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Title: Spinal cord proliferation and differentiation: the role of neural tube closure
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Introduction.

Our research is focused on studying the development of the central nervous system. Specifically, we are interested in the developmental role played by the closure of the neural tube, the structure that gives rise to the brain and spinal cord. Spina bifida, or failure of the neural tube to close, is the model we are using to study the developmental role of neural tube closure. Spina bifida in humans is characterized by overgrowth of neural tissue in the open spinal cord, and this overgrowth leads to incontinence and paralysis of the lower limb (Northrup & Volcik, *Curr Probl Pediatr* 2000, 30(10): 313-32). To determine the role that neural tube closure plays in regulating this overgrowth, and how this overgrowth relates to the regulation of the production of different cell types within the nervous system, we are analyzing the mouse mutant *Splotch-delayed* (Sp^d). Sp^d mice carry a spontaneous mutation within the gene *Pax3,* which causes a spina bifida-associated overgrowth very similar to the human form of the defect.

Original Aims of the project:

There were 3 aims of the original project.

1. To examine how the failure of the neural tube to close affects proliferative and differentiated populations of the spinal cord.
2. To determine whether an imbalance in the normal proliferative and cell death processes underlies the overgrowth seen in spina bifida, and how such an imbalance occurs.
3. To evaluate whether rescue of neural tube closure in Sp^d embryos using Folic Acid (FA) is sufficient to rescue these neuronal defects.

Project successes.

We have demonstrated that the failure of the neural tube to close results in the overgrowth of both differentiated and proliferative regions, in both the dorsal and ventral halves of the neural tube. We have also established that this overgrowth is directly related to an increase in proliferation, and is not mitigated by an increase in cell death. Interestingly, we have also shown that closure of the Sp^d neural tube, using FA supplementation, is sufficient to rescue these defects.

The following data support our conclusion that closure of the neural tube is an important step in regulating neurogenesis in the neural tube:

1. The overgrowth of the Sp^d neural tube in the spina bifida region is evenly distributed along the dorso-ventral axis of the neural tube, and present in both proliferative and differentiated regions at E12.5. These changes are in comparison to wild type littermate controls, and are not present in the unaffected, anterior Sp^d neural tube. These data suggest that closure regulates neurogenesis in the neural tube.
2. Despite the appearance of spina bifida in Sp^d at E10.5, when the neural tube has just failed to close, preliminary data indicates that the overgrowth of the proliferative and differentiated regions has not yet occurred. Thus, the disregulation of neurogenesis occurs at a time point after the neural tube has failed to close, suggesting that neural tube closure is an important step in the regulation of neurogenesis.

3. We have demonstrated that all spinal cord populations examined, including proliferative and differentiated cells, are equally affected by an increase in cell number in the spina bifida region of *Sp*^{d} at E12.5. These changes are in comparison to wild type controls, and are not present in the unaffected, anterior *Sp*^{d} neural tube. These data suggest that failure of the neural tube to close results in overproduction of cells in the neural tube.
4. The observed increases in cell number are not present in the spina bifida region of *Sp*^{d} at E10.5, when the neural tube has just failed to close. These data suggest that the overproduction of cells in the neural occurs after the neural tube has failed to close, and no earlier.
5. To determine whether cell death is increased in response to increased neurogenesis in the spina bifida region of *Sp*^{d}, we chose to examine the levels of cell death. We have found that cell death does not play a substantial role in the functional or cellular deficits found in the *Sp*^{d} neural tube at E12.5. These data suggest that where neural tube closure regulates neurogenesis, it does not appear to act on cell death at E12.5.
6. Examination of a pro-proliferative molecule, Sonic Hedgehog (Shh), secreted by the ventral neural tube structure known as the floor plate, shows a significant increase in area of expression in the open *Sp*^{d} neural tube at E10.5 (Fig.1). These data are in comparison to wild type littermate controls. These results are in agreement with the finding that the number of cells labeled with the marker HNF3β, also a floor plate marker, is increased in the open *Sp*^{d} neural tube at E11.5, as compared to wild type littermate controls. Together, these data suggest that the failure of the neural tube to close results in the expansion in size and cell number of the potent ventral signaling center, the floor plate.
7. FA supplementation during the period of neural tube closure is sufficient to allow the *Sp*^{d} neural tube to close. Analyses similar to those described above at E12.5 have revealed that the gross appearance and cellular components of the overgrowth that characterize spina bifida in *Sp*^{d} are also rescued with FA supplementation. These data suggest that closure of the neural tube is sufficient to regulate the overgrowth phenotypes.

These findings suggest that neural tube closure acts to regulate the proliferation and differentiation of neuronal cells in the neural tube. Our finding that closure of the *Sp*^{d} neural tube induced by FA administration is sufficient to prevent the formation of these defects, demonstrates that closure of the neural tube plays an important role in the proper specification of cells in the developing spinal cord. Consequently, we propose that the failure of the neural tube to close perturbs this process of cell specification, resulting in increased numbers of proliferating and differentiated cells.

This research will not only greatly enhance our understanding of the normal process of cell specification in the developing nervous system, but will also open new avenues for cell-based therapies in terms of improving function in adult nervous system impairments.

Project challenges.

Previous data have indicated that the proliferative and differentiated regions of the neural tube are expanded in the spina bifida region of Sp^d at E12.5. Furthermore, this expansion was equally distributed along the dorso-ventral axis of the neural tube. To determine whether this expansion occurs prior to E12.5, we initially proposed a set of experiments to analyze these regions along the dorso-ventral axis of the neural tube at E10.5. We chose to do this analysis at E10.5, as this time point is immediately after the neural tube has failed to close in the Sp^d neural tube.

The original experiments that showed an expansion were immunohistochemical studies on E12.5 frozen sections using three markers to detect cells in the S phase of the cell proliferation cycle, differentiated neurons, and the dorsal proliferative region of the neural tube. The combination of these markers divides the neural tube into proliferative and differentiated regions, with respect to the dorso-ventral axis. Subsequent to completion of the immunohistochemical experiments, the sections were photographed using a confocal microscope (courtesy of Noriko Kane-Goldsmith and the Keck Center for Collaborative Neuroscience); captured images were then analyzed using computer software, and the area of each region measured.

As there was great technical difficulty involved with analyzing tissue from open neural tube using multiple markers at once, we determined that this difficulty could be avoided by using one marker found throughout the neural tube at E10.5. For this analysis we chose the neuronal cell adhesion molecule N-cadherin. Immunohistochemical labeling with N-cadherin combined with microscopy and image analysis (described above) allows area measurements to be taken of the entire neural tube at E10.5. As many specific cell populations fail to show significant increases in cell number at E10.5, we expect that analysis of the neural tube *in toto* will yield similar results. The results of this experiment are currently being analyzed.

Implications for future research.

Our results indicate that upon neural tube closure, there is a signal that acts to down-regulate the rates of proliferation and differentiation in the neural tube. Furthermore, our results indicate that when this signaling event fails to occur (i.e. in spina bifida) the normal process of neurogenesis becomes disregulated, resulting in the production of too many cells. Exactly how the overproduction of cells in the developing neural tube ultimately results in the defects of bowel and urinary incontinence, and paralysis in the human form of the disorder has yet to be established. Our working hypothesis has been that the overproduction of cells in spina bifida produces a neural tube with too many neurons competing for support in a congested embryonic environment, and that as a consequence of this congestion, the appropriate developmental connections are never made, thus resulting in paralyses. Understanding how neurogenesis is regulated under normal and lesioned conditions is important not only in determining the cause of these deficits, but also in developing new treatments that utilize advances made in the emerging field of stem cell biology.

Plans to continue research.

Future research in our laboratory on this model will evaluate candidate molecules that might underlie the overgrowth seen in spina bifida. Elucidating this mechanism will provide us with the signal that disrupts neurogenesis in the open neural tube, leading to over-proliferation and ultimately, paralysis or dysfunction. To determine the mechanism behind this overgrowth, we will examine known molecules that act to regulate proliferation and are produced in the dorsal neural tube. These experiments are similar to those described above which analyzed Sonic Hedgehog in the ventral neural tube. These experiments are currently underway.

To evaluate other signaling molecules using a wide-net approach, we are currently investigating techniques such as microarray analyses which will allow us to evaluate the activity of a wide variety of molecules that would be likely candidates (e.g. BMPs, WNTs, cell cycle proteins, etc.). Such experiments would be completed within the next 4-6 months, and would evaluate the activity of these molecules in wild type and Sp^d neural tube, as well as FA-treated wild type and Sp^d neural tube.

Publications and presentations.

Abstracts:

1. Analysis of Neural Tube Overgrowth in Spina Bifida Using the Splotch-Delayed Mouse Mutant. Nancy Vranich, Tanuja Kulkarni, Andrew Caprariello, and James H. Millonig. The Society for Neuroscience 34th Annual Meeting, San Diego, California, October 27, 2004.
2. Spina Bifida and Neural Tube Development: Exploring the Role of Neural Tube Closure in the Mouse Mutant *Splotch-delayed.* N Vranich, M Shah, A Caprariello, J Millonig. 2006 Northeast Regional Meeting of the Society for Developmental Biology, Marine Biological Laboratories, Woods Hole, Massachusetts, April 28th, 2006.
3. Spina Bifida and neural tube development: The role of neural tube closure in the mouse mutant Splotch-d. Nancy E. Vranich, Mayank A. Shah, Andrew Caprariello, James H. Millonig. Society for Developmental Biology 65th Annual Meeting, Ann Arbor, Michigan, June 17-18, 2006.

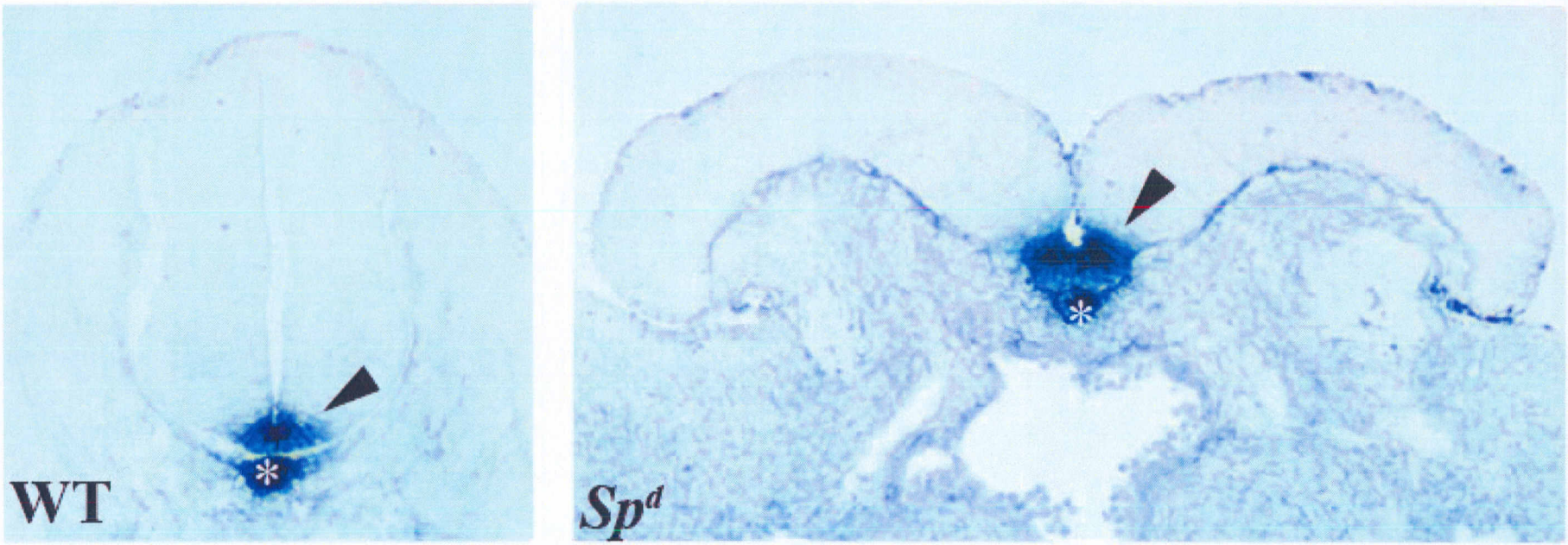

Figure 1. In Situ hybridization showing Sonic Hedgehog mRNA espression in the posterior neural tube of wild-type (WT) and Splotch-delayed (Sp^d) at E10.5. Note the increase in total area of expression in the floor plate (arrows) of the Sp^d neural tube. Notochord (*)