GENETIC REGULATION OF THE GENERATION OF NEURONAL DIVERSITY IN THE DEVELOPING MAMMALIAN BASAL FOREBRAIN

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience

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Washington, DC
October 18th, 2010
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ABSTRACT

The forebrain is the structure that is most implicated in complex mammalian behaviors: consciousness, reason, language, memory, and emotionality are all the result of cellular and molecular processes in this region of the brain. The telencephalon is the embryonic precursor to the forebrain; through several developmental mechanisms, including the patterning of progenitor domains, and the proliferation, migration, and specification of neuronal subtypes, the telencephalon gives rise to a complex and highly ordered functional network made up of a diversity of neuronal subtypes that process and integrate information and control behavior. The molecular mechanisms by which this brain region is formed during embryonic development are still being explored. We sought to examine the mechanisms used in the development of forebrain neuronal diversity by examining several important developmental questions using a combination of genetic fate-mapping, mutagenesis, cell birth-dating, migration assays, immunohistochemistry, and electrophysiology. We examined the genetic regulation of border formation in the telencephalon, and the impact that the correct patterning of this structure has on the fate of cells in the forebrain. We found that genetic regulation of border formation is critical for dorsal ventral patterning and is necessary for the correct generation of excitatory and inhibitory neuronal subpopulations in the amygdala and olfactory bulb. We also examined the importance of embryonic development on the fate of cells in the postnatal
forebrain, and found that the generation of neuronal diversity in the amygdala, striatum, and olfactory bulb is regulated by interactions between several biological processes, including the origin and timing of progenitor cell birth, combinatorial codes of transcription factor expression, and diverse migratory pathways.
Acknowledgements

I would foremost like to thank my mentor Dr. Joshua Corbin for his support, expertise, and guidance throughout my time as a graduate student in his lab; he has helped me to grow and develop into a successful independent scientist.

I would also like to thank the members of my committee, Drs. Elena Casey, Maria Donoghue, Tarik Haydar, Dan Pak, and Stefano Vicini, for helping me to refine and strengthen my research project and advance my dissertation research.

A number of people have been kind in sharing their knowledge, providing technical instruction, and experimental guidance, without which many of my experiments would not have been possible; for this I thank Berenice Alfonso, Drs. Tarik Haydar, Rosalind Carney, and Esumi Shigeyuki, and my instructors at the MBL.

I also thank Dr. Jean-Marie Mangin for his expertise, technical assistance, experimental guidance, and generosity of spirit; his collaboration has been invaluable.

Finally, my family has also been incredibly supportive of me throughout my graduate career; I would especially like to thank Ron and Jeri Cocas for their love and encouragement of my life and my work.
This research is dedicated to Patricia Phillips.
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Chapter 1

Introduction
INTRODUCTION

*Forebrain Development: Perspectives*

Over one hundred years ago, Golgi and Ramon y Cajal, through their analyses of anatomy and cellular staining, provided the first insights into the complexity of neural anatomy and the structure of neuronal circuits in the brain. Their initial work provided the first evidence that individual neurons were the basic signaling component of the nervous system (Puelles, 2009). Neuronal diversity is immediately evident when one examines the enormous variability in morphology, surface receptor expression, neurotransmitter content, axonal trajectory, synaptic specificity, and firing properties present in different neuronal subtypes. It is now estimated that there are over 100 billion neurons in the brain, and that each of these neurons forms several thousands of synapses with other neurons (Serafini, 1999). It has been postulated that over one thousand different neuronal subtypes are present in the brain. The field of neurodevelopment today is currently attempting to understand how this neuronal diversity is generated and how neuronal circuits are formed during development.

While hundreds of different neuronal subtypes are purported to exist throughout the brain, one of the fundamental categorical distinctions in the forebrain is the division of cells into excitatory and inhibitory neuron subtypes. Excitatory neurons generally release the neurotransmitter glutamate, which leads to excitation, and, if threshold is reached, transmission of signal through the firing of an action potential in postsynaptic neurons, while inhibitory neurons generally release the neurotransmitter GABA, which tends to dampen and inhibit the signal in a postsynaptic cell, decreasing the likelihood
that the threshold for an action potential will be reached. While other major classifications exist, based upon morphology (bipolar, multipolar, pyramidal, granule), length of projection (interneurons make local connections while projection neurons send axons to distant locations), or direction of information sent (afferent, or sensory, vs. efferent, or motor), our focus was on the classification of excitatory and inhibitory interneurons and their subtypes. Inhibitory interneurons are sub-classified by many different properties (axon branch points, dendritic morphology, ion channels expressed, postsynaptic targets, etc), but morphology, neurotransmitter, neuropeptide, and calcium binding proteins are the common means of dividing interneurons broadly into the main subtypes found in the forebrain (DeFelipe, 1993). Neuropeptide Y (NPY), somatostatin (SOM), parvalbumin (PV), calretinin (CR), calbindin (CB), vasointestinal peptide (VIP) and cholecystokinin (CCK) are the major interneuron subtypes; these neurons also have unique firing properties, morphologies, and spatial distributions (Ascoli, et al., 2008). In contrast, excitatory neuron subtypes have been delineated with less ease: few markers have been found that allow for easy division into excitatory neuron subtypes. The main classes of excitatory neurons are based upon morphology (pyramidal, spiny stellate) and firing properties (fast-spiking, rebound firing, pyramidal-like) (Chen, et al., 2006; Molnar and Cheung, 2006). We used the above classifications as a framework to examine the generation of neuronal diversity in the developing forebrain.

While the adult brain is classified into numerous distinct structures based upon anatomy and function, during early embryonic development, the brain is divided into six major developmental domains. The telencephalon gives rise to the cerebral cortex and basal ganglia, the diencephalon to the thalamus and hypothalamus, and the
mesencephalon gives rise to the midbrain. The rhombencephalon gives rise to the hindbrain, which is sub-divided into the metencephalon (pons and cerebellum), and the myelencephalon (medulla), and the caudal neural tube gives rise to the spinal cord (see Figure 1); reviewed in (Rubenstein and Beachy, 1998; Wilson and Houart, 2004). Adult structures are defined by the type of information processed, the cell subtypes they contain, the overall anatomy, including distribution of cell bodies and processes, and the type of synaptic connections made between cells inside and outside of the structure. Similarities between anatomy and structure indicate a conserved mechanism for creating functionally diverse brain regions. For example, the amygdala is a nuclear structure and contains sub-nuclei that are in composition both cortical-like and striatal-like: the baso-lateral amygdala is mainly an excitatory output nucleus, containing excitatory pyramidal neurons and local inhibitory interneurons much like in the cortex, while the central nucleus is composed entirely of inhibitory neurons whose outputs resemble more closely the inhibitory projection neurons found in the striatum (Swanson and Petrovich, 1998). These interesting relationships between structure and function in brain regions that were derived from adjacent progenitor domains during development led us to examine whether the embryonic origins of these structures are important for determining their neuronal subtype composition, structure, and function in the adult forebrain.

Several important questions are as yet unresolved in the field of developmental neurobiology. How do different regions of the CNS, and the individual neuronal subtypes they contain, establish their identity? How are uniform pools of progenitor cells separated into compartments that have very different functions? What are the genetic and environmental interactions used in the developing brain and how are they integrated in
space and time to specify this cellular diversity? Related to this question, how does an individual progenitor cell integrate multiple genetic signals over time to become specified into a particular cell type? How is tissue patterned in a three dimensional space, across time? We chose to address aspects of these broad questions by asking three very specific questions about embryonic development: 1. Is there transcriptional regulation of the formation and patterning of the telencephalic dorsal ventral border? 2. Is the patterning of the telencephalic dorsal-ventral border important for the correct formation of adjacent progenitor cell compartments and therefore for the correct specification of neuronal cell subtypes in adult brain target regions, specifically, the amygdala and olfactory bulb? 3. What is the relationship between embryonic origin and timing of birth in the determination of cell fate in the striatum and amygdala? The results from these studies inform our current understanding about the genetic regulation of patterning during telencephalic development and the importance of embryonic origin for the generation of neuronal diversity in the adult forebrain.

**Early Patterning of the Developing Brain**

Patterning of the brain allows the compartmentalization of tissue into sub-domains based upon unique combinations of transcription factor expression that confer progenitor cells with different neuronal fates. Early patterning events are critical for establishing the initial tissue polarity, cell positioning, and compartmentalization necessary for subsequent steps in development. Patterning also requires the integration of intrinsic and extrinsic factors that provide cells with information about their location in time and space, and ultimately their identity. Many neuronal progenitors are born at a
precise time and location in the developing brain, migrate in a predicted manner, and settle in a particular region of the adult brain. Therefore, the correct patterning of progenitor cells during development is critical for the generation of neuronal diversity in the mature brain. Understanding how different regions of the developing brain are patterned, and therefore, how cellular identity and diversity arises, is a fundamental question in the field of neurobiology today. While the focus of our studies was on patterning events in the developing telencephalon, which begin around E9.5 in the mouse, (embryonic day 9.5; mouse gestation is 19 days), patterning of the neural axis begins earlier in development with the formation of the neural plate around E8.0. Formation of the neural plate and neural tube and the parcellation of the neural tube into anterior/posterior, dorsal/ventral, and rostral/caudal axes provide the initial framework upon which the telencephalon is formed. A brief discussion of early patterning therefore provides a useful introduction, as early patterning events share many common biological mechanisms with later telencephalic patterning events.

The developing nervous system in all vertebrates becomes compartmentalized during early embryonic development. Tissue is initially polarized by expression of morphogens, secreted molecules that spread from a localized source and act in a concentration dependent manner, creating a gradient of the secreted molecule across the neural epithelium (Dessaud, et al, 2008; Liem, et al., 2000). Morphogens serve as initial inducing factors by acting as signaling molecules that bind to receptors on the surface of the cells and activate internal transcription factor cascades. Expression of specific subsets of transcription factors in turn regulate the expression of downstream genes that are critical for the production of specific proteins necessary for the specialized cellular
machinery present in different cell subtypes. These molecular processes lead to the patterning of initially homogeneous tissue into distinct cellular compartments with unique cell subpopulations and are reviewed in (Rubenstein and Beachy, 1998; Wilson and Houart, 2004). Further refinement of functional domains occurs as cells make and receive synaptic contacts with other cell or tissue targets, leading to refinement in the receptor subtypes that are expressed, the number and type of synaptic contacts formed, and the types of information that the cell processes. This combination of intrinsic factors and extrinsic cues allows an initially uniform group of cells to proliferate and differentiate into a highly specialized network responsible for a diversity of function in the behaving animal, be it a spinal motor reflex or a freezing response in a frightened animal.

Neural Induction and the Formation of the Spinal Cord

While telencephalic patterning has only recently been examined in detail, the patterning events that lead to the induction of nervous tissue and the formation of the spinal cord have been well elucidated (reviewed in Tanabe and Jessell, 1996; Wilson and Maden 2005); these events are summarized in Figure 2. The nervous system is derived from the ectoderm in the developing embryo. This ectodermal sheet begins to acquire neural properties when BMP signaling is inhibited, inducing progenitors to differentiate into neural plate cells. The neural plate buckles at its midline, and the dorsal folds fuse together to form the neural tube. The rostral neural tube gives rise to the brain and the caudal neural tube gives rise to the spinal cord. Spinal cord differentiation occurs ventrally via signals from the mesoderm below the ventral neural tube (the notochord, which secretes Sonic Hedgehog, (SHH)) and dorsally via ectodermal cells above the
neural tube that secrete BMPs. Subsequently, Shh is induced in the glial floor plate cells of the neural tube, and SHH from the floor plate and notochord induces the formation of motorneurons and ventral interneurons in the ventral spinal cord via a concentration-dependent mechanism, with lower concentrations of SHH producing interneurons and higher concentrations producing motorneurons. SHH binding in these cells relieves inhibition of the protein Smoothened, which activates Gli transcription factors that in turn induce expression of Hnf3B, Ptc, and other target genes that mediate the specification of different motorneuron and ventral interneuron subclasses. Dorsally, BMPs from the ectoderm initiate BMP signaling in the glial cells in the roof plate of the neural tube; BMPs in turn induce production of dorsal sensory commissural interneurons. BMPs induce these different dorsal cell types by activating serine-threonine kinase receptors that phosphorylate SMAD proteins, which induce transcription of Wnt-1 and other genes that mediate sensory interneuron specification in the dorsal spinal cord; these events are reviewed in (Jessell, 2000; Ingham and McMahon, 2001). BMPs and SHH provide important and similar roles in the dorsal-ventral patterning of the telencephalon, discussed below.

Initial Patterning of the Telencephalon

The telencephalon is the embryonic correlate of the rostral adult forebrain in mammals, which includes the cerebral cortex, hippocampus, olfactory bulb, basal ganglia, and amygdala. It is the most complex aspect of the central nervous system and is critical for all higher functions, including sensorimotor integration, language, learning, memory, emotionality, and cognition. It is also critical for more subtle aspects of
behavior in humans: creativity, critical thinking, and personality are all the result of information processing in the forebrain. It is also a region often implicated in human brain diseases and disorders that involve cognition, including schizophrenia, autism, Down’s syndrome, epilepsy, and Alzheimer’s disease. Understanding the biological mechanisms that regulate the development of the forebrain is therefore critical to our understanding of human cognitive function and dysfunction.

Transcription factors and secreted molecules establish dorsal-ventral and anterior-posterior polarity in the developing forebrain and are important for conferring neuronal identity upon these different progenitor pools; these events are reviewed in (Fishell and Hebert, 2008; Rubenstein and Beachy, 1998; Wilson and Houart, 2004). Initially, at E8.5, Foxg1 expression is initiated in the telencephalic primordium, which at this stage is one cell-length in thickness. After Foxg1 is expressed, several additional transcription factors are expressed that begin to separate the forebrain into the dorsal and ventral telencephalon. This division is regulated by the dorsalizing transcription factor Gli3 and the morphogen Shh, which is expressed ventrally, and represses Gli3 in the ventral telencephalon; together, they set up the initial dorsal-ventral polarity; these initial patterning events are summarized in Figure 3. The dorsal telencephalon is subsequently separated further into the anterior/lateral area that will contribute to the cerebral cortex and the posterior/medial area that will contribute to the hippocampus, and the ventral telencephalon is separated into the lateral ganglionic eminence (LGE), which will contribute to the striatum and olfactory bulb, and the medial ganglionic eminence (MGE), which will contribute to the globus pallidus and also give rise to the interneurons of the basal ganglia, cortex, and amygdala.
Initial induction of telencephalic character begins with the onset of Foxg1 expression in anterior plate cells. Foxg1, in concert with SHH expression, induces the extracellular signal and morphogen FGF8 at E9, which is expressed rostrally and is necessary for the anterior identity of the telencephalon, as well as for the induction of ventral transcription factors necessary to pattern the MGE and LGE, such as Nkx2.1 and Gsx2 (Fuguchi-Shimogori and Grove, 2001; Shimamura and Rubenstein, 1997). Pax6 and Lhx2 are expressed at E9.5 in the dorsal telencephalon, and Pax6 expression extends down into the ventral telencephalon where it meets Nkx2.1 expression. It is not known what regulates the initial activation of Pax6, although Gli3 appears to be important for its maintenance. Downstream from these initial patterning genes, several additional homeodomain transcription factors are expressed in distinct sub domains that are postulated to further parcellate discrete sub-domains (Puelles, et al., 2003). By E10.5, Gsx2 is expressed and occupies the territory between Pax6 and Nkx2.1 at the level of the LGE (Corbin, et al., 2003; Puelles, et al., 2000). Beginning at E10.5, Emx1/2 and Tbr1 are expressed in the pallium. Emx1/2 is important for the formation of the medial pallium into the hippocampus and Lhx2 and Pax6 are required for the correct formation of the dorsal pallium into the neocortical primordium (Mangale, et al, 2007; Muzio, et al., 2002; Stoykova, et al., 1996; Talamillo, et. al, 2003; Warren, et al; 1999; Yoshida, et al., 1997). Also at E10.5, Dlx1/2 and Lhx6 are expressed, and further parcellate GE sub domains. Nlx2.1 is required for formation of the MGE and Gsx2 and Dlx genes are required for correct LGE formation (Anderson, et al., 1997; Cobos, et al., 2005; Corbin, et al., 2001, 2003; Marin, et al., 2000; Nery. et al., 2003; Yun, et al., 2001, 2003). In the dorsal medial telencephalon, at E10.5, medial-lateral specification is regulated by bone morphogenic
protein (Bmp) and Wnt family secreted signaling molecules, which are expressed at the dorsal midline and are critical for the specification of the cortical hem, an organizer that specifies the hippocampus (Hebert, et al., 2002; Machon, et al., 2007; Mangale, et al., 2008). These transcription factor expression patterns are illustrated in Figure 4.

**Borders as Important Mediators of Patterning**

Compartmentalization occurs after tissue polarity is established and the tissue is refined into domains containing similar types of progenitor cells. These domains are separated by distinct boundaries, or borders, that are typically established by the genetic cross-repressive interactions of transcription factors that are expressed in adjacent progenitor cell domains (Kiecker and Lumsden, 2005; Blair, 2003). These borders are often transient structures, and typically pattern tissue through a combination of intrinsic factors and extrinsic cues, via nested expression of specific genes at the border and local sources of secreted molecules that continue to provide cells with information about their position and their identity. Once established, borders in the developing nervous system often also act as signaling centers to pattern surrounding tissue via the actions of secreted molecules, which act to maintain cell position and identity in cells that are distant from the physical border (Blair, 2003; Irvine, 2001).

Particular borders in the brain have well characterized patterning functions, for example, at the midbrain-hindbrain boundary (the MHB, or Isthmic organizer). This organizer forms both a morphological and a molecular boundary between the midbrain and hindbrain and patterns surrounding tissue via several key developmental events. Initially, genetic cross repression of the homeobox-domain containing transcription
factors *Otx2* and *Gbx2* establish initial anterior-posterior polarity (Joyner, et al., 2000). Subsequently, secretion of the factors Fgf8 and wingless int (*Wnt1*) further refine the expression of *Otx2* and *Gbx2* (Liu, et al., 1999; Irving, et al., 2000; Martinez, et al., 1999). This refinement of gene expression patterns leads to the segregation of cells across the border, such that cells expressing *Otx2* do not mix with cells expressing *Gbx2*. As indicated by loss of function experiments, activation of *Wnt1* is critical for patterning this region; in its absence there is a loss of midbrain and hindbrain structures (McMahon and Bradley, 1990). FGF8 expressed at the MHB specifically serves as an organizing factor of the midbrain and hindbrain, as indicated by gain of function experiments showing that ectopic expression of FGF8, but not *Wnt1*, leads to the formation of additional midbrain and hindbrain structures (Liu, et al., 1999; Irving, et al., 2000; Martinez, et al., 1999). These secreted molecules serve to specify cells at the MHB by activating key transcription factors such as *Nkx2.2* and *Lmx1b*, which lead to the generation of separate domains of dopaminergic and serotonergic progenitors on opposite sides of the border (Joyner, et al, 2000). This mechanism of extrinsic cues and intrinsic factors working in concert to establish a boundary between compartments that ultimately become separate brain regions is likely a common means of patterning molecularly distinct but spatially confluent regions of the developing brain. While the mechanisms by which the MHB regulates patterning of the presumptive midbrain and hindbrain have been well explored, the mechanisms underlying patterning at borders elsewhere in the brain are just beginning to be understood. One of the aims of this thesis was to elucidate the role of borders by examining the genetic regulation of the formation and maintenance of the pallial
subpallial boundary (PSB) and its role in the patterning and specification of cells in the developing forebrain.

During embryogenesis, the PSB divides two compartments in the telencephalon: the pallium, which will give rise to the cerebral cortex and hippocampus, and the subpallium, which will give rise to the basal ganglia (Mastick, et al., 1997; Toresson, et al., 2000). Dorsal to the PSB is the pallium, which can be broken down into smaller progenitor domains: the medial pallium (mP), which will contribute to the three-layered hippocampus, the dorsal pallium (dP), which will contribute to excitatory neurons of the six-layered neocortex, the lateral pallium (lP), which will contribute to the limbic cortex, and the ventral pallium (vP), which will contribute to the amygdala and claustrum.

Ventral to the PSB is the subpallium, which includes the LGE, MGE, and CGE (caudal ganglionic eminence). The MGE and CGE produce the majority of inhibitory interneurons that populate the cortex and basal ganglia (Anderson, et al., 2001; Corbin, et al., 2001; Marin, et al., 2000; Nery, et al., 2002; Wichterle, et al., 2001; Xu, et al., 2004). The dLGE gives rise to inhibitory interneurons destined for the olfactory bulb, and the vLGE gives rise to inhibitory medium spiny neurons, which are the main neuronal subtype found in the striatum (Marin and Rubenstein, 2003; Stenman, et al., 2003).

Previous work has shown that via the lateral cortical stream, cells derived from the PSB contribute to portions of the basal telencephalic limbic system, specifically, the developing amygdala (Carney, et al., 2006). These progenitor domains are summarized in Figure 5.

**Genetic Regulation of the Pallial-Subpallial Boundary**
The PSB forms at embryonic day 10.5 (E10.5) in the mouse from the genetic cross repression of the homeobox gene Gsx2 ventrally in the subpallium and the paired homeobox gene Pax6 dorsally in the pallium (Corbin, et al., 2000; Toresson, et al., 2000; Yun, et al., 2001). The paired homeobox gene Pax6 and the bHLH genes Neurogenin 1 (Ngn1) and Ngn2 are expressed broadly in the dorsal compartment (Medina, et al., 2004; Puelles, et al., 2000). The homeobox gene Dbx1 and the secreted frizzled protein Sfrp2 are expressed more narrowly, specifically, at the dorsal side of the PSB (the vP) (Stoykova, et al., 2000). The empty spiracles drosophila homologues Emx1 and Emx2 are also expressed broadly in the dorsal compartment but are excluded from the vP (Puelles, et al., 2000). Pax6 expression has been shown to activate Sfrp2, which abuts Wnt expression at the border in the ventricular zone (VZ) (Stoykova, et al., 2000). At the ventral side of the PSB (the dLGE) progenitor cells express the homeobox genes Gsx1/2 and Dlx1/2 in the LGE and MGE VZ, Nkx2.1 in the MGE, Isl1 in the LGE/MGE SVZ (subventricular zone) and mantle, and the bHLH gene Mash1 in the LGE and MGE VZ and SVZ (Medina, et al., 2004; Puelles, et al., 2000). These expression patterns form a nested map of transcription factor expression that largely corresponds to developing progenitor domains; this is summarized in Figure 5. These transcription factors provide a useful readout of border patterning and were used in our studies as a means of determining the nature of the patterning defects present after conditional mutagenesis.

Gain and loss of function experiments have provided insight into how these expression patterns are regulated at the PSB. In Pax6 (sey/sey) mutant mice, the dorsal side of the PSB, (the vP), is lost and the dLGE is expanded (Mastick, et al., 1997; Stoykova, et al., 1996; Yun, et al., 2001). At E12.5, the ErbB ligand Tgfα, as well as
Sfrp2 expression is lost from the vP and Ngn1/2 is decreased in the pallium, and there is an expansion of ventral gene expression, with Gsx2, Dlx1/2, and Mash1 expression extending across the border into the vP and dP (Assimacopoulos, et al., 2003; Carney, et al., 2009; Chapouton, et al., 1999; Stoykova, et al., 1996; 1997; 2000; Mastick, et al., 1997; Stenman, et al., 2003; Toresson, et al., 2000; Yun, et al., 2001). Pax6 expression is lost in the domain where Gsx2 is expanded, and severely reduced throughout the pallium. Er81, which is expressed on both sides of the PSB, has a more complicated regulation by Gsx2 and Pax6: Er81 shows decreased expression in Gsx2 mutants, and is misplaced in Pax6 mutants, ventralized further into the LGE, suggesting that both Pax6 and Gsx2 are important for maintenance of this transcription factor (Carney, et al., 2003; Toresson, et al., 2000). These differences are summarized in Figure 6. Pax6 mutants, in addition to defects in dorsal-ventral patterning at the border, have increased proliferation in the pallium, abnormal migration of cortical precursors, loss of eye and nasal structures, and a number of cortical layer, patterning, and arealization defects, consistent with the indispensable role of Pax6 in multiple aspects of telencephalic development (Caric, et al., 1997; Dellovade, et al., 1998; Hill, et al., 1991; Jimenez, et al., 2002; Manuel, et al., 2005; Schmahl, et al., 1993; Simpson, et al., 2009; Stoykova, et al., 1996; Warren, et al., 1999).

In Gsx2/- embryos, a concomitant dLGE defect is apparent (Corbin, et al., 2000; Szucsik, et al., 1997; Toresson, et al., 2000; Yun, et al., 2001; 2003). At E12.5, the subpallial progenitor region shrinks and a dorsalization of the dLGE is apparent, as indicated by the expansion of Pax6, Sfrp2, and Dbx1 expression across the border into the dLGE. A decrease in Mash1 and Dlx1/2 in the dLGE and a loss of Isl1 in LGE SVZ and
mantle indicates that the LGE is reduced. The dLGE proliferative zone is also reduced, as are the numbers of cells in the LCS migrating towards the amygdala. Interestingly, *Emx1* expression is not altered in these mutants, indicating that it is not likely to be regulated by *Pax6* or *Gsx2*. Later in development, at E16.5, many of the border defects are restored: *Dlx1/2* and *Mash1* expression are present in the dLGE. However, Islt1 expression is still decreased, consistent with the large-scale striatal defects present in these mutants. This partial rescue is likely due to an up-regulation of *Gsx1* that is able to compensate in part for the absence of *Gsx2* (Toresson and Campbell, 2001). These changes are summarized in Figure 6. No changes in cell death are observed in these mutants, indicating that these changes in patterning do not result in a change in cell survival. Finally, in *Pax6*−/−; *Gsx2*−/− double mutants, *Dlx1/2* and *Mash1* are expanded dorsally as in Sey/Sey mutants, and *Pax6* is expanded ventrally as in *Gsx2* mutants, indicating that dorsal *Pax6* is required to repress a ventral fate in the pallium, and ventral *Gsx2* is required to repress a dorsal fate in the LGE (Carney, et al., 2009; Yun, et al., 2001).

Importantly, *Pax6* and *Gsx2* are not the only important players in dorsal-ventral patterning at the PSB. Loss of *Ngn2* leads to loss of dorsal genes in the vP and expansion of ventral genes dorsally (Fode, et al., 2000). Mis-expression of *Mash1* or *Gsx2* in the cortical VZ can drive ectopic expression of other ventral genes, suggesting that correct dorsal specification requires the repression of ventral genes (Fode, et al., 2000; Waclaw, et al., 2009). In addition to transcriptional regulation of patterning at the PSB, several molecules have been implicated during the development of the embryonic CNS that are important for cell sorting. Previous work examining cadherins in the developing telencephalon has shown that R-cadherin expression is important for the sorting of dorsal...
and ventral cells at the PSB, and that the adhesive properties of cells at the PSB are also altered in \textit{Pax6} mutants, implicating cadherin mediated adhesion as an important means of segregating cells at the PSB (Stoykova, et al., 1997). In addition, ephrin signaling has been previously shown to be important for repulsion and adhesion in a number of different developmental contexts, including axonal guidance, cell sorting within rhombomeres in the developing hindbrain, and stop signal cues for cells at the PSB (Nomura, et al., 2006; reviewed in Cooke and Moens, 2002; Tessier-Levine, 1995). In \textit{Pax6} mutants, ephrin A5 expression is decreased and dorsal-lateral olfactory tract cells ectopically invade the ventral side of the PSB (Nomura, et al., 2006). Blockade or over expression of EphARs and ephrinA5 leads to disrupted migration of these cells at the PSB (Nomura, et al., 2006). Further, ephrinA5 mutants have a phenotype similar to \textit{Pax6} mutants, with dorsally derived cells normally destined for the olfactory tract ectopically migrating into the ventral telencephalon. While not directly examined in this thesis, Eph/ephrin and cadherin-mediated sorting and migration is likely critical in maintaining PSB patterning of progenitor cell compartments downstream of the \textit{Pax6} and \textit{Gsx2} transcriptional regulation of the PSB examined in Chapter 2.

\textit{Migration and Neural Diversity}

Ventricular zones in the developing telencephalon proliferate rapidly during embryogenesis to expand the progenitor pool to the number of cells necessary for the composition of a given brain region. However, these cells are often born in locations distant from their ultimate destination. Migration allows common progenitor subtypes to contribute to multiple brain regions and increase the diversity of cells present without
requiring each local germinal zone to produce the multitude of cell types found in each mature brain region (reviewed in Corbin, et al., 2003; Kriegstein and Noctor, 2004; Marin and Rubenstein, 2003). The pallial VZ gives rise to cells that migrate radially in an inside-first, outside-last fashion to produce the excitatory pyramidal neurons that make up 85% of the neuronal composition of the cortex. The pallium also gives rise to a small number of cells that migrate tangentially and ventrally into the developing striatum to become inhibitory MSNs (Williame-Morawek, et al., 2008; Cocas, et al., 2009). Pallial derived cells are also known to migrate rostrally to become inhibitory neurons in the OB (Kohwi, et al., 2007; Williame-Morawek, et al., 2008). Our recent work and those of others also suggests that pallial-derived cells migrate tangentially and ventrally to contribute to the excitatory neuron pool in the amygdala (Waclaw, et al., 2010). The ganglionic eminences are responsible for producing most of the inhibitory neurons of the forebrain. The vLGE gives rise to the medium spiny neurons of the striatum, while the dLGE gives rise to the inhibitory interneurons in the olfactory bulb (Stenman, et al., 2003). The MGE gives rise to PV+ and SOM+ interneurons and the CGE gives rise to CR+ and NPY+ interneurons that migrate dorsally and tangentially into the cortex, striatum, and amygdala (Anderson, et al., 2001; Corbin, et al., 2001; Marin, et al., 2000; Nery, et al., 2002; Wichterle, et al., 2001; Xu, et al., 2004) (Figure 7). Understanding the origins and migratory routes of progenitor domains in the forebrain and their role in the generation of neuronal diversity was an important question examined in this thesis. Specifically, we examined the contribution of the LGE and vP to the amygdala and olfactory bulb in Chapter 2. We also sought to elucidate the contribution of the pallium to the amygdala and striatum in Chapter 3.
Development of Basal Forebrain Structures

We chose to analyze how genetic disruption of the PSB impacted the fate of progenitor pools that are adjacent to the PSB. We also sought to examine the differential progenitor origins in the domains near and adjacent to the PSB, particularly the vP, dLGE, and dP, contributed to the generation of neural diversity in the basal forebrain. As these domains give rise to cells that contribute to the amygdala, striatum, and olfactory bulb, a brief discussion of the development of these structures follows.

Striatum Development

The striatum is the major source of input to the basal ganglia and connects the cerebral cortex with the thalamus, globus pallidus, and substantia nigra. It is critical for motor behavior, including coordinated and planned movement and sensorimotor processing, and has also been implicated in emotionality and cognition (for review, see Gerfen, et al., 1992). Its impairment in Huntington’s disease leads to uncontrolled and excessive motor behavior and slurred speech. During embryonic development, multiple ventral progenitor pools contribute to the developing striatum; these progenitor pools and migratory routes are summarized in Figure 7. The majority (90-95%) of the striatum is composed of GABAergic medium spiny neurons (MSNs), and a minority (5-10%) of inhibitory interneurons that express a diverse number of neurotransmitters and peptides (Kemp and Powell, 1971; Gerfen, 1992; Kawaguchi, 1993; Kawaguchi et al., 1995; Tepper and Bolam, 2004). Early in embryogenesis, these interneurons are born in the MGE and migrate tangentially into the striatal primordium to provide connections
between the MSNs locally (Hamasaki, et al., 2003, Sussel et al., 1999; Wichterle et al., 1999; Marin, et al., 2000). Preplate neurons, which proliferate in the piriform cortex adjacent to the striatum, migrate inward and may be important “pioneer” cells that are important in axon guidance and cell positioning (Hamasaki, et al., 2001). In contrast, MSNs are generated locally from Dlx1/2+ progenitors in the LGE. Once specified in the VZ, these cells migrate radially and separate into the two primary striatal compartments, the patches, which are formed from early migrating MSNs precursors, beginning at E12.5, and the surrounding matrix, formed from later migrating MSN precursors, beginning at around E14.5 (Deacon et al., 1994; Olsson et al., 1995, 1999; Wichterle et al., 2001; van der Kooy and Fishell, 1987; Krushel et al., 1989, 1995; Song and Harlan, 1994).

Analysis of mutants null for key transcription factors provides information about striatal specification. Loss of Mash1 or Gsx2, which are expressed in the LGE, results in a severe reduction of early born patch MSNs, while loss of Dlx1/2, which is expressed in the SVZ, results in a severe reduction of later born matrix neurons, indicating that timing of birth and early transcription factor expression in the LGE is likely important for determining striatal neuron diversity (Anderson, et al., 2007; Wang, et al., 2009). Recent work also indicates that Notch signaling may be important for regulating the production of patch vs. matrix neurons. Loss of Notch signaling during embryonic development leads to a reduction in the number of early-born patch but not later-born matrix neurons, indicating that Notch may mediate a patch vs. matrix MSN fate choice in striatal VZ progenitors (Mason, et al., 2005). Striatal patch and matrix neurons send information to the thalamus via two pathways. In the direct pathway, MSNs project to the internal
segment of the globus pallidus, which then projects to the thalamus; in the indirect pathway, MSNs project to the external segment of the globus pallidus, which then projects to the subthalamic nucleus, which in turn projects back to the globus pallidus, which then projects to the thalamus (Gerfen, et al., 1992). We examined the contribution of dorsal lineage neurons to patch vs. matrix striatal domains and to neurons that participated in the direct and indirect striatal pathways in more detail; these data are described in Chapter 3.

**Amygdala Development**

The amygdala is a key structure in the limbic system that functions in several important behaviors, including distinct components of motivation, memory, and emotionality. In addition, the amygdala is also involved in feeding, reproductive, and defensive behaviors, and, in conjunction with the nucleus accumbens and prefrontal cortex, regulates aspects of drug-seeking behavior in animals (Stoykova et al., 1996; Swanson and Petrovich, 1998; Sah et al., 2003; Maren and Quirk, 2004). Dysfunction of the amygdala is also postulated to be an important component in autism, a developmental disorder that is characterized by impaired social reciprocity and dysregulation of emotionality (Amaral, et al., 2008; Markram, et al., 2008). The amygdala is a complex nucleated structure comprised of at least 11-15 distinct sub-nuclei, although the basolateral amygdala complex (BLC) is the most well studied circuit. The BLC circuitry includes the excitatory and inhibitory neurons in the baso-lateral (BLA) and lateral (LA) nuclei, which receive cortical and thalamic input, and the central (CA) amygdala nucleus, which provides output to the hypothalamic and brainstem regions (Sah, et al., 2003).
These nuclei are functionally connected by the inhibitory intercalated cell mass (ICM) that is adjacent to the BLA and LA; specifically, excitatory neurons in the BLC synapse onto inhibitory neurons in the ICM, which in turn synapse on inhibitory neurons in the CA nucleus, whose inhibitory output is important for both the initiation and the extinction of fear responses (Kaoru, et al., 2009; Likhtik, et al., 2008) (see Figure 8).

Previous research indicates that cells at the PSB contribute to the mature amygdala. Birth dating studies from 30 years ago illustrated that a population of cells migrate from the PSB to the presumptive amygdala (Bayer, et al., 1983). This path of cell migration, the LCS, as evidenced by work from our lab and others using DiI and retroviral tracing experiments, is comprised of two populations of cells that arise from the dorsal and ventral sides of the PSB and migrate to the baso-lateral amygdala (Bayer et al., 2001; Medina, et al., 2004; Bai, et al., 2007, Carney, et al., 2006). Like the striatum, multiple progenitor pools contribute to the amygdala, and evidence from RNA expression profiles of Emx1, Pax6, and Dlx2 indicate that this structure is derived from both pallial and subpallial progenitors, some of which are derived locally in the ventral pallium and amygdala primordium (Nery et al., 2002; Puelles, et al., 2000). In addition, recent work fate-mapping amygdala progenitor pools indicate that both pallial and subpallial structures contribute to the baso-lateral amygdala complex (Cocas, et al., 2009; Hirata, et al., 2009; Waclaw, et al., 2010). Using Dlx5/6Cre mice to fate-map the LGE, MGE, and CGE progenitor domains, Isl1-Cre mice to label the ventral LGE domain, and Dbx1-Cre mice to label the vP, the authors found multiple sources for amygdala domains. Specifically, Dlx5/6-lineage progenitors from the ganglionic eminences gave rise to the inhibitory interneurons in the baso-lateral and lateral nuclei, the central nucleus, and the
intercalated cell mass. Isl1-lineage progenitors from the ventral LGE gave rise to inhibitory neurons in the central nucleus and striatum but not cells in the baso-lateral complex. Dbx1-lineage progenitors from the vP gave rise to excitatory neurons in the lateral and baso-lateral complex but did not give rise to inhibitory interneurons in any amygdala nuclei. These data indicate that the baso-lateral amygdala is derived from progenitor cell domains that also comprise the cortex: excitatory neurons from the pallium and inhibitory neurons from the ganglionic eminences produce the majority of the neurons fated to become both structures.

Recent work has also shown that a caudal progenitor pool derived from the ventral pallium contributes cells that migrate specifically to the NLOT2 nucleus of the amygdala, indicating that at least part of this structure also shares origins with the cortex (Remedios, et al., 2007). Our recent work has also revealed that there is a preoptic area progenitor pool that appears to be solely dedicated to the medial amygdala (Hirata et al., 2009). Additionally, it has been shown through in vivo transplantation studies that the caudal ganglionic eminence (CGE) contributes cells to the baso-lateral amygdala (Nery, et al., 2002). These data indicate that multiple progenitor domains are responsible for neuronal diversity in the mature amygdala, and that some of these progenitor pools are shared with the cortex. We examined the pallial and subpallial components of the amygdala progenitor pools by genetic fate mapping of Emx1, Dbx1, and Gsx2 transcription factor domains; the results are discussed in Chapters 2 and 3.

*Olfactory Bulb Development*
The olfactory bulb is responsible for the processing of odor information that is then relayed to limbic system, specifically, the piriform cortex, amygdala and hypothalamus, which are involved in the integration of these signals into the conscious perception of smell and the concomitant processing of the emotional and reproductive salience of smells (Mori, et al., 1999). Odor information is relayed from highly ordered sensory neurons in the peripheral olfactory epithelium. Sensory neurons that express a common receptor send their axons to a common target in the olfactory bulb, a glomerulus, which is made up of sensory neuron axons and the mitral and tufted cell dendrites with which they form synapses (Linster and Cleland, 2009). Subsequent processing and integration of olfactory information is regulated by numerous inhibitory interneuron subtypes found in the glomeruli and also in deeper regions of the bulb.

Mitral cells are generated first, beginning at E11.5, while tufted cells are born later, beginning around E13.5, and granule cells begin proliferating at E18.5 and continue postnatally (Hinds, 1968). OB interneurons are generated during development beginning at E11.5, and are derived from the dLGE and pallial (most likely, the vP and lP) primordium and migrate rostrally along the RMS to reach the OB. All interneuron subtypes begin to be generated during embryogenesis, with temporal differences in proliferation for different subtypes: TH+ CB+ and PV+ interneurons are generated during neurogenesis, while CR+ interneuron proliferation peaks around birth (Batista-Brito, 2008). Olfactory bulb neurons continue to be produced in the adult SVZ, and postnatal neurogenesis generates the full diversity of olfactory bulb interneurons, indicating that the dLGE is likely to be the embryonic precursor to the adult SVZ (Lledo, et al., 2008). Embryonic olfactory neurogenesis appears to be critical for establishing the initial
interneuron progenitor pools and for the formation of the initial structure of the bulb, as
Gsx2 conditional mutants with loss of Gsx2 only in embryogenesis have severe defects in
the formation of the OB and the generation of OB inhibitory neurons (Waclaw, 2009). To
test whether cells at the PSB contribute to the mature olfactory bulb, we conducted the
complementary conditional mutagenesis experiment, removing Pax6 in Gsx2-lineage
cells, and found that cells at the PSB also contribute to the inhibitory interneuron pools in
the OB; our findings are discussed in detail in Chapter 2.

Summary

While the findings outlined above provide us with a solid foundation about the
early developmental events that underlie patterning, migration, and fate in the developing
forebrain, many aspects of embryonic development remain to be elucidated.
Understanding how early patterning events at the PSB are important for the specification
of cells in progenitor pools adjacent to the boundary is critical to advancing our
knowledge of forebrain patterning; we chose to examine this by analyzing the formation
and refinement of the PSB and the consequences of genetic disruption of progenitor cells
at the PSB on dorsal-ventral patterning (Chapter 2). We find that the correct specification
of the PSB is the result of a combination of local changes in transcription factor
expression and cell motility at the PSB. We also determined that Pax6 and Gsx2 work in
concert to specify not only the correct establishment of the PSB, but also cell
subpopulations generated at the PSB that give rise to excitatory and inhibitory neurons in
the amygdala and inhibitory interneurons in the OB (Chapter 2). Understanding how
different progenitor pools differentially contribute to the amygdala, striatum, and
olfactory bulb also provides insight into the genetic regulation of the generation of neuronal diversity, and so we used genetic fate mapping of embryonic progenitor domains to analyze cell fate in the basal forebrain (Chapters 2 and 3). We also find that multiple progenitor domains at and around the PSB give rise to a diversity of neuronal subtypes in the amygdala, striatum, and olfactory bulb, and that this neuronal diversity is generated by a combination of differential transcription factor expression, timing of birth, and migration trajectory (Chapters 2 and 3). Together, these studies provide important insight into the genetic mechanisms that regulate the generation of neuronal diversity in the developing basal forebrain.
Figure 1. The major subdivisions of the embryonic central nervous system and their corresponding adult structures.
Figure 1. The major subdivisions of the embryonic central nervous system and their corresponding adult structures.

During embryonic development the neural tube is divided into six major subdivisions. Rostrally, the prosencephalon is divided into the telencephalon, which will become the cerebral cortex, basal ganglia, hippocampus, amygdala, and olfactory bulb, and the diencephalon, which will become the hypothalamus, thalamus, epithalamus, and retina. The mesencephalon will give rise to the midbrain. The rhombencephalon is divided into the metencephalon, which will give rise to the pons and cerebellum, and the myelencephalon, which will give rise to the medulla. The caudal portion of the neural tube gives rise to the spinal cord. Adapted from (Kiecker and Lumsden, 2005).
Figure 2. Dorsal-ventral patterning of the neural tube and the formation of the spinal cord.
Figure 2. Dorsal-ventral patterning of the neural tube and the formation of the spinal cord.

A. The neural plate is formed from the ectoderm. Inducing signals include BMPs, which are expressed laterally and SHH, which is secreted from the ventral mesoderm-derived Notochord. B. The neural plate buckles and the two folds fuse together to form the dorsal midline. C. The neural plate is now the neural tube, and BMPs expressed in the glial cells of the roof plate of the neural tube pattern the dorsal neural tube; SHH from the notochord and the glial cells of the floor plate patterns the ventral neural tube. D. SHH works in a concentration-dependent manner to induce different transcription factors that specify cell identity in the spinal cord. High levels of Shh induce floor plate cells, V3 interneurons, and motorneurons. Lower concentrations of SHH induce V1 and V2 interneurons. Abbreviations: BMPs, Bone Morphogenetic Proteins, D, dorsal, FP, floor plate, N, Notochord, MN, Motoneuron, SHH, Sonic Hedgehog, V1, Ventral Interneuron1, V2, Ventral Interneuron 2, V3, Ventral Interneuron 3. Adapted from (Gammill and Bronner-Fraser, 2003).
Figure 3. Initial polarity and patterning of the developing telencephalon.
Figure 3. Initial polarity and patterning of the developing telencephalon.

At E8.5, Foxg1 induces the initial regionalization of the forebrain. By E9.5 Fgf8 expression rostralizes the anterior forebrain; Gli3 expressed in the anterior telencephalon repressed Fgf8 and is itself repressed by Shh in the ventral forebrain. By E10.0, expression of BMPS and WNTS establish medial-lateral identity. Pax6 and Emx1/2 are expressed in the dorsal domain and Gsx2 and Nkx2.1 are expressed in the ventral domain and are responsible for downstream dorsal-ventral patterning of progenitor subdomains. Modified from Fishell and Hebert, 2008.
Figure 4. Transcription factor cascades regulate patterning of the telencephalon.
Figure 4. Transcription factor cascades regulate patterning of the telencephalon.

Initial patterning is established by cross-repressive interactions between Fgfs, Gli3, and Shh. Refinement of progenitor domains by Pax6, Lhx2, Gsx2, and Nkx2.1 determine pallial and subpallial subdivisions. The pallium gives rise to the cortex and hippocampus, and the subpallium gives rise to the olfactory bulb, striatum, pallidum, and the inhibitory interneurons of the forebrain. Modified from Fishell and Hebert, 2008.
Figure 5. Progenitor domains and transcription factor expression patterns of the telencephalon.

Pax6, Emx1, Ngn2
Dbx1, Pax6, Sfrp2
Gsx2, Sp8
Gsx2, Dlx1/2, Mash1
Figure 5. Progenitor domains and transcription factor expression patterns of the telencephalon.

*Pax6, Emx1, and Ngn2* are expressed in the dP and mP. *Dbx1, Sfrp2*, and *Pax6* are expressed in the vP. The PSB divides the vP from the dLGE. *Gsx2* and *Sp8* are expressed in the dLGE. *Gsx2, Dlx1/2*, and *Mash1* are expressed in the MGE. Abbreviations: dP, dorsal pallium, dLGE, dorsal LGE, lP, lateral pallium, MGE, medial ganglionic eminence, mP, medial pallium, PSB, pallial subpallial boundary, vLGE, ventral LGE, vP, ventral pallium.
Figure 6. Summary of gene expression changes in *Pax6* and *Gsx2* mutants.

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Figure 6. Summary of gene expression changes in *Pax6* and *Gsx2* mutants. (A-A’)

*Pax6* expression is lost from the vP and reduced in the pallium in *Sey/Sey* mutants and is expanded across the PSB into the dLGE in *Gsx2/-* mutants. (B-B’) *Ngn2* expression reduced in the pallium in the *Sey/Sey* mutants and is expanded into the vP in *Gsx2/-* mice. (C-C’) *Dbx1* and *Sfrp2* expression is lost from the vP in *Sey/Sey* mutants and is expanded across the PSB into the dLGE in *Gsx2/-* embryos. (D-D’) *Er81* expression is expanded at the PSB in *Sey/Sey* mutants and is reduced in the dLGE in *Gsx2/-* mutants. (E-E’) *Gsx2* expression is expanded across the PSB into the vP in *Sey/Sey* mutants and is reduced in the dLGE in *Gsx2/-* mutants. (F-F’) *Dlx1/2* and *Mash1* expression is expanded in the SVZ and across the PSB into the vP in *Sey/Sey* mutants and is reduced in the VZ dLGE in *Gsx2/-* embryos. (G-G’)*Sp8* expression is expanded in the SVZ and across the PSB into the vP in *Sey/Sey* mutants and is reduced in the SVZ dLGE in *Gsx2/-* mutants.
Figure 7. Migratory routes in the developing telencephalon.
Figure 7. Migratory routes in the developing telencephalon.

Left side, excitatory progenitor cell migratory routes: Cells from the vP, IP, and dP migrate tangentially to contribute to excitatory neurons in the amygdala. Cells from the vP and IP migrate rostrally to contribute to excitatory neurons in the olfactory bulb and cells from the dP may also contribute to this population. Cells from the mP migrate radially to contribute to the hippocampus. Cells from the dP migrate radially in an inside-first outside-last fashion to populate the layers of the cortex. Right side, inhibitory progenitor cell migratory routes: Cells from the dLGE and MGE migrate tangentially to contribute to inhibitory interneurons in the amygdala. Cells from the MGE (and CGE, not shown) give rise to inhibitory interneuron progenitors that migrate tangentially to inhibitory interneurons in the cortex and striatum. Cells from the dLGE give rise to inhibitory interneuron progenitors that migrate rostrally to seed to OB. vLGE cells migrate radially to contribute to the MSNs of the striatum; some vP cells may also migrate ventrally to contribute to this population. Preplate cells migrate medially from the IP into the striatum. Abbreviations: AMY, Amygdala, CX, Cortex, HIP, Hippocampus, OB, Olfactory Bulb, STR, Striatum.
Figure 8. Baso-lateral Amygdala Complex Circuitry.
Figure 8. Baso-lateral amygdala complex circuitry.

The basolateral amygdala complex (BLC) is composed of the LAT and BLA nuclei. These nuclei contain pyramidal-like excitatory neurons that receive excitatory projections from the cortex and send excitatory projections to the inhibitory neurons of the ICM and CA nucleus. Local inhibitory interneurons synapse on these excitatory cells in the basolateral amygdala. The ICM cells receive excitatory input from the BLA and the cortex. In addition to receiving excitatory input from the BLA, the CA also receives inhibitory input from ICM cells. Abbreviations: BLA, basolateral amygdala nucleus, CA, Central nucleus of the amygdala, ICM, Intercalated cell mass, LAT, lateral amygdala nucleus. Adapted from Waclaw, 2010.
Chapter 2

*Pax6 is required at the telencephalic pallial-subpallial boundary for the generation of neuronal diversity in the post-natal limbic system*


*In revision, October, 2010, Journal of Neuroscience*
ABSTRACT

During embryogenesis, the pallial-subpallial boundary (PSB) divides the two main progenitor domains in the telencephalon: the pallium, the major source of excitatory neurons, and the subpallium, the major source of inhibitory neurons. The PSB is formed at the molecular interface between the pallial (high Pax6+) and subpallial (high Gsx2+) ventricular zone (VZ) compartments. Initially, the PSB contains cells that express both Pax6 and Gsx2, but during later stages of development, this boundary is refined into two separate compartments. In this study we examined the developmental mechanisms underlying PSB boundary formation and the post-natal consequences of conditional loss of Pax6 function at the PSB on neuronal fate in the amygdala and olfactory bulb, two targets of PSB-derived migratory populations. Our cell fate and time-lapse imaging analyses reveal that the sorting of Pax6+ and Gsx2+ progenitors during embryogenesis is the result of a combination of changes in gene expression and cell movements. Interestingly, we find that in addition to giving rise to inhibitory neurons in the amygdala and olfactory bulb, Gsx2+ progenitors generate a subpopulation of amygdala excitatory neurons. Consistent with this finding, targeted conditional ablation of Pax6 in Gsx2+ progenitors results in discrete local embryonic patterning defects that are linked to changes in the generation of subsets of post-natal excitatory and inhibitory neurons in the amygdala and inhibitory neurons in the olfactory bulb. Thus, in PSB progenitors, Pax6 plays an important role in the generation of multiple subtypes of neurons that contribute to the amygdala and olfactory bulb.
INTRODUCTION

The amygdala and olfactory bulb are ancient limbic structures in the relatively evolutionarily youthful region of the forebrain. Both have an important function in circuits that process memory, respond to fear and fear extinction, and are implicated in several developmental disorders, including autism spectrum disorders (Rodrigues et al., 2004; Amaral et al., 2008; Herry et al., 2008; Markram et al., 2008; Monk, 2008). Unraveling the genetic and cellular mechanisms that drive the formation and specification of these structures is therefore necessary to understand function and dysfunction in the adult forebrain.

Interestingly, major neuronal sub-populations in the amygdala and olfactory bulb (OB), two key interconnected structures of the limbic system, are derived from a common origin in the embryonic forebrain. This region is the PSB, a transient molecular border where telencephalic pallial and subpallial gene expression meet (Toresson et al., 2000; Yun et al., 2001; Corbin et al., 2003; Carney et al., 2006; Carney et al., 2009). Subsets of cells generated at the PSB give rise to neurons in the OB via the rostral migratory stream (RMS), and to subsets of excitatory neurons in the baso-lateral complex (BLC) of the amygdala via the lateral cortical stream (LCS) (Stenman et al., 2003a; Carney et al., 2006; Lledo et al., 2008; Waclaw et al., 2009; Hirata et al., 2009). In addition, progenitors from the dorsal lateral ganglionic eminence (dLGE) aspect of the PSB contribute to the amygdala intercalated cell mass (ICM), a specialized subpopulation of BLC inhibitory neurons that are essential for fear conditioning (Likhtik et al., 2008; Kaoru et al., 2009; Waclaw et al., 2010).
The PSB is characterized by the convergence of expression of two transcription factors in the ventricular zone, *Pax6* in the ventral aspect of the pallium (vP), and *Gsx2* in the subpallium at the dorsal aspect of the LGE (dLGE) (Toresson et al., 2000; Yun et al., 2001; Corbin et al., 2003; Carney et al., 2006; Carney et al., 2009). Analyses of mouse mutants have revealed that *Pax6* and *Gsx2* function in a cross-repressive and combinatorial manner for proper patterning of the PSB (Mastick et al., 1997; Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001; Nomura et al., 2006; Carney et al., 2009). During embryogenesis, *Pax6* and *Gsx2* expression patterns at the PSB are dynamic, initially overlapping at E10.5, and subsequently refining by mid-neurogenesis into two separate compartments (Corbin et al., 2003). However, the cellular mechanisms that regulate the sorting of progenitors to their respective compartments at the PSB remain unexplored. Further, little is known about the link between the genetic regulation of PSB patterning and the generation of neuronal diversity in the amygdala and the OB.

In this study we utilized genetic fate mapping and conditional mutagenesis to address the above questions. We found that refinement of the PSB is a dynamic process regulated by changes in gene expression and cell movements. Further, *Gsx2*-lineage cells gave rise to multiple inhibitory interneuron subtypes in the OB and in the BLC of the amygdala. Unexpectedly, *Gsx2*-lineage cells also generated a subpopulation of excitatory neurons in the BLC. Moreover, conditional ablation of *Pax6* at the PSB resulted in focal defects in patterning during embryogenesis. Corresponding with this mis-patterning were alterations in the generation of neuronal subpopulations in the post-natal brain: fewer *Gsx2*-lineage excitatory neurons and inhibitory intercalated cells were present in the mutant amygdala, and fewer inhibitory interneurons were found in the outer layers of the
olfactory bulb. The formation and maintenance of the PSB by \textit{Pax6} and \textit{Gsx2} is therefore not only important for the correct patterning of progenitor domains during embryonic development; it is also critical for the establishment of subsets of excitatory and inhibitory neuron populations in the post-natal limbic system.

**METHODS**

\textit{Animal Use}. Swiss Webster (Taconic Farms, Albany, NY), \textit{ROSA-YFP} (Jackson Laboratory, Bar Harbor, Maine, \textit{Gsx2}^{\text{Cre}} (N. Kessaris, University College London, (Kessaris et al., 2006)), \textit{Dbx1}^{\text{Cre}} (A. Pierani, Wassef, 2003), \textit{Pax6-GFP} (GENSAT, (Gong et al., 2003)) and \textit{Pax6}^{\text{flax/flax}} mice (D. Price, University of Edinburgh (Simpson et al., 2009)) used in these studies were maintained according to the protocols approved by Children’s National Medical Center and the University of Edinburgh. \textit{Gsx2}^{\text{Cre}} and \textit{Pax6}^{\text{flax/flax}} mice were maintained on a mixed C57Bl/6 x SW background at Children’s Hospital; \textit{Pax6}^{\text{flax/flax}} and \textit{Gsx2}^{\text{Cre} \text{; } \text{RYFP}}; \textit{Pax6}^{\text{flax/flax}} mice were maintained on C57/BL6 background at the University of Edinburgh. For staging of the embryos, midday of vaginal plug detection was considered as embryonic day 0.5 (E0.5). For post-natal animals, the day of birth was considered as post-natal day 0 (P0). The genotyping of animals was performed as described previously (Carney et al., 2009; Simpson et al., 2009). \textit{Gsx2}^{\text{Cre} +/ -}; \textit{Pax6}^{+/+}; \textit{ROSA-YFP}^{+/+} were used as controls while \textit{Gsx2}^{\text{Cre} +/ -}; \textit{Pax6}^{\text{cKO}}; \textit{ROSA-YFP}^{+/+}(referred to as \textit{Pax6}^{\text{cKO}}) mice were used for analysis of conditional knockouts.
**Tissue Preparation and Histology.** For immunofluorescence and *in situ* hybridization at embryonic ages, brains were fixed in 4% paraformaldehyde (PFA) for 2 hours or overnight, respectively. Brains were cryoprotected by sucrose immersion, embedded in Histoprep (Fisher Scientific, Pittsburgh, PA) and frozen. Serial coronal sections of embedded tissue were cut at 20-30 µm thickness using a cryostat and mounted on glass slides. Post-natal animals were transcardially perfused at P21 with 4% PFA, post-fixed for 2-4 hours, and processed as with the embryonic tissue.

**Immunohistochemistry.** Cryostat mounted sections were air-dried and rinsed 3 times in PBS before blocking for 1 hour in 10% normal donkey serum diluted in PBS with 0.2% Triton to prevent non-specific binding. Primary antibodies were diluted in 1% serum diluted in PBS with 0.2% Triton; sections were incubated in primary antibody overnight at 4°C. The primary antibodies used were as follows: goat anti-Pax6 (1:200; Santa Cruz, Santa Cruz, CA), rabbit anti-Pax6 (gift of V. van Heyningen), mouse anti-NeuN (1:500; Covance, Princeton, NJ), rat anti-GFP (Nacala, Japan, 1:2000), rabbit anti-Gsx2 (1:1500, gift of K. Campbell), rabbit anti-Tbr1 (1:1000; gift of R. Hevner); guinea-pig anti-panDlx (1:1500; gift of K. Yoshikawa), rabbit anti-TH (1:1000; Chemicon, rabbit anti-Mef2c (1:1,000) rat anti-Somatostatin (1:1000; Millipore); rabbit anti-parvalbumin (1:1000), rabbit anti-calbindin (1:1000, Sigma), rabbit anti-calretinin, (1:2000). To detect primary antibodies, secondary antibodies raised in mouse, rat, goat, guinea pig, and rabbit were used (Cy3 and Cy5 at 1:200, FITC at 1:50; all from Jackson Immunoresearch, West Grove, PA, USA). Sections were incubated for 2 hours in 1% serum in PBS with 0.2%
Triton and were washed and cover slipped with gel mount (Sigma) or Vectashield with DAPI (Vector Labs, Burlingame, CA).

In situ hybridization. Tissue was prepared as described above. Non-radioactive dioxygenin-labeled RNA in situ hybridization was carried out as described previously (Carney et al., 2009). The probes used in this study were Islt1, Ngn2, Tsh1, Sp8, and Sfrp2.

Time Lapse Imaging. Time lapse imaging experiments were carried out as described previously, (Gal et al., 2006). Briefly, E11.5 and E13.5 brains were dissected in ice cold HBSS (Invitrogen), the skull and meninges were removed, and brains were placed in 3% low melt agarose (Fisher Scientific, Pittsburgh, PA, USA). 250-300 µm slices were cut using a vibratome (VT1000S; Leica, Nussloch, Germany), and sections were allowed to recover at 37°C in MEM supplemented with L-glutamine (1:100), penicillin/streptomycin (1:100) and 10% fetal bovine serum (all from Invitrogen). Slices were then mounted on an inverse Zeiss (Thornwood, NY) LSM 510 confocal microscope mounted with a custom-made incubation chamber and perfusion system. Slices were continuously perfused with Neurobasal medium supplemented with B27, N2, penicillin/streptomycin, l-glutamine, and CO2 and maintained at 370C. Z-stack images of GFP+ cells were taken using the Covalent multiphoton laser scanning at 790nm every 6 minutes for 16-21 hours. The time series Z stacks were compiled and processed using Velocity imaging software. For analysis of cell movements, Image J cell tracking software was used to calculate distance traveled and velocity of individual cells.
Electrophysiological recordings were performed from fluorescent $Gsx2$-lineage YFP+ cells located in the lateral nucleus of the amygdala. Briefly, animals were deeply anesthetized (with isoflurane) until non-responsive and then decapitated. Brains were removed and immediately immersed for 2-3 minutes in an ice-cold-oxygenated (95% O$_2$/5% CO$_2$) sucrose slicing solution (mM): 87 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 7 MgCl$_2$, 0.5 CaCl$_2$, 25 NaHCO$_3$, 25 Glucose, 75 Sucrose (347 mOsmol) at pH 7.4. Coronal slices containing the amygdala were cut on a vibratome (VT1000S, Leica) at 250 µm. Slices were collected and placed in a clean chamber containing oxygenated sucrose slicing solution at room temperature and incubated for one hour, then transferred to a chamber containing oxygenated artificial cerebral spinal fluid (ACSF) (mM): 119 NaCl, 26 NaHCO$_3$, 10 glucose, 2.5 KCl, 1.25 NaH$_2$PO$_4$·H$_2$O, 1.3 MgSO$_4$, and 2.5 CaCl$_2$·2H$_2$O; pH 7.4, room temperature. Neurons were located and visualized with a fixed staged, upright microscope (BX51WI, Olympus) equipped with a 4x objective and a 63x insulated objective, infrared (IR) illumination, Nomarski optics, an IR-sensitive video camera (Oly-150) and fluorescent lamp. Glass pipettes (borosilicate glass with filament, WPI, FL) were pulled with a Flaming/Brown Micropipette Puller (PP 830, Narishige, Japan) to a resistance of 3-6 MOhms. For all recordings the intracellular pipette solution consisted of (in mM): 130 K-gluconate, 20 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl$_2$, 2 Na$_2$ATP, 0.4 NaGTP and 3 mg/ml biocytin (pH adjusted to 7.2, 290 mOsmol). Recordings were performed at room temperature with continuous perfusion (2 ml/minute) of ACSF. Cells were recorded in current clamp mode using a multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and a Digidata 1322A data acquisition system.
(Molecular Devices) and PC running pClamp 9.2 (Molecular Devices). For all cells, membrane potential and input resistance values were recorded. Cells were then characterized on the response to depolarizing and hyperpolarizing current pulses for a duration of 1000 msec in 16 consecutive sweeps. Off-line analysis was performed using Clampfit 9.2 (Molecular Devices). In all experiments, data were filtered at 10 kHz during capacitance compensation and 5 kHz during subsequent data recording. The traces were digitized at 10 kHz. All voltage measurements and steps were corrected for a junction potential offset.

Microscopy. In situ hybridization photographs were taken using an Olympus (Center Valley, PA) BX51 microscope. Fluorescent photographs were taken using a Zeiss (Thornton, NY) LSM 510 confocal microscope. For confocal image analysis, each fluorophore was scanned sequentially and Z-stacks of the images obtained were collapsed into a single projection image or presented as individual optical sections. Figures were prepared using Image J and Adobe Illustrator; brightness and contrast adjustments were applied equally across all images.

Data Analysis. Post-natal sections from Gsx2$^{Cre}$; ROSA-YFP; Pax6$^{+/+}$ and Gsx2$^{Cre}$; Pax6$^{flax/flax}$, ROSA-YFP brains were photographed as described above. For each immunohistochemical marker, coronal sections of the lateral and baso-lateral amygdala at Bregma levels -1.6 to -2.4 and of the olfactory bulb at Bregma levels 3.5 to 4.3 were examined from $n=/>3$ Gsx2$^{Cre}$; RFYP; Pax6$^{+/+}$ and Gsx2$^{Cre}$; Pax6$^{flax/flax}$ brains. The following criteria were applied to determine co-localization of cell-subtype markers with
YFP fluorescence: 1. Cells were counted from individual optical sections, not collapsed projection images. 2. Cells were counted as double positive if an immuno-positive YFP cell body was clearly co-localized with the fluorophore of interest and contained a nucleus that was also DAPI-positive. All statistical analyses of differences between groups were conducted using a two-tailed type two student’s t test, and the null hypothesis was rejected if p<0.05.

RESULTS

Mechanisms of PSB refinement

The PSB is a region in the ventricular zone of the developing telencephalon where high pallial-expressed Pax6 in the vP converges with high subpallial-expressed Gsx2 in the dLGE (Toresson et al., 2000; Yun et al., 2001; Corbin et al., 2003; Carney et al., 2006; Carney et al., 2009). In addition, our previous work has revealed that at the onset of Gsx2 expression (27 somites, ~E10.0), Gsx2 and Pax6 expression intermingle, and this overlap is largely resolved by 3-4 days later (Corbin et al., 2003). However, how this border is refined remains unexplored. Therefore, we sought to further investigate the timing and the cellular mechanisms underlying PSB refinement. Previous studies of boundary refinement in other brain regions indicate that there are two main non-mutually exclusive mechanisms by which this may occur: 1. Cells expressing patterning genes not shared by their neighbor cells down-regulate the “incorrect” genes and up regulate the “correct” genes expressed by their neighbors 2. Cells expressing patterning genes not shared by neighboring cells move into the territory of like-expressing cells.
To explore whether PSB VZ progenitor cells change gene expression during the period of PSB refinement, we indelibly labeled Gsx2-expressing progenitors and their descendants in Gsx2\textsuperscript{Cre}; ROSA-YFP embryos (Kessaris et al., 2006). Thus, by analysis of Gsx2+, Pax6+ and YFP+ (Gsx2-lineage) cells during the window of PSB refinement, we were able to determine if Gsx2+ cells changed their fate to become Pax6+ cells. Consistent with our previous studies (Corbin et al., 2003) we found that at E11.5, the PSB is a mixed boundary, with Pax6+ cells present in the Gsx2+ domain, and Gsx2+ cells present in the Pax6+ domain, and a third population of cells expressing both Pax6 and Gsx2 (Fig. 9A,B). At earlier embryonic ages, YFP+ (Gsx2-lineage) cells in the more ventral aspect of the PSB (dLGE) co-label largely with Gsx2 protein (Fig. 9C,D). Additionally, a sub-population of YFP+ cells express Pax6 (Fig. 9E,F). In contrast, by E15.5, the PSB is largely refined, as these two progenitor domains are no longer intermixed. This is evident by the absence of Gsx2+ cells in the Pax6+ domain and Pax6+ cells in the Gsx2+ domain. In addition, with the exception of a few cells at the border, Pax6 and Gsx2 protein expression is no longer overlapping (Fig. 10A,B). In contrast, YFP+, Gsx2-, Pax6+ cells are present beyond the refined Gsx2+ domain, revealing that they have down-regulated expression of Gsx2 and up-regulated expression of Pax6 (Fig. 10C-F,G). Thus, at least in case of dLGE progenitors, changes in gene expression are a mechanism employed to refine the PSB.

We next wanted to determine whether the cell movements of progenitors across the PSB during development also contributed to refinement of the PSB. To accomplish this we carried out time-lapse imaging experiments using Pax6-GFP mice (Gong et al., 2003) to label the complementary dorsal side of the PSB (the vP) (Figures 11-13). Using
two-photon microscopy to image E11.5 and E13.5 brain slices for at least 18 hours, we observed that cell movements across the PSB are much more frequent at E11.5, with numerous cells moving both dorsally and ventrally in the VZ and SVZ (subventricular zone). In contrast, at E13.5, cell movements are much less frequent and largely constricted to the SVZ (Figures 11-13). These data indicate that as the border is refined by changes in transcription factor expression during development, a concomitant change in cell movement across the boundary between the pallial and subpallial domains occurs. Thus, our data indicate that both gene expression changes and cell movements contribute to refinement of the PSB.

**Fate of Gsx2-derived cells**

Cell transplantation and genetic fate-mapping studies (Stenman et al., 2003a; Carney et al., 2006; Waclaw et al., 2006; Brill et al., 2008; Hirata et al., 2009; Waclaw et al., 2009; Waclaw et al., 2010) have shown that cells derived from the PSB contribute to the amygdala and olfactory bulb. We sought to determine the specific sub-types of neurons that Gsx2-lineage progenitors gave rise to in these two structures by examining the distribution of YFP+ cells in Gsx2^{Cre}; ROSA-YFP mice at post-natal day 21 (P21). We found that in the lateral (LAT) and baso-lateral (BLA) amygdala the majority of neurons express inhibitory markers (data not shown and Figure 20). However, as revealed by expression of Tbr1, Gsx2-lineage cells also unexpectedly gave rise to a subpopulation of excitatory neurons (Fig. 14A-C). This represented 38% (257/740, N=3) of the total Tbr1+ population in the LAT nucleus and 11% (80/756, N=3) of the total Tbr1+ population in the BLA nucleus. In addition, Mef2c, which is enriched in the LAT nucleus
of the amygdala (Waclaw et al., 2010), also marked a subpopulation of Gsx2-lineage cells (35%, 186/835, N=3) (Fig. 14D,E). Gsx2-lineage cells also comprised the lateral and medial intercalated cell mass (ICM) (Fig. 14F), a group of inhibitory neurons likely derived from the PSB (Waclaw et al., 2010). The ICM is important in modulating inhibitory input between the baso-lateral complex and the central nucleus, an important circuit in mediating fear responses.

Previous studies have shown that LAT/BLA pyramidal-like excitatory neurons can be distinguished from interneurons based on their large soma and specific firing properties (Sah et al., 2003; Sah and Lopez De Armentia, 2003; Cocas et al., 2009). We therefore performed patch clamp recordings of Gsx2-lineage cells in P21 brain slices to examine whether they would exhibit pyramidal-like functional properties. These large, pyramidal-shaped Gsx2-lineage neurons had an average membrane capacitance of 120 ± 44 pF and an average membrane resistance of 278 ± 89 Mohms (n = 18) (Table 1). They all exhibited a regular but accommodating firing pattern (Frequency = 16.8 ± 3.8 Hz; Accommodation ratio = 0.29 ± 0.12; n = 18) and a large and slow after-hyperpolarization (AHP) (I_{AHP} = -8.3 ± 2.0 mV; Half-width_{AHP} = 132 ± 39 ms; n = 18), both features typical of pyramidal-like neurons in the lateral amygdala (Fig. 14G,H, Table 1) (Sah et al, 2003; Cocas et al, 2009). These neurons could easily be distinguished from the few Gsx2-lineage interneurons we also recorded (n = 3), (Fig. 14I-K). These interneurons exhibited a significantly lower membrane capacitance (Cm = 40 ± 21), higher membrane resistance (Rm = 790 ± 290 Mohms), a higher firing frequency (Frequency = 36 ± 5 Hz), a biphasic AHP and a shorter action potential (Half-spike duration = 1.5 ± 0.3 ms).
We also examined the fate of Gsx2-lineage cells in the P21 olfactory bulb. Consistent with previous studies (Young et al., 2007), we found that this lineage gives rise to inhibitory neurons throughout the olfactory bulb, as indicated by co-labeling with Dlx and Foxp2 (Fig. 15L-N and Figure 19). Interestingly, a small (4%, 8/326, N=3) population of Gsx2-lineage cells in the periglomerular layer also expressed the excitatory neuronal marker Tbr1 (Fig. 15L and Figure 19). Together these data demonstrate that within two limbic structures, the amygdala and the olfactory bulb, Gsx2-lineage cells contribute to both excitatory and inhibitory neuronal populations.

We wanted to analyze the differential contribution of dorsal and ventral PSB populations to the adult basal forebrain. Dbx1 marks the vP, the dorsal side of the PSB, and therefore examining the Dbx1-lineage population allowed us to fate-map the complementary dorsal PSB domain to compare to the Gsx2-lineage analysis of cell fate in the amygdala and olfactory bulb. We found that in the amygdala, like Gsx2-lineage progenitors, Dbx1-lineage progenitors gave rise to excitatory neurons; unlike Gsx2-lineage progenitors, Dbx1-lineage progenitors did not give rise to inhibitory neurons in the BLA and LAT nuclei (Fig 15A-F). In the OB, Dbx1-lineage cells did not give rise to inhibitory interneurons but instead contributed to Reelin+ neurons and Tbr1+ neurons and in the external plexiform and mitral cell layers (Fig. 15G-K). These analyses indicate that cells generated on opposite sides of the PSB give rise to common and separate cell subpopulations in the amygdala and olfactory bulb.

Effects of conditional Pax6 mutagenesis
Our previous work (Corbin et al., 2003) and this study (Figures 9 & 10) reveal that during a distinct window of development a subpopulation of cells at the PSB express both Pax6 and Gsx2, and that Gsx2-lineage cells give rise to both excitatory and inhibitory neurons in the amygdala and olfactory bulb (Figure 14). Furthermore, homozygous Small eye (Sey/Sey) Pax6 mutants exhibit disruptions of the PSB, with expansion of subpallial cells into the pallial domain, a loss of pallial-specific gene expression, and abnormal cell migration (Stoykova et al., 1996; Mastick et al., 1997; Dellovade et al., 1998; Toresson et al., 2000; Jimenez et al., 2002; Stenman et al., 2003b; Talamillo et al., 2003; Kroll and O’Leary, 2005; Nomura et al., 2006; Carney et al., 2009). However, Sey/Sey mutants are embryonic lethal, which has confounded analysis of the post-natal consequences of Pax6 loss of function. To examine the consequences of PSB mis-patterning on the generation of neuronal diversity in the post-natal brain, we sought to examine neuronal fate in Pax6 mutants. To overcome embryonic lethality, we conditionally knocked out Pax6 in Gsx2-derived cells using Pax6^floxflox; Gsx2^Cre mice. This is, to our knowledge, the first in-depth analysis of Pax6 loss of function and its impact on neuronal subpopulations in the post-natal forebrain.

We first examined putative embryonic patterning defects in Gsx2-driven Pax6 conditional mutants (Gsx2^Cre; Pax6^floxflox; ROSA-YFP mice- hereafter referred to as Pax6^cKO mice) compared to control mice (Gsx2^Cre; Pax6^+/+; ROSA-YFP). Consistent with PSB changes previously observed in Sey/Sey mutants (Mastick et al., 1997; Yun et al., 2001; Carney et al., 2009), analysis at E15.5 revealed patterning defects specifically at the PSB. Expression of the vP marker Sfrp2 was substantially decreased in Pax6^cKO mutants (Fig. 16A,F). In addition, expression of the dLGE markers Sp8 and Tsh1
persisted and appeared slightly expanded (Fig. 16B,C,G,H). This was accompanied by a retraction of expression of pallial marker Ngn2 at the PSB; however, its expression in the remainder of the pallium was normal (Fig. 16D,F). Further, the expression pattern of the subpallial marker Isl1, which marks the developing striatum, was unaffected (Fig. 16I,J).

We next analyzed whether Pax6cKO embryos displayed an alteration of marker expression locally at the PSB. In E15.5 Pax6cKO embryos, Pax6 expression was decreased at the PSB and vP, compared to controls (Fig. 17A,B,G,H). Interestingly, Pax6 expression was also decreased in Pax6cKO embryos in the developing amygdala (Fig. 17C,I). The Gsx2-lineage (YFP+) domain was expanded, encroaching closer to the sulcus (Fig. 17D,J). In addition, the domain of Gsx2 expression was expanded, with more Gsx2+ cells scattered ectopically in the vP (Fig. 17E,K). Expression of Ngn2 was also decreased at the vP in E13.5 Pax6cKO mutants, indicating that the vP domain was abnormal in these embryos (Fig. 17F,L). Collectively, these results indicate that, rather than the global patterning defects previously observed in Sey/Sey mutants, Pax6cKO embryos have focal patterning defects at the PSB, characterized by a loss of the vP and an expansion of the dLGE domain.

We next examined whether Pax6cKO embryos had interneuron migratory defects similar to the defects observed in Pax6 null embryos (Stoykova). At E15.5, we observed an increased density of Gsx2-lineage interneurons in the SVZ of the dorsal pallium, far beyond the expanded domain of Gsx2 expression (Figure 18A,B,F,G). In the control embryo, Gsx2-lineage cells were sometimes found in radially-dispersed groups, resembling clones of cells (Figure 18 C, D). However, in Pax6cKO embryos, Gsx2-lineage
cells frequently formed heterotopias of cells that were never observed in control mice; further, these Gsx2-lineage cell clusters often contained Gsx2+ cells, which were not observed in control mice (Figure 18C-E, H-J). These data indicate that loss of Pax6 in Gsx2+ cells at the PSB results in interneuron migration defects similar to those observed in the global Pax6 mutant.

We next sought to examine the long-term consequences of PSB disruption on neuronal diversity in two regions known to be derived from the PSB: the amygdala and the OB. In the amygdala, we found that the overall structure of the BLC appeared normal (Fig. 19A,D). We also observed that the numbers and distribution of calbindin+ (CB+), calretinin+ (CR+), and NOS+ inhibitory interneuron subpopulations in the BLA and LAT nuclei, and CB+ interneurons in the central nuclei were not altered in Pax6cko mice (Figure 20). In contrast, Foxp2+ inhibitory interneuron numbers were significantly decreased in the lateral and medial ICM domains (Fig. 19A,B,E,F,I). Additionally, the number of Gsx2-lineage Tbr1+ excitatory neurons was significantly decreased in Pax6cko mice in the LAT nucleus (Fig. 19A,C,E,G,I). However, there were not significant decreases in the number of Gsx2-lineage Tbr1+ neurons in the BLA nucleus (Fig. 19A,D,E,H,I). These data indicate that, in the BLC, Pax6 is required in the Gsx2-lineage for generation of a subpopulation of excitatory neurons and inhibitory ICM neurons, but not inhibitory interneurons.

Next, we examined the consequences of the conditional loss of Pax6 on the generation of neuronal diversity in the post-natal olfactory bulb. Previous work has shown post-natal olfactory bulb interneuron defects in Sey heterozygotes, but analysis of Sey homozygotes has not been possible due to embryonic lethality (Dellovade et al.,
Despite the fact that many OB cells are derived from the Gsx2-lineage, the general structure of the OB appeared normal in P21 \textit{Pax6}\textsuperscript{cKO} mutants (Figure 21). However, the total percentage of Gsx2-lineage cells in the glomerular layer was decreased in \textit{Pax6}\textsuperscript{cKO} mice (44\%, in control mice compared to 37\%, in \textit{Pax6}\textsuperscript{cKO} mice, \textit{p}=0.03, \textit{N}=3). When we examined interneuron subtypes, we found significant decreases in the numbers of TH+,

PV+, and CR+ interneuron subtypes (Fig. 21C-F,I,J). In contrast, there were no significant differences in CB+ interneurons or Tbr1+ excitatory neurons (Fig. 21A,B,G,H,I). These data reveal that \textit{Pax6} functions in Gsx2+ cells to generate the correct numbers of OB interneurons, but not excitatory neurons.

As we observed that \textit{Pax6}\textsuperscript{cKO} embryos had abnormal interneuron migration and cortical heterotopias in the developing pallium, we wanted to determine whether these embryonic defects led to changes in the interneuron populations in the postnatal cortex. We analyzed the distribution of Gsx2-lineage interneurons in the layers of the sensorimotor cortex at P21, and found no changes in distribution or total cell number in \textit{Pax6}\textsuperscript{cKO} mutants (Figure 22A-C). We also examined the number and distribution of Gsx2-lineage PV+ and SST+ interneurons in \textit{Pax6}\textsuperscript{cKO} mice, and found that while the number of Gsx2-lineage PV+ and SST+ interneurons were reduced, particularly in the lower layers of the cortex, these decreases were not significant (Figure 22D). These data suggest that the embryonic abnormalities in migrating cortical interneurons do not lead to major defects in interneuron populations in the mature cortex.

In summary, our results reveal that the pallial-subpallial boundary is a dynamic border, with refinement of the expression of Pax6 and Gsx2 occurring via changes in
gene expression (Figures 9 and 10) and a complementary change in cell motility, with fewer cell movements occurring in the VZ of the PSB as development proceeds (Figures 11-13). Using genetic fate mapping to label the Gsx2+ dLGE, we found that cells from this domain gave rise to both excitatory and inhibitory lineages in the amygdala and olfactory bulb (Figure 14). We also found that the Dbx1+ vP gives rise to different populations than the Gsx2+ dLGE: while both domains give rise to excitatory neurons in the amygdala, the Dbx1-lineage but not the Gsx2-lineage gives rise to excitatory neurons in the OB (Figure 15). The conditional ablation of Pax6 from cells that also express Gsx2 resulted in abnormal patterning at the PSB, an ectopic expansion of the dLGE, and cortical heterotopias (Figures 16-18). This PSB disruption resulted in differential alterations in neuronal cell subpopulations in the amygdala and olfactory bulb, regions that are seeded by cells from the PSB. Specifically, the numbers of inhibitory interneurons (but not excitatory neurons) were decreased in the olfactory bulb and the numbers of excitatory neurons and ICM interneurons but not other interneuron subtypes were decreased in the LAT nucleus of the amygdala (Figures 19-21). Interestingly, the disruption of the PSB did not alter cortical interneuron populations in the adult (Figure 22), suggesting that disruption of the PSB does not impact migrating interneurons from the subpallium. These findings provide an important link between the essential function of Pax6 during the development and patterning of the embryonic brain and the generation of neurons that give rise to the post-natal limbic system.

**DISCUSSION**
One important facet of nervous system development is the establishment of cell-lineage compartments from which disparate neuronal cell types are born. These compartments are separated by discrete borders, which act to maintain gene expression domains and also as a physical barrier to prevent cell mixing. Borders in the embryonic nervous system are typically formed by cross-repressive interactions between opposing transcription factors that regulate the expression of additional secreted and cell adhesion molecules, which in turn function to establish and maintain lineage compartments (for review, see (Kiecker and Lumsden, 2005)). The most well characterized border is the mid-hindbrain boundary (MHB), which is marked by the convergence of expression of the transcription factors Otx2 and Gbx2, as well as the expression of secreted Wnts and Fgf8 (Rhinn and Brand, 2001). These intrinsic and extrinsic factors act in a combinatorial manner to generate different neuronal populations on either side of the border. Disruption of this molecular cascade results in abnormal cell specification and structural abnormalities (Liu et al., 1999; Martinez et al., 1999).

While the establishment and importance of the MHB has been well-characterized, major aspects of the regulation of the development of the telencephalic PSB, which separates the pallial and subpallial domains of the telencephalon, remain unknown. Our current and previous work has shown that the PSB is a dynamic border in which expression of Pax6 and Gsx2, whose convergence defines the PSB, changes over neurogenesis from an early overlapping stage to a later period of refinement. In addition, previous studies from our laboratory and others have shown that cells born at the PSB contribute extensively to the limbic system, most prominently to the amygdala and the olfactory bulb (Stenman et al., 2003a; Carney et al., 2006; Lledo et al., 2008; Hirata et al.,
In this study we found that the refinement of the PSB, as defined by separation of high Pax6 and Gsx2 protein-expressing progenitors, is complete by approximately mid-neurogenesis (E15.5), a time when progenitor pools are largely specified. By genetically fate mapping subpallial PSB (dLGE) progenitors, we reveal that a major mechanism of PSB refinement is via changes in gene expression, as many Gsx2+ cells change their fate to express Pax6. We also find that the permeability of the border, as evidenced by cell movements in the ventricular zone, decreases with development. With these data we argue that the refinement of the PSB is driven by both changes in gene expression in individual cells and by changes in the fluidity of cell movements at the PSB. These developmental mechanisms for refining the boundary are likely utilized to prevent cells from pallial and subpallial compartments from mixing with each other later in development. Consistent with previous studies, which have shown differences in cell adhesion and cell adhesion molecule expression at the PSB (Stoykova et al., 1997; Nomura et al., 2006), these genetic and cellular mechanisms of PSB refinement are also most likely coordinated with changes in cell-adhesion between pallial and subpallial domains.

Although our findings show that the PSB is largely refined by E15.5, the question arose as to which role, if any, Pax6 plays in Gsx2+ cells during the relatively prolonged period of gene expression overlap (approximately 4 days). We addressed this question by conditionally knocking out Pax6 only in cells that co-express Gsx2, thus specifically manipulating this progenitor pool. At embryonic stages, we observe an expansion of the dLGE at the expense of the vP. This result is consistent with the PSB patterning defects observed in studies of full Pax6 (Sey/Sey) mutants (Stoykova et al., 1996; Yun et al.,
2001; Stenman et al., 2003b; Talamillo et al., 2003; Carney et al., 2009). As the PSB is the origin of cells that will populate the amygdala and olfactory bulb, the well-characterized patterning defects in Sey/Sey mutants are predictive of limbic system disruptions. However, as these mice are embryonic lethal, it has not been possible to explore the effects of embryonic disruption of the PSB in the mature brain. Our conditional mutagenesis approach has allowed us to overcome this major hurdle and directly examine the long-term consequences on cell specification in the amygdala and olfactory bulb, two major migratory targets of the PSB. Our major finding from this analysis is that Pax6 is necessary in Gsx2+ cells for the correct formation of both excitatory and specific inhibitory neurons in the post-natal amygdala and subsets of interneurons in the post-natal olfactory bulb. Thus, our data provide novel insight into the function of Pax6 in the developing brain and link embryonic PSB mis-patterning to changes in post-natal neuronal diversity in two key limbic system structures.

Previous gene expression and fate mapping studies have revealed that the amygdala is derived from a combination of progenitor pools from disparate regions of the brain, including the PSB (Nery et al., 2002; Tole et al., 2005; Carney et al., 2006; Cocos et al., 2009; Hirata et al., 2009; Waclaw et al., 2010). The dLGE and vP components of the PSB generate subpopulations of inhibitory and excitatory neurons that largely populate the baso-lateral complex (BLC) of the amygdala. Previous work from our laboratory and others has shown that both pallial- and subpallial-derived progenitor pools, as well as diencephalic sources, contribute to neuronal diversity in numerous nuclei of the amygdala (Cocos et al., 2009; Hirata et al., 2009; Garcia-Moreno et al., 2010; Waclaw et al., 2010). With regard to the BLC, genetic fate mapping studies have
revealed that dorsal pallial-derived *Emx1*+ and vP-derived *Dbx1*+ populations generate excitatory neurons (Cocas et al., 2009; Hirata et al., 2009). In contrast, it appears that the *Gsx2*+ dLGE may be a major source of the specialized intercalated cell masses (ICM) (Waclaw et al., 2010).

Consistent with these studies, our fate mapping of *Gsx2*-lineage neurons in the amygdala reveals a significant contribution to the ICM, as well as a number of other interneuron subtypes. While the generation of inhibitory neurons from the *Gsx2* lineage is consistent with the dLGE and broader subpallial expression of this gene, our finding that *Gsx2*-lineage neurons also generate a significant population of excitatory neurons was surprising. The most straightforward interpretation of this finding is that this population is derived from the PSB *Gsx2*/*Pax6*+ progenitor pool that is present during early neurogenesis. Thus, a broader picture is emerging in which amygdala excitatory neurons are generated from sets of genetically identifiable pallial-derived progenitor pools. However, the full extent of overlap between *Emx1*, *Dbx1*+ *Pax6*+ and *Gsx2*+ progenitors and how these genes act in concert to specify different functional excitatory neuronal subtypes in a combinatorial manner remains to be fully elucidated.

In this respect, our *Pax6* conditional mutagenesis data provide novel insight into the function of *Pax6* in amygdala formation. The loss of *Pax6* specifically in *Gsx2*+ cells results in a significant decrease in the number of BLC *Gsx2*-derived excitatory neurons. Interestingly, this effect is only observed in the lateral and not the baso-lateral nucleus, revealing that *Pax6* expression in *Gsx2*-expressing progenitors is differentially required for neurogenesis in these two amygdala sub-domains. Complementing our findings is the analysis of conditional *Gsx2* mutants which display the opposite result-- an
increase in excitatory neurons in the amygdala, also specifically in the lateral and not the baso-lateral nucleus (Waclaw et al., 2010). Together, these results reveal that there is an absolute requirement for Pax6, but not Gsx2, in Gsx2-expressing progenitors, in the generation of lateral, but not baso-lateral, amygdala excitatory neurons. Also interesting is the effect of the conditional loss of Pax6 on the generation of neurons in the intercalated cell masses (ICM). These neurons regulate feed-forward inhibition and are an essential component of fear circuitry. Recent studies have revealed that Gsx2 and Sp8 are required for the specification of these neurons, as the conditional loss of either gene results in deficiencies in ICM generation (Waclaw et al., 2009; Waclaw et al., 2010). As we reveal that the conditional loss of Pax6 results in an expansion of Gsx2 at the PSB during embryogenesis, we would predict a complementary finding: an increase in ICM numbers. In contrast to this prediction, we find that conditional loss of Pax6 in Gsx2+ cells results in a significant decrease in cells in the ICM. Therefore, our results show that Pax6 expression in Gsx2+ cells is also indispensable for the correct specification of ICM cells, however, in contrast to specification of lateral amygdala excitatory neurons, which can be specified in the absence of Gsx2, both Pax6 and Gsx2 are required for ICM generation.

In the olfactory bulb, our Pax6 conditional mutants show specific losses of CR+, tyrosine hydroxylase (TH+) and parvalbumin (PV+) subclasses of interneurons. The decrease in TH+ and PV+ populations is consistent with previous studies of Pax6 function (Dellovade et al., 1998; Kohwi et al., 2005; Brill et al., 2008; Haba et al., 2009). Reduction of gene dosage in Sey heterozygotes, which are not embryonic lethal, results in fewer TH+ and PV+ olfactory bulb interneurons (Haba et al., 2009). In addition,
transplantation of Sey/Sey cells into the post-natal SVZ has directly demonstrated that Pax6 is required for specification of TH+ interneurons (Brill et al., 2008). Thus, the decrease in TH+ and PV+ interneurons in the Pax6 conditional mutants is due to a direct loss of function of Pax6 in populations that express Gsx2. In contrast, the decrease in CR+ neurons, similar to changes in amygdala ICM neurons, is not predicted by previous analyses. Previous studies have shown that specification of CR+ neurons requires normal function of the zinc finger gene, Sp8 (Waclaw et al., 2006; Waclaw et al., 2009). Moreover, Sp8 is regulated by Gsx2, as Sp8 expression is lacking in Gsx2 mutants (Waclaw et al., 2009). In contrast, our conditional Pax6 mutants display a maintained expression of Sp8 and expanded Gsx2 expression at the PSB. Thus, since disruption of either Gsx2 or Pax6 results in CR+ interneuron loss in the olfactory bulb it appears that similar to specification of ICM amygdala neurons, both Pax6 and Gsx2 are required to specify the olfactory bulb CR+ population, and indeed may work in concert. Such a result is reminiscent of the combinatorial role of Pax6 and Dlx in the post-natal generation of dopaminergic (TH+) periglomerular neurons in the olfactory bulb (Brill et al., 2008).

While we cannot rule out the possibility that some adult-born cells in the SVZ express Pax6 and Gsx2, and are therefore also affected in our Pax6cKO mice, TH+, PV+, and CB+ olfactory bulb interneurons have a peak of generation early in embryogenesis, at the time that Pax6 and Gsx2 are overlapping at the PSB, and are therefore affected during embryogenesis (Batista-Brito et al., 2008). Regardless, results from our analyses of Pax6 function in Gsx2+ neurons combined with the results of others greatly informs a model in which Pax6 and Gsx2 are differentially required for the specification of unique sub-
populations of OB inhibitory neurons and of excitatory neurons and specialized ICM inhibitory neurons in the amygdala.

In summary, our studies reveal novel insight into the relationship between embryonic telencephalic patterning and limbic system formation, with a specific focus on the amygdala and olfactory bulb. As disruption of the development of the limbic system is a hallmark feature of numerous neuro-developmental disorders, most notably autism spectrum disorders (Rodrigues et al., 2004; Amaral et al., 2008; Herry et al., 2008; Markram et al., 2008; Monk, 2008), it will be interesting to determine if disrupted development of the telencephalic pallial-subpallial boundary is a common feature of disorders with a limbic system component. Indeed, Tlx mutant mice, which have a disrupted vP PSB domain and amygdala abnormalities, have increased aggression, suggesting that the correct development of this boundary is necessary for the formation of amygdala fear circuitry and, correspondingly, with modulation of fear response behaviors in the adult animal (Roy et al., 2002; Stenman et al., 2003b). Toward this end, understanding the long-term behavioral consequences of other specific genetic disruptions of this embryonic boundary will prove highly informative.
Figure 9. The Pallial Subpallial Boundary (PSB) at E11.5 is a mixed domain.
Figure 9. The Pallial Subpallial Boundary (PSB) at E11.5 is a mixed domain.

(A), In Gsx2-Cre; ROSA-YFP embryos, Pax6 and Gsx2 protein expression overlaps in the ventricular zone at the PSB. Arrowheads delineate the region of Pax6-Gsx2 overlap. 

(B-C) Higher magnification of boxed area in (A), Pax6 (B), Gsx2 (C), and Overlay (D) illustrate intermingling of Pax6+ and Gsx2+ cells, along with a population that express both Pax6 and Gsx2 (Arrowheads). (E) Schematic illustrates Pax6 and Gsx2 overlap. (F) YFP expression marks Gsx2-lineage cells with faithfully recapitulation of Gsx2 protein expression; not all cells are recombined at this early age. Arrowheads reference the boundary of overlap of Pax6 and Gsx2 in (A). (G-I) Higher magnification of boxed area in (F), YFP (G), Gsx2 (H), and Overlay (I) shows double YFP+/Gsx2+ cells. Arrowheads mark the edge of the YFP and Gsx2 protein domains. (J) Schematic illustrates YFP and Gsx2 expression. (K) Pax6 and YFP expression overlap at the PSB. Arrowheads delineate the region of overlap. (L-N) Higher magnification of boxed area in (K), YFP (L) Pax6 (M) and Overlay (N) show Pax6+/YFP+ cells in the PSB domain. Arrowheads mark Pax6+/YFP+ cells. All images are taken from triple immunostaining of the same section, representative of sections from N=4 brains. Scale bar in B, D, F, 25um. 

Abbreviations: dLGE, dorsal Lateral Ganglionic Eminence, vP, Ventral Pallium.
Figure 10. By E15.5, the PSB is refined into separate pallial and subpallial compartments.
Figure 10. By E15.5, the PSB is refined into separate pallial and subpallial compartments.

(A) In the VZ of the PSB, Pax6 and Gsx2 protein overlap is reduced to only a few cell diameters. Arrowheads delineate the extent of Pax6-Gsx2 protein overlap. (B-C) Higher magnification of boxed area in (A), Pax6 (B), Gsx2 (C), and Overlay (D) illustrate that there are very few cells co-expressing Pax6 and Gsx2 and no Pax6+/Gsx2+ cell mixing in the VZ. (E) Schematic illustrating refinement of Pax6 and Gsx2 expression. (F) YFP expression in the Gsx2-lineage embryo shows that Gsx2-lineage cells are present beyond the boundary of Gsx2 protein expression. Arrowheads delineate the boundary of Gsx2 and YFP protein expression. (G-I) Higher magnification of boxed area in (F), YFP (G), Gsx2 (H), and overlay (I) shows the extended YFP+ domain; arrowheads mark the edge of the Gsx2 and YFP protein domains. (J) Schematic illustrates YFP expansion beyond Gsx2 expression. (K) Pax6 and YFP expression overlap at the refined PSB. Arrowheads mark the extent of overlap, which corresponds to the Gsx2 protein-negative domain and indicates that Gsx2-lineage cells, which are now Gsx2 protein-negative, have become Pax6+. (L-N) Higher magnification of boxed area in (K), YFP (L), Pax6 (M), and Overlay (N). (O) Schematic illustrates the YFP and Gsx2 overlapping domain at the refined PSB. Arrowheads mark the domain of Pax6+/YFP+ overlap in the Gsx2 protein-negative PSB domain. Scale bar in B, D, F, 25um. G. Schematic summarizing the refinement of Pax6-Gsx2 protein expression at the border between E11.5 and E15.5, and the subsequent formation of a Gsx2-lineage (YFP+) Gsx2 protein-negative, Pax6 protein-positive domain.
Figure 11. Time-lapse imaging of cell motility at the PSB at E11.5 and E13.5.
Figure 11. Time-lapse imaging illustrates that cell motility decreases between E11.5 and E13.5 at the PSB.

(A) E11.5 300um Pax6-GFP slice; cells are labeled throughout the vP and pallium. Boxed area indicates VZ area analyzed for cell movements in (C-E). (B) E13.5 300um Pax6-GFP slice; cells are labeled throughout the vP and pallium. Boxed area indicates VZ area analyzed for cell movements in (C, D, and F). (C). Cells were tracked through time until their cell bodies disappeared outside of the z plane of the section, and average velocity was calculated for each cell. Cell velocity was compared for N=50 cells in the VZ at E11.5 and at E13.5; E11.5 cells moved significantly faster than E13.5 cells; p=0.0059. Each point represents the average velocity of an individual cell. (D) Total distance traveled was calculated for N=50 cells at E11.5 and at E15.5; each cell is plotted as a single point on the graph. (E, F) While the total distance traveled was not significantly different between the two groups, the number of cells that moved 1-10um was higher at E13.5 compared to E11.5, and the number of cells that moved farther than 10um was higher at E11.5 than E13.5; cells at each age are pooled by total distance traveled.
Figure 12. Cells are highly motile in the VZ of the PSB at E11.5. E11.5 18 hour 2Photon time-lapse slice imaging movie, using Pax6-GFP embryos, illustrates cell motility in the VZ and SVZ at this age.

Figure 13. Cells are more motile in the SVZ and mantle of the PSB E13.5. E13.5 16 hour 2Photon time-lapse slice imaging movie, using Pax6-GFP embryos, illustrates cell motility in the VZ and SVZ at this age.
Figure 14. Multiple neuronal subtypes in the post-natal baso-lateral complex of the amygdala and olfactory bulb are derived from the Gsx2-lineage cells.
Figure 14. Multiple neuronal subtypes in the post-natal baso-lateral complex of the amygdala and olfactory bulb are derived from the Gsx2-lineage cells.

(A) Low power magnification of the post-natal brain showing YFP+ Gsx2-lineage cells throughout the baso-lateral amygdala complex (BLC) in P21 brains. (B and C) Many YFP+ cells co-express the excitatory neuron marker Tbr1 in the LAT and BLA nuclei (arrowheads). Higher magnification of boxed area in (B and C) highlight single channel and overlay images. (D, E) Mef2c, a transcription factor enriched in the LAT nucleus and implicated in neuronal differentiation, co-labels with YFP cells in the LAT and BLA amygdala (arrowheads). Side panels show higher magnification single channel and overlay images of double positive cells from boxed regions in (D and E). (F) Gsx2-lineage YFP+/Foxp2+ inhibitory neurons are present in the lateral intercalated cell mass (LICM); (arrowheads). Adjacent high-magnification images show single channel and overlay of Foxp2+/YFP+/DAPI+ cells. (G) Trace from recording of YFP+ cell recorded in current clamp from fate mapped Gsx2-lineage P21 brain slices from the LAT amygdala. Steps below the trace show corresponding current steps, at hyperpolarizing, rheobase, and maximum firing frequency levels. These cells have slower frequencies and higher amplitudes characteristic of amygdala pyramidal excitatory cells. (H) Post-hoc immunohistochemistry of a biocytin-filled cell post-recording (red) also expressing YFP (green) with characteristic pyramidal cell morphology. (I) Trace of GFP+ cell from the LAT amygdala that is typical of an inhibitory interneuron, faster frequency and smaller amplitudes are characteristic of inhibitory interneurons. (J) Post-hoc immunohistochemistry of a biocytin-filled cell post-recording (red) also expressing YFP (green) with bipolar cell morphology characteristic of inhibitory neurons (K) Example of
a single trace showing an action potential (AP) from a pyramidal-like neuron in (G) (black) and an interneuron in (I) (red); the pyramidal-like AP is significantly broader than the interneuron AP. (L) Gsx2-lineage cells are present throughout the post-natal olfactory bulb; Dlx labels inhibitory cells throughout the OB and co labels with many YFP+ cells. (M) Higher magnification of the glomerular layer, arrowheads point to Dlx+/YFP+ inhibitory neurons. (N) Some YFP+ cells in the glomeruli co-localize with Tbr1. Foxp2 labeling also marks inhibitory cells in the glomerular layer; single arrowheads point to YFP+ cells that are also Foxp2+; double arrowheads point to YFP+ cells that are also Tbr1+. Abbreviations: CE, Central Nucleus, BLA, Baso-lateral Nucleus, EPL, External Plexiform Layer, GL, Glomerular Layer, IPI, Internal Plexiform Layer, LAT, Lateral Nucleus, LICM, Lateral Intercalated Cell Mass, MICM, Medial Intercalated Cell Mass, ML, Mitral cell Layer.
Figure 15. Fate-map of cells derived from the dorsal PSB using $Dbx1^{Crs}$; ROSA-YFP mice indicates that this progenitor domain gives rise to excitatory neurons in the amygdala and olfactory bulb.
Figure 15. Fate-map of cells derived from the dorsal PSB using $Dbx1^{Cre}$; ROSA-YFP mice indicates that this progenitor domain gives rise to excitatory neurons in the amygdala and olfactory bulb. (A) 10x of $Dbx1$-lineage cells in the BLA. (B) $Dbx1$-lineage cells are not part of the ICM interneuron population. (C-F) $Dbx1$-lineage cells colabel for the excitatory neuron marker Tbr1 and also Mef2c in the LAT (C,E) and BLA (D,F) nuclei. (G,H) $Dbx1$-lineage cells give rise to Tbr1+ excitatory neurons and Reelin+ neurons in the periglomerular and external plexiform layers (I-K) $Dbx1$-lineage cells do not express the interneuron markers CR, CB, Dlx.
Figure 16. Embryonic PSB-specific patterning defects in E15.5 $Pax6^{cKO}$ mutants.

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<tr>
<th>Pallial Subpallial Boundary Markers</th>
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<tr>
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Control

$Pax6^{cKO}$
Figure 16. Embryonic PSB-specific patterning defects in E15.5 $Pax6^{cKO}$ mutants. (A) Expression of $Sfrp2$ marks the vP as shown in control (arrowhead). (F) In $Pax6^{cKO}$ embryos, $Sfrp2$ expression is severely reduced (arrowhead). (B) $Sp8$ marks the dLGE, (G) In $Pax6^{cKO}$ $Sp8$ persists and appears expanded. Arrowheads show domain of $Sp8$ expression. (C) $Tsh-1$ also marks the dLGE as well as the LCS as shown in control. (H) Similar to $Sp8$, $Tsh1$ remains expressed in $Pax6^{cKO}$ embryos. Arrowheads point to $Tsh-1^+$ LCS. (D) $Ngn2$ is expressed throughout the pallium VZ in control embryos. (I) In $Pax6^{cKO}$ embryos, $Ngn2$ expression decreased at the vP but not lost in the pallium. (E) The subpallial expressed transcription factor $Islt1$ labels the differentiating zone of the LGE in control embryos. (J) $Islt1$ expression is not affected in $Pax6^{cKO}$ embryos.
Figure 17. The PSB in Pax6<sup>cko</sup> mutants is expanded dorsally during embryogenesis.
Figure 17. The PSB in *Pax6*^cKO^ mutants is expanded dorsally during embryogenesis.

(A) Pax6 is expressed in the pallium and vP in E15.5 control embryos. (B) Higher magnification of PSB from (A) showing high expression of Pax6 in the VZ (Arrowheads). (C) Higher magnification of amygdala primordium from (A), with numerous Pax6+ cells. (D) In E15.5 control embryos, the Gsx2-lineage YFP+ cell domain (arrowhead) extends beyond the Gsx2 protein domain (double arrowhead); asterisk marks the sulcus. (E) Single channel of Gsx2 protein expression is also shown; asterisk marks the sulcus; arrowheads are as in (D). (F) Ngn2 expression in *Pax6*^cKO^ control brain at E13.5; Ngn2 expression is present throughout the pallium and stops at the vP. (G) In E15.5 *Pax6*^cKO^ mice, Pax6 expression is decreased in the vP (Arrowheads). (H) Higher magnification of PSB of (G) illustrates that there are fewer Pax6+ cells in the vP. (I) In the *Pax6*^cKO^ amygdala primordium, few cells are labeled with Pax6. (J) In E15.5 *Pax6*^cKO^ embryos, the Gsx2 protein domain and *Gsx2*-lineage domain is ectopically expanded dorsally towards the sulcus (as evidenced by the decrease in distance between the arrowheads and the asterisk). (K) Gsx2 protein expression alone; Gsx2+ cells are ectopically dorsally expanded towards the sulcus. (L) In E13.5 *Pax6*^cKO^ embryos, Ngn2 expression is decreased at the PSB, with fewer Ngn2+ cells in the vP.
Figure 18. Defects in migrating interneurons in $Pax6^{cKO}$ embryos.
Figure 18. Defects in migrating interneurons in *Pax6<sup>cKO</sup>* embryos.

At E15.5, loss of *Pax6* in *Gsx2*-lineage cells results in alterations in tangential migration and cortical heterotopias. (A,C) More migrating YFP+ interneuron progenitors are present in the SVZ corridor in the *Pax6<sup>cKO</sup>* cortex. (B,D) In control mice, some *Gsx2*-lineage cells are present in clonal stripes extending from the VZ. However, in *Pax6<sup>cKO</sup>* embryos, these stripes are more frequent and heterotopic YFP+ cell masses, which are not found in controls, are present in the SVZ and mantle of the cortex. (C,E) *Gsx2+* cells are present in the heterotopias; *Gsx2+* cells are never found in migrating interneurons in control cortex.
Figure 19. Alterations of Gsx2-derived excitatory and inhibitory cell subpopulations in the Pax6\textsuperscript{cKO} post-natal amygdala.
Figure 19. Alterations of Gsx2-derived excitatory and inhibitory cell subpopulations in the Pax6cKO post-natal amygdala.

(A-E) Low-power magnification of the Baso-lateral Amygdala Complex (BLC), illustrating numerous of Gsx2-lineage cells and localization of Tbr1+ excitatory neurons and Foxp2+ ICM inhibitory neurons in control (A) and Pax6cKO mice (E) at P21. (B) Higher magnification of boxed LICM region in (A) reveals Gsx2-lineage neurons (YFP+) co-expressing Foxp2 (arrowheads) but not Tbr1 in control mice. (F) Higher magnification of boxed region in E, shows fewer cells co-express Foxp2 and YFP in the LICM region in Pax6cKO brains (arrowheads mark YFP+/Foxp2+ cells). (C, G) Higher magnification of boxed region of the LAT nucleus from (A, E) is shown. Tbr1+/Gsx2-lineage cells in the LAT nucleus are numerous in control (C) and greatly decreased in Pax6cKO mutants (G); arrowheads point to Tbr1+/YFP+ cells. Boxed region in (C, G) is magnified in right panels: YFP (green), Tbr1 (red), DAPI (blue), and Overlay. (D, H) Higher magnification of the BLA region shows less Tbr1+ Gsx2-lineage in the LAT nucleus, in both control (C) and Pax6cKO (G) brains. Arrowheads mark double+ cells. Boxed region in (D, H) is magnified in right panels: YFP (green), Tbr1 (red), DAPI (blue), and Overlay (I).

Quantification of cell counts in control and Pax6cKO amygdala, in the LAT, BLA, and LICM sub regions. In the LAT nucleus, 44% of DAPI+ cells expressed Tbr1 in control mice, (740/1642, N=3) vs. 34% in Pax6cKO mice (594/1510. N=3 p=0.08). The number of Gsx2-lineage Tbr1+ cells in the LAT nucleus was significantly decreased from 16% in control mice, (257/1642, N=3), to 3%, (55/1769, N=3), in Pax6cKO mice, (p=0.002). In the BLA nucleus, Tbr1+ cell numbers or YFP+/Tbr1+ cells were not significantly different in Pax6cKO mice; 5% (756/1643, N=3) of control Gsx2-lineage cells and 3%
(566/1450, N=3) of $Pax6^{cKO}$ Gsx2-lineage cells were double positive (p=0.92). In the LICM, 69% (158/232, N=4) of Gsx2-lineage cells were Foxp2+ in control mice, vs. 56% (136/237, N=4) in $Pax6^{cKO}$ mice, a significant decrease (p=0.02); in the medial ICM, in control mice, 69% of Gsx2-lineage cells were Tbr1+, (173/335, N=4); whereas in $Pax6^{cKO}$ mice, 51% were double-positive, (115/280; N=4) also a significant decrease (p=0.02.) * Demarcates p<0.05; ** demarcates p>0.01.
Figure 20. P21 $Pax6^{cKO}$ mice do not exhibit defects in the generation of Gsx2-lineage inhibitory interneurons in the Baso-lateral Amygdala Complex.

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Figure 20. P21 *Pax6<sup>cKO</sup>* mice do not exhibit defects in the generation of Gsx2-lineage inhibitory interneurons in the Baso-lateral Amygdala Complex.

(A, E) CB and YFP staining in the Central nucleus are not different between Control and *Pax6<sup>cKO</sup>* brains. (B, F) CR and YFP labeling of Control and *Pax6<sup>cKO</sup>* BLA nucleus, and of CCI, an oligodendrocyte marker; no differences in labeling for CR or CCI were found in *Pax6<sup>cKO</sup>* brains. (C, D, G, H) (Arrowheads point to CR+/YFP+ cells). Labeling for the inhibitory interneuron markers NOS and CB in the LAT nucleus; no differences in staining were found between Control and *Pax6<sup>cKO</sup>* brains (Arrowheads point to YFP+/NOS+ and YFP+/CB+ cells).
Figure 21. Inhibitory but not excitatory cell subpopulations are altered in the Pax6<sup>cko</sup> post-natal olfactory bulb.
Figure 21. Inhibitory but not excitatory cell subpopulations are altered in the
\(Pax6^{cKO}\) post-natal olfactory bulb.

(A, B) CB and NeuN mark inhibitory neurons in the Glomerular layer, which co-localize
with YFP expression. No change in CB+ cells was observed between control and
\(Pax6^{cKO}\) mice. Panels to the right are from boxed region in A and B: YFP (green), CB
(red), NeuN (blue), and Overlay (arrowheads mark CB+/YFP+ cells). (C, D) CR+ and
TH+ interneurons also co-localize with YFP in the glomerular layer. Both interneuron
markers are reduced in \(Pax6^{cKO}\) glomeruli. Panels to the right are from boxed regions in
C and D: YFP (green), CR (red), TH (blue), and Overlay (arrowheads mark CR+/YFP+
cells, double arrowheads mark TH+/YFP+ cells). (E, F) PV+ cells in the EPL co-express
YFP in control mice. In the \(Pax6^{cKO}\) OB, these numbers are greatly reduced. Panels to the
right are from boxed regions in E and F: YFP (green), PV (red), DAPI (blue), and
Overlay (arrowheads mark PV+/YFP+ cells). (G, H) Tbr1+ cells in the PGL and EPL
rarely co-express YFP. There is no change in these \(Gsx2\)-lineage Tbr1+ cell in the
\(Pax6^{cKO}\) mutant OB. I, Graph of the total number of inhibitory interneuron subtypes in
Control and \(Pax6^{cKO}\) mice. (J) Quantification of the number of \(Gsx2\)-lineage TH+, PV+, and
CR+ interneurons in control and \(Pax6^{cKO}\) mutant OB. The numbers of these three
cell subtypes were significantly decreased in mutants while CB+ and Tbr1+ numbers
were not affected. These changes were observed in both their total numbers and the
number \(Gsx2\)-lineage populations. Changes in the total numbers were as follows: TH: 5%
(85/1546, N=3) in control mice vs. 2% (32/1843, N=3) in \(Pax6^{cKO}\) mice (p=0.04); PV:
8% (60/624, N=3) in control mice, 1% (10/877, N=3) in \(Pax6^{cKO}\) mice (p=0.002); CR:
24% (360/1546, N=3) in Control mice vs. 16% (284/1843, N=3) in \(Pax6^{cKO}\) mice
Changes in each marker in Gsx2-lineage numbers are as follows: the number of CB+ Gsx2-lineage interneurons was not changed 14% (98/992, N=3) in Control, 19% (102/806, N=3) in Pax6\textsuperscript{cKO} mice (p=0.06), but TH+, PV+, and CR+ Gsx2-lineage interneurons were decreased: TH: 9% (63/718, N=3) in Control mice vs. 1% (7/652, N=3) in Pax6\textsuperscript{cKO} mice, (p=0.01); PV: 40% (34/80, N=3) in Control mice vs. 0% (0/71, N=3) in Pax6\textsuperscript{cKO} mice (p=0.008); CR: 27% (192/718, N=3) in Control mice vs. 21% (135/652, N=3) (p=0.04). The number of Tbr1+ excitatory neurons in the periglomerular layers were not altered in Pax6\textsuperscript{cKO} mice: 25% (200/802, N=3), of DAPI+ cells were Tbr1+ in Control mice vs. 29% (233/780, N=3) of Tbr1+ cells in Pax6\textsuperscript{cKO} mice (p=0.053). * Demarcates p<0.05; ** demarcates p>0.01.
Figure 22. Loss of Pax6 at the PSB in embryogenesis does not cause changes in Gsx2-lineage interneuron subtypes in the postnatal cortex.
Figure 22. Loss of *Pax6* at the PSB in embryogenesis does not cause changes in *Gsx2*-lineage interneuron subtypes in the postnatal cortex.

(A) By P21, *Gsx2*-lineage cells give rise to PV+ and SST+ interneurons in the cortex (arrowheads). (B) SST+/YFP+ and PV+/YFP+ expression in the *Pax6<sup>cKO</sup>* cortex. Distributions of SST+ and PV+ as well as *Gsx2*-lineage neurons were not different in *Pax6<sup>cKO</sup>* mutant cortices. (C) All analyses were done in sensorimotor cortex. Total number of *Gsx2*-lineage neurons is not changed in upper, middle, or lower layers of *Pax6<sup>cKO</sup>* cortex. (D) While the number of PV+/YFP+ and SST+/YFP+ cells were decreased, particularly in lower motor cortex, these differences were not significantly different between *Pax6<sup>cKO</sup>* and control cortices.
Table 1. Electrophysiological Properties of Gsx2-lineage neurons in the LAT nucleus.

<table>
<thead>
<tr>
<th></th>
<th>Pyramidal-like (n = 18)</th>
<th>Interneuron (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane resistance (MΩ)</td>
<td>278 ± 89</td>
<td>790 ± 290</td>
</tr>
<tr>
<td>Membrane capacitance (pF)</td>
<td>120 ± 44</td>
<td>40 ± 21</td>
</tr>
<tr>
<td>Membrane potential (mV)</td>
<td>-60 ± 4.5</td>
<td>-55 ± 5</td>
</tr>
<tr>
<td>Peak amplitude (mV)</td>
<td>51 ± 6.0</td>
<td>41 ± 4.0</td>
</tr>
<tr>
<td>Half-spike duration for 1st</td>
<td>2.2 ± 0.4</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Spike (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum frequency (Hz)</td>
<td>16.8 ± 3.8</td>
<td>36.2 ± 5.7</td>
</tr>
<tr>
<td>Accommodation ratio</td>
<td>0.29 ± 0.12</td>
<td>0.54 ± 0.2</td>
</tr>
<tr>
<td>Sag (mV)</td>
<td>4.9 ± 2.5</td>
<td>4.4 ± 3.0</td>
</tr>
<tr>
<td>I_T-like current (mV)</td>
<td>6.7 ± 3.7</td>
<td>15.3 ± 6.5</td>
</tr>
<tr>
<td>AHP amplitude (mV)</td>
<td>-8.3 ± 2.0</td>
<td>7.9 ± 3.4</td>
</tr>
<tr>
<td>AHP decay half-time (ms)</td>
<td>132 ± 39</td>
<td>biphasic</td>
</tr>
</tbody>
</table>
Chapter 3.

*Emx1*-lineage progenitors differentially contribute to neural diversity in the striatum and amygdala


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ABSTRACT

In the developing mammalian basal telencephalon, neural progenitors from the subpallium generate the majority of inhibitory medium spiny neurons (MSNs) in the striatum, while both pallial and subpallial-derived progenitors contribute to excitatory and inhibitory neuronal diversity in the amygdala. Using a combination of approaches, including genetic fate-mapping, cell birth-dating, cell migration assays, and electrophysiology, we find that cells derived from the $Emx1$- lineage contribute to two distinct neuronal populations in the mature basal forebrain: inhibitory MSNs in the striatum, and functionally distinct subclasses of excitatory neurons in the amygdala. Our cell-birth dating studies reveal that these two populations are born at different times during early neurogenesis, with the amygdala population born prior to the MSNs. In the striatum, $Emx1$-lineage neurons represent a unique subpopulation of MSNs: they are disproportionally localized to the dorsal striatum, found in dopamine receiving, reelin-positive patches, and are born throughout striatal neurogenesis. In addition, our data suggest that a subpopulation of these $Emx1$-lineage cells originate in the pallium and subsequently migrate to the developing striatum and amygdala. Our intersectional fate-mapping analysis further reveals that $Emx1$-lineage cells that co-express $Dlx$ exclusively generate MSNs, but do not contribute to the excitatory neurons in the amygdala. Thus, both the timing of neurogenesis and differential combinatorial gene expression appear to be key determinants of striatal versus amygdala fate decisions of $Emx1$-lineage cells.
INTRODUCTION

Neuronal diversity in the mature telencephalon is generated during embryogenesis, where telencephalic progenitor pools are formed from the restricted regional gene expression patterns of key transcription factors. Multiple ventral telencephalic (subpallial) progenitor pools contribute to the developing striatum, which is the inhibitory center of the telencephalon vital for movement and impulse control (Wichterle et al., 1999; Marin et al., 2000; Wichterle et al., 2001; Nery et al., 2002). The majority (90-95%) of the striatum is composed of GABAergic medium spiny neurons (MSN)(Gerfen, 1992). MSN progenitors are derived primarily from the lateral ganglionic eminence (LGE) (Wichterle et al., 1999; Wichterle et al., 2001). These cells migrate radially and separate into the two primary striatal compartments, distinguished by their cortical laminar inputs and by neurochemical markers: the patches, which are formed from MSN precursors, the majority of which are born early (in the mouse between E12.5-E13.5), and the surrounding matrix, the majority of which are born later (in the mouse between E13.5-E15.5) (Fishell and van der Kooy, 1987, 1991; Gerfen, 1992; Krushel et al., 1993; Mason et al., 2005).

Another major basal telencephalic structure is the amygdala, a complex nuclear structure that is a key component of the limbic system. The amygdala plays an important role in modulating fear, aggression, and emotionality (Stoykova et al., 1996; Swanson and Petrovich, 1998; Sah et al., 2003; Maren and Quirk, 2004). Similar to the striatum, multiple progenitor pools contribute to the amygdala, and evidence from cell transplantation, genetic fate-mapping, and mRNA expression profiles indicate that this structure is derived from dorsal (pallial) and ventral (subpallial) progenitors, at least some
of which are born early in embryonic neurogenesis (Nery et al., 2002; Carney et al., 2006; Remedios et al., 2007; Bai et al., 2008; Hirata et al., 2009; Soma et al., 2009)

Previous work has revealed that progenitor populations marked by the pallial-expressed transcription factor, *Emx1*, appear to contribute to numerous telencephalic structures, including the striatum (Willaime-Morawek et al., 2006; Willaime-Morawek and van der Kooy, 2008), olfactory bulb (Fogarty et al., 2007; Kohwi et al., 2007; Young et al., 2007) and amygdala (Puelles et al., 2000; Gorski et al., 2002; Medina et al., 2004; Tole et al., 2005). However, the relationship between the origin and timing of the generation of *Emx1*+ progenitors and their ultimate fate in the mature striatum and amygdala remains unknown.

In this study, we utilized a multidisciplinary approach to investigate several key developmental aspects of the striatum and amygdala, including the genetic heterogeneity and origin of the *Emx1*-lineage, the timing of their genesis, and their ultimate fate in these two structures. Our data reveal a relationship between the timing of the generation and the postnatal fate of *Emx1*-lineage progenitors in the striatum and amygdala. We also find that *Emx1*-lineage cells populate the striatum and amygdala at earlier ages than previously recognized. By use of an intersectional genetic fate-mapping strategy, our data also intriguingly reveal that, in addition to timing, differential combinatorial gene expression within *Emx1*-lineage cells may be an important determinant of their distinct fates in the striatum versus the amygdala. These findings provide novel insight into the plasticity of a subset of telencephalic neural progenitors from a broadly common lineage, wherein their ultimate neuronal fate in the postnatal brain appears to be related to both the timing of their birth and combinatorial gene expression during embryogenesis.
METHODS

Animal Use. Swiss Webster (Taconic Farms, Albany, NY), Z/EG, ROSA-YFP, ROSA-LacZ (Jackson Laboratory, Bar Harbor, Maine) (Novak et al., 2000; Srinivas et al., 2001), Emx1-Cre (K. Jones, U. of Colorado, Boulder) (Gorski et al., 2002), and Small eye (Sey) (Hill et al., 1991) mice used in these studies were maintained according to the protocols approved by Children’s National Medical Center and Georgetown University Medical Center. Emx1-Cre, ROSA-YFP, and Sey+/−; Emx1-Cre mice were maintained on a mixed C57Bl/6 x SW background; Z/EG mice were maintained on a SW background. For the intersectional fate mapping studies (Figure 7) Emx1-Cre, Dlx5/6-Flpe, and RCE: dual mice were maintained according to the protocols approved by the New York University School of Medicine, NY. For these experiments, the neo cassette from Emx1-Cre driver mice (T. Iwasato, BSI RIKEN, Japan) was removed before crossing. Dlx5/6-Flpe driver mice (G.M. and G.F., manuscript submitted) expressed the Flpe site-specific recombinase under control of the intergenic enhancer residing between Dlx5 and 6 (Stenman et al., 2003). To generate RCE: dual reporter mice, a CAG promoter with floxed- and flrked-stop cassettes followed by EGFP reporter was targeted to the R26R locus (Sousa et al., 2009). For staging of the embryos, midday of vaginal plug detection was considered as embryonic day 0.5 (E0.5). For postnatal animals, the day of birth was considered as postnatal day 0 (P0). The genotyping of animals were performed as described previously (Gorski et al., 2002; Carney et al., 2009).
Tissue Preparation and Histology. For immunofluorescence and in situ hybridization at embryonic ages, brains were fixed in 4% paraformaldehyde (PFA) for 2 hours or overnight, respectively. Brains were cryoprotected by sucrose immersion, embedded in Histoprep (Fisher Scientific, Pittsburgh, PA) and frozen. Serial coronal sections of embedded tissue were cut at 20-30 μm thickness using a cryostat and mounted on glass slides.

Immunohistochemistry. Cryostat mounted sections were air-dried and rinsed 3 times in PBS before blocking for 1 hour in 10% normal donkey serum diluted in PBS with 0.2% Triton to prevent non-specific binding. Primary antibodies were diluted in 1% serum diluted in PBS with 0.2% Triton; sections were incubated in primary antibody overnight at 4°C. The primary antibodies used were as follows: goat anti-Pax6 (1:200; Santa Cruz, Santa Cruz, CA), mouse anti-NeuN (1:500; Covance, Princeton, NJ), rabbit anti-Ng2 (1:400, Covance), mouse anti-GFAP (1:200, Sigma, St. Louis, MO), rabbit anti-DARPP-32 (1:1000, Covance), rat anti-BrdU (1:250, Serotec, Oxford, UK), mouse BrdU (1:50, Sigma), rat anti-GFP (Nacala, Japan, 1:2000), rabbit anti-Gsx2 (1:1500, gift of K. Campbell), mouse anti-CC1 (1:50, Calbiochem, San Diego, CA), rabbit anti-Tbr1 (1:1000; gift of R. Hevner); guinea-pig anti-Dlx2 (1:1500; gift of K. Yoshikawa), rat anti-CTIP2 (1.300; Abcam); rabbit anti-TH (1:1000; Chemicon, mouse anti-reelin (1:1000, Millipore Bioscience Research Reagents, Billerica, MA), rabbit anti-MOR1-β (1:10,000; Millipore); rat anti-Somatostatin (1:1000; Millipore). To detect primary antibodies, secondary antibodies raised in mouse, rat, goat, guinea pig, and rabbit were used (Cy3 and Cy5 at 1:200, FITC at 1:50; all from Jackson Immunoresearch, West Grove, PA,
Sections were incubated for 2 hours in 1% serum in PBS with 0.2% Triton and were washed and cover slipped with gel mount (Sigma) or Vectashield with DAPI (Vector Labs, Burlingame, CA). Sections cover slipped with gel mount were incubated first with To-Pro-3 iodide (1:100, Invitrogen, Carlsbad, CA) for 10 minutes and then washed and cover slipped.

**BrdU Labeling.** To label cells in S-phase, E9.5, E11.5, E13.5, E14.5 and E15.5 Emx1-Cre; ROSA-YFP pregnant dams were administered BrdU (Sigma), dissolved in PBS, by intraperitoneal injection at a dose of 100 mg/kg. To achieve short-term labeling, the dams were sacrificed after 2 hours and embryos were prepared for processing as described above. Cryostat sections were processed for immunohistochemical labeling of YFP using rat anti-GFP, as described above, and post-fixed with 4% PFA for 15 minutes. A sodium citrate pretreatment was performed before processing sections for BrdU immunohistochemistry, as described previously (Tang et al., 2007). Briefly, a food steamer (Oster 6.1 quart model) was preheated to 100°C, slides were placed horizontally in the incubator, covered with 500ul 10mM sodium citrate, and incubated for 15 minutes. Slides were allowed to cool for 2 minutes before continuing with standard immunohistochemistry procedures.

**In situ hybridization.** Tissue was prepared as described above. Non-radioactive dioxygenin-labeled RNA in situ hybridization was carried out as described previously (Carney, et al., 2009). The probes used in this study were Emx1 (Yoshida et al., 1997) and Cre (Hirata et al., 2009).
**Dil Cell Migration Assay.** E11.5 and E13.5 brains were dissected in ice cold HBSS (Invitrogen), the skull and meninges were removed, and brains were placed in 3% low melt agarose (Fisher Scientific, Pittsburgh, PA, USA). 250-300 µm slices were cut using a vibratome (VT1000S; Leica, Nussloch, Germany), and sections were allowed to recover at 37°C in MEM supplemented with L-glutamine (1:100), penicillin/streptomycin (1:100) and 10% fetal bovine serum (all from Invitrogen). Dil crystals were placed in the lateral pallium and the slices were cultured in Neurobasal media supplemented with L-glutamine (1:100), penicillin/streptomycin (1:100), and B-27 (1:50; Invitrogen) for 2 days in vitro (2 DIV). After culture, sections were washed in PBS, post-fixed in 4% PFA for 15 minutes, and washed again in PBS. Sections were then cover slipped using concave slides (Fisher Scientific), and slices where Dil was targeted to the ventral or lateral pallium and Dil+ cells were observed migrating ventrally were immediately photographed.

**In Utero Electroporation.** Electroporations were carried out as previously described (Gal et al., 2006). CAG-RFP was used at a concentration of 4µg/µl, diluted in PBS and Fast-Green. 1µl of RFP was injected into the lateral ventricles of E12.5-E14.5 embryo brains, and micro tweezertrodes were oriented with the positive electrode over the cortical primordium; 4 pulses of 33mV of current were then applied, with 50msec intervals. Embryos were then allowed to survive for 1 day, 3 days, or to full term, and sacrificed postnatally at P15 or P30. Brains were processed as in preparation for immunohistochemistry. The location of electroporation was determined by examining the
brain in serial coronal sections. Only animals with RFP+ electroporated cells in the pallium or cortex were analyzed.

**Electrophysiology.** Electrophysiological recordings were performed from fluorescent Emx1-lineage cells located in the striatum and basolateral nucleus of the amygdala. Briefly, animals were deeply anesthetized (with CO2) until non-responsive and then decapitated. Brains were removed and immediately immersed for 2-3 minutes in an ice-cold - oxygenated (95% O₂ / 5% CO₂) sucrose slicing solution (mM): 234 sucrose, 11 glucose, 24 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 10 MgSO₄ and 0.5 CaCl₂. Coronal slices containing the striatum or amygdala were cut on a vibratome (Leica) at 250 µm. Slices were collected and placed in an oxygenated incubation chamber containing pre-heated (32°C), oxygen equilibrated, artificial cerebral spinal fluid (ACSF) (mM): 126 NaCl, 26 NaHCO₃, 10 glucose, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 2 MgCl₂·6H₂O, and 2 CaCl₂·2H₂O; pH 7.4. The slices were incubated for one hour at 32°C, and then allowed to cool to room temp until transferred to the recording chamber. Neurons were located and visualized with a fixed staged, upright microscope (E600 FN Nikon, Melville, NY) equipped with a 4x objective and a 60x insulated objective, infrared (IR) illumination, Nomarski optics, an IR-sensitive video camera (COHU) and fluorescent lamp (D-FI universal epi-fluor illuminator, Nikon) equipped with a 450-490 λ filter. Glass pipettes (non-filament borosilicate glass, Garner Glass Company, Claremont, CA) were pulled with a Flaming / Brown Micropipette Puller (Model, P-97, Sutter Instruments, Novato, CA) to a resistance of 3-5 MΩ. For all recordings the intracellular pipette solution consisted of (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 EGTA, and 2 MgCl₂.
Recordings were performed at room temperature with continuous perfusion (2 ml/minute) of ACSF. Cells were recorded in current clamp mode using a multiclamp 700A amplifier (Axon) and digitizer (DigiDATA, Axon). For all cells, membrane potential and input resistance values were recorded. For all current clamp recordings, current was injected into the cell in order to keep the cell at -60 mV. Cells were then characterized on the response to depolarizing and hyperpolarizing current pulses for a duration of 600 ms in 12 consecutive sweeps. For striatal cells, threshold responses were measured and Rheobase currents were recorded. Cells were analyzed off-line using pClamp software (Axon) and specialized graphing software (Origin).

*In vitro differentiation assay.* E13.5 *Emx1-Cre; ROSA-YFP* brains were dissected out in ice cold HBSS, and divided into medial pallium, dorsal pallium, ventral/lateral pallium, and LGE regions. Cells were disassociated and plated in MEM with supplements on PDL-coated coverslips, described in detail previously (Costa et al., 2008). After 1 DIV, 100 µl B27 was added to the media. Cells were cultured for 10 days, fixed, and immunostained with antibodies to GAD65/67 and CTIP2, and subsequently counterstained with DAPI.

*Microscopy.* *In situ* hybridization photographs were taken using an Olympus (Center Valley, PA) BX51 microscope. Fluorescent photographs were taken using a Zeiss (Thornwood, NY) LSM 510 confocal microscope. For confocal image analysis, each fluorophore was scanned sequentially and Z-stacks of the images obtained were collapsed into a single projection image or presented as individual optical sections. Figures were
prepared using Image J and Adobe Illustrator; brightness and contrast adjustments were applied equally across all images.

*Data Analysis.* Postnatal sections from *Emx1-cre; ROSA-YFP* brains were photographed as described above. For each immunohistochemical marker, 3 coronal sections of dorsal striatum at Bregma levels 0.14-0.98 were examined from *n*=3 animals, except where noted. The following criteria were applied to determine co-localization of cell-subtype markers with YFP fluorescence: 1. Cells were counted from individual optical sections, not collapsed projection images. 2. Cells were counted as double positive if an immunopositive YFP cell body was clearly co-localized with the fluorophore of interest and contained a nucleus that was also DAPI-positive. 3. For BrdU quantification, cells were counted as BrdU+ if they contained medium to strong labeling with BrdU, defined as labeling at least 50% of the DAPI nuclear stain.

**RESULTS**

*Fate of Emx1-lineage cells in the postnatal striatum and amygdala*

To determine the fate of *Emx1*-lineage cells in the postnatal striatum and amygdala, we crossed previously generated *Emx1-Cre* mice (Gorski et al., 2002) to *ROSA-YFP* reporter mice. We observed that YFP+ *Emx1*-lineage cells were distributed preferentially in the dorsal striatum (Fig. 23A), a region that receives layer V cortical inputs, primarily from neocortical areas. To determine the cellular fate of *Emx1*-lineage cells in the striatum, we carried out immunohistochemical analyses with antibodies to YFP and all mature striatal cell types, including inhibitory neuronal populations and glia.
Additionally, we labeled with markers for the patch and matrix domains of the striatum. When labeled with neuronal markers, we found that 44% (89/198, n=3) of the Emx1-lineage cells in the striatum co-localized with NeuN, and that most (96%, 67/70 n=2) YFP+/NeuN+ cells also co-expressed DARPP-32 (Fig. 23B). YFP+/DARPP32+ cells in the dorsal striatum revealed represented approximately 4% (105/2713, n=3) of the total DARPP-32+ MSN population. These Emx1-lineage cells also co-labeled with CTIP2 (Fig. 23C), which along with DARPP-32, labels medium spiny neurons (MSNs) (Gerfen, 1992; Arlotta et al., 2008). In contrast, immunolabeling with several markers for interneuronal subtypes (parvalbumin (PV), calretinin (CR), somatostatin (SST), neuropeptide Y (NPY), and neuronal nitric oxide synthase (NOS)), revealed that approximately only 1 YFP+ cell per section also expressed CR, with no YFP+ cells co-localizing with other markers of inhibitory neurons (data not shown). In addition, we found that many Emx1-lineage cells expressed CC1 (26%, 49/188, n=3), GFAP (9%, 17/179, n=3) or NG2 (21%, 43/240, n=3), markers for mature oligodendrocytes, astrocytes, and glial progenitors, respectively (Figure 24, Table 2).

As the largest population of striatal neurons derived from the Emx1-lineage were DARPP-32+/CTIP2+ MSNs, we wanted to determine whether these neurons contributed to both major subdivisions of the striatum, patch (also called striosomes) and matrix. We labeled the patch domains with reelin and MOR1-β, and the matrix domains with somatostatin (SST) and CB, as SST+ fibers from the cortex allow demarcation of the matrix and CB labels projection neurons in the matrix (Gerfen, 1985, 1992; Alcantara et al., 1998; Arlotta et al., 2008). Few Emx1-lineage MSNs were found in the SST+ fiber-rich matrix, and a small number of Emx1-lineage co-localized with CB (Fig. 23D, E). In
contrast, we found that the majority (91%, 98/108, \(n=2\)) of Emx1-lineage MSNs were in reelin-positive patches, while only 44% (1153/2630, \(n=2\)) of YFP-negative MSNs were present in reelin-positive patches (Fig. 23F). This result was further supported by the co-localization of YFP+ cells with \(\mu\)-opioid receptor-\(\beta\) (MOR1- \(\beta\)) expression (Fig. 23G).

In addition, we found that 100% (28/28, \(n=2\)) of Emx1-lineage MSNs were in tyrosine hydroxylase (TH)+ domains (Fig. 23H). TH labeling marks dopaminergic projections from the substantia nigra, also referred to as dopaminergic islands. Thus, Emx1-lineage progenitors gave rise to patch MSNs innervated by dopaminergic inputs from the substantia nigra (SN).

In addition to patch/matrix subdivisions, striatal MSNs can also be classified based on their output projections. MSNs receive excitatory projections primarily from layer V of the cortex, and send inhibitory projections out of the striatum through two primary pathways (Gerfen, 1985). Direct pathway MSNs project directly to the substantia nigra, express D1 dopamine receptors (D1 nigral MSNs of the direct pathway), and facilitate movement (Gerfen et al., 1990). Indirect pathway MSNs project to the substantia nigra via the globus pallidus, and express D2 dopamine receptors (D2 pallidal MSNs of the indirect pathway), and inhibit movement. To determine whether Emx1-lineage MSNs were part of one or both of these circuits, we carried out whole-cell current-clamp recordings using previously established physiological criteria to differentiate between D1 and D2 MSN subtypes (Ade et al., 2008; Gerfen et al., 2008).

Based on the spiking threshold of YFP+ cells, we found that Emx1-lineage neurons were both high threshold D1-like MSNs (Fig. 23I; 2/8 cells, Rheobase = 90-100 pA) and low-threshold D2-like MSNs (Fig. 23J; Table 1; 6/8 cells; Rheobase = 15-30 pA). As D1
MSNs make up 35% of striatal neurons, and D2 MSNs account for 40% of striatal neurons (Gerfen, 1992), our findings suggest that Emx1-lineage neurons disproportionately contribute to the D2 MSN subtype, as 75% of Emx1-lineage cells were D2-like MSNs. Thus, collectively these data reveal that Emx1-lineage progenitors gave rise to MSNs that preferentially localized to the patch domains, which receive cortical input from deep layer V neurons and subcortical input from ventral tier dopaminergic neurons from the substantia nigra. Furthermore, we found that Emx1-lineage derived MSNs provide output to the substantia nigra via both direct and indirect pathways, although more Emx1-lineage MSNs contributed to the D2 MSN subpopulation that is responsible for inhibition of movement.

In addition to contributing to the postnatal striatum, consistent with previous studies (Gorski et al., 2002; Medina et al., 2004), we observed that Emx1-lineage cells also contributed to the postnatal amygdala. However, in previous studies the cellular fate of Emx1-lineage cells was not determined. We therefore examined whether Emx1-lineage cells in the mature amygdala gave rise to excitatory neurons similar to their pallial counterparts in the cerebral cortex, or to inhibitory neurons similar to their subpallial counterparts in the striatum. We found that, in contrast to the striatum, Emx1-lineage YFP+ cells in the mature basolateral complex (BLC) co-localized exclusively with Tbr1 in the both the basolateral and lateral nuclei (Fig. 25A-C), with no YFP+ cells expressing inhibitory neuronal markers. Previous studies (for review, see (Sah et al., 2003)) have revealed that, based on measures of firing properties, excitatory neurons in the lateral and basolateral amygdala comprise two distinct electrophysiological subtypes, pyramidal-like and burst-firing neurons. To examine the physiological fate of amygdala
Emx1-lineage cells, we carried out patch-clamp slice recordings in the lateral and basolateral amygdala nuclei. This analysis revealed that Emx1-lineage cells generated these two cell types, which were interestingly segregated to separate nuclei. Emx1-lineage cells in the lateral nucleus exclusively gave rise to pyramidal-like excitatory neurons ($n=7$), whereas Emx1-lineage cells in the basolateral nucleus only gave rise to burst-firing excitatory neurons ($n=7$) (Fig. 25I-K, Table 2).

**Differences in the timing of the generation of striatal and amygdala Emx1-lineage neurons**

As our above data revealed that the Emx1-lineage generates different cell types in the striatum and the amygdala, we sought to determine whether developmental differences in timing of the birth of Emx1+ progenitors is related to the generation of these two disparate neuronal populations. To accomplish this, we used BrdU birth-dating to label cells in S phase at E9.5, E11.5, E13.5, E14.5, and E15.5. In the striatum, a minority of Emx1-lineage MSNs were labeled with BrdU at early ages of BrdU administration (4%, (4/103, $n=3$) at E9.5; 15%, (9/59, $n=2$) at E11.5) (Fig. 26A, D). BrdU administration at later ages resulted in more labeling of Emx1-lineage MSNs (23%, (15/63, $n=2$) at E13.5, 29%, (21/78, $n=3$) at E14.5, and 27% (14/50, $n=2$) at E15.5 (Fig. 26B, C, D). These birthdates were also coincident with the timing of neurogenesis of Emx1-negative MSNs, in which fewer Emx1-negative MSNs are born early ((3% (86/3339, $n=3$) at E9.5, and 11% (355/3221, $n=2$) at E11.5). Birth-dates of later born Emx1-negative MSNs were also similarly distributed (33% at E13.5, (670/2032, $n=2$), 31% at E14.5 (1272/4093, $n=3$), and 27% at E15.5 (703/2261, $n=2$) (Fig. 26D).
We next examined whether *Emx1*-lineage neurons in the amygdala are generated at different times than *Emx1*-lineage neurons in the striatum. Similar to the striatum, BrdU administration at E9.5, resulted in a small (7%, 48/731, \(n=6\)) population of heavily labeled BrdU+/Tbr1+ *Emx1*-lineage excitatory neurons in the lateral and basolateral amygdala (Fig. 26E,F). In contrast, administration of BrdU two days later at E11.5 resulted in a larger population (14%, 89/741, \(n=6\)) of strongly BrdU-labeled excitatory neurons of the *Emx1*-lineage in both the postnatal lateral and basolateral amygdala, as compared to the striatum. These cells were distributed throughout the lateral and basolateral nuclei (Fig. 26G, H'). Additionally, also in contrast to the striatum, only very few (2%, 9/555, \(n=3\)) amygdala neurons were heavily BrdU-labeled when BrdU was administered at E13.5 (Fig. 26I, J). Interestingly, however, BrdU administration at this age selectively labeled intercalated cells, which are located at the boundary of the lateral and basolateral nuclei (Fig. 26I). These specialized inhibitory neurons regulate feed-forward cortical inputs and fear circuitry outputs (Marowsky et al., 2005; Hefner et al., 2008; Likhtik et al., 2008). Very few neurons were born in the amygdala at later ages, with 1% labeled at E14.5 (7/279, \(n=3\)), and at E15.5, with 0.1% labeled (1/608, \(n=3\)) (data not shown). These differences in timing, in which *Emx1*-lineage cells that contribute to the amygdala precedes the generation of *Emx1*-lineage cells that contribute to the striatum is graphically shown in Fig. 26K.

*Origins of basal telencephalic Emx1-lineage neurons*

Previous expression studies (Puelles et al., 2000; Gorski et al., 2002) revealed that *Emx1* is expressed throughout the lateral and dorsal pallial domains, beginning during
early neurogenesis. This restricted expression pattern of *Emx1* raised the question as to whether striatal and amygdala *Emx1*-lineage cells were pallial-derived. To test this we took a number of approaches. We first examined expression of *Emx1* during early neurogenesis, beginning at E9.5, when *Emx1* is first present, and compared this expression pattern to the pattern of *Cre* expression and the location of *Emx1*-lineage cells as revealed from *Emx1-Cre; ROSA-LacZ* crosses. We reasoned that recombined *Emx1*-lineage cells observed outside the domain of *Emx1* and *Cre* expression most likely migrated from an *Emx1*+ source. Indeed, at E11.5 and E13.5, β-gal staining in *Emx1-Cre; ROSA-LacZ* brains revealed that *Emx1*-lineage cells were present in the ventral pallium and dLGE, beyond the domain of *Emx1* and *Cre* mRNA expression (Fig. 27C, D, G, H, K, and L). This result suggested that *Emx1*+ cells had migrated from the pallium to the subpallium during early neurogenesis. In fact, late in embryogenesis (older than E15.5) and early in postnatal development, cortically-derived *Emx1*-lineage cells have been shown to migrate ventrally into the developing striatum (Willaime-Morawek et al., 2006; Willaime-Morawek and van der Kooy, 2008).

To directly examine whether pallial-derived *Emx1*-lineage cells indeed migrate from the pallium to the subpallium early in development, we placed DiI in the lateral and dorsal-lateral pallium of E11.5 (Fig. 28A-F) and E13.5 (Fig. 28G-L) *Emx1-Cre; ROSA-YFP* brains and cultured for 36–48 hours *in vitro*. DiI+ cells were observed migrating into the dLGE, thus confirming that pallial progenitors had the capacity to migrate ventrally. We also observed DiI+/YFP+ *Emx1*-lineage cells migrating ventral-laterally from the dorsal-lateral pallium into the ventral pallium and LGE (Fig. 28D-F, J-L).
To further examine this migratory path using an *in vivo* assay, we electroporated the dorso-lateral pallium *in utero* at E12.5 with a construct that constitutively expresses RFP in electroporated cells. Three days later, at E15.5, we observed RFP+ cells that had migrated away from the site of electroporation in the dorso-lateral pallium and toward the ventral telencephalon (Fig. 28M,N), consistent with previous work showing pallial-to-subpallial migration using *in utero* electroporations (Bai et al., 2008; Soma et al., 2009). This observation was also consistent with our *in vitro* findings of an early pallial to subpallial migratory wave. From our long-term analysis of cortically electroporated brains, we also found a small number of RFP+ cells in the dorsal striatum (Fig 28O-Q).

Although the above evidence revealed that *Emx1*-lineage cells migrate from the pallium to the subpallium, we wanted to examine whether some of these cells might also be locally derived. To accomplish this, we labeled *Emx1-Cre; ROSA-YFP* embryos with a short pulse of BrdU at either E11.5 or E13.5, and sacrificed after 2 hours (Figure 29). We reasoned that any BrdU+ cells in the LGE would most likely be derived locally as 2 hours would not be enough time for a pallial-derived cell population to migrate ventrally into the LGE. Interestingly, a few *Emx1*-lineage cells in the LGE were also heavily labeled with BrdU at E13.5, although not at E11.5, suggesting that at E13.5 there may also be a locally-derived *Emx1*+ population. Thus, in addition to arising from the pallium, it is likely that a small subpopulation of *Emx1*+ cells present in the LGE at E13.5 and later were locally derived. This finding is also consistent with a recent study revealing that *Emx1-Cre* mice have the capacity to recombine in subpallial Gsx2-positive cells (Waclaw et al., 2009).
Generation of MSNs from Emx1+ pallial progenitors in vitro

Given the above evidence that dorsal Emx1-lineage cells were able to migrate ventrally, and that pallial RFP-labeled cells were found in the postnatal striatum, we next sought to determine whether pallial-derived Emx1-lineage cells had the capacity to generate striatal MSNs. To accomplish this, we dissected distinct domains of the E13.5 Emx1-Cre; ROSA-YFP pallium and LGE (schema shown in Figure 30) and allowed them to differentiate into neurons in vitro. Differentiated MSNs were identified by triple immunostaining with YFP, CTIP2 and GAD65/67 (Figure 30). We found that Emx1-lineage cells from the medial pallium were inefficient at producing MSNs, while cells taken from either the dorsal pallium or the region of the ventral/lateral pallium were able to produce MSNs with a greater efficiency (Fig. 30A-C; F). In contrast, Emx1-lineage cells in the LGE were not as efficient at making MSNs as cells taken from the dorsal or ventral/lateral pallium (Fig. 30D, F). Thus, this in vitro data revealed that embryonic Emx1-lineage cells derived from the pallium have the capacity to generate MSNs.

Differential combinatorial gene expression in striatal and amygdala Emx1-lineages

Once we established that Emx1-lineage cells were able to migrate ventrally from the pallium and had the potential to become inhibitory MSNs, we sought to determine whether subpallial Emx1-lineage cells were expressing genes characteristic of their putative pallial origin or of their new subpallial location. To accomplish this, sections from Emx1-Cre; ROSA-YFP embryos were co-immunostained for YFP and individually for the subpallial markers Gsx2 and Dlx2, or the pallial markers Pax6 and Tbr1. At E11.5 Emx1-lineage cells in the LGE were predominately found in the dLGE, and were also
present near the pallial-subpallial border (PSB) (Fig. 31A). These cells co-expressed the subpallial ventricular zone (VZ) homeobox transcription factor Gsx2 (Fig. 31B). By E13.5, many more cells were present throughout the LGE, and these cells had leading processes extending in multiple directions, suggesting that they were moving between the LGE mantle, SVZ, and VZ (Fig. 31C,D, D’). These Emx1-lineage cells also expressed Gsx2 in the VZ, and in addition, co-expressed Dlx2, a homeobox transcription factor expressed in the VZ and SVZ of the subpallium (Fig. 31E-F). These data indicate that, in contrast to the Emx1-lineage cells that remain in the pallium, the subpopulation of Emx1-lineage cells that are present in the LGE express subpallial genes characteristic of their new environment.

We further examined whether Emx1-lineage cells located in the subpallium continued to express pallial genes consistent with their probable pallial origin. We immuno-labeled E11.5 and E13.5 sections with Pax6, and found that some Emx1-lineage cells at the ventral pallial aspect of the PSB continued to express Pax6 protein. However, consistent with their expression of subpallial markers as shown above, cells in the LGE VZ and SVZ did not express Pax6 (Fig. 31G,J,K). We also examined sections more caudally at the level of the CGE and the developing amygdala. While Pax6 was not expressed at this plane of section in the amygdala primordium at detectable levels (data not shown), Tbr1, a marker of the excitatory neuronal lineage, was highly expressed, and most Emx1-lineage cells at this age expressed Tbr1 in the amygdala primordium and piriform cortex, but not in the dLGE or VP (Fig. 31H, I, L, M and data not shown).

As our above evidence collectively indicates that pallial-derived Emx1+ progenitors contribute to both excitatory and inhibitory neuronal populations in the basal
telencephalon, we next sought to determine whether interaction with the PSB, a region through which many of these cells presumptively migrate, is important in the regulation of this differential fate. To accomplish this, we examined the status of Emx1-lineage cells in loss of Pax6 function (Sey/Sey; Small eye) mice in which the PSB is severely disrupted (Fig. 32). In these mutants, proliferation is increased in the cortex, differentiation is impaired, migration out of the pallial VZ is reduced, and migration at the PSB is abnormal (Schmahl et al., 1993; Caric et al., 1997; Chapouton et al., 1999; Warren et al., 1999; Quinn et al., 2007; Carney et al., 2009). We found an increased number of Emx1-lineage progenitors in the LGE of Emx1-Cre; Sey/Sey mice at E13.5, although this difference was not apparent at earlier ages (Fig. 32A-D). Analysis at E17.5, when many LGE progenitors have already differentiated into MSNs, revealed that there were increased numbers of CTIP2+/Emx1-lineage cells in the striatal primordium in Emx1-Cre; Sey/Sey mice compared to control mice (Fig. 32E, F). In contrast, in the developing amygdala, the number of Tbr1+/YFP+ neurons was not different between controls and Sey/Sey mice at either E13.5 or E17.5 (Fig. 32G-J). These data indicated that there was not an obvious defect in proliferation, migration, or fate of Emx1-lineage cells in the amygdala. Therefore, disruption of the PSB results in an increase in the numbers of Emx1-derived MSNs in the striatum, but not the amygdala, indicating a role for Pax6 in one or more of several developmental processes: 1) determination of the dorsal to ventral migratory route of Emx1-lineage cells to the LGE, 2) regulation of the fate of these cells in the striatum, or 3) regulation of the size of the Emx1-derived MSN progenitor pool.

Our above analyses of gene expression profiles of Emx1-lineage cells during embryogenesis (Fig. 31) revealed what appeared to be two distinct pools of basal
telencephalic Emx1-lineage cells: ones that express subpallial markers and presumably contribute to the striatum to generate MSNs, and ones that maintain expression of pallial markers and contribute to excitatory neurons in the amygdala. To directly examine whether Emx1-lineage cells that also expressed subpallial transcription factors had a different fate in the basal telencephalon than Emx1-lineage cells, we used an intersectional genetic fate-mapping approach (Kim and Dymecki, 2009) in which Emx1-Cre mice were crossed to Dlx5/6-Flpe mice (G.M. and G.F., manuscript submitted). Their progeny were crossed to RCE: dual reporter mice, which expresses EGFP only when both Cre and Flpe have been expressed in the same cell (Sousa et al., 2009). During embryogenesis, we found that Emx1; Dlx-lineage cells were remarkably restricted to the LGE and not present in other telencephalic regions (Fig. 33A, B). Many of these cells were labeled with the neuronal progenitor marker TUJ1 (Fig. 33C, D). Consistent with the restricted pattern of cell distribution in the embryonic brain, we observed that in the postnatal brain Emx1; Dlx-lineage cells were found almost exclusively in the postnatal striatum (Fig. 33E), and were notably absent from the amygdala (data not shown). Furthermore, Emx1; Dlx-lineage cells expressed DARPP-32 and NeuN, but did not express markers for glial cells or interneurons, indicating that they were also restricted in their cellular fate (Fig. 33F-I, and data not shown). Thus, Emx1-lineage neurons that express Dlx genes during development exclusively give rise to an MSN subpopulation in the striatum.

DISCUSSION

Emx1-lineage contribution to the striatum and amygdala
In this study, using a combination of approaches, we investigated the timing, fate and origins of $Emx1$-lineage cells in the striatum and amygdala, two major structures of the basal telencephalon. In relation to fate, most prominently, our immunohistochemical and electrophysiological characterization in the striatum reveals that $Emx1$-lineage neurons in the striatum comprise a subset of striatal MSNs. $Emx1$-lineage striatal neurons display a number of specific characteristics distinct from non-$Emx1$ lineage MSNs. First, striatal $Emx1$-lineage cells, while present throughout the dorsal-ventral and rostral-caudal axis, are disproportionately localized in the dorsal portion of the striatum, which receive connections from neurons in deep layer V of the cortex. $Emx1$-lineage MSNs are also preferentially localized to the patch (striosome) compartment of the striatum. Patch neurons receive inputs primarily from the limbic cortex, in contrast to matrix neurons, which receive inputs primarily from neocortical areas (Gerfen, 1984; Donoghue and Herkenham, 1986). Previous work has also shown that one of the targets of patch neurons is the basolateral nucleus of the amygdala, indicating that this $Emx1$-lineage subpopulation may be an important subcomponent of a limbic circuit that modulates information between the cortex and amygdala (Ragsdale and Graybiel, 1988). It is interesting to note that while $Emx1$-lineage neurons contribute mainly to patches in the striatum our BrdU analysis indicates that this cell population is born throughout striatal development. As previous work has shown that patch neurons are born earlier than matrix neurons (Fishell and van der Kooy, 1987; Fishell et al., 1990; Krushel et al., 1993; Song and Harlan, 1994; Krushel et al., 1995; Olsson et al., 1997; Mason et al., 2005) our data interestingly suggest that $Emx1$-lineage neurons may produce patch neurons over a longer period of time than the $Emx1$-negative MSN progenitor pool.
Intriguingly, our results also indicate that, in addition to the LGE, at least a subpopulation of the $Emx1$-lineage MSNs of the striatum appear to be derived from the embryonic pallium, a region not previously recognized as a source of basal telencephalic inhibitory projection neurons. The LGE origin is supported by our short-term BrdU birth-dating experiments which reveal some $Emx1$-lineage dividing cells in the VZ of the LGE at E13.5 (but not E11.5), as well as recent work has shown that $Emx1$-$Cre$ mice can be used to recombine cells in the LGE (Waclaw et al., 2009). However, we suggest that an early-generated $Emx1^+$ progenitor pool from the developing cerebral cortex is also an important source of striatal neurons. Our evidence for this is as follows: 1) at embryonic stages we find that $Emx1$-lineage cells are present in regions that extend beyond the pallial $Emx1$ mRNA expressing domain, suggestive of an active migration ventrally, 2) our in vitro and in vivo migration assays reveal that $Emx1$-lineage cells from the pallium migrate ventrally into the LGE and striatum, respectively, 3) our in vitro differentiation assay indicates that the E13.5 pallium has the capacity to generate MSNs 4) our embryonic $Sey/Sey$ mutant analysis shows increased numbers of $Emx1$-lineage MSNs in the striatal primordium. This putative novel source of striatal inhibitory neurons indicates a greater flexibility in telencephalic dorsal-ventral patterning than previously recognized.

In addition to the striatum, the other major target of $Emx1$-lineage cells is the amygdala. Similar to other telencephalic structures, our work and the work of others have revealed that numerous spatially separate progenitor populations contribute to the developing amygdala (Puelles et al., 2000; Medina et al., 2004; Tole et al., 2005). Gene expression and fate-mapping studies have suggested one such population is the cortically-derived $Emx1$-lineage, which is expressed in the developing amygdala; analysis of $Emx1$-
CRE mice has revealed recombined cells in the mature amygdala (Fig 2.; (Puelles et al., 2000; Gorski et al., 2002; Medina et al., 2004). Our fate-mapping analysis in this study demonstrates definitively that Emx1-lineage progenitors give rise to excitatory neurons in the amygdala. Our electrophysiological analyses extend the above findings, revealing that these Emx1-lineage cells generate two subclasses of excitatory neurons: pyramidal-like neurons in the lateral nucleus of the amygdala, and burst-firing neurons in the basolateral nucleus of the amygdala.

**Differential cell birth date and genetic heterogeneity of Emx1-derived striatal and amygdala neurons**

Our fate mapping data reveal that Emx1-lineage progenitors generate strikingly disparate neuronal subtypes depending on which structure they will eventually populate. The factors that regulate the decision of Emx1-lineage cells to contribute to one of these specific pools is not known, however, it is likely contingent upon one of three non-mutually exclusive mechanisms: 1) differential timing of progenitor cell birth, 2) location along the anterior-posterior and/or dorsal-ventral axis of the developing cerebral cortex, or 3) local heterogeneity within the cortical VZ. Our analyses here indicate that both timing and genetic heterogeneity may play a causal role in cell fate decisions within the Emx1-lineage in determining their differentiation into striatal inhibitory neurons or amygdala excitatory neurons. The differences in the timing of the generation of Emx1-lineage amygdala neurons versus striatal neurons is quite striking, with Emx1-lineage amygdala excitatory neurons generated between E9.5 and E11.5 and Emx1-lineage MSNs generated between E11.5 and E15.5. This early generation of amygdala neurons is
consistent with other studies that have indicated that the amygdala is a relatively early-born structure (McConnell and Angevine, 1983; Carney et al., 2006; Hirata et al., 2009; Soma et al., 2009). This differential timing for the generation of neuronal diversity is also highly reminiscent of the mechanisms for the generation of neuronal diversity in numerous regions of the neuraxis including the developing layers of the cerebral cortex, the patch-matrix of the striatum, and dorsal-ventral diversity in the spinal cord (Miller and Nowakowski, 1988; Song and Harlan, 1994; Brittis et al., 1995; McConnell, 1995; Lai et al., 2008).

In addition, our gene expression and intersectional fate mapping analyses indicates that genetic heterogeneity of Emx1-lineage cells also likely informs cell fate decisions. In support of this, Emx1-lineage cells in the embryonic LGE express Gsx2 and Dlx2, transcription factors required for inhibitory neuronal fate, while Emx1-lineage cells in the developing amygdala primordium exclusively express Pax6, a transcription factor required for pallial fate (Stoykova et al., 1996; Toresson and Campbell, 2001; Yun et al., 2001; Corbin et al., 2003; Stenman et al., 2003; Cobos et al., 2005; Carney et al., 2009). Our Emx1; Dlx intersectional fate-mapping provides direct insight into the putative combinatorial code for the differential specification of striatal and amygdala neurons, as the subpopulation of Emx1-lineage neurons in the striatum appear to require Dlx expression to become inhibitory neurons. This is consistent with previous work using Emx1 and Dlx genetic fate-mapping to analyze the origins of tyrosine-hydroxylase+ and CR+ interneurons olfactory bulb neurons, which in part appear to be generated from the ventral pallium and the LGE (Kohwi et al., 2007; Batista-Brito et al., 2008), regions where overlap between Emx1 and Dlx lineages occur (Kohwi et al., 2007). Consistent
with our findings, previous work has indicated that *Dlx* genes are necessary for the initiation of GABA production, and so it may be that *Emx1*-lineage cells must also express *Dlx* family members in order to become specified to an inhibitory neuronal phenotype (Stuhmer et al., 2002; Cobos et al., 2005).

Although our data are strongly suggestive that both timing and genetic heterogeneity are key determinants in the differential fate of striatal versus amygdala *Emx1*-lineage neurons, the exact mechanisms of this determination are unclear. It is possible that *Emx1*-lineage neurons in the developing cerebral cortex (or LGE) up-regulate *Dlx* genes (and other genes that specify the ventral program) before they become post-mitotic and migrate to the striatum. In support of this, previous analyses have revealed that a subpopulation of cortical progenitors express *Dlx2* in the ventricular zone (Nery et al., 2003), and our *in vitro* differentiation assays shown here reveal that *Emx1*+ cortical progenitors have the capacity to generate MSNs. Alternatively, the differential fates of ventrally-migrating *Emx1*-lineage cells whose ultimate destination is the striatum or amygdala may also be influenced by external cues at the boundary between the developing cortex and striatum (the PSB). This area expresses a number of molecules that are secreted locally, including *Sfrp2* and *Tgfα*, and the more caudally expressed *Fgf7* (Stoykova et al., 1996; Toresson and Campbell, 2001; Carney et al., 2009). As *Emx1*-lineage cells from the pallium migrate ventrally through the region at the PSB, and more caudally, at the level of the developing amygdala, it is possible that these migrating *Emx1*+ cells up-regulate different transcription factors that endow them with the capacity to migrate to different locations and differentiate into different cell subtypes when compared to *Emx1*+ cells that remain in the cerebral cortex. Indeed, our observed
increase in the numbers of *Emx1*-derived neurons in the LGE in *Sey/Sey* mice, which display a disrupted PSB, is consistent with such a role for the PSB in fate and/or migratory route determination.
Figure 23. *Emx1*-lineage cells give rise to patch MSNs in the mature striatum.
Figure 23. *Emx1*-lineage cells give rise to patch MSNs in the mature striatum.

(A) Low-power image of P30 *Emx1*-Cre; *ROSA*-YFP striatum shows YFP+ *Emx1*-lineage cells present at high levels in the dorsal striatum. In this panel, lateral is to the right and medial is to the left. (B-C) Higher magnification of a single confocal optical section of the medio-dorsal striatum shows triple labeled YFP+/DARPP-32+/NeuN+ neurons (B, arrowheads). YFP+ cells are also labeled by the MSN marker CTIP2 (C, arrowheads).

(D) Few *Emx1*-lineage neurons are labeled by the matrix projection neuron marker calbindin (arrowheads point to positive cell, double arrowheads point to negative cell).

(E) YFP+ cells are found outside of the domain of somatostatin (SST)+ fibers, which demarcates the matrix (arrowheads). (F-G) Immunolabeling with patch markers μ-opioid receptor-1β (F, arrowheads) and reelin (G, arrowheads) reveal co-localization with YFP+ *Emx1*-lineage neurons. YFP+ cells also are surrounded by tyrosine hydroxylase (TH)+ fibers (H, arrowheads).

(I-J) Patch-clamp recordings of YFP+ cells in the striatum reveal that *Emx1*-lineage neurons are both D1- and D2- type MSNs. (I) Trace from a single D1-like YFP+ cell; current held at -60mV. Average resting membrane potential was -87.2mV (*n*= 2). (J) Trace from a single D2-like YFP+ cell; current held at -60mV. Average resting membrane potential was -85.4mV (*n*=6). Abbreviations: CC, Corpus callosum, Str, Striatum. Scale bars in A, 500 µm; B-H, 100µm.
Figure 24. *Emx1*-lineage cells also give rise to glia in the mature striatum.
Figure 24. *Emx1*-lineage cells also give rise to glia in the mature striatum.

(A) YFP+ cells co-expressing the mature oligodendrocyte marker CC1 are shown (arrowheads) (B) Some YFP+ cells are also labeled by the astrocyte marker GFAP (arrowheads) (C) Some YFP+ cells also express the oligodendrocyte progenitor marker NG2 (arrowheads). (D) Quantification of *Emx1*-lineage cells in the striatum at P30 reveals that 44% of YFP+ cells are NeuN+ mature neurons (Figure 1B), 21% are oligodendrocyte precursors, 26% are mature oligodendrocytes, and 9% are astrocytes.
Figure 25. *Emx1*-lineage cells give rise to excitatory neurons in the basolateral complex of the amygdala.
Figure 25. *Emx1*-lineage cells give rise to excitatory neurons in the basolateral complex of the amygdala.

(A) As shown at P30, YFP+ *Emx1*-lineage cells are present throughout the basolateral and lateral nuclei of the amygdala. (B-C) Higher magnification optical sections of boxed regions of the lateral nucleus and basolateral nucleus shown in (A) reveals that YFP+ cells in both of these nuclei also express Tbr1, a marker of excitatory neurons. (Arrowheads in B, C show YFP+/Tbr1+ cells; DAPI is shown in blue). (D-K) Cells of the amygdala in *Emx1-Cre; Z/EG* animals were also analyzed by immunohistochemistry (D, E) and by patch-clamp slice recordings at P21 (F-K) *Emx1*-lineage cells in *Z/EG* reporter animals (D) also express Tbr1 in the basolateral amygdala complex (BLC) (E). Low magnification of a bright-field view of the amygdala (F) shows location of recording. (G, H) Higher magnification of bright field (G) and fluorescent images (H) illustrate a GFP+ cell prior to patching. (I) Coronal section from an *Emx1-Cre; ROSA-LacZ* brain at the level of the basolateral complex shows many recombined cells in the lateral (J) and basolateral (K) amygdala. Colored circles illustrate the differential distribution of *Emx1*-lineage excitatory neurons identified by recordings in J & K. (J) Traces from a representative GFP+ cell in the lateral nucleus of the amygdala; all GFP+ cells recorded in this nucleus exhibited a pyramidal-like firing pattern of action potentials (*n* = 7). (K) Traces from a representative GFP+ cell recorded in the basolateral nucleus; this population generated action potentials in a burst-firing pattern (*n* = 7). BL, Basolateral nucleus of the amygdala; L, Lateral nucleus of the amygdala; DEn, Dorsal endopiriform nucleus. Scale bars in A, 300 µm; B, C, 100 µm.
Figure 26. Differential birth dates of *Emx1*-lineage cells in the striatum and amygdala.
Figure 26. Differential birth dates of *Emx1*-lineage cells in the striatum and amygdala.

(A) BrdU at administration at E11.5 labels *Emx1*-lineage neurons in the dorsal striatum at P15 (arrowheads point to a BrdU+/YFP+/DARPP32+ cell; double arrowheads point to a BrdU-/YFP+/NeuN+ cell). (B) Administration of BrdU at E13.5 results in more YFP+ cells double-labeled. (Arrowheads point to a BrdU+/YFP+/NeuN+ cell; double arrowheads point to a BrdU-/YFP+/NeuN+ cell). (C) BrdU administration at E14.5 results in many co-labeled *Emx1*-lineage cells at P15. (Arrowheads point to BrdU+/YFP+/DARPP-32+ cells; double arrowheads point to BrdU-/YFP+/DARPP-32+ cells). (D) Quantification of striatal birthdates, *Emx1*-lineage MSNS: 4%, (4/103, \( n = 3 \)) at E9.5; 15%, (9/59, \( n = 2 \)) at E11.5, 23%, (15/63, \( n = 2 \)) at E13.5, 29%, (21/78, \( n = 3 \)) at E14.5, and 27% (14/50, \( n = 2 \)) at E15.5. *Emx1*-negative MSNs: 3% (86/3339, \( n = 3 \)) at E9.5, 11% (355/3221, \( n = 2 \)) at E11.5, at E13.5, 33%, (670/2032, \( n = 2 \)), at E14.5, 31% (1272/4093, \( n = 3 \)), and at E15.5, 27% (703/2261, \( n = 2 \)). (E-F) In the amygdala, BrdU labels 7% of Tbr1+/YFP+ cells at E9.5. (D) BrdU alone; few cells are labeled in the BL at E9.5. (E) Low-power image of the basolateral nucleus; (F) Higher magnification single optical sections of boxed section in (E); single arrowheads point to triple-labeled BrdU+/Tbr1+ YFP+ cells; double arrowheads point to BrdU- YFP+/Tbr1+ cells. (G-H), BrdU administration at E11.5; BrdU+ cells are present throughout the BLA. BrdU- labeled *Emx1*-lineage cells are present throughout both the BL and L nuclei (26.4% of YFP+/Tbr1+ cells were also heavily labeled with BrdU); arrowheads in H point to BrdU+/YFP+/Tbr1+ cells. (I-J) BrdU at E13.5; in the BLA very few cells are BrdU-labeled. However, intercalated cell masses both medially and laterally are heavily labeled.
by BrdU (I). Low power and higher power optical sections show that few Enx1-lineage cells are labeled with BrdU at E13.5 in the BL (1.5% of YFP+/Tbr1+ cells were heavily labeled); (arrowheads point to the only BrdU+/YFP+/Tbr1+ cell in the section). (M) Graph of the percentages of striatal and amygdala fated Enx1-lineage BrdU+ cells born at different times during embryogenesis. In the amygdala, 7% were born at E9.5, (48/731, n=6), 14% at E11.5 (89/741, n=6), 2% at E13.5 (9/555, n=3), 1% at E14.5, (7/279, n=3), at 0.1% at E15.5 (1/608, n=3). Abbreviations: BLC, basolateral complex; LITC, lateral intercalated cells; MITC, medial intercalated cells; PCx, piriform cortex. Scale bars in A, C-D, 100 µm; B, E, 100 µm, B’, E’, 50 µm.
Figure 27. Expression of *Emx1* and *Emx1*-derived cells in the embryonic telencephalon.
Figure 27. Expression of *Emx1* and *Emx1*-derived cells in the embryonic telencephalon.

(A-D) At E9.5 and E10.5, *Emx1* is expressed throughout the dorsal pallium, and in the dorsal and lateral pallium at E11.5 and E13.5. *Emx1* is not expressed in the ventral pallium or the ganglionic eminences at any age. (E-H) *Cre* expression is comparable to *Emx1* expression at these stages and is not expressed in the LGE. (I-L) LacZ staining of *Emx1-Cre*; *ROSA-LacZ* embryos show that at E9.5 *Emx1* recombination is just beginning to occur in the pallium (I). By E10.5 *Emx1*-lineage cells are observed just beyond the domain of *Emx1* mRNA expression (J, arrowheads). *Emx1*-lineage cells are present in the VP and LGE at E11.5 and E13.5 (K-L; arrowheads point to cells in the LGE and double arrowheads mark recombined cells more ventrally). Abbreviations: Ctx, Cortex, DP, Dorsal Pallium; LGE, Lateral ganglionic eminence, LP, Lateral Pallium; MGE, Medial Ganglionic Eminence, SP, Sub-pallium, VP, Ventral Pallium.
Figure 28. A subpopulation of *Emx1*-lineage progenitors migrates ventrally from the developing cerebral cortex into the LGE during early neurogenesis.
Figure 28. A subpopulation of *Emx1*-lineage progenitors migrates ventrally from the developing cerebral cortex into the LGE during early neurogenesis.

(A-C) Low power images of a coronal *Emx1*-Cre; *ROSA*-YFP slice show DiI placement at E11.5 in the lateral pallium (arrowheads mark cells that have migrated). (C’) Illustrates DiI crystal location. (D-F) Higher magnification of the boxed region in (C) reveals that a subpopulation of DiI+ cells have migrated from the lateral pallium to the ventral pallium and LGE. Arrowheads point to several cells that have migrated ventrally that are also YFP+. 83% (5/6) of the slices had ventrally migrating DiI- labeled that also co-labeled with YFP. (G-I, I’) DiI placed in the lateral pallium at E13.5 also reveals cells migrating ventrally into the VP and LGE. (J-L) Higher magnification of the boxed region in (I); arrowheads point to DiI+/YFP+ cells in the LGE. 67% (4/6) of the slices had ventrally migrating DiI- labeled cells that also co-labeled with YFP. (M-Q) Embryos were electroporated *in utero* with 4µg/µl CAG-RFP plasmid. The lateral pallium was specifically targeted at embryonic ages as indicated and sacrificed 3 days later (E15.5), at P15, or at P30. (M-N) Short-term analysis of electroporated brains shows that the electroporation was targeted specifically in the lateral pallium. (N) Higher magnification of the boxed region in (M) shows cells with ventrally oriented leading processes (arrowheads) (n=5 electroporations) (O) Coronal sections of a P15 animal electroporated at E13.5; RFP+ cells are present in the dorsal striatum near the SVZ (n=3) (P) Coronal section of a P30 animal electroporated at E14.5; cells are present in the dorsal striatum. (Q) Image of the adjacent cortex; RFP+ cells are present in the upper layers of the cortex, indicating correct embryonic dorsal pallium targeting (n=2). Scale Bars in A-C, 250 µm; D-F, 50 µm; G-I, 250 µm; J-L, 125 µm.
Figure 29. Short-pulse BrdU-labeling of Emx1-Cre; ROSA-YFP embryos.
Figure 29. Short-pulse BrdU-labeling of Emx1-Cre; ROSA-YFP embryos.

(A) 2-hour BrdU pulse at E11.5; most subpallial Emx1-lineage cells are not labeled by BrdU (arrowhead). (B) Few Emx1-lineage cells are labeled with BrdU administration at E13.5, arrowheads point to 2 BrdU-labeled, YFP+ cells in the VZ of the LGE.
Figure 30. A subpopulation of pallial-derived Emx1-lineage cells is competent to produce inhibitory medium spiny neurons \textit{in vitro}.
Figure 30. A subpopulation of pallial-derived Emx1-lineage cells is competent to produce inhibitory medium spiny neurons in vitro.

(A) Most YFP+ cells dissected from the medial pallium (MP) do not express either CTIP2 or GAD65/67. (B-D) In contrast, more YFP+ cells dissected from the dorsal pallium (DP) (B), ventral/lateral pallium (VP/LP) (C) and LGE (D) co-express CTIP2 and GAD65/67 (arrowheads point to YFP+/CTIP2+/GAD65/67+ cells). (E) Schematic illustrating the four regions that were dissected and differentiated in vitro. (F) Quantification of percentage of YFP+ cells that co-expressed GAD65/67 and CTIP; the DP and VP/LP produced greater percentages of MSNs from the Emx1-lineage (DP: 12%, 165/1403, n=6; VP/LP: 15%, 22/164, n=4) compared to cells taken from either the MP (5%, 26/515, n=6) or LGE (5%, 4/84, n=4).
Figure 31. Expression of subpallial and pallial markers in *Emx1*-lineage cells during embryogenesis correspond to their basal telencephalic location.
Figure 31. Expression of subpallial and pallial markers in *Emx1-*lineage cells during embryogenesis correspond to their basal telencephalic location.

(A) Confocal images from sections from *Emx1*-Cre; *ROSA*-YFP brains illustrate that several *Emx1-*lineage cells are present at E11.5 in the dLGE in both the VZ and SVZ (arrowheads). (B) YFP+ cells in the dLGE express Gsx2, which marks the VZ of the subpallium. Arrowheads in B show YFP+/Gsx2+ cells. (C-D) By E13.5, many *Emx1-*lineage cells are present in the LGE (D) shows a higher magnification of the boxed region in (C). (E-F) Confocal optical images reveal that a subpopulation of YFP+ cells in the LGE express the subpallial transcription factors Gsx2 (E) and Dlx2 (F) (arrowheads show double labeled cells and double arrowheads show YFP+ cells only). (G) Optical sections reveal that *Emx1-*lineage cells in the dLGE at E11.5 do not express the pallial marker Pax6 (double arrowheads). However, *Emx1-*lineage cells in the VP are also Pax6+ (arrowheads). (H-I) Low (H) and higher power optical section (I) of boxed region in (H) showing recombined cells are also present more caudally in the region of the developing piriform cortex and amygdala. Many of these cells are Tbr1+. (J-K) By E13.5 many YFP+ cells are present beyond the Pax6+ ventral pallium and are located in the LGE in both the VZ and the SVZ (K) Higher magnification optical section of boxed region in J reveals that in the LGE, YFP+ cells do not co-label with Pax6 (arrows). (L-M) In the more caudal regions, including the presumptive amygdala, many *Emx1-*lineage cells are present, and most express Tbr1. Higher magnification optical section of boxed region in (L) is shown in (M). Scale bar in B corresponds to 300 µm in A, C; in B, G, 200 µm; E, F, I, M, 100 µm. Abbreviations: AMY, Amygdala, CGE, Caudal ganglionic eminence;
PSB, Pallial subpallial boundary; PCx, Presumptive piriform cortex; Str, Striatal primordium.
Figure 32. Disruption of the PSB in Sey/Sey mice alters the number of Emx1-lineage in the striatal primordium but not in the amygdala primordium.
Figure 32. Disruption of the PSB in Sey/Sey mice alters the number of Emx1-lineage in the striatal primordium but not in the amygdala primordium.

(A) WT E11.5 Emx1-Cre; ROSA-YFP section labeled with Gsx2; a few YFP+/Gsx2+ cells are present in the LGE. (B) In the Sey mutant, Gsx2 protein expands dorsally and few Emx1-lineage cells are present in the LGE (C) WT E13.5 mouse; YFP+ cells co-label with Gsx2 in the VZ of the LGE. (D) In the E 13.5 Sey mutant, the number of YFP+ cells is ectopically expanded past the PSB (arrowheads), and Gsx2 expression is expanded dorsally, but there is no change in the number of YFP+ cells or their position in the LGE (110 CTIP2+/YFP+ cells in n=3 WT, 108 CTIP2+/YFP+ cells in n=3 mutants). (E) At E17.5 in the WT mouse, scattered YFP+/CTIP2+ cells are present in the dorsal-lateral striatum and many YFP+ cortical fibers are visible in the dorsal-medial striatum. Insets illustrate that few CTIP2+ cells are YFP+ (arrowheads). (F) In the Sey mutant, significantly more CTIP2+/YFP+ cells (arrowheads) are present, see inset, and the YFP+ cortical fibers are markedly diminished; CTIP2+ YFP+ cells in mutants are 182% of CTIP2/YFP+ cell numbers in controls, p<.05 (95 CTIP2+/YFP+ cells in n=3 WT, 173 CTIP2+/YFP+ cells in n=3 mutants. (G) At E13.5, the WT amygdala primordium contains many YFP+/Tbr1+ cells. (H) In the Sey mutant, numerous YFP+ cells are still present. (I-J) In addition, YFP+/Tbr1+ cells are present in both WT and mutants at E17.5. Quantification of E13.5 and E17.5 revealed no significant differences in the numbers of YFP+ cells in the amygdala primordium (Sey mutants had 102% of control Tbr1+/YFP+ cell numbers at E13.5 (415 Tbr1+/YFP cells in Sey/Sey mice, n=3, 405 Tbr1+/YFP cells in WT mice, n=3) and 115% of controls at E17.5 (233 Tbr1+/YFP cells in Sey/Sey mice,
$n=3$, 202 Tbr1+/YFP cells in WT mice, $n=3$); these differences were not statistically significant).
Figure 33. Cells derived from *Emx1* and *Dlx* combinatorial lineages are present in the embryonic LGE and contribute exclusively to the postnatal striatum.
Figure 33. Cells derived from *Emx1* and *Dlx* combinatorial lineages are present in the embryonic LGE and contribute exclusively to the postnatal striatum.

(A-B) At E13.5, GFP+ *Emx1*; *Dlx*-lineage cells are present throughout the SVZ and mantle of the LGE. Higher magnification of boxed region in (A) is shown in (B). (C, D) Many GFP+ *Emx1*; *Dlx*-lineage cells express TUJ1 (arrowheads), an early neuronal marker, as shown at lower power in (C) and high-power in (D). (E, F) Low-(E) and high-(F) power images of a P30 *Emx1-Cre; Dlx5/6-Flpe; RCE: dual brain shows the distribution of intersectional lineage cells (arrowheads) in the striatum. (G-I) Optical sections of GFP+ cells immunolabeled with NeuN (G) or DARPP-32 (I) reveals these *Emx1*; *Dlx*-lineage cells are NeuN+ and DARPP32+ (G,I), but are not positive for the glial lineage marker Ng2 (H). Arrowheads show double labeled cells for each marker while double arrowheads show non-double labeled cells. Scale bars in A, 100 µm, B-C, 50 µm, E, 500 µm, F, 100 µm.
Table 2. *Emx1*-lineage cells contribute to multiple neural subtypes in the basal forebrain.

<table>
<thead>
<tr>
<th>Striatal Cell Subtypes</th>
<th>Amygdala Cell Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>44 %</td>
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<tr>
<td>Astrocytes</td>
<td>9%</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td>25%</td>
</tr>
<tr>
<td>NG2 cells</td>
<td>100%</td>
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**Neuronal Subtypes**

<table>
<thead>
<tr>
<th>DARPP-32+ MSNs</th>
<th>Calretinin+ Interneurons</th>
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<td>&lt;1%</td>
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**MSN subtypes**

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<th>91%</th>
<th>Matrix neurons</th>
<th>9%</th>
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</thead>
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<tr>
<td>D1-like Nigral MSNs</td>
<td>25%</td>
<td>D2-like Pallidal MSNs</td>
<td>75%</td>
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</table>

**Amygdala Cell Subtypes**

<table>
<thead>
<tr>
<th>Neuronal Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Nucleus</td>
</tr>
<tr>
<td>Pyramidal-like excitatory neurons</td>
</tr>
<tr>
<td>100%</td>
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</table>
Chapter 4

Implications for Forebrain Development and Neuronal Diversity
CONCLUSIONS

Summary of Findings

The development of the rostral forebrain is driven by embryonic patterning of the telencephalon into sub-domains based upon unique combinations of transcription factor expression that confer progenitor cells with different neuronal fates. The PSB divides two compartments in the telencephalon: the pallium, which will give rise to the cerebral cortex and hippocampus, and the subpallium, which will give rise to the striatum, amygdala, and olfactory bulb. We used a combination of approaches, including genetic fate mapping, conditional mutagenesis, cell birth dating, cell migration assays, and electrophysiology to link neuronal fate in the postnatal brain with progenitor cell origins during embryogenesis (Chapters 2 and 3). We also examined the developmental mechanisms underlying PSB boundary formation and the post-natal consequences of conditional loss of Pax6 function at the PSB on neuronal fate in the amygdala and olfactory bulb, two targets of PSB-derived migratory populations (Chapter 2). We found that the sorting of dorsal Pax6+ and ventral Gsx2+ progenitors during embryogenesis is the result of a combination of changes in gene expression and cell movements. Interestingly, we found that in addition to giving rise to inhibitory neurons in the amygdala and olfactory bulb, Gsx2-lineage progenitors generate a subpopulation of amygdala excitatory neurons. Targeted conditional ablation of Pax6 in Gsx2-lineage progenitors resulted in discrete local embryonic patterning defects that are linked to changes in the generation of subsets of post-natal excitatory and inhibitory neurons in the amygdala and inhibitory neurons in the olfactory bulb. Thus, in PSB progenitors, Pax6 plays an important role in the generation of multiple subtypes of neurons that contribute
to the amygdala and olfactory bulb. We also found that cells derived from the pallial Emx1-lineage contribute to two distinct neuronal populations in the mature basal forebrain: inhibitory MSNs in the striatum, and functionally distinct subclasses of excitatory neurons in the amygdala (Chapter 3). Our cell-birth dating studies reveal that these two populations are born at different times during early neurogenesis, with the amygdala population born prior to the MSNs. In conclusion, the embryonic origin and timing of neurogenesis and differential combinatorial gene expression appear to be key determinants of neuronal fate decisions important for the establishment of neuronal diversity in the basal forebrain.

**The role of borders in the developing brain**

The studies presented in this thesis inform our understanding of how borders function in the developing brain. Like the MHB, the telencephalic PSB is a domain formed by cross-repressive interactions between homeobox transcription factors, that, when disrupted by loss of function experiments, cause shifts in the expression of patterning genes into the domains normally occupied by expression of the missing transcription factors. Also like the MHB, the PSB contains domains of nested gene expression that are highest at the border region, suggesting that cells at the PSB are exposed to different extrinsic cues than their neighbors located farther from the border. Unlike the MHB and the rhombomere boundaries in the hindbrain, which do not allow mixing between cell lineage compartments (Jimenez-Guri, et al., 2010), the PSB is a semi-permeable boundary: it serves to separate developing progenitor zones of the cortex and striatum in the VZ, but importantly allows dorsally migrating interneuron progenitors
from the MGE and CGE and ventrally migrating MSN precursors from the pallium in the SVZ. Further, our time-lapse imaging studies show that some cell movements occur between the dorsal and ventral halves of the PSB in the VZ. It is likely that the PSB functions largely an active border for the majority of cortical and striatal precursors immediately adjacent to it, while the few progenitors destined to migrate through it express receptors or adhesive/repulsive molecules that allow them to migrate through it when their cortical and striatal progenitor neighbors cannot. This selective permeability would be problematic in areas that require more regimented cell segregation, such as the hindbrain. However, such mechanisms for generating diversity are critical in the forebrain, which contains more subtypes than any other brain region; in the cortex alone, it is estimated that there are 500 different neuronal subtypes, based upon transcription factor expression, morphology, neurotransmitter and receptor subtypes, and connectivity (Serafini, 1999).

**The PSB is a Unique Proliferative Domain**

Unlike other borders, the PSB also appears to be a unique proliferative domain, with cells at and immediately adjacent to the border generating multiple neuronal subtypes, contributing to non-adjacent, even distant, structures, notably giving rise to excitatory and inhibitory neurons in the amygdala and olfactory bulb. Disruption of the PSB therefore does not simply alter patterning and formation of the cortex and striatum, as would be expected if its only purpose was to segregate these two developing brain regions. We find that border disruption leads to mis-specification of multiple neuronal subpopulations, notably, excitatory neurons in the amygdala and inhibitory neurons in the
olfactory bulb. Interestingly, we also find that disruption of the PSB does not appear to disrupt the ultimate specification of cortical interneuron pools in the cortex, despite the presence of interneuron heterotopias in Pax6\(^{KO}\) cortices. As the cortical heterotopias occur prior to the normal apoptosis that refines cell subpopulations in development, perhaps these interneuron pools are correctly specified and so can compensate for abnormal migration early in development.

The question remains whether the PSB is really a unique proliferative zone, or whether it is simply immediately adjacent to two proliferative zones that generate many different cell subtypes. At the MHB, the boundary between the developing midbrain and hindbrain is formed by a group of non-neuronal “border” cells that inhibit proliferation at the boundary using nested BHLH transcription factor expression (Geling, et al., 2003). The PSB, in contrast, has no clear non-neuronal border cell population, and there is no direct evidence for inhibition of proliferation at the PSB; short-term labeling with BrdU does not show an absence of proliferation at the PSB (Sahara, et al., 2009). The most likely source of “border” cells that are not of vP or dLGE character is the Pax6+/Gsx2+ cell population that we observe at the overlap between the two domains. We show that while the PSB is refined into largely separate domains by mid-neurogenesis, the two domains never completely refine into entirely separate compartments: even at E15.5, there are a small number of Pax6+ Gsx2+ cells remaining in the PSB VZ. It is not known whether cells that maintain Pax6 and Gsx2 may have a unique fate compared with cells that ultimately down-regulate one of the two transcriptional programs. The most likely candidates for cells that require both Pax6 and Gsx2 to produce a single cell type are the intercalated cells in the baso-lateral amygdala. The intercalated cells receive input about
fearful stimuli from both limbic cortex and the baso-lateral amygdala, and provide inhibitory input to the central nucleus; recently, these cells have been shown to be required for fear extinction in conditioned fear response paradigms (Lihtik, et al., 2008). This is the only cell population that appears to maintain Gsx2 and Pax6 expression in some cells migrating in the LCS (and indeed, some migrating cells in the LCS also express both Dlx and Pax6). Intercalated cell numbers are also significantly decreased when either Pax6 or Gsx2 is lost. Further, we show in our Emx1-lineage birth-dating of the amygdala that the ICM cells are born at around E13.5, at a time when both Pax6 and Gsx2 still overlap at the PSB. However, as dual Pax6-Gsx2 fate-mapping has not been completed, nor has the loss of both Pax6 and Gsx2 exclusively in border cells has not been experimentally tested, it is not known for certain whether these dual expressing border cells have any meaningful and separate biological function from their neighbors that express only Pax6 or Gsx2.

Further, while there is strong evidence that Pax6 and Gsx2 cross-repress each other to establish the PSB, it is not clear which transcriptional program is dominant in an individual cell. It may be that gene dosage is important, and that cells that express Pax6 and Gsx2 down-regulate the factor that is expressed at lower levels to become a pallial or a subpallial progenitor cell. The cellular mechanism of border refinement is also not completely clear. While we know that cell movements are possible early in the ventricular zone of the PSB, and we know that cells that once expressed subpallial genes are able to express pallial transcription factors once the boundary is refined, we do not know the mechanism by which a Pax6+ cell in Gsx2+ territory chooses to down-regulate Pax6 and up-regulate Gsx2 or to migrate back into the Pax6+ territory. There may be
additional cell-cell interactions that dictate whether $Pax6$-driven or $Gsx2$-driven transcriptional programs are ultimately initiated in an individual cell: neighboring cells expressing only $Pax6$ or $Gsx2$ may signal dual expressing cells or cells expressing transcriptional programs not in common with their neighbors to down-regulate the “wrong” transcription factor that is not expressed by surrounding cells. Indeed, Ephrin A5 is expressed throughout the LGE and its receptor, EphA4 is expressed throughout the telencephalon (Nomura, et al., 2006). Eph-Ephrin interactions mediate contact-dependent repulsion in cells expressing the ligand and the complementary receptor, and therefore may be a useful mechanism for down regulating either $Pax6$ or $Gsx2$ in dual expressing cells.

The MHB serves as a border during development, but its presence is transient: it does not appear to contribute directly to any adult structure: the mesencephalon and metencephalon immediately adjacent to it gives rise to the midbrain and hindbrain, but the MHB itself does not produce either of these structures. Likewise, the pallium and ganglionic eminences are the primordial domains of the adult cortex and basal ganglia. It is of interest to postulate whether the PSB is also an embryonic precursor to an adult structure, or functions simply as a boundary like the MHB. The adult SVZ is the most likely candidate structure to be derived from the PSB. The adult SVZ is located adjacent to the corpus callosum at the border between the cortex and striatum in the adult brain. Work from numerous labs has shown that the adult SVZ, like the embryonic dLGE and vP, provides progenitor cells that migrate along the rostral migratory stream to seed the adult OB (Lois and Alvarez-Buylla, 1993, Luskin, et al., 1993). The SVZ is derived from progenitors in the embryonic VZ: $Emx1$-, $Dbx1$-, and $Gsx2$-lineage fate-mapping reveals
that these lineages give rise to SVZ cells in sub-domains of the SVZ that correspond to their dorsal-ventral expression patterns during development (Young, et al., 2008). Further, Emx1-, Dbx1-, and Gsx2-lineage cells continue to proliferate in the OB, indicating that these embryonic progenitor domains remain active in the adult SVZ. Finally, the SVZ maintains dorsal and ventral domains that produce different types of neurons in the adult olfactory bulb: dorsally-derived SVZ cells disproportionally produce CR+ interneurons, whereas ventrally-derived SVZ cells disproportionally produce CB+ interneurons in the OB. Further investigations into the embryonic origins of this adult proliferative zone would therefore be informed by analysis of embryonic disruption of the PSB. It would be interesting, in particular, to know whether the correct formation of the PSB during development is necessary for the correct generation of different precursor cell types in the different regions of the adult SVZ and OB.

**Genetic Regulation of Neuronal Fate**

The general principles that dictate dorsal-ventral patterning are conserved in the forebrain; Shh serves as a morphogen to confer a ventral identity on cells in the ventral forebrain, and Bmps and Wnts confer dorsal-medial identity, with Fgf8 expressed in a rostral-caudal gradient to establish rostral polarity in the forebrain. Similar to the spinal cord, induction of transcription factors by Shh leads to the activation of a number of downstream transcription factors whose expression, a combinatorial code including Nkx2.2, Pax6/7, Dbx1/2, Nkx6.1 and Irx3, dictate the major progenitor cell subdivisions in the spinal cord (Reviewed in Jessell, 2000). However, unlike the spinal cord, Shh does not seem to be required for the activation of a combinatorial code of transcription factors.
that specify each cell subtype: in the absence of Shh and Gli3, much of the defects in patterning and cell specification in the forebrain of Shh mutants is rescued (Rallu, et al., 2002). It also appears that the combinatorial code of gene expression that is a major mechanism of the generation of neural diversity in the spinal cord does not have as simple a correlate in the forebrain: Pax6, Emx1, Dbx1, Gsx1/2, Nkx2.1, Mash1, and Dlx transcription factors are all expressed in unique subdomains, but knockout and overexpression studies have not yielded clear transcriptional codes for cell identities. Further, parcellating the developing forebrain into sub-compartments based upon gene expression have yielded maps that resemble spinal cord expression patterns, but with many additional domains of nested and overlapping gene expression, consistent with the additional complexity and neuronal diversity present in the forebrain.

Data from our studies informs this question of transcriptional regulation of progenitor domains and the generation of neuronal diversity in the forebrain. Our data suggest that there are many exceptions to the general principle that one unique combination of transcription factors specifies one unique cell subpopulation, based upon two pieces of evidence: 1. Many transcription factors give rise to several different neuronal subtypes. For example, Emx1, which is expressed in progenitors that give rise to inhibitory MSNs in the striatum, inhibitory neurons in the olfactory bulb, excitatory neurons in the cortex and amygdala, and glia in several brain regions. 2. Many transcription factors expressed in either the dorsal or ventral telencephalon can produce the same lineage. For example, our work indicates that Dlx-lineage, Gsx2-lineage, and Emx1-lineage progenitors are all able to give rise to MSNs in the striatum. We also find that both Gsx2-lineage progenitors and Dbx1-lineage progenitors can give rise to
excitatory neurons in the olfactory bulb. These lineage maps are summarized in Figure 34. *Dbx1*, *Gsx2*, and *Emx1* each mark a different sub-domain along the dorsal-ventral axis, yet we find that all three transcription factors are expressed in progenitors that give rise to excitatory neurons in the amygdala. It is possible that all of these transcription factors are expressed in the same cell, as we are only able to label one of the transcription factors at a time. Our combinatorial fate-map of cells from the *Emx1-Dlx* dual lineage may inform this issue: dual-expressing cells made up only a fraction of the cells marked by either *Emx1* fate-mapping or *Dlx* fate-mapping alone in the striatum, and importantly did not mark cells in the amygdala. This suggests that the *Emx1*, *Gsx2*, and *Dbx1*-lineage progenitors that give rise to excitatory neurons in the amygdala are likely to be separate progenitor pools, and also indicates that combinatorial expression of dorsal and ventral genes is quite rare in the forebrain.

While the presence of multiple transcription factors in multiple distinct cell types does not negate the possibility of a combinatorial code, the fact that none of these transcription factors label a unique cell subtype and that many transcription factors label the same cell subtype suggests that either 1. Additional unidentified transcription factors are required to specify these unique cell subtypes or 2. We are currently grouping different cell subtypes into the same category of neuron subtype, as we do not yet have the tools to identify functional differences between all of the neuronal subtypes. It is probable that both possibilities are true. For example, it is likely that our current criteria for defining excitatory neuron subtypes in the amygdala are not stringent enough to tease apart the neuronal subtypes that are marked by each of the *Gsx2*, *Emx1*, and *Dbx1* fate-mapped populations. At this time, there are not meaningful categories of subtypes of
excitatory cells similar to the Petilla nomenclature existing for the characterization of inhibitory interneuron subtypes (Ascoli, et al, 2008). It may be that there are not a large number of discrete excitatory neuron subtypes, as is the case for interneurons, rather, excitatory neurons may have different spiking properties, ion channel compositions, and morphologies that place cell differences along a continuum rather than into discrete categories of excitatory neuron subtypes. However, it is also possible that there is a common molecular signal or program that can be used to integrate intrinsic factors and extrinsic signals, to specify the same cell subpopulation despite its multiple origins and disparate combinations of homeobox transcription factors expression. Indeed, our findings using Emx1; Dlx dual-fate mapping indicate that this is the case for at least a subpopulation of striatal MSNs. This would suggest that the excitatory amygdala progenitor pool fate is not specified by any of these early expressed transcription factors alone. This flexibility in the genetic program required to specify a given cell fate is in contrast to more regimented genetic programming of fate in other brain regions, particularly in the spinal cord. This is interesting, given that specific cell fates in the spinal cord are determined by expression of many of the same homeobox transcription factors that are expressed in forebrain progenitors.

There is evidence, then for both convergence and divergence in the transcriptional regulation of cell fate: in addition to multiple transcription factors producing common cell subtypes, the same transcription factor appears to be able to generate multiple cell subtypes. Two explanations are possible: 1. There is redundancy in genetic specification of cell fate in the forebrain. 2. These transcription factors are not always required to directly determine neuronal fate, but may work as selector genes, able to activate
numerous transcriptional programs that activate or repress many different target genes, depending upon other available cofactors, the chromatin state of the target genes, and the enzymatic activity of the cell (presence of methylases and demethylases, etc.), and thus lead to the specification of many different cell fates.

**Generation of Neuronal Diversity**

If progenitors from the same region at the PSB are able to give rise to a diversity of excitatory and inhibitory neuronal subtypes in multiple and distant forebrain regions that are not entirely explainable by initial transcription factor expression, what might be additional mechanisms for creating this diversity? Our data suggest that a combination of differences in cell motility, (perhaps dictated by expression of surface molecules important for signaling and adhesion/repulsion), regulation by multiple transcription factors, and the timing of origin of the cell (birth date) are all likely to provide additional cues that allow a progenitor cell in a mixed domain to migrate through the PSB to the cortex or the striatum, or to the olfactory bulb or amygdala. Indeed, evidence from our work has shown a change in cell motility as development proceeds, with cells at the PSB early in development, at E11.5, moving longer distances and faster than cells later in development, at E13.5. This may be driven by changes in adhesion molecules; while there is no evidence for changes in cell fate after the loss of adhesive/repulsive cues, cell sorting at the PSB is disrupted in *Sey/Sey* mutants and patch/matrix segregation is disrupted in Ephrin mutants (Nomura, et al., 2006; Passante, et al., 2008). Pax6 regulates expression of EphrinA5, and part of the disruption of cells at the PSB in *Sey* mutants may be a result of defects in cell sorting meditated by loss of Eph/Ephrin signaling at this
boundary. The calcium binding proteins R-cadherin and Cadherin 6 are expressed at the dorsal and ventral sides of the PSB and are known to be important in maintaining cell adhesion at this boundary (Stoykova, et al., 1997).

Cells derived at and around the PSB give rise to neuronal subtypes in multiple structures; progenitor cells that are destined for the amygdala and become excitatory neurons are labeled by genes expressed in the pallium, ventral pallium, and dorsal LGE. Inhibitory intercalated cells of the amygdala, inhibitory neurons in the baso-lateral nuclei, inhibitory interneurons in the olfactory bulb, excitatory neurons in the OB, and striatal MSNs are all derived in part from the vP or dLGE, the two sides of the PSB. Nested gene expression may help to parcellate this small domain into multiple progenitor domains that migrate to many locations and give rise to many diverse neuronal subtypes, and target-derived extrinsic cues may confer additional neuronal subtype specificity. Gene expression patterns and their corresponding cell subtype/brain region are summarized in Figure 35. Further, timing of birth-date may be useful for separating these progenitor pools. Within the Emx1-lineage, in the amygdala, progenitors born at earlier ages, at E9.5 and E11.5, give rise to neurons in the baso-lateral nucleus, while progenitors born at E13.5 give rise to cells in the intercalated cell mass of the amygdala. In the striatum, Emx1-lineage progenitors are born throughout neurogenesis, with peak proliferation occurring between E13.5- E15.5. Work from other groups has shown that cells derived from the dLGE that are born at earlier ages, at E11.5, populate the amygdala, while cells born at later ages, at E13.5, give rise to cells in the olfactory bulb (Waclaw, et al, 2009). This link between timing of birth and cell fate and location is reminiscent of cortical layer specification, where early-born cortical progenitors give rise to neurons in deep layers.
and later-born progenitors give rise to neurons in superficial layers of the cortex (for review, see McConnell, 1995). Therefore, the unique combination of timing, gene expression, and location of origin in progenitor domains around the PSB are all important in generating neural diversity in the adult forebrain.

The generation of neuronal diversity in the forebrain also appears to be conferred by a number of different signals. For example, the transcription factors Pax6 and Emx2 are expressed in a rostral to caudal and caudal to rostral gradient, respectively, and are important for arealization of the developing brain, similar to graded Engrailed expression in the hindbrain (Muzio, et al., 2002). Pax6 is clearly important in establishing aspects of dorsal-ventral patterning beyond what was previously known: its removal even in the small domain of overlap with Gsx2 during early development causes similar disruptions to dorsal-ventral patterning of the border as are present in the full Pax6 mutant. Further, its removal leads to an expansion of ventrally migrating Emx1-lineage cells in the striatum, suggesting that Pax6 normally represses Emx1+ cell migration into the basal telencephalon in the pallium. Importantly, Pax6 and Gsx2 are not the only important players in dorsal-ventral patterning. Loss of Ngn2 leads to loss of dorsal markers in the vP and expansion of ventral markers dorsally (Fode, et al., 2000). Mis-expression of Mash1 or Gsx2 in the cortical VZ can drive ectopic expression of other ventral markers, suggesting that correct dorsal specification requires the repression of ventral genes (Fode, et al., 2000; Waclaw, et al, 2009).

Generating neuronal diversity by creating separate progenitor pool compartments that supply multiple brain regions with specified cell subtypes is clearly not the only possible means of specifying different cell subtypes in the brain. Why has this
mechanism been frequently utilized during mammalian development, rather than generating the full diversity of subtypes locally in each brain sub-region, perhaps by a stochastic mechanism of proliferation from a multipotent parent progenitor cell? The answer may lie in the changes in brain morphology and function across evolution. Reusing existing progenitor domains in evolutionarily older regions of the brain, and adding complexity by using new migratory routes may be more efficient than creating entire compartments with multiple cell subtypes de novo. Further, using secreted molecules and transcriptional regulation of cell differentiation in concert allows plasticity in cell fate determination that would allow more diversity than stochastic mechanisms alone. Each additional transcriptional program or secreted molecule or afferent input would activate many different downstream programs, with possibilities for many more diverse cell fates.

**Intrinsic vs. Extrinsic Determination of Cell Fate**

A protracted debate in the field centers around the degree to which progenitor cells are intrinsically specified with their mature identities and functions early in development, or are molded by afferent input that subsequently dictate the mature cellular and physiological features and connectivity of the cell and determine its role in a network of cells that process a certain type of information. A great deal of evidence indicates that specification of cell identity occurs early and is regulated by transcription factors expressed early in a cell’s life. For example, genetic studies of cell fate in the MGE and CGE indicate that many cortical interneurons have shared mature electrophysiological, morphological, and immunohistochemical features that correlate
with their much earlier spatial-temporal origins in the developing basal telencephalon (Butt, et al., 2005; Miyoshi, et al., 2010). Further, clonal analysis of excitatory progenitors indicates that sister cells have a tendency to be synaptically connected to their sister clones in their adult location in the cortex (Yu, et al, 2009). Additionally, removal of genes important in early patterning of the telencephalic primordium, such as in the case of Fgf8-/- mutants, results in defects in cortical arealization, leading to abnormal formation of cortical sensory cortex sub domains and abnormal thalamic innervation (Huffman, et al., 2004).

Our work also supports early genetic specification of neuronal identity. Our genetic fate mapping studies in the amygdala indicates that neuronal progenitors with different origins in embryogenesis have shared excitatory neuron fates in the lateral amygdala. Further, our work examining the impact of embryonic disruption of Pax6 progenitor domains at the PSB suggests that early Pax6 expression is important for the correct specification of mature cell subtypes in the amygdala and olfactory bulb, although the impact of this disruption on network function remains to be explored. We also found that disruption of Pax6 led to increases in the numbers of Emx1-lineage MSNs in the developing striatum, suggesting that Pax6 represses proliferation or MSN fate choice in these cells. Our conditional mutagenesis data provide evidence that early genetic specification in progenitor cells is important for the determination of mature cell subtype identity.

Early transcriptional regulation of neuronal fate likely provides the cellular framework for the composition of the mature brain structure and basic neuronal morphology and function, and target-derived cues and sensory experience then refine this
pattern, producing functional networks of interconnected neurons that process a specific type of information. A number of studies that disrupt interactions between sensory neurons and their targets, alter sensory information during critical periods of input during postnatal development, or alter thalamic innervation to the cortex, indicate that afferent input is important for the formation of mature cellular properties and regional cellular architecture (for reviews, see Hensch, 2005; Penn and Schatz, 1999; O’Leary et al., 1994). These studies indicate that there are aspects of both intrinsic and extrinsic specification of neuronal subtypes; the question of how these two types of cues are integrated across development to produce a mature functional neuron and define its connectivity remains an unresolved research question.

**Evolutionary Perspectives**

It is interesting to note that developmental signals and programs for patterning and cell specification in the brain are evolutionarily conserved across species, despite the diversity of body form and function. Similar molecular mechanisms allow for the correct development of drosophila wings, zebrafish hindbrains, human cortices, and murine telencephalic development: intrinsic and extrinsic mechanisms are employed in concert to specify a diversity of progenitor pools from an initially uniform population of progenitor cells, the formation of borders is used to separate adjacent but different progenitor domains, and the same genetic and cellular programs are reiterated at different times and places in development to produce incredibly different groups of cells with diverse functions that are reliably similar across species. Indeed, many of the same transcription factors are used in flies and mammals: the Drosophila Gsx2 homologue, *ind*, is expressed
in the ventral nerve cord, and when mutated, leads to a reduction of ventral neuroblasts, while the remaining neuroblasts appear to have acquired a dorsal fate, similar to the changes in dorsal-ventral transcription factor expression seen at the PSB in Gsx2 and Pax6 mutants. Further, during CNS development, ind is known to repress eyeless, the Drosophila Pax6 homologue, much like its function in the mammalian telencephalon (Von Ohlen, et al., 2007).

**Final Remarks**

The work presented in this thesis provides important new insight into the genetic mechanisms regulating the generation of neuronal diversity in the developing mammalian basal forebrain. We found that origin, timing, and transcription factor expression are important for determining whether an Emx1-lineage cell becomes an excitatory neuron in the amygdala or an inhibitory neuron in the striatum. We found that the refinement of the PSB during development is driven by a combination of changes in transcription factor expression and cell motility. We also found that Pax6 is required locally for correct patterning of the PSB, and that removal of Pax6 exclusively in border cells also expressing Gsx2 is sufficient to confer patterning defects at the border. Finally, we found that disruption of the PSB via Pax6 deletion leads to cell fate defects in the amygdala and olfactory bulb. These data implicate early genetic regulation of patterning as critical for the determination of neuronal fate and the generation of neuronal diversity in the mature forebrain. Elucidating the mechanisms through which transcriptional regulation of target genes affects developmental programs subsequent to the determination of cell fate,
including axon outgrowth, synaptogenesis, and the refinement of connectivity, will be important and interesting future research directions.
Figure 34. Fate map summarizing our findings from our genetic fate-mapping experiments analyzing *Emx1*-*, Dbx1*-*, and *Gsx2*-lineage neuronal populations in the basal forebrain.
Figure 34. Fate map summarizing our findings from our genetic fate-mapping experiments analyzing *Emx1*-*, Dbx1*-*, and *Gsx2*-lineage neuronal populations in the basal forebrain. Left, OB. *Emx1*- and *Gsx2*-lineage progenitors give rise to inhibitory neurons in the glomerular layer of the OB. *Dbx1*-lineage progenitors give rise to Tbr1+ excitatory mitral cells and reelin+ neurons in the periglomerular, external plexiform, and mitral cell layers, with rare *Gsx2*-lineage excitatory neurons also found in this layer. *Gsx2*-lineage progenitors also give rise to inhibitory neurons in the periglomerular, internal plexiform, and granule cell layers. Right, Sratum and Amygdala. *Emx1*- and *Gsx2*-lineage cells give rise to MSNs in the striatum; the majority are from the *Gsx2*-lineage. *Gsx2*-lineage cells also label inhibitory interneurons in the striatum. *Emx1*-*, *Dbx1*-*, *Gsx2*-lineage cells label excitatory neurons in the LAT and BLA. *Gsx2*-lineage cells also become inhibitory interneurons in the BLA and LAT, the CE, and the ICM of the amygdala.
Figure 35. Embryonic progenitor domains are correlated with neuronal fate in the adult basal forebrain.
Figure 35. Embryonic progenitor domains are correlated with their cell fate in the 
adult basal forebrain. Based upon fate-mapping data, the embryonic progenitor domains 
are correlated with specific cell subtypes in their adult structure. X indicates that there is 
evidence that cells from a particular embryonic progenitor domain have been shown to 
give rise to a particular neuronal subtype in that brain structure, - indicates that there is no 
evidence for that particular embryonic structure giving rise to the indicated cell subtype 
and region.
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