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The Notch Signaling Pathway Drives Astrogliogenesis by Regulating the Expression of the Transcription Factors NFIA and Sox9

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The Notch Signaling Pathway drives astrogliogenesis by regulating the expression of the transcription factors NFIA and Sox9.

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Abstract: In the developing nervous system the Notch signaling pathway has recently been shown to be necessary and sufficient for neural stem cells to differentiate into astroctyes, a process called astrogliogenesis. However, the mechanism of how Notch signaling regulates this process has not yet been characterized. Our lab is examining whether Notch signaling controls the expression of the genes *NFIA* and *Sox9* which are known to regulate astogliogenesis. These candidate genes were selected because their DNA sequences have characteristics that indicate their expression could be controlled by Notch through a transcriptional complex of Notch and other proteins, including the DNA binding protein RBP/J. We developed a low-cost, effective method of qPCR to study the relative abundance of gene expression in mice that underwent selective deletion of the RBP/J gene in neural progenitors (including neural stem cells). We found that disrupting Notch signaling in neural progenitors of the developing central nervous system caused a significant decrease in both *NFIA* and *Sox9* mRNA expression. In order to ensure that this trend was reflected at the protein level we performed fluorescence microscopy utilizing antibodies targeting the NFIA protein. To determine if Notch directly regulates *NFIA* and *Sox9* expression we are currently optimizing a Chromatin immuno-Precipitation assay to establish whether the Notch-RBP/J transcriptional complex binds to the *NFIA* and *Sox9* promoter regions that are important to drive their expression. These novel findings offer insight into this putative signaling pathway, advancing our understanding of how neural stem cell differentiation is regulated.

Keywords: Gliogenesis, Notch Signaling Pathway, NFIA, Sox9, qPCR home-mix.

Introduction:

During development neural progenitors, including neural stem cells, are capable of dividing and maturing, or differentiating, into the variety of cells that make up an organism. An important question in stem cell biology is how these cells coordinate the differentiation and development of tissues and organ systems at precisely the right time and place. In the developing central nervous system (CNS) neural stem cells (NSCs) are responsible for giving rise to several kinds of specialized tissue including neurons, and glia or astrocytes (a specific type of glia). During the early stages of the formation of the CNS neural stem cells reside mainly in the ventricular zone and preferentially give birth to neurons (neurogenesis). As development progresses these stem cells begin to undergo glial-fate specification and migrate outward, predominantly giving rise to glia (gliogenesis). The Notch signaling pathway is known to have multiple critical roles in the regulation of NSCs throughout this

neurogenic to gliogenic "switch." The Notch receptor, which spans the plasma membrane, is activated by ligands such as Delta 1. Upon ligand binding, an inner cellular fragment of the receptor known as the Notch intercellular domain (NICD) is cleaved and translocates to the nucleus where it binds to its transcriptional cofactor RBP/J (a DNA binding protein). The Notch-RBP/J complex then binds to the promoter region of a variety of different genes and drives their expression. Prior to and during neurogenesis the Notch signaling pathway functions to regulate stem cell maintenance by directly regulating the expression of *Hes* transcriptional repressor genes (Ohtsuka et al. 1999). The *Hes* genes inhibit proneural genes such as *neurogenins* (reviewed in Louvi and Artavanis-Tsakonas, 2006) and allow NSCs to undergo asymmetric division, simultaneously giving rise to neurons as well as more NSCs. Conversely, later in development the Notch signaling pathway is both necessary and sufficient to promote NSC differentiation into astroctyes and peripheral glia in the central and peripheral nervous systems (Taylor et al. 2007, Morrison et al. 2000). The mechanism of how Notch regulates this process has not yet been characterized. Although the up-regulation of the *Hes* genes has been shown to promote gliogenesis in the retina (Hojo et al. 2000), similar experiments examining the role of *Hes 1* and *Hes 5* in the developing spinal cord found that the misexpression of these genes alone is not sufficient to promote gliogenesis in the spinal cord (Ohtsuka et al. 2001). This indicates that Notch regulates the expression of an alternative astrogliogenic gene in the developing CNS.

In order to address this issue we compiled a list of genes known to be involved in the regulation of astrogliogenesis. The transcription factor *NFIA* has been shown to promote glial-fate specification in the ventricular zone during early astrogliogenesis, and to promote the terminal differentiation of progenitors into astrocytes later in development (Deneen et al. 2006), the loss of NFIA expression has been shown to result in a loss of glial cells in the ventricular zone (Deneen et al. 2006) similar to results seen when Notch signaling is disrupted just prior to astrogliogenesis (Taylor et al., 2007). These observations suggest that *NFIA* could be a novel effector of Notch regulated gliogenesis. A second transcription factor *Sox9* is also necessary for astrogliogenesis in the CNS and functions to regulate the proper timing of glial differentiation (Stolt, et al. 2003). Loss-of-function studies have shown that disrupting Notch signaling in neural progenitors results in a loss of *Sox9* expression (Taylor et al. 2007). However, it was not shown whether Notch regulates *Sox9* gene directly. We sought to define a more complete expression profile of *Sox9* in order to establish if it is indeed directly regulated by the Notch signaling pathway. Using a bio-informatic approach we identified putative binding sites of the Notch-RBP/J complex on the promoter region of both *NFIA* and *Sox9*. This information establishes the possibility of a direct link between Notch signaling and these two astrogliogenic genes.

To determine if Notch signaling is necessary for *NFIA* and *Sox9* expression, we conditionally deleted *Rbpsuh* from NSC in the developing nervous system in mice. To assay the gene expression in the mutant mice, we developed a low-cost effective approach using quantitative PCR to measure mRNA expression, and standard immunohistochemical techniques for protein expression. We found that disruption of Notch signaling by deletion of the Rbpsuh gene significantly decreased the expression of the proastrocyte genes *NF1A* and *Sox9*. Thus these genes are likely effectors of Notch signaling known to regulate astrogliogenesis.

Materials and Methods

Mice

Mice were housed in the animal facilities at Grand Valley State University. The care and treatment of these animals followed those outlined in Application for Animal Use in Research or Teaching and were approved by the Grand Valley State University's Institutional Animal Care and Use Committee (IACUC). All animals used in this study were genotyped with PCR on genomic DNA to verify

whether tissue samples contained the Rbpsuh gene deletion. The *Cre* insert gene was amplified using forward primer ATTGCTGTCACTTGGTCGTGG and reverse primer GAAAATGCTTCTGTCCGTTTGC. In *Nestin-Cre* tissue this reaction yields a 210 bp product. For detection of the wild type *Rbpsuh* (*Rbpsuh* ^{wt}) allele reactions contained forward primer GTTCTTAACCTGTTGGTCGGA and reverse primer GCTTGAGGCTTGATGTTCTGTAATGC yielding a 487 bp PCR product. To detect the *Rbpsuh* floxed (*Rbpsuh* ^{fl}) inserts on the Rbpsuh allele the forward primer GCAATCCATCTGTTCAATGGCC and reverse primer GAAGGTCGGTTGACACCAGATAGC were used, these primers yield a 598bp product. Samples in which the PCR reactions were positive for *Nestin-Cre* and showed that both *Rbpsuh* alleles were floxed (*Rbpsuh* $^{fl/fl}$) qualified as *Rbpsuh* cKO tissue. Tissue samples extracted from animals of all other genotypes were used as control tissue: the *Cre* insert alone, *Rbpsuh* $^{fl/fl}$ alone, or *Cre* insert along with the *Rbpsuh* fl/wt heterozygote have never been observed to deviate from the wild type phenotype.

Total RNA Isolation and cDNA Generation

Tissue was obtained from the developing nervous system at a variety of different developmental time points from both Nes-Cre^{+} Rbpsuh $^{fl/fl}$ mice and wild type mice. This tissue was then dissociated and a cell count was determined using a hemocytometer. 18,000 to 90,000 cells of the extracted tissue was then added to TriZol and stored at -80^{0}C. Once thawed, glycogen was added to the TriZol before separating the solution into an aqueous and organic layer in order to increase the yield of RNA. Chloroform was then added to the solution and it was centrifuged at 10,500 rpm for 15min at -4^{0}C. The top aqueous layer was then transferred to an RNase free microcentrifuge tube and 500 □L of Isopropanol was added to precipitate the RNA. The solution was centrifuged at 10,500 rpm for 15 minutes at -4^{0}C and the supernatant was removed. The RNA pellet was then washed with 1000□L of 80% ice cold ethanol and centrifuged again at 8,500 rpm for 15 minutes at -4^{0}C. Again the supernatant was removed and the pellet was allowed to air dry. The RNA was then resuspended in Nuclease free H_2O and treated with RQ-1 RNase-Free DNase (Promega, Madison, WI). 80 units of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen). RNA clean up was preformed using an RNeasy Mini Kit (Qiagen) following manufacturer's specifications with two exeptions (1) the initial flow-through of the RLT-BME-Ethanol buffer was saved and run through the column a second time, and (2) a second 30 □L of H_2O was added to the column to increase the RNA elution yield. The total RNA was then treated with random primers and Multiscribe Reverse Transcriptase (Applied Biosystems) in order to generate cDNA. This solution was diluted in H_20 to a concentration of 100 cells/□L to be used for qPCR. All cDNA solutions were stored at -20^{0}C.

Quantitative Polymerase Chain Reaction

A low cost effective qPCR mix was developed. Our final reaction mix consisted of 0.15 □L or 0.75 Units of HotStar Taq Plus DNA Polymerase (Qiagen), 2□L 10X Hot start buffer containing 15mM $MgCl_2$, 2□L of solution containing 10mM each dNTP, 2.4 □L of 25 mM MgCl2, 1 □L SYBR green I (Invitrogen) working stock prepared by a dilution series of 1:100 in DMSO then 1:50 in H_20 (final dilution of 1:100,000). 2□L of 5uM Forward primer solution and 2□L of 5□M Reverse primer solution were added to the reaction mix along with 2□L of cDNA solution (100cells /□L), the appropriate amount of H_20 was then added for a final reaction volume of 20 □L. Optical 8X tube strips were used with Optical 8X cap strips (Stratagene). Initial denaturation was carried out at 95^{0}C for 5 minutes, followed by 40 cycles of 95^{0}C for 30seconds, 58^{0}C for 30seconds, and 72^{0}C for 30seconds. A dissociation curve was run following each reaction. The *NFIA* gene was amplified using the forward primer AAGCCTCCAACCACATCAAC and the reverse primer TTTACAAAGCTTGGATCCCG, this reaction amplifies a product 124 base pairs in length. The *Sox9* gene was amplified using the forward primer AGGAAGCTGGCAGACCAGT and the reverse

primer CGAAGGGTCTCTTCTCGCT, this reaction amplifies a product 102 base pairs in length. □-actin was used as the normalizer gene and was amplified with the forward primer GATATCGCTGCGCTGGTCGTC and the reverse primer TCTCTTGCTCTGGGCCTCGTCAC, this reaction produces a 176 bp product. The efficiency of these reactions was tested using a cDNA serial dilution and determined to be within the acceptable range 90-110%. All reactions were carried out using the Mx3000p qPCR machine (Stratagene).

Immunohistochemistry and tissue preparation

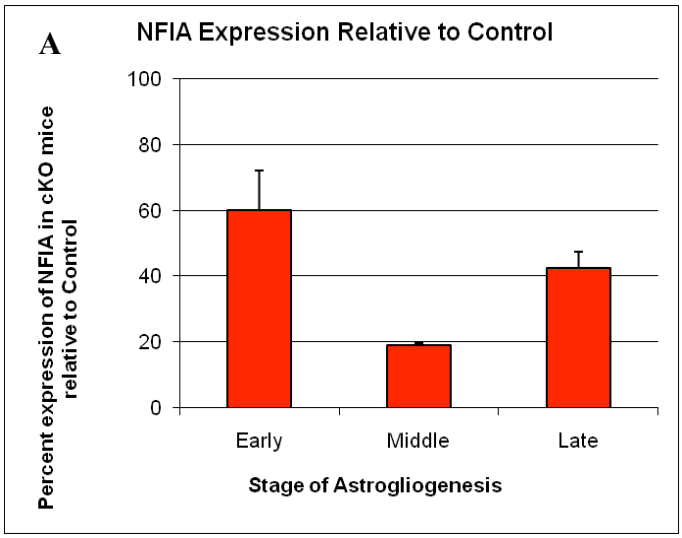
The spinal cords of e18.5 embryos were dissected and fixed in 4% paraformaldehyde overnight. The fixed neural tissue was then washed in PBS, cryoprotected in 15% sucrose, and mounted in Tissue-Tek OCT (VWR, West Chester, PA) prior to snap-freezing and sectioning into 12□m sections. Tissue sections were collected using a Leica cryostat and mounted on glass slides. Tissue slides were stored at -80^{0}C.

Tissue sections were blocked in modified GSS (PBS containing 5% goat serum and 0.5% Triton X-100). The primary antibody targeting NFIA, NF-1A pAb (Active Motif), was diluted 1:2000 in modified GSS and applied to the neural tissue slide followed by a cover slip. This was allowed to incubate overnight at 4^{0}C. The tissue sections were then washed and a diluted 1:500 of Cy3 goat anti rabbit antibody (Jackson Immunoresearch, Baltimore) was applied and incubated for 1 hour at room temperature. Nuclei were counterstained with 2.5 □g/ml DAPI for 10 minutes at room temperature and then mounted using ProLong antifade solution (Molecular Probes, Eugene, OR).

Results

To determine if Notch signaling is necessary for *NFIA* and *Sox9* expression the first step was to establish if disrupting Notch signaling causes a loss of *NFIA* expression. The complete loss-of-function of Notch signaling, such as in the case of *Notch1* or *Rbpsuh* deletion, results in premature neural differentiation and depletion of the progenitor pool (Lutof et al., 2002) prior to the onset of astrogliogenesis. This makes these animals unsuitable for investigating the role of Notch signaling in both the early and late stages of astrocyte differentiation. To circumvent this issue we used a Cre-recombinase-LoxP conditional gene deletion approach in mice. Mutant mice (described as *Rbpsuh* cKO or *Rbpsuh* conditional knockout mice) express *Cre recombinase* under a *Nestin* promoter, which drives expression of Cre recombinase in neural stem cells in the developing central nervous system. These mutant mice also have recognition sites for *Cre recombinase* engineered in the exons for the gene *Rbpsuh*, which encodes the RBP/J protein. When *Cre recombinase* is expressed in NSCs of *Rbpsuh* cKO mice, it excises the *Rbpsuh* gene so that it can no longer be expressed and disrupts the formation of the Notch transcriptional complex and Notch signaling. Gene deletion in NSCs occurs shortly before astrogliogenesis begins, thus blocking all canonical Notch signaling in these NSCs, and rendering them unable to undergo astrogliogenesis (Taylor et al., 2007).

We used the quatitative Polymerase Chain Reaction (qPCR) to compare the relative abundance of *NFIA* and *Sox9* gene expression in the *Rbpsuh* cKO mice to control littermates. We found that the conditional deletion of the gene *Rbpsuh* from NSCs of the CNS results in a decrease in the amount of *NFIA* and *Sox9* expression at all stages of astrogliogenesis. The data obtained from qPCR shows that at E14.5 *NFIA* expression is 60% that of the control (sd 12%). At E15.5 the expression of *NFIA* in cKO mice is 19.15% that of the control (sd 0.46%) and at E.19.5 *NFIA* expression is 42.5 % that of the control (sd 4.95%). In the *Rbpsuh* cKO mice *Sox9* expression is 49% that of the control (sd 3%). At E15.5 it is 12.30% that of the control (sd 0.99%) and at E 19.5 *Sox9* expression is 38.5 % of the control (sd 3.54%) (Figure 1). The results obtained from the qPCR analysis of *Sox9* expression at early stages of astrogliogenesis in *Rbpsuh* cKO mice where consistent with previous findings (Taylor et al. 2007).



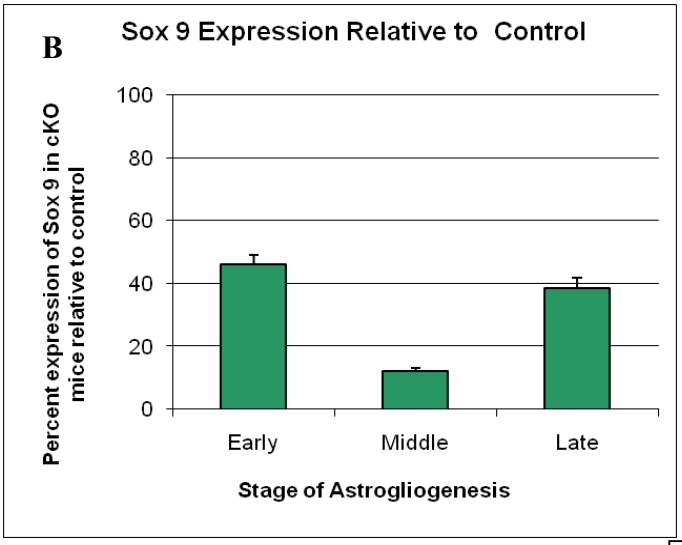


Figure 1: The conditional KO of RBP/J in NSC results in a decrease in the relative abundance of both *NFIA* and *Sox9* mRNA expression in the developing CNS
(A) qPCR analysis of *NFIA* mRNA expression in developing neural tube of *Rbpsuh* cKO mice as compared to the control. Three different stages of astrogliogenesis were assessed (Early = E14.5, Middle = E15.5 and Late = E19.5) N = 2 for all stages of astrogliogenesis.
(B) qPCR analysis of *Sox9* mRNA expression in developing neural tube of *Rbpsuh* cKO mice as compared to the control. Three different stages of astrogliogenesis were assessed (Early = E14.5, Middle = E15.5 and Late = E19.5) N=2 for all stages of astrogliogenesis.

The immunofluorescence assay of NFIA protein expression showed that at E18.5 fewer cells expressed NFIA in the *Rbpsuh* cKO mice then in the control mice. As expected in the control condition cells staining positive for NFIA were concentrated in the regions in which astocytes arise, dorsal to the ventricular zone surrounding the neural tube and radiating outward from this position to both the left and right ventral horns of the CNS. In the spinal cords of the cKO mice there is a striking decrease in the number

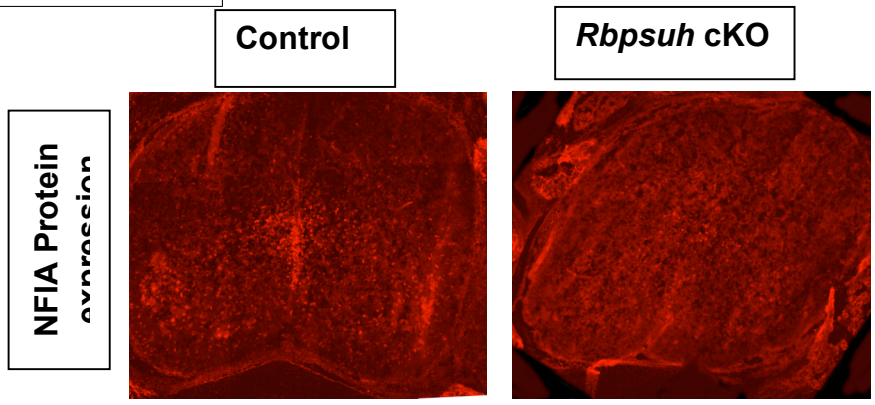


Figure 2: The conditional KO of RBP/J in NSCs results in a decrease in the relative abundance of NFIA protein expression primarily in the regions surrounding the neural tube. These images show a transverse cross section of the E18.5 developing spinal cord stained with an antibody targeting NFIA.

of cells that are brightly stained for NFIA in the region surrounding the neural tube (which has collapsed, a trait common to this model) as well as the ventral regions of the CNS (Figure 2).

We found that blocking Notch signaling results in a significant decrease of both *NFIA* and *Sox9* expression in the developing CNS at all stages of astrogliogenesis prior to birth. Pups born with this mutation die at birth, so later time-points could not be tested.

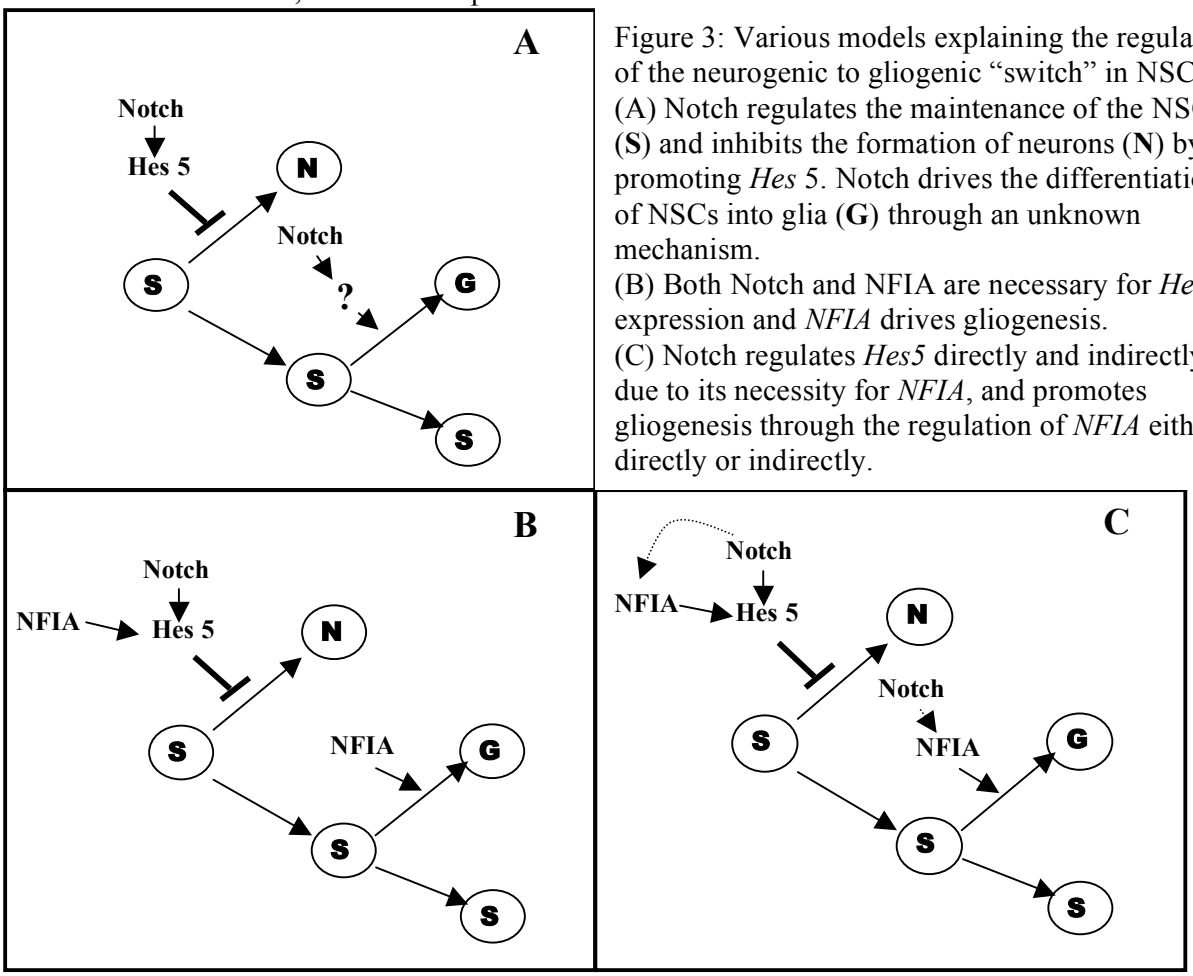


Figure 3: Various models explaining the regulation of the neurogenic to gliogenic "switch" in NSCs. (A) Notch regulates the maintenance of the NSCs (**S**) and inhibits the formation of neurons (**N**) by promoting *Hes* 5. Notch drives the differentiation of NSCs into glia (**G**) through an unknown mechanism.
(B) Both Notch and NFIA are necessary for *Hes* 5 expression and *NFIA* drives gliogenesis.
(C) Notch regulates *Hes5* directly and indirectly due to its necessity for *NFIA*, and promotes gliogenesis through the regulation of *NFIA* either directly or indirectly.

Discussion

These findings indicate that Notch signaling is necessary for the expression of both *NFIA* and *Sox9* at multiple stages of astrogliogenesis in the developing CNS (Figs. 1 & 2), unveiling a new function of the Notch signaling pathway. However, it is not know whether Notch directly or indirectly regulates the expression of these genes. Notch could regulate a signaling cascade which in turn regulates *NFIA* and *Sox9* expression. We are currently performing a Chromatin Immuno-precipitation assay to determine if the Notch-RBP/J complex directly drives *NFIA* and *Sox9* expression by binding to the promoter region of these genes at sites identified by our bioinfomatic approach. These ongoing experiments will determine how Notch regulates these astrogliogenic genes.

The results of this study provide evidence in support of a unitary mechanism that regulates the neurogenic to gliogenic "switch" of NSCs in developing spinal cord. Currently two separate theories of how this process is regulated exist. The first is that developing neuroblasts present Notch ligand feedback signals that activate Notch signaling in the surrounding NSCs and promote differentiation into glia (Morrison et al. 2000). This theory garnered support from the finding that

disruption of Notch signaling "*in vivo"* results in a severe defect of astrogliogenesis (Taylor et al., 2007) indicating that Notch is both necessary and sufficient for glial differentiation (Figure 3 (A)). A second explanation is that the Notch signaling pathway and its effectors function to suppress neurogenesis, while the *NFIA* gene, in conjunction with another transcription factor *NFIB*, promote glia-fate specification (Deneen et al., 2006). Interestingly, it has been found that *NFIA* is also required for the inhibition of neurogenesis due to its necessity for the maintenance of the Notch effector *Hes5* (Deneen et al. 2006) (Figure 3 (B)). While these two explanations of the "switch" of NSCs to glial lineages could exist contemporaneously, our data suggest that they are in the same pathway (Figure 3 (C)).

These findings may also yield insight into several pathologies that afflict the nervous system including multiple sclerosis, and the glial scarring that is associated with damage to the spinal cord. Both of these pathologies have been linked to an astrocyte hypertrophy (John et al., 2002, Ridet et al 1997) making the Notch signaling pathway and its effectors potential targets for novel therapies.

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