Department of Biological Sciences

A Study on the Role of SOX1 in the regulation of Glial Specification in the Developing Spinal Cord of Mice

by

Nicholas Genethliou

A Thesis Submitted for the degree of the

Doctor of Philosophy

at the

University of Cyprus

March 2009

The Cyprus Institute of Neurology and Genetics,

Developmental and Functional Genetics Group

Στην αγαπημένη μου σύζυγο και στους γονείς μου

ΣΕΛΙΔΑ ΕΓΚΥΡΟΤΗΤΑΣ

Υποψήφιος Διδάκτορας: Νικόλας Γενεθλίου

Τίτλος Διατριβής: "A study on the role of SOX1 in the regulation of glial specification in the developing spinal cord of Mice."

Η παρούσα Διδακτορική Διατριβή εκπονήθηκε στο πλαίσιο των σπουδών για απόκτηση Διδακτορικού Διπλώματος στο Τμήμα Βιολογικών Επιστημών και εγκρίθηκε στις 19 Μαρτίου 2009 από τα μέλη της Εξεταστικής επιτροπής.

Εξεταστική Επιτροπή:

Ερευνητικός Σύμβουλος: Δρ. Σταύρος Μαλάς, Διευθυντής Ομάδας Αναπτυξιακής και Λειτουργικής Γενετικής, Ινστιτούτο Νευρολογίας και Γενετικής Κύπρου.

_____

Πρόεδρος: Νιόβη Σανταμά, Αναπληρώτρια Καθηγήτρια

Άλλα μέλη:

Παντελής Γεωργιάδης, Επίκουρος Καθηγητής
Πάρης Σκουρίδης, Λέκτορας
Ρεβέκα Μάτσα, Καθηγήτρια
Δόμνα Καραγωγέως, Καθηγήτρια

Abstract

The transition from neurogenesis to gliogenesis in the developing spinal cord, known as the neuron-glial fate switch, requires the coordinated function of patterning factors, pro-glial factors and Notch signaling. How this is regulated within molecularly distinct progenitor domains of the developing neural tube is poorly understood. Here I show in mice that the transcription factor SOX1 plays a key role in this process. Sox1 is expressed in a dynamic manner in the ventral spinal cord during gliogenesis and that PAX6, NKX2.2 and Notch signalling are required to maintain Sox1 expression in that region. Loss of SOX1 leads to enhanced production of oligodendrocyte precursors from the pMN domain and is associated with stage-specific regulation of Hes1 expression. I also present genetic evidence suggesting a requirement for Notch signalling to initiate the neuron-glial fate switch in a HES-independent manner. These data integrate functional roles of neural patterning, Notch signalling and SOX1 and propose a genetic mechanism that links different pathways known to affect gliogenesis.

Περίληψη

Η μετάβαση από τη νευρογένεση στη γλοιογένεση στον αναπτυσσόμενο νωτιαίο μυελό, χρειάζεται τη συντονισμένη λειτουργία μεταξύ των παραγόντων δημιουργίας προτύπου, τους προ-γλοιακούς παράγοντες και του συστήματος μεταγωγής σήματος Notch. Το πώς αυτό ρυθμίζεται μέσα στις διάφορες περιοχές πρόδρομων κυττάρων του αναπτυσσόμενου νευρικού σωλήνα δεν είναι πολύ κατανοητό. Σε αυτή τη μελέτη δείχνω ότι στα ποντίκια ο μεταγραφικός παράγοντας SOX1 παίζει ένα σημαντικό ρόλο σε αυτή τη διαδικασία. Το Sox1 κατά τη διάρκεια της γλοιογένεσης εκφράζεται με ένα δυναμικό τρόπο στο κοιλιακό μέρος του νωτιαίου μυελού και οι παράγοντες PAX6 και NKX2.2 καθώς και το σήμα του Notch απαιτούνται για να διατηρηθεί η έκφραση του Sox1 σε αυτή την περιοχή. Απώλεια του SOX1 οδηγεί στην αυξημένη παραγωγή ολιγοδενδριτικών πρόδρομων κυττάρων από την περιοχή pMN και συνδέεται με την ρύθμιση της έκφρασης του γονιδίου Hes1 που συμβαίνει ειδικά στο συγκεκριμένο στάδιο. Επίσης, παρουσιάζω γενετικά δεδομένα τα οποία προτείνουν ότι ο μηχανισμός Notch είναι αναγκαίος για την έναρξη της μετάβασης από τη νευρογένεση στη γλιογένεση, με ένα τρόπο που είναι ανεξάρτητος από τους παράγοντες HES. Αυτά τα αποτελέσματα συνενώνουν λειτουργικούς ρόλους της νευρικής δημιουργίας προτύπου, του συστήματος μεταγωγής σήματος Notch και της λειτουργίας του SOX1 και υποστηρίζουν ένα γενετικό μηχανισμό που συνδέει διαφορετικά μονοπάτια που είναι γνωστό ότι επηρεάζουν τη γλοιογένεση.

Acknowledgements

First I would like to thank my beloved wife **Antigoni** for her constant support, love and patience. I would also like to thank my parents **Thomas** and **Despina** and my brother **Genethlios** for all their help and love.

I would like to further thank my supervisor **Dr Stavros Malas**, without whom this thesis would have never been made. I will always be grateful for all the help and encouragement during these years.

To my co-supervisor **Professor Andreas Constantinou**: thanks for all the help.

All past and present members of the DFGG are acknowledged for their contribution to this thesis and for creating the nice working atmosphere. Special thanks to the person that spent a lot of his time to guide me and help me, **Dr Michael Orford**.

I am very grateful to **Dr Nikoleta Kessaris** and **Professor William D Richardson** for their hospitality and their advice.

Finally, the **Research Promotion Foundation of Cyprus** for funding a large part of my PhD.

Table of Contents:

List of Figures:

List of Tables:

List of abbreviations:

AP	Anterior-Posterior
AS	Astrocyte
ASP	Astrocyte Precursor
BAC	Bacterial Artificial Chromosome
BMP	Bone Morphogenetic Protein
CNS	Central Nervous System
DEPC	Diethyl Pyrocarbonate
dNTP	2'-Deoxynucleoside 5'-Triphoshate
dSC	Dorsal Spinal Cord
D-V	Dorso-Ventral
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
ES cell	Embryonic Stem cell
EtOH	Ethanol
FGF	Fibroblastic Growth Factor
Fgfr3	Fibroblast Growth Factor Receptor-3
FP	Floor Plate
GFAP	Glial Fibrillarly Acidic Protein
GM	Grey Matter
GOF	Gain-of-function
GRP	Glial Restricted Precursor
GS	Glutamine Synthetase
HD	Homeodomain
Hes	Hairy/Enhancer of Split
HH stage	Hamburger and Hamilton stage
Hh	Hedgehog
HLH	Helix Loop Helix
HMG	High Mobility Group
ICM	Inner Cell Mass
Id	Inhibitor of differentiation
ISH	In Situ Hybridisation
LOF	Loss-of-function
MN	Motor Neuron
MNOP	Motor Neuron/Oligodendrocyte Precursor Cell
N/G	Neuron/Glial
NEP	Neuroepithelial Precursor Cell
NFIA	Nuclear Factor I A
NFIB	Nuclear Factor I B
NICD	Notch Intracellular Domain
NLS	Nuclear Localisation Signal
NRP	Neuronal Restricted Precursor
NS	Nervous System
NSC	Neural Stem Cells
OL	Oligodendrocyte

OLP	Oligodendrocyte Precursor
OT	Olfactory Tubercle
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PFGE	Pulse Field Gel Electrophoresis
PNS	Peripheral Nervous System
PS-1	Presenilin-1
RA	Retinoic Acid
RG	Radial Glial
RP	Roof Plate
SC	Spinal Cord
SCZ	Stem Cell Zone
SDS	Sodium Dodecyl Sulfate
Shh	Sonic Hedgehog
Smo	Smoothened
SSC	Saline Sodium Citrate
Sulf1	Sulfatase 1
SVZ	Subventricular Zone
TBE	Tris Borate
TF	Transcription Factor
TGFβ	Transforming Growth Factor-β
TM	Tamoxifen
VS	Ventral Striatum
vSC	Ventral Spinal Cord
VZ	Ventricular Zone
WM	White Matter
WMA	White-Matter Astrocytes
Wnt	Wingless/Int
Wt	Wild Type

Chapter 1

Introduction

1.1 General features of the Central Nervous System in mammals

The mammalian Nervous System (NS) controls all the biological processes and movements of the body. It has the ability to receive information from peripheral organs and interpret it via electrical signals transmitted through the nerve cells. In mammals, it consists of the Central Nervous System (CNS) which consists of the brain and spinal cord and the Peripheral Nervous System (PNS) which transmits and receives electrical impulses to and from the CNS. The CNS has the ability to send out signals to the PNS either consciously or unconsciously.

The NS consists primarily of two types of cells, neurons and glia and is the most complex organ of the body. This complexity arises primarily from the fact that it contains approximately 200 different types of neurons making up around 10% of the NS mass, while the rest of the NS mass is made up of glial cells and their derivatives. Neurons are involved in transmitting information whereas glial cells are responsible for providing physical support to neurons and may also be involved in some aspects of signal transmission.

The initial complexity of the NS is established very early in development through the activity of morphogens, molecules that are released from tissues surrounding the CNS, which determine the anterior-posterior and dorso-ventral identity of different domains of the NS.

In order for the NS to develop proper connectivity during development, neurons are generated first followed by the production of glial cells. The temporal and spatial regulation of this process, known as the neuron-glial (N/G) fate switch is very crucial for the correct organisation of the NS. An early onset of the N/G switch will probably result in a deficit of neurons. On the other hand, if this switch is delayed, then the most likely outcome will be an excess of neurons and a delay of the development of glial cell, as shown by some genetic studies (Stolt et al., 2003).

During development all neurons and glial cells are specified by progenitors lining the germinal layer of the developing NS, known as the ventricular zone (VZ). In the developing spinal cord, the VZ is divided into progenitor domains along the dorso-ventral (D-V) axis by a process known as neural patterning (Jessell, 2000). Although progenitors within each domain are considered to have stem cells properties, they express different combinations of transcription factors making them different from their neighbouring progenitor cells. These molecularly dissimilar progenitors generate different types of neurons. The positional identity of progenitor cells with seemingly common stem cell properties is the organising feature of the developing CNS.

1.2 General aspects of murine CNS development

The CNS in the mouse begins to develop through a process known as neural induction at about embryonic day 6.5 (E6.5). At this stage the gastrulating embryo consists of three layers, an outer ectodermal, an intermediate mesodermal and an inner endodermal layer. The ectoderm is destined to form both neural and epidermal tissue. The first neural structure that forms is the neural plate that forms around the node in the ectodermal layer of the embryo. The neural plate then folds and forms a tubular structure, the neural tube, which will be divided into four distinguishable areas. The first three areas in the rostral part form the brain vesicles (forebrain, midbrain and hindbrain). The fourth area, which forms in the caudal part, is a uniformly narrow tube that will generate the future spinal cord. Each of these four initial regions will be further subdivided into distinct D-V domains that dictate the position and nature of cell types that are generated in each area. This progressive regionalization is due to integration of differential signals along the rostro-caudal and D-V axis. The signalling factors that orchestrate neural development are the Fibroblastic growth factors (FGF), Wingless/Int (Wnt), Hedgehogs, Transforming growth factor-βs/Bone morphogenetic proteins (TGFβ/BMP), Notch, and finally the small molecule retinoic acid (RA) (Briscoe and Novitch, 2008; Jessell, 2000).

1.2.1 Neural Induction

The process leading to the formation of the first neural tissue, the early neural plate, is known as neural induction. Spemann and Mangold were the first to show with classic transplantation experiments that the dorsal lip of the blastopore of the Xenopus embryo has the ability to induce neural character in adjacent cells. They named this region 'the Organizer' because it had the ability to induce a second embryonic axis when repositioned in the embryo (Spemann and Mangold, 1924). This 'Organizer' is the equivalent of the node in the amniotes, a mesodermal group of cells that forms in the most distal part of a 'cup-shaped' murine embryo (Rallu et al., 2002), (Fig. 1). The neural plate forms in a narrow strip of cells above the node and over the last 15 years many studies have attempted to understand how the overlying medial ectoderm transform to neural tissue. A series of experiments led to the proposition of the 'default model'. This model proposes that neural induction occurs as a result of the inhibition of Bone Morphogenetic Proteins (BMP) function in the embryonic ectoderm. In the prospective neural plate the inhibition of BMP activity in ectodermal cells will help them adopt a neural fate (Hansen et al., 1997; Hawley et al., 1995; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Lamb et al., 1993; Sasai et al., 1995). The ectoderm normally produces BMPs and it has been proposed that the underlying node produces the BMP inhibitors Noggin, Chordin and Follistatin, that inhibit in the overlying ectoderm BMPs via protein sequestering. However, although these experiments demonstrated that the organizer/node is sufficient to induce ectopic neural cells, genetic studies showed that even in the absence of a node neural induction could still take place. Mouse embryos lacking the transcription factor HNF3β or the Arkadia protein, which acts upstream of Hnf3β, fail to generate node and node derivatives and yet these embryos develop anterior neural plate with an initial rostro-caudal pattern. These data suggest that the generation of anterior neural cells in the mouse embryo does not require a functional node or node derivatives (Ang and Rossant, 1994; Episkopou et al., 2001; Klingensmith et al., 1999). Collectively, these findings indicated that the necessary neural inducing signals derive from tissues other than or in addition to the node/organizer (Wilson and Edlund, 2001).

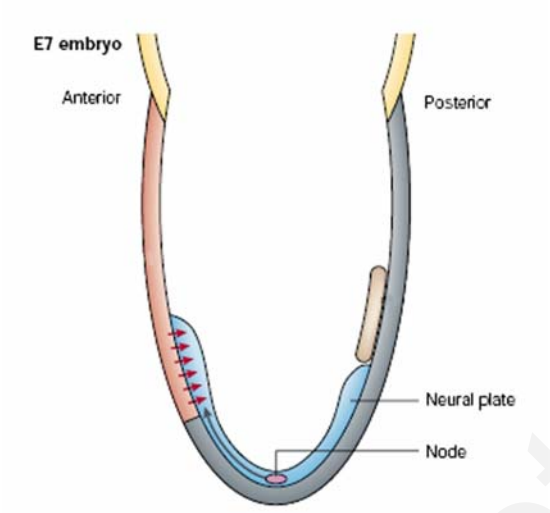

Figure 1. The murine embryo during neural induction.

The embryo has a 'cup-shape' and in the most distal part of it the node is formed. The node releases inductive signals in the overlying ectoderm in order to transform it to neural tissue. Adopted by Rallu et al., 2002. Red arrows indicate signals released from the primitive endoderm (pink layer) that influence the overlying ectoderm (blue). The anterior is to the left and the posterior to the right.

The lack of a requirement of the node for the generation of the anterior neural cells in the mouse embryo, left open the possibility that the specification of neural cells is initiated before the formation of the node. Studies in chick have shown that neural induction is initiated already at the blastula stage (Streit et al., 2000). Medial epiblast cells isolated from chick blastula stage embryos and grown *in vitro* generate cells of neural character in an FGF-dependent manner, whereas epiblast cells isolated from a more lateral region generate cells of epidermal character, in response to Wnt and BMP signals (Wilson and Edlund, 2001). These results coupled with additional data in the chick and Xenopus suggested that a pre-gastrula FGF signalling is required for neural induction (Delaune et al., 2005; Kuroda et al., 2005; Wilson and Edlund, 2001). These observations led to the re-evaluation of the 'default model'. Thus a more updated model suggests that the initial neural specification of epiblast cells occurs in response to FGF signalling during the blastula stage. The role of the node is to protect these cells from high levels of BMP and Wnt signals –which promote the generation of epidermal ectoderm– thus to maintain and stabilize neural fate by secretion of BMP inhibitors. In

the absence of the node or the BMP inhibitors the neural plate develop smaller (Fekany-Lee et al., 2000; Hammerschmidt et al., 1996) and decreased levels of BMP signals lead to massive expansion of the neural plate (Dick et al., 2000; Hild et al., 1999; Nguyen et al., 1998; Reversade et al., 2005).

1.2.2 Rostro-caudal patterning of the CNS

The newly formed neural plate has initially rostral (forebrain) features but gradual regression of the organizer leads to an elongation of neural plate leading to the formation of more caudal regions such as the midbrain, hindbrain and spinal cord. The process of diversifying the rostro-caudal character of the neural plate is regulated by anterior-posterior (AP) patterning mechanisms. In 1938 Speman suggested the 'two inducer' model for the induction of caudal neural tissue. He proposed that the anterior and posterior neural tissue is generated through the activities of two distinct neural inducers. Another model, supported by transplantation experiments, is the 'activation and transformation' model that was initially proposed by Nieuwkoop (Nieuwkoop et al., 1952). This model suggests that presumptive neural cells in the mid-gastrula neural plate are first 'activated' to become neural and then some of them are 'transformed' into caudal neural cells by exposure to caudalizing signals (Doniach, 1995; Harland, 2000; Stern, 2001). A fate map of caudal neural plate in a mid-gastrula (HH stage 3) (Hamburger and Hamilton, 1992) chick embryo shows that these cells will contribute to the caudal nervous system, but not to the forebrain. Yet, HH stage 3 prospective caudal explants, maintained in isolation from extrinsic signalling input, generate cells characteristic of the forebrain, but not of caudal neural derivatives (Muhr et al., 1999). A number of different signalling factors are required to generate the different positional identities along the AP axis of the neural tube. These factors include the FGFs, Wnts and the Retinoic Acid (RA). Different combinations of the activity of these signals lead to the positional specification along the AP axis.

RA is produced from the paraxial mesoderm (somites) that flanks the caudal neural plate (prospective spinal cord). RA is a small molecule that enters the recipient

cell and binds to nuclear RA receptors (RARs and RXRs) which in turn bind to gene promoters containing RA response elements (RARE). Such genes include the Hox (Homeodomain Box) genes and some members of the Otx homeobox gene family, such as Otx2 that is responsible for the specification of the anterior brain (Acampora et al., 1995). Some of Hox genes are responsible for specifying caudal fates of the CNS and have RAREs in their promoter regions (Dupe et al., 1997; Gould et al., 1998; Marshall et al., 1994). Since RA is not produced by neural cells, it is regarded as a morphogen acting in a graded manner leading, initially, to posteriorization of the neural plate (Maden, 1999). Some loss-of-function studies argue that the posteriorizing role of RA may be restricted to the head-trunk boundary instead of having a graded influence throughout the CNS. The FGF signalling may also provide a posteriorizing influence in the more caudal regions (Isaacs et al., 1998; Pownall et al., 1998; Pownall et al., 1996), and also Wnt signalling is a strong candidate mechanism for the specification of caudal neural characters (Bang et al., 1999; Domingos et al., 2001; Erter et al., 2001; Fekany-Lee et al., 2000; Fredieu et al., 1997).

1.2.3 Dorso-ventral Patterning of the Spinal Cord

Within a given axial level of the developing CNS the acquisition of the A-P identity is followed by the specification of the dorso-ventral (D-V) identity of neural progenitors. This D-V patterning has been studied very extensively in the spinal cord and will be described in some detail as it is very relevant to this study (Jessell, 2000).

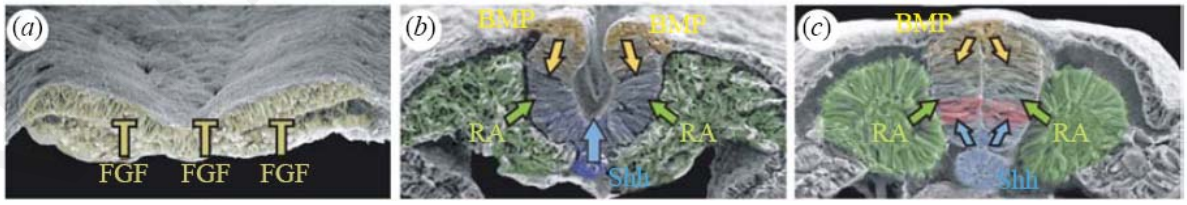

Figure 2. Stages of posterior neural tube development and the signalling pathways taking part.

Scanning electron micrographs of the developing neural tube at (a) neural plate, (b) neural folds and (c) neural tube stages. Images display sources of FGF (yellow), retinoic acid (RA, green), Shh (blue-produced by the Notochord and then Floor Plate) and BMPs (orange). Adopted from Bricsoe and Novitch, 2008.

As the caudal neural plate emerges, the region very close to the node is known as the stem cell zone (SCZ) (Figs. 2-3). When the node regresses and continues to produce FGFs, neural differentiation begins rostral (anterior) to this zone at the region immediately adjacent to the emerging paraxial mesoderm (somites). The elongating neural plate thus comes under the influence of lateral signals from the somites and signals released from the notochord (believed to be an extension of the node).

The initial D-V patterning of the spinal cord is established through the action of mainly four extracellular signalling molecules as summarized in Figure 3. As neural cells move out of the SCZ they must switch off the FGFs (especially FGF8). The FGFs are responsible for the maintenance of undifferentiated progenitors in the caudal SCZ. Application of FGF antagonists such as SU5402, that blocks FGF signalling, induces movement of cells out of the SCZ and into the neural tube where they differentiate prematurely (Henrique et al., 1997; Storey et al., 1998).

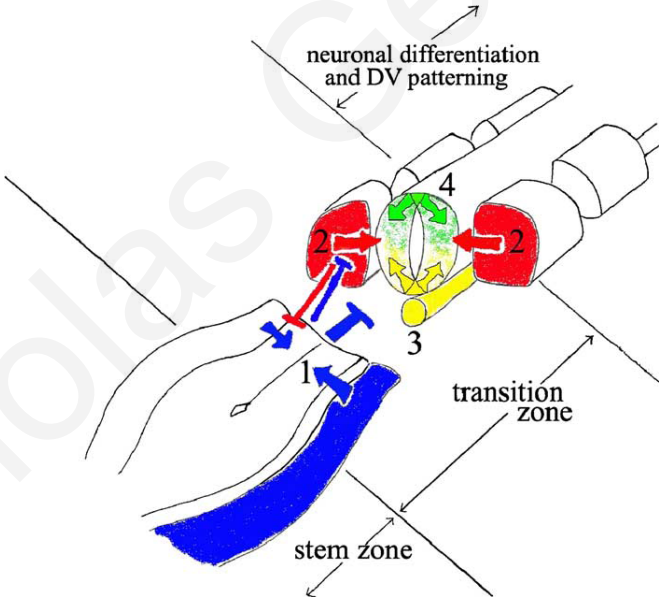

Figure 3. Summary diagram showing the extrinsic signals influencing the establishment of the D-V patterning.

In the stem zone, FGFs (signal 1) prevent neural differentiation in the neural plate. In the transition zone, the notochord differentiates and starts to express Shh (signal 3) while the differentiated somites begin to express RALDH which synthesizes RA (signal 2). BMPs start to be produced from the RP (signal 4). Adopted from Wilson and Maden, 2005.

Just rostral to the SCZ, somites begin to differentiate from paraxial mesoderm that flanks the neural tube. Paraxial mesoderm produces RA that represses Fgf8 expression (Diez del Corral et al., 2002). At this stage most neural cells begin to express a set of homeodomain transcription factors known as **Class I genes** (Pax6, Pax7, Dbx1, Dbx2 and Irx3) and also switch on Sox1. RA is generated by the enzyme RALDH2 (Berggren et al., 1999; Blentic et al., 2003; Niederreither et al., 1997; Swindell et al., 1999). In Raldh2 null embryos, Fgf8 expression is prolonged, it becomes stronger in the pre-neural tube and neural differentiation is prevented (Molotkova et al., 2005). Fgf8 down-regulates Raldh2 in the paraxial mesoderm, demonstrating mutual repressive interaction between these two extracellular signals (Fig.3) (Wilson and Maden, 2005).

Many studies have demonstrated that the expression of Class I genes (and Sox1; Eumorphia Remboutsika, unpublished data) is initiated by RA. In the absence of RA signalling, the expression of these genes is reduced (Novitch et al., 2003). Conversely, addition of RA to intermediate neural plate explants resulted in the induction of these genes (Pierani et al., 1999). In the presence of FGF, the low level of expression of Pax6, Irx3, Dbx1 and Dbx2 in these neural explants is extinguished (Novitch et al., 2003). Conversely, in the absence of FGF in the chick embryo premature activation of Pax6 expression is observed (Bertrand et al., 2000). These data demonstrate that Class I genes are induced and repressed by RA and FGF, respectively (Briscoe and Novitch, 2008; Wilson and Maden, 2005).

Upon neural tube closure, the developing spinal cord begins to come under the influence of signals that determine the D-V position of progenitors that express Class I genes. In the ventral spinal cord neural patterning is primarily determined by Sonic Hedgehog (Shh) signalling. SHH is a glycoprotein encoded by the vertebrate orthologue of the Drosophila segment-polarity gene Hedgehog (HH), (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). Shh is expressed by the underlying notochord and later the floor plate and is believed to act in a concentration-dependent fashion to induce the expression of different progenitor markers, depending on the distance of each progenitor cell relative to the floor plate (Fig. 3). SHH

is more concentrated in the most ventral SC and gradually gets less concentrated in more dorsal regions (Chamberlain et al., 2008).

Progenitor cells respond to this concentration gradient and begin to express a series of transcription factors, known as **Class II** genes (Shh-induced). Class II proteins include Nkx6.1, Nkx6.2, Nkx2.2 and Olig2. All genes are members of the homeodomain protein (HD) family, except Olig2 which is a bHLH protein (Briscoe et al., 2000; Novitch et al., 2001). These transcription factors function as transcriptional repressors and mediate, at progenitor level, the interpretation of Shh signalling (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997b; Gotz et al., 2005; Novitch et al., 2001; Vallstedt et al., 2001).

The expression of each Class I gene is repressed at distinct thresholds of Shh activity; consequently their ventral limits of expression are determined directly by Shh signalling. But, the response to Shh signalling, is not sufficient to explain the sharp domains of gene expression of Class I and Class II proteins observed. Gain- and loss-of-function studies provided direct evidence for the existence of selective cross-repressive interactions between complementary pairs of Class I and Class II proteins expressed in neighbouring domains that are responsible for establishing the sharp progenitor boundaries (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997b; Gotz et al., 2005; Novitch et al., 2001; Vallstedt et al., 2001). These cross-repressive interactions ensure that each progenitor expresses a distinct combination of Class I and Class II proteins (Fig. 4). Class I and Class II proteins are all transcriptional repressors and directly interact with the Groucho family of co-repressors (Muhr et al., 2001), except PAX6 that appears to repress Nkx2.2 indirectly, presumably via the induction of an unknown repressor. PAX6 has been shown to regulate Wnt signalling in the intermediate spinal cord and Wnt signalling has been proposed to regulate the p3/pMN boundary (Lei et al., 2006). In this study I will provide genetic evidence showing that Nkx2.2 does not repress Pax6 at any developmental stage (Genethliou et al., 2009).

A fully patterned spinal cord is subdivided into eleven D-V progenitor domains (Fig. 5). Each domain expresses a unique combination of transcription factors. The Roof Plate (RF) and Floor Plate (FP) do not generate any neurons but only glial cells. This combinatorial expression pattern of many transcription factors within each progenitor domain underlies the diversity of neurons generated in the spinal cord. The dorsal half of the spinal cord includes six domains (dI1-dI6) that produce six types of dorsal interneurons. The ventral half gives rise to five domains, which in a ventral-to-dorsal direction are denoted as p3-pMN-p2-p1-p0 (Fig. 4 and Fig. 5; Jessell, 2000). The pMN domain will generate the motor neurons (MN), while the p3, p1 and p0 domains will generate the V3, V1 and V0 interneurons respectively. The p2 domain is known to generate at least two types of interneurons (V2a and V2b) (Wilson and Maden, 2005). Studies in our laboratory have indentified a third type (V2c; Personal communication with H. Panayi).

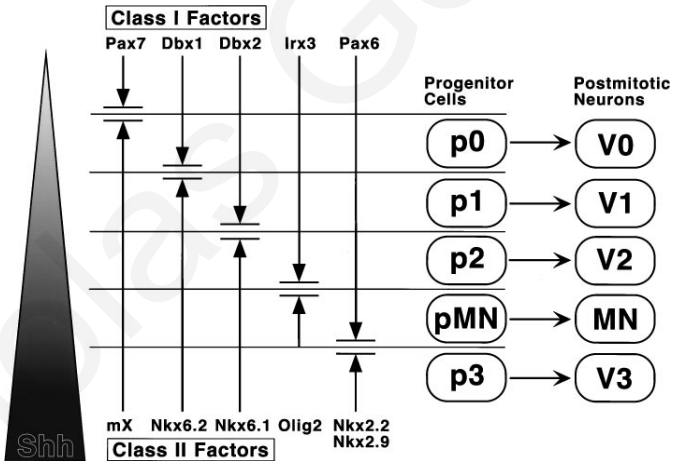

Figure 4. **Shh activates the expression of Class II genes and represses Class I genes.**

Different Class II and Class I factors are activated or repressed, respectively, by Shh in a concentration-dependent manner. The Class II factors are shown on the bottom of the figure while Class I factors are shown at the top of the figure. Oppossing bars indicate the respective pairs of Class I and Class II factors. Cross-repressive interactions between each pair results in the establishment of sharp progenitor boundaries. The Class II factor that antagonises Pax7 expression is still unknown and is referred to as mX. Adopted from Shirasaki and Pfaff, 2002.

A vast amount of data has been provided to suggest that the initial patterning of the ventral spinal cord (vSC) into five progenitor domains is responsible for generating the correct type of neurons at the appropriate place and that SHH is ultimately responsible for determining neuronal diversity in this region (reviewed by Briscoe and Novitch, 2008). It is generally believed that neurons generated in progressively more ventral regions of the neural tube require correspondingly higher Shh concentrations for their induction. Blocking Shh signalling either by Shh inhibitors or by deletion of the Shh gene abrogates the differentiation of MNs (Ericson et al., 1997a; Ericson et al., 1996; Marti et al., 1995; Roelink et al., 1995), the generation of FP cells, V3 and V2 interneurons (Briscoe and Ericson, 2001; Jessell, 2000). V0 and V1 interneurons still develop, albeit in reduced numbers, so their specification does not totally depend on Shh (Litingtung and Chiang, 2000). Genetic studies suggest that the development of these two

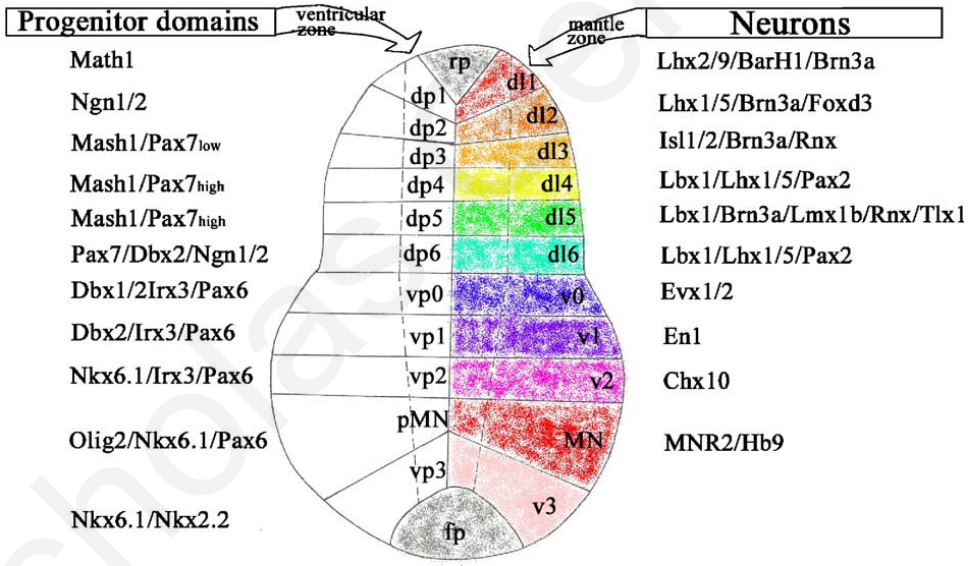

Figure 5. The organisation of the early embryonic the spinal cord.

The dorso-ventral patterning of the spinal cord results in the generation of 6 dorsal (dp1-dp6) and 5 ventral (p3-p0) progenitor regions which will eventually give rise of different types of neurons. In the VZ the different progenitor domains are indicated between dotted vertical lines. Different transcription factors expressed in each domain are shown to the left. The mantle zone, coloured region outside the dotted lines, shows the neuronal output of each domain and the neuronal markers assosiated with each neuronal subtype are shown to the right. Adopted from Wilson and Maden, 2005.

types of interneurons is dependent upon RA signalling (Pierani et al., 1999; Wilson et al., 2004). Another hypothesis, based on loss-of-function studies using $Shh^{-/-}$ embryos, is that residual HH signalling coming from the Indian Hedgehog which is expressed nearby in gut ectoderm is sufficient to specify some V1/V0 interneurons. Consistent with this hypothesis, embryos that lack the Shh receptor Smoothened (Smo) do not form FP and fail to develop four of the five progenitor domains, except the p0 domain. Although some V0 neurons are formed in Smo mutant embryos, these form at abnormally ventral positions, suggesting that specification of all vSC fates, with the possible exception of some V0 neurons, requires HH signalling (Lupo et al., 2006; Wijgerde et al., 2002).

1.2.3.1 Intracellular interpretation of Shh signalling

Since SHH is a diffused glycoprotein it requires an intracellular mechanism for signal transduction. SHH binds to its receptors (Patched and Smothend) and the response is mediated by Gli transcription factors (orthologues of the Drosophila gene Cupitus interuptus). In mice there are three Gli genes, Gli1-3 (Hui et al., 1994). Only Gli2 and Gli3 have critical roles in mediating Shh response. Both proteins have C-terminal transcriptional activator and N-terminal transcriptional repressor domains (Aza-Blanc et al., 2000; Dai et al., 1999; Ruiz i Altaba, 1999; Sasaki et al., 1999). Gli3 is expressed in a dorsal-to-ventral direction whereas Gli2 is expressed in a ventral-to-dorsal direction. Since the concentration gradient of SHH also takes place in a ventral-to-dorsal direction in the vSC, Gli3 appears to antagonise HH signalling whereas Gli2 protein positively modulates HH response. The function of both Gli2 and Gli3 is modulated by HH signalling such that it enhances the activator function of Gli2 and inhibits the repressor activity of Gli3 (Aza-Blanc et al., 2000; Bai et al., 2004; Lei et al., 2004; Ruiz i Altaba, 1999; Wang et al., 2000).

Gli2 mutant embryos have severe defects in the generation of FP and V3 neurons and there is concomitant ventral expansion of motor neurons (Matise et al., 1998), but all other neurons are generated at their appropriate D-V positions. This phenotype can be rescued by replacing Gli2 with a Gli1 cDNA, suggesting that the inductive effects of Gli2

are mediated through its transcriptional activator function (Bai and Joyner, 2001). In Gli3 knockout embryos, patterning is normal in the ventral regions of the SC, but there is a dorsal expansion of cell types typical for the intermediate neural tube (p1-p0). This means that these cells that normally fail to respond to Shh signalling now are able to do so and acquire a more ventral character (Persson et al., 2002). These defects are rescued by a truncated allele of Gli3 that encodes the repressor domain, suggesting that only the repressor activity of Gli3 is normally required in antagonising Shh signalling SC (Persson et al., 2002).

Despite the fact that Shh signalling has been proposed to be the key mechanism for generating ventral cell fates, in the Gli3/Shh double mutant mouse, there is a recovery of MNs, V2, V1 and V0 neurons but not V3 neurons (Litingtung and Chiang, 2000; Wijgerde et al., 2002). These data suggest that, in the absence of Shh signalling, Gli3 normally represses ventral neuronal fates. Thus, an important role of Shh signalling in the ventral neural tube is the inhibition of Gli3 repressor activity (Briscoe and Novitch, 2008). In contrast, in Gli3/Shh double mutants FP cells and V3 neurons are absent, suggesting that Shh signalling is primarily required for the induction of these two domains. This phenotype is similar to that observed, in Gli2 mutants (Matise et al., 1998), showing that the generation of FP cells and V3 neurons depends on the transcriptional activator functions of Gli2, induced by Shh signalling. As expected Gli2/Gli3 double mutants develop a neural tube similar to the Gli3/Shh double mutants and still lack the FP and p3 domain, reinforcing the view that the induction of these two fates critically depends on Shh signalling (Bai et al., 2004; Lei et al., 2004). The takehome message of these results is that Gli3 represses ventral neural fates and Shh signalling acts by relieving this repression and promoting the transcriptional activity of Gli2 (Briscoe and Novitch, 2008).

1.2.3.2 BMP and Wnt signalling in dorso-ventral patterning

BMPs are secreted proteins that are expressed by the dorsal ectoderm overlying the spinal cord and the Roof Plate (RP). They act in a concentration-dependent manner,

equivalent to SHH in the vSC. The most common BMPs that are expressed from the RP are Bmp4, Bmp5 and Bmp7 (Wilson and Maden, 2005). The secretion of BMPs is required for the induction of neural crest cells and dorsal interneurons (Liem et al., 2000). Gain- and loss-of-function experiments favour the view that BMPs are responsible for dorsal patterning of the spinal cord. For instance, electroporation of BMPs in the chick neural tube resulted in the ectopic expression of Pax7 and the repression of Pax6. Dorsal neuronal subtypes were induced and ventral subtypes were reduced (Timmer et al., 2002). In contrast, when both of the BMP receptors are absent there is a loss of dl1 interneurons and a dorsal shift of dl2 interneurons (Wine-Lee et al., 2004).

BMP signalling seems to act upstream of yet another extracellular signal that is present in the dorsal spinal cord, the Wnt signalling. In embryos mutant for BMP receptors, Wnt signalling is attenuated (Wine-Lee et al., 2004). Wnts are mitogens responsible for inducing proliferation in a concentration-dependent manner rather than being involved in D-V patterning (Megason and McMahon, 2002). However, recent evidence in chick have provided evidence that Wnt signalling might be involved in regulating dorsal spinal cord patterning (Alvarez-Medina et al., 2008) and Wnt1-Wnt3a double mutant mouse embryos generate more dI3 neurons at the expense of dl1 and dl2 neurons (Muroyama et al., 2002).

1.3 Glial specification

The spinal cord, like all regions of the CNS, first generates neurons and then switches to generating glial cells, that is astrocytes (AS) and oligodendrocytes (OLs) (Rowitch, 2004). The switch from neurogenesis to gliogenesis is known as the neuron-glial (N/G) fate switch. A key question in developmental neuroscience is to understand how this switch is initiated. This is particularly important since glial cells in the spinal cord are produced from VZ progenitors that continue to express the same repertoire of patterning factors that are involved in neuronal specification.

One way of producing different cells types from neural progenitors is to have a reservoir of tripotent neural stem cells (NSC) always in place and direct their differentiation at different times points. Although many studies in the developing and adult CNS have demonstrated that tripotent NSCs can be isolated and expanded *in vitro* there is no yet direct evidence that these cells exist *in vivo*. The idea that NSCs exist *in vivo* in the ventricular zone (VZ) of the spinal cord has been challenged based on direct *in vivo* assays of the differentiation potential of putative NSC without prior *in vitro* manipulation (Gabay et al., 2003; Mukouyama et al., 2006). These observations imply that neural progenitors from the same region of the CNS might have a dynamic developmental potential at different time points and that the CNS might be formed though sequential lineage restriction of neural progenitors. Therefore, any model trying to understand the N/G switch needs to:

1. Explain how patterning factors, which continue to be expressed both during neurogenesis and gliogenesis integrate their function during the N/G switch to determine the spatial control of gliogenesis, just like they do during neurogenesis.
2. Explain if neural patterning regulates sub-type identity of glial cells, in the same manner as it regulates neuronal subtype identity.
3. Identify the intrinsic and extrinsic factors that regulate the temporal control of gliogenesis, that is factors that direct neurogenic progenitors to become gliogenic.

4. Explain how the production of glial fates is regulated in progenitor domains that generate both OLs and ASs.

1.3.1 Spatial control of Gliogenesis

1.3.1.1 Spatial control of Oligodendrogenesis in the spinal cord

In the embryonic murine spinal cord, Oligodendrocyte precursors (OLPs) are initially positive for PDGFRα and Sox10 (Hall et al., 1996; Zhou et al., 2000) and migrate from the VZ in the grey matter (GM) where they continue to proliferate until they differentiate terminally in the white matter (WM) and begin to produce myelin around the axons. The spatial specification of OLs has been extensively studied over the last fifteen years. In the spinal cord, these cells are generated from two regions. The main source is the pMN domain (Fig. 5; page 17). This domain first produces motor neurons and then switches to generating OLP and AS precursors (ASP) (Lu et al., 2002; Masahira et al., 2006; Pringle and Richardson, 1993; Takebayashi et al., 2002; Zhou and Anderson, 2002). OL are also specified from the dorsal spinal cord (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). For some years it was believed that the pMN domain generates only neurons and then OL but it is now accepted that both OLs and ASs are generated from the pMN con-currently (Masahira et al., 2006). Although the dorsal Oligogenic domains have not been determined by fate mapping, by way of consensus these are believed to be generated in three $Pax3^{+}$ domains, that is dI5-dI3 (Kessaris et al., 2008).

While the pMN domain expresses Olig2 before OLP specification takes place, in the dorsal spinal cord Olig2 is expressed only after OLPs are specified. Hence in the pMN domain OLP specification takes place in an OLIG2-dependent manner while in the dorsal spinal cord OLP specification takes place in an OLIG2-independent manner. The mechanism that specifies dorsal OLPs is currently unknown, but it has been proposed that attenuation of BMP signalling might contribute to the specification of dorsal OLPs (Vallstedt et al., 2005).

Descriptive gene expression studies in mice (Fu et al., 2002) and in the chick (Agius et al., 2004; Danesin et al., 2006) suggest that the p3 domain, that expresses NKX2.2, also generates OLs. However, direct fate mapping studies have not been provided to corroborate these findings. Nevertheless Nkx2.2 is important during the maturation of OLs since it is expressed by terminally differentiating OLs (Kessaris et al., 2008; Qi et al., 2001; Richardson et al., 2006).

Intrinsic determinants of OLPs in the pMN domain

In the pMN domain the OLIG2 transcription factor is absolutely critical for both MN and OL production (Lu et al., 2000; Novitch et al., 2001; Zhou and Anderson, 2002; Zhou et al., 2001). Since a single protein is involved in the production of very different cell types it must function in a context-dependent manner. For instance ectopic expression of Olig2 in early stage chick embryos fails to initiate OLP specification and induces the motor neuron fate (Novitch et al., 2001), suggesting that the induction of the OL fate must be stage- or context-dependent.

One observation which sparked a series of investigations was that in the chick spinal cord, before the emergence of OLP from the pMN, the p3 domain expanded dorsally and some Nkx2.2^{+} cells co-expressed Olig2, creating a hybrid pMN/p3 domain (Agius et al., 2004; Fu et al., 2002; Zhou et al., 2001). This phenomenon has led to the proposition that OLP specification takes place in this hybrid domain and not in progenitors expressing only Olig2 (Zhou and Anderson, 2002). This argument has been supported by studies in the chick where the co-expression of both NKX2.2 and OLIG2 induced ectopic OLP. NKX2.2 alone led to the repression of neurogenesis and so it has been postulated that in this hybrid domain, Olig2 specifies the OLP fate in a context of repressed neurogenesis. However, soon after this model was not supported by genetic evidence using mice that lack Nkx2.2. In these embryos, OLP specification was normal, suggesting that the formation of a hybrid Nkx2.2/Olgi2^{+} domain is not a prerequisite to generate OLPs and that OLIG2 alone could specify OLPs *in vivo* (Qi et al., 2001).

Regardless of the mechanism that specifies the OL fate, the spatial control of OL specification from the pMN depends on mechanisms that maintain the integrity of the pMN (Olig2) domain. Several genetic studies have demonstrated this requirement. In embryos lacking Pax6, Nkx2.2 expression expands dorsally due to proposed repressive functions of PAX6 on Nkx2.2 (see Fig. 4; page 16). NKX2.2, however also represseses Olig2 (Novitch et al., 2001) and thus Olig2 expression is almost abolished in Pax6 mutant embryos (Sun et al., 1998). This leads to a dramatic reduction in the production of OLPs from the pMN domain which by e18.5 is restored to normal levels (Sun et al., 1998). Furthermore, Nkx6.1 (Liu et al., 2003) and Gli2 (Qi et al., 2003) mutant mice, which display a dramatic transient decrease in Olig2 expression, exhibit a delay and transient reduction in PDGFRα expression (marker for OLPs) (Liu et al., 2003; Qi et al., 2003). Surprisingly, in Nkx6.1 and Nkx6.2 double mutant mice Nkx2.2 expression also expands dorsally leading to the abrogation of Olig2 expression in the pMN and a concomitant lack of PDGFRα expression in the pMN (Cai et al., 2005; Vallstedt et al., 2005). This latter observation was in fact the direct demonstration that there must be a dorsal source of OLPs other than the pMN. Collectively these studies suggest that any molecular re-specification of the pMN domain that leads to loss of OLIG2 abolishes the specification of OLPs.

1.3.1.2 Spatial control of Astrogenesis in the spinal cord

In contrast to OLs, it is generally believed that ASs are generated in all domains of the developing spinal cord (Kessaris et al., 2008). So far only one factor has been identified that affects the spatial control of astrogenesis. Deletion of the Tal1 (SCL) gene, which is normally expressed by some progenitors in the p2 domain, leads to loss of ASs produced in this domain (Muroyama et al., 2005). SCL is a bHLH transcription factor that is specifically expressed in the p2 progenitor domain during neurogenesis and gliogenesis (Briscoe et al., 2000). At E14.5 it is also expressed in Fgf3-, Id3- and S100-positive immature astrocyte progenitors (Muroyama et al., 2005). Tissue specific deletion of the Scl gene resulted in severe reduction in p2-associated $S100\beta^+$ astrocyte progenitors and an increase of $Olig2^+$ OLPs compared to the wild type embryos. Conversely, forced expression of mouse SCL in the chick SC resulted in the suppression of endogenous

Olig2 and the ectopic expression of s100β, Fgfr3 and Id3 suggesting that SCL is necessary and sufficient for p2-associated astrocyte development (Muroyama et al., 2005).

1.3.2 Genetic regulation of glial subtype identity

In spite of the existence of at least two sources of OLPs in the spinal cord, there is not yet evidence of any functional differences between these two classes of OLPs. On the other hand very recent evidence suggests that the organisation of the spinal cord into progenitor domains might regulate AS subtype identity. So far AS have been classified as protoplasmic or fibrous based on morphological differences and their location in the grey and white matter (GM/WM), respectively (Miller and Raff, 1984). The major marker for WM fibrous astrocytes is the glial fibrillarly acidic protein (GFAP) (Antanitus et al., 1975), while the calcium-binding protein S100β (Ludwin et al., 1976) labels mainly protoplasmic GM astrocytes. Other markers for astrocytes are the enzyme glutamine synthetase (GS) (Norenberg and Martinez-Hernandez, 1979), the glutamate transport GLAST (Schmitt et al., 1997) and fibroblast growth factor receptor-3 (Fgfr3) (Pringle et al., 2003). However, not all these are completely specific. GLAST of instance is expressed by radial glial cells as well (Deneen et al., 2006; Ogawa et al., 2005).

The first evidence of AS heterogeneity in the SC comes from *in vitro* studies (Miller et al., 1994). Also, morphologically distinct astrocyte subtypes have been identified in different layers of the olfactory bulb (Bailey and Shipley, 1993), and astrocytes with different electrophysiological properties have been described in distinct regions of the hippocampal area (D'Ambrosio et al., 1998).

Recently the first genetic evidence has been provided of the existence of three different AS subtypes in the WM of the vSC that are specified by a homedomain code, like neuronal specification (Hochstim et al., 2008). These three AS subtypes were named VA1-3 (VA- Ventral Astrocytes) and have been described on the basis of the expression in the WM of the guidance molecules Reelin and Slit1. All three types are specified in the vSC and migrate only to the WM of the vSC. VA1 ASs are $Reelin^{+}/Slit1^{-}$ and they

occupy the dorso-lateral WM, VA2 ASs are Reelin^{+}/Slit1^{+} and they occupy the ventro-lateral WM and finally VA3s ASs are Reelin^{-}/Slit1^{+} and they occupy the ventro-medial WM (Fig. 6). In the VZ of E13.5 embryos three expression domains have also been identified based on Reelin and Slit1 expression, named pA1-A3. They have been proposed to be the corresponding progenitor domains where these three three AS subtypes are specified. Based on gene expression comparison between patterning factors expressed in these domains and Slit/Reelin expression, the pA1-A3 domains have been mapped to correspond to: pA3-p3; pA2 (pMN-p2); pA1 (p1) (Fig. 6).

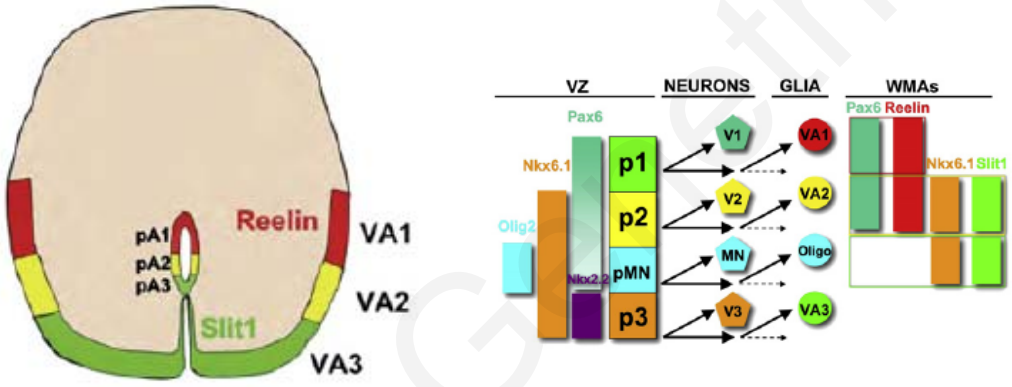

Figure 6. Composite schematic of the spinal cord illustrating the positions of VA1-VA3 astrocytes in the WM at E18.5 and corresponding progenitor domains (pA1-3) in the VZ at E13.5. Adopted from Hochstim et al., 2008.

It has been proposed that the positional specification of these AS subtypes is controlled by the combinatorial function of PAX6 and NKX6.1 during AS specification in the VZ, analogous to the control of neuronal sub-type specification that takes place in the VZ during neurogenesis. In Pax6 mutant embryos there is a complete conversion of all AS to a VA3 (Slit1^{+}) subtype (green WM in Fig. 6 expands). This was interpreted to suggest that PAX6 must repress Slit1 in the VZ. Indeed, in the chick spinal cord PAX6 is sufficient to induce and repress Reelin and Slit1 expression, respectively. On the other hand NKX6.1 was shown to be sufficient to induce Slit1 and repress Reelin, respectively. The problem with this model is that PAX6 and NKX6.1 do not have cross-repressive interactions (See Fig. 4; page 16) and are co-expressed in the pMN and p2 domains (Fig.

5; page 17). To explain the functional implications of this co-expression in the pA2 domain where both PAX6 and NKX6.1 are expressed, Hochstim et al proposed that NKX6.1 somehow overrides the repression of PAX6 on Slit1 thus allowing Slit1 to be expressed in progenitors that express PAX6. NKX2.2 has also been proposed to function in the same manner as NKX6.1 in promoting Slit1 expression. In the p3 domain, where NKX2.2 and NKX6.1 are both expressed, Hochstim et al. predicted that NKX2.2 must simply repress Pax6 from this domain based on the proposed cross-repression between PAX6 and NKX2.2 (See Fig. 4; page 16; Muhr et al., 2001). In this study I will provide direct genetic evidence suggesting that NKX2.2 never represses Pax6 and that NKX2.2 and not NKX6.1 is the key HD factor regulating the expression of Slit1 in the vSC in a Pax6-independent manner (Genethliou et al., 2009). Nevertheless, the Hochstim et al (2008) study represents the first demonstration that neural patterning not only determines neuronal subtype identity but also AS subtype identity.

1.3.3 Temporal control of gliogenesis

1.3.3.1 Intrinsic regulators of the temporal control of gliogenesis

In addition to understanding how the domain-specific organization of the spinal cord affects glial subtype identity, certain molecular changes of the N/G switch affect all progenitor domains and thus regulate the N/G fate switch in a temporal manner. In the mouse spinal cord, the N/G fate switch starts at embryonic day 11.5 (e11.5) and progresses in a ventral-to-dorsal direction (Kessaris et al., 2008). This fate switch is marked by progressive extinction of neurogenesis (Sugimori et al., 2007), up-regulation of radial glial (RG) markers such as Glast (Ogawa et al., 2005), up-regulation of some Sox genes, like Sox9 and Sox5/6/8 (Stolt et al., 2004; Stolt et al., 2003; Stolt et al., 2006; Wegner, 2008), up-regulation of the pro-glial Nuclear Factors I A and B (NFIA/B; Deneen et al., 2006) and dynamic changes in the expression of the Notch effectors Hes1 and Hes5 (Sugimori et al., 2007; Wu et al., 2003).

Despite these descriptive gene expression changes, there is not yet a consensus view what exactly triggers gliogenesis. One idea is that the abrogation of neurogenesis is sufficient to elicit gliogenesis. This argument has been rehearsed extensively and

proposes that the N/G switch represents the manifestation of a 'default' state that begins to take place once neurogenesis stops. This argument has been put forward based on the analysis of Neurogenin 1 and 2 (Ngn1/2) double mutant embryos. NGN1 and NGN2 are very important proneural factors that instruct progenitors to produce neurons. However, in these embryos only late astroglial markers are prematurely expressed and not AS progenitor markers. Furthermore, these embryos express ectopically another proneural gene, Mash1 (Scardigli et al., 2001) which, in addition to its proneural function biases OLP specification at the expense of AS (Sugimori et al., 2007). Accordingly, mouse embryos expressing Mash1 from the Ngn2 locus, in an $Ngn2^{-/-}$ background, show an increase in $PDGFR\alpha^{+}$ OLP numbers compared to the Ngn1/2 double mutant embryos. Conversely, $Mash1^{-/-}$ embryos and transgenic embryos expressing Ngn2 from the Mash1 locus show a decrease in the OLP cells (Sugimori et al., 2007). These data shows that Mash1 plays an important role in determining the timing of differentiation of OLs.

Another factor proposed to affect the temporal control of the N/G switch is the transcription factor SOX9. The gene coding for SOX9 is expressed in the VZ of embryonic spinal cord at a time point that coincides with the emergence of gliogenic radial glia (e11.0; Ogawa et al., 2005). Conditional inactivation of Sox9 led to prolonged generation of ventral neurons and concomitant inhibition of gliogenesis that partially recovered at late embryonic stages (Stolt et al., 2003), probably due to redundant functions between SOX9 and SOX8 (Stolt et al., 2003). This phenotype was interpreted to suggest that SOX9 instructs progenitors to begin to make glial and in its absence this switch does not take place and neurogenesis is prolonged. However SOX9 has not been shown to be sufficient to induce the N/G fate switch (Deneen et al., 2006).

For a number of years now, the debate over the origin of glial cells centred on the issue whether the N/G switch is characterised by the emergence of glial-restricted progenitors (GRPs) irrespective of the progenitor domain that they originate (Rao, 1999; Rao and Mayer-Proschel, 1997; Rao et al., 1998). This debate has primarily been fuelled by the general belief that NSC must exist in the developing CNS, but the data supporting the existence of these cells was primarily obtained from *in vitro* studies, as discussed

earlier. The GRP hypothesis stipulates that glial cells are generated indirectly via restricted precursors that are mono- (OL or AS only) or bi-potent (AS and OL). In cell culture assays using precursors isolated during the onset of gliogenesis, only neuronal restricted progenitors (NRP) and glial restricted progenitors (GRP) were observed (Rao, 1999). On the other hand, the fact that pMN domain generates motor neurons, oligodendrocytes and astrocytes (Pringle et al., 1998; Pringle and Richardson, 1993) led some researchers to propose that these cells may arise from the same precursor cells at different times in development. However direct evaluation of the potency of pMN progenitors, expressing Olig2, isolated from e9.5 (neurogenic) and e13.5 (gliogenic) spinal cords failed to support the existence of lineage-restricted progenitors or even tripotent NSC (Mukouyama et al., 2006). More recently, and in view of the fact that the GRP-hypothesis has not gained strong support, two additional models have been proposed to explain glial lineage specification in progenitor domains.

The first model postulates that the induction of gliogenesis and the repression of neurogenesis are controlled by a single transcription factor NFIA (Deneen et al., 2006). This gene begins to be expressed at around e11.5 in the VZ of the mouse spinal cord, the precise time point that the N/G switch takes place. Using gain-of-function studies in the chick embryo it has been proposed that NFIA is sufficient to induce a pro-astrocytic program of glial specification in all progenitor cells. NFIA is expressed ia lla pMN cells however during gliogenesis. In order to explain the pro-astrocytic function of NFIA Deenen et proposed that the pMN domain, contrary to fate mapping data (Masahira et al., 2006), does not generate any ASs and that OLIG2, physically interacts with NFIA and neutralizes its pro-astrocytic effect, driving these cells towards the OL lineage. In support of this model, in Olig2$^{-/-}$ embryos additional ASs are generated at the expense of OLPs (Zhou and Anderson, 2002). However, one complicating factor is that in Olig2$^{-/-}$ embryos, the transcription factor SCL, which is normally expressed in the neighboring p2 progenitor domain and which controls a pro-astrocytic program of differentiation in this domain, expands ventrally in the prospective pMN and might also contribute to this OLP-AS fate switch (Muroyama et al., 2005). While this model proposes an important function for NFIA in the VZ once it is expressed, the genetic mechanism initiating this first

gliogenic switch by inducing NFIA expression in neural progenitors is unknown. I will provide the first genetic evidence suggesting that this pan-gliogenic switch is regulated by Notch signalling.

The second model tries to explain how patterning factors regulate gliogenesis in the the vSC and focuses in the p3-p2 domains (Sugimori et al., 2007). This model makes lineage predictions on the basis of combinatorial expression of a large set of transcription factors including the patterning factors NKX2.2, OLIG2 and PAX6, proneural helix-loop helix (HLH) factors NGN1-3 and MASH1 and inhibitory HLH factors such as ID1 and HES1 (Sugimori et al., 2007). According to this model neurons are generated in all progenitor domains, primarily through the combinatorial expression of patterning and proneural HLH factors. OLPs are predicted to be generated in the p3 and pMN domains through combinatorial expression of NKX2.2/MASH1 and OLIG2/MASH1, respectively while ASs are predicted to be generated in all domains by progenitors that have switched off patterning factors (i.e Nkx2.2, Olig2 and Pax6) but continue to maintain ID1/HES1 expression in the VZ. Certain elements of this model however have already been challenged. For instance both Nkx6.1 and Pax6 have been shown to be expressed by AS progenitors (Hochstim et al., 2008). Furthemore, the suggestion that the p3 domain generates OL awaits formal verification from fate mapping studies.

1.3.3.2 Extrinsic Signals that regulate the acquisition of the glial fates

1.3.3.2.1 Shh signalling in OL specification

In the previous section I have described various findings that implicated transcription factors in the initiation of the N/G switch. Because this switch takes place during a time window that extrinsic factors are also expressed, their involvement has also been studied. The Shh signalling pathway again has been proposed to be required for OL specification in the ventral spinal cord, but in a different way to its earlier function during neuronal specification (Orentas et al., 1999; Pringle et al., 1996). In the absence of Shh signalling, ventrally derived OLPs fail to be generated (Nery et al., 2001; Orentas et al., 1999; Tekki-Kessaris et al., 2001). On the other hand, transplantation experiments with Shh-expressing tissue adjacent to the dorsal neural tube *in vivo* (Orentas and Miller,

1996; Pringle et al., 1996) or the ectopic expression of Shh in the dorsal midline of the spinal cord induces ectopic specification of OLPs (Lu et al., 2000). This effect of Shh on OLP specification is likely to be due to the up-regulation of Olig2 and the concomitant repression of neurogenesis (Danesin et al., 2006).

More recently in the chick embryo SHH has been proposed to regulate the production of p3-derived OL progenitors in a dose-dependent manner. During the N/G switch in the chick, the p3 ($Nkx2.2^{+}$) domain in the chick spinal cord expands dorsally while the pMN domain retracts ventrally. This boundary shift has been proposed to be caused by sudden changes in the concentration of SHH protein that during this boundary shift concentrates heavily in the p3 domain (Danesin et al., 2006). Concomitant with the accumulation of SHH, the gene coding for the enzyme Sulfatase 1 (Sulf1) begins to be expressed by p3 progenitors while during neurogenesis this gene is only expressed by Floor Plate (FP) cells. Ectopic expression of Shh led to ectopic production of OLPs while ectopic expression of Sulf1 led to accumulation of SHH in ectopic sites. Shh has been shown to inhibit neurogenesis at a concentration much higher than that required to induce neural patterning. Thus is the p3 domain, Sulf1 has been proposed to mediate the repression of neurogenesis, through regulating the local concentration of SHH protein, thereby allowing OL specification to take place (Danesin et al., 2006). The role of SULF1 in OL specification in mice has not been tested and I will provide genetic evidence in this study to suggest that Sulf1 expression is regulated by patterning factors specifically during OL specification and that this regulation may have implications on the maintenance of the patterning of the neural tube during gliogenesis (Genethiou et al., 2009).

1.3.3.2.2 BMP signalling in OL specification

BMP signalling is known to repress Olig2 expression (Mekki-Dauriac et al., 2002) probably by indirect enhancement of Irx3 expression (Nicolay et al., 2007). This should result in the repression of OLP specification. Indeed, neuro-epethilial progenitors (NEPs) cultured in the presence of BMPs failed to generate OLs (Gross et al., 1996; Mekki-Dauriac et al., 2002). Also, ectopic expression of BMPs in chick ventral spinal

cord inhibits the development of $O4^+$ cells (Mekki-Dauriac et al., 2002) [O4 is an antibody that detects sulfatide and other glycolipids, expressed in the surface of immature and mature OLs (Ono et al., 1995; Sommer and Schachner, 1981)]. In contrast, inhibition of BMP signalling with Noggin causes an increase of $O4^+$ cells (Mekki-Dauriac et al., 2002). Hence, BMP signalling negatively regulates OL specification. BMP also inhibit differentiation of OLPs by inducing expression of the inhibitors of differentiation (Id) bHLH genes (Samanta and Kessler, 2004), which act as dominant negative inhibitors of OLIG1/2 (Kessaris et al., 2008).

1.3.3.2.3 Notch signalling in Glial specification

Notch pathway is another mechanism that is involved in glial specification but its function in OL specification has been controversial, depending on the organism under study (Appel et al., 2001; Park and Appel, 2003; Taylor et al., 2007). Notch is a transmembrane receptor that is activated by Delta ligands presented on the surface of the neighbouring cells. Ligand binding leads to the proteolytic cleavage of part of Notch intracellular domain (NICD) which then enters the nucleus. There it creates an activator transcriptional complex with the protein RBP-J leading to activation of the transcriptional repressors of Hairy/Enhancer of Split (Hes) gene family (Justice and Jan, 2002). HES factors are the main effectors of Notch signalling. During glial specification only Hes1 and Hes5 are expressed. During neurogenesis HES genes repress the expression of proneural genes like Ngn1-3 and Mash1 thereby maintaining neural progenitors in an undifferentiated state (reviewed by Louvi and Artavanis-Tsakonas, 2006). In zebra fish in the absence of Notch signalling excess neurogenesis takes place in the spinal cord at the expense of OLPs (Appel et al., 2001; Park and Appel, 2003). Conversely, when Notch signalling is activated there is an excess of OLPs at the expense of MNs (Park and Appel, 2003). More recently two other HES-like genes have been described, named Hey1 and Hey2 that are also believed to be Notch effectors (Sakamoto et al., 2003). Interestingly in the spinal cord these latter genes are expressed specifically during gliogenesis as I will show in this study genetic evidence about their regulation by PAX6.

In mice, however, the role of Notch appears to have the opposite effect to that proposed for zebrafish. In a recent study, Nestin Cre^{+} $Rbpsuh^{fl/fl}$ embryos were generated to conditionally delete the Rbpsuh (RBP-J) gene which mediates the function of all Notch receptors. As a result canonical Notch signalling was abolished in the SC. These embryos compared to their wild type littermates showed no differences in the number of neurons, but they had a significant increase of OLPs and mature OLs in later stages, at the expense of ASs (Taylor et al., 2007). This was the first demonstration in mice that attenuation of Notch signalling favours the OL fate at the expense of the AS fate. This study however, could not explain why the OL lineage was favoured at the expense of the AS lineage but nevertheless reinforced the view that Notch may be required both for OL specification as well as OL maturation (Genoud et al., 2002; Givogri et al., 2002; Wang et al., 1998). I will provide genetic evidence suggesting that Notch regulates SOX1 and NFIA as part of a mechanism to regulate OL and AS specification in the pMN.

AS are generated from all progenitor domains and are thought to be generated from the transformation of residual un-differentiated radial glia. Immunohistochemical studies showed cells with antigenic and morphological phenotypes intermediate between radial glial and AS (Barry and McDermott, 2005; Voigt, 1989) and also the fact that radial glial cells begin to disappear during development at about the same time as AS appear (Misson et al., 1988) suggest that AS arise from radial glia cells. Earlier studies showed that AS and OLs are generated preferentially from different part of the VZ of the embryonic spinal cord (Lu et al., 2002; Zhou and Anderson, 2002). However, more recently, lineage-tracing studies have shown that some domains, like the pMN domain, generate both AS and OLs (Sugimori et al., 2007).

Notch signalling is also involved in astrocyte specification per se. When adult rat hippocampal progenitors were transduced by a constitutively active form of Notch, astrocyte production was induced (Tanigaki et al., 2001). Also, viral infection of the constitutively active intracellular domain of Notch1 in E14.5 spinal cord cells resulted in promotion of the astrocytic fate and inhibition of OLs (Wu et al., 2003). These Notch signalling effects seem to be mediated through HES1 and HES5 activity. In cultured

NEPs, Hes1 over-expression was unable to block AS or OL specification. In contrast in cultured GRPs, Hes1 specifically blocked the production of OLs and favoured the production of AS while Hes5 blocked both lineages in GRPs (Wu et al., 2003). These observations suggest that, HES1 could mediate the OL vs AS fate choice *in vivo* but genetic evidence suggesting such as stage-specific requirement has not been provided. I will provide such evidence in the context of this study.

Concluding remarks and unresolved issues

As mentioned earlier the N/G fate switch is marked by progressive extinction of neurogenesis (Sugimori et al., 2007), up-regulation of RG markers such as Glast (Ogawa et al., 2005), up-regulation of some Sox genes (Stolt et al., 2004; Stolt et al., 2003; Stolt et al., 2006; Wegner, 2008), up-regulation of the pro-glial Nuclear Factors I A and B (NFIA/B; Deneen et al., 2006) and dynamic changes in the expression of the Notch effectors Hes1 and Hes5 (Sugimori et al., 2007; Wu et al., 2003). During the N/G switch uncommitted neuroepithelial progenitors continue to express a repertoire of patterning factors (Jessell, 2000), posing the question of how temporal and spatial specification of neural lineages is regulated within each progenitor domain.

Overall the picture that is emerging from various studies requires genetic studies that will help integrate the function of neural patterning, Notch signalling and perhaps other signalling mechanisms in order to develop a more coherent picture of the spatial and temporal regulation of the N/G switch. This study provides strong genetic evidence linking the function of neural patterning and Notch signalling through SOX1 function and also provides genetic evidence suggesting that Notch signalling regulates the initial pro-astrocytic gliogenic switch in all progenitor domains. Before describing the findings, some description of current knowledge on SOX1 function is merited.

1.4 SOXB protein function during CNS development

The first member of the Sox gene family to be identified was the Sry gene. Sry is the mammalian testis-determining gene (Berta et al., 1990; Sinclair et al., 1990). Today, more than 20 Sox genes have been identified on the basis of their homology to the high mobility group (HMG) box of the Sry gene (Gubbay et al., 1990). The HMG box is a 79 amino acid DNA binding domain which is present in a large number of proteins belonging to the HMG box super family.

Based on this HMG box amino acid sequence, SOX proteins are grouped into seven groups (Bowles et al., 2000). Many proteins within each group share conserved structural domains outside the HMG box. The SoxB group is divided to SoxB1 subgroup which includes SOX1-3 that function as transcriptional activators and to SoxB2 subgroup which includes SOX14 and SOX21 that function as transcriptional repressors (Bylund et al., 2003; Malas et al., 1999; Sandberg et al., 2005; Uchikawa et al., 1999). Unlike most transcription factors, the SOX proteins bind in the minor groove of the DNA resulting in the induction of a dramatic bend within the DNA helix (Giese et al., 1992; van de Wetering and Clevers, 1992). Members of the Sox family are found to be expressed in a wide variety of tissues during development such as neural tissue, lens, gut epithelium, B-cell, muscle, hair follicle, blood vessel etc (Bowles et al., 2000).

Sox1-3 are all expressed in the developing CNS. In the neural tube, Sox1 is expressed from the onset of somitogenesis while Sox2 and Sox3 are already expressed prior to gastrulation in the ectoderm (Wood and Episkopou, 1999). Sox1 deficient mice are viable but develop micropthalmia (Nishiguchi et al., 1998). This is due to a requirement for SOX1 for the regulation of γ-crystallins, which is essential for lens development. In the absence of Sox1 the expression of γ-crystallins in the developing lens is severely down-regulated (Kamachi et al., 1998; Nishiguchi et al., 1998). Sox1 mutant mice also suffer from spontaneous (and invariably lethal) seizures between 4 and 6 weeks of age. Seizures can be induced by stress such as excessive noise or even routine handling but they also occur spontaneously in all mutant animals (Malas et al., 2003). In

the forebrain Sox1 is expressed in the VZ and subventricular zone (SVZ) and also in neurons of the striatal bridges that are linked with the olfactory tubercle (OT) of the ventral striatum (VS). The neurons that contribute to the OT are $Sox1^{+}$ and during early development they fail to differentiate and migrate properly to the prospective OT region (Ekonomou et al., 2005). In the absence of Sox1, the VZ and SVZ are not affected, while the striatal bridges and the OT are missing. Proliferation assays showed that this phenotype is not associated with any proliferation defect and that the reason of this neuronal loss is the failure of these prospective OT neurons to differentiate and migrate to the appropriate position (Ekonomou et al., 2005). The epilepsy has been proposed to be linked to the absence of the OT neurons (Malas et al., 2003).

Several *in vitro* studies have proposed that SOX1 unlike SOX2/3 is sufficient to induce neuronal differentiation in uncommitted progenitors. In contrast *in vivo* studies in the chick suggested that all SOXB1 genes block neuronal differentiation (Bylund et al., 2003). Pevny et al (1998) studied the function of Sox1 using the P19 neuroectodermal cell line. These cells are embryonic carcinoma cells with the ability to differentiate into all three germ lineages (McBurney, 1993). With the addition of RA, P19 cells differentiate into neuroepithelial-like cells (Jones-Villeneuve et al., 1982) and 15% of these differentiate into mature neurons. In undifferentiated P19 cells Sox1 mRNA is very low but addition of RA induces strong expression of Sox1 (Pevny et al., 1998). Interestingly, over-expression of SOX1 promoted neural differentiation in P19 cells and down-regulation of Sox1 was necessary for these cells to differentiate further into mature neurons (Pevny et al., 1998). Kan and colleagues (Kan et al., 2004), used telencephalic progenitor cells transfected with a Sox1-expressing plasmid also suggested that SOX1 promotes neuronal differentiation of neural stem or progenitor cells. This study revealed a very interesting observation in that functional SOX1 binding sites have been reported to exist on the Hes1 promoter. However, SOX1 has been proposed to function as a repressor of Hes1 expression, which is in contrast to other studies suggesting that SOX1 functions as an activator (Bylund et al., 2003).

A very recent study examined the effects of over-expression or down-regulation of SOX1 in embryoid bodies (EBs). This study also suggested that SOX1 favours the development and maintenance of the neuroectoderm and needs to be down-regulated for further neurogenesis to proceed as proposed by Penvy and colleagues (Suter et al., 2008). However, genetic loss of function studies failed to show a specific requirement for SOX1 in the VZ at any developmental stage.

Two studies in the chick embryos proposed a model in an attempt to explain at what specific time point SOXB1 proteins block neurogenesis (Bylund et al., 2003; Sandberg et al., 2005). According to this model, over-expression of Sox1-3 in the chick SC blocked neuronal differentiation downstream of the function of proneural genes, like Neurogenins 1-3. Thus over-expression of SOX1-3 did not affect proneural gene expression, therefore the commitment to the neuronal lineage was not affected, but the progression from the Ngn^{+} status to the committed neuronal status ($NeuroM^{+}$) was blocked. Conversely, over-expression of Ngn2 down-regulated Sox1-3 expression suggesting that proneural bHLH proteins need to down-regulate SOXB1 gene expression to allow neurogenesis to proceed (Bylund et al., 2003). A follow up study showed that Sox21 over-expression in the chick SC had the opposite results to that of SOXB1 factors and promoted neuronal differentiation. SOX21 was proposed to be a target for proneural bHLH proteins in that over-expression of proneural proteins resulted in up-regulation of the level of Sox21 expression. Because both SOX1-3 and SOX21 are expressed in all neural progenitors it has been proposed that the outcome of the opposing activities between SOX21 vs SOX1-3 is regulated by the SOXB1/SOX21 protein ratio present in each cell (Sandberg et al., 2005). One prediction from this study is that SOX21 is a critical component of neurogenesis but again loss of function studies do not support such a role (unpublished data communicated from Dr Jonas Muhr and also data obtained in this study). Therefore the role of SOX1 during neurogenesis is likely to be modulated by context-dependent mechanisms that cannot be recapitulated in gain-of-function studies. This may explain the discrepancies between the results obtained from different laboratories using different experimental approaches.

Chapter 2

Results

2.1 Scientific and technological objectives

Scientific Objectives

The major scientific objective of this study was to analyse the function and genetic regulation of SOX1 during the N/G switch. As the project progressed it became apparent that Sox1 was regulated by the patterning factors NKX2.2 and PAX6 in a region-dependent manner and this investigation expanded further to study mouse mutants of these two genes in order to establish if certain glial defects associated with loss of these genes were due to SOX1 function. In the latter parts of this study and as more data were being evaluated, I decided that I needed to use Presenilin-1 mutant embryos that manifest severely attenuated Notch signalling. Therefore, this study constitutes an integrated attempt to assign specific functions during glial specification not only to SOX1 but to NKX2.2, PAX6 and Notch signalling that, as it will become apparent, function upstream of Sox1-mutant embryos.

The investigation to study the role of Sox1 during glial specification was initially sparked by two observations made at the start of this project in relation to the expression of Sox1. Using a 'knock-in' line where the Sox1 gene was replaced by the β-galactosidase gene (Malas et al., 2003), Dr Malas, observed that Sox1 expression, as reported by β-gal staining was not uniform in all regions of the developing spinal cord from e11.5 onwards. Since at this stage the N/G switch takes place I reasoned that SOX1 might have a role to play in this process. The dynamic expression of Sox1 at this stage of spinal cord development was also confirmed using a GFP 'knock-in' line made available to us (Aubert et al., 2003).

In parallel to studying the function of SOX1, I have been developing mice that are deficient for SOX21. These mice have been successfully produced but the analysis of the phenotype has not been undertaken. In Appendix II some data are presented on the expression of SOX21 but no further analysis was carried out since there was no discernable glial defect in these mice that merited further investigation to tie in with the phenotype of SOX1.

Technological Objectives

When this study was undertaken the most pressing problem that needed to be resolved was the development of a well-functioning anti-SOX1 antibody. Even the published antibody (Pevny et al., 1998) was not sufficiently specific in my studies and in my view the images reported in the original study show non-specific staining. The same issue applies for SOX21 and SOX3 against which no antibodies are available so far. Despite the fact that the transgenic knock-in lines that were available to me at the start of the project expressed reporter genes and could be used to define the expression Sox1, neither β-gal nor eGFP could be used for precise domain mapping of Sox1 expression. B-gal is a cytoplasmic protein while eGFP is also cytoplasmic and the protein itself is very stable and is detected in cells that switch off the expression of the gene even after 36 hours.

Hence the first major technological objective was to develop a good anti-SOX1 antibody. In the course of this study two anti-SOX1 antibodies were developed and in addition, all commercial antibodies available were tested. Two antibodies proved to give reasonable staining, one developed by me and one commercial, but the staining could not always be reproduced and the fixation conditions that were required did not work with other antibodies.

Hence a major technological objective was to develop a reporter line that expressed a nuclear form of GFP under the control of the promoter/enhancer region of Sox1, in order to circumvent the problem with the anti-SOX1 antibodies and be able to map precisely the expression of Sox1 in the ventral spinal cord. Having achieved this task, and as the project progressed, I used a Sox1-Cre^{ERT2} transgenic line for fate mapping studies that was developed by Professor William Richardson and Dr Nicoletta Kessaris at the Wolfson Institute of Biomedical Research, London.

2.2 Generation of a transgenic mouse line expressing nuclear GFP from the Sox1 promoter

In order to achieve this task a Bacterial Artificial Chromosome (BAC) was obtained from a publically available BAC library. This clone contained approximately 120 kb upstream and 100 Kb downstream of the Sox1 exon. I reasoned that this clone should contain all the regulatory elements of the endogenous gene. I used BAC recombination (see Materials and Methods) to replace the open reading frame of Sox1 with a cassette coding for a nuclear version of eGFP. This GFP gene was made by Dr Stavros Malas and consisted of a commercial Nuclear Localisation Signal (NLS; Invitrogen) retrofitted in the eGFP gene. Two founder transgenic lines were generated that carried the recombinant BAC and both expressed in an identical manner. I used one of these lines throughout the course of this study. Where necessary I crossed this allele, denoted Sox1^{-GFPn}, in different genetic backgrounds to monitor Sox1 expression. All embryos expressing Sox1^{-GFPn} could be easily scored in whole mount preparations using an epifluorescent UV light source so their genotyping was very easy and had similar expression pattern to the β-geo knock-in embryos (not shown).

2.2.1 Sox1^{-GFPn} faithfully recapitulates Sox1 expression

In order to test if the Sox1^{-GFPn} allele recapitulates the expression of the endogenous gene we correlated the expression of Sox1^{-GFPn} against the expression of the endogenous protein using two antibodies and by in situ hybridisation. One commercial antibody was raised in goat (Santa Cruz, 17318) and one that we developed using a commercial company against the peptide (AGGRHPHAHPAHPHPHHPHAHPHNPQP). Both antibodies were specific as they showed no cross-reactivity in Sox1$^{-/-}$ embryos but nevertheless both required special fixation conditions that could not always allow their use with other antibodies. The important point for this expression pattern analysis was to precisely map which region in the vSC switched off Sox1 and at what developmental time point. The GFPn protein did not show prolonged stability and its expression disappeared when the gene was switched off hence it was a very good reporter for my

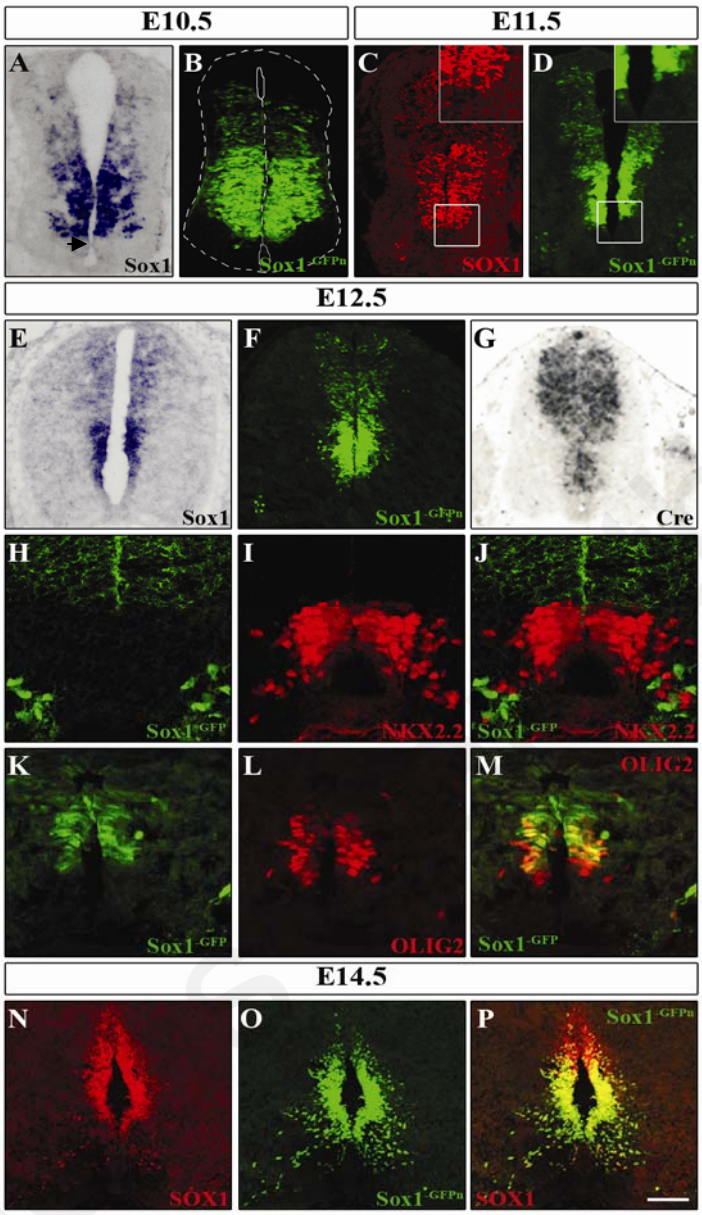

Figure 7. Comparison of SOX1, Sox1 and Sox1^{GFPn} expression at e10.5-e14.5 in the spinal cord

Sox1^{-GFPn} expression correlated precisely with Sox1 expression at e10.5 (A,B). Note the dorsal^{-low} and ventral^{-high} gradient of Sox1 and Sox1^{-GFPn} expression. Note also that Sox1 is very weak in the most ventral part of the spinal cord (arrow in A). By e11.5 SOX1 and Sox1^{-GFPn} expression is switched off in the ventral spinal cord (boxed area in C, D and insets). (E) At e12.5 the dorsal^{-low} and ventral^{-high} expression domain of Sox1 is fully resolved and this is precisely reflected by the expression of the Sox1^{-GFPn} allele (F). (G) Shows the expression of Cre from the Sox1-Cre^{ERT2} BAC line that I will describe later showing that this line too expresses the transgene very similar to the endogenous gene. The Cre probe is very good hence the differences in the intensity of staining between E and G. Panels H to M show comparison between the expression of NKX2.2 (I) and OLIG2 (L) and a GFP knock-in line to reinforce the point that Sox1 is switched off from the p3 domain (Nkx2.2^{+}) and is partly expressed in the pMN domain (Olig2^{+}). (N-P) Comparison between an anti-SOX1 antibody and Sox1^{-GFPn} at e14.5 in the spinal cord. Panel C shows staining of the guinea pig anti-SOX1 antibody and panel N staining with a goat (commercial anti-SOX1 antibody).

studies. As shown in Fig. 7 the Sox1^{-GFPn} line recapitulated the spatial expression of Sox1 accurately.

2.3 Sox1 expression

2.3.1 Expression of Sox1 in the mouse embryonic spinal cord

I first carried out a gross analysis of Sox1^{-GFPn} expression at most developmental stages in the spinal cord (Fig. 8). This analysis showed that Sox1 is expressed in a very dynamic manner in the VZ. For instance between e9.5 and e12.5 the VZ expression of Sox1^{-GFPn} was very dynamic and not uniform (Fig. 8A-C). Gradually the VZ expression of Sox1 was diminishing and by e17.5-e18.5 only a subset of VZ cells expressed Sox1. Migrating GFP^{+} cells shown in Fig. 8C (arrow) represent a new type of interneurons that derive from the p2 domain (Personal communication with H. Panayi). All migrating cells outside the VZ from e14.5 onwards represented AS progenitors as will be described later.

Having established that Sox1^{-GFPn} expression was dynamic in the VZ during early stages of neural development, it was necessary to map the expression boundaries of the VZ expression using several markers that define distinct progenitor domains. This analysis was performed between e9.5-e12.5 (Figs. 9-10).

At e9.5 Sox1^{-GFPn} expression was uniform in all regions of the VZ but was excluded from the floor plate (FP) expressing Shh, roof plate (RP) and a region ventral to the RP that is negative for Gsh2 expression (Fig. 9A-C). However, starting at e10.5 and culminating at e11.5, this mostly uniform expression displayed dynamic changes resolving into three domains: a ventral Sox1^{-ve} expressing Nkx2.2, an intermediate Sox1^{high} expressing Glast and a dorsal Sox1^{low} expressing Gsh2 (Fig. 9D-F). Dorsal Sox1^{-GFPn} progenitors were mostly negative for Ngn2 and Mash1, particularly from e11.5 onwards, but some cells co-expressed both Sox1^{-GFPn} and either proneural gene (Fig. 9G-L). These data suggest that as the N/G switch begins at e11.5 the expression of Sox1 becomes dynamic and is specifically switched off from the p3 (Nkx2.2^{+}) domain.

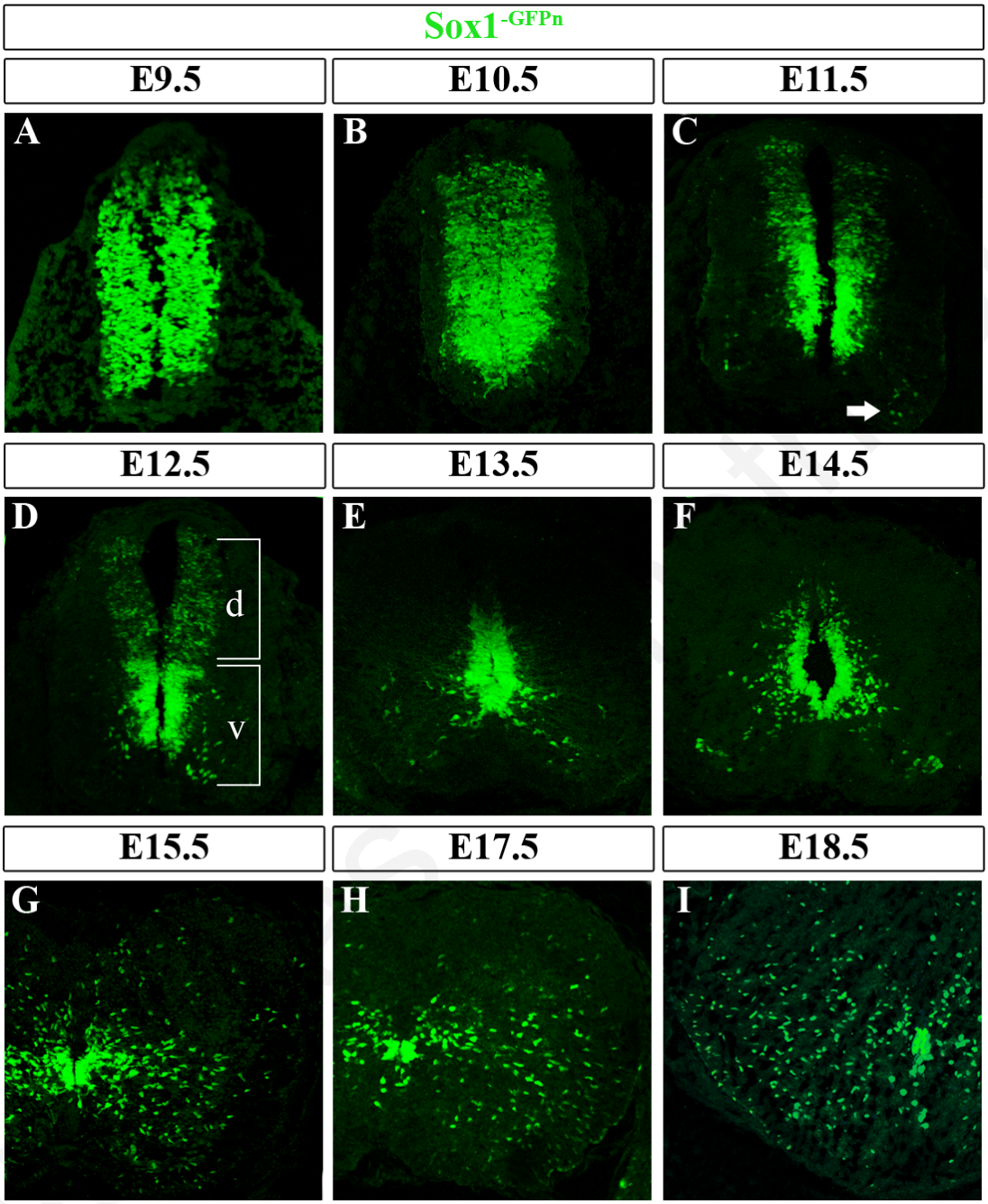

Figure 8. Gross analysis of Sox1^{-GFPn} expression in the developing spinal cord

All embryonic spinal cord sections taken from carrier embryos of the Sox1^{-GFPn} allele analysed at the indicated developmental stage. Note that between e10.5 and e12.5 there is a change in the intensity of GFP expression resolving into a ventral^{-high} (v-ventral) and dorsal^{-low} (d-dorsal) domain (panel D). The dorsal domain gradually lost Sox1^{-GFPn} expression and by e15.5 only a small domain in the VZ expressed GFP. Numerous migrating GFP^{+} cells are seen after e13.5 while a small number of neurons (arrow in C) retain Sox1^{-GFPn} expression.

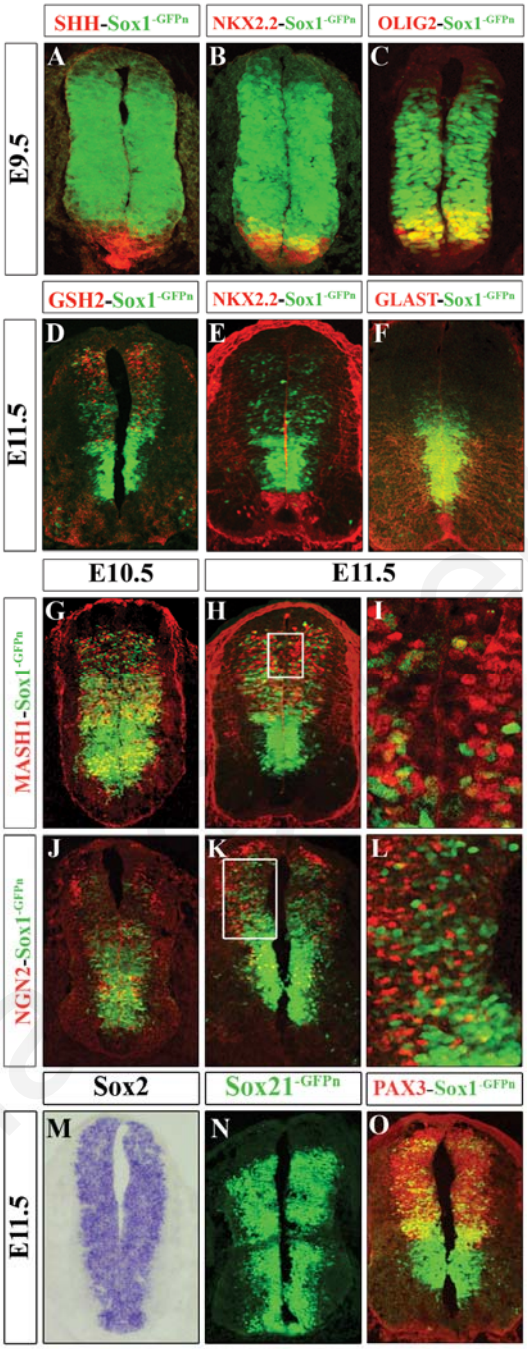

Figure 9: Expression of Sox1-^{GFPn} between e9.5 and e11.5 in the spinal cord

The markers used and embryos stage are shown on the side and the top of each panel. Note the uniform expression of Sox1 at e9.5 (A-C) that transforms to domain-specific expression by e11.5 (D-F). The Sox1^{low} domain co-localizes with Gsh2 and Pax3 (D, O) and the Sox1-^{high} domain co-localizes with Glast^{+} radial glia (F) while the Nkx2.2^{+} domain switches off Sox1^{-GFPn} expression (E). In the vSC both Mash1 and Ngn2 are co-expressed with Sox1^{-GFPn} while in the dorsal SC, Sox1^{-GFPn} expression is mostly mutually exclusive with Mash1 and Ngn2 expression, except very close to the lumen, insets (I,L) in H,K. Note that neither Sox21 nor Sox2 show such domain specific expression (M, N).

2.3.1.1 Changes in Sox1 expression coincide with re-patterning of the p3 and pMN domain

The timing of this change in Sox1 expression in the VZ of the vSC is equivalent to the developmental stage in the chick embryo that boundary shifts in the p3/pMN domain have been observed (Fu et al., 2002; Agius et al., 2004). This boundary shift signals the neuron-glial (N/G) fate switch that begins in the vSC and progresses more dorsally. In the chick embryo the p3 domain expands dorsally while the ventral limit of the Olig2 domain retracts ventrally. This re-patterning also takes place in mice between e10.5 and e12.5 and coincided precisely with the temporal changes in Sox1 expression (Fig. 10). Specifically, from e11.5 the Nkx2.2 expression begun to expand dorsally while the ventral limit of Olig2 and Pax6 expression retracted. Concomitant with the expansion of the Nkx2.2 domain, Sox1^{-GFPn} expression was gradually switched off from this domain and by e12.5 it precisely matched the ventral limit of Pax6 (Fig. 10L, M-O). Within the pMN, Sox1^{-GFPn} expression was weaker in the ventral part similar to Pax6 (Fig. 10F brackets). Note that Pax6 expression overlaps with Olig2 in the dorsal pMN (Fig. 10P-R) and is not extinguished as recently suggested (Sugimori et al., 2007). These data demonstrated that, at the onset of the N/G fate switch and coincident with the re-patterning that takes place in the VZ of the p3 and pMN domains, Sox1 expression is switched off in the p3 domain, remains strong in all intermediate Glast^{+} progenitors and is expressed in a subset of dorsal Gsh2^{+} progenitors. This expression pattern is unique to Sox1 and no other transcription factor reported to date shows similar expression pattern changes. The fact the Sox1^{-GFPn} precisely matched the expression of Pax6 by e12.5 suggested that this transgene must respond appropriately to the developmental mechanism that regulates Sox1 expression and is not a fortuitous coincidence. The next question was to understand how such a dynamic expression pattern is genetically regulated.

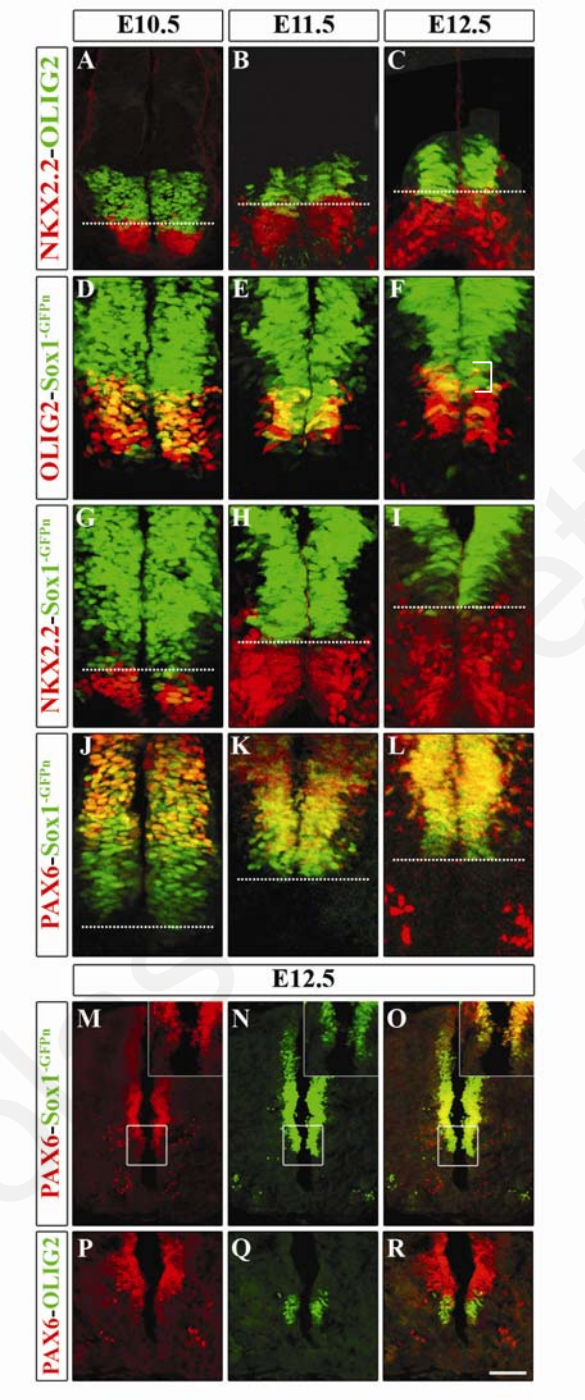

Figure 10: Expression of Sox1^{-GFPn} between e10.5 and e12.5 during vSC re-patterning

Note the expansion of the Nkx2.2^{+} domain and the retraction of the ventral limit of Olig2, Pax6 and *Sox1^{-GFPn}* between e10.5 and e12.5. Dotted lines show the boundary shifts. By e12.5 Sox1^{-GFPn} becomes very weak in the ventral pMN (brackets in 11F) and precisely coincides with Pax6 expression (J-L; M-O). Note also that Pax6 and Olig2 overlap in the dorsal pMN (P-R) unlike what is being suggested that Pax6 is switched off from the pMN domain altogether at this stage (Sugimori et al., 2007).

2.3.2 SHH is not sufficient for down-regulating Sox1 in the ventral Spinal Cord

In the vSC of chick embryos the expansion and retraction of the p3 and pMN domains, respectively, has been attributed to the accumulation of Sonic Hedgehog (SHH) protein in the p3 domain. This phenomenon is believed to be caused by the enzyme SULF1, whose gene expression expands from the floor plate (FP) to the p3 domain during this developmental switch (Danesin et al., 2006). SULF1 is an enzyme which removes sulfate groups from heparin sulphate proteoglycans and activates them to concentrate morphogens, such as SHH, in a specific domain (Ai et al., 2003; Lin, 2004). In mice, this phenomenon has not been investigated and hence I tested if SHH protein accumulates in the p3 domain when Sulf1 expression expands in this region. Similar to the chick spinal cord, both SHH protein accumulation and Sulf1 expression take place in the mouse p3 domain and that Sulf1 and Sox1^{-GFPn} expression showed complementary spatial changes in their expression patterns (Fig. 11).

The accumulation of SHH in the p3 domain raised the possibility that increased Shh signalling might be contributing to the down-regulation of Sox1 in this domain. Therefore it was necessary to investigate this possibility in the chick embryo using gain-of-function (GOF) experiments. The expression of all SOXB genes (except Sox14) was analysed in the chick spinal cord to confirm that chicken Sox1 (cSox1) is also repressed in the p3 domain between E5 and E7, the equivalent stage in the chick when the N/G switch take place. As anticipated, cSox1 was also repressed in a domain close to the vSC which most certainly includes the p3 domain (Fig. 12A, B). cSox2 did not change while cSox3 and cSox21 expression was down-regulated in the vSC to a greater extent. Their mouse counterparts however do not show such expression pattern changes (not shown). However, miss-expression of a Shh-expressing plasmid at E2 had no effect on Sox1 expression analysed at E4 (Fig. 12K, L). These data suggest that changes in the strength of SHH signalling are unlikely to be responsible for the repression of Sox1 in the p3 domain.

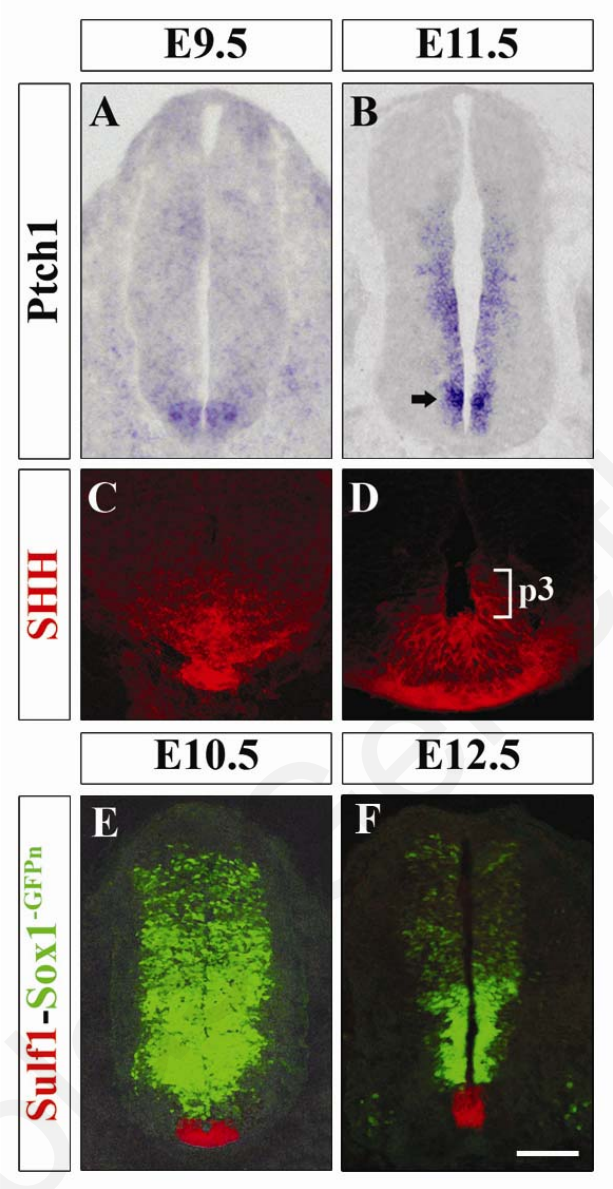

Figure 11: Changes in Shh signalling activity and SHH protein distribution correlate with down-regulation of Sox1^{-GFPn} in the p3 domain

At e9.5 Ptch1 expression, reporting the response of cells to Shh signalling, is mostly confined to a few ventral progenitors (possibly p3) while at e11.5 it expands to most VZ progenitors and becomes strong close to the p3/pMN region (A vs B; arrow). This up-regulation coincides with the accumulation of SHH protein in the p3 domain (C vs D; brackets), up-regulation of Sulf1 and down-regulation of Sox1^{-GFPn}, respectively (E, F).

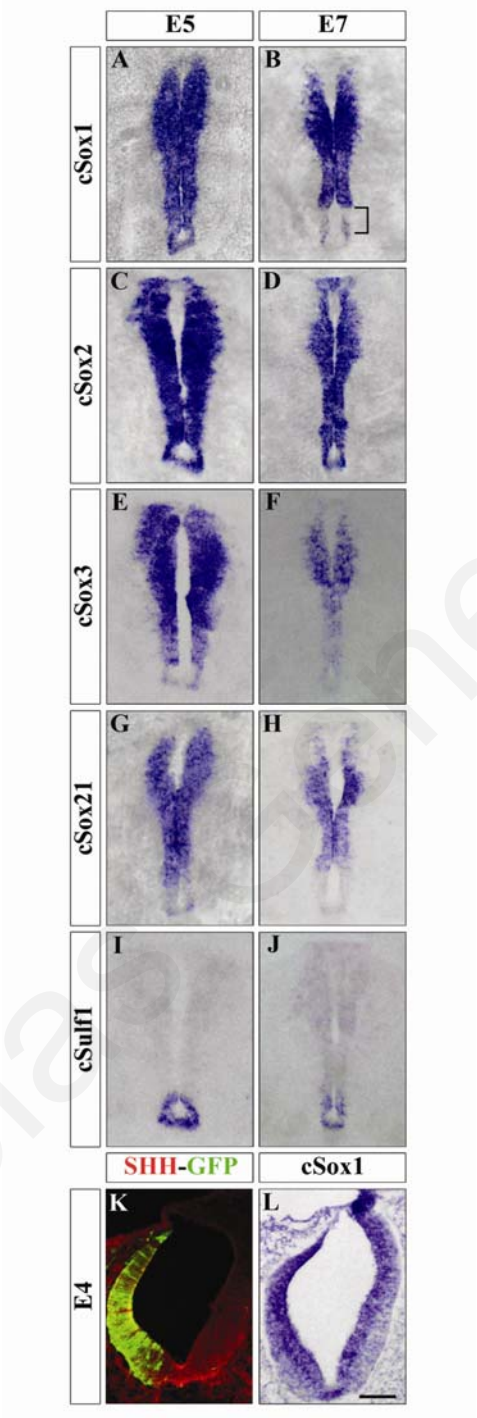

Figure 12: Shh signalling is not responsible for down-regulating Sox1 in the p3 domain in the chick embryo

Comparative expression of all SoxB1 (and Sox21) genes during the neuron-glial switch in the chick spinal cord. Note that cSox1 and cSox21 are specifically repressed in the ventral-most spinal cord between E.5 and E.7 (A,B,G,H) while cSox2 does not change (C,D) and cSox3 is widely reduced (E,F). Sulf1 expands in the p3 area (I, J). Brackets in B indicate the p3 domain. Electroporation of Shh-expressing plasmid does not repress cSox1 expression in the VZ (K,L).

2.3.3 Sox1 expression in the ventral Spinal Cord is regulated by Pax6 and Nkx2.2 during gliogenesis

The foregoing analysis suggested that SHH may not be directly responsible for regulating Sox1 expression in the vSC, raising the question which other factors are responsible for regulating Sox1 expression in such a dynamic manner. I reasoned that NKX2.2 and PAX6 might be implicated in this process since they are primarily responsible for regulating the boundaries of the p3 and pMN domains (Jessell, 2000; Lei et al., 2006). Also the fact that Sox1 was repressed from the p3 domain specifically during the N/G switch was an additional reason for testing this possibility. In order to address this question, I crossed the Sox1^{-GFPn} allele in mice carrying either the naturally occurring mutation on the Pax6 gene, know as *small eye* (*Sey*) (Hill et al., 1991) or mice carrying a targeted deletion of the Nkx2.2 gene (Sussel et al., 1998). These mice were obtained from Dr Anastasia Stoykova and Dr Johan Ericson, respectively.

In embryos lacking PAX6 function analysed at e10.5 the expression of Sox1^{-GFPn} was completely abolished, except in a few scattered cells in the vSC (Fig. 14A, B). These cells have been extensively analysed by another PhD candidate and represent the progenitors of some V2 interneurons (Panayi et al., submitted). However, at e12.5 the expression of Sox1^{-GFPn} reappeared only in the dorsal ventricular zone and was still absent in the vSC (Fig. 14C, D). By e12.5 the expression in the V2 progenitors was extinguished as neurogenesis finishes in this domain (Ogawa et al., 2005). It is well known that in Pax6 mutant embryos Nkx2.2 expression expands dorsally (Briscoe at al., 1999). However, the expansion of Nkx2.2 expression did not coincide with the area where Sox1^{-GFPn} expression was lost (Fig. 14C, D), suggesting that it is the loss of PAX6 and not the expansion of NKX2.2 that caused the failure of Sox1^{-GFPn} to be expressed in the vSC.
The changes in the expression of Sox1 in Pax6-mutant embryos were confirmed by in situ hybridisation as well.

I then turned to analysing Sox1^{-GFPn} expression in Nkx2.2 mutant embryos. It has been widely predicted that in these embryos Pax6 should expand ventrally and occupy

the p3 domain (Nkx2.2^{+}; Briscoe et al., 1999; Muhr et al., 2001; Hochstim et al., 2008). Contrary to these predictions, Pax6 never expanded ventrally at e12.5 (Fig. 13E, F). Despite the fact the Sox1^{-GFPn} depended on PAX6 function (Fig. 13B, D), in the absence of NKX2.2 function Sox^{-GFPn} expression did not follow Pax6 expression and expanded ventrally but not in the entire prospective p3 domain (Fig. 13G, H). Olig2 expression expanded in the prospective p3 domain as reported (Novitch et al., 2003) since NKX2.2 represses Olig2 (Fig. 13G, H). Very importantly, Sox1^{-GFPn} and Olig2 expression maintained the same dynamic expression relationship as shown in Figure 10F (page 47). That is in wt embryos Sox1^{-GFPn} is expressed mostly in the dorsal pMN domain while in Nkx2.2-mutant embryos, when the pMN domain becomes larger, Sox1^{-GFPn} is expressed in more ventrally located cells. These data clearly suggest that PAX6 regulates the expression of Sox1 in the ventral-to-intermediate spinal cord (pMN-p0) in an NKX2.2-independent manner while NKX2.2 prevents Sox1 expression from the entire pMN domain specifically during the N/G switch in a PAX6-independent manner. Finally, they suggest that NKX2.2 does not repress Pax6 in the p3 domain as widely predicted thus allowing these two genetic models to be used to assign cell-autonomous functions to either factor because the expression changes observed in the respective null backgrounds cannot be explained by cross-repressive interactions. The functional implications of these observations will be further elaborated with more data.

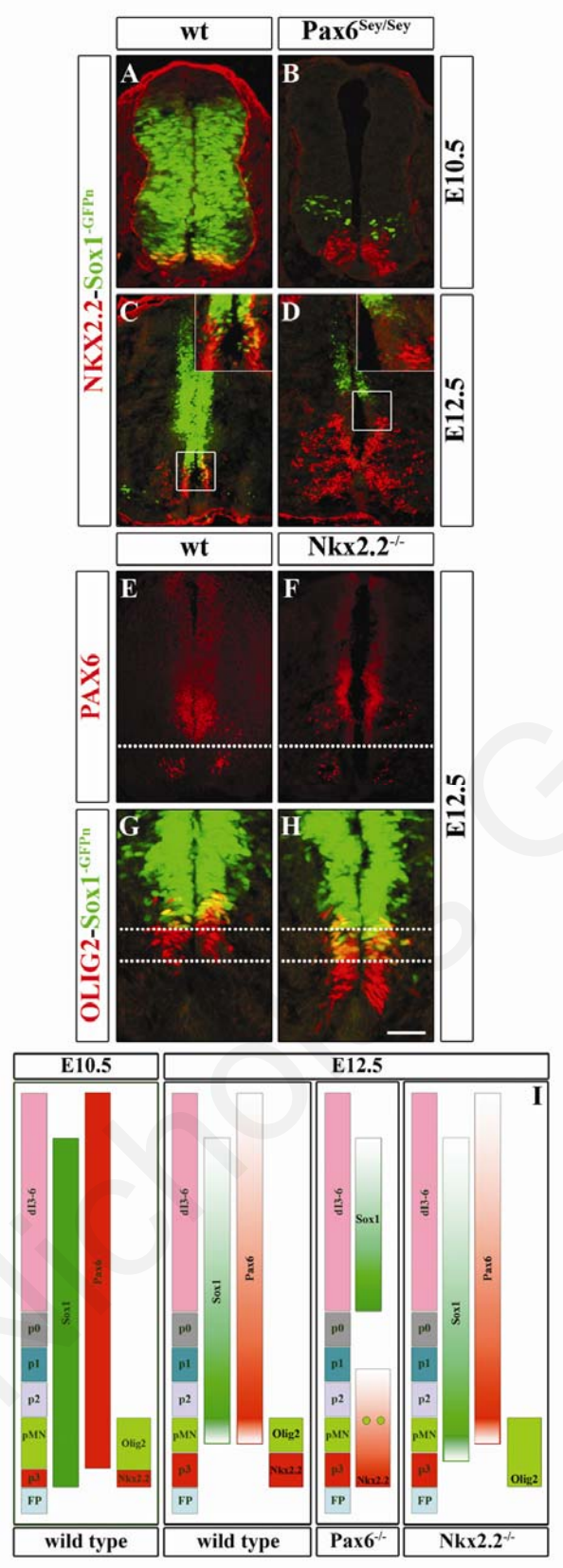

Figure 13: Genetic requirement for PAX6 and NKX2.2 to regulate the expression of Sox1^{-GFPn}

(A,B) Shows the expression of Sox1^{-GFPn} and NKX2.2 in wt and Pax6-mutant embryos at e10.5. Note that in mutant embryos Nkx2.2 expands mildly while Sox1^{-GFPn} expression is lost in the entire spinal cord except is some cells in the vSC. These cells represent V2 interneuron progenitors and express Mash1 (Panayi et al., under review). (C, D) Shows the expression of Sox1^{-GFPn} and NKX2.2 in wt and Pax6-mutant embryos at e12.5. Note that a dorsal domain of Sox1^{-GFPn} expression reappears but in the vSC NKX2.2 does not expand to the domain that still does not express Sox1^{-GFPn}, suggesting that it is the loss of PAX6 and not the expansion of NKX2.2 represses the expression of Sox1^{-GFPn}. (E, F) Shows the expression of PAX6 in wt and Nkx2.2-mutant embryos that does not change. (G, H). Shows the expression of OLIG2 and Sox1^{-GFPn} in wt and Nkx2.2-mutant embryos. Note that OLIG2 expands ventrally and reaches the FP whereas Sox1^{-GFPn} becomes up-regulated in more OLIG2-expressing cells and expands ventrally as well but does not reach all the p3 domain. (I) Shows diagrammatically all the gene expression changes between wt at e10.5 and e12.5 and the gene expression changes at e12.5 in Pax6- and Nkx2.2-mutant embryos. Fading colours representing diminishing expression.

2.3.4 NKX2.2 and PAX6 regulate Slit1 and Sulf1 expression in the ventral Spinal Cord during gliogenesis

While analysing the expression of Sox1 in these two genetic backgrounds, I became aware of the study by Hochstim et al (2008) showing that PAX6 regulates Slit1 expression in white matter ASs in the vSC. Because I have established that Pax6 is required to regulate Sox1 expression in the pMN-p0 domain, I tested if SOX1, PAX6 and NKX2.2 regulate Slit1 expression in the VZ in an analogous manner. At the same time I analyzed the expression of Sulfatase 1 (Sulf1) that show temporal expression changes precisely at the same time point of spinal cord development as the changes noted for Sox1 (Fig. 11 page 49). To achieve a comparative expression pattern map, I used in situ hybridization on serial sections from the same embryo in order to map the expression boundaries of Sulf1/Slit1 relative to known molecular markers, like Pax3 that labels progenitor domains dorsal to the p0 domain (Fig. 14).

In both Nkx2.2$^{-/-}$ and Pax6$^{Sey/Sey}$ embryos analyzed at e10.5, Sulf1 and Slit1 were expressed in the FP like control embryos (not shown), suggesting that neither PAX6 nor NKX2.2 had any influence on their FP expression. However, at e12.5 in Pax6$^{Sey/Sey}$ embryos the expression of both Slit1 and Sulf1 was up-regulated in the VZ and the dorsal limit of their expression reached the ventral limit of Pax3 expression (Fig. 14A-D). In Nkx2.2$^{-/-}$ embryos both Slit1 and Sulf1 expression failed to expand in the VZ and were only expressed in the FP (Fig. 15E, F). Again I reconfirmed in this experiment that Pax6 expression did not expand in the prospective p3 domain of Nkx2.2$^{-/-}$ embryos (Fig. 14G, H). At this stage of development Nkx2.9 which is also expressed in the p3 domain at e10.5 and has been proposed by Briscoe et al., (1999) to repress Pax6 as well, is switched off (not shown).

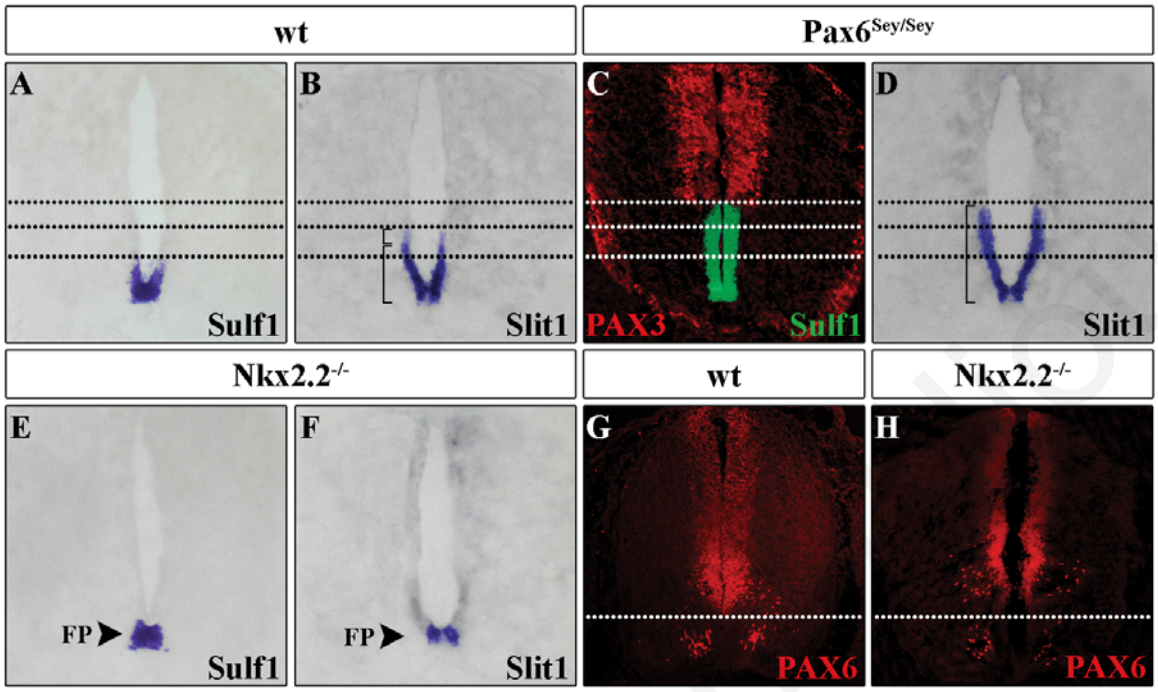

Figure 14: Domain-specific requirement for NKX2.2 and PAX6 to regulate Sulf1 and Slit1 expression

Note that the expression of Slit1 extends beyond that of Sulf1 (A, B) whereas both genes are expressed in the same manner in $Pax6^{Sey/Sey}$ embryos (C, D; brackets). The precise dorsal limit of Slit1 in wt embryos has been reported to be the Nkx6.1 expression boundary (Hochstim et al., 2008). Note the diminishing Slit1 expression in the domain dorsal to the Sulf1 boundary (brackets in B). In $Nkx2.2^{-/-}$ embryos both Slit1/Sulf1 are only expressed in the floor plate (FP; E, F), whereas Pax6 expression does not expand in the p3 domain (G, H). Dotted lines indicate the limits of expression boundaries. In panels G and H the dotted lines indicate the prospective p3 domain.

These data suggest that under physiological conditions and in a stage- and region-specific manner, NKX2.2 controls the induction of Slit1 and Sulf1 expression in the vSC in a PAX6-independent manner. Conversely, loss of PAX6 led to co-extensive up-regulation of both Slit1/Sulf1 in the entire vSC despite the initial differences in their expression limits (Fig. 14A, B).

I then tested whether the ventral expansion of Sox1 in Nkx2.2$^{-/-}$ embryos was the cause of the repression of Sulf1/Slit1 from the p3 domain. Analysis of Sox1$^{-/-}$ or Nkx2.2$^{-/-}$ /Sox1$^{-/-}$ double mutant embryos established that it is the absence of NKX2.2 and not the upregulation of SOX1 in the some p3 domain progenitors that repressed Slit1/Sulf1 expression (not shown). Nevertheless, in embryos lacking SOX1, I noted a minor dorsal shift in the expression limit of Sulf1 leading to merging of the p3/pMN boundary but SOX1 function was otherwise not mediating the regulation of Slit1/Sluf1 expression by either PAX6 or NKX2.2 (Fig. 15).

All the expression pattern changes described so far in different genetic models are shown in Figure 16. Altogether these data suggest that the VZ of the vSC during the N/G fate switch is divided into progenitor domains that can be visualized by changes in the expression of Sox1, Slit1 and Sulf1 and this expression pattern is regulated by NKX2.2 and PAX6. Importantly, the function of NKX2.2 and PAX6 in regulating the expression of each gene seems to be cell-autonomous, since boundary re-specification in the respective null backgrounds cannot explain all the changes in the expression patterns observed. For instance PAX6 has been proposed to repress Slit1 expression in the VZ (Hochstim et al., 2008) but I show here that in the absence of NKX2.2, Slit1 expression is lost from the p3 domain but Pax6 expression does not expand in this domain. Equally, Sox1 expands partly in the p3 domain of Nkx2.2$^{-/-}$ embryos and does not follow Pax6 expression. Conversely, in Pax6$^{-/-}$ embryos, Nkx2.2 expands modestly but does not reach the p0 domain. Slit1 and Sulf1 do not follow the dorsal limit of Nkx2.2 expansion in Pax6 mutant embryos. Similarly Sox1 is lost in the vSC up to the limit of the p0 domain,

where Nkx2.2 never reaches. This domain where Sox1 expression is lost coincides with the Sox1^{high} domain described in Figure 10.

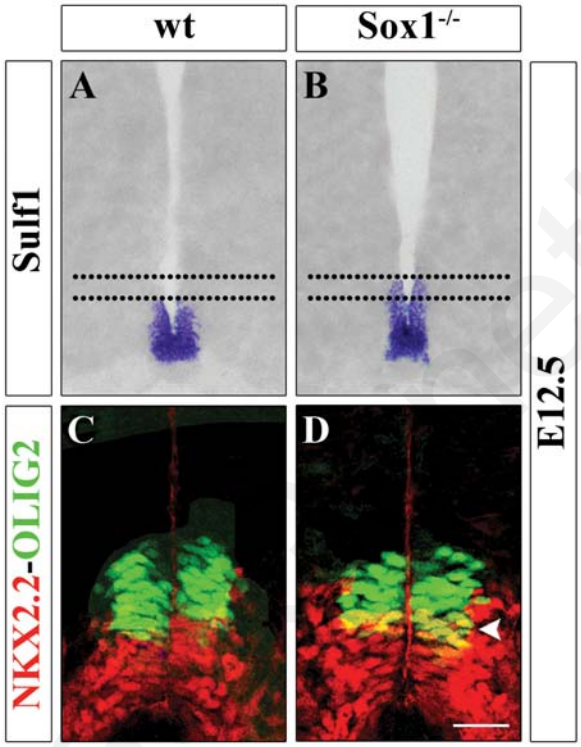

Figure 15: SOX1 is required to maintain the precise spatial expression of Sulf1

Loss of Sox1 leads to minor expansion of Sulf1 expression beyond its normal limit (A, B) and merging of the p3/pMN domain (C, D; arrow head). The merging of the p3/pMN domains occurs before OLPs begin to migrate but I considered this inconsequential to the accelerated production of OLs (see later) since the co-expression of Olig2/Nkx2.2 has no consequences on OL specification (Qi et al., 2001).

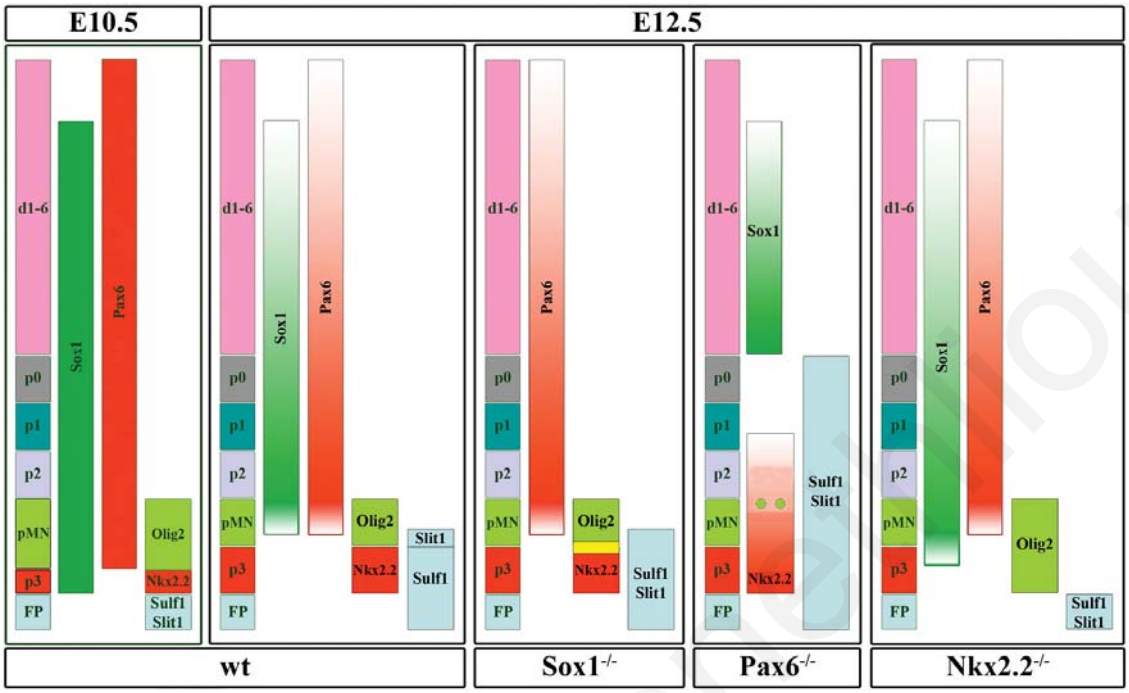

Figure 16: Diagrammatic representation of the temporal and spatial changes in the expression of the markers analyzed

Progenitor domains are represented with labeled colored boxes. Gene expression domains are shown as continuous bars. Fading colour shows reduced expression. The dorsal limit for Sulf1 stops in the vpMN domain (where some Olig2/Nkx2.2 overlap is noted) at e12.5 in Wt embryos. Note that Slit1 expression is diminishing as it reaches the p2 domain. The deregulation of Slit1/Sulf1 expression was monitored in serial sections using Pax3 and Dbx1 expression for comparison. Note the merging of p3/pMN domains in $Sox1^{-/-}$ embryos and the co-expression of Nkx2.2/Olig2. In wt embryos the dorsal limit of Sulf1 expression at e12.5 was strong in the p3 domain and negligible in the vpMN domain.

Finally, I asked if the requirement for PAX6 function to regulate Sox1 expression was specific only to this gene or affected other SOX genes, particularly from the SOXB group. Neither loss of PAX6 or SOX1 had any effect on the expression of other SOX genes, some of which have also been implicated in gliogenesis (Figure 17). This distinct genetic regulation of Sox1 expression by PAX6 and NKX2.2 revealed a regulatory mechanism that regulates Sox1 expression that was never suspected before for a gene that is expressed in most neural progenitors and suggests a specific function of this gene during the N/G switch.

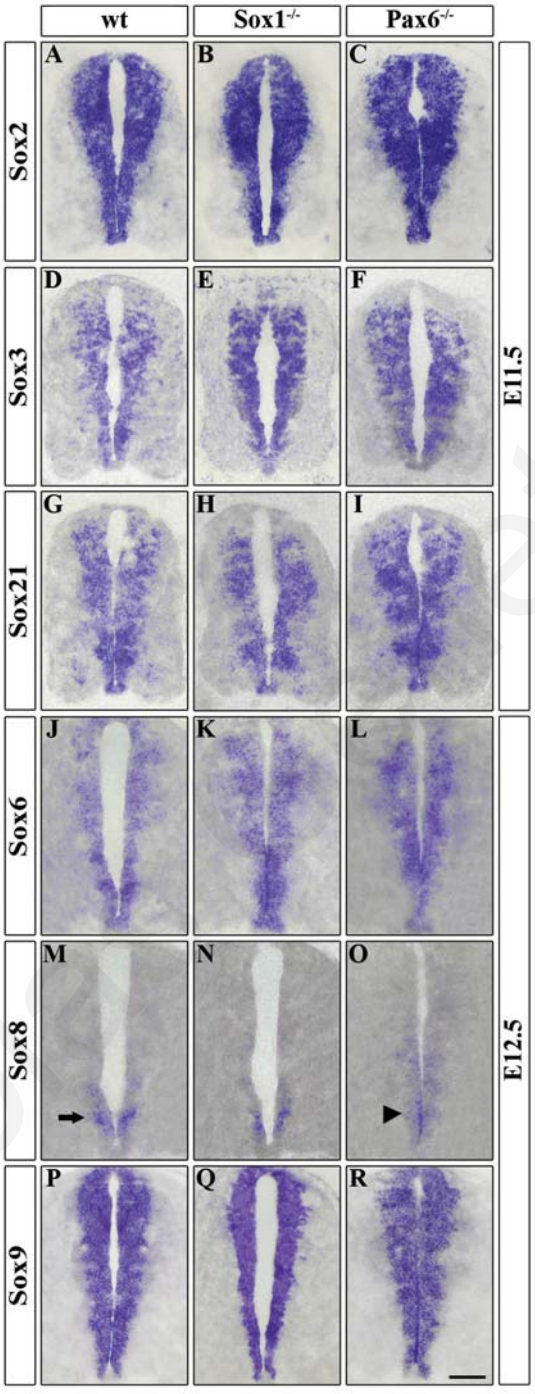

Figure 17: Expression of Sox2/3/6/8/9/21 in Sox1 and Pax6 null embryos during the N/G switch

All Sox genes tested were not affected in either genetic background (A-L, P-R) except Sox8 (M-O) which normally shows strong expression in the pMN region (arrow) that is lost in Pax6-mutant embryos, most likely due to the expansion of Nkx2.2 and re-specification of the pMN domain.

2.3.5 Implications of the domain-specific expression of Slit1 and Sulf1 PAX6 during the N/G switch

The foregoing analysis revealed two important points in relation to PAX6 and NKX2.2 function that merited further investigation:

1. They suggest that PAX6 regulates the domain specific of expression of Sulf1 and Slit1 and
2. That NKX2.2 regulated the induction of Slit1/Sulf1 expression in the p3 domain VZ.

Why are these observations important? As discussed in the introduction (page 31; first paragraph) Sulf1 has been proposed to regulate the local concentration of SHH in the p3 domain where it is physiologically expressed. The deregulation of Sulf1 expression in $Pax6^{-/-}$ embryos beyond the p3 domain raised the possibility that it might cause an increase in the level of Shh signalling in domains dorsal to the p3 domain leading to deregulation of neural patterning.

In relation to Slit1, as discussed in the introduction (page 25), the expression of this gene in the VZ of the vSC is believed to correlate with specification of the two most ventral AS subtypes recently identified, namely VA2/3, that continue to express Slit1 in the respective white matter (Hochstim et al., 2008). I thus investigated if loss of PAX6 had an effect on Shh signalling at e12.5 embryos and if loss of NKX2.2 had an effect on positional specification of ASs at e18.5 embryos.

In order to analyse Shh response in the VZ of the spinal cord, I used Patched (Ptch1) expression that gives a read-out of the response of neural progenitors to SHH (Danesin et al., 2006). At e10.5 Ptch1 expression did not change in either the wild type (wt) or mutant embryos (Fig. 18 A, B) consistent with the fact the Sulf1 expression does not expand beyond the FP at this stage (not shown). However at e12.5, I noted a dramatic

enhancement of Ptch1 in the entire vSC (Compare Fig. 18C, D), consistent with the observed up-regulation of Sulf1 expression in the vSC (Fig. 11F; page 49). However, enhanced accumulation of SHH protein could not be detected directly, presumably due to the limitation of immunohistochemical detection of SHH protein in domains away from the source of Shh gene expression, which continued to be the FP at all stages of spinal cord development.

To examine if the enhanced Ptch1 expression had consequences on Shh signalling other than up-regulation of Ptch1, I compared the expression pattern of four Class I (Shh-repressed) and three Class II (Shh-induced) genes between e10.5 and e12.5 in *Sey*/*Sey* embryos. As expected, Nkx2.2 expression expanded dorsally at both e10.5 and e12.5 stages (Fig. 19E-H) (Briscoe et al., 1999). At e12.5, I noted peri-ventricular staining of $Nkx2.2^{+}$ cells dorsal to the presumptive pMN domain (Fig. 18G, H asterisks). These cells that expressed Nkx2.2 outside the VZ, have been identified as $O4^{+}$ OLPs (Sugimori et al., 2007), but it is doubtful if this assignment is correct (Prof. William Richardson, unpublished data). As expected Olig2 expression that is normally repressed by NKX2.2 (Novitch et al., 2001) was almost extinguished both at e10.5 and e12.5 (Fig. 18F, H). Some $Olig2^{+}$ cells, that escaped Nkx2.2-repression were found at e13.5 (not shown; Sun et al., 1998, Sugimori et al., 2007). Nkx6.1 expression did not change at either stage (Fig. 18I-L). The analysis of the expression of Class I genes (Shh-repressed) was more informative. I first noted a dorsal shift of the ventral limit of Pax6 mRNA expression that was not apparent at e10.5 despite the expansion of Nkx2.2 at both stages (Fig. 18M-P). Furthermore, Irx3 expression was reduced despite the fact that OLIG2 expression, the repressor of Irx3 (Muhr et al., 2001), was present in only very few cells (Fig. 18Q-T). Finally, expression of Dbx1 and Dbx2 was reduced, with Dbx2 being more affected than Dbx1 (Fig. 18U-Z2). These data suggest that the stage-specific expression changes of several Class I genes in $Pax6^{-Sey/Sey}$ embryos are likely to be due to the ventralization of p3-p0 progenitors brought about by expansion of Sulf1 expression and consequent elevation of SHH activity. These gene expression changes argue for a direct effect of higher levels of SHH activity on neural progenitors rather than by changes in the expression pattern of homedomain Class II factors with reported cross-repressive

interactions with Class I factors. For instance loss of Olig2 expression was expected to lead to ventral expansion of Irx3 like that observed in Olig2 mutant embryos (Zhou and Anderson, 2002). Instead Irx3 expression was reduced along with Pax6 expression that was only reduced at e12.5 and not at e10.5 despite the fact that Nkx2.2 expands dorsally at both stages. The same applies for Dbx1/2 whose expression is reduced only at e12.5.

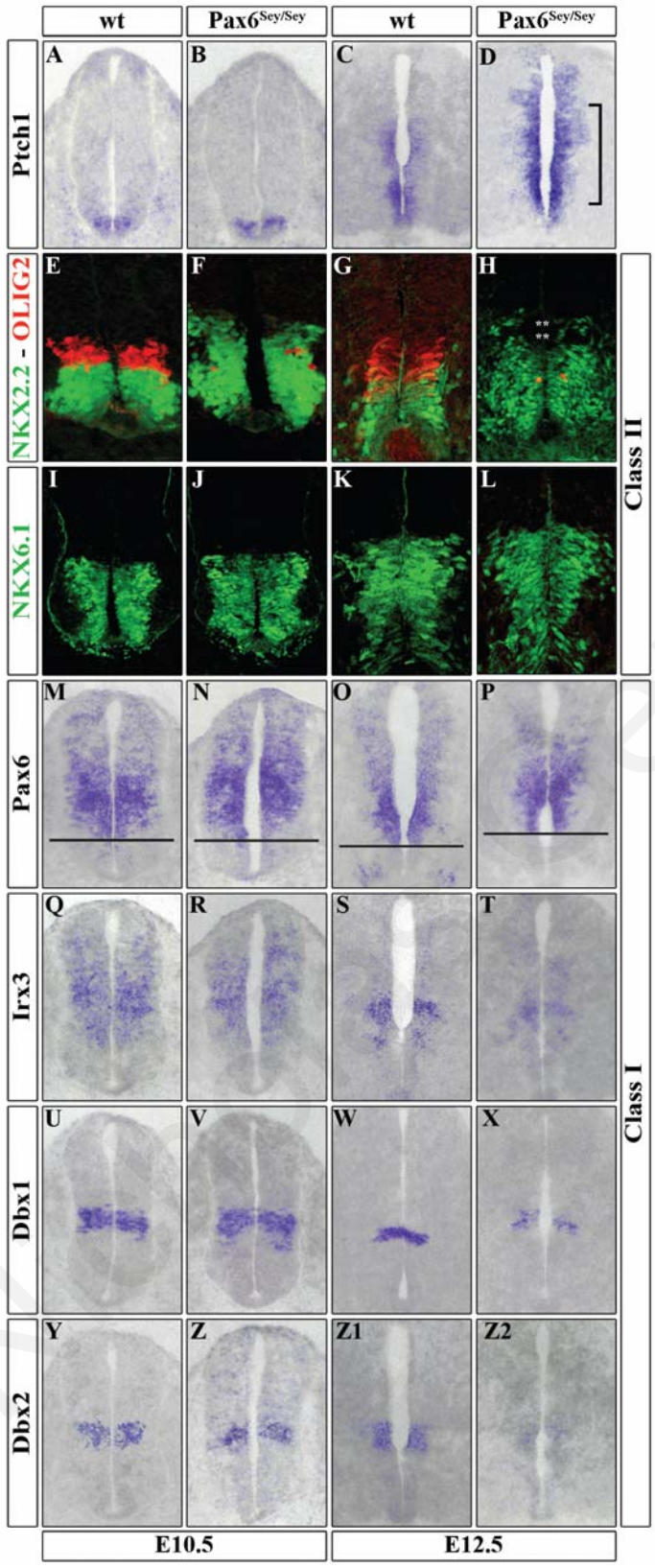

Figure 18: Stage-specific changes in neural patterning in Pax6$^{Sey/Sey}$ embryos

Comparative expression analysis of Class I and Class II genes in Pax6$^{Sey/Sey}$ embryonic spinal cords between e10.5 and e12.5. Note the strong up-regulation of Ptch1 in Pax6$^{Sey/Sey}$ in the entire VZ specifically at e12.5. At e12.5 Nkx2.2^{+} cells dorsal to the pMN are located in peri-ventricular location (asterisks represent the p2-VZ). Olig2 expression is almost lost at both stages (E-H) and Nkx6.1 was not affected (I-L). Note that at e10.5 Pax6 expression does not change while at e12.5 it shifts slightly dorsally (M-P). Irx3 expression is attenuated only at e12.5 (S,T) and Dbx2 expression is almost lost at e12.5 (Z1, Z2) and Dbx1 expression is weaker only at e12.5 (W, X). Bars indicate boundary shifts. Brackets in D indicate the expanded Ptch1 expression.

I then turned to analysing AS sub-type specification in wt, Pax6-, Sox1- and Nkx2.2-mutant embryos at e18.5, the stage that white matter ASs begin to express mature markers like GFAP and region-specific markers like Slit1, expressed by VA2/3 AS subtypes (Hochstim et al., 2008). The expression of Slit1 and GFAP was normal in Sox1-mutant embryos (Fig. 19B, F) consistent with fact that no changes in Slit1 expression were noted at e12.5 in the VZ of these embryos.

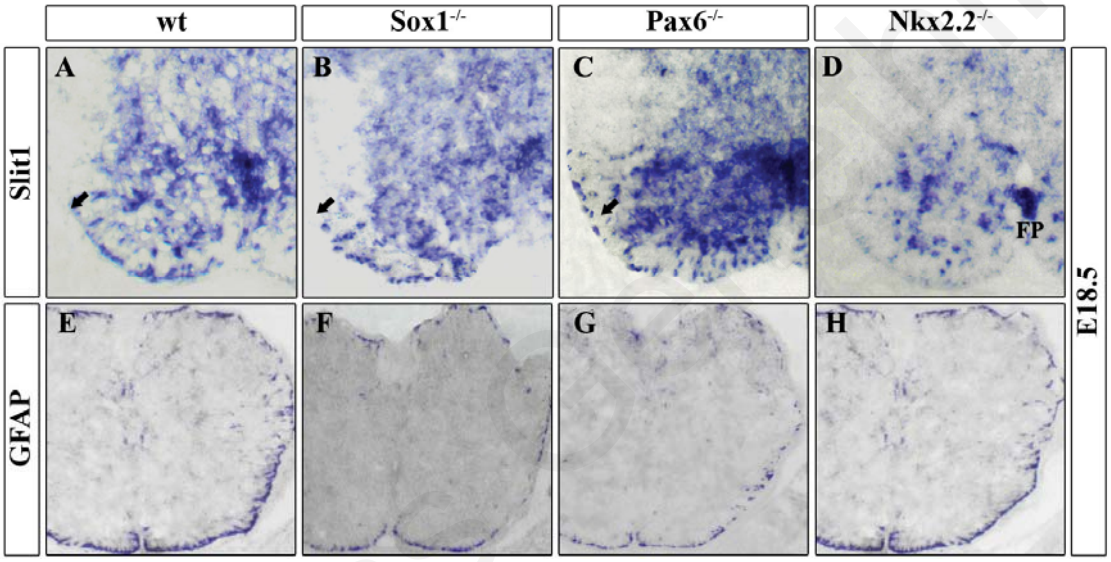

Figure 19: AS development in Sox1-, Pax6- and Nkx2.2-mutant spinal cords at e18.5

The development of mature AS in the spinal cord was normal in Sox1-mutant embryos (B, F) as assessed by Slit1, that marks VA2/3 AS subtypes and GFAP that labels all ASs in the WM. In *Sey/Sey* embryos the Slit1-expression domain expanded in the WM but GFAP expression was normal as reported (Hochstim et al., 2008). Note the dramatic loss of Slit1 expression in the WM in Nkx2.2-mutant embryos (D) but no changes in GFAP expression (H). Arrows depicts the VA2/VA3 boundary according to Hochstim et al (2008).

As reported by Hochstim et al (2008), the Slit1 expression domain in the WM expanded in Pax6-mutant embryos (Fig. 19C, G), consistent with the dorsal expansion of Slit1 in the VZ of *Sey/Sey* embryos as shown in Fig. 14M (page 55). In line with the prediction that Slit1 expression in the VZ, correlated with Slit1 expression in WM ASs,

in Nkx2.2- mutant embryos Slit1 expression was dramatically reduced in the WM (Fig. 19D), but GFAP expression was normal (Fig. 19H; Qi et al., 2001). Hence the reduced expression of Slit1 in the VZ of Nkx2.2-mutant embryos (Fig. 14F) reflected on the expression of the same gene in the WM.

Collectively, these data suggest that the VZ expression of Slit1 directly predicts the regional specification of AS subtypes. Importantly, we show that in the absence of Nkx2.2 Slit1 expression is greatly reduced in the WM, suggesting that NKX2.2 is the primary determinant of VA2/3 AS subtypes ($Slit1^{+}$) and not Nkx6.1 as suggested. It should be emphasised that Briscoe and colleagues (1999) have shown that Nkx6.1 is not affected in Nkx2.2 mutant embryos thus even in the presence of NKX6.1, NKX2.2 regulates AS subtype identity in VA2/3 AS subtypes in a PAX6-independent and not in a PAX6- dependent manner as suggested by Hochstim et al (2008). This part of my work has now been reported (Genethliou et al., 2009).

2.4 Sox1-expressing progenitors contribute to a subset of ventral glial cells

The foregoing section has dealt with three important issues.

1. First, I described the dynamic expression of Sox1 in the vSC.
2. Second, I showed that Sox1 is regulated by two patterning factors namely PAX6 and NKX2.2 in a spatially distinct manner.
3. Third, I showed that the latter two factors not only regulate Sox1 expression but also regulate Slit1 and Sulf1 expression in the VZ. Despite the fact SOX1 does not mediate these regulatory activities, I have investigated further the functional implications of deregulating Sulf1 and Slit1 expression and have shown that these have direct and stage-specific implications on neural patterning and AS subtype identity, respectively.

I then turned to a more detailed analysis on the function of SOX1 during glial specification. The dynamic expression of Sox1^{-GFPn} in the gliogenic neuroepithelium, particularly the fact that its expression was very weak in the ventral pMN (Fig. 11F; page 47), prompted me to investigate the relationship between Sox1-expressing progenitors and glial lineages, particularly OLs. Despite the fact the pMN-domain is undoubtedly the main source of ventral OLs, both Sox1 and Pax6 expression showed weak expression in this domain particularly the ventral half. I needed, therefore, to establish if these residual Sox1-expressing cells could, at any time in development, give rise to OLs or they simply represented cells that never contribute to the OL lineage. To address this question I took advantage of the existence of a BAC transgenic line expressing Tamoxifen (TM)-inducible Cre recombinase (*CreERT2*) from Sox1 regulatory sequences developed by Prof. William Richardson and Dr Nikoletta Kessaris, at the Wolfson Institute of Biomedical Research, University College London. This line allowed temporal activation of Cre activity at the appropriate developmental window and was an excellent tool to specifically answer this question. The following data were obtained by me after spending five weeks in the laboratory of Prof. Richardson.

The first task was to confirm that the expression boundaries of the Sox1$^{-CreERT2}$ allele, revealed by TM induction at e10.5 and analysis at e12.5, precisely matched those of the Sox1^{-GFPn} allele in relation to Nkx2.2 and Olig2 expression in that YFP was detected mostly in the dorsal pMN and not in the ventral pMN (Fig. 20A, B). At this induction window, dorsal spinal cord Sox1^{+} progenitors contributed primarily to neurons (Fig. 20C), consistent with the ongoing neurogenesis in this region (Ogawa et al., 2005). Induction at e10.5 and e11.5 and harvesting at e14.5 and e15.5, respectively, identified several Olig2^{+} and Sox10^{+} OLPs originating from the Sox1-lineage primarily located in the ventral GM (Fig. 20E,F,H,I). These cells are most-likely pMN-derived OLs since dorsal OLs are specified at a later stage (e15.5; Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). Importantly, at no stage have I observed migrating OLPs or maturing OLs in the WM to express Sox1, monitored by comparing Sox1^{-GFPn} and Olig2 expression (Fig. 20D,G,M,N).

Induction at e14.5 and analysis at e17.5, also failed to show any OLPs originating from the Sox1-lineage (Fig. 20K). By this time most Olig2 expression in the pMN is switched off and the Sox1^{-GFPn} allele is also switched off from the dorsal Pax3^{+} oligogenic domain (Cai et al., 2005; Vallstedt et al., 2005; Fig. 20J), suggesting that the down-regulation of Sox1^{-GFPn} and also that of Sox1$^{-CreERT2}$ allele, is likely to coincide with the production of dorsal OLPs. By e15.5, the VZ expression of Sox1^{-GFPn} coincided with an S100β expression domain (Fig. 20L, O, P), proposed to define p2 progenitors (Stolt et al., 2003).

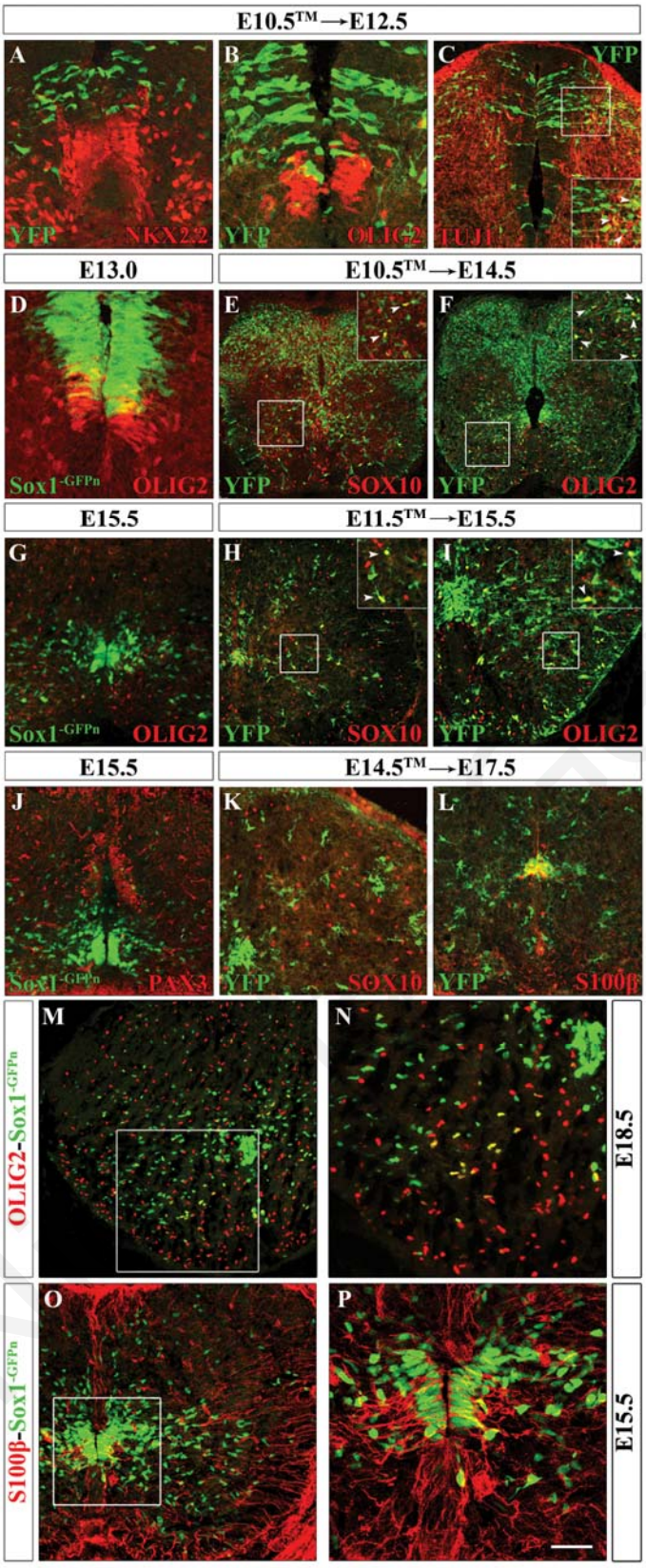

Figure 20: Stage-specific fate mapping of the Sox1-lineage

Analysis of YFP staining of Sox1$^{-CreERT2}$; R26-YFP embryos. The stage of Tamoxifen (TM) administration is denoted as E.X^{TM} followed by the stage harvested. TM induction at e10.5 and harvesting at e11.5 revealed the temporal and spatial down-regulation of the transgene in the p3 domain and vpMN domain (A-B) and that dorsal Sox1 progenitors contributed to neurons (C). Note that at the stages indicated Sox1^{-GFPn} is not expressed in the OL lineage (Olig2^{+}; D, G, M, N) but Sox1-progenitors contribute to ventral OL (E, F, H, I). Very few Olig2^{+}/ Sox1^{-GFPn+} positive cells were consistently seen in some sections at e18.5 close to the pMN domain (N); The down-regulation of Sox1^{-GFPn} in the Pax3 domain (J) is consistent with the failure of Sox1-progenitors to contribute to dorsal OL (K) by which stage Sox1-progenitors are confined to an S100β expressing domain (L,O,P). Arrowheads indicated cells co-expressing YFP and the marker studied. YFP expression is only seen in some cells because TM induction dos not activate Cre in all Sox1$^{-CreERT2}$ expressing cells.

Recently Hochstim et al (2008) showed that from the $Pax6^{+}$ expression domain in the VZ of the developing spinal cord, migrating cells retained Pax6 expression and contributed to VA1 and VA2 AS subtypes. Since the expression of $Sox1^{-GFPn}$ in the VZ precisely matched that of Pax6, I asked if the migrating $Sox1^{-GFPn+}$ cells retained Pax6 expression as well. Consistent with this prediction, I noted a continuum of $Pax6^{+}/Sox1^{GFPn+}$ cells leaving the VZ, migrating in the GM and settling in the WM (Fig. 21A-F). The expression of Pax6 was lower in the GM than in WM cells. White matter $Pax6^{+}$ ASs transiently retained $Sox1^{-GFPn}$ expression that was rapidly down regulated upon terminal differentiation (Fig. 21C-F), consistent with a previous report showing that Sox1 is not expressed in mature $GFAP^{+}$ ASs (Kan et al., 2007).

The data presented in Figures 20 and 21 clearly suggest that at the stages analysed the Sox1-lineage contributes only to ventral and not to dorsal OLPs and that Sox1 is expressed in a subset of AS progenitors expressing Pax6, most likely the VA1/2 subtype. All dorsal progenitors switch off Sox1 prior to dorsal OLP specification while ventral OLPs switch off Sox1 once they leave the VZ. From e15.5 onwards Sox1 is expressed only in a sub-domain of the VZ that also expresses S-100β, a domain that has been previously determined to be the p2 domain (Stolt et al., 2003). This expression pattern is unique to Sox1 as no other transcription factor has ever been reported to have such an expression pattern during the specification and migration of glial lineages.

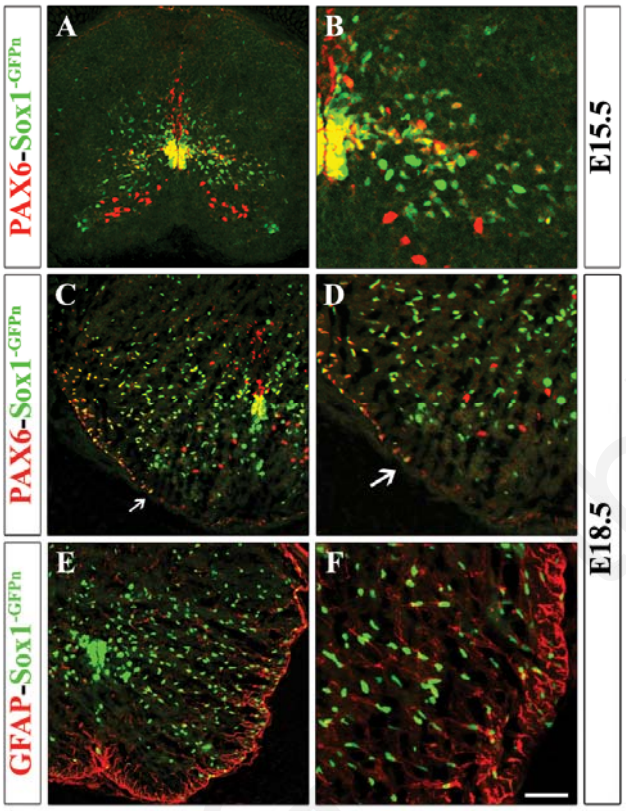

Figure 21: Expression of Sox1 in VA1/2 AS progenitors

$Sox1^{-GFPn}$ progenitors migrating from the VZ express low level of PAX6 (A,B) and when these cells settle in the white matter PAX6 expression is stronger and some transiently retain $Sox1^{-GFPn}$ (C,D). Some $GFAP^{+}$ ASs also express transiently $Sox1^{-GFPn}$ (E, F). Arrows define the VA1/2 boundary in the WM (Hochstim et al., 2008).

2.5 Accelerated oligodendrocyte specification in the vSC of Sox1-deficient embryos

In the preceding section I have shown that Sox1-expressing cells in the pMN generate OLs but when these OLs are specified they immediately switch off Sox1. This observation suggested that SOX1 may inhibit OL specification. Hence I began to analyse OL specification in embryos lacking SOX1.

In the pMN domain both uncommitted neural progenitors and committed OLPs express Olig2 while OLPs express Sox10 as well. I thus performed quantitative analysis at the forelimb level of pre-migrating, pMN-derived OLPs expressing $Sox10^+$ and migrating $Sox10^+$ and $PDGFR\alpha^+$-expressing cells between e12.5 and e14.5. At the same time, I quantified $Mash1^+$ cells in the pMN, since Mash1 has been shown to be expressed in the pMN by OLPs once they get specified (Sugimori et al., 2007). This analysis showed that in the mutant pMN domain, there was a significantly higher number of $MASH1^+$ and $SOX10^+$ cells compared to controls (Fig. 22A-D; O). A similar result was obtained for migrating $Sox10^+$ and $PDGFR\alpha^+$ OLPs at e13.5 and e14.5 (Fig. 22E-J, O; data not shown). Importantly at e13.5, I consistently observed that $Olig2^+$ progenitors in the pMN domain were rapidly being depleted in mutant embryos and the number of migratory $Olig2^+$ progenitors was also significantly greater (Fig. 22K, L; data not shown). While OLP numbers increased at early stages of development, the expression of MBP^+ at e15.5 and e18.5 was normal suggesting that loss of Sox1 did not cause a premature expression of mature OL markers (Fig. 22M, N).

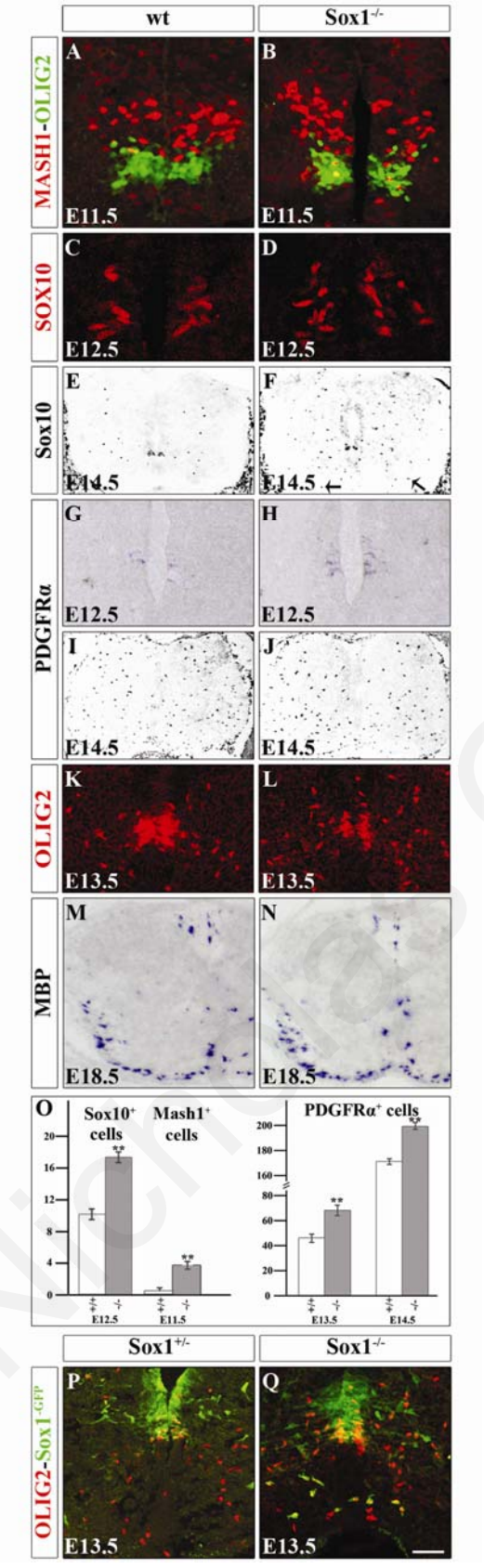

Figure 22: Oligodendrocyte development in Sox1 null embryos

Premigratory MASH1^{+} and SOX10^{+} OLPs (A-D) and post-migratory (E, F) OLP stained with anti-SOX10 and anti-MASH1 antibody (A, B; C, D) and *in situ* hybridization (E, F). Post-migratory Pdgfrα^{+} OLP revealed by *in situ* hybridization (G-J). Note the rapid depletion of pMN-residing Olig2^{+} progenitors in Sox1-null embryos (K, L). White matter MBP^{+} OL appear normally distributed (M,N). (O) Pre-migrating Sox10^{+} and Mash1^{+} OLs and post-migrating Pdgfrα^{+} pMN-derived OLPs show statistically significant increase in mutant spinal cords (40 sections of 3 pairs of embryos). Statistical significance is indicated above respective bars (** $P \leq 0{,}001$). (P, Q) Note that OLIG2 is expressed in several GFP^{+} mutant cells (yellow cells in Q).

The number of $HB9^+$ motor neurons was also unaffected (not shown) and I did not observe premature Olig2 or Sox10 expression in the dorsal spinal cord, suggesting that loss of SOX1 did not affect the timing of dorsal OLP specification. It is important to note that the temporal window of this analysis was carried out between e12.5 and e14.5 before any dorsal OLPs begin to be specified, thus only pMN-derived OLPs were counted.

At e13.5 in mutant spinal cords I have noted that several GFP^+ cells migrating in the ventral GM retained GFP and these cells also expressed OLIG2. I have never observed these cells in heterozygote embryos (Fig. 22P, Q). Also in the mutant cells GFP expression was eventually switched off as the cells moved well into the GM. This was not due to simply "GFP-carryover" since in no other region of the spinal cord, even in mutant embryos that have two copies of GFP, have I observed short-term labelling of migrating cells.

On the basis of these data and in view of the fact that Sox1 is not expressed in migrating OLPs, I conclude that the production of OLPs in the pMN domain is enhanced in Sox1 mutant spinal cords. This phenotype is similar to the one reported for Sox6-mutant embryos (Stolt et al., 2006). However, as shown in Fig. 17 (page 59), Sox6 expression was normal in $Sox1^{-/-}$ spinal cords. Furthermore loss of Sox6 led to premature expression of MBP, a terminal differentiation marker suggesting that the enhanced expression of Sox10 in the absence of Sox6 most likely reflected premature terminal differentiation rather than enhanced production of OLs. Therefore SOX1 most likely regulated the production of OLPs within the pMN domain, a conclusion supported by the observation that Olig2-expressing cells were rapidly being depleted from the pMN domain. Finally, the co-expression of OLIG2 and GFP in mutant cells, even at low exposure for GFP suggested that $Sox1^+$ cells in the VZ of the pMN destined to generate other cells types, most likely ASs, changed fate and reverted to an OL fate. Because of lack of specific markers for pMN-derived ASs, I cannot directly test this possibility.

2.6 Stage-specific requirement for SOX1 to maintain Hes1 expression in the vSC

The pMN domain for sometime now was thought to generate only motor neurons and OLPs but not ASs. However, stage-specific lineage tracing have suggested that Olig2-expressing progenitors give rise to AS progenitors (Masahira et al., 2006). Since Olig2 expression is extinguished from the pMN domain by e14.5 (not shown), this implies that both AS and OL progenitors must be generated from the pMN domain concurrently before Olig2 expression is lost. This is also supported, indirectly, by the observation that AS subtype identity in the vSC is determined as early as e12.5 in mice (Hochstim et al., 2008) and also by the fact that NFIA, a pro-astrocytic transcription factor (see introduction page 29) begins to be expressed at e11.5-e12.5 (Deneen et al., 2006; see below). Hence, in order to explain the molecular basis of the enhanced production of OLPs, it was necessary to address pathways that are likely to regulate the binary fate choice (OL vs AS) in the pMN domain.

I was prompted to study the expression of the all Notch effectors, Notch ligands and their receptors for three reasons. First, the Notch effector HES1, has been proposed to specifically block OL specification and promote AS specification *in vitro* (Sugimori et al., 2007; Wu et al., 2003). Second, a functional SOX1 DNA binding site has been reported to exist in the Hes1 promoter very close to an RBP-J binding site (Kan et al., 2004). Third, conditional inactivation of RBP-J, coding for a trascription factor that activates all Hes (and Hey) genes upon Notch activation, led to enhanced production of OLPs from the pMN at the expense of ASs, at the appropriate temporal window (Taylor et al., 2007). Hence attenuation of Notch signalling can enhance OL production.

The effectors analysed were Hes1, Hes5, Hey1 and Hey2, the latter two are only expressed specifically during gliogenesis (Iso et al., 2003). Hes3 is not expressed at this stage. Also I analysed the expression of Notch1-3, Dll1, Dll3 and Jagged 1. Dll4, also a Notch ligand is only expressed in the p2 domain (Peng et al., 2007) and was not tested.

I first analysed Hes1 and Hes5 expression at e10.5 before the N/G switch sets in both in $Sox1^{-/-}$ and $Pax6^{-/-}$ embryos. The analysis on $Pax6^{-/-}$ was necessary since Sox1 expression is downstream of PAX6. I found that the expression of Hes1 and Hes5 in the spinal cord at e10.5 was largely complementary (Wu et al., 2003) and was not affected by either loss of SOX1 or PAX6 (Fig. 23A-F). At e12.5 in wt embryos, Hes1 expression became very strong in the vSC while Hes5 expression begun to recede from the vSC (Fig. 23G, J). In $Sox1^{-/-}$ embryos, Hes1 expression was dramatically reduced in all regions of the SC while Hes5 was unchanged (Fig. 23H, K). Consistent with the fact that Sox1 is genetically downstream of PAX6 only in the vSC, Hes1 expression was also reduced in the vSC of Pax6-mutant embryos – but only in the region of vSC where Sox1 expression requires PAX6 function- while Hes5 expression was also slightly reduced in the vSC (Fig. 23I, L). The expression of Hey1 and Hey2 was analysed at e12.5. None of these genes was affected in $Sox1^{-/-}$ embryos whereas in Pax6-mutant embryos Hey1 expression was almost extinguished while Hey2 was not significantly affected (Fig. 23M-R). The expression of all Notch ligands and all receptors studied was normal in both genetic backgrounds (Fig. 24).

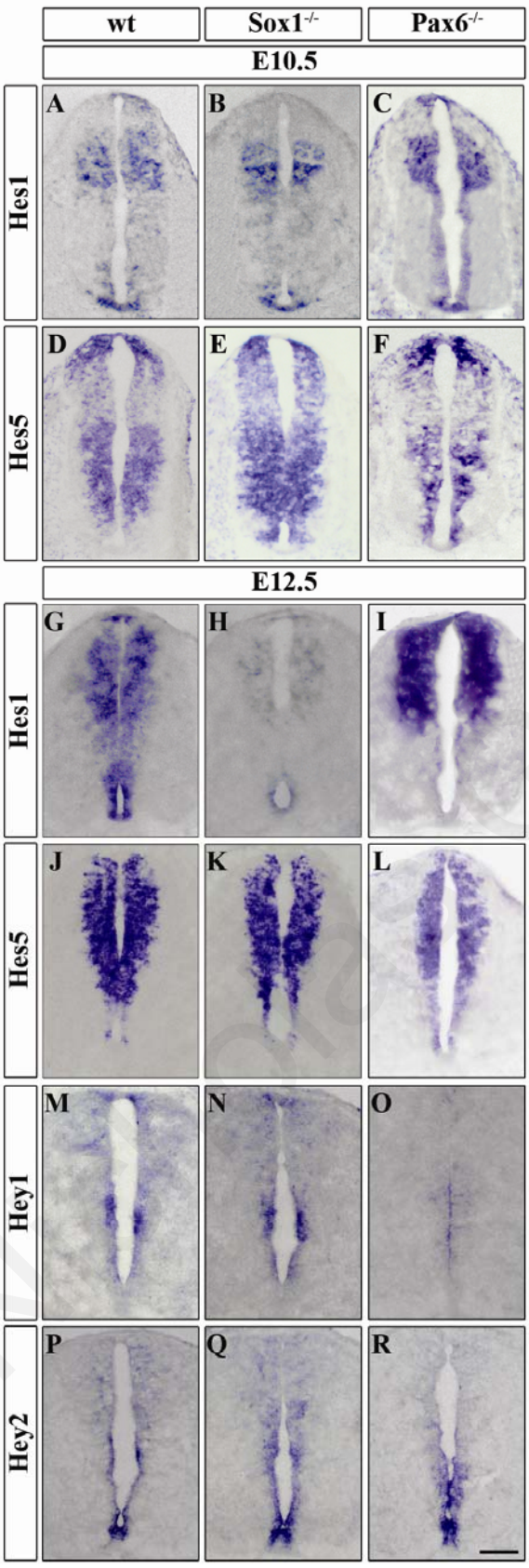

Figure 23: Stage-specific requirement for PAX6 and SOX1 to maintain Hes1 expression

Hes1 and Hes5 expression was compared in the two genetic backgrounds at e10.5 and e12.5. Note that the expression of neither gene is affected at e10.5 (A-F). At e12.5 in both genetic backgrounds Hes1 expression is reduced in the vSC but in $Sox1^{-/-}$ embryos the reduction extends to all regions of the spinal cord (G-I) while some reduction in Hes5 expression in the vSC is noted in $Pax6^{-/-}$ (L). Hey1 expression was detected in an intermediate region of the vSC and was abolished in Pax6-mutant embryos (M-O) while Hey2 expression was largely unaffected (P-R).

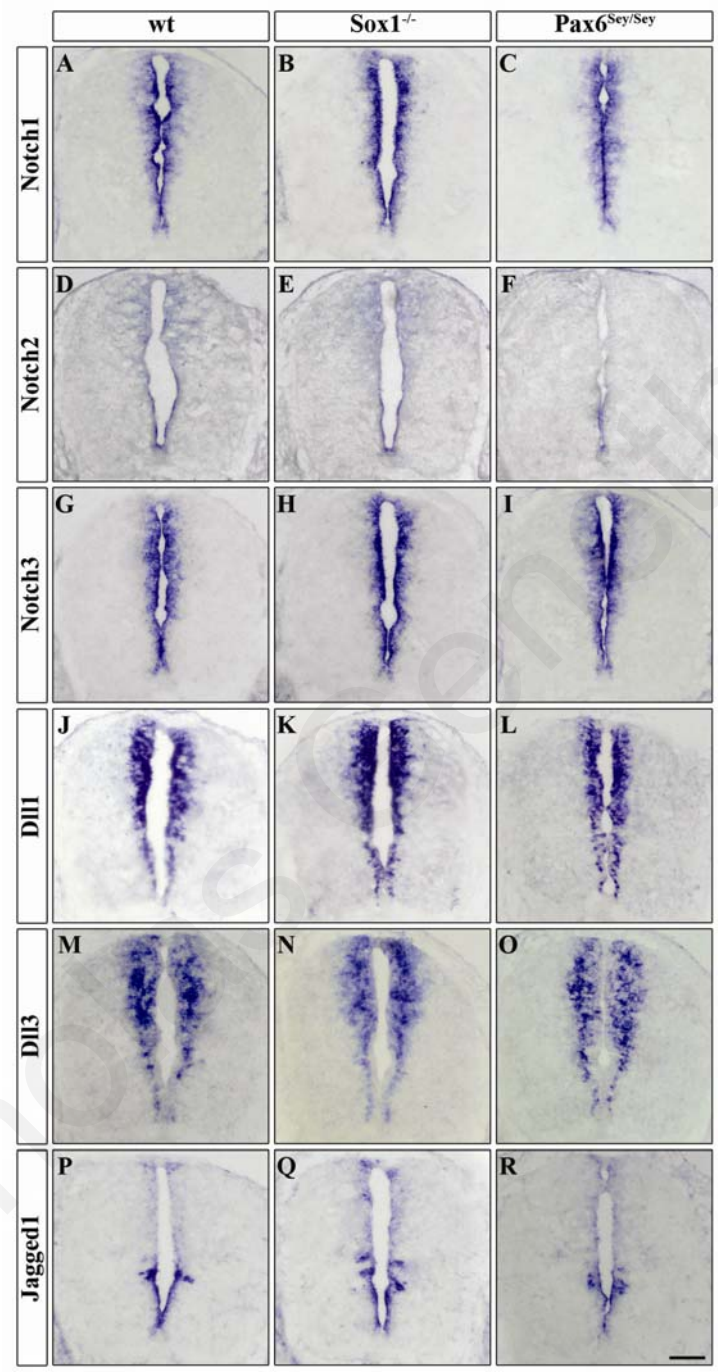

Figure 24: Expression of the Notch ligands (Dll1/3, Jagged1) and receptors (Notch1-3) at e12.5 in Sox1- and Pax6-mutant spinal cords

In situ hybridisation analysis of e12.5 spinal cords from the indicated genetic backgrounds. Notch2 detection was not optimal but I could not discern any differences between the two genetic models.

The data presented in Figures 24 and 25 reveal two important genetic mechanisms:

1. Specifically during the temporal window in which OLPs are specified, Hes1 expression in the vSC critically depends on the genetic hierarchy PAX6→SOX1.
2. Hey1 expression is also regulated by PAX6 in a SOX1-independent manner.

Both observations reveal that there is a link between neural patterning and the regulation of Notch effectors. At the top of this hierarchy is PAX6 which regulates two Notch effectors, Hes1 and Hey1. So far it was always assumed that Hes gene expression is regulated by Notch signalling, but these data suggest a context-dependent regulation of Hes1 by PAX6 and SOX1 and another PAX6-dependent pathway regulating Hey1 expression.

2.7 Genetic requirement for Notch signalling to maintain Sox1 expression in the vSC

The data presented in the preceding section suggested that HES1, a factor with strong anti-oligogenic function (Wu et al., 2003), was positively regulated by SOX1. Therefore one likely explanation for the enhanced production of OLPs from the pMN of Sox1-mutant embryos is the loss of Hes1 expression. Hes1 expression also depends on Notch signalling and upto now it has been assumed that there is no mediating factor, other than RPBJ, to affect Hes1 expression once Notch signalling is activated (reviewed by Kageyama et al., 2008). For instance conditional inactivation of Notch1 affects the neural expression of both Hes1 and Hes5 (Yang et al., 2006).

I wondered, therefore, if Notch signalling was required to maintain Hes1 expression at the same temporal window as the requirement for SOX1 to maintain Hes1 expression. Also I needed to examine if Sox1 expression itself was influenced by Notch signalling. Since Notch signalling affects neural progenitors per se (Yang et al., 2006) and Sox1 is expressed in neural progenitors, it was vital to choose a genetic model with attenuated Notch signalling but normal expression of progenitor markers. To address this question I have chosen the Presenilin-1 (PS-1) mutant embryos as a model (Shen et al., 1997), for the following reasons. All Notch receptors are cleaved intra-cellularly to release the NICD, which translocates into the nucleus and forms a complex with RBP-J in order to activate Hes and Hey genes. PS-1 and PS-2 are involved in this cleavage and therefore loss of PS-1 or PS-2 affects Notch signalling from all receptors. Because of some redundancy between PS-1 and PS-2 function, PS-1-mutant embryos show attenuated Notch signalling in the forebrain (Handler et al., 2000) that does not affect neural progenitors in the vSC (Peng et al., 2007; see below).

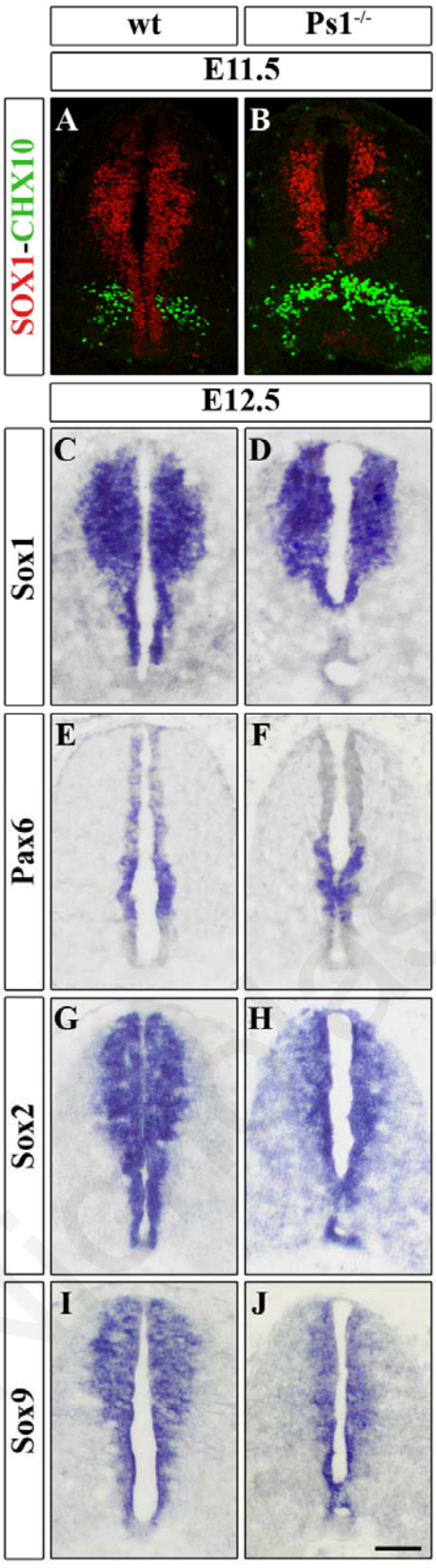

Figure 25: Genetic requirement of Notch signalling to maintain Sox1 expression in the vSC

Analysis of PS-1$^{-/-}$ at e11.5 and 12.5 shows that SOX1 is specifically down-regulated in the vSC up to the p2 domain as shown by Chx10 staining (A-D). Pax6, Sox2 and Sox9 expression was not affected (E-J), suggesting that progenitor indentities are maintained in PS-1 at this developmental window.

In PS-1$^{-/-}$ embryos at e11.5 and e12.5 Sox1 expression was dramatically down-regulated in a domain-specific manner such that it was almost abolished in the p3-p2 domains as judged by Chx10 expression (Fig. 25A-D). Furthermore, loss of PS-1 did not affect Pax6, Sox2, Sox9 and Olig2 expression, despite the thinner VZ in PS1$^{-/-}$ spinal cords (Fig. 25E-J; data not shown). Since Sox1 was only down-regulated in the most ventral domains of the spinal cord, I asked if Hes1 expression was affected in a similar manner. Despite the partial loss of Sox1 expression, Hes1 expression at e12.5 was drastically reduced in all regions of the spinal cord while Hes5 expression was not affected (Fig. 26A-D).

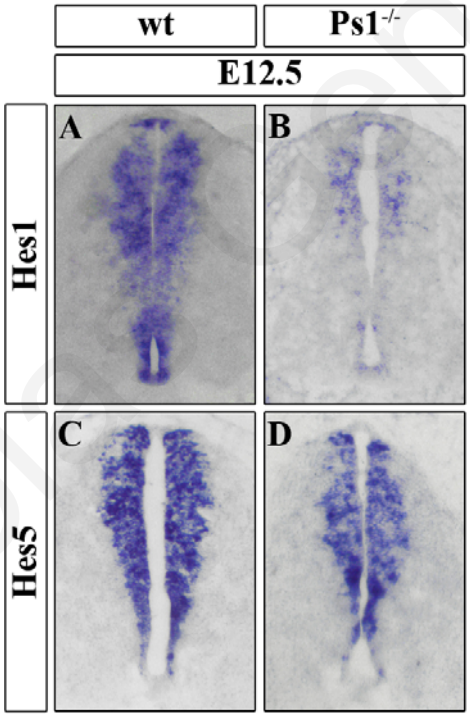

Figure 26: Genetic requirement for Notch signalling to maintain Hes1 expression in the spinal cord

In PS-1$^{-/-}$ spinal cords Hes1 expression was strongly down-regulated (A-B) while Hes5 expression was not affected (C-D).

These data revealed important findings in relation to Sox1 regulation.

1. They show that Notch signalling affects Hes1 expression in a global manner while Sox1 expression is only affected in a region-specific manner.
2. Since Pax6 expression is not affected in PS-1$^{-/-}$ embryos, the requirement for Notch signalling to maintain Sox1 expression must be PAX6-independent.
3. The fact that in PS-1 mutant embryos the expression of several progenitor markers is not affected, indicates that loss of Sox1 expression is not merely due to the depletion of progenitors in the vSC.

The foregoing analysis suggested a complex genetic mechanism to ensure that the expression of both Sox1 and Hes1 is maintained in the vSC precisely when OLP are specified. One mechanism involves SOX1 function and the other mechanism involves Notch signalling. Since there are SOX1 and RBP-J binding sites on the Hes1 promoter, loss of either SOX1 or Notch signalling is likely to lead to the same outcome. Thus Sox1$^{-/-}$ embryos display features of embryos showing attenuated Notch signalling during OLP specification and may contribute to the enhanced production of OLPs in mutant embryos similar to the enhanced production of OLPs in RBP-J mutant spinal cords (Taylor et al., 2007).

2.8 Requirement for Notch signalling to initiate gliogenesis in the spinal cord in a HES-independent manner

In the previous section I have shown a requirement for PAX6, SOX1 and Notch signalling to maintain the expression of Hes1. One issue that has been debated over the last decade is whether Notch signalling is sufficient to induce the gliogenic switch in all regions of the spinal cord. All experiments performed so far to test if Notch activates gliogenesis used gain-of-function (GOF) constitutive activation of Notch signalling which invariably leads to increased AS specification. However, increased AS

specification can be caused indirectly by an increase in the pool of undifferentiated progenitors that activated Notch invariably induces. Constitutive activation of Notch signalling can be achieved either by constitutive expression of a Hes gene or by constitutive expression of the NICD but the outcome is not always the same. Notch signalling can function either through a HES-mediated manner or through a HES-independent manner, as in the case of neurogenesis (Holmberg et al., 2008).

Since I found that three genetic models, namely Sox1-, Pax6- and PS-1 have similarities in the regulation of Hes1, I decided to test directly if Notch signalling was necessary to initiate the gliogenic switch per se in all regions of the spinal cord. As I explained on page 29, GOF studies in the chick spinal cord have shown that Nuclear Factor IA (NFIA) is the most upstream marker indentified to date that marks the onset of the N/G switch and regulates the specification of Glast^{+} radial glia (Deneen et al., 2006). Since I have found that PAX6, SOX1 and PS-1 regulate components of Notch signalling without affecting progenitor markers, these three genetic models offered an unparalleled opportunity to study the astrogenic switch by monitoring the expression of NFIA in the VZ.

I first tested if NFIA expression was affected in Sox- and Pax6-mutant spinal cords at e12.5 the earliest stage of the N/G switch (Deneen et al., 2006). Despite the down-regulation of Hes1 in both Pax6 and Sox1-mutant spinal cords at e12.5, NFIA and the early AS progenitor markers Fgfr3 and Glast were normal in both genetic backgrounds suggesting that the regulation of NFIA expression was SOX1-, PAX6- and HES1-independent (Fig. 27).

I then asked if loss of PS-1 affected NFIA expression. In PS-1$^{-/-}$ embryos the expression of NFIA was drastically reduced (Fig. 28A, B). In parallel, the expression of its downstream target Glast was also reduced in extent (Fig. 28C, D) while some reduction in B-FABP, an astrocyte progenitor marker (Taylor et al., 2007), was also noted in the vSC (Fig. 28E, F). Finally, by e13.5 the expression of the AS marker Fgfr3 was almost abolished suggesting that AS specification is severely compromised in these embryos (Fig. 28G, H).

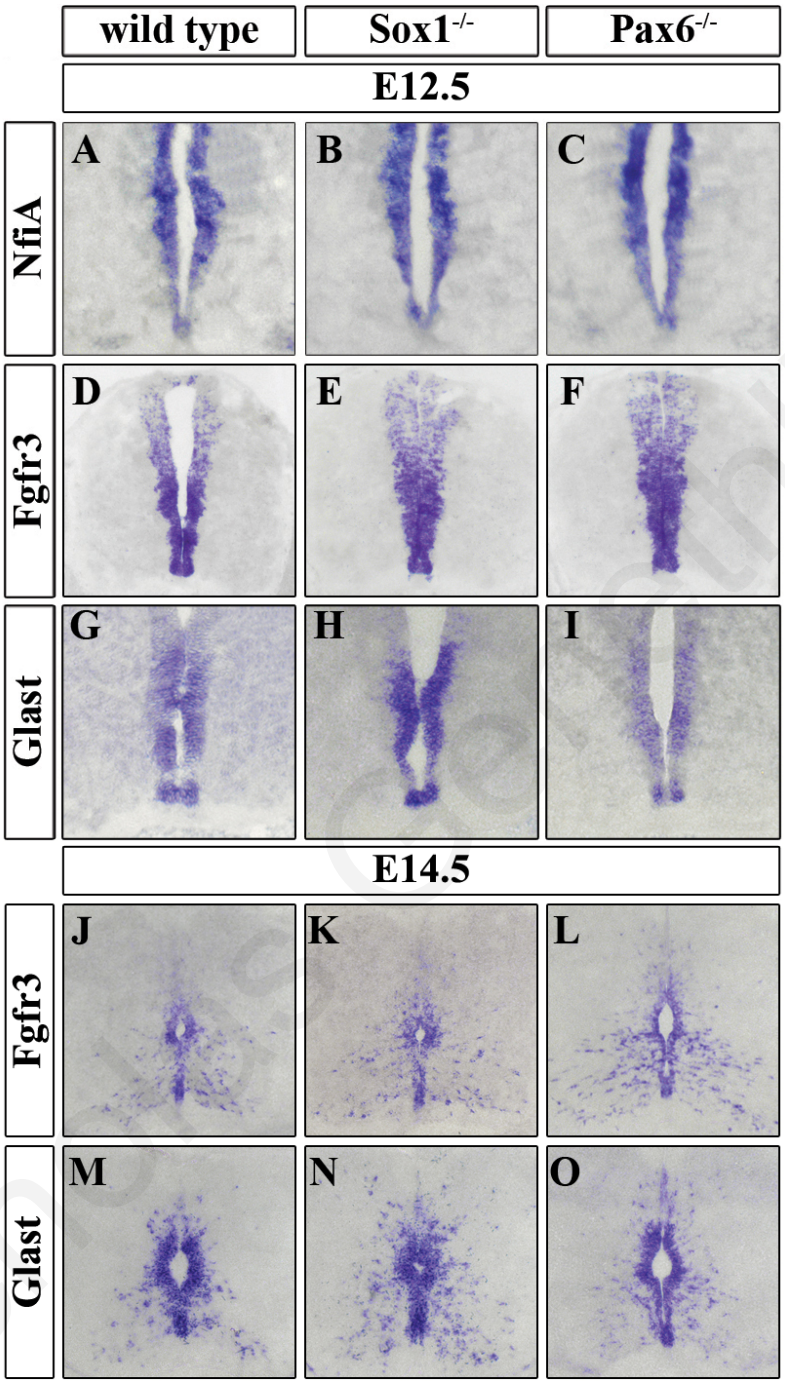

Figure 27: Astrocyte specification in Sox1- and Pax6-mutant spinal cords

At e12.5 the expression of NFIA, Fgfr3 and Glast was not affected in either genetic background (A-I) and as expected at e14.5 neither Fgfr3 nor Glast expression was affected at e14.5 (J-O).

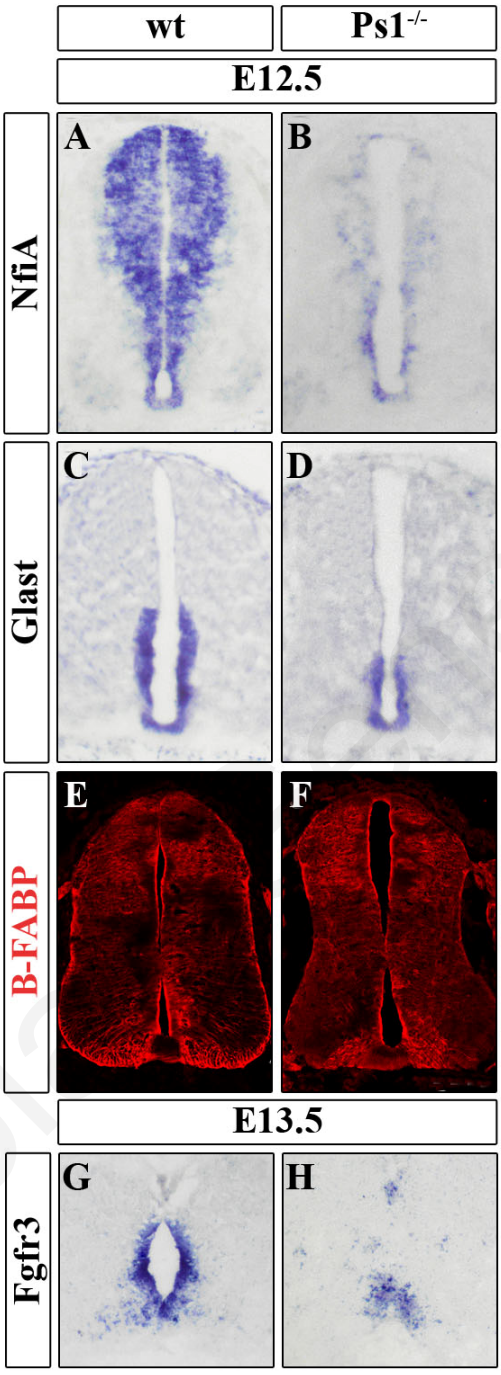

Figure 28: Genetic requirement of Notch signalling to initiate NFIA expression in the spinal cord

In PS-1$^{-/-}$ spinal cords NFIA expression was almost abolished (A-B) while Glast expression was strongly reduced except in a small region in the vSC where some residual NFIA expression is still maintained (C-D). Also B-FABP expression was reduced except in the ventral-most region, possibly because of residual NFIA expression (E, F). (G, H). Note the very strong reduction in AS specification in the entire spinal cord except in the most ventral part where some Fgfr3 expression is retained.

Therefore, despite changes in the expression of Hes1 in all genetic backgrounds studied, NFIA expression was only affected in the PS-1$^{-/-}$ embryos. In view of the fact that Hes5 expression was not affected in PS-1$^{-/-}$ embryos, I conclude that the initiation of NFIA and Glast expression, that is gliogenesis pe se, primarily depends on intact Notch signalling and that the requirement for Notch in this process is likely to take place in a HES1/5-independent manner. Also despite the fact that Hes5 expression is less sensitive to the level of Notch signalling, NFIA function is unlikely to be required for maintaining Hes5 expression, as seems to be the case in the chick embryo (Deneen et al., 2006).

The foregoing analysis of glial specification and the relationship between Notch signalling, Sox1 expression and PAX6 function, suggests that Notch signalling is not only involved in the N/G switch *per se* but also has a key role to play in the pMN domain where it regulates the expression of Sox1. In order to comprehend the implications of the different genetic pathways all gene expression changes noted in each progenitor domain in Sox1-, Pax6- and PS-1 mutant embryos are tabulated in Table 1.

Table 1: Expression matrix of Hes1, Sox1, NFIA and Hey1 at e12.5 in three genetic models analyzed at e12.5.

Progenitor Domain	Hes1				Sox1				NFIA				Hey1			
	Wt	Sox1$^{-/-}$	Pax6$^{-/-}$	PS-1$^{-/-}$	Wt	Sox1$^{-/-}$	Pax6$^{-/-}$	PS-1$^{-/-}$	Wt	Sox1$^{-/-}$	Pax6$^{-/-}$	PS-1$^{-/-}$	Wt	Sox1$^{-/-}$	Pax6$^{-/-}$	PS-1$^{-/-}$
dI1	***	*	***	*					***	***	***	*				
dI2	***	*	***	*					***	***	***	*				
dI3	***	*	***	*	**	**	**	**	***	***	***	*				
dI4	***	*	***	*	**	**	**	**	***	***	***	*				
dI5	***	*	***	*	**	**	**	**	***	***	***	*				
dI6	***	*	***	*	**	**	**	**	***	***	***	*				
p0	***	*	*	*	***	***	*	***	***	***	***	*	***	***	*	N/T
p1	***	*	*	*	***	***	*	***	***	***	***	*	***	***	*	N/T
p2	***	*	*	*	***	***	*	*	***	***	***	*	***	***	*	N/T
pMN	***	*	*	*	**	**	*	*	***	***	***	*	**	**	*	N/T
p3	***	*	*	*			*	*	***	***	***	*	**	**	*	N/T

The expression level of the wild type has been set to ***. The relative expression levels have not been determined by software analysis they are presented for descriptive purposes. Reduced or absent expression is denoted by * while the thinner VZ in PS-1$^{-/-}$ is reflected with **. Important gene expression changes are coloured in red and highlighted in yellow. The precise limits of Hey1 expression could not be determined with confidence. The domain where Sox1/Hes1 expression is lost in Pax6$^{-/-}$ embryos has been determined by correlating relative to Pax3 expression.

Summary of all major findings:

In this study I have presented a comprehensive analysis of the genetic regulation and function of SOX1 during the N/G fate switch in the spinal cord in mice. In doing so, I have revealed various genetic pathways affecting gliogenesis that so far have not been reported in relation to patterning and Notch signalling. My major findings are summarized as follows:

1. I have shown that Sox1 is expressed in a dynamic manner in the VZ during the N/G switch and that this expression pattern is regulated by PAX6 and NKX2.2 in a cell autonomous manner and that no other SOXB gene is regulated in the same manner by these patterning factors.

2. I have shown that SOX1 is never expressed in OLPs, regardless of their origin, and is only expressed in a subset of AS progenitors.
3. I have shown that while NKX2.2 and PAX6 regulate Sox1 expression they also regulate AS subtype identity by regulating Slit1 expression, in contrast to recent data suggesting that NKX6.1 is the major determinant of Slit1 expression.
4. I have also shown that PAX6 regulates the domain specific expression of Sulf1, coding for an enzyme involved in the local concentration of SHH, a process which has been implicated in OL specification in the vSC. The data presented suggest that PAX6 may have a role to play in regulating the distribution and hence strength of Shh signalling in the VZ of the gliogenic neuroepethilium.
5. I have shown that loss of SOX1 leads to accelerated production of OLPs from the pMN domain and based on short-term lineage tracing it is very likely that there is an AS to OL fate switch in the pMN of Sox1-mutant mice.
6. I have shown that SOX1 regulates Hes1 expression downstream of PAX6, in a temporal window when pMN-derived OLPs are specified, providing for the first time a link between neural patterning and the regulation of a Notch effector. In this context I have found that PAX6 regulates Hey1 another glial-specific Notch effector.
7. I have also shown that Notch signalling regulates Sox1 expression specifically during the N/G switch in a domain-specific manner, including the pMN, reinforcing my findings that Notch and SOX1 have a key role to play during the production of glial lineages from the pMN.
8. I have finally shown, for the first time, that Notch signalling not only regulates the expression of Sox1 but also regulates the onset of the most upstream gliogenic marker identified, NFIA, suggesting that Notch signalling is involved in initiating the N/G fate switch per se. In this context, the genetic data presented suggest that the requirement of Notch signalling to initiate NFIA expression is likely to involve a HES1/5- independent process. This observation provides the first genetic, LOF, data of an instructive role of Notch in promoting the N/G switch.

I will discuss these findings in the general context of current knowledge and will propose a genetic model interlinking various genetic factors implicated in gliogenesis on the basis of my findings.

Chapter 3

Discussion

Introductory remark

During the last few years, factors like SOX9, NFIA (Deneen et al., 2006; Stolt et al., 2003) and Notch signalling (Taylor et al., 2007) have emerged as having a critical function during glial cell specification but how their function is coordinated has not been determined. In spite of the fact that this study was focused on SOX1 function, as my worked progressed I have uncovered different regulatory mechanisms that function upstream and downstream of SOX1 all of which converge to regulate the N/G switch in multiple ways. I will discuss each of the findings in the appropriate context.

The expression of SOX1 during glial specification

The expression of SOX1 during gliogenesis has unique features. I have shown using both expression studies and fate mapping that Sox1-expressing progenitors contribute to pMN-derived OLPs which rapidly switch off Sox1 once they leave the VZ. In the dorsal spinal cord, Sox1 is not expressed in the VZ when dorsal OLPs are specified (around e15.5). It is presently unclear why Sox1 expression is switched off in dorsal progenitors and what mechanism is responsible for its regulation. Loss of Sox1 was not sufficient to elicit premature OL specification from the dorsal spinal cord, suggesting that an upstream factor/mechanism is required to initiate OL specification in this region and once this mechanism sets in, SOX1 needs to be down-regulated.

During AS specification in the spinal cord, Sox1 is expressed in an S100β^+ domain and is maintained in ventral AS progenitors that express Pax6. I have identified these cells to be of the VA1 and VA2 subtype (Fig. 21; page 70) (Hochstim et al., 2008). The fact that all ASs, develop normally in Sox1 in mutant embryos is most likely due to the fact that Sox2, is also expressed in all AS progenitors (Dr Remboutsika, S. Malas, not shown) and could compensate for the loss of SOX1 function. Also the expression of HES1, proposed to have a pro-astrocytic role (Sugimori et al., 2007), is restored in both Sox1- and Pax6- mutant embryos by e15.5.

The role of PAX6 and NKX2.2 in glial fate specification

By studying the genetic regulation of Sox1 in the spinal cord I have uncovered very important functions for NKX2.2 and PAX6. Up to now it has been difficult to determine the precise role of PAX6 during gliogenesis *in vivo* due to the dorsal expansion of the Nkx2.2 expression domain in Pax6 mutant spinal cords. By studying $Pax6^{-/-}$, $Sox1^{-/-}$ and PS-$1^{-/-}$ embryos, I was able to attribute a stage-specific requirement for PAX6, through SOX1 function, on the regulation of Hes1 expression. Thus for the first time a stage-specific link is established between the function of neural patterning and Notch signalling, mediated by SOX1. I was also able to show that Pax6 again in the vSC regulates the expression of Hey1, another Notch effector (Fig. 23; page 76).

The genetic studies that I report on Pax6-mutant embryos revealed that in the vSC and specifically in the pMN-p0 domains PAX6 has multiple functions. First, it positively regulates Sox1, Hey1 and Hes1 only in this region. Second, it prevents Sulf1 and Slit1 to be expressed in these progenitors (Figures 13, 14, 16; pages 53-58). In relation to the latter two genes I have shown that the deregulation of Slit1 and Sulf1 affects AS subtype identity and Shh signalling (Genethliou et al., 2009).

The data presented argue that PAX6 functions in a stage-specific manner in the pMN-p0 domains. I have shown that at e10.5 Sox1 expression in the VZ depends on PAX6 in the entire spinal cord (Fig. 13; page 53). However, at e12.5 a dorsal domain of Sox1 expression reappears in mutant embryos. This domain coincides with the $Sox1^{-low}$ and the $Pax6^{-low}$ domains that I described in Fig. 9 (page 45) (see also Fig. 10M-N; page 47).

How can these multiple molecular alterations in Pax6 mutant embryos be reconciled with the reported defects in glial specification? Sugimori and colleagues suggested that PAX6 represses both OL and AS specification and that loss of PAX6 is predicted to accelerate both processes (Sugimori et al., 2007). However, Sun et al (1998) previously showed that in Sey/Sey embryos there is a dorsal shift in the origin of pMN-

derived OLPs and a delay, leading to a significant but transient reduction in the number of PDGFRa^{+} OLPs. Eventually the number of OLs is restored in these embryos.

In this study I have not observed accelerated expression of AS progenitor markers in Sey/Sey embryos as reported by Sugimori et al. (2007) but only an expansion of Slit1 in both the VZ and the WM. The reason for this discrepancy is presently unclear, but could be due to genetic background differences since the reported accelerated AS specification phenotype was evident only at e14.5 and not e15.5. AS specification is reported to take place at e12.5 (Hochstim et al., 2008; this study).

The delay in OL production observed by Sun et al., can now be re-evaluated in the light of my data. This phenotype is most likely due to the severe reduction of Olig2 expression in the pMN at e12.5. However, this delay could be exacerbated by the dorsal expansion of Sulf1 (Fig. 14; page 55). SULF1 has been shown to concentrate high levels of SHH protein where it is expressed (Danesin et al., 2006) and therefore an expanded Sulf1 domain is likely to induce more Nkx2.2 (and thus repress more Olig2^{+} cells) as a consequence of enhanced Shh signalling. Indeed at e12.5 I have observed expression changes in Class I genes that are consistent with enhanced Shh signalling at e12.5 and not at e10.5 (Fig. 18; page 63).

The role of NKX2.2 in AS specification is complicated by the ventral expansion of Olig2 in Nkx2.2$^{-/-}$ spinal cord (Qi et al., 2001). I have shown however that PAX6 does not contribute to the re-specification of the p3 domain during the N/G switch since it does not expand ventrally in Nkx2.2$^{-/-}$ embryos as widely predicted by other groups (Briscoe et al., 1999; Hochstim et al., 2008). Interestingly, in Nkx2.2$^{-/-}$ embryos Slit1 expression is not only lost in the VZ but is also dramatically reduced in the WM at e18.5 (Fig. 19; page 64). Thus, despite the proposition that NKX6.1 is the transcription factor primarily regulating Slit1 expression in the VZ (Hochstim et al., 2008), my data argue that NKX2.2 and not NKX6.1 is the key regulator of Slit1 expression (Genethliou et al., 2009).

Altogether my findings suggest that PAX6 and NKX2.2 regulate several important genes, including Sox1, during glial specification that so far remained unexplored. The reason for that is most likely due to the general belief by many researchers that cross-repressive interactions between these factors would not allow someone to assign factor-specific functions by studying the respective mutant mice. Clearly this is not the case.

The role of NKX2.2 in regulating Sox1 expression

My studies on the regulation of Sox1 by NKX2.2 provide some clues about the likely function of SOX1 in the pMN. I have shown that Sox1 expands ventrally in the absence of NKX2.2. This observation suggests a context-dependent mechanism. At e9.5 (Fig. 9B; page 45) Sox1 and Nkx2.2 are co-expressed whereas by e12.5 their expression is mutually exclusive (Fig. 10C; page 47). One possibility that I considered is the increase in SHH activity as a consequence of expression of SULF1 in the p3 domain. However, using the chick embryo as a model, I have shown that an increase in SHH is unlikely to cause the repression of Sox1 from the p3 domain. Hence the mechanism that NKX2.2 regulates to affect Sox1 expression in the p3 domain is at present unknown. The reason why Sox1 needs to be down-regulated from the p3 domain during gliogenesis also remains unclear. One simple explanation is that the p3 domain generates a special type of OLs that are repressed by SOX1. At present this scenario cannot be tested since there are no specific markers for p3-derived OLs.

The analysis of Nkx2.2 mutant embryos, however, gave an important clue as to the possible function of SOX1 in the pMN. In these embryos Sox1 expression expands ventrally but its does not cover the entire prospective p3 domain. Only dorsal cells from this enlarged 'pMN-like' domain express Sox1, while Olig2 is expressed in the entire p3 domain. Hence Sox1 maintains the same dynamic expression in relation to Olig2 as it does in wt embryos (Fig. 13; page 53). In Nkx2.2 mutant embryos, Pax6 does not expand ventrally. This observation suggests that despite the co-extensive expression of PAX6 and Sox1 in the pMN (Fig. 10; page 47), it is not PAX6 that maintains this dynamic

expression of Sox1 in the pMN but another mechanism that depends on NKX2.2 function.

I propose that in the pMN domain SOX1 is most likely the primary factor that regulates the AS vs OL fate. Since fate mapping studies have shown that Olig2-progenitors make both OLs and ASs (Masahira et al., 2006), then there must be a factor that operates at progenitor level to promote one fate and repress the other. This is exactly what happens during neurogenesis, some cells commit to neurogenesis and some remain undifferentiated. I propose that SOX1 is switched off in cells that commit to the OL fate and remains on in the cells that will commit to the AS fate. This conclusion is supported by the observation that in Sox1-mutant spinal cords I have observed GFP^+ cells expressing Olig2 (Fig. 22Q; page 72). I propose that these cells were destined to make AS but loss of SOX1 caused them to switch fate and become OLs.

There are two additional, although indirect, reasons why I propose that the enhanced production of OLs in Sox1 mutant embryos is caused by an AS to OL fate switch in the pMN. First, in the overlying p2 domain SOX1 regulates the V2b vs V2c interneuron fate switch (Panayi et al., under review). Second, and most important, in Olig2 mutant embryos the functional output of the pMN domain is reversed and all Olig2-expressing cells make AS instead of OLs (Zhou and Anderson., 2001). In these mutant embryos the markers of the overlying p2 domain expand in the pMN but Olig2 expression is not lost as expected, since Irx3 that expands in the pMN is a potent repressor of Olig2 (Muhr et al., 2001). Pax6 expression in Olig2 mutant cords does not expand to cover the entire pMN (note the relationship between Pax6 and Olig2 in Fig. 10P-R; page 47). Therefore despite the patterning defects observed in Olig2 mutant embryos the pMN domain is not re-specified but only the cell fates are reversed. This is fundamentally important and suggests that a gene that is not involved in patterning must be causing the OL to AS fate switch in the absence of Olig2.

Deneen et al., (2006) suggested that this factor is NFIA in order to explain the pro-astrocytic function of NFIA. Although NFIA is upregulated in migrating Olig2-

expressing mutant cells, NFIA is also normally expressed in some OLPs in wt embryos. Furthermore NFIA is expressed in all Olig2-expressing cells in the pMN. Deneen et al., suggested that OLIG2 physically interacts with NFIA and neutralises its pro-astrocytic effect in the pMN. This model predicts that the pMN cannot make any AS from Olig2-expressing progenitors and clearly this is not the case in the light of fate mapping data (Masahira et al., 2006). So NFIA cannot be the cause for the OL to AS fate conversion in $Olig2^{-/-}$ embryos. Since Sox1 is never expressed in OLPs, it emerges as a strong candidate of causing this fate switch. This scenario raises the exciting prospect of testing Olig2/Sox1 double mutant embryos for OL specification.

Data communicated to Dr Malas by Dr David Rowitch also reinforce this hypothesis. In Olig2-mutant embryos Pax6 is expressed in migrating mutant cells that converted to AS. I have shown in Fig. 21 (page 70) that Sox1 and Pax6 are co-expressed in all migrating AS progenitors. So I anticipate that since PAX6 is expressed in Olig2-mutant cells it is almost certain that SOX1 will be expressed too. Dr Kessaris has tried to analyse Sox1 expression in Olig1/2 double mutant embryos by in situ hybridisation but the results were not of good quality to draw any conclusions. The absence of a well-functioning anti-SOX1 antibody clearly precluded me from testing this directly and verification of this hypothesis will require a longer-term genetic study.

The preceding discussion naturally leads to one simple question. Why did I not test if SOX1 blocks OL specification? I have not attempted this experimentally for several reasons. All gain-of-function (GOF) studies reported for SOX1-3 claim that these factors promote the undifferentiated state. Hence over-expression of SOX1-3 leads to block of differentiation. Therefore, one cannot assign a factor-specific effect if the outcome of such manipulations will be the same regardless of the SOX1-3 factor used. Second, Sox2, which appears to have a redundant function with SOX1 using GOF approaches, has a different expression pattern during the N/G switch. Using a Sox2-GFP knock-in line, it has been found that Sox2 is expressed in OLPs (Dr E. Remboutsika and Dr S. Malas). Also Sox2 is expressed in the entire pMN whereas Sox1 is not. Therefore, SOX1 and SOX2 must function in a context-dependent manner and any GOF approach

perceived must try to recapitulate the appropriate context of ectopic Sox1 expression. This is why the suggestion to look at Olig2/Sox1 double mutant embryos for OL specification is of paramount importance and serves as the best context-dependent experiment to test if SOX1 blocks OL specification, under physiologically regulated conditions.

The role of Notch signalling in glial cell specification

Notch signalling is a universal mechanism for regulating cell fate decisions in many different systems (Louvi and Artavanis-Tsakonas, 2006; Radtke et al., 2005). During neurogenesis, Notch signalling inhibits the acquisition of the neuronal fate and promotes the progenitor state, ensuring in this manner that progenitors are not prematurely depleted and some are left to make glial cells. While many studies have addressed genetically the role of Notch during neuronal lineage commitment, the role of Notch during gliogenesis is less clear, primarily because the acquisition of the astro-glial fate is a late event and progenitor depletion complicates phenotypic analysis (reviewed by Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006).

It is generally believed that the gliogenic switch *per se* is comprised by two independent processes: the inhibition of neurogenesis and the activation of gliogenesis (Deneen et al., 2006). Over the years it has been assumed that Notch signalling promotes astro-gliogenesis, an inference drawn primarily though GOF approaches. However, constitutive activation of Notch signalling leads to results that may not reflect the physiological role of Notch. More importantly, GOF experiments cannot distinguish between an instructive role of Notch in promoting astro-gliogenesis and the well known role for Notch in the maintenance of the progenitor state. For instance, transient constitutive activation of Notch in the developing cortex in mice leads to an increased in the number of late born neurons, despite the inhibitory effect that Notch has on neurogenesis, simply due to an increase in progenitor numbers that can differentiate to late-born neurons once the artificial activation of Notch attenuates (Sakamoto et al., 2002). Likewise, constitutive activation of Notch in the cortex prior to the onset of astro-

gliogenesis led to the overproduction of astrocytes, again due to an expansion of the progenitor pool (Sakamoto et al., 2002).

So far loss-of-function (LOF) experiments failed to provide convincing evidence that Notch signalling instructs the gliogenic switch due to premature depletion of progenitors. For instance conditional inactivation of Notch1, thought to be the most important Notch receptor in the CNS (Yoon and Gaiano, 2005), caused premature depletion of progenitors thus not allowing the evaluation of the role of Notch during gliogenesis (Yang et al., 2006).

The first indication that Notch signalling may have an instructive role in astro-gliogenesis has been provided by the conditional inactivation of the Rbpj gene. The product of this gene codes for RBP/J or CBF1 which is a constitutive co-factor of all Notch receptors. Thus upon ligand-receptor interactions between adjacent cells, Notch receptors are cleaved by the γ-secretase complex (involving PS-1 and PS-2) to release the NICD. This in turn forms a complex with CBF-1 and converts the latter into an activator, promoting the transcription of target genes including Hes and Hey genes (Iso et al., 2003; Kageyama et al., 2008). Hence inactivation of Rpbj is thought to affect the Notch pathway mediated by all receptors. These embryos had only minor defects in neurogenesis but severe depletion of astrocyte progenitors as judged by the expression of the radial glial marker B-FABP. The loss of AS progenitor markers was associated with significant increase of OLs that are specificed from the pMN (Taylor et al., 2007).

Taylor et al (2007) also showed that loss of RBP/J caused loss of Sox9 expression at around e14.5 but not earlier. They suggested that the reduction in AS observed was caused by loss of Sox9 since ablation of Sox9 in the spinal cord also leads to severe reduction of AS (Stolt et al., 2003). However, as these authors acknowledged, loss of Sox9 also led to severe reduction of OLs and hence loss of Sox9 could not explain the increase in OLs observed in Rbpj mutant spinal cords.

The genetic data that I provide using the PS-1 mutant embryos suggest a molecular framework to explain how Notch signalling regulates both AS and OL specification. As shown in Fig. 28 (page 85) the most upstream progliogenic factor indentified to date, NFIA (Deenen et al., 2006) directly depends on Notch signalling. I anticipate that NFIA must also be lost in Rpbj mutant embryos and is very likely the cause of the severely reduced ASs. I have shown that the expression of this gene is lost specifically and was not associated with a mere loss of progenitors. These data uncouple the pro-astrocytic role of Notch with its role in maintaining progenitors, a function that is demonstrated for the first time using LOF data.

In relation to OL specification again the work presented here provides important clues. I have shown that in the vSC, Sox1 expression depends on intact Notch signalling (Fig. 25C-D; page 80). Therefore, Notch signalling in all progenitor domains maintains the expression of NFIA, a key pro-astrogenic factor while at the same time ensures that the expression of SOX1, an anti-oligogenic factor is maintained, particularly in the vSC where most OLs are specified. Hence the overproduction of OLs observed in Rpbj mutant embryos is likely to be caused by the combined loss of SOX1 and NFIA and not by loss of Sox9.

Since Notch signalling uses effectors to achieve its function, using the PS-1, Sox1- and Pax6-mutant embryos I was able to determine that NFIA is induced by Notch signalling in a Hes1/5-independent. This is because in the latter two genetic models, NFIA expression is not altered whereas Hes1 expression decreases. In PS-1 mutant embryos both NFIA and Hes1 expression is reduced suggesting a Hes1/5-independent regulation of NFIA (see table 1; page 87).

The data that I have presented leave unanswered one key question. If Notch signalling instructs the gliogenic switch by inducing NFIA then how is this achieved molecularly at the right time? The simple experiment will be to over-express the NICD fragment of Notch (constitutive Notch activation) in the chick spinal cord and look for upregulation of Glast and NFIA expression. Deneen et al (2006) reported that this does

not take place, nor does Hes1 over-expression upregulated Glast expression. However, such GOF experiments are done at the neurogenic phase (HH stage E2). Notch seems to function in a context-dependent manner and GOF studies cannot always reveal its function at the appropriate developmental window.

One strong candidate for mediating NFIA induction is HEY2. This factor is a Notch effector but it is only expressed when the N/G switch takes place. The expression of Hey2 shows a ventral-to-dorsal gradient (Fig. 23; page 76), similar to the direction of gliogenesis. It is thus possible that Hey2 is mediating NFIA induction.

The relationship between SOX1 and Hes1 expression

In this study I have presented strong evidence to suggest that SOX1 is implicated in the genetic regulation of the Notch effector Hes1 in a stage-specific manner. I have shown also that both Notch signalling and SOX1 function converge on the regulation of Hes1. At this point I should stress that Hes1-mutant embryos show premature depletion of progenitors before the N/G switch and thus OL specification cannot be assessed (Ishibashi et al., 1995). A previous study has reported functional SOX1 binding sites on the Hes1 promoter (Kan et al., 2004). Importantly, HES1 and not HES5 has been proposed to inhibit OL specification only in glial-restricted progenitors *in vitro* and not in neuroepithelial progenitors, arguing for a context- and stage-specific function of HES1 during OL specification (Wu et al., 2003). Hence, the value of the Sox1-mutant model is unique in this context because its effect on Hes1 expression only manifests at a developmental window that OL specification from the pMN domain peaks, which is e12.5-e13.5.

Is the reduced expression of Hes1 caused by a cell-autonomous function of SOX1? I was unable to obtain clear data on the immuno-histochemical localisation of HES1 and SOX1. Even if this effect is not cell-autonomous loss of Hes1 may contribute to the enhanced production of OLs. Data communicated to Dr Malas by Dr Masato Nakafuku (senior author in Sugimori et al, 2007) suggests that HES1 is not expressed in

OLPs. Hence if neither SOX1 nor HES1 are expressed in OLPs it is reasonable to assume that one function of SOX1 is probably to maintain Hes1 expression in prospective AS progenitors in the pMN.

A genetic model for glial specification

The data presented in this study in relation to glial specification leads me to propose a genetic model to explain how the N/G is genetically regulated both globally and specifically in the pMN domain. This genetic model integrates genetic data presented here and also elements of previous models addressing the N/G switch (Deneen et al., 2006; Sugimori et al., 2007; Fig. 29). This model assumes that Sox9 expression, shown to be required for the correct timing of the N/G fate switch (Stolt et al., 2003) is regulated by an as yet unknown mechanism since none of the mutant mice I have analysed show changes in Sox9 expression. The elements of this model are:

1. Notch signalling induces pro-astrocytic glial specification in all progenitor domains by inducing NFIA expression in the VZ.
2. In the pMN domain where a binary fate choice needs to be regulated, SOX1 regulates the AS vs OL fate.
3. In parallel pathways, both PAX6 and Notch maintains Sox1 expression in the pMN domain, thus ensuring that this binary fate choice is maintained.
4. Since Notch signalling is believed to inhibit precursor cell differentiation (Sugimori et al., 2007; Wu et al., 2003), the effect of Sox1 mutation might be expected to cause accelerated production of OLPs and rapid depletion of the neuroepithelial precursors, as observed.

This is probably why in Nkx2.2$^{-/-}$ embryos both Sox1 and Olig2 expression is dynamically maintained to ensure that the regulation of this binary fate choice is still operational in the expanded pMN domain. This model predicts that in PS-1$^{-/-}$ embryos OLP production should also be enhanced while in both Sox1$^{-/-}$ and PS-1$^{-/-}$ embryos ASs production should be reduced. For the Sox1$^{-/-}$ embryos, we cannot test specifically for pMN-derived ASPs since no marker exists for these cells. In PS-1$^{-/-}$ embryos the

transcription factor SCL, that is expressed in the overlying p2 domain is also lost (Peng et al., 2007) and this will most likely lead to dorsal expansion of Olig2 (Muroyama et al., 2005) causing over-production of OLPs. This is why the value of Sox1 mice is unique because it is a target of Notch and the enhanced production of OLPs takes place in the absence of any changes in SCL expression.

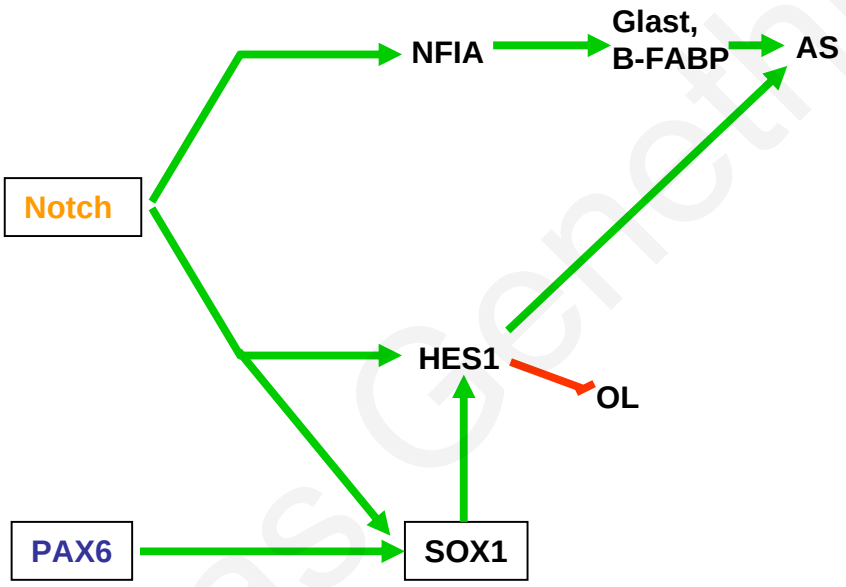

Figure 29: Genetic model showing the epistatic relationship between intrinsic determinants of gliogenesis

This model postulates that: (1) the initiation of NFIA expression is achieved by a Notch-dependent but Hes-independent mechanism and is required for inducing RG markers (Deneen et al., 2006; this study). (2) PAX6 is required for the initiation (SM unpublished) and maintenance of Sox1 expression in the vSC which in turn regulates Hes1 expression only during gliogenesis. Finally, SOX1 may regulate the AS vs OL fate in the pMN. Green arrows indicate induction and red arrows indicate repression of expression or of a mechanism. OL-Oligodendrocytes; AS-Astrocytes.

Concluding remarks

This study leaves several unresolved issues and I will mention just some. First, how can both Notch signalling and PAX6 regulate Sox1 expression in the vSC, albeit in incompletely overlapping domains? One possibility is that both PAX6 and PS-1 regulate Sox1 indirectly through Wnt signalling, known to be activated by PAX6 and PS-1 (Kim et al., 2001; Lei et al., 2006; Soriano et al., 2001). However, the expression of the Wnt inhibitor SFRP2 in $Pax6^{-/-}$ spinal cords is maintained at axial levels that already lost SOX1 expression (not shown). Also Dr Malas has tested the expression of Sox1 in SFRP2 mutant embryos and this was normal. Another possibility is that PAX6 may regulate the expression of Sox1 specifically in the pMN-p0 domain via Hey1. This possibility is currently being investigated by Dr Malas as part of another study.

This study is a good paradigm of dissecting a genetic pathway using only loss of function data that are likely to show only physiological effects. The literature in relation to the N/G switch is full of GOF data that have not been substantiated with loss of function studies.

Appendix A

Materials and Methods

Part 1: General Techniques

Sterilisation Key: a- sterilised by autoclaving at 15 Ib/sq for 30 minutes.
f - filter sterilised by filtration through a 0.22-micron filter.

1.1 Bacterial media, strains, antibiotics

1.1.1 Liquid and solid bacteriology media (w/v)

LB (Luria-Bertani) Medium[a]:

1 % Bacto-tryptone

0.5% Bacto-yeast extract

0.5% NaCI (Sodium Chloride)

LB Agar[a]:

Liquid LB medium plus 1% agar.

SOC (Super Optimal broth with Catabolite repression) Medium[a]:

2% bacto-tryptone

0.5% bacto-yeast extract

10mM NaCl

2mM KCl

10mM $MgCl_2$

Supplemented with 20mM sterile glucose after sterilisation.

1.1.2 Bacterial Strains

Strain	Genotype	Use
DH5α	F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1 λ^-	Recombination-deficient strain used for propagating recombinant bacterial plasmids. Heat-shock transformation.
DH10b	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG	A non-methylation sensitive strain used for cloning, propagation of large DNA plasmids and BACs and for BAC recombination. DNA transformation performed by elecroporation.

1.1.3 Antibiotic stock solutions and working concentrations

Name	Stock	Working concentration	
		High copy plasmids	BAC recombination
Ampicillin (Amp)	50mg/ml in dd* H_2O	50μg/ml	50μg/ml
Kanamycin (Kan)	50mg/ml in dd H_2O	50μg/ml	15μg/ml
Tetracycline (Tet)	10mg/ml in 75% ethanol	10μg/ml	3μg/ml
Chloramphenicol (Cm)	30mg/ml in ethanol	50μg/ml	15μg/ml
Streptomycin	50mg/ml in dd H_2O	50μg/ml	N/A

* - deionised distilled water N/A: not applicable

1.2 Growth of bacterial cultures, storage media

1.2.1 Growth in liquid media

All liquid bacterial cultures were set up in a purpose-designated area. A single colony was grown overnight in LB medium containing the appropriate antibiotic and left shaking at 200 rpm in a 37°C incubator.

1.2.2 Growth on solid media

Single bacterial colonies were obtained by streaking a small amount of a stock culture onto hardened LB agar; the plate sealed using Parafilm and incubated overnight at 37°C. Bacteria stocks were stored in 20% glycerol at -80°C.

1.2.3 Preparation of DH10b electro-competent cells

Solutions used:

a. dd H_2O
b. 10% glycerol in dd H_2O[a]
c. LB medium[a]

Strain DH10b was grown on LB/streptomycin agar plate overnight at 37^oC. A single colony was expanded overnight in a 5ml LB/streptomycin medium. The resulting culture was further expanded through transfer into 200 ml LB/streptomycin medium. The culture was grown until the absorbance at 600 nm reached 0.7 (usually 2 hours). For the preparation of the competent cells, all subsequent steps were carried out in a 4^oC chamber. The cell suspension was collected in pre-cooled 50 ml Falcon tubes in 50 ml aliquots and placed on ice for 15 minutes. The cells were collected by centrifugation at 5000 rpm for 15 minutes at 4^oC and gently resuspended in 10 ml of ice-chilled dd H_2O. The suspension was spun to collect the cells at 5000 rpm for additional 15 minutes at 4^oC. This step repeated once and then the suspension was gently resuspended in 10%

glycerol and was spun for another 15 minutes at 5000 for 4°C. Finally the cells were resuspended in 2 ml 10% glycerol, aliquoted 40 µl in each pre-cooled 0.5 ml polypropylene tube and stored at -80°C.

1.2.4 Preparation of DH5α competent cells

Solutions used:

a. 0.1M $CaCl_2$[f]
b. 10% Glycerol in 0.1M $CaCl_2$ (dilute 50% glycerol[a] in 1M $CaCl_2$[f])
c. LB medium[a]

DH5α strain was streaked and grown on LB agar plate overnight at 37°C. A single colony was picked and expanded overnight on a 2ml LB medium. 1ml of saturated overnight culture inoculated 500 ml of LB medium. The fresh culture was incubated in a flask at 37°C until the absorbance at 600 nm reached 0.5 (2 to 3 hours). For the preparation of the competent cells, all subsequent steps were carried out in a 4°C chamber. The culture was transferred in pre-chilled 50 ml Falcon tubes. Bacteria cells were collected by centrifugation at 5000 rpm for 15 minutes at 4°C. The bacterial cell pellet was then resuspended in 10 ml cold 0.1M $CaCl_2$ and pooled together into one pre-chilled 50ml Falcon tube. The suspension was spun at 5000 rpm for 15 minutes at 4°C and the pellet was resuspended in 10ml ice-cold 0.1M $CaCl_2$ and set on ice for 30 minutes. Then the pellet was resuspended in 2 ml 10% glycerol in 0.1M $CaCl_2$ and left on ice overnight at 4°C. The next day the suspension was dispensed into 50µl aliquot in pre-chilled sterile 0.5ml polypropylene tubes and stored at -80°C.

1.3 DNA Extraction protocols

1.3.1 Extraction of Total mouse genomic DNA

Solutions used:

a. **TNES (v/v):**
   10 mM Tris, pH 7.5
   400 mM NaCl
   100 mM EDTA (Ethylenediaminetetraacetic Acid)
   0.6% SDS (Sodium Dodecyl Sulfate)
   dd H_2O
b. **6M NaCl**
c. **10 mg/ml Proteinase K** (in dd H_2O)
d. **TE**[f]
   10 mM Tris, pH 8.0
   1 mM EDTA
   dd H_2O

Mouse genomic DNA was extracted from the tail, ear, embryos or mammalian cells such as ES cells. The tissue biopsies were placed in 600 µl TNES solution with 35 µl Proteinase K in polypropylene tubes and incubated overnight at 55^{o}C. After the digestion 166.7 µl 6M NaCl were added in the tube and they were shaken vigorously for 15 seconds, followed by centrifugation at 14000 g for 5 minutes at room temperature. The supernatant was collected into a clean tube and the DNA precipitated by the addition of one volume of ice cold 95% ethanol (EtOH). The DNA was spooled out and rinsed with 70% EtOH. The 70% EtOH was removed and the DNA pellet was allowed to dry for 5 minutes. Then the DNA was resuspended in 100-500 µl of TE depending on the size of the pellet. The dissolved DNA was then stored at 4^{o}C until needed.

1.3.2 Extraction of high copy plasmids from E. coli cells

Plasmid DNA was extracted using a procedure based on alkaline lysis of bacterial cells and the reagents used are commercially available (Eppendorf Perfectprep® Plasmid Mini Kit for small quantities of plasmid DNA and Qiagen® QIAfilter Plasmid Maxi Kit for larger amounts of DNA). The protocols followed were as recommended by the suppliers.

1.3.3 Extraction of Bacterial Artificial Chromosome (BAC) from E. coli cells

Solutions used:

a. **P1[f]:**
   15 mM Tris, pH 8
   10 mM EDTA
   100 μg/ml RNase A
   dd H_2O
b. **P2[f] (v/v):**
   0.2 N NaOH
   1% SDS
   dd H_2O
c. **P3[a]**
   3 M KOAc, pH 5.5
   dd H_2O
d. **TE[f]**
   10 mM Tris, pH 8.0
   1 mM EDTA
   dd H_2O

BAC DNA was extracted from the bacterial cells by an alkaline lysis procedure. A 2ml overnight culture of BAC containing E. coli cells spun down at 3000 rpm for 10 minutes at room temperature. The supernatant was discarded and each pellet was

resuspended in 0.3 ml of P1 solution. Then 0.3 ml of P2 solution was added and the tube was inverted gently 4 to 5 times to mix the contents allowed to sit at room temperature for 5 minutes until its appearance changed from very turbid to almost translucent. Then 0.3 ml of chilled P3 solution was added slowly and was gently inverted. The tubes were placed on ice for at least 5 minutes and they were centrifuged at 10000 rpm for 10 minutes at 4^{o}C. From this point onwards we used cut tips in order to prevent shearing of the high molecular weight BAC DNA. The supernatant was then transferred to a 1.5 ml polypropylene tube that contained 0.8 ml ice-cold isopropanol and were mixed by gentle inverting. After 5 minutes that was left to set on ice we spun it for 15 minutes. The DNA pellet was washed with 70% EtOH twice and left to air-dry at room temperature for 5 minutes. Finally, the BAC DNA resuspended in 10-40 μl TE depending on the size of the pellet. The dissolved DNA was then stored at 4^{o}C until needed.

1.3.4 Quantification of DNA

The concentration of DNA in solution was determined spectrophotometrically at wavelength 280 nm in a Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer. For pure DNA the ratio of (OD_{260}/OD_{280}) was between 1.8 and 2.0

1.4 DNA Modification reactions

1.4.1 Cleavage of DNA with bacterial endonucleases type II

The endonucleases used were all purchased from New England Biolabs (NEB) and used according to the suppliers protocols.

1.4.1.1 Cleavage of low molecular weight DNA

Plasmid DNA or DNA produced by the PCR were restricted in 20 μl reaction volume using 5-10 units of the enzyme at the appropriate reaction temperature, in the case of diagnostic digestions; and in 100 μl reaction volumes with 20-30 units of the

enzyme, if the DNA was going to be used for cloning. The digestion time varied from 2 hours to overnight depending on the restriction enzyme properties. In the case of combination of two restriction enzymes in one reaction we used the suggesting buffer from the supplier's catalogue. After the reaction the DNA was loaded on an agarose gel for electrophoresis. For cloning the appropriate DNA band was then cut with a scalpel and DNA was purified with the Eppendorf® Perfectprep® Gel Cleanup Kit. The protocol followed was as recommended by the supplier.

1.4.1.2 Cleavage of BAC DNA

BAC DNA was cleaved in 20 μl reaction volume using 10 units of enzyme in the presence of 5 mM Spermidine at the appropriate reaction temperature. Everything else was the same as the restriction of low molecular weight DNA.

1.4.2 Ligation of DNA

All the ligation reactions were carried out at 14^{o}C overnight.

1.4.2.1 Directional cloning

Two DNA fragments were digested with compatible restriction enzymes and purified. Then we calculated the amount of each fragment to put in the ligation reaction. The calculation was based in the formula:

ng of insert = (ng vector x size of insert) / size of insert

The total amount of DNA should not exceed 100 ng per reaction. The DNA fragment were ligated in a 10 μl reaction volume with 1x ligation buffer and 1 unit of T4 DNA ligase.

1.4.2.2 Cloning of PCR products

All the PCR products cloned ranged in size between 400 and 1000 bp. A single set of conditions was followed irrespective of the size of the DNA fragments. The vector and other reagents used are commercially available (pGEM®-T Easy Vector System I; Promega). Always three microlitres of non-purified PCR product (about 25 ng) was ligated to 50 ng of pGEM®-T Easy Vector in the presence of 1X ligase buffer and 1 unit of T4 DNA ligase in a 10 μl total reaction volume.

1.4.3 BAC Recombination

BAC Recombination is a technique for the genetic alteration of BAC DNA which is based on the homologues recombination that happens in some bacterial strains. Homologues recombination is the exchange of genetic information between two DNA molecules in a precise and specific manner. It occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine.

We used the Red/ET Recombination Kit from Gene Bridges. Redα is a phage derived protein with a 5'-3' exonclease activity and Redβ is a DNA annealing protein. A double-stranded break repair is initiated by the recombinase protein pairs, Redα/Redβ. First Redα digests one strand of the DNA, leaving the other strand as a 3' ended, single-stranded DNA overhang. Then Redβ binds and coats the single strand. The protein-nucleic acid filament aligns with homologues DNA. Once, aligned, the 3' end becomes a primer for DNA replication (Fig. 30).

The Red/ET Recombination Kit includes the plasmid pSC101-BAD-gbaA-tet and the Redα/Redβ proteins are under the control of an arabinose-inducible promoter (Zhang et al., 1998).

The first step for the BAC recombination was to introduce 3' and 5' end homology to the DNA cassette that would recombine the BAC. For this purpose we

amplify by PCR the targeted cassette using a 3' oligonucleotide which contained an overhang with a 3' homology and a 5' oligonucleotide with a 5' homology to the precise sites of recombination. The PCR product was precipitated with 100% Ethanol, washed with 70% Ethanol, dried and dissolved in a small amount of ddH_2O.

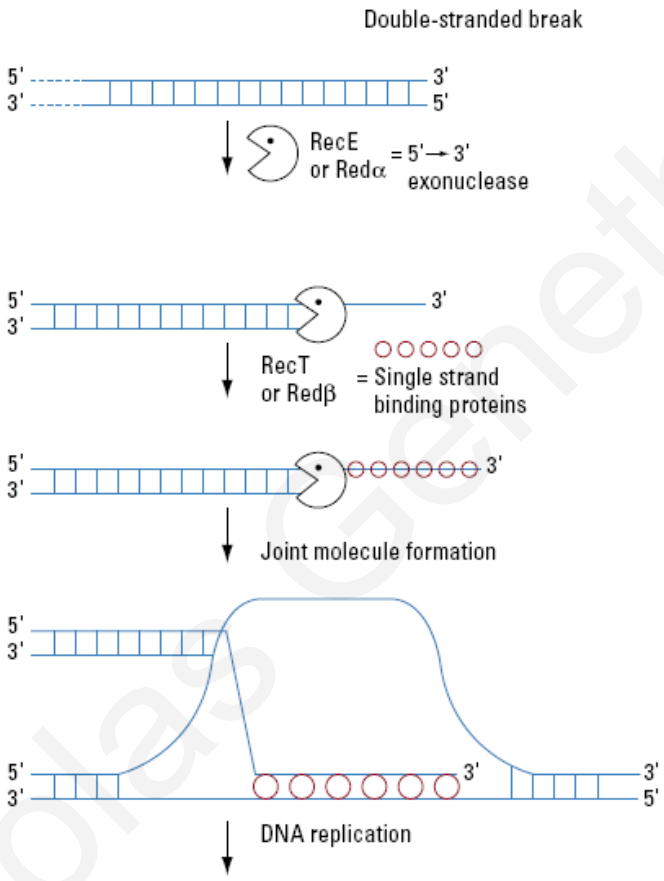

Figure 30. Mechanism of Red/ET recombination. Adopted by the Technical Protocol of BAC Subcloning kit By Red®/ET® Recombination (Gene Bridges, Version 2.4, February 2005).

1.5 Transformation of competent E. coli cells

1.5.1 Heat-shock transformation of DH5α

A small amount of ligation or plasmid DNA (1 to 1.5 μl) was incubated on ice with 50μl of DH5α competent cells for 45 minutes, heat-shocked at 42^{o}C for 30 seconds and placed again on ice for 5 minutes. To this 1 ml of pre-warmed (at 37^{o}C) SOC medium was added and incubated at 37^{o}C for 1 hour, to allow the transformed cells time to express the antibiotic-resistance gene. A fraction of the culture (50-200 μl) was spread onto LB-agar plates containing the appropriate antibiotic. The plates were incubated at 37^{o}C overnight.

1.5.2 Electroporation of DH10b

An aliquot of DH10b bacterial cells were mixed with 1 μl of ligation while were incubated on ice. The cells were put in a pre-chilled Bio-Rad® 0.1 cm electroporation cuvette and electroporated by a Bio-Rad® Gene Pulser II electroporator. The settings of the electroporator were 1.7 KV, 10 μF, 600 Ohms. The cells were resuspended in 1 ml of SOC medium without antibiotic, returned in a 15 ml Falcon tube and incubated for at least one hour at 37^{o}C (except the pSC101 containing plasmid that were incubated at 30^{o}C; see below) shaking. A fraction of the culture was plated onto LB-agar plates with the appropriate antibiotic. The plates were incubated at 37^{o}C (or 30^{o}C for the pSC101 containing cells) overnight.

1.5.3 Screening of the cells that carry the recombinant plasmids

Single colonies were picked and resuspended in one well each of a 96-well plate. 5 μl of each resuspended colony was used for a PCR reaction. The forward primer was located upstream of the insert in the vector and the reverse primer on the insert, or the reverse primer was on the vector but downstream of the insert and the forward was on the insert. The clones that gave positive PCR reactions were expanded in mini-cultures and DNA was isolated to confirm correct cloning by preparative DNA analysis.

1.6 In vitro DNA replication

1.6.1 Exponential amplification using the Polymerase Chain Reaction (PCR)

Reagents used:

1. **Thermits aquaticus (Taq)** thermostable DNA polymerase (Qiagen).
2. **10X reaction buffer**, consisting of 200 mM $(NH4)_2SO_4$ (Ammonium Phosphate), 75Q mM Tris-HCI pH 9.0 (at 25°C), 0.1% (w/v) Tween.
3. **25 mM $MgCI_2$** (Magnesium Chloride)
4. **100 mM solution of 2'-Deoxynucleoside 5'-Triphoshate** (dNTPs; Pharmacia).
5. **Synthetic oligonucleotides or primers** (20-30 bases long; purchased from MWG).

All PCR reactions were carried out in an automated thermocycler (Biometra T1 Thermocycler; Omnigene). All reactions were prepared in either 25 µl or 50 µl reaction volume in the presence of 1X reaction buffer, 200 mM of each dNTP, 1.5-2.0 mM $MgCl_2$, 20 or 40 pmole of each primer and 25 ng of DNA (genomic or cloned). This mixture was overlaid with mineral oil and reactions were carried out for 30-35 cycles of 94/95°C for 20-30 seconds, 53-62°C for 30 seconds, and 74°C for 30 seconds - 2 minutes, depending on the primer set. Amplification of high G+C regions needed the addition of 1x Q solution (Qiagen).

1.6.2 Digoxigenin labelling of RNA probes

Digoxigenin (Dig) is a small molecule with high immunogenicity conjugated to the Uridine RNA nucleotide. An RNA probe labelled with Dig can then be recognized by an anti-Dig antibody so a visualisation and detection of this RNA can take place.

Reagents used:

1. **T7 or T3 RNA Polymerase** (Roche Applied Science)
2. **10X Transcription Buffer**, consisting of 0.4 M Tris-HCl pH 8.0 (at 20°C), 60 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 20 mM spermidine.

3. **10X Dig RNA labelling mix** (Roche applied Science), consisting of 10 mM ATP, 10mM CTP, 10 mM GTP, 6.6 mM UTP, 3.5 mM DIG-11-UTP pH 7.5 (at 20^{o}C).
4. **0.75 M DTT**
5. **RNaseOUT^{TM} Recombinant Ribonuclase Inhibitor** (Invitrogen)
6. **Diethyl Pyrocarbonate (DEPC) treated ddH_2O.** ddH_2O was mixed with DEPC (Sigma) in a final concentration (v/v) 0.1%, stand at room temperature for 1 hour and then autoclaved. The DEPC-treated ddH_2O (DEPC-H_2O) is RNase free.

All RNA labelling reactions were carried out at 37^{0}C in RNase-free 0.2ml polypropylene tubes. All reactions were prepared in 20 µl reaction volume in the presence of 1X transcription buffer, 1X Dig RNA labelling mix, 0.225 M DTT, 40 units RNaseOUT^{TM} and 200ng-1 µg of linear template DNA. To this, 40 units of T7 or T3 RNA polymerase were added and the reaction was incubated at 37^{o}C for 2 hours and then 80 µl of DEPC-H_2O was added and the product analysed by gel electrophoresis to verify RNA synthesis. The product was dispensed in 5 µl aliquots and stored at -80^{o}C.

1.6.3 Radioactive labelling of DNA

Preparation of polynucleotide DNA probes

The DNA used for radioactive labelling was prepared by preparative gel electrophoresis in 1% (1X TBE) agarose gel electrophoresis. The fragment of interest was excised from the gel and purified by Eppendorf® Perfectprep® Gel cleanup Kit.

The DNA was labelled radioactively using the commercially available kit Amersham Rediprime^{TM} II DNA labelling system (GE Healthcare Life Sciences) which contains all the appropriate reagents in a crystallised state in a single tube (except the ^{32}P-dCTP). Approximately 25 ng of DNA was denatured by heating at 99°C for 5 minutes in a total volume of 45 µl, placed for 5 minutes on ice and mixed with

the crystallised reaction reagents. Usually 50 μCi (Curie) of alpha-^{32}P-dCTP (10 μCi/μl) per reaction was used. The reaction was incubated at 37°C for 20 minutes and the unincorporated nucleotides removed by centrifugation through an illustra^{TM} MicroSpin G-50 column for 3 minutes at 1500g. Prior to use, the DNA probes were denatured by boiling for 5 minutes and added to the appropriate hybridisation mixture.

1.6.4 DNA sequencing

DNA sequencing was performed using a kit for Dye Terminator Cycle DNA sequencing from Beckman Coulter (CEQ DTCS – Quick Start Kit, England) and fractionated on an automated DNA sequencer (CEQ2000XL-Beckman Coulter).

1.7 Electrophoretic analysis of the DNA

Buffers used:

- Running Buffer (w/v):
  5X Tris-borate (TBE) [445 mM Tris base; 445 mM boric acid; 10 mM EDTA].
- 6X Loading buffer (w/v):
  0.25% bromophenol blue, 0.25% xylene cyanol FF, 50 mM EDTA, 30% glycerol.

1.7.1 Agarose gel electrophoresis of low molecular weight DNA

All agarose gel electrophoresis was performed using 0.7%-2% agarose gels (1X TBE; 0.5 μg/ml of ethidium bromide) in a horizontal gel apparatus. For the analysis of PCR products, the gels used were between 1-2%. The gels were run at 7V/cm in a BRL 'midi-gel' set up (30 cm x 12 cm; model H5) or a Hybaid Electro-4 gel tank (27cm x 11 cm; HB-E4-GT). Plasmid DNA was analysed in 0.8%-1% midi-gels at 4V/cm. Restricted genomic DNA was analysed in 0.8% agarose gels cast in a 25 x 20 cm moult and electrophoresed in a BRL apparatus [35 x 20 cm (model H4)] at 0.875 V/cm. All agarose gels were run in 1X TBE buffer containing 0.5 μg/ml ethidium bromide. The

DNA samples were mixed with loading buffer before loaded onto the gel. The DNA was visualised by exposing the gels to ultraviolet light of 300 nm wavelength.

1.7.2 Pulse field gel electrophoresis (PFGE) of high molecular weight DNA

Pulse field gel electrophoresis was performed using a CHEF-DR II electrophoresis system (Bio-Rad®). The electrophoresis was carried out in a 4°C chamber and the running buffer temperature was maintained constant through continuous circulation using a pump which was attached to the gel chamber. The gel was cast in 14 cm x 12.7 cm casting stand using 1% agarose in 0.5X TBE buffer at 5 mm thickness. Before loading, the blocks were placed on ice for 10 minutes to increase the rigidity of the agarose and reduce the risk of damage while handling. It was very important to avoid trapping air-bubbles within the wells. After loading, the wells were filled with melted agarose (same one as that used to pour the gel) and the gel equilibrated at 4°C for 20 minutes in the running buffer. The electrophoretic conditions were 8 v/cm using 70 seconds switch times on a Pulsewave 760 Switcher (Bio-Rad®). After the run, the gel was stained in 0.5 μg/ml of ethidium bromide and visualised at 300 nm ultraviolet light.

1.8 Southern blotting

1.8.1 Alkaline DNA transfer

Solutions used:

1. **Depurination solution:** 0.25 M HCl
2. **Denaturation buffer:** 1.5 M NaCl, 0.5 M NaOH
3. **20X Saline sodium citrate (SSC):** 3 M NaCl, 0.3 M Sodium citrate
4. **ddH_2O**

Mouse genomic DNA was digested with an appropriate restriction enzyme and the DNA fragments were separated by agarose gel electrophoresis, visualised and photographed at 300 nm ultraviolet light. Then, the gel was placed in Depurination solution for 30 minutes with constant shaking, rinsed twice in ddH_2O and placed in Denaturation solution for 30 minutes to 1 hour. For the blotting we used Amersham Hybond-N+ membrane (GE Healthcare Life Sciences). The transfer apparatus was a standard capillary blotting set-up using Denaturation buffer as the transfer buffer. After the overnight transfer, the membrane was washed twice in 5X SSC for 15 minutes each time to neutralise it. If storage was required the membrane was dried on sheet of Whatman 3mm filter paper.

1.8.2 Hybridisation of radiolabelled probes to immobilised DNA

Solutions used:

1. **Solution A (for Modified Church Buffer):** 1 M $Na_2HPO_4.2H_2O$
2. **Solution B (for Modified Church Buffer):** 1 M $NaH_2PO_4.H_2O$
3. **Modified Church Buffer (v/v):**

Solution A	342 ml
Solution B	158 ml
SDS	7%
EDTA	10 mM
ddH_2O	up to 1 L

4. **Sonicated Salmon Sperm DNA 10 mg/ml**
5. **Rinse buffer (v/v):** 2X SSC, 0.1 % SDS
6. **ddH_2O**

The membrane was placed in a hybridisation bottle and rinsed with modified Church Buffer. All hybridisations were carried out in modified Church Buffer. The prehybridisation was done in the presence of 100 μg/ml of denatures sheared salmon sperm DNA for at least 2 hours at 65^{o}C before adding the radiolabelled probe (prepared from mouse genomic DNA). The hybridisation was performed for about 20 hours at

65°C. Then, the membrane was washed with 2X SSC/0.1% SDS twice for 30 minutes each time at 65°C. If it was necessary we could wash more to reduce background. Autoradiography was done for between 1 day to 1 week using Kodal Biomax Autoradiography film and Kodak intensifying screens.

1.9 In situ hybridisation

1.9.1 Preparation of template DNA for the generation of RNA probes

The probes that were donated from other laboratories (Table 2) were sent as plasmid DNA. We then transformed them to competent cells and we stored them as glycerol stocks at -80°C. When we needed the plasmid we grew the E. coli cells and isolated the plasmid DNA. Then using a restriction enzyme we linearised the DNA and we separated it by Agarose gel Electrophoresis and purified it using the Eppendorf® Perfectprep® Gel Cleanup Kit. The purified DNA was then dissolved in DEPC-treated H_2O and it was then used as template for the Digoxigenin labelling of the anti-sense RNA probes.

The rest of the probes we used where generated by PCR (Table 3). First, a region usually in the 3'UTR of each gene was selected and amplification of between 350-1800 bp. Products were cloned either by direct PCR cloning (pGEM T-easy Promega) or by directional cloning into the Bluesctript plasmid using PstI and EcoRI restriction sites engineered on the primers. Directional PCR from the ligation using a primer upstream of the T7 promoter and the antisence primer always produced the template for antisense RNA synthesis. The PCR product was purified by Agarose gel Electrophoresis and the Eppendorf® Perfectprep® Gel Cleanup Kit followed by RNA synthesis. The primers used for each probe are listed below.

Table 2. ISH probes donated.

Gene name	Source
mSox3	M. Wegner
mSox6	M. Wegner
mSox8	M. Wegner
mSox9	M. Wegner
cSox1	H. Kondo
cSox2	H. Kondo
cSox3	H. Kondo
cSox21	H. Kondo
mDbx1	T. Jessell
mDbx2	T. Jessell
mSlit1	M. Tessier-Lavigne
mPax6	P. Gruss
mHes1	R. Kageyama
rSox10	W. D. Richardson
mPDGFRα	W. D. Richardson
mOlig2	W. D. Richardson
Dll1	Ryo Kageyama
Dll3	Ryo Kageyama
Jagged	Ryo Kageyama
Hey1	Ryo Kageyama
Hey2	Ryo Kageyama

Table 3. ISH probes generated.

Gene name	Forward oligo	Reverse oligo	Size (bp)
cGlast	aatattctgcagcagcccaaacctatcg	cctctatcacagaattcccc	760
cSulf1	gcactgcaggtagaaggcagcgcctcacc	aatgaattcccccatgtgccttttgcatc	1150
cSulf2	gcactgcagaggaagcacagtgtacagag	ttaagaattcacttcttacatagacgttcc	1050
mFgfr3	gtcgacctgcagaaattacgggtacctgaagg	tcgaggaattctgaaaggtagcagtctaggc	912
mGFAP	attctgcagctagaacttgggtggg	aattgaattctctgcctcagggtgccgagg	940
mGlast	gtcgacctgcagcagcccaaacctatcg	tcgaggaattcgggaagtgtaccccagaagg	839
mHes5	cgacctgcaggaggcggtacag	aattgaattctgcttgcccagcagcaacgg	710
mIrx3	aactgcagtgcttccgttctcttccagg	ggaattcagacaggcttgttctctccg	470
mMBP	cgacctgcagccctccccgctcagccttcc	aattgaattcgtcccacttccagatgcgcc	960
mNfia	tactacagcatgagtccagg	aatagaattctcccgtggtccaacactgac	885
mNkx6.1	gcctgcagctcccctccgcttccctctc	aattgaattcctcatcagcgcaccgtgc	600
mPtch1	aactgcagactggcagccgagac	cggaattcgagcggtccctattgctagg	1500
mS100β	ctgcaggaagaataagaagc	aatagaattcggtctcactcatgttcaaag	490
mSfrp2	aactgcagtgcacctgtgaggag	ggaattctgtagctgggatgggaacgc	810
mSox1	Wood and Episkopou., 1999		
mSox2	cgacctgcagtacaactccatgac	aattgaattccctccagatctatacatggtcc	780
mSox5	aactgcagcagttctatgctgc	cggaattcccaggtgctgtttgctgagg	1000
mSox21	gctagcgaattcgtataggtgtcaggcagagg	agatccgggctgtgttctgc	500
mSulf1	aacactgcagctgagaagcagataggatgg	aatgaattcatgttgacagactcaccagg	690
mSulf2	aacactgcagctgggagacctgacagaagg	aatgaattcgcaggaacactgtaagatgg	860

1.9.2 Hybridisation of DIG-labelled RNA probes to tissue mRNA

Solutions used:

a. RNase free 0.2M Phosphate Buffer (PB), pH 7.2

15 mM	$Na_2HPO_4.7H_2O$
5 mM	$NaH_2PO_4.H_2O$

Disolve in DEPC-treated ddH_2O.

b. 4% PFA in 0.1 M RNase free PB[f] (w/v).

c. RNase free Phosphate Buffered Saline (PBS)

0.1 M	PB
125 mM	NaCl

Disolve in DEPC-treated ddH_2O.

d. RNase free 20% Sucrose[f] (w/v).

20g	Sucrose
50ml	RNase free 0.2 M PB

Bring the volume up to 100ml with DEPC-treated ddH_2O.

e. 10X "Salts"[a]:

2 M	NaCl
100 mM	Tris.HCl pH 7.5
50 mM	$NaH_2PO_4.2H_2O$
50 mM	Na_2HPO_4
50 mM	0.5M EDTA

DEPC-treated and then autoclaved.

f. Hybridisation Buffer:

1X "Salts"

50% (v/v) deionised formamide

10% (w/v) dextran sulphate (Sigma)

0.1 mg/ml yeast tRNA (Roche)

1X Denhardt's solution (Invitrogen)

All the reagents must be RNase free.

g. Washing solution (v/v):

1X SSC

50% Formamide

0.1% Tween-20

h. 5X MABT (v/v):

0.5 M Maleic Acid

0.75 M NaCl

0.5% Tween-20

pH to 7.5 with NaOH

i. Blocking solution

2% (w/v) Blocking reagent (Roche)

1.25X MABT

10% (v/v) Heat inactivated Sheep Serum

j. Alkaline-phosphatase staining buffer (without NBT/BCIP) (v/v):

100 mM NaCl

50 mM $MgCl_2$

100 mM Tris.HCl pH 9.5

0.1% Tween-20

k. Nitroblue tetrazolium salt (NBT - Roche) 100 mg/ml dissolved in 70% Dimethylformamide.

l. 5-bromo-4-chloro-3-indolyl-phosphate (BCIP - Roche) 50 mg/ml dissolved in Dimethylformamide.

m. 10% (w/v) Polyvinyl alcohol (PVA).

n. Staining Buffer with NBT/BCIP (v/v):

50% Staining buffer
25 mM $MgCl_2$
0.1 mg/ml BCIP
0.1 mg/ml NBT
5% PVA

o. Fluka DPX mounting medium

Mouse embryos were fixed overnight in 4% PFA (0.1 M PB), rinsed in 0.1 M PBS, cryoprotected in 20% Sucrose (o.1 M PB) overnight and embedded in Tissue-Tek® OCT Compound and stored in -80°C until required. Embryos were sectioned in a cryostat (10-15 μM) and collected on a Menzel-Gläser Superfrost Ultra Plus slide. The slides were let to dry in a dust-free environment for two hours and then they were put on a humidified chamber with a 3 sheets of Whatman paper soaked with washing solution without Tween-20. The probes were diluted in hybridisation buffer and warmed at 70°C for 10 minutes. Then they were added on the slides and cover slips were put on top of the probe. The humidified chamber was sealed with tape and incubated at 65°C overnight.

The next day slides were placed in a coplin jar containing pre-warmed washing solution and let them washed at 65°C for about 30 minutes or until the cover slips fell off. Slides were washed for two more times, 30 minutes each at 65°C with washing solution

and twice with 1X MABT at room temperature for 20 minutes each. After the washes slides were put on an immunohistochemistry humidified box and blocking solution was added to the sections for an hour at room temperature, followed by the addition of the anti-Digoxigenin antibody conjugated with Alkaline Phosphatase (Roche Anti-Digoxigenin-AP, Fab fragments) diluted in 1:1000 in blocking solution. The slides were incubated overnight at 4^{o}C. Slides were then washed for 3 times in 1X MABT for 20 minutes each and staining buffer without NBT/BCIP for 2 times 10 minutes each. Finally the slides were put in coplin jars with staining buffer, wrapped in aluminium foil and incubated at 37^{o}C for 6-48 hours. When the enzymatic reaction was deemed completed the slides were washed in water dehydrated in 60%, 80%, 95%, and 100% Ethanol and finally placed in Xylene. The slides were then mounted with DPX mounting medium and were visualised with a white-light microscope.

1.10 Immunohistochemistry

Solutions used:

a. 0.2M Phosphate Buffer (PB), pH 7.2

15 mM	$Na_2HPO_4.7H_2O$
5 mM	$NaH_2PO_4.H_2O$

Disolved in ddH_2O.

b. 4% PFA in 0.1 M PB[f] (w/v).

c. MEMFA Fixative, pH 7.4 (v/v)

0.1 M	MOPS
2 mM	EGTA
1 mM	$MgSO_4$
3.7 %	formaldehyde

Dissolved in ddH_2O.

d. Phosphate Buffered Saline (PBS)

0.1 M	PB
125 mM	NaCl

Disolved in ddH_2O.

e. 20% Sucrose[f] (w/v).

20g	Sucrose
50ml	0.2 M PB

Bring the volume up to 100ml with ddH_2O.

f. Blocking solution

0.1 M	PB
0.1%	Triton-X
0.1%	Azide
1%	Heat inactivated Donkey Serum[f]

Dissolved in ddH_2O.

Mouse embryos were fixed depending on antibody requirements and/or embryo stage in either 4% PFA or MEMFA fixative between 30 minutes to overnight for 4% PFA and 20 minutes to 3 hours for MEMFA, and rinsed in 0.2 M PB three times for 30 minutes each. Then they were cryoprotected in 20% Sucrose overnight and embedded in Tissue-Tek® OCT Compound and stored at -80^{o} until required. Embryos were sectioned in a cryostat (12-15 μM) and collected on a Menzel-Gläser Superfrost Ultra Plus slide. The slides were let to dry for one hour and then they were put on a humidified chamber with some water on the bottom. A line with PAP Pen was drawn around the sections and the sections were blocked with blocking solution for 10 minutes. The primary antibodies were then diluted in blocking solution and they were put on the sections. The sections incubated overnight at 4^{o}C. The dilutions of the primary antibodies are shown in Table 3.

Table 4. Antibodies used.

Antigen name	*Antibody code*	*Source*	*Species raised in*	*Dilution*	*Fixation conditions*
GFP	A11122	Invitrogen	Rabbit	1:4000	PFA or MEMFA
Glast	AB1782	Chemicon	guinea pig	1:500	30-45 minutes MEMFA
Mash1		gift from F. Guillemot	mouse	1:500	30-45 minutes MEMFA
Ngn2	sc-19234	Santa Cruz	goat	1:200	30-45 minutes MEMFA
Nkx2.2	74.5A5	Hybridoma	mouse	1:50	20-40 minutes MEMFA
Nkx6.1	F55A12	Hybridoma	mouse	1:200	30-45 minutes MEMFA
Olig2	sc-19967	Santa Cruz	goat	1:500	30-45 minutes MEMFA
Olig2		gift from B. Novitch	goat	1:200	30-45 minutes MEMFA
Pax3	Pax3	Hybridoma	mouse	1:20	30-45 minutes MEMFA
Pax6	AB5409	Chemicon	rabbit	1:2000	30-45 minutes MEMFA
s100β	s2657	Sigma	mouse	1:500	overnight PFA
Sonic hedgehog (SHH)	5E1	Hybridoma	Mouse	1:50	30-45 minutes MEMFA fixation and 10 minutes Aceton Post-fixation
Sox1	sc-17318	Santa Cruz	goat	1:200	30-45 minutes MEMFA
Sox1		generated in house	guinea pig	1:8000	
Sox9	sc-20095	Santa Cruz	rabbit	1:500	30-45 minutes MEMFA
Sox10		gift from M. Wegner	guinea pig	1:1000	overnight PFA

The primary antibodies were removed in the next day and the sections were washed with PBS three times for 10 minutes each time. After the PBS washes we put the secondary antibodies that were diluted in blocking solution. The secondary antibodies were chosen by the species that the primary antibody was raised in. All the secondary antibodies were Invitrogen Alexa and their dilution was 1 in 2000. The sections were incubated at room temperature for 60 to 75 minutes and then the secondary antibodies were removed and the sections washed two times with PBS for 10 minutes each wash. We, then, applied 1 in 10000 dilution of Hoechst in PBS, for 15 seconds and immediately we washed with PBS for another 10 minutes. At the end, we covered the slides with cover slips that had three drops of DEKO fluorescent mounting medium and we put nail varnish at the edges of the cover slips. The sections were visualised and photographed using a Leica Confocal Microscope (model TSL).

1.11 LacZ Staining

LacZ is an Escherichia coli gene which is part of the Lac operon and is responsible for the production of the enzyme β-galactosidase. The physiological function of β-galactosidase is to cleave the disaccharide lactose into glucose and galactose. In molecular biology, LacZ is used as a reporter gene because it has the ability to cleave X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and release a blue colour. Another form of the LacZ gene that is used in molecular biology is the β-geo gene which is a fusion between LacZ and Neomycin resistance gene, which is used for antibiotic selection for the cell clones that carry the transgene.

Solutions used:

a. 0.1M Sodium Phosphate Buffer, pH 7.2

0.5M Na_2HPO4
0.5M NaH_2PO_4

b. Fixation solution

0.2%	Glutaraldehyde
2%	Formaldehyde
5mM	EGTA, pH 7.3
2mM	$MgCl_2$

Dissolve in 0.1M Sodium Phosphate buffer

c. Rinse solution

2mM	$MgCl_2$
0.1%	Sodium Deoxycholate
0.2%	NP 40

Dissolve in 0.1 M Sodium Phosphate buffer

d. Stain solution

1 mg/mL	X-Gal
5mM	K Ferricyanide
5mM	K Ferrocyanide

Dissolve in Rinse Buffer

Mouse embryos were put in Fixation Buffer for one hour, in room temperature, shaking. Then, the embryos were washed with Rinse buffer twice over one hour. After that, the rinse was replaced by the Stain solution. The tube was covered with Aluminium foil and incubated shaking for 5 to 7 hours at 37^{o}C. At the end of the reaction, the embryos were washed with Rinse buffer and fixed in Formalin at 4^{o}C overnight, embedded in Tissue-Tek® OCT Compound and stored at -80^{o}C until required. The frozen embryos were sectioned in a cryostat, collected on a Menzel-Gläser Superfrost Ultra Plus slide and visualised and photographed with a light microscope.

1.12 Chick in ovo electroporation

Fertilised chick eggs were incubated at 38^{o}C for about 40 hours, until the chick embryos reach the E2.0 stage. A 50-ml syringe was used in order to remove 10 ml of the egg white. Then the top of the egg was taped and opened by curled scissors in order to open to expose the embryo. A Shh expression construct (a gift from James Briscoe) was electroporated in E2.0 embryos at 100 ng/μl and electroporation was carried out using 0.2 mm electrodes (Nepa Gene Corporation, Chiba, Japan) and four pulses of 20V (Intracel, TSS20). After the electroporation we covered the embryos with some egg white to chill them and continued incubation for two more days (stage E4.0) at 38^{o}C and they were harvested in PBS and processed for in situ hybridisation and immunohistochemisty.

1.13 Animal handling

1.13.1 Breeding

All the animals were bred in an animal house facility with controlled temperature, humidity and light hours. The hygiene standards of the animal house were very high and blood samples were sent for virus and bacterial tests every six months. For the breeding of the different mouse lines used in this project we used wild type mice CD1 and C57BL6. All the animals were breed in reasonable numbers for the needs of the experiments.

1.13.2 Genotyping

Genotyping was carried out by PCR analysis. The set of primers for each genotyping is shown in Table 3. Pax6 heterozygous animals were identified morphologically by the small eye and the absence of the eye lens. The embryos were also genotyped by PCR analysis using the same set of primers shown in Table 3 with the exception of Sox1$^{GFP/GFP}$ embryos that were identified using GFP intensity on whole

mount embryos using an epi-fluorescent UV light source and scored for microphthalmia. Pax6 mutant embryos were identified morphologically by the absence of the eye and the shape of the telencephalon.

1.13.3 Time mating

Female mice were selected to be in the oistro-cycle (oistro-selection) and were put for mating in the male cages. The females that were found to be plugged were considered to be pregnant for E0.5 embryos and were separated from the male cage.

1.13.4 Euthanasia

The number of the sacrificed animals was limited to the absolutely necessary for the needs of the project. All the animals were killed by cervical dislocation. .

1.13.5 Embryo harvesting

The pregnant mother was killed and the uterus horns were removed and placed in cold PBS. With the help of a stereoscope, the embryos were harvested from the uterine horns and placed in cold PBS. The guts and organs of embryos older than E13.5 were removed in order to have better penetration of the fixatives.

Part 2: Genetic tools used in this study

2.1 Generation of the Sox1^{-GFPn} transgenic mouse

The Sox1^{-GFPn} transgenic mouse is a reporter mouse line, which expresses a nuclear form of GFP under the control of the Sox1 promoter. The transgenic mouse was generated by modifying a Sox1 BAC using genetic recombination to replace the Sox1 ORF with a GFP expressing cassette.

2.1.1 GFP expressing cassette

The cassette which replaced the Sox1 ORF was a part of the 63GNeoKan plasmid which was generated by colleagues. The cassette had a GFP gene (Invitrogen) retrofitted with an NLS, obtained from commercial YFP-NLS (Invitrogen). In front of the GFP gene is an internal ribosome entry site (IRES). After the IRES-GFP-NLS there was an antibiotic resistance gene for Neomycine and Kanamycine under the control of both eukaryotic (PGK) and prokaryotic (EM7) promoters. The antibiotic resistance gene and the promoters were flanked between FRT recombination recognition sites (Fig. 31)

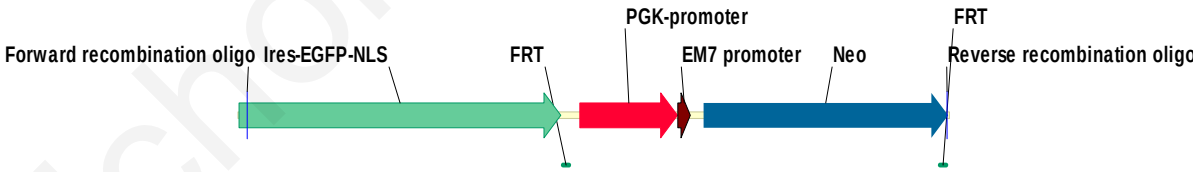

Figure 31. The GFP-NLS expressing cassette.

2.1.2 Modification of Sox1 BAC

The GFP-NLS expressing cassette was amplified by PCR. The oligos used for this PCR was the following:

Forward oligo: *cctggagcccgtcagtatcggcggaattccagctgagcgccggtcgctaccattaccagttggtctggt gtcaaaaataa***ccccctaacgttactggccg**

Reverse oligo: *acatgaccttccactcggccccgaggcgcttgctgatctccgagttgtgcatcttggggttttcctgggcc atcttgcgtttggcc***ggcctttggtacc**

In **bold** is the sequence that anneals for the PCR and in *italics* is the homology with the Sox1 locus.

The ORF of the Sox1 BAC (clone RP23-118F24) was replaced with the GFP-NLS expressing cassette by the BAC homologues recombination. The bacterial clones which survived the Kan (for the GFP-NLS cassette) and Cm (for the Sox1 BAC) selection were screened by PCR using a forward oligo in the 5' Sox1 region (ctcaacttggccacgactgc) and a reverse oligo in the GFP gene (tgtcggccatgatatagacg). The positive clones gave a 1300bp PCR product. In order to confirm if these clones were positives we isolated the BAC DNA from them and digested it with XhoI and NotI and checked if the restriction pattern of the BAC DNA was correct.

After the modification of the Sox1 BAC we removed the antibiotic resistance gene and the promoters by FLP-FRT recombination. FLP-FRT recombination is a site-directed recombination technology. FLPe recombinase recognises the two identical FRT sides and deletes everything between them. The FLP-FRT recombination was performed by the 706-FLP expression plasmid kit (Gene Bridges).

2.1.3 Preparation of the BAC DNA

A big quantity of the positive BAC DNA was isolated from bacterial cultures. This BAC DNA was then linearised by restriction with the endonuclease enzyme NotI.

Then the linear BAC DNA was cleaned by PFGE, resuspended in microinjection buffer (10mM Tris, 0.1 mM EDTA, 30μM spermine, 70μM spermidine, 100mM NaCl) and provided to the transgenic unit for pronucleus injections.

2.2 Mutant lines used in this study

Other lines used but not generated in this study are:

a. $Sox1^{KO}$ (Aubert et al., 2003; Malas et al., 2003)
b. Pax6 (Sey; Hill et al., 1991)
c. $Nkx2.2^{KO}$ (Sussel et al., 1998)
d. $Sox2^{RSox1}$ (Ekonomou et al., 2005)
e. $Rosa26^{stopYFP}$ (Srinivas et al., 2001)
f. $PS\text{-}1^{KO}$ (Shen et al., 1997)

Appendix B

Work generated but not included in this thesis

Part of my initial project plan was to generate a conditional allele for Sox21, a gene that was thought to be a critical mediator of all proneural genes in regulating the onset of neurogenesis. During the course of developing this line we were informed by a group in Sweden, headed by Dr Jonas Muhr at the Karolinska Institute, that they had generated these mice and surprisingly they had no neurological defect. I nevertheless continued to generate these mice as part of my training in gene targeting and have successfully completed this task. Below I will briefly describe the targeting allele made and will present a photo of adult mice bearing a germ line deletion of the Sox21 gene.

Sox21 targeting construct:

The Sox21 targeting construct had the Sox21 ORF between two LoxP repeat sites which are recognised by the CRE recombinase. Next to the 5' LOX P site was the eYFP gene which would be activated after the deletion of the Sox21 ORF. The construct also contained a selectable marker (Kan/Neo) under the control of a PGK and an EM7 promoter (Fig. 32).

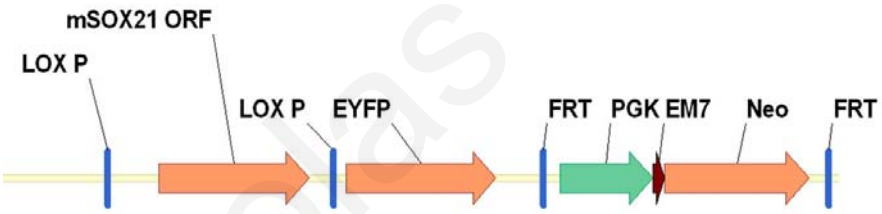

Figure 32. The Sox21 targeting construct.

Phenotyping characteristics of homozygous Sox21 mutant mice

These mice are born normal but fail to develop any hair and are considerably smaller than their litter-mate controls (Fig. 33). Although we have been informed by Dr Jonas Muhr that Sox21-null embryos have no neurological defects, it is possible that glial lineages may be affected, but I did not analyse any glial markers. I have however, generated a BAC-transgenic line that expresses nuclear GFP from a Sox21 BAC and used

this line for a very selective analysis of its expression in the developing spinal cord to get a rough idea what the expression pattern is. I found that all Olig2^{+} OLPs express the transgene but additional GFP^{+} cells were noted in the GM migrating from the VZ after e12.5. These cells are most likely AS progenitors since they do not seem to concentrate preferentially in any progenitor domain and their late expression suggests that they maybe glial progenitors (Fig. 34).

Figure 33. Homozygous mutant Sox21 mouse.

The Sox21 null mouse is born normal and can survive, but fails to develop any hair and it is smaller than its littermate controls.

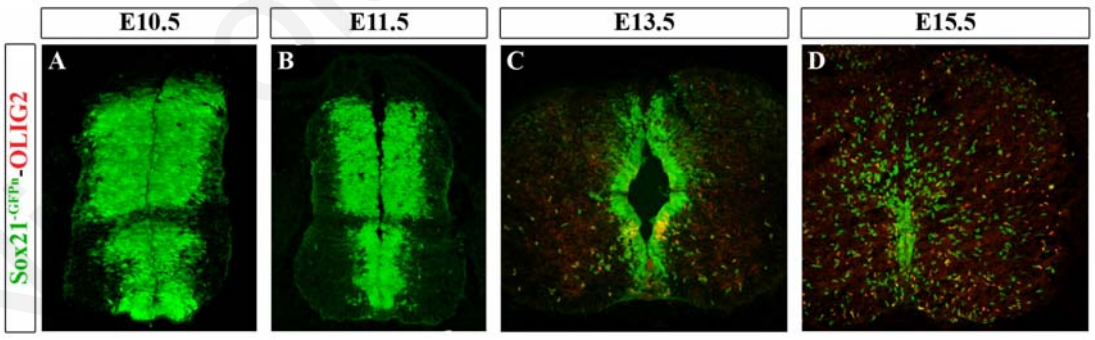

Figure 34. Gross analysis of Sox21^{-GFPn} expression in the developing spinal cord

Sox21 expression is reported here using a BAC transgenic line that I have generated expressing GFPn. The expression pattern is shown only in relation to Olig2 to show that all OLIg2 expressing cells (OLPs) are Sox21^{+}.

References

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., and Brulet, P. (1995). Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. Development *121*, 3279-3290.

Agius, E., Soukkarieh, C., Danesin, C., Kan, P., Takebayashi, H., Soula, C., and Cochard, P. (2004). Converse control of oligodendrocyte and astrocyte lineage development by Sonic hedgehog in the chick spinal cord. Dev Biol *270*, 308-321.

Ai, X., Do, A. T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U., and Emerson, C. P., Jr. (2003). QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J Cell Biol *162*, 341-351.

Ai, X., Kitazawa, T., Do, A. T., Kusche-Gullberg, M., Labosky, P. A., and Emerson, C. P., Jr. (2007). SULF1 and SULF2 regulate heparan sulfate-mediated GDNF signalling for esophageal innervation. Development *134*, 3327-3338.

Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S., and Marti, E. (2008). Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression. Development *135*, 237-247.

Ang, S. L., and Rossant, J. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. Cell *78*, 561-574.

Antanitus, D. S., Choi, B. H., and Lapham, L. W. (1975). Immunofluorescence staining of astrocytes in vitro using antiserum to glial fibrillary acidic protein. Brain Res *89*, 363-367.

Anthony, T. E., Klein, C., Fishell, G., and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron *41*, 881-890.

Appel, B., Givan, L. A., and Eisen, J. S. (2001). Delta-Notch signalling and lateral inhibition in zebrafish spinal cord development. BMC Dev Biol *1*, 13.

Aubert, J., Stavridis, M. P., Tweedie, S., O'Reilly, M., Vierlinger, K., Li, M., Ghazal, P., Pratt, T., Mason, J. O., Roy, D., and Smith, A. (2003). Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice. Proc Natl Acad Sci U S A *100 Suppl 1*, 11836-11841.

Aza-Blanc, P., Lin, H. Y., Ruiz i Altaba, A., and Kornberg, T. B. (2000). Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. Development *127*, 4293-4301.

Bai, C. B., and Joyner, A. L. (2001). Gli1 can rescue the in vivo function of Gli2. Development *128*, 5161-5172.

Bai, C. B., Stephen, D., and Joyner, A. L. (2004). All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev Cell *6*, 103-115.

Bailey, M. S., and Shipley, M. T. (1993). Astrocyte subtypes in the rat olfactory bulb: morphological heterogeneity and differential laminar distribution. J Comp Neurol *328*, 501-526.

Bang, A. G., Papalopulu, N., Goulding, M. D., and Kintner, C. (1999). Expression of Pax-3 in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. Dev Biol *212*, 366-380.

Barry, D., and McDermott, K. (2005). Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. Glia *50*, 187-197.

Berggren, K., McCaffery, P., Drager, U., and Forehand, C. J. (1999). Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. Dev Biol *210*, 288-304.

Berta, P., Hawkins, J. R., Sinclair, A. H., Taylor, A., Griffiths, B. L., Goodfellow, P. N., and Fellous, M. (1990). Genetic evidence equating SRY and the testis-determining factor. Nature *348*, 448-450.

Bertrand, N., Medevielle, F., and Pituello, F. (2000). FGF signalling controls the timing of Pax6 activation in the neural tube. Development *127*, 4837-4843.

Blentic, A., Gale, E., and Maden, M. (2003). Retinoic acid signalling centres in the avian embryo identified by sites of expression of synthesising and catabolising enzymes. Dev Dyn *227*, 114-127.

Bowles, J., Schepers, G., and Koopman, P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev Biol *227*, 239-255.

Braquart-Varnier, C., Danesin, C., Clouscard-Martinato, C., Agius, E., Escalas, N., Benazeraf, B., Ai, X., Emerson, C., Cochard, P., and Soula, C. (2004). A subtractive approach to characterize genes with regionalized expression in the gliogenic ventral neuroepithelium: identification of chick sulfatase 1 as a new oligodendrocyte lineage gene. Mol Cell Neurosci *25*, 612-628.

Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol *11*, 43-49.

Briscoe, J., and Novitch, B. G. (2008). Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. Philos Trans R Soc Lond B Biol Sci *363*, 57-70.

Briscoe, J., Pierani, A., Jessell, T. M., and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell *101*, 435-445.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L., and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature *398*, 622-627.

Bylund, M., Andersson, E., Novitch, B. G., and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat Neurosci *6*, 1162-1168.

Cai, J., Qi, Y., Hu, X., Tan, M., Liu, Z., Zhang, J., Li, Q., Sander, M., and Qiu, M. (2005). Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. Neuron *45*, 41-53.

Chamberlain, C. E., Jeong, J., Guo, C., Allen, B. L., and McMahon, A. P. (2008). Notochord-derived Shh concentrates in close association with the apically positioned basal body in neural target cells and forms a dynamic gradient during neural patterning. Development *135*, 1097-1106.

Chang, D. T., Lopez, A., von Kessler, D. P., Chiang, C., Simandl, B. K., Zhao, R., Seldin, M. F., Fallon, J. F., and Beachy, P. A. (1994). Products, genetic linkage and limb patterning activity of a murine hedgehog gene. Development *120*, 3339-3353.

D'Ambrosio, R., Wenzel, J., Schwartzkroin, P. A., McKhann, G. M., 2nd, and Janigro, D. (1998). Functional specialization and topographic segregation of hippocampal astrocytes. J Neurosci *18*, 4425-4438.

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J Biol Chem *274*, 8143-8152.

Danesin, C., Agius, E., Escalas, N., Ai, X., Emerson, C., Cochard, P., and Soula, C. (2006). Ventral neural progenitors switch toward an oligodendroglial fate in response to increased Sonic hedgehog (Shh) activity: involvement of Sulfatase 1 in modulating Shh signalling in the ventral spinal cord. J Neurosci *26*, 5037-5048.

Delaune, E., Lemaire, P., and Kodjabachian, L. (2005). Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. Development *132*, 299-310.

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C. J., Gronostajski, R. M., and Anderson, D. J. (2006). The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. Neuron *52*, 953-968.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A. F., Talbot, W. S., Bouwmeester, T., and Hammerschmidt, M. (2000). Essential role of Bmp7 (snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. Development *127*, 343-354.

Diez del Corral, R., Breitkreuz, D. N., and Storey, K. G. (2002). Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development *129*, 1681-1691.

Domingos, P. M., Itasaki, N., Jones, C. M., Mercurio, S., Sargent, M. G., Smith, J. C., and Krumlauf, R. (2001). The Wnt/beta-catenin pathway posteriorizes neural tissue in Xenopus by an indirect mechanism requiring FGF signalling. Dev Biol *239*, 148-160.

Doniach, T. (1995). Basic FGF as an inducer of anteroposterior neural pattern. Cell *83*, 1067-1070.

Dupe, V., Davenne, M., Brocard, J., Dolle, P., Mark, M., Dierich, A., Chambon, P., and Rijli, F. M. (1997). In vivo functional analysis of the Hoxa-1 3' retinoic acid response element (3'RARE). Development *124*, 399-410.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. Cell *75*, 1417-1430.

Ekonomou, A., Kazanis, I., Malas, S., Wood, H., Alifragis, P., Denaxa, M., Karagogeos, D., Constanti, A., Lovell-Badge, R., and Episkopou, V. (2005). Neuronal migration and ventral subtype identity in the telencephalon depend on SOX1. PLoS Biol *3*, e186.

Episkopou, V., Arkell, R., Timmons, P. M., Walsh, J. J., Andrew, R. L., and Swan, D. (2001). Induction of the mammalian node requires Arkadia function in the extraembryonic lineages. Nature *410*, 825-830.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V., and Jessell, T. M. (1997a). Graded sonic hedgehog signalling and the specification of cell fate in the ventral neural tube. Cold Spring Harb Symp Quant Biol *62*, 451-466.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signalling required for the specification of motor neuron identity. Cell *87*, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M., and Briscoe, J. (1997b). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell *90*, 169-180.

Erter, C. E., Wilm, T. P., Basler, N., Wright, C. V., and Solnica-Krezel, L. (2001). Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. Development *128*, 3571-3583.

Fekany-Lee, K., Gonzalez, E., Miller-Bertoglio, V., and Solnica-Krezel, L. (2000). The homeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. Development *127*, 2333-2345.

Fogarty, M., Richardson, W. D., and Kessaris, N. (2005). A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. Development *132*, 1951-1959.

Fredieu, J. R., Cui, Y., Maier, D., Danilchik, M. V., and Christian, J. L. (1997). Xwnt-8 and lithium can act upon either dorsal mesodermal or neurectodermal cells to cause a loss of forebrain in Xenopus embryos. Dev Biol *186*, 100-114.

Fu, H., Qi, Y., Tan, M., Cai, J., Takebayashi, H., Nakafuku, M., Richardson, W., and Qiu, M. (2002). Dual origin of spinal oligodendrocyte progenitors and evidence for the cooperative role of Olig2 and Nkx2.2 in the control of oligodendrocyte differentiation. Development *129*, 681-693.

Gabay, L., Lowell, S., Rubin, L. L., and Anderson, D. J. (2003). Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. Neuron *40*, 485-499.

Genethliou, N., Panayiotou, E., Panayi, H., Orford, M., Mean, R., Lapathitis, G., and Malas, S. (2009). Spatially distinct functions of PAX6 and NKX2.2 during gliogenesis in the ventral spinal cord. Biochem Biophys Res Commun.

Genoud, S., Lappe-Siefke, C., Goebbels, S., Radtke, F., Aguet, M., Scherer, S. S., Suter, U., Nave, K. A., and Mantei, N. (2002). Notch1 control of oligodendrocyte differentiation in the spinal cord. J Cell Biol *158*, 709-718.

Giese, K., Cox, J., and Grosschedl, R. (1992). The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell *69*, 185-195.

Givogri, M. I., Costa, R. M., Schonmann, V., Silva, A. J., Campagnoni, A. T., and Bongarzone, E. R. (2002). Central nervous system myelination in mice with deficient expression of Notch1 receptor. J Neurosci Res *67*, 309-320.

Gotz, K., Briscoe, J., and Ruther, U. (2005). Homozygous Ft embryos are affected in floor plate maintenance and ventral neural tube patterning. Dev Dyn *233*, 623-630.

Gould, A., Itasaki, N., and Krumlauf, R. (1998). Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoid pathway. Neuron *21*, 39-51.

Gross, R. E., Mehler, M. F., Mabie, P. C., Zang, Z., Santschi, L., and Kessler, J. A. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron *17*, 595-606.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature *346*, 245-250.

Hall, A., Giese, N. A., and Richardson, W. D. (1996). Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors. Development *122*, 4085-4094.

Hamburger, V., and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. 1951. Dev Dyn *195*, 231-272.

Hammerschmidt, M., Serbedzija, G. N., and McMahon, A. P. (1996). Genetic analysis of dorsoventral pattern formation in the zebrafish: requirement of a BMP-like ventralizing activity and its dorsal repressor. Genes Dev *10*, 2452-2461.

Handler, M., Yang, X., and Shen, J. (2000). Presenilin-1 regulates neuronal differentiation during neurogenesis. Development *127*, 2593-2606.

Hansen, C. S., Marion, C. D., Steele, K., George, S., and Smith, W. C. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. Development *124*, 483-492.

Harland, R. (2000). Neural induction. Curr Opin Genet Dev *10*, 357-362.

Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W., and Cho, K. W. (1995). Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. Genes Dev *9*, 2923-2935.

Hemmati-Brivanlou, A., Kelly, O. G., and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell *77*, 283-295.

Hemmati-Brivanlou, A., and Melton, D. A. (1994). Inhibition of activin receptor signalling promotes neuralization in Xenopus. Cell *77*, 273-281.

Henrique, D., Tyler, D., Kintner, C., Heath, J. K., Lewis, J. H., Ish-Horowicz, D., and Storey, K. G. (1997). cash4, a novel achaete-scute homolog induced by Hensen's node during generation of the posterior nervous system. Genes Dev *11*, 603-615.

Hild, M., Dick, A., Rauch, G. J., Meier, A., Bouwmeester, T., Haffter, P., and Hammerschmidt, M. (1999). The smad5 mutation somitabun blocks Bmp2b signalling during early dorsoventral patterning of the zebrafish embryo. Development *126*, 2149-2159.

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D., and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature *354*, 522-525.

Hochstim, C., Deneen, B., Lukaszewicz, A., Zhou, Q., and Anderson, D. J. (2008). Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. Cell *133*, 510-522.

Holmberg, J., Hansson, E., Malewicz, M., Sandberg, M., Perlmann, T., Lendahl, U., and Muhr, J. (2008). SoxB1 transcription factors and Notch signalling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation. Development *135*, 1843-1851.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R., and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. Dev Biol *162*, 402-413.

Isaacs, H. V., Pownall, M. E., and Slack, J. M. (1998). Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3. Embo J *17*, 3413-3427.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev *9*, 3136-3148.

Iso, T., Kedes, L., and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signalling pathway. J Cell Physiol *194*, 237-255.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet *1*, 20-29.

Jones-Villeneuve, E. M., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol *94*, 253-262.

Justice, N. J., and Jan, Y. N. (2002). Variations on the Notch pathway in neural development. Curr Opin Neurobiol *12*, 64-70.

Kageyama, R., Ohtsuka, T., and Kobayashi, T. (2008). Roles of Hes genes in neural development. Dev Growth Differ *50 Suppl 1*, S97-103.

Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R., and Kondoh, H. (1998). Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. Development *125*, 2521-2532.

Kan, L., Israsena, N., Zhang, Z., Hu, M., Zhao, L. R., Jalali, A., Sahni, V., and Kessler, J. A. (2004). Sox1 acts through multiple independent pathways to promote neurogenesis. Dev Biol *269*, 580-594.

Kessaris, N., Pringle, N., and Richardson, W. D. (2008). Specification of CNS glia from neural stem cells in the embryonic neuroepithelium. Philos Trans R Soc Lond B Biol Sci *363*, 71-85.

Kim, A. S., Anderson, S. A., Rubenstein, J. L., Lowenstein, D. H., and Pleasure, S. J. (2001). Pax-6 regulates expression of SFRP-2 and Wnt-7b in the developing CNS. J Neurosci *21*, RC132.

Klingensmith, J., Ang, S. L., Bachiller, D., and Rossant, J. (1999). Neural induction and patterning in the mouse in the absence of the node and its derivatives. Dev Biol *216*, 535-549.

Krauss, S., Concordet, J. P., and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell *75*, 1431-1444.

Kuroda, H., Fuentealba, L., Ikeda, A., Reversade, B., and De Robertis, E. M. (2005). Default neural induction: neuralization of dissociated Xenopus cells is mediated by Ras/MAPK activation. Genes Dev *19*, 1022-1027.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D., and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. Science *262*, 713-718.

Lei, Q., Jeong, Y., Misra, K., Li, S., Zelman, A. K., Epstein, D. J., and Matise, M. P. (2006). Wnt signalling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signalling in the neural tube. Dev Cell *11*, 325-337.

Lei, Q., Zelman, A. K., Kuang, E., Li, S., and Matise, M. P. (2004). Transduction of graded Hedgehog signalling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord. Development *131*, 3593-3604.

Liem, K. F., Jr., Jessell, T. M., and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. Development *127*, 4855-4866.

Lin, X. (2004). Functions of heparan sulfate proteoglycans in cell signalling during development. Development *131*, 6009-6021.

Litingtung, Y., and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. Nat Neurosci *3*, 979-985.

Liu, R., Cai, J., Hu, X., Tan, M., Qi, Y., German, M., Rubenstein, J., Sander, M., and Qiu, M. (2003). Region-specific and stage-dependent regulation of Olig gene expression and oligodendrogenesis by Nkx6.1 homeodomain transcription factor. Development *130*, 6221-6231.

Liu, Z., Hu, X., Cai, J., Liu, B., Peng, X., Wegner, M., and Qiu, M. (2007). Induction of oligodendrocyte differentiation by Olig2 and Sox10: evidence for reciprocal interactions and dosage-dependent mechanisms. Dev Biol *302*, 683-693.

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. Nat Rev Neurosci *7*, 93-102.

Lu, Q. R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C. D., and Rowitch, D. H. (2002). Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell *109*, 75-86.

Lu, Q. R., Yuk, D., Alberta, J. A., Zhu, Z., Pawlitzky, I., Chan, J., McMahon, A. P., Stiles, C. D., and Rowitch, D. H. (2000). Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron *25*, 317-329.

Ludwin, S. K., Kosek, J. C., and Eng, L. F. (1976). The topographical distribution of S-100 and GFA proteins in the adult rat brain: an immunohistochemical study using horseradish peroxidase-labelled antibodies. J Comp Neurol *165*, 197-207.

Lupo, G., Harris, W. A., and Lewis, K. E. (2006). Mechanisms of ventral patterning in the vertebrate nervous system. Nat Rev Neurosci *7*, 103-114.

Maden, M. (1999). Heads or tails? Retinoic acid will decide. Bioessays *21*, 809-812.

Malas, S., Duthie, S., Deloukas, P., and Episkopou, V. (1999). The isolation and high-resolution chromosomal mapping of human SOX14 and SOX21; two members of the SOX gene family related to SOX1, SOX2, and SOX3. Mamm Genome *10*, 934-937.

Malas, S., Postlethwaite, M., Ekonomou, A., Whalley, B., Nishiguchi, S., Wood, H., Meldrum, B., Constanti, A., and Episkopou, V. (2003). Sox1-deficient mice suffer from epilepsy associated with abnormal ventral forebrain development and olfactory cortex hyperexcitability. Neuroscience *119*, 421-432.

Marshall, G. M., Cheung, B., Stacey, K. P., Norris, M. D., and Haber, M. (1994). Regulation of retinoic acid receptor alpha expression in human neuroblastoma cell lines and tumor tissue. Anticancer Res *14*, 437-441.

Marti, E., Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature *375*, 322-325.

Masahira, N., Takebayashi, H., Ono, K., Watanabe, K., Ding, L., Furusho, M., Ogawa, Y., Nabeshima, Y., Alvarez-Buylla, A., Shimizu, K., and Ikenaka, K. (2006). Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. Dev Biol *293*, 358-369.

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A., and Joyner, A. L. (1998). Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development *125*, 2759-2770.

McBurney, M. W. (1993). P19 embryonal carcinoma cells. Int J Dev Biol *37*, 135-140.

Megason, S. G., and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development *129*, 2087-2098.

Mehler, M. F., Mabie, P. C., Zhu, G., Gokhan, S., and Kessler, J. A. (2000). Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. Dev Neurosci *22*, 74-85.

Mekki-Dauriac, S., Agius, E., Kan, P., and Cochard, P. (2002). Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord. Development *129*, 5117-5130.

Miller, R. H., and Raff, M. C. (1984). Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. J Neurosci *4*, 585-592.

Miller, R. H., Zhang, H., and Fok-Seang, J. (1994). Glial cell heterogeneity in the mammalian spinal cord. Perspect Dev Neurobiol *2*, 225-231.

Misson, J. P., Edwards, M. A., Yamamoto, M., and Caviness, V. S., Jr. (1988). Mitotic cycling of radial glial cells of the fetal murine cerebral wall: a combined autoradiographic and immunohistochemical study. Brain Res *466*, 183-190.

Molotkova, N., Molotkov, A., Sirbu, I. O., and Duester, G. (2005). Requirement of mesodermal retinoic acid generated by Raldh2 for posterior neural transformation. Mech Dev *122*, 145-155.

Muhr, J., Andersson, E., Persson, M., Jessell, T. M., and Ericson, J. (2001). Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell *104*, 861-873.

Muhr, J., Graziano, E., Wilson, S., Jessell, T. M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. Neuron *23*, 689-702.

Mukouyama, Y. S., Deneen, B., Lukaszewicz, A., Novitch, B. G., Wichterle, H., Jessell, T. M., and Anderson, D. J. (2006). Olig2+ neuroepithelial motoneuron progenitors are not multipotent stem cells in vivo. Proc Natl Acad Sci U S A *103*, 1551-1556.

Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H., and Takada, S. (2002). Wnt signalling plays an essential role in neuronal specification of the dorsal spinal cord. Genes Dev *16*, 548-553.

Muroyama, Y., Fujiwara, Y., Orkin, S. H., and Rowitch, D. H. (2005). Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. Nature *438*, 360-363.

Nery, S., Wichterle, H., and Fishell, G. (2001). Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain. Development *128*, 527-540.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M., and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. Dev Biol *199*, 93-110.

Nicolay, D. J., Doucette, J. R., and Nazarali, A. J. (2007). Transcriptional control of oligodendrogenesis. Glia *55*, 1287-1299.

Niederreither, K., McCaffery, P., Drager, U. C., Chambon, P., and Dolle, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. Mech Dev *62*, 67-78.

Nieuwkoop P. D., Boterenbrood E. C., Kremer A., Bloemsma F. F. S. N., Hoessels E. L. M. J., Meyer G. and Verheyen F. J. (1952). Activation and organization of the central nervous system in amphibians. J Exp Zool 120, 1–108.

Nishiguchi, S., Wood, H., Kondoh, H., Lovell-Badge, R., and Episkopou, V. (1998). Sox1 directly regulates the gamma-crystallin genes and is essential for lens development in mice. Genes Dev *12*, 776-781.

Norenberg, M. D., and Martinez-Hernandez, A. (1979). Fine structural localization of glutamine synthetase in astrocytes of rat brain. Brain Res *161*, 303-310.

Novitch, B. G., Chen, A. I., and Jessell, T. M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. Neuron *31*, 773-789.

Novitch, B. G., Wichterle, H., Jessell, T. M., and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron *40*, 81-95.

Ogawa, Y., Takebayashi, H., Takahashi, M., Osumi, N., Iwasaki, Y., and Ikenaka, K. (2005). Gliogenic radial glial cells show heterogeneity in the developing mouse spinal cord. Dev Neurosci *27*, 364-377.

Ono, K., Bansal, R., Payne, J., Rutishauser, U., and Miller, R. H. (1995). Early development and dispersal of oligodendrocyte precursors in the embryonic chick spinal cord. Development *121*, 1743-1754.

Orentas, D. M., Hayes, J. E., Dyer, K. L., and Miller, R. H. (1999). Sonic hedgehog signalling is required during the appearance of spinal cord oligodendrocyte precursors. Development *126*, 2419-2429.

Orentas, D. M., and Miller, R. H. (1996). The origin of spinal cord oligodendrocytes is dependent on local influences from the notochord. Dev Biol *177*, 43-53.

Park, H. C., and Appel, B. (2003). Delta-Notch signalling regulates oligodendrocyte specification. Development *130*, 3747-3755.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M., and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signalling in combination with a Gli2 mutation. Development *127*, 1593-1605.

Peng, C. Y., Yajima, H., Burns, C. E., Zon, L. I., Sisodia, S. S., Pfaff, S. L., and Sharma, K. (2007). Notch and MAML signalling drives Scl-dependent interneuron diversity in the spinal cord. Neuron *53*, 813-827.

Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Bose, J., Ruther, U., Ericson, J., and Briscoe, J. (2002). Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes Dev *16*, 2865-2878.

Pevny, L. H., Sockanathan, S., Placzek, M., and Lovell-Badge, R. (1998). A role for SOX1 in neural determination. Development *125*, 1967-1978.

Pierani, A., Brenner-Morton, S., Chiang, C., and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell *97*, 903-915.

Pownall, M. E., Isaacs, H. V., and Slack, J. M. (1998). Two phases of Hox gene regulation during early Xenopus development. Curr Biol *8*, 673-676.

Pownall, M. E., Tucker, A. S., Slack, J. M., and Isaacs, H. V. (1996). eFGF, Xcad3 and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus. Development *122*, 3881-3892.

Pringle, N. P., Guthrie, S., Lumsden, A., and Richardson, W. D. (1998). Dorsal spinal cord neuroepithelium generates astrocytes but not oligodendrocytes. Neuron *20*, 883-893.

Pringle, N. P., and Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. Development *117*, 525-533.

Pringle, N. P., Yu, W. P., Guthrie, S., Roelink, H., Lumsden, A., Peterson, A. C., and Richardson, W. D. (1996). Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog. Dev Biol *177*, 30-42.

Pringle, N. P., Yu, W. P., Howell, M., Colvin, J. S., Ornitz, D. M., and Richardson, W. D. (2003). Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains. Development *130*, 93-102.

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J., and Qiu, M. (2001). Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. Development *128*, 2723-2733.

Qi, Y., Tan, M., Hui, C. C., and Qiu, M. (2003). Gli2 is required for normal Shh signalling and oligodendrocyte development in the spinal cord. Mol Cell Neurosci *23*, 440-450.

Radtke, F., Wilson, A., and MacDonald, H. R. (2005). Notch signalling in hematopoiesis and lymphopoiesis: lessons from Drosophila. Bioessays *27*, 1117-1128.

Rallu, M., Corbin, J. G., and Fishell, G. (2002). Parsing the prosencephalon. Nat Rev Neurosci *3*, 943-951.

Rao, M. S. (1999). Multipotent and restricted precursors in the central nervous system. Anat Rec *257*, 137-148.

Rao, M. S., and Mayer-Proschel, M. (1997). Glial-restricted precursors are derived from multipotent neuroepithelial stem cells. Dev Biol *188*, 48-63.

Rao, M. S., Noble, M., and Mayer-Proschel, M. (1998). A tripotential glial precursor cell is present in the developing spinal cord. Proc Natl Acad Sci U S A *95*, 3996-4001.

Reversade, B., Kuroda, H., Lee, H., Mays, A., and De Robertis, E. M. (2005). Depletion of Bmp2, Bmp4, Bmp7 and Spemann organizer signals induces massive brain formation in Xenopus embryos. Development *132*, 3381-3392.

Richardson, W. D., Kessaris, N., and Pringle, N. (2006). Oligodendrocyte wars. Nat Rev Neurosci *7*, 11-18.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. Cell *75*, 1401-1416.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M., and et al. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. Cell *76*, 761-775.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. Cell *81*, 445-455.

Rowitch, D. H. (2004). Glial specification in the vertebrate neural tube. Nat Rev Neurosci *5*, 409-419.

Ruiz i Altaba, A. (1999). Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development *126*, 3205-3216.

Sakamoto, K., Ohara, O., Takagi, M., Takeda, S., and Katsube, K. (2002). Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification. Dev Biol *241*, 313-326.

Samanta, J., and Kessler, J. A. (2004). Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development *131*, 4131-4142.

Sandberg, M., Kallstrom, M., and Muhr, J. (2005). Sox21 promotes the progression of vertebrate neurogenesis. Nat Neurosci *8*, 995-1001.

Sasai, Y., Lu, B., Steinbeisser, H., and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature *377*, 757.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development *126*, 3915-3924.

Satow, T., Bae, S. K., Inoue, T., Inoue, C., Miyoshi, G., Tomita, K., Bessho, Y., Hashimoto, N., and Kageyama, R. (2001). The basic helix-loop-helix gene hesr2 promotes gliogenesis in mouse retina. J Neurosci *21*, 1265-1273.

Scardigli, R., Schuurmans, C., Gradwohl, G., and Guillemot, F. (2001). Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. Neuron *31*, 203-217.

Schmitt, A., Asan, E., Puschel, B., and Kugler, P. (1997). Cellular and regional distribution of the glutamate transporter GLAST in the CNS of rats: nonradioactive in situ hybridization and comparative immunocytochemistry. J Neurosci *17*, 1-10.

Shen, J., Bronson, R. T., Chen, D. F., Xia, W., Selkoe, D. J., and Tonegawa, S. (1997). Skeletal and CNS defects in Presenilin-1-deficient mice. Cell *89*, 629-639.

Shirasaki, R., and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. Annu Rev Neurosci *25*, 251-281.

Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A. M., Lovell-Badge, R., and Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature *346*, 240-244.

Sommer, I., and Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev Biol *83*, 311-327.

Soriano, S., Kang, D. E., Fu, M., Pestell, R., Chevallier, N., Zheng, H., and Koo, E. H. (2001). Presenilin 1 negatively regulates beta-catenin/T cell factor/lymphoid enhancer

factor-1 signalling independently of beta-amyloid precursor protein and notch processing. J Cell Biol *152*, 785-794.

Spemann, H. and Mangold, H. (1924). Über induktion von Embryonalagen durch Implantation Artfremder Organisatoren. Roux' Arch. Entw. Mech 100, 599–638.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol *1*, 4.

Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A., and Briscoe, J. (2005). A gradient of Gli activity mediates graded Sonic Hedgehog signalling in the neural tube. Genes Dev *19*, 626-641.

Stern, C. D. (2001). Initial patterning of the central nervous system: how many organizers? Nat Rev Neurosci *2*, 92-98.

Stolt, C. C., Lommes, P., Friedrich, R. P., and Wegner, M. (2004). Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. Development *131*, 2349-2358.

Stolt, C. C., Lommes, P., Sock, E., Chaboissier, M. C., Schedl, A., and Wegner, M. (2003). The Sox9 transcription factor determines glial fate choice in the developing spinal cord. Genes Dev *17*, 1677-1689.

Stolt, C. C., Schlierf, A., Lommes, P., Hillgartner, S., Werner, T., Kosian, T., Sock, E., Kessaris, N., Richardson, W. D., Lefebvre, V., and Wegner, M. (2006). SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. Dev Cell *11*, 697-709.

Storey, K. G., Goriely, A., Sargent, C. M., Brown, J. M., Burns, H. D., Abud, H. M., and Heath, J. K. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. Development *125*, 473-484.

Streit, A., Berliner, A. J., Papanayotou, C., Sirulnik, A., and Stern, C. D. (2000). Initiation of neural induction by FGF signalling before gastrulation. Nature *406*, 74-78.

Sugimori, M., Nagao, M., Bertrand, N., Parras, C. M., Guillemot, F., and Nakafuku, M. (2007). Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. Development *134*, 1617-1629.

Sun, T., Pringle, N. P., Hardy, A. P., Richardson, W. D., and Smith, H. K. (1998). Pax6 influences the time and site of origin of glial precursors in the ventral neural tube. Mol Cell Neurosci *12*, 228-239.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L., and German, M. S. (1998). Mice lacking the homeodomain

transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development *125*, 2213-2221.

Suter, D. M., Tirefort, D., Julien, S., and Krause, K. H. (2008). A Sox1 to Pax6 switch drives neuroectoderm to radial glia progression during differentiation of mouse embryonic stem cells. Stem Cells.

Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M., and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. Dev Biol *216*, 282-296.

Takebayashi, H., Nabeshima, Y., Yoshida, S., Chisaka, O., Ikenaka, K., and Nabeshima, Y. (2002). The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Curr Biol *12*, 1157-1163.

Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H., and Honjo, T. (2001). Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. Neuron *29*, 45-55.

Taylor, M. K., Yeager, K., and Morrison, S. J. (2007). Physiological Notch signalling promotes gliogenesis in the developing peripheral and central nervous systems. Development *134*, 2435-2447.

Tekki-Kessaris, N., Woodruff, R., Hall, A. C., Gaffield, W., Kimura, S., Stiles, C. D., Rowitch, D. H., and Richardson, W. D. (2001). Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. Development *128*, 2545-2554.

Timmer, J. R., Wang, C., and Niswander, L. (2002). BMP signalling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. Development *129*, 2459-2472.

Uchikawa, M., Kamachi, Y., and Kondoh, H. (1999). Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken. Mech Dev *84*, 103-120.

Vallstedt, A., Klos, J. M., and Ericson, J. (2005). Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. Neuron *45*, 55-67.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M., and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. Neuron *31*, 743-755.

van de Wetering, M., and Clevers, H. (1992). Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. Embo J *11*, 3039-3044.

Voigt, T. (1989). Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol *289*, 74-88.

Wang, B., Fallon, J. F., and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell *100*, 423-434.

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., and Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. Neuron *21*, 63-75.

Wegner, M. (2008). A matter of identity: transcriptional control in oligodendrocytes. J Mol Neurosci *35*, 3-12.

Wijgerde, M., McMahon, J. A., Rule, M., and McMahon, A. P. (2002). A direct requirement for Hedgehog signalling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. Genes Dev *16*, 2849-2864.

Wilson, L., Gale, E., Chambers, D., and Maden, M. (2004). Retinoic acid and the control of dorsoventral patterning in the avian spinal cord. Dev Biol *269*, 433-446.

Wilson, L., and Maden, M. (2005). The mechanisms of dorsoventral patterning in the vertebrate neural tube. Dev Biol *282*, 1-13.

Wilson, S. I., and Edlund, T. (2001). Neural induction: toward a unifying mechanism. Nat Neurosci *4 Suppl*, 1161-1168.

Wine-Lee, L., Ahn, K. J., Richardson, R. D., Mishina, Y., Lyons, K. M., and Crenshaw, E. B., 3rd (2004). Signalling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. Development *131*, 5393-5403.

Wood, H. B., and Episkopou, V. (1999). Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech Dev *86*, 197-201.

Wu, Y., Liu, Y., Levine, E. M., and Rao, M. S. (2003). Hes1 but not Hes5 regulates an astrocyte versus oligodendrocyte fate choice in glial restricted precursors. Dev Dyn *226*, 675-689.

Xin, M., Yue, T., Ma, Z., Wu, F. F., Gow, A., and Lu, Q. R. (2005). Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. J Neurosci *25*, 1354-1365.

Yang, X., Tomita, T., Wines-Samuelson, M., Beglopoulos, V., Tansey, M. G., Kopan, R., and Shen, J. (2006). Notch1 signalling influences v2 interneuron and motor neuron development in the spinal cord. Dev Neurosci *28*, 102-117.

Yoon, K., and Gaiano, N. (2005). Notch signalling in the mammalian central nervous system: insights from mouse mutants. Nat Neurosci *8*, 709-715.

Zhang, Y., Buchholz, F., Muyrers, J. P., and Stewart, A. F. (1998). A new logic for DNA engineering using recombination in Escherichia coli. Nat Genet *20*, 123-128.

Zhou, Q., and Anderson, D. J. (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell *109*, 61-73.

Zhou, Q., Choi, G., and Anderson, D. J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron *31*, 791-807.

Zhou, Q., Wang, S., and Anderson, D. J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron *25*, 331-343.

Genethliou et al., 2009

Biochemical and Biophysical Research Communications 382 (2009) 69–73

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Spatially distinct functions of PAX6 and NKX2.2 during gliogenesis in the ventral spinal cord

Nicholas Genethliou [a,b], Elena Panayiotou [a,b], Helen Panayi [a], Michael Orford [a], Richard Mean [a], George Lapathitis [a], Stavros Malas [a,*]

[a] The Cyprus Institute of Neurology and Genetics, Airport Avenue, No. 6, Agios Dometios 2370, Nicosia, Cyprus
[b] Department of Biological Sciences, University of Cyprus, P.O. Box 20537, 1678 Nicosia, Cyprus

ARTICLE INFO

Article history:
Received 15 February 2009
Available online 1 March 2009

Keywords:
Pax6
Nkx2.2
Slit1
Sulfatase I
Sulfatase II
Oligodendrocytes
Astrocytes
Neural patterning

ABSTRACT

During ventral spinal cord (vSC) development, the p3 and pMN progenitor domain boundary is thought to be maintained by cross-repressive interactions between NKX2.2 and PAX6. Using loss-of-function analysis during the neuron-glial fate switch we show that the identity of the p3 domain is not maintained by the repressive function of NKX2.2 on Pax6 expression, even in the absence of NKX2.9. We further show that NKX2.2 is necessary to induce the expression of Slit1 and Sulfatase 1 (Sulf1) in the vSC in a PAX6-independent manner. Conversely, we show that PAX6 regulates Sulf1/Slit1 expression in the vSC in an NKX2.2/NKX6.1-independent manner. Consequently, deregulation of Sulf1 expression in Pax6-mutant embryos has stage-specific implications on neural patterning, associated with enhancement of Sonic Hedgehog activity. On the other hand, deregulation of Slit1 expression in gliogenic neural progenitors leads to changes in Astrocyte subtype identity. These data provide important insights into specific functions of PAX6 and NKX2.2 during glial cell specification that have so far remained largely unexplored.

Introduction

The ventral spinal cord (vSC) in mice is organized into five progenitor domains that generate different subtypes of neurons and glia [1]. The two most ventral progenitor domains, known as p3 and pMN, are defined by the specific expression of Nkx2.2 and Olig2 during neurogenesis [2]. The p3/pMN progenitor boundary is believed to be maintained by cross-repression between PAX6 and NKX2.2 homedomain (HD) factors [3,4]. At the onset of the N/G switch, the pMN domain switches from generating motor-neurons to generate mostly Oligodendrocytes (OLs) and some Astrocytes (ASs) [5–8]. This process is preceded by a dorsal expansion of the p3 domain and a ventral retraction of the pMN domain [8–10]. In the chick embryo this boundary shift is characterized by a sharp increase of Sonic Hedgehog (SHH) protein in neuroepithelial cells located in the p3 domain [11,12]. The enzyme Sulfatase 1 (SULF1) has been proposed to mediate this process since Sulf1 expression expands from the Floor Plate (FP) to the p3 domain [12]. The functional significance of this phenomenon remains unknown, despite the suggestion that it may regulate the specification of p3-derived OLs in the chick embryo [12]. The genetic regulation of Sulf1 expression is also unknown.

More recently the domain-specific organization of the vSC has been proposed to govern the positional specification of three different subtypes of ventral astrocytes (VA), named VA1–3 ASs [13], that express Reelin and Slit1. White matter (WM) VA1 ASs express only Reelin and are specified in Pax6$^+$ progenitors located dorsal to the p2 domain. VA2 ASs express both Reelin and Slit1 and are specified in Nkx6.1/Pax6$^+$ progenitors (pMN–p2) and VA3 ASs are specified in Nkx2.2/Nkx6.1$^+$ (p3) progenitors and express only Slit1. It has been proposed that the combinatorial expression of PAX6 and NKX6.1 in ventricular zone (VZ) progenitors regulate the expression of both Reelin and Slit1 in the VZ and by doing so govern the subtype identity of the respective ASs [13]. Thus PAX6 induces Reelin and represses Slit1, while NKX6.1 (and NKX2.2) induce Slit1 and repress Reelin.

In this study, we considered NKX2.2 and PAX6 as primary candidates for regulating both Slit1 and Sulf1 in the vSC. We first show that under physiological *in vivo* conditions the proposed repressive function of NKX2.2 on Pax6 expression in the p3 domain is not supported by loss-of-function data even when NKX2.9 is switched off. This observation allowed us to establish a specific genetic requirement for NKX2.2 to initiate both Slit1 and Sulf1 expression in the vSC in a PAX6-independent manner. Conversely, we show that PAX6 has an NKX2.2/NKX6.1-independent function in regulating Sulf1/Slit1 expression in the pMN–p0 domains. Finally, we demonstrate that deregulation of Sulf1 and Slit1 expression has functional

* Corresponding author. Fax: +357 22 392641.
E-mail address: smalas@cing.ac.cy (S. Malas).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.02.134

implications on the response of progenitors to SHH signaling and on AS subtype identity, respectively.

Materials and methods

Transgenic mice. The mouse mutant lines used were: $Pax6^{Sey/Sey}$ [14] and $Nkx2.2^{-KO}$ [15]. Genotyping was performed by PCR for Nkx2.2 and by morphological examination of the telencephalon and absence of eye development for $Pax6^{Sey/Sey}$ embryos. All animal procedures were performed in accordance with a license issued by the Chief of Veterinary Services of the Republic of Cyprus, as stipulated by National Law.

In situ hybridization and immunohistochemistry. Embryos were fixed overnight in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), cryo-protected overnight in 20% (w/v) sucrose in PB and sectioned on a cryostat (14 μm). In situ hybridization was performed as described [16,17]. The probes used were: Nkx2.2 and Olig2 (Gift from Bill Richardson), Dbx1, Dbx2 (gift from Tom Jessell), Slit1 (gift from Marc Tessier-Lavigne), Pax6 (Gift from Peter Gruss), Nkx2.9 (gift from Johan Ericson). The rest of the probes were generated by PCR amplification with cDNA-specific primers for the gene of interest, ligated in pGEM-T Easy (Promega) and amplified by directional PCR to generate the template for antisense probe synthesis (T7 end). More information on the sequence is available on request. All images were captured using an Olympus microscope (Olympus SZX12) and digital camera (Olympus DP70). For domain-specific comparisons of gene expression, in situ hybridisation was carried out on consecutive sections and images captured were photo-converted using Adope Photoshop and aligned. Immunohistochemistry was performed using MEMFA-fixed embryos. The length of fixation was 20–40 min, depending on the stage and antibodies used. Sections were cut at cryostat (10–12 μm). Immunohistochemistry was performed according to standard procedures using the following primary antibodies: Shh, Nkx2.2, Nkx6.1, Pax3 (Hybridoma), Olig2 (Santa Cruz), Pax6 (Millipore). Images were captured on a TCSL confocal microscope (Leica, Germany).

Results

Temporal gene expression changes in the p3 and pMN domains

The neuron-glial (N/G) fate switch in the vSC in mice takes place between embryonic day 11.0 (e11.0) and e12.0 [18,19]. In agreement with previous data [8], we confirmed that between e10.5 and e12.5 the expression domain of Nkx2.2 expanded dorsally while the ventral limit of Olig2 and Pax6 retracted ventrally (not shown). Concomitant to this boundary shift, the expression of Sulf1 and Slit1 expanded from the FP in the vSC. Slit1 expression expanded beyond the pMN to reach the p2 domain as reported [13] while Sulf1 expanded to overlap partly with the ventral pMN (vpMN) domain (Supplementary Fig. 1A–D). At the same time Sulf2 expression, coding for an enzyme related to SULF1 [20], expanded to overlap with the p0 domain ($Dbx1^{+}$) while Nkx2.9 expression was switched off from the p3 domain (Supplementary Fig. 1E–H). Consistent with a role of SULF1 in regulating the local concentration of SHH in the p3 domain in the chick [11,12], we detected enhanced accumulation of SHH protein in the p3 domain at e11.5 that was not evident at e9.5 (Supplementary Fig. 1I–J). At the same developmental window the expression of Patched 1 (Ptch1), providing readout for Shh signaling [21], expanded from the p3 domain to reach more than two thirds of the VZ, suggesting that neuroepithelial progenitors became more exposed to SHH at e11.5 than at e9.5 (Supplementary Fig. 1K and L). These data suggest that during the N/G fate switch the boundary shifts observed in the p3/pMN domains affecting Nkx2.2/Pax6/Olig2 expression, are temporally associated with changes in the expression of Slit1, Sulf1 and Sulf2 and correlate with changes in the response of VZ progenitors to SHH activity.

Stage- and region-specific requirement for PAX6 and NKX2.2 to regulate the expression of Slit1 and Sulf1

We then asked how the expression of Sulf1/Sulf2 and Slit1 is genetically regulated. To test this we analyzed their expression in $Pax6^{Sey/Sey}$ and $Nkx2.2^{-/-}$ embryos. In both $Nkx2.2^{-/-}$ and $Pax6^{Sey/Sey}$ embryos analyzed at e10.5, Sulf1/2 and Slit1 were expressed in the FP like control embryos (not shown), suggesting that neither PAX6 nor NKX2.2 had any influence on their FP expression. However, at e12.5 in $Pax6^{Sey/Sey}$ embryos the expression of both Slit1 and Sulf1 was up-regulated in the VZ and the dorsal limit of their expression reached the ventral limit of Pax3 expression (Fig. 1A–D). In $Nkx2.2^{-/-}$ embryos both Slit1 and Sulf1 expression failed to expand in the VZ and were only expressed in the FP (Fig. 1E and F). One likely reason for this is the predicted ventral expansion of PAX6. However, Pax6 expression did not expand in the prospective p3 domain (Fig. 1G and H) despite the extinction of Nkx2.9 expression by this stage (Supplementary Fig. 1H). The expression of Sulf2 was not affected in any genetic background (not shown).

These data suggest that under physiological conditions in a stage- and region-specific manner, NKX2.2 controls the induction of Slit1 and Sulf1 expression in the vSC in a PAX6-independent manner. Conversely, loss of PAX6 led to co-extensive up-regulation of both Silt1/Sulf1 in the entire vSC despite the initial differences in their expression limits (Supplementary Fig. 2). They also suggest that, under physiological *in vivo* conditions, the proposed repressive function of NKX2.2 on Pax6 expression could not be corroborated by loss-of-function analysis at e12.5.

Stage-specific enhancement of Shh signaling in the absence of PAX6

Since SULF1 has been shown to promote accumulation of SHH in ectopic sites of expression [12], we wondered if the deregulation of Sulf1 expression in $Nkx2.2^{-/-}$ and $Pax6^{Sey/Sey}$ embryos had implications on Shh signaling and/or neural patterning. In $Nkx2.2^{-/-}$ embryos neural patterning was normal except from the ventral expansion of Olig2 as predicted by the repressive effects of NKX2.2 on Olig2 expression (not shown) [2,22]. In $Pax6^{Sey/Sey}$ embryos where both Sulf1 and Sulf2 were expressed in the p3–p0 region we found that at e10.5 Ptch1 expression did not change (Fig. 2A and B). However, at e12.5 we observed strong up-regulation of Ptch1 expression in the VZ of $Pax6^{Sey/Sey}$ embryos (Fig. 2C and D). To examine the consequences on neural patterning we compared the expression pattern of four Class I and three Class II genes at e10.5 and e12.5. As expected, Nkx2.2 expression expanded dorsally at both stages in $Pax6^{Sey/Sey}$ (Fig. 2E–H). The expansion of Nkx2.2 caused repression of Olig2 expression [2] (Fig. 2E–H) while Nkx6.1 expression did not change at any developmental stage (Fig. 2I–L). The analysis of the expression of Class I genes, which are directly repressed by SHH, was more informative. We first noted a dorsal shift of the ventral limit of Pax6 expression at e12.5. Importantly, this shift was not apparent at e10.5 despite the expansion of Nkx2.2 at both stages (Fig. 2M–P). Furthermore, Irx3 expression was reduced only at e12.5 despite the fact that Olig2 expression was almost abolished at both stages (Fig. 2Q–T) and loss of Olig2 should lead to ventral expansion of Irx3 in the pMN domain [8]. Finally, the expression of Dbx1 and Dbx2 was only reduced at e12.5, with Dbx2 being more affected than Dbx1 (Fig. 2U–Z2).

These data suggest that the stage-specific expression changes of several Class I genes in $Pax6^{Sey/Sey}$ embryos are likely to be due to the ventralization of p3–p0 progenitors brought about by expan-

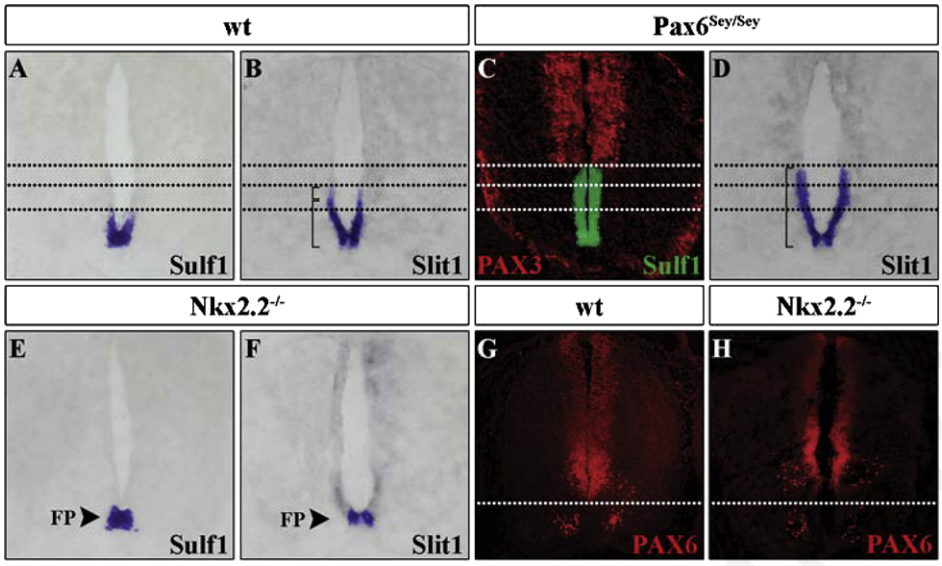

Fig. 1. Domain-specific requirement for NKX2.2 and PAX6 to regulate Sulf1 and Slit1 expression. Note that the expression of Slit1 extends beyond that of Sulf1 (A,B) whereas both genes are expressed in the same manner in $Pax6^{Sey/Sey}$ embryos (C,D; brackets). The precise dorsal limit of Slit1 in wt embryos has been reported to be the Nkx6.1 expression boundary [13]. Note the diminishing Slit1 expression in the domain dorsal to the Sulf1 boundary (brackets in B). In $Nkx2.2^{-/-}$ embryos both Slit1/Sulf1 are only expressed in the floor plate (FP; E,F), whereas Pax6 expression does not expand in the p3 domain (G,H). Dotted lines indicate the limits of expression boundaries. In (G,H), the dotted lines indicate the prospective p3 domain.

sion of Sulf1 (and Sulf2) expression and consequent elevation of SHH activity. These gene expression changes argue for a direct effect of higher levels of SHH activity on neural progenitors rather than by changes in the expression pattern of homedomain Class II factors with reported cross-repressive interactions with Class I factors [3,23].

Astrocyte subtype specification in $Nkx2.2^{-/-}$ embryos

The loss of Slit1 expression from the VZ in $Nkx2.2^{-/-}$ embryos prompted us to examine the consequences on Slit1 expression in the WM in spinal cords of e18.5 embryos, where it labels VA3 and VA2 ASs. Consistent with the fact that Pax6 expression does not expand ventrally in these embryos (Fig. 2), Reelin expression in the VZ, that depends on PAX6 function [13], did not change either at e13.5 (Supplementary Fig. 3A–D). However, the expression of Slit1 in the WM was drastically reduced (Supplementary Fig. 3E and F). The expression of NKX6.1 was normal in $Nkx2.2^{-/-}$ embryos at both e10.5 and 12.5 (not shown; [4]) and the expression of the generic AS marker GFAP was also normal (Supplementary Fig. 3G and H) [22]. These data suggest that loss of Slit1 expression in the VZ had a direct impact on Slit1 expression in the WM. The fact that GFAP expression was normal suggests that AS subtype identity, as judged by Slit1 expression, was altered but AS specification per se was not affected. It is possible that the functional re-specification of the p3 domain to a 'pMN-like' domain, imparts this region with astrogenic capacity normally specific to the pMN domain [6].

Discussion

In this study we report four important observations. (1) That the genetic data do not support the view that NKX2.2 represses Pax6 expression in the p3 domain, even when Nkx2.9 is switched off. (2) That the induction of Sli1/Sulf1 expression in the vSC is solely regulated by NKX2.2, in a PAX6-independent. (3) That the domain-specific expression of Silt1/Sulf1 is primarily regulated by PAX6 and neither NKX2.2 nor NKX6.1 contributes to the deregulation of their expression in the absence of PAX6. (4) That deregulation of Slit1 and Sulf1 expression has functional implications on AS differentiation and neural patterning.

What is preventing PAX6 expression from expanding in the p3 domain of $Nkx2.2^{-/-}$ embryos? We were intrigued also by the fact that even at e10.5 in $Pax6^{Sey/Sey}$ embryos the dorsal expansion of Nkx2.2 did not cause repression of Pax6 transcription (Fig. 2M and N) raising doubts if NKX2.2, under physiological conditions, is able to repress Pax6 at any stage. It is possible that Pax6 expression is directly repressed by Shh in the vSC and ectopic expression of NKX2.2 (even at a low level) in the chick embryo may induce Sulf1 expression, that is regulated by Nkx2.2 (this study), leading to an increase in the local concentration of SHH as shown [12] and direct repression of Pax6. In $Nkx2.2^{-/-}$ embryos such mechanism could still be operational and mediated by SULF2 that is expressed normally and seems to have a redundant function with SULF1 in other systems [26].

In relation to the stage-specific induction of Slit1/Sulf1 in the vSC our findings support a context- and stage-dependent mechanism that directly implicates NKX2.2, in a PAX6-independent manner. Nkx2.2 and Nkx2.9 are both expressed in the p3 domain before Sulf1/Slit1 expression expands in the vSC (not shown). Once Nkx2.9 is switched off (around e11.5) Sulf1/Slit1 begin to be expressed dorsal to the FP. Despite the redundant function between NKX2.2 and NKX6.1 to induce Slit1 expression in the chick embryo, NKX6.1 cannot compensate for the loss of NKX2.2. Since NKX2.2 acts as a repressor and is believed to have a dominant function over NKX6.1 in the p3 domain [23], the requirement for NKX2.2 to activate both genes may be achieved via the de-repression of a repressor, a mechanism believed to operate in all progenitor domains in the vSC [3,23]. OLIG2, is an unlikely repressor in this context since there is overlap between Slit1/Sulf1/Olig2 in the pMN domain.

The differential dorsal expansion of Silt1 (p3–p2) and Sulf1 (p3–vpMN) may suggest different mechanisms for their regulation. In the case of Slit1, despite the repressive effect of PAX6 on Slit1 expression [13], the physiological level of its expression may be determined by the dose of NKX2.2/NKX6.1. However, in the absence of PAX6, the expansion of NKX2.2 expression does not reach the ventral limit of PAX3, like Slit1 and Sulf1 do. Thus, it is most

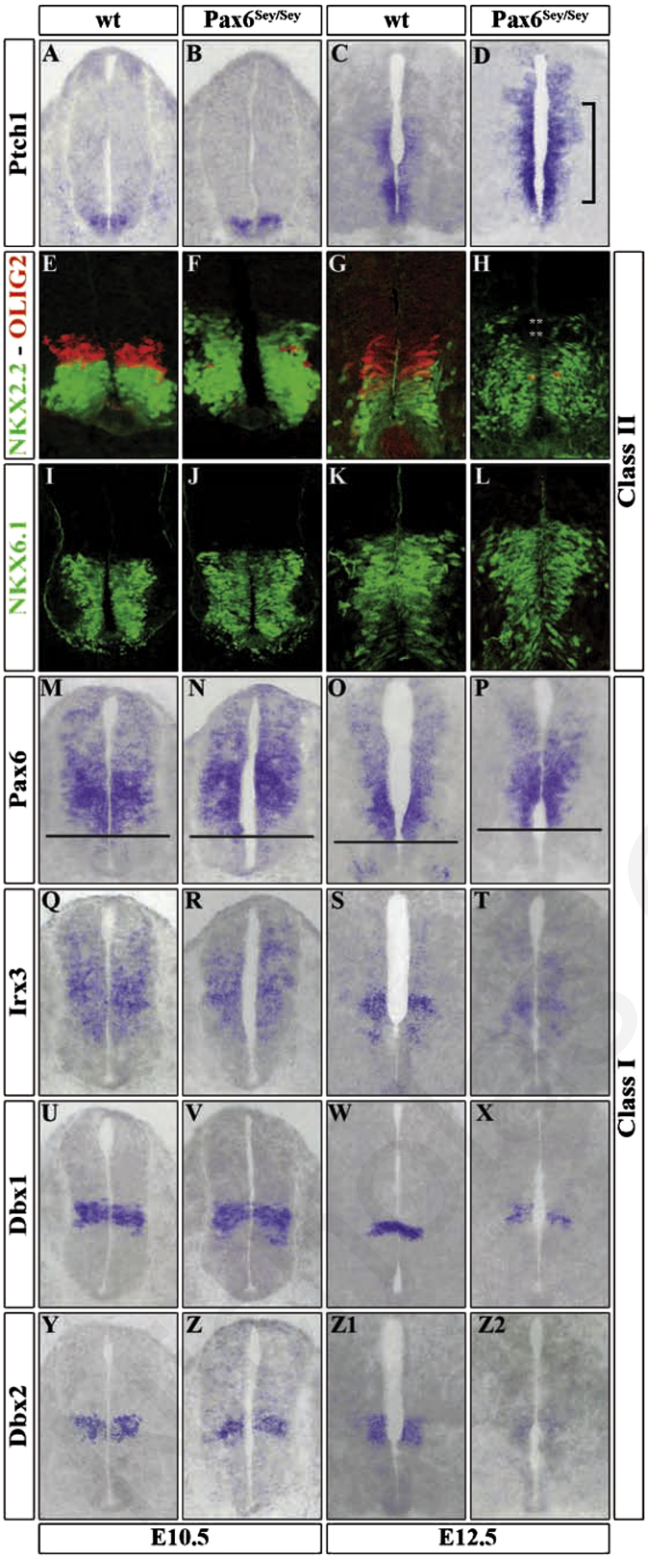

Fig. 2. Stage-specific changes in neural patterning in Pax6$^{Sey/Sey}$ embryos. Comparative expression patterns of Class I and Class II genes in Pax6$^{Sey/Sey}$ embryonic spinal cords between e10.5 and e12.5. Note the strong up-regulation of Ptch1 in Pax6$^{Sey/Sey}$ in the entire VZ specifically at e12.5. At e12.5 Nkx2.2^{+} cells dorsal to the pMN are located in peri-ventricular location (asterisks represent the p2–VZ). Olig2 expression is almost lost at both stages (E–H) and Nkx6.1 was not affected (I–L). Note that at e10.5 Pax6 expression does not change while at e12.5 it shifts slightly dorsally (M–P). Irx3 expression is attenuated only at e12.5 (S,T) and Dbx2 expression is almost lost at e12.5 (Z1,Z2) and Dbx1 expression is weaker only at e12.5 (W,X). Bars indicate boundary shifts. Brackets in (D) indicate the expanded Ptch1 expression.

likely the absence of PAX6 that caused the deregulation of Sulf1/Slit1 expression in the vSC of Pax6$^{Sey/Sey}$ embryos. In the pMN–p0 domain PAX6 regulates the expression of the Wnt inhibitor SFRP2 [24] and the transcription factor SOX1 expression. However, neither factor mediates the domain-specific regulation of Slit1/Sulf1 by PAX6 (SM; unpublished data).

Finally, we show that the deregulation of Slit1 and Sulf1 have functional implications. In the case of Sulf1 we find that in Pax6-$^{Sey/Sey}$ embryos Shh signaling is enhanced leading to stage-specific changes in the expression of Class I genes (Shh-repressed). These gene expression changes were stage-specific and were not associated with changes in Class II genes, with reported cross-repressive activities with Class I factors. In Nkx2.2$^{-/-}$ embryos, despite the loss of Sulf1 expression in the VZ, neural patterning is maintained correctly, most likely due to the normal expression of Sulf2 and the redundant function between SULF1/2 [26]. One function of PAX6 is to repress Sulf1 expression beyond the p3 domain. In retrospect, this seems important for maintaining the correct functional output of the pMN domain during gliogenesis. Expansion of Sulf1 across the pMN domain most likely will enhance Shh signaling [12], induce further expansion of Nkx2.2^{+} domain leading to more repression of Olig2 expression and reduction in OL specification. This is probably why in Pax6$^{Sey/Sey}$ embryos the origin of pMN-derived OLs shifts dorsally and the initial production of OLs is severely reduced [25]. In the case of Slit1 we show that, in agreement with previous findings, deregulation of Slit1 expression affects the expression of Slit1 in WM ASs [13]. In Nkx2.2$^{-/-}$ embryos the VZ expression of Slit1 directly correlates with Slit1 expression in WM ASs. Likewise in Pax6$^{Sey/Sey}$ spinal cords all ventral ASs acquire a VA3 (Slit1^{+}) subtype [13] (not shown) and this is reflected in the changes of Slit1^{+} expression in the VZ. Thus our data agree with the proposition that the specification of VAs take place in the VZ but we find that our data support a mechanism that primarily implicates NKX2.2 in the induction of Slit1 expression in the VZ, that is not antagonized, cell-autonomously, by PAX6. They also suggest that the gliogenic neuroepithelium is divided up to specific domains of Sulf1 and Sulf2 expression and NKX2.2 and PAX6 regulate some aspects of this organization.

Acknowledgments

We thank Dr. Tom Jessell for in situ probes, Dr. Johan Ericson and Dr. Anastasia Stoykova for the mice and Georgia Philippou for the excellent animal care. This work was supported by grants to S.M. (FP5; Acronym: Stembridge; RPF of Cyprus).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.134.

References

[1] T.M. Jessell, Neuronal specification in the spinal cord: inductive signals and transcriptional codes, Nat. Rev. Genet. 1 (2000) 20–29.
[2] B.G. Novitch, A.I. Chen, T.M. Jessell, Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2, Neuron 31 (2001) 773–789.
[3] J. Muhr, E. Andersson, M. Persson, T.M. Jessell, J. Ericson, Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube, Cell 104 (2001) 861–873.
[4] J. Briscoe, L. Sussel, P. Serup, D. Hartigan-O'Connor, T.M. Jessell, J.L. Rubenstein, J. Ericson, Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling, Nature 398 (1999) 622–627.
[5] Q.R. Lu, T. Sun, Z. Zhu, N. Ma, M. Garcia, C.D. Stiles, D.H. Rowitch, Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection, Cell 109 (2002) 75–86.
[6] N. Masahira, H. Takebayashi, K. Ono, K. Watanabe, L. Ding, M. Furusho, Y. Ogawa, Y. Nabeshima, A. Alvarez-Buylla, K. Shimizu, K. Ikenaka, Olig2-positive

progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells, Dev. Biol. 293 (2006) 358–369.
[7] N.P. Pringle, W.D. Richardson, A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage, Development 117 (1993) 525–533.
[8] Q. Zhou, D.J. Anderson, The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification, Cell 109 (2002) 61–73.
[9] E. Agius, C. Soukkarieh, C. Danesin, P. Kan, H. Takebayashi, C. Soula, P. Cochard, Converse control of oligodendrocyte and astrocyte lineage development by Sonic hedgehog in the chick spinal cord, Dev. Biol. 270 (2004) 308–321.
[10] H. Fu, Y. Qi, M. Tan, J. Cai, H. Takebayashi, M. Nakafuku, W. Richardson, M. Qiu, Dual origin of spinal oligodendrocyte progenitors and evidence for the cooperative role of Olig2 and Nkx2.2 in the control of oligodendrocyte differentiation, Development 129 (2002) 681–693.
[11] C. Braquart-Varnier, C. Danesin, C. Clouscard-Martinato, E. Agius, N. Escalas, B. Benazeraf, X. Ai, C. Emerson, P. Cochard, C. Soula, A subtractive approach to characterize genes with regionalized expression in the gliogenic ventral neuroepithelium: identification of chick sulfatase 1 as a new oligodendrocyte lineage gene, Mol. Cell. Neurosci. 25 (2004) 612–628.
[12] C. Danesin, E. Agius, N. Escalas, X. Ai, C. Emerson, P. Cochard, C. Soula, Ventral neural progenitors switch toward an oligodendroglial fate in response to increased Sonic hedgehog (Shh) activity: involvement of Sulfatase 1 in modulating Shh signaling in the ventral spinal cord, J. Neurosci. 26 (2006) 5037–5048.
[13] C. Hochstim, B. Deneen, A. Lukaszewicz, Q. Zhou, D.J. Anderson, Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code, Cell 133 (2008) 510–522.
[14] R.E. Hill, J. Favor, B.L. Hogan, C.C. Ton, G.F. Saunders, I.M. Hanson, J. Prosser, T. Jordan, N.D. Hastie, V. van Heyningen, Mouse small eye results from mutations in a paired-like homeobox-containing gene, Nature 354 (1991) 522–525.
[15] L. Sussel, J. Kalamaras, D.J. Hartigan-O'Connor, J.J. Meneses, R.A. Pedersen, J.L. Rubenstein, M.S. German, Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells, Development 125 (1998) 2213–2221.
[16] M. Fruttiger, L. Karlsson, A.C. Hall, A. Abramsson, A.R. Calver, H. Bostrom, K. Willetts, C.H. Bertold, J.K. Heath, C. Betsholtz, W.D. Richardson, Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice, Development 126 (1999) 457–467.
[17] N.P. Pringle, W.P. Yu, S. Guthrie, H. Roelink, A. Lumsden, A.C. Peterson, W.D. Richardson, Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog, Dev. Biol. 177 (1996) 30–42.
[18] B. Deneen, R. Ho, A. Lukaszewicz, C.J. Hochstim, R.M. Gronostajski, D.J. Anderson, The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord, Neuron 52 (2006) 953–968.
[19] Y. Ogawa, H. Takebayashi, M. Takahashi, N. Osumi, Y. Iwasaki, K. Ikenaka, Gliogenic radial glial cells show heterogeneity in the developing mouse spinal cord, Dev. Neurosci. 27 (2005) 364–377.
[20] D.H. Lum, J. Tan, S.D. Rosen, Z. Werb, Gene trap disruption of the mouse heparan sulfate 6-*O*-endosulfatase gene, Sulf2, Mol Cell. Biol. 27 (2007) 678–688.
[21] L.V. Goodrich, R.L. Johnson, L. Milenkovic, J.A. McMahon, M.P. Scott, Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog, Genes Dev. 10 (1996) 301–312.
[22] Y. Qi, J. Cai, Y. Wu, R. Wu, J. Lee, H. Fu, M. Rao, L. Sussel, J. Rubenstein, M. Qiu, Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor, Development 128 (2001) 2723–2733.
[23] J. Briscoe, A. Pierani, T.M. Jessell, J. Ericson, A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube, Cell 101 (2000) 435–445.
[24] Q. Lei, Y. Jeong, K. Misra, S. Li, A.K. Zelman, D.J. Epstein, M.P. Matise, Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube, Dev. Cell 11 (2006) 325–337.
[25] T. Sun, N.P. Pringle, A.P. Hardy, W.D. Richardson, H.K. Smith, Pax6 influences the time and site of origin of glial precursors in the ventral neural tube, Mol. Cell. Neurosci. 12 (1998) 228–239.
[26] X. Ai, T. Kitazawa, A.T. Do, M. Kusche-Gullberg, P.A. Labosky, C.P. Emerson Jr., SULF1 and SULF2 regulate heparan sulfate-mediated GDNF signaling for esophageal innervation, Development 134 (2007) 3327–3338.