Endogenous Stem Cells in the Adult Murine Spinal Cord

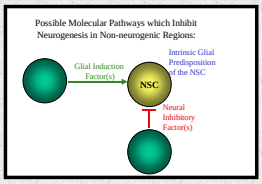
J. Rowlett , D. Imrie, T. Miller, and J.D. Flax*

Dept. of Neurology, The University of Arizona, Tucson, Arizona

BACKGROUND & SIGNIFIGANCE

Neural stem cells (NSCs) are cells capable of self-renewal and differentiation into both neurons and glia. These cells have be isolated from all regions of the neuroaxis in the adult central nervous system, suggesting that these endogenous NSC could be utilized for replacement of degenerated neurons. However, new neurons appear to be born *in vivo* in only a select few germinal regions of adult mammals. For instance, the subventricular zone [SVZ] surrounding the lateral ventricles of the brain continuously generates the neurons of the olfactory bulb while NSCs in the spinal cord fail to produce new neurons *in vivo*. Interestingly, when grow in culture, adult spinal cord-derived NSCs do give rise to neurons, suggesting that local microenvironmental cues present in the mature spinal cord may suppress neural differentiation, or alternatively, glial inducing signals may predominate in the spinal cord. While much is known about the molecular networks that regulate NSC differentiation, little is known about how these pathways are utilized to regulate endogenous stem cell differentiation in a non-germinal zone such as the spinal cord. As most of the central nervous system inhibits neural differentiation of NSCs, it is crucial that we understand the mechanisms that regulate this block. Conceivably, by overcoming this block to neurogenesis it may be possible to direct endogenous NSCs to become neurons, replacing those neurons lost to disease and injury. In order to pursue these objectives we are exploring the mechanisms that thwart neuronal differentiation of NSC in the adult murine spinal cord.

HYPOTHESIS



While a glial predisposition may be a component of the block to NSC neurogenesis in the adult spinal, exogenous glial inductive or neural inhibitory signals, produced by cells in the NSC niche, are more likely to encourage gliogenesis. We are exploring the role of two such factors in regulating NSC fate in the adult murine spinal cord: Notch1 and Bone Morphogenic Protein family members(BMP).

AIMS

- Develop markers for identification of NSCs in the adult spinal cord *in situ*.
- Determine if the neural inhibitory factors Notch and BMP are active in NSCs in the adult spinal cord.
- Develop an organotypic culture system for testing the effect of modulating Notch and BMP activity on stem neurogenesis.

METHODS

Neural stem cell culture: Spinal cords were isolated from either 5-7 week old adult or 12.5 days gestation embryonic C57BL/6J animals, dissociated with papain, and plated in neurobasal medium, N2 supplement, 20 ng/ml bFGF, 10 ng/ml EGF (Invitrogen), and 2 µg/ml heparin (Sigma). Neurospheres were expanded from these cultures for 2 weeks and processed for immunocytochemistry.

Intact spinal cords: Spinal cords were isolated from either 5-7 week old adult or 12.5 days gestation embryonic C57BL/6J animals and processed for immunocytochemistry.

Immunofluorescence for neural stem cells and progeny markers: Cryosections from neurospheres and intact sections derived from adult and embryonic were spinal cord were labeled with panels of primary antibodies potentially identifying neural stem cells; nestin, Sox1, Sox2, & GFAP (Chemicon) and detected with cy2, cy3 and cy5 conjugated secondary antibodies (Jackson ImmunoResearch). Sections were scanned using a Zeiss LSM 510 NLO Metaconfocal microscope.

Immunohistochemistry for cleaved Notch1 and anti-phospho SMAD: Intact adult or embryonic spinal cord was paraffin embedded and labeled with antibodies which identify activated Notch and Bone Morphogenic Protein pathways (anti-cleaved Notch1, Anti-phospho SMAD 1, 5 & 8, and anti-phospho STAT3, Cell Signaling Technologies) and detected using a Vector ABC, peroxidase kit.

Organotypic cultures of adult spinal cord: Spinal cords from 5-7 week old C57BL/6J animals was removed and sectioned into 300 µm thick coronal sections. These were placed on a 24mm transwell insert (0.4 µm pore, polycarbonate membrane, Costar). Medium was either basal medium (50% MEM, 25% HBSS, 25% horse serum plus 6 mg/ml D-glucose) or medium supplemented up to 50% native bovine hemoglobin (Biopure) and 10 µM CNQX and 10 µM MK-801 (Sigma). Sections were cultured for up to 14 days prior to processing for cell viability or immunohistochemistry.

Cell viability assay: Propidium Iodide (7.5 µM final, Molecular Probes) was added to cultures one hour prior to viability assessment. Cultured sections were removed for culture chambers, washed in PBS and observed using confocal microscopy.

Section processing and immunofluorescence: Sections will be immersed in 4% paraformaldehyde, paraffin embedded, and sectioned, and processed for anti-cleaved Notch1 and anti-phospho SMAD 1, 5 & 8 immunohistochemistry.

RESULTS: NSC MARKERS

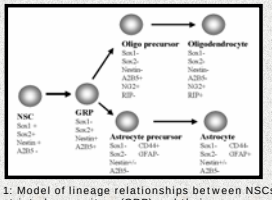


Figure 1: Model of lineage relationships between NSCs, glial restricted progenitors (GRP) and their progeny.
This model is based on the one developed by Liu et al., 2002 for lineage relationships during spinal cord development.

- No single marker identifies NSCs and GRPs.
- A panel of partially overlapping markers can identify each of these stages along this developmental pathway.

RESULTS: NSC MARKERS cont.

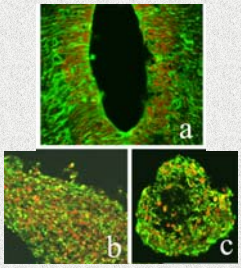


Figure 2. Development of a panel of markers for NSCs in the adult spinal cord.

- **Strategy:** test various panels of NSC markers (see Figure 1) for co-expression in known NSCs (embryonic spinal cord and adult and embryonic-derived NSCs grown in culture).
- **Results:** Here tissue was double-labeled with two stem cell/progenitor markers; the transcription factor Sox2 (red) and the intermediate filament Nestin (green) and captured with confocal microscopy. a) gestational day E12.5 spinal cord neuroepithelium, note that Sox2 is only present cells in the neuroepithelium around the central canal and not in the differentiated cells in the mantle. The nestin positive radial glia continue more laterally, sending processes to the pia. b) E12.5 day-derived spinal cord NSCs expanded *in vitro* and c) Adult spinal cord-derived NSCs expanded *in vitro*. In both cases at least a portion of the NSCs containing neurospheres co-express Sox2 & nestin.
- **Conclusion:** Both embryonic and adult stem cells express the NSCs markers Sox1 & 2 and Nestin, and at least a portion of these co-express these markers. Therefore these markers may be used to identify NSCs in the adult spinal cord.

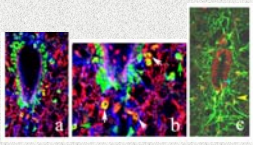


Figure 3. Identification of presumptive NSCs and committed precursors in the SVZ of the adult murine spinal cord.

- **Strategy:** test various panels of NSC marker for co-expression in the adult murine spinal cord.
- **Results:** a) Illustrates the central canal region, with b) being a higher magnification image of the ventral portion of the SVZ. Sox2 (green) labels both ependymal cells flanking the central canal as well as cells in the SVZ and immediate area. Sox1 (red) labels neurons and their processes but also co-localizes with Sox2 in a small number of cells primarily in the ventral portion of the SVZ (white arrows). Sox1 & 2 double positive cells are not observed elsewhere in the cord suggesting that these cells may represent NSC. Interestingly, The Sox1 & 2+ cells are nestin negative (blue), however as NSCs in the lateral ventricular SVZ express GFAP we examined the co-expression of GFAP (green) and Sox2 (red) (Figure 3c). A significant number of GFAP/Sox2+ cells were identified in the ventral region (yellow arrow). Of note, we observed that a portion of these cells extended processes through the ependyma into the central canal (blue arrow). This behavior has been observed in a portion of the NSCs present in the SVZ of the adult lateral ventricle , again suggesting that these are NSCs (Lim et al., 2000).
- **Conclusion:** NSCs appear to be located in the adult spinal cord in the ventral SVZ. As NSCs are found in the SVZ of the brain, it suggests that this region is specialized NSC niche, essential for NSC regulation.

RESULTS: ORGANOTYPIC CULTURES

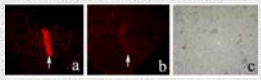


Figure 4. Development of an organotypic model of the adult spinal cord.
An organotypic culture of spinal cord would facilitate the rapid testing of agonists and antagonists of pro-glial and anti-neuronal factors in a three dimensionally intact, cytoarchitecturally appropriate environment. As inhibition of neurogenesis is likely to involve local cell communication, preservation of the microenvironment is essential.

While traditional organotypic cultures have been derived from postnatal animals, there appear to be abnormalities in glial development, and it is unknown if the germinal zone in the SVZ forms appropriately. Thus, initial studies have been aimed at developing an organotypic culture from adult mice. As the region is fully mature at the time of culture, the limitations observed in postnatal cultures are not a concern. However, hypoxia is a significant problem in adult derived cultures.

- **Strategy:** Minimize hypoxia by using bovine hemoglobin would efficiently transfer oxygen to the center of the tissue slice. In addition, we attempted to circumvent the exitotoxic effects of hypoxia by using glutamate antagonists.
- **Results:** Using a dose response curve, we found that high levels of hemoglobin in combination with glutamate antagonists greatly enhanced survival of neurons and glia within the cultures. Slices were cultured for 6 days, treated with the cell death marker dye propidium iodine (PI). Live, PI treated slices were then observed using confocal microscopy. With low levels of hemoglobin significant cell death is observed, including the ependyma and its environs (a, arrow is dead ependymal cells). Alternatively as seen in b), when 50% v/v of hemoglobin are used in the culture medium, significantly less death is observed (including the ependyma, arrow). Histologic analysis of these 50% hemoglobin containing culture illustrates significant survival of neurons and glia (Fig. 4c).
- **Conclusion:** Initial results suggest significant cell viability, however the presence of NSCs and the preservation of the SVZ stem cell niche must be assessed.

RESULTS: NOTCH & BMP ACTIVITY

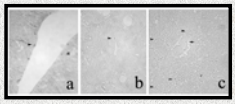


Figure 5. BMP signaling absent from the periependymal region of the intact, but present in the hypoxic adult murine spinal cord. BMP has been demonstrated to be a key inhibitor of neurogenesis in the SVZ of the brain (Lim et al., 2000). We hypothesize that BMP signaling will similarly act as an inhibitor of neurogenesis in the adult cord.

- **Strategy:** As a first step in exploring the role of BMP in regulating neurogenesis in the cord, the activity and location of BMP signaling in the intact or hypoxic adult spinal cord was assessed. Antibodies which recognize the presence of BMP's down stream activated signal cascade phospho-SMAD 1, 5, & 8 were used for this purpose.
- **Results:** (a) BMP signaling (as expected) is operational in the E 12.5 da. embryonic neuroepithelium (arrows indicate some cells actively responding to BMP). (b) Little or no BMP signaling is observed in the intact adult periependymal region, (c) however in adult spinal cord organotypic cultures, cultured for 24 hours with no hemoglobin or glutamate antagonists, so as to induce hypoxic injury, some nuclei are labeled with the anti-phospho-SMAD. This suggests that injury may activate the BMP system. We are currently exploring if BMP signaling is activated in NSCs or committed progenitors, and thus gain insight into to timing and possible roles of this signaling in NSC behavior.
- **Conclusion:** This data suggests that BMP signaling may not be present in quiescent NSCs, but that injury may activate the BMP system. We are currently exploring if BMP signaling is activated in NSCs or committed progenitors, and thus gain insight into to timing and possible roles of this signaling.

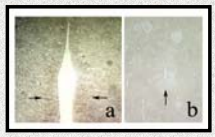


Figure 6. Notch1 signaling is present in the embryonic neuroepithelium of the spinal cord but absent from the periependymal region of the intact adult murine spinal cord. Like BMP, Notch1 signaling has been demonstrated to be a key inhibitor of neurogenesis during development (reviewed in Panchision & McKay, 2002). We hypothesize that Notch1 signaling will similarly act as an inhibitor of neurogenesis in the adult cord.

- **Strategy:** As a first step in exploring the role of Notch1 in regulating neurogenisis in the cord, the activity and location of Notch1 signaling in the intact adult spinal cord was assessed. Antibodies which recognize the presence of cleaved, intracellular fragment of the Notch1 receptor, an indicator of Notch activity, was used for this purpose.
- **Results:** Significant levels of activated Notch1 is present in the E12.5 day embryonic spinal cord neuroepithelium (the region bounded by the arrows in a), but not in the SVZ of an intact adult mouse (b, arrow indicates the ependyma).
- **Conclusion:** As Notch plays a critical role in NSC renewal and differentiation it is not surprising that a high level of activity is observed at E12.5, however given the presence of both Notch and its ligand delta mRNA in the adult periependymal region, it is surprising that Notch signaling is not observed. It is conceivable that injury may be required to activate Notch signaling (as per BMP signaling above).

CONCLUSIONS & FUTURE AIMS

- We have identified presumptive markers of NSCs in the adult murine spinal cord. However lineage studies are required for confirmation of the veracity of these markers.
- NSCs appear to be located in the adult spinal cord in the ventral SVZ. As NSCs are found in the SVZ of the brain, it suggests that this region is specialized NSC niche, essential for NSC regulation.
- BMP and Notch1 activity are not present in the periependymal region of the intact adult spinal cord, but at least BMP appears to be activated in this region following hypoxic injury. As almost no mitosis (and presumably NSC differentiation) is occurring in the intact spinal cord SVZ, it would be parsimonious for the system to activate BMP and Notch only when it is required for stem cell renewal and lineage determination (e.g. during injury).
- We are currently exploring whether BMP signaling in occurring in NSCs or in other cells within the NSC niche. This may help determine whether this factor is acting directly or indirectly on NSCs.
- Preliminary studies on organotypic culture suggest reasonable survival of cellular constituents. We are currently evaluating the preservation of the NSC niche in these cultures.
- Future studies will be aimed at assessing the effect of BMP blockade on NSCs.

REFERENCES

Lim et al., Noggin Antagonizes BMP Signaling to Create a Niche for Adult Neurogenesis. Neuron, 2000, 28: 713–726.

Liu et al., Oligodendrocyte and astrocyte development in rodents: an in situ and immunohistological analysis during embryonic development. Glia, 2002, 40:25-43.

Panchision & McKay, The control of neural stem cells by morphogenic signals. Current Opinion in Genetics and Development, 2002, 12:478–487

ACKNOWLEDGEMENTS

This work was supported by the Himelic Neuromuscular Research Foundation.