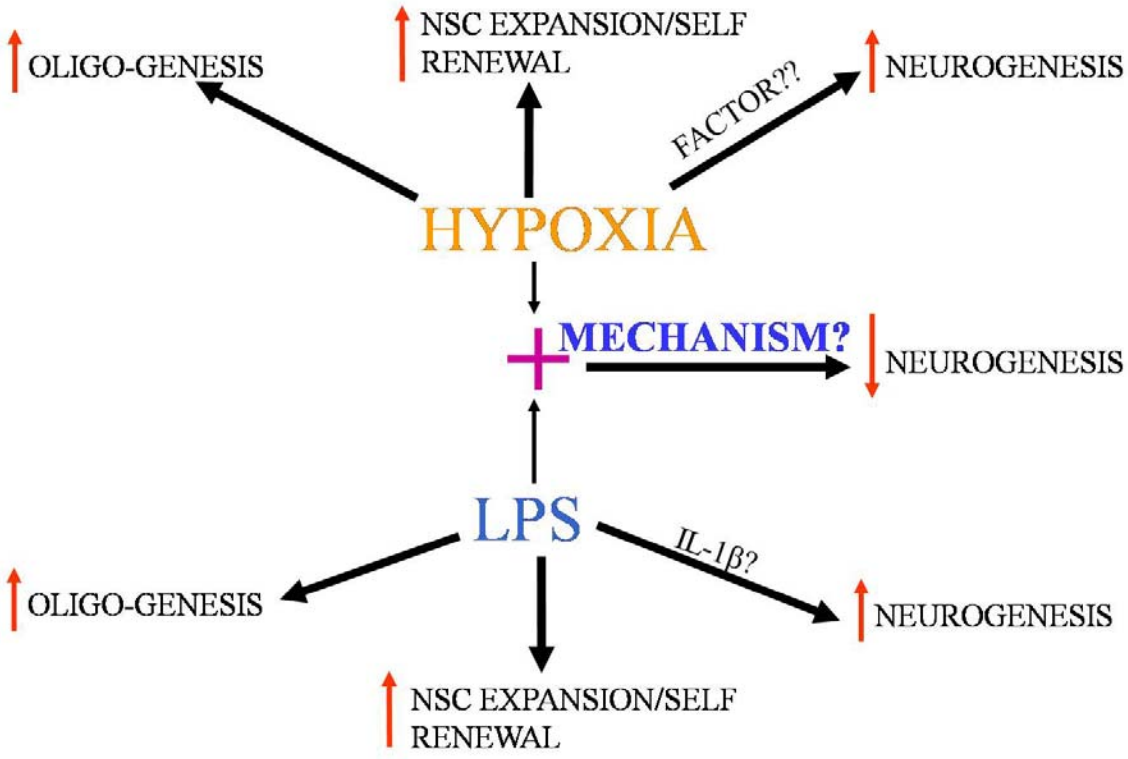

Figure 5.1: Summary of effects of inflammation and hypoxia on postnatal SVZ NSCs.

Exposure to hypoxia or inflammation (LPS) increases SVZ NS expansion, neurogenesis and oligodendrogliogenesis. Exposure to the combined insult decreases neurogenesis. The effects on SVZ neurogenesis and SVZ NS expansion appear to be mediated by IL-1β.

Condition	Timing	Self-Renewal	Migration	Process Outgrowth	Glial Effects
EC +NS (primary ECs)	co-culture	↑	----------	↓↓↓	+ +
ECCM (primary ECs)	differentiation	----------	----------	↓↓↓	(*↑↑oligos) + +
ECCM (bEND3)	during expansion	↑↑↑	NO CHANGE	↓↓↓	+ +
OGD-ECCM (bEND3)	during expansion	NO CHANGE	↑↑↑	↑↑↑	+/-

Figure 5.2: Summary of the effects of endothelial cells on postnatal SVZ NSCs.

Endothelial cell-secreted factors increased postnatal SVZ NS self-renewal and altered neuron and glial morphology. Endothelial cell-secreted factors decreased process outgrowth and increased oligodendrocyte production. OGD-exposed endothelial cells secreted factors that increased neuroblast migration, process outgrowth and altered glial morphology.

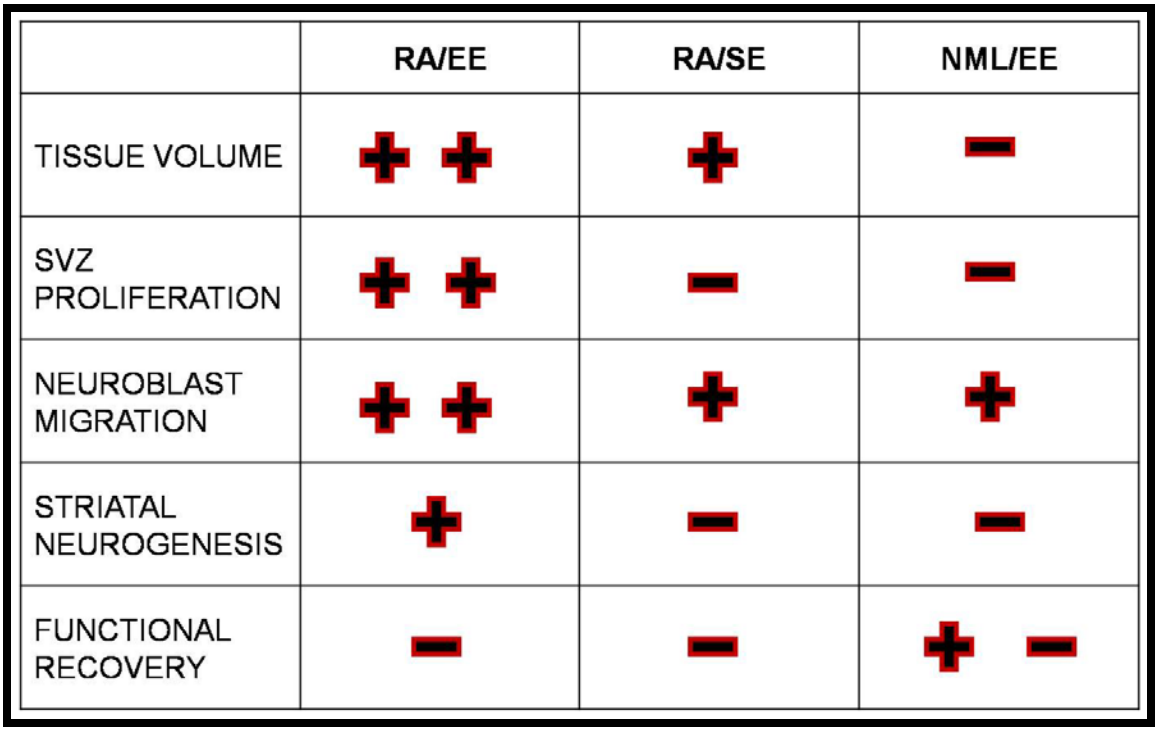

Figure 5.3: Summary of RA/EE effects on neurogenesis and recovery after stroke.

Combined RA/EE treatment provided the greatest enhancement of neurogenesis compared to other conditions. No treatment significantly improved functional recovery.

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Appendix

Primary Antibodies	Species	Concentration	Target	Company
β-Tubulin (TUJ1)	Mouse	1:400	Neuronal marker	Covance
β-III-Tubulin	Rabbit	1:1000	Neuronal marker	Epitomics
BrdU	Mouse	1:1000	Proliferating cells	Roche
BrdU	Rat	1:25	Proliferating cells	Serotec
BrdU	Rat	1:250	Proliferating cells	Accurate Chem.
Caspase-3	Rabbit	1:1000	Apoptotic cells	BD Biosciences
CD-31	Rat	1:200	Endothelial Cells	Chemicon Int.
CD-68	Mouse	1:100	Microglia	Serotec
Doublecortin (C-18)	Goat	1:2500	Immature neurons	Santa Cruz
Doublecortin	Rabbit	1:1000	Immature neurons	Private
GFAP	Rabbit	1:500	Astrocytes	DakoCytomation
GFAP	Mouse	1:500	Astrocytes	Sigma
Glut-1	Rabbit	1:400	Endothelial Cells	Chemicon Int.
Ki67	Rabbit	1:500	Proliferating Cells	Vector Labs
Kip1/p27	Mouse	1:1000	Cell Cycle	BD Biosciences
MAP2	Mouse	1:500	Neurons (A, B and C)	Sigma
MAP2	Mouse	1:500	Neurons (2A +2B)	Sigma
MAP2	Mouse	1:500	Neurons(2A +2B)	Sigma
MBP	Rat	1:1000	Oligodendrocytes	Chemicon Int.
Nestin	Mouse	1:100	Progenitors	Chemicon Int.
Nestin (Rat-401)	Mouse	1:10	Progenitors	Hybridoma
NeuN	Mouse	1:1000	Mature neuron	Chemicon Int.
NG2	Rabbit	1:500	Glial progenitors	Chemicon Int.
RC2	M(IgM)	1:50	Radial Glia	Hybridoma
Reca-1	Mouse	1:100	Endothelial Cells	Serotec
Rip	Mouse	1:200	Oligodendrocytes	Chemicon
Sox2	Rabbit	1:5000	Neural Stem Cells	Chemicon
ZRF-1	Mouse	1:200	Radial Glia	Zfin

Secondary Antibodies	Species	Concentration	Company
Alexa 488	Goat-anti-Mouse	1:400	Molecular Probes
Alexa 488	Goat-anti-Rabbit	1:400	Molecular Probes
Alexa 488	Goat-anti-Rat	1:400	Molecular Probes
Alexa 488 (IgM)	Goat-anti-Mouse	1:400	Molecular Probes
Alexa 594	Goat-anti-Rabbit	1:400	Molecular Probes
Alexa 594	Goat-anti-Mouse	1:400	Molecular Probes
Alexa 594	Goat-anti-Rat	1:400	Molecular Probes
Biotinylated IgG	Donkey-anti-Goat	1:200	Molecular Probes
Biotinylated IgG	Goat-anti-Rabbit	1:200	Vector
Biotinylated IgG	Horse-anti-Mouse	1:200	Vector