

● INVITED REVIEW

Unmasking the responses of the stem cells and progenitors in the subventricular zone after neonatal and pediatric brain injuries

Mariano Guardia Clausi, Ekta Kumari, Steven W. Levison*

Department of Pharmacology, Physiology and Neuroscience, Rutgers-New Jersey Medical School, Newark, NJ, USA

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Abstract

There is great interest in the regenerative potential of the neural stem cells and progenitors that populate the subventricular zone (SVZ). However, a comprehensive understanding of SVZ cell responses to brain injuries has been hindered by the lack of sensitive approaches to study the cellular composition of this niche. Here we review progress being made in deciphering the cells of the SVZ gleaned from the use of a recently designed flow cytometry panel that allows SVZ cells to be parsed into multiple subsets of progenitors as well as putative stem cells. We review how this approach has begun to unmask both the heterogeneity of SVZ cells as well as the dynamic shifts in cell populations with neonatal and pediatric brain injuries. We also discuss how flow cytometric analyses also have begun to reveal how specific cytokines, such as Leukemia inhibitory factor are coordinating SVZ responses to injury.

Key Words: CNS regeneration; cytokines; glial progenitors; gliogenesis; inflammation; cerebral palsy; traumatic brain injury; stroke

*Correspondence to:
Steven W. Levison, Ph.D.,
levisosw@rutgers.edu.

orcid:
0000-0002-5531-5216
(Mariano Guardia Clausi)
0000-0001-8409-3942
(Ekta Kumari)
0000-0002-1264-7309
(Steven W. Levison)

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Introduction

Over the past decade many studies have reported increased cell proliferation in the subventricular zone (SVZ) during recovery from neonatal and pediatric brain injuries. Both neural stem cells (NSCs) and neural progenitors (NPs) are proliferative cells that populate the SVZ niche. NPs have limited self-renewal capacity and most NPs divide ~8 times before they become postmitotic (Raff et al., 1988). NSCs by definition are self-renewing cells that are competent to proliferate across the lifespan. They are multipotential, whereas NPs are either multipotential, bipotential or unipotential (Buono et al., 2012). There has been little consensus on which cells are proliferating in response to neonatal and pediatric brain injuries. For example, using a rat model of perinatal hypoxia-ischemia (H-I), Felling et al. (2006) reported increased proliferation of periventricular Nestin⁺ cells in the SVZ 3 days after injury. They also established that there was an increase in the number of neurospheres formed, which was at that time regarded as index of the number of NSCs (Felling et al., 2006). Thus, these data suggested that this neonatal injury was increasing the number of NSCs in the SVZ. However, in a mouse model of perinatal stroke, Spadafora et al. (2010), found that most of the new neurons produced after injury were not derived from NSCs, but were arising from more restricted NPs (Spadafora et al., 2010). In this study, the authors used intra-ventricular injection of lentivirus-expressing green fluorescent protein (GFP) to label the NSCs. An increase in the numbers of doublecortin-positive

(DCX⁺) immature neurons in the striatum after the stroke was observed, but those cells did not express GFP. Based on these data, they concluded that these new neurons were produced from NPs that were activated in response to the stroke. More recently, using a model of pediatric traumatic brain injury, Goodus et al. (2015) reported that there was a strong proliferative response of cells that are Nestin⁺ and achaete-scute homolog 1-negative (ASCL-1⁻) (presumptive NSCs) that preceded the production of new neurons (Goodus et al., 2015). Goodus et al. (2015) also showed that there was an increase in the numbers of neurospheres produced from the injured SVZ.

There were numerous differences in these 3 studies including, animal species, ages of the animals evaluated, and criterion applied to define the NSCs, each of which could have contributed to the different interpretations of the results reported. Alternatively, the NSCs and NPs could be responding differently to each type of injury. Felling et al. (2006) investigated the cell proliferation after perinatal H-I using double immunofluorescence for proliferating cell nuclear antigen (PCNA) (a marker for cell proliferation) and Nestin (a marker frequently used to identify NSCs). That particular study showed a significant increase in PCNA/nestin double positive cells in the injured hemisphere. They also restricted their analyses to cells found within 30 μm of the ventricular wall, which is considered the NSC niche. Whereas they observed increased numbers of Nestin⁺/PCNA⁺ cells in the medial aspect of the SVZ, there were no significant differences

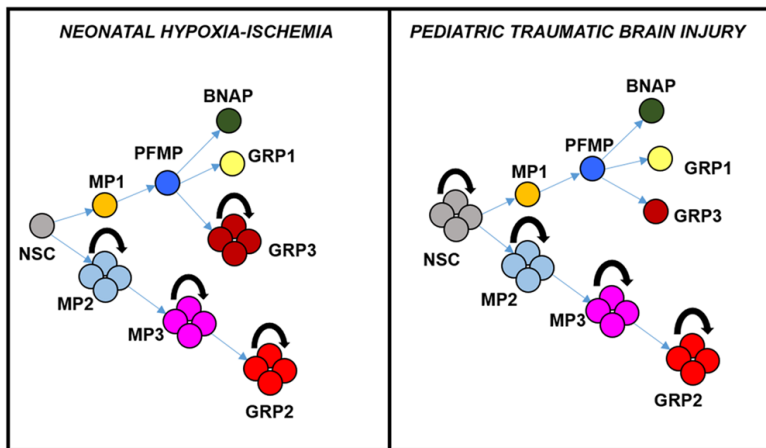


Figure 1 Distinctive responses of subsets of subventricular zone (SVZ) cells to brain injuries.

As detailed in the text, a variety of immature cells reside in the SVZ that can be delineated using flow cytometry. This lineage model summarizes how these different cell types respond to neonatal hypoxia-ischemia (H-I) vs. traumatic brain injury. Whereas neonatal H-I increases the proportion of MP2, MP3/GRP2 and GRP3s, traumatic brain injury increases the numbers of NSCs, MP2, and MP3/GRP2s. BNAP: Bipotential neuronal-astrocytic progenitor; GRP: glial-restricted progenitor; NSC: neural stem cell; PFMP: PDGFR-FGF2-responsive MP cell.

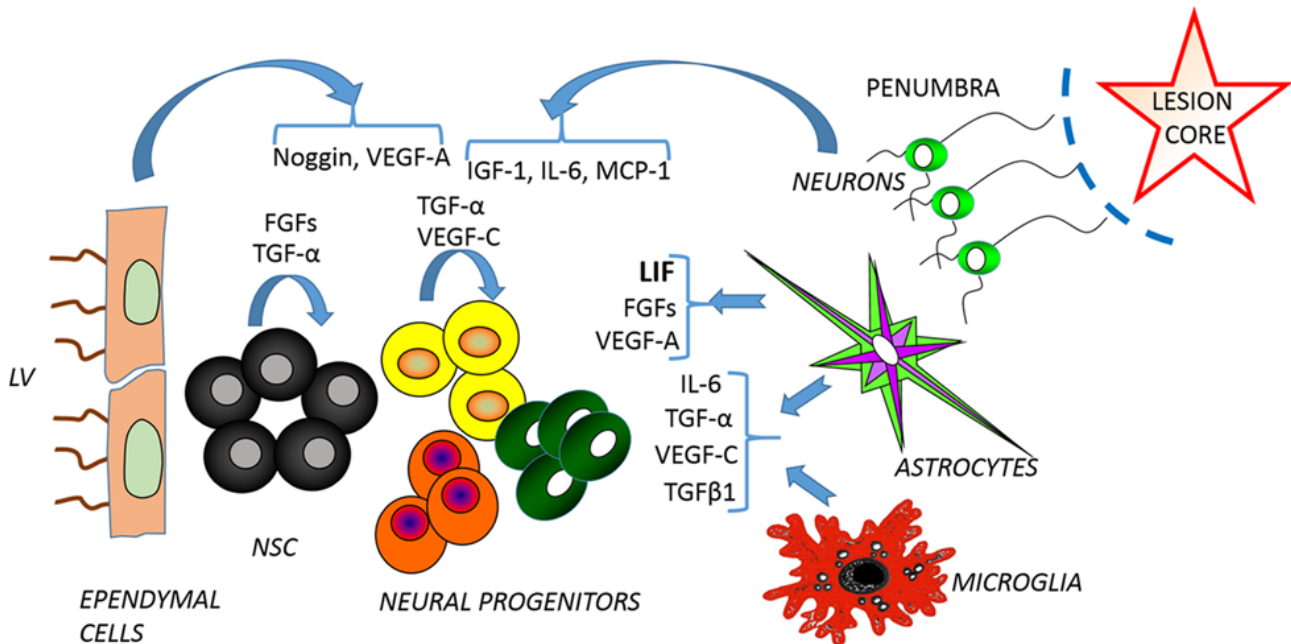


Figure 2 Multiple signals affect subventricular zone (SVZ) cell responses to brain injury.

Neural stem cells (NSCs) and progenitors are constantly exposed to multiple extracellular signals. These signals come from multiple sources; they are produced by local cells that form the specialized microenvironment of the SVZ, other signals produced by neurons, astrocytes, microglia and macrophages diffuse into the SVZ from the injured brain tissue. The magnitude and types of signals produced depend on the degree and type of brain damage. FGFs: Fibroblast growth factors; IGF-1: insulin-like growth factor 1; IL-6: interleukin 6; LIF: leukemia inhibitory factor; MCP-1: monocyte chemoattractant protein-1; TGF- α : transforming growth factor alpha; TGF β 1: transforming growth factor beta 1; VEGF-A: vascular endothelial growth factor A.

throughout the entire dorsolateral SVZ in the number of PCNA/Nestin double positive cells, indicating a differential response by the putative NSCs vs. progenitors of the SVZ.

In another study, Wang et al. (2007) asked whether hyperbaric oxygen therapy could promote NSC proliferation after H-I in neonatal rat (Wang et al., 2007). They exposed the animals to 60 minutes of hyperbaric oxygen beginning 3 hours after neonatal H-I, and then administered bromodeoxyuridine (BrdU) 5 consecutive times every 8 hours, euthanizing the animals at 7 days of recovery. They then quantified the numbers of BrdU⁺/Nestin⁺ cells across the entire dorsolateral SVZ. They found that there were more BrdU⁺/

Nestin⁺ cells in the hyperbaric group compared to controls, which they interpreted as an increase in the numbers of proliferating NSCs. However, their approach was flawed both in that they did not restrict their analyses of BrdU⁺/Nestin⁺ cells to the NSC niche, and they relied solely upon Nestin as their NSC marker. While Nestin is an intermediate filament protein that is expressed by neuroepithelial stem cells, it also is present in multiple immature cells in the CNS as well as in reactive astrocytes (Johansson et al., 2002).

In the most recent study cited above, Goodus et al. (2015) used a variety of approaches to unmask the identity of the cells that were proliferating in the SVZ after neonatal and

adolescent traumatic brain injuries (Goodus et al., 2015). Using immunofluorescence for Nestin, Ascl-1 (a marker not present in NSCs but expressed by multipotential progenitors) and Ki67 (to identify the proliferating cells) they found that there was an increase in the numbers of Nestin⁺/Ascl-1⁻/Ki-67⁺ cells residing immediately adjacent to ependymal cells in the medial aspect of dorsolateral SVZ (presumptive NSCs) subsequent to a controlled cortical impact. In contrast, there was less proliferation of the Nestin⁺/Ascl-1⁺/Ki-67⁺ cells (multipotential progenitors) residing throughout the SVZ, reminiscent of the studies by Felling et al. (2006).

Reconciling the results of these studies requires both precision in how NSCs and NPs are defined and also the use of more sensitive measures to distinguish NSCs from transit amplifying neural progenitors. Given the intense interest in the precursors of the SVZ, many labs have been characterizing the types of cells that reside within this germinal matrix using a variety of antigenic features and sometimes morphological and geographical criteria. Alphabetic lexicon has become popular that parses the cells into A, B and C cells. While this classification scheme is widely used, it is far too simplistic, as it is clear that the SVZ is comprised of NSCs, multipotential progenitors (MPs), bipotential progenitors (BPs) and unipotential precursors (Levison and Goldman, 1997). Indeed, because neurobiologists have not have the tools needed to distinguish NSCs from the MPs and BPs residing in the SVZ many investigators have resorted to calling the primitive cells of the SVZ, neural stem/progenitors (NSPs).

A New Approach to Study the Cells Residing within Stem Cell Niches

Fairly recently, investigators have moved towards using multicolor flow cytometry to character the cells of the SVZ and this approach has uncovered the true complexity of the cellular make-up of this germinal zone. The advantage of flow cytometry is that the expression of multiple markers can be analyzed simultaneously to study heterogeneous populations of cells. Several years ago we used 2-color flow cytometry to characterize the responses of SVZ cells to neonatal H-I and demonstrated that epidermal growth factor receptor (EGFR) expression increased in different populations of NPs (identified as NG2⁺ and polysialylated neuronal cell adhesion molecule-positive (PSA-NCAM⁺) cells) as well as the NSCs (identified as Lex⁺ cells) (Alagappan et al., 2009). To more precisely distinguish the different populations of progenitors within the neonatal SVZ, Buono et al. (2012) developed a 4 marker flow cytometry panel comprised of CD133, LeX, NG2 and CD140a to parse the cells of the neonatal SVZ into 8 distinct SVZ subtypes (Buono et al., 2012). The multipotential progenitors included: NSC (CD133⁺LeX⁺NG2⁻CD140a⁻), MP1 (CD133⁺LeX⁺NG2⁻CD140a⁻), MP2 (CD133⁺LeX⁺NG2⁺CD140a⁻), MP3 (CD133⁻LeX⁻NG2⁺CD140a⁻), MP4 (CD133⁺LeX⁺NG2⁺CD140a⁺) and PDGFR-FGF2-responsive MP cell (PFMP) (CD133⁻LeX⁻NG2⁺CD140a⁺). There were 4 types of bipotential progenitors identified that included the bipotential neuronal-astro-

cytic progenitor (BNAP) (CD133⁻LeX⁺NG2⁺CD140a⁻) and 3 glial-restricted progenitors (GRP1) (CD133⁻LeX⁺NG2⁺CD140a⁻), GRP2 (CD133⁻LeX⁻NG2⁺CD140a⁻) and GRP3 (CD133⁻LeX⁻NG2⁺CD140a⁺).

This tool has allowed more detailed analyses of SVZ cell responses to brain injury. As reported recently by Buono et al. (2015), neonatal H-I increased the proportion of several MPs and BPs that included MP2s, MP3/GRP2s as well as GRP3s (Buono et al., 2015). However, to their surprise, the numbers of NSCs in the SVZ decreased. In contrast, Goodus et al. (2015) reported an increase in MP2s, GRP2/MP3s and an increase in the NSCs in response to pediatric traumatic brain injury (**Figure 1**). To further evaluate whether the increase in these specific subsets of neural precursors in response to brain injury could be attributed to changes in their proliferation; the incorporation of the thymidine analogue EdU was added to the flow cytometry protocol. Unlike the commonly used BrdU, Edu detection requires no acid treatment (Buck et al., 2008) which makes it compatible with the staining of the other four makers of the flow panel. In fact, we found increased incorporation of EdU in the MPs and GRPs 24 hours after neonatal H-I and after pediatric traumatic brain injury, which correlated with their increased frequency at 48 hours after injury (Buono et al., 2015). On the other hand, the strong trend toward fewer EdU⁺ NSCs after neonatal H-I suggested that these cells are proliferating more slowly or that they are dividing symmetrically to expand the population of MP.

Factors Modulating the Response of the Stem Cell Niche to Brain Injury

NSCs reside within specific compartments in the brain that provide signals to maintain their stemness. The signals present within these niches can change subsequent to brain injuries (**Figure 2**). Studies profiling the cytokines that increase after injury have established that leukemia inhibitory factor (LIF) is significantly increased within 1 day in several models of neonatal and pediatric injury and thus correlates with the increase in NSCs and progenitors (Covey and Levison, 2007). Loss-of-function studies using mice heterozygous for LIF in the neonatal H-I model revealed that the expansion of MP3/GRP2s, GRP3s and MP2s were blunted, indicating that these progenitors require LIF signaling to expand after injury (Buono et al., 2015). But, these responses become blunted with age. Using a model of pediatric traumatic brain injury (TBI), we demonstrated that there is a strong proliferative response of the SVZ which decreases with the age of the animals, being almost null in 60 days old rats (an age equivalent to young adults) (Goodus et al., 2015). Studies have found that the intrinsic properties of the stem cells and progenitors change with age. For example, levels of telomerase decline in the SVZ with aging (Conover and Shook, 2011). Additionally, changes to the niche occur with age. For example, transforming growth factor beta (TGF- β) levels increase during aging which correlates with decreased neurogenesis (Buckwalter et al., 2006; Pineda et al., 2013) and levels of IGF-II decrease with aging, and IGF-II is an important stem

cell maintenance factor (Ziegler et al., 2015).

Concluding Remarks and Future Perspectives

A successful regenerative response to injury requires not only neural progenitor cell proliferation but also migration, maturation and functional integration into the existing neural circuitry. While we have begun to uncover the complexity of the SVZ response to neonatal and pediatric brain injuries, more studies are necessary to understand the type of cells that each neural precursor generates and how these cells interact with the damaged tissue. One concern is that it is not clear whether the most appropriate types of new cells are formed. For example, the majority of the newly generated cells differentiate into astrocytes with fewer becoming mature neurons or new oligodendrocytes. Moreover, after H-I virtually all of the new neurons produced are calretinin⁺ interneurons (Yang et al., 2008). This is further compounded when it becomes apparent that most of the new neurons have disappeared by 30 days after they were produced (Goodus et al., 2015). Using flow cytometry we now have the opportunity to glean new insights into which cells are expanding after injury and which signaling molecules are coordinating their response. By understanding the endogenous mechanisms of cell replacement it is conceivable that interventions can be designed to enhance repair of the damaged brain.

Many research labs are presently working to identify therapeutics that will stimulate the proliferation of oligodendrocyte progenitors to promote myelination in those pathologies where the white matter is primarily affected such as in diffuse white matter injury accompanying preterm birth. Other laboratories are trying to identify therapeutics to promote neuroblast proliferation and migration so that SVZ cells can be coerced to replace neurons that have died subsequent to a stroke or after a traumatic brain injury. The studies reviewed above indicate that it will be important to understand why the younger brain elicits a stronger proliferative response than the adult brain. Revealing the underlying mechanisms should enable interventions to revive the adult neurogenic niche. All of these are important goals to pursue so that more productive regeneration can occur after brain injuries.

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