# PAX6 CONTROLS ASTROCYTE POSITIONAL IDENTITY IN THE SPINAL CORD

Thesis by

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In Partial Fullfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2007

(Defended May 23, 2007)

*To my parents and to Joana*

# Acknowledgements

I would like to thank my advisor David Anderson for his unstoppable passion for new data and results and his contagious drive to learn and discover. Equally important was his insistence on excellence and doing things the right way. I soon learned how experiments without proper controls would be received! Importantly, he always led by example, demanding more of himself than he demanded of those of us in the lab. For all of these reasons he will always have my utmost respect and my gratitude for the experience of training in his lab.

I would also like to thank the other members of my thesis committee: Marianne Bronner-Fraser, Paul Sternberg, and Kai Zinn. They each offered a different perspective in our meetings and I thank them for their constructive pragmatic approach and valuable insights and ideas.

Several other members of the Caltech community have been very helpful. Rochelle Diamond of the Sorting Facility was an enormous help and a pleasure to work with. Jennifer Alex, Shirley Pease, Bruce Kennedy and all of the technical straff of the Caltech Animal Facility were invaluable with maintaining mouse lines and with timed matings. Peter Lwigale was very helpful in my ability to dramatically improve survival to late stages with chick electroporations. Lisa Taneyhill taught me QPCR, which has since spread throughout the Anderson lab.

I owe an enormous debt of gratitude to many past and present members of the laboratory. Jae Kim first trained me in Neural Crest Biology and many techniques in embryology and cell culture when I rotated and first joined the lab. Qiao Zhou was

an important mentor and collaborator from whom I learned a great deal. His contribution to my education and progression cannot be understated. Zhou and I worked together on the Olig Microarray experiments and early analysis of candidate genes. Gloria Choi was a great friend whose intelligence and wise instincts helped guide me along. I also thank Yosuke Mukoyama for many important discussions and for always taking the time and interest to advise me about my project and helping instruct me in the art of FACS sorting and many other techniques. I thank LiChing Lo who is an authority on many techniques in the lab and who helped me on many occasions with valuable insights based on her vast experience. Donghun Shin, Laurent Van Trigt, Kenji Orimoto, Xinzhong Dong, Limor Gabay, Sally Lowell, Emma Dormand and Sebastian Gerety were also very helpful past lab members whom I count as friends.

More recently, Ben Deneen has been an essential collaborator and mentor in all of my efforts and a good friend. Ben has gone out of his way to provide a lot of very helpful practical guidance with performing more decisive experiments to really nail down results, an area in which he leads by example. In addition to advice, Ben has helped me out a great deal experimentally by sharing reagents and along with our subgroup mate, Agnes Lukaszewicz, we have enjoyed an outstanding collaborative approach especially with chick experiments. “There’s no ‘I’ in team.” Agnes and Sophia Vrontou have been helpful roommates in the lab and good friends, whom I have engaged in many animated discussions and both of whom have helped me on several occasions. Sophia was my last line of defense, when I would forget something she was always there late to take care of it! Joana Yamada (now

Hochstim), Peng Wu, and Monica Martinez have all been a very big help with genotyping mouse tails. Celso Perez, Jung Sook Chang, and Ritchie Ho have been very helpful with cell culture support and maxipreps. I also thank my two great rotation students, Melanie Lee and Janet Chow who were very productive and helpful to me and a pleasure to work with. And thanks to all of the Anderson Lab Members past and present with whom I have overlapped, since all of you have helped me in one way or another.

I can't thank Gaby enough for the many, many occasions on which she has so readily helped me with anything I needed. I have never met someone who works harder and with more energy to get things done than Gaby. Problems with lab equipment, orders or anything were always rapidly and decisively addressed which allowed me to focus on experiments and made my time so much easier. Likewise Gina Mancuso was a big help with administrative issues, scheduling, etc, and I thank her so much for always being so ready to help and so good at taking care of things.

I want to thank my parents for always making education the only priority besides family and for leading me to believe in myself and embrace challenges. I thank my sister and the rest of my extended family for their unconditional love and support, which I have never doubted. Finally, I especially want to thank another former member of the lab, my wife Joana, for her continual patience, love and support which brings me great sense of happiness and stability despite any stresses or difficulties in life. And also, or course, for our beautiful little girl ☺

## Abstract

**While astrocytes play many diverse and important roles in the vertebrate CNS, little is known about the molecular diversity of these cells or the factors specifying such diversity. We found that the secreted signaling molecules Reelin and Slit1 mark 3 positionally defined subsets of astrocytes in the ventral white matter of the spinal cord: Reelin+/Slit1- astrocytes in the lateral white matter (L type), Reelin+/Slit1+ astrocytes in the ventral-lateral white matter (VL type), and Slit1+/Reelin- astrocytes in the ventral-medial white matter (VM type). The homeodomain transcription factor Pax6 is specifically expressed in Reelin+ astrocytes (L and VL type). We found that Pax6 plays a necessary and instructive role in specifying these populations via its actions of promoting Reelin and repressing Slit1 expression. We additionally show that the homeodomain transcription factor Nkx6.1 specifically marks Slit1+ astrocytes (VM and VL type), and provide evidence that VL type astrocytes (Pax6+/Nkx6.1+) are derived from the p2 domain of the ventricular zone (Pax6+/Nkx6.1+). These data are consistent with a model whereby these astrocyte populations are prespecified in the ventricular zone. Importantly, we provide the first evidence that molecularly distinct subtypes of astrocytes are produced during development, each with a defined positional identity in the white matter. While positional identity is an important property of many neuronal subtypes, it has not been previously described in glial cells and may be important for region specific functions of these astrocyte subpopulations.**

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# Chapter1

## Introduction

All multicellular organisms begin from a single cell which proliferates and gives rise to several distinct cell types organized in a complex three-dimensional architecture. This cellular diversity and organization enables multicellular organisms to achieve a wide degree of functional capabilities. A striking example is illustrated by the vertebrate central nervous system where a vast array of neuronal and glial subtypes, organized and interconnected together, provide the necessary substrate for fascinating higher order functions such as perception, behavior, cognition and emotion.

It is estimated that there are thousands of different kinds of neurons in the vertebrate central nervous system (Hall 1992). These neurons can be distinguished by different morphologies as well as molecular criteria such as neurotransmitter/neuropeptide secretion and expression of different ion channels, receptors or molecular markers (Hall 1992). Different neurons are also spatially organized so as to have a positional identity. Positional identity is particularly important property of these neuronal subtypes because it establishes a spatial organization which allows for neurons to find and connect with their proper partners to form circuits. This is evident in the cerebral cortex where different types of neurons are arranged in both layers and columns, as well in the spinal cord where subclasses of motor neurons are arranged in columns and pools in the ventral horn (Jessell, 2000).

In addition to neurons, the central nervous system is also comprised of two major classes of glial cells: astrocytes and oligodendrocytes. Glial cells outnumber neurons by a 10 to 1 margin and perform critical functions (Kettenmann 1995).

Oligodendrocytes have been primarily associated with one critical supportive role, that of wrapping axons with myelin sheaths to enable rapid saltatory conduction. By contrast, astrocytes have been associated with several diverse functions including both passive supportive functions such as balacing pH and ion concentrations, recycling neurotransmitters, storing energy, controlling blood vessels and forming the blood-brain barrier, as well as actively modulating CNS function by communicating with neurons at synapses, providing trophic signals for adult neurogenesis and serving as a source of stem cells (Dani et al., 1992; Doetsch, 2003; Gee and Keller, 2005; Parpura and Haydon, 2000; Song et al., 2002). Given this functional diversity an important question is whether there are molecularly distinct subsets of astrocytes and whether these astrocyte subtypes have different functional responsibilities. Furthermore, since spatial segregation and organization are important for the proper function of neuronal subtypes, an important question is whether different types of astrocytes are also spatially organized so as to have positional identity and whether postional identity is important for their functions.

In the introduction, I will discuss existing evidence for astrocyte diversity and highlight unresolved questions. I will next examine the strategies and molecular mechanisms which underly the generation of neuronal and glial cell diversity, focusing primarily on the spinal cord where these concepts have been well characterized. Finally, I will introduce our findings regarding the role of Pax6 in specifying the molecular and positional identity of astrocytes in the ventral spinal cord.

## Astrocyte Diversity

### *Diversity of Astrocyte functions*

Astrocytes are the most abundant cell type in the central nervous system (CNS) and perform a wide variety of diverse roles. Many of these functions are involved with passively providing a supportive environment for neurons. These include balacing pH and ion concentrations, recycling neurotransmitters, storing energy, controlling blood vessels and forming the blood-brain barrier (Gee and Keller, 2005). However, there is also increasing evidence that, like neurons, astrocytes may play an active role in information processing in the CNS (Fields and Stevens-Graham, 2002). Hippocampal astrocytes respond to glutamatergic firing with actively propagating $Ca^{2+}$ waves in organotypic slice cultures (Dani et al., 1992). A higher resolution version of this experiment, where photo-release of glutamate onto single astrocytes resulted in calcium elevations in some of the neighboring astrocytes, suggests that astrocytes might have specific connectivity with one another (Sul, Orosz et al. 2004). The full functional significance of such astrocytic networks or circuits as well as their specificity and prevalance within various regions of the CNS remains to be elucidated.

Astrocytes can also impact neuronal function at the synapse. Perisynaptic astrocytes modulate the activity of adjacent neurons by releasing glutamate (Parpura et al., 1994; Parpura and Haydon, 2000). Astrocyte-derived glutamate is thought to promote neuronal synchrony, enhance neuronal excitability and to modulate synaptic transmission (Haydon and Carmignoto, 2006). Astrocytes can also regulate synaptic

plasticity by releasing D-serine which acts on the glycine binding site of NMDA receptor, and contribute to heterosynaptic depression by their release of ATP which is extracellularly converted to the inhibitory molecule adenosine (Haydon and Carmignoto, 2006).

In addition to affecting synaptic function, astrocytes impact ongoing development and remodeling in the adult CNS. It is well established that astrocytes proliferate in response to CNS injury leading to formation of scars and inhibiting proper axonal regeneration (Cafferty et al., 2007). However, it has also been shown that astrocytes can provide a substrate for neurite outgrowth in organotypic cultures of rat ventral mesencephalon (Elisabet Berglöf, 2007). Additionally, co-culture experiments demonstrate that astrocytes increase the proliferation and neuronal commitment of adult CNS stem cells. Coupled with the close physical association of astrocytes with stem cells in the dentate gyrus of the hippocampus, this suggests astrocytes provide important trophic signals instructive for adult neurogenesis (Song et al., 2002). Although very limited in scope, adult neurogenesis yields functional neurons which may be important in circuits involving learning and memory (Paton and Nottebohm, 1984; van Praag et al., 2002). GFAP+ astrocytes have been implicated as the source of adult CNS stem cells in the subventricular zone and dentate gyrus of the hippocampus (Doetsch et al., 1999; Laywell et al., 2000; Seri et al., 2001). Thus astrocytes play many diverse and important functional roles in the CNS ranging from providing support, to actively participating in information processing and guiding ongoing development and remodeling in the adult.

***Molecular, Morphological and Positional Diversity***

Several morphologically distinct subclasses of astrocytes have been described both in vitro (Miller and Szigeti, 1991) and in vivo (Mary S. Bailey, 1993). Additionally, the morphologically distinct type 1 (protoplasmic) and type 2 (fibrous) astrocytes observed in rat optic nerve and cortical cultures have been shown to have different antigenic profiles. Type 1 astrocytes express lower levels of GFAP and are negative for A2B5 and tetanus toxin, while type 2 astrocytes express high levels of GFAP and bind both A2B5 and tetanus toxin (Miller and Raff, 1984). There is also evidence that type 1 and type 2 astrocytes may have different distribution between the white and grey matter: cultures from developing gray matter generate only type 1 astrocytes, while white matter derived cultures generate both type 1 and type 2 astrocytes (Miller and Raff, 1984). However because these types are characterized after in vitro culture, it is not clear whether they are truly differentially distributed in vivo. Hippocampal astrocytes in the CA1 and CA3 layers have different electrophysiological properties, suggesting they may represent functionally distinct subtypes. (D'Ambrosio et al., 1998). Astrocytes within the CA1 layer display heterogeneous expression of glutamate transporter and AMPA receptor (Zhou and Kimelberg, 2001). More recently, gene expression profiling experiments were conducted on various *in vitro* astrocyte cultures and astrocyte-rich CNS tissues in an effort to uncover information about the molecular diversity of astrocytes (Bachoo et al., 2004).

While there is evidence supporting the notion of morphological, molecular and regional heterogeneity among astrocytes, it is not clear whether astrocyte subtypes

with distinct molecular and positional identities are specified during development. An alternative explanation for the observed heterogeneity is that a single type of astrocyte is produced initially from a uniform population of progenitors but that astrocytes can subsequently alter their phenotypes (including gene expression, morphology, electrophysiological properties, etc.) in response to local signals in the region in which they settle. Genetic studies and lineage tracing experiments can distinguish between these possibilities. If a gene can instructively promote a particular astrocyte identity this argues against a model of passive acquisition of heterogeneity due to regional cues and for a model of cell intrinsic identity specification. Furthermore if cells derived from a particular domain of progenitors, as marked with a lineage marker, give rise to astrocytes with a distinct molecular and positional identity this also argues that these astrocytes are pre-specified.

## Generation of Cellular diversity in the Spinal cord

### *Spatial patterning and Neuronal subtype specification*

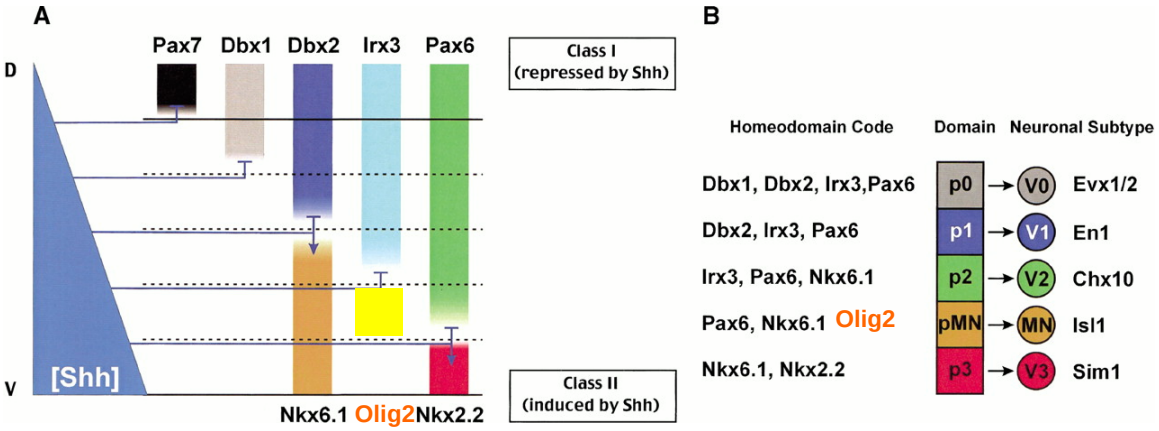
In the ventral spinal cord, molecularly distinct subtypes of neurons are generated from spatially segregated domains of progenitor cells along the dorsoventral axis. A schematic of how this strategy of spatial patterning is achieved is shown in Figure 1. Dorsoventral patterning of progenitors is initially established by the action of the morphogen Sonic Hedgehog (Shh) which diffuses from its ventral point of origin in the floorplate and notochord (Briscoe et al., 2000). Sonic Hedgehog induces the expression of Class II transcription factors such as Nkx2.2, Nkx6.1 and Olig2 and represses the expression of Class I transcription factors such as Pax6, Dbx2

and Irx3 (Briscoe et al., 2000; Jessell, 2000; Novitch et al., 2001). Each of these transcription factors is differentially sensitive to Shh concentration, which leads to each gene having a unique and restricted expression pattern along the dorsoventral axis. For example, a higher concentration of Shh is required to induce Nkx2.2 compared to that required to induce Nkx6.1, thus Nkx6.1 expression extends more dorsally where Shh concentrations are lower. This logic also holds true for Class I genes, for example higher concentrations of Shh are needed to repress Pax6 compared with those required to repress Dbx2, thus Pax6 expression extends more ventrally into regions with higher Shh concentration (Jessell, 2000). In addition to the Shh gradient, dorsally derived Bone Morphogenic Proteins (BMPs) exert an opponent influence on these patterning factors (Jessell, 2000).

Pairs of Class I and Class II genes exert cross repressive interactions on one another which allows for the refinement of these extracellular signals into sharp boundaries. For example Pax6 and Nkx2.2 cross repress each other, and Nkx6.1 and Dbx2 cross repress each other (Ericson et al., 1997; McMahon, 2000) These boundaries partition the ventricular zone into domains each of which is defined by a combinatorial code of homeodomain and bHLH transcription factor expression and each of which generates a particular class of neurons (Jessell, 2000). In the ventral spinal cord there are five discrete progenitor domains: p0, p1, p2, pMN, and p3. These domains give rise to 4 distinct classes of interneurons (V0, V1, V2 and V3) as well as motor neurons. Each of these neuronal subtypes are distinguished both by their expression of specific molecular markers and by their positional identity. The molecular markers identifying each neuronal subclass as well as the combinatorial

code of patterning molecules expressed in each progenitor domain are illustrated in Figure 1.

**Figure 1 – Neural Patterning in the Ventral Spinal Cord**




- modified from: (McMahon, 2000)

In addition to the general classes of neurons specified by this spatial pattering, there is a more detailed hierarchical determination of neuronal subtype identity within the motor neuron lineage. LIM homeodomain proteins act to further sub-specify motor neurons into distinct columns, divisions and pools, each of which is distinguished by a distinct cell body positioning within the motor column and by projections to distinct targets (Jessell, 2000). This further subdivision of neuronal subtypes is also seen with V2 interneurons, where GATA 2 and GATA 3 mark a population designated V2b, which is distinct from the previously characterized Chx10+ V2a interneurons (Karunaratne et al., 2002). These additional subdivisions

result in an even greater diversity of neurons generated just within the ventral spinal cord.

***Neuron-glia transition***

In all vertebrates in all regions of the CNS, neurons are generated prior to glial cells. In the spinal cord, this involves a temporal transition in ventricular zone precursors from producing neurons to producing glial cells. This "Gliogenic" switch involves both the inhibition of neurogenesis and the induction of gliogenesis. Recently, the NFI family of transcription factors was found to be both necessary and sufficient for this transition (Deneen et al., 2006). GLAST is a marker of radial-glia / astrocyte precursors which is not expressed in ventricular zone progenitors during neurogenesis but which turns on at the onset of the transition to gliogenesis (Shibata et al., 1997). Like GLAST, NFI genes are also upregulated in ventricular zone at the onset of gliogenesis. Moreover, NFIA/B gain of function drives precocious expression of GLAST, and conversely the normal temporal induction of GLAST fails to occur in the presence of NFIA siRNA (Deneen et al., 2006). Thus the pro-Glial aspect of this transition appears to be controlled by NFI genes.

The neuron-glial transition also requires the inhibition of neurogenesis. Notch signaling is necessary for this to occur and the mechanism involves Notch effectors such as Hes genes which are able to block the expression of proneural genes (Louvi and Artavanis-Tsakonas, 2006). Interestingly, NFI genes also play a role in inhibiting neurogenesis, as extended neurogenesis and loss of progenitors were observed in

NFIA siRNA experiments, and it was discovered that Notch signaling is dependant on NFI gene function during gliogenesis (Deneen et al., 2006).

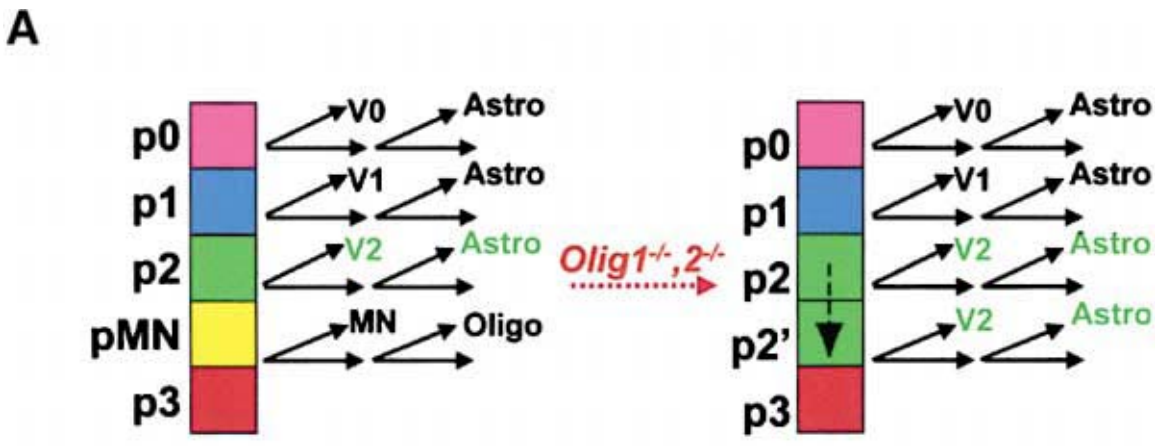
Another important aspect of the transition from neurogenesis to gliogenesis is that it appears to be an irreversible transition, at least within ventricular zone progenitors from the pMN domain. While isochronic transplantation of neurogenic stage (mouse E9.5) FACS isolated Olig2-GFP + progenitors into the neurogenic environment of the E2 chick embryonic spinal cord, these cells made neurons. By contrast, heterochronic transplantation of gliogenic stage (mouse E13.5) Olig2-GFP+ progenitors into the neurogenic E2 chick spinal cord did not yield any detectable neurons (Mukouyama et al., 2006). These in vivo experiments provide a more accurate assessment of the intrinsic potential of these cells than can be obtained by in vitro experiments. In fact these results contradict the in vitro observation that E13.5 glial stage Olig2-GFP+ cells can produce neurospheres which subsequently yield neurons (Mukouyama et al., 2006). The in vitro neurogenic capacity of these cells is likely due to reprogramming in neurosphere culture which has been previously observed with regard to patterning information (Gabay et al., 2003).

***Spatial patterning and Gliogenesis***

The strategy of spatial patterning which is essential in the generation of neuronal diversity is also to specify oligodendrocyte vs. astrocyte fate during the glial phase of development. Oligodendrocytes are generated from the pMN domain of the ventral ventricular zone, which is specifically marked by the expression of the bHLH transcription factor Olig2 during bothe the neurogenic and gliogenic phases of

development (Lu et al., 2000; Zhou et al., 2000). This domain generates motor neurons during neurogenesis and Olig2 is necessary and sufficient for both motor neuron and oligodendrocyte fate (Lu et al., 2002; Novitch et al., 2001; Zhou and Anderson, 2002; Zhou et al., 2001). In Olig1,2 -/- embryos, the progenitors of the erstwhile pMN domain acquire the identity of the immediately dorsal p2 domain, as Irx3 expands ventrally during the phase of ventral patterning (Zhou and Anderson, 2002). This transformed ectopic p2 domain generates V2 interneurons and then subsequently astrocytes. This fate transformation is illustrated in Figure 2. Olig2 controls the specification of a neuronal subtype and glial subtype from the same progenitor domain during different phases of development.

**Figure 2: Cell Fate Transformation in the Olig1,2 -/- mutant**




(Zhou and Anderson, 2002)

Whether other factors involved in neuronal patterning are also important in glial subtype specification is less clear. While Nkx2.2 represses Olig2 during neurogenesis, it is co-expressed with Olig2 in the oligodendrocyte lineage and collaborates with Olig2 in promoting oligodendrocyte fate (Zhou et al., 2001). Additionally, Nkx2.2 is required for oligodendrocyte differentiation (Qi et al., 2001). Pax6 is required for the normal onset and positioning of oligodendrocyte formation, which may be a result of dysregulated dorsal expansion of Nkx2.2 into pMN during normal patterning (Sun et al., 1998). While Pax6 is expressed in progenitors dorsal to pMN which give rise to astrocytes, Pax6 mutant mice do not show deficits in GLAST expression indicating normal astrocyte precursor formation (Ogawa et al., 2005).

The fact that glial progenitors convert to astrocyte formation in the Olig mutant supports the notion that astrocytes are generated as the "default" glial fate. Consistent with this it has been shown that the pro-glial NF1 genes are sufficient to promote astrocyte differentiation in the absence of Olig2 antagonism (Deneen et al., 2006). Also, astrocytes appear to be broadly generated from VZ progenitors outside of pMN, as assayed by the migration of NFIA+, GLAST+, and FGFR3+ cells (Deneen et al., 2006; Pringle et al., 2003; Shibata et al., 1997). Thus it appears that gliogenesis carries an intrinsic astrocytic bias upon which Olig2 acts (Deneen et al., 2006). This is evident in the fact that Olig2+ oligodendrocyte precursors require the pro-glial function of NFI genes and are co-expressed in ventricular zone precursors. Olig2 is able to block the pro-astrocytic effects of NFI genes once cells have exited the ventricular zone. Olig2+ cells in the pMN domain express GLAST, yet once they migrate out of the ventricular zone they no longer express GLAST. Furthermore

Olig2 antagonizes the ability of NFI genes to induce astrocyte differentiation and GFAP expression (Deneen et. al., 2006). Thus the pro-glial program of NFI genes is coupled to their later pro-astrocytic functions, and while Olig2 is dependant on the early pro-glial function it is also sufficient to override the later pro-astrocytic functions.

The above results suggest that astrocyte specification can proceed under the control of NFI genes so long as Olig2 is repressed in astrocyte precursors. Further evidence of this comes from the discovery that the bHLH transcription factor SCL is a p2 domain specific factor which promotes astrocyte fate and represses oligodendrocyte generation within this domain by repressing Olig2 expression (Muroyama et al., 2005). These studies also show that Olig2 can repress SCL. Such cross-repressive interactions can ensure the silencing of Olig2 expression in astrocyte precursors and allow for astrocye specification and differentiation.

Recently it has been shown that some dorsal progenitors can give rise to oligodendrocytes (Cai et al., 2005; Vallstedt et al., 2005) and conversely some pMN progenitors can give rise to astrocytes or ependymal cells (Masahira et al., 2006). Despite these alternative sites of origin, it still remains universally true that Olig2 function is required for oligodendrocyte formation and conversely the absence of Olig2 expression is required for astrocytic differentiation. These experiments suggest that there may be more plasticity in glial cell fate specification than is observed in the case of neurons. This could possibly be due to the rapid exit from the cell cycle and terminal differentiation in newly generated neurons which restricts any opportunity for switching fates. Interestingly, the dorsal site of origin of some oligodendrocytes

has led to speculation over whether there are distinct subtypes of oligodendrocytes (Richardson et al., 2006).

***Outline of the Thesis***

This thesis aims to address the how astrocyte diversity is specified in the developing spinal cord. Chapter 2 describes Microarray experiments comparing Olig -/- and Olig +/- populations during early gliogenesis which identified genes differentially expressed in early astrocyte vs. oligodendrocyte progenitor populations. I will discuss how these microarray data were analyzed and how among the candidate genes with increased in the Olig -/- astrocytic population we identified Reelin, Slit1 and Pax6, which we confirmed to be expressed in subsets of white matter astrocytes but not in oligodendrocytes. In Chapter 3, I will discuss our finding that the partially overlapping pattern of Reelin and Slit1 expression defines 3 molecularly and positionally distinct subsets of astrocytes in the ventral white matter and that this positional identity is controlled by Pax6. Additionally, I will discuss our discovery that while Pax6 specifically marks Reelin expressing astrocytes, Nkx6.1 specifically labels Slit1 expressing astrocytes. I will present our evidence for the prespecification of astrocyte identity using these markers along with the Olig2-GFP in the Olig -/- mutant as marker of p2 derived astrocytes. Importantly, the regulation of astrocyte positional identity in the spinal cord by Pax6 provides the first evidence that different types of astrocytes are produced during development.

Finally in the Appendix, I will discuss various other experiments involved with testing the function of genes identified in the microarray screen. These include:

examining the role of Pax6 in pMN progenitors during motor neurogenesis, cloning and testing Tet inducible RCAN retroviral vectors for inducible misexpression of Pax6 in ovo, investigating the role of Shox2 in motor-neuron vs. V2 interneuron fate and oligodendrocyte precursor induction, and functional studies of HFH4 during gliogenesis.

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# Chapter 2

## Microarray Screen for Olig target genes in gliogenesis

**Christian Hochstim, Qiao Zhou and David Anderson**

## Introduction

The bHLH transcription factor Olig2 is the first gene identified which regulates glial subtype specification. Olig2 is necessary and sufficient for oligodendrocyte formation and in the absence of Olig2, cells marked by a knockin *Olig2*-GFP generate astrocytes (Zhou and Anderson, 2002; Zhou et al., 2001). This fate conversion suggests that in addition to promoting oligodendrocyte fate, Olig2 plays an important role in suppressing astrocyte fate (Lu et al., 2002; Zhou and Anderson, 2002). Given evidence that Olig2 acts as a transcriptional repressor (Zhou et al., 2001), we postulated that important astrocyte regulatory genes might be targets of Olig2 repression and designed a screen to identify these target genes.

Our strategy was to use Olig2-GFP to FACS isolate glial progenitors from Olig +/- and Olig -/- embryos and to compare their gene expression profiles on Affymetrix cDNA microarrays. We hoped to identify new pro-astrocytic or anti-oligodendrocytic genes as genes upregulated in the Olig -/- population. At the time we intitiated this screen, no instructive factors had been identified which could drive precocious astrocyte development or repress oligodendrocyte development. Genes with both of these functions have since been identified (Deneen et al., 2006; Muroyama et al., 2005). Fortunately, among the genes upregulated in the Olig -/- 'astrocytic' population, we identified markers specific to positionally defined subsets of astrocytes, which enabled us to discover the first evidence of astrocyte subtype specification during development.

## Results

### *Isolation and Microarray comparison of E14.5 Olig +/- and Olig -/- glial progenitors*

We performed our first microarray comparison with cells isolated from E14.5 embryos. The major reason for selecting this stage was to avoid contamination of our samples by neurons. Olig2 is normally expressed in motor neuron precursors but is rapidly downregulated as precursors migrate out of the ventricular zone and differentiate into motor neurons. Expression of Olig2 protein is not detectable in postmitotic motor neurons. By contrast, due to the stability of the GFP protein, Olig2-GFP expression perdures into postmitotic motor neurons (+/-) or V2 interneurons (-/-). Some of this GFP protein perdurance in neurons is still detectable in the early stages of gliogenesis (E12-E13). By E14.5, there is no detectable GFP expression in neurons and we were confident that all GFP+ cells isolated for comparison would be glial precursors.

We performed 2 independent experiments at E14.5 including dissection and dissociation of spinal cords, FACS isolation, aRNA amplification and hybridization to Affymetrix mouse A, B and C chips (see Experimental Procedures). Sample FACS profiles for the Olig +/- and Olig -.- populations are illustrated in Figure 1.

The major caveat with the E14.5 screen is that in heterozygotes the majority of GFP+ oligodendrocyte precursors have migrated out of the ventricular zone while the majority of GFP+ ‘astrocyte precursors’ in homozygotes remain in the ventricular zone (Figure 1). This fundamental difference in cellular position could result in

significant differences in gene expression between these two populations which are unrelated to the question of oligodendrocyte vs. astrocyte fate.

***A Second Microarray comparison of Olig +/- and Olig -/- progenitors: E13.0 Screen***

To complement our analysis and address our concerns, we performed a second microarray comparison with cells isolated at E13.0. At this stage we were able to obtain very comparable populations of ventricular zone cells for the comparison, by using PDGFRa staining as a negative selection marker to exclude the few migrating oligodendrocyte precursors in the Olig1,2 +/- heterozygote (see FACS plots, Figure 1). The possibility of neuronal contamination due to GFP perdurance, as discussed above, was the potential caveat at this earlier stage. We performed independent replicates at this timepoint, as was done at E14.5.

***MAS4 and Rosetta Resolver Analysis: selection of candidate genes***

The results of the E14.5 and E13.0 screens revealed several interesting candidates common to both screens. For both screens we initially analyzed the data using Affymetrix Microarray Suite software (MAS4). We set our threshold for differential expression at a minimum 3 fold change in expression between samples and a minimum average difference change between samples of 50 (with the target intensity normalized to 200). In the E14.5 screen of the A, B, and C chips over 1300 genes/ESTs were scored as differentially expressed, while in the E13.0 screen around 400 genes/ESTs were differentially expressed. Many cell adhesion molecules and

cell cycle factors were differentially expressed in the E14.5 screen, and this background (presumably due to fundamental differences in ventricular vs. migrating cells) was eliminated in the E13.0 screen. Likewise, factors involved in neurogenesis such as Irx3, Chx10 and Islet1 were identified as differentially expressed in the E13.0 screen but not the E14.5 screen. Importantly, both screens found oligodendrocyte precursor markers (PDGFRa, sox10 and NG2) to be strongly downregulated in the Olig1,2 -/- mutant, while the astrocyte marker glutathione transferase Yb is strongly upregulated in the Olig 1,2 -/- mutant. The consistency of these genechip results with our previous observations in the Olig1,2 -/- mutant is an important positive control and indicates that our screens can identify expected differences in gene expression between the cell types being compared.

In order to eliminate likely false positives and focus on genes whose differential expression is really due to the presence or absence of Olig expression, we decided to focus on genes differentially expressed in both screens. For this we performed analysis with Rosetta Resolver, which enabled us to perform a 4 way cross comparison, using both replicates of both the E14.5 and E13.0 screens. This analysis yielded around 350 genes across the A, B and C chips with at least a 3 fold change and a p value less than .01. The log intensity plot for the A chip is shown in Figure 2. Genes with higher expression in the Olig -/- population are shown in blue, while genes with higher expression in the Olig +/- population are shown in green. The lines represent the boundary for a 3 fold change. Thus all genes outside these lines have a greater than 3 fold intensity difference between the 2 samples. One of the genes increased in the Olig -/- mutant population is Pax6 which is highlighted in red. A

total of 41 transcription factors were differentially expressed across the A, B and C chips in this analysis and these are shown in Table 1.

***Candidate gene validation***

We next began using in situ hybridization to examine the expression of candidate genes through different stages of gliogenesis. Priority for follow up analysis by in situ was given to transcription factors and genes with known functions in the nervous system with strong fold changes and average difference changes. 35 candidate genes were tested by in situ hybridization of Olig1,2 +/- heterozygous and Olig1,2 -/- homozygous embryos during early gliogenesis (E12.5), mid gliogenesis (E14.5) and late gliogenesis (E18.5). Sample in situ panels are shown for 2 candidate transcription factors: HFH4 (Figure 3) and Shox2 (Figure 4). From these analyses we selected 5 genes whose expression patterns suggest they may be regulated by Olig and are expressed in glial cells or their precursors: HFH4, Shox2, Pax6, Reelin and Slit1. We performed further analysis and experiments with these genes. Experiments with Pax6, Reelin and Slit1 are discussed in Chapter 3, while experiments involving HFH4 and Shox2 are discussed in the Appendix. All of these genes were upregulated in the Olig1,2 -/- mutant, which is interesting given Olig2 appears to act as a transcriptional repressor.

## Discussion

Among the interesting candidate genes we identified in this screen were the secreted signaling molecules Reelin and Slit1 and the homeodomain transcription

factor Pax6, all of which were upregulated in the Olig -/- “astrocyte progenitor” population. Our expression data confirmed that these genes are expressed in specific subsets of astrocytes in the ventral white matter and are not expressed in oligodendrocytes. Studies with these genes led to our discovery of how astrocyte positional identity is regulated in the spinal cord (Chapter 3).

Functional experiments were also performed 2 other candidate genes upregulated in the Olig -/- population, both of which were expressed in a restricted subset of ventricular zone progenitors during early gliogenesis. First, the homeodomain transcription factor Shox2, which upon closer examination was expressed in the pMN domain at the onset of oligodendrocyte formation, as well as in V2 interneurons and whose gain of function phenotypes include repression of MN fate, promotion of V2 interneuron fate, and promoting precocious oligodendrocytes together with Olig2. Second, was the forkhead transcription factor HFH4, whose gain of function and knockout analysis had no phenotypes with respect to general oligodendrocyte and astrocyte specification markers. These experiments are discussed in further detail in the Appendix.

Interestingly, we did not succeed in our original goal to identify a pro-astrocytic regulatory gene with this screen. One possible reason for this lack of success is the finding by our lab that the pro-astrocytic NFI family of transcription factors are also pan-glial regulators during early specification (Deneen et al., 2006) and thus are not transcriptionally regulated by Olig2 or differentially expressed in our screen. Rather Olig2 and NFI genes appear to antagonize each others function via biochemical interaction (Deneen et al., 2006). The bHLH transcription factor SCL

was recently shown to play a role in both V2b interneuron and astrocyte generation from the p2 domain via cross-repressive interactions with Olig2 (Muroyama et al., 2005). Unfortunately, SCL was identified as upregulated in the Olig -/- mutant population in our screen (see Table I), but we chose not to pursue it due to published data at the time that it was only expressed in V2 interneurons and not in any other cells at stages of early gliogenesis (Emma Smith, 2002). Despite our inability to find a pro-astrocytic regulator, we were able to take advantage of the identification of Pax6, Reelin and Slit1 expression in subsets of astrocytes to uncover the first evidence of astrocyte subtype specification during development.

**Experimental Procedures**

*Spinal cord dissociation and FACS*

We devised a method to rapidly genotype embryos using Xgal staining of the head. This was done to speed up the process of dissection and sorting with minimal wait time, thus minimizing cell death. Olig1,2 -/- homozygotes show very bright Xgal staining (due to the Olig1-lacZ knockin allele), Olig1,2 +/- heterozygotes show weak staining, and wild type embryos have no staining. PCR was then done retrospectively to confirm the genotype. We dissected out the spinal cords and used enzymatic dissociation with Papain and DNAse (Worthington) to generate a single cell suspension for FACS. The E13.0 sample was stained with anti-PDGFRa antibody (eBioscience, 1:50) followed by PE conjugated anti-Rat secondary to exclude migrating oligodendrocyte precursors

*RNA isolation, amplification and preparation*

Immediately following FACS, RNA was isolated from the cells using the Stratagene microRNA isolation kit. The RNA was then subjected to two rounds of amplification using the MessageAmp aRNA kit (Ambion). The aRNA was biotin labeled during the second round and fragmented to an average size of 80-100 bp, as recommended by Affymetrix. Using these procedures we could generate around 100 ug of aRNA probe from a starting material of 3000-5000 FACS isolated cells.

*In situ hybridization*

Non-radioactive in situ hybridization using DIG-labelled probes was performed on frozen sections of mouse spinal cord, as previously described (Zhou et al., 2000).

**Acknowledgements**

We thank Rochelle Diamond and the Caltech Flow Cytometry Facility for FACS purification of our samples. We also thank Elizabeth Zhou (Stanford) for performing our microarray hybridization. We thank Kayla Smith, Jose Luis Reichmann and the Millard and Muriel Jacobs Genetics and Genomics Laboratory for assistance with Microarray analysis. We thank R. Blaschke for Shox2 in situ probe.

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**Figure 1: Isolation of Olig +/- and - / - cells for Gene Expression Profiling**

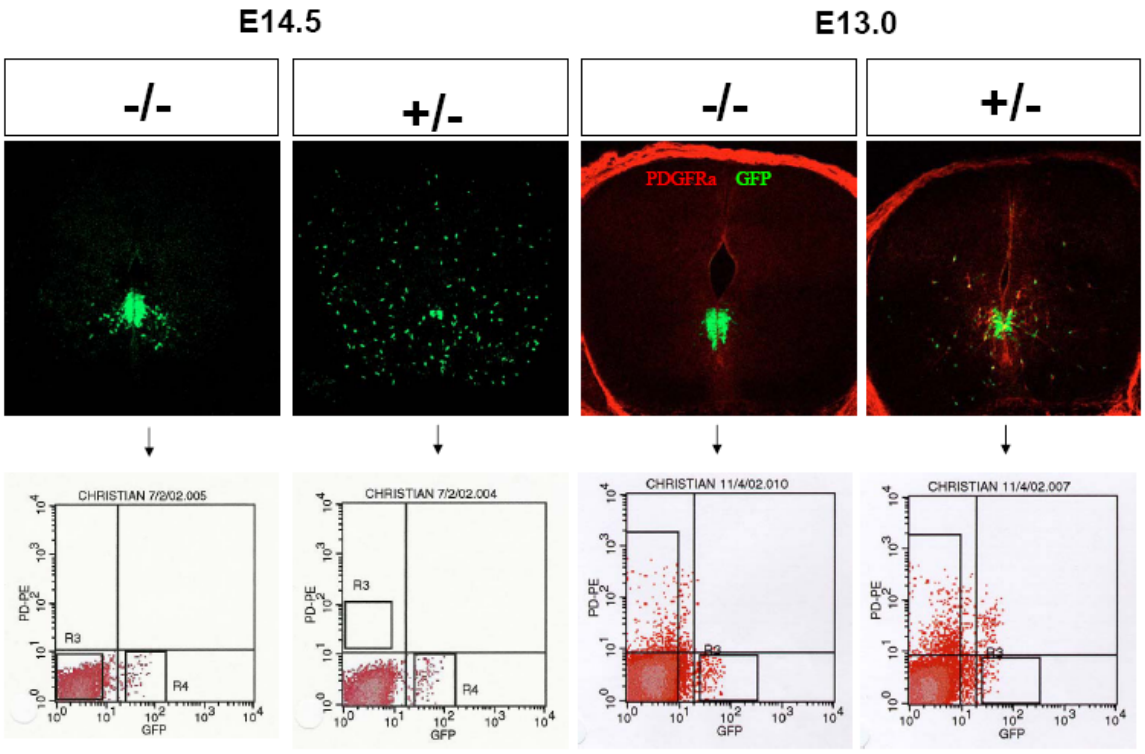


**Figure 2: Analysis of Differential Gene Expression**
**(Rosetta Resolver: E13.0 and E14.5 Data)**

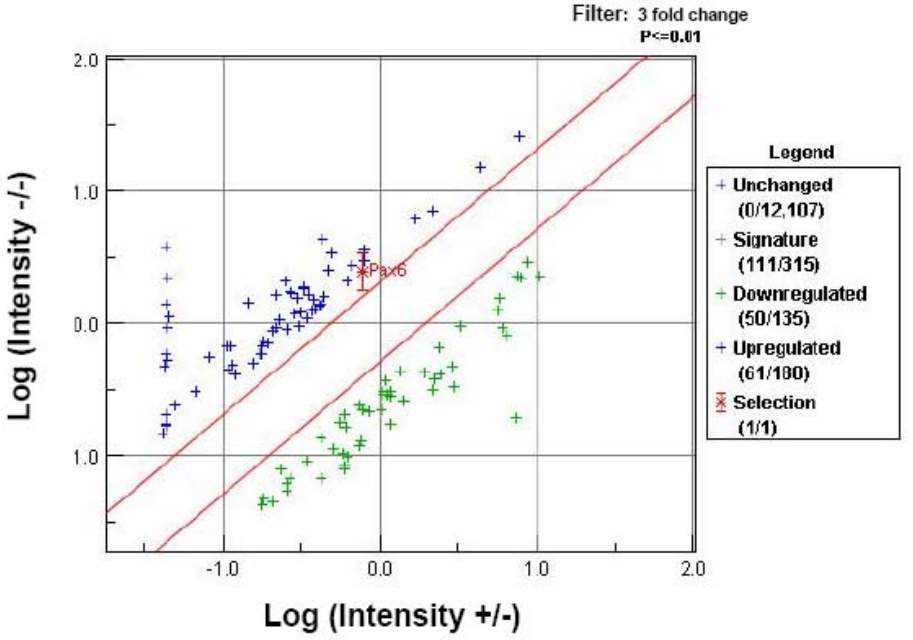


Table 1 – Differentially expressed Transcription Factors E13/E14.5 combined analysis

| Transcription Factor | Affymetrix Probe Set ID | Chip | Fold Change | P value |
|---|---|---|---|---|
| thrsp (??) | 160306_at | A | 38.64526 | 9.70E-11 |
| Gbx2 (homeodomain) | 94200_at | A | 15.63335 | 5.00E-03 |
| shox2 (paired homeodomain) | 99042_s_at | A | 13.8363 | 5.00E-05 |
| GATA 3 (Zn finger) | 100924_at, 111479_at (B), 130316_f_at [C] | A, B, C | 13.68944 | 3.40E-09 |
| tal1 (bHLH) | 97973_at | A | 12.48429 | 8.70E-13 |
| Gli4 (Kruppel family Zn Finger) | 166968_f_at | C | 11.71575 | 1.50E-10 |
| Gli2 (Kruppel family Zn finger) | 100395_at, 166589_at [C] | A, C | 7.65802 | 1.40E-03 |
| Six5 (homeobox) | 97122_at | A | 5.62276 | 1.70E-03 |
| St18 / neural Zn finger 3 | 167829_r_at | C | 5.4754 | 4.00E-05 |
| Teashirt 3 (Zn finger, homeobox) | 104948_at | B | 5.43652 | 1.00E-07 |
| Retinoid X receptor gamma (Zn finger) | 92237_at | A | 5.31652 | 3.20E-03 |
| Foxj1 / HFH4 (forkhead, winged-helix) | 98831_at | A | 5.29319 | 3.70E-09 |
| Myeloid leukemia factor 1 (?) | 102061_at | A | 5.17993 | 2.70E-13 |
| myt1-like (Zn finger) | 96496_g_at | A | 5.12931 | 5.90E-04 |
| sterol regulatory binding element factor 1 (bHLH) | 93264_at | A | 4.82305 | 9.00E-05 |
| Beta 3 (bHLH) | 129880_s_at, 129881_r_at | C | 4.76351 | 2.50E-03 |
| tgif (TALE homeobox) | 101502_at | A | 4.13782 | 2.30E-04 |
| RIKEN cDNA 2810028A01 (Zn finger) | 135902_at | C | 3.77185 | 4.20E-03 |
| chx10 (paired HD) | 101127_at | A | 3.74129 | 5.80E-03 |
| NM 172690 (Zn finger PHD finger ??) | 135758_at | C | 3.64518 | 2.00E-03 |
| atf3 (bZIP) | 104155_f_at | A | 3.36385 | 7.20E-03 |
| Pax6 (paired homeodomain) | 92271_at | A | 3.31469 | 5.70E-07 |
| leucine zipper transcription factor-like 1 | 163330_at | B | 3.28556 | 6.90E-13 |
| Estogen receptor 1 alpha (Zn finger C4 type) | 115428_at | B | 3.22464 | 3.30E-03 |
| RIKEN cDNA 8030486J21 (Zn Finger ??) | 141005_at | C | -3.09476 | 3.60E-03 |
| Hmga2 (high mobility AT hook) | 99058_at | A | -3.25315 | 2.80E-07 |
| limb bud and heart (activator) | 107954_at | B | -3.28543 | 5.90E-04 |
| Asf1b (asf-like, p53-like) | 97364_at | A | -3.69187 | 7.30E-04 |
| myc (leucine zipper) | 104712_at | A | -4.19229 | 9.20E-03 |
| Tox (thymus HMG box) | 105907_at, 112175_at | B | -4.21586 | 7.50E-04 |
| cDNA BC005471 (kruppel assoc. box, Zn finger) | 105184_at | B | -4.52709 | 4.50E-04 |
| Zfp 365 (Zn Finger ??) | 166820_at | C | -4.56918 | 2.70E-06 |
| AI552443 Soares cDNA (putative leucine zipper TF) | 111147_at | B | -4.84154 | 4.00E-05 |
| Creb5 (basic leucine zipper) | 115835_at | B | -6.82533 | 3.20E-04 |
| sox10 (Hmg) | 102856_at, 100283_at | A | -6.95795 | 6.50E-04 |
| NM 172578 (Myb DBD, homeodomain-like ??) | 167265_at | C | -7.22663 | 7.30E-07 |
| RIKEN cDNA 2610040E16 (Zn finger C2H2) | 109430_at | B | -7.27139 | 1.90E-03 |
| Irx1 (iroquois class homeobox) | 111554_i_at, 111555_f_at | B | -14.05651 | 1.00E-05 |
| Olig1 (bHLH) | 165742_r_at | C | -17.28564 | 6.40E-29 |
| myt1 (Zn finger) | 95347_at, 169294_s_at [C] | A, C | -27.63643 | 2.90E-39 |

Figure 3: HFH4/Foxj1 in situ on Olig +/+ and Olig -/- spinal cord

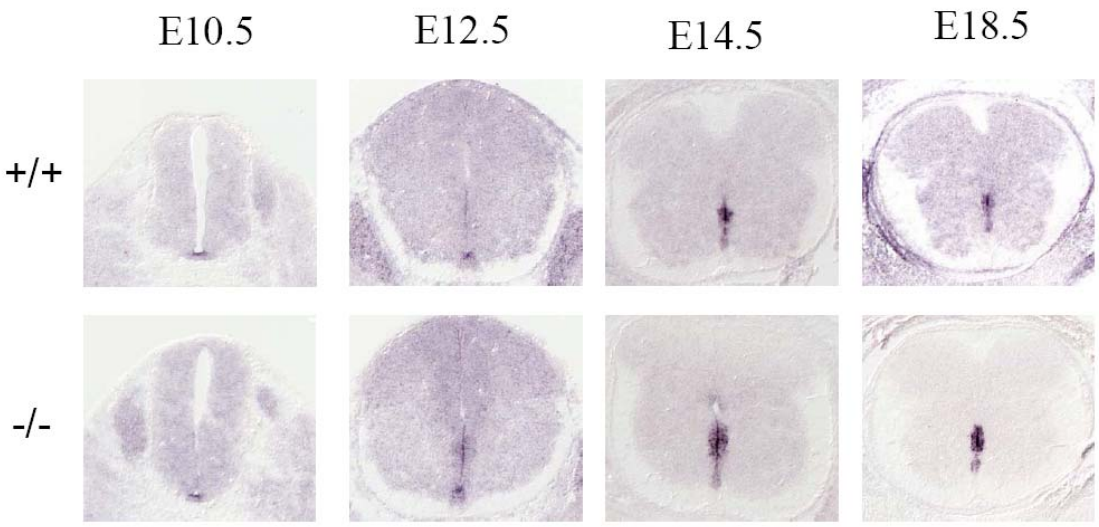
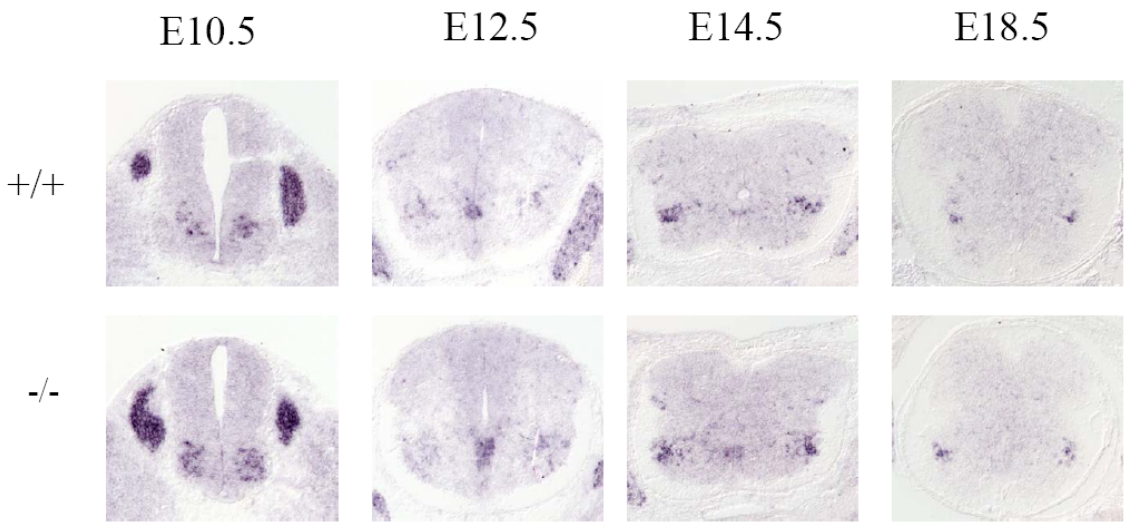


Figure 4: Shox2 in situ analysis on Olig ++ and Olig -/- spinal cord



# Chapter 3

## Pax6 controls astrocyte positional identity in the spinal cord

**Christian Hochstim, Ben Deneen, Qiao Zhou, and David Anderson**

## Abstract

**While astrocytes play many diverse and important roles in the vertebrate CNS, little is known about the molecular diversity of these cells or the factors specifying such diversity. We found that the secreted signaling molecules Reelin and Slit1 mark 3 positionally defined subsets of astrocytes in the ventral white matter of the spinal cord: Reelin+/Slit1- astrocytes in the lateral white matter (L type), Reelin+/Slit1+ astrocytes in the ventral-lateral white matter (VL type), and Slit1+/Reelin- astrocytes in the ventral-medial white matter (VM type). The homeodomain transcription factor Pax6 is specifically expressed in Reelin+ astrocytes (L and VL type). We found that Pax6 plays a necessary and instructive role in specifying these populations via its actions of promoting Reelin and repressing Slit1 expression. We additionally show that the homeodomain transcription factor Nkx6.1 specifically marks Slit1+ astrocytes (VM and VL type), and provide evidence that VL type astrocytes (Pax6+/Nkx6.1+) are derived from the p2 domain of the ventricular zone (Pax6+/Nkx6.1+). These data are consistent with a model whereby these astrocyte populations are prespecified in the ventricular zone. Importantly, we provide the first evidence that molecularly distinct subtypes of astrocytes are produced during development, each with a defined positional identity in the white matter. While positional identity is an important property of many neuronal subtypes, it has not been previously described in glial cells and may be important for region specific functions of these astrocyte subpopulations.**

## Introduction

Astrocytes are the most abundant cell type in the central nervous system (CNS) and perform a wide variety of diverse roles. Many of these functions are involved with passively providing a supportive environment for neurons, including balacing pH and ion concentrations, recycling neurotransmitters, storing energy, controlling blood vessels and forming the blood-brain barrier (Gee and Keller, 2005). However, there is also evidence suggesting that, like neurons, astrocytes may play an active role in information processing in the CNS (Fields and Stevens-Graham, 2002). Hippocampal astrocytes respond to glutamatergic firing with actively propagating $Ca^{2+}$ waves in organotypic slice cultures (Dani et al., 1992). Perisynaptic astrocytes can also modulate the activity of adjacent neurons by releasing glutamate (Parpura et al., 1994; Parpura and Haydon, 2000). Additionally, astrocytes provide trophic signals for adult neurogenesis (Song et al., 2002), and some astrocytes have been implicated as a source of CNS stem cells (Doetsch, 2003; Laywell et al., 2000).

Several morphologically distinct subclasses of astrocytes have been described both in vitro (Miller and Szigeti, 1991) and in vivo (Mary S. Bailey, 1993). Additionally, the morphologically distinct type 1 (protoplasmic) and type 2 (fibrous) astrocytes observed in rat optic nerve and cortical cultures have been shown to have different antigenic profiles. Type 1 astrocytes express lower levels of GFAP and are negative for A2B5 and tetanus toxin, while type 2 astrocytes express high levels of GFAP and bind both A2B5 and tetanus toxin (Miller and Raff, 1984). There is also evidence that type 1 and type 2 astrocytes may have different distribution between the white

and grey matter: cultures from developing gray matter generate only type 1 astrocytes, while white matter derived cultures generate both type 1 and type 2 astrocytes (Miller and Raff, 1984). However because these types are characterized after in vitro culture, it is not clear whether they are truly differentially distributed in vivo. Hippocampal astrocytes in the CA1 and CA3 layers have different electrophysiological properties, suggesting they may represent functionally distinct subtypes. (D'Ambrosio et al., 1998). Astrocytes within the CA1 layer display heterogeneous expression of glutamate transporter and AMPA receptor (Zhou and Kimelberg, 2001). More recently, gene expression profiling experiments were conducted on various *in vitro* astrocyte cultures and astrocyte-rich CNS tissues in an effort to uncover information about the molecular diversity of astrocytes (Bachoo et al., 2004). While there is evidence supporting the notion of morphological, molecular and regional heterogeneity among astrocytes, it is not clear whether this is indicative of distinct pre-specified astrocyte subtypes or merely passively acquired phenotypic differences due to regional cues acting on a single uniformly specified astrocyte population.

By contrast, the mechanisms generating neuronal diversity and for specifying oligodendrocyte and astrocyte fate have been better characterized. In the ventral spinal cord, molecularly distinct subtypes of neurons are generated from spatially segregated domains of progenitor cells along the dorsoventral axis. These spatially discrete progenitor domains are generated by a combinatorial code of homeodomain and bHLH transcription factors which interpret the graded morphogen signal of Shh and refine this signal by cross-repressive interactions (Briscoe et al., 2000; Jessell,

2000). The neuronal subtypes generated from this spatial patterning are distinguished both by their expression of specific molecular markers and by their positional identity along the dorsoventral axis. There is a hierarchical determination of neuronal subtype identity in the motor neuron lineage, where LIM homeodomain proteins act to further sub-specify motor neurons into distinct columns, divisions and pools, each of which is distinguished by a distinct cell body positioning within the motor column and by projections to distinct targets (Jessell, 2000).

Oligodendrocytes and astrocytes are generated from ventricular zone progenitors following a cell intrinsic temporal switch from neurogenesis to gliogenesis. In the spinal cord, this switch involves the upregulation of the pro-glial NFI family of transcription factors as well as the inhibition of neurogenesis by Notch signalling (Deneen et al., 2006; Louvi and Artavanis-Tsakonas, 2006). Oligodendrocytes are generated from the pMN domain of the ventral ventricular zone, which is marked by the expression of the bHLH transcription factor Olig2 (Lu et al., 2000; Zhou et al., 2000). This domain generates motor neurons during neurogenesis and Olig2 is necessary and sufficient for both motor neuron and oligodendrocyte fate (Lu et al., 2002; Novitch et al., 2001; Zhou and Anderson, 2002; Zhou et al., 2001). In the absence of Olig2, the progenitors acquire the identity of the immediately dorsal p2 domain and they generate V2 interneurons and then astrocytes. Thus, Olig2 controls the specification of a neuronal and glial subtype from the same progenitor domain during different phases of development.

The fact that glial progenitors convert to astrocyte formation in the Olig mutant supports the notion that astrocytes are generated as the “default” glial fate. Consistent

with this it has been shown that the pro-glial NF1 genes are sufficient to promote astrocyte differentiation in the absence of Olig2 antagonism (Deneen et al., 2006). Also, astrocytes appear to be broadly generated from VZ progenitors outside of pMN, as assayed by the migration of NFIA+, GLAST+, and FGFR3+ cells (Deneen et al., 2006; Pringle et al., 2003; Shibata et al., 1997). Further evidence that repression of Olig2 is essential in astrocyte specification comes from the discovery that the bHLH transcription factor SCL is a p2 domain specific factor which promotes astrocyte fate and represses oligodendrocyte generation within this domain by repressing Olig2 expression (Muroyama et al., 2005).

We sought to investigate the issue of astrocyte diversity and its developmental specification in the spinal cord. As a first step in addressing this question it was necessary to identify molecular markers which label specific populations of astrocytes. Here we describe 3 molecularly and positionally distinct subpopulations of astrocytes in the spinal cord using the secreted signaling molecules Reelin and Slit1 as molecular markers: Reelin+/Slit1- in the lateral white matter, Reelin+/Slit1+ in the ventral-lateral white matter, and Slit1+/Reelin- in the ventral-medial white matter.

The homeodomain transcription factor Pax6 is specifically expressed in Reelin+ astrocytes. Pax6 has been previously shown to play an essential role as fate determinant in the development of many organs including cortex, spinal cord, eye and pancreas (Ashery-Padan et al., 2000; Gotz et al., 1998; Marquardt et al., 2001; Muzio et al., 2002; St-Onge et al., 1997) In the spinal cord, Pax6 is part of the combinatorial code specifying ventral neuronal subtypes, and it is required for V1 and

V2 interneuron generation (Ericson et al., 1997). We found that Pax6 is necessary and sufficient to promote Reelin and repress Slit1 in astrocytes of the ventral white matter. These results suggest that, like Olig2, Pax6 plays a dual role and in regulating both neuronal and glial subtype specification and provides additional evidence that the same factors can impact the specification of cell types from a particular pool of VZ progenitors in both the neurogenic and gliogenic phases.

## Results

*Reelin and Slit1 mark subpopulations of astrocytes in ventral white matter*

During gliogenesis, the bHLH transcription factor Olig2 controls the oligodendrocyte vs. astrocyte fate decision, with progenitors in the Olig2 mutant mouse generating astrocytes instead of oligodendrocytes (Zhou and Anderson, 2002). We used Affymetrix cDNA microarrays to compare the gene expression profiles of FACS isolated Olig2-GFP expressing glial progenitors from the Olig1,2 -/- and Olig1,2 +/- spinal cord. Reelin and Slit1 were among the candidate genes with higher expression in the Olig1,2 -/- 'converted astrocyte progenitor' population. As our initial analysis, we performed in situ hybridization on spinal cord sections and found Reelin and Slit1 were expressed in the white matter at E18.5, indicating these genes were likely expressed in glial cells (Figure 1 A, F). We then examined the expression of these genes more closely in double labeling studies using Reelin antibody and a *Slit1*-GFP reporter from mice previously described (Plump et al., 2002). As expected, both Reelin and Slit1 were found to be expressed in GFAP+ and

NFIA+ astrocytes (Figure 1) and were not expressed in Olig2+ oligodendrocytes (data not shown). Both Reelin and Slit1 were also expressed in many neurons in the grey matter, but in the white matter their expression was astrocyte specific. Morphologically, both Reelin+ and Slit1+ astrocytes are characterized by their cell bodies localized at the subpial surface (as identified by NF1A+ nuclei) with radially oriented GFAP+ processes projecting inward. This morphology has been previously characterized as the most common for astrocytes in the white matter (Liuzzi and Miller, 1987).

Interestingly, we found that Reelin and Slit1 did not label all astrocytes, but each was spatially restricted to a subset of astrocytes in the ventral white matter. Reelin was expressed in astrocytes of the lateral and ventral-lateral white matter, but not in astrocytes close to the ventral midline (Figure 1). Slit1 on the other hand is expressed in astrocytes in the ventral-medial and ventral-lateral white matter but not in astrocytes of the lateral white matter. Double labelling of Reelin and Slit1 shows that their expresion overlaps in the ventral-lateral white matter (Figure 1 K). Quantification of this overlap shows that around 50% of Reelin+ astrocytes are also Slit1+, and around 50% of Slit1+ astrocytes are also Reelin+. Thus astrocytes in the ventral white matter can be divided into 3 positionally and molecularly defined subpopulations which are approximately equal in abundance: Slit1+ only astrocytes in the ventral-medial, Slit1+/Reelin + co-expressing astrocytes in the ventral-lateral, and Reelin + only astrocytes in the lateral white matter (see Figure 1 L). For simplicity, we will designate these molecularly and positionally distinct populations

as L type (lateral white matter – Reelin+), VL type (ventral-lateral white matter – Reelin+, Slit1+), and VM type (ventral-medial white matter – Slit1+).

*Reelin expressing astrocytes co-express Pax6*

Pax6 was also upregulated in the Olig1,2 -/- population in our microarray screen. We found that Pax6 marked a subpopulation of astrocytes in the ventral-lateral white matter very similar in distribution to that of Reelin. The Pax6+ fraction represents approximately 40% of GFAP+ and NF1A+ white matter astrocytes (Figure 2 A-D, I). Double labeling confirmed that Pax6 and Reelin are colocalized and mark L and VL type astrocytes (Figure 2 E,F,I). We confirmed that, like Reelin, Pax6 was co-expressed with Slit1 in the VL type astrocyte population (Figure 2 G,H, I). Since Pax6 has been shown to be a cell fate determinant in many systems, including during neurogenesis in the ventral spinal cord, we next sought to examine whether Pax6 played any role in regulating the identity of these astrocyte subpopulations.

*Pax6 is required for Reelin expression in astrocytes*

Pax6 knockout mice have been previously generated with LacZ knocked into the Pax6 locus (St-Onge et al., 1997). We examined E18.5 spinal cord sections from Pax6 -/- and +/+ mice and found that there were a normal number of NF1A and GFAP+ astrocytes in the Pax6 -/- spinal cord (Figure 3). Thus Pax6 does not appear to be required for generic migration and differentiation of astrocytes.

We examined Reelin expression in the Pax6 mutant. While astrocyte development *per se* was not affected, we found a significant loss of Reelin expression in astrocytes

in the Pax6 mutant. This loss of astrocytic Reelin was evident both by in situ hybridization (Figure 3 A,B,E,F) and immunostaining (Figure 3 C,D,G,H). We found that the total number of Reelin+/GFAP+ and Reelin+/NFIA+ cells was significantly reduced, as were the percentages of GFAP+ and NFIA+ cells which are Reelin+ (Figure 3 I, J). One possible explanation for the loss of Reelin+ astrocytes is that the loss of Pax6 leads to the selective cell death of this subpopulation or to a failure to migrate or differentiate normally, coupled with a non cell-autonomous compensation for the number of GFAP+ and NFIA+ astrocytes. This is not the case because there were a normal number of Bgal+ astrocytes in the white matter of the Pax6 -/- mutant. The percentage of Bgal+ cells expressing Reelin in the Pax6 -/- mutant was severely reduced compared to the percentage of Pax6+ cells which normally express Reelin in the wild-type (Figure 3J). Thus Pax6 is required for the expression of Reelin in L and VL type astrocytes, but not for their formation and localization.

*Astrocyte subtype conversion in the absence of Pax6*

Since L type astrocytes express Pax6 and Reelin but not Slit1, we sought to examine whether Pax6 might also play a role in preventing Slit1 expression in these cells. To this end, we crossed the Pax6-lacZ mice with Slit1-GFP mice and analyzed the expression of *Slit1*-GFP in Pax6 mutants. We found that in addition to the loss of Reelin, GFAP+ and NFIA+ astrocytes in the lateral white matter of the Pax6 mutant upregulate Slit1 expression. While around 50% of Pax6+ cells normally co-express Slit1 (VL type), nearly all of the Bgal+ cells in the Pax6 -/- co-express Slit1, indicating upregulation of Slit1 in L type astrocytes (Figure 4). Therefore, Pax6 is

required for the normal repression of Slit1 in L type astrocytes. The expansion of Slit1 expression together with the loss of Reelin expression in astrocytes of the Pax6 -/- mutant results in the molecular conversion of the L and VL type astrocytes (marked by Bgal expression) to the VM identity (Figure 4 H). Taken together these data demonstrate that Pax6 is required for the generation of L and VL type astrocytes in the white matter of the ventral spinal cord via its regulation of Reelin and Slit1 expression.

*Pax6 is sufficient to promote Reelin and repress Slit1 expression in astrocytes*

The loss of function data suggest that Pax6 has pro-Reelin and anti-Slit1 functions. In order to determine whether Pax6 plays an instructive role in regulating the expression of these genes and therefore astrocyte identity, we performed in vivo gain of function experiments in the embryonic chick neural tube. We examined Reelin and Slit1 expression in the embryonic chick spinal cord to confirm that these genes labeled subsets of white matter astrocytes and found Reelin+/NFIA+ astrocytes in the lateral white matter and Slit1+/NFIA+ astrocytes in the ventral white matter of the E12 chick spinal cord (data not shown). We sought to examine the effect of Pax6 misexpression on these populations. The spinal cord of E2 chick embryos was electroporated with replication competent RCAS B retroviruses carrying either the chick Pax6 or GFP genes. Electroporated embryos were harvested and analyzed at E12, following 10 days of development in ovo. We found that Pax6 misexpression significantly increased the percentage of Reelin+ /NFIA+ cells in the white matter compared with control GFP misexpressing embryos (56% vs. 20%) (Figure 5). At

least some of this increase can be attributed to positionally ectopic Reelin expression in ventral astrocytes (Figure 5 C). The total number of NFIA+ astrocytes was not affected by Pax6 electroporation (data not shown). Thus Pax6 is sufficient to promote Reelin expression in astrocytes while not affecting general astrocyte fate.

Interestingly, we only detected a mild increase in astrocytic Reelin when comparing the electroporated (56% Reelin+/NFIA+) vs. control sides (42% Reelin+/NFIA+) of Pax6 electroporated embryos despite a visibly much higher level of Pax6 expression on the electroporated side. One possible explanation for this finding is that low levels of Pax6 misexpression on the control side, due to secondary infection by the replication competent virus, are sufficient to promote an increase in Reelin expression.

We also examined the effects of Pax6 misexpression on Slit1. We found a significant reduction in the percentage of Slit1+/NFIA+ astrocytes with Pax6 misexpression compared with the GFP control (Figure 5). Thus Pax6 is also sufficient to repress Slit1 expression in astrocytes. Interestingly, the reduction of Slit1 expression was only seen on the electroporated side of Pax6 electroporated embryos. This suggests that the low levels of Pax6 misexpression on the control side, due to viral spread, are not sufficient to repress Slit1. Taken together these results show that Pax6 is sufficient to promote Reelin expression and repress Slit1 expression in astrocytes. Through these functions Pax6 plays an instructive role in regulating astrocyte identity.

*Slit1 expressing astrocytes co-express Nkx6.1*

Given our results with Pax6, we hypothesized that another regulatory gene may specifically mark Slit1+ astrocytes. Such a gene would also partially overlap with Pax6 and Reelin in type VL astrocytes, and represent the functional antagonist of Pax6 in regulating the identity of these populations. Nkx6.1 is a homeodomain transcription factor expressed in the ventral ventricular zone which normally partially overlaps with Pax6 in the pMN and p2 domains. The ventral p3 domain only expresses Nkx6.1 but not Pax6, while the more dorsal p0 and p1 domains express Pax6 but not Nkx6.1 (Jessell, 2000). This partially overlapping pattern of Pax6 and Nkx6.1 expression in the ventricular zone bears an intriguing resemblance to the partially overlapping pattern of Reelin and Slit1 expressing astrocytes in the white matter. We examined Nkx6.1 expression in E18.5 spinal cord and found that Nkx6.1 specifically marks Slit1 expressing astrocytes (Figure 6 A-C). Double labeling with Pax6 and Nkx6.1 revealed that these genes partially overlap in astrocytes of the ventral-lateral white matter, recapitulating the pattern of Reelin and Slit1 expression (Figure 6 D-F). Thus Pax6 partially overlaps with Nkx6.1 both in the ventricular zone and in ventral white matter astrocytes, such that these factors mark 3 populations of glial progenitors in the ventricular zone and 3 positionally defined types of astrocytes along the subpial surface of the ventral white matter.

*Pax6+/Nkx6.1+ astrocytes are derived from the p2 domain*

The correlation between the pattern of Nkx6.1 and Pax6 expression in ventricular zone and the white matter suggests a simple combinatorial model for the generation of these astrocyte subpopulations (Figure 6 K). Nkx6.1+ astrocytes of the

ventral-medial white matter (VM type) could be derived from Nkx6.1+ progenitors from the p3 domain, Nkx6.1+, Pax6+ astrocytes of the ventral-lateral white matter (VL type) could be derived from the Nkx6.1+, Pax6+ progenitors of the p2 domain, and Pax6+ astrocytes of the lateral white matter (L type) could be derived from the Pax6+ progenitors of the p0 and p1 domains. Although Pax6 and Nkx6.1 also overlap in the pMN domain in the ventricular zone, the pMN domain is unlikely to contribute significantly to these astrocyte subtypes since pMN progenitors give rise almost exclusively to oligodendrocytes, under the direction of Olig2 (Zhou and Anderson, 2002).

In order to test our hypothesis that these astrocyte subpopulations may be prespecified from particular ventricular zone domains, we took advantage of the fact that Olig2-GFP expressing cells in the Olig1,2 -/- mutant are respecified to p2 domain identity and generate p2 derived astrocytes (Zhou and Anderson, 2002). We sought to test our prediction that the Nkx6.1+, Pax6+ VL type astrocyte population was derived from the p2 domain, using Olig2-GFP in the Olig1,2 -/- mutant as a p2 lineage marker. We performed triple-labeling with Nkx6.1, Pax6 and Olig2-GFP on E18.5 Olig1,2 -/- mutant spinal cord. In this assay we found that the p2 derived Olig2-GFP+ astrocytes are Nkx6.1+ and Pax6+ (Figure 6 G-J). Not all of the Nkx6.1+, Pax6+ cells are Olig2-GFP+ since Olig2-GFP only marks the super-numerary copy of p2 replacing pMN.

These data suggest that the p2 domain gives rise to the Pax6+/Nkx6.1+ VL type astrocyte population of the ventral-lateral white matter. The patterning of astrocytic progenitors in the ventricular zone could specify the molecular and

positional identity of astrocytes in the ventral white matter. Further lineage tracing experiments are necessary to formally determine whether the Pax6+, Reelin+ astrocytes of the lateral white matter are derived from p0-p1 progenitors and whether the Nkx6.1+, Slit1+ astrocytes of the ventral medial white matter are derived from the p3 domain.

## Discussion

While astroycte diversity has been described in various contexts of the adult CNS, the generation of this diversity has not been examined in vivo during development. We identified 3 molecularly and positionally defined astrocyte subpopulations in the ventral white matter of the E18.5 mouse spinal cord. We found that the homeodomain transcription factor Pax6 is an essential factor and plays an instructive role in specifying the positional identity of these populations. We additionally provide evidence that at least one of these populations is prespecified in the ventricular zone, and propose a general model of how these subtypes are generated based on the correlation between the partially overlapping patterns of expression of Pax6 and Nkx6.1 in both the ventricular zone and the white matter.

### *Molecular and Positional Identity of Astrocytes in the ventral white matter of the spinal cord*

It has been shown that progenitors of the ventral ventricular zone give rise to at least 5 general classes of neurons, each arising from distinct domain of VZ progenitors (Briscoe et al., 2000; Jessell, 2000). During gliogenesis, cells from the

pMN domain generate oligodendrocytes (Zhou and Anderson, 2002; Zhou et al., 2000), while progenitors from other ventral domains make astrocytes (Deneen et al., 2006; Pringle et al., 2003; Shibata et al., 1997). Prior to this study, it was not clear whether a single type of astrocyte was generated from all of the domains outside of pMN or whether molecularly distinct subtypes of astrocytes are produced. Microarray analysis of Olig mutant mice led us to identify Reelin and Slit1 as new markers which are specific for astrocytes and not oligodendrocytes. Importantly, Reelin and Slit1 were expressed in subsets of astrocytes. We found that astrocytes of the ventral white matter of the spinal cord are divided into 3 positionally and molecularly defined subpopulations which are approximately equal in abundance: VM type (Slit1+, Nkx6.1+, ventral-medial white matter), VL type (Slit1+,Reelin +, Pax6+, Nkx6.1+, ventral-lateral white matter), and L type (Reelin +, Pax6+, lateral white matter).

Importantly these astrocyte populations are restricted to defined positional identities along the subpial surface of the ventral white matter. This is particularly interesting since although neuronal subtypes in the spinal cord are specified to discrete positional identities, positional identity has not previously been identified in the generation of spinal cord glia. While oligodendrocytes are primarily generated from a defined subset of progenitors in the pMN domain, they become widely distributed throughout the gray and white matter of the spinal cord (Zhou et al., 2000). Previous studies of astrocyte development in the spinal cord with markers such as GLAST, FGFR3 and NFI genes similarly revealed a broad migration and distribution of astrocytes (Deneen et al., 2006; Pringle et al., 2003; Shibata et al.,

1997). Likewise, markers of differentiated astrocytes such as GFAP and S100B are expressed broadly throughout the white matter. Using the molecular markers Reelin and Slit1, along with Pax6 and Nkx6.1, we were able to identify the L, VL and VM type astrocyte populations distributed along the arc of ventral white matter. The positional identity of these astrocytes may be important for region specific functions and represents a new level of both molecular diversity and organizational complexity of spinal cord glia.

*Pax6 is necessary for the positional identity of spinal cord white matter astrocytes*

Analysis of Pax6 -/- mutant embryo spinal cord revealed that Pax6 plays 2 roles which allow for the specification of astrocyte positional identity. First, Pax6 is required for Reelin expression in both VL and L type astrocytes. Second, Pax6 is required for the repression of Slit1 expression in L type astrocytes. These functions may or may not be mechanistically related. Since VL type astrocytes are Reelin+/Slit1+, the functions of Pax6 in repression of Slit1 and the promotion of Reelin need not be coupled in a fate switching mechanism. On the contrary, the fact that Pax6 fails to repress Slit1 in VL type astrocytes, yet still serves to promote Reelin in this population suggests that these 2 functions can be uncoupled.

There are a few possible explanations that could explain why Pax6 apparently represses Slit1 expression in L type astrocytes but not in VL type astrocytes. One possibility is that there is a gene or factor present in the VL type astrocytes but not in the L type population which interferes with the ability of Pax6 to repress Slit1. Another possibility is the converse, that is that there is a cofactor required for Pax6 to

be able to repress Slit1 which is only present in L type but not VL type astrocytes. Finally, a dose dependant mechanism is possible where repression of Slit1 is highly sensitive to Pax6 concentration and where hypothetically, higher levels of Pax6 expression in L type astrocytes compared with VL type astrocytes could lead to Slit1 repression in only the L type population. While there is no evidence of such a difference in Pax6 levels of expression by immunostaining, such a model has precedent since it has been shown that Pax6 concentration can be critical in other developmental systems with a high degree of haploinsufficiency being observed in Pax6 +/- mice with respect to eye development (Davis-Silberman et al., 2005). However this possibility is unlikely as we did not detect any happloinsufficiency in Pax6 +/- spinal cord with regard to either loss of Reelin or upregulation of Slit1 (data not shown).

*Pax6 is sufficient to promote Reelin and repress Slit1 expression in white matter astrocytes*

In order to gain a better understanding of the role of Pax6 in Reelin promotion and Slit1 repression, we misexpressed Pax6 in embryonic chick neural tube and assayed for the effects on Reelin and Slit1 expression on NFIA+ astrocytes. We found that Pax6 was sufficient to promote an increase in the percentage of NFIA+ cells that are Reelin+, as well as to reduce the percentage of NFIA+ cells that are Slit1+ within the white matter. Importantly these results demonstrate that Pax6 does indeed play instructive roles with respect to both its function to promote Reelin and repress Slit1. The fact that Pax6 alone is sufficient to repress Slit1 expression in this

experiment argues against a model where Pax6 requires a cofactor to repress Slit1, which is normally present in L type but not VL type astrocytes.

The most likely explanation for why Pax6 normally represses Slit1 in lateral but not ventral-lateral white matter astrocytes is the presence of a factor in VL but not L type astrocytes which interferes with the ability of Pax6 to repress Slit1. This interference could be overridden in a gain of function experiment due to the high levels of Pax6 misexpression. Nkx6.1 is a candidate factor for this role, as it is expressed in VL but not L type astrocytes. Additionally, while Nkx6.1 and Pax6 do not transcriptionally repress each other during patterning, there is precedent for their functional antagonism in the determination of $\alpha$ and $\beta$ cell fates in the pancreas (Hill et al., 1999; Schisler et al., 2005). A competition experiment in which Nkx6.1 is misexpressed together with Pax6 would reveal if indeed Nkx6.1 can block the ability of Pax6 to repress Slit1 expression.

*Prespecification of the ventral-lateral astrocyte subpopulation*

An important issue regarding the generation of these astrocyte subtypes is whether each of the positionally defined populations is prespecified from a particular domain of progenitors in the ventricular zone. To address this issue we used the Olig2-GFP in Olig1,2 -/- mice as a lineage marker of p2 derived astrocytes and used triple-labeling together with Pax6 and Nkx6.1 to determine whether any of the 3 astrocyte subpopulations are derived from p2. We found that the Olig2-GFP + astrocytes of Olig1,2 -/- mice had the Pax6+ and Nkx6.1+ phenotype of VL type astrocytes, suggesting that this population is derived from the p2 domain. Not all of

the Pax6+/Nkx6.1+ astrocytes in this experiment were Olig2-GFP+, however, since the Olig2-GFP only marks the pMN → p2 converted domain and not the endogenous p2 domain.

*Spatial patterning during gliogenesis*

Interestingly, the expression of Pax6 and Nkx6.1 overlaps in the p2 and pMN domains of the ventricular zone. While the pMN domain is specialized to form oligodendrocytes, the astrogenic progenitors of the ventral ventricular zone are characterized by the p0-p1 domains expressing Pax6 but not Nkx6.1, the p2 domain expressing both Pax6 and Nkx6.1, and the p3 domain expressing Nkx6.1 but not Pax6. Given the evidence that the VL type astrocytes (Pax6+/Nkx6.1+) are derived from p2, a simple model can be proposed based on further correlating the expression of Pax6 and Nkx6.1 in the ventricular zone with their expression in white matter astrocytes. In such a model, in addition to the Pax6+/Nkx6.1+ progenitors of the p2 domain giving rise to the Pax6+/Nkx6.1+ astrocytes of the ventral-lateral white matter (for which we have short term lineage tracing evidence), the Pax6+/Nkx6.1- progenitors of the p0 and p1 domains would give rise to the Pax6+/Nkx6.1- L type astrocytes of the lateral white matter and the Nkx6.1 only expressing progenitors of the p3 domain would give rise to the Nkx6.1 only expressing astrocytes of the ventral-medial white matter. Futher lineage tracing and functional experiments are necessary to prove the specific origins of the ventral-medial and lateral populations. Nevertheless our findings illustrate that astrocytes of the ventral spinal cord are specified into at least 3 molecularly and positionally distinct subtypes and that Pax6 is

a critical regulator of this process. The full extent and functional significance of this new aspect of glial diversity remains to be explored, yet given the genetic investment in specifying these distinct astrocyte subtypes it is likely that they have some region specific functions.

## Experimental Procedures

### *Mouse mutants*

Olig1,2 -/- mice (Zhou and Anderson, 2002), Pax6-LacZ mice (St-Onge, Sosa-Pineda et al., 1997) and Slit1-GFP mice (Plump, Erskine et al. 2002) were genotyped by PCR using lacZ and GFP primers. Pax6-LacZ mice were crossed into the Slit1-GFP background to generate Pax6 -/-, Slit1-GFP +/- and Pax6 +/-, Slit1-GFP +/- embryos for analysis.

### *In situ hybridization / Immunohistochemistry*

In situ hybridization was performed on frozen sections as previously described (Zhou, Wang et al 2000). Antibodies were used against Pax6 (rabbit polyclonal, Covance and IgG1 monoclonal, DHSB), Reelin (G10 IgG1 monoclonal, Novus and 142 IgG1 monoclonal, Novus), GFP (chick polyclonal, Abcam), GFAP (rabbit polyclonal, DAKO and IgG1 monoclonal, Chemicon), NFIA (rabbit polyclonal, Active Motif), S100B (IgG1 monoclonal, Sigma), Olig2 (rabbit polyclonal, a kind gift of Tom Jessell), Nkx6.1 (IgG1 monoclonal, DHSB)

### *Chick embryo electroporation*

Chick embryos were electroporated at E2 with either RCAS B Pax6 or RCAS B GFP replication competent avian retroviruses, using established methods (Zhou, Choi et al. 2001). Embryos were incubated in ovo in a humidified 37 C incubator until E12.

## Acknowledgements

We thank Anastasia Stoykova, Peter Gruss and Guillermo Lanuza for sharing Pax6-LacZ mice and embryos, and Marc Tessier-Lavigne for the Slit1-GFP mice. We also thank Ed Laufer for chick Slit1 in situ probe. We thank Shirley Pease, Jennifer Alex and the staff of the Caltech animal facility for assistance with mouse breeding and timed matings; Peter Lwigale for advice and assistance with electroporation and egg husbandry techniques; Monica Martinez for genotyping mouse lines; Ritchie Ho and Jung Sook Chang for technical assistance; Gabriele Mosconi for labratory management; Gina Mancuso for administrative assistance; D.J.A. is an Investigator of the Howard Hughes Medical Institute.

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## Figure Legends

**Figure 1. Reelin and Slit1 mark subsets of astrocytes**

(A, F) In situ hybridization with probes for mouse Reelin and Slit1 showing their expression in ventral white matter. (B-E) Double labeling with Reelin and astrocyte markers GFAP and NFIA by immunohistochemistry. Arrowheads show astrocytes expressing Reelin in the lateral white matter. Arrows point out astrocytes in the ventral white matter which do not express Reelin. (G-J) Double labeling with Slit1 and either GFAP or NFIA. Arrowheads indicate Slit1+ astrocytes in the ventral white matter, while arrows denote Slit1 – astrocytes in the lateral white matter. (K) Double labeling with Reelin and Slit1 in the ventral white matter reveals 3 populations: L – lateral white matter, Reelin+ only, VL – ventral-lateral white matter Reelin+/Slit1+, and VM – ventral-medial. (L) Quantification of the percentage of these 3 populations out of the total white matter astrocytes demonstrates they are nearly equal in relative abundance (n = 3 embryos).

**Figure 2. Pax6 is coexpressed with Reelin in astrocytes**

**(**A-D) Pax6 double-labeling with GFAP and NFIA reveals its expression in astrocytes in the lateral white matter. (E-H) Pax6 double-labeling with Reelin and Slit1-GFP reveals that Pax6 is co-expressed with nearly all Reelin+ astrocytes but only partially overlaps with Slit1+ astrocytes. Positionally, Pax6 expression like that

of Reelin marks both the L and VL astrocytes but not the VM population. (I) Quantification of the percentage of various astrocytes expressing Pax6. Pax6 is expressed in around 40% of total white matter astrocytes as marked by GFAP or NFIA expression. Around 97% of Reelin+ astrocytes express Pax6, while around 50% of Slit1+ astrocytes express Pax6. The Slit1+/Pax6+ astrocytes are the VL population (n = 5 embryos).

**Figure 3. Loss of Reelin+ astrocytes in Pax6 -/- mice**

Analysis of Pax6 -/- embryonic spinal cord in comparison to wild-type littermates. (A, B) In situ hybridization shows Reelin expression in ventral lateral white matter of wild-type E18.5 spinal cord. (E, F) By contrast, in situ of Pax6 -/- littermates shows a loss of Reelin expression in the white matter at this stage. (C, G) Double-labeling of Reelin and GFAP shows Reelin expressing astrocytes in lateral white matter in the wild-type. In the Pax6 -/- spinal cord, GFAP+ astrocytes are present in the lateral white matter but they are not co-expressing Reelin. (D, H) Double-labeling of Reelin and NFIA reveals that NFIA+ astrocytes are present in both the wild-type and Pax6 -/- white matter, but Reelin expression is lost in these cells in the Pax6 -/-. (I, J) Quantification of white matter astrocytes in the Pax6 -/-. Wild-type counts are represented as blue bars, Pax6 -/- counts are represented by red bars, Pax6 +/- heterozygote is represented with a purple bar. 5 Wild type and 6 mutant embryos from 3 independent litters were counted. (J) The total number of astrocytes in the white matter as assessed by GFAP or NFIA is not changed in the Pax6 -/- spinal cord, assessed as the number of positive cells per section. However, there are significant

reductions in the total number of Reelin+, GFAP+ and Reelin+, NFIA+ cells in the Pax6 -/-, compared to wildtype (p values = 0.000007, and .00000008, respectively). Importantly there is no difference between the number of Bgal+, GFAP+ astrocytes in the Pax6 -/- and the number of Pax6+, GFAP+ astrocytes in the wild-type. (I) The percentage of GFAP+ or NFIA+ astrocytes expressing Reelin is significantly reduced in the Pax6 -/- mutant. Nearly 100% of the Bgal+ cells in the Pax6 -/- and the Pax6+ cells in the wildtype are GFAP+ and NFIA+. By contrast, there is a significant loss of Reelin expression among Bgal expressing cells in the Pax6 -/- compared to the normal amount of Reelin expression in Pax6+ cells. Nearly all Bgal+ cells in Pax6 +/- are also Pax6+, confirming Bgal is a faithful approximation of Pax6 expressing cells in this assay.

**Figure 4. Astrocyte subtype conversion in Pax6 -/- mice**

(A,D) Double labeling with Slit1-GFP and GFAP in Pax6 +/+ and Pax6 -/- embryos which are also heterozygous for Slit1-GFP. Slit1-GFP is normally not expressed in L astrocytes in wildtype, but is expressed in these GFAP+ cells in the Pax6 -/-. (B, E) Double labeling with Slit1-GFP and NFIA shows upregulation of Slit1 in L astrocytes in Pax6 -/-. (C, F) Double labeling with Bgal and Slit1-GFP in the Pax6 -/- shows that Slit1 expression is upregulated in the cells which have lost Pax6 expression, and where it is normally not expressed as evidenced by Pax6 and Slit1-GFP double labeling in the wild-type.

(G) Quantification of Slit1-GFP expression among astrocyte populations. Slit1 expression is significantly increased among total astrocytes marked by NFIA and

GFAP in the Pax6 -/-. Additionally nearly all Bgal+ astrocytes in the Pax6 -/- are Slit1+, while only around 50% of Pax6+ astrocytes are Slit1+ in the wildtype. (n = 3 embryos each, ++ and --, from 2 independent litters). (H) Model of astrocyte phenotype in the Wildtype and Pax6 -/- spinal cord. Due to the loss of Reelin and upregulation of Slit1, all of the astrocytes in the ventral white matter are Slit1+ and are thus phenotypically converted to the VM type, while the L type and VL type are lost.

**Figure 5. Pax6 promotes Reelin and represses Slit1 in astrocytes**

(A) Pax6 immunostaining reveals misexpression of Pax6, primarily on the right side (electroporated side) of the E12 spinal cord section. (F) GFP misexpression, was also confirmed by immunostaining. (B, C, G, H) Reelin in situ hybridization followed by NFIA immunostaining. An increase in the percentage of Reelin+/NFIA+ cells was detected with Pax6 overexpression relative to the GFP control. The arrow denotes an ectopically a Reelin+/NFIA+ cell which appears ectopically ventral. (D, E, I, J) Slit1 in situ hybridization followed by NFIA immunostaining. The percentage of Slit1+/NFIA+ cells is reduced with Pax6 misexpression compared to GFP control. This reduction is apparent between the electroporated and control sides (D). (K) Quantification of Pax6 and GFP gain of function. The percentage of Reelin+/NFIA+ was measured by counting all NFIA+ cells in the white matter and then the Reelin+, NFIA+ double positive cells. The percentage of Reelin+/NFIA+ cells is significantly increased between the electroporated side of Pax6 vs. the electroporated side of GFP ($p = .00000013$), however there was only slightly significant increase in the Pax6

electroporated vs. contralateral sides (p=.02). Similarly, the percentage of Slit1+/NFIA+ cells was quantified and it was found that there was a significant reduction in Slit1+ astrocytes on the electroporated side with Pax6 relative to the either the contralateral side (p = .002) or GFP control electroporated side (p = .009).

**Figure 6. Model of ventral astrocyte specification**

(A-C) Double-labeling reveals Nkx6.1 expression marks Slit1-GFP+ astrocytes in the white matter (arrow). Thus Nkx6.1 marks VM and VL type astrocytes in the white matter. (D-E) Pax6 and Nkx6.1 double labeling shows partial overlap in the both the ventricular zone (arrowhead) and white matter (arrow). (G-J) Olig2 -/- astrocytes (derived from supernumery copy of p2) are marked by Olig2-GFP. These astrocytes are both Nkx6.1+ and Pax6+, indicating a VL type phenotype. (K) The correlation between the partial overlapping pattern of Nkx6.1 and Pax6 in the ventricular zone and white matter, along with the evidence that the double positive population is derived from p2, suggest a model whereby each subtype of astrocyte is derived from ventricular zone domains with corresponding Pax6 and Nkx6.1 expression.

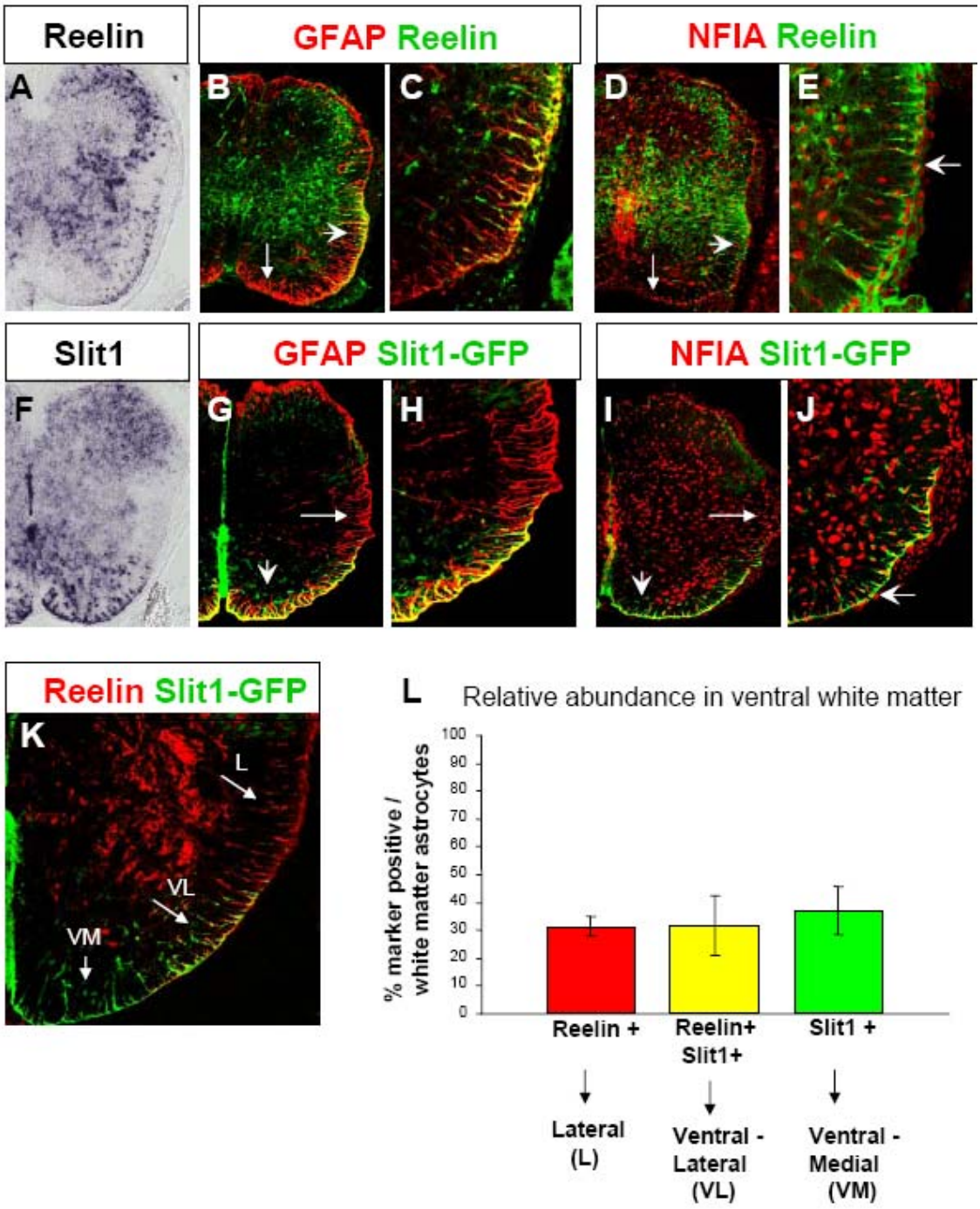
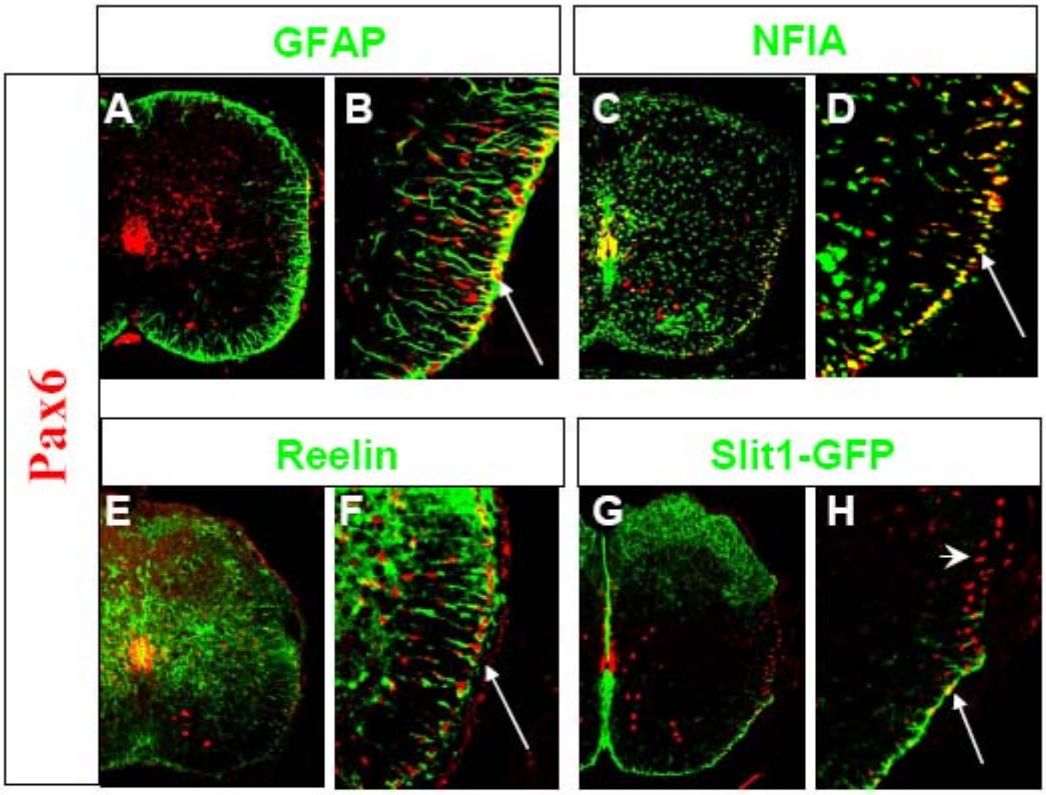



**Figure 1. Reelin and Slit1 mark subsets of astrocytes**



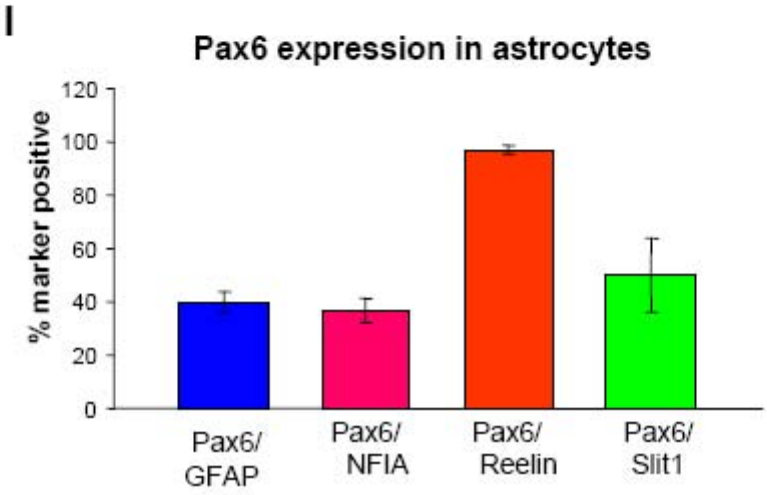




**Figure 2. Pax6 is coexpressed with Reelin in astrocytes**

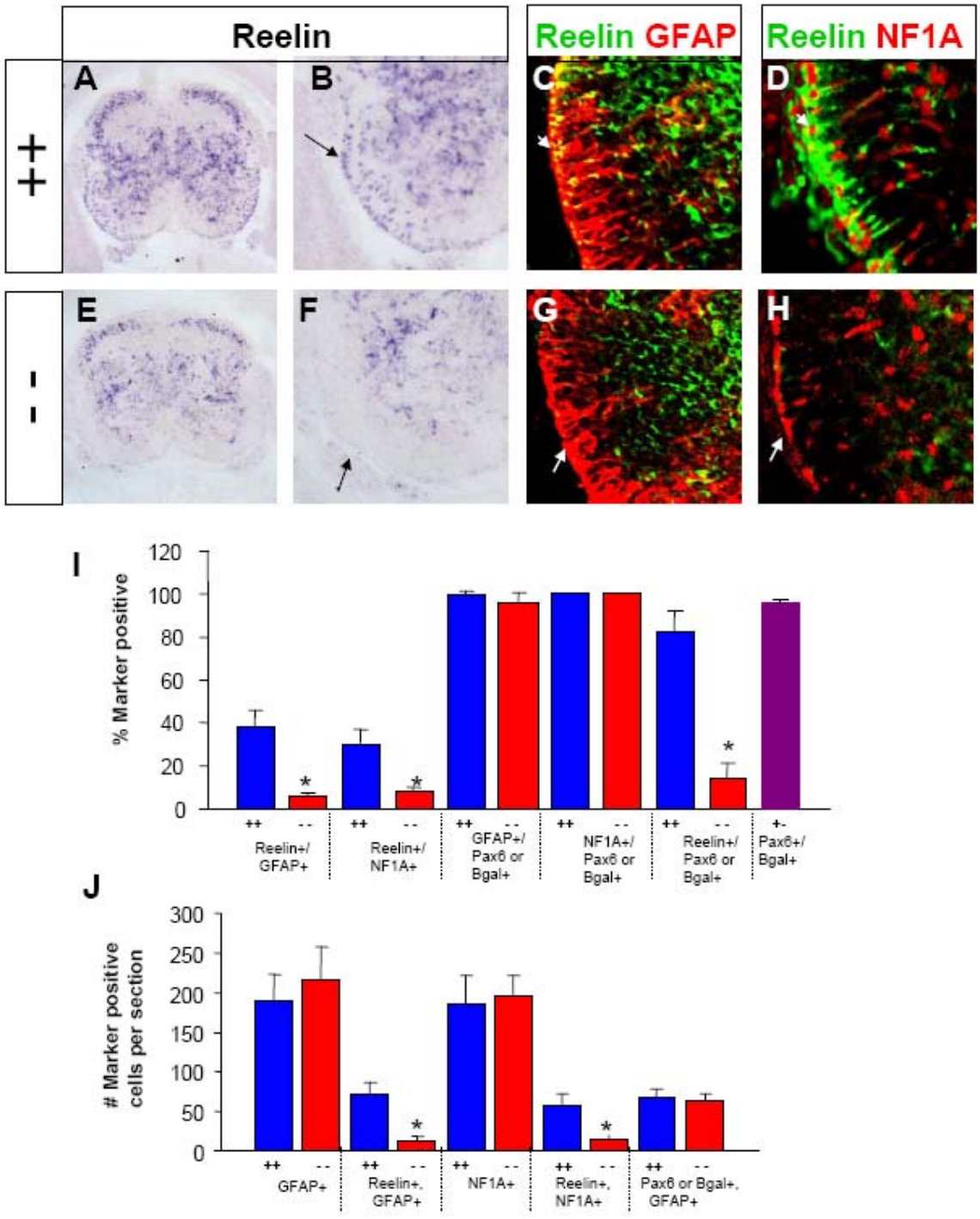



**Figure 3. Loss of Reelin+ astrocytes in Pax6 -/- mice**

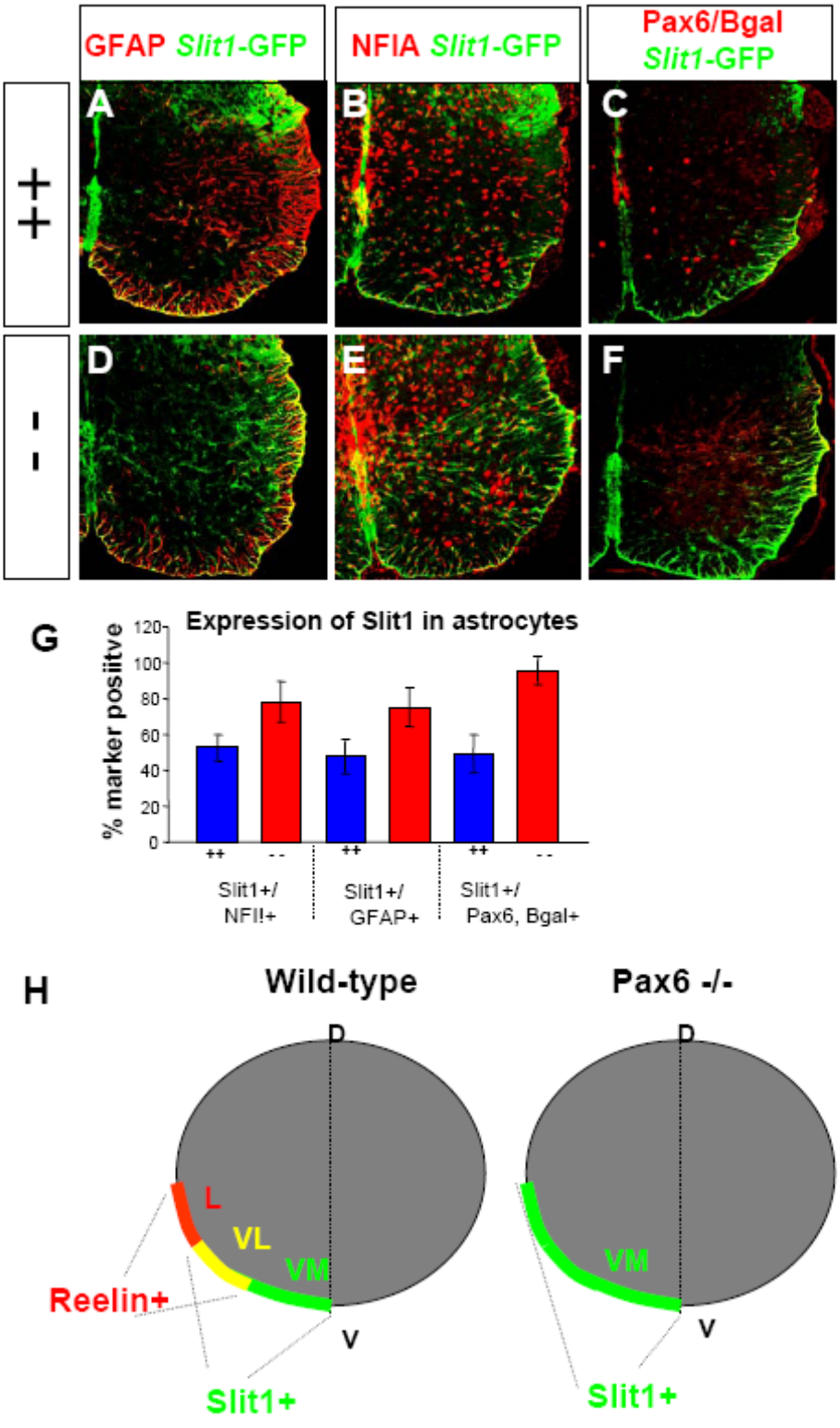



**Figure 4. Astrocyte subtype conversion in Pax6 -/- mice**

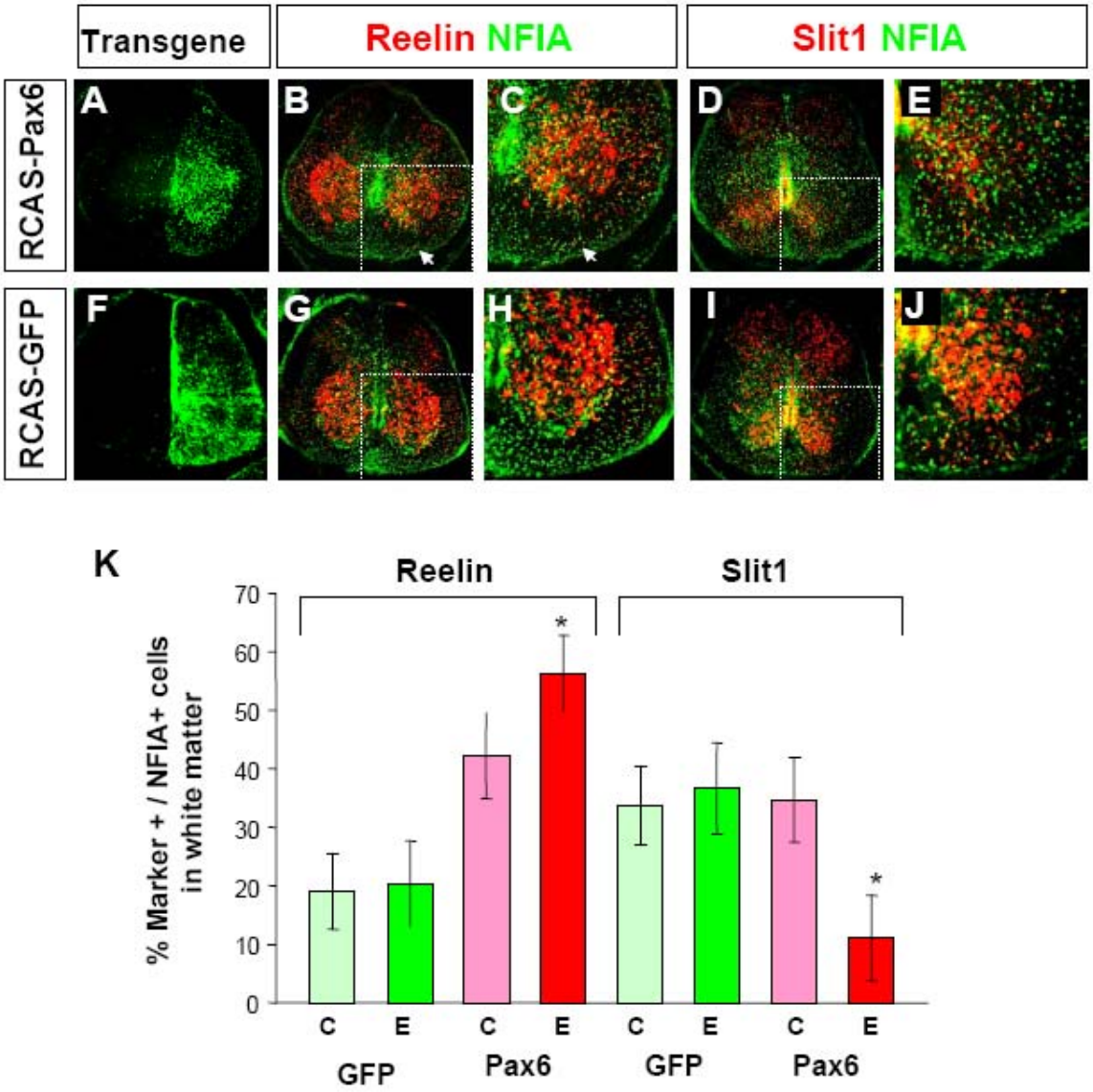



**Figure 5. Pax6 promotes Reelin and represses Slit1 in astrocytes**

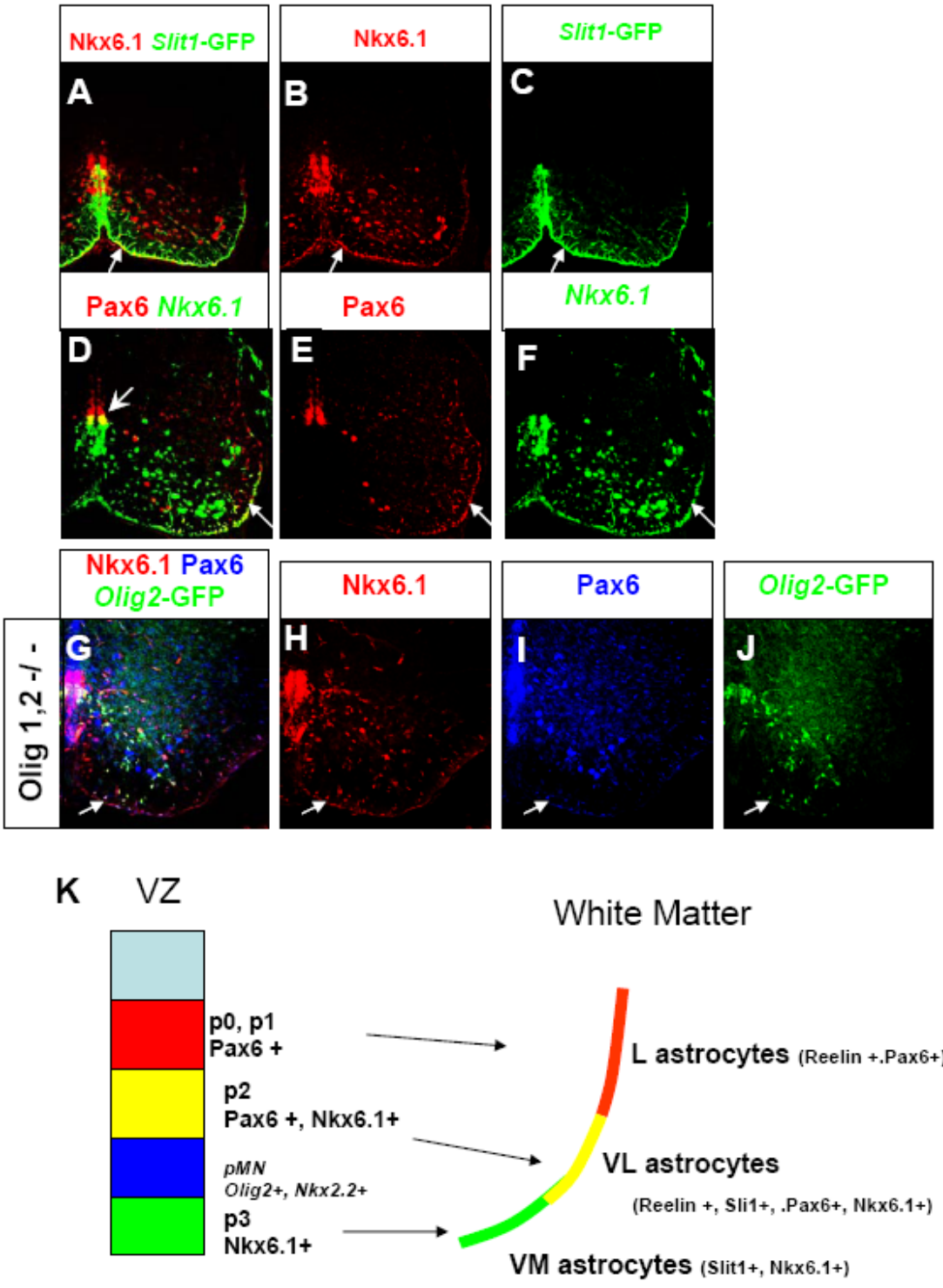



**Figure 6. Model of ventral astrocyte specification**

# Appendix

# I. The role of Pax6 in pMN progenitors

*Christian Hochstim, Melanie Lee and David Anderson*

We generated, tested and identified a functional RCAS-U6-shRNA for chick Pax6 as previously described (Deneen et al., 2006). We were intending to study the effects of Pax6 knockdown during early gliogenesis by electroporating this construct in E2 chick neural tube. However, we noticed a dramatic loss of progenitors in the pMN domain which was evident prior to gliogenesis at E4. In figure I-1, Pax6 is knocked down in the pMN region on the electroporated side which is identified by AMV staining. Olig2 expression is lost in the presence of Pax6 siRNA and there is a corresponding loss of the progenitor marker PCNA in this region. By contrast both the differentiated motor neuron marker HB9 and the postmitotic neuron marker NeuN are ectopically expressed in the ventricular zone. Importantly, these effects were not observed when electroporated with and RCAS carrying a mutated Pax6 shRNA sequence.

The loss of pMN progenitors was quantified with double labeling of Olig2 and Hb9. The expression of these 2 genes was counted as a function of spatial bins of distance from the ventricle. In the presence of the pax6 siRNA there was a medial displacement of HB9 and a loss of Olig2. Two possible explanations could explain this loss of progenitors and their spatial replacement with differentiated neurons. First, the progenitor cells could precociously differentiate into neurons in situ, depleting the progenitor pool. Alternatively, the progenitor cells could die and neurons move into the ventricular zone to fill the space left by the loss of progenitors. We observed increased cell death with Pax6 siRNA electroporations relative to both

the mutant siRNA or GFP controls by staining for activated Caspase 3 (data not shown). This cell death was not adequately rescued by co-electroporation with p35 anti-apoptotic protein (data not shown). Thus it is difficult to tell whether these data suggest that Pax6 is required for the survival of progenitors, or for keeping them in the undifferentiated state, or both. It has been reported that the Pax6 target, Fabp7 plays an essential role in blocking precocious progenitor differentiation in the cortex (Arai et al., 2005). However, we did not see this loss of progenitor cell phenotype in the pMN domain of Pax6-LacZ -/- mouse spinal cord. Given this negative result in the mouse, and the confounds with cell death, it is difficult to conclude whether anything can be concluded from these results about Pax6 function in these progenitors.

## II. Tet-inducible Avian retroviruses

*Christian Hochstim, Janet Chow and David Anderson*

One of the confounds of performing electroporation experiments in chick neural tube with respect to studying phenotypes during gliogenesis is that the optimal time for injection and electroporation is at E2, prior to neurogenesis. Thus it is difficult to determine whether a gene function yielding a glial phenotype is due to its function in glial cells or secondary to an earlier role in patterning or neurogenesis. To circumvent this problem reagents for conditional misexpression are needed. Tet inducible RCAN retroviral vectors have been developed as a two virus system: one constitutively expressing rtTA, and the other virus with the gene of interest downstream of TREtight promoter. This system has been shown to be effective along

with in ovo administration of Dox (Sato et al., 2002). We developed these vectors for the misexpression of Pax6 and GFP (Figure II-1). We tested the responsiveness and tightness of the system by coelectroporating the rtTA and TRET-GFP viruses along with a dsRED reporter (to demonstrate successful electroporation) added Dox to some of the embryos. In this initial test it is clear that the system is both robust and is not leaky in the absence of Dox (Figure II – 2). However in subsequent testing with Dox administration at E4-E6, we stuggled to efficient induction of gene expression

## III. The role of Shox2 in V2/MN Identity and oligodendrocyte precursor specification

*Christian Hochstim, Qiao Zhou and David Anderson*

Shox2 was upregulated in the Olig2 -/- mutant population in our Microarray experiments. It was found to be expressed in a subset of ventral ventricular zone progenitors specifically during glial stages (see Chapter 2, figure 3). However it was also persistently expressed on a population of neurons, likely V2 interneurons based on their position and distribution. Double in situ hybridization of E13 spinal cord revealed that the ventricular zone expression of shox2 colocalizes with Olig2 in the pMN domain (Figure III – 1). This is suprising since we expected based on its upregulation in the Olig mutant array sample, that it might be repressed by Olig2. It is most likely that we identified shox2 as a false positive due to its expression on V2 interneurons in which Olig2-GFP expression can perdure, as discussed in Chapter 2. Nevertheless, we were intrigued by this glial specific pMN expression.

We performed gain of function experiments with RCAS-Shox2 electroporation at E2 and analyzed at E5. We found that Shox2 overexpression strongly repressed motor neurogenesis as evidenced by strong reductions in MNR2/HB9, Lim3 and Isl1,2 expression (Figure III-2). Shox2 did not reduce motor neurogenesis by depleting pMN progenitors. There were a normal number of Olig2+ cells and Sox9 staining was normal throughout the ventricular zone. Shox2 promoted an increase in Chx10+ V2a interneurons, including ectopic V2 interneurons within the motor column (Figure III-2, arrow). No increase was detected in GATA3+ V2b interneurons. Quantification of the relative levels of HB9 and Chx10 revealed a 64% decrease in Hb9+ motor neurons coupled with a 54.5% increase in Chx10+ V2a interneurons. Because motor neurons are nearly 8 fold more numerous than V2a interneurons, the effect to repress motor neurogenesis is numerically much more significant. These experiments show that Shox2 can repress motor neurogenesis in the presence of normal Olig2 expression. This is intriguing because since Shox2 turns on in the pMN domain at the time of the neuron-glia transition, it suggests that Shox2 might play a role in downregulating motor neurogenesis within Olig2+ cells and play a role in their transition to making oligodendrocytes.

Shox2 misexpression alone was not sufficient to promote precocious oligodendrocyte formation, however co-electroporation of RCAS-Shox2 together with RCAS-Olig2 was sufficient to promote ectopic and precocious oligodendrocytes (Figure III -3). An attractive hypothesis from all of these data is that the function of Shox2 in repressing motor neurogenesis is important both in pMN progenitors as they transition to making oligodendrogyes and in newly born V2a interneurons by

repressing the alternative motor neuron fate. Loss of function experiments are required to test these ideas. Shox2 knockout mice have been generated (Yu et al., 2005) and we hope to receive embryos for analysis. If our hypothesis is correct, we would predict 2 phenotypes: a delay in the onset of oligodendrocyte precursor specification, coupled with extended motor neurogenesis in the pMN domain, and loss of V2a interneurons and their conversion to ectopic motor neurons.

## IV. HFH4 is not essential for gliogenesis

*Christian Hochstim, Qiao Zhou and David Anderson*

HFH4 had the most specific expression pattern of any gene we tested from our microarray candidates. It was upregulated in the Olig -/- mutant population. It was only expressed during gliogenesis in a specific subset of ventricular zone progenitors in the ventral spinal cord (see Chapter 2, Figure 4). This was intriguing because it appeared specific for glial progenitors, but unlike most glial markers, it was not expressed on any migrating or differentiating cells but remained completely ventricular zone specific even at E18.5. We verified that there is increased overlap between HFH4 and Olig2-GFP in the Olig mutant (Figure IV – 1).

We examined whether misexpresion of RCAS-HFH4 would affect early oligodendrocyte or astrocyte precursor specification and saw no effects (data not shown). Additionally we obtained and analyzed HFH4 -/- knockout embryos (You et al., 2004) to see if there were any defects in early glial development but we didn't see any difference in the expression of FGFR3 of GLAST in the ventricular zone or migrating precursors (Figure IV – 3). Thus it appears that HFH4 is not involved in

oligodendrocyte or astrocyte specification. Its restricted expression to only a subset of ventral progenitors, suggests that it has a highly specialized role, perhaps in ependymal cells or some late-generated ventricular zone population.

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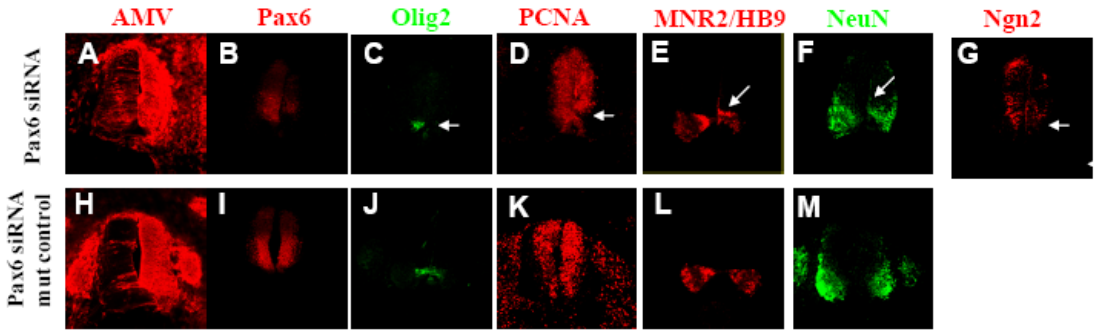
**Figure I-1 Loss of Motor Neuron progenitors with Pax6 siRNA knockdown**



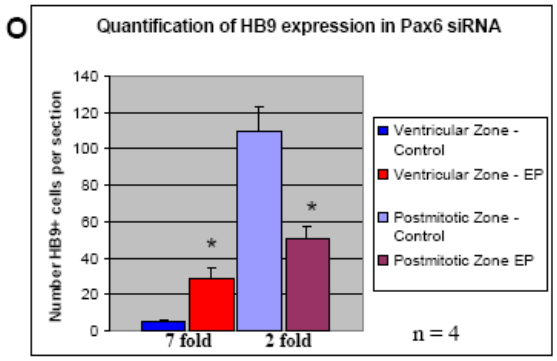
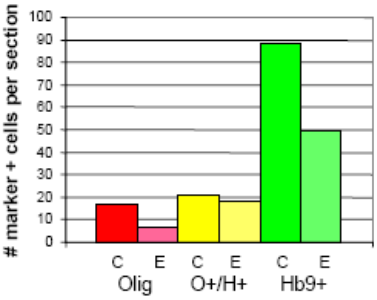
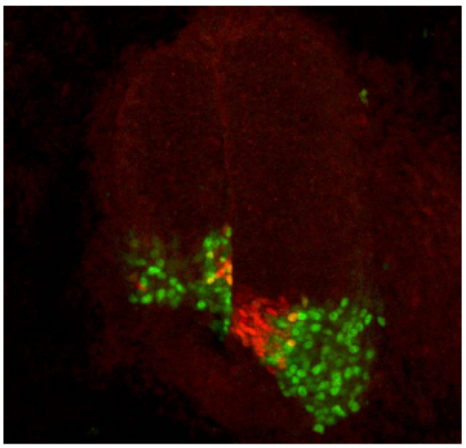
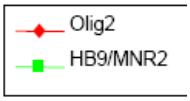



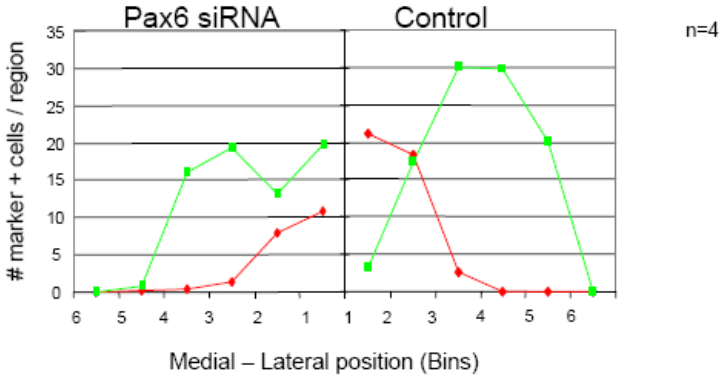
**Figure I-2 Pax6 knockdown alters Spatial Distribution of MN lineage markers**






The medial – lateral axis of the spinal cord was divided into bins 1 through 6, where bin 1 is adjacent to the ventricle and each bin is approximately 23 uM wide. The ventricular zone corresponds approximately to bins 1 and 2 and the postmitotic zone to bins 3 through 6



## Figure II-1 Inducible Gain of Function (Tet ON system)

**Regulatory protein:**
rtTA expressed constitutively from CMV promoter; binds to TRE-tight and **activates transcription in the presence of Dox**


Cloning: CMV-rtTA from Clontech pTet ON → Slax shuttle → RCAS

**Response plasmids:**
Gene expression controlled by TRE-tight promoter which is silent unless activated by rtTA bound to Dox. RCAN viral LTR cannot affect gene expression due to mutation of the splice acceptor site upstream of promoter-gene cassette


Cloning: Pax6 and GFP cloned into Clontech pTRE-tight;
promoter-gene casette → Slax shuttle → RCAN

**Figure II-2 Activation of Tet-inducible GFP by Dox: E3 (18 hour)**

3 constructs: RCAN-TREtight-GFP
pTet-ON (CMV-rtTA)
pCS-dsRED

Single administration of 80 ug Dox (sub-vittelline)
Harvest 18 and 48 hours post-electroporation

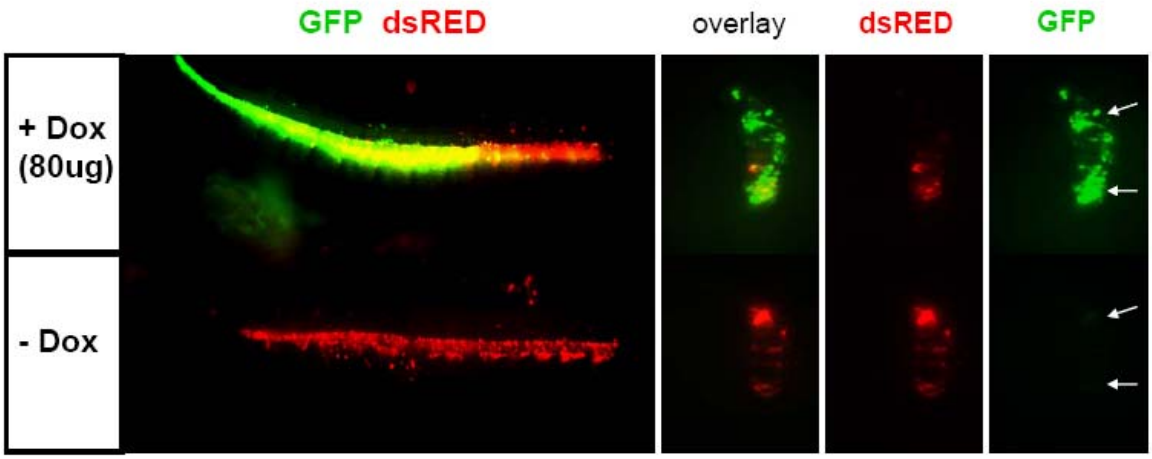


**Figure III – 1 Shox2 is co-expressed with Olig2 in pMN domain during early gliogenesis**

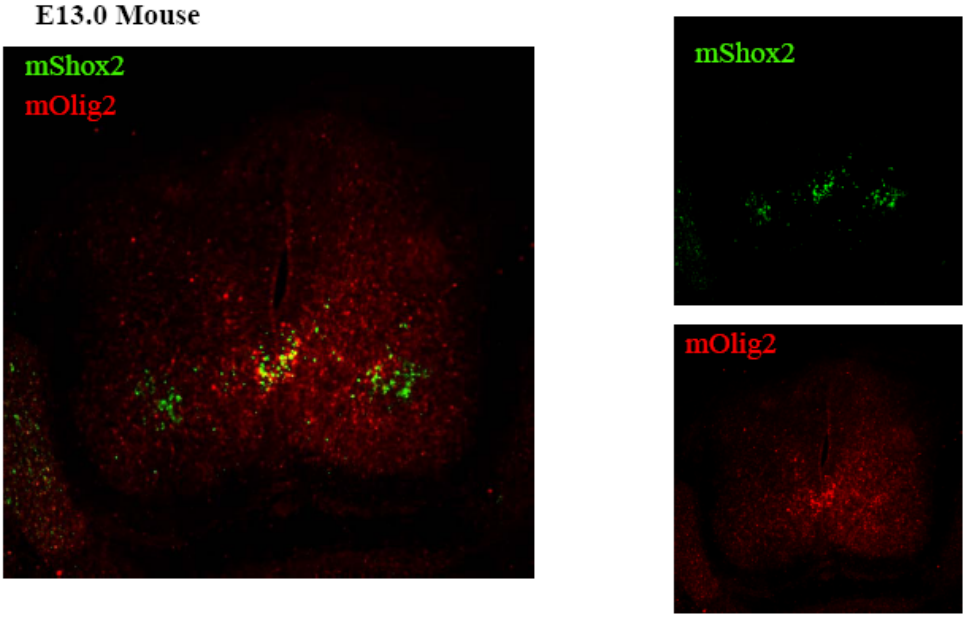
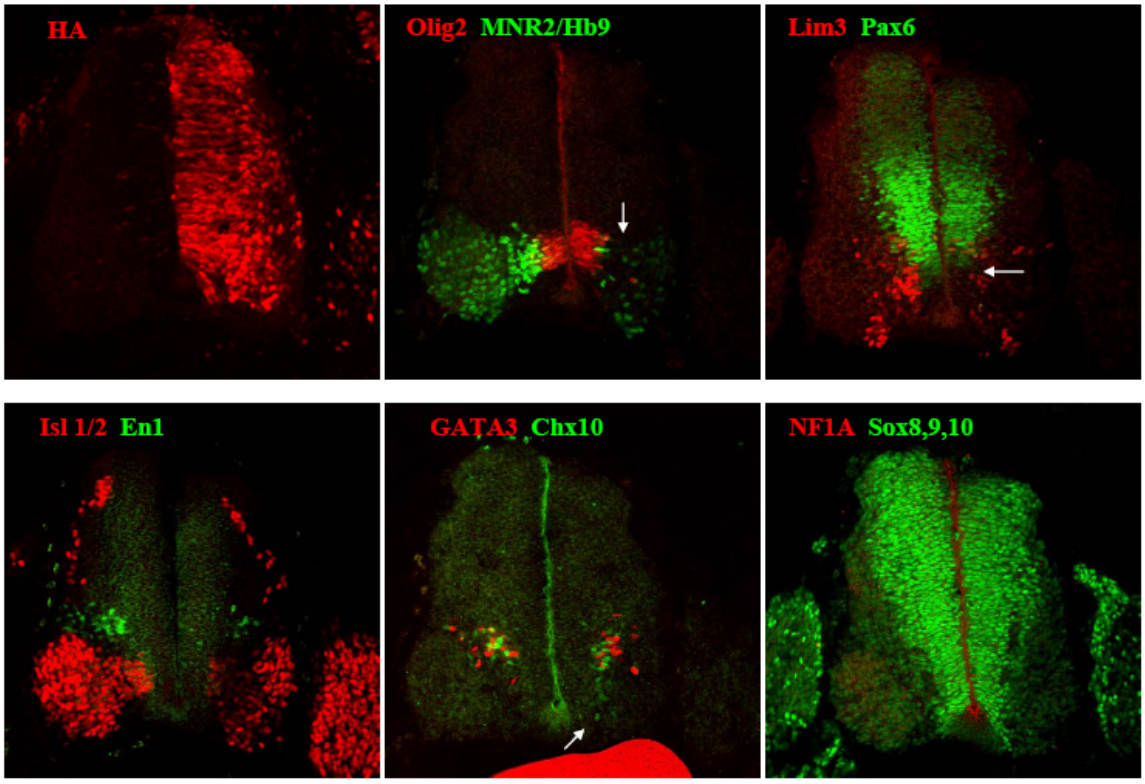
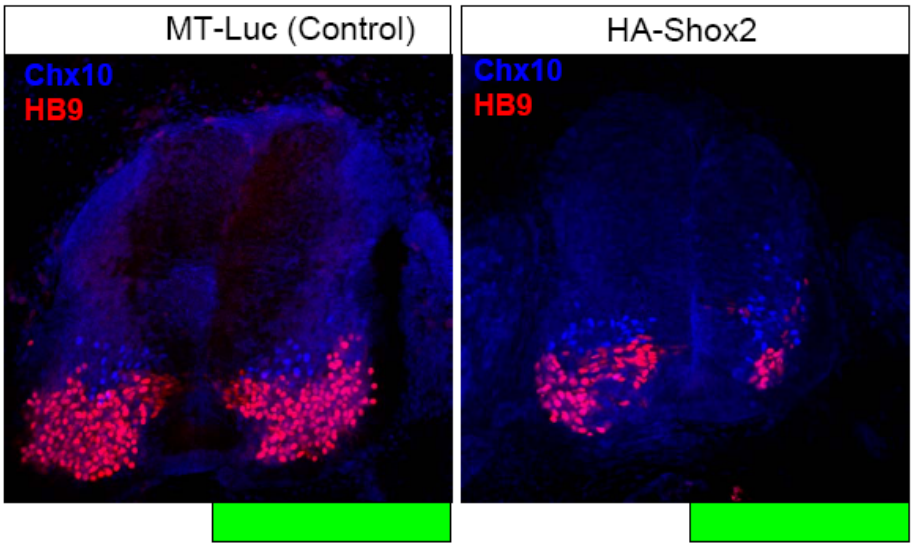


**Figure III-2 HA-Shox2 GOF is sufficient to represses motor neurogenesis**




| | HB9 analysis | | | Chx10 analysis | | |
|---|---|---|---|---|---|---|
| | # HB9 (U) | # HB9 (E) | % Change [(U – E)/U] | #Chx10 (U) | #Chx10 (E) | % Change [(U – E)/U] |
| MT-luc | 170 | 155 | 8.8% decrease | 22 | 21 | 4.5% decrease |
| HA-shox2 | 111 +/- 11.6 | 39 +/- 5 | 64% decrease | 22 +/- 2.6 | 34 +/- 5 | 54.5% increase |

**Figure III – 3 Co-misexpression of RCAS mShox2 + RCAS MT-cOlig2 promotes ectopic and precocious oligodendrocyte generation**

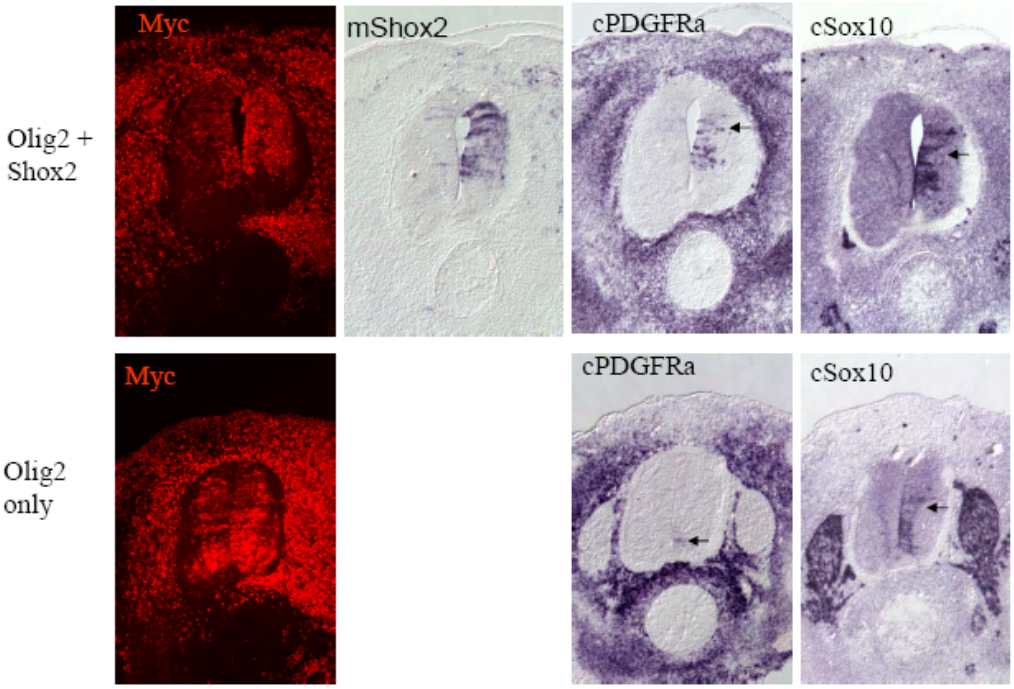


**Figure IV-1 Increased overlap of HFH4 with *Olig2-GFP* in Olig -/- spinal cord**

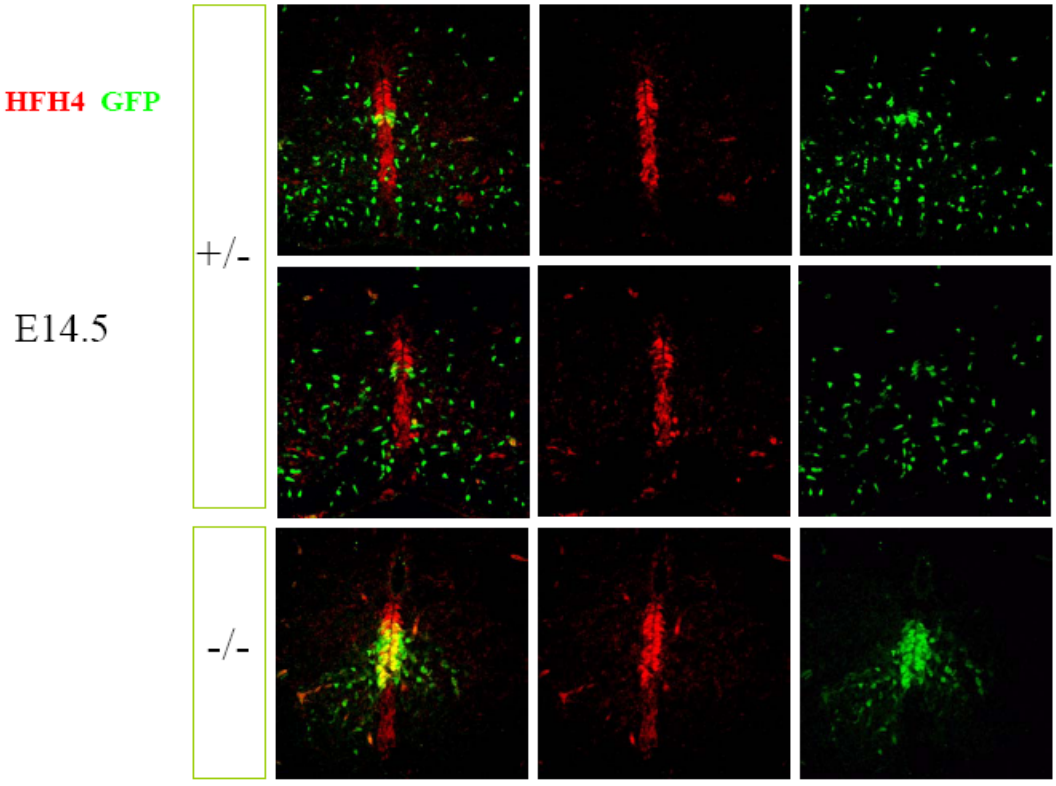


**Figure IV-3 HFH4 mutant - E14.5 early astrocyte markers**