THE ROLE OF PTF1A IN SPINAL CORD DEVELOPMENT

APPROVED BY SUPERVISORY COMMITTEE

Jane E. Johnson, Ph.D., Mentor
Associate Professor, Neuroscience

Keith Parker, M.D., Ph.D., Chair
Professor, Internal Medicine-Endocrinology

Daniel Garry, M.D.
Assistant Professor, Internal Medicine-Cardiology

Raymond MacDonald, Ph.D.
Professor, Molecular Biology

This dissertation is dedicated to my family and friends

who have supported me throughout my life and in loving

memory of Alton F. Wilbur

THE ROLE OF PTF1A IN SPINAL CORD DEVELOPMENT

by

STACEY MARIE GLASGOW

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June 2006

ACKNOWLEDGEMENTS

My deep gratitude goes to my mentor, Jane E. Johnson, for her guidance and support during my graduate school career. In particular, I would like to thank her for giving me a lab "home" when I was in need of a new one. Jane's concern for her students and her approach to science are a model to follow. It has been my honor to learn from her.

I would like to thank my committee members, Dr. Keith Parker, Dr. Daniel Garry, and Dr. Raymond MacDonald for their comments on my dissertation and for the time they have spent discussing and thinking about my project. I would especially like to thank Dr. Raymond MacDonald for his collaboration on this project. His resources, comments, insights, and especially his enthusiasm have been a great benefit and are deeply appreciated.

My thanks and appreciation goes out to the past and present members of the Johnson lab, their presence alone made lab more fun. I would like to particularly thank Mike Henke who has not only been my bay-mate but also my partner in this project. I have greatly valued his comments and opinions regarding the project and enjoyed his friendship. In addition I would like to send a special thanks to Amy Helms. She has been my sounding board and cherished friend. I would also like to thank Carrie Weldon, Judy Dumas, and Trisha Savage for their technical help and friendship.

I would like to acknowledge Ying Liu and Tina Matos for the steadfast friendship. The conversations we have had, regardless of topic, have kept me sane. I have made many friends over my graduate school career and I have appreciated everyone.

I whole heartedly thank my family. I have missed them dreadfully during school, but I have not once doubted their love and support. It has been their constant encouragement that

has given me the courage to forge on when I felt like I would rather not. I would like to thank my sister, and best friend, for always being there for me and telling me silly things when I needed to hear them.

Finally, I would like to thank God for answering my prayers for strength to continue on in graduate school.

THE ROLE OF PTF1A IN SPINAL CORD DEVELOPMENT

Publication No. _____

Stacey Marie Glasgow, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Supervising Professor: **Jane E. Johnson, Ph.D**.

Mutations in the human and mouse Ptf1a genes result in permanent diabetes mellitus and cerebellar agenesis. We show that PTF1a is present in precursors to GABAergic neurons in both the cerebellum and the spinal cord dorsal horn. A null mutation in *Ptf1a* reveals its requirement for the dorsal horn GABAergic neurons. Specifically, PTF1a is required for the generation of early born (dI4, E10.5) and late born (dIL^A, E12.5) dorsal interneuron populations identified by homeodomain factors Lhx1/5 and Pax2. Furthermore, in the absence of PTF1a, the dI4 dorsal interneurons trans-fate to dI5 ($Lmx1b^+$), and the dIL^A to dIL^B ($Lmx1b^+$;$Tlx3^+$). This mis-specification of neurons results in a complete loss of

inhibitory GABAergic neurons and an increase in the excitatory glutamatergic neurons in the dorsal horn of the spinal cord by E16.5. Thus, PTF1a function is an essential determinant for selecting the GABAergic over the glutamatergic neuronal cell fate in the developing spinal cord, and provides an important genetic link between inhibitory and excitatory interneuron development. Furthermore, Ptf1a appears to exert its functions within the neural tube, at least in part, as a component of a heterotrimeric complex composed of Ptf1a, E-protein, and Rbpsuh. Over-expression assays with Ptf1a and mutant forms of Ptf1a support a model in which the activity of Ptf1a is mediated by a balance between its participation in a trimeric complex, which includes Rbpsuh, and its participation as part of a heterodimer with E-proteins.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Stacey Glasgow, R.M. Henke, Raymond J. MacDonald, Christopher V.E. Wright, Jane E. Johnson. PTF1a Specifies Dorsal Horn GABAergic Over Glutamatergic Cell Fate. (2005) Development.

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF DEFINITIONS

bHLH- basic Helix-Loop-Helix

BMP- Bone Morphogenetic Protein

CNS- Central Nervous System

dI- dorsal interneuron

dIL- dorsal interneuron late

dP- dorsal progenitor

DRG- Dorsal Root Ganglion

DTA- diphtheria toxin

Esp- Enhancer of split

HD- Homeodomain

HES- Hairy Enhancer of split

Kuz- Kuzbanian

M- Mantle

MN- Motor Neuron

NPS- Nail Patella Syndrome

NICD- Notch intracellular domain

PNS- Peripheral Nervous System

PTF1- Pancreatic transcription factor complex

Ptf1a- Pancreatic transcription factor 1a

Shh- Sonic hedgehog

SuH- Suppressor of Hairless

TF- transcription factor

CHAPTER ONE

Introduction

Interaction with the physical environment is a fundamental part of life. Sensory information from the periphery, including pain, temperature, and mechanoreception, is all processed by the central nervous system. These somatosensory inputs are processed in a hierarchal fashion; sensory information is relayed from the periphery to the dorsal spinal cord, then to higher brain centers, and ultimately back to the spinal cord to supply the motor output that yields a desired physical response. These processes are highly coordinated and rely on a diverse neuronal population to execute these functions. The foundation of these functions is the precisely connected neural circuits of the brain and spinal cord.

The mature spinal cord is composed of thousands of neuronal cell types, which are classified by location, morphology, connections, and neurotransmitter phenotype (Kandel et al., 1991). The majority of cells within the grey matter of the spinal cord are motor neurons and interneurons. Sensory neuronal cell bodies themselves sit in the dorsal root ganglia (DRG), while the interneurons serve to integrate and process information. The dorsal spinal cord itself is organized into five laminae, which are comprised of unique sets of neurons (Altman and Bayer, 1984). The marginal zone or lamina I, is primarily comprised of four cell types: pyramidal neurons, flattened aspiny neurons, multipolar neurons, and fusiform spiny neurons. Lamina II-III or substantia gelatinosa, is composed of small short-axoned golgi cells (central cells) and larger stalked and islet cells. Lamina IV-V contain larger pyramidal neurons and medium sized neurons (Altman and Bayer, 2001). The organization of the mature spinal cord is a reflection of the diverse cell types, patterns, and migration trajectories established during neural development. Moreover, generating the precise

connections between spinal interneurons and peripheral sensory neurons is dependent upon the proper specification of these cells early in development.

The specification of neuronal cell-types has been an area of intense scrutiny. The types of cells that will be generated from the neural epithelium are determined both by extrinsic factors and intrinsic factors. Extrinsic factors from the environment are important for setting up the anterior-posterior and dorsal-ventral patterning of the neural tube; while intrinsic factors, such as transcription factors, appear to be responsible for the further specification into specific neuronal populations. Through the use of gain- and loss-of function experiments, significant progress has been made in unraveling the genetic code of transcription factors that determine the fate of a neuron (Briscoe and Ericson, 2001; Caspary and Anderson, 2003; Helms and Johnson, 2003; Jessell, 2000). Understanding the molecular basis of the combinatorial code consisting of homeodomain (HD) and basic Helix-Loop-Helix (bHLH) transcription factors and how this relates to cell identity and ultimately to circuitry formation is a fundamental issue in developmental neuroscience.

General Neural Development

During development a dramatic rearrangement of blastula cells occurs in a process termed gastrulation. As gastrulation proceeds three layers are created: the ectoderm, the mesoderm, and the endoderm. The endoderm will produce digestive structures, the liver, and pancreas; the mesoderm gives rise to the skeletal system, muscular system, and the vascular system; lastly, the ectoderm will produce the epidermis, the peripheral nervous system (PNS) and central nervous system (CNS). Specifically, the nervous system is derived from a flat sheet of cells that receive signals from an underlying mesoderm derived structure, the

notochord (Fig 1.1). In a process called neural induction, signals from the notochord and flanking ectoderm trigger the cells of the dorsal midline to change and begin to bend creating the neural groove. The edges continue to move upward creating the neural fold. The neural folds converge and fuse, forming the neural tube (Fig 1.1). During this process cells are actively migrating from the dorsal fusion points. This population of migratory cells, the neural crest cells, gives rise to most of the craniofacial structures and PNS.

As development proceeds, the rostral portion of the neural tube develops into the hindbrain, midbrain, and forebrain. The caudal neural tube, on the other hand, progresses to form the mature spinal cord. Within the developing spinal cord, cells are categorized as actively proliferating and are situated closer to the midline (ventricular zone-VZ); or cells are differentiated and have settled in the lateral region of the spinal cord (mantle layer). As cells become committed to a certain neuronal lineage they exit the cell cycle, detach from the ventricular surface, and migrate to the mantle layer. During this process the cell expresses specific transcription factors, which are determinants of differentiation. The combination of transcription factors which a cell expresses will set into motion the cellular machinery that will determine the cell's eventual fate (Tanabe and Jessell, 1996).

The neural tube can also be divided into dorsal and ventral halves. Cells that are derived from the ventral half of the neural tube are motor neurons and ventral interneurons (Briscoe and Ericson, 2001; Jessell, 2000). These ventrally derived cells will eventually populate the mature ventral horn where they will be involved in motor outputs. Cells derived from the dorsal half of the developing spinal cord are predominately interneurons that will become the cells of the dorsal horn (Caspary and Anderson, 2003; Helms and Johnson,

2003). The dorsal horn is a vital component of the somatosensory system, where sensory information is processed and relayed to higher brain centers.

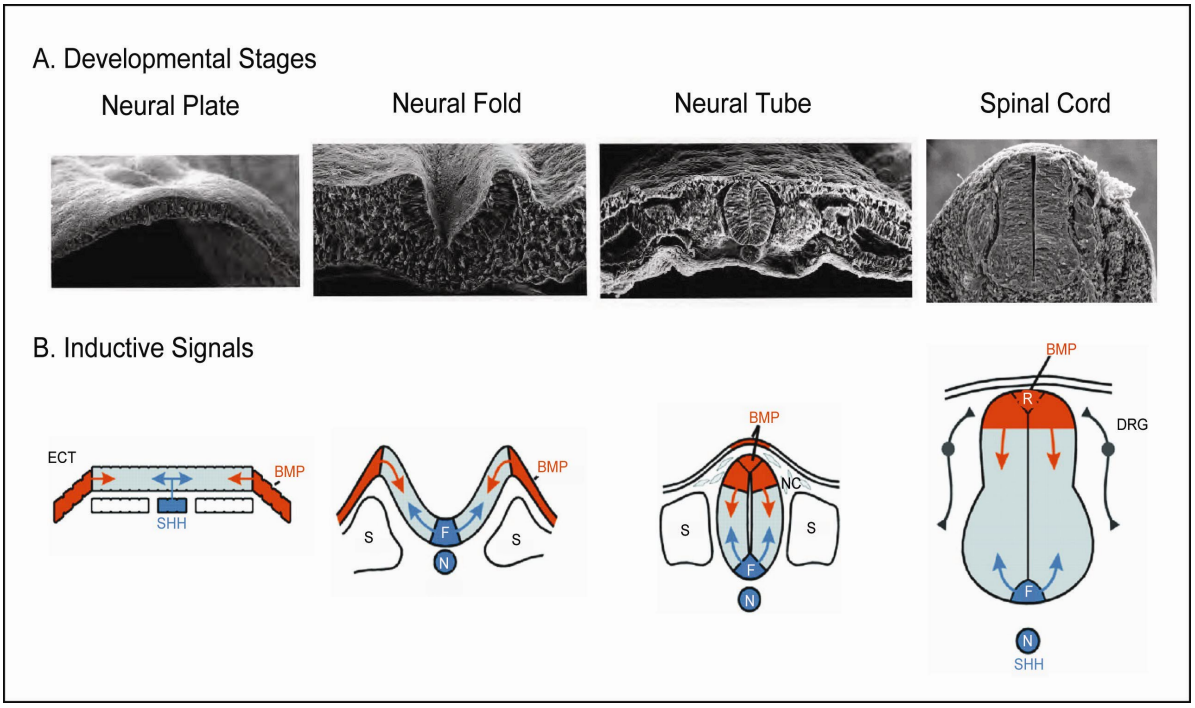


Figure 1.1 Neural Development

(A) Representative scanning EM images of different stages of neural tube formation. The neural plate is located above the notochord and is flanked by the epidermal ectoderm. The neural plate begins to fold forming the neural groove. The folding edges continue to move upward and fuse creating the neural tube. Within the neural tube, cells located at the most dorsal end comprise the roof plate, while at the most extreme ventral end, floor plate cells are found. (B) Two major classes of proteins provide the inductive signals that control differentiation along the dorsal-ventral axis of the developing spinal cord. Bone morphogenetic proteins (BMP) and Sonic hedgehog (Shh) are expressed in the roof plate and floor plate respectively, upon

neural tube closure. The location of expression of these factors is represented at different developmental stages. Adapted from Kandel, 2001 and http://www.med.unc.edu/embryo_image.

Inductive Signaling

The ultimate patterning of the spinal cord and neural circuitry connections depends on the early subdivision of the neural epithelium into discrete domains and the generation of a variety of neuronal cell types. Upon closure of the neural tube two organizing centers – the roof plate and the floor plate, establish distinct regions of the developing spinal cord. These two signaling centers produce the signals that establish a dorsal-ventral pattern to the spinal cord, Bone Morphogenetic Proteins (BMP) and Sonic Hedgehog (Shh) (Fig 1.1)(Briscoe and Ericson, 2001; Jessell, 2000; Lee and Jessell, 1999). Shh is the primary signaling molecule produced by the floor plate; while the roof plate produces Wnt1 and Wnt3a, as well as several members of the TGF-β signaling families, including Bone Morphogenetic Protein (BMP)-4, BMP-5, BMP-7, GDF-6, GDF-7, and Activin-B.

Patterning of the ventral neural tube and the role of Shh, a secreted glycoprotein in this process has been well documented (Jessell, 2000; Wilson and Maden, 2005). Loss of Shh signaling, either by gene targeting or via the use of blocking antibodies, prevents the differentiation of the notochord and floor plate; in addition motor neurons are lost and ventral interneurons are either not generated or are diminished in number (Chiang et al., 1996; Ericson et al., 1996; Marti et al., 1995; Roelink et al., 1995). Furthermore, *in vitro* experiments demonstrate that different ventral interneuron populations require various

amounts of Shh to be induced. Gradually increasing Shh concentrations by two- or three-fold results in the generation of five neuron populations (Ericson et al., 1997a; Ericson et al., 1997b). Thus the position in which a neuron lies in the ventral neural tube is predicted based upon its responsiveness to Shh, so that cells situated more ventrally require higher levels of Shh for induction. (Ericson et al., 1997a).

While data had demonstrated a clear role for Shh in specification, how a gradient of Shh achieved this induction was not understood. Several HD transcription factors, such as Pax7, Pax3, Pax6, Nkx2.2, Dbx1, and Dbx2, are expressed in the ventral neural tube. Consistent with the idea that HD factors are involved in refining expression domains in response to a gradient of Shh activity, mice mutant for Nkx2.2 and Pax3, Pax7, and Pax6 lead to the loss of specific ventral neuron populations (Briscoe et al., 1999; Ericson et al., 1997b; Mansouri and Gruss, 1998). Furthermore, these factors are regulated by Shh signaling (Briscoe et al., 1999; Ericson et al., 1996; Ericson et al., 1997b; Goulding et al., 1993). The HD factors can be classified into two categories: Class I, which are repressed by Shh; and Class II, which are induced by Shh signaling. The Class I and II proteins display selective cross-repressive activities which subdivide the ventral neural tube into discrete expression domains (Briscoe et al., 2000). The combination of graded Shh signal and expression of HD factors defines discrete domains of progenitor populations with a specific transcription factor signature.

Patterning of the dorsal neural tube is not as well understood. However, a similar signaling center and activity gradient appears to be at work in dorsal patterning. Genetic ablation of the roof plate, by expressing diphtheria toxin (DTA) under the control of the Gdf7

promoter, resulted in the loss of dorsal Pax7 expression with the expansion of the ventral Pax6 domain. Importantly, the absence of the roof plate resulted in the loss of dI1-dI3 interneuron populations and an expansion of dI4, while ventral makers were unaffected; demonstrating a clear role for the roof plate in patterning and specifying the neural tube(Lee et al., 2000). Moreover, these effects could be recapitulated by expression of the BMP antagonists Follistatin and Noggin, suggesting that this effect is elicited by BMP signaling (Lee et al., 1998; Liem et al., 1997).

Several BMPs are expressed in the tissue adjacent to the neural plate and in the roof plate of the neural tube (Fig1.1). BMPs are members of the TGF-β signaling superfamily. TGF-β family members have clear roles in patterning and organogenesis. BMPs and Activins, like other TGF-β family members, signal through the type I and type II Serine/Threonine kinase transmembrane receptors (Heldin et al., 1997; Massague, 1998). Ligand induced heterodimer assembly of the receptor triggers a series of phosphorylation events that culminate in the activation of a group of signaling molecules, the Smads. The Smad signal transduction pathway relays a extracellular signal to the nucleus where the Smads themselves act as transcriptional regulators (Attisano and Wrana, 2000; Whitman, 1998).

Attempts to determine if BMP molecules participate in patterning and specification of the neural tube are limited by functional redundancy among the members of the TGF-β super-family and the early embryonic lethality of the certain BMP mutants (Dudley and Robertson, 1997; Winnier et al., 1995; Zhang and Bradley, 1996). However, several mouse mutants have underscored the importance of the roof plate in patterning the neural tube and

demonstrated a role for BMPs in the process. The *dreher* mouse which lacks a roof plate produces a reduced number of dI1 (Lhx2/9+) cells but retains the dI2 (Lim1/2) and dI3 (Iset1/2) populations. These *dreher* mice, which are mutated for the homeodomain transcription factor Lmx1a, differ from the Gdf-7-DTA mice, which lacks dI1-3; this suggests that the *dreher* mice retain some signaling lost in the Gdf-7-DTA roof plate ablated mice (Lee et al., 2000; Manzanares et al., 2000; Millonig et al., 2000). This signaling, most likely BMP signaling, could have derived from the flanking ectoderm which expresses several BMPs. Indeed, recent studies have shown that BMP-4 is necessary for the expression of Lmx1a in the chick neural tube, where Lmx1a functions to repress Cath1 (chick homolog of Math1) expression and impair dI1 formation, thereby defining the roof plate as separate from the dI1 interneuron population (Chizhikov and Millen, 2004; Millen et al., 2004). The neuronal polarity of dI1 neurons of *derher* embryos is disrupted, resulting in aberrant axonal projections which presumably would impede the proper formation of certain neuronal circuits (Millen et al., 2004). Together these studies point to a definite role for the roof plate in patterning, and strongly imply a role for BMP signaling in the process.

Gdf-7 mutant mice fail to form dI1 interneurons or to express appropriate levels of Math1 (Lee et al., 1998). Addition of Gdf-7 or BMP7 to neural tube explants restores the ability to generate Lhx2/9 (dI1) and Math1 positive cells. Furthermore, Gdf-7 and BMP7, are sufficient to induce the Lhx2/9 and Math1 expression in wildtype explants and this activity could be blocked by the addition of BMP inhibitors (Lee et al., 1998). These results demonstrate the importance of BMPs in the generation of dorsal interneuron populations. Moreover, in undifferentiated chick neural tube explants, the addition of BMP-4, -5, and

BMP-7 induced dI1 interneurons. As in the mouse, addition of BMP inhibitors attenuated this activity (Liem et al., 1997). Addition of Activin A to explants increased the number of dI1 cells. More importantly, the addition of high levels of Activin A to explants resulted in decreased dI1 neurons, while dI3 neurons increased. This result implies that differences in the concentration of BMPs leads to different cell fates (Liem et al., 1997).

Loss of function experiments, while providing evidence that BMP signaling was important in the dorsal neural tube, were unable to establish a precise role for BMP signaling within the developing spinal cord. Timmer, et al. approached this issue by over-expressing constitutively active BMP receptors in the chick neural tube (Timmer et al., 2002). Utilizing this approach it becomes apparent that BMP signaling is important for generating discrete boundaries of expression, not only on early patterning genes like Pax6 and Pax7 but also in proneural genes like Cath1 and Cash1, and that this activity is dependent upon different concentrations of BMP. For instance, high levels of BMP signaling induced the expression of Cath1 and repressed Cash1, while completely abolishing expression of cNgn1 in the dorsal neural tube. Consequently, development of the post-mitotic neurons dI1 was induced while dI2-dI3 neurons were repressed. In contrast, low levels of BMP signaling induced cNgn1 and increased the dI3 marker Isl1 (Timmer et al., 2002). Thus, the counterpart to the concentration dependence on Shh in the ventral neural tube is the difference in the levels of BMP signaling acting to pattern the dorsal neural tube.

The Msx family of transcription factors has been reported to be downstream effectors of BMP signaling in neural tissue. The expression of the three mouse Msx factors (Msx1-3) in regions of BMP signaling is suggestive of their roles as mediators of BMP signaling

(Furuta et al., 1997; Liem et al., 1995; Shimeld et al., 1996; Timmer et al., 2002). Also, BMP signaling induces the expression of Msx in various CNS regions (Davidson, 1995). Similar to genetic studies for BMPs in mouse, Msx knockout studies have not uncovered a role for Msx factors in spinal cord development as there appears to be some functional redundancy among the family members. Gain-of-function experiments reveal differential temporal activities for BMP signaling and Msx factor functions (Liu et al., 2004). Early (HH10-12) forced expression of a constitutively active form of BMP receptor 1a or 1b (caBMPr) in the chick neural tube represses neuronal differentiation, increases neural crest cells and roof plate markers, while inducing apoptosis. Late expression of caBMPr (HH14-16) did not promote the neural crest or roof plate fates, instead dI1 and dI3 interneurons were formed in increased numbers (Liu et al., 2004). Electroporation of Msx1 mimicked the effects of early caBMPr over-expression in the chick; while the induction of dI1 and dI3 interneurons observed with late caBMPr expression was phenocopied by introduction of Msx3 (Liu et al., 2004). Therefore, Msx1 and 3 have distinct functions in the developing neural tube and are likely downstream effectors of BMP signaling.

Together BMP and Shh work in opposing concentration gradients to subdivide the neural tube into distinct expression domains (dorsal, medial, and ventral) with different developmental potentials. These potentials are further defined by the expression of bHLH transcription factors, dorsally, and homeodomain transcription factors ventrally. Consequently, these factors serve to restrict the fate of cells in the initially homogenous progenitor zone of the early neural tube. Further signals are required, whether instructive or

permissive, to further compartmentalize the neural tube and to determine the eventual fate of a cell.

Notch/Delta Signaling

A central question in developmental biology is how extrinsic and intrinsic signals are coordinated and executed to specify cell fates in developing embryos. In neural development two specific questions arise: 1) how is neural diversity achieved? and 2) what factors lead to cell fate determination? As mentioned above, a transcription factor signature determines the fate of a particular cell, and the role of bHLHs and HD factors in this process is well established. Yet, in several neural contexts different cell types are generated from the same region, but their births are separated temporally. For example, in the ventral neural tube motor neurons are born early, while glia are generated at later stages. It is thought that this is accomplished for the most part, by lateral inhibition. Lateral inhibition is a process in which a dividing cell must decide whether to differentiate into a post-mitotic neuron or remain in a progenitor state. The process of lateral inhibition has most extensively been studied in the nervous system, particularly during *Drosophila melanogaster* development. However components of the lateral inhibition pathway are conserved among species including mouse, chick, *Xenopus*, and zebrafish . This conservation argues for a similar lateral inhibition process across species and places importance on Notch signaling in this process (Austin et al., 1995; Chitnis and Kintner, 1996; Chitnis, 1999; de la Pompa et al., 1997; Haddon et al., 1998; Henrique et al., 1997).

In *Drosophila*, cells are progressively determined by a hierarchy of regulators. This type of determination allows for the progressive restriction of developmental potentials

within a certain field of cells. First, localized factors must set up a cellular field in which cells are equally competent to adopt a certain cell fate, called an equivalence group (Greenwald and Rubin, 1992). The expression of localized factors will further restrict the fate of a cell. For instance the selection of a neuron from a group of uniform ectodermal cells is initiated by the recruitment of a small cluster of cells that have neuronal potential. This cluster is called the proneural cluster (Fig 1.2). Within this cluster neuronal fate is determined by cell-cell interactions between neighboring cells (Greenwald and Rubin, 1992; Simpson, 1990). At the center of this process are the receptor Notch and its ligands Delta, Jagged, and Serrate (Artavanis-Tsakonas et al., 1999).

Initially cells in the equivalence group express comparable amounts of Notch and Delta. A slight imbalance between neighboring cells occurs, where one cell expresses more Delta. This increased Delta then activates Notch more strongly in its neighboring cell. Notch activation in this cell inhibits the production of ligand, so that a cell that produces more ligand is forcing its neighbor to produce less. Consequently, the cell will receive less inhibitory signal from the neighbor cell and produce more ligand. In this manner minor imbalances are amplified and result in divergent cell fate decisions (Fig 1.2). Thus, a cell that expresses more Delta will differentiate into a neuron, while the cell that expresses more Notch will remain in a progenitor state (Baker, 2000; Lewis, 1998; Portin, 2002).

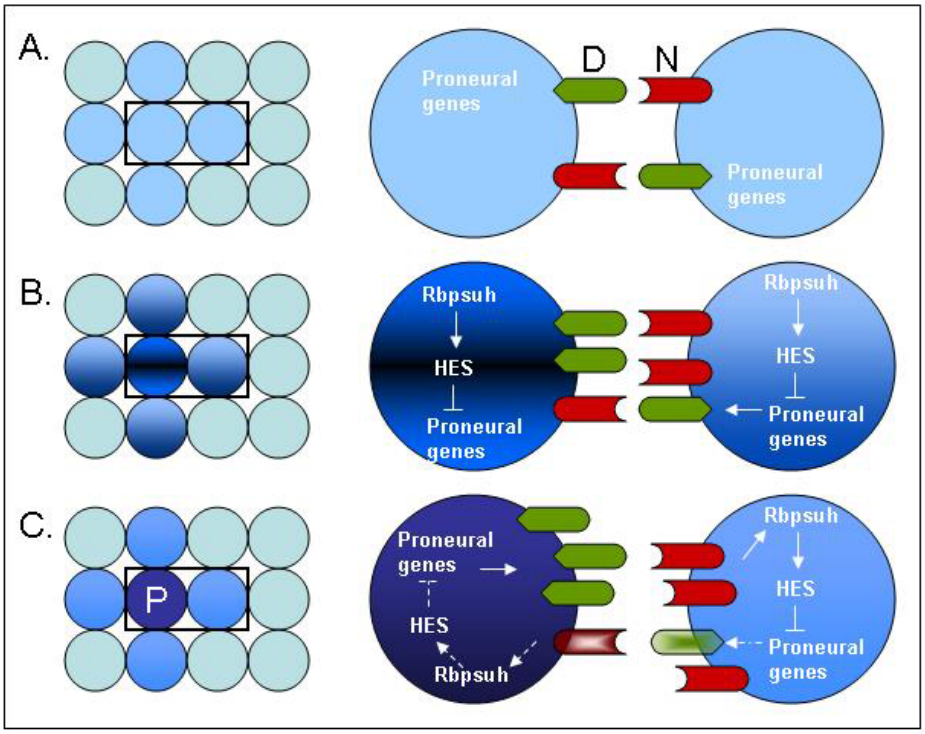


Figure 1.2 Notch signaling in the neural cell identity.

(A) Initially Notch signaling between cells in a group is equivalent. Each cell in the proneural region has an equal capacity to differentiate. (B) A cell within the equivalence group begins to express more Delta (Green) than its neighboring cells (dark blue cell verses light blue cell within boxed region). Delta signals to the neighboring cell activating Notch (Red) in that cell. (C) The slight imbalance in Notch signaling is quickly amplified. The transcription factor Rbpsuh is activated by Notch signaling, which in turn increases HES transcription factor levels. HES inhibits the transcription of proneural genes such as achaete-scute in the neighboring cell (light blue); while in the initial signaling cell (dark blue) the opposite occurs, less Notch signaling results in the de-repression of the proneural genes, represented in the panels to the right. Consequently, the cell that expresses more ligand (Delta in this case) differentiates, while the cell that expresses more Notch remains in the undifferentiated state. Notch (N); Delta (D); neuronal progenitor cell (P). Adapted from Kandel, 2000.

Notch was first identified as a result of a haploinsufficiency which led to notches at the wing margins in *Drosophila* (Moohr, 1919). Loss-of-function studies in *Drosophila* later demonstrated the neurogenic phenotype of Notch mutants; loss of Notch resulted in the switch from epidermis fate to neural fate (Poulson, 1937). Notch is a single-pass transmembrane protein with epidermal growth factor repeats in the extracellular domain and an intracellular domain comprised of ankyrin repeats (Wharton et al., 1985a). In addition the intracellular domain contains a PEST domain and a glutamine-rich region (Wharton et al., 1985b).

The Notch signaling pathway is initiated when the ligand interacts with the extracellular surface of the Notch receptor. This leads to cleavage of the intracellular domain of Notch by a protein complex that contains Presenilin; translocation to the nucleus also requires Presenilin (Struhl and Greenwald, 1999; Struhl and Greenwald, 2001). In addition, a metalloprotease, Kuzbanian (Kuz) is also required for the processing of the NICD (Pan and Rubin, 1997). NICD does not contain a DNA binding domain, but does have a transcription activation domain. Consequently, NICD interacts with other factors to affect transcription. Once in the nucleus NICD interacts with Rbpsuh (also called RBPJκ, CBF1, CSL- in vertebrates; Su(H)- in *Drosophila*) from the Rbpsuh binding site GTGGGAA(Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000; Mumm et al., 2000). Rbpsuh unbound by NICD acts in a repressor complex to inhibit transcription (Kao et al., 1998; Ling et al., 1994). The Rbpsuh-NICD complex, however, activates transcription of the Notch signaling target Hairy and Enhancer of split complex genes, or HES (Enhancer of split [E(spl)] in

Drosophila) (Artavanis-Tsakonas et al., 1999; Greenwald, 1998). The HES family of bHLHs, in turn, repress the expression of proneural genes such as Mash1; thus enabling cells to remain in a progenitor state (Chen et al., 1997; van Doren et al., 1994).

HES proteins can act in two ways to regulate transcription. Active repression by HES proteins is a DNA binding dependent mechanism in which HES proteins form homodimers and bind to a sequence CACNAG called an N-Box (Ohsako et al., 1994; Sasai et al., 1992; van Doren et al., 1994). HES recruits the co-repressor Groucho (TLE in mammals)(Grbavec and Stifani, 1996); Groucho, in turn, recruits a histone deacetylase (HDAC) which is thought to repress transcription via chromatin modification (Chen et al., 1999). The second mechanism is passive, where HES proteins can form non-functional heterodimers with other bHLH factors. For example, HES1 has been shown to form heterodimers with E47 (Sasai et al., 1992). Many bHLH factors, like Mash1 and MyoD form heterodimers with E47; consequently the sequestration of E47 by HES1 leaves these bHLHs unable to execute normal functions. The requirement of HES proteins for proper neuronal differentiation is established by loss-of-function phenotypes. In the absence of HES1 or HES5 premature neuronal differentiation occurs, while HES1/5 double mutants had a more severe neuronal differentiation phenotype (Ishibashi et al., 1995; Ohtsuka et al., 1999; Tomita et al., 1996a). Mutation of the effector of HES expression, Rbpsuh, results in embryonic lethality before E10.5(Oka et al., 1995). Rbpsuh mutant embryos demonstrate an absence of HES5 expression and an increase in Mash1 expression (de la Pompa et al., 1997). Premature neuronal differentiation was seen in Rbpsuh mutant mice. These phenotypes were more severe than those observed in Notch1 mutant embryos (de la Pompa et al., 1997).

It is clear that Notch signaling links the fate of a cell to that of its neighbors and has prominent affects on neuronal differentiation (Artavanis-Tsakonas et al., 1999). While the decision to continue dividing or to begin a differentiation program is fundamental, it does not determine the identity of the cell. The identity of a multipotent progenitor cell within the developing CNS begins with the choice between neuron or glia. Increasing evidence demonstrates that Notch signaling not only functions to inhibit neuronal differentiation, but also plays a critical role in the cell fate decisions between neurons and glia.

Several studies in retina and cortex suggest that Notch signaling is sufficient to direct the glial fate (Chambers et al., 2001; Gaiano et al., 2000; Louvi and Artavanis-Tsakonas, 2006; Morrison et al., 2000; Nieto et al., 2001; Scheer et al., 2001; Yoon and Gaiano, 2005). In the retina, activation of Notch signaling also promotes the glial fate. In zebrafish retina expression of activated Notch receptor not only inhibits neuronal differentiation, keeping a population of cells in a progenitor state, it also promotes a subset of cells to become glia (Scheer et al., 2001). Similar results were obtained with rodent models (Bao and Cepko, 1997; Dorsky et al., 1995). Coincident with these studies, activation of Notch, or its downstream effectors Hes1 and Hes5, in rodents promotes gliogenesis at the expense of neurons (Bao and Cepko, 1997; Furukawa et al., 2000; Hojo et al., 2000). Mouse telencephalic cells infected with NICD (to mimic constitutive Notch1 signaling) acquired radial glia fate. Interestingly, temporal difference in Notch1 activation had divergent effects. During neuronal differentiation NICD infected cells attenuated proliferation. Conversely, during gliogenesis, Notch1 activated cells increased proliferation accompanied by the subsequent generation of more glia (Chambers et al., 2001). These studies and others suggest

that Notch has an instructive role in gliogenesis and points to a role for Notch signaling in the temporal separation of neurogenesis and gliogenesis.

Taken together, the environment in which a cell finds itself is central to its eventual fate. The expression gradients established by BMP and Shh signaling begin to set up differences between cell populations. Cell "state" decisions are mediated through cell-cell interactions, or lateral inhibition mediated by Notch signaling, such that a cell will decide to remain a progenitor or begin to differentiate. Instructive signals like Notch or a specific transcription factor signature then refines these fates and establishes discrete neuronal populations.

Basic Helix-Loop-Helix Transcription Factor Family

The transcription factor combinatorial code which governs the progressive determination of fate includes the Basic Helix-Loop-Helix (bHLH) proteins, which are transcriptional regulators of a variety of developmental processes including cellular differentiation and lineage commitment. The bHLH proteins mediate their effects by protein dimerization and DNA recognition (Murre et al., 1989a). The bHLH transcription factors function by binding to a core hexa-nucleotide E-Box, CANNTG (Ephrussi et al., 1985). The E-box consensus sequences are found in the enhancers of a variety of tissues including muscle, neuronal, and pancreatic tissues (Efstratiadis et al., 1980; Jaynes et al., 1988; Murre et al., 1989b). DNA binding is mediated by the ~60 amino acid basic bHLH motif. The bHLH crystal structure of E47, bound to an E-box, was solved demonstrating that the bHLH region is composed of two alpha-helices separated by a loop. The basic region contacts DNA in the major groove, while the HLH domain is involved in protein dimerization (Ferre-

D'Amare et al., 1993; Voronova and Baltimore, 1990). Furthermore, the stability of the HLH interaction with DNA is re-enforced by van Der Waals interactions with conserved hydrophobic residues (Ellenberger et al., 1994).

The bHLH factors are classified by three criteria: 1) dimerization capabilities; 2) DNA binding specificities; and 3) tissue distribution (Murre et al., 1994). Class I proteins are ubiquitously expressed and are called E-Proteins. These E-Proteins, including E12, E47, HEB, and E2-2, form homodimers and heterodimers with class II proteins (Massari and Murre, 2000; Murre et al., 1989b). Class II is composed of numerous bHLHs including Mash1, Math1, MyoD, and NeuroD, are tissue specific and generally prefer to form heterodimers. Class III contains a leucine zipper motif adjacent to the HLH and include the Myc protein; while Class IV proteins dimerize with Class III proteins or with each other (Mad, Max, Mxi) (Ayer et al., 1993; Blackwood and Eisenman, 1991). Class V factors on the other hand, negatively regulate Class I and Class II proteins. Class V (Ids) proteins lack a basic region and function by forming non-DNA binding heterodimers that act in a dominant negative fashion, in a form of passive repression (Benezra et al., 1990). Class VI proteins are defined by a characteristic proline motif in their basic region and include the HES transcription factors, negative regulators of neurogenesis (Jan and Jan, 1993; van Doren et al., 1994).

The bHLH Transcription factors in Neurogenesis

Class II bHLH factors can be divided into two categories those which are determination factors and those that are differentiation factors (Lee, 1997a). Differentiation factors including NeuroD, Math2, and NSCL-1 and -2 are primarily expressed at later stages

of development than determination factors, mainly in post-mitotic cells (Fig 1.3), and are often expressed in adulthood (Bartholoma and Nave, 1994; Lipkowitz et al., 1992; Schwab et al., 1998). This subclass of bHLH proteins can induce neural specific markers and cell-cycle arrest, suggesting they function to maintain the differentiated state (Farah et al., 2000; Lee, 1997b). Determination factors are expressed earlier in development. In the spinal cord, in particular, bHLH factors are expressed in mostly discrete domains within ventricular zone of the developing neural tube (Fig 1.3) (Gowan et al., 2001; Helms et al., 2005). Expression of these bHLH factors can be detected starting ~E9.5 in mouse embryo. Expression is transient and regulated spatially and temporally. Expression of these factors is generally extinguished as cells exit the cell cycle and differentiate.

The importance of bHLHs in neural development was originally established in *Drosophila*. For example, the Achaete-Scute complex (AS-C) and Atonal (ato) are required for the proper formation of components of *Drosophila* PNS and CNS (Campuzano and Modolell, 1992; Jarman et al., 1993). AS-C endows neuroectodermal cells with the capacity to form neuroblasts, as AS-C mutants fail to form proper neuroblasts and instead become epidermis. Conversely, over-expression of AS-C leads to increased neuronal hyperplasia (Campos-Ortega and Knust, 1990; Jimenez and Campos-Ortega, 1990). Atonal is required for the proper formation of chordotonal organs and eye development, as mutants fail to form these structures and over-expression leads to an increased number of chordotonal organs (Jarman et al., 1994; Jarman et al., 1995). The Neurogenins (Ngn), on the other hand, were first identified in rodents and then in *Xenopus* where over-expression of Ngn1 led to ectopic neurogenesis at the expense of ectodermal cells and activation of X-NeuroD, another neural

bHLH factor (Ma et al., 1996). Moreover, X-ngn-1 is responsive to Notch signaling (Chitnis et al., 1995; Ma et al., 1996).

Mammalian homologs of the Ato and AS-C families have been identified. Math1 and Math5 are the homologs of Ato, Ngn1and Ngn2 are homologs of TAP (target of poxn, also called biparous), while Mash1 is the homolog of AS (Gautier et al., 1997) (Akazawa et al., 1995; Bush et al., 1996; Gradwohl et al., 1996; Guillemot and Joyner, 1993; Johnson et al., 1990). Math1 is expressed in the developing neural tube, brain stem, granule cells of the cerebellum, and rhombic lip of the brain's fourth ventricle, in addition to precursors to inner ear hair cells, and Merkel cells of the skin (Ben-Arie et al., 1997; Ben-Arie et al., 2000; Bermingham et al., 1999; Helms et al., 2000). Expression of Math1 is also seen in the whisker vibrissae and the developing pontine nuclei (Ben-Arie et al., 2000; Helms et al., 2001). Loss-of-function studies have shown Math1 to be essential for the formation of the external granule layer of the cerebellum, the pontine nuclei, and hair cells of the inner ear and dorsal commissural interneurons (Ben-Arie et al., 1997; Bermingham et al., 1999; Gowan et al., 2001).

During development Mash1 is expressed in the neural tube giving rise to specific region of the brain and spinal cord, and in progenitors of autonomic neurons and the olfactory epithelium (Guillemot and Joyner, 1993; Johnson et al., 1990). In the absence of Mash1, progenitors to primary olfactory neurons fail to form, as do autonomic neurons and some enteric neurons (Blaugrund et al., 1996; Cau et al., 1997; Guillemot et al., 1993). In addition, progenitors of the sub-ventricular zone in the telencephalon do not form and cell fail to express HES5 or Delta1 (Casarosa et al., 1999; Hirsch et al., 1998; Horton et al.,

1999). Ngn1 and 2 are expressed in the neural tube and in the dorsal dorsal root ganglia (DRG) (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996). Ngn1 and 2 share many of the same expression domains including the olfactory epithelium, cranial and trigeminal ganglia. Olfactory sensory neurons fail to differentiate in Ngn1 mutants as do proximal cranial sensory ganglia (Cau et al., 2002). Ngn2 mutants fail to form distal cranial sensory ganglia that are derived from epibranchial placodes. Additionally, in the absence of Ngn2 no expression of differentiation factors or Delta-1 is observed (Fode et al., 1998). Ngn1/2 double mutants lack spinal sensory ganglia and some ventral interneurons. Together, evidence points to a vital role for bHLH factors in the formation and proper specification of various neuronal lineages. Yet, the question of how these factors affect differentiation, especially in the developing spinal cord, and how they fit into the transcription factor code are not answered by these experiments.

bHLH Factors in Cell Type Specification of the Dorsal Spinal Cord

The establishment of precise cell numbers and cell types with the spinal cord involves concerted mechanisms that influence neural progenitor pool size and differentiation into specific neuronal cells types. Several lines of evidence, including gain- and loss-of-function studies, have determined roles for bHLH factors, particularly Math1, Mash1, and Ngn1 in neurogenesis and cell-type specification. The ability of bHLH factor to induce neuronal differentiation has been established through gain-of-function studies in several systems including retina, cortex, and spinal cord (Cai et al., 2000; Cepko, 1999; Farah et al., 2000; Jan and Jan, 1993; Kim et al., 1997; Kim et al., 2004; Lee, 1997a; Nakada et al., 2004). Differentiation within the developing spinal cord is defined by several criteria including cell-

cycle exit, translocation out of the ventricular zone into the mantle layer, and expression of neuronal specific markers. Utilizing these definitions in the chick system, Nakada *et al*. identified the specific regions of the Math1 and Mash1 proteins which are responsible for the differentiation and specification properties of these factors (Nakada et al., 2004). Mash1 and Math1 are able to induce neuronal differentiation, while the muscle specific bHLH MyoD, lacked this activity in the chick neural tube. Capitalizing on these activities, chimeric constructs where created in which regions of Math1 or Mash1 HLH were swapped with analagous regions of MyoD. The chimeras revealed that the differentiation activity of Mash1 lies in Helix1 of the HLH motif; furthermore, Helix1 was sufficient to confer differentiation activity to MyoD. Specification activity, on the other hand, was restricted to Helix2 of Math1, and both Helix1 and Helix2 of Mash1 (Nakada et al., 2004). Roles for bHLH factors in specification of neurons have been extensively studied (Ben-Arie et al., 1997; Bermingham et al., 1999; Bermingham et al., 2001; Casarosa et al., 1999; Fode et al., 1998; Fode et al., 2000; Gowan et al., 2001; Guillemot et al., 1993; Parras et al., 2002; Tomita et al., 1996b)(this dissertation). In the spinal cord the expression domains of the bHLH factors and the populations which they specify are tightly linked.

Expression of the neuronal bHLHs is in mostly non-overlapping domains within the developing neural tube. For example, the expression boundaries of Mash1, Math1, and Ngn1 in the neural tube are sharply defined (Fig 1.3) (Gowan et al., 2001), as are the boundaries between Mash1 and Ngn1 in the developing forebrain (Ma et al., 1997). These boundaries extend to the single cell level, so that a cell does not express two of each of these bHLHs. Loss-of-function mutations in an individual bHLH result in disruption of the expression

boundary of the remaining bHLHs. In the absence of Math1, the expression domains of Ngn1 and Ngn2 are expanded dorsally. Similarly, in the absence of Ngn1/2 the boundary of Math1 expression broadens filling in the Ngns domain (Gowan et al., 2001). Together these data point to a model in which bHLHs cross-inhibit each others expression to uphold sharp expression boundaries. Further evidence is provided by over-expression assays, where mis-expression of Ngn1 in chick neural tube led to the suppression of Cath1 and Cash1 (chick homologs); while mis-expression of Math1 resulted in the reduction of Cash1 and cNgn1 (Gowan et al., 2001).

This pattern of non-overlapping expression domains is analogous to that of Homeodomain (HD) factors in the ventral spinal cord where Class I and Class II HD factors appear to rule specification (Briscoe et al., 2000). This progressive patterning by the creation of distinct expression domains is a fundamental theme in cell-type specification. Indeed, in addition to the timing of a neuron's birth, the position along the dorsal-ventral axis from which a cell is derived certainly dictates the path of its fate by virtue of the factors it expresses. Accordingly, the transcription factors that a progenitor cell expresses and the factors it later expresses in the post-mitotic state have been used to define eight distinct interneuron populations in the developing dorsal spinal cord (Gross et al., 2002; Helms and Johnson, 2003; Jessell, 2000; Müller et al., 2002). The eight populations are divided by birth date: six early born (dI1-dI6) interneurons, which are born E10-E11.5, and two late born (dIL^A and dIL^B) interneurons born E11-E13 (Fig 1.3). These populations can be further subdivided by their dependence upon roof plate signals. Class A neurons require roof

plate signaling to form properly, while Class B and dIL$^{A/B}$ neurons form independently of roof plate signals (Lee, 1997a; Müller et al., 2002).

Gene knockout studies have established a requirement for bHLH and HD factors in the proper formation of these specific neuronal populations. *Math1* is required for dI1 neurons (Bermingham et al., 2001) (Gowan et al., 2001). In the absence of Math1 neurons no longer express the dI1 marker Lhx2/9; instead cells now express the dI2 marker Lhx1/5. Embryos mutant for Ngn1 display a decrease in the dI2 markers Brn3a/Lhx1/5 but they are not completely missing. Ngn2, on the other hand, is not strictly required for any neuronal population. However, compound mutants (Ngn1/2) lack dI2 interneurons, suggesting there is some functional redundancy between Ngn1 and Ngn2 (Gowan et al., 2001). Mash1 null embryos lack dI3 and dI5 as evident by the decrease in Islet1 and Lmx1b expression, respectively.

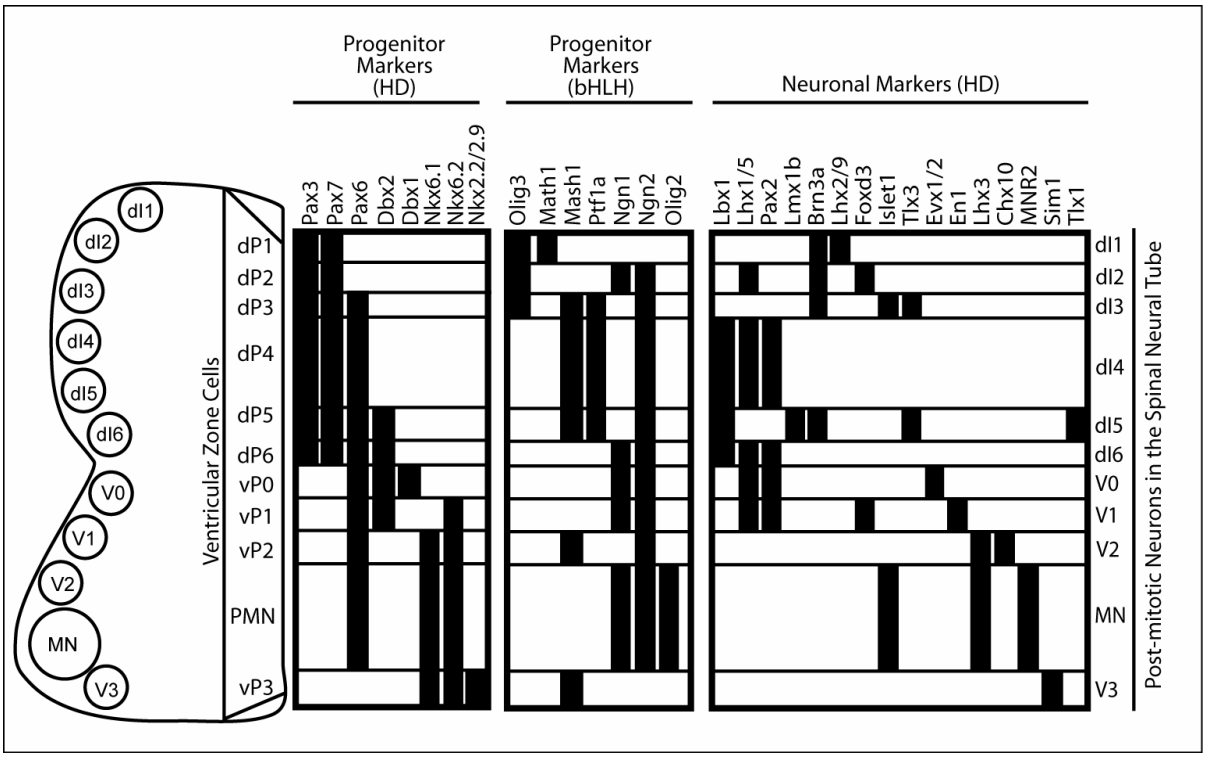


Figure 1.3 Summary of Populations**.**

Shown is a schematic representation of the homeodomain (HD) and bHLH transcription factors expressed in the early developing neural tube. Six early born (dI1-dI6) and five ventral populations (V0-V3 and MN) are derived from their respective ventricular zone progenitor domains shown to the left of the image. The HD and bHLH factor expression domains are represented by solid bars (middle). Similarly, the HD factors expressed in the post-mitotic neurons are displayed to the right. It is a combinatorial transcription factor code that progressively determines the identity of a spinal cord neuron. For, instance the unique transcription factor signature of a dI1 interneuron is: Pax3, Pax7, Olig3, Math1, Brn3a, and Lhx2/9.

Homeodomain and bHLH Transcription Factors in Cell Type Specification

A group of recent papers has greatly contributed to the understanding of the transcription factor combinatorial code, which progressively refines the identity of

differentiating (and maturing) neurons. As a whole, these papers have further refined the definitions of cell-types and established roles for bHLHs and HD factors in specification of both early and late interneurons. Moreover, these studies begin to establish roles for some of these factors as molecular switches and in the formation of neural circuits, where the neurons are not well defined at the molecular level.

Two studies on the HD factor Lbx1 have divided cells into two distinct classes, A and B (Gross et al., 2002; Müller et al., 2002). Class A neurons, dI1-dI3, do not express Lbx1 and are dependent upon roof plate signals (Lee et al., 2000; Müller et al., 2002). Class B neurons, dI4-dI6 are Lbx1 positive. In early stage embryos (E10.5) the absence of Lbx results in an increase in dI2 (Lhx1/5^{+}, Foxd3^{+}) and dI3 (Islet1/2^{+}) neurons; and a loss of the dI4, 5, and 6 marked by Pax2 and Lmx1b. Instead, cells of the neural tube are patterned dI1-dI2-dI3-dI2-dI3-dI4 (Gross et al., 2002; Müller et al., 2002). Thus, Lbx1 is required for the proper specification of dI4-6 interneurons. At later stages Lbx1 is expressed in both dIL^{A} and dIL^{B} interneurons, and is required for the formation of dIL^{B} neurons, as is evident by the loss of Lmx1b (dIL^{B}) expression in the Lbx1 mutant. Loss of Lhx1/5^{+} (dIL^{A}) and Brn3a^{+} in the Lbx1 null embryo indicate that Lbx1 may also be important for the proper regulation of dIL^{A} neurons; however, the loss of Lhx1/5+ cells is observed after the onset of cell death associated with this mutant (Gross et al., 2002; Müller et al., 2002). As in the early neural tube, in the absence of Lbx1 there is a marked increase in the expression of Islet1/2^{+} cells. Interestingly, at E13 cell death occurs and progresses so that by P0, cell numbers, specifically the cells of the substantia gelatinosa, are decreased by 30% (Gross et al., 2002; Müller et al., 2002).

Lbx1 defines a class of neurons, Class B, while the lack of expression delineates Class A neurons. A bHLH factor, Olig3, is expressed in the progenitor domain adjacent to dI1-dI3 (Fig 1.3)(Muller et al., 2005). Olig3 overlaps with Math1, Ngn1, Ngn2, and a subset of the most dorsal Mash1 expressing cells at E10.5. Olig3 mutant embryos lack dI2 and dI3 neurons and dI1 cells are generated at a reduced level. Importantly, Lbx1 expression was increased along with Pax2 and Lhx1/5, markers of dI4 neurons. Compound Olig3 and Lbx1 mutants rescued the decrease in dI2, but the dI3 phenotype was not changed (Muller et al., 2005). These data suggest that Olig3 provides important information for the specification of dI3 interneurons. Furthermore, the authors propose that the main function of Olig3 may be to suppress the generation of Class B neurons by suppressing Lbx1 expression.

The HD factors Gsh1 and Gsh2 have been implicated in the specification of dorsal interneurons (Kriks et al., 2005). Gsh2 is expressed in the progenitors to dI3-dI5, where its expression overlaps with a subset of $Pax7^{+}$ and $Pax3^{+}$ cells at E10.5. Gsh2 loss-of-function mutants lose $Islet1^{+}/Tlx3^{+}$, or dI3 interneurons, while gaining dI2 neurons. No changes in Lbx1, Pax2, or Olig3 were observed. Ngn1 and Ngn2 expression was increased, consistent with the gain of dI2 neurons in the absence of Gsh2. Mash1 was decreased in the most dorsal region of its expression domain (dP3 area). Taken together, Gsh2 is required for the formation of dI3 neurons and the suppression of Ngn1/2. The suppression of Ngn1(thereby dI2 cells) may permit the expression of Mash1 in the most dorsal aspect of its expression domain allowing the formation of dI3 neurons (Kriks et al., 2005).

Indeed, Mash1 has been shown to give rise to dI3 and dI5 populations (Helms et al., 2005; Kriks et al., 2005). In the absence of Mash1, $Islet1/2^{+}$ (dI3) cells are greatly reduced,

and dI4 ($Pax2^{+}/Lim1/2^{+}$) are formed in increased numbers. Mash1 is sufficient to induce dI3 and dI5 neurons when over-expressed in the chick neural tube (Helms et al., 2005; Kriks et al., 2005). Mash1 is expressed in a broad domain within the ventricular zone of the developing neural tube. Cross-inhibitory regulation as demonstrated for Mash1 and Ngn1 is not observed between Mash1 and Ngn2 (Gowan et al., 2001; Helms et al., 2005). Instead Mash1 and Ngn2 are co-expressed in a subset of cells in the dP3-dP5 region. The role of Ngn2 in these cells is to function downstream of Mash1 to modulate the number of cells generated from Mash1 expressing progenitors (Helms et al., 2005).

In addition to its role in early cell-type specification, Mash1 has recently been shown to have an important function in the generation of late born interneuron populations. The progenitor domain from which late interneurons are born is a broad domain and populations that arise are not segregated into discrete regions as seen with the dI1-6; instead cells are intermingled. Mash1 cells expressed within this broad progenitor domain give rise to not only dIL^{A} but also to dIL^{B} neurons (Battiste, Helms, Johnson unpublished). In the absence of Mash1, dIL^{A} cells were dramatically reduced, while dIL^{B} neurons appeared unaffected (Helms and Johnson unpublished; Müller and Birchmeir, personal communication)**.** Mash1 null embryos have a reduction in neurogenesis in combination with impairment in the ability of progenitor cells to exit the cell cycle and differentiate (Helms et al., 2005)(Helms, unpublished) . Curiously, these cells expressed markers of differentiation but do not express a specific lineage transcription factor signature; suggesting that in the absence of Mash1 cells cannot retain progenitor character and these cells are subsequently eliminated.

Müller and Gross defined two late $dIL^{A/B}$, which are characterized by the expression of Lhx1/5 and Pax2 (dIL^{A}) and Lmx1b (dIL^{B}). Neurons of the $dIL^{A/B}$ subtype give rise to GABAergic and Glutamatergic neurons, respectively (Cheng et al., 2004). HD factors also have specificity functions in the dorsal neural tube, but these are largely restricted to post-mitotic cells. Recently, the requirement for specific HD transcription factors, such as Tlx1, Tlx3, Lbx1 and Pax2 in post-mitotic cells, has been demonstrated in generating glutamatergic versus GABAergic neurons in the dorsal horn (Cheng et al., 2004; Cheng et al., 2005). Pax2 is required for the formation of GABAergic neurons; however, in the absence of Pax2 a concomitant increase in glutamatergic neurons is not observed. Tlx1/3 double mutants, on the other hand, lose glutamatergic cells of the dorsal horn while GABAergic neurons increase in number. In this mutant, $Pax2^{+}$ cells which normally do not co-express Lmx1b, are found to be $Lmx1b^{+}$ suggesting a switch in cell fate (Cheng et al., 2004). Furthermore, over-expression of Tlx3 in chick neural tubes yields an increase in glutamatergic neurons. The authors suggest that Tlx1/3 acts as a post-mitotic molecular switch between GABAergic and glutamatergic cell fates within the developing spinal cord.

Lbx1 is expressed in differentiating excitatory and inhibitory neurons, as well as in the precursors to these cells, the Class B neurons (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002). Loss of Lbx1 leads to reduction in GABAergic cells of the dorsal horn (Cheng et al., 2005; Gross et al., 2002). Loss of GABAergic markers is apparent before cell death begins in this mutant (E12.5). In addition markers for glutamatergic neurons are increased in the absence of Lbx1 (Cheng et al., 2005). Embryos mutant for Lbx1 lose Pax2 expression which is consistent with the loss of GABAergic neurons (Gross et al., 2002). Yet,

this mutant retains the dIL^A marker Lhx1/5. Müller, *et al*. determined these cells to be $Brn3a^+/Lhx1/5^+$, suggesting that these were "dI2-like" cells. Cheng *et al*. observed that these $Lhx1/5^+$ cells were now expressing VGLUT2 (glutamatergic marker), an event that rarely occurs in wildtype embryos. Surprisingly, no change was observed in Tlx3 expression in the absence of Lbx1, suggesting that this phenotype is independent of Tlx3 (Cheng et al., 2005). Lbx1/Tlx3 double mutants lacked expression of GABAergic markers and had a surprising increase in VGLUT2 expression (Cheng et al., 2005). The authors conclude that Tlx3 acts upstream of Lbx1 to antagonize its function, thereby permitting the glutamatergic cell fate in $Tlx3^+$ cells. They further speculate that its regulation is not transcriptional, but may be at the protein level as Lbx1 and Tlx3 are co-expressed in glutamatergic neurons.

The neurons that comprise the adult spinal cord are a diverse population of cell-types with complex functions. These cells receive and process information from the environment, then integrate and relay the information to higher brain centers, and later coordinate the appropriate motor response. The neuronal diversity needed to execute these functions is great. Yet, little is known about the molecular characteristic of these dorsal horn neurons and even less is known about the developmental pathways which yield them. Intriguingly, these recent studies imply that post-mitotic cells retain some plasticity in relation to neurotransmitter phenotype. Increasing focus is being applied to this problem and developmental mechanisms are beginning to be elucidated in the generation of neuronal circuits.

The bHLH Factor Ptf1a in Development

Ptf1a encodes a bHLH transcription factor most closely related to the Twist subclass of bHLH genes (Krapp et al., 1996; Obata et al., 2001). Twist family members are categorized as Class II bHLH proteins what can form heterodimers with Class I bHLH factors, like E47 (Castanon and Baylies, 2002). Twist proteins have been shown to play roles in the specification of mesodermal derivatives. Indeed, mutation of Twist family members across species lead to defects in muscle development and abnormalities of the face and limb (Chen and Behringer, 1995; Howard et al., 1997; Verzi et al., 2002). In mice, Ptf1a was first identified as one of three subunits of the PTF1 transcription factor complex required for expression of pancreatic digestive enzyme genes (Cockell et al., 1989). Ptf1a is a 48kDa protein, also known as P48 (Krapp et al., 1996). Gene knockout studies have demonstrated a requirement for Ptf1a in pancreatic development. In the absence of Ptf1a exocrine lineages fail to develop, instead cells adopt a duodenal fate (Kawaguchi et al., 2002; Krapp et al., 1998). Suggesting that Ptf1a is required for endodermal cells to acquire a pancreatic fate and that Ptf1a descendents are found in exocrine, endocrine, and pancreatic ducts (Kawaguchi et al., 2002).

As a bHLH factor, PTF1a is notable in that it not only heterodimerizes with the E-protein, E47, but it also complexes with Rbpsuh (RBP-Jk, CBF1), an intercellular mediator of Notch signaling, and thus, may directly impact Notch signaling pathways (Beres et al., 2006; Obata et al., 2001). Ptf1a can bind to DNA as a heterodimer with an E-Protein, a complex that requires an E-box for binding or as a heterotrimer with Rbpsuh that requires both an E-box and a TC-box (TTTCCCA) (Beres et al., 2006). The trimer can activate transcription in reporter assays. This activation requires a tryptophan (W298) residue in

Ptf1a that is required for Rbpsuh binding (Beres et al., 2006). Moreover, Ptf1a interacts with HES1 in the yeast-two-hybrid assay; this interaction can inhibit the activity of the PTF1 complex on pancreatic targets(Ghosh and Leach, 2006). However, functional studies remain to be undertaken to determine the in vivo function of the trimer and whether the binding of HES1 is meaningful.

In nervous tissue, Ptf1a transcripts can be detected as early as E9.0 by PCR, prior to the onset of exocrine transcription in the developing pancreas. Furthermore, Ptf1a mRNA transcript has been observed in the dorsal neural tube at E9.0 (Krapp et al., 1998) a structure that gives rise to dorsal horn of the mature spinal cord. Obata, et al. showed that Rbpsuh bound Ptf1a in the yeast-two-hybrid assay using an E9.5 cDNA library. Expression analysis by *in situ* hybridization of early embryos revealed that Ptf1a is expressed in the developing NT at E9.5 and E10.5. When P19 embryonic carcinoma cells were induced to differentiate by treatment with retinoic acid, Ptf1a transcript was induced suggesting that Ptf1a plays a role in neurogenesis. Although no phenotype had been reported in the neural tube of Ptf1a knockout mice, over-expression of Ptf1a in *Xenopus* oocytes suggested that Ptf1a suppresses neuronal differentiation of primary sensory neurons while not affecting motor neurons (Obata et al., 2001).

Supporting a role for Ptf1a in nervous system development, null mutation of *Ptf1a* in mouse not only leads to the loss of pancreatic tissue but also to cerebellar agenesis (Hoshino et al., 2005; Krapp et al., 1998; Rose et al., 2001; Sellick et al., 2004). Furthermore, null mice die within three hours after birth. Truncation of the human *PTF1A* gene leads to permanent neonatal diabetes mellitus and cerebellar hypoplasia, culminating in death within

three months of age (Sellick et al., 2004). The phenotype is further characterized by intrauterine growth retardation, dysmorphic head structure including triangular face and dysplastic ears, and joint stiffness. Moreover, neuronal activity was impaired as was evident by abnormal movements, hyper- cerebral excitability, and irregular breathing patterns. The respiratory dysfunction resulted in apnea and required ventilation (Hoveyda et al., 1999; Sellick et al., 2004).

Thesis rationale and goals

Somatosensory inputs from the periphery, including pain, temperature, and mechanoception, are processed in the central nervous system in a hierarchal fashion. Sensory information is relayed from the periphery to the dorsal spinal cord, then to higher brain centers, and ultimately back to the spinal cord to supply the motor output that yields a desired physical response. These processes are accomplished via a network of neuronal circuits that comprise diverse neuronal cell types that include excitatory and inhibitory interneurons. While substantial progress as been made in identifying transcription factors that are required for, or at the very least define, a range of early neural populations within the developing spinal cord; however, little is known about the developmental mechanisms for the generation of later born excitatory and inhibitory neural cell types.

The bHLH factors have well established functions in neural differentiation and specification. Recent studies have determined a role for Ptf1a in pancreatic lineage decisions and others have suggested a role for Ptf1a in neural differentiation. In addition, mutations in the human PTF1A gene result in neural defects, including the loss of the cerebellum and respiratory abnormalities. These abnormalities may reflect improperly formed neural circuits

such that an imbalance occurs between excitatory and inhibitory activity. Consequently, the goals of this thesis work are to: 1) characterize the expression of Ptf1a in the CNS; 2) determine the requirement for Ptf1a in early and late neural populations of the spinal cord; 3) evaluate the effects of loss of Ptf1a function on neurotransmitter phenotype in the spinal cord; and 4) attempt to understand the molecular mechanism by which Ptf1a exerts its function.

I showed that Ptf1a expression in the developing mouse nervous system is restricted to the dorsal neural tube from hindbrain caudally to the tail. Within the caudal neural tube, Ptf1a has partial overlap with other bHLH factors and is required for the formation of dI4 interneurons. At later stages, Ptf1a expressing progenitors form dIL^A neurons, which are GABAergic neurons. Mice null for Ptf1a lack GABAergic neurons in the dorsal spinal cord. Furthermore, at all three stages tested, lack of Ptf1a led to a switch in cell fate from dI4 to dI5; dIL^A to dIL^B; and GABAergic to glutamatergic. This work suggests that Ptf1a further refines the transcription factor signature network that determines cell fate by acting as a molecular switch for the specification of two opposing cell fates of the dorsal horn - GABAergic versus Glutamatergic. This is supported by recent work in which specific loss of Ptf1a in the cerebellum results in loss of GABAergic neurons (Hoshino et al., 2005). Additionally over-expression of Ptf1a in the dorsal telencephalon, a region that normally produces glutamatergic neurons and is devoid of Ptf1a expression, led to the production of GABAergic neurons with the proper migration patterns and projections for GABAergic neurons (Hoshino et al., 2005).

In addition, Ptf1a appears to exert its functions within the neural tube, at least in part, as a component of a heterotrimeric complex composed of Ptf1a, an E-protein, and Rbpsuh. Over-expression assays with Ptf1a and mutant forms of Ptf1a supports a model in which the activity of Ptf1a is mediated by a balance between its participation in a trimeric complex, which includes Rbpsuh, and its participation as part of a heterodimer with E-proteins.

CHAPTER TWO

Materials and Methods

Animal Husbandry

The *Ptf1a*^{Cre} mutant mice have been previously described; these mice have Cre-recombinase replacing the *Ptf1a* protein coding region (Kawaguchi et al., 2002). The reporter strains *R26R-YFP* (Srinivas et al., 2001) and *R26R-LacZ* (Soriano, 1999) (Gt(ROSA)26Sor^{tmlSor}) were used to visualize cells and their progeny that were exposed to Cre recombinase activity. Genotyping was performed using PCR with primers 5'-AACCAGGCCCAGAAGGTTAT-3' and 5'-TCAAAGGGTGGTTCGTTCTC-3' for wild type *Ptf1a* locus, and with 5'-GCATAACCAGTGAAACAGCATTGCTG-3' and 5'-GGACATGTTCAGGGATCGCCAGGCG-3' for *Cre* in the mutant allele. PCR conditions for Ptf1a and Cre reactions were: 95°C-5min; (95°C-30sec, 60°C-30sec, 72°C-1min) X 4 cycles; (94°C-30sec, 57°C-1min, 72°C-1min) X 36 cycles; 72°C-10min; 4°C hold. R26R mice were genotyped with 5'-AAAGTCGCTCTGAGTTGTTAT-3'; 5'-GCGAAGAGTTTGTCCTCAACC-3'; 5'-GGAGCGGGAGAAATGGATATG-3'. PCR conditions for R26R reactions were: 98°C-2min; (95°C-30sec, 60°C-1min, 72°C-1min) X 4 cycles; (94°C-30sec, 57°C-1min, 72°C-1min) X 36 cycles; 72°C-10min; 4°C hold.

Tissue Preparation

Appropriately staged embryos were dissected in ice-cold 0.1M Sodium Phosphate buffer pH7.4 and fixed at 4°C in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4. E10.5-12.5 embryos were fixed for 2 hours. Embryos staged E15.5-E18.5 were more thoroughly dissected by removal of skin, visceral tissue, and most bone including the ribs so that spinal cords were contained solely within the spinal column. E15.5-E18.5 spinal cords

were fixed overnight in 4% formaldehyde at 4°C, washed twice in 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes and then again overnight. Brains were severed and completely stripped of extraneous tissue and fixed overnight in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4 and washed twice for 30 minutes, followed by an overnight wash. Tissues were then sunk in 30% sucrose overnight.

Adult tissue was obtained by perfusion with 4% formaldehyde. Mice were anesthetized with Avertin (0.024mls/gm). The perfusion apparatus was flushed with roughly 100ml of cold 0.1M Sodium Phosphate buffer pH7.4. An incision was made in anesthetized mice across the lower abdomen and up the ribcage to expose the chest cavity. A butterfly needle connected to the perfusion apparatus was inserted into the lower left quadrant of the heart and an incision was made in the upper right quadrant of the heart. 0.1M Sodium Phosphate buffer pH7.4 was circulated through the mouse until the heart turned white (10 minutes). 4% para-formaldehyde was then circulated through the mouse for 10 minutes. Spinal cords and brains were then fully dissected away from the spinal column and post-fixed overnight at 4°C in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4, and washed three times in 30ml of 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes. All tissues were sunk in 30% sucrose in 0.1M Sodium Phosphate buffer pH7.4 overnight, embedded in OCT compound (Tissue Tek) and cryo-sectioned.

Chick in ovo electroporation

White leghorn eggs were obtained from Texas A&M University-College Station poultry farm. Eggs were incubated at 38°C and 70% humidity in a Kuhl Corporation incubator. A small hole was made in the egg and 2-3ml of amniotic fluid was removed. A

window was opened on the top of the egg to allow for visualization of the embryo. The hole was covered with clear packing tape and incubated further until the embryo reached the appropriate stage for injection. A General Valve Corporation Picospritizer II (Fairfield, NJ) was used to inject DNA solutions into the lumen of the developing chick neural tube. 1XPBS (prepared from store bought 10X PBS stock pH 7.4) was added on top of injected chicks and electroporations were performed using a BTX ECM 830 power supply and gold electrodes (San Diego, CA); electroporation was done with three 50ms pulses at 25V. Electroporation readings of 18-25V were considered good. Chick embryos were injected with 2μg/μl DNA (unless otherwise noted) between chick stages HH13and HH15 for non-reporter constructs. For reporter constructs stage HH16-18 were electroporated. Embryos were harvested either 24 hours, 48 hours, or 72 hours post-electroporation depending upon experiment and processed for immunohistochemistry.

Electroporations used the expression vector pMiwIII, which drives expression through a chick β-actin promoter (Matsunaga et al., 2001; Suemori et al., 1990). PCR fragments containing Ptf1a and various mutants of Ptf1a were cloned into the Nco1 and Xba1 sites of the pMiwIII vector containing five Myc tag repeats. Expression was verified with antibodies to Myc and Ptf1a. See Appendix for Ptf1a expression vector maps.

β-gal staining of mouse and chick embyros

Mouse embryos were fixed for 30 minutes in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4 at room temperature and washed three times in 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes each. Embryos were incubated at 30°C for 24 hours in X-gal staining solution (PBS/5mM Kferricyanide, 5mM Kferrocyanide, 2mM $MgCl_2$,

1mg/ml X-gal), washed three times in 0.1M Sodium Phosphate buffer pH7.4 for 20 minutes, and post-fixed in 4% paraformaldehyde. Chick embryos were fixed for 30 minutes in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4 at room temperature and washed three times in 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes each. Embryos were incubated at 30°C for 24 hours in X-gal staining solution (PBS/35mM Kferricyanide, 35mM Kferrocyanide, 2mM $MgCl_2$, 1mg/ml X-gal), washed three times in 0.1M Sodium Phosphate buffer pH7.4, and post-fixed in 4% para-formaldehyde.

For staining of β-gal on cryo-sections, mice or chicks were fixed, 2 hours and 1hour respectively, in 4% para-formaldehyde at 4°C then processed and sectioned. Sections were collected on pre-warmed slides. A Fisher slide warmer was used to maintain the temperature of the slide between 37°C and 40°C throughout the sectioning process. If Histobond slides were used, sections were incubated on the slide warmer for an additional 10-15min post-collection to achieve proper adhesion of the sections to the slides. Slides containing sections were washed twice in 0.1M Sodium Phosphate buffer pH7.4 for 10 minutes and incubated overnight in X-gal staining solution as above. Slides were then washed in 1XPBS and coverslipped for imaging.

Counterstaining

Nuclear fast red staining was performed on cryo-sections and vibratome sections. Slides were air dried for 30 minutes at room temperature. The slides were dipped 5 times in water, incubated for 5 minutes in filtered nuclear fast red stain, dipped 7 times in water, dipped 7 times in fresh water, and dehydrated stepwise in ethanol (30%, 50%, 75%, 95%,

and 100%) for 2 minutes each. Slides were treated three times for 2 minutes in xylene and mounted with permount.

Nissl staining was performed on cryo-sections. Sections were incubated in 70% ethanol for 1minute, 50% ethanol for 1minute, dipped 10 times in water, and incubated in cresyl violet for 1-5 minutes. Slides were then rinsed in water (10 dips), rinsed again in fresh water, and treated with 95% ethanol plus glacial acetic acid (3 dips). Slides were passed through ethanol baths, 95%, 100%, and 100% for 5 dips each and cleared in xylene for 3-5 minutes. Permount was used to mount slides.

Immunohistochemistry

Single and double immunohistochemistry were performed under the same conditions. Slides were washed three times for 8 minutes in 1X PBS to remove OCT. Slides were then incubated in 1XPBS/1%goat serum/0.1%Triton-X-100 for 30 minutes. The blocking solution was removed and the appropriate primary antibody, diluted in 1XPBS/1%goat serum/0.1%Triton-X-100, was added to slides for incubation overnight at 4°C. Primary antibody was removed and saved for future use; slides were washed three times for 8 minutes in 1XPBS; and diluted secondary antibodies were added for incubation at room temperature for one hour in the dark. Slides were then washed three times in 1XPBS for 8 minutes and mounted with Vectashield for imaging. Double immunohistochemistry was performed by co-incubation with both primary antibodies. Embryos processed for immunohistochemistry were cryosectioned at 30μm.

Immunofluorescence was performed using the following primary antibodies: Mouse anti-BrdU (Becton Dickinson), guinea pig anti-VGLUT2 (Chemicon), mouse anti-Lhx1/5

(4F2) (Developmental Studies Hybridoma Bank), rabbit anti-PTF1a (Li and Edlund, 2001), mouse anti-Mash1 (Lo et al., 1991), rabbit anti-GFP (Molecular Probes), chicken anti-GFP (Chemicon), guinea pig anti-Lmx1b (Müller et al., 2002), mouse anti-GAD67 (Sigma), rabbit anti-Islet1/2 (Tsuchida et al., 1994), rabbit anti-Pax2 (Zymed), rabbit anti-Tlx3 (gift from T. Müller and C. Birchmeier), and mouse anti-GABA (Sigma). For a more detailed summary see Table 2.1.

Immunofluorescence analysis was performed using a Bio-Rad MRC 1024 confocal microscope. Images were processed using Adobe Photoshop. Specific neuronal cell types were counted using images from a minimum of 3 different animals on 3 or more sections from forelimb level.

Brdu incorportation

For mouse BrdU experiments, BrdU (200μg/g body weight) was injected into pregnant mothers for one hour before sacrifice. For chick BrdU experiments, 100μl of BrdU (10mg/ml) was injected in 4- 25μl spots into the yolk surrounding the neural tube of the developing chick. Sections from BrdU injections were treated with 2N HCl for 15 minutes, and 0.1M sodium borate PH 8.5 for 20 minutes prior to incubation with primary BrdU antibody. For double immunofluorscence involving BrdU, BrdU treatment was performed after the first primary antibody had undergone the entire immunofluorscence protocol.

In Situ Hybridization

Probe was prepared by cutting 10μg of plasmid DNA at the 5' end with the appropriate enzyme. The cut plasmid was purfied using a Qiagen PCR clean up kit and eluted in 40μl of nuclease-free water. Probes were synthesized as run-off transcripts using

bacteriophage RNA polymerases T3, T7 or SP6. A 50µl reaction was setup to synthesize RNA probe: 1X synthesis buffer, DNA template (2.5µg), RNA polymerase PLUS (90units), water, and lastly 10mM NTPs/digoxygenin-UTP. Probes were synthesized for 2 hours at 37°C, then 10 units of RNase-free DNAse was added for an additional 30 minutes at 37°C to digest DNA template. Un-incorporated ribonucleotides were separated on a mini-Biospin 6 spin column (Bio-Rad) as per manufacturer's instructions. For a summary of probes used see Table 2.2.

mRNA in situ hybridization was carried out as described (Birren et al., 1993; Cheng et al., 2004). Slides were dried at 50°C for 15 minutes. Slides were then fixed in 4% para-formaldehyde in DepC-PBS at room temperature for 20 minutes and washed twice in DepC-PBS at room temperature for 5 minutes. Proteinase K (10µg/mL; Roche) in PK buffer (50mM Tris-HCl pH7.5/5mM EDTA) treatment followed for 8-15 minutes depending upon the age of the embryos. Next slides were washed with DepC-PBS for 5 minutes and fixed again in 4% para-formaldehyde for 15 minutes. Slides were rinsed in DepC-water (2 dips). Slides were incubated in 0.1M triethanolamine for 10 minutes, washed in DepC-water for 5 minutes, and treated with hybridization buffer (50% formamide, 5X SSC, 0.3mg/mL yeast RNA, 0.1mg/mL heparin, 1X Denhardt's solution, 0.1% Tween-20, 5mM EDTA) for 1-4 hours at 65°C. Probe was added at 2ng/µl in hybridization buffer for incubation overnight at 65°C.

On the second day, slides were washed once with 2XSSC at 65°C for 15 minutes. Slides were rinsed with 2XSSC at room temperature for 5 minutes, treated for 45 minutes at 37°C with 10mg/mL of RNaseA and 1U/mL RNase T1, and rinsed in 2XSSC at room

temperature. Slides were then washed twice in 0.2XSSC for 30 minutes at 65°C, washed twice in PBT (1XPBS, 2mg/mL BSA, 0.1% Triton X-100) for 20 minutes at room temperature, and blocked for one hour at room temperature (PBT + 10% sheep serum). A humidity chamber was used to incubate slides with 250µl of anti-digocygenin antibody (1:1000 in PBT+ sheep serum) overnight at 4°C.

On day three, slides were washed 3 times in PBT for 30 minutes each at room temperature, and washed twice in alkaline-phosphatase buffer (100mM Tris pH 9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween-20) for 5 minutes at room temperature. Probes were developed in the dark by incubation with reaction solution (alkaline phoshpatase buffer, 1µl NBT, 3.5µl of BCIP) for 2-24 hours. Reactions were ceased by washing slides with PBS followed by fixation with 4% para-formaldehyde in PBS for 15 minutes at room temperature. Slides were mounted with Permount.

Antibody	Species	Cell type marked	Dilution	Source	Notes
BrdU	Mouse	Replicating DNA (S-phase)	1:25	Becton-Dickinson	
Brn3a*	Guinea pig	dI1-3, dI5 interneurons	1:250	E. Turner	Do not use blue channel
Calbindin	Mouse	Purkinje cells of the cerebellum	1:1000	Sigma	
GABA	Rabbit	GABAergic neurons	1:1000	Sigma	
GAD67	Mouse	GABAergic neurouns	1:1000	Chemicon	Does not stain nuclei
GFP	Chicken	Green fluorescent protein	1:250	Chemicon	weak
GFP	Mouse	Green fluorescent protein	1:500	Molecular Probes	Weak
GFP	Rabbit	Green fluorescent protein	1:500	Molecular Probes	Yields the best results
Glyt2	Guinea pig	Glutamatergic neurons	1:1500	Chemicon	Do not use blue channel
Islet1/2	Mouse	dI3 and motor neurons	1:50	DSHB	
Islet1/2*	Rabbit	dI3 and motor neurons	1:10,000	Tsuchida et al.	
Lhx1/5	Mouse	dI2, dI4, dI6, dIL^{A}	1:50	DSHB	
Lhx2/9	Rabbit	dI1	1:4000	T. Jessell	
Lmx1b*	Guinea pig	dI5 and dIL^{B}		T. Muller	Use green or red channel

Mash1*	Rabbit	Mash1	1:500	Johnson Lab	
Mash1	Mouse	Mash1	1:100	Anderson Lab	
Myc	Mouse	Myc tag	1:1000	Santa Cruz	
Myc	Rabbit	Myc tag	1:1000	Santa Cruz	
NeuN	Mouse	Neurons	1:1000	Chemicon	0.2%NP40
Ngn2*	Mouse	Ngn2	1:10	Anderson Lab	Mix 2 sources 1:20 each
Pax2	Rabbit	dI4 and dIL^{A}	1:500	Zymed	
Ptf1a*	Rabbit	Ptf1a	1:500	H. Edlund	
Tlx3*	Rabbit	dI5 and dILB	1:10,000	T. Muller	
Tuj1	Mouse	Neural β-tubulin	1:5000	Covance	
VGLUT2	Guinea pig	Glutamatergic neurons	1:2500	Chemicon	NP40; wash with 0.1%NP40/PBS; No serum with the secondary antibodies; Staining is found in terminals

Table 2.1. Summary of Antibodies

ntibodies used are listed in alphabetical order. Also listed is the neuronal cell type labeled by the antibody the source of the antibody and any special conditions that were required for a particular antibody.

* indicates antibodies that should be conserved.

Probe	**Species**	**Polymerase**	**Antisense**
Cash	chick	T3	Xho1
cGad1	chick	T7	BamH1
cNgn1	chick	T7	Sac1
cNgn2	chick	T7	Sac1
cPtf1a	chick	T3	EcoR1
cVGLUT2	chick	Sp6	EcoRV
Gad1	mouse	T7	Xba1
Glyt2	mouse	T7	BamH1
Gria/Glur2	mouse	T7	Xba1
Grik	mouse	T3	EcoR1
Ptf1a	mouse	T3	BamH1
VGLUT2	mouse	Sp6	EcoRV

Table 2.2. Summary of In Situ Hybridization Probes.

A listing of the probes used for *In Situ* is shown. The appropriate RNA polymerase and restriction enzyme utilized for creating antisense probe are listed.

CHAPTER THREE

CHARACTERIZATION OF PTF1A EXPRESSION IN THE NERVOUS SYSTEM

Introduction

The bHLH family of transcription factors is expressed in a wide variety of locations across the developing nervous system. Knockout studies have determined a vital role for these factors in the generation of their relative structures including regions of the brain, the olfactory epithelium, some enteric neurons, and the inner ear hair cells (Ben-Arie et al., 1997; Ben-Arie et al., 2000; Bermingham et al., 1999; Guillemot and Joyner, 1993; Helms et al., 2000; Johnson et al., 1990) (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996). In addition to these extra-neural tube regions of the CNS members of the bHLH family are expressed in the developing neural tube where the expression of Math1, Mash1, Ngn1, and Ngn2 are distinct and in most cases non-overlapping (Gowan et al., 2001).

Ptf1a transcripts can be detected as early as E9.0 in the neural tube by PCR (Krapp et al., 1998). Expression analysis by *in situ* hybridization of early embryos revealed that Ptf1a is expressed in the developing NT at E9.5 and E10.5 (Obata et al., 2001). However, these data do not precisely define Ptf1a expression within the developing neural tube nor do they establish expression boundaries with respect to other bHLH factors expressed at specific developmental times. As Ptf1a expression is transient, lineage tracing techniques must be applied to determine the eventual fate of Ptf1a expressing progenitor cells. In this manner, adult cells that are derived from $Ptf1a^{+}$ progenitors can be identified.

PTF1a is present in the developing nervous system

We used recombination based lineage tracing in vivo to characterize regions of the nervous system derived from *Ptf1a* expressing cells. The mouse strain $Ptf1a^{Cre/+}$, which has

the *Ptf1a* protein coding region replaced by that of Cre recombinase (Kawaguchi et al., 2002), was crossed with the Cre reporter strain *R26R-stop-lacZ*$^{+/-}$. The *R26R-stop-lacZ*$^{+/-}$ allele provides a permanent lineage marker for the cells with Cre recombinase activity (Soriano, 1999). ß-gal staining in a *Ptf1a*$^{Cre/+}$*;R26R-stop-lacZ*$^{+/-}$ embryo at E11.5, and dissected brain and spinal cord at E16.5, illustrate that *Ptf1a* prior to these stages is expressed in the neural tube, and the *Ptf1a* lineage extends from the hindbrain caudally to the tail (Fig 3.1A,C). ß-gal staining in the pancreas is also detected at E11.5 (Fig 3.1A, arrow). Within the neural tube, ß-gal staining is restricted to dorsal regions (Fig 3.1A-C), and in restricted regions in the ventral diencephalon (Fig 3.1C, inset) that include the preoptic nucleus and ventral hypothalamus. To identify the cell types in the adult nervous system that are derived from *Ptf1a*-expressing cells, we examined ß-gal staining in adult brains and spinal cords from these mice. A majority of stained cells in the spinal cord reside in the dorsal horn primarily in laminae I-IV, with a few scattered cells found in ventral laminae (Fig 3.2A,B). In the cerebellum, *Ptf1a*-expressing cells give rise to multiple GABAergic cell types including at least a subset of Purkinje cells, stellate, basket, and Golgi cells (Hoshino et al., 2005) (Fig. 3.2C). A population of cells in the ventral hypothalamus is also detected (Fig 3.2D). The requirement for PTF1a for the development of GABAergic neurons in the cerebellum has recently been reported (Hoshino et al., 2005).

PTF1a is restricted to post-mitotic cells within the ventricular zone of the dorsal neural tube in an overlapping pattern with Mash1

The ß-gal staining in the dorsal neural tube resembled the expression pattern of another bHLH transcription factor, Mash1 (Gowan et al., 2001; Helms et al., 2005). To

characterize the expression pattern of *Ptf1a* in more detail in this region, we used double label immunofluorescence with rabbit anti-Ptf1a (Li and Edlund, 2001) and mouse anti-Mash1 (Lo et al., 1991), or mouse anti-BrdU in BrdU-pulsed embryos. At E10.5, Ptf1a is detected within the central portion of the dorsal Mash1 domain (Fig. 3.3A-C). In this region, Mash1 levels are low relative to levels in adjacent dorsal and ventral regions (Fig. 3.3B). Because Mash1 is present in ventricular zone cells adjacent to dI3-dI5 neurons (Helms et al., 2005), this pattern suggests that Ptf1a may be in the dI4 precursor domain. Within the domain common to both factors, Ptf1a and Mash1 co-label a subset of cells located on the ventricular side (Fig 3.3C'). The pattern of Ptf1a is dynamic and by E11.5, the dorsal and ventral boundaries of Ptf1a become identical with those of Mash1 (Fig 3.3E-G). At this stage of neural tube development, the dorsal ventricular zone gives rise to the two late born neurons, dIL^A and dIL^B. Ptf1a marks cells that appear to have exited the cell cycle since they rarely co-label with BrdU incorporation at either E10.5 or E11.5 (Fig 3.3 D,D',H,H'). This cell-cycle status of Ptf1a cells contrasts to the Mash1 population where a subset of $Mash1^+$ cells are still $BrdU^+$ (Helms et al., 2005). Taken together, Ptf1a is largely restricted to post-mitotic cells in the ventricular zone of the dorsal neural tube in a pattern that suggests it may be in the precursors to dI4, dIL^A and/or dIL^B neurons.

SUMMARY

Ptf1a, like other bHLH proteins, is expressed in multiple areas of the developing nervous system. Ptf1a is expressed in the E10.5 developing neural tube in a domain that is

flanked by two high expressing Mash1 regions. This region corresponds to dP4 from which dI4 neurons are derived. At E11.5 the Ptf1a expression domain expands to match to Mash1 expression boundaries. This expanded domain gives rise to the $dIL^{A/B}$ neurons. In the adult dorsal horn, neurons that at one time expressed Ptf1a were located in laminae I-IV, regions that are known to function in modulating somatosensory information including pain responses. In addition, Ptf1a progenitors give rise to several cell-types of the cerebellum including some Purkinje cells. Finally, Ptf1a progenitors give rise to a population of cells within the ventral hypothalamus.

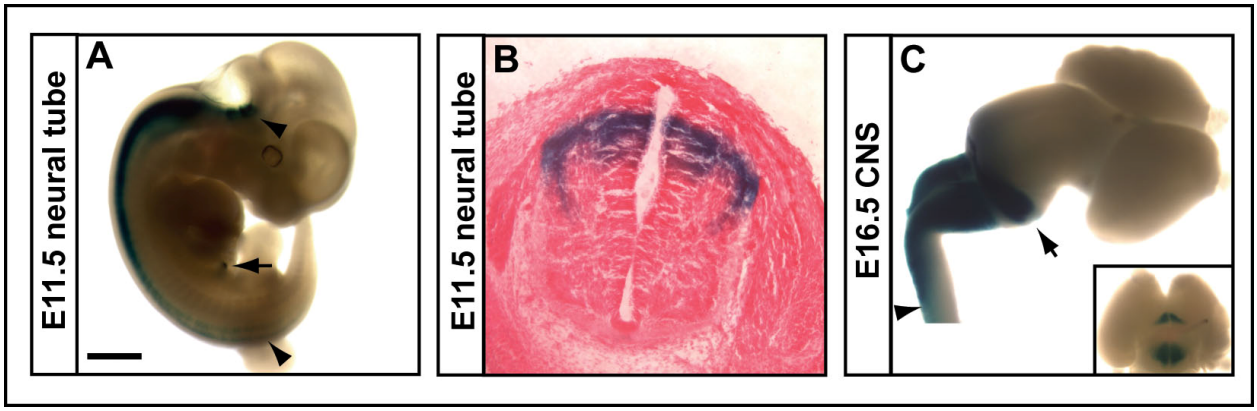


Figure 3.1 PTF1a is in precursors to multiple neural tissues.

The PTF1a lineage was visualized by ß-gal staining of *Ptf1a*$^{Cre/+}$;*R26R-stop-lacZ*$^{+/-}$ mice. (A) E11.5 whole mount showing ß-gal staining in the dorsal neural tube from the mid-hindbrain to the tail (arrowheads). Staining in the pancreas is indicated by the arrow. (B) Cross section of E11.5 showing ß-gal staining in a discrete domain within the developing spinal cord. (C) brain and spinal cord dissected from an E16.5 embryo, stained for ß-gal, showing expression in cerebellum (arrow) and dorsal spinal cord (arrowhead). Ventral diencephalon also reveals cells from the Ptf1a lineage (inset). Scale bar indicates 1mm.

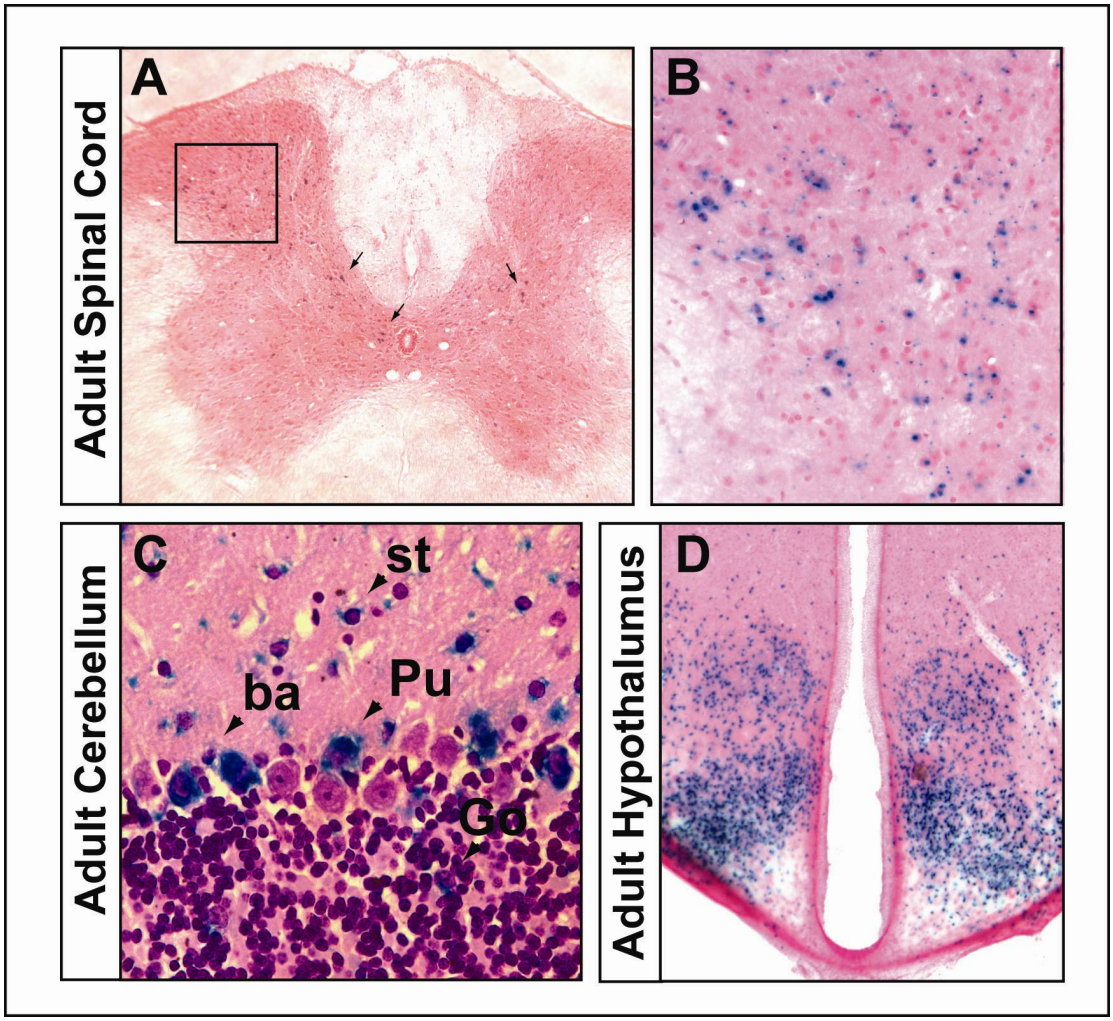


Figure 3.2 Cells which have expressed PTF1a become neurons of the dorsal horn, cerebellum, and hypothalamus.

(A,B) adult cervical spinal cord showing ß-gal stained cells primarily in laminae I-IV. The boxed area in (A) is shown at higher magnification in (B). Arrows (A) highlight labeled cells found ventral to boxed area. (C) adult cerebellum with ß-gal stained Purkinje (Pu), stellate (st), basket (ba), and Golgi (Go) cells. (D) coronal section of the adult brain reveals ß-gal stained cells in the ventral hypothalamus. Tissue is counterstained with nuclear fast red (A,B,D) and Nissl (C).

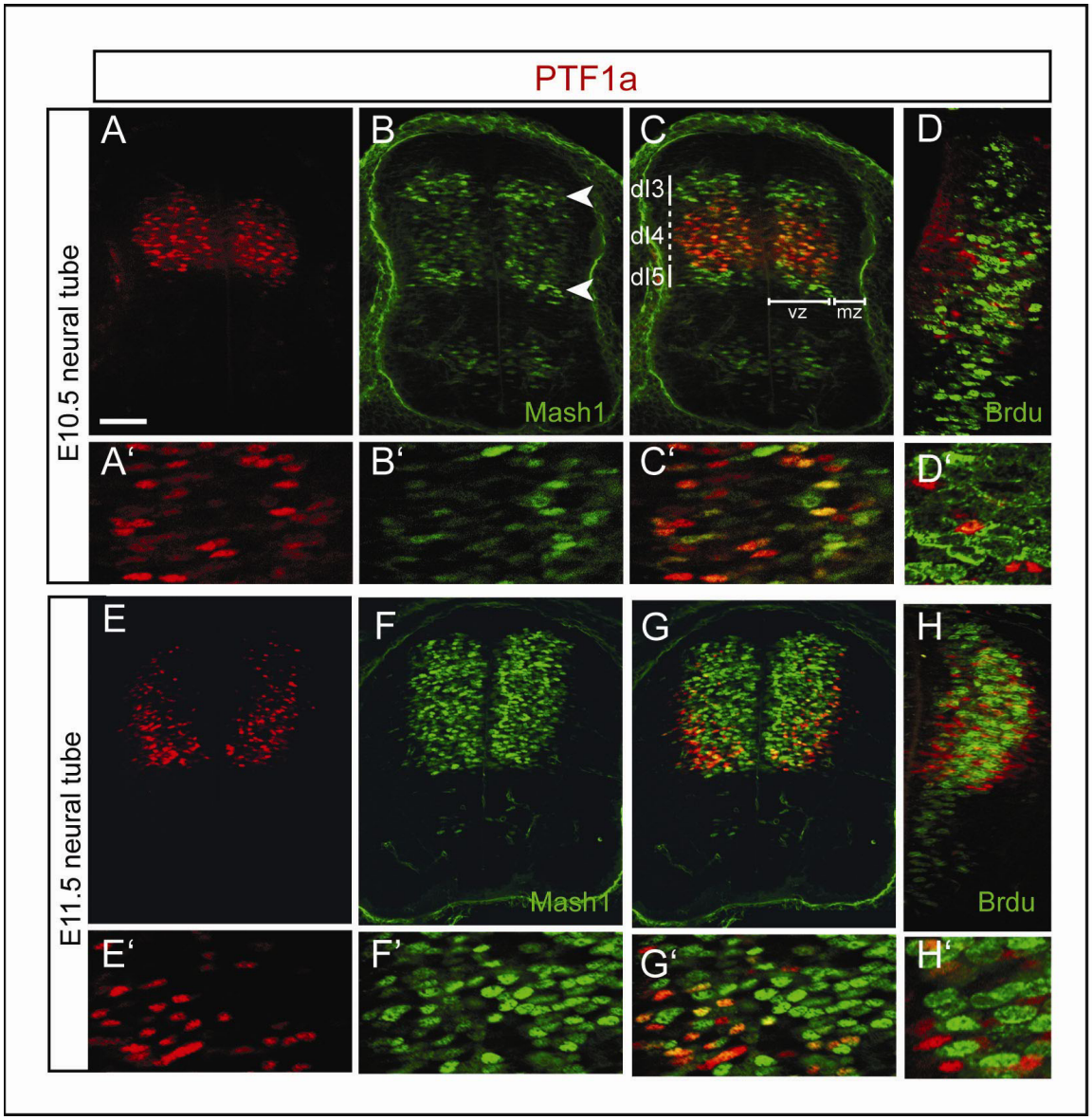


Figure 3.3 PTF1a is restricted to post-mitotic cells within the ventricular zone of the dorsal neural tube in an overlapping pattern with Mash1.

Figure 3.3. Ptf1a is restricted to post-mitotic cells within the ventricular zone of the dorsal neural tube in an overlapping pattern with Mash1. Immunofluorescence on transverse neural tube sections of E10.5 (A-D') and E11.5 (E-H') wildtype mice. (A-C;E-G) PTF1a (red), Mash1 (green), and their co-labeling (yellow) in a subset of cells. (D,H) PTF1a (red) and BrdU incorporation (green) are largely non-overlapping. Abbr: VZ, ventricular zone; MZ, marginal zone; dI3-dI5, dorsal interneurons 3-5. Scale bar indicates 50μm (A-H); 12.5 μm (A'-H').

CHAPTER FOUR

PTF1A IS REQUIRED FOR THE SPECIFICATION OF SPECIFIC NEURONAL POPULATIONS

Introduction

The requirement for bHLH transcription factors, Math1 (dI1), Ngn1 (dI2), Ngn2, and Mash1 (dI3 and dI5), for the formation of specific neuronal subtypes defined by the HD factors has been demonstrated (Bermingham et al., 2001; Gowan et al., 2001; Helms et al., 2005; Muller et al., 2005). In addition, the six early born neurons (dI1-6) can be divided into those that require Olig3, dI1-3, and those that require Lbx (dI4-dI6) (Muller et al., 2005; Müller et al., 2002). While some interneuron populations have been shown to rely on bHLH factors for proper development, specification of the dI4 and dI6 populations have not been correlated with a specific bHLH. Mash1 is required to generate dI3 and dI5 interneurons but not the dI4 neurons. Within the E10.5 ventricular zone, Mash1 is expression in the dP4 region is relatively low while Ptf1a expression is high; suggesting that Ptf1a may influence the specification of neurons derived from this progenitor domain.

PTF1a is required to generate dI4 and to suppress dI5 dorsal interneurons

Embryos null for PTF1a (*Ptf1a*$^{Cre/Cre}$) were examined at E10.5 for markers that distinguish interneuron populations dI2-dI6. These early neuronal populations are defined by several criteria that include birthdate (prior to E11) and the expression of HD transcription factors (Helms and Johnson, 2003) (Fig 1.3). In the absence of PTF1a (*Ptf1a*$^{Cre/Cre}$), the dI4 population is absent as demonstrated by the loss of Pax2;Lhx1/5 double positive cells (Fig 4.1 A,B; arrow). Conversely, dI5 neurons, marked by Lmx1b expand dorsally into the domain normally containing dI4 neurons (Fig 4.1A-D; arrow). No change in cell number was observed in the dI2 (Lhx1/5) and dI3 (Isl1) populations (Fig 4.1B,D; see counts in Table 4.1).

No change in cell death was observed using TUNEL or Caspase3 immunocytochemistry at this stage (data not shown). Thus, PTF1a is essential for the generation of dI4 interneurons and the suppression of dI5. This is in contrast to recent studies showing Mash1 is required for dI5 while suppressing dI4 neurons (Helms et al., 2005).

In the *Ptf1a* null mutant embryos, the number of cells increased in the dI5 cells complements the number of dI4 neurons lost (Table 4.1). These results suggest that in the absence of PTF1a, the dI4 cells trans-fate into dI5. To test this directly, we crossed the *Ptf1a*$^{Cre/+}$ mice to the *R26R-stop-YFP* Cre reporter strain, which will express YFP in cells with Cre recombinase and in all descendants of these cells (Srinivas et al., 2001). In *Ptf1a*$^{Cre/+}$*; R26R-stop-YFP*$^{+/-}$ mice, the dI4 marker Lhx1/5 co-localized with YFP, demonstrating that dI4 neurons are derived from PTF1a precursor cells, and thus, the loss of dI4 neurons in the Ptf1a null embryos is cell-autonomous (Fig 4.1E,F). In these embryos, YFP did not co-label with the dI5 marker Lmx1b (Fig 4.1G). In contrast, in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/-}$ embryos, the dI4 neurons were lost and YFP now co-localized with Lmx1b (dI5)(Fig 4.1G,H). Thus, in the absence of PTF1a, the cell generates a dI5 neuron rather than a dI4.

At all stages examined, the YFP expression was higher in the *Ptf1a* mutant than in the *Ptf1a* heterozygous embryos. This is at least partly due to the presence of two Cre alleles in *Ptf1a*$^{Cre/Cre}$ versus one allele in *Ptf1a*$^{Cre/+}$. However, it could also reflect a component of a negative feedback loop in the regulation of the *Pft1a* locus.

PTF1a is required to generate late born dIL^{A} and to suppress dIL^{B} interneurons

A second round of neurogenesis occurs in the developing spinal cord between E11-E13 to form the dIL^A and dIL^B populations of dorsal interneurons (Gross et al., 2002; Müller et al., 2002). The *Ptf1a* mutants were examined at E12.5 with Pax2 and Lhx1/5, which mark dIL^A, and Lmx1b and Tlx3, which mark dIL^B, to determine if these two late-born populations require PTF1a. In the absence of PTF1a, Pax2 was completely lost and Lhx1/5 was dramatically reduced specifically in the dorsal half of the neural tube, revealing a loss of dIL^A neurons (Fig 4.2A,B). The number of cells expressing Lmx1b or Tlx3 (dIL^B) was significantly increased in the absence of PTF1a, while cells expressing Isl1 were unaffected (Fig 4.2C,D,I,J, see Table 4.2 for cell counts). No increase in cell death was detected using TUNEL or Caspase3 immunocytochemistry at this stage (data not shown). These results demonstrate that PTF1a is required for the formation of the dIL^A neurons and normally suppresses the formation of dIL^B neurons.

Although not as complete as the dI4/dI5 switch at E10.5, there was a concomitant increase in dIL^B neurons when dIL^A were lost, suggesting there is a fate switch from dIL^A to dIL^B in *Ptf1a* mutant embryos. To visualize the switch in cell fate, we again utilized *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. In *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/-}$ embryos, Lhx1/5 (dIL^A) co-localized with YFP demonstrating that the loss of dIL^A in the *Ptf1a* null is a cell-autonomous effect (Fig 4.2E,E',F,F'). The dIL^B markers Lmx1b and Tlx3 are not co-expressed with YFP in embryos heterozygous for *Ptf1a* (Fig 4.2G,G',I). However, in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/-}$ embryos, there is a dramatic increase in the number of YFP/Lmx1b and YFP/Tlx3 double positive cells, consistent with a cell fate switch from dIL^A to dIL^B (Fig 4.2H,H',J). Interpretation of this result is tempered by the increase in YFP

expression in the *PTF1a* mutant (Fig 4.2F,H), as stated earlier. Also, the increase in the markers for dIL^B in the mutant cannot completely account for the number of dIL^A cells lost. This could reflect differences in temporal characteristics or detection efficiency of the individual markers. Regardless, the overlap in YFP and dIL^B markers in the mutant, combined with PTF1a being largely restricted to postmitotic cells, the increase in the number of dIL^B, and the lack of apoptosis, together suggest that PTF1a serves as a switch between the dIL^A and dIL^B interneuron subtypes.

PTF1a is required for the formation of GABAergic neurons in the dorsal horn

In the absence of Pax2, expression of the GABAergic marker gene *Gad1* (encoding glutamic acid decarboxylase, GAD67) is lost in the dorsal horn of E13 mouse embryos (Cheng et al., 2004). As the *Ptf1a* null embryos completely lose expression of Pax2 in the dorsal neural tube, we predicted that the absence of PTF1a would also result in loss of GABAergic neurons. To test this hypothesis, *Ptf1a* mutants were analyzed at E16.5 for expression of GABAergic markers. In situ hybridization with a *Gad1* probe demonstrates there is a complete loss of *Gad1* in the dorsal horn in the absence of PTF1a, while the most ventral expression appears largely unaffected (Fig 4.3A,B). The protein encoded by *Gad1*, GAD67, is also absent in the dorsal horn of PTF1a deficient embryos (Fig 4.3F), as is GABA, the neurotransmitter itself that defines a GABAergic interneuron (Fig 4.3H).

Although the level of PTF1a decreases by E16.5, we were able to use $Ptf1a^{Cre}$*;R26R-stop-YFP* embryos to map the fate of *Ptf1a* expressing cells into E16.5 dorsal horns to verify that the loss of GABAergic neurons is cell-autonomous. The vast majority of YFP in $Ptf1a^{Cre/+}$*;R26R-stop-*$YFP^{+/-}$ embryos is restricted to the dorsal spinal cord at E16.5 (Fig

4.3C). Co-localization of YFP with GAD67 and GABA indicates that the loss of GABAergic neurons in PTF1a deficient embryos is at least in part a cell-autonomous effect as expected (Fig 4.3E,G). These co-localization experiments are not as clear as the analysis with the transcription factor markers at earlier stages since the neurotransmitter proteins tend to localize in the distal processes of the neurons, while the YFP is mainly cytoplasmic with some signal reaching distal processes. In Fig. 4E, arrows indicate regions outside the cell body where YFP and GAD67 co-localize. GABA is easier to detect in cell bodies and thus, the overlap with YFP is clearer (Fig 4.3G, arrows). It is also important to note that the in vivo recombination system used here to trace the lineage of PTF1a cells is not 100% efficient and is not expected to indicate every PTF1a descendent. Regardless, together the data demonstrate that PTF1a is in cells fated to become GABAergic neurons of the dorsal horn and that it is essential for this neuronal subtype to form.

YFP in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/-}$ embryos, which are deficient in PTF1a, is also largely restricted to the dorsal horn, but it is detected in more cells and encompasses a broader medial-lateral area compared to heterozygous embryos (Fig 4.3C,D). Besides suggesting PTF1a may normally be in a negative autoregulatory loop, the aberrant location of YFP labeled cells on the lateral edges that appear to stream ventrally are consistent with mis-specification of neuronal subtype in the mutant.

PTF1a suppresses glutamatergic neuronal differentiation in the dorsal horn

In the absence of PTF1a there is a neuronal subtype switch from dI4/dIL^{A} to dI5/dIL^{B} (Figs. 4.1,2). The dIL^{B} neurons form the glutamatergic neurons in the dorsal horn (Cheng et al., 2004). To determine if the aberrantly formed dIL^{B} neurons continue to mature with

glutamatergic characteristics, we examined *Ptf1a* mutant embryos for the VGLUT2 (vesicular glutamate transporter2) and GluR2/3 (glutamate receptor). mRNA in situ hybridization and immunofluorescence detected an increase of VGLUT2 and GluR2/3 in the dorsal horn of $Ptf1a^{Cre/Cre}$ embryos at E16.5, when compared to $Ptf1a^{Cre/+}$ embryos (Fig 4.4A-D, and data not shown). This increase is clearly indicated by an increase in VGLUT2 in the more superficial laminae as indicated by the arrows in Fig 4.4C,D. Likewise, an increase in the density of Tlx3 expressing cells is also seen at E16.5 (Fig 4.4G,H), consistent with the importance of Tlx1/3 in generation of dorsal horn glutamatergic neurons (Cheng et al., 2004). In contrast, no increase was detected in the glycinergic neuronal marker GlyT2 by mRNA in situ hybridization (data not shown).

Just as at the earlier embryonic stages, we examined $Ptf1a^{Cre/+}$;*R26R-stop-YFP*$^{+/-}$ and $Ptf1a^{Cre/Cre}$;*R26R-stop-YFP*$^{+/-}$ embryos for co-localization of VGLUT2 with YFP to address the question of a neurotransmitter fate switch of the *Ptf1a* mutant cells. Significant co-expression of VGLUT2 with YFP is observed only in the *Ptf1a* null embryos (Fig 4.4E,F). As with the GABAergic markers, the colocalization of VGLUT2 with YFP has caveats due to the enrichment of VGLUT2 in distal processes. However, taken together with the loss of Pax2 expression and increase in Tlx3, these results demonstrate PTF1a functions as a switch; it is required for the generation of GABAergic neurons and suppresses generation of glutamatergic neurons in the dorsal horn of the spinal cord (Fig 4.4).

Summary

In the absence of Ptf1a, dI4 interneurons marked by Lhx1/5^{+} Pax2^{+} fail to form; instead dI5 neurons (Lmx1b^{+}) are formed. In a cell-autonomous manner, Ptf1a null embryos undergo a trans-fating event where dI4 become dI5. Similarly, late born dIL^{A} interneurons that give rise to GABAergic neurons of the dorsal horn are lost in the Ptf1a mutant. Again, in a cell autonomous fashion dIL^{A} neurons instead become dIL^{B} in the absence of Ptf1a. Correspondingly, lack of Ptf1a results in the complete loss of GABAergic neurons in the dorsal horn accompanied by a concomitant increase in glutamatergic neurons. Therefore, Ptf1a is strictly required for the formation of dI4, dIL^{A}, and GABAergic interneurons and suppression of the alternative dI5, dIL^{B}, and glutamatergic cell fates, respectively.

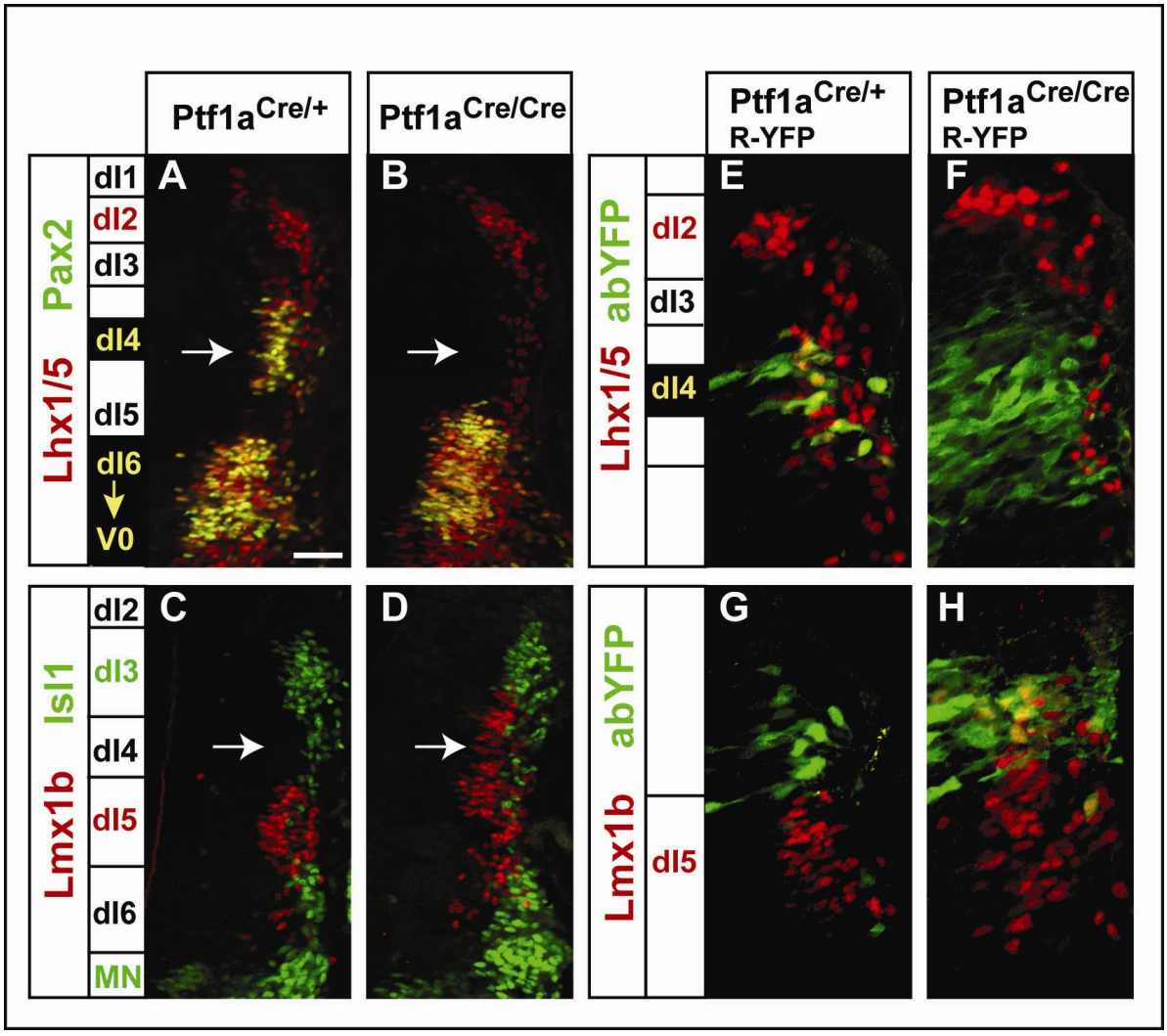


Figure 4.1 dI4 are trans-fated to dI5 interneurons in PTF1a deficient embryos.

Figure 4.1 dI4 are trans-fated to dI5 interneurons in PTF1a deficient embryos.

Immunofluorescence on neural tube transverse sections of *Ptf1a*$^{Cre/+}$ (A,C) and *Ptf1a*$^{cre/cre}$ (B,D) mouse E10.5 embryos. (A,B) dI4 neurons marked in yellow by co-labeling of Lhx1/5 (red) and Pax2 (green) are lost in the *Ptf1a* null (arrows). (C,D) Lmx1b (red) labeling dI5 is expanded in *Ptf1a* null embryos, while Isl1 (green) labeling dI3 are not affected. (E-H) Anti-GFP antibody was used to detect YFP in E10.5 *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. YFP (green) co-localizes with the Lhx1/5 (dI4;red), but fails to do so in *Ptf1* null embryos (compare E and F). (G) Lmx1b (dI5;red) does not co-localize with YFP in the presence of PTF1a, but they do co-localize in the null (H). The ventricle is to the left in all panels as shown. Scale bar equals 50µm (A-D); 25µm (E-H).

Interneuron Population	# cells per quadrant	
	$Ptf1a^{Cre/+}$	$Ptf1a^{Cre/Cre}$
dI2(Lhx1/5+ Pax2-)	58+/-10	50+/-11
dI3(Isl1+)	86+/-8	75+/-13
dI4(Pax2+)	51+/-11	0**
dI5(Lmx1b+)	67+/-12	113+/-16**

** p<0.001

Table 4.1. Neuronal population cell counts for E10.5 embryos.

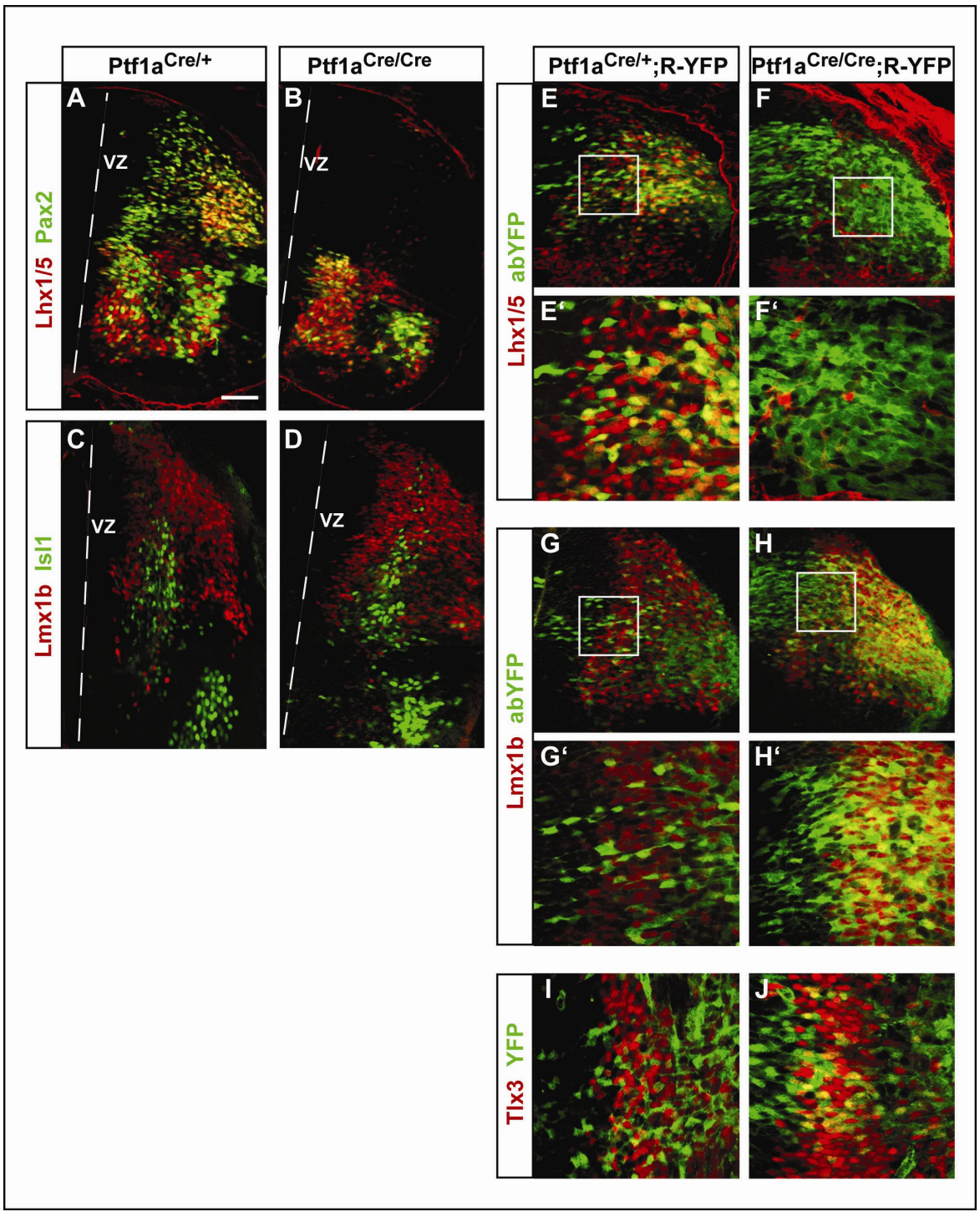


Figure 4.2 dIL^{A} trans-fate to dIL^{B} interneurons in PTF1a null embryos.

Figure 4.2 dILA trans-fate to dILB interneurons in PTF1a null embryos.

Immunofluorescence on neural tube transverse sections of *Ptf1a*$^{Cre/+}$ (A,C) and *Ptf1a*$^{Cre/Cre}$ (B,D) mouse E12.5 embryos. (A,B) dIL^{A} neurons, marked by Lhx1/5 (red) and Pax2 (green), are lost specifically in the dorsal neural tube, but not the ventral neural tube in the *Ptf1a* null (dashed line indicates the position of the ventricle). (C,D) dIL^{B} neurons, marked by Lmx1b (red) increase in the *Ptf1a* null, but there is no change in the number of Isl1 (green) cells. (E-J) Anti-GFP antibody was used to detect YFP at E12.5 in *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. (E-F') In the presence of PTF1a, YFP (green) co-localizes (yellow) with the dIL^{A} marker, Lhx1/5 (red), but it does not do so in *Ptf1a* null embryos. (G-H') Lmx1b (dIL^{B}; red) and (I-J) Tlx3 (dIL^{B}; red) rarely co-localizes with YFP in embryos with PTF1a, while substantially more Lmx1b/YFP and Tlx3/YFP double positive cells appear in *Ptf1a* deficient embryos (yellow). The boxed regions in (E-H) are shown at higher magnification in (E'-H'). Scale bar equals 50μm (A-H); 25μm (E'-J').

	# of cells per dorsal quadrant	
Interneuron Population	$Ptf1a^{Cre/+}$	$Ptf1a^{Cre/Cre}$
dIL^{A} (Lhx1/5+)	300+/-16	36+/-12**
dIL^{A} (Pax2+)	363+/-22	0**
dIL^{B} (Lmx1b+)	307+/-12	476+/-45**
dIL^{B} (Tlx3+)	334+/-12	469+/-49**
		** p<0.001

Table 4.2. Neuronal population cell counts for E12.5 embryos.

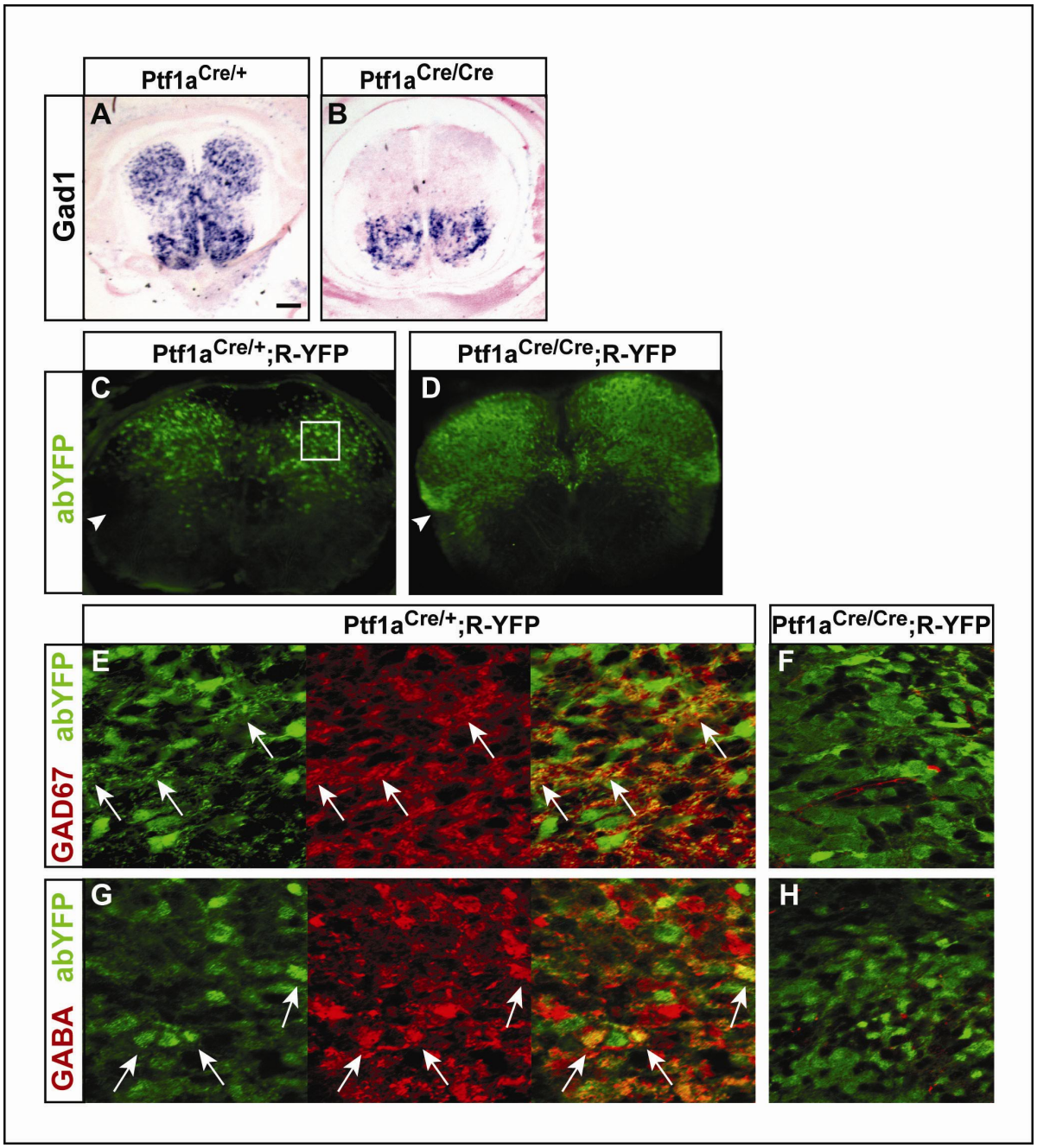


Figure 4.3 PTFla is required for generation of dorsal horn GABAergic neurons.

Figure 4.3 PTFla is required for generation of dorsal horn GABAergic neurons. Transverse sections through spinal cord cervical regions of *Ptf1a*$^{Cre/+}$ and *Ptf1a*$^{Cre/Cre}$ mouse E16.5 embryos were processed for mRNA in situ hybridization with the GABAergic marker gene Gad1 (A,B).Note the complete absence of Gad1 in the dorsal regions in the absence of PTF1a. Loss of ventral Gad1 was not consistently observed. (C-H) Anti-GFP antibody was used to detect YFP in *Ptf1a*^{Cre}*;R26R-stop-YFP* E16.5 embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. (C) The lineage reporter YFP is detected largely in the dorsal regions in wildtype. (D) In contrast, in the mutant, YFP is detected at higher levels in more cells and with a different organization (arrowheads). Boxed area in (C) is the approximate area shown in (E-H). Immunofluorescence for GAD67 (E) and the neurotransmitter GABA (G) co-localize with YFP in embryos heterozygous for *Ptf1a* (arrows) but not in embryos lacking *Ptf1a* (F,H). Panels (E-H) are all from dorsal horn regions. Scale bar equals 110μm (A,B); 13 μm (E-H).

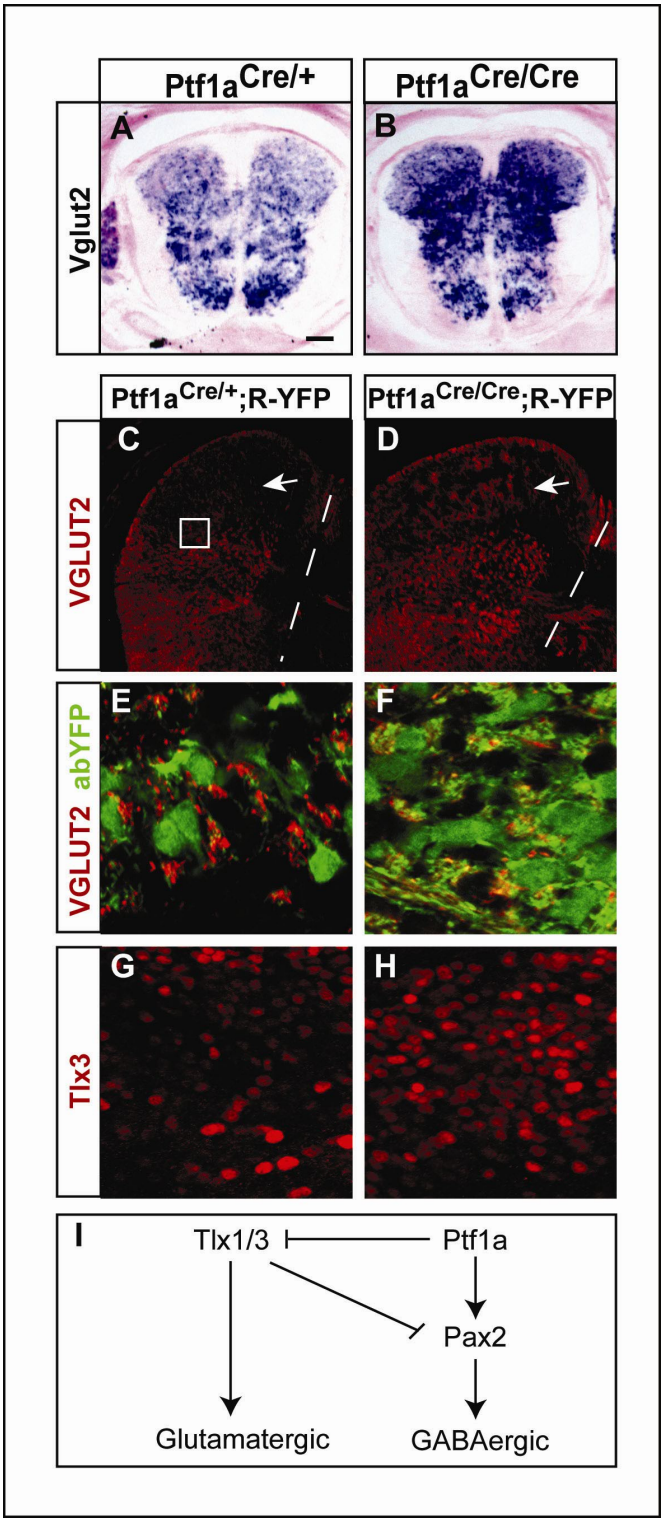

Ptf1a^Cre/+
Ptf1a^Cre/Cre
Vglut2
A
B
Ptf1a^Cre/+;R-YFP
Ptf1a^Cre/Cre;R-YFP
VGLUT2
C
D
VGLUT2 abYFP
E
F
Tlx3
G
H
I
Tlx1/3
Ptf1a
Pax2
Glutamatergic
GABAergic

Figure 4.4 PTFla is required for suppression of dorsal horn glutamatergic neurons**.**

Figure 4.4 PTFla is required for suppression of dorsal horn glutamatergic neurons. Transverse sections through spinal cord cervical regions of *Ptf1a*$^{Cre/+}$ and *Ptf1a*$^{Cre/Cre}$ mouse E16.5 embryos were processed for mRNA in situ hybridization for the glutamatergic marker Vglut2 gene (A,B) or for immunofluorescence for the protein VGLUT2 (C,D). Note the increase in Vglut2 specifically in the dorsal regions in the absence of PTF1a. The arrows in (C,D) indicate superficial laminae that have substantial increase in VGLUT2 in the mutant. The dashed line in (C,D) indicates the midline. (E,F) Anti-GFP antibody was used to detect YFP in *Ptf1a*^{Cre}*;R26R-stop-YFP* E16.5 embryos. VGLUT2 and YFP seldom co-localize in embryos containing PTF1a (E), however, co-localization of VGLUT2 and YFP is detected in distal processes in the *Ptf1a* null (F). The density of Tlx3+ cells is increased in dorsal regions in the mutant (G,H). (E-H) are all from dorsal horn regions indicated by the box in (C). (I) a model for the a role of PTF1a in GABAergic and glutamatergic neurons in the dorsal horn. PTF1a acts in two ways: determination of GABAergic neurons by inducing Pax2, which is required for the expression of the GABAergic phenotype in these cells, and suppressing Tlx3, which is required for specifying glutamatergic neurons by inducing glutamatergic specific genes and suppressing Pax2 (Cheng et al., 2004). Scale bar equals 110μm (A,B); 50μm (C-D), 13μm (E-H).

CHAPTER FIVE

THE PTF1 COMPLEX IN NEURAL TUBE DEVELOPMENT

Introduction

Utilizing Ptf1a deficient embryos we were able to demonstrate an absolute requirement for Ptf1a in the specification of early born dI4 and GABAergic neurons in the dorsal horn. Moreover, loss of Ptf1a expression led to the derepression of the dI5 and glutamatergic neurons. The lineage tracing experiments determined that this activity was cell-autonomous. Pancreatic studies have determined that Ptf1a acts as a component of a trimeric complex, the PTF1 complex (Beres et al., 2006). Consequently, we were interested in determining in what capacity Ptf1a functioned in the neural tube and if this function was a result of Ptf1a participating in a complex similar to that found in pancreas.

PTF1 complex is an active complex in the chick neural tube

To investigate the mechanism of Ptf1a action in neural tube development, Ptf1a and several control Ptf1a mutant constructs were electroporated into chick neural tubes. Ptf1a expression plasmid (pMiWIII Ptf1a) (Fig 5.1 and appendix) was electroporated into HH15 chick embryos; embryos were harvested 24 hours later. No changes in expression of Lhx1/5 or Pax2 (dI4) were observed between control and electroporated embryos; although not significant statistically, some embryos displayed an increase of Lhx1/5 expression (Fig 5.2). Similarly, embryos harvested 72 hours post-electroporation and assayed for the GABAergic marker Gad1, showed no change in expression of the marker (data not shown; see appendix for summary of results). However, Hoshino *et al.* overexpressed Ptf1a in the developing dorsal telencephalon, a region that usually gives rise to glutamatergic neurons which is devoid of Ptf1a expression, and found that Ptf1a induced expression of GABAergic markers

(Hoshino et al., 2005). At this point we do not understand why Ptf1a can induce GABAergic neurons in the cortex but not in the neural tube. Some possibilities include the absence of a co-factor in the neural tube, an imbalance with competing complexes, and differences in expression level between mouse-chick over-expression assays. Another possibility is the timing of over-expression in the chick assay. Ptf1a is normally expressed in post-mitotic cells while electroporated cells tend to be mitotic.

Although wildtype Ptf1a did not alter cell types in the chick neural tube, over-expression of a control Ptf1a mutant, Ptf1a^{EG} (Fig 5.1), that has been shown to disrupt dimer and trimer function on pancreatic targets, resulted in a striking increase in Lhx1/5, a dI4 cell population marker that requires Ptf1a for expression (Fig 5.3A-E, Fig 5.5 C, F). Interestingly, many of the ectopic Lhx1/5 positive cells were found within the VZ, a region where actively dividing cells reside (Fig. 5.3A-E). NeuN and BrdU, two markers used to determine a cell's differentiation state were not affected by over-expression of Ptf1a^{EG}, suggesting that the cells were not prematurely differentiating (Fig 5.4). These cells were not co-positive for Brn3a or Lbx1b indicating that they could not be assigned to dI2 or dI4-like populations. Instead it appears that Ptf1a^{EG} exclusively induces the expression of Lhx1/5.

The striking increase in Lhx1/5 resulting from over-expression of Ptf1a^{EG} could be attributed to two events: 1) the sequestration of E-protein; or 2) the titration of some other unknown co-factor. A similar mutation in Mash1 resulted in what appeared to be a dominant negative phenotype when over-expressed, possibly due to sequestering of E-proteins (Nakada et al., 2004). To test this possibility Ptf1a^{EG} was electroporated in decreasing amounts in an attempt to alleviate the competition. Decreasing the amount of vector did not temper the

increase in Lhx1/5 (Fig 5.4). Furthermore, co-expressing Ptf1a^{EG} with the E-protein E47 did not ameliorate the phenotype (Figure 5.4 C,D), suggesting that Ptf1a^{EG} is not acting to sequester E-protein.

Recently, RM Henke identified Ngn2 as a neuronal downstream target of Ptf1a (unpublished). Ngn2 is co-expressed with Ptf1a in a subset of cells in the dP4 region of the dorsal spinal cord. The identification of a neural target for Ptf1a enabled us to determine if Ptf1a^{EG} was indeed a DNA binding mutant on neural targets as it was on the elastase1 enhancer. Astoundingly, Ptf1a^{EG} bound the Ngn2 enhancer as a trimer complex with Rbpush in a robust fashion; while it failed to bind as a heterodimer with E-protein (RM Henke, unpublished), suggesting that the increase in Lhx1/5 was a result of PTF1 complex activity. To test the requirement of Rbpsuh in the PTF1 complex, a Ptf1a mutant construct, Ptf1a^{W298A}, was over-expressed in chick (Fig.5.1). The tryptophan (W) residue mutated in this construct is located in the C-terminus of Ptf1a and has been shown to be critical for the formation of the trimer complex on pancreatic targets (Beres et al., 2006). Over-expression of Ptf1a^{W298A} did not result in ectopic expression of Lhx1/5, indicating that Rbpsuh is critical for this activity (Fig 5.5). As Ptf1a^{EG} exclusively forms the trimer complex these data suggests the activity resulting in the ectopic expression of Lhx1/5 requires trimer formation. Consistent with this, the Lhx1/5 phenotype was ablated when a compound mutant, Ptf1a$^{EG/W298A}$, was tested in this assay (Fig 5.1 and 5.5). One interpretation of these results is that the ratio of trimer to dimer formation for each Ptf1a variant is important for the Lhx1/5 phenotype. Excess trimer relative to dimer, as is the case when Ptf1a^{EG} is over-expressed, is the activity required to activate expression of Lhx1/5.

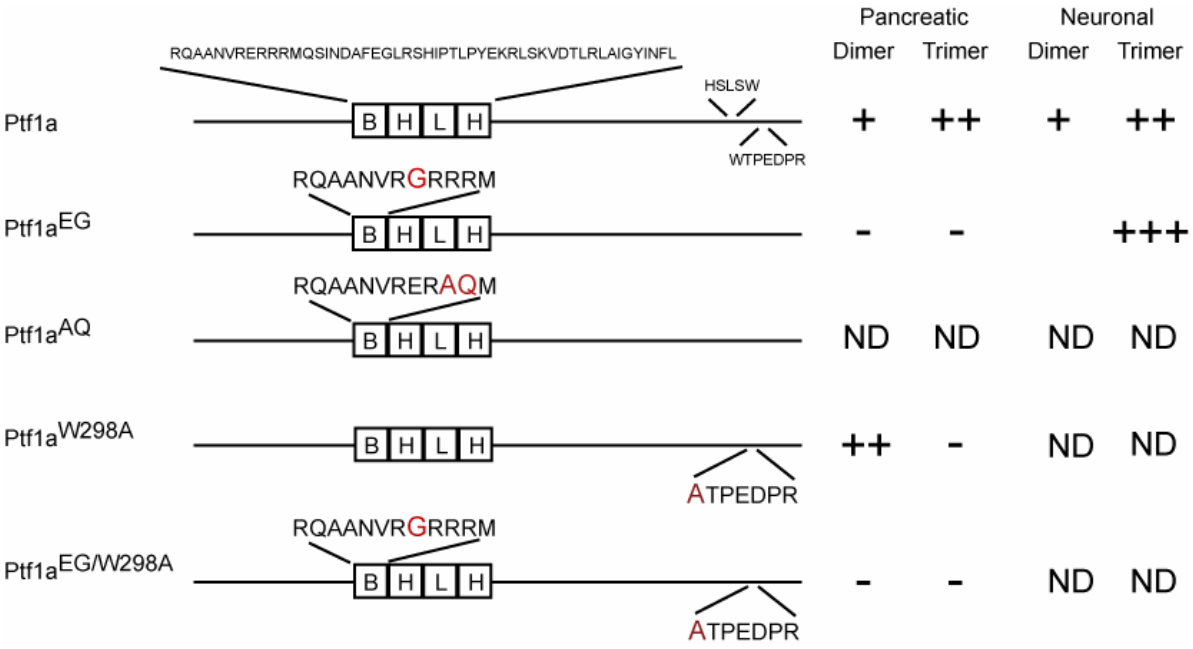


Figure 5.1 Summary of Ptf1a mutant constructs and DNA binding activity on pancreatic and neuronal targets**.**

Shown are schematic representations of the Ptf1a protein sequence and modifications made to Ptf1a amino acids. For Ptf1a the entire bHLH amino acid sequence is depicted. Also shown are the conserved residues surrounding the two tryptophan residues identified to be important for trimer formation with Rbpsuh. Ptf1a mutant constructs were made and the amino acids modified are shown in red. The ability of each protein to form a dimer or trimer is summarized to the right. Pancreatic target is for elastase1 only (T. Beres). Neuronal target is Ngn2 (RM Henke, unpublished).

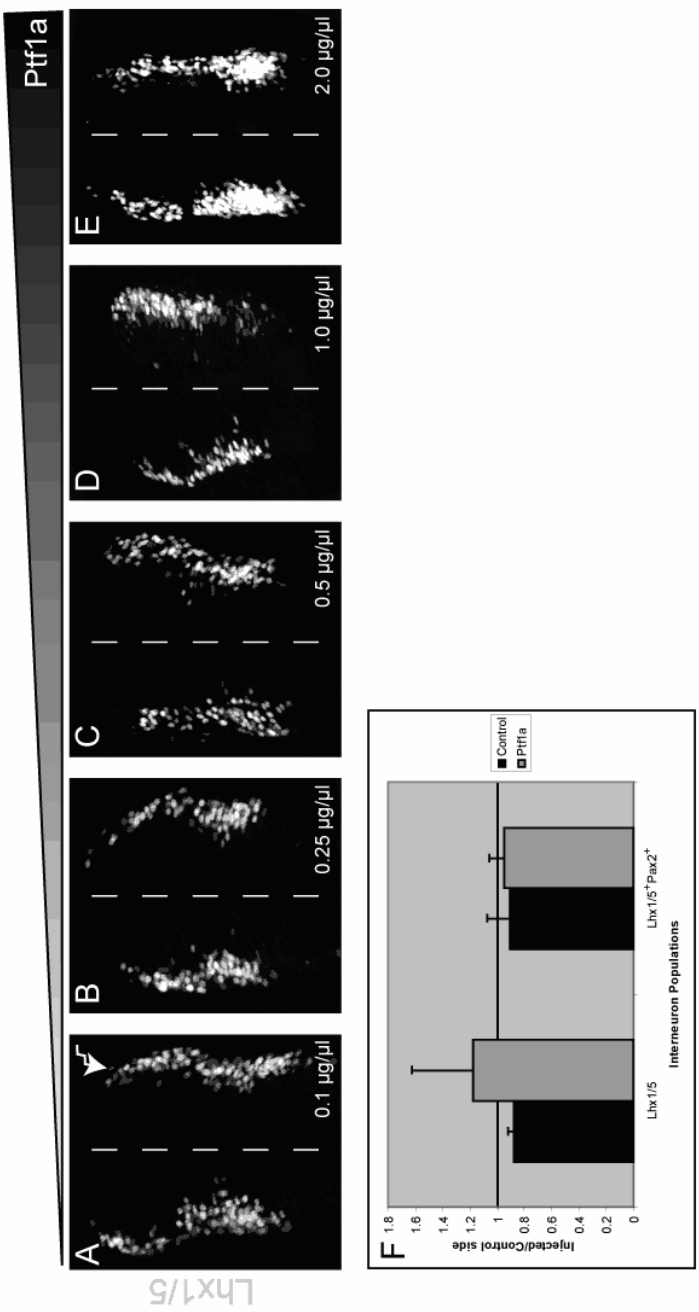


Figure 5.2 Ptf1a is not sufficient to specify dI2 or dI4 interneurons.

Figure 5.2 Ptf1a is not sufficient to specify dI2 or dI4 interneurons. HH 13-15 stage chick embryos were electroporated with the Ptf1a expression vector pMiWIII-Myc-Ptf1a and harvested 24 hours later. Ptf1a was electroporated in increasing concentrations shown from left to right (A-E). Expression of Ptf1a was determined by immunofluorescence with Myc and Ptf1a antibody (data not shown). Electroporated side is to the right in all panels. No obvious change was observed in Lhx1/5 expression or Pax2 (not shown). (F) Quantification of the Lhx1/5 positive cells. A ratio between injected versus control side is graphed so that a value above one is an increase in expression while a value less that one is a decrease in expression. No statistical differences were detected.

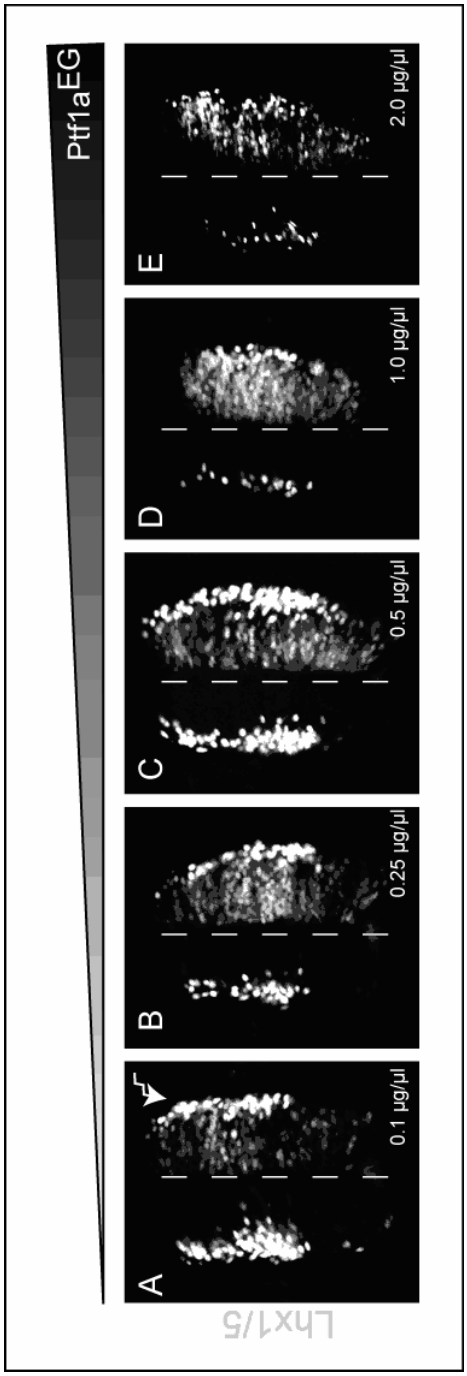


Figure 5.3 The DNA binding mutant Ptf1a^{EG} induces ectopic Lhx1/5 expression.

Figure 5.3 The DNA binding mutant Ptf1a^{EG} induces ectopic Lhx1/5 expression. HH 13-15 stage chick embryos were electroporated with the Ptf1a^{EG} expression vector pMiWIII-Myc-Ptf1a^{EG} and harvested 24 hours later. Ptf1a^{EG} was electroporated in increasing concentrations shown from left to right (A-E). Electroporated side is to the right in all panels. Ectopic Lhx1/5 positive cells were found even at the lowest concentration of Ptf1a^{EG} tested. Lhx1/5 positive cells were located throughout the dorsal ventral axis and found within the ventricular zone, a region that normally does not express markers of differentiation.

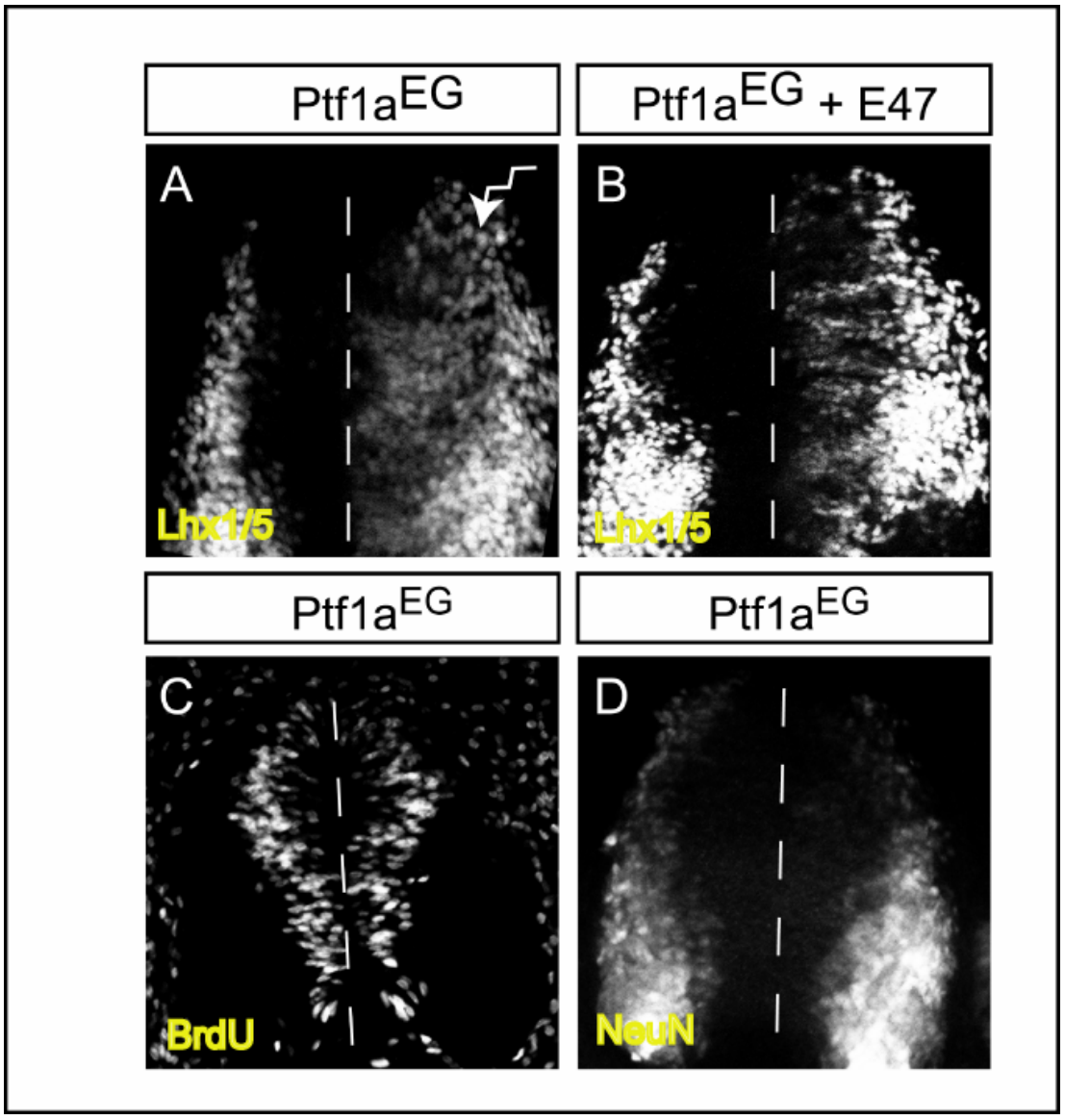


Figure 5.4 Ptf1a^{EG} activity is not a result of sequestering E-proteins.

Figure 5.4 Ptf1a^{EG} activity is not a result of sequestering E-proteins. HH 13-15 stage chick embryos were electroporated with the Ptf1a^{EG} expression vector pMiWIII-Myc-Ptf1a^{EG} and harvested 72 hours later. Electroporated side is to the right in all panels. Later stage embryos electroporated with Ptf1a^{EG} show the same phenotype as embryos harvested at 24 hours (A). (B)Addition of an E-protein, E47, did not alleviate the ectopic expression of Lhx1/5, indicating that Ptf1a^{EG} does not sequester E-proteins in this assay. No difference in BrdU or NeuN expression was observed between electroporated and control sides of the embryos (C-D).

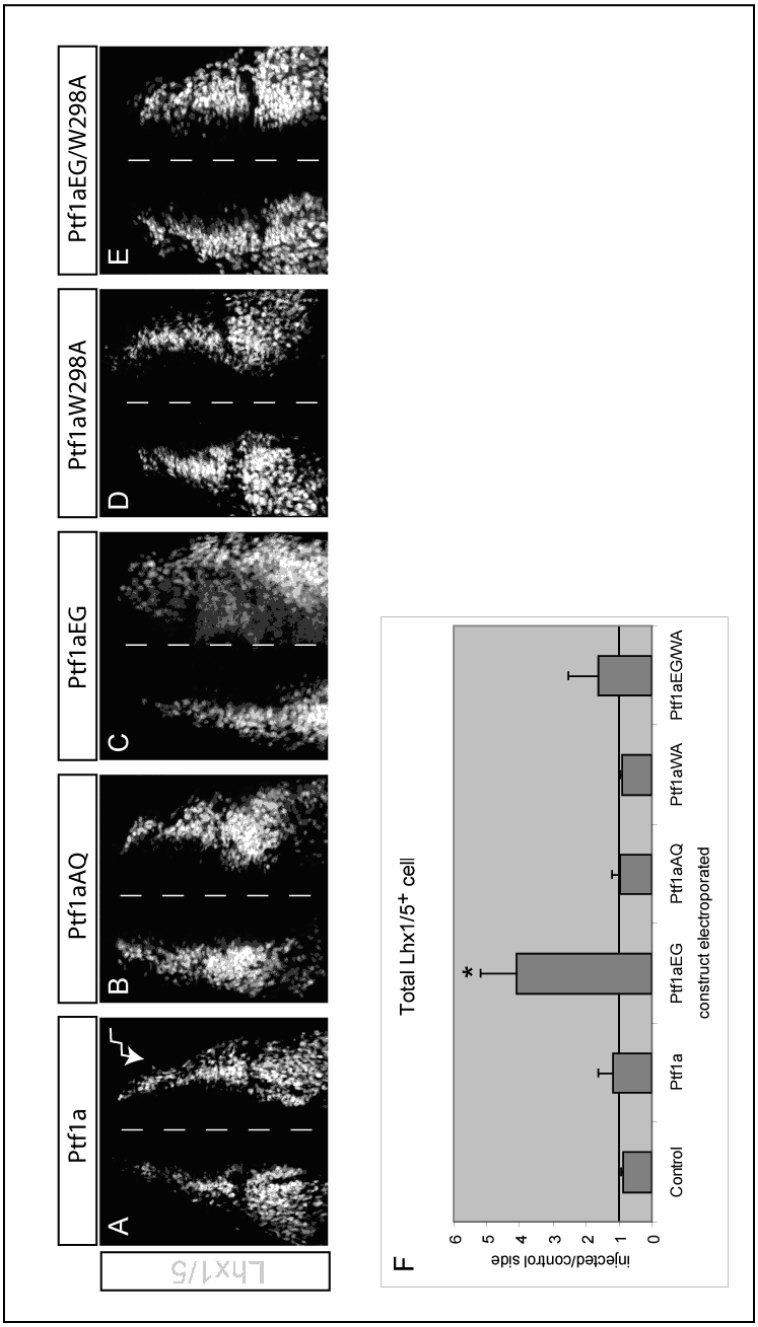


Figure 5.5 PTF1 trimer formation is required for ectopic Lhx1/5.

Figure 5.5 PTF1 trimer formation is required for ectopic Lhx1/5. HH 13-15 stage chick embryos were electroporated with the various Ptf1a expression vectors and harvested 72 hours later. Electroporated side is to the right in all panels. (A) Over-expression of Ptf1a had no affect on Lhx1/5 expression. (B) Electroporation of a DNA binding mutant, $Ptf1a^{AQ}$ did not affect Lhx1/5 expression. $Ptf1a^{EG}$, on the other had dramatically increased expression of Lhx1/5 (C). Over-expression of a Ptf1a mutant that cannot bind Rbpsuh, $Ptf1a^{W298A}$, did not result in a change in Lhx1/5 expression; indicating that the binding of Rbpsuh is critical to Ptf1a activity. Forced expression of a compound mutant, $Ptf1a^{EG/W298A}$, which cannot bind DNA or Rbpsuh abolished $Ptf1a^{EG}$ activity (E). (F) Quantification of the Lhx1/5 positive cells. A ratio between injected versus control side is graphed so that a value above one is an increase in expression while a value less that one is a decrease in expression. * indicated $p<0.001$.

CHAPTER SIX

Discussion

Ptf1a in Neurotransmitter Phenotype

This work demonstrates the requirement of the bHLH transcription factor, PTF1a, for the overwhelming majority of GABAergic neurons present in the dorsal spinal cord (Fig 4.3). GABAergic neurons in the dorsal horn produce presynaptic inhibition of primary sensory afferents, and thus, represent a major gatekeeper for the strength of sensory input to the spinal cord (Dickenson, 2002; Lu and Perl, 2003; Lu and Perl, 2005; Rudomin and Schmidt, 1999). The functional importance of these local circuit inhibitory neurons is exemplified in disorders such as those seen in peripheral nerve injury in which unregulated pain responses (allodynia) are associated with the loss of inhibitory activity of GABAergic neurons (Drew et al., 2004; Ibuki et al., 1997; Wiesenfeld-Hallin et al., 1997). The imbalance of inhibitory and excitatory neuronal activity can result in sensory disorders such as hyper- or hypo-algesia. This concept is illustrated by studies which perturb the balance of inhibition to excitation in the spinal cord. For example, wildtype animals pharmacologically treated with GABA antagonist develop allodynia (Drew et al., 2004). In addition, transgenic mice which lack the GABA receptor, $GABA_A$, display hyperalgesia and symptoms of allodynia. In both cases the pain disorders could be ameliorated with administration of GABA agonist; demonstrating these aberrant pain response are due to a deregulation of dorsal horn inhibition (Drew et al., 2004). The inhibitory, or GABAergic, neurons of lamina I-III are thought to contribute to neuropathic pain (Polgar et al., 2005). Loss of GABAergic activity upon neuropathic injury increases dorsal horn excitability in rat models. The mechanical

hypersensitivity observed upon nerve injury is alleviated with GABA treatment (Baba et al., 2003).

A delicate balance between the inhibitory and excitatory circuitry is clearly exemplified in pain disorders. However, maintaining a proper balance, or more precisely the tempering of glutamatergic excitatory activity, has been suggested to play an important role in brain disorders such as epilepsy, schizophrenia, autism, and anxiety (Levitt, 2005). Mice which have a 50% reduction in GABAergic interneurons in the cortex have increased anxiety and are less likely to socialize with other mice (Eagleson et al., 2005; Levitt, 2005). Moreover, the decrease in GABA expression and the concomitant increase in excitability lead to spontaneous seizures in these mice (Eagleson et al., 2005). While Ptf1a expression is not found in the developing telencephalon (this study) other bHLH factors, Mash1 and Ngn2, are expressed in this region and have been shown to play important roles in the specification of telencephalic GABAergic and glutamatergic neurons, respectively (Casarosa et al., 1999; Fode et al., 2000; Schuurmans et al., 2004). The role of bHLH factors in specification of NT phenotype has been reported in the telencephalon, cerebellum, PNS, and now in the spinal cord. This suggests that bHLHs are constituents of a conserved developmental pathway for the specification of NT phenotype.

Human patients which express a truncated form of Ptf1a have increased cerebral excitability, suggesting that Ptf1a has an important developmental role in suppressing this activity (Hoveyda et al., 1999; Sellick et al., 2004). As we have shown, Ptf1a is strictly required for the formation of GABAergic neurons of the dorsal horn and Hoshino *et al*

demonstrated the importance of Ptf1a in the formation of GABAergic neurons of the cerebellum - it is therefore likely that the hyper-excitability observed in Ptf1a mutant patients is a reflection of the loss of GABAergic activity (Glasgow et al., 2005; Hoshino et al., 2005). Lmx1b, a homeodomain transcription factor involved in the specification of glutamatergic neurons of the dorsal horn, has recently been identified as the causative mutation in Nail Patella Syndrome (NPS) (Cheng et al., 2004; Dunston et al., 2005a; Dunston et al., 2005b). While NPS is characterized by dysplasia of the nails and elbow, combined with the absence of patellae, patients often describe burning sensations of the limbs and present with diminished pain responses; suggesting that deregulation of glutamatergic activity is involved in these dysfunctions. Cumulatively these findings re-enforce the idea that embryonically expressed bHLH and HD transcription factors are vital to the proper formation and connectivity of the somatosensory system.

The transcription factors, including Ptf1a, that mediate the development of NT phenotype in the dorsal spinal cord are expressed in post-mitotic cells. Moreover, the switch in NT phenotype observed in Tlx1/3 double mutants and the Ptf1a mutant suggests that the neurons are retaining some plasticity. Yet the question is how much plasticity do they retain? Most neurons express a restricted set of neurotransmitters and at times may release more then one neurotransmitter. Some neurons of the nervous system, particularly in the PNS, have been shown to switch their NT phenotype. For example, sympathetic neurons in the rodent sweat glands have been shown to switch their NT from noradrenergic to cholinergic during development (Habecker et al., 2002). In the developing auditory pathway, GABAergic

neurons of the lateral superior olive switch to glycinergic transmission (Nabekura et al., 2004). Thus, it appears that neurons do retain the ability to switch the neurotransmitter they release. Evidence for this phenomenon in the CNS is just beginning to emerge, providing evidence that NT switching can occur in this system.

Data presented in this study and the work of many others provide definitive evidence that NT fate is "hard wired" or directly tied to a genetic program (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005; Goridis and Brunet, 1999; Goulding, 2004; Hendricks et al., 2003; Pattyn et al., 1999). However, recent evidence challenges these views. Borodinsky *et al* provide provocative evidence that calcium spike activity is required for the correct expression of neurotransmitters in the Xenopus developing spinal cord (Borodinsky et al., 2004; Spitzer et al., 2004). In this study the level of Ca^{2+} signaling was modified by the expression of different ion channels. The modification in Ca^{2+} patterns within the cells caused the cells to switch their NT; surprisingly, the markers of cell identity were unaltered (Borodinsky et al., 2004). Whether the expression of ion channels is directly linked to transcription factors like Ptf1a and Tlx1/3 that specify neurons which are GABAergic or glutamatergic, or if channel expression is completely unrelated to these transcription factors remains to be determined. Interpretations should be cautioned as these experiments are performed in *Xenopus* which undergo a primary and secondary neurogenesis. Primary neurogenesis is involved in swimming and escape reflexes in the tadpole. These processes are important for survival therefore circuits must be refined rapidly. Higher order vertebrates do not undergo this process. Nevertheless, it appears that neuronal activity can regulate

neurotransmitter expression and suggests that transcription factors progressively refine the developmental potential of neuronal populations, which can be further modulated by ion levels within the cells.

Neuronally expressed ion channels and the resultant influx or efflux of ions can have profound affects on neurotransmitters. Many developing regions of the CNS have been shown to have episodes of spontaneous activity including the brain, spinal cord, and retina (Ben-Ari, 2002; Crair, 1999; Feller, 1999; Goulding and Pfaff, 2005; Penn and Shatz, 1999; Rivera et al., 2005; Zhang and Poo, 2001). This work has culminated in the current view that activity is vital to the wiring of neuronal circuits and many believe that this activity is mediated through GABA. Early in development GABA acts in an excitatory fashion. As development progresses GABA assumes inhibitory activity (Wu, 1992). This functional switch from depolarizing to hyperpolarizing GABA activity was found to take place as episodes of spontaneous activity receded in the rat hippocampus (Ben-Ari et al., 1989). In the rodent hippocampus the change of GABAergic cells from excitatory to inhibitory is believed to be modulated by Cl^- levels (Rivera et al., 2005). Many speculate that the GABAergic excitation is the driving force behind the spontaneously generated activity in developing neuronal networks.

Within the spinal cord, locomotion and respiration are the primary focus of rhythmic spontaneous activity study. Administration of $GABA_A$ receptor antagonist resulted in aberrant motor neuron pathfinding and a decrease in spontaneous activity; demonstrating a role for GABA in spontaneous activity and pathfinding in this system (Hanson and

Landmesser, 2003; Hanson and Landmesser, 2004; Milner and Landmesser, 1999). Spontaneous activity has also been observed in the hindbrain, a region important for respiration. This activity is critical for proper development of respiratory circuits (Goulding and Pfaff, 2005). Respiratory failure has been cited as the cause of death, within hours of birth, for Tlx1/3 double mutants. Electrophysiological recordings determined that there was increased inhibition in the hindbrains of Tlx1/3 null mice resulting from the lack of glutamatergic neurons in this mutant (Cheng et al., 2004). Ptf1a is also expressed in the hindbrain (data not shown) and by extension of the dorsal spinal cord results, Ptfe1a is required for the development of GABAergic neurons of the hindbrain. Ptf1a null mice, like other bHLH mutant mice, die within three hours of birth. Patients born with mutant PTF1a have difficulty breathing, have episodes of apnea, and must be placed on ventilators shortly after birth (Hoveyda et al., 1999). It is probable that Ptf1a mutant mice, Ptf1a patients, and Tlx1/3 null mice all share a common dysfunction - an imbalance between GABAergic and glutamatergic activity. Little is known about the circuits involved in spontaneously generated activity. Likewise, knowledge of neural circuit formation within the spinal cord is just beginning to flourish. Determining how the different types of neurons that comprise mature circuits form, in particular the role that bHLH factors play in this process is fundamental to understanding the development of neuronal circuits that modulate responses to both sensory input and various CNS disorders. Thus, it appears that transcription factors render cells competent to proceed down a specific developmental pathway while local signals

and electrical activity propagate the cell fate decision and modulate the eventual NT phenotype of the cell.

Ptf1a is a binary switch between GABAergic and Glutamatergic cell fates

GABAergic and glutamatergic neurons appear to be alternative fate choices in the dorsal neural tube, and their development is genetically linked through the function of transcription factors such as PTF1a, Tlx1/3, Lbx1, and Pax2 (Cheng et al., 2004; Gross et al., 2002; Müller et al., 2002). Results presented here demonstrate that PTF1a is in post-mitotic cells of the dorsal neural tube. Therefore, PTF1a has selector function opposite to Tlx1/3; it is required for the generation of dI4 and dIL^{A} fates, which largely mature into GABAergic neurons, and it suppresses the alternative fates, dI5 and dIL^{B}, which largely form glutamatergic neurons. Recently it was proposed that the HD transcription factors Tlx1 and Tlx3 serve as selector genes biasing choice of glutamatergic over GABAergic cell fates in the dorsal horn (Cheng et al., 2004). This may at least be in part due to their inhibition of *Pax2* expression since Pax2 is required for the formation of GABAergic neurons in the dorsal horn, and in the Tlx1/3 double mutant, Pax2 is dramatically increased (Cheng et al., 2004). Pax2 does not appear to have selector function as there is no concomitant increase in glutamatergic neurons in *Pax2* null embryos (Cheng et al., 2004).

Another HD factor Lbx1, is also required for generating the correct numbers of GABAergic neurons (Gross et al., 2002; Müller et al., 2002). Cheng *et al* speculate that Lbx1 itself is the opposing binary switch (together with Tlx1/3) between glutamatergic and GABAergic cell fates in the dorsal horn (Cheng et al., 2005). The authors confirmed that

GABAergic neurons are markedly reduced in Lbx1 null embryos, and showed that glutamatergic neurons were increased (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002). However, in the absence of Lbx1 the Tlx genes are not affected leading the authors to concluded that the suppressive activity of Lbx1 on glutamatergic neurons is independent of Tlx 1/3(Cheng et al., 2005). Yet, this argument inherently implies that Lbx1 and Tlx1/3 are not directly opposing switches as Lbx1 lacks the ability to suppress Tlx1/3 expression; this is in contrast to Ptf1a which does possess the ability to negatively influence Tlx3 expression (this study) making it a more likely candidate for a binary switch with Tlx1/3 in the specification of NT phenotype. Notably, the absence of Lbx1 has been shown to result in an increase in the HD factors Islet1/2 (Müller et al., 2002). While Islet1/2 have not been attributed a transmitter phenotype, the increase in this population in Lbx1 null embryos could conceivably be the source of increased glutamatergic neurons in this mutant. Interestingly, Lbx1 mutant mice have increased cell death beginning at E13.5, with an apparently larger proportion of dIL^{B} than dIL^{A} neurons undergoing programmed cell death (Gross et al., 2002). Moreover, the post-mitotic factors Ptf1a and Tlx3 are co-expressed with Lbx1 in dIL^{A} and dIL^{B}, respectively. Perhaps the role of Lbx1 in the embryonic spinal cord is two-fold: to initially participate in specification of both dIL^{A} and dIL^{B} neurons and then later to maintain their survival. Equally probable is that mutant cells are simply eliminated as a consequence of incorrect connections and aberrant migration patterns; although these concepts have not been tested. Moreover, the effect of loss of Tlx3 or Lbx1 (or in combination) on Ptf1a expression has not been investigated and may provide important information on the

relationship between these factors. In combination, these data suggest that Ptf1a and Tlx3 act as opposing switches to specify two cell fates within the dorsal spinal cord which can be distinguished by their neurotransmitter phenotype.

The function of Ptf1a in switching cell fates in the dorsal spinal cord is similar to the role attributed to Ptf1a in pancreatic development. Inactivation of Ptf1a switches progenitor cells from pancreatic lineages to duodenal lineages (Kawaguchi et al., 2002). The identification of a bHLH factor, Ptf1a, which specifies GABAergic neurons while suppressing the alternative glutamatergic fate, is a novel finding for the dorsal neural tube. In the cortex the ability of bHLH factors to specify neurotransmitter phenotype as been established. In the developing ventral telencephalon, Mash1 is necessary and sufficient to specify GABAergic neurons (Casarosa et al., 1999; Fode et al., 2000). Ngn1 and Ngn2 on the other hand, are required for the formation of glutamatergic neurons in the dorsal telencephalon (Fode et al., 2000; Schuurmans et al., 2004). Furthermore, Mash1 and Ngn2 have defined roles in the specification of serotonergic neurons (5-HT), and dopaminergic neurons, respectively (Hirsch et al., 1998; Kele et al., 2006; Pattyn et al., 1999).

The avalanche of recent attention focused on the relationship between transcription factors and neurotransmitter phenotype has revealed roles for both homeodomain and bHLH factors in the specification of this vital component of neuron maturity. The identification of the dIL^A and dIL^B populations in the dorsal neural tube has unleashed a plethora of studies on the development of neurotransmitter phenotype. Yet, it is prudent to remember that the identification of populations which give rise to GABAergic and glutamatergic neurons is

rudimentary and relatively new in the dorsal neural tube. Many cells within the developing dorsal horn have not been attributed a neurotransmitter phenotype, for example Islet1/2 remain uncoupled to a NT phenotype. Furthermore, early born neurons (dI1-dI6) are equally lacking in NT assignment. Nor is the eventual location of these neurons in the dorsal horn clearly defined. The gap in knowledge pertaining to the settlement of dorsal interneurons in the dorsal horn is more striking with respect to the dI1-dI6 populations. This disparity is made more apparent by the discovery that the V1 interneuron population in the ventral spinal cord gives rise to four different groups of cells which express four different NTs in the adult ventral horn (Alvarez et al., 2005); demonstrating the diversity of neurotransmitter phenotypes that are derived from a single cell HD factor defined population. Consequently, studies are limited by the current knowledge of the field; in this case, we are restricted by the definition/identification of neuron populations. Nonetheless, significant progress has been made in identifying transcription factors which drive cells down a developmental program leading to a particular neurotransmitter phenotype.

PTF1a in the specification of early born interneurons

Efforts to unravel the transcription factor code for the specification of spinal cord neurons have revealed a complex interplay of spatial and temporal control between bHLH and HD transcriptions factors (Caspary and Anderson, 2003; Lee and Pfaff, 2001). The bHLH transcription factors present in the ventricular zone form the basis for the code for dI1 to dI5 in the dorsal neural tube. Math1 and Ngn1/2 specify the two most dorsal interneuron populations, dI1 and dI2 (Bermingham et al., 2001; Gowan et al., 2001). Mash1 specifies dI3

and dI5 neurons (Helms et al., 2005), while PTF1a is required for dI4 (Glasgow et al., 2005). The function of Mash1 and PTF1a in dI3-dI5 is consistent with their expression patterns. PTF1a is present in the central region of the Mash1 domain, a region that contains cells with the lowest Mash1 expression. Indeed, using an in vivo recombination based lineage tracing paradigm Mash1 efficiently traces to dI3 and dI5, but not to dI4 neurons (Helms et al., 2005), while PTF1a traces to dI4. Further refinement of the code is seen with co-expression of bHLH factors such as Mash1 and Olig3 (Muller et al., 2005), which specify the dI3 fate, while Mash1 alone, or with another factor such as the HD factor Lbx1, specify dI5 (Helms et al., 2005). The fate switch of the dI4 to dI5 rather than dI3 in the PTF1a null may reflect the presence of Mash1 and Lbx1 in these cells. HD factors in addition to Lbx1, including Msx3 and Gsh1/2, also influence the transcription factor code, likely through regulation of bHLH expression (Kriks et al., 2005; Liu et al., 2004). Taken together, it is clear that neural specific bHLH factors, combined with HD factors, play an important role in obtaining the neuronal diversity seen in the mature spinal cord.

bHLH factors are required for the formation of several structures of the nervous system, including the spinal cord (Bertrand et al., 2002). As discussed above, bHLH factors are required for there formation of specific interneurons populations within the spinal cord, but also in other regions of the nervous system (Ben-Arie et al., 1997; Bermingham et al., 2001; Bertrand et al., 2002; Fode et al., 1998; Fode et al., 2000; Gowan et al., 2001; Guillemot et al., 1993). There roles in cell-type specification have been well documented. In addition to their roles in specification, bHLH factors also have a well established role in

neural differentiation (Akazawa et al., 1995; Cai et al., 2000; Cepko, 1999; Farah et al., 2000; Gradwohl et al., 1996; Guillemot et al., 1993; Lee et al., 1995; Ma et al., 1996; Shimizu et al., 1995). Emerging evidence has suggested that the two bHLH factor functions, differentiation and specification, can be attributed to different regions of the protein (Huang et al., 2000; Nakada et al., 2004; Talikka et al., 2002). Studies in *Xenopus* illustrate that specific targets are differentially activated by Helix1 of the HLH domain of Xash1 (Mash1 homolog) and X-Ngnr1 (Ngn1 homolog) (Talikka et al., 2002). Expanding upon this idea, Nakada *et al*. demonstrated that different regions of the HLH region modulate different functional outputs of the bHLHs Mash1 and Math1. For instance, Helix1 of Mash1 confers neuronal differentiation activity to the protein, while Helix1 and Helix2 are necessary for specification activity in the developing chick neural tube (Nakada et al., 2004). The HLH region of these factors is commonly involved in protein-protein interactions, typically with E-proteins. Therefore, it is likely that the HLH domain confers specificity by interacting with cofactors in a context dependent manner. In the neocortex, pyramidal neurons are specified by Ngn2 (Schuurmans et al., 2004). The ability of Ngn2 to promote the migration by inducing a polarized leading process is dependent on phosphorylation of tyrosine 241(Y421) in the c-terminal of the protein (Hand et al., 2005). Eliminating this phosphorylation activity, however, does not affect the ability of Ngn1/2 to induce differentiation of these neurons. Taken together, new evidence is emerging that points to the importance of regions outside of the bHLH region, and likely protein-protein interactions, in the activity of these factors.

While Ptf1a has a role in specification of neuronal identity in the cerebellum, pancreas, and neural tube, Ptf1a has not been shown to drive differentiation in these systems (Glasgow et al., 2005; Hoshino et al., 2005; Kawaguchi et al., 2002; Rose et al., 2001). The inability of Ptf1a to induce differentiation is likely to involve the cell-cycle status of the Ptf1a expressing progenitors. Ptf1a is not detected in actively proliferative cells in the neural tube and pancreas but is in post-mitotic cells. As Ptf1a is co-expressed with Mash1 in the neural tube and Mash1 is required but not sufficient to induce Ptf1a expression, Mash1 is likely a differentiation factor in cells that express Ptf1a(Glasgow et al., 2005). In addition, Ptf1a has a unique protein-protein interaction ability to interact with Rbpsuh and Hes family members Hey1 and Hey2 (Ghosh and Leach, 2006; Obata et al., 2001). This interaction with Rbpsuh is mediated by information provided in the C-terminal region, outside of the bHLH domain (Beres et al., 2006). Ptf1a, similar to other bHLH factors, likely has differential functions mediated by various regions of the protein.

The Ptf1 complex in CNS development

PTF1a is a Twist-like bHLH factor that can heterodimerize with E-proteins such as E47, and bind e-box containing DNA (Beres et al., 2006; Obata et al., 2001). However, it is unique in the Class A bHLH family in that it also directly interacts with Rbpsuh, a transcriptional effector of the Notch signaling pathway (Beres et al., 2006; Obata et al., 2001). These different protein-protein and protein-DNA interactions suggest a variety of mechanisms of action for PTF1a, including many of those mechanisms previously described

for selector gene function (Mann and Carroll, 2002). For example, similar to Twist function, heterodimerization with E-proteins could sequester this shared partner from other class A bHLH factors such as Mash1. Depending on the transcriptional activity of these different heterodimers, the consequence could be either an increase or decrease in specific target expression. Furthermore, by forming a trimer with Rbpsuh and E-protein (Beres et al., 2006), this complex not only has altered DNA target recognition, but may also directly impact Notch signaling through an as yet unidentified mechanism. The bioinformatical characterization of neuronal targets of the Ptf1a complex suggests that different target have divergent recognition capabilities; where one target may exclusively bind the PTF1 complex while an other target may bind PTF1 complex and Rbpsuh. Combining these different protein-protein interactions together with complex arrangement of cis-elements on transcriptional targets, make PTF1a nicely suited for complex interactions required for generating the appropriate number of specific neuronal cell-types.

Overexpression of PTF1a in the chick neural tube is not sufficient to specify a neuronal population. Nonetheless, mis-expression of PTF1a in the dorsal telencephalon conferred a GABAergic phenotype to the neurons generated (Hoshino et al., 2005) supporting a role for PTF1a in determining GABAergic neuronal cell fates. The possible competing roles for Ptf1a in different complexes make overexpression paradigms difficult to interpret. Wildtype Ptf1a has been show to bind to both pancreatic and neural target genes as a dimer and also as a trimer in gel shift assays. In an over-expression assay, a factor like Ptf1a which can participate in different complex would form both complexes. E-protein

levels are abundant allowing for many dimer complexes to form. Heterotrimeric complex may form at lower levels if Rbpsuh is limiting, thereby shifting the balance toward dimer formation rather than trimer formation. Dimer function has not been elucidated; however a likely event is that bound dimer blocks the binding of the trimeric complex to the target sequence. Consequently, the relative proportion of trimer to dimer would dictate the outcome of the assay (Fig 6.1A). While not statistically significant, the variablitiy between embryos when Ptf1a was over-expressed with respect to an increase in Lhx1/5 expression may reflect variations in the balance between the trimer and dimer complex

Intriguingly, over-expression of Ptf1a with a mutation in the basic region ($Ptf1a^{EG}$) led to the ectopic expression of Lhx1/5 (Fig 5.3). The induction of Lhx1/5 by $Ptf1a^{EG}$ is not likely to result from sequestering either E-protein as overexpression of $Ptf1a^{EG}$ in conjunction with E-protein does not alleviate the phenotype (Fig 5.3A-B). Moreover, overexpression of $Ptf1a^{EG/W298A}$ did not affect the expression of Lhx1/5 making it unlikely that $Ptf1a^{EG}$ is exerting its activity by preventing Rbpsuh from binding its normal targets. Furthermore, gel shift data demonstrate that $Ptf1a^{EG}$ is unable to bind as a dimer with E-protein; instead it preferentially forms a trimeric complex. In fact, $Ptf1a^{EG}$ appears to bind as a trimer more efficiently than wildtype Ptf1a on a neural target, Ngn2 (RM Henke, unpublished). Therefore, $Ptf1a^{EG}$ is free from competition from the heterodimer and can exert its function without impediment (Fig 6.1B). Together these data indicate that the PTF1 complex acts on neural targets and that the outcome of this activity is likely tied to a balance between Ptf1a as a component of a trimer or dimer complex.

Future Directions

These studies determined the absolute requirement for Ptf1a in the specification of dI4, dIL^{A}, and GABAergic neurons. Moreover, over-expression assays in the chick have begun to decipher the mechanism in which Ptf1a exerts its functions in the neural tube. Nonetheless, these data raise several questions about the functional role of Ptf1a in neural development.

While studies in pancreas have determined an important role for the PTF1 complex in the regulation of several pancreatic targets, the identification of neuronal downstream targets will be vital to understanding PTF1 complex function in the nervous system. This is particularly important since preliminary evidence with $Ptf1a^{EG}$ indicates that the $Ptf1a^{EG}$-E-protein-Rbpsuh complex has different binding properties depending on the target sequence (RM Henke, unpublished). Specifically, the $Ptf1a^{EG}$-E-protein-Rbpsuh complex binds efficiently an enhancer element in the neural target Ngn2 (Ch.5).

Current studies in the lab are now focused on Ngn2 as a downstream target of the PTF1 complex. Ngn2 is a particularly attractive target as it is co-expressed with Ptf1a in a subset of cells in the neural tube. Furthermore, a Ngn2 enhancer has been previously characterized and the predicted PTF1 binding sites fall within this enhancer segment. Mutational analysis of PTF1 binding sites within the enhancer, both in transgenic mice and gel shift assays together with Ptf1a construct over-expression studies in chick, will provide evidence for an important role for the PTF1 complex in regulating Ngn2 expression.

RM Henke has bioinformatically identified several other putative down stream targets of the PTF1 complex including Pax2, Lhx1, and Lhx5. Further studies of these three HD transcription factors is warranted as they are dependent on Ptf1a for expression as determined by Ptf1a loss of function studies (Ch 3 and 4). While the enhancer regions for these factors have not been fully characterized, it will be important to map PTF1 binding sites to regions flanking these genes and determine if they are conserved across multiple species. The binding properties of the PTF1 complex lend themselves to a bioinformatical approach to identify downstream targets; as more targets are identified and characterized refinements can be made to the bioinformatics searches to improve the reliability of predicting targets.

This study and those in the pancreas have provided evidence that Ptf1a acts together with E-proteins and Rbpsuh in a trimeric complex to affect transcription of downstream targets. Recently, Ray MacDonald's laboratory has generated a Ptf1a^{W298A} knockin mouse. The Ptf1a^{W298A} (Fig 5.1) mutation leads to a severe reduction in the binding of Rbpsuh to Ptf1a. These mutant mice are being utilized to further characterize the PTF1 complex in the nervous system. Many avenues can be pursued with this mutant line but of particular interest are the dependency of the dI4, dIL^{A}, and GABAergic neurons in the dorsal spinal cord and cerebellum, on the interaction of Ptf1a with Rbpsuh. Furthermore, analysis of this mutant will provide insight into whether the Ptf1a-E protein heterodimer is sufficient to specify any of these populations as the heterodimer would be the predominant complex formed in the Ptf1a^{W298A} mice.

In summary, several avenues exist for further elucidation of the role of the PTF1 complex in neural development. Studies of this kind will further refine the transcription factor code which determines the fate of differentiating cells. Ultimately these studies defining the molecular determinants of neuronal cell type specification are vital to stem cell biology and may have important implications in the regeneration (repopulation) of neurons in patients with spinal cord injury.

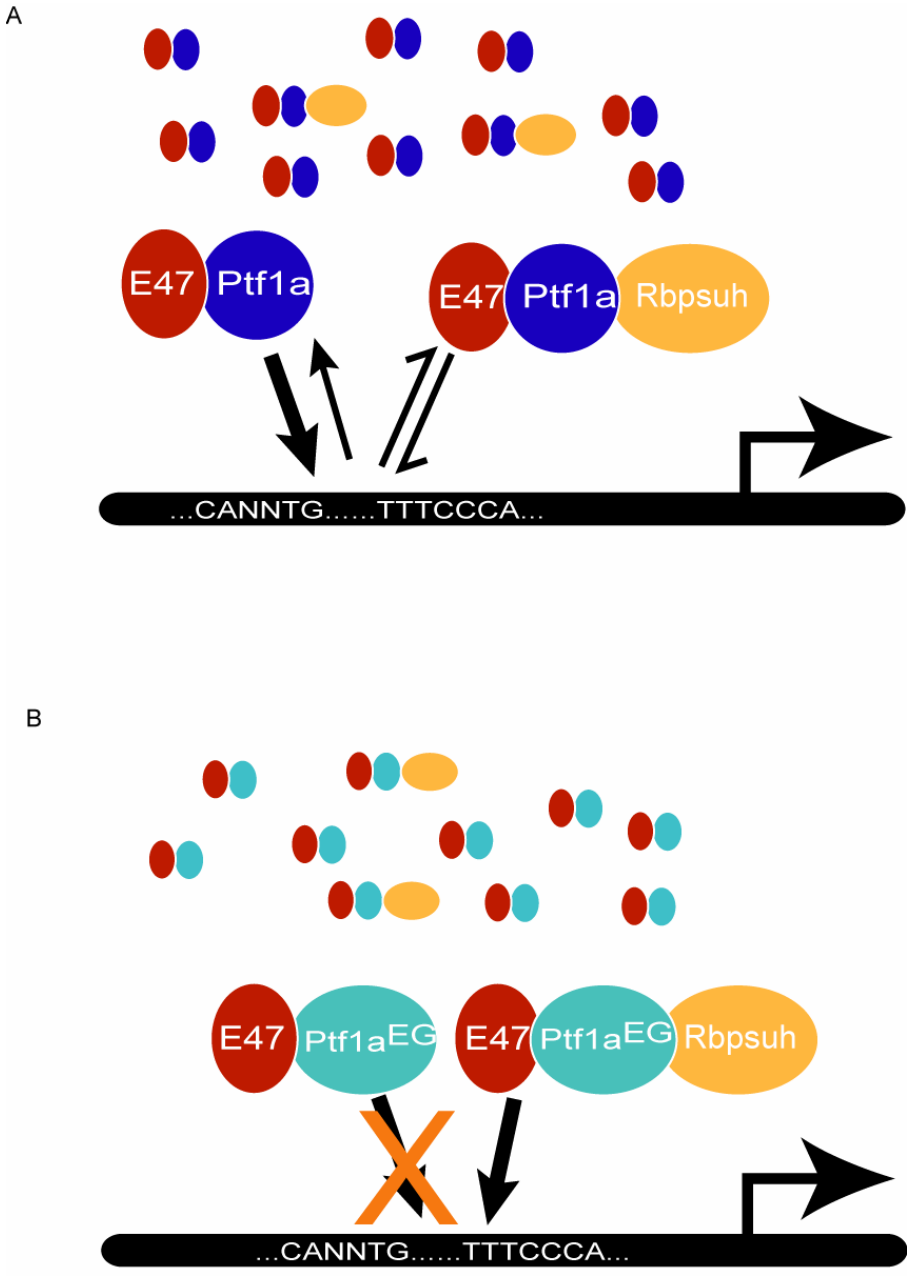


Figure 6.1 Model for PTF1a binding

Figure 6.1 Model for Ptf1a binding. (A) Wildtype Ptf1a can bind DNA as a heterodimer with E-protein or as a trimer with E-protein and Rbpsuh. When the site is occupied by the dimer it can block the binding of the trimeric complex. This competition creates a situation in which activity is dependent upon the ratio of dimer and trimer within the cell. (B) $Ptf1a^{EG}$ is unable to bind DNA in the heterodimer form. However, $Ptf1a^{EG}$ can bind to DNA as a trimer. Therefore, $Ptf1a^{EG}$ likely represents PTF1 trimer activity.

APPENDIX A

Ptf1a Expression Vectors

Glasgow Plasmids: Ptf1a

Plasmid Name: pMiWIII-Ptf1a

Gene: Ptf1a

Vector size: pMiWIII/6.1kb

Insert size: 984

Resistance: Amp

Cloning sites: Nco1/Xba1

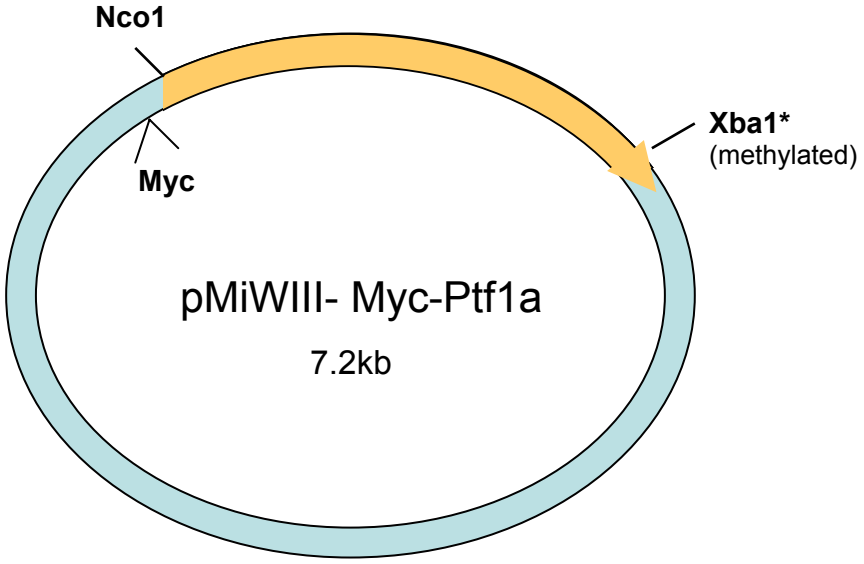
Primers: p485B: 5'-CTTTGGCCATGGACGCCGTACTCCTGGAGC-3'
P483C: 5'-GACCGGCTCTAGAGCCCGATGTGAAGCTGTC-3'

Template: psp73-P48

Constructed by: Stacey Glasgow, TB

Features: chick β-actin promoter, SV40 poly A, pUC18 backbone, Myc, (see a map of pMiWIII for more details).

Plasmid Use: chick electroporation



MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGDE
QAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHALAPPPLGDPGEP
EDNVSYCCDAGAPLAAFPYSPGSPPSCLAYPCAAVLSPGARLGGLN
GAAAAAAARRRRRVRSEAELQQLRQAANVRERRRMQSINDAFEGL
RSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAGGCG
GPGGSRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPLAGHS
LSWTDEKQLKEQNIIRTAKVWTPEDPRKLNSKSFDNIENEPP

Glasgow Plasmids: Ptf1aE169→G

Plasmid Name: pMiWIII -Ptf1a-E169→G

Gene: Ptf1a

Vector size: pMiWIII/6.1kb

Insert size: 984

Resistance: Amp

Cloning sites: Nco1/Xba1

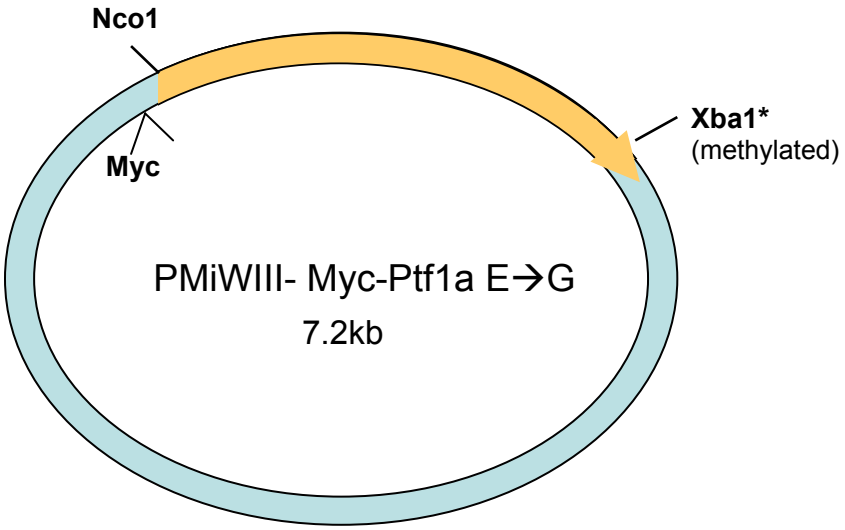
Primers: p485B: 5'-CTTTGGCCATGGACGCCGTACTCCTGGAGC-3'
P483C: 5'-GACCGGCTCTAGAGCCCGATGTGAAGCTGTC-3'

Template: psp73-P48E→G

Constructed by: SG, TB

Features: chick β-actin promoter, SV40 poly A, pUC18 backbone, Myc (see a map of pMiWIII for more details).

Plasmid Use: chick electroporation



MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGD
EQAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHALAPPPLGDPG
EPEDNVSYCCDAGAPLAAFPYSPGSPPSCLAYPCAAVLSPGARLG
GLNGAAAAAAARRRRRVRSEAELQQLRQAANVR**G**RRRMQSIND
AFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGS
GAGGCGGPGGSRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYG
LPPLAGHSLSWTDEKQLKEQNIIRTAKVWTPEDPRKLNSKSFDNIE
NEPP

Note: aa#169: gag → ggg. Disrupts dimer formation but not trimer formation when on DNA.

Glasgow Plasmids: Ptfa1 RR171-AQ

Plasmid Name: pMiWIII -Ptf1a-AQ

Gene: Ptf1a

Vector size: pMiWIII/6.1kb

Insert size: 984

Resistance: Amp

Cloning sites: Nco1/Xba1

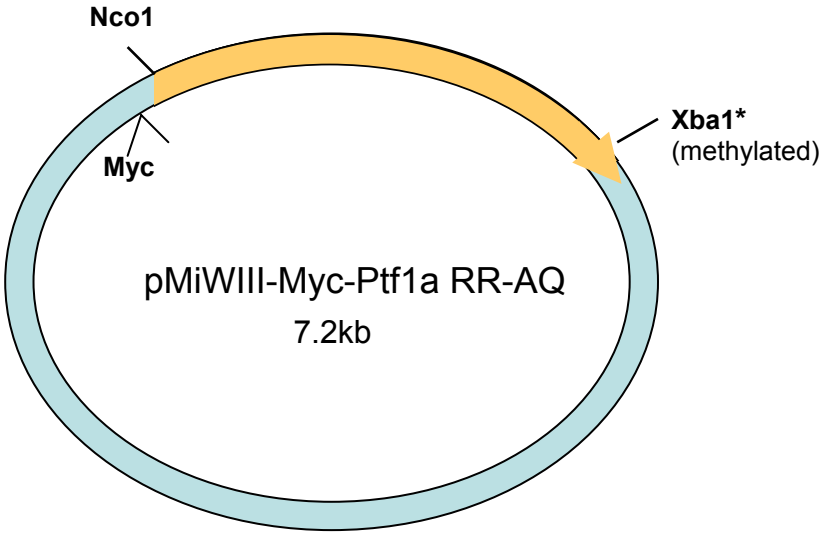
Primers: p485B: 5'-CTTTGGCCATGGACGCCGTACTCCTGGAGC-3'
P483C: 5'-GACCGGCTCTAGAGCCCGATGTGAAGCTGTC-3'

Template: psp73-P48AQ

Constructed by: SG, TB

Features: chick β-actin promoter, SV40 poly A, pUC18 backbone, Myc (see a map of pMiWIII for more details).

Plasmid Use: chick electroporation



MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGDE
QAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHALAPPPLGDPGE
PEDNVSYCCDAGAPLAAFPYSPGSPPSCLAYPCAAVLSPGARLGG
LNGAAAAAAARRRRRVRSEAELQQLRQAANVRER**AQ**MQSINDAF
EGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAG
GCGGPGGSRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPL
AGHSLSWTDEKQLKEQNIIRTAKVWTPEDPRKLNSKSFDNIENEPP

Note: aa#171,172. Considered a DNA binding mutation and protein-protein interaction mutant

Glasgow Plasmids: Ptf1aW280A

Plasmid Name: pMiWIII-Ptf1a-W280A

Gene: Ptf1a

Vector size: pMiWIII-NLS/6.1kb

Insert size: 984

Resistance: Amp

Cloning sites: Nco1/Xba1

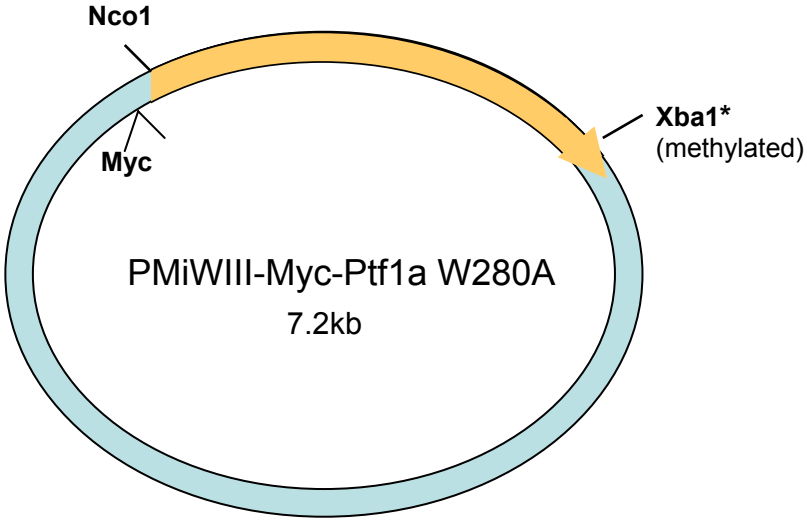
Primers: p485B: 5'-CTTTGGCCATGGACGCCGTACTCCTGGAGC-3'
P483C: 5'-GACCGGCTCTAGAGCCCGATGTGAAGCTGTC-3'

Template: psp73-P48 W280A

Constructed by: SG, TB

Features: chick β-actin promoter, SV40 poly A, pUC18 backbone, Myc (see a map of pMiWIII for more details).

Plasmid Use: chick electroporation



MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGDE
QAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHALAPPPLGDPGEP
EDNVSYCCDAGAPLAAFPYSPGSPPSCLAYPCAAVLSPGARLGGLN
GAAAAAAARRRRRVRSEAELQQLRQAANVRERRRMQSINDAFEGL
RSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAGGCG
GPGGSRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPLAGHS
LS**A**TDEKQLKEQNIIRTAKVWTPEDPRKLNSKSFDNIENEPP

Notes: aa#280: tgg → gcg. Cannot bind RBP-J-L and has roughly 50% reduced ability to bind RBP-Jκ.

Glasgow Plasmids: Ptf1a W298A

Plasmid Name: pMiWIII-Ptf1a-W298A

Gene: Ptf1a

Vector size: pMiWIII-NLS/6.1kb

Insert size: 984

Resistance: Amp

Cloning sites: Nco1/Xba1

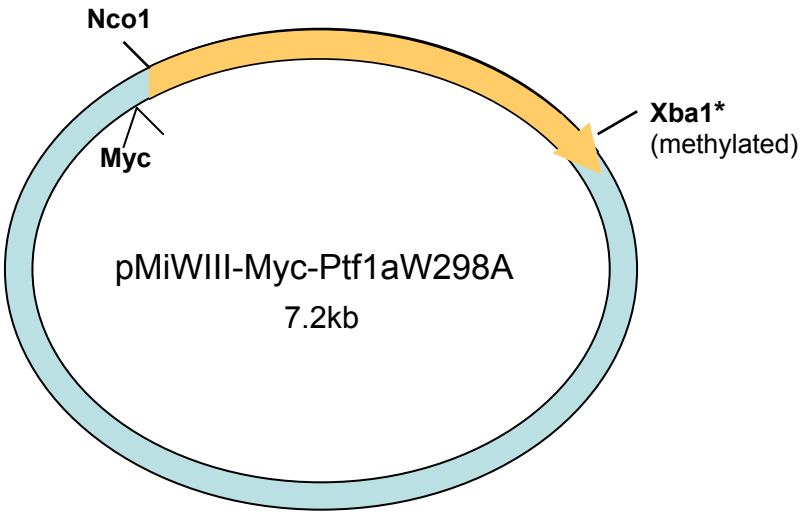
Primers: p485B: 5'-CTTTGGCCATGGACGCCGTACTCCTGGAGC-3'
P483C: 5'-GACCGGCTCTAGAGCCCGATGTGAAGCTGTC-3'

Template: psp73-P48 W298A

Constructed by: Stacey Glasgow, Tom Beres

Features: chick β-actin promoter, SV40 poly A, pUC18 backbone, Myc, (see a map of pMiWIII for more details).

Plasmid Use: chick electroporation



MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGDE
QAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHALAPPPLGDPGEP
EDNVSYCCDAGAPLAAFPYSPGSPPSCLAYPCAAVLSPGARLGGLN
GAAAAAAARRRRRVRSEAELQQLRQAANVRERRRMQSINDAFEGL
RSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAGGCG
GPGGSRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPLAGH
SLSWTDEKQLKEQNIIRTAKV**A**TPEDPRKLNSKSFDNIENEPP

Note: aa#298: tgg → gcg. Cannot bind RBP-Jκ but can still bind RBP-J-L.

APPENDIX B

Summary of Ptf1a Overexpression data

component1	component 2	component 3	Lhx1/5	Pax2	Gad1
Ptf1a			No change	No Change	No change
Ptf1aAQ	E47		No change	No change	No change
Ptf1a(Dll1)	E12		No change	No change	No change
Ptf1a(Dll1)	E47		No change	No change	No change
Ptf1a	E12	NICD	No change	No change	No change
Ptf1a	E12	VP-16 Rbpsuh	No change	No change	No change
Ptf1a	E47	VP-16 Rbpsuh	No change	No change	No change
Ptf1a	E47	NICD	No change	No change	decrease
Ptf1a	Mash1		No change	No change	No change
Ptf1a	Hes1		No change	No change	—
Ptf1a	Lbx		No change	No change	No change
Ptf1aEG			increase	—	—
Ptf1aEG	E47		increase	No change	—
Ptf1aEG	E47	VP-16 Rbpsuh	—	No change	—
Ptf1aEG/WA			No change	—	—
Ptf1aEG/WA	E47	VP-16 Rbpsuh	—	No change	—
Ptf1aEG/WA	E47		—	No change	—
Ptf1aEG/WA	VP-16 Rbpsuh		—	No change	—
Ptf1aWA			No change	—	—
Ptf1aWA	E47		No change	No change	—
Ptf1aAQ			No change	—	—

Ptf1a overexpression study summary. The plasmids injected whether single or in combination are listed. The chicks were injected HH 13-HH 15 and harvested 72 hours later. Pax2 and Lhx1/5 were analyzed by immunofluorscence while Gad1 expression was determined by in situ hybridization.

BIBLIOGRAPHY

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S., and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of the Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. J Biol Chem *270*, 8730-8738.

Altman, J., and Bayer, S. A. (1984). The development of the rat spinal cord. Advances in Anatomy Embryology and Cell Biology *85*, 1-166.

Altman, J., and Bayer, S. A. (2001). Development of the Human Spinal Cord, Oxford University Press).

Alvarez, F. J., Jonas, P. C., Sapir, T., Hartley, R., Berrocal, M. C., Geiman, E. J., Todd, A. J., and Goulding, M. (2005). Postnatal phenotype and localization of spinal cord V1 derived interneurons. J Comp Neurol *493*, 177-192.

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Attisano, L., and Wrana, J. L. (2000). Smads as transcriptional co-modulators. Curr Opin Cell Biol *12*, 235-243.

Austin, C. P., Feldman, D. E., Ida, J. A. J., and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development *121*, 3637-3650.

Ayer, D. E., Kretzner, L., and Eisenman, R. N. (1993). Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell *72*, 211-222.

Baba, H., Ji, R. R., Kohno, T., Moore, K. A., Ataka, T., Wakai, A., Okamoto, M., and Woolf, C. J. (2003). Removal of GABAergic inhibition facilitates polysynaptic A fiber-mediated excitatory transmission to the superficial spinal dorsal horn. Mol Cell Neurosci *24*, 818-830.

Baker, N. E. (2000). Notch signaling in the nervous system. Pieces still missing from the puzzle. Bioessays *22*, 264-273.

Bao, Z. Z., and Cepko, C. L. (1997). The expression and function of Notch pathway genes in the developing rat eye. J Neurosci *17*, 1425-1434.

Bartholoma, A., and Nave, K. A. (1994). NEX-1: a novel brain-specific helix-loop-helix protein with autoregulation and sustained expression in mature cortical neurons. Mech Dev *48*, 217-228.

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci *3*, 728-739.

Ben-Ari, Y., Cherubini, E., Corradetti, R., and Gaiarsa, J. L. (1989). Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol *416*, 303-325.

Ben-Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M., and Zoghbi, H. Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. Nature *390*, 169-172.

Ben-Arie, N., Hassan, B. A., Bermingham, N. A., Malicki, D. M., Armstrong, D., Matzuk, M., Bellen, H. J., and Zoghbi, H. (2000). Functional conservation of *atonal* and *Math1* in the CNS and PNS. Development *127*, 1039-1048.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell *61*, 49-59.

Beres, T. M., Masui, T., Swift, G. H., Shi, L., Henke, R. M., and Macdonald, R. J. (2006). PTF1 Is an Organ-Specific and Notch-Independent Basic Helix-Loop-Helix Complex Containing the Mammalian Suppressor of Hairless (RBP-J) or Its Paralogue, RBP-L. Mol Cell Biol *26*, 117-130.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A., and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. Science *284*, 1837-1841.

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B., and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on Math1. Neuron *30*, 411-422.

Bertrand, N., Castro, D. S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neuroscience *3*, 517-530.

Birren, S. J., Lo, L., and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development *119*, 597-610.

Blackwood, E. M., and Eisenman, R. N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science *251*, 1211-1217.

Blaugrund, E., Pham, T. D., Tennyson, V. M., Lo, L., Sommer, L., Anderson, D. J., and Gershon, M. D. (1996). Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence. *122*, 309-320.

Borodinsky, L. N., Root, C. M., Cronin, J. A., Sann, S. B., Gu, X., and Spitzer, N. C. (2004). Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. Nature *429*, 523-530.

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

Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. Current Opinion in Neurobiology *11*, 43-49.

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

Bush, A., Hiromi, Y., and Cole, M. (1996). Biparous: a novel bHLH gene expressed in neuronal and glial precursors in Drosophila [published erratum appears in Dev Biol 1999 Jan 15;205(2):332]. Developmental Biology (Orlando) *180*, 759-772.

Cai, L., Morrow, E. M., and Cepko, C. L. (2000). Misexpression of basic helix-loop-helix genes in the murine cerebral cortex affects cell fate choices and neuronal survival. Development *127*, 3021-3030.

Campos-Ortega, J. A., and Knust, E. (1990). Molecular analysis of a cellular decision during embryonic development of *Drosophila melanogaster*: epidermogenesis or neurogenesis. Eur J Biochem ***190***, 1-10.

Campuzano, S., and Modolell, J. (1992). Patterning of the *Drosophil*a nervous system: the *achaete-scute* gene complex. Trends Genet *8*, 202-208.

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525-534.

Caspary, T., and Anderson, K. V. (2003). Patterning cell types in the dorsal spinal cord: what the mouse mutants say. Nat Rev Neurosci *4*, 289-297.

Castanon, I., and Baylies, M. K. (2002). A Twist in fate: evolutionary comparison of Twist structure and function. Gene *287*, 11-22.

Cau, E., Casarosa, S., and Guillemot, F. (2002). *Mash1* and *Ngn1* control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development *129*, 1871-1880.

Cau, E., Gradwohl, G., Fode, C., and Guillemot, F. (1997). *Mash1* activates a cascade of bHLH regulators in olfactory neuron progenitors. Development *124*, 1611-1621.

Cepko, C. L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Current Opinion in Neurobiology *9*, 37-46.

Chambers, C. B., Peng, Y., Nguyen, H., Gaiano, N., Fishell, G., and Nye, J. S. (2001). Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. Development *128*, 689-702.

Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. Genes Dev *13*, 2218-2230.

Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B., and Ball, D. W. (1997). Conservation of the Drosophila lateral inhibition pathway in

human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proceedings of the National Academy of Sciences of the United States of America *94*, 5355-5360.

Chen, Z. F., and Behringer, R. R. (1995). twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes Dev *9*, 686-699.

Cheng, L., Arata, A., Mizuguchi, R., Qian, Y., Karunaratne, A., Gray, P. A., Arata, S., Shirasawa, S., Bouchard, M., Luo, P.*, et al.* (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci *7*, 510-517.

Cheng, L., Samad, O. A., Xu, Y., Mizuguchi, R., Luo, P., Shirasawa, S., Goulding, M., and Ma, Q. (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat Neurosci *8*, 1510-1515.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature *383*, 407-413.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Dorsophila neurogenic gene Delta. Nature *375*, 761-766.

Chitnis, A., and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. Development *122*, 2295-2301.

Chitnis, A. B. (1999). Control of neurogenesis--lessons from frogs, fish and flies. Curr Opin Neurobiol *9*, 18-25.

Chizhikov, V. V., and Millen, K. J. (2004). Control of roof plate formation by Lmx1a in the developing spinal cord. Development *131*, 2693-2705.

Cockell, M., Stevenson, B. J., Strubin, M., Hagenbuchle, O., and Wellauer, P. K. (1989). Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas. Mol Cell Biol *9*, 2464-2476.

Crair, M. C. (1999). Neuronal activity during development: permissive or instructive? Curr Opin Neurobiol *9*, 88-93.

Davidson, D. (1995). The function and evolution of Msx genes: pointers and paradoxes. Trends Genet *11*, 405-411.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J., and Conlon, R. A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. Development *124*, 1139-1148.

Dickenson, A. H. (2002). Gate control theory of pain stands the test of time. Br J Anaesth *88*, 755-757.

Dorsky, R. I., Rapaport, D. H., and Harris, W. A. (1995). Xotch inhibits cell differentiation in the Xenopus retina. Neuron *14*, 487-496.

Drew, G. M., Siddall, P. J., and Duggan, A. W. (2004). Mechanical allodynia following contusion injury of the rat spinal cord is associated with loss of GABAergic inhibition in the dorsal horn. Pain *109*, 379-388.

Dudley, A. T., and Robertson, E. J. (1997). Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. Dev Dyamics *208*, 349-362.

Dunston, J. A., Lin, S., Park, J. W., Malbroux, M., and McIntosh, I. (2005a). Phenotype severity and genetic variation at the disease locus: an investigation of nail dysplasia in the nail patella syndrome. Ann Hum Genet *69*, 1-8.

Dunston, J. A., Reimschisel, T., Ding, Y. Q., Sweeney, E., Johnson, R. L., Chen, Z. F., and McIntosh, I. (2005b). A neurological phenotype in nail patella syndrome (NPS) patients illuminated by studies of murine Lmx1b expression. Eur J Hum Genet *13*, 330-335.

Eagleson, K. L., Bonnin, A., and Levitt, P. (2005). Region- and age-specific deficits in gamma-aminobutyric acidergic neuron development in the telencephalon of the uPAR(-/-) mouse. J Comp Neurol *489*, 449-466.

Efstratiadis, A., Posakony, J. W., Maniatis, T., Lawn, R. M., O'Connell, C., R.A., S., DeRiel, J. K., and al., e. (1980). The structure and evolution of the human beta-globin gene family. Cell *21*, 653-668.

Ellenberger, T., Fass, D., Arnaud, M., and Harrison, S. (1994). Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. Genes & Development *8*, 970-980.

Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985). B-lineage-- specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science *227*, 134-140.

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

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

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

Farah, M. H., Olson, J. M., Sucic, H. B., Hume, R. I., Tapscott, S. J., and Turner, D. L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development *127*, 693-702.

Feller, M. B. (1999). Spontaneous correlated activity in developing neural circuits. Neuron *22*, 653-656.

Ferre-D'Amare, A. R., Prendergast, G. C., Ziff, E. B., and Burley, S. K. (1993). Recognition of Max of its cognate DNA through a dimeric bHLH/Z domain. Nature *363*, 38-45.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C., and Guillemot, F. (1998). The bHLH protein NEUROGENIN2 is a detemination factor for epibranchial placode-derived sensory neurons. Neuron *120*, 483-494.

Fode, C., Ma, Q., Casarosa, S., Ang, S.-L., Anderson, D. J., and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev *14*, 67-80.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M., and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Müller glia by postnatal retinal progenitor cells. Neuron *26*, 383-394.

Furuta, Y., Piston, D. W., and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development *124*, 2203-2212.

Gaiano, N., Nye, J. S., and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron *26*, 395-404.

Gautier, P., Ledent, V., Massaer, M., Dambly-Chaudiere, C., and Ghysen, A. (1997). tap, a Drosophila bHLH gene expressed in chemosensory organs. Gene *191*, 15-21.

Ghosh, B., and Leach, S. D. (2006). Interactions between hairy/enhancer of split-related proteins and the pancreatic transcription factor Ptf1-p48 modulate function of the PTF1 transcriptional complex. Biochem J *393*, 679-685.

Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V., and Johnson, J. E. (2005). Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. Development *132*, 5461-5469.

Goridis, C., and Brunet, J. F. (1999). Transcriptional control of neurotransmitter phenotype. Curr Opin Neurobiol *9*, 47-53.

Goulding, M. (2004). Neurobiology: a matter of balance. Nature *429*, 515-517.

Goulding, M., and Pfaff, S. L. (2005). Development of circuits that generate simple rhythmic behaviors in vertebrates. Curr Opin Neurobiol *15*, 14-20.

Goulding, M. D., Lumsden, A., and Gruss, P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. Development *117*, 1001-1016.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R., and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron *31*, 219-232.

Gradwohl, G., Fode, C., and Guillemot, F. (1996). Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. Dev Biol *180*, 227-241.

Grbavec, D., and Stifani, S. (1996). Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. Biochemical & Biophysical Research Communications *223*, 701-705.

Greenwald, I. (1998). LIN-12/Notch signaling: lessons from worms and flies. Genes Dev *12*, 1751-1762.

Greenwald, I., and Rubin, G. M. (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. Cell *68*, 271-281.

Gross, M. K., Dottori, M., and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron *34*, 535-549.

Guillemot, F., and Joyner, A. (1993). Expression of murine Achaete-Scute and Notch homologues in the developing central nervous system. Mech Dev *42*, 171-185.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463-476.

Habecker, B. A., Klein, M. G., Sundgren, N. C., Li, W., and Woodward, W. R. (2002). Developmental regulation of neurotransmitter phenotype through tetrahydrobiopterin. J Neurosci *22*, 9445-9452.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D., and Lewis, J. (1998). Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. Development *125*, 359-370.

Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, J. I., Guerrier, S., Boutt, E., Peters, E., Barnes, A. P., Parras, C*., et al.* (2005). Phosphorylation of Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. Neuron *48*, 45-62.

Hanson, M. G., and Landmesser, L. T. (2003). Characterization of the circuits that generate spontaneous episodes of activity in the early embryonic mouse spinal cord. J Neurosci *23*, 587-600.

Hanson, M. G., and Landmesser, L. T. (2004). Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. Neuron *43*, 687-701.

Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature *390*, 465-471.

Helms, A. W., Abney, A., Ben-Arie, N., Zoghbi, H. Y., and Johnson, J. E. (2000). Autoregulation and multiple enhancers control *Math1* expression in the developing nervous system. Development *127*, 1185-1196.

Helms, A. W., Battiste, J., Henke, R. M., Nakada, Y., Simplicio, N., Guillemot, F., and Johnson, J. E. (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. Development *132*, 2709-2719.

Helms, A. W., Gowan, K., Abney, A., Savage, T., and Johnson, J. E. (2001). Overexpression of MATH1 disrupts the coordination of neural differentiation in cerebellum development. Mol Cell Neurosci *17*, 671-682.

Helms, A. W., and Johnson, J. E. (2003). Specification of dorsal spinal cord interneurons. Curr op in Neurobiology *13*, 42-49.

Hendricks, T. J., Fyodorov, D. V., Wegman, L. J., Lelutiu, N. B., Pehek, E. A., Yamamoto, B., Silver, J., Weeber, E. J., Sweatt, J. D., and Deneris, E. S. (2003). Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron *37*, 233-247.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signaling in the embryonic chick retina. Curr Biol *7*, 661-670.

Hirsch, M.-R., Tiveron, M.-C., Guillemot, F., Brunet, J.-F., and Goridis, C. (1998). Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development *125*, 599-608.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F., and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. Development *127*, 2515-2522.

Horton, S., Meredith, A., Richardson, J. A., and Johnson, J. E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Mol Cell Neurosci *14*, 355-369.

Hoshino, M., MNakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y., Fukuda, A., Fuse, T., Matsuo, N., Sone, M*., et al.* (2005). Ptf1a, a bHLH Transcriptional Gene, Defines GABAergic Neuronal Fates in Cerebellum. Neuron *47*, 201-213.

Hoveyda, N., Shield, J. P., Garrett, C., Chong, W. K., Beardsall, K., Bentsi-Enchill, E., Mallya, H., and Thompson, M. H. (1999). Neonatal diabetes mellitus and cerebellar hypoplasia/agenesis: report of a new recessive syndrome. J Med Genet *36*, 700-704.

Howard, T. D., Paznekas, W. A., Green, E. D., Chiang, L. C., Ma, N., Ortiz de Luna, R. I., Garcia Delgado, C., Gonzalez-Ramos, M., Kline, A. D., and Jabs, E. W. (1997). Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. Nat Genet *15*, 36-41.

Huang, M. L., Hsu, C. H., and Chien, C. T. (2000). The proneural gene amos promotes multiple dendritic neuron formation in the Drosophila peripheral nervous system. Neuron *25*, 57-67.

Ibuki, T., Hama, A. T., Wang, X. T., Pappas, G. D., and Sagen, J. (1997). Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. Neuroscience *76*, 845-858.

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

Jan, L., and Jan, Y. N. (1993). HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell *75*, 827-830.

Jarman, A. P., Grau, Y., Jan, L. Y., and Jan, Y. N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell *73*, 1307-1321.

Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y., and Y.N., J. (1994). *atonal* is the proneural gene for *Drosophila* photoreceptors. Nature *369*, 398-400.

Jarman, A. P., Sun, Y., Jan, L. Y., and Jan, Y. N. (1995). Role of the proneural gene, *atonal*, in formation of *Drosophila* chordotonal organs and photoreceptors. Development *121*, 2019-2030.

Jaynes, J. B., Johnson, J. E., Buskin, J. N., Gartside, C. L., and Hauschka, S. D. (1988). The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. Molecular and Cellular Biology *8*, 62-70.

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

Jimenez, F., and Campos-Ortega, J. A. (1990). Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster. Neuron *5*, 81-89.

Johnson, J. E., Birren, S. J., and Anderson, D. J. (1990). Two rat homologues of *Drosophila achaete-scute* specifically expressed in neuronal precursors. Nature *346*, 858-861.

Kandel, E. R., Schwartz, J. H., and Jessell, T. M. (1991). Principles of Neural Science, Third edn (Norwalk, CT, Appleton and Lange).

Kao, H. Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C. R., Evans, R. M., and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev *12*, 2269-2277.

Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J., and Wright, C. V. E. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genetics *32*, 128-134.

Kele, J., Simplicio, N., Ferri, A. L., Mira, H., Guillemot, F., Arenas, E., and Ang, S. L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. Development *133*, 495-505.

Kim, P., Helms, A. W., Johnson, J. E., and Zimmerman, K. (1997). *XATH1*, a vertebrate homolog of *Drosophila atonal*, induces neuronal differentiation within ectodermal progenitors. Dev Biol *187*, 1-12.

Kim, S., Yoon, Y. S., Kim, J. W., Jung, M., Kim, S. U., Lee, Y. D., and Suh-Kim, H. (2004). Neurogenin1 is sufficient to induce neuronal differentiation of embryonal carcinoma P19 cells in the absence of retinoic acid. Cell Mol Neurobiol *24*, 343-356.

Krapp, A., Knofler, M., Frutiger, S., Hughes, G. J., Hagen-buchle, O., and Wellauer, P. K. (1996). The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. EMBO J *15*, 4317-4329.

Krapp, A., Knofler, M., Ledermann, B., Burki, K., Berney, C., Zoerkler, N., Hagenbuchle, O., and Wellauer, P. K. (1998). The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes & Development *12*, 3752-3763.

Kriks, S., Lanuza, G. M., Mizuguchi, R., Nakafuku, M., and Goulding, M. (2005). Gsh2 is required for the repression of Ngn1 and specification of dorsal interneuron fate in the spinal cord. Development *132*, 2991-3002.

Lee, J. E. (1997a). Basic helix-loop-helix genes in neural development. Curr Opin Neurobiol *7*, 13-20.

Lee, J. E. (1997b). NeuroD and neurogenesis. Developmental Neuroscience *19*, 27-32.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic-helix-loop-helix protein. Science *268*, 836-844.

Lee, K. J., Dietrich, P., and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. Nature *403*, 734-740.

Lee, K. J., and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci *22*, 261-294.

Lee, K. J., Mendelsohn, M., and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev *12*, 3394-3407.

Lee, S. K., and Pfaff, S. L. (2001). Transcriptional networks regulating neuronal identity in the developing spinal cord. Nat Neurosci *4 Suppl*, 1183-1191.

Levitt, P. (2005). Disruption of interneuron development. Epilepsia *46 Suppl 7*, 22-28.

Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol *9*, 583-589.

Li, H., and Edlund, H. (2001). Persistent expression of Hlxb9 in the pancreatic epithelium impairs pancreatic development. Dev Biol *240*, 247-253.

Liem, K. F., Tremml, G., and Jessell, T. M. (1997). A role for the roof plate and its resident TGF-β-related proteins in neuronal patterning in the dorsal spinal cord. Cell *91*, 127-138.

Liem, K. F., Tremml, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell *82*, 969-979.

Ling, P. D., Hsieh, J. J., Ruf, I. K., Rawlins, D. R., and Hayward, S. D. (1994). EBNA-2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1. J Virol *68*, 5375-5383.

Lipkowitz, S., Gobel, V., Varterasian, M. L., Nakahara, K., Tchorz, K., and Kirsch, I. R. (1992). A comparative structural characterization of the human NSCL-1 and NSCL-2 genes. Journal of Biological Chemistry *267*, 21065-21071.

Liu, Y., Helms, A. W., and Johnson, J. E. (2004). Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development *131*, 1017-1028.

Lo, L.-C., Johnson, J. E., Wuenschell, C. W., Saito, T., and Anderson, D. J. (1991). Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially-restricted subsets of early neuroepithelial and neural crest cells. Genes Dev *5*, 1524-1537.

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

Lu, Y., and Perl, E. R. (2003). A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. J Neurosci *23*, 8752-8758.

Lu, Y., and Perl, E. R. (2005). Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). J Neurosci *25*, 3900-3907.

Ma, Q., Kintner, C., and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. Cell *87*, 43-52.

Ma, Q., Sommer, L., Cserjesi, P., and Anderson, D. J. (1997). Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. J Neurosci *17*, 3644-3652.

Mann, R. S., and Carroll, S. B. (2002). Molecular mechanisms of selector gene function and evolution. Curr Opin Genet Dev *12*, 592-600.

Mansouri, A., and Gruss, P. (1998). *Pax3* and *Pax7* are expressed in commissural neurons and restrict ventral neuronal identity in the spinal cord. Mech Dev *78*, 171-178.

Manzanares, M., Trainor, P. A., Ariza-McNaughton, L., Nonchev, S., and Krumlauf, R. (2000). Dorsal patterning defects in the hindbrain, roof plate and skeleton in the dreher (dr(J)) mouse mutant. Mech Dev *94*, 147-156.

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

Massague, J. (1998). TGF-beta signal transduction. Annu Rev Biochem *67*, 753-791.

Massari, M. E., and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol *20*, 429-440.

Matsunaga, E., Araki, I., and Nakamura, H. (2001). Role of Pax3/7 in the tectum regionalization. Development *128*, 4069-4077.

Millen, K. J., Millonig, J. H., and Hatten, M. E. (2004). Roof plate and dorsal spinal cord dl1 interneuron development in the dreher mutant mouse. Dev Biol *270*, 382-392.

Millonig, J. H., Millen, K. J., and Hatten, M. J. (2000). The mouse *Dreher* gene *Lmx1a* controls formation of the roof plate in the vertebrate CNS. Nature *403*, 764-769.

Milner, L. D., and Landmesser, L. T. (1999). Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. J Neurosci *19*, 3007-3022.

Moohr, O. (1919). Genetics, Vol 4).

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G., and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell *101*, 499-510.

Muller, T., Anlag, K., Wildner, H., Britsch, S., Treier, M., and Birchmeier, C. (2005). The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. Genes Dev *19*, 733-743.

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M., and Birchmeier, C. (2002). The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron *34*, 551-562.

Mumm, J. S., and Kopan, R. (2000). Notch signaling: from the outside in. Dev Biol *228*, 151-165.

Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J., and Kopan, R. (2000). A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol Cell *5*, 197-206.

Murre, C., Bain, G., van Dijk, M. A., Engel, I., Furnari, B. A., Massari, M. E., Matthews, J. R., Quong, M. W., Rivera, R. R., and Stuiver, M. H. (1994). Structure and function of helix-loop-helix proteins. Biochim Biophys Acta *1218*, 129-135.

Murre, C., McCaw, P. S., and Baltimore, D. (1989a). A new DNA binding and dimerization motif in immunoglobin enhancer binding, *daughterless, MyoD* and *myc* proteins. Cell *56*, 777-783.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B.*, et al.* (1989b). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537-544.

Nabekura, J., Katsurabayashi, S., Kakazu, Y., Shibata, S., Matsubara, A., Jinno, S., Mizoguchi, Y., Sasaki, A., and Ishibashi, H. (2004). Developmental switch from GABA to glycine release in single central synaptic terminals. Nat Neurosci *7*, 17-23.

Nakada, Y., Hunsaker, T. L., Henke, R. M., and Johnson, J. E. (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus cell-type specification. Development *131*, 1319-1330.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401-413.

Obata, J., Yano, M., Mimura, H., Goto, T., Nakayama, R., Mibu, Y., Oka, C., and Kawaichi, M. (2001). p48 subunit of mouse PTF1 binds to RBP-Jk/CBF-1, the intracellular mediator of Notch signalling, and is expressed in the neural tube of early stage embryos. Genes to Cells *6*, 345-360.

Ohsako, S., Hyer, J., Panganiban, G., Oliver, I., and Caudy, M. (1994). hairy function as a DNA-binding helix-loop-helix repressor of *Drosophila* sensory organ formation. Genes Dev *8*, 2743-2755.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO Journal *18*, 2196-2207.

Oka, C., Nakano, T., Wakeham, A., de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W., and Honjo, T. (1995). Disruption of the mouse RBP-J kappa gene results in early embryonic death. Development *121*, 3291-3301.

Pan, D., and Rubin, G. M. (1997). Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. Cell *90*, 271-280.

Parras, C. M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D. J., and Guillemot, F. (2002). Divergent functions of the proneural genes *Mash1* and *Ngn2* in the specification of neuronal subtype identity. Genes & Development *16*, 324-338.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature *399*, 366-370.

Penn, A. A., and Shatz, C. J. (1999). Brain waves and brain wiring: the role of endogenous and sensory-driven neural activity in development. Pediatr Res *45*, 447-458.

Polgar, E., Hughes, D. I., Arham, A. Z., and Todd, A. J. (2005). Loss of neurons from laminas I-III of the spinal dorsal horn is not required for development of tactile allodynia in the spared nerve injury model of neuropathic pain. J Neurosci *25*, 6658-6666.

Portin, P. (2002). General outlines of the molecular genetics of the Notch signalling pathway in Drosophila melanogaster: a review. Hereditas *136*, 89-96.

Poulson, D. F. (1937). Chromosomal deficiences and the embryonic development of Drosophila melanogaster. Proc Nat Acad Sci *23*, 133-137.

Rivera, C., Voipio, J., and Kaila, K. (2005). Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII. J Physiol *562*, 27-36.

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

Rose, S. D., Swift, G. H., Peyton, M. J., Hammer, R. E., and MacDonald, R. J. (2001). The role of PTF1-P48 in pancreatic acinar gene expression. Journal of Biological Chemistry *276*, 44018-44026.

Rudomin, P., and Schmidt, R. F. (1999). Presynaptic inhibition in the vertebrate spinal cord revisited. Exp Brain Res *129*, 1-37.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev *6*, 2620-2634.

Scheer, N., Groth, A., Hans, S., and Campos-Ortega, J. A. (2001). An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development *128*, 1099-1107.

Schuurmans, C., Armant, O., Nieto, M., Stenman, J. M., Britz, O., Klenin, N., Brown, C., Langevin, L. M., Seibt, J., Tang, H.*, et al.* (2004). Sequential phases of cortical specification involve Neurogenin-dependent and -independent pathways. Embo J *23*, 2892-2902.

Schwab, M. H., Druffel-Augustin, S., Gass, P., Jung, M., Klugmann, M., Bartholomae, A., Rossner, M. J., and Nave, K. A. (1998). Neuronal basic helix-loop-helix proteins (NEX, neuroD, NDRF): spatiotemporal expression and targeted disruption of the NEX gene in transgenic mice. Journal of Neuroscience *18*, 1408-1418.

Sellick, G. S., Barker, K. T., Stolte-Dijkstra, I., Fleischmann, C., Coleman, R. J., Garrett, C., Gloyn, A. L., Edghill, E. L., Hattersley, A. T., Wellauer, P. K.*, et al.* (2004). Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat Genet *36*, 1301-1305.

Shimeld, S. M., McKay, I. J., and Sharpe, P. T. (1996). The murine homeobox gene Msx-3 shows highly restricted expression in the developing neural tube. Mech Dev *55*, 201-210.

Shimizu, C., Akazawa, C., Nakanishi, S., and Kageyama, R. (1995). MATH-2, a mammalian helix-loop-helix factor structurally related to the product of the *Drosophila* proneural gene *atonal*, is specifically expressed in the nervous system. European Journal of Biochemistry *229*, 239-248.

Simpson, P. (1990). *Notch* and the choice of cell fate in *Drosophila* neuroepithelium. Trends Genet *6*, 343-345.

Sommer, L., Ma, Q., and Anderson, D. J. (1996). Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogenity in the developing CNS and PNS. Mol Cell Neurosci *8*, 221-241.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genetics *21*, 70-71.

Spitzer, N. C., Root, C. M., and Borodinsky, L. N. (2004). Orchestrating neuronal differentiation: patterns of Ca2+ spikes specify transmitter choice. Trends Neurosci *27*, 415-421.

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

Struhl, G., and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. Nature *398*, 522-525.

Struhl, G., and Greenwald, I. (2001). Presenilin-mediated transmembrane cleavage is required for Notch signal transduction in Drosophila. Proc Natl Acad Sci U S A *98*, 229-234.

Suemori, H., Kadodawa, Y., Goto, K., Araki, I., Kondoh, H., and Nakatsuji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous beta-galactosidase expression. Cell Differ Dev *29*, 181-186.

Talikka, M., Perez, S. E., and Zimmerman, K. (2002). Distinct patterns of downstream target activation are specified by the helix-loop-helix domain of proneural basic helix-loop-helix transcription factors. Dev Biol *247*, 137-148.

Tanabe, Y., and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. Science *274*, 1115-1123.

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

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S. L., Nakanishi, S., Guillemot, F., and Kageyama, R. (1996a). Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron *16*, 723-734.

Tomita, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (1996b). *Mash1* promotes neuronal differentiation in the retina. Genes to Cell *1*, 765-774.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M., and S.L., P. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell *79*, 957-970.

van Doren, M., Bailey, A. M., Esnayra, J., Ede, K., and Posakony, J. W. (1994). Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of *achaete*. Genes Dev *8*, 2729-2742.

Verzi, M. P., Anderson, J. P., Dodou, E., Kelly, K. K., Greene, S. B., North, B. J., Cripps, R. M., and Black, B. L. (2002). N-twist, an evolutionarily conserved bHLH protein expressed in the developing CNS, functions as a transcriptional inhibitor. Dev Biol *249*, 174-190.

Voronova, A., and Baltimore, D. (1990). Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proc Natl Acad Sci USA *87*, 4722-4726.

Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas, S. (1985a). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell *43*, 567-581.

Wharton, K. A., Yedvobnick, B., Finnerty, V. G., and Artavanis-Tsakonas, S. (1985b). opa: a novel family of transcribed repeats shared by the Notch locus and other developmentally regulated loci in D. melanogaster. Cell *40*, 55-62.

Whitman, M. (1998). Smads and early developmental signaling by the TGFbeta superfamily. Genes Dev *12*, 2445-2462.

Wiesenfeld-Hallin, Z., Aldskogius, H., Grant, G., Hao, J. X., Hokfelt, T., and Xu, X. J. (1997). Central inhibitory dysfunctions: mechanisms and clinical implications. Behav Brain Sci *20*, 420-425; discussion 435-513.

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

Winnier, G., Blessing, M., Labosky, P. A., and Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev *9*, 2105-2116.

Wu, S. M. (1992). Functional organization of GABAergic circuitry in ectotherm retinas. Prog Brain Res *90*, 93-106.

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

Zhang, H., and Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development *122*, 2977-2986.

Zhang, L. I., and Poo, M. M. (2001). Electrical activity and development of neural circuits. Nat Neurosci *4 Suppl*, 1207-1214.

VITAE

Stacey Marie Glasgow was born in San Antonio, Texas, on June 15, 1978, the daughter of Maria Zelma Glasgow and James Edward Glasgow. After completing her work at Southside High School, San Antonio, Texas in 1996, she entered Trinity University in San Antonio, Texas. She received the degree of Bachelor of Science with a major in Biology from Trinity University in May 2000. In August of 2000 she entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Dallas. She joined the Integrative Biology program and joined the laboratory of Jane E. Johnson. During her tenure at UT Southwestern, she was awarded a Minority Supplement Award from the National Institutes of Heath, which supported her research for 3 years.