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Late Wave of Neurogenesis Generates Novel Dorsal and Ventral Neurons in the Spinal Cord and Central Canal

by

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ABSTRACT

During embryogenesis the embryonic spinal cord is organized into eleven progenitor domains that express different combinations of transcription factors (TF) and generate different subsets of neurons and glia. One of the ventrally located domains, known as the p2-domain, generates three subtypes of interneurons (INs), V2a ($Chx10^+$), V2b ($Gata3^+$) and V2c ($Sox1^+$). The allocation of these early lineages takes place in common neuronal progenitors and involves Notch signalling. We have attempted to identify the origin and character of three populations of late born neurons in order to possibly shed some light on their probable function and ultimate task within their system. Here we show that the p2-domain is a source of two additional IN subtypes that are generated after these early neuronal subtypes are generated. One group is a subset of V2b INs which express the TFs Pax6 and Gata3 and are generated in a Notch-dependent manner. The other group of neurons transiently expresses GATA3 and migrate in the dorsal spinal cord but do not express any known marker of dorsal INs. A separate group of late born neurons contacting the cerebrospinal fluid surrounding the central canal exhibits interplay between the nuclear and cytoplasmic expression of GATA3 and SOX1 during post natal development. Additionally, the number of GATA3 positive cells is reduced by 40% in the absence of Sox1. Therefore, Sox1 appears to be imperative in ensuring the generation and preservation of this group of late born cells.

ΠΕΡΙΛΗΨΗ

Κατα τη διαρκεια της εμβρυογένεσης ο νωτιαίος μυελός αποτελείται από έντεκα προγονικές (αρχέγονες) περιοχές οι οποίες εκφράζουν διαφορετικούς συνδυασμούς μεταγραφικών παραγόντων και παράγουν διαφορετικές υποομάδες νευρώνων και γλοιακών κυττάρων. Μια από τις περιοχές που βρίσκεται στην κοιλιακή μοίρα του νωτιαίου μυελού, γνωστή ως p2, είναι υπεύθυνη για την παραγωγή των ενδιάμεσων νευρώνων, V2a ($Chx10^{+}$), V2b ($Gata3^{+}$) και V2c ($Sox1^{+}$). Ο διαχωρισμός αυτών των αρχικών κυτταρικών πληθυσμών γίνεται από ένα κοινό πληθυσμό νευρικών προγονικών κυττάρων και καθοδηγείται από το σηματοδοτικό μονοπάτι Notch. Έχουμε προσπαθήσει να αναγνωρίσουμε την καταγωγή και τις ιδιότητες τριών πλυθησμών μεταγενέστερων κυττάρων. Στην παρούσα εργασία δείχθηκε ότι η p2 περιοχή των μυών (ποντικών) είναι πηγή παραγωγής ακόμα δύο υποτύπων διάμεσων νευρώνων οι οποίοι παράγονται μετά την παραγωγή των πρώιμων νευρικών υποτύπων που αναφέρθηκαν ήδη. Η μια ομάδα αποτελεί υποομάδα των V2b διάμεσω νευρώνων και εκφράζει τους μεταγραφικούς παράγοντες Pax6 και Gata3 και η παραγωγή τους εξαρτάται από το σηματοδοτικό μονοπάτι Notch. Η άλλη ομάδα νευρώνων παροδικά εκφράζει GATA3 και μεταναστεύει στη ραχιαία μοίρα του νωτιαίου μυελού χωρίς όμως να εκφράζει οποιουσδήποτε δείκτες που εκφράζονται από τους ραχιαίους διάμεσους νευρώνες. Μια επιπλέον ξεχωριστή ομάδα νευρώνων οι οποίοι παράγονται σε κατοπινά στάδια και οι οποίοι έρχονται σε επαφή με το εγκεφαλονωτιαίο υγρό που περιβάλλει τη κενρική αύλακα, παρουσιάζουν εναλλαγή μεταξύ της πυρηνικής και της κυτταροπλασματικής έκφρασης του Gata3 και του Sox1 κατά τα μετεμβρυικά στάδια ανάπτυξης του οργανισμού. Επιπλέον, κατά την απουσία του Sox ο αριθμός των GATA3 θετικών κυττάρων μειώνεται περίπου κατά 40 %. Το Sox1 παρουσιάζεται να είναι αναγκαίο για την δημιουργία και σωστή διατήρηση αυτής της ομάδας κυττάρων.

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TABLE OF CONTENTS

LIST OF FIGURES

Appendix I - Materials and Methods

LIST OF TABLES

LIST OF ABBREVIATIONS

BMP – Bone morphogenetic proteins

BrdU – 5-bromo-2'-deoxyuridine

CNS – Central Nervous System

CSF – Cerebrospinal fluid

EGF – Epidermal Growth Factor

FGF – Fibroblast Growth Factor

FP – Floor Plate

GFAP – Glial fibrillary acidic protein

HL – Hodgkin's lymphoma

IN – Interneuron

MN – Motor Neuron

NFkB – Nuclear Factor kappa B

Ngn2 – Neurogenin 2

NLS – Nuclear Localization Signal

NTD – Neural Tube Defect

OL – Oligodendrocyte

MAPK – Mitogen-activated protein kinase

PNS – Peripheral Nervous System

PSA-NCAM – Polysialylated Neuronal cell Adhesion Molecule

PSEN – Presenilin

Ptch – Patched

Sey – Small eye

Shh – Sonic Hedgehog

Shh-N – Sonic Hedgehog modified N-terminus

Smo – Smoothened

TF – Transcription Factor

TGF-β – Transforming Growth Factor beta

Th cells – T-helper cells

vSC – Ventral Spinal Cord

CHAPTER 1

INTRODUCTION

1.1 MAMMALIAN NERVOUS SYSTEM-GENERAL INFORMATION:

The nervous system is made up of the central nervous system (CNS) and the peripheral nervous system (PNS). The central nervous system itself is composed of the brain and the spinal cord, whereas the peripheral system is comprised of ganglia (clusters of neuron) which are in turn coupled on to sensory neurons. The afferent (sensory) function of the PNS allows information to be relayed from within the system and the external environment to the CNS. The efferent function of the CNS on the other hand is responsible for conveying information (alternatively instructions) from the CNS to the rest of the system (Nieuwenhuys, 2002).

The nervous system draws its name from the nerves which are essentially fibrous cylindrical shaped bundles that radiate from the central cord and the brain and branch out to innervate the entire body. At the cellular level the CNS is principally composed of neurons and glial cells. The human brain typically contains approximately 86 billion neurons and 85 billion glial (Figure 1: Main Cellular Classification of the Central Nervous System) (Azevedo *et al.*, 2009).

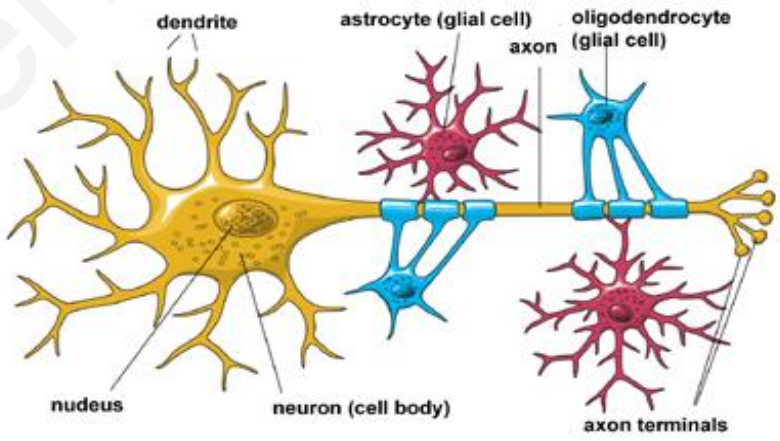


Figure 1: Main Cellular Classification of the Central Nervous System-the cells making up the CNS and their Relative Arrangement and Architecture. (Office of Communications and Public Liaison NIH, October 2012)

Neurons represent the core constituent of the entire nervous system and there are many different types, in morphology and function. Many of them are highly specialized, such as sensory and motor neurons (MNs). Sensory neurons are in charge of translating external stimuli into internal stimuli. Following the activation by external sensory contribution, these neurons send projections to the CNS allowing information to be relayed either to the brain or the spinal cord. MNs reside within the CNS and their projections extend outside of the CNS and subsequently influence muscles, thus been associated with an efferent neuron (Kandel & Squire, 2000; Nieuwenhuys, 2002).

Interneurons (INs) are specialized in connecting neurons to other neurons within the same sector of the brain or the spinal cord. INs themselves are a multipolar type of neuron connecting the afferent and efferent neurons within neural conduits (Ascoli *et al.*, 2008).

Glial cells are divided up to neuroglia and astroglia. Neuroglia, oligodendrocytes (OLs) in the CNS and Schwann cells in the PNS, are a non-neuronal cell population responsible for producing myelin which is a dielectric layer crucial for the proper function of neurons. Astroglia, or astrocytes, are involved with homeostasis and providing neuron nourishment, as well as being vital in facilitating signal transmission within the entire nervous system. Within the human brain the number of neuron cells is approximately the same as the number of glial cells even though their relative proportions at various regions within the brain greatly differ (Bushong *et al.*, 2002).

In the CNS there exists another type of supporting glial cells called microglia. Microglia originate from hematopoietic precursors instead of ectodermic tissue. They make up 15% of the entire CNS cell population and become activated following brain

damage. In essence they are highly specialized macrophages, capable of phagocytosis when CNS neurons need to be protected (Ferrer *et al.*, 1990; Bushong *et al.*, 2002). .

In addition to astrocytes and OLs, in the CNS there exist specialized glial called ependymal cells and radial glial. **Astrocytes** are the most abundant of macroglia and they are classified in two distinct categories; the **protoplasmic** and **fibrous** types. The distinction arises from their morphology and localization. Protoplasmic astrocytes are contained in the grey matter and possess pronged short and thick processes. Fibrous astrocytes however are found in the white matter and instead have very long and thin, slightly less branched processes. Their function is to adjust the neurons' surrounding environment by recovering neurotransmitters and surplus ions, as well as establishing the blood-brain barrier. **OLs** produce myelin which insulates neuronal axons in the form of a sheath so as to facilitate the propagation and transmission of electrical impulses. **Ependymal cells** produce and secrete the cerebrospinal fluid (CSF) whilst circulating it giving rise to the blood-CSF barrier. These are found along the ventricular and cavity walls. Ependymocytes are also believed to possess neural stem cell character. **Radial glial** originate from neuroepithelials following the start of neurogenesis. During development they form the scaffold which allows the newly formed neurons to migrate to their appropriate location, as well as acting as neural progenitors. In the cerebellum of mature brains, radial glial are called the Bergmann glial and are responsible for adjusting the synaptic plasticity between neurons. In the mature retina, radial glial are the Müller cells, involved with the facilitation of neuronal bidirectional communication (Baumann & Pham-Dinh, 2001; Bushong *et al.*, 2002; Jessen & Mirsky, 2005).

1.2 EMBRYONIC DEVELOPMENT, FROM OOCYTE TO NEURULA:

Fertilization spawns the creation of the zygote. Following the cleavage, mitotic divisions convert the zygote into the blastula –essentially a ball made up of a collection of cells, more specifically three layers. The blastula subsequently undergoes gastrulation where the three layers of cells are reorganized into three distinct domains, the endoderm, mesoderm and ectoderm. During the process of organogenesis, organs and tissues transpire from within each of the layers (Greene & Copp, 2009).

As shown in figure 2, as soon as fertilization takes place the embryo undergoes cleavage within the zona pellucida. At the third cellular division the cells are compacted into a structure identified as the morula. During the blastocyst stage the inner cell mass and the trophoblast are visible. The inner cell mass contributes to the actual embryo as well as some of the extraembryonic tissue. Inner cell mass cells that are found facing the blastocoel will give rise to the primitive ectoderm which will in turn formulate the visceral and epidermal endoderm. The remaining cells from the inner cell mass will form the epiblast. During the late blastocyst stage the epiblast will be hollowed out and in turn be enclosed by the visceral endoderm thus creating the egg cylinder. The primitive streak will be initiated at the caudal end and will give rise to the mesoderm and the endoderm so that during the neurula stage the head folds and the neural plate will be completely formed (Mok & Sweetman, 2011).

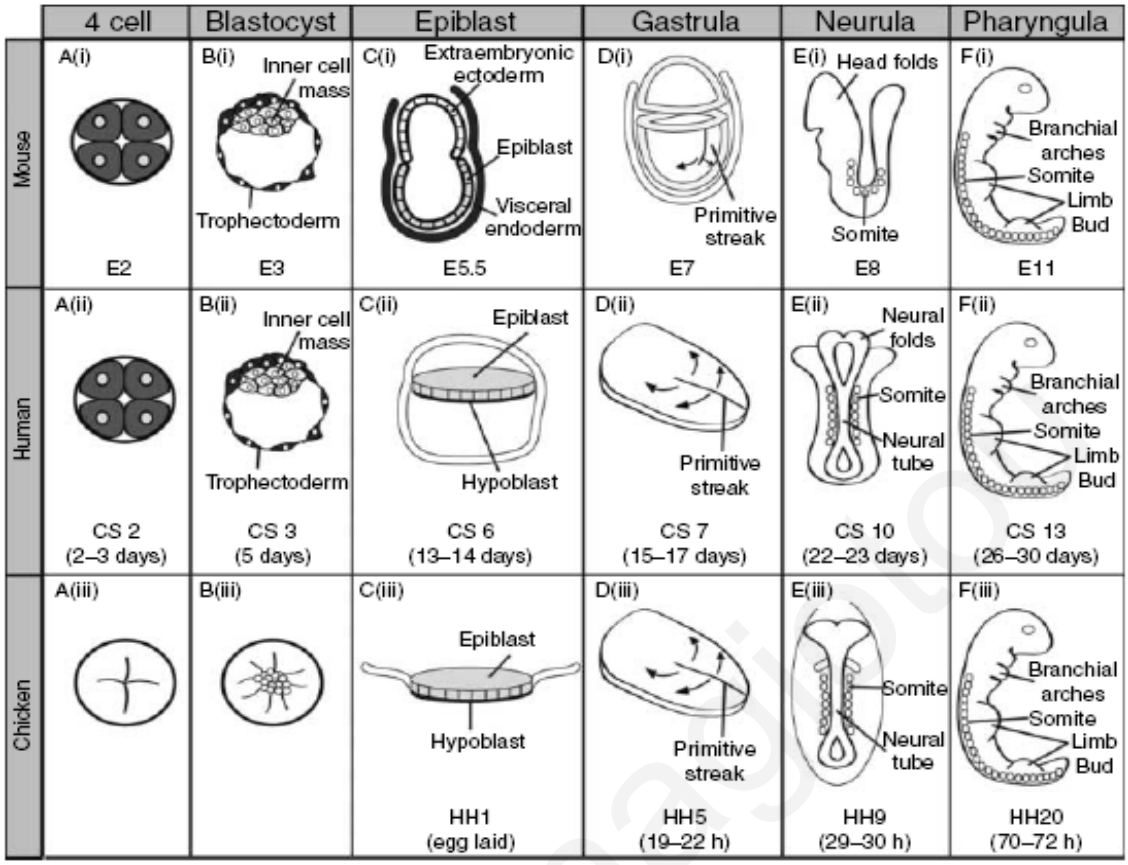


Figure 2: Mouse, human and chick Embryo Developmental Stages (Mok & Sweetman, 2011)

The ectoderm gives rise to the nervous system, the epidermis and the neural crest. The mesoderm forms somites which in turn will generate muscles, the dermis, the notochord (transient embryonic structure), blood and blood vessels, bones, connective tissue as well as the ribs and the vertebrae's cartilage. Finally, the endoderm will give rise to the digestive and respiratory system's epidermis as well as the organs associated with these systems. Bone Morphogenetic Protein (BMP) signalling is responsible for segregated neural from non-neural ectoderm. Specifically, BMP-4 is a transforming growth factor which induces ectodermal cells to differentiate into skin cells. However, in the absence of BMP-4, ectodermal cells would automatically differentiate into neural cells. Underneath the ectoderm, the mesodermal cells secrete chordin, noggin and follistatin, which inhibit the action of BMP-4, thus allowing the cells exposed to the aforementioned factors to differentiate into neural cells (Greene & Copp, 2009).

When the zygote reaches the 16-cell stage it is considered to become a morula. Most notably, the cells located at the outermost reach begin differentiating and give rise to the trophoectroderm, while the cells facing inwards will become the inner cell mass cells of the blastocyst. The inner cells mass cells through the action of multiple paracrine factors such as fibroblast growth factor 4 (FGF-4) ultimately generate all the organs and tissues of the embryo. The trophoectroderm will contribute to the placenta by differentiating into the chorion's trophoblast cells. Proper placenta establishment is crucial during development since disruption to the placental organization may even lead to spontaneous abortion. The main gene identified to affect the placenta's development is Mash2 which becomes activated directly after uterine transplantation. In Mash2 knockout mice the placenta is completely abolished causing the embryos to die at E10.5.

As soon as the embryo reaches the third day after fertilization, an invagination develops (the blastocoel) which fills with fluid secreted from the trophoectodermal cells. As a result, the inner cell mass cells are clearly separated from the trophoectroderm transforming into the blastocyst.

At E4.0 the blastocyst reaches the uterus where the dissolution of the zona pellucida allows for the late stage blastocyst to be implanted in the uterine wall. This blastocyst is the source of embryonic stem (ES) cells which contains the inner cell mass (ICM) which will later on go on to generate all the organs and tissues. Even though the pluripotency and immortality of *in vivo* obtained ES cells is still been questioned, it should be noted that ES cells attained at this point from *in vitro* grown blastocysts are more similar to epiblast cells. Epiblast cells occur at E4.5 and are also pluripotent. However, epiblast cells *in vitro*, while having the ability to differentiate into all the different cell types cannot be used to generate an entire fully formed organism as they

would in the *in vivo* scenario. This may be a result of the absence of the appropriate conditions or environment required for the establishment of cellular polarity. In addition, in the absence of the trophoectroderm and other supporting structures, the ES cells may not have the ability to form the required interactions, so that they will not be exposed to the necessary factors which would in turn enable the cells to generate a fully formed organism.

After 4.5 days the mouse embryo has already been implanted in the uterus and the ICM has differentiated into two distinct layers. One is the hypoblast (situated directly next to the blastocoel) which will eventually differentiate into the embryonic endoderm and the other layer is the epiblast. The primitive ectoderm will subsequently differentiate to generate the endoderm, mesoderm and ectoderm, the three primary layers during the stage of gastrulation. The primitive endoderm will in turn differentiate into the visceral and parietal endoderm which is instrumental in establishing the final development body plan.

Gastrulation has been shown to start from the posterior end of the embryo due to the presence of the node (related to Hensen's node found in chick embryos). At the opposite end, the anterior visceral endoderm (AVE) regulates the embryo's patterning at the anterior end. These two signalling centres are of immense significance since the embryo cannot proceed with development in their absence. Later on, at E6.5 (14-16 days in humans) the primitive streak arises starting from the node so that eventually these migrating cells will connect the node to the AVE. During this migration the notochord and mesoderm will be clearly established. The notochord is the structure that will connect the two ends. Thus by the end of gastrulation at E8.0, the late stage blastocyst has given rise to the endoderm, the mesoderm and the ectoderm of the mature gastrula.

1.3 NEURAL PLATE CREATION:

Formation of the neural plate marks the beginning of the process of neural induction or neurulation, during which the neural tube transforms into the primordial structures which will later on give rise to the CNS.

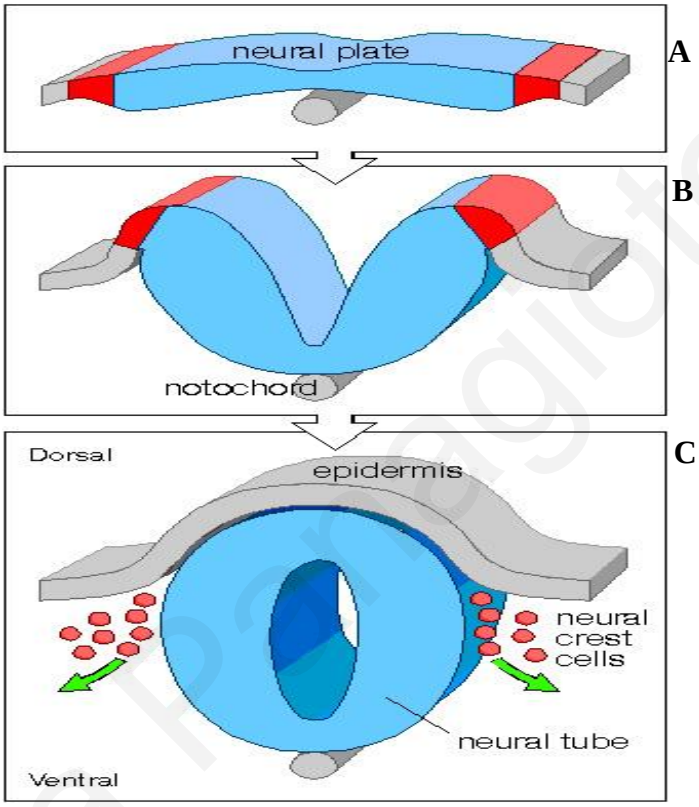


Figure 3: Neural tube formation through neural plate folding (Memorial University, CA, BIOL3530; http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_Ch11/fig11_9.jpg)

The notochord initiates the creation of the CNS by signalling the ectodermal germ layer above it to create the neural plate. The neural plate (Figure 3: A) will in turn fold inwards, inevitably giving rise to the neural tube. The neural tube will then on differentiate into the spinal cord and the brain. Neurulation is composed of two distinct stages, the primary and secondary stage.

During primary neurulation the plate will fold inwards until the edges come together and ultimately fuse (Figure 3: B). The adaptation of the primitive ectoderm to the neuronal ectoderm relies on the factors secreted from the mesodermal layer.

Throughout the beginning of gastrulation, cells from the mesoderm will migrate up the length of the dorsal midline and thus generate the notochord. The cells which make up the ectoderm and lie directly on top of the mesoderm will come into being the neural plate as a response to the factors that they will be exposed to. Neurulation is essentially initiated by BMP-4 signals secreted by the ectoderm. The cells exposed to BMP-4 will differentiate into epidermal cells. Chordin, noggin and follistatin are the inhibitory signals which are required to counteract the effects of BMP-4. The notochord secretes these factors so that the overlying ectodermal cells will be exposed to them and will transform into the anterior neuro-ectodermal tissue of the neural plate. In addition, some cells will not only be exposed to the three inhibitory signals but also to FGF so that they will develop into the posterior neural plate. The rest of the ectodermal population which will not be exposed to either the inhibitory signals or FGF will formulate the epidermis (Wurst & Bally-Cuif, 2001).

Finally, in secondary neurulation (Figure 3: C) the final structure of the neural tube forms by inducing the hollowing out of the interior. The cells from the neural ectoderm and the endoderm constitute the innermost (medullary) cord that will eventually contract and divide to generate cavities. The cavities will then fuse in order to give rise to one single tube.

The tubes ensuing from the primary and secondary neurulation processes ultimately fuse to form one continuous structure. However, it is evident that the folding of the neural tube does not occur in one step. Instead, many postulations have been put forward proposing that the actual neural tube closure occurs in parts. This is supported by the high occurrence of neural tube defects (NTDs), as well as the different nature of them. For instance, failure of the hindbrain neuropore to the anterior neuropore closure will result in anencephaly, whereas, failure of complete closure of the hindbrain to the

cervical boundary will result in craniorachischisis. NTDs occur in about one in five hundred live births, and they do result in debilitating effects (De Wals *et al.*, 2007).

1.4 NEURAL INDUCTION:

Spemann and Mangold published a key paper in 1924, where they had discovered the node, the organizer as they termed it, a region found in the embryo during the gastrula stage (as explained above). In their chimera experiment, two types of newt embryos were used, a lighter coloured one (Triton cristatus) and a darker coloured one (Triton taeniatus) in order to distinguish between the host from the transplanted cells (Figure 4). Due to the differently coloured varieties of newt it was possible to distinguish between the two within the resulting chimeric embryo, both macroscopically and microscopically. When they were analysed histologically it was evident that the darker pigmented transplanted tissue had given rise to the development of an ectopic notochord whist the rest of the neural tissue surrounding it was formed by the lighter coloured host embryo (Spemann & Mangold, 2001).

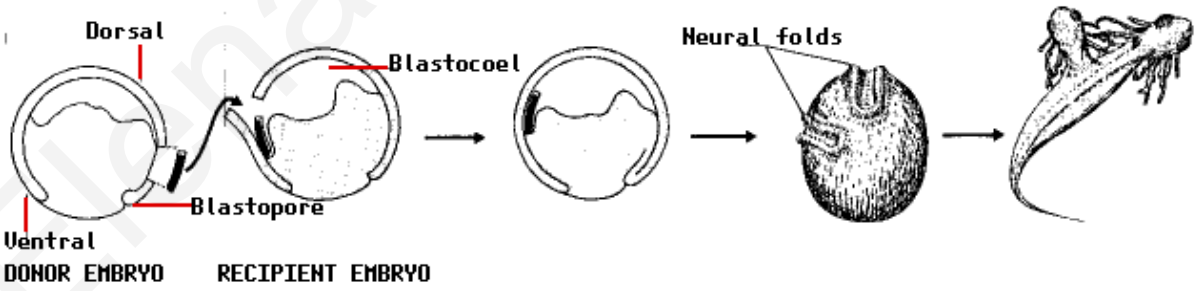


Figure 4: The Spemann-Mangold experiment (Spemann & Mangold, 2001)

Therefore, the transplantation of an ectopic node induced the formation of another notochord which in turn induced ectopic neural tissue. The implications of this experiment exemplified the fact that the organizer has the innate ability to affect the differentiation of cells found in proximity to it in a non-cell autonomous fashion

(Niehrs, 2004). Also, in the absence of the node, the graft will assume a somite fate. The greater implication of these observations is that cells follow a regulative mode of development, meaning that cells may change their fate depending on their relative position next to other cells.

1.5 SONIC HEDGEHOG PATHWAY:

Many of the intrinsic components and aspects of the Sonic Hedgehog (Shh) transduction pathway are highly conserved through species and evolution. A great deal of the information collected on the pathway results from studies carried out in the *Drosophila* and most recently in chicks (Briscoe *et al.*, 2000).

Prior to the secretion of the mature Shh protein, the molecule needs to undergo a series of modifications. Initially the molecule is produced as a 45 kDa pre-protein which needs to go through an initial cleavage which results in the addition of a cholesterol molecule to the C- terminus initially anchoring the entire molecule to the plasma membrane. This will eventually act as a cholesterol transferase. In addition another hydrophobic molecule is added to the N-terminus, a palmitate. This palmitylated moiety increases the potency of the entire Shh protein by 30-fold. However, it is the autoproteolytic cleavage of the inactive Shh protein which bestows the molecule its biologically active form, Sonic hedgehog modified N-terminus (Shh-N). These modifications are crucial in ensuring the appropriate cell signalling abilities of the molecule (Cayuso *et al.*, 2006).

Patched (Ptch) is the 12-pass Trans membrane receptor for Shh-N. In the absence of Shh-N, Patched inhibits the Smoothened (SMO) protein, which regulates the actual signal transduction of the Shh signal (Figure 5). However, the inhibition of Smoothened by Patched is lifted in the presence of Shh-N, so that the Shh signal can

then be conveyed on to the Gli TFs, the nuclear carriers of the signal (Poh *et al.*, 2002). This suggests that varying the levels of activated Gli factors regulates the activation and suppression of the class I and II proteins (Cayuso *et al.*, 2006).

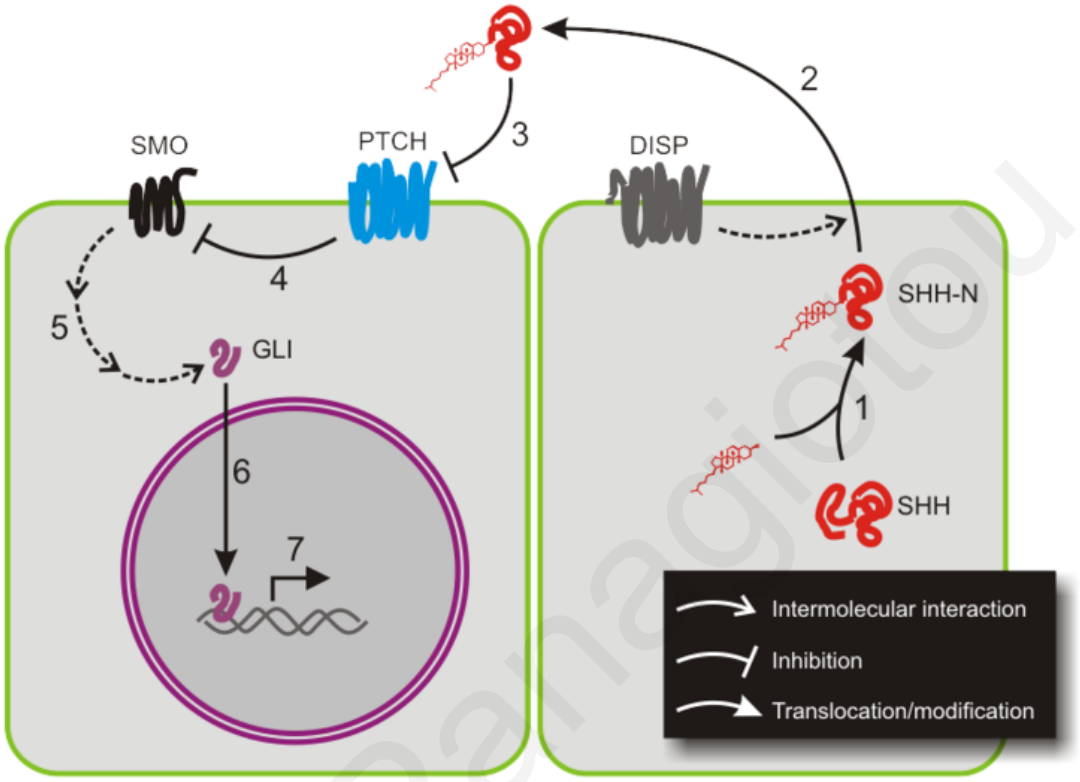


Figure 5: Sonic hedgehog transduction pathway. 1 The Shh molecule becomes palmitylated and is thus biologically active. 2-4 The modified Shh signal, Shh-N requires Dispatched (DISP) in order to be secreted and binds to its receptor PTCH, in receiving cells, which then relieves the inhibition of SMO. 5-7 The Gli nuclear effector in activated and thus enters the nucleus where it influences the transcription of related genes. (Peter Znamenskiy; donated to public domain)

1.6 VENTRAL PATTERNING:

Patterning is defined as the generation of varying cellular subtypes from their respective progenitors as a result of their spatial positioning down the ventral-dorsal and anterior-posterior axes (Ericson *et al.*, 1995).

It is generally considered that most species attain patterning solely based on the egg's position which will ultimately determine the cellular fate and establishment of the various cell types and organs. However, mammalian embryos are thought to receive

different cues other than the ones innately set forth. Instead it is firmly believed that in the premature embryo the fate of one cell depends on its interactions with the surrounding cells and the signals they receive from secreted morphogens. Thus, it is crucial for appropriate development to set up the precise pattern of gene expression which will in turn ensure the correct cellular differentiation and subsequent organ formation (McMahon *et al.*, 1998).

In most organisms a continuous interplay of information is exchanged between a respective morphogen and its target cells. This information relay not only enables the cells to regulate their own intracellular reaction to the graded signal but also allows for the gradient to be altered accordingly.

A morphogen is a signal which has the ability to affect cells located away from it and at the same time induces a variable effect depending on the relative concentration of the signal the cells are exposed to. Shh is one of the factors which act as morphogens during embryonic development since it has been explicitly shown that even when the signal has been shut down or even interrupted further away from the secretion site the effects are detrimental to the expected pattern (Teillet *et al.*, 1998). Furthermore, introduction of modified Shh of varying concentrations will once again induce an unusual neural progenitor differentiation.

The developing neural tube initially contains cells that have equivalent properties irrespective of their dorso-ventral position. However even before neural tube closes, the notochord initiates the secretion of Shh, in response; this triggers the floor plate to also begin producing Shh. Thus resulting in the establishment of a Shh gradient across the neural tube ends, which affects the cells exposed to it in a concentration dependent manner. Following the folding of the neural plate and the subsequent formation of the neural tube, a floor plate region and a roof plate region are

created due to the secretion of Shh from the most ventral portion of the plate (adjacent to the notochord) and BMP-4 from the most dorsal portion of the tube. Ultimately, the ventral neural plate will generate most of the ventral nervous system, as well as the motor segment, and the brain stem. The roof plate is involved in patterning the dorsal (sensory portion) of the spinal cord (Briscoe *et al.*, 1999; Ribes & Briscoe, 2009).

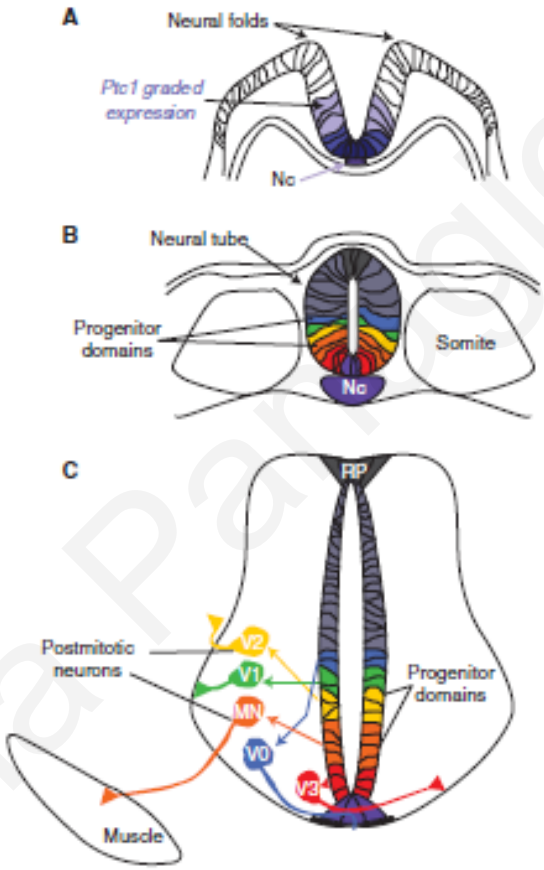


Figure 6: Ventral Spinal Cord Patterning; A: Gradient induced expression of Ptc1 (Shh receptor) resulting from Shh secreted solely from the notochord. B: Following the closure of the spinal cord the six domains are established along the ventral axis (FP, p3, pMN, p2, p1 and p0). C: The progenitor domains give rise to individual types of interneurons (V0-V3) or motor neurons. These distinct cell types travel via the regular migration pathways in order to reach their ultimate positions within the spinal cord, whereas their extended axons will produce the required neuronal circuits. (Ribes & Briscoe, 2009)

Regarding the ventral spinal cord, the varying Shh concentration controls the expression of a battery of TFs which are in turn expressed from different neuronal progenitors. Specifically, the combined expression and regulation of those TFs gives

rise to six distinct domains along the ventral spinal cord, the floor plate (FP), p3, pMN, p2, p1 and p0 (Figure 6). The sequence with which the TFs are expressed is based upon the requirement for an increased concentration of Shh over an extended period of time, given that the greater the Shh concentration or the longer the cells are exposed to it the domains will assume a more ventral character (Ericson *et al.*, 1995).

As has been aforementioned, Shh is initially secreted from the notochord. This centre however progressively degenerates so that another centre for Shh secretion can be established from the floor plate. It should be noted though that this second centre only comes about following the establishment of the six distinct domains, which would indicate that the domains are only created depending on the Shh secreted from the notochord (Figure 6B,) (Briscoe & Ericson, 2001; Ribes & Briscoe, 2009).

Using recombinant, biologically active, fusion Shh^{-GFP} protein (Chamberlain *et al.*, 2008) in order to observe and quantify the accumulation and distribution of Shh along the ventral spinal cord showed that Shh concentration decreases in an exponential manner, moving from the floor plate to the roof plate. Unsurprisingly, the floor plate, along with the p3 domain, experience the highest exposure to the morphogen, whereas the intensity dramatically decreases across the pMN domain and finally becomes almost undetectable across the p2 domain. Furthermore, it was observed that the intensity of expression of Shh increases as the neural tube matures through the developmental process. The implication of this is the fact that progenitors situated along the halfway point of the ventral spinal cord will be exposed to increasingly higher concentrations of Shh for a prolonged period as compared to progenitor cells found at the dorsal domains. This observation is in agreement with the expression of genes generally associated with higher concentration of Shh. Specifically, Olig2 (induces MN production) is expressed during short pulses of low morphogen exposure, while when

Shh expression reaches more dorsal levels the OLIG2 expressing domain shifts along the gradient. At the same time, the progenitors previously expressing OLIG2 at low Shh concentrations will instead be induced to produce NKX2.2 following longer exposure (Ribes & Briscoe, 2009). This relationship between the levels of the Shh morphogen and the gene expression profile is explained in detail in figure 7.

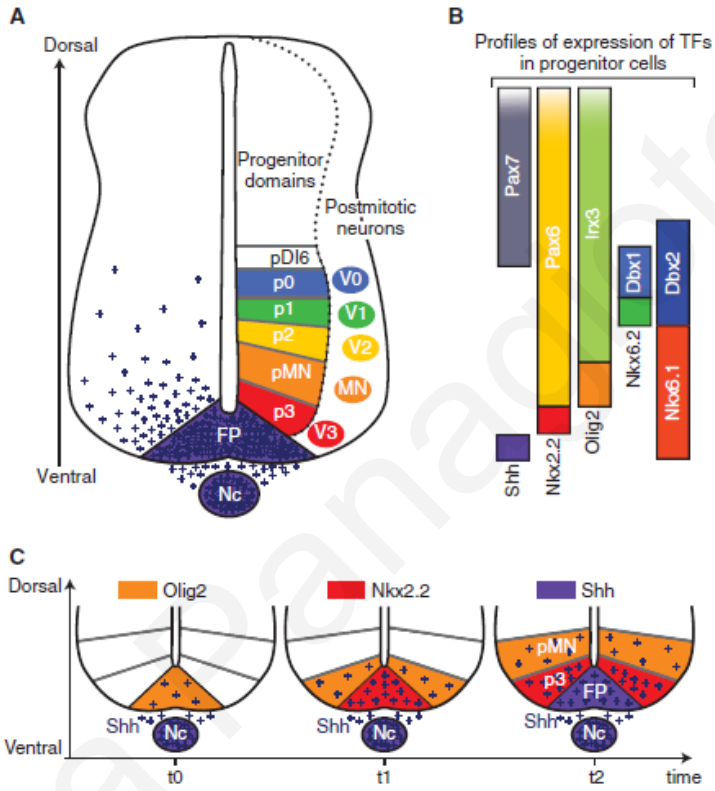


Figure 7: Gene expression coupled to relative Shh concentration; A: The Shh gradient resulting from production of the signal from the notochord and the floor plate gives rise to the six ventral domains and consequently to the distinct interneurons and motor neurons. B: Each domain expresses a distinct combination of transcription factors which in turn grant each domain is own unique characteristics. C: As explained in the text, during different developmental stages and as the concentration of Shh increases, the pMN domain migrates dorsally in response to the increases expression of Shh while other combinations of gene expression are induced. (Ribes & Briscoe, 2009)

Patterning in the ventral neural tube has been identified to occur in three distinct phases. Initially, a dorso-ventral gradient is established through the Shh-N been secreted by the notochord, thus establishing the floor plate. Secondly, this directly impacts the expression of homeodomain TFs which are either activated or suppressed in the presence of Shh-N. The TFs, whose expression is inhibited (also referred to as

the class I TFs), are Pax6, Pax7, Dbx2 and Irx3. On the other hand class II TFs activated by Shh signalling, include Nkx2.2, Nkx6.1, Nkx6.2 and the basic helix-loop-helix factor, Olig2 (Novitch *et al.*, 2001; Vallstedt *et al.*, 2001) Cross-repressive interactions between pairs of class I and class II proteins regulate the respective boundaries and establish sharp progenitor domain borders which are maintained throughout development. The demarcation of progenitor domains into regions of unique TF profile results in the generation of different cell types, such as INs and MNs, along the dorso-ventral axis.

Studies suggest that initially a low concentration of the Shh-N is required to initiate the acquisition of differentiated cellular character. Furthermore, the signal 'priming/activation' had to be delivered at a specific time interval or else it would be inconsequential. However, this initial concentration is not enough to actually propagate the effects of the signal and induce the generation of the different cell types. Instead, the concentration has to be increased by ten times in order to bring about the desired effects (Ericson *et al.*, 1996; Poh *et al.*, 2002). Furthermore, following a certain period in their development, a cell's fate was no longer influenced by the presence or maintenance of the Shh-N signal. This decisive time point, though, varied depending on the respective cell type. Experiments carried out by Poh *et al.*, 2002 exemplified that whereas MNs remain dependent upon the continuation of the Shh-N signal for as long as twenty-four hours following their generation, the removal of the signal from V2 INs (just dorsal to MNs) twelve hours after the generation appears to be insignificant.

Pax6, Nkx6.1 and Olig2 define the pMN domain which will in turn produce MNs. Olig2 thereafter relieves the repression of MNs inducing factors (Mizuguchi *et al.*, 2001; Sun *et al.*, 2001) as well as Neurogenin 2 (Ngn2), thus encouraging the progenitors to exit the cell cycle and instead differentiate to take on the required

neuronal attributes. In the chick embryo an increase in the concentration of Shh-N induces the expansion of the Nkx2.2 domain, overlapping with the Olig2 domain so that OL production is favoured over MNs production (Marquardt & Pfaff, 2001; Soula *et al.*, 2001). In the mouse however such mechanism has not been proven and the MN to OL switch mechanism has not been fully established.

Despite the fact that Shh-N is in its own right one of the most critical determinants of early development and has accordingly been conserved throughout evolution, other molecules also play an immensely significant role. Pierani *et al.*, (1999) have shown that even though Shh-N may be able to instigate the terminal differentiation of V0 and V1 spinal INs, in its absence retinoid signalling can independently induce the production of both of these sub populations. Furthermore, Noggin-deficient mice (the BMP 2/4 repressor mostly thought to be associated with dorsal patterning) exhibit an incomplete floor plate structure as well as reduction in the number of ventral INs and MNs ((McMahon *et al.*, 1998). Evidently, BMP factors are as significant as Shh-N in establishing the appropriate patterning profile, as well as ensuring all cell types' specification is achieved.

1.7 VENTRAL INTERNEURONS:

Four classes for INs (INs) arise from the ventral spinal cord (vSC), the V3, V2, V1 and V0 INs. They are classified in such a way as to reflect their origin and the TF combination they express (Briscoe *et al.*, 2000; Jessell, 2000). Each of these classes is further divided into smaller subpopulations of more specialized INs (Figure 8).

All V0 IN progenitors are marked by the expression of DBX1 whereas mature V0 INs express the neurotransmitters GABA and GLUTAMATE. The category is further sub-divided into $V0_{D\ (dorsal)}$ and $V0_{V\ (ventral)}$ INs which express Evx1/2. This class

of INs has also been shown to initiate contralateral projections to MNs, as well as being involved with left/right alteration (Lanuza *et al.*, 2004).

V1 IN progenitors are marked by the expression of EN1 and give rise to Renshaw cells and Ia inhibitory INs. Renshaw cells express calbindin, while Ia inhibitory INs express PARVALBUMIN. V1 INs express the neurotransmitters GABA and GLYCINE while both subpopulations establish ipsilateral projections to MNs, as they've been proposed to be involved in locomotion cycle speed (Gosgnach *et al.*, 2006) .

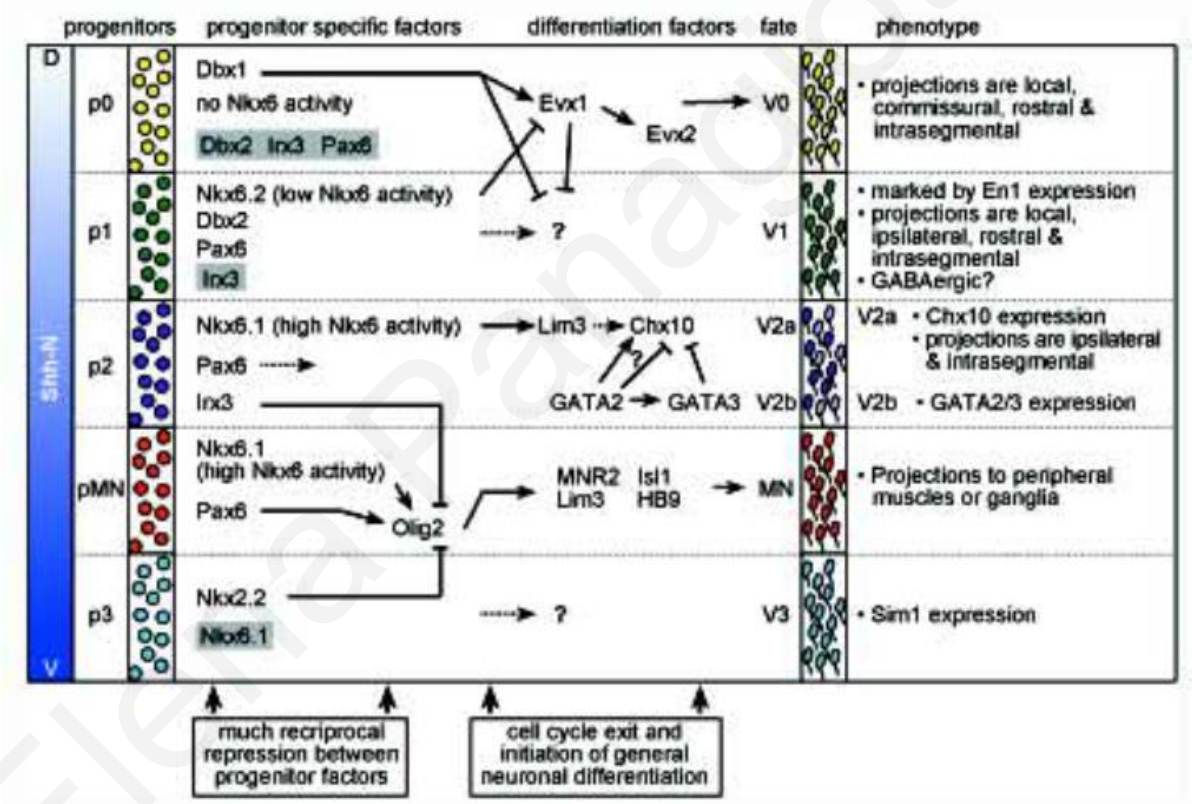


Figure 8: Molecular events which ultimately give rise to ventral interneuron subtypes. (Poh *et al.*, 2002)

Lhx3 positive progenitors generate V2 INs. This class is further divided into the Chx10/Sox14 positive V2a INs, the Gata3 positive V2bs and the Sox1 positive V2c INs (Panayi *et al.*, 2010). Whereas V2a INs secrete the neurotransmitter Glutamate, V2bs are instead inhibitory GABAergic and Glycinergic neurons. Regarding information concerning their known projections and proposed functions, conclusive data is only

available for the V2a INs. The Chx10 positive subclass form ipsilateral projections networking the INs to both MNs and V0 INs, they've been proposed to be involved with burst robustness and left-right alteration (Crone *et al.*, 2008).

The Glutamatergic V3 INs are derived from Sim1 positive progenitors which in turn are generated from the most ventral Nkx2.2^{+} p3 domain. Zhang *et al.*, used a Sim1^{-Cre} mouse model crossed to a reporter model expressing membrane bound EGFP to establish that approximately 85% of the V3 INs are commissural while the rest establish ipsilateral projections (Zhang *et al.*, 2008). The same study also concluded that the V3 INs are heavily implicated with burst robustness. Finally, these INs were found to associate with MNs, Renshaw cells, Ia inhibitory INs and V2 INs (Stepien & Arber, 2008).

1.8 GATA3 PHYSIOLOGICAL AND ABERANT EXPRESSION:

As part of this study concentrates on a unique expression pattern of Gata3 in the murine spinal cord, a more detailed introduction into the Gata family of TFs is warranted. The Gata family of TFs is known to contain six members in vertebrates. Gata1, Gata2 and Gata3 are heavily implicated in the specification and terminal differentiation of the hematopoietic system (Orkin, 1996; Ting *et al.*, 1996). Gata4, Gata5 and Gata6 have been shown to be involved with the differentiation of the cardiac system and viscera (Kuo *et al.*, 1997). However, only Gata2 and Gata3 have been found to be expressed in the central nervous system (Nardelli *et al.*, 1999).

Gata3 is made up of two zinc finger motifs which bind to the six-nucleotide consensus sequence {A/T}GATA{A/G} (Racz *et al.*, 2011). Gata3 knockout mice die in their embryonic stage, whereas Gata3 expression levels are immensely significant since haplo-insufficiency of Gata3 causes the rare abnormal dominant disease HDR

syndrome (OMIM: 146255) (Muroya *et al.*, 2001; Nesbit *et al.*, 2004). Gata3 is expressed in the skin, in the developing central nervous system, inner ear, eyes, mammary glands, thymus and the embryonic kidney. It is also expressed in cells of the hematopoietic system, the T-cells, Natural killer cells and Natural killer T cells (Kim *et al.*, 2006; Hosoya-Ohmura *et al.*, 2011; Eifan *et al.*, 2012). Furthermore, GATA3 is expressed in the V2b ventral spinal cord inhibitory INs as well as been initially expressed transiently in the newly identified Sox1 positive V2c INs (Panayi *et al.*, 2010).

Cytokines secreted from the T helper type 2 (T_h2) control rhinitis, atopic dermatitis, allergic inflammation in asthma, as well as parasitic humoral immunity (Maneechotesuwan *et al.*, 2007). T_h2 lymphocytes are in turn regulated by Gata3, which also manages the transcription of IL-4, IL-5 and IL-13 via chromatin remodelling (Lee *et al.*, 2001; Takemoto *et al.*, 2002).

The size of GATA3 exceeds the limit for passive nuclear diffusion; therefore it encompasses a nuclear localization signal (NLS) sequence so that it needs to be actively transported through the nuclear membrane in order to reach its target genes. Importin-α (karyopherin-α) has been shown to be involved in the transport of GATA3 from the cytoplasm to the nucleus. The affinity of importin-α to the GATA3 NLS is heavily affected by GATA3 phosphorylation (Yang *et al.*, 1994; Ohno *et al.*, 1998).

Maneechotesuwan *et al.*, have shown that the p38 MAPK (Mitogen-activated protein kinase) pathway facilitates serine phosphorylation which triggers the transport of the GATA3 protein from its cytoplasmic location where it is in an inactive state, into the nucleus through interaction of its NLS sequence with importin-α. Anti-CD28 and anti-CD3 antibodies were used to co-stimulate and thus simulate the activation of T cells by antigen presenting cells, thus inducing the activation of the p38 MAPK

pathway (Figure 9). As a result of this cascade been initiated GATA3 becomes phosphorylated, then interacts with importin-α and is escorted into the nucleus where it binds to the promoters of the T_h2 cytokine genes, resulting in an increase in the genes' expression, thus ultimately inducing T-cell stimulation. This effect was again replicated when 40% of the T-cells isolated from flesh blood were shown to have cytoplasmic GATA3 expression, whereas following phosphorylation GATA3 was nuclear following anti-CD3/CD28 stimulation (Maneechotesuwan *et al.*, 2007).

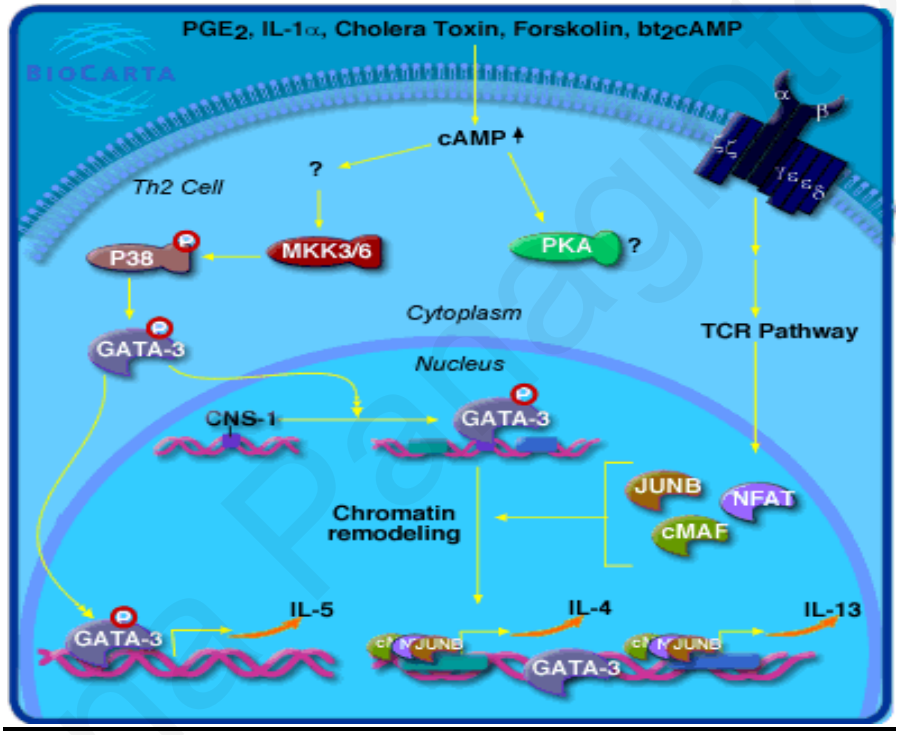


Figure 9: Gata3 involved in the T_h2 cytokine gene expression through the p38 MAPK pathway. http://www.biocarta.com/pathfiles/h_gata3pathway.asp

GATA3 has also been found to be expressed in some breast tumours (West *et al.*, 2001) as well as been related with the estrogen receptor alpha protein (ERS1) (van de Rijn & Rubin, 2002). Usary *et al.*, reported that in one of the breast tumour cases they had examined, GATA3 exhibited complete cytoplasmic expression whereas the adjoining unaffected ductal cells had a nuclear expression pattern. This was found to be caused be a somatic mutation which induced a shift in the NLS sequence this altering the purpose and decreasing the operation of GATA3 (Usary *et al.*, 2004) .

Hodgkin lymphoma (HL) is a high prevalence malignant lymphoma which routinely presents Hodgkin and Reed/Sternberg (HRS) tumour cells along with other mixed cellular infiltrate (Kuppers *et al.*, 2003). The majority of HRS cells originally from B-cells which demonstrate an altered expression profile which also includes the Gata3 gene which is physiologically strictly expressed in T-cells (Stanelle *et al.*, 2010). Notch1 has also been reported to affect the development of T_h2 cells since in murine CD4 positive cells, Gata3 was shown to be activated by Notch1 through direct promoter transactivation, where their joint activity has been proposed to promote the suppression of B-cell genes in HRS cells and thus induce enhanced growth and limited apoptosis in those cells (Stanelle *et al.*, 2010) . The Gata3 transcriptional start site and promoter region has previously been found to be occupied by both Notch1 and NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells complex) in HL cells so that NFkB inhibition or Notch1 suppression lead to the down regulation of Gata3 nuclear expression in these cells, thus restricting GATA3 action (Stanelle *et al.*, 2010). It should be noted however that Gata3 expression was found to be greatly enhanced in human oesophageal cancer cells when compared to the normal oesophageal mucosal cells (Shiga *et al.*, 1993). As has been aforementioned, Gata3 expression appears to vary depending on the type of cancer, possibly reflecting the TF's mode of action.

Finally, Gata3 has been identified in human pancreatic cancers, where it was found to be overexpressed, both in the mRNA and protein level. Gata1 and Gata2 were not expressed at all in these cells, so that Gata3 is selectively and specifically expressed (Gulbinas *et al.*, 2006). Gata3 was found to be only expressed exclusively in the cytoplasm and not in the nucleus. These results are in contrast to the primarily nuclear expression pattern observed in most of the other tumours, as well as breast tumours (Hoch *et al.*, 1999).

GATA3 overexpression affects the proper differentiation of neurons and adipocytes (Lim *et al.*, 2000; Tong *et al.*, 2000) where their differentiation seems to be halted and severely de-programmed. In these cells GATA3 expression appears to disturb the TGF-β (Tumour growth factor beta) pathway (Gulbinas *et al.*, 2006). There is also evidence linking the dedifferentiation process to the primal stages of pancreatic cancer development (Real, 2003). Therefore, this information indicates that disruption in the TGF-β pathway by the increased cytoplasmic GATA3 expression may be involved in dedifferentiation. In addition, GATA3 deficiency in adult mice has been shown to induce dedifferentiation of the luminal epithelium (Zheng *et al.*, 2011). Finally, GATA3 has been proposed to act as a tumour suppressor so that abolishing its nuclear function may lead to cancer development.

1.9 THE EPENDYMA AND THE CENTRAL CANAL:

Neurogenesis was believed to be completed soon after birth. Glial and neuronal cellular populations are the result of the wide-spread proliferation of cells from the ventricular zone of the brain and spinal cord early in development (Shihabuddin *et al.*, 1997). The skin epithelium, small intestine epithelium and bone marrow are systems known for their proliferative capacities, as a response to the appropriate signalling mechanisms (Potten & Loeffler, 1990). Similar, self-renewing stem cell-like neurons were initially described in the brain's sub ependymal layer.

Cells lining the ependyma are remnants of progenitors originating from the primitive neuroepithelium which eventually formulate the lining of the spinal cord central canal and the cerebral ventricles (Del Bigio, 1995). The ependyma has been shown to possess proliferative capabilities (Martens *et al.*, 2002) as well as forming a selective barrier between the neural tissue and the CSF (Prothmann *et al.*, 2001).

Interruption or disruption of the ependymal layer has been reported to induce hydrocephaly as well as disturbance of the proper course of the CSF throughout the spinal cord since this layer has been credited with eliminating endogenous and exogenous toxic molecules from the central nervous system (Brody *et al.*, 2000; Kobayashi *et al.*, 2002).

Proliferating cells in the spinal cord parenchyma have been shown to originate and migrate from the ependyma to the grey matter (Kojima & Tator, 2002). Self-renewing neurospheres produced from tissue including the central canal demonstrated that neural stem cells populate the proximity of the central canal (Martens *et al.*, 2002).

Ependymal cells have been shown to initiate production of neurons in the rat brain in the third ventricle on E16 (which according to the Carnegie stage comparison chart corresponds to E14.5 in mouse development). Ependymocyte proliferation has been shown to occur along a rostral to caudal gradient in the spinal cord (Altman & Bayer, 1984). The time point at which proliferation stops postnatally has been reported at many varying stages, with one study reporting no apparent proliferation after the postnatal stage P4 (Spassky *et al.*, 2005) whereas it has also been reported that lateral ventricle proliferation was detected as far as five to seven weeks postnatally (Korr, 1980). Using homeodomain TF profiling and fate mapping studies in rodents, ependymocytes have thus far been reported to originate from the p3, pMN and p2 domains (Fu *et al.*, 2003b; Masahira *et al.*, 2006).

The area surrounding the central canal of juvenile turtles is comprised of cells which share many of the molecular and functional attributes of immature neurons found in neurogenic niches (Fernandez *et al.*, 2002; Russo *et al.*, 2004). In the mammalian adult brain, neurons which retain the ability to proliferate and differentiate throughout life have been found to originally express the polysialylated neural cell adhesion

molecule (PSA-NCAM) and doublecortin. As these cells mature and become assimilated into established neuronal circuits they begin expressing NEUN and instead become negative for doublecortin and PSA-NCAM, (Horner *et al.*, 2000; van Praag *et al.*, 2002) thus attaining the molecular profile of mature neurons. Cells with a similar molecular profile and attributes have also been identified in the adult mammalian ependyma (Marichal *et al.*, 2009). Some central contacting cells in the adult rat spinal cord express HUC/D, PSA-NCAM and doublecortin, thus indicating that they have an immature character, similar to the neuroblasts in the adult rodent brain (Seki, 2002). However, non-neuronal cellular populations have also been found to co-express PSA-NCAM and doublecortin. Central canal contacting cells though also possess electrophysiological properties associated with neuronal populations. Ependymal cells of non-neuronal character exhibit glial like readings such as broad gap junction coupling, low input resistance and high resting membrane potential, as well as expressing S100-β (Bruni, 1998). Central canal contacting cells however are uncoupled with high input resistance, as well as exhibiting regenerative potential (Marichal *et al.*, 2009). These data suggest that central canal contacting cells are distinct from ependymocytes, as well as been part of the neuronal lineage.

Sevc's group, using BrdU (5-bromo-2'-deoxyuridine) data reported two subpopulations of ependymocytes in the spinal cord of purportedly distinct origins. One subpopulation was shown to originate from radial glial progenitors during embryogenesis. The second population was reported to occur during the postnatal stages which were linked to two bundles of glial fibres emerging from the floor and roof plate. The time point for the second wave's emergence was found to be between P8-P15, as shown by BrdU injection assays at different spinal cord segments (Sevc *et al.*, 2011). Spinal cord radial glial form a platform required to assist in the relocation of

neurons which will ultimately populate the grey matter (McMahon & McDermott, 2002).

In the brain, the B1 cells which are periventricular astrocytes have been widely postulated to be neural and OL progenitors in the adult forebrain (Menn *et al.*, 2006; Mirzadeh *et al.*, 2008). It has been proposed that central canal cells are not related to the conventional multiciliated ependymal cells but are instead more closely related to the B1 forebrain cells (Martens *et al.*, 2002).

The Notch pathway is known to maintain cells in an undifferentiated state and thus be involved in cell cycle management and regulation (Yamamoto *et al.*, 2001a). Notch1 has been shown to be expressed in ependymal cells following spinal cord injury. Cells which express NOTCH1 were also observed to differentiate into radial glial and postnatally differentiate into glial fibrillary acidic protein (GFAP) positive perivascular cells and astrocytes (Gaiano *et al.*, 2000). These cells have been previously postulated to be the adult neuronal progenitors or even neural stem cells.

The adult rodent spinal cord ependyma has been morphologically thoroughly described, even though the functions of most of the cells have not been fully elucidated yet. Morphological studies have shown that CSF contacting neurons adjoining the central canal have dendritic processes which run into the lumen (Bruni & Reddy, 1987). Amongst the earliest documented descriptions of this region is Ramon y Cajal's in his "Textbook on the nervous system of man and the vertebrates". Three different cellular populations were identified, the posterior, anterior and lateral cells. Lateral cells were recognized by short basal processes often branching out and reaching into the sub-ependymal grey matter (Oliviero *et al.*, 2012). Posterior and anterior cells have long radial processes. Some of the cells with the long radial processes have been further described as tanycytes (radial ependymocytes). These ependymocytes extend their

processes into the ventricular area and are positive for GFAP (Mothe & Tator, 2005). Furthermore, bi-nucleated ependymocytes have been recently identified. Numerous pleomorphic vesicles were found in the globular extensions of the central canal contacting neurons. These globular extensions were also found to contain a great number of mitochondria. Additionally, the neurons' cytoplasm was found to be less electron-dense than the rest of the ependymal cells', as well as contain free ribosomes; but lacking lipid droplets and intermediate filaments. Furthermore, small chromatin clumps were noticed to be associated with the nuclear envelope, whereas the cells only have one single cilium and a rectilinear situated centriole (Alfaro-Cervello *et al.*, 2012). The presence of various neuronal markers and the neurotransmitters GABA and VIP, as well as the existence of synaptic vesicles are indicative of the fact that central canal contacting cells are indeed operational neurons (Barber *et al.*, 1982; LaMotte, 1987; Vigh *et al.*, 2004). It has been suggested that CSF-contacting neurons may secrete compounds or factors into the central canal or they may utilize their contact to Reissner's fibre and sense the spinal cord's motion in chordates (Alfaro-Cervello *et al.*, 2012).

1.10 SPINAL CORD INJURY AND REGENERATION:

In lower vertebrates, lizards and amphibians, the spinal cord the ependyma is crucial during regeneration following spinal cord injury. In some low vertebrates the plasticity of the central canal is so developed that the central canal coordinates the reconnection of the spinal cord, even after it has been severed (Dervan & Roberts, 2003). Whereas in adult mammals it appears that not only is the regenerative capacity extremely limited but even when these cells are activated following injury they

predominantly differentiate into astrocytes which relocate to the site of injury and build the glial scar (Mothe & Tator, 2005) .

Spinal cord injury has been shown to elicit the regeneration capabilities of the ependymal cells, the same effect observed when a mixture of FGF2 and epidermal growth factor (EGF) were intrathecally introduced following clip compression injury (Kojima & Tator, 2000; 2002). Following spinal cord injury the ependymal cells have been shown to multiply and differentiate in order to stimulate spinal cord regeneration. The number of ependymal cells radically declines postnatally, even though ependymal cell proliferation is quite standard during the embryonic and even early postnatal stages. The ependyma does retain some diminished regenerative capabilities in adult mammals (Bruni, 1998).

Using Dil, the fluorescent carbocyanine tracer, *in vivo* labelling of ependymal cells allowed them to be tracked after injury. Following minimal injury these cells could be distinguished from other cells infiltrating the central canal region after the injury so that three days after the injury the BrdU labelling index of ependymal cells increased by 7% (Mothe & Tator, 2005).

Ependymal cell proliferation was considered to occur as a reaction to injury where the ependymocytes terminally differentiate into astrocytes which eventually travel to the injury site and form part of the glial scar (Mothe & Tator, 2005). Furthermore, when the response of ependymal cells to spinal cord injury and haemorrhage was investigated in rats, the cells were shown to achieve maximum proliferative activity two days following the injury and return to physiological levels two days later, thus four days after the initial injury (Bruni & Anderson, 1987; Bruni & Reddy, 1987). Also, NESTIN was found to be actively expressed, not only following the injury but at later time points as well (Shibuya *et al.*, 2002). NESTIN expression

was found to vary in magnitude according to the severity of the injury. Additionally, the longer the expression of NESTIN was maintained the greater the improvement of motor limb function was observed (Yamamoto *et al.*, 2001a).

Takahashi's study in 2003 showed that non-reactive astrocytes were marked by the absence of NESTIN but were GFAP positive, whereas active astrocytes were both GFAP and NESTIN positive. An increased number of non-reactive astrocytes in the ependyma lining was accompanied by the termination of the repair process, whereas repair proceeded only when active astrocytes were present (Takahashi *et al.*, 2003). Glial cells secrete nitric oxide, cytokines and neurotrophins which instigate and propagate the repair mechanism (Lipton *et al.*, 1993; Sawada *et al.*, 1995; Miyazaki *et al.*, 1999). Therefore, it appears to be crucial to enable reactive astrocytes to persist along the site of injury in order to prolong the repair mechanism, as well as to encourage injury site neurogenesis instead of gliogenesis.

Functional rehabilitation following spinal cord injury in rodents and primates has been observed after stem cell transplantation. The advantages observed by stem cell transplantation may arise from a number of processes, such as the ability to replenish lost cells while allowing for more neurotrophic factors to be secreted. However, the most significant effect may stem from the re-myelination of damaged neurons near the site of injury, as a result of oligodendrogenesis (Enzmann *et al.*, 2006; Thuret *et al.*, 2006). Even though ependymal cells have a very restricted proliferative ability, following spinal cord injury they generate astrocytes which will eventually migrate to the site of injury and incorporate into the glial scar as well as generating a small number of OLs (Meletis *et al.*, 2008).

In general, central nervous system injuries are characterized by slow and incomplete functional recovery. This slow recovery is party caused by the inability of

the damaged axonal processes to re-grow and re-innervate their respective targets since production of astrocytes is favoured over the production of OLs and neurons, in addition to demyelinated axons which inhibit proper signal propagation (Schwab, 2004). In the event of demyelination axons become incapable of allowing the transmission of electrical impulses, as well as becoming more prone to chronic degeneration. Generation of OLs enables the myelin sheath to be renewed, even though this is a lengthy process. Ependymocytes not only participate in the regeneration of astrocytes but also to a lesser degree OLs and thus contribute to the re-myelination of axons along the site of spinal cord injury (Blakemore & Patterson, 1978; Yang *et al.*, 2006a).

1.11 OBJECTIVES:

We have previously shown that the p2 domain generates at least three types of ventral INs from common $FoxN4^{+}$ progenitors, namely V2a, V2b and V2c (Del Barrio et al., 2007; Li et al., 2010; Panayi et al., 2010). These cells are first detectable in the marginal zone at around e10.5-e11.00 and express Chx10, Gata3 and Sox1, respectively. Pax6 is a transcription factor that is first expressed by most neural progenitors of the spinal cord and is required for correct neural patterning during neurogenesis (Ericson et al., 1997b) and gliogenesis (Genethliou et al., 2009; Hochstim et al., 2008). At thoracic level of the spinal cord at e12.0-e12.5 Pax6 expression extends to a small group of ventral neurons whose origin and development has not been investigated.

In addition, another group of cerebrospinal fluid contacting neurons has been observed. In general, CSF contacting neurons have been purported to be involved in an early response mechanism to injury and trauma (Conte et al., 2008) even though a great

deal of speculation and lack of information surrounds these cells. Therefore, we have attempted to further characterize the nature of this group of late born cells and thus gain some insight into their expression profile.

In order to be able to achieve the overall objective the following technological objectives have been identified:

1. Use transgenic mice lines and protein markers to characterize the ventral late born Pax6 positive neurons.
2. Use protein expression markers to characterize a small dorsal population of Gata3 positive neurons.
3. Use protein expression markers to characterize the Gata3 and Sox1 positive CSF contacting cells

CHAPTER 2

RESULTS

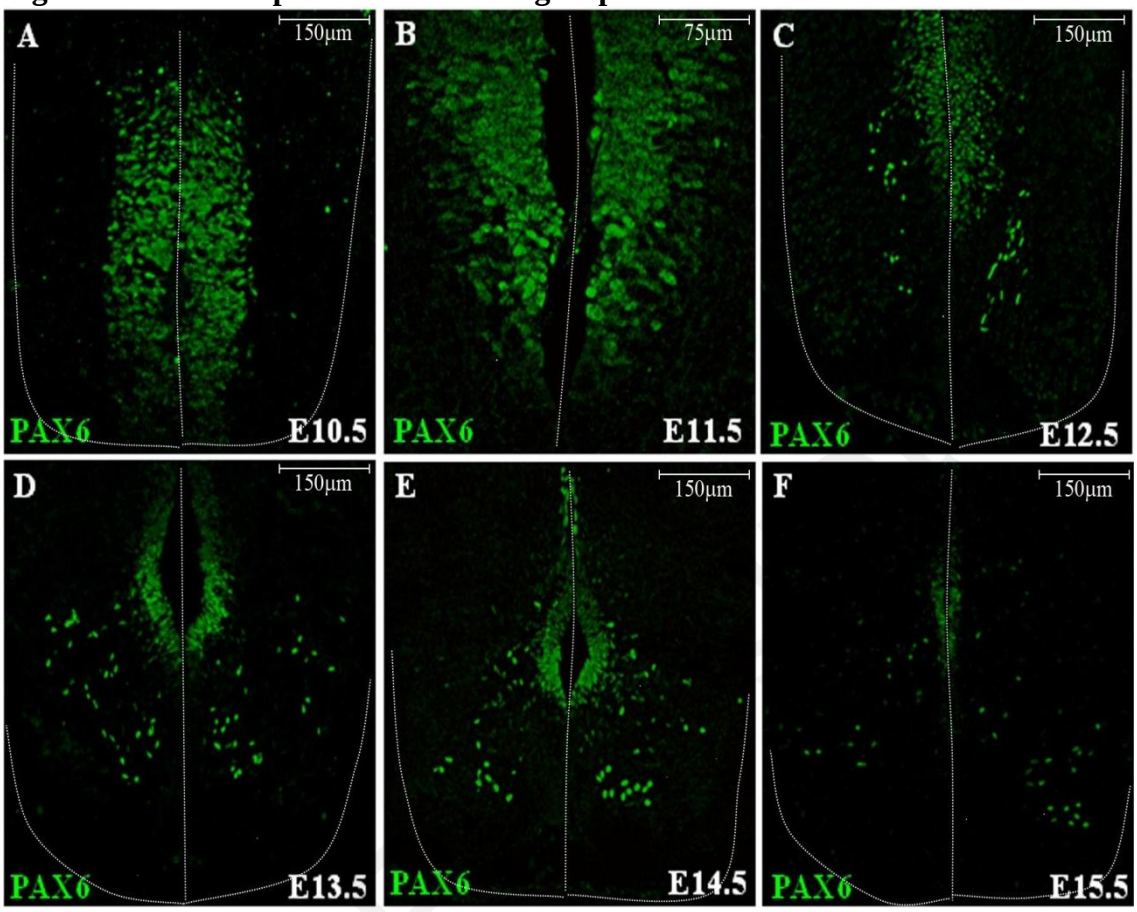
2.1 LATE WAVE NEUROGENESIS IN THE VENTRAL SPINAL CORD

The scope of this study was to study late neurogenesis in the ventral spinal cord during embryogenesis. We focused our study predominantly on neurogenic patterns of ventral INs. In addition, using reporter mouse lines and protein expression analysis we characterized the molecular composition of the adult central canal of the spinal cord. We first begin with our data on embryonic late born neurons in the vSC. *Note that dotted lines were added to the figures to indicate the section's localization where necessary and/or possible.*

2.1.1 PAX6 is expressed in a late-born class of V2 and V0 INs

Different groups, including ours, have previously shown that the p2 domain generates at least three types of ventral INs from common FoxN4^{+} progenitors, namely V2a, V2b and V2c. These cells are first detectable in the marginal zone at around E10.5-E11.00 and express CHX10, GATA3 and SOX1, respectively (Del Barrio *et al.*, 2007; Li *et al.*, 2010; Panayi *et al.*, 2010). Pax6 is a TF that is first expressed by most neural progenitors of the spinal cord and is required for correct neural patterning during neurogenesis (Ericson *et al.*, 1997) and gliogenesis (Hochstim *et al.*, 2008; Genethliou *et al.*, 2009b). At the thoracic level of the spinal cord at E12.0-E12.5 PAX6 expression extends to a small group of ventral neurons whose origin and development has not been investigated (Fig 10).

Figure 10: PAX6 expression in a small group of ventral neurons.



*Spinal cord sections from wild type embryos at the denoted stages were immunostained with anti-PAX6 antibody. Panels **A** and **B** depict the expression of PAX6 along the ventricular zone. Panels **C-F** show the migration of PAX6 positive neurons, particularly a smaller neuron population resting at the ventral region of the developing spinal cord.*

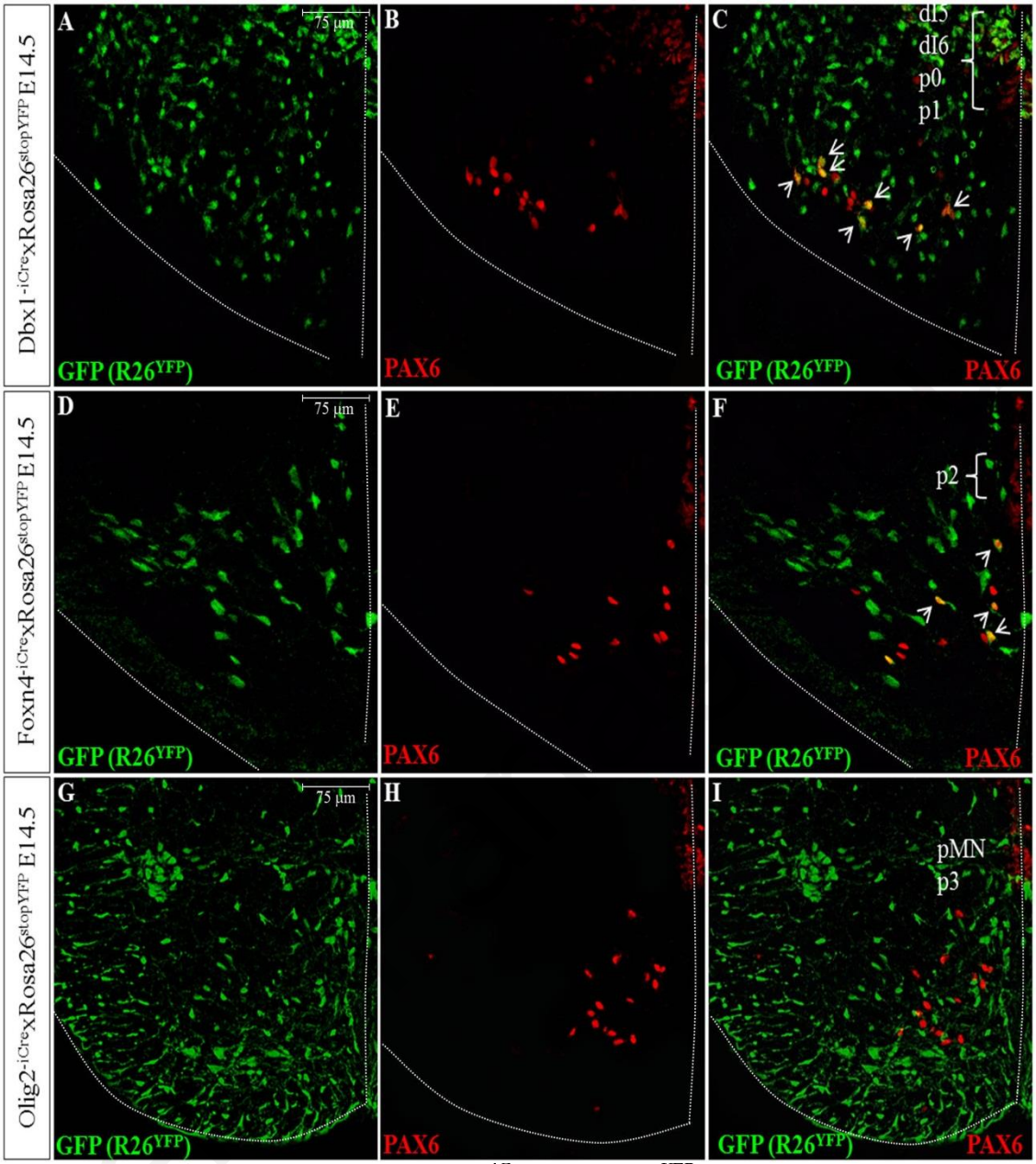
2.1.2 PAX6^{+} cells originate from the Dbx1 and FoxN4 lineages

Based on their final resting position, it seemed likely that the PAX6^{+} neurons originated from ventral progenitor domains. To determine from which progenitor domain(s) they originate, we conducted genetic fate mapping using three Cre–expressing transgenic mice which, all together, represent all six ventral progenitor domains. These were Olig2^{-iCre} (labelling p3- and pMN-derived cells), Foxn4^{-iCre} (labelling p2-derived neurons) and Dbx1^{-iCre} (labelling p0 and p1- derived cells). Males

from each line were crossed to Rosa26^{stopYFP} females and embryos or adult progeny were analysed at the appropriate stages.

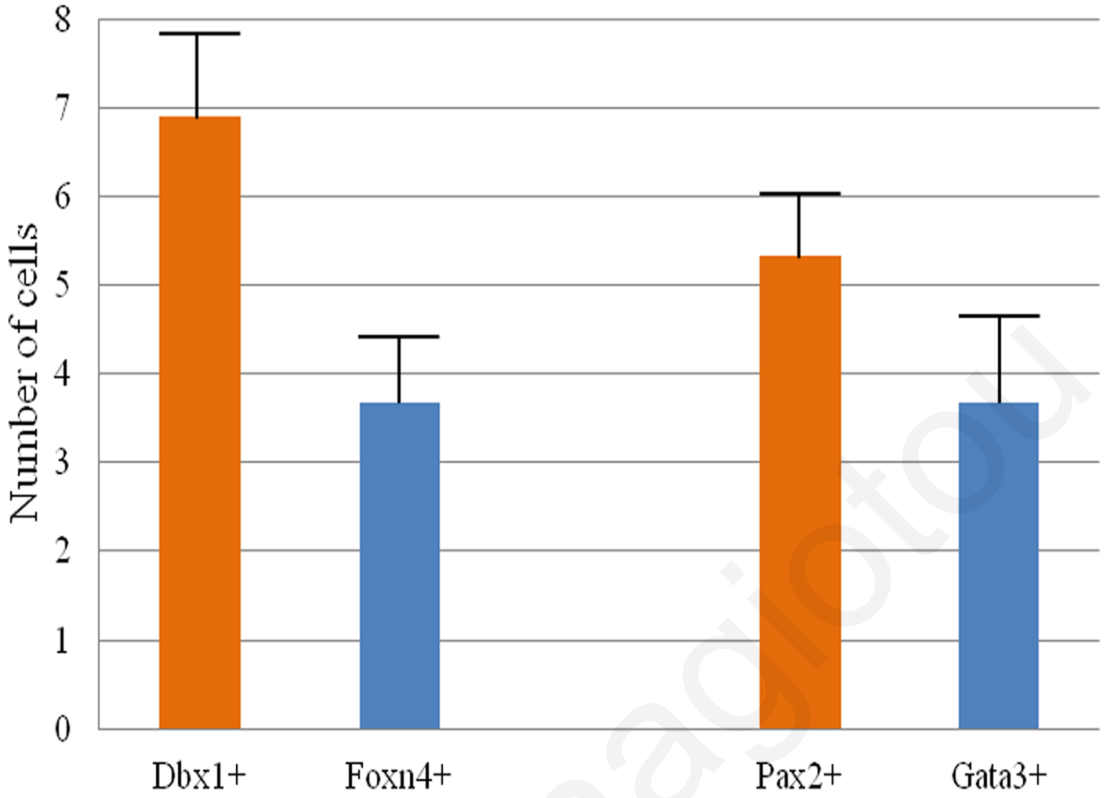
Analysis of embryonic cords derived from a Dbx1^{-iCre} x Rosa26^{stopYFP} cross showed that the majority of the PAX6^{+} neurons derive from Dbx1 lineage suggesting origin from progenitors residing in the p0 – p1 region (Fig. 11A-C and Fig. 12). However, an additional group of PAX6^{+} neurons originated from Foxn4^{+} progenitors suggesting origin from the p2 domain (Fig. 10D-F and Fig. 12). The lineage relationship between FoxN4-expressing progenitors continued to hold true over a range of developmental stages (Fig 13). Finally, analysis of the progeny of the Olig2^{+} lineage in Olig2^{-iCre}xRosa26^{stopYFP} embryos at E14.5 failed to establish a lineage relationship between the p3/pMN domain and the PAX6^{+} neurons (Fig. 11G-I). These data demonstrate that PAX6^{+} neurons originate from several domains of the vSC. A small group originates from the p2 domain and the remaining from Dbx1^{+} progenitors. Taken together our data suggest that an additional type of PAX6^{+} INs is generated in the p2 domain at a later stage and represent V2 neurons.

Figure 11: Lineage analysis of the PAX6^{+} neurons.



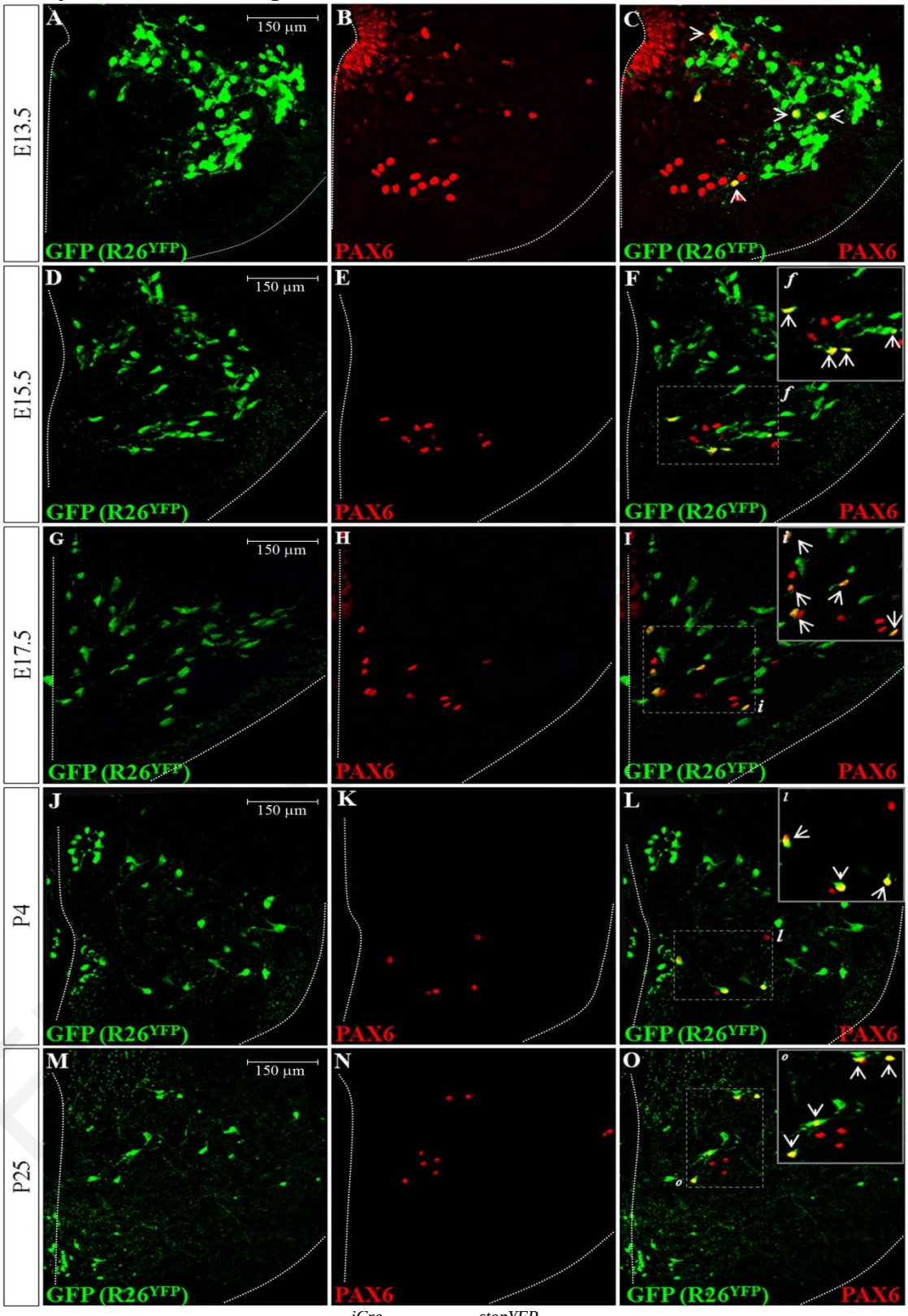
*Spinal cord sections from Dbx1^{-iCre}xRosa26^{stopYFP} (panels **A-C**), Foxn4^{-iCre}xRosa26^{stopYFP} (panels **D-F**) and Olig2^{-iCre}xRosa26^{stopYFP} (panels **G-I**). E14.5 embryos were stained with anti-GFP (R26^{YFP}) and anti-PAX6 antibodies. Arrows in panel **C** indicate neurons from the Dbx1 lineage that co-express PAX6. Arrows in panel **F** show neurons resulting from the FoxN4 lineage that also express PAX6, whereas the overlaid image from Olig2^{-iCre}xRosa26^{stopYFP} (GFP) and PAX6, panel **I**, indicates that no neurons resulting from the Olig2 lineage express PAX6.*

Figure 12: Cell count of PAX6 $^{+}$ cells.



Spinal cord sections from E14.5 Dbx1^{-iCre}xRosa26^{stopY}and Foxn4^{-iCre}xRosa26^{stopYFP}embryos were stained with anti-PAX6 antibody and the double positive cells were counted. Wild type E14.5 embryos' spinal cord sections were double-stained with anti-PAX6 and anti-Pax2 or anti-Gata3 antibodies. Again, the double positive cells were counted.

Figure 13: Lineage analysis of p2-derived neurons relative to PAX6 expression in embryonic and adult stages.

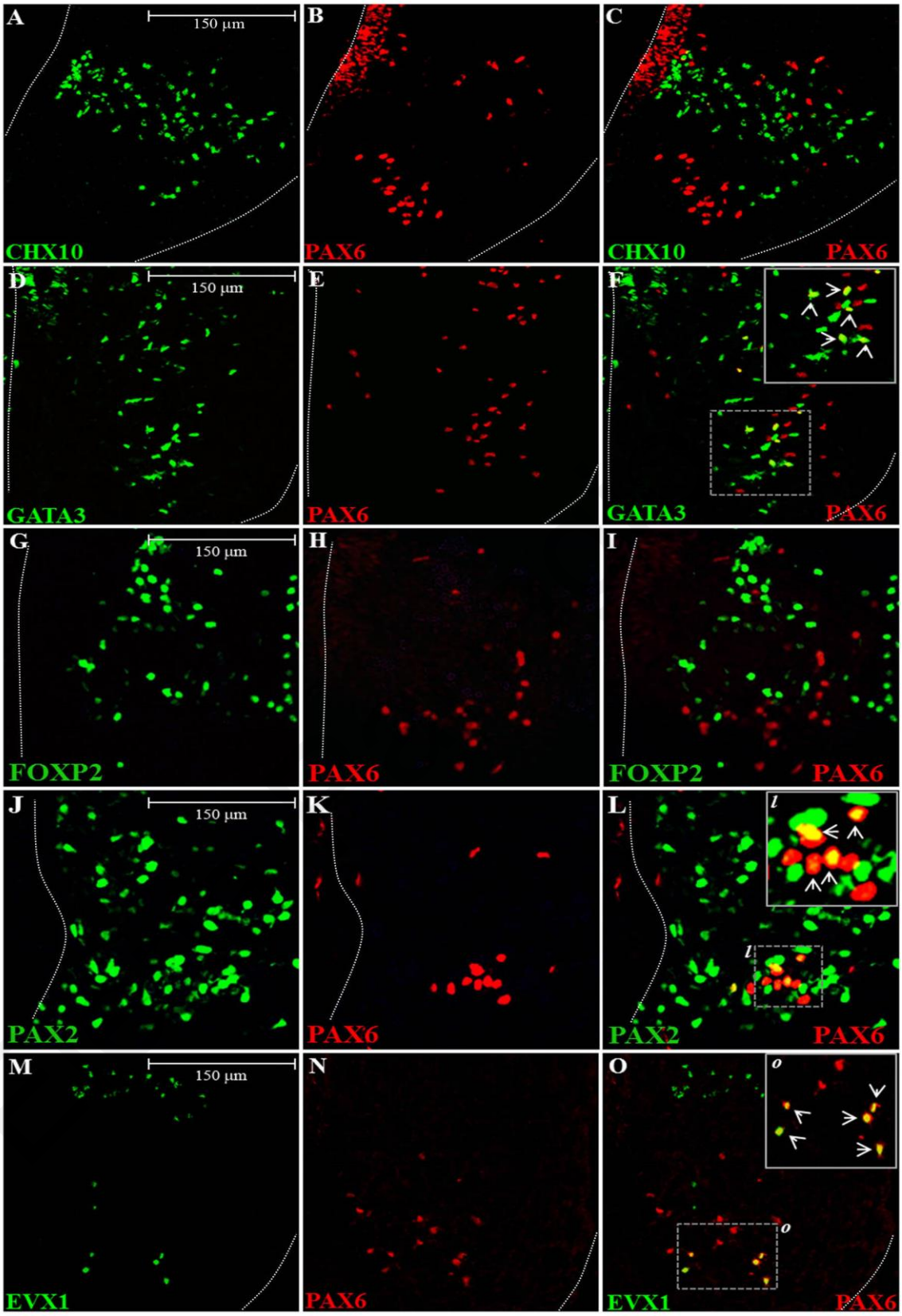


Spinal cord sections from FoxN4^{-iCre}x Rosa26^{stopYFP} embryos at the denoted stages were stained with anti-GFP (R26^{YFP}) and anti-PAX6 antibodies. Insets include higher magnification images of the dotted areas in the respective images and arrows indicate cells from the FoxN4 lineage which also express PAX6.

2.1.3 PAX6^{+} V2 interneurons represent a subset of V2b INs

We next analyzed if PAX6^{+} cells express other markers of the V2 IN sub lineages. We have previously shown that these cells are distinct from V2c INs (Panayi *et al.*, 2010). We thus tested if they express CHX10 or GATA3, markers of V2a and V2b INs respectively. Our data showed that none of these PAX6^{+} cells express CHX10 (Fig.14 A-C) while at least some cells express GATA3 (Fig 14D-F and Fig. 12). Given that GATA3 is not expressed by any other neuronal subtype of the vSC, we conclude that at least some of the p2-derived PAX6^{+} INs represent a subclass of late-born V2b INs .

Figure 14: Expression of V0, V1 and V2 markers relative to PAX6 in the vSC.



*Spinal cord sections of E14.5 wild type embryos were stained with anti-CHX10 antibody (**A**) and anti-PAX6 antibodies (**B**). The overlay of the two images shows that no PAX6 positive interneurons co-express CHX10 (**C**). Anti-GATA3 (**D**) and anti-PAX6 (**E**) antibodies were used to stain E14.5 sections. The overlaid image (**F**) illustrates*

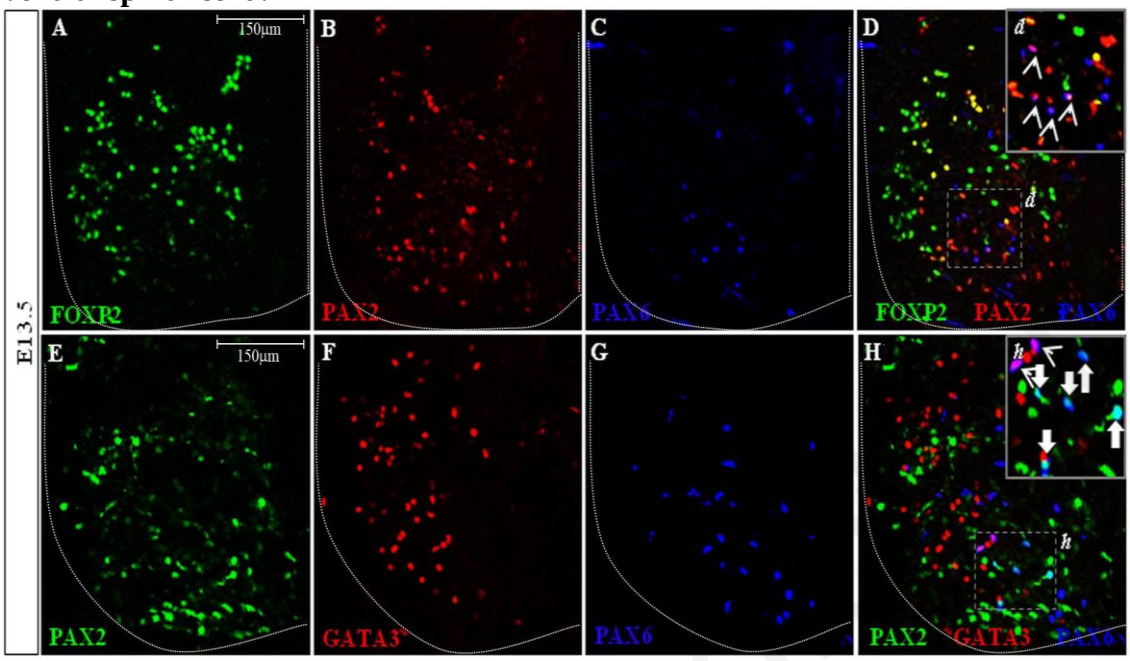
*that some PAX6 positive interneurons also express GATA3, shown by arrows. E13.5 embryos were stained with anti-FOXP2 (**G**), anti-PAX2 (**J**), Anti-EVX1 **(M)** and anti-PAX6 (**H** and **K**) antibodies. Note that none of the PAX6$^+$ express FoxP2 (**I**) while the majority express Pax2 (**L**; arrowheads) and some are positive for EVX1 (**O**; arrowheads).*

2.1.4 The p0 domain generates the majority of PAX6$^+$ neurons

Our fate mapping data suggested that the majority of PAX6$^+$ neurons derive from a region encompassing two domains (p1and p0). We then analyzed the marker expression profile of those PAX6$^+$ neurons that are not V2 INs using antibodies that label subsets of V0 and V1 INs. We found that some PAX6$^+$ neurons express EVX1, the hallmark marker for V0 INs (Fig 14M-N), PAX2 (Fig 14J-L and Fig. 12), a marker for V0 ventral (V0v), while none express FOXP2 (Fig 14G-I), a marker for V1 INs (Pierani *et al.*, 1999; Lanuza *et al.*, 2004; Morikawa *et al.*, 2009).

Having established that some PAX6$^+$ neurons express PAX2 (and are therefore V0v INs) and some express GATA3 (and are a subset of V2b INs) we used triple staining using PAX6/PAX2/GATA3 to find out if PAX6$^+$/PAX2$^+$ and PAX6$^+$/GATA3$^+$, represent the total of PAX6$^+$ neurons (Fig 15H). We found that some neurons co-expressed either PAX2/PAX6 or GATA3/PAX6. In addition some cells expressed only PAX6. These data suggest that the majority of PAX6$^+$ neurons are V0v neurons while the remaining represent a subset of V2b neurons and some PAX6$^+$ cells that express only PAX6 and whose origin cannot be determined but may possibly originate from the Dbx1 lineage (Fig. 12).

Figure 15: Expression of PAX6 relative to FOXP2, PAX6 and GATA3 in the ventral spinal cord.



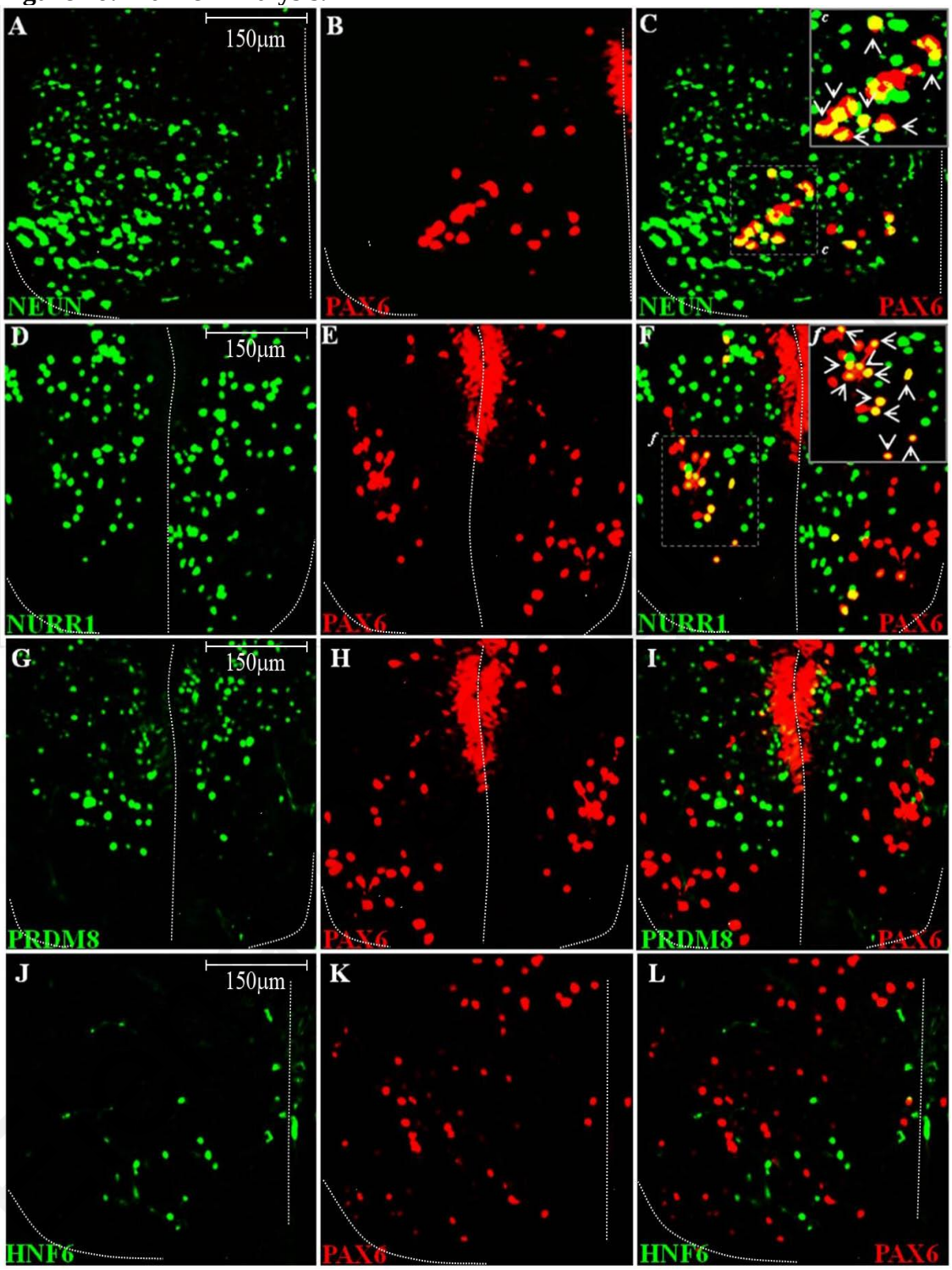
*Spinal cord sections from wild type E13.5 embryos were triple stained with FOXP2, PAX2 and PAX6 (panels **A-D**). Panels **d** and **h** show enlarged views of the dotted areas. Note in panel **H** that there are cells (blue) that do not express either PAX2 or GATA3 while some express PAX2-PAX6 (indicated by arrows) and some express PAX6-GATA3 (arrows).*

2.1.5 PAX6$^+$ neurons, heterogeneous population of excitatory and inhibitory INs

To further characterize the expression profile of the late born PAX6$^+$ INs we stained wild type embryo sections using anti-NEUN (Fig 16A-C) and anti-NURR1 (Fig 16D-F) antibodies relative to PAX6. NEUN, the neuron-specific nuclear protein, which has been shown to be expressed in the majority of neuronal cells of the nervous system (Mullen *et al.*, 1992), clearly labels the PAX6+ cells, indicating that these are indeed neurons. NURR1, known to be expressed in glutamatergic neurons (Stam *et al.*) was also found to label many of the PAX6+ INs. The PAX6+ INs were however negative for PRDM8 (Fig 16G-I), which is known to label in V1 and some V2 INs (Komai *et al.*, 2009) and HNF6 (Fig 16J-L) which labels V1 INs (Stam *et al.*).

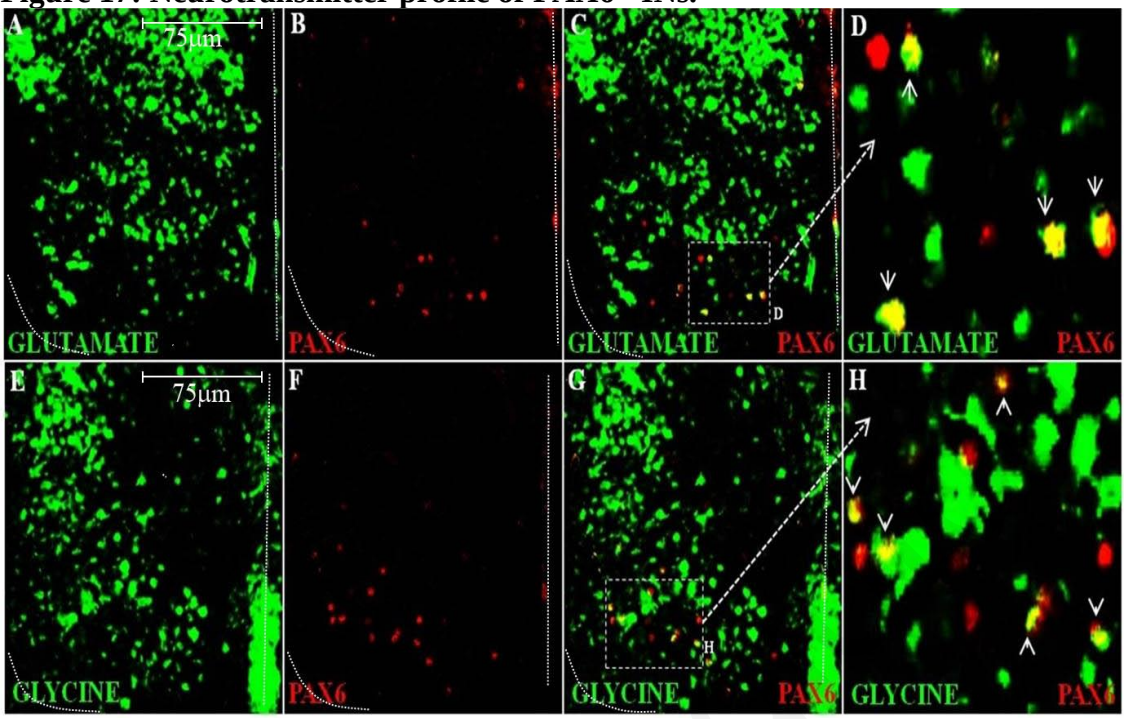
Furthermore, we found that some of the $PAX6^{+}$ INs are excitatory glutamatergic INs (Fig. 17A-D), while some are inhibitory Glycinergic INs (Fig. 17E-H). It is interesting to note that V2b INs are inhibitory glycinergic neurons (Joshi *et al.*, 2009) while some V0 INs were shown to be excitatory (Lanuza *et al.*, 2004), further indicating that the PAX6+ INs are a heterogeneous population originating from various domains.

Figure 16: Marker Analysis.



*Spinal cord sections of wild type embryos at E14.5 were double stained with anti-PAX6 antibody and various markers labelling different classes of cells. Anti-NEUN antibody (**A-C**) labelling neuronal nuclei shows that most of the PAX6 positive interneurons do co-express NEUN (**c**, enlarged panel). Anti-NURR1 antibody (**D-F**), which labels V0 interneurons, confirms that most of the late born PAX6 interneurons are positive for NURR1 (panel **f**, arrows). None of the PAX6 positive cells were labelled with PRDM8 which labels label V1, V2 and motor neurons (**G-I**). Also, none of the PAX6 cells were positive for HNF6, which labels V1 interneurons. Note: Panels D-I are pictures from the same section, triple staining NURR1/PRDM8/PAX6*

Figure 17: Neurotransmitter profile of PAX6+ INs.



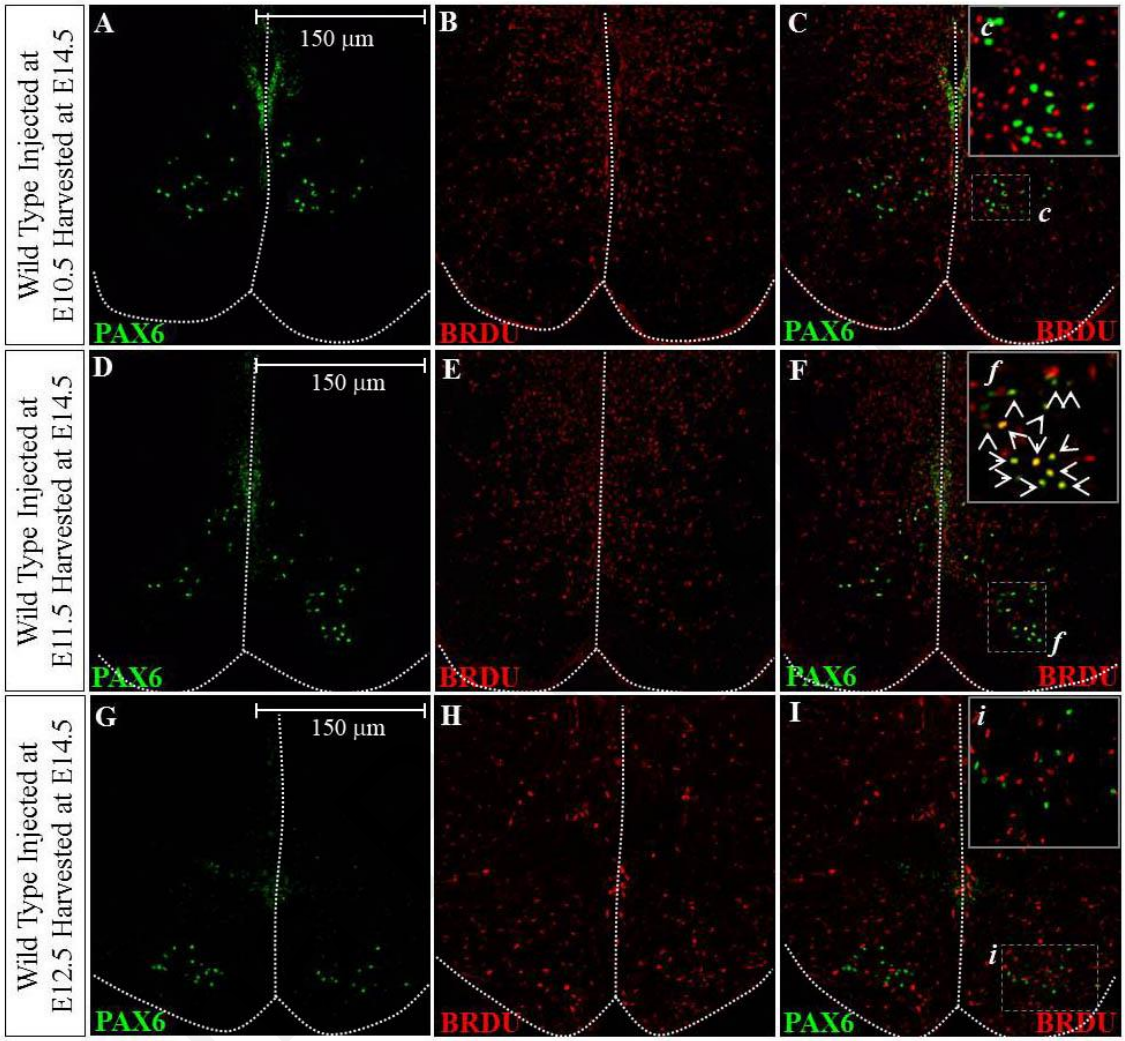
*Spinal cord sections of wild type embryos at E14.5 were stained with anti-GLUTAMATE and anti-PAX6 antibodies (**A-D**). Panel **D** is the enlarged section of the dotted square from panel **C**, illustrating the cells' neurotransmitter co-expression. There are two cell populations, a few PAX6 positive cells which do not co-express GLUTAMATE. The white arrows indicate that the rest of the PAX6 positive cells all co-express GLUTAMATE. Panels **E-H** are images of sections immunostained with GLYCINE and PAX6. Panel **H** is a magnified part of the section from panel **G**, showing that some of the PAX6 positive cells are inhibitory glycinergic neurons.*

2.1.6 PAX6 INs are generated at a narrow developmental window

It has been previously shown that V2 INs acquired post-mitotic fate at around E9.5-E10 (Nardelli *et al.*, 1999). Given that $PAX6^{+}$ INs are seen to migrate out of the ventricular zone at E12.0, we reasoned that they must be generated at a later stage of neurogenesis compared to the V2 neurons described so far. To determine the precise developmental window during which these cells are born we used *in vivo* BrdU incorporation. We injected pregnant mice with BrdU solution at E10.5, e11.5 and E12.5 and harvested the embryos at E14.5 (Fig. 18). We found that the $PAX6^{+}$ neurons are primarily specified at around E11.5, two days later compared to the specification of

V2a-V2c INs. These data confirm that there is a temporal distinction of the two waves of neurogenesis that take place in the p2 domain.

Figure 18: Birth-dating of PAX6+ INs.



*Pregnant mice were injected with BrdU at E10.5 (panels **A-C**) and E11.5 (panels **D-F**) and then the embryos were sacrificed at E14.5 and immunostained with anti-PAX6 and anti-BRDU antibodies. Panel **c** is an enlarged section of **C**, showing that none of the PAX6 positive interneurons were born at E10.5. Panel **f** (the enlarged part of image **F**) shows that most of the ventral PAX6+ cells are also positive for BRDU and were thus born at E11.5.*

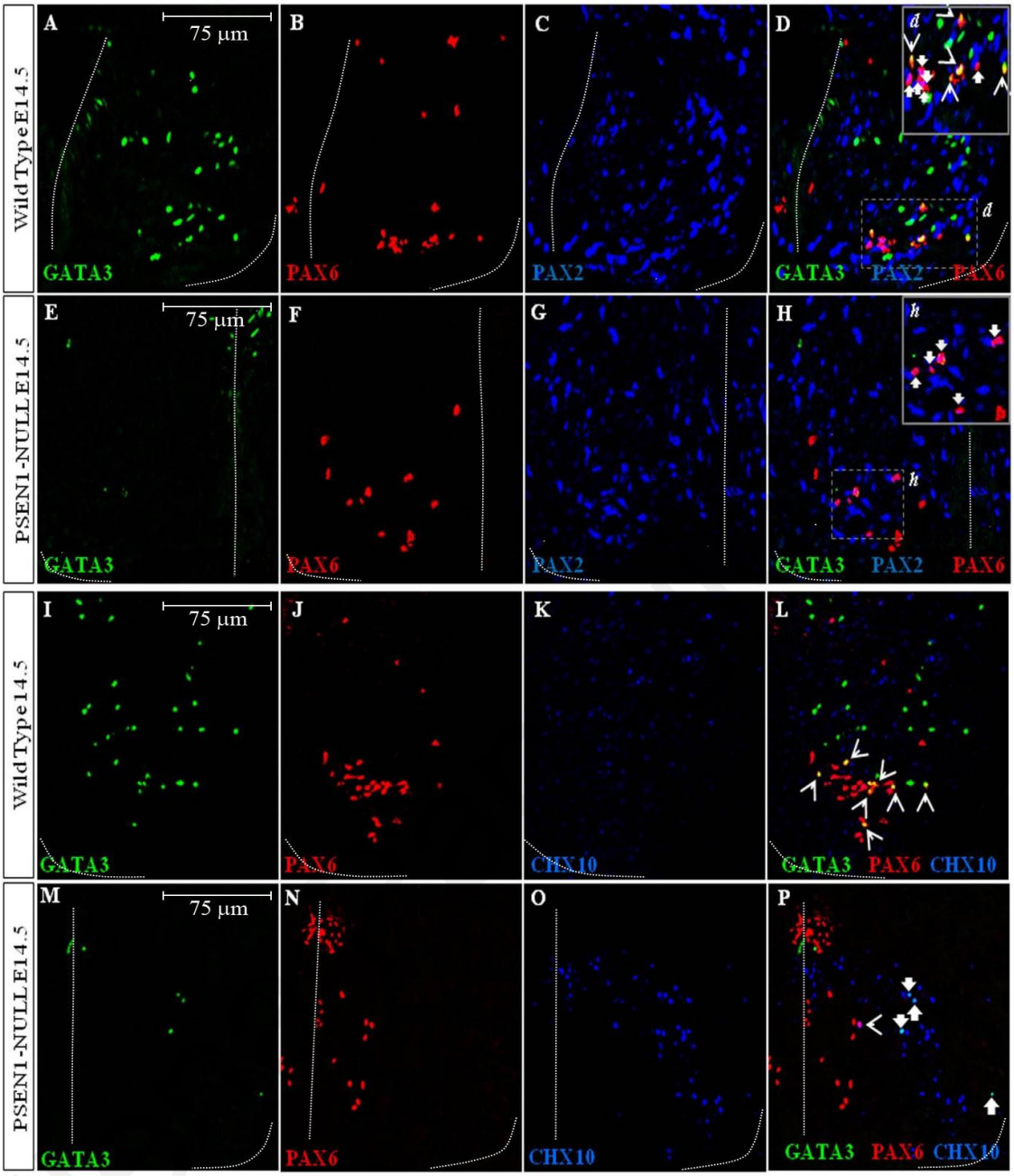
2.1.7 Notch signaling is responsible for the specification of PAX6$^+$ neurons

Previous work has shown that Notch signaling is responsible for the allocation of neuronal subtype specification in V2 INs operating in post-mitotic progenitors (Yang *et al.*, 2006b). More specifically loss of Notch signaling leads to overproduction of V2a

INs at the expense of V2b (and possibly V2c INs). These studies have been carried out at early stage embryos using Presenilin I (Psen1) and FoxN4-deficient embryos (Li *et al.*, 2005; Yang *et al.*, 2006b; Del Barrio *et al.*, 2007). We thus asked if loss of Psen1 function affected the production of $PAX6^+$ neurons that also express GATA3 (which we know must derive from the p2 domain).

Analysis of spinal cord sections from PSEN1-deficient and wild type embryos revealed that although the production of $PAX6^+/PAX2^+$ neurons was not affected, the production of $PAX6^+/GATA3^+$ neurons was abolished (Fig 19A-H). However, unlike early stages of development when GATA3 expression was completely abolished in PSEN1-deficient embryos, at later stages analyzed some expression of GATA3 was preserved. Importantly, however, in PSEN1-deficient embryos the very few PAX6-expressing cells produced had a confused phenotype and also expressed CHX10, while the few $GATA3^+$ cells produced were also positive for CHX10 (Fig 19P). Furthermore, a couple of the $PAX6^+$ cells expressed CHX10, seemingly the same effect observed with the V2b INs which also switch fate to the V2a IN subset in the absence of Notch signaling. These results indicate that the late born $GATA3^+/PAX6^+$ neurons, like the early born $GATA3^+/PAX6^-$ cells, are specified in a Notch-dependent manner, while the production of $PAX6^+/PAX2^+$ neurons was essentially unaffected.

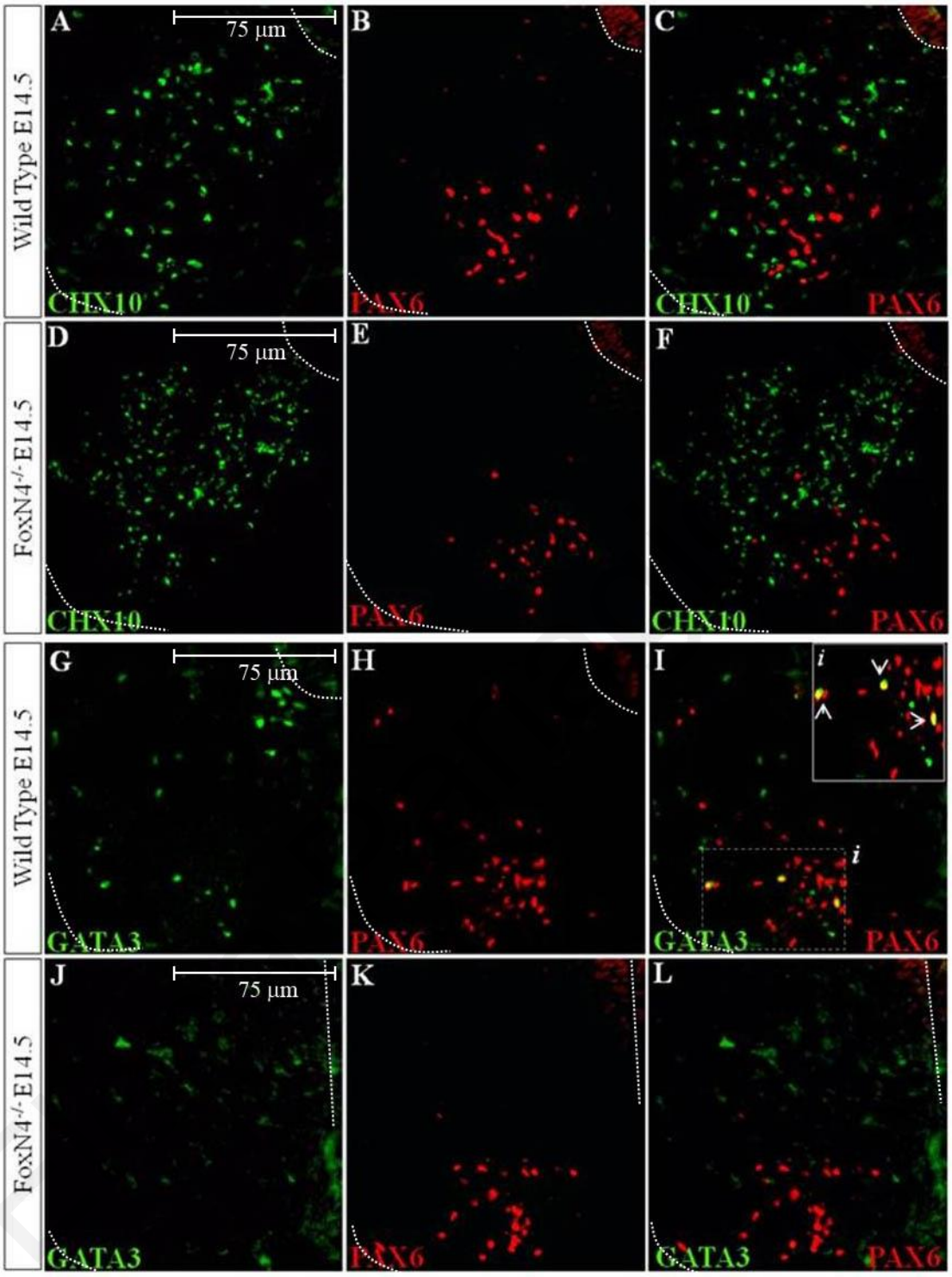
Figure 19: Presenilin1 Knock-out Experiments.



*Spinal cord sections of E14.5 embryos were immunostained with GATA3, PAX6 and PAX2. Wild type embryos were used for panels **A-D** and PSEN1 KO embryos for **E-H**. Panel **d** shows that some PAX6 positive neurons are also positive for GATA3 (white arrows) and the rest of the PAX6 cells are positive for PAX2 (white bold arrows). The PSEN1 KO section (panel **h**) indicates that the PAX6-PAX2 neurons were not affected (white bold arrows), however, since the GATA2 interneurons are not born in the PSEN KO model there are no PAX6-GATA double positive interneurons. Panels **I-L** (wild type sections) and **M-P** (PSEN1 KO sections) were immunostained with anti-GATA3, anti-PAX6 and anti-CHX10 antibodies. The wild type embryos' sections show that some of the PAX6 positive cells co-express GATA3 (white arrows, panel **L**), whereas, none of the PAX6 positive cells co-express CHX10. Panel **P** shows that some cells from the KO sections in the absence of GATA3 expression acquired a confused phenotype where a few PAX6 positive cells co-expressed CHX10 (panel **P**, bold arrows) and some cells co-expressed GATA3 and CHX10 (panel **P**, white arrows).*

FoxN4 knockout embryos were also used to examine whether the PAX6 interneuron production was affected when FoxN4 expression was abolished. Wild type and FoxN4 knock-out E14.5 embryo spinal cords were double stained with antibodies against PAX6 and either GATA3 or CHX10. CHX10 expression was apparently not affected, whereas GATA3 expression was severely diminished in the knock-out cords. And as with the PSEN-1 knock-outs, PAX6 positive cells were produced as expected even though none of the cells exhibited a confused phenotype (Fig. 20).

Figure 20: FoxN4 Knock-out Experiments.



Spinal cord sections of E14.5 wild type and FoxN4 KO embryos were immunostained with GATA3, PAX6 and CHX10. Panels A-C are wild type sections stained with anti-CHX10 and anti-PAX6 antibodies, none of the PAX6 positive cells expressed CHX10 (panel C). The same observation holds true for the FoxN4-/- sections (panels D-F) and none of the markers' expression appeared to be affected by the absence of FoxN4. Wild type embryo sections (panels G-I) were immunostained with anti-GATA3 and anti-PAX6 antibodies. Panel i shows that a few PAX6 positive cells are also positive for GATA3. The number of GATA3 cells in the FoxN4 KO decreased considerably (G) and no PAX6 positive cells were positive GATA3 (L).

2.2 LATE WAVE NEUROGENESIS IN THE DORSAL SPINAL CORD

2.2.1 Gata3 expression extends to a dorsal population persisting in the adult stages

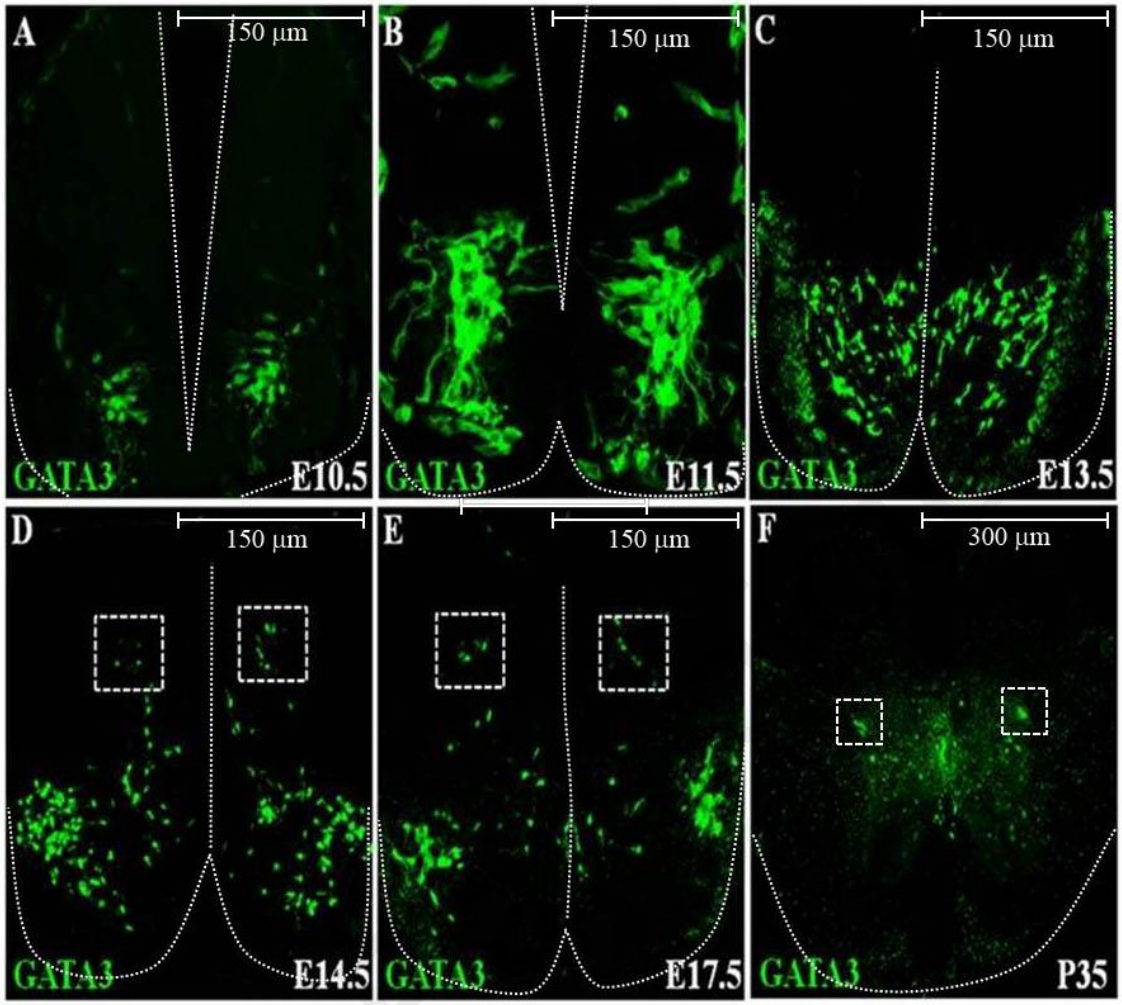
While studying the expression of GATA3 in the vSC we identified a dorsal population of Gata3 positive cells which only become apparent after E14.5.

GATA3, as has been aforementioned, is known to be expressed in several regions of the developing central nervous system and direct the development and terminal differentiation of numerous cellular populations. In order to carry out a detailed analysis of the development of this population, our previously published $Gata3^{-}$ ^{eGFP} mouse reporter line was used, (Panayi *et al.*, 2010) thus eliminating the need to use GATA3 antibody and only utilize anti GFP. Pre-natal stages; as early as E10.5 were used, as well as the post-natal stages P10 and P20 and extending up to P35.

The obtained results from the marker analysis (Figure 21) indicate that before E14.5 these dorsal $GATA3^{+}$ cells cannot be distinguished in their perceived position or even migrating into their dorsal ultimate location. However, at E14.5 the cells are already found at this dorsal region and seem to remain at this position throughout development, while their numbers appear to be unchanged during this period (Figure 22). This population also appears to persist postnatally, even though the cells appear to be more tightly packed together.

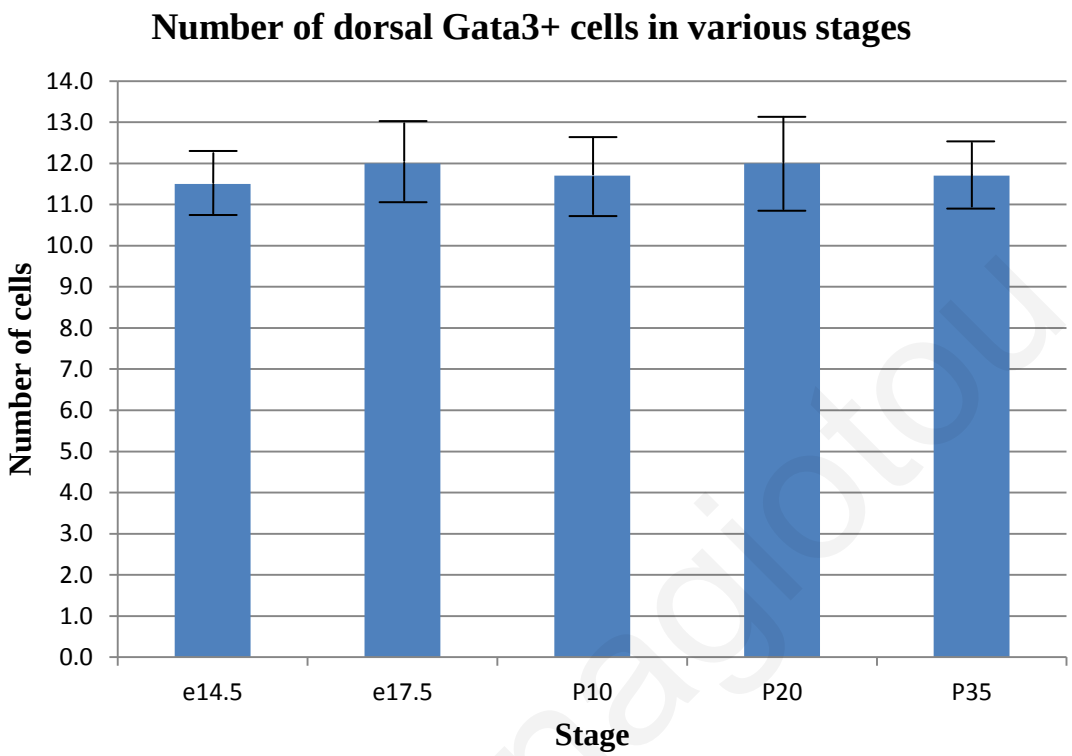
Thus this population not only persists through the developmental stages but also postnatally, therefore indicating that the expression of GATA3 is possibly imperative in the maintenance of this population's character.

Figure 21: GATA3 expression in a small group of dorsal neurons.



*Spinal cord sections from GATA3-^{eGFP} embryos at the denoted stages were immunostained with anti-GFP antibody. Panels **A** and **B** depict the expression of Pax6 along the marginal. Panels **C-E** show the migration of GATA3 positive neurons, particularly a smaller neuron population resting at the dorsal region of the developing spinal cord. Panel **F** depicts the expression of the Gata3 at the postnatal stage P35.*

Figure 22: Number of dorsal GATA3+ cells throughout various embryonic and post-natal stages



A minimum of five sections from each stage were stained with α-GFP antibody and then the number of dorsal Gata3 positive cells were counted and tabulated. There was no significant difference between stages.

2.2.2 GATA3 dorsal cells marker analysis

Having explored the time-line of this Gata3 positive population's development by looking at the various stages, a variety of markers was used in order to provide further insight into their properties and function. Markers which label dorsal interneuron populations were initially used (Figures 23 and 24).

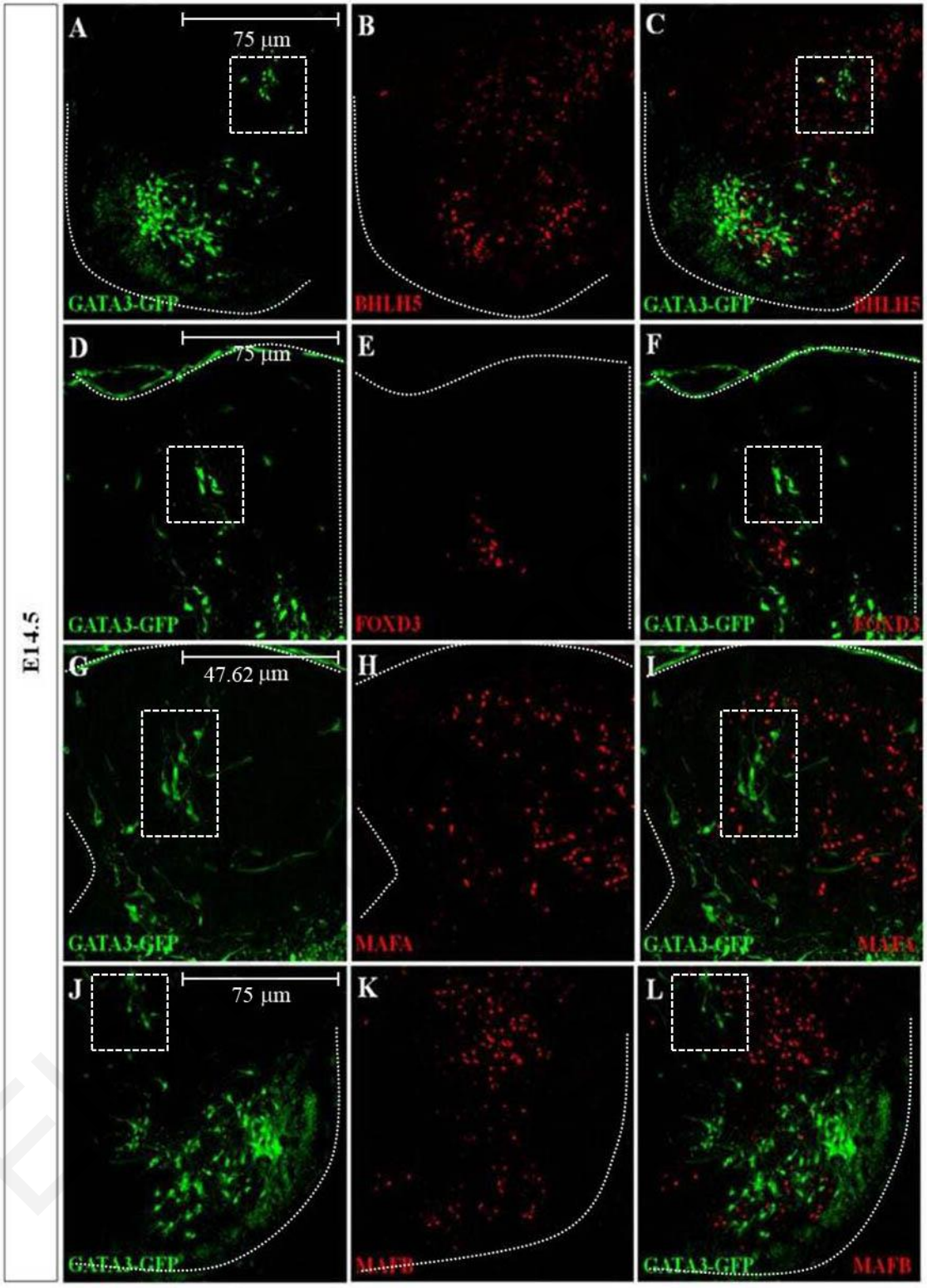
The BHLHB5 antibody was used, which amongst other populations is known to label dI6 interneurons, as well as some V1 and V2 interneurons in the ventral spinal cord (Liu *et al.*, 2007). FOXD3 antibody was also employed, which has been shown to label dI2 interneurons (Wine-Lee *et al.*, 2004). Furthermore, Foxd3 is a transcriptional regulator which promotes the development of neural tube progenitors into neural crest cells while inhibiting their differentiation into interneurons (Kos *et al.*, 2001). The MAF antibodies, MAF A and MAF B were also used to stain spinal cord sections in order to detect possible co-localization with the dorsal GATA3 positive cells. This family of TFs have been shown to be expressed in Renshaw cells (Stam *et al.*, 2012). Renshaw cells are inhibitory interneurons which reside in the spinal cord's grey matter and regulate the firing of α-motor neurons projecting from the ventral horn while releasing GLYCINE (Benito-Gonzalez & Alvarez, 2012). Previous studies have shown that Pax6 is involved in the development of Maf A+ and Maf B+ cells (Sapir *et al.*, 2004).

In addition, the markers BRN3A, LBX1 and PAX2 were correspondingly used to counter stain the spinal cord sections obtained from the Gata3^{-eGFP} reporter embryos. BRN3A (the brain specific homeobox domain protein 3a) is specifically expressed in the developing central nervous system and is heavily implicated in the maintenance of sub populations of cells in the PNS, the retina and the developing embryonic brain.

Furthermore, this protein has been shown to be expressed in the dorsal interneuron subpopulations, dI1, dI2, dI3 and dI5 (Caspary & Anderson, 2003; Iulianella *et al.*, 2009). Finally, the LBX1, PAX2, SOX1 and SOX14 antibodies were used. Lbx1 is one of the TFs required for the specification and maintenance of many dorsal populations specifically the dI4, dI5 and dI6 interneurons, whereas PAX2 is only expressed in the dorsal populations dI4 and dI6 (Iulianella *et al.*, 2009; Andersson *et al.*, 2012). Finally, the expression of the SOX1 and SOX14 TFs was investigated. Sox1 and Sox14 are implicated in the determination and terminal differentiation of a number of cellular populations in the developing mammalian nervous system while it is also known to be involved in the determination and maintenance of p2 derived interneurons (V2c and V2a respectively) (Panayi *et al.*, 2010).

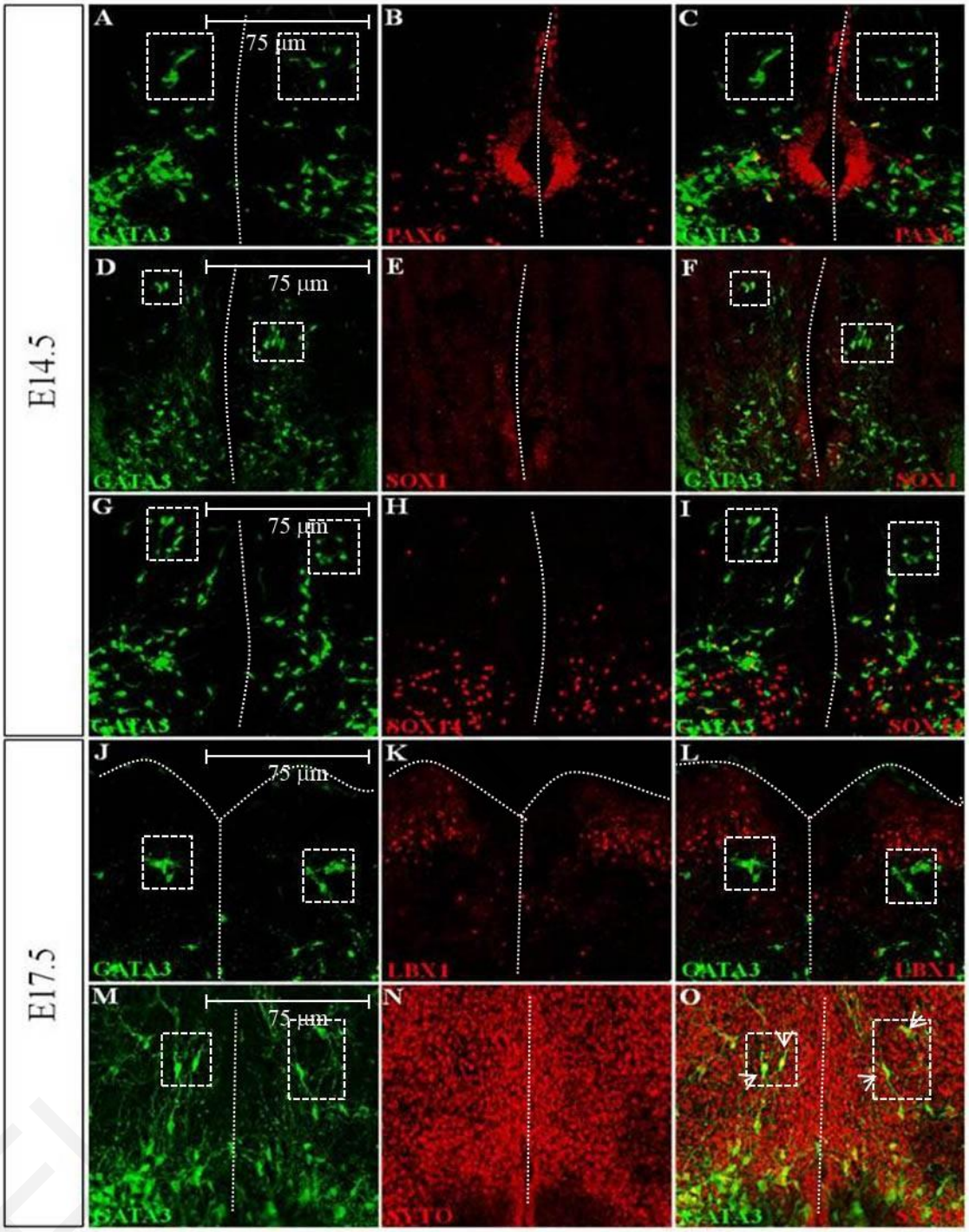
As is evident by figures 23 and 24, none of the markers used co-localized with the dorsal GATA3 positive cells. Therefore, it has not been possible to obtain further information on these cells' identity and characteristics by using the aforementioned markers.

Figure 23: Marker Analysis



Spinal cord sections of GATA3^{YFP} embryos at E14.5 were double stained with anti-GFP antibody and various markers labeling different classes of interneurons. Anti-BHLHB5 antibody (A-C). Anti-FOXD3 antibody (D-F). Anti-MAFA (G-I) and anti-MAFB (J-L). None of the dorsal GATA3 positive cells express any of the aforementioned dorsal markers.

Figure 24: Marker Analysis Cont.

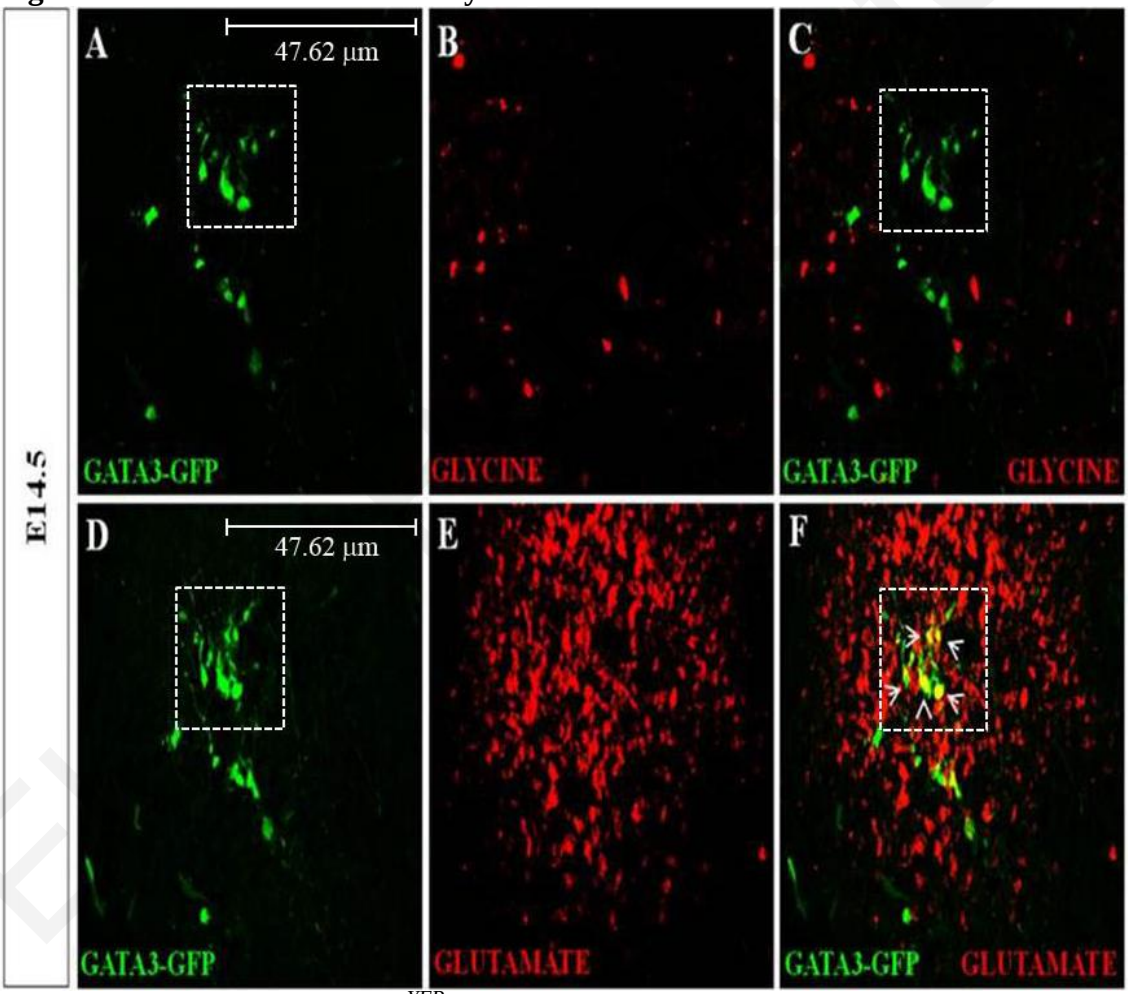


*Spinal cord sections of GATA3^{YFP} embryos at E14.5 were double stained with anti-GFP antibody and anti-Pax6 (**A-C**), anti-SOX1 (**D-F**) and anti-SOX14 (**G-I**) antibodies. None of the dorsal GATA3 positive cells co-expressed the antibodies used. E17.5 GATA3^{YFP} spinal cord sections were as before double stained with anti-GFP and anti-LBX1 (**J-L**) and SYTO red stain (**M-O**).The GATA3 positive cells were not positive for LBX1, they were positive for SYTO red however, which stains nucleic acids indicating that the dorsal GATA3 positive population is active.*

2.2.3 The dorsal Gata3+ population are excitatory Glutamatergic cells

Since none of the expression markers were useful in determining the identity of the dorsal Gata3 positive cells, the neurotransmitter profile of the cells was established by using antibodies against GABA, GLYCINE and GLUTAMATE. Whereas the GABA and GLYCINE markers did not co-localize with the cells, GLUTAMATE labelled the entire population (Figure 25). This indicates that this dorsal Gata3 cells have an excitatory glutamatergic profile.

Figure 25: Neurotransmitter Analysis



*Spinal cord sections of GATA3^{YFP} embryos at E14.5 were stained with anti-GLYCINE antibody (**A-C**). Panels **D-F** are images of sections immunostained with anti-GLUTAMATE. Panel **F** shows that all of the GATA3 expressing cells are excitatory Glutamatergic neurons.*

2.3 CENTRAL CANAL CONTACTING NEURONS

2.3.1 Gata3 and Sox1 expression varies depending on the post-natal stage

Another population of late born cells has been observed along with the ventral late born PAX6 positive cells and the GATA3 derived dorsal migrating cells. These cells were initially observed during the post-natal stages and appear to be clustered around the central canal as well as be in direct contact with the CSF. The most intriguing aspect of this cell population however is the fact that during the P0 stage GATA3 staining was nuclear, whereas at later stages GATA3 expression was entirely cytoplasmic.

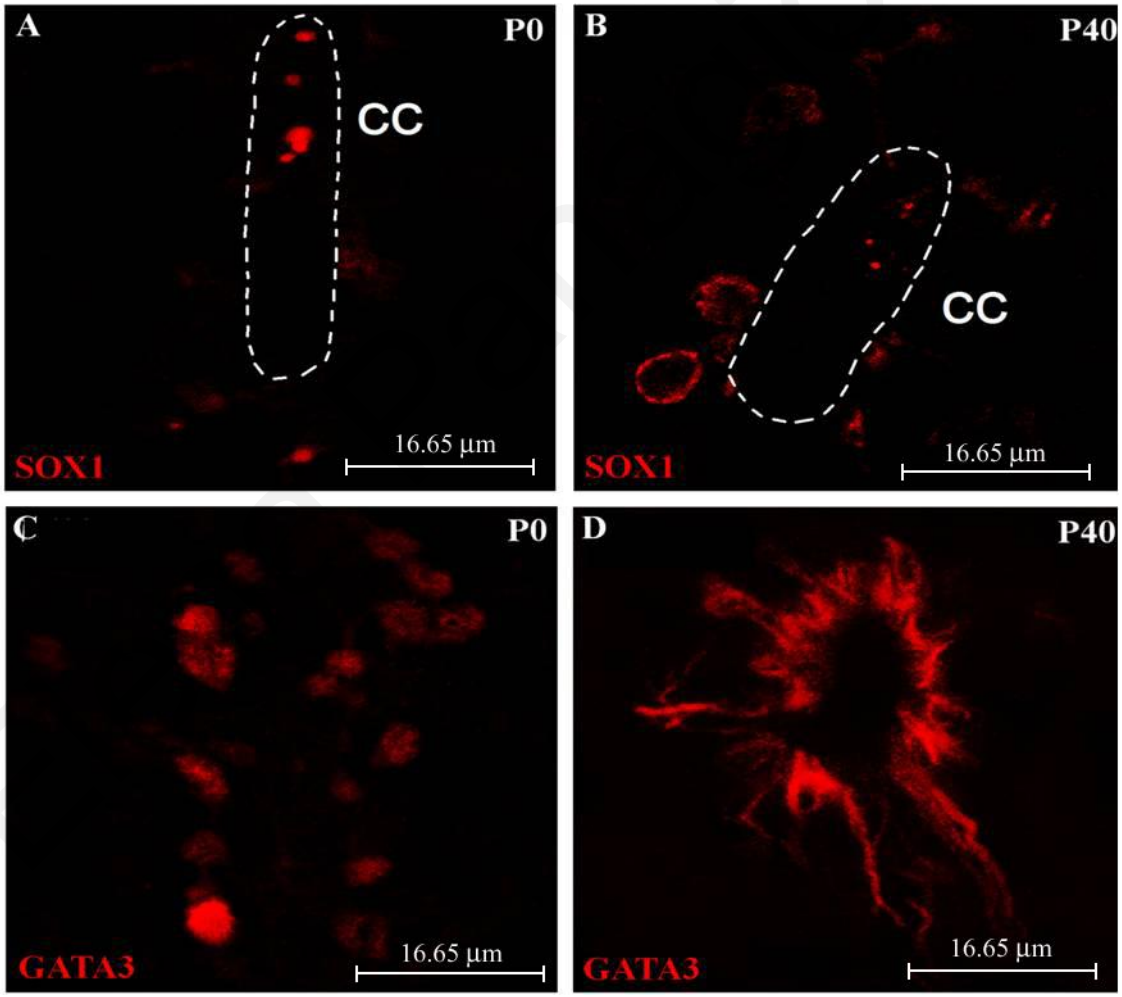
Furthermore, SOX1 protein expression appeared to be secreted from the neurons' dendritic processes and into the central canal. This is the first time CSF contacting neurons have been shown to express the TF Gata3. As is evident in Figure 26 from wild type sections stained with GATA3 and SOX1 at P0 and P40, the expression of these two TFs is variable depending on the stage the sections were taken at.

At P0, staining with anti-SOX1 antibody indicates that SOX1 does not stain any of the cell's nuclei but instead is found at the base of the dendritic processes. GATA3 at this stage is found to be expressed only in the cells' nuclei. At P40 though, SOX1 still appears to be expressed at the dendritic processes, even though not as prominently, as well as surrounding the nuclei, GATA3 however is entirely cytoplasmic. Even though CSF contacting neurons have been previously described (Alfaro-Cervello *et al.*, 2012) GATA3 has not previously been shown to be expressed in these cells, especially in such a variable expression pattern where GATA3 is entirely cytoplasmic since Gata3 is

a TF and thus has a nuclear action where the protein needs to be transported into the nucleus where it can regulate target genes' transcription.

Cytoplasmic expression of GATA3 would suggest that the protein's function is hindered. Previous studies in certain types of cancers (especially pancreatic cancer) indicated that even though Gata3 action in the nucleus is hindered, the actual level of transcription found in the cytoplasm is not affected at all since mRNA expression assays have shown that transcription levels do not decrease when the protein is located in the cytoplasm (Stanelle *et al.*, 2010).

Figure 26: Sox1 and Gata3 Variable Expression at Progressing Post-natal Stages.



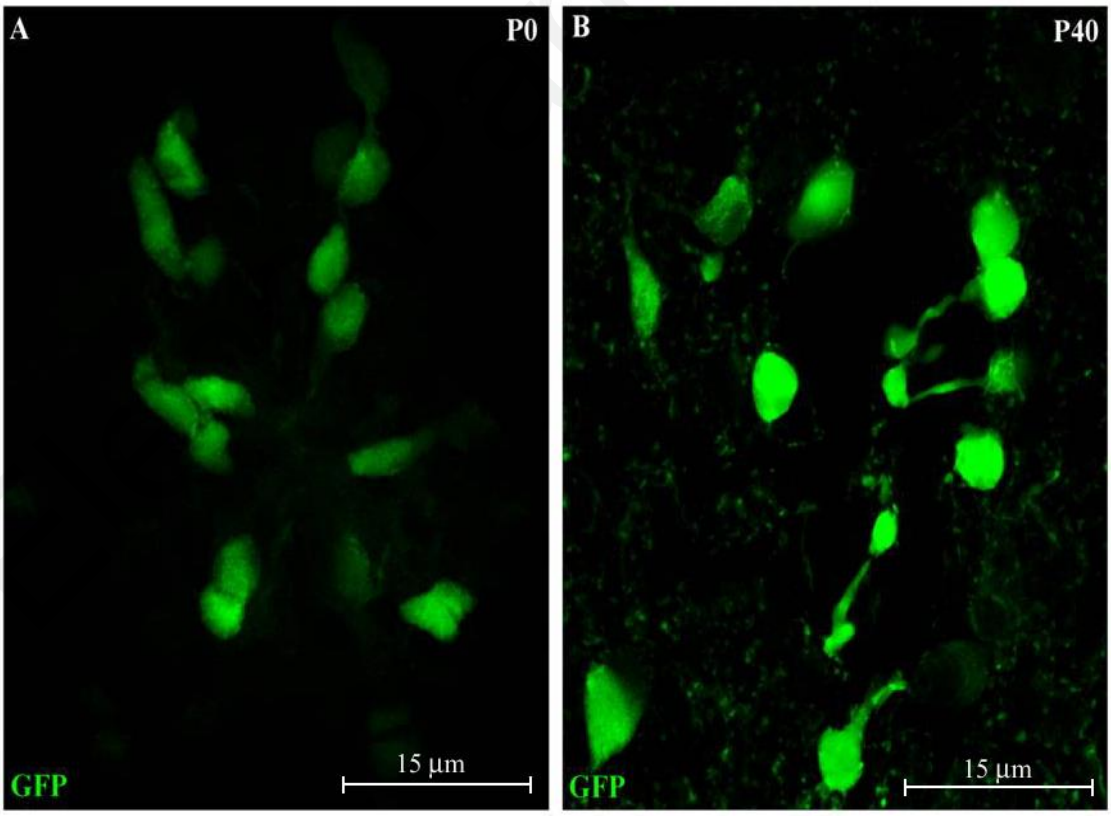
*Spinal cord sections from wild type pups at P0 and mice at P40 were immunostained with anti-SOX1 and anti-GATA3 antibodies. Panels **A** and **B** relate to the SOX1 expression. P0 sections stained with anti-SOX1 show that SOX1 is found mostly in the central canal, at P40 SOX1 appears more prominently labelling cells. GATA3 expression (**C**) at P0 appears to be nuclear, though at P40 GATA3 expression appears to be cytoplasmic (**D**).*

2.3.2 Gata3^{-eGFP} reporter correlates to the protein's true expression profile

Since GATA3's variable manner of expression will be examined in correlation with other markers at different stages, it is appropriate to employ our previously published Gata3^{-eGFP} mouse reporter line without having to stain using the anti-GATA3 antibody, thus making staining with other markers more convenient and cost effective. However, in order to be able to use this reporter line we need to ensure that this line will accurately reflect the protein's true expression at the required stages.

Figure 27 exhibits that GATA3 expression can be traced using the reporter line, both at P0 and P40, even though the cytoplasmic expression of GATA3 is not reflected in the GFP expression pattern since the reporter employed has a nuclear localization.

Figure 27: GATA3^{-eGFP} Reporter Expression



Spinal cord sections of P0 pups and P40 mice from the GATA3-GFP reporter line. At both stages GATA3 appears to be nuclear as well as labelling dendritic processes.

2.3.3 Gata3+ central canal contacting cells are born from E14.5-E16.5

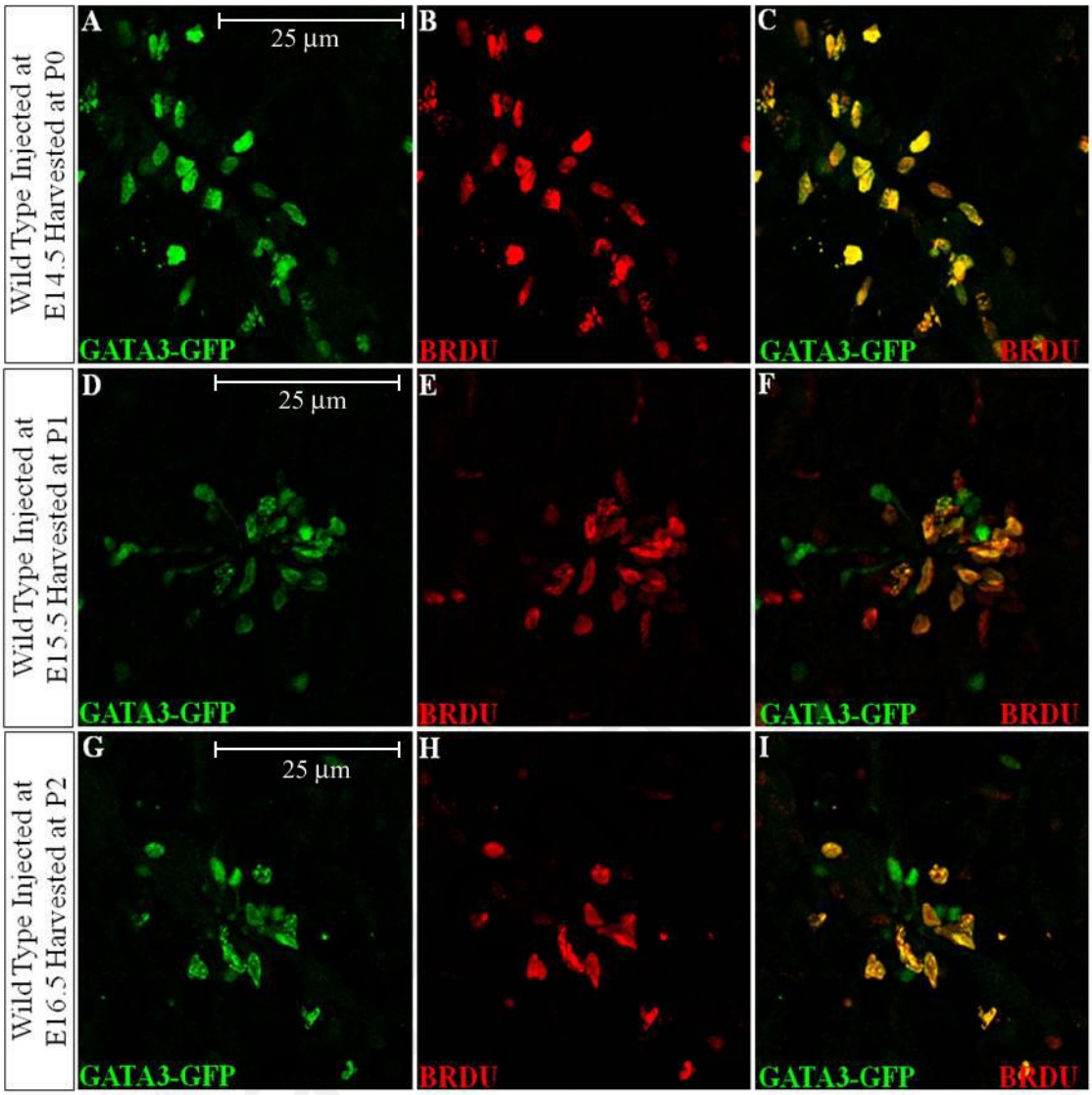
In order to carry on with further analyses into the nature of the CSF contacting neurons and investigate whether GATA3 expression completely correlates with these cells, BrdU birth dating experiments were carried out at various embryonic stages including the postnatal stages P0 and P1.

5-bromo-2-deoxyuridine, BrdU is an exogenous S-phase marker which is incorporated into the replicating DNA of dividing cells. Bioavailability of BrdU following intraperitoneal injections has not been definitively determined; however pre-existing data approximate this period to be two hours post injection, so that BrdU will be integrated into actively replicating DNA in the first two hours after the injection has been administered (Sevc *et al.,* 2011). BrdU will strictly be incorporated into cells undergoing the S-phase of the cell cycle. Thus, even if an absolute number of cells cannot be established it is undeniably indicative of a trend.

Therefore, pregnant Gata3^{-eGFP} reporter mice were injected with BrdU at the various aforementioned stages and the pups were then collected and sacrificed. Spinal cord sections from these pups were stained for BrdU and GFP, in order to discern whether any of the Gata3 positive cells were born/actively-proliferating at the respective stages BrdU injections were delivered.

Obtained results indicate that the Gata3+ CSF contacting cells are born and continue to proliferate from embryonic stage E14.5 up to E16.5 (Figure 28). However, none of the cells seemed to be born after E16.5 and similarly none were born postnatally. There results correlate with previous studies in rats relating to ependymocytes which have been shown to be born at E16 in the brain -e14.5 in mice- (Altman & Bayer, 1984).

Figure 28: Birth dating analysis of Central Canal Contacting GATA3+ cells



*Pregnant Gata3^{YFP} mice were injected with BrdU at E14.5 (panels **A-C**), E15.5 (panels **D-F**) and E16.5 (panels **G-I**); then the pups were sacrificed postnatally and immunostained with anti-GFP and anti-BRDU antibodies. Gata3^{+} cells are born from E14.5-E16.5.*

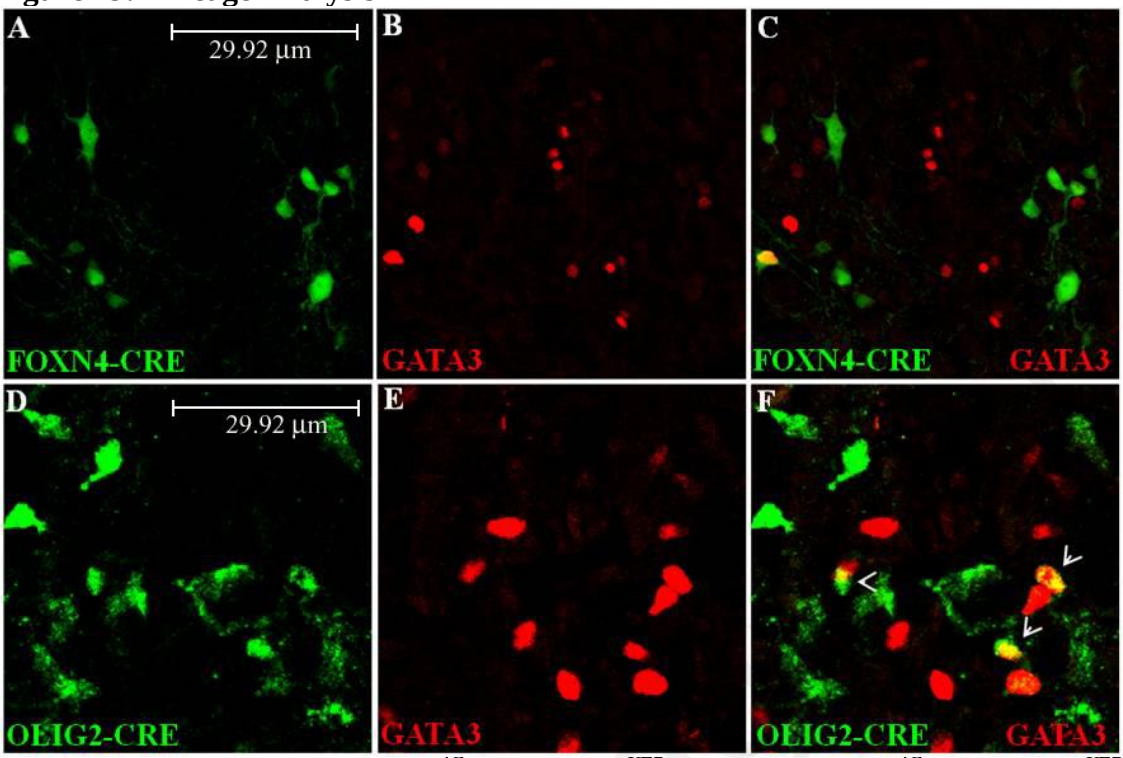
2.3.4 Some Gata3+ central canal originate Olig2^{+} progenitors

As has been previously discussed, lineage experiments utilize the transgenic Cre/loxP system which is required when investigating the origins of a particular cellular population. The population of CSF contacting cells under investigation express GATA3, one of the markers for p2 derived interneurons which originate from FoxN4 positive progenitors. Accordingly, the FoxN4^{-iCre} line was used to determine whether these GATA3 positive cells also originate from the same domain as the rest of the spinal cord GATA3 positive cells, or whether they begin expressing GATA3 later on in their development. Additionally, the Olig2^{-iCre} line will be also be used, which labels all cells deriving from the pMN and p3 domains.

P0 spinal cord obtained sections from the FoxN4^{-iCre} and Olig2^{-iCre} lines were stained with anti-GFP and anti-GATA3 antibodies. Interestingly, as is indicated by the results (Figure 29), none of the GATA3 positive CSF contacting cells originated from the p2 domain, whereas, approximately 50% of the cells originate from Olig2 positive progenitors (figure 30).

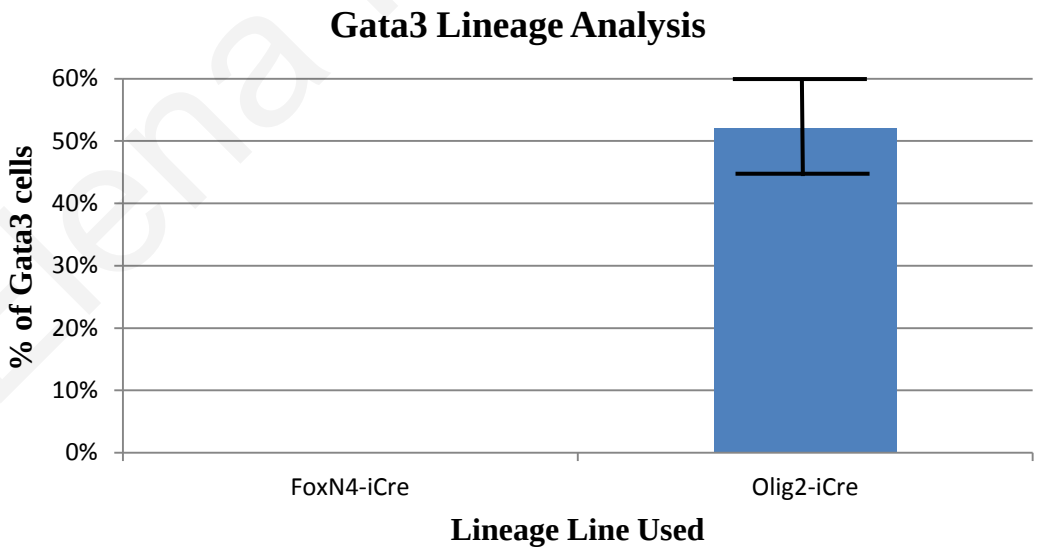
It has been previously reported in rodents that ependymocytes originate from the pMN, p3 or p2 domains (Fu *et al.*, 2003a; Masahira *et al.*, 2006). Our data however indicate that none of the Gata3 central canal contacting neurons originate from p2 progenitors.

Figure 29: Lineage Analysis



Spinal cord sections from Foxn4^{-iCre}xRosa26^{stopYFP} (A-C) and Olig2^{-iCre}xRosa26^{stopYFP} (D-F). Spinal cord sections from P0 pups were stained with anti-GFP (R26^{YFP}) and anti-GATA3 antibodies. Arrows in panel F indicate some neurons from the Olig2 lineage that co-express PAX6.

Figure 30: Lineage Analysis Cell Count



Percentage of Gata3 positive cells which co-labeled with the respective line, exhibiting that about 50% of the GATA3+ cells originate from Olig2+ progenitors.

2.3.5 Gata3 CSF contacting cells are capable of producing primary and secondary neurospheres

Due to the location of these central canal cells, which are found so near to the ependymal cells, and due to the interplay of variable expression profile between the Gata3 and Sox1 factors, a protocol to obtain primary and secondary neurospheres with was set up. Therefore, in order to investigate whether these GATA3+ cells have any stem cell or proliferative abilities as other ependymal cells, the neurospheres assay was employed.

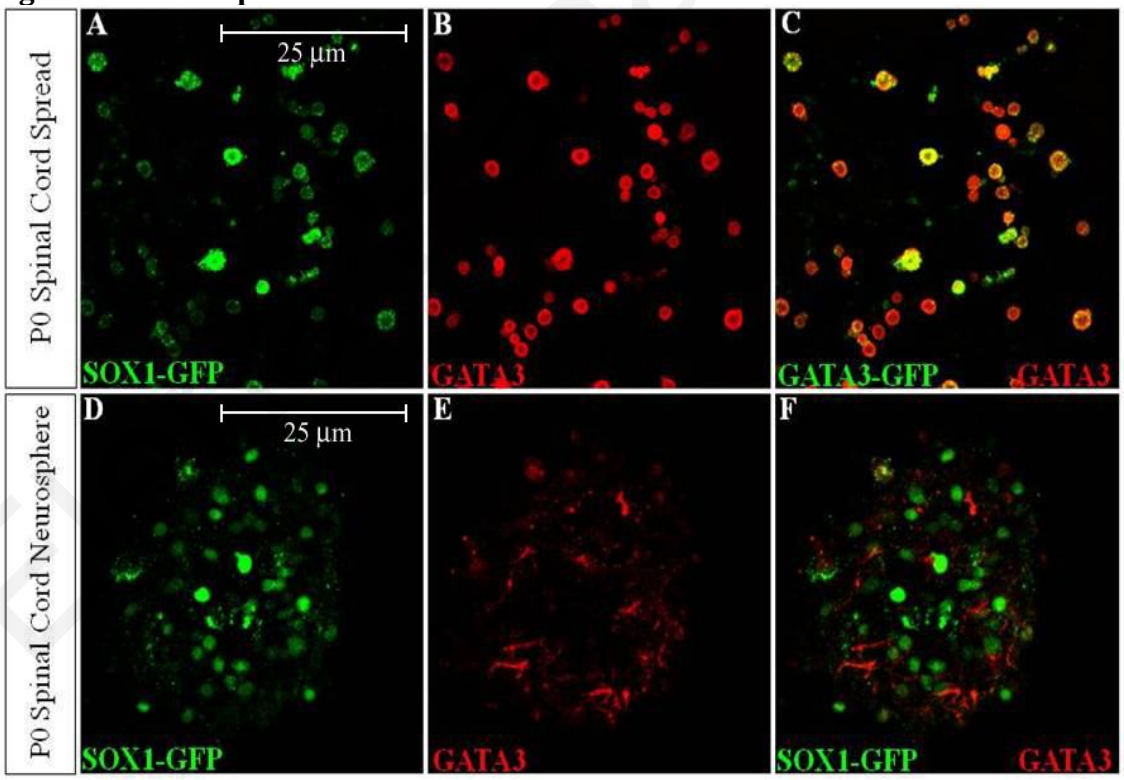
The neurospheres assay, also known as the stemness assay, was used to evaluate whether the $GATA3^{+}/SOX1^{+}$ central canal contacting cells possess the three main properties of stem cells which are: proliferation, multipotency and self-renewal. Following the establishment of primary neurospheres, these may be dissociated and re-suspended; provided that the primary neurospheres contain neural stem cells, secondary neurospheres will also be formed.

Neurospheres are an artificial culture system providing a means of investigating neural stem cells or progenitors *in vitro*. Purported neural stem cells were allowed to suspend in a medium containing the required growth factors, such as FGF and EGF, however, the medium lacks any attachment substrates in order to ensure that the single cell suspension does not form clusters amongst them but instead are allowed to form the artificial environment. Fully mature neurospheres will eventually contain a few neural stem cells, as well as astrocytes and oligodendrocytes.

Thus, a cell spread was obtained from the central canal portion of P0 $Sox1^{-Bac}$ pups' spinal cord. This spread was then dispersed as a single cell suspension and treated with appropriate growth factors and nutrients, eventually forming primary neurospheres (Figure 31 A-C). Mature established neurospheres were then re-suspended again and

allowed to re-formulate once more so that secondary neurospheres could be obtained (Figure 31 D-F). The primary spread staining with anti-GFP and anti-GATA3 antibodies indicates that all of the of the SOX1 positive cells were also GATA3 positive, even though most of the GATA3 positive cells were not also positive for SOX1, which would correlate the fact that the artificial environment found in a neurosphere only contains a small number of neural stem cells. The secondary neurospheres were again stained with the same antibodies; however, GATA3 expression was both nuclear and cytoplasmic. The ability of the initial central canal spread to form primary and then secondary neurospheres indicates to the presence of neural stem cells and most importantly made it possible to observe the nuclear and cytoplasmic expression of GATA3 *in vitro*.

Figure 31: Neurospheres



*Sections from Sox1^{GFP} spread from P0 obtained spinal cord tissue (**A-C**) and Sox1^{GFP} secondary neurospheres from the same tissue (**D-F**) double stained with anti-GFP and GATA3 antibodies.*

2.3.6 Gata3^{-eGFP} reporter P0 and P40 marker analysis

As has been aforementioned, the Gata3^{-eGFP} reporter line was used after having established that it correctly represents the protein's expression at the stages which will be examined. P0 and P40 Gata3^{-eGFP} pups' spinal cord sections were used in order to carry out a marker analysis and explore the cells' characteristics further.

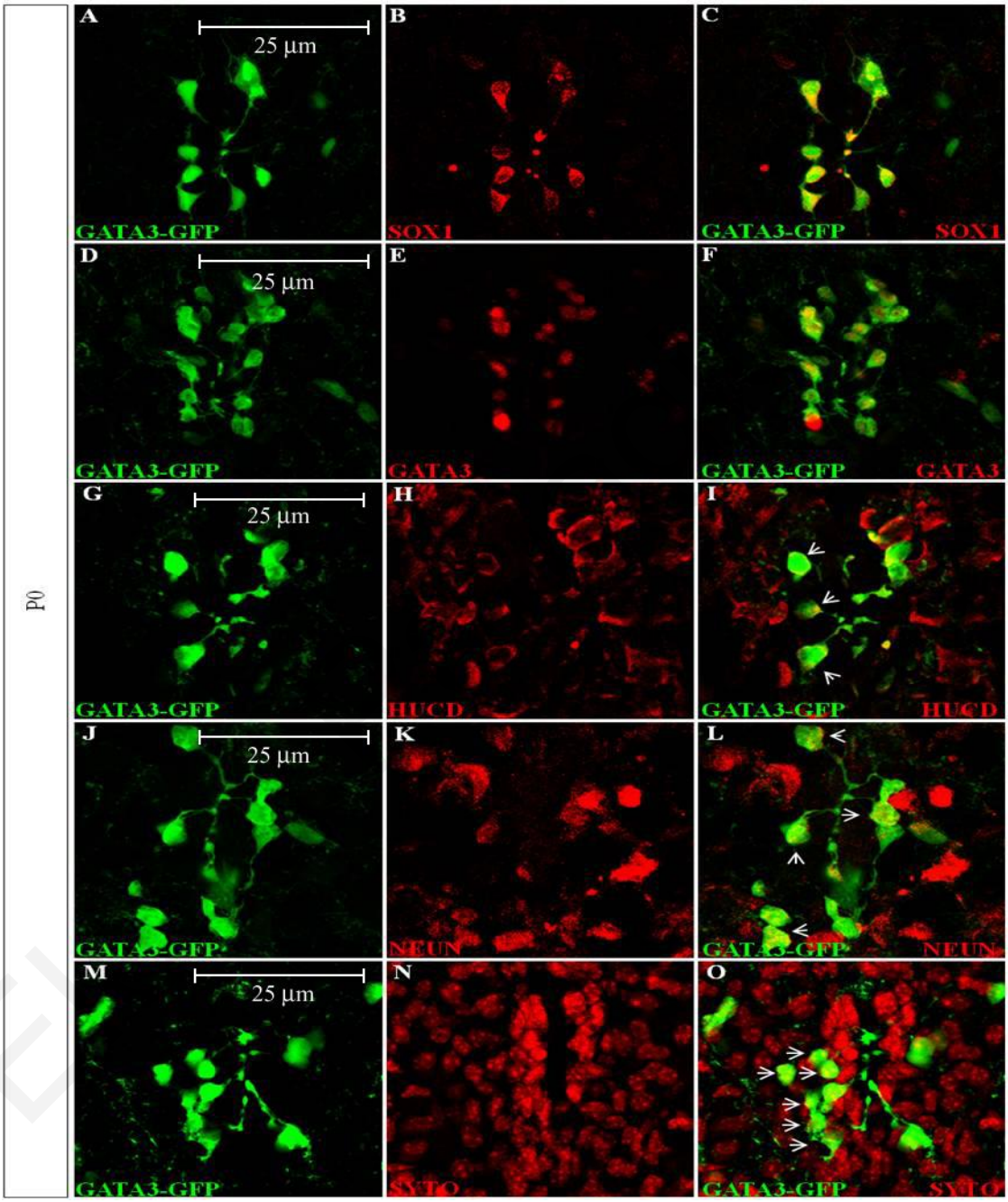
The P0 sections which were stained with anti-SOX1 and anti-GATA3 antibodies (Figure 32 A-C and D-F respectively) demonstrate that GATA3 and SOX1 expression was nuclear, even though SOX1 seems to be quite prominently expressed from the dendritic processes. Furthermore, some GATA3^{+} cells were also positive for HUC/D, an immature neuronal fate determinant, while some were positive for NEUN, a mature neuron marker (Figure 32 G-I and J-L). Finally, sections were stained with SYTO dye, which labelled all of the GATA3 positive cells, this indicating that the cells are alive.

A similar staining procedure was carried out on P40 sections (Figures 33 and 34) showed that even though SOX1 staining was still nuclear (albeit more prominent along the processes), while GATA3 was entirely cytoplasmic (Figure 33 A-C and D-F respectively). Furthermore, some GATA3 positive cells were also positive for HUC/D while none were positive for NEUN, nor SOX2 and PAX6 (known stem cell markers) and S100-β which specifically labels GFAP negative protoplasmic astrocytes. It should also be noted that the GATA3 positive cells were also negative for GFAP, another astrocyte marker (Young *et al.,* 2010; Gomez-Lopez *et al.,* 2011).

These results suggest that the Gata3 positive CSF contacting neurons are found in a variable state of neural differentiation, so that some are in a premature stage (HuC/D^{+}) whereas others are in a terminally differentiated stage (NeuN^{+}). The fact that

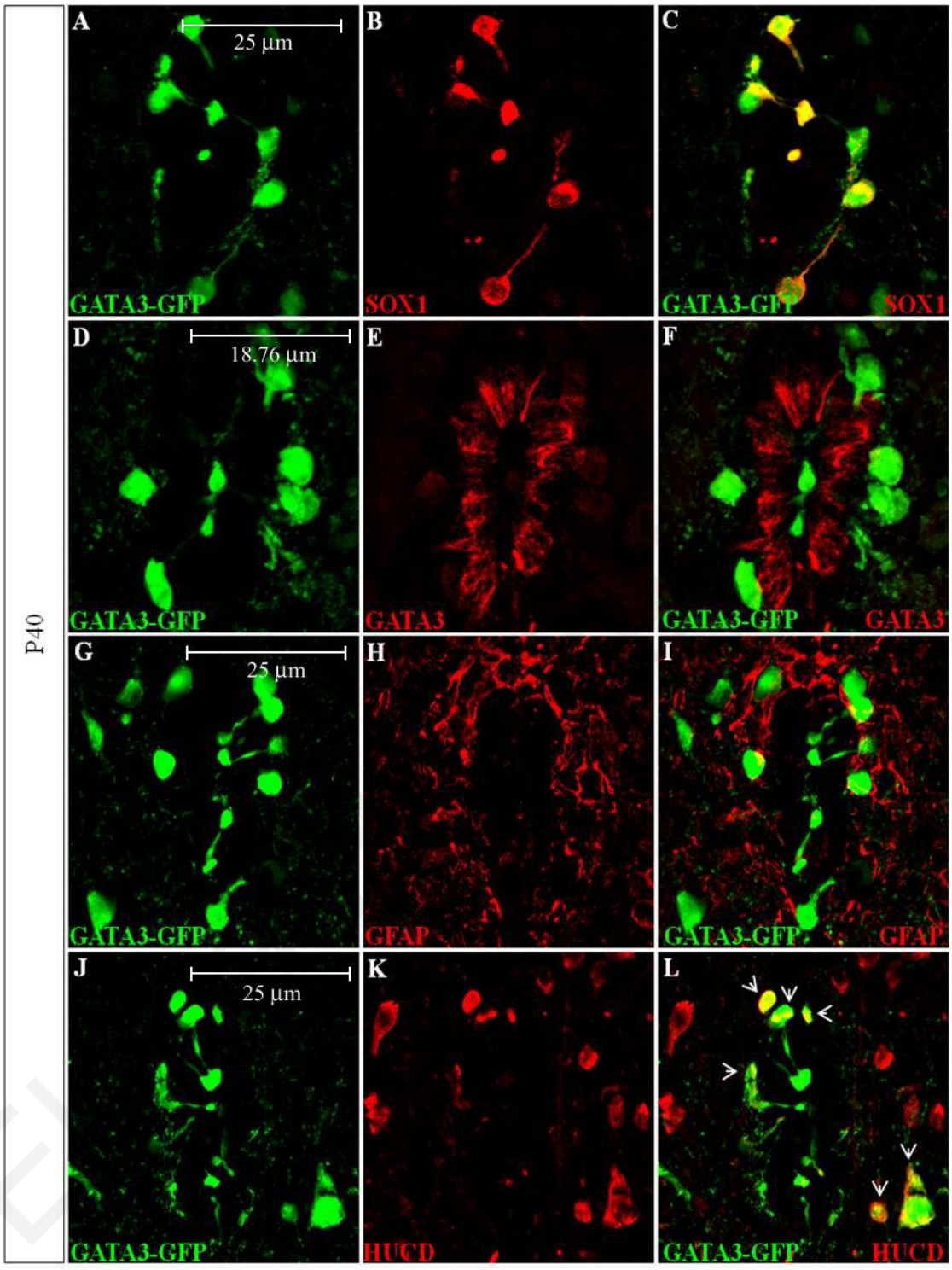
at P40 none of the neurons are NeuN^{+} possibly indicates that the cytoplasmic expression of GATA3 may be inhibiting the neurons' ability to reach maturity and instead are maintained in a perpetually undifferentiated state.

Figure 32: P0 Gata3^{-eEFP} reporter



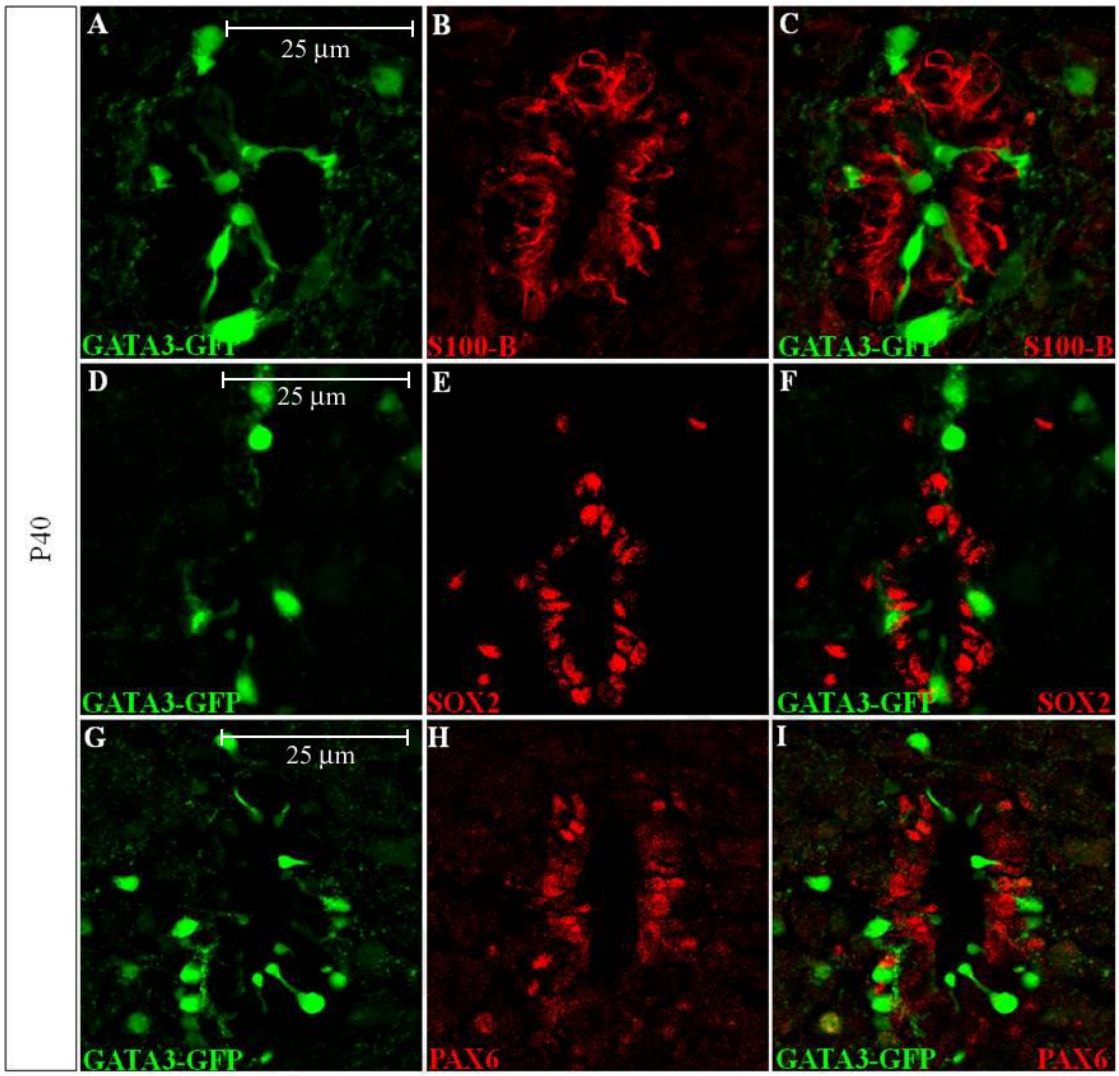
*Spinal cord sections from the Gata3^{-eEFP} reporter line from P0 pups stained with anti-SOX1 (**A-C**), anti-GATA3 (**D-F**), anti-HUC/HUD (**G-I**), anti-NEUN (**J-L**), and SYTO stain (**M-O**). The CSF contacting cells are positive for SOX1 which is nuclear and expressed through the dendridic processes. GATA3 is exhibits a nuclear expression, they are also positive for HUC/D which labels neuronal progenitors, as well as NEUN. SYTO stain labels the DNA.*

Figure 33: P40 Gata3^{-eEFP} reporter



*Spinal cord sections from the Gata3^{-eEFP} reporter line from P40 young adult mice stained with anti-SOX1 (**A-C**), anti-GATA3 (**D-F**), anti-GFAP (**G-I**), anti-HUC/D (**J-L**). Gata3 staining is entirely cytoplasmic, while SOX1 is nuclear. Some of the GATA3 positive cells co-express the immature neuron marker HUC/D, while the cells are negative for GFAP.*

Figure 34: P40 Gata3^{-eEFP} reporter cont.



*Spinal cord sections from the Gata3^{-eEFP} reporter line from P40 young adult mice stained with anti-S100-β (**A-C**), anti-SOX2 (**D-F**) and anti-PAX6 (**G-I**). None of the GATA3 positive cells were co-labelled with the aforementioned antibodies.*

2.3.7 Decrease in Sox1 affects the number of Gata3+ central canal contacting cells formed

The marker analysis was also repeated using the previously published Sox1^{-BAC} mouse line (Genethliou *et al.*, 2009a). Again P0 pups and P40 young adults' spinal cord sections were used to carry out this analysis (Figures 35-41).

The P0 sections were stained with anti-GATA3 antibody which co-labelled all of the GFP-positive Sox1 cells, even though not all GATA3 positive cells were also

positive for SOX1 (Figure 35 A-C). Staining with anti-SOX1 antibody indicates that even though SOX1 nuclear expression is well represented by the BAC line, the staining observed along the processes can only be obtained when using the antibody (Figure 36 D-F). Furthermore, the GFP positive cells did not co-label with OLIG2, PAX6 and S100-b (Figure 35 G-I, J-L and Figure 36 A-C respectively). As expected from previous experiments, some of the SOX1 positive cells were also positive for HUC/D.

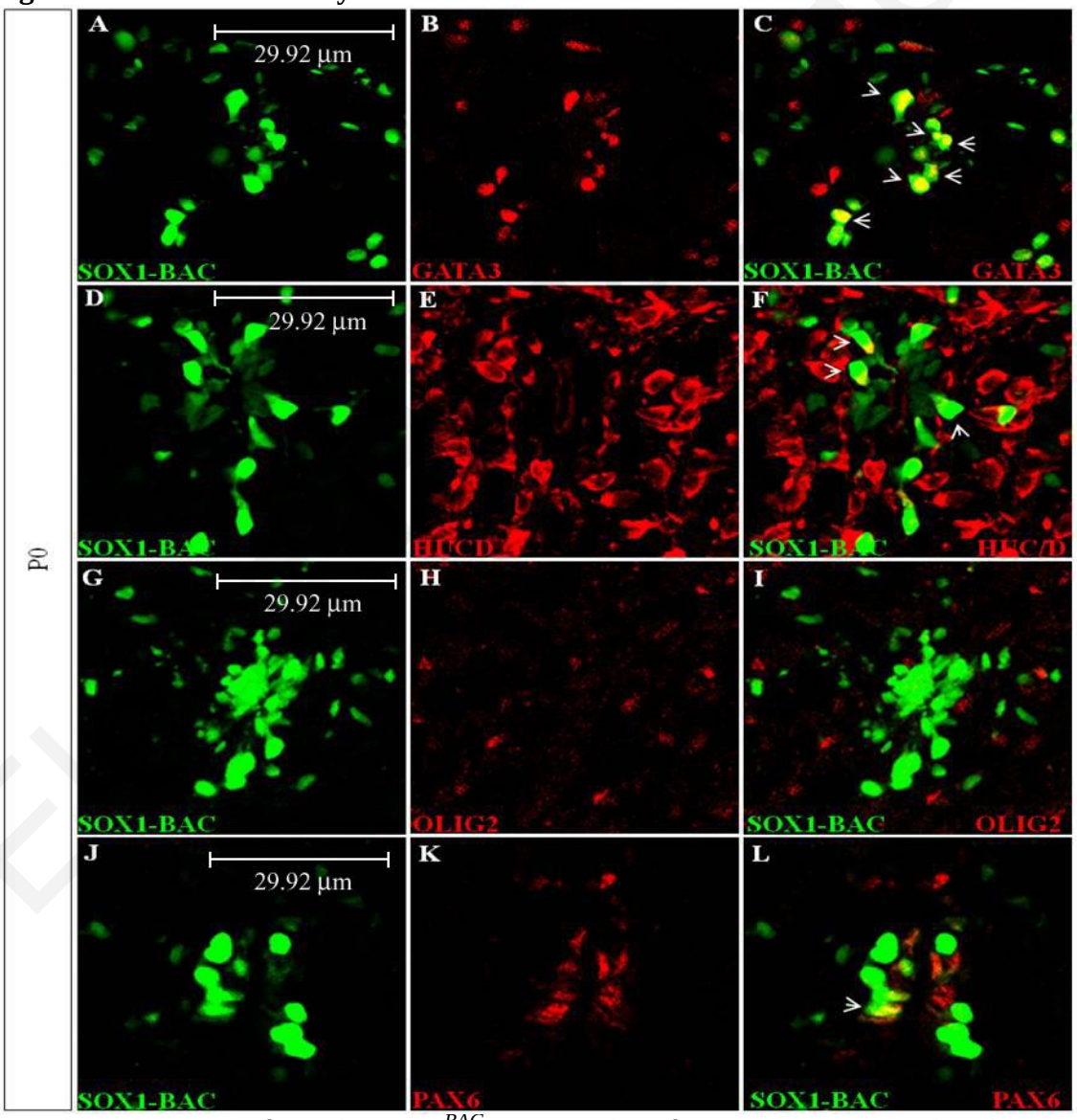
The P40 sections which were stained with anti-GATA3 and anti-SOX1 antibodies again showed that GATA3 expression was entirely cytoplasmic, whereas SOX1 expression was nuclear. Staining with GFAP, S100-β, and NEUN was again negative, whereas some cells were stained with α-HuC/D antibody.

In addition, the $Sox1^{-BAC}$ line was interbred with the $Pax6^{+/-}$ (*Sey*) line which would result in decreasing the amount of SOX1 produced, as Pax6 has been shown to regulate the expression of SOX1 during development in a domain specific manner (Genethliou *et al.,* 2009a). Therefore, in order to investigate whether reducing the expression of SOX1 would affect the production of the $GATA3^{+}$ central canal contacting cells, the two lines were interbred and P0 pups' and P40 young adults' spinal cords were extracted and used to stain against SOX1 and GATA3. Our results (Figure 39) show that the expression of SOX1 is affected as expected and at the same time, at P0, nuclear expression of GATA3 appears diminished, since less Gata3 positive cells were observed (Figure 40). At P40, GATA3 expression was again cytoplasmic and thus could not accurately be evaluated.

Finally, following this observation, the $Gata3^{-eGFP}$ reporter line was interbred with our $Sox1^{-bgeo}$ knock out line, so that the effect of the absence of SOX1 on the $GATA3^{+}$ CSF contacting population could be further investigated (Figure 41). The number of GATA3+ cells was reduced compared to the wild type sections by

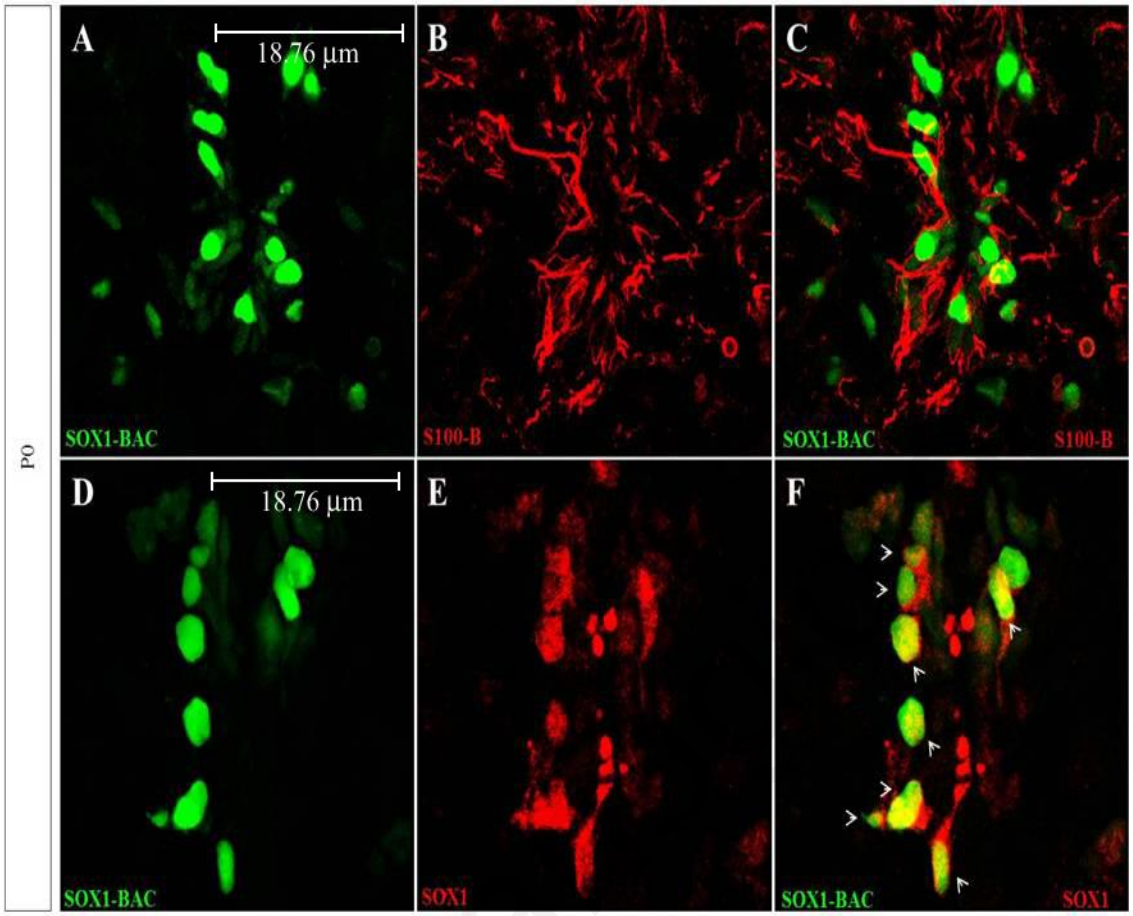
approximately 40% (Figure 40). Furthermore, even though at this stage some GATA3 positive cells should co-label with NEUN (as observed in the other experiments) none were observed to do so in the Sox1^{-bgeo} knock out line. Cell counts indicated that the number of GATA3 central canal contacting cells significantly decrease in the absence of Sox1, while none of the cells appear to be positive for NEUN. Therefore, these data signify that SOX1 expression is essential for the production and maintenance of the central canal GATA3 positive cells.

Figure 35: Sox1-BAC analysis



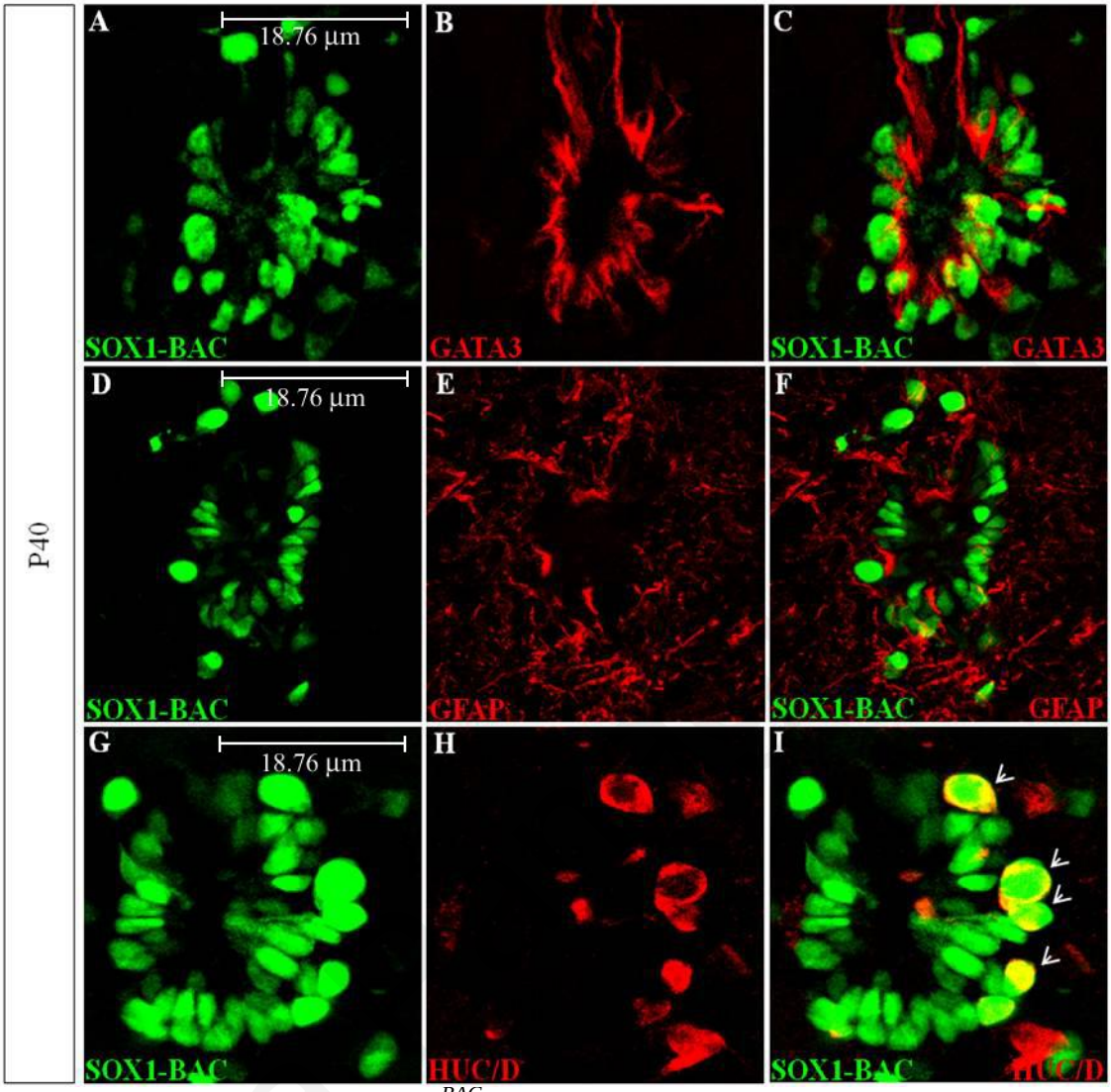
*Spinal cord sections from the Sox1^{-BAC} reporter line from P0 pups stained with anti-GATA3 (**A-C**), anti-HUC/D (**D-F**), anti-OLIG2 (**G-I**) and anti-PAX6 (**J-L**). Gata3 expression was nuclear, whereas some Sox1 positive cells were also positive for HUC/D.*

Figure 36: P0 Sox1^{-BAC} analysis cont.



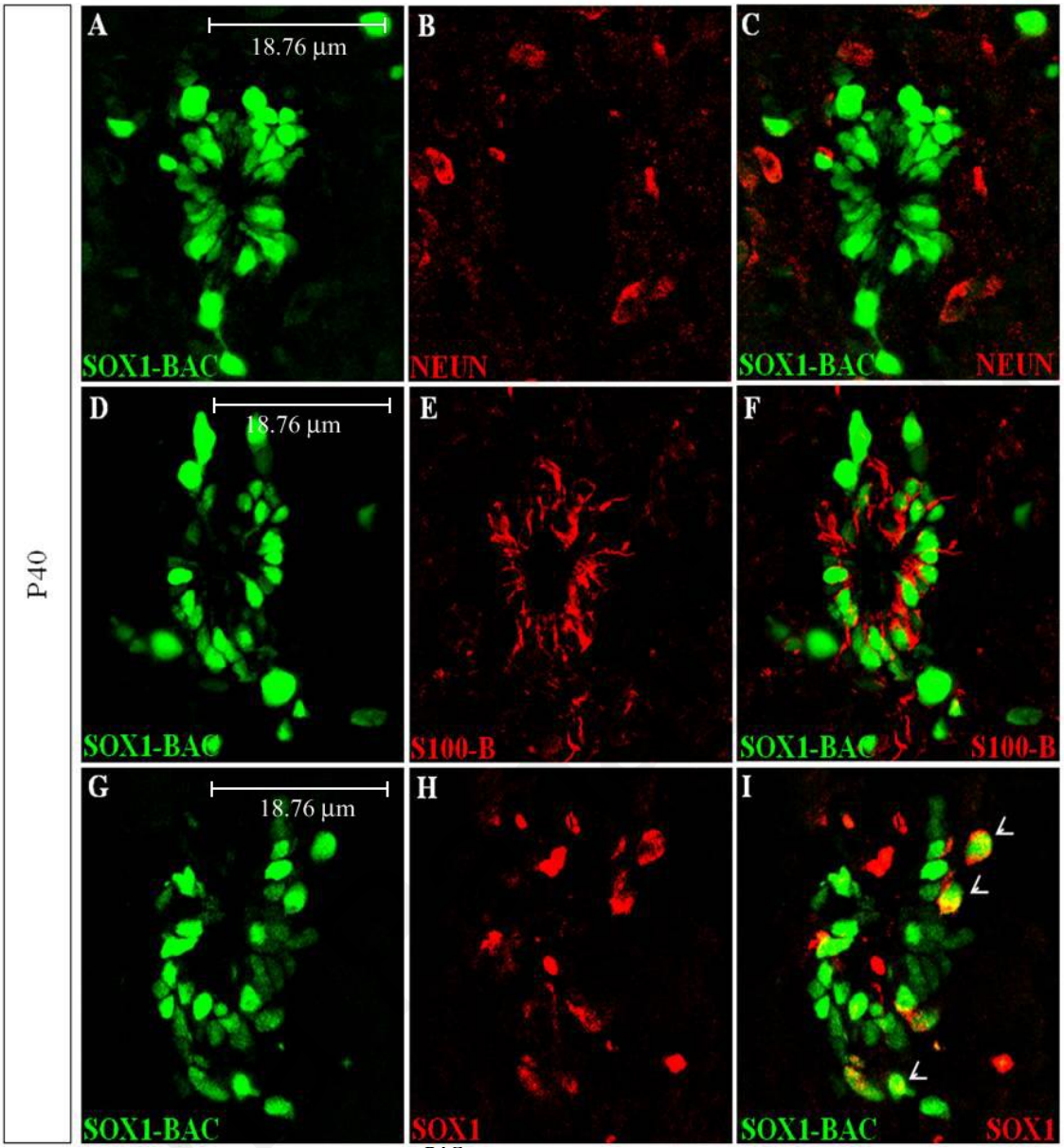
*Spinal cord sections from the Sox1^{-BAC} reporter line from P0 pups stained with anti-S100-β (**A-C**), anti-SOX1 (**D-F**) antibodies. None of the SOX1+ cells expressed S100-β.*

Figure 37: P40 Sox1^{-BAC} analysis



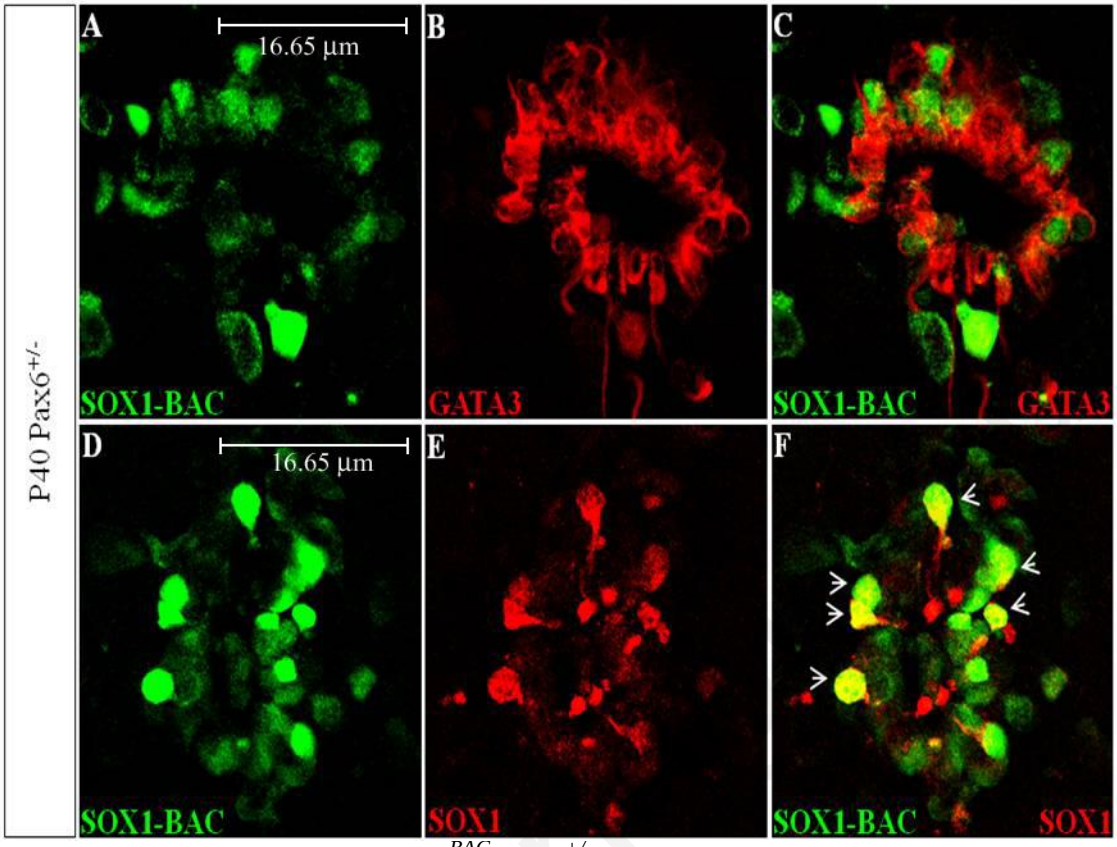
*Spinal cord sections from the Sox1^{-BAC} reporter line from P40 young adult mice stained with anti-GATA3 (**A-C**), anti-GFAP (**D-F**) and anti-HUC/D antibodies. Some of the SOX1 positive cells were positive for HUC/D, whereas Gata3 expression was entirely cytoplasmic.*

Figure 38: P40 Sox1^{-BAC} analysis cont.



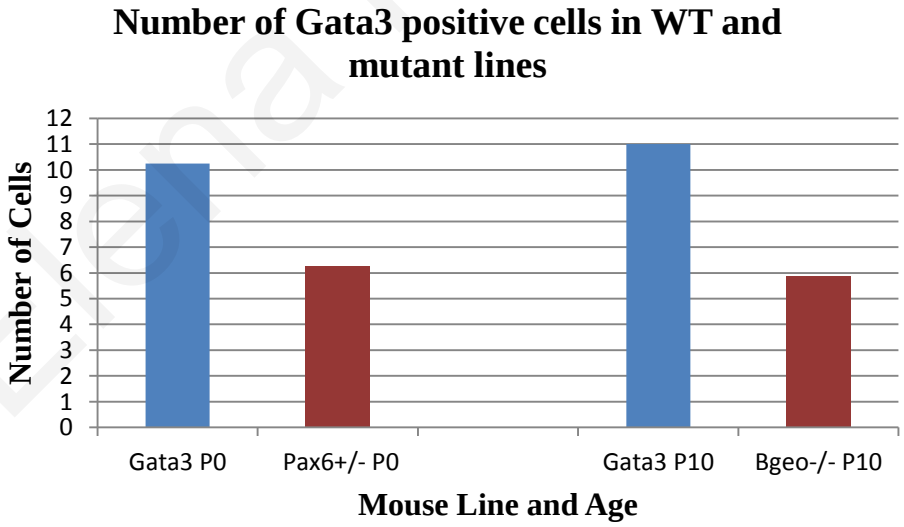
*Spinal cord sections from the Sox1^{-BAC} reporter line from P40 young adult mice stained with anti-NEUN (**A-C**), anti-S100-β (**D-F**) and anti-SOX1 (**G-I**) antibodies. The SOX1 positive cells were not positive for NEUN or S100-β.*

Figure 39: Sox1^{-BAC} PAX6$^{+/-}$ Cross



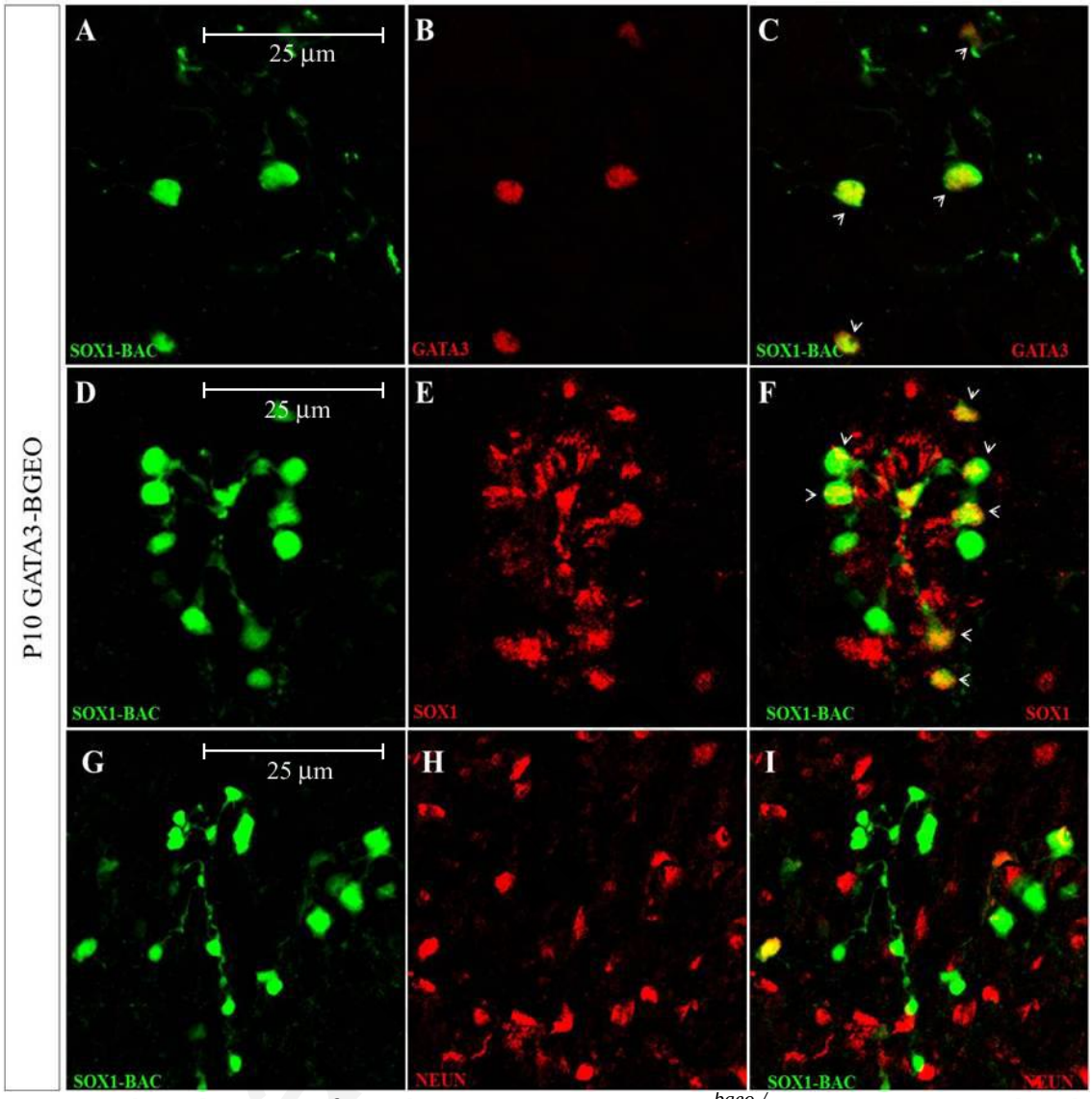
*Spinal cord sections from the Sox1^{-BAC} Pax6$^{+/-}$ P40 young adult mice stained with anti-GATA3 (**A-C** and anti-SOX1 (**D-F**) antibodies. The Sox1^{-Bac} cells correlated to the antibody's expression, while again GATA3 expression was cytoplasmic.*

Figure 40: Gata3 cell count



The number of GATA3 positive cells was counted in the Gata3^{-eGFP} reporter line at stages P0 and P10 (blue bars) whereas the brown bars correspond to the expression of GATA3 in Pax6$^{+/-}$ background and the Sox1$^{Bbgeo\ -/-}$ background.

Figure 41: P10 GATA3 and Sox1$^{Bgeo-/-}$

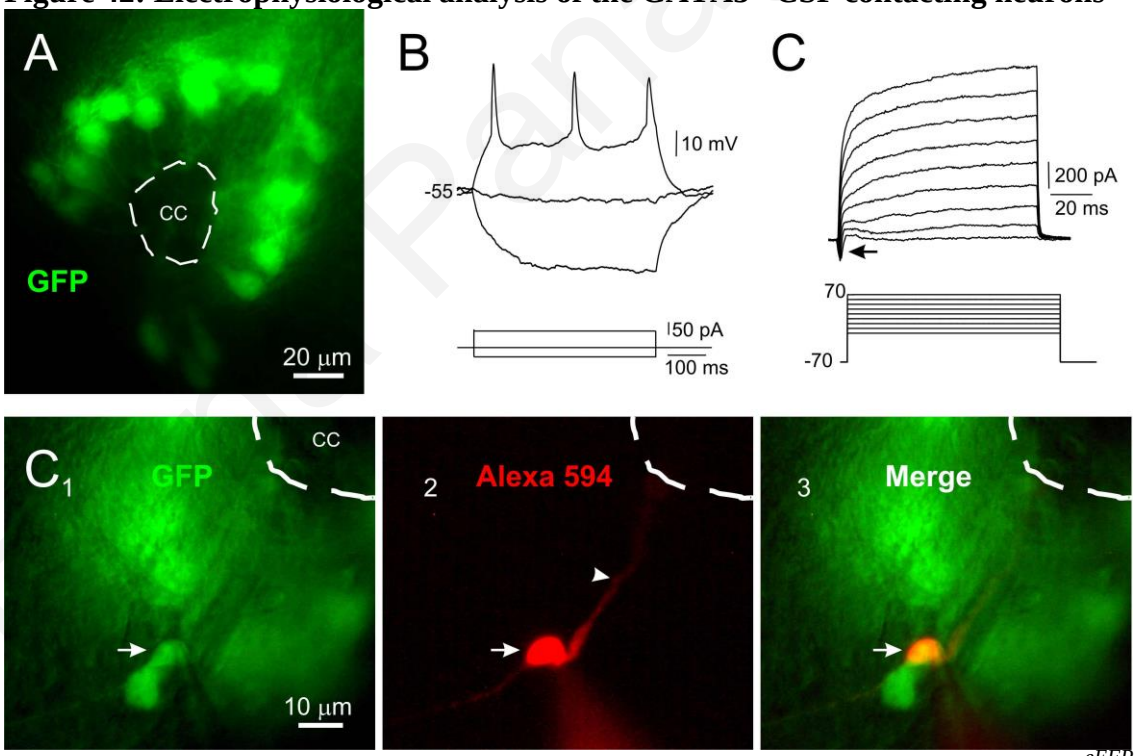


*P10 Spinal cord sections from the Gata3 reporter Sox1$^{-bgeo-/-}$ cross were stained with anti-SOX1 (**A-C**), anti-GATA3 (**D-F**) and anti-NEUN (**G-I**) antibodies. GATA3 and SOX1 expression was nuclear, whereas none of the GFP positive cells were also positive for NEUN. The number of GATA3 positive cells does appear to be decreased though compared to wild type sections.*

2.3.8 Electrophysiological data provide further insight on the nature of the Gata3+ CSF contacting neurons

Further information on the nature of the CSF contacting GATA3+ cells was obtained by some preliminary electrophysiological experiments carried out by Dr Raul E. Russo (Departamento de Neurofisiología Celular y Molecular IIBCE Montevideo, Uruguay) on the Gata3^{-eGFP} reporter line. Results show that the cells have inward currents, suggesting that these are indeed active neurons. However, they appear to lack A-type K+ currents, usually found in other central canal cells. There needs to be further experimentation however before reaching to any conclusions on the meaning of this observation.

Figure 42: Electrophysiological analysis of the GATA3+ CSF contacting neurons



Preliminary Electrophysiology Experiments. Spinal cord sections from the Gata3^{-eEFP} reporter line from P5 pups. ***A*** *Central canal region living slice.* ***B*** *GFP positive cell able to fire small action potential.* ***C*** *Underlying currents voltage clamp mode. C_{1-3} GFP cell recorded and injected with Alexa 594.*

CHAPTER 3

DISCUSSION

3.1 General Conclusions

This thesis has concentrated on studying late waves of neurogenesis in the murine spinal cord. These late waves were observed to give rise to a population of Pax6 positive cells which migrate ventrally, whereas another population of Gata3 positive cells migrated and persisted in the dorsal region of the spinal cord. Finally, CSF contacting cells which expressed both GATA3 and SOX1 postnatally in both a nuclear and cytoplasmic pattern.

Late born PAX6 positive cells which migrate ventrally and have not previously been studied were identified. The main conclusions which have arisen from this study are:

- Neurogenesis in the p2 domain appears to be in two waves and not in one as originally thought.
- A new type of neuron, deriving from the p2 domain, was discovered in the ventral spinal cord expressing PAX6 as a subset of the V2b interneurons.
- The p0 domain also generates neurons expressing PAX6 that migrate ventrally, a V0v subset.

Another late born population not previously studied was identified, expressing GATA3 throughout their embryonic development and persisted even after they reached their final resting position in the dorsal portion of the cord. Several factors and antibodies generally expressed in the region were used to further investigate these cells'; however none of those factors were found to be co-expressed. Nevertheless they were found to release GLUTAMATE, indicating that these have an excitatory nature. Therefore, the principal conclusions obtained from this study are the following:

- GATA3 positive cells appear at their dorsal position at E14.5 and remain throughout embryonic development and as well as postnatally.
- Do not express any known marker of dorsal interneurons.
- All of the dorsal GATA3 positive cells studied are excitatory Glutamatergic neurons.

Central canal cells have been previously studied, resulting in identifying various subpopulations of ependymal cells. One of those subpopulations are the CSF contacting neurons which have been shown to have dendritic processes which extending into the central canal. Ependymal cells lining and surrounding the central canal have been previously purported to possess regenerative properties as well as respond to injury attempting to assist in replenishing neural and glial cells. However in this study we've shown for the first time that the CSF contacting neurons express GATA3 and SOX1 in an exceptional manner. GATA3 is initially found to be cytoplasmic at P0 and SOX1 is expressed in the dendritic processes, at later adult stages however GATA3 is cytoplasmic. Considering that GATA3 is a TF and thus acts in the nucleus where it controls the transcription of other genes, its presence and persistence in the nucleus suggests that the TF's action is purposely inhibited. Furthermore, using a Sox1 knock out line exhibited a decrease in the number of CSF contacting Gata3 positive cells. Therefore, this functional analysis suggests that Sox1 is imperative in either maintaining the character of these cells or prohibiting them from migrating away from the central canal lumen. In summary the main conclusions from this part of the study are:

- GATA3 expression was found to be nuclear at P0 but was converted to cytoplasmic at P40

- SOX1 appears to be more prominently expressed in the nucleus at P40 rather than at P0

- 40% decrease of GATA3 expressing CSF contacting neurons in the absence of Sox1

3.2 Late wave neurogenesis in the ventral spinal cord

The combined expression of various TFs directs the differentiation and fate of cells found in the nervous system. The p0 domain (expressing-Dbx1) gives rise to two subtypes of INs, the excitatory $V0_V$ INs and the inhibitory $V0_D$ INs (Hori & Hoshino). Our previous work has shown that the p2 domain generates at least three types of neurons namely V2a, V2b and V2c which are generated before mid-gestation (Panayi *et al.*, 2010). In that study we showed that V2c INs are lineally related to V2b INs and acquire a distinct specification through the action of SOX1.

3.2.1 Late born PAX6+ INs are a heterogeneous population of cells originating from both the p0 and the p2 domain

In this study we show that a late born group of $Pax6^+$ neurons is a heterogeneous population of INs which originate from both the p0 domain, constituting a subset of the $V0_V$ INs, and the p2 domain as a subset of the V2b INs. This diverse population of $Pax6^+$ cells was shown to include both inhibitory and excitatory neurons. This finding is in accordance with our lineage data, which specifies that these cells are derived from diverse domains which produce INs with different properties.

PAX2 expression in the spinal cord has been shown to begin at stage 15 in chick embryos, thus E12 at the mouse embryo, and marks two ventral IN subsets which are distinctly discernible by the expression of EN1 and EVX1 (Burrill *et al.*, 1997).

Our data illustrates that a subpopulation of the $Pax6^+$ neurons, approximately 60%, derive from the Dbx1 lineage, express both PAX2 and EVX1 (Figures 11 and 12). V0 commissural neurons are separated into inhibitory (70%) and excitatory (30%) INs .Our data indicates that the late born $Pax6^+$ cells are both inhibitory Glycinergic and excitatory Glutamatergic INs (Figure 17), thus corresponding to the origin of these cells.

3.2.2 The p2 domain gives rise to an additional late born subpopulation of PAX6+ cells

We also show that the p2 domain generates an additional interneuron subtype which expresses PAX6. Unlike the other three types of neurons generated, these cells are born later in development and represent a subset of V2b INs, co-expressing PAX6 and GATA3. It is noteworthy that as $PAX6^+$ cells migrated out of the ventricular zone they did not express GATA3. However as they settled in their ventral position they switched on Gata3. This sequential expression of PAX6 followed by GATA3 clearly points to a different mechanism that drives the expression of GATA3 in these cells compared to other $GATA3^+$ neurons which acquire GATA3 expression on the borders of the VZ through a mechanism controlled by Notch signaling. What switches on Gata3 in these cells remains to be determined.

3.2.3 Varying mechanisms drive the specification of p2 derived INs

Previous studies have shown that the Notch pathway determines the specification of V2 INs (Del Barrio *et al.*, 2007). Specifically loss of Notch at e9.5 converts V2b INs to V2a INs. Our present data, combined with our previous work, suggest that there must be at least two additional mechanisms that determine V2b sub

lineages. One mechanism acting early and driving expression of SOX1 in a small subset of V2b neurons while at least two days later an additional mechanism sets in and drives expression of GATA3 in another small subset of V2b neurons that initially express only Pax6. We name these $PAX6^{+}/GATA3^{+}$ neurons V2d neurons to distinguish them from $PAX6^{-}/GATA3^{+}$ cells. We cannot exclude the possibility that a $PAX6^{+}/GATA3^{-}$ V2 derived cellular subpopulation also exists but we cannot determine if this is the case using our current data.

Our data pose two fundamental questions in relation to V2 IN development. First what drives the expression of PAX6 late in development in a small subset of neurons? Second, Pax6 is a patterning factor which, after mid-gestation, is responsible for the specification of different types of astrocytes in the p2 domain. How the neuronal and glial specification roles segregate is yet another unresolved question. Regarding neuronal specification, it is conceivable that signals from the surrounding environment might play a role. What is striking, however, is that the number of V2c and V2d INs, compared to the total number of V2b INs, is very small (no more than five cells either side of the tube) and thus whatever signals operate on these two classes of neurons must be highly selective.

Whatever the underlying mechanism operating to specify V2d INs is, our data reveal that loss of Notch signaling converts these cells to V2a neurons. We noted that in the absence of NOTCH some GATA3 expression was maintained (unlike early stages) but $PAX6^{+}/GATA3^{+}$ cells converted to $PAX6^{+}/CHX10^{+}$ cells. While this fate switch is a universal characteristic of all V2b INs, the expression of Pax6 in post-mitotic V2d INs (unlike that of Gata3), is unlikely to be regulated by Notch as it is maintained in the mutant cells. Therefore, the specification of V2d INs is likely to be regulated by a combination of factors and not just PAX6. This is in contrast to the specification of V2c

INs where the expression of SOX1 alone is necessary for the acquisition of the V2c neuronal fate.

3.3 Late wave neurogenesis in the dorsal spinal cord

The dorsal GATA3 positive cells identified at E14.5 were also found to persist postnatally. Several dorsal cell markers were used to investigate these cells' identity (Figures 23 and 24).

3.3.1 Dorsal GATA3 positive cells do not express any of the known dorsal cell markers used but are excitatory glutamatergic neurons

Several markers of dorsal cells and interneurons were used to investigate their relationship to the dorsal GATA3 positive cells. Apart from utilizing Sox1 and Sox14, dorsal interneuron factors marking the dorsal populations dI2, dI3, dI4, dI5 and dI6, as well as the V1 and V2 interneurons. However, none of those factors co-localized with the dorsal GATA3 positive cells. Finally, the entire population was shown to be labelled by GLUTAMATE, the most common neurotransmitter in vertebrates' spinal cord, even though the distribution of many glutamatergic circuits is still highly unknown (Fernandez-Lopez *et al.*, 2012).

Further analysis needs to be carried out in order to identify other markers identified by this dorsal GATA3 positive populations.

3.4 Late wave neurogenesis in the central canal

Neurons have been shown to come in contact with the CSF by extending their perikarya, dendrites or axons. The majority of the CSF contacting neurons extend their processes into the ventricular fissure, the processes end to ciliated terminals as with sensory cells (Vigh & Vigh-Teichmann, 1998). The majority of neural cells in the

vertebrate central nervous system remain sequestered from the CSF by means of the ependymal lining. However, a few cells do manage to send their processes through the lining and interact with the external CSF space. These neurons' function is most probably related or influenced by the CSF. In the central canal of the mouse and rat, CSF contacting neurons have been described but no marker has so far been discovered and no genetic data exist about their development. In this study we show that in the mouse CSF-contacting cells express SOX1 and GATA3 in a dynamic manner relative to their cellular location and in a time-dependent manner.

TFs undergo nuclear localization where they bind onto promoter regions of genes they control and affect their expression, either increasing or decreasing their level of transcription. Gata3 is a TF of major significance; both Gata2 and Gata3 are involved in the development of numerous systems. Gata3 is notably involved in the specification of the V2b spinal interneurons. FoxN4 positive progenitors give rise to the p2 interneurons. The Notch pathway is involved in the bifurcation of the Chx10 positive V2a interneurons and the Gata3 positive V2b interneurons. Recently we have been able to identify another subpopulation of V2b interneurons, the V2c Sox1 positive population, which similarly to the V2b interneurons which originally express Gata3. The Gata3 TF however was initially identified to be expressed in T helper cells where it was found to be implicated in the specification of T-helper type 2 cells which operate alongside B-cells and are associated with humoral immunity, thus instigating the transcription of IL-3, IL-4 and IL-13.

3.4.1 GATA3 expressed by CSF contacting neurons in the central canal

In our study we found, for the first time, that CSF-contacting cells express GATA3. Our experiments have indicated that Gata3 expression profile varies depending on the stage at which analysis was carried out. During the early post-natal

stages (P0 to P30), GATA3 was nuclear as expected, however at the early adulthood stages the protein appears to be kept outside the nucleus. In other systems, GATA3 was also found to be variable in different forms of cancer. In breast cancer and Hodgkin lymphoma it was found to be located in the nucleus, whereas it has been found to be completely cytoplasmic in pancreatic cancers. GATA3 has been implicated in de-differentiation, a process purported to occur in the initial stages of pancreatic cancer development.

3.4.2 GATA3 switches from nuclear to cytoplasmic expression in a time-dependant manner

Dedifferentiation is a process most often observed in amphibians, worms and plants so that partially differentiated cells or even terminally differentiated cells will regress into a less differentiated stage. This process is usually found to occur in relation to a recovery mechanism. Cultured cells observed to undergo dedifferentiation have lost their physiological expression profile and even changed shape or properties. Dedifferentiation is either viewed as been part of the immune response which has through evolution disappeared in humans, or it may be viewed as an abnormal effect which forces the cells to depart from their customary developmental program and become cancerous.

Therefore, considering that GATA3 is found to be completely cytoplasmic in pancreatic cancers where it has been linked to dedifferentiation and having observed a similar expression pattern in the P40 CSF contacting spinal cord neurons of adult mice may be indicative of the function of these CSF contacting cells. GATA3, as has been aforementioned, is a TF, or a sequence-specific DNA-binding factor, demonstrating the fact that this protein's function is to bind to specific DNA sequences in the nucleus and thus summon or obstruct the action of RNA polymerase. GATA3 is found in the

cytoplasm where its NLS signal is phosphorylated and thus transported into the nucleus via importin-a (karyopherin-α). Increased nuclear expression of GATA3 has been observed after activation of the T-cell receptors, both in humans as well as mouse models where GATA3 has been shown to determine Th2 cell differentiation and specification. (Suzuki *et al.*, 2002; Maneechotesuwan *et al.*, 2007). Thus, it is plausible to propose that inhibiting the action of GATA3 and instead restricting it to the cytoplasm may be a mechanism of inhibiting differentiation and maintaining the cells in an immature state - expression of HUC/D, immature neuronal marker instead of NEUN, a mature neuronal marker- (Figures 23 and 24). Especially when coupling this to the more prominent expression SOX1 within the nucleus, a factor which instead inhibits differentiation and promotes neurogenesis.

3.4.3 Neurospheres obtained from central canal cells exhibit the same GATA3 nuclear to cytoplasmic expression switch observed in vivo

Two distinct locations in the adult rodent spinal cord have been proposed as purported stem cell niches, the peripheral region: parenchymal NG2+ pre-cursors and central canal region cells. Neurospheres generated from the peripheral region were very restricted regarding their proliferation, as well as self-renewal and multipotency (Yamamoto *et al.*, 2001b). On the other hand, neurospheres derived from tissue containing the central canal portion were not limited and instead generated true neurospheres with the ability to re-generate while giving rise to neurons and glia (Martens *et al.*, 2002). The central canal of the spinal cord is surrounded by a heterogeneous population of pre-cursor cells which possess the ability to generate both neurons and glia. As well as been a unique neuronal niche, regarding the types of cells contained there as well as their distinct properties and probable functions; since they

maintain their neurogenic capacity and ability to replicate (Garcia-Ovejero *et al.*, 2013). Therefore, it is imperative to fully understand and document the nature and characteristics of the adult ependyma and assess its potential in disease, injury and therapy.

The CSF contacting cells we are investigating have also proven capable of generating primary neurospheres, thus indicating that the cells possess stem cell-like characteristics in the aspect that they hold regenerative abilities. The primary neurospheres were dissociated and re-plated, and then gave rise to secondary neurospheres. Having stained the neurospheres with anti-GATA3 antibody, we were able to observe both nuclear and cytoplasmic GATA3 expression, once more noting the dynamic expression of the protein in these central canal contacting cells. Bearing in mind that a neurosphere system is in reality an artificial system which encourages the production of neurons, oligodendrocytes and astrocytes (thus validating the multipotency of the cells used to establish the neurosphere initially) which also contains a very small percentage of neural stem cells. This small percentage if contained within the neurosphere system can be used to set up secondary neurospheres. The most significant outcome from this experiment however, apart from the fact that we were able to establish both primary and secondary neurospheres from central canal tissue, is the fact that the same effect observed *in vivo* was observed *in vitro*. GATA3 was both nuclear and cytoplasmic, even though the cytoplasmic expression was much more perceptible than the nuclear one. This however would substantiate the initial observation and validate the cytoplasmic nature of GATA3 at specific time points as observed *in vivo*.

3.4.4 CSF contacting GATA+ neurons are born from E14.5 until E16.5 and some originate from the pMN domain

Birth-dating experiments show that the central canal contacting cells were born starting at e14.5 during embryonic development, until the e16.5 stage. It appears that the majority of the cells are born on e14.5 when sections were stained at P0, while fewer cells were BRDU positive during stages e15.5 and e16.5 when stained at P1 and P2 respectively. This is probably indicative of the fact that some of these cells migrate away from the central canal postnatally.

Furthermore, previous studies have proposed that ependymal cells originate from the progenitors which constitute the pMN, p3 or p2 domains (Fu *et al.*, 2003b; Masahira *et al.*, 2006). To investigate this and narrow down the source for the CSF contacting cells of the ependymal population the $FoxN4^{-iCre}$ (labelling the p2 domains) and $Olig2^{-iCre}$ (labelling the pMN and p3 progenitor domains) were used. Our results show that approximately half of the CSF contacting cells population originate from $Olig2^{+}$ progenitors from the pMN/p3 domains whereas none of the cells originate from the p2 domain.

3.4.5 GATA3+ CSF contacting cells are a mixed population of mature and immature neurons

Even though there's no firm evidence on most of the central canal's cells' functions some of the cell types have been more heavily studied and reported on than the rest. Some central canal contacting cells have been reported as retaining both mature and immature neuron characteristics since they express neuronal markers such as NEUN, PSA-NCAM, DOUBLECORTIN and HUC/D (Marichal *et al.*, 2009;

Sabourin & Pollack, 2009). Similarly, the spinal cord Gata3^{-eGFP} reporter sections analysed at P0 and P40 show that some of the CSF contacting GATA3 positive cells express HUC/D and some express NEUN (Figures 32-34). These factors are found in immature and mature neurons respectively.

3.4.6 Sox1 is necessary for the proper development of the GATA3+ CSF contacting cells

The Gata3^{-GFP} reporter line was crossed with the Sox1 knock out line (Bgeo) in order to study the effects if any of the absence of Sox1 on the expression of the GATA3 positive CSF contacting cells. Therefore, spinal cord sections were analysed using antibodies against GATA3, SOX1, HUC/D and NEUN. GATA3 and SOX1 expression was nuclear, whereas some GATA3 positive cells also co-expressed HUC/D, but none expressed NEUN. Since NEUN, the neuronal nuclear antigen, is found to be expressed more prominently as the cells switch off immature markers such as HUC/D and doublecortin, it is considered to signify more mature neurons (Dredge & Jensen, 2011). This fact would wither indicate that the absence of Sox1 impedes the production of terminally differentiated neuronal cells. However, when considering the nature of SOX1 in maintaining cells' undifferentiated state and thus promoting neurogenesis, may suggest that cells mature and migrate away from the central canal so that they would not be detected. The presence of cells expressing HUC/D would also reflect that Sox3 may offer some functional redundancy and allow for the neuronal progenitor identity to be maintained.

Finally, and perhaps most notably, functional analysis using the Sox1 knock out line (Bgeo line) showed that the number of GATA3 positive central canal contacting cells was significantly decreased in the absence of Sox1 by approximately 40%, when

compared to wild type sections. This phenotype suggests that SOX1 is imperative in these cells' productions and maintenance.

Neuronal stem cells from the spinal cord could be instigated to regenerate in vivo, thus inducing recovery from spinal cord injury. This strategy may be a means of replacing invasive stem cell transplantation which would erase the need for immunosuppression therapy. Therefore, been able to regulate the proliferation of ependymal cells and their progeny would encourage functional and motor recovery of injured spinal cord sites.

Even though stem cell-like cells lining the central canal can be stimulated to migrate and differentiate following spinal cord injury they do not appear to be able to re-generate the spinal cord. It may be possible however to employ neurotrophic agents to augment the stimulation of central canal lining cells thus enhancing their ability to proliferate following injury. Spinal cord ependymocytes possess neural stem cell abilities *in vitro* and *in vivo* following spinal cord injury.

The capacity to replace/replenish damaged cells in order to restore spinal cord function by utilizing undifferentiated or progenitor cells would appear to be a very 'promising' approach (Garcia-Ovejero *et al.*, 2013). However, the practical significance of the central canal cells is quite perplexing. They have been proposed to be sensory neurons, responsible for regulating the central canal's composition (Vigh & Vigh-Teichmann, 1998) Furthermore, the varying stages of neuronal maturation may either be indicative of the fact that the cells' retain distinct electrophysiological properties, serving different purposes, or the fact that they may originate at diverse time points.

3.4.7 Final points

At very early stages of embryonic development the spinal cord consists of neuroepithelial cells (NEPs) that progressively acquire dorso-ventral identity and become organised into eleven progenitor domains each one exhibiting unique genetic marker profiles. The acquisition of domain-specific characteristics by NEPs is called patterning and is caused by signalling molecules released from dorsal and ventral midline structures. The ventral spinal cord (vSC) in is organized into five progenitor domains (ventral-p3-pMN-p2-p1-p0-dorsal), specified through the graded activity of Sonic Hedgehog (SHH) released from the notochord and floor plate (Ericson et al., 1997a). SHH either induces or represses the expression of homedomain (HD) and basic helix-loop-helix (bHLH) transcription factors (TFs) creating a unique expression pattern of these factors in the five most ventral progenitor domains {Jessell, 2000 #2}. The dorsal spinal cord (dSC) is organised into six progenitor domains. Members of the bone morphogenetic protein (BMP) family, produced from dorsal midline structures, create a gradient of BMP-dependent activity that is responsible for patterning dorsal progenitor domains, along with other signalling molecules. Similar to the vSC, in the dSC patterning by BMPs and other factors create a unique expression pattern of bHLH and HD TF factors in each progenitor domain. The TFs expressed in each progenitor domain are primarily responsible for specifying the neural and glial output of that domain.

While neural patterning is the overarching mechanism for establishing cellular diversity in the spinal cord, additional mechanisms operate in each domain that further enhance cellular diversity. For example, the pMN domain generates different types of motor neurons (MNs) while the p2 domain generates at least three different types of interneurons (INs), named V2a, V2b and V2c. In the p2 domain the specification of

V2a and V2b IN fates is determined in post-mitotic progenitors through the action of Notch/Delta signalling, both in mice (Del Barrio et al., 2007; Peng et al., 2007) and zebrafish (Batista et al., 2008; Kimura et al., 2008). The mechanism underlying the specification of V2c INs is yet to be determined (Panayi et al., 2010). Downstream of Notch, the action of the LIM-only protein (LMO4) either favours or inhibits transcriptional complexes in subsets of post-mitotic progenitors and consolidates the V2a and V2b IN fates (Joshi et al., 2009).

In this study we investigated the neuronal output of the p2 domain after the production of V2a, V2b and V2c INs. We discovered two additional IN subtypes that derive from $GATA3^{+}$ progenitors. One subgroup migrates ventrally and expresses Pax6. The other group migrates in the dSC and derives from the V2b lineage. Unlike early-born V2 INs, these late-born $Pax6^{+}$ INs are generated in a Foxn4- and Notch-independent manner. These data reveal that the p2-domain generates even more IN subtypes than originally appreciated and suggest that additional, temporally distinct, mechanisms operate in this domain to govern the production of different neuronal IN subtypes.

Furthermore, a late born population which arises between E14.5 and E16.5 which surrounds the central canal and contacts the CSF has been heavily investigated. These cells appear to be unique in the sense that their expression profiles change postnatally by initially co-expressing Gata3 and Sox1 in a manner where Gata3 is nuclear and Sox1 seems to be expressed by the dendridic processes which contact the central canal. Later on though as the animal grows, Gata3 expression around the central canal appears to be entirely cytoplasmic and Sox1 is almost exclusively nuclear.

APPENDIX I
MATERIALS AND METHODS

1. DNA EXTRACTION AND QUANTIFICATION

1.1 Extraction of Total mouse genomic DNA

Reagents used:

Reagent	Recipe
TNES	10 mM Tris, pH 7.5 400 mM NaCl 100 mM EDTA (Ethylenediaminetetraacetic Acid) 0.6% SDS (Sodium Dodecyl Sulfate) dd H_2O
6M NaCl	
10 mg/ml Proteinase K	in dd H_2O
TE (filter sterilized)	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.

1.2 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

2. DNA AMPLIFICATION AND ELECTROPHORESIS

2.1 DNA amplification using the Polymerase Chain Reaction (PCR)

Reagents used:

Reagent	Recipe
Taq thermostable DNA polymerase	
10X reaction buffer	200 mM $(NH4)_2SO_4$ (Ammonium Phosphate) 75Q mM Tris-HCI pH 9.0 (at 25°C) 0.1% (w/v) Tween.
25 mM $MgCI_2$	
100 mM solution of 2'-Deoxynucleoside 5'-Triphoshate (from Sigma)	
Synthetic oligonucleotides (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).

2.2 Electrophoretic analysis of the DNA

Buffers used:

Reagent	Recipe
Running Buffer 5X Tris-borate (TBE)	445 mM Tris base 445 mM boric acid 10 mM EDTA
6X Loading buffer	0.25% bromophenol blue 0.25% xylene cyanol FF 50 mM EDTA, 30% glycerol.

2.3 Aqarose 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.

3. IMMUNOHISTOCHEMISTRY

Reagents used:

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

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.
Phosphate Buffered Saline (PBS)	0.1 M PB 125 mM NaCl Disolved in ddH_2O.
20% Sucrose	20g Sucrose 50ml 0.2 M PB Bring the volume up to 100ml with ddH_2O.
Blocking solution	0.1 M PB 0.1% Triton-X 0.1% Azide 1% Heat inactivated Donkey Serum 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 1. Primary Antibodies used.

Antigen	Source	Antibody Code	Species Antibody was raised in	Dilution used	Fixative used
Bhlhb5	Gift from Dr Frédéric Clotman	N/A	Goat	1/500	PFA or Memfa
BrdU	Sigma	B8434	Mouse	1/50	PFA
Chx10	Santa Cruz	Sc-21690	Goat	1/500	PFA or Memfa
Evx1	Developmental Studies Hybridoma Bank	99.1-3A2	Mouse	1/50	Memfa
Foxd3	Gift from Dr Frédéric Clotman	N/A	Guinea Pig	1/5,000	PFA
FoxP2	Abcam	ab1307	Goat	1/1,000	PFA
GABA	Immunosolution	IG1004	Rabbit	1/5,000	PFA or Memfa
Gata3	Gift from Dr Frank Grosveld	N/A	Rat	1/100	PFA or Memfa
GFAP	Millipore	AB5804	Rabbit	1/200-1/400	PFA or Memfa
GFP	Invitrogen	A11122	Rabbit	1/4,000-1/6,000	PFA or Memfa
GFP	Nacalai	GF090R	Rat	1/2,000	Memfa
Glutamate	Immunosolution	IG1006	Rabbit	1/10,000	PFA or Memfa
Glycine	Immunosolution	IG1002	Rat	1/5,000	PFA or Memfa
HNF6	Laboratory of Neural Differentiation / LPAD Unit	4079C	Guinea Pig	1/5,000	PFA
HuC/D	Invitrogen	A21271	Mouse	1/500	PFA or Memfa
Lbx1	Gift from Dr Müller	N/A	Guinea Pig	1/2,000	Memfa
MafA	Gift from Dr Frédéric Clotman	N/A	Guinea Pig	1/5,000	PFA or Memfa
MafB	Gift from Dr Frédéric Clotman	N/A	Guinea Pig	1/2,000	PFA or Memfa
NeuN	Millipore	MAB377	Mouse	1/400	Memfa
Nurr1	Provided by Dr. Yuichi Ono	N/A	Rat	1/2,000	PFA
Olig2	Santa Cruz	sc-19967	Goat	1/500	PFA or Memfa
Pax2	Sigma	AV100859	Rabbit	1/1,000	Memfa
Pax6	Developmental Studies Hybridoma Bank	PAX6	Mouse	1/50	Memfa
Pax6	Chemicon	AB5409	Rabbit	1/2,000	Memfa
Prdm8	Provided by Pr. Yoichi Shinkai	045R2	Rabbit	1/200	PFA
S100-b	Sigma	s2657	Mouse	1/500	PFA or

					Memfa
Sox1	Santa Cruz	sc-17318	Goat	1/400	PFA or Memfa
Syto Dye	Invitrogen	S11356 45 Blue Fluorescent	N/A	1/100	PFA or Memfa

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).

Table 2. Secondary Antibodies used.

Reactivity	Host	Code	Excitation Wavelength (nm)	Emission Wavelength (nm)
Goat	Donkey	A-11055	488	519
	Donkey	A-21432	555	565
	Rabbit	A-21086	632	647
Guinea Pig	Goat	A-21435	555	565
Mouse	Donkey	A-21202	488	519
	Donkey	A-31570	555	565
Rabbit	Donkey	A-21206	488	519
	Donkey	A-31572	555	565
	Goat	A-21070	632	647

Rat	Donkey	A-21208	488	519
	Goat	A-21434	555	565

4. 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.

Reagents used:

Reagent	Recipe
0.1M Sodium Phosphate Buffer, pH 7.2	0.5M Na_2HPO4 0.5M NaH_2PO_4
Fixation solution	0.2% Glutaraldehyde 2% Formaldehyde 5mM EGTA, pH 7.3 2mM $MgCl_2$ Dissolve in 0.1M Sodium Phosphate buffer
Rinse solution	2mM $MgCl_2$ 0.1% Sodium Deoxycholate 0.2% NP 40 Dissolve in 0.1 M Sodium Phosphate buffer
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.

5. ANIMAL HANDLING

5.1 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.

5.2 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.

5.3 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.

5.4 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. .

5.5 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.

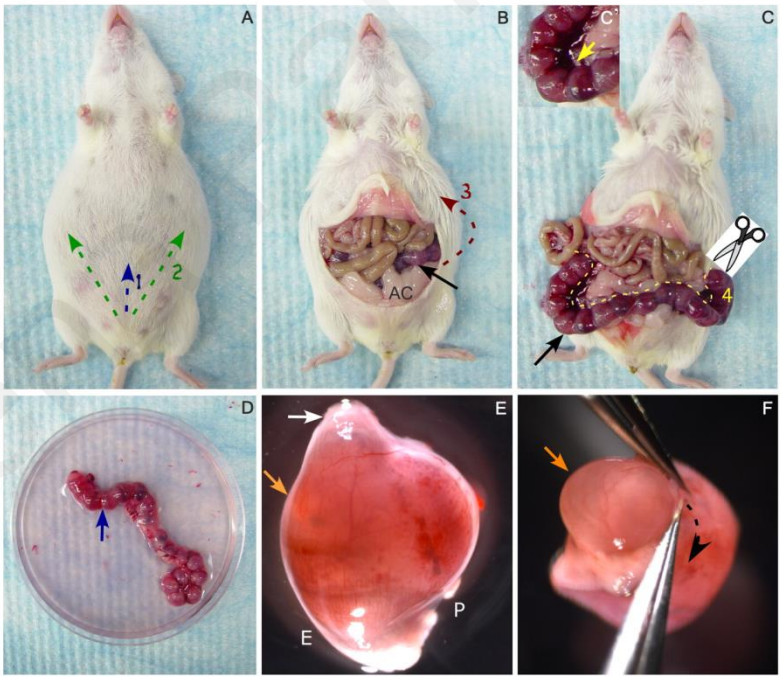


Figure 45: Embryo harvesting from pregnant mice. A-D Location and cuts on the abdominal cavity and uterine horn removal. E&F Embyos removed from the embryonal sac to release the embryos.
(http://cshprotocols.cshlp.org/content/2011/1/pdb.prot5558/F2.expansion.html)

6. LIQUID AND SOLID BACTERIOLOGY MEDIA

Medium	Recipe
LB (Luria-Bertani) Medium[autoclaved]	1 % Bacto-tryptone 0.5% Bacto-yeast extract 0.5% NaCI (Sodium Chloride)
LB Agar[autoclaved]	Liquid LB medium plus 1% agar.
SOC (Super Optimal broth with Catabolite repression) Medium[autoclaved]	2% bacto-tryptone 0.5% bacto-yeast extract 10mM NaCl 2mM KCl 10mM $MgCl_2$ Supplemented with 20mM sterile glucose after sterilisation.

6.1 Antibiotic stock solutions and working concentrations

Table 3. Antibiotic Stock Solutions.

Name	Stock Concentration	Working Concentration	
		High Copy Plasmids	Bacs
Ampicillin	50mg/ml in dd H_2O	50µg/ml	50µg/ml
Kanamycin	50mg/ml in dd H_2O	50µg/ml	15µg/ml
Tetracycline	10mg/ml in 75% ethanol	10µg/ml	3µg/ml
Chloramphenicol	30mg/ml in ethanol	50µg/ml	15µg/ml
Streptomycin	50mg/ml in dd H_2O	50µg/ml	-

7. GROWTH OF BACTERIAL CULTURES, STORAGE MEDIA

7.1 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.

7.2 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.

7.3 Preparation of DH10b electro-competent cells

Reagents used:

Reagent	Recipe
LB (Luria-Bertani) Medium$^{\text{autoclaved}}$	1 % Bacto-tryptone 0.5% Bacto-yeast extract 0.5% NaCI (Sodium Chloride)
10% glycerol in dd H_2O $^{\text{autoclaved}}$	
dd H_2O	

Strain DH10b was grown on LB/streptomycin agar plate overnight at 37^{o}C. 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^{o}C 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^{o}C 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^{o}C. 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^{o}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^{o}C.

7.4 Preparation of DH5α competent cells

Reagents used:

Reagent	Recipe
LB (Luria-Bertani) Medium$^{\text{autoclaved}}$	1 % Bacto-tryptone 0.5% Bacto-yeast extract 0.5% NaCI (Sodium Chloride)
10% Glycerol in 0.1M $CaCl_2$	dilute 50% glycerol[a] in 1M $CaCl_2$
0.1M $CaCl_2$ $^{\text{filter sterilized}}$	

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.

8. DNA EXTRACTION FROM E. COLI

8.1 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.

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

Reagents used:

Reagent	Recipe
P1[filter sterilized]	15 mM Tris, pH 8 10 mM EDTA 100 µg/ml RNase A

	dd H_2O
P2[filter sterilized]	0.2 N NaOH 1% SDS dd H_2O
P3[autoclaved]	3 M KOAc, pH 5.5 dd H_2O
TE[filter sterilized]	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.

9. DNA CLONING

9.1 Ligation of DNA

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

9.2 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.

9.3 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.

10. TRANSFORMATION OF COMPETENT E. COLI CELLS

10.1 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.

10.2 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°C (except the pSC101 containing plasmid that were incubated at 30°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°C (or 30°C for the pSC101 containing cells) overnight.

10.3 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.

11. IN SITU HYBRIDISATION

11.1 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. 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.

11.2 Hybridisation of DIG-labelled RNA probes to tissue mRNA

Reagents used:

Reagent	Recipe
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.
4% PFA in 0.1 M RNase free PB$^{\text{filter sterilized}}$	
RNase free Phosphate Buffered Saline (PBS)	0.1 M PB 125 mM NaCl Disolve in DEPC-treated ddH_2O.
RNase free 20% Sucrose$^{\text{filter sterilized}}$	20g Sucrose 50ml RNase free 0.2 M PB Bring the volume up to 100ml with DEPC-treated ddH_2O.
10X "Salts"$^{\text{autoclaved}}$	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.
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

Washing solution	1X SSC 50% Formamide 0.1% Tween-20
5X MABT	0.5 M Maleic Acid 0.75 M NaCl 0.5% Tween-20 pH to 7.5 with NaOH
Blocking solution	2% (w/v) Blocking reagent (Roche) 1.25X MABT 10% (v/v) Heat inactivated Sheep Serum
Alkaline-phosphatase staining buffer (without NBT/BCIP)	100 mM NaCl 50 mM $MgCl_2$ 100 mM Tris.HCl pH 9.5 0.1% Tween-20
Nitroblue tetrazolium salt (NBT - Roche) 100 mg/ml dissolved in 70% Dimethylformamide	
5-bromo-4-chloro-3-indolyl-phosphate (BCIP - Roche) 50 mg/ml dissolved in Dimethylformamide.	
10% (w/v) Polyvinyl alcohol (PVA)	
Staining Buffer with NBT/BCIP	50% Staining buffer 25 mM $MgCl_2$ 0.1 mg/ml BCIP 0.1 mg/ml NBT 5% PVA
Fluka DPX mounting medium	

Mouse embryos were fixed overnight in 4% PFA (0.1 M PB), rinsed in 0.1 M PBS, cryo-protected in 20% Sucrose (o.1 M PB) overnight and embedded in Tissue-Tek® OCT Compound and stored in -80^{o}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 hybridization buffer and warmed at 70^{o}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^{o}C overnight.

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

12. CHICK IN OVO ELECTROPORATION

Fertilized 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°C and they were harvested in PBS and processed for in situ hybridization and immunohistochemistry.

13. NEUROSPHERES

The neurospheres were set up using the protocol published by *Protocol Exchange* (2006). Neural Stem Cell Culture: Neurosphere generation, microscopical analysis and cryopreservation. Pacey et al., 2006

The feeding mixture was prepared as follows from the stock solutions:

	20mL	6mL	4mL	2mL
DMEM.F12	19,200	5790	3864	1932
N2	0.200	0.060	0.040	0.020
Pen/Strep	0.200	0.060	0.040	0.020
Insulin	0.040	0.012	0.008	0.004
Glucose	0.240	0.046	0.024	0.012
FGF	0.040	0.012	0.008	0.004
EGF	0.040	0.012	0.008	0.004
Heparin	0.040	0.012	0.008	0.004

14. BrdU DETECTION

Reagents used:

Reagent	Recipe
Brdu STOCK (50µg/Kg body weight)	7.5mg BrdU 500µl 0.9 NaCl (sterile Saline) 3.5µl 1M NaOH Vortex to mix. Filter to sterile. *Inject 100µl per 30g body weight*

Pregnant mice are injected with Brdu (50µg/Kg body weight). The mice need to be injected accordingly every 3-4 hours at the required stage. The mice will subsequently be euthanized at the appropriate time.

The embryos will be dissected, fixed and cryoprotected as previously described. However, before incubating the slides with the primary anti-BrdU antibody, the slides need to be treated with 2M HCl for 1 hour at 37°C. Afterwards they need to be thoroughly washed with HBSS (neutralise) before proceeding with the aforementioned immunohistochemistry protocol.

APPENDIX II
WORK NOT INCLUDED IN THESIS

A few independent projects were also undertaken during the process of this thesis. Even though these are not described here they do relate to this work in the scope that the methods used are similar to the ones described here. These projects have contributed in the deeper understanding of the techniques, as well as provide an enhanced appreciation of the field.

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Generation of an ABCG2$^{GFPn\text{-}puro}$ transgenic line – A tool to study ABCG2 expression in mice

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ABSTRACT

The ATP-binding cassette (ABC) transporter 2 (ABCG2) is expressed by stem cells in many organs and in stem cells of solid tumors. These cells are isolated based on the side population (SP) phenotype, a Hoechst 3342 dye efflux property believed to be conferred by ABCG2. Because of the limitations of this approach we generated transgenic mice that express Nuclear GFP (GFPn) coupled to the Puromycin-resistance gene, under the control of ABCG2 promoter/enhancer sequences. We show that ABCG2 is expressed in neural progenitors of the developing forebrain and spinal cord and in embryonic and adult endothelial cells of the brain. Using the neurosphere assay, we isolated tripotent ABCG2-expressing neural stem cells from embryonic mouse brain. This transgenic line is a powerful tool for studying the expression of ABCG2 in many tissues and for performing functional studies in different experimental settings.

Introduction

Reporter mouse strains expressing the Green Fluorescent Protein (GFP) in a manner that faithfully recapitulates the expression of a gene of interest dramatically enhance our ability to study gene function. Global efforts are now underway to develop such lines on a very large scale (e.g. http://www.gensat.org/index.html; http://www.mmrrc.org/).

The ABCG2 protein is regarded as a universal stem cell marker in many tissues [1]. Its expression and function have been directly correlated with the SP phenotype, a Hoechst 3342 dye efflux property shared by many stem cells [2]. This phenotype is also shared by stem cells residing in solid tumors and ABCG2 is believed to protect these tumor-initiating cells from cytotoxic drugs [3,4]. ABCG2 is also expressed in endothelial cells of the brain vasculature and is believed to be responsible for mediating the blood–brain-barrier (BBB) [5].

Unlike other tissues, prospective isolation of neural stem cells (NSC) does not rely on the SP phenotype. This is because fresh neural cells displaying the SP phenotype are endothelial cells that do not posses NSC properties [6]. Thus, while ABCG2 is regarded as a universal stem cell marker in many tissues it is not possible to use the SP analysis to assess if ABCG2-expressing neural progenitors behave as NSC *in vitro*. This is also compounded by the low level of ABCG2 mRNA in neural progenitors [7].

In order to directly visualize and isolate ABCG2-expressing cells we developed transgenic mice that express a nuclear form of GFP (GFPn) coupled to the Puromycin (Puro) resistant gene, under the control of ABCG2 promote/enhancer sequences. Using these mice we demonstrate that ABCG2, in addition to being expressed in several tissues, is also expressed in tripotent NSC of the developing Central Nervous System (CNS) and in endothelial cells of the brain vasculature. This line is thus an invaluable tool for isolating and manipulating ABCG2-expressing cells without using cytotoxic reagents.

Materials and methods

Generation of ABCG2$^{GFPn\text{-}puro}$ and ABCG2^{d2EGFPuro} alleles. The nuclear GFP (GFPn) was generated by fusing a commercially available Nuclear Localisation Signal (NLS; Invitorgen) to the 3′ of Ires-eGFP ORF to generate Ires-GFPn. This was cloned upstream of an Ires-Puromycin cassette (Invitrogen) to generate Ires-GFPn-Ires-Puro. We used Bacterial Artificial Chromosome (BAC) recombination [8] to insert this cassette in the 3′UTR of clone RP23-327P13 (210 Kb encompassing the ABCG2 gene). Homology sequences were added to the cassette using a PCR-based strategy with long oligonucleotide overhangs. After recombination clone integrity was verified by DNA fingerprinting and pronuclear injection was carried out using standard procedures. Three lines were generated that expressed ABCG2$^{GFPn\text{-}puro}$ in the same manner in all tissues

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

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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 motorneurons 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

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SOX1 links the function of neural patterning and Notch signalling in the ventral spinal cord during the neuron-glial fate switch

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ABSTRACT

During neural development the transition from neurogenesis to gliogenesis, known as the neuron-glial (N/G) fate switch, requires the coordinated function of patterning factors, pro-glial factors and Notch signalling. How this process is coordinated in the embryonic spinal cord is poorly understood. Here, we demonstrate that during the N/G fate switch in the ventral spinal cord (vSC) SOX1 links the function of neural patterning and Notch signalling. We show that, SOX1 expression in the vSC is regulated by PAX6, NKX2.2 and Notch signalling in a domain-specific manner. We further show that SOX1 regulates the expression of Hes1 and that loss of Sox1 leads to enhanced production of oligodendrocyte precursors from the pMN. Finally, we show that Notch signalling functions upstream of SOX1 during this fate switch and is independently required for the acquisition of the glial fate *per se* by regulating Nuclear Factor I A expression in a PAX6/SOX1/HES1/HES5-independent manner. These data integrate functional roles of neural patterning factors, Notch signalling and SOX1 during gliogenesis.

Introduction

During embryonic development spinal cord (SC) neuro-epithelial progenitors (NEPs) are located in the ventricular zone (VZ). The VZ of the vSC is organised into five progenitor domains known as p3-pMN-p2-p1-p0. NEPs in each domain express different sets of homedomain (HD) transcription factors [1] and first produce neurons and then switch to producing glial cells. This N/G fate switch starts around embryonic day 12 (e12) and progresses in a ventral-to-dorsal direction [2]. At this stage NEPs transform to radial glial (RG) cells [3] and begin to express several Sox genes [4,5] and pro-astrocytic factors [6] while the expression of the Notch effectors HES1 and HES5 changes dynamically [7,8]. How the expression and function of this new set of RG-specific genes is regulated is not well understood.

One domain where glia specification has been extensively studied is the pMN progenitor domain. This domain first produces motor neurons (MNs) and then switches to generating oligodendrocyte precursors (OLPs) together and some astrocyte precursors (ASPs) [9–12]. It is the major source of spinal Oligodendrocytes (OL) but the interplay between different factors expressed in the pMN during OL specification is not well understood. The HD factor PAX6 has been proposed to block OL specification from the pMN [7] and is selectively expressed only in a subset of ASPs [13,14]. However, PAX6 target genes that potentially mediate this function are not known.

Another mechanism that affects glial cell specification is Notch signalling [15]. In the forebrain Notch signalling is sufficient to induce the NEP to RG transformation [16]. In the spinal cord reduced Notch signalling leads to enhanced production of pMN-derived OLPs while ASPs specification is severely reduced [17]. However, the molecular basis of this phenotype has not been convincingly resolved [4].

Here we have studied the function and genetic regulation of the high mobility group (HMG) transcription factor SOX1. We show that SOX1 links the function of neural patterning and Notch signalling specifically during gliogenesis. We further show that Notch signalling is genetically required to initiate the N/G fate switch *per se* independent of its role to regulate progenitor identities or Sox1 expression.

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Development/Plasticity/Repair

Sox1 Is Required for the Specification of a Novel p2-Derived Interneuron Subtype in the Mouse Ventral Spinal Cord

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During mouse development, the ventral spinal cord becomes organized into five progenitor domains that express different combinations of transcription factors and generate different subsets of neurons and glia. One of these domains, known as the p2 domain, generates two subtypes of interneurons, V2a and V2b. Here we have used genetic fate mapping and loss-of-function analysis to show that the transcription factor Sox1 is expressed in, and is required for, a third type of p2-derived interneuron, which we named V2c. These are close relatives of V2b interneurons, and, in the absence of Sox1, they switch to the V2b fate. In addition, we show that late-born V2a and V2b interneurons are heterogeneous, and subsets of these cells express the transcription factor Pax6. Our data demonstrate that interneuron diversification in the p2 domain is more complex than previously thought and directly implicate Sox1 in this process.

Introduction

The ventral spinal cord (vSC) in mice is organized into five progenitor domains (ventral-p3-pMN-p2-p1-p0-dorsal), specified through the graded activity of sonic hedgehog released from the notochord and floor plate (Ericson et al., 1997a). Progenitor cells in each domain express different sets of homeodomain and basic helix–loop–helix transcription factors, which are primarily responsible for specifying the neuronal and glial lineages characteristic of that domain (Briscoe et al., 2000; Muhr et al., 2001; Hochstim et al., 2008). For example, the pMN domain generates different types of motor neurons (MNs), whereas the p2 domain generates at least two different types of interneurons (INs), named V2a and V2b. Neuronal diversity in the pMN domain is achieved through the action of extrinsic and intrinsic factors that influence the fates of postmitotic progenitors (Dalla Torre di Sanguinetto et al., 2008). Similarly, neuronal subtype specification in the p2 domain is determined in postmitotic progenitors through the action of Notch/Delta signaling in both mice (Del Barrio et al., 2007; Peng et al., 2007) and zebrafish (Batista et al., 2008; Kimura et al., 2008). Thus, postmitotic Notch $^{-high}$ progenitors give rise to V2b INs, whereas Notch $^{-low}$ progenitors acquire a V2a fate. Accordingly, attenuation of Notch signaling leads to overproduction of V2a INs at the expense of V2b INs (Yang et al., 2006; Del Barrio et al., 2007; Peng et al., 2007; Batista et al., 2008). Downstream of Notch, the action of the Lim-only protein (Lmo4) either favors or inhibits transcriptional complexes in subsets of postmitotic progenitors and consolidates the V2a and V2b IN fates (Joshi et al., 2009).

Sox1 is a transcription factor belonging to Group B of the Sox gene family. It is expressed by most neural progenitors in the embryonic spinal cord (Pevny et al., 1998; Wood and Episkopou, 1999; Genethliou et al., 2009) and forebrain in which it is required for correct differentiation of postmitotic GABAergic neurons that contribute to the ventral striatum (Malas et al., 2003; Ekonomou et al., 2005).

In this study, we investigated the expression and function of Sox1 in a new group of INs that are generated in the vSC. We show that these cells derive from the p2 domain but are distinct from V2a and V2b INs. We named these cells V2c INs and show that they are lineally related to V2b INs. In the absence of Sox1, V2c INs become reprogrammed toward the V2b cell fate, suggesting that SOX1 is necessary for regulating the V2b versus V2c fate choice. Finally, we show that the transcription factor Pax6 is expressed in subsets of late-born V2a and V2b INs and possibly in a fourth type of p2-derived INs. These data reveal that the p2 domain generates more IN subtypes than originally appreciated and that Sox1 has an essential role in this diversification.

Materials and Methods

Transgenic mice. We used the following established mouse lines: Sox1 ^{KO} (Aubert et al., 2003), SOX1 $^{\beta\text{-geo}/+}$ (Malas et al., 2003), Pax6 (*Sey* allele) (Hill et al., 1991), Rosa26 ^{stopYFP} (Srinivas et al., 2001), and Foxn4 ^{KO} (Li et al., 2004). We generated two additional lines, Foxn4 $^{-iCre}$ and GATA3 $^{-eGFP}$, using bacterial artificial chromosome (BAC) recombination as described previously (Zhang et al., 2000). The BACs used to generate Foxn4 $^{-iCre}$ and GATA3 $^{-eGFP}$ mouse lines were 150 and 180 kb long, respectively. To generate the BACs, we first made a purpose-built cassette containing

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STEM CELLS
TISSUE-SPECIFIC STEM CELLS

Sox1 Maintains the Undifferentiated State of Cortical Neural Progenitor Cells via the Suppression of Prox1-Mediated Cell Cycle Exit and Neurogenesis

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Key Words. *Sox* genes • Neural stem cells • Neurogenesis • Cell division • Cortex

ABSTRACT

Neural stem/progenitor cells maintain their identity via continuous self-renewal and suppression of differentiation. Gain-of-function experiments in the chick revealed an involvement for Sox1-3 transcription factors in the maintenance of the undifferentiated neural progenitor (NP) identity. However, the mechanism(s) employed by each factor has not been resolved. Here, we derived cortical neural/stem progenitor cells from wild-type and *Sox1*-null mouse embryos and found that Sox1 plays a key role in the suppression of neurogenic cell divisions. Loss of Sox1 leads to progressive depletion of self-renewing cells, elongation of the cell cycle of proliferating cells, and significant increase in the number of cells exiting the cell cycle. In proliferating NP cells, Sox1 acts via a prospero-related homeobox 1 (Prox1)-mediated pathway to block cell cycle exit that leads to neuronal differentiation in vivo and in vitro. Thus, our results demonstrate that Sox1 regulates the size of the cortical NP pool via suppression of Prox1-mediated neurogenic cell divisions. STEM CELLS *2011;29:89–98*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Neuroepithelial (NE) cells reside in the ventricular zone (VZ) of the developing brain and spinal cord and give rise to all cell lineages of the mature nervous system in response to extrinsic and intrinsic determinants [1]. During cortical neurogenesis, NE cells transform to a related cell type, the radial glial (RG) cells, which in vivo are restricted to the generation of neurons at this stage (reviewed by ref [2]) in response to Notch signaling [3]. Uncommitted RG cells express Notch receptors and upregulate the expression of Notch effectors, such as Hes1 and Hes5, after activation by Notch ligands, which become upregulated in the cell membrane of adjacent progenitor cells, committed to the neuronal lineage. Commitment of neuronal progenitors is manifested by the upregulation of the proneural basic-helix-loop-helix (bHLH) genes, *Neurogenins 1-3 (Ngns)* and *Mash1*, which are sufficient to initiate a cell autonomous program of neuronal differentiation [4]. In response to Ngn function, specified neuronal progenitors switch on another class of bHLH factors, such as *NeuroD* and *Nscl1*, and homeodomain factor, *Prox1* [5, 6], which consolidate neuronal specification and facilitate terminal differentiation [7, 8]. Overexpression of *Prox1* in neuronal progenitors can drive cells out of the cell cycle, but terminal differentiation is not fully achieved as it requires the function of *Ngns* or *Mash1* [9].

Neuronal commitment is also regulated by factors belonging to SoxB group of transcription factors [10], divided into SoxB1 (Sox1-3) and SoxB2 subgroups (Sox14 and Sox21) [11, 12]. Based on gain-of-function analysis, SoxB1 factors have been proposed to have a redundant function and block neurogenesis, whereas Sox21 (and Sox14; S.M. unpublished data) can drive NE cells out of the cell cycle and promote neurogenesis [13]. Although both Notch signaling and SoxB1 activities have a similar function and suppress neurogenesis, they appear to do so using distinct mechanisms; Notch signaling mainly represses the expression of proneural genes (*Ngns* and *Mash1*), whereas SoxB1 factors antagonize neurogenesis downstream of proneural factors [14]. As Sox21 counteracts SoxB1 function and promotes neurogenesis downstream of proneural factors [15], it has been proposed that neuronal cell

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