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Abstract

Our results demonstrate for the first time novel coordinated expression of Hoxa2, Hoxd1 and Pax6 proteins that coincide with the three developmental stages of the diencephalic mouse brain. During the first stage of diencephalic development (embryonic day (E)10-12) Hoxa2, Hoxd1 and Pax6 (an early marker of the diencephalon) were expressed as early as E10.5 in prosomeres (p), p2 and p3. All three proteins continue to exhibit overlapping domains of expression at the E12.5-13 (beginning of the second stage) when the primitive cell layer begins to differentiate into the internal germinal, external germinal and mantle layers. Towards the end of the second stage (E15), Pax6 expression was down-regulated whereas Hoxa2 and Hoxd1 continued to exhibit overlapping domains of expression for both protein and mRNA. Hoxd1 expression decreased significantly in the third stage of diencephalic development (E16-postnatal) such that only Hoxa2 expression persisted in the diencephalon of newborn mice. The temporal and spatial expression of these three proteins imply that coordinated waves of Hoxa2, Hoxd1 and Pax6 expression may be required to provide positional information for the specification of the diencephalon.

We have also used immunohistochemical and in situ hybridization histochemistry to demonstrate novel Hoxa2 gene expression in the pallium and subpallium of the developing telencephalon. Pax6, which is expressed in the pallium and delineates the pallium/subpallium boundary is co-localized with both Hoxa2 and Hoxd1 in the pallium. As development progresses, Hoxa2 expression within the pallium becomes more restricted to the cortical plate of the telencephalon. Analysis of E11.5 Hoxa2/- embryos exhibits in some embryos loss of the pallium/subpallium boundary, ectopic Pax6
expression within the subpallium, and the subsequent enlargement and positional shift of
the medial ganglionic eminence. Furthermore, an up-regulation of Islet1, a marker for
striatal projection neurons as well as the marker of developing oligodendrocytes, Olig2
was observed in Hoxa2 -/- mutants. Hence, in addition to the prospective role of Hoxa2
in the dorsal-ventral patterning of the telencephalon, these results implicate a role for
Hoxa2 in the specification of striatal neurons and oligodendrocytes.

Furthermore, immunohistochemistry, in situ hybridization and RT-PCR analysis
was also employed to demonstrate novel Hoxa2 expression within the developing and
postnatal murine eye. Hoxa2 is initially expressed within the surface ectoderm and
proximal portion of the optic vesicle. During embryonic development, Hoxa2 expression
continues in the developing lens and within a subset of differentiating retinal cells. In the
postnatal retina, Hoxa2 is expressed in the inner region of the inner nuclear layer as well
as in the ganglion cell layer, and gradually ceases in the lens as lens fiber cells
differentiate and lose their nuclei. Analyses with cell specific markers, revealed
expression of Hoxa2 within the ganglion and amacrine neuronal cells.
Objectives

1. Characterization of the anti-F92 antibody to determine its specificity for embryonic and recombinant Hoxd1 protein (Appendix A).

2. Using the anti-F92 (anti-Hoxd1 antibody), a comparative immunohistochemical analyses with anti-Hoxa2 antibodies was conducted on consecutive adjacent tissue sections to determine the spatial and temporal protein expression patterns of Hoxa2, Hoxd1 and Pax6 in the developing mouse CNS (E9.5, E10, E12, E14, E16, E18 NB, PND 3). Focus was given toward the diencephalon, telencephalon and the developing neural retina.

Hypothesis

Hoxa2 plays an important role in morphogenesis and cell specification in the developing murine diencephalon, telencephalon and neural retina.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Adil Nazarali for his guidance throughout the course of my thesis, as well as my committee members, Dr. X-M Li, Dr. Phyllis Paterson, Dr. Wolfgang Walz, Dr. A. Jourio and the graduate chairs, Dr. Fred Remillard and Dr. Marianna Foldvari. I would like to also thank Dr. Kathryn Todd, my external examiner for attending my defence. I am indebted to my parents for their huge financial contributions throughout the pursuit of my thesis and to Zeynep Akin for being patient and tolerant throughout my insufferable moods. I would also like to thank Sheng Yu for her technical assistance and support. Finally, I would like to thank my room-mate, Colin Wirl for all the times he cooked me dinner when I was working late.


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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>Bmps</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>CGE</td>
<td>caudal ganglionic eminence</td>
</tr>
<tr>
<td>CH</td>
<td>cortical hem</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COR</td>
<td>cornea</td>
</tr>
<tr>
<td>CP</td>
<td>choroid plexus</td>
</tr>
<tr>
<td>CSB</td>
<td>cortical striatal boundary</td>
</tr>
<tr>
<td>CX</td>
<td>cortex</td>
</tr>
<tr>
<td>d</td>
<td>diencephalon</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctade-cyl-3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>dLGE</td>
<td>dorsal lateral ganglionic eminence</td>
</tr>
<tr>
<td>DOV</td>
<td>dorsal optic vesicle</td>
</tr>
<tr>
<td>DP</td>
<td>dorsal pallium</td>
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<tr>
<td>dt</td>
<td>dorsal thalamus</td>
</tr>
<tr>
<td>DV</td>
<td>dorsal-ventral</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ep</td>
<td>epithalamus</td>
</tr>
<tr>
<td>FgFs</td>
<td>fibroblast growth factors</td>
</tr>
<tr>
<td>Fgf8</td>
<td>fibroblast growth factor 8</td>
</tr>
<tr>
<td>Gcl/GCL</td>
<td>ganglion cell layer</td>
</tr>
<tr>
<td>hb</td>
<td>hindbrain</td>
</tr>
<tr>
<td>hp</td>
<td>hypothalamus</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
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<tr>
<td>L/LE</td>
<td>lens</td>
</tr>
<tr>
<td>LFC</td>
<td>lens fiber cells</td>
</tr>
<tr>
<td>lge/LGE</td>
<td>lateral ganglionic eminence</td>
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<tr>
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<td>lateral pallium</td>
</tr>
<tr>
<td>LV</td>
<td>lens vesicle</td>
</tr>
<tr>
<td>mb</td>
<td>midbrain</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>mge/MGE</td>
<td>medial ganglionic eminence</td>
</tr>
<tr>
<td>MP</td>
<td>medial pallium</td>
</tr>
<tr>
<td>NB</td>
<td>newborn</td>
</tr>
<tr>
<td>NR</td>
<td>neural retina</td>
</tr>
<tr>
<td>ON</td>
<td>optic nerve</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OS</td>
<td>optic stalk</td>
</tr>
<tr>
<td>OV</td>
<td>optic vesicle</td>
</tr>
<tr>
<td>p(1-6)</td>
<td>prosomeres 1-6</td>
</tr>
<tr>
<td>pINL</td>
<td>presumptive inner nuclear layer</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
</tr>
<tr>
<td>POV</td>
<td>proximal optic vesicle</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SE</td>
<td>surface ectoderm</td>
</tr>
<tr>
<td>Sey</td>
<td>small eye</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>t</td>
<td>thalamus</td>
</tr>
<tr>
<td>v</td>
<td>third ventricle</td>
</tr>
<tr>
<td>vLGE</td>
<td>ventral lateral ganglionic eminence</td>
</tr>
<tr>
<td>VP</td>
<td>ventral pallium</td>
</tr>
<tr>
<td>vt</td>
<td>ventral thalamus</td>
</tr>
<tr>
<td>V/VZ</td>
<td>ventricular zone</td>
</tr>
</tbody>
</table>
1.0 GENERAL INTRODUCTION

The homeobox gene family function as transcriptional regulators through the coding of a 60 amino acid motif, the homeodomain (reviewed in Mark, 1997). They are believed to act at the top of the genetic hierarchy playing crucial roles in specifying positional information along the antero-posterior axis of the developing embryo. This was first exemplified by spontaneous mutations in Drosophila which resulted in the transformation of one body part into the likeness of another (Lewis, 1978).

Distinct families of homeobox genes are present which differ in the type of homeodomain they encode. The two groups of homeobox genes are the clustered or Hox genes and the non-clustered or divergent homeobox genes. In vertebrates, there are 39 clustered Hox genes that exhibit differing anterior domains of expression along the developing embryo (reviewed in Mark et al, 1997, Akin and Nazarali, 2004). Early on in development their expression domains vary anywhere from the posterior tail, along the spinal cord up to their anterior limit of expression in the hindbrain of the central nervous system. Hence, it has been well established that Hox genes are generally not found in the rostral regions of the brain early on in development. The divergent homeobox genes on the other hand are expressed in the rostral forebrain early on in development. Mutation analysis resulting in the loss of these rostral brain structures has demonstrated the essential role of these genes in patterning the forebrain and midbrain.

The Hox genes which we are investigating are Hoxa2 and Hoxd1 belonging to the HoxA and HoxD clusters, respectively. Amongst the Hox gene family, Hoxa2 exhibits the most anterior domain of expression along the neural tube (Krumlau, 1993) and
continues to be expressed in regions such as the spinal cord, cerebellum, lungs, liver, and palate during the later stages of development (Tan et al., 1992). However, while the expression of Hoxa2 has been documented both at the early and later stages of embryonic development, expression analysis in the developing brain in older staged embryos has not previously been reported. As for Hoxd1, little information exists regarding its domain of expression along the neural tube early on in development or at the later stages of development. Therefore the aim of this thesis was to examine for the first time the expression of the Hox genes, Hoxa2 and Hoxd1 within the developing murine forebrain, including the diencephalon, telencephalon and neural retina.
2.0 LITERATURE REVIEW

2.1 Introduction to Homeobox Genes

Evolutionarily conserved homeobox genes were initially discovered in the fruit fly Drosophila where spontaneous homeotic mutations were found to transform one segment into the likeness of another. This led to the speculation that these homeotic genes provide segmental identity and positional information along the anterior-posterior (AP) body axis of the developing Drosophila (Lewis, 1978).

Many Drosophila homeotic genes contain a characteristic 180 bp sequence referred to as the homeobox (Gehring, 1987). This homeobox encodes an evolutionarily conserved 60 amino acid DNA binding motif, the homeodomain, that forms a helix-turn-helix protein structure (Laughon and Scott, 1984; Qian et al., 1989; Kissinger et al., 1990). The homeodomain proteins act as transcriptional factors through recognition of specific regulatory sequences within gene promoters or enhancers, resulting in the activation or repression of downstream target genes (Gehring et al., 1990; reviewed in Akin and Nazarali, 2004).

2.2 Structure and Function of Homeobox Genes

Homologous homeobox genes have since been identified in various metazoa, ranging from nematodes (Costa et al., 1988), to mice (McGinnis et al., 1984) and humans (Levine et al., 1984). The homeotic genes in Drosophila are characterized by a clustered chromosomal organization. They are arranged into two clusters, the Bithorax and Antennapedia complexes collectively referred to as the homeotic complex (HOM-C) located on chromosome 3 (Lewis, 1978; Kaufman et al., 1990). In vertebrates the homeobox family is comprised of over 1000 homeodomain proteins (http://research.nhgri.nih.gov/homeodomain/) divided into two subfamilies: the clustered and the “divergent” homeobox genes (reviewed in Mark et al., 1997; Akin and Nazarali,
Thirty-nine clustered vertebrate homeobox genes, which are referred to as the \textit{Hox} genes, have been identified to date by virtue of their homology to the \textit{Drosophila} HOM-C genes. Based on their sequence similarity with the HOM-C genes and their chromosomal position, the vertebrate \textit{Hox} genes are organized into four separate clusters \textit{(HoxA, HoxB, HoxC, HoxD)}, located on chromosomes 6, 11, 15, 12 in mice, and on chromosomes 7, 17, 12, 2 in humans, respectively (Duboule and Dolle, 1989; Graham \textit{et al.}, 1989) (see Fig. 1). These clusters of \textit{HOM-C/Hox} genes display a 5' to 3' orientation along the chromosome that corresponds to their direction of transcription (Fig. 1).

The remaining homeobox genes have traditionally been referred to as "dispersed" or "divergent" since they are not organized in large chromosomal clusters, but instead are dispersed throughout the genome. Also, many "divergent" homeobox genes have additional conserved domains or motifs that may be used to classify them into smaller subfamilies, such as the \textit{Pax} family of transcription factors (Mark \textit{et al.}, 1997). Many of these genes also play a vital role in embryogenesis, especially in regards to the development of the eye.

\textbf{2.2.1 Colinearity}

\textit{Hox/HOM-C} genes display a characteristic correlation between the rostral limits of expression along the AP axis, and their position along the chromosome. This relationship is referred to as "spatial colinearity" (Duboule and Dolle, 1989; Graham \textit{et al.}, 1989). The most 3' genes in the chromosomal cluster generally have the most anterior limits of expression and are expressed earlier than their 5' neighbours along the chromosome (Izpisua-Belmonte \textit{et al.}, 1991; Dekker \textit{et al.}, 1993) (Fig.1). \textit{In-situ} hybridization illustrates the colinear manner in which \textit{Hox} genes are expressed in each tissue, suggesting an important role for these genes in AP patterning of the embryo (Duboule and Dolle, 1989; Graham \textit{et al.}, 1989; reviewed in Akin and Nazarali, 2004).
Figure 2.1 Schematic illustration of the colinear expression of the paralog cluster of *Drosophila* homeotic genes (HOM-C) and the four mammalian Hox gene clusters. Each paralog gene represented in the different mammalian clusters and its related *Drosophila* HOM-C complex is denoted by the same color box. The expression domains of the various HOM-C/Hox genes are depicted in the fly and human schematic. Abbreviations: lab, labial; pb, proboscipedia; Dfd, Deformed; Scr, Sex comb reduced; Ant, Antennapedia; Ubx, Ultrabithorax; abd-A, abdominal-A; Abd-B, Abdominal-B (taken from Mark et al., 1997 with permission from Lippincott Williams & Wilkins).
2.2.2 Paralog Groups

Sequence similarity and organization of the genes indicate that the vertebrate Hox clusters have arisen by duplication and divergence from a common ancestor predating the organization of homeobox containing genes in insects (Duboule and Dolle, 1989; Graham et al., 1989). The linear arrangement of Hox genes in the clusters have been evolutionarily maintained; subsequently Hox genes located in different clusters are related in DNA structure and organization to members of other Hox clusters as well as to the Drosophila HOM-C genes. For example, those Hox genes positioned at the most 3' end of their respective clusters (Hox1, Hoxb1, Hoxd1) in mice are highly homologous with each other, and as well they bear high sequence similarity with the most anterior HOM-C gene, labial in Drosophila (Fig. 1). Thirteen groups of these related genes, referred to as paralogs, have been identified in mouse and human Hox clusters (Scott, 1992) (Fig.1). The genes in paralog groups 1-4 are expressed in the hindbrain, while groups 5-13 are present in the spinal cord (Krumlauf et al., 1993). Paralog genes are located in the same relative position in each respective cluster, however it should be noted that not all paralog groups have a gene present in all of the clusters. It is suggested that some members of the clusters were not duplicated, or they were lost during the evolution of vertebrates (Krumlauf, 1992). Double and triple mutant analyses of Hox paralog genes in mice revealed an increase in the penetrance and severity of the phenotype displayed in comparison to a single mutation of the same genes (Gavalas et al., 1998; Manley and Capecchi, 1997). These studies reveal the functional redundancy of Hox paralog genes.
3.0 Introduction to the Forebrain

The telencephalon develops from the anterior most region of the neural plate and is the seat of higher cognitive functions such as language, memory, emotion and voluntary motor control. Various tissues such as the cerebral cortex, basal ganglia, thalamus, hypothalamus, as well as the eye are all components of the telencephalon (as reviewed in Campbell, 2003; Corbin et al., 2001).

Upon closure of the neural tube, the embryonic vertebrate brain is initially regionally divided into three vesicles, the prosencephalon (forebrain), mesencephalon (midbrain) and the rhombencephalon (hindbrain). These vesicles are then subdivided as follows: the prosencephalon is further divided into the telencephalon and diencephalon, and the rhombencephalon into the metencephalon and the mylencephalon.

The telencephalon consists of dorsal (pallial) and ventral (subpallial) domains which differ significantly morphologically and functionally. The dorsal pallium is subdivided into the medial, dorsal, lateral and ventral pallium that gives rise to the hippocampus, the neocortex, the olfactory cortex and the claustrum, respectively (Puelles et al., 1999; Puelles et al., 2000). During development of the murine telencephalon the ventral subpallium at embryonic stage 12.5 (E12.5) forms bulge like protrusions referred to as the medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminences (Fig 2). These medial, lateral and caudal ganglionic eminences give rise to the striatum, globus pallidus, and the amygdala of the basal ganglia, respectively (Deacon et al., 1994; Olsson et al., 1995).
Figure 3.1 Coronal schematic of an E12.5 mouse embryo illustrating the subdomains of the telencephalon. (a) Schematic illustrating the primary divisions of the telencephalon. (b) Division of the pallial telencephalon into further subdomains. Abbreviations: dLGE, dorsal lateral ganglionic eminence, DP: dorsal pallium, LGE: lateral ganglionic eminence, LP: lateral pallium, MGE: medial ganglionic eminence, SVZ: subventricular zone, VP: ventral pallium, VZ: ventricular zone. (taken from Campbell, 2003 with permission from Elsevier).
3.1 Lamination of the Neocortex

The adult cerebral cortex is characterized by six layers (I-VI) of neurons displaying distinct morphological and functional properties. According to birth dating analyses the cortical neurons develop from a pseudostratified epithelial layer, the dorsal ventricular zone, in a sequential manner (Takahashi et al., 1999). During mouse corticogenesis postmitotic cells migrate from the ventricular zone (VZ) at embryonic stage 11 (E11) and accumulate at the pial surface of the neocortex to form the preplate layer. The preplate contains afferent and efferent fibers as well as some early generated neurons such as the Cajal-Retzius and prospective subplate neurons. Subsequently, at E13 the migration of a second generation of postmitotic neurons from the VZ subdivides the preplate layer into a superficial marginal zone (MZ) and the deep subplate layer to form the cortical plate. This superficial MZ forms the first cortical layer containing Cajal-Retzius neurons. The Cajal-Retzius neurons secrete a protein, Reelin, which is imperative for positioning of cortical plate neurons (D'Arcangelo et al., 1995). As development progresses between E14-E18 postmitotic neurons continue to emerge from the VZ and migrate through the subplate layer toward the cortical plate forming the six layered neocortex in an “inside out” manner (layers II-VI). Hence, early-born neurons occupy the deep layers of the cortical plate whereas neurons found superficially within the neocortex are generated later in development (Bayer and Altman, 1991). The subplate then degenerates upon completion of cortical plate formation to ultimately generate the six layered neocortex.
3.2 Cell Migration

Amongst the many types of neurons present in the cortex, the glutamatergic projections neurons are important in forming synaptic connections with distant targets while the inhibitory γ-aminobutyric acid (GABA-ergic) interneurons form local synaptic connections. The most common mode of neuronal migration in the neocortex is via radial migration (Marin and Rubenstein, 2001). Postmitotic cells migrate from the VZ of the dorsal telencephalon to the cortical plate using the processes of radial glial cells as a scaffold. However, recent birthdating, genetic and fate mapping analyses has demonstrated that not all cortical neurons are generated in the VZ of the dorsal telencephalon as previously assumed (Gotz and Bolz, 1994; Parnavelas, 2000; Corbin et al., 2001; Marin and Rubenstein, 2001). The medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminences of the ventral telencephalon contribute many interneurons of the intermediate zone of the neocortex which migrate tangentially to the neocortex (De Carlos et al., 1996, Anderson et al., 1997, Tamamaki et al., 1997). While radially migrating neurons comprise the vast majority of glutamatergic projection neurons, the tangentially migrating interneurons make up the GABA-ergic cells in the neocortex (Marin and Rubenstein, 2001; Corbin et al., 2001). The tangential migration of cells from the MGE has been found to give rise to the majority of interneurons in the cortex and the hippocampus (Wichterle et al., 1999; Sussel et al., 1999; Anderson et al., 2001). The CGE also populates the neocortex and the hippocampus (Nery et al., 2002) while the LGE has been shown to generate interneurons that migrate to the olfactory bulbs (Wichterle et al., 1999). Genetic analysis also suggests that the LGE may also contribute to the cortical interneuron population (Anderson et al., 2001). However, it still remains
unclear whether the ganglionic eminences are the only contributors to the cortical interneuron cell population or if the neocortex also generates some interneurons (Gotz and Bolz, 1994).

3.3 Models of Cortical Patterning

Currently, the two established models regarding the regionalization of the telencephalon into discrete functional subunits are the protocortex model (O'Leary, 1989) and the protomap model (Rakic, 1988). According to the protocortex model of cortical patterning the cortical primordium is initially a homogenous sheet of epithelial tissue that becomes patterned later in development due to extrinsic cues from the cortical innervation of axons from the thalamus. The functional and structural plasticity of the cortex has been demonstrated by studies in the ferret in which retinal axons misdirected to auditory centers led to changes in the organization of the auditory centers to that of retinal centers (O'Leary, 1989; Sharma et al., 2000). However, recently numerous studies advocate support for the protomap model and suggest that thalamocortical innervation may be required later in development for arealization of the telencephalon (Pallas, 2001).

The protomap model views intrinsic regional differences to be due to molecular determinants in the cortical primordium which play crucial roles in neocortical regionalization. Hence, early on in development as neurons are being generated and migrating they are programmed by these molecular determinants to have regional identity and proliferative capacity. Support for this model is evident from mutation analysis in mice lacking the transcription factor Gbx2 which display severe thalamic defects such
that cortical innervation is disrupted (Miyashita-Lin et al., 1999). However, this loss of cortical innervation by thalamic axons did not perturb the laminar organization nor the regionalization of the cortex as revealed by gene expression analysis. Similarly, the disruption of thalamocortical axons in Mash1 homozygous embryos (Tuttle et al., 1999) did not have any effects on the graded expression of various genes within the cortical plate (Nakagawa et al., 1999). Furthermore, a topographic shift of thalamocortical axons in Dlx1/2 mutant mice also did not affect the neocortex regionalization (Garel et al., 2002). Hence, these studies provide evidence of intrinsic factors governing regionalization of the telencephalon.

### 3.4 Signaling Molecules and Telencephalon Regionalization

Various signaling centers located in the telencephalon or within its vicinity provide positional information and establish cortical domains. These diffusible cues emanate from these signaling centers and induce various homeodomain and basic helix-loop-helix (bHLH) transcription factors to generate specific cellular identities. Anterior-posterior (AP) patterning is influenced by the anterior neural ridge, while dorsal-ventral (DV) patterning is influenced by such signaling centers as the cortical hem, the roof plate, and the prechordal plate. The murine anterior neural ridge is located at the rostral most end of the neural tube between the non-neural ectoderm and the neural plate (Couly and Le Douarin, 1988; Eagleson et al., 1995) (Fig. 3a,b). The anterior neural ridge secretes an early signaling molecule fibroblast growth factor 8 (Fgf8) that has been found to mediate the expression of the transcription factor Foxg1 (formally referred to as BF1) which is critical for regionalization and growth of the telencephalon (Shimamura and
Rubenstein, 1997). Foxg1 is pivotal in telencephalic induction as loss of expression results in cerebral hypoplasia (Xuan et al., 1995). Furthermore, the telencephalon of mice (Meyers et al., 1998) and zebrafish (Shanmugalingam et al., 2000) is significantly smaller in Fgf8−/− mutants.

The second signaling center, the cortical hem, lies between the hippocampus and the choroid plexus providing dorsal signaling via the expression of numerous Wnt and Bmp genes (Grove et al., 1998; Furuta et al., 1997 (Fig. 3a,b,c). Wnt factors have been found to be involved in the specification of the hippocampus which is evident in mutation analysis of Wnt3a in mice which demonstrate a deletion of the hippocampus (Lee et al., 2000). The dorsal roof plate also produces bone morphogenic proteins (Bmps) in the dorsal medial telencephalon which are involved in the morphogenesis of the choroid plexus. Mutation analysis of the Bmp receptor 1a in the telencephalon resulted in a marked reduction of cells in the choroid plexus (Hebert et al., 2002), whereas the choroid plexus epithelium expands into the forebrain with constitutively activated Bmp receptor 1a (Panchision et al., 2001).

Ventral signaling centers such as the prechordal plate express Sonic hedgehog (Shh) which is vital in ventral patterning of the telencephalon and repression of dorsal telencephalic fates (Erickson et al., 1995; Shimamura et al., 1995) (Fig. 3a,c). Lack of Shh in mice has demonstrated a loss of the MGE, LGE and CGE of the ventral telencephalon, expansion of the dorsal specific marker Emx1, as well as cyclopia and holoprosencephaly (Chiang et al., 1996). In contrast, in vitro (Shimamura and
Figure 3.2 Schematic illustration of the signaling centres in the rat telencephalon. (a) External location of the signaling centres. (b) Internal location of signaling centres. The prechordal mesoderm (Shh) (orange), anterior neural ridge (Fgf8) (red), the roof plate (Bmp) and the cortical hem (Wnt) (brown outlined by white dashes). Abbreviations: CX: cortex, lge: lateral ganglionic eminence, mge: medial ganglionic eminence. (taken from Monuki and Walsh, 2001 with Nature Publishing Group's permission [http://www.nature.com/]).
Fig. 3.3 Schematic illustration of an E12.5 coronal section of the mouse telencephalon. The choroid plexus (CP), and the cortical hem (CH) secrete various BMPs, and Wnts. The medial ganglionic eminence (MGE) secretes the signalling molecule Sonic hedgehog (Shh). CSB is the cortical striatal boundary (taken from Zaki et al., 2003 with permission from author).
Rubenstein, 1997) and in vivo (Barth and Wilson, 1995; Corbin et al., 2000) analysis of ectopic Shh expression has induced ventral telencephalic specific markers in the dorsal telencephalon. Studies conducted by Rallu et al., (2002) indicate that despite the abolishment of the Shh gene in mice, the expression of some ventral markers could still be detected albeit at reduced levels. This dorso-ventral telencephalic patterning in the absence of Shh suggested that in addition to Shh dependent pathways, Shh independent pathways may also be involved in telencephalic patterning which at this time remain unknown (Rallu et al., 2002).

3.5 Transcription Factors and Regionalization of the Cortex

The aforementioned signaling centers act on various transcription factors involved in cortical regionalization. Two such transcription factors which have been implicated in arealization of the cortex are Emx, the vertebrate homolog of Drosophila empty spiracles, and Pax6 homeobox genes (O’Leary et al., 1994). During embryonic development, the homeobox gene Pax6 displays graded mRNA and protein expression along the VZ of the cortex with higher levels of expression evident in the rostral lateral regions and lower levels in the medio-caudal cortex (Bishop et al., 2000; Walther and Gruss, 1991). Conversely, the expression of the homeodomain protein Emx2 is complementary to that of Pax6, with expression predominantly higher at the caudal-medial region of the cortex and lower in the rostral-lateral cortex (Gulisane et al., 1996; Mallamaci et al., 1998). Embryos displaying a loss of Emx2 demonstrated an expansion of the anterior-lateral cortex (motor and somatosensory) and the subsequent reduction of the caudal-medial regions of the cortex (visual) (Mallamaci et al., 2000; Bishop et al., 2000; Bishop et al.,
2002). However, loss of Pax6 had the opposite effect, the caudal-medial cortical domains were expanded while the anterior-lateral cortical domains were reduced (Bishop et al., 2000; Bishop et al., 2002). Although the cortical defects of Pax6 and Emx2 mutant mice cannot be analyzed postnatally due to the lethality of these mice, the expansion and reduction of various gene expression domains in Pax6 and Emx2 knockout mice clearly demonstrate the pivotal role of these genes in cross-regulation during the regionalization of the cortex.

3.6 Dorsal-Ventral Patterning of the Telencephalon

The mammalian telencephalon is divided into dorsal pallial and ventral subpallial domains. The boundary spanning these domains is referred to as the pallial/subpallial border or pallio-subpallial boundary (Fig. 3.1) and various genes expressed within the progenitor cells of the developing telencephalon display either dorsal (pallial) or ventral (subpallial) expression. As mentioned previously, researchers have subdivided the pallium into medial, dorsal, lateral and ventral domains (Fig. 3.1) (Puelles et al., 1999; Puelles et al., 2000). Although Pax6 displays primarily dorsal expression in the telencephalon, it is also expressed at a very low level in the dorsal LGE (Stoykova et al., 2000). Hence, the expression of various genes in this area led to the subdivision of the LGE into dorsal and ventral domains (Yun et al., 2001). Hence the pallial/subpallial boundary is the region situated between the ventral pallium (VP) and the dorsal LGE of the subpallium (Fig. 3.1).

Several homeobox genes such as Pax6 (Walther and Gruss, 1991), Emx1, Emx2 (Simeone et al., 1992), as well as the basic helix-loop-helix genes Neurogenin 1 (Ngn1)
and Neurogenin 2 (Ngn2) (Sommer et al., 1996) are dorsal telencephalic markers. Pax6, Emx1 and Emx2 demonstrate complementary graded expression throughout the pallium with Pax6 displaying high levels of expression in the VP spanning the pallial/subpallial boundary. Conversely, Emx1 and Emx2 display high levels of expression in the medial pallium (MP). Ngn1 also displays graded expression in the pallium with higher levels in the VP while Ngn2 displays uniform expression throughout the pallium (Fode et al., 2000).

Genetic regulators which have been implicated in ventral telencephalic specification include the homeobox genes Gsh1 and Gsh2 (Valerius et al., 1995), Dlx1, Dlx2, (Simeone et al., 1992), Nkx2.1 (Shimamura et al, 1995), and the basic helix-loop-helix gene Mash1 (Guillemot and Joyner, 1993). The subpallial markers Gsh2, Dlx2 and Mash1 are expressed in progenitor cells of the MGE and the LGE as well as the dorsal LGE. Nkx2.1 expression however is restricted to the progenitors of the MGE.

The importance of these genes in either dorsal or ventral specification of the telencephalon has been exemplified by various mutation analyses. The expression patterns of the Ngn genes and Mash1 are complementary in the pallium and subpallium, respectively (Gradwohl et al., 1996; Ma et al., 1997). Absence of Ngn2 in embryos resulted in the ectopic expansion of the ventral telencephalic marker Mash1 in the dorsal pallium, which is intensified in homozygous Ngn1/Ngn2 double mutants (Fode et al., 2000). Hence, it appears that the dorsoventral identity in the telencephalon is maintained by the repression of Mash1 by the Ngns (Fode et al., 2000). Genetic interactions between Pax6 and Gsh2 have also been found to be involved in the regionalization of the telencephalon. Pax6 mutants display numerous cortical defects including thinning of the
cortex, and loss of pallial/subpallial boundary with subsequent expression of subpallial markers in the pallium (Stoykova et al., 1996; Stoykova et al., 1997). Similarly, the lack of Gsh2 in murine embryos resulted in the loss of the pallial/subpallial boundary as demonstrated by the ventral expansion of cortical markers (Pax6 and Ngn2) in the LGE (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001; Corbin et al., 2003) and subsequent respecification of the dorsal LGE into the VP (Yun et al., 2001). In addition, further analysis of Pax6 mutants also revealed that the VP was respecified into a dorsal LGE like structure (Yun et al., 2001). However, in Pax6/Gsh2 double homozygous mice the molecular markers of the striatum (LGE) are restored and subsequently the striatum is much larger in size than that of the Gsh2-/- mice (Toresson et al., 2000). In regards to the cortex, Pax6/Gsh2 double mutants continued to ectopically express Mash1, Dlx, Ngn1 and Ngn2, and as previously demonstrated in Sey/Sey mutant mice there was an increase in the number of progenitor cells in the cortex resulting in an enlarged ventricular/subventricular zone in the cortex. Despite this the severity of abnormalities in the cortical plate and intermediate zone of Sey/Sey mice were greatly reduced in Pax6/Gsh2 double knock out mice (Toresson et al., 2000). Hence, ectopic Pax6 expression in Gsh2-/- may result in abnormal development of the striatum while ectopic Gsh2 in Pax6 Sey/Sey mice may suppress normal cortical development. Therefore Pax6 and Gsh2 genetically cross repress each other to establish the pallial/subpallial border (Corbin et al., 2000; Torresson et al., 2000; Yun et al., 2001). Furthermore, Pax6 may also regulate Ngn1, Ngn2, Mash1 and Dlx1 in the telencephalon (Toresson et al., 2000). Pax6, Gsh2 and Nkx2.1 are imperative in maintaining the cortical, LGE and MGE subdivisions of the telencephalon respectively. The globus pallidus in Nkx2.1 mutants is
respecified into the dorsal LGE derivative of the striatum (Sussel et al., 1999), while disruption of *Pax6* led to the expansion of the MGE expressed *Nkx2.1* into the LGE (Stoykova et al., 2000). Previous analysis also revealed a ventral expansion of *Pax6* into the LGE of *Nkx2.1* -/- mice (E10.5-E12.5) (Sussel et al., 1999). Hence, it was suggested that *Pax6* and *Nkx2.1* also cross repress each other in the maintenance of the LGE and MGE, respectively. However, mutation analysis of younger staged embryos (E9.5) revealed that *Nkx2.1* expression is not expanded dorsally in *Pax6* -/- mice and conversely, *Pax6* is not ventrally expressed in *Nkx2.1* mice (Corbin et al., 2003). So while *Pax6* and *Nkx2.1* may co-repress each other at later stages (E11-E13) (Stoykova et al., 2000), this is not the case at earlier stages (E9.5) of telencephalic development (Corbin et al., 2003).

Complementary expression of *Gsh2* and *Nkx2.1* is also observed in the MGE and LGE, respectively. Recent studies conducted in homozygous *Nkx2.1* and *Gsh2* double knock out mice revealed that unlike *Pax6* -/-, *Gsh2* -/- double mutants; *Nkx2.1* -/-, *Gsh2* -/- double homozygous mutants exhibit an increased severity in the phenotype as compared to their respective single mutants (Corbin et al., 2003). A similar dorsal expansion of *Ngn2* and *Pax6* as well as the ventral expansion of LGE specific markers (*Crbp1, Ebf1*) was observed compared to that which was reported with single *Pax6* and *Gsh2* mutants, respectively. These molecular modifications in expression were also accompanied by a significant reduction in the size of the ventral telencephalon (Corbin et al., 2003). Hence, unlike *Pax6* and *Gsh2*, *Gsh2* and *Nkx2.1* cooperatively interact in ventral telencephalic patterning (Corbin et al., 2003).

Overall, mutation analysis has revealed that a multitude of homeodomain containing transcription factors as well as bHLH transcription factors are imperative in
the regionalization of the developing telencephalon. The interplay of these genes is essential for the dorsoventral patterning of the developing murine telencephalon.

3.7 Prosomeric Model of Forebrain Development

Similar to the transient segmentation of the hindbrain into rhombomeric units (Lumsden and Keynes, 1989), the vertebrate forebrain has also been proposed to be subdivided into six prosomeric units (Puelles and Rubenstein, 1993; Bulfone et al., 1993). According to the prosomeric model the telencephalon is subdivided into a longitudinal domain which extends as far rostrally as the optic stalks but does not divide the telencephalic vesicles. This longitudinal axis is then divided transversely into domains termed prosomeres. Morphological constrictions along the neural tube as well as spatial and temporal expression analysis of various homeodomain regulatory genes in the mouse and chick forebrain (Bulfone et al., 1993) has divided the forebrain into the diencephalon and the secondary prosencephalon. Also analogous to the rhombomeres, cell migration across these prosomeric boundaries is limited (Figdor and Stern, 1993; Neyt et al., 1997).

Prosomeres 1-3 (p1-p3) comprise the diencephalon, while the secondary prosencephalon which consists of the ventral hypothalamus and dorsal telencephalic vesicles is composed of prosomeres 4-6 (p4-p6). More recently, the number of prosomeres originally proposed and the nomenclature of the remaining prosomeres have been revised (Puelles and Rubenstein, 2003). Although p1-p3 of the diencephalon remain, the p4-p6 have been abolished due to discrepancies in gene expression patterns and mutation analysis suggesting these prosomeres act as a single unit. Hence, the
secondary prosencephalon is now divided into the telencephalon and hypothalamus. In the caudal diencephalon, p1 continues to contain the pretectum, whereas p2 has been modified to contain the thalamus (previously dorsal thalamus) and epithalamus. p3 consists of the prethalamus (previously ventral thalamus) and recent expression analysis has included the eminentia thalami (originally part of p4) in this prosomere (Puelles and Rubenstein, 2003). Despite these recent changes to the prosomeric model, throughout the course of my thesis the literature cited remains in the context of the original prosomeric model of the telencephalon.

3.8 Diencephalon

Unlike the cortex the diencephalon is not a laminated structure but rather is organized similarly to the midbrain and the hindbrain in that it contains discrete functional nuclei. The diencephalon functions to relay information from caudal regions such as the midbrain, hindbrain, cerebellum and spinal cord to the cortex (Jones, 1985). In addition, the reciprocal connections between the cortex and the thalamus are important for the relay and processing of sensory, auditory, visual and motor information (O’Leary et al., 1994).

During development the primary prosencephalon is divided into the secondary prosencephalon (the telencephalon) and the diencephalon. Diencephalon development is often described in the context of the prosomeric model of telencephalic development. According to the prosomeric model, the nested expression of various genes such as Pax6, as well as the morphological constrictions in the tissue have divided the diencephalon into three distinct prosomeres (p1-p3) (Puelles and Rubenstein, 1993; Bulfone et al.,
p1 gives rise to the pretectum, while p2 and p3 forms the dorsal thalamus and ventral thalamus, respectively.

The pretectum which connects the forebrain and midbrain vesicles is characterized by a group of axon bundles in its dorsal portion, the posterior commissure. The posterior commissure is an important landmark as it defines the forebrain-midbrain boundary (Mastick et al., 1997) and also coincides with the caudal limit of expression of an important candidate gene in prosencephalon patterning, \textit{Pax6} (Stoykova and Gruss, 1994; Stoykova et al., 1996; Mastick and Easter, 1996; Mastick et al., 1997). The dorsal and ventral thalamus of the diencephalon is divided by the zona limitans interthalamica. The zona limitans interthalamica is a strip of cells which lies between the dorsal and ventral thalamus (Shimamura et al., 1995).

3.9 \textit{Pax6} and the Diencephalon

\textit{Pax6} is an early marker of the diencephalon with mRNA expression first being detected at E8.0 in the presumptive forebrain (Walther and Gruss, 1991). At E9.0-E9.5 \textit{Pax6} is initially intensely expressed throughout the presumptive prosomeres of the diencephalon (Grindley et al., 1997; Mastick et al., 1997). However as the postulated prosomeres of the diencephalon develop at E11.5, the levels of \textit{Pax6} expression change with strong expression being detected in p1 and p3, with weaker \textit{Pax6} levels of expression in p2 (Grindley et al., 1997; Mastick et al., 1997). There are variations in the level of expression within these prosomeric domains with high levels of \textit{Pax6} expression being evident in p1 in the region spanning the diencephalic/mesencephalic border, as well as near the epiphysis border in p2, and in the zona incerta in p3. This differential \textit{Pax6}
expression suggests that Pax6 may be involved in maintaining these prosomeric boundaries (Grindley et al., 1997).

The Small eye (Sey) mutation is the original naturally occurring point mutation of Pax6 which results in a dysfunctional protein production (Hill et al., 1991). The Pax6 Sey-INeu allele on the other hand is an insertional mutation present within the transactivation domain resulting in the truncation of the protein product (Grindley et al., 1997). Homozygous Pax6 Sey-INeu display abnormalities in the formation of the hemispheric sulcus situated between the telencephalon and diencephalon (Grindley et al., 1997). Similarly, in Pax6 say/seymice the hemispheric sulcus was also less prominent (Mastick et al., 1997) Furthermore, analysis of various expression markers in Pax6 Sey-INeu indicated that the regionalization of the diencephalon was disrupted with mutants displaying a blurring of the p1 and p2 boundary as well as the p2 and p3 boundary, the dorsal expansion of the ventral p2 and p3, the expansion of the zona limitans and a smaller posterior commissure tract at the diencephalon/mesencephalon border (Grindley et al., 1997). However, other studies in Pax6 say/seymutant mice revealed that although anatomically the diencephalon was abnormal (Stoykova et al., 1996) gene expression and morphological analysis revealed that the prosomeres of the diencephalon were formed somewhat normally (Stoykova et al., 1996; Mastick et al., 1997; Warren and Price, 1997; Kawano et al., 1999; Pratt et al., 2000). More recent studies of Pax6 say/seymice demonstrated a dorsal expansion of ventral genes suggesting that the differentiation of the dorsal thalamus is effected in Pax6 say/seymutants (Pratt et al., 2000). In addition, researchers have also reported the enlargement of the third ventricle (Warren and Price,
1997; Mastick et al., 1997) and a reduction in the number of cells in the diencephalon (Warren and Price, 1997).

Studies have indicated that Pax6 also plays a role in defining the di-mesencephalic boundary. The caudal limit of Pax6 expression is found at the posterior commissure which marks the forebrain-midbrain boundary (Stoykova and Gruss, 1994; Stoykova et al., 1996; Mastick et al., 1997). Mutational analysis of mice lacking Pax6 demonstrate a loss of the pl/mesencephalic boundary (Mastick et al., 1997; Warren and Price, 1997) and the subsequent fate change of the diencephalon to the mesencephalon which is exemplified by the rostral expansion of the mesencephalic gene marker (Dbx) and reduction of caudal p1 (pretectum) markers (Pax6, Lim1, Gsh1) (Mastick et al., 1997). Similarly, loss of Pax2/Pax5 expression in the mesencephalon resulted in the caudal expansion of Pax6 expression and the lack of mesencephalon formation (Schwartz et al., 1999). Misexpression of the mesencephalic genes En1 and Pax2 in the diencephalon also changed the fate of the diencephalon into the mesencephalon (Araki and Nakamura, 1999; Okafuji et al., 1999), while Pax6 overexpression in the mesencephalon resulted in the caudal shift of the di-mesencephalic boundary. Hence, these studies demonstrate that repressive interactions between Pax6, En1 and Pax2 are crucial for di-mesencephalic boundary formation (Matsunaga et al., 2000).

3.10 Pax6 and Thalamocortical Development

In addition to the crucial role of Pax6 in telencephalic, and diencephalic morphogenesis (Walter and Gruss, 1991; Stoykova, et al., 1996; Stoykova, et al., 1997; Grindley et al., 1997), Pax6 has also been found to be involved in axonal pathfinding.
The thalamocortical axon projection, which is one of the major tracts in the forebrain is generated in the dorsal thalamus (Angevine, 1970). These axonal fibers originate in the dorsal thalamus, extend into the ventral thalamus, and enroute to the hypothalamus (prior to entry) suddenly exit the diencephalon and proceed dorsolaterally through the ventral telencephalon to the neocortex. Conversely, the corticothalamic axons originate in the neocortex and take the opposite path to their target, the dorsal thalamus. These thalamocortical and corticothalamic projections converge in the ventral telencephalon to form the internal capsule enroute to the cortex and thalamus, respectively (Miller et al., 1993; De Carlos and O’Leary, 1992). Although Pax6 mutant mice (Pratt et al., 2000; Hevner et al., 2002) and rats (Kawano et al., 1999) develop the dorsal thalamus, studies demonstrate that they fail to form a thalamocortical tract that projects into the cortex. In addition to the loss of thalamocortical projections to the cortex, corticothalamic (corticofugal) projections are also disrupted in Pax6-/- mice (Hevner et al., 2002; Jones et al., 2002). Similarly, thalamocortical and corticofugal projection defects have also been demonstrated in mutant mice lacking transcription factors such as Mash1 (Tuttle et al., 1999), Gbx2 (Hevner et al., 2002) and Dlx1/2 double mutants which also display a loss in the number of thalamic projections to the cortex (Garel et al., 2002). Thalamocortical and corticofugal projections enroute to the cortex and thalamus respectively, transverse the pallial/subpallial (Figure 2) and diencephalic-telencephalic boundaries which bear distinct molecular properties (Puelles et al., 2000). The expression of multiple transcription factors such as Pax6, Emx2, Dlx2 and Mash1 in the pallial/subpallial boundary (Puelles et al. 2000) suggests these genes may modulate
molecular patterning and play a role in axonal pathfinding (Jones et al., 2002, Lopez-Bendito and Molnar, 2003). The aforementioned defects in axonal pathfinding exemplify the importance of normal expression patterns of these genes within the pallial/subpallial boundary as well as within the diencephalic-telencephalic boundary through which axonal projections navigate (Lopez-Bendito and Molnar, 2003).

3.11 Axonal Pathfinding

Although the exact modes governing axonal pathfinding are unknown at this time, several mechanisms have been proposed. Studies have indicated that chemoattractants (Braisted et al., 2000; Braisted et al., 1999) and chemorepellents (Braisted et al., 1999) may act as cues for thalamocortical pathfinding. Co-culture studies have indicated that failure of thalamocortical axonal entry in the hypothalamus is due to the release of chemorepellent activities from the hypothalamus (Braisted et al., 1999). Similarly, in vitro analysis demonstrated that the release of the diffusible chemoattractant Netrin-1 from the ventral telencephalon influenced axonal growth (Braisted et al., 1999; Braisted et al., 2000). In addition, the disorganization and thinning of the thalamocortical projections in the ventral telencephalon of Netrin-1 -/- mice, as well as, the significant reduction in the number of projections reaching the cortex, further demonstrates that Netrin-1 plays a role in thalamocortical pathfinding. However, thalamocortical projections continue to target the neocortex in Netrin-1 -/- mice (albeit at lower numbers) which is indicative that other guidance cues play a role in axonal pathfinding (Braisted et al., 2000).

Studies have also suggested that correct thalamocortical pathfinding is dependent on transient thalamic efferents generated in the medial ventral telencephalon, whose
axons act as a scaffold for guiding axons from the dorsal thalamus to the ventral telencephalon (Metin and Godement, 1996; Tuttle et al., 1999). Mash1 knock out mice lack the ventral telencephalon and hence the thalamocortical axonal projections in these mice do not proceed beyond the diencephalon/telencephalon boundary, and do not innervate the ventral telencephalon (Tuttle et al., 1999). These results suggest that Mash1 may be required for the projection of this transient axonal scaffold. Analysis of Pax6-/- embryos revealed that this transient ventral telencephalic tract is absent although Mash1 mRNA and protein expression is not altered (Pratt et al., 2002). However, as previously mentioned, the dorsal thalamus in Pax6-/- mice exhibits abnormal differentiation (Pratt et al., 2000), and proliferation (Warren and Price, 1997). As well, recent Pax6 chimera studies also indicate that Pax6 is required for dorsal thalamus development (Pratt et al., 2002). Hence, it has been suggested that the loss of thalamocortical formation in Pax6-/- mice may be due to the inability of the dorsal thalamus to receive transient ventral telencephalic afferents (Pratt et al., 2002).

According to the "handshake" model of axonal pathfinding, the convergence of thalamocortical and the reciprocal corticofugal axons in the internal capsule provides the thalamocortical axons with a preplate and subplate scaffold to guide them to the cortex. Conversely, the corticofugal axons use the scaffold provided by the thalamocortical axons to target the dorsal thalamus (Molnar and Blakemore, 1995; Molnar et al., 1998). Although Pax6 mutant mice (Pratt et al., 2000) and rats (Kawano et al., 1999) develop the dorsal thalamus, studies demonstrate that they fail to form a thalamocortical tract that projects into the cortex. In addition to the loss of thalamocortical projections to the cortex, further studies conducted demonstrate that corticothalamic projections are also
disrupted in Pax6−/− mice (Hevner et al., 2002; Jones et al., 2002). Hence, the corticofugal and thalamocortical pathway are not independent of each other and upon reaching the ventral telencephalon, signaling via reciprocal axons may direct them to their subsequent targets (Hevner et al., 2002). Furthermore, it has also been suggested that evaluation of corticofugal defects in Mash1 mutant mice may provide support for this “handshake model” of axonal pathfinding (Hevner et al., 2002).

4.0 Introduction of the Eye

The vertebrate eye is an outgrowth of the central nervous system that has been well characterized and studied. The eye is easily accessible making it an ideal model system to study the intricacies of cell fate determination and cellular patterning within the central nervous system (Cepko et al., 1996). The mammalian eye originates from coordinated interactions between distinct sources of primordial tissue. These sources include the wall of the diencephalon, the surrounding mesenchyme and the overlying surface ectoderm. Eye development initially begins at embryonic day E8.5 with the bilateral evagination of the diencephalic wall to form the optic vesicle. Subsequent contact between the optic vesicle and the overlying surface ectoderm at E9.0 is essential for the thickening of the surface ectoderm in the formation of the lens placode. The invagination of this lens placode forms the lens pit, which subsequently results in the invagination of the optic vesicle to develop a bilayered optic cup at E10.0. At E10.5 the lens pit is completely invaginated forming the optic vesicle, which becomes separated from the overlying ectoderm. The inner layer of the optic cup develops into the neural retina, while the outer layer will form the retinal pigmented epithelial layer. The
proximal portion of the optic vesicle gives rise to the optic stalk (reviewed in Graw, 1996; Ashery-Padan and Gruss, 2001) (Fig. 4.1).

Interactions between these tissues of distinct embryological origin are crucial for development of the vertebrate eye. Failure of the optic vesicle and the overlying ectoderm to juxtapose has been found to impede lens specification (Li et al., 1994; Porter et al., 1997; Mathers et al., 1997; Nguyen and Arnheiter, 2000). Consequently, formation of the optic cup is dependent on the invagination of the lens placode. An ablation in lens placode development further perturbs optic cup formation resulting in rudimentary development of the neural retina and pigmented epithelial layer (Hyer et al., 1998; Ashery-Padan et al., 2000). Hence, it has been suggested that extrinsic and intrinsic factors within these tissues are involved in eye development.

4.1 Specification of the Neural Retina and the Pigmented Epithelial Layer

The neuroepithelium of the optic vesicle is initially bipotential, consequently the region capable of generating the distal inner neural retina and the proximal outer pigmented epithelial layer can be reversed (Pittack et al., 1997; Desire et al., 1998). Several extrinsic factors emanating from the surface ectoderm are influential in regulating intrinsic factors (transcription factors) in the optic vesicle that ultimately specify the development of these two layers. One such family of extrinsic factors imperative in neural retina specification is the fibroblast growth factors (Fgfs) (Pittack
Figure 4.1 Mouse vertebrate eye development. The optic vesicle is an outgrowth of the diencephalon at E8.5 that contacts the surface ectoderm at E9.0. Signals from the optic vesicle induce the formation of the optic placode and subsequent invagination at E10. By E11 the invagination of the lens placode has led to the development of the lens vesicle. Consequently, this invagination also leads to the formation of the optic cup which is comprised of the inner neural retina and the outer retinal pigmented epithelial layer. By E15 the lens vesicle has differentiated into the lens, while differentiation of the neural retina and pigmented epithelial layer continues. Abbreviations: COR, cornea; LE, lens epithelium, LFC, lens fiber cells; LP, lens placode, LV, lens vesicle; NR, neural retina; ON, optic nerve; OS, optic stalk; OV, optic vesicle; RPE, retinal pigmented epithelium; SE, surface ectoderm (taken from Ashery-Padan and Gruss, 2001 with permission from Elsevier).
et al., 1997; Desire et al., 1998). Several studies indicate that inhibition of FGF signalling via the use of neutralizing antibodies or antisense oligonucleotides inhibited neural retina development (Pitack et al., 1997; Desire et al., 1998). However, other studies indicate that removal of the surface ectoderm resulted in the reversal in orientation of these layers with respect to the lens. Replacing the surface ectoderm with FGF's alone was found to restore the development of the neural retina and prevent the distal retinal pigmented epithelium (RPE) formation (Nguyen and Arnheiter, 2000).

One transcription factor speculated to have a role in partitioning the optic vesicle into the neural retina and the RPE, through which FGF’s act is Mitf. In mice, the basic-helix-loop-helix (bHLH) transcription factor gene Mitf is initially expressed throughout the dorsal optic vesicle and later during development becomes restricted to the presumptive RPE (Nguyen et al., 1997; Nguyen and Arnheiter, 2000). Studies conducted with FGF coated beads placed near the presumptive RPE in retinal explants prevented the formation of RPE and expression of RPE specific genes such as Mitf. Instead this region adopted characteristics of the neural retina with the expression of neural retina specific genes (Nguyen and Arnheiter, 2000). Furthermore, Mitf homozygous mutant mice also exhibit transformation of the presumptive RPE into a neural retina expressing neural retinal specific genes, indicating the importance of Mitf for RPE specification (Nguyen and Arnheiter, 2000). Consequently, these results suggest that the neuroepithelium expresses both neural retinal and RPE specific genes and upon contact of the optic vesicle with the overlying surface ectoderm, extrinsic factors (FGF’s) released from the surface ectoderm downregulate the pigmented epithelial specific gene Mitf in the neural retina. Hence, the region not expressing Mitf develops into the neural retina while regions where
Miff expression is not downregulated develop into the RPE (Nguyen and Arnheiter, 2000).

Moreover, explant studies conducted in the chick have shown that the extraocular mesenchyme surrounding the optic vesicle is also involved in the specification of the RPE layer (Fuhrmann et al., 2000). The extraocular mesenchyme activates Miff, the RPE specific gene, and subsequently confines the neural retinal specific genes to the neural retina. Hence, the overlying surface ectoderm and the extraocular mesenchyme have opposing functions in patterning the optic vesicle into the neural retina and the pigmented epithelial domains.

4.1.1 The Neural Retina

The retina is an ideal model system to study the molecular mechanisms regulating cellular differentiation in the vertebrate central nervous system (CNS) (Cepko et al., 1996). It is attributed to the fact that the retina is a laminated structure that is easily accessible, has a simple structure containing a limited number of cell types, and is autonomous of the CNS. Retinal cellular differentiation results in the formation of this laminar structure from a pseudostratified neuroepithelium (Fig.4.2). The mature retina consists of three layers of neuronal and glial cell types: the outer nuclear layer contains the rod and cone photoreceptor cells; the inner nuclear layer contains bipolar, horizontal, amacrine and Müller glia cells; and the ganglion cell layer contains ganglion cells and displaced amacrine cells (Sidman, 1961; Young, 1985). During retinogenesis these
Figure 4.2 Schematic representation of the vertebrate retinal cellular arrangement prior to and during differentiation of the ganglion cells. (a) Prior to neuron development, uncommitted optic cup cells are located throughout the optic cup. This is represented by the white and green dots in the figure. During mitosis, the cells move to the scleral side of the optic cup as illustrated by the blue cells. (b) Proliferation continues at the scleral side of the optic cup as neurogenesis occurs. However, at this time postmitotic cells begin to migrate (pink cells) and mature ganglion cells accumulate in the ganglion cell layer (taken from Vetter and Brown, 2001 with permission from author).
Various cell types do not differentiate from distinct separate cell lineages but rather differentiate in a conserved sequential manner from a dividing neuroblast layer of multipotent progenitor cells (Turner and Cepko, 1987). Birth dating analysis has demonstrated that although there is some overlap in retinal birth order, ganglion cell differentiation invariably is the first to occur followed by horizontal, cone photoreceptors, amacrine, rod photoreceptors, bipolar and Müller glia cells (Cepko et al., 1996). Interestingly, this order of histogenesis is largely conserved in the other vertebrates such as the cat (Zimmerman et al., 1988), chick (Prada et al., 1991), and fish (Sharma and Ungar, 1980). However, retinogenesis is not complete at birth but rather continues postnatally to develop rod photoreceptors, bipolar, Müller glia cells and a small number of ganglion and amacrine cells (Sidman, 1961; Young, 1985) (Fig.4.3).

4.1.2 Retinal Cell Fate Determination

The mechanisms governing this conserved histogenesis and cellular diversity are largely unknown at this time, although it is speculated that extrinsic environmental factors as well as intrinsic factors play a dual role. To explore the role of environmental factors, rat co-aggregate experiments have been performed in which E16 retinal progenitor cells were labeled and co-cultured with postnatal day 0 (PND0) cells (Belliveau and Cepko, 1999). The environment from the PND0 cells substantially inhibited the number of amacrine cells produced by the E16 progenitor cells. To determine whether the inhibitory signal was emitted by the amacrine cells in the PND0 cells, further analysis was conducted co-culturing purified PND0 amacrine cells with E16 retinal cells. Once again amacrine cell genesis was inhibited in these E16 cells.
Figure 4.3 Illustration of the over-lapping birth-dates of the various retinal cell types during murine neurogenesis. Ganglion cells are the first to initiate differentiation, followed by horizontal, cone photoreceptors, amacrine, rod photoreceptors, bipolar and lastly Müller glia cells (taken from Marquardt and Gruss, 2002 with permission from Elsevier).
suggesting that amacrine cell number is controlled by feedback inhibition (Belliveau and Cepko, 1999). Furthermore, studies conducted in the frog (Reh and Tully, 1986) and fish (Negishi et al., 1982) revealed a similar amacrine inhibitory feedback mechanism. Explant studies in the chick suggest that ganglion cells also are capable of providing feedback inhibition and that ganglion cell genesis is regulated by soluble factors produced by earlier born ganglion cells (Waid and McLoon, 1998).

There are however limitations to the extent to which extrinsic factors play a role in cell fate determination. Several studies have indicated that an early progenitor cell is incapable of generating all the cell types that are made by later progenitor cells, and late progenitor cells cannot be forced into making early retinal cell types (Austin et al., 1995; Belliveau and Cepko, 1999; Belliveau et al. 2000). Extrinsic cues therefore are important in regulating the number of temporally appropriate cell types but they do not force a cell to generate inappropriate cell types (Livesey and Cepko, 2001). It has been proposed that in order for a retinal cell to be responsive to extrinsic signals these cells must be in a competent state (Cepko et al., 1996). This competence theory refers to the temporal expression of a repertoire of genes within a cell at particular times making this cell competent to respond to environmental cues to produce specific cell types. A classic in vitro study conducted by Watanabe and Raff (1990) examined the effect of environmental cues on rod photoreceptor development, which for the first time demonstrated the importance of intrinsic factors. Rod photoreceptor cells express opsin, a specific marker of differentiated rod cells. However, there is a delay in the onset of opsin expression, with expression commencing approximately 8 days after a rod cell is generated in the embryonic rat eye (Treisman et al., 1988) to more than 4 days in the
postnatal rat eye (Watanabe and Raff, 1990). When E15 retinal rat cells were mixed with an excess of PND1 cells there was no change in the time at which opsin was expressed, suggesting that these cells were not in a competent state to express opsin. However, when the cells were “competent” to express opsin, levels of expression were significantly higher than cells that were not mixed with PND1 cells. This suggests that in order for a cell to respond to environmental cues, intrinsically it must be in a state of competence (Cepko et al., 1996). This state of cell competence is transient, where a mitotic retinal cell passes through multiple competent states during which the generation of a specific cell type is regulated by positive and negative extrinsic factors (Cepko et al., 1996). Concurrently, extrinsic as well as intrinsic factors play a role in directing retinal cell fate determination.

4.2 Transcription Factors and the Retina

Mutation analysis has revealed a multitude of transcription factors involved in the various stages of eye development including specification of the eye field, retinal proliferation and differentiation. Several gene families have been found to play critical roles both in vertebrate and invertebrate ocularogenesis, including those encoding homeodomain proteins.

4.2.1 Pax6

One such gene believed to be crucial to eye development is the transcription factor Pax6, a member of the paired homeobox gene family. Much of our knowledge today regarding eye development has been acquired through studies of this gene. Pax6,
initially isolated in the mouse (Walther and Gruss, 1991), was found to display a high degree of homology through a wide variety of species ranging from humans (Ton et al., 1991) to *C. elegans* (Chisholm and Horvitz, 1995) and *Drosophila* (Quiring et al., 1994).

The dosage of the *Pax6* gene has been shown to have a profound effect on eye development. Mutations in the *Pax6* gene of mice referred to as *Small eye* (*Sey*), result in the reduction in eye size, lens and iris abnormalities in heterozygotes, and a complete loss of eye formation in homozygous mice (Hill et al., 1991). In humans, *PAX6* haploinsufficiency is characterized by similar phenotypes known as aniridia (Glaser et al., 1992) and Peter's anomaly (Hanson, 1994), while homozygous *PAX6* mutations result in anophthalmia. These similarities in mutant phenotypes suggest that *Pax6* may play similar roles in murine and human ocular development. Furthermore, a mutation in *Drosophila* gene *eyeless* (*ey*), which is homologous to *Pax6*, also results in the partial or complete absence of compound eyes (Quiring et al., 1994).

In addition to the eye formation defects observed following the loss of *Pax6* gene function, abnormalities are also observed upon overexpression of *Pax6*. While transgenic mice with a human *PAX6* locus were found to rescue *Sey* heterozygous and homozygous mutants, mice carrying more than two copies of the *Pax6* locus displayed multiple abnormalities such as microphthalmia and lamination defects (Schedl et al., 1996). Misexpression studies in *Drosophila* (Halder et al., 1995) and the frog (Chow et al., 1999) demonstrate ectopic eye formation. Pax6 protein hence has been found to be essential for eye development in many species studied, which has led *Pax6* to be considered a “master regulatory gene” for eye organogenesis (Halder et al., 1995). The
precise role of Pax6 in eye vertebrate and non-vertebrate development is unknown at this time.

Pax6 expression is initially detected in the anterior neural plate, including the optic pit and the presumptive diencephalon of vertebrates (Walther and Gruss, 1991; Grindley et al., 1995; Macdonald and Wilson, 1997). As development progresses Pax6 is expressed throughout the optic vesicle, the optic stalk and the surface ectoderm. Formation of the optic cup results in Pax6 expression in the presumptive neural retina and the RPE, as well as in the developing lens (Grindley et al., 1995; Macdonald and Wilson, 1997). As differentiation ensues Pax6 expression decreases and becomes restricted in the mature retina to the ganglion and amacrine cells, the lens and the cornea epithelium. This pattern of expression has been evolutionarily conserved in the zebrafish, Xenopus, mouse and chick, and is therefore indicative of conserved Pax6 function (Macdonald and Wilson, 1997) (Fig. 4.4 and 4.5).

In order to determine the role of Pax6 in eye development various mutation analyses have been carried out. Conditional Pax6 knock-out studies in the developing lens primordium demonstrated that the lens was not required for the development of the laminated retina (Ashery-Padan et al., 2000). However, similar knock-out studies in the neuroretina have suggested that Pax6 may be required during later stages of development in undifferentiated retinal cells to maintain their pluripotency, allowing them to generate the various cell types comprising the retina (Marquardt et al., 2001). Inactivation of Pax6 in the neuroretina resulted in the generation of only one retinal cell type, the amacrine cell. Hence, Pax6 may control the multipotency of retinal cells (Marquardt et al., 2001).
Figure 4.4 Illustration of a subset of transcription factors initially co-expressed within the progenitor cells of the retina. As these progenitor cells become postmitotic during development, expression of the different transcription factors becomes restricted to their respective layers. It is unclear whether these factors co-localize in all retinal progenitor cells. Abbreviations: gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigmented epithelium (taken from Marquardt and Gruss, 2002 with permission from Elsevier).
Figure 4.5 Expression of transcription factors in vertebrate retinal progenitor and differentiated cells. Initially during retinal development, chx10 and pax6 are expressed throughout the neural retina, but as differentiation ensues their expression becomes restricted to specific retinal neurons in the mature retina. Also note expression of Crx and Brn3 in a subset of retinal neurons (taken from Fuhrmann et al., 2000b).
4.2.2 Rx/rax

Mutation analysis of another paired-type homeobox gene, Rx/rax, suggests that this gene may also play a role in retinal development. Like Pax6, Rx/rax is also conserved across the phyla with homologs identified in humans, mouse, zebrafish and Drosophila (Mathers et al., 1997). However, Rx/rax is expressed much earlier than Pax6 in the developing eye and its expression is restricted (Furukawa et al., 1997). In the mouse, Rx/rax expression is first detected at E7.5 in the anterior head fold region and later is specified to the developing optic stalk, optic vesicles, and the ventral diencephalon. As development proceeds to E10.5, Rx/rax is expressed within the mitotic progenitor cells of the retina. However, as differentiation ensues, Rx/rax expression becomes significantly reduced and is no longer apparent in the differentiated adult retina (Furukawa et al., 1997). Hence, Rx/rax expression in the mouse (Furukawa et al., 1997), as well as similar expression analysis in retinal proliferating cells of the fish (Mathers et al., 1997) and Xenopus (Casarosa et al., 1997), suggest that Rx/rax plays a role in retinal progenitor cell proliferation. Misexpression analysis of Xrx, a Rx/rax homolog in Xenopus, revealed formation of ectopic retinal tissue, along with hyperproliferation of both the neural and pigmented retina (Mathers et al., 1997; Andreazzoli et al., 1999). This provides further evidence that rax plays a pivotal role in the regulation of retinal proliferation.

While rax heterozygous mice displayed no abnormalities, null mutants homozygous for the Rx/rax gene resulted in a complete loss of visible eye structures (Mathers et al., 1997). Unlike that of homozygous Pax6/small eye mutant embryos in which morphologically abnormal optic vesicles do form but subsequently degrade
resulting in anophthalmia (Hogan et al., 1986; Grindley et al., 1995), *Rx/rax* -/- mutants fail to form optic vesicles, optic cups and optic sulci (Mathers et al., 1997).

Furthermore, expression analysis in *Rx/rax* -/- mutants of the homeobox genes *Pax6*, *Otx2* and *Six3* that are normally located in retinal progenitor cells demonstrated a lack of expression of these genes in the primordium of the optic vesicle (Zhang et al., 2000). However, similar analysis in *Pax6* -/- mutant mice revealed expression of *Rx/rax*, *Otx2* and *Six3* homeobox genes in the rudimentary optic cup (Zhang et al., 2000). Hence, the ability of homozygous *Pax6* mutants to form optic vesicles and the expression of these retinal eye specific genes suggests *Pax6* is not required for optic vesicle formation.

However, *rax* expression and mutational analysis indicates that in addition to regulating retinal progenitor proliferation, *rax* plays an important role in the initial specification of the retinal primordia (Zhang et al., 2000).

### 4.2.3 Chx10

Other homeobox genes are involved in cell type specification of retinal progenitor cells. The mouse paired class homeobox gene *Chx10* has been implicated in retinal cell proliferation and bipolar cell differentiation (Burmeister et al., 1996). Several *Chx10* homologs are expressed in the developing eye of *C.elegans* (Hawkins and McGhee, 1990), goldfish (Levine and Schechter, 1993), and humans (Percin et al., 2000). Human *CHX10* has a similar pattern of expression to other vertebrate homologs in the retina; with *CHX10* expression restricted to the developing progenitor cells in the developing neuroretina and later during cell differentiation to the inner cell layer of the mature retina (Percin et al., 2000) (Fig. 4.4 and 4.5). Immunohistochemical analysis revealed these
cells in the inner nuclear layer to be bipolar cells (Burmeister et al., 1996). However, mice also display occasional Chx10 expression in the ganglion cells (Liu et al., 1994; Burmeister et al., 1996).

Spontaneous mutations in both the mouse (Burmeister et al., 1996) and human (Percin et al., 2000) Chx10/CHX10 genes, as well as targeted disruption of Alx via antisense oligonucleotides in the zebrafish (Barabino et al., 1997), results in an ocular retardation phenotype. Microphthalmia, cataracts, and an increased thinning of the retina characterize this homozygous phenotype. In mice and humans, homozygous Chx10 null mutations also displays loss of bipolar cell differentiation and lens defects (Burmeister et al., 1996; Percin et al., 2000). While thinning of the retina and a loss of bipolar cells may be directly attributed to the role of Chx10 in cell proliferation and bipolar differentiation, other characteristics are present in mouse Chx10 mutants. These include failure to form an optic nerve, rod photoreceptor defects and degeneration of the lens. At this time it is unknown whether these abnormalities are directly or indirectly related to the loss of Chx10, however it has been suggested that these are secondary effects (Burmeister et al., 1996).

4.2.4 Crx

The generation of cone and rod photoreceptor cells is dependent on another homeobox gene, Crx. This Otx family member, which is a paired-like homeodomain protein, has been found to be specifically expressed in mouse developing and adult retinal photoreceptor cells (Furukawa et al., 1997; Chen et al., 1997). Crx transcripts are first detected in the murine embryo at E12.5 in the outer region of the neural retina, which will
give rise to the photoreceptor layer. While *Crx* expression prior to birth corresponds to cone cell genesis, persistent *Crx* expression postnatally correlates with rod photoreceptor genesis. *Crx* expression peaks at PND4-6, which is approximately around the time of maximum rod differentiation (Carter and LaVail, 1979), and is maintained in the outer nuclear layer of the adult retina (Furukawa *et al.*, 1997; Chen *et al.*, 1997) (Fig. 4.5). A similar restricted pattern of *CRX* expression has also been demonstrated in the adult human retina (Freund *et al.*, 1997).

Retrovirus mediated *Crx* overexpression analysis in the PND0 rat retina resulted in an increase in rod photoreceptor cell number while inhibiting amacrine cell genesis and substantially decreasing the number of Müller glia cells (Furukawa *et al.*, 1997). Furthermore, while *in vivo* analysis of a retroviral dominant negative *Crx* variant in the rat did not show an impediment in photoreceptor development, the outer segment and rod terminal formation was inhibited (Furukawa *et al.*, 1997). Similarly, homozygous *Crx* generated mutants in mice also demonstrated disrupted photoreceptor outer segment formation, as well as subsequent degeneration of the outer nuclear layer and a loss of normal cone and rod activity as determined by electroretinography (Furukawa *et al.*, 1999). Biochemical analysis has indicated that *Crx* is involved in transactivating various photoreceptor-specific genes such as *rhodopsin*, *arrestin*, *β-phosphodiesterase* and *interphotoreceptor retinoid-binding protein* (Chen *et al.*, 1997; Furukawa *et al.*, 1997). Expression analysis of various photoreceptor-specific genes in PND10 *Crx* -/- mutant mice demonstrated a reduction of many photoreceptor-specific genes such as *rhodopsin*, *cone opsin*, *cone arrestin*, and *rod B-phosphodiester* (Furukawa *et al.*, 1999). *Rhodopsin* mutant mice also exhibit an absence of outer segments (Humphries *et al.*, 1997)
suggesting this similar phenotype in Crx -/- mutant mice is possibly due to a reduction of rhodopsin and opsin expression (Furukawa et al., 1999). Hence, these results indicate that while Crx plays an important role in regulating photoreceptor-specific genes in the retina, Crx may not be required for retinal differentiation. Many human retinal degenerative diseases such as retinitis pigmentosa (Sohocki et al., 1998), cone-rod dystrophy (Freund et al., 1997; Sohocki et al., 1998) and Leber congenital amaurosis (Sohocki et al., 1998) all of which subsequently lead to loss of vision have been attributed to mutations in CRX.

4.2.5 Basic Helix-Loop-Helix Genes

*Drosophila* and vertebrate homologs of proneural basic helix-loop-helix (bHLH) transcription factors are important regulators of neuronal cell type specification. Vertebrate proneural genes are grouped into four subfamilies containing highly related bHLH domains, three of which are *Drosophila* orthologs of the aschaete-scute, atonal, and tap/biparous genes (Guillemot, 1999).

Other bHLH genes have also been isolated which have an antagonistic function during retinal neurogenesis. This vertebrate class of antagonistic genes is referred to as Hes/Her, and has sequence and function similarity with the *Drosophila* genes hairy and enhancer-of-split (Sasai et al., 1992). These genes inhibit neurogenesis by acting as transcriptional repressors of proneural bHLH spatial and temporal gene expression (Ishibashi et al., 1995; Tomita et al., 1996b).

Several bHLH genes have been found to be important in cellular determination and differentiation in the developing invertebrate and vertebrate retina. Vertebrate bHLH
genes include positive regulators such as *Mash1* (Guillemot and Joyner, 1993), *Math3* (Takebayashi *et al.*, 1997), *Math5* (Brown *et al.*, 1998), *NeuroD* (Morrow *et al.*, 1999), as well as negative regulators such as *Hes1* (Tomita *et al.*, 1996b) and *Hes5* (Hojo *et al.*, 2000).

### 4.2.6 Homeobox Genes and Basic Helix-Loop-Helix Genes

The combination of different classes of transcription factors, such as homeobox genes and basic helix-loop-helix (bHLH) factors are found to play a role in retinal cell fate specification. One such case is with the specification of the bipolar cell. Mutation analyses of the homeobox gene *Chx10* (Burmeister *et al.*, 1996) and the double mutant *Mash1/Math3* (Tomita *et al.*, 1996a; Tomita *et al.*, 2000) revealed a complete loss of bipolar cells. Also, the *Mash1/Math3* double mutant displayed a substantial increase in Müller glia cell number (Tomita *et al.*, 1996a; Tomita *et al.*, 2000). However, misexpression studies have demonstrated that neither the homeobox gene *Chx10* nor the bHLH genes *Mash1* and *Math3* alone can induce bipolar cell specification. Misexpression of *Chx10* demonstrated the ability to generate all immature cell types (neuronal and glial) in the inner nuclear layer, while *Mash1* and *Math3* predominately formed rod photoreceptors. Instead, misexpression of either *Chx10* and *Mash*, or *Chx10* and *Math3*, promotes the generation of bipolar cells while decreasing Müller glia genesis (Hatakeyama *et al.*, 2001). Similarly, recent studies have also demonstrated that the *Math3* and *NeuroD* double-mutants have a complete loss of amacrine cells (Inoue *et al.*, 2002). These double mutants along with retinal explants from *NeuroD* null mice also exhibit an increase in Müller glia cell fate, as well as ganglion genesis in the double
mutant (Inoue et al., 2002; Morrow et al., 1999). Once again, while the misexpression of each of these genes alone was not adequate to induce amacrine cell genesis the combination of either Math3 or NeuroD with the Six3 or Pax6 homeobox genes did induce amacrine cell specification (Inoue et al., 2002). Although amacrine cells were generated with the combination of Math3 and Pax6/Six3, a larger number of horizontal cells were also induced (Inoue et al., 2002). The homeobox gene Crx and the bHLH gene NeuroD have been found to be required for photoreceptor cell type specification (Furukawa et al., 1999; Morrow et al., 1999).

These studies demonstrate that bHLH genes confer neuronal versus glial fate determination. In addition, misexpression studies have revealed that bHLH genes preferentially induce genesis of the neuronal subtype in which they are expressed suggesting that they, along with homeobox genes, are also involved in neuronal subtype specification (Tomita et al., 2000; Hatakeyama et al., 2001; Inoue et al., 2002).

Studies have also demonstrated Pax6 binding to the promoter regions of various bHLH genes such as Ngn2, Mash1 and Math5 (Marquardt et al., 2001). Furthermore, conditional Pax6 mutations in the neuroretina of mice resulted in reduced proliferation of progenitor cell and the generation of only one type of retinal cell, the amacrine cell. Subsequent analyses revealed a loss of Math5, Mash1, and Ngn2 expression in the retinal progenitor cells. These results demonstrate that Pax6 plays a role in proliferation and maintaining progenitor cell multi-potency. Also, transcriptional activation of bHLH factors via Pax6 is required for mediating the generation of the various retinal cell types in the developing eye (Marquardt et al., 2001).
However, misexpression studies have also indicated that only certain combinations of bHLH and homeobox genes are important for retinal cell specification (Inoue et al., 2002). Furthermore, not all bHLH genes are involved in regulating neuronal cell fate, as Hes1, Hes5 and Hesr2 have been found to promote glial cell fate (Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001). In fact misexpression analysis has demonstrated that rax, Hes1 and Notch1 gave rise to cells expressing Müller glia markers, while a dominant negative Hes1 gene reduced the number of glia cells in postnatal mice (Furukawa et al., 2000).

4.2.7 Brn-3b

Brn-3, a subfamily of the POU-IV domain is found to play critical roles in mammalian sensory neuron development (Xiang, 1998). Although all three members of the Brn-3 subfamily (Brn-3a, Brn-3b and Brn-3c) are expressed in the retinal ganglion cells (Xiang et al., 1993; Xiang et al., 1995), Brn-3b in particular is thought to be essential for retinal ganglion cell development. This is demonstrated in Brn-3b null mutant mice, which display defective retinas and a 70% loss of ganglion cells (Gan et al., 1996; Erkman et al., 1996). To determine whether Brn-3b was involved in ganglion cell fate specification, cell migration or ganglion survival, knock-in studies were conducted. Expression analysis of the knock-in reporter gene during ganglion cell fate specification and migration coincided with wild-type Brn-3b expression, indicating initial ganglion genesis is unaltered in Brn-3b deficient mice (Gan et al., 1999). However, neonate knock-in mice displayed thinning of the retina and a progressive loss of reporter gene expression. These results suggest that although Brn-3b may not be required for ganglion
cell fate specification and migration, it is fundamental for ganglion cell maintenance and survival (Gan et al., 1999). Like the Brn-3b adult null mutant mice (Gan et al., 1996), the knock-in Brn-3b mutant mice also exhibited a thinner optic nerve (Gan et al., 1999). Further analysis revealed that this results from atypical coalescing of the ganglion cell processes (Gan et al., 1999), ultimately leading to an increase in apoptotic ganglion cell death in the perinatal and early postnatal stages of mouse development (Xiang, 1998).

Unlike the other transcription factors reported to play a role in cell type specification, these results suggest Brn-3b is involved in the later stages of ganglion cell differentiation, playing a crucial role in retinal ganglion axon formation (Gan et al., 1999). Hence, Brn-3b has been regarded as one of the key intrinsic factors required for ganglion cells to acquire competence for ganglion cell differentiation (Cepko et al., 1996).

4.3 Signalling Molecules and the Retina

The control of retinal cellular competence has been found to involve various signalling molecules and pathways such as the Notch pathway. The neurogenic gene Notch was initially discovered in Drosophila and has been found to be an important regulator of eye development (Fortini et al., 1993; Cagan and Ready, 1989). Notch signalling is highly conserved in both invertebrates and vertebrates, showing similarities in ligands, receptors and downstream transcription factors (Weinmaster, 1997). Notch gene homologs have been identified in various vertebrate species such as the rodent (Bao and Cepko, 1997; Lindsell et al., 1996), chick (Austin et al., 1995; Henrique et al., 1995), frog (Coffman et al., 1990; Chitnis et al., 1995), fish (Sullivan et al., 1997) and humans (Ellisen et al., 1991). Each mammalian species has also been found to contain multiple
Notch homologs, such as the mouse where three Notch genes have been identified (Weinmaster et al., 1992; Lardelli et al., 1994).

In Drosophila, Notch signalling pathways are involved in arresting cell determination during retinal neurogenesis (Fortini et al., 1993; Cagan and Ready, 1989). Various studies have been conducted to address the role of Notch in vertebrate retinogenesis and to determine if the function of Notch in Drosophila function is also conserved. Expression analysis detected Notch1 transcripts within undifferentiated retinal cells in the chick (Austin et al., 1995), as well as in the mouse retina (Guillemot and Joyner, 1993). Furthermore, expression of Notch1 and its ligand Delta were found to have highly overlapping spatial and temporal expression patterns within the rat (Lindsell et al., 1996; Bao and Cepko, 1997) and chick (Ahmad et al., 1997) neural retina during the period of cellular determination and differentiation. As neurogenesis progresses with ensuing differentiation the level of Notch1 and Delta expression diminishes.

To examine the function of Notch genes, antisense oligonucleotides were administered in the retina of the chick during retinal development (Austin et al., 1995). This diminished Notch activity and subsequently increased the amount of progenitor cells, which differentiated into ganglion cells (Austin et al., 1995). Similarly, overexpression of a dominant negative form of Delta^{STU} in Xenopus resulted in a bias toward early retinal differentiation of ganglion and cone photoreceptor cells (Dorsky et al., 1997). However, studies using retroviral injection of exogenous Notch1 had the opposite effect. In Xenopus (Dorsky et al., 1995) and in the rat (Austin et al., 1995; Bao and Cepko 1997), exogenous in vitro and in vivo addition of X-Notch1/Notch1
respectively inhibited differentiation of retinal neurons and diminished ganglion cell
differentiation.

These studies suggest that Delta/Notch signalling pathways are involved in
regulating molecular retinal competence by limiting the number of cells that adopt a
particular cell fate. High levels of Delta/Notch signalling prevents cellular differentiation
while its absence enables the progenitor cells to be in a “competent state” to respond to
environmental and extracellular cues. Like Drosophila, it has been suggested that
vertebrates also utilize lateral inhibition by Delta/Notch signalling to regulate retinal
competence, ultimately generating the correct number of retinal cell types at the
appropriate stages of development (Rapaport and Dorsky, 1998).

5.0 Clinical Implications

Although at this time all of the complexities underlying the development of the
brain and eye remain unknown the use of animal models in research has provided insight
into the establishment of these regions in humans. In particular the expression analyses
of various transcription factors such as homeobox genes during embryonic development
as well as mutation analysis in mice has provided valuable insight into the genetic
mechanisms underlying the development of the CNS. In fact, in some cases an abnormal
phenotype, which has been identified in mice through mutation analysis has been found
to also display a comparable disease phenotype in humans. Cortical abnormalities such
as schizencephaly (a cleft in the cerebral cortex) in mice (Yoshida et al., 1997) and
humans (Brunelli et al., 1996; Granata et al., 1997) has been shown to be associated with
mutations in the EMX2 gene. Furthermore, failure of the telencephalic vesicle to
undergo cleavage or holoprosencephaly has also been identified in patients displaying mutations in the sine oculis (SIX3) homeobox transcription factor (Wallis et al., 1999). The heterozygous mutations in PAX6 leads to the clinical conditions of aniridia (Glaser et al., 1992) and Peters’ anomaly (Hanson, 1994) in humans with a similar disease phenotype also observed in the mouse (Hill et al., 1991). Additionally, mutation of the homeobox gene CRX has also been associated with cone-rod dystrophy (Freund et al., 1997; Sohocki et al., 1998), retinitis pigmentosa (Sohocki et al., 1998), and Leber congenital amaurosis (Sohocki et al., 1998) in the human retina. Hence, a comprehensive expression and mutation analysis of homeobox genes in animal models is key to deciphering the genetics governing human conditions such as Parkinson’s disease and Huntington’s disease, as well as various retinal diseases.
6.0 Coordinated Expression of Hoxa2, Hoxd1 and Pax6 in the developing diencephalon (Manuscript published)

6.1 Introduction

Several developmental regulatory genes have temporal and spatial patterns of expression in the murine forebrain (prosencephalon) and this in conjunction with early anatomical descriptions of the forebrain has given rise to the prosomeric model of forebrain development (Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Shimamura, et al., 1995). According to this model, the forebrain is divided into six transverse domains termed prosomeres. The primary prosencephalon is subdivided into the secondary prosencephalon (which includes prosomeres p4 to p6) and the diencephalon (p1 to p3), the subject of this study. The prosomeres p1, p2 and p3 give rise to the pretectum, dorsal thalamus and ventral thalamus, respectively. Dorsal of p2 is the domain of the epithalamus and the hypothalamus is localized to the ventral region of the secondary prosencephalon.

The molecular mechanisms involved in the patterning and specification of the diencephalon are unclear. We have examined the spatial and temporal expression patterns of Hoxa2, Hoxd1 and Pax6 proteins in the diencephalon of embryonic, fetal and newborn mice. The diencephalon develops in three stages (Niimi et al., 1961). In mice the first stage lasts from E10 to E12, during which the diencephalon consists of a dense cell layer (Fig. 1a-c). In the second stage of development, from E13 to E15, this primitive dense cell layer differentiates into the internal germinal, external germinal and mantle layers. In the third stage, from E16 to postnatally in mice, differentiation of functional neuronal nuclei occurs with the distinction between the three layers no longer being apparent.
Hox genes in the developing embryo are expressed in large rostral-caudal domains, with differing anterior boundaries (reviewed in McGinnis and Krumlauf, 1992; Rubenstein and Puelles, 1994; Keynes and Krumlauf, 1994; Mark et al., 1997). Among the Hox cluster gene family, the Hoxa2 gene has the most anterior expression domain along the mouse neural tube at the 25-somite stage (E9.5), being expressed as far rostral as the r1/r2 rhombomere boundary (Krumlauf et al., 1993; Prince and Lumsden, 1994). Little information is available on Hoxa2 mRNA expression profile in older embryos (Tan et al., 1992; Hao et al., 1999; Nazarali et al., 2000). Hunt et al. (1991) reported the expression of Hoxd1 transcripts in the ectoderm overlying the hindbrain and in posterior regions of the embryo. However, studies by Frohman and Martin (1992) documented initial transient Hoxd1 expression along the lateral plate mesoderm up to the level of the posterior hindbrain boundary. The dorsal-ventral domain of Pax6 mRNA expression in the CNS varies along the anteroposterior axis, and by E8 the prospective forebrain exhibits Pax6 expression but the prospective midbrain does not (Walther and Gruss, 1991). The generation of Hoxa2 (Gendron-Macguire et al., 1993; Rijli et al., 1993; Gavalas et al., 1997; Barrow and Capecchi, 1999) and Pax6 (Grindley et al., 1997; Warren and Price, 1997) mutants illustrate the important role these genes play in the normal development of the CNS. Recent findings also implicate an important role for Hoxa2 in specifying motor neuron identity (Hao et al., 1999; Davenne et al., 1999; Jungbluth et al., 1999). However, the morphology of Hoxd1 mutant mice has not been reported. There is a paucity of available information on the expression profile of the protein products of Hoxa2 or Hoxd1 (Hao et al., 1999) as well as on the expression profile of Hoxa2 or Hoxd1 transcripts in the fetal, neonatal or adult mice (Tan et al.,
1992; Hao et al., 1999; Nazarali et al., 2000; Nazarali et al., 1992). We have performed immunohistochemical and in situ hybridization histochemical analyses on consecutive sections and our findings reveal coordinated expression of Hoxa2, Hoxd1, and Pax6 in the developing diencephalon.

6.2 Results and Discussion

The E10.5 embryos exhibit intense Hoxa2 expression as well as weaker Hoxd1 expression in the diencephalic region of p2 and p3 (Fig. 6.1 a-c). Furthermore, both Hoxa2 (Fig. 6.1 a,b) and Hoxd1 (Fig. 6.1 c) were co-localized with Pax6 (an early marker of the diencephalon (Grindley et al., 1997; Warren and Price, 1997), in many cells within p2 and p3 (Fig. 6.2). Hence, all three transcriptional factors (Hoxa2, Hoxd1 and Pax6) show expression during the primary stage of diencephalic development. In addition, in E12.5 embryos, Pax6 had overlapping domains of expression with the Hoxa2 and Hoxd1 proteins in the thalamus (p2 and p3) and in the hypothalamus of the developing diencephalon (Fig. 6.1 d-l). Double-labeling studies confirmed co-localization of Hoxa2 and Hoxd1 with Pax6 in numerous cells in the hypothalamus although many nuclei in this region were also only Hoxa2 +ve (Fig. 6.1 h) or Hoxd1 +ve (Fig. 6.1 l). In situ hybridization histochemistry revealed analogous Hoxa2 and Hoxd1 mRNA profiles with their respective protein products in the diencephalon (Fig. 6.1 m-o; Fig. 6.3 d,g,j). Hoxa2 transcripts have also previously been detected within the diencephalic and telencephalic region in E12.5 mouse embryos (Fig. 5b in Tan et al., 1992) as well in the head region in embryos from E12 to E15 (Nazarali et al., 2000). Since Pax6 is expressed in the early
Figure 6.1 Immunohistochemical staining of sections from 10.5-day-old (a-c) and 12.5-day-old (d-l) mouse embryos. The figures are labeled in the bottom right hand corner with the stage of embryonic development. On the top right hand corner the molecular probes used are indicated. Hence if the figure is labeled Hoxa2/Pax6 as in (b), this section is doubled-labeled with these two markers and Hoxa2 was visualized with diaminobenzidine (DAB) thus marking expressing cells black and Pax6 was visualized with Vector Nova RED (Vector Laboratories, Inc., Burlingame, CA) marking these cells orange (Hao et al., 1999). In contrast, figures labeled Pax6/Hoxa2 as in h, then Pax6 expressing cells were visualized with DAB marking Pax6-expressing cells black and Hoxa2-expressing cells were visualized with Vector Nova RED, marking Hoxa2-expressing cells orange (Hao et al., 1999). (a) shows Hoxa2/Pax6 expression in prosomere p2; (b) is a higher magnification of p2 in (a). Cells that exhibit black nuclear staining are Hoxa2 positive cells (closed arrows in b) and those that demonstrate red-orange staining are Pax6. An open arrow in (b) is pointing to a cell that does not express Hoxa2 or Pax6. Many cells within p2 and p3 exhibit co-localization of Hoxa2 and Pax6 which is indicated by the orange-brown staining. (c) is from the same diencephalic region (p2) as that shown in (b), exhibiting Hoxd1 (black)/Pax6 (red-orange) staining. Fewer cells express Hoxd1 in comparison to Pax6 or Hoxa2. Double-labeled immunohistochemical stained transverse sections of the diencephalon from 12.5-day-old mouse embryos are shown in d-l. Figures in d-h exhibit Hoxa2 (red-orange) and Pax6 (black) labeled nuclei or reddish orange-brown if Pax6/Hoxa2 positive. A closed arrow in (h) is pointing to a cell expressing Hoxa2 (red-orange) and an open arrow is pointing to a cell expressing Pax6 (black). Figures in i-l exhibit Hoxd1 (red-orange) and Pax6 (black) or Pax6/Hoxd1 staining (reddish orange-brown). A closed arrow in (l) is pointing to a cell expressing Hoxd1 (red-orange) and an open arrow is pointing to a cell expressing Pax6 (black). A smaller arrow in (l) to the right side is pointing to a cell co-expressing Pax6 and Hoxd1 (reddish orange-brown). (e) and (f) are higher magnifications of the diencephalon (thalamus and hypothalamus) in (d). (g) is the higher magnification of the thalamus in (f) and (h) is a higher magnification of the hypothalamic region within the closed box in (f). (j) is higher magnification of the diencephalon (thalamus and hypothalamus) in (i) and (k) is a higher magnification of the thalamus in (j). (l) is a higher magnification of the hypothalamic region in the closed box in (j). (m-o) are in situ hybridization probed sections of 12.5-day-old mouse embryos. (m) is probed with Hoxa2 and (o) is probed with Hoxd1. (n) is higher magnification of the thalamic region in (m) and (o) is from a similar thalamic region as shown in (n), probed with Hoxd1. hp: hypothalamus; mge: medial ganglionic eminence; p2: prosomere 2; t: thalamus; v, third ventricle. Bar = 100 μm, except for (a) where bar = 200 μm.
Figure 6.2 Schematic representation of a parasagittal section of 10.5-day old mouse forebrain (similar to the plane of section shown in Fig. 1a) exhibiting Hoxa2 (red), Hoxd1(green) and Pax6 (black) protein expression. The three prosomeric transverse domains, p1-p3; epithalamus, ep; midbrain, mb and hindbrain, hb are illustrated.
diencephalic region at E8.5 (Puelles and Rubenstein, 1993; Walther and Gruss, 1991) prior to the Hoxa2 or Hoxd1, continued expression of Pax6 may be required for the expression of Hoxa2 and Hoxd1 at E10.5-E12.5 in the first stage (E10-E12) of diencephalon development.

At E13, the onset of the second stage of diencephalic development, Hoxa2 and Hoxd1 continued to exhibit overlapping domains of expression in this region (Fig. 6.3 b,c). Pax6 is also expressed in the developing diencephalon at this stage (Warren and Price, 1997) and co-ordinated expression of Hoxa2, Hoxd1 and Pax6 may be contributing to an inductive signal for the primitive dense cell layer to differentiate into the internal germinal, external germinal and mantle layers. By this stage of development, the diencephalon is divided by sulci (Fig. 6.3 a) into Herrick's four zones, the epithalamus, dorsal thalamus (p2), ventral thalamus (p3) and hypothalamus (ventral region of p4-p6) (Niimi et al., 1961; Coggeshall, 1964; Salinas and Nusse, 1992). During the later half of the second stage (E15), Hoxa2 and Hoxd1 continued to exhibit overlapping expression domains of mRNA (Fig. 6.3 d-f) and protein (Fig. 6.3 g-k). Double-labeling with Pax6/Hoxa2 (Fig. 6.3 j,k) and Pax6/Hoxd1 (Fig. 6.3 g-i) in transverse sections of the diencephalic region from E15 staged embryos reveal the presence of mostly Hoxa2 and Hoxd1 expressing cells, respectively. Consequently, downregulation of Pax6 at E15 (Fig. 6.3 g-k) appeared to precede the transition into the third stage of development when differentiation of functional neuronal nuclei occurs. Significant downregulation of Hoxd1 in the diencephalon was first observed at E16 (Fig. 6.3 n,q). Hoxa2 protein expression continued throughout the second (Fig. 6.3 b,j,k) and into the third (Fig. 6.3 l,m,o,p,r) stages of diencephalon development.
6.3 Conclusion

The temporal expression of the three transcriptional factors, Hoxa2, Hoxd1 and Pax6, appeared to coincide with the three stages of diencephalon development (Niimi et al., 1961), thus exhibiting three distinct waves of expression. *In situ* hybridization histochemistry as well as double-labeling studies with Pax6, a marker for cells within the early developing diencephalon, confirmed the presence of Hoxa2 and Hoxd1 mRNA and protein in the diencephalon. In the first wave, beginning at E10.5-12, during the primary stage of diencephalic development, Hoxa2, Hoxd1 and Pax6 expression was co-localized (Fig. 6.1 a-c). In addition, all three proteins exhibited overlapping domains of expression at E12.5 (Fig. 6.1 d-l) when the primitive dense cell layer begins to differentiate into the internal germinal, external germinal and mantle layers. Subsequently, downregulation of Pax6 (Fig. 6.3 g-k) in the later part of the second stage of diencephalon development (E15) appeared to precede the transition into the third stage of development. In the third wave, from E16 into postnatal life, decreased Hoxd1 expression (Fig. 6.3 n,q) coincided with the differentiation of functional neuronal nuclei with the distinction between the three layers no longer apparent. Hoxa2 continued to be expressed in the mouse diencephalon at least until birth (Fig. 6.3 o,r). These results support the view that coordinated expression of Hoxa2, Hoxd1 and Pax6 genes may play a role in specifying the cytoarchitecture of the developing diencephalon.
Figure 6.3 (a-c) Parasagittal sections and (d-r) transverse sections. (a) Cresyl violet stained section of the forebrain from a 13-day-old mouse embryo. (b,c) Higher magnifications of the ventral thalamic region indicated in the closed box in (a). Immunohistochemical staining of sections from 13-day-old (b,c), 15-day-old (g-k), 16-day-old (m,n,p,q) and 18-day-old (l) mouse embryos as well as newborn mice (o,r). (b,j-m,o,p,r) exhibit Hoxa2 expression; (c,g,h,i,n,q) exhibit Hoxd1 expression. (h) and (k) are higher magnifications of the diencephalon in (g) and (j), respectively; (p) and (q) are higher magnifications of the diencephalon in (m) and (n), respectively; (r) is a higher magnification of the diencephalon in (o). (i) is a higher magnification of the diencephalon in (h). (g-i) were double-labeled with Pax6 and Hoxd1, where Pax6 expressing cells were stained with DAB (black) and Hoxd1-expressing cells stained (red-orange) with Vector Nova RED. Many nuclei exhibit Hoxd1 (red-orange) expression in the diencephalon at E15, whereas Pax6 (black) expression in the same section was not observed. Hoxd1-expressing cells projecting away from the third ventricle (indicated by arrows) can be seen in (g) and (h). A similar pattern of Hoxa2-expressing cells projecting away from the diencephalon can be observed in (d) and (j). (j) and (k) were doublelabeled with Pax6 (DAB, black) and Hoxa2 (Vector Nova RED, red-orange). Only Hoxa2-expressing nuclei were observed in the E15 diencephalon (j,k). The nuclei in (j) and (k) that appear dark are strongly stained red-orange for Hoxa2 when observed under higher magnification. (d-f) in situ hybridization probed sections of 15-day-old mouse embryos. (d) is probed with Hoxa2 and (e) is higher magnification of the diencephalic region in (d). (f) is probed with Hoxd1. The expression of Hoxa2 mRNA (e) and protein (k) is higher respectively, when compared to that of Hoxd1 (f,i) from a similar region of the diencephalon. cp: choroid plexus; d: diencephalon; dt: dorsal thalamus; hp: hypothalamus; NB: new born embryo; t: thalamus, v:third ventricle, vt: ventral thalamus. Bar - 100 µm except for (a) where bar = 200 µm.
6.4 Materials and Methods

6.4.1 Immunohistochemical analysis

The Hoxa2 and Hoxd1 rabbit polyclonal antibodies were generated using oligopeptides, SPLTSNEKNLKHFOHQ (J-3) and ATAASVASIKLPRSETS (J-92), respectively (Hao et al., 1999; Nazarali et al., 2000) (data not shown). The Hoxa2 and Hoxd1 polyclonal antibodies recognized a single protein band from mouse embryonic tissues. In addition, both antibodies exhibited nuclear staining in tissue sections which is indicative of Hox transcriptional factors (Fig. 1b,c) and further specificity was also observed in competition experiments where addition of excess peptide resulted in background staining (Hao et al., 1999) (data not shown). Embryos were staged according to Kaufmann (1992). Swiss CD-1 mice were considered E0 days pregnant on the day the vaginal plug was found. Immunohistochemical methodology was adopted from Hao et al. (1999). The primary antibodies (Hoxa2 (1:1000); Hoxd1 (1:200); monoclonal Pax6 antibody (BAbCO, CA) 1:500) and the secondary antibody (biotinylated goat anti-rabbit IgG (1:500), Vector Laboratories Inc., Burlingame, CA), were diluted in 1% skim milk in PBS containing 0.1% Triton X-100.

6.4.2 In situ hybridization histochemistry

A modification of the method of Strahle et al. (1994) described in Hao et al. (1999) was employed. Swiss CD-1 mouse embryos were fixed in 4% paraformaldehyde, embedded in OCT medium (Tissue- Tek, Miles Inc., Elkhart, IN) and sectioned at 8 μm with a cryostat. A 518 bp segment of the Hoxa2 gene from nucleotide residues 1681 to 2199 (Tan et al., 1992) a region outside of the homeobox domain and a 273 bp segment
of the Hoxd1 gene from nucleotide residues 1024 to 1297 (Frohman and Martin, 1992), also outside the homeobox domain, were independently subcloned into pBluescript II SK(+) (Stratagene) and used as the templates for the synthesis of RNA probes. Digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN) was incorporated into the RNA synthesized to yield digoxigenin-labeled RNA probes. The reaction was catalysed by T7 or T3 RNA polymerase.
7.0 Hoxa2 expression in the pallium and subpallium of the developing murine forebrain

7.1 Introduction

The telecephalon is comprised of two distinct morphological regions, the pallium (cortex) and the subpallium (basal ganglia). The pallium is further subdivided into the dorsal, medial, lateral and ventral pallium, each respectively giving rise to the neocortex, primordia of the hippocampus (archicortex), paleocortex and the claustrum-endopiriform nucleus, piriform and insular cortex (Bayer and Altman, 1991; Puelles et al., 1999; Puelles et al., 2000; Stoykova et al., 2000). Portions of the amygdala are also derived from the lateral and ventral pallium (Puelles et al., 1999; Puelles et al., 2000; Stoykova et al., 2000). The subpallium consists of the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) from which the striatum and globus pallidus develop respectively, that ultimately constitute the major components of the basal ganglia (Smart and Sturrock, 1979; Deacon et al., 1994). Several developmental genes (Emxl, Dlx1, Ngn2, Vax1, Tbr1, Six3, Mash1, Pax6) have defined dorsal or ventral expression domains, some of which delineate the pallium/subpallium boundary (Stoykova et al., 2000). Pax6 expression is restricted to the neuroepithelial progenitor cells of the pallium with notably intense expression in the region of the ventral pallium (Walther and Gruss, 1991; Stoykova et al., 1996; Stoykova et al., 1997; Puelles et al., 1999; Stoykova et al., 2000).

Pax6 mutants have been found to display profound cortical defects including a reduction in the depth of the cortical plate, loss of the pallial/subpallial boundary and ectopic dorsal expansion of ventral subpallial markers (Warren et al., 1999; Stoykova et
al., 2000; Yun et al., 2001) where the ventral pallium is partially re-specified to express molecular characteristics of the dorsal LGE (Yun et al., 2001). Morphological deformities also occur within the claustrum, piriform, endopiriform nucleus, and insular cortex that arise from the ventral pallium (Stoykova et al., 2000).

It has been suggested that the genes involved in the dorsal-ventral patterning of the spinal cord may also play a parallel role in the regionalization of the developing telencephalon (Toresson et al., 2000; Wilson and Rubenstein, 2000). Previous expression analysis in the developing mouse has demonstrated initial Hoxa2 protein expression in the ventral horn of the spinal cord with expression extending to the dorsal horn as development progresses (Hao et al., 1999). Hoxa2 expression predominantly remains in the dorsal horn of the adult spinal cord (Hao et al., 1999) (Appendix C). Moreover, our recent analysis in the diencephalon, a constituent of the forebrain revealed coordinated expression of Hoxa2, Hoxd1 and Pax6 in the developing thalamus and hypothalamus (Wolf et al., 2001). This along with the aforementioned data has led us to examine the prospective role of Hoxa2 in the developing murine telencephalon.

### 7.2 Results and Discussion

Immunohistochemistry along with in situ hybridization histochemistry was used to determine the expression of Hoxa2 and Hoxd1 during the neurogenesis of embryonic (E) 12.5-16.0 day mice. At E12.5 the LGE emerges, during which time Hoxa2 and Hoxd1 transcripts were found in the pallium within the ventricular and subventricular zone of the LGE and the sulcus between MGE and LGE. Weaker expression was also evident in the ventricular zone of the MGE (Fig. 7.1 A-C). Immunohistochemical analysis on
consecutive adjacent sections revealed congruent Hoxa2 and Hoxd1 patterns of expression (Fig. 7.1 D-F). Furthermore, double-labeling with Pax6 antibody demonstrated co-localization of both Hoxa2 and Hoxd1 with Pax6 throughout the pallium. However, unlike other telencephalic markers, that either demonstrate pallial or subpallial expression, Hoxa2 and Hoxd1 expression at E12.5 was evident in both of these regions (Fig. 7.1 A-F). Since most of the interneurons of the olfactory bulb and cortex arise from the LGE and MGE, respectively, expression of Hoxa2 and Hoxd1 in these regions may play a role in the generation of these cells (for review, see Parnavelas, 2000).

As embryogenesis progresses to E16.0, Hoxa2 and Hoxd1 protein expression became more restricted to the cortical plate of the developing neocortex (Fig. 7.1 G-I). However, weak expression was also evident in the striatum. At this stage, Hoxd1 expression was significantly down regulated in comparison to that of Hoxa2 in this region (Fig. 7.1 G-I). Hoxa2 continued to be weakly expressed in the cortical plate and striatum at E18.0, however Hoxd1 expression was no longer evident at this stage (data not shown).

In some Hoxa2 -/- E11.5 embryos, Pax6 expression extended further in the medial pallium and into the subpallium thus obscuring the pallial/subpallial boundary (Fig. 7.2 B, C, E, F) typically seen in the wildtype embryos (arrow in Fig. 7.2A, D). In addition, aberrations were evident in regards to the size and position of the MGE within the telencephalon (Fig. 7.2 B, C, E, F). In other Hoxa2 -/- embryos at E11.5, up-regulation of Pax6 was evident in the pallium (Fig. 7.5 C), but no gross abnormalities were observed. Surprisingly, at E12.5 of development the Pax6-demarcated pallial/subpallial boundary was re-established. However, we observed an increase in Pax6 staining in the
Figure 7.1 Transverse sections of the forebrain of E12.5 (A-F) and E16 (G-I) mouse embryos. In A-D and G, dorsal is to the right. Intense Hoxa2 mRNA expression is seen throughout the cortex extending into the LGE and sulcus between MGE and LGE (A,B); Figure (B) is a higher magnification of (A). There is a similar pattern of Hoxd1 expression (C) in the telencephalon, but in comparison to Hoxa2, staining is weaker for Hoxd1 in the LGE and in the sulcus between the MGE and LGE. Figures D-F are immunohistochemically double-labeled with Pax6 and Hoxa2 (D, E) and with Pax6 and Hoxd1(F) antibodies. Cells stained with Pax6, exhibit black nuclear staining, whereas Hoxa2 and Hoxd1 display red nuclear staining. Co-localization of Pax6 with either Hoxa2 or Hoxd1 results in a brownish-red stain. Note the congruency of Hoxa2 protein expression (D) with mRNA expression (A, B) in the pallium (P) and in the LGE and MGE. Similar results were also observed with Hoxd1 protein expression (data not shown). Figure (E) is a higher magnification of the pallium enclosed in box (D), showing intense single-labeled red Hoxa2 cells (arrow) and co-localization of Pax6 and Hoxa2 indicated by the brownish-red nuclear staining (arrowhead). Hoxd1 expression is much lower in this region (F); however single-labeled Hoxd1 cells (arrow) and co-localized Hoxd1 and Pax6 cells were also present (arrowhead). G, H, I are immunohistochemically stained for Hoxa2 (G, H) and Hoxd1 (I). Note the strong Hoxa2 expression (H) and down-regulated Hoxd1 expression (I) in the cortical plate. Abbreviations: CP, cortical plate; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; P, pallium; SVZ, subventricular zone; VZ, ventricular zone. Bar = 100 μm, except for (A) where bar = 200 μm.
Figure 7.2 Transverse (slightly frontal) sections of E11.5 Hoxa2-/- mouse embryos reveal developmental abnormalities within the telencephalon. Figures (A, D) are wild-type and (B, C, E, F) are mutant embryos immunostained with Pax6. Figure (A) exhibits typical wild-type Pax6 expression throughout the pallium delineating the pallial/subpallial boundary (arrow). Figures D-F are higher magnifications of A-C, respectively. The pallial/subpallial boundary is no longer apparent in Hoxa2-/- embryos (B, C, E, F) due to the ectopic expression of Pax6 in the subpallium. Note the thickening of the neuroepithelium in these embryos (arrow in B, E). Expression of Pax6 in the medial pallium is also extended in the Hoxa2-/- embryos (compare arrowheads in A, D with that in E, F). Also note the expression of Pax6 in the MGE of the mutant mice (B, C, E, F), which normally is devoid of Pax6 (A, D). Abbreviations: MGE, medial ganglionic eminence. Bar = 200 µm for A-C and Bar = 100µm for D-F.
thalamus and hypothalamus of the diencephalon in these Hoxa2 -/- mutant embryos (Fig. 7.3 A,B) in comparison to their wild-type littermates (Fig. 7.3 C). As development progressed to E13.5, Pax6 expression was decreased in the diencephalon of both heterozygote (Fig. 7.3 I) and Hoxa2 -/- embryos (Fig. 7.3 F), however staining was enhanced in the posterior horn of the lateral ventricles of these mice (Fig. 7.3 E). Furthermore, the mutant embryos also displayed increased Pax6 expression throughout the pallium but particularly within the lateral and ventral pallium (compare Fig. 7.3 D,E with Fig.7.3 G, H). No obvious differences were observed between the heterozygotes and their wild-type litter-mates at all developmental stages that were examined.

To further characterize the loss of Hoxa2, immunofluorescence was conducted on coronal telencephalic sections utilizing the pallial marker Pax6 as well as the subpallial proneural bHLH marker Mash1. Mash1 expression begins at E9.5 and is restricted to the progenitor cells of the MGE and presumptive LGE of the subpallium (Yun et al., 2001). In addition to the generation of the interneuronal population of cells within the ventral subpallium, a large number of oligodendrocytes also arise from this region of the telencephalon (Corbin et al., 2001; Marin and Rubenstein, 2001). The bHLH transcription factor Olig2 is an early putative oligodendrocyte marker which displays expression in the ventral telencephalon of the mouse embryo at E9.5. Strong Olig2 expression has been reported in the VZ of the MGE with weaker levels of expression in the VZ of the LGE (Nery et al., 2001). Since Hoxa2 expression at E12.5 has also been found within these regions of the subpallium (Fig. 7.1 A,B,D) and Hoxa2 has also been previously demonstrated to be expressed in the oligodendrocytes of the spinal cord (Hao et al.,1999) the protein expression of Olig2 in potential developing oligodendrocytes was
Figure 7.3 Transverse (slightly frontal) sections through the forebrain of wild-type or hetero (C, G, H, I) and Hoxa2 -/- embryos (A, B, D, E, F) stained with Pax6 antibody. Figures (A-C) are E12.5 and (D-I) are E13.5 staged embryos. Arrow in (A) shows Pax6-demarcated pallial/subpallial boundary. At E12.5, Pax6 expression in the Hoxa2 -/- embryos is up-regulated in the thalamus and hypothalamus of the diencephalon (A, B). Figure (C) shows normal level of staining in the wild-type diencephalon at E12.5. Expression of Pax6 is also enhanced in E13.5 Hoxa2 -/- mice in the region surrounding the posterior horn of the lateral ventricle (compare D with G). Figure (F) is a higher magnification of (D) illustrating an increase in Pax6 staining in this region (arrow, compare F with I). An increase in Pax6 expression is also evident in the LP and VP of Hoxa2 -/- embryos (E), while the same displayed much weaker staining in the hetero forebrain (H). Abbreviations: DP, dorsal pallium; H, hypothalamus; LP, lateral pallium; LV, lateral ventricle; MP, medial pallium; T, thalamus; V, third ventricle; VP, ventral pallium. Bar = 100µm for all figures except for A, D, G where Bar = 200µm.
also analyzed in the telencephalon of *Hoxa2* −/− mutants. Additionally, analysis of the LIM homeobox protein Islet1, a marker of striatal projection neurons was also conducted (Toresson et al., 2000; Toresson and Campbell, 2001; Wang and Liu, 2001). While it is known that Islet1 is a marker of striatal projection neurons in the LGE at E13.5 in mouse, the onset of Islet1 expression in the telencephalon has not been reported in the mouse. However, expression analysis conducted in the developing rat has demonstrated Islet1 expression to initiate at E13 when the MGE is apparent, but the LGE has not formed (Wang and Liu, 2001). Islet1 expression was observed in the lateral regions of the MGE and presumptive LGE, with expression being absent in the VZ (Wang and Liu, 2001).

At E10.5 there is a marked reduction in the size of the telencephalon of *Hoxa2* mutants (Fig. 7.4 G,J) as compared with that of their heterozygote wild-type littermates (Fig. 7.4 A,D) as well as morphological abnormalities in the overall appearance of the telencephalon. In addition to these morphological abnormalities, changes were also observed in the expression patterns of Pax6, Mash1, Olig2 and Islet1 in *Hoxa2* −/− mice. Expression analysis of these *Hoxa2* −/− mutants revealed loss of the characteristic Pax6 expression in the pallium of these mutants (Fig. 7.4 H) as compared to that normally seen in the wildtype pallium (Fig. 7.4 B). For the most part the domain of expression of the subpallial marker Mash1 appeared somewhat normal in these *Hoxa2* mutants (Fig. 7.4 I). However, due to morphological abnormalities within the forebrain it is difficult to assess the variance of expression for Mash1. Also, the pallium seemed to exhibit a few cells expressing Mash1 (Fig. 7.4 I). While typical Olig2 expression is seen in the ventral telencephalon of E10.5 wildtype embryos (Fig. 7.4 E), expression was not detected in the
Figure 7.4 Immunofluorescence of E10.5 coronal sections of the mouse telencephalon. Figures (A-F) are consecutive adjacent sections of heterozygote embryos (+/-) and Hoxa2 mutant (-/-) embryos are shown in figures (G-K). Hoescht staining revealed morphological differences between the heterozygote embryos (A,D) and the Hoxa2 mutant embryos (G,J). The characteristic staining of Pax6 in the pallium of the telencephalon is evident in heterozygote embryos (B), however expression is absent in the mutant littermate (H). Similar to the heterozygote embryo in (C), Mash1 staining in the Hoxa2 -/- (I) also appears to display a dorsal limit of expression (arrow) despite morphological abnormalities. Figure (E) demonstrates the domain of Olig2 expression evident at this time within the subpallium of heterozygote embryos. Conversely, Olig2 expression was not evident in the mutant at this time (data not shown). A higher magnification of the enclosed box region in (D) is shown in figure (F). Note Islet1 expression along the lattermost region of the developing MGE. Islet1 expression is upregulated within the subpallium of the mutant telencephalon (K). Arrows indicate the dorsal or ventral limits of expression. Abbreviations: MGE, medial ganglionic eminence.
mutant littermates at this time (data not shown). Islet1 expression was also weakly detected at E10.5 in the wild-type ventral mouse telencephalon (Fig. 7.4 F). The temporal and spatial pattern of Islet1 in the telencephalon was similar to that previously reported in the developing rat (Wang and Liu, 2001). However, levels of Islet1 expression in Hoxa2 mutants at this time were significantly increased and Islet1 expression was observed in the VZ of the MGE (Fig. 7.4 K). It should be noted that the sample size used at this E10.5 stage was n=1 and therefore further expression analyses of Hoxa2 -/- mice should be conducted.

As development progresses increased levels of Pax6 expression is observed in the ventral, lateral and dorsal pallium of Hoxa2 -/- mutants at E11.5 (Fig. 7.5 C). Similarly this up-regulation of Pax6 in the Hoxa2 -/- mutants also continues at later stages of development which is similar to that observed in transverse sections of E13.5 embryos (Fig. 7.3 E). Expression analysis of Mash1 in E11.5 (Fig. 7.5 F), E12.5 (Fig. 7.6 C) and E13.5 (data not shown) Hoxa2 -/- mice indicated that there were no apparent changes regarding either the level or limits of expression within the ventral telencephalon. However, while the domain of Olig2 in E11.5 mutant embryos continues to be expressed along the subpallium up to the pallial/subpallial border there is an increase in Olig2 protein levels in the ventricular zone of the MGE (Fig. 7.5 I). This up-regulation of Olig2 continues at E12.5 (Fig. 7.6 F) and E13.5 (Fig. 7.6 I) in the MGE and LGE of the ventral telencephalon of Hoxa2 -/- mice as compared to the wild-type embryos (Fig. 7.6 E, H).

In summary our results have revealed new expression domains of Hoxa2 and Hoxd1 within the pallium and subpallium. Previously, we reported coordinated
Figure 7.5 Immunofluorescence analysis of coronal telencephalic sections of E11.5 Hoxa2 -/- mice reveals changes in pallial and subpallium expression. Heterozygote embryos are shown in figures (A,B,D,E,G,H) while figures (C,F,G) illustrate the mutant embryos. Pax6 levels of expression in the pallium of E11.5 Hoxa2 mutants (C) is increased in comparison to the staining evident in the heterozygote embryo (B). No apparent changes were observed either in the level or dorsal limit of Mash1 expression within heterozygote (E) and the mutant (F) embryos. However, an up-regulation of Olig2 expression is evident within the VZ of the MGE in Hoxa2 mutants (I) as compared to that of the heterozygote (H). Abbreviations: MGE, medial ganglionic eminence; VZ, ventricular zone. Arrows indicate the dorsal or ventral limits of expression.
Figure 7.6 Coronal sections of E12.5 and E13.5 embryos. Similar patterns of Mash1 expression is evident in both E12.5 heterozygote (B) and mutant (C) embryos. Conversely, Olig2 continues to demonstrate increased levels of expression in the MGE of E12.5 (F) and E13.5 (I) mutant embryos, as compared to wildtype E12.5 (E) and E13.5 (H) embryos. Abbreviations: MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence.
expression of Hoxa2, Hoxd1 and Pax6 to coincide with the three stages of diencephalon development (Wolf et al., 2001). All three transcriptional factors exhibited overlapping domains of expression in the developing diencephalon at E12.5 when the primitive dense cell layer begins to differentiate into the internal germinal, external germinal and mantle layers (Wolf et al., 2001). At this same stage of development (E12.5) we also found an increase in Pax6 staining in the developing diencephalon of the Hoxa2-/- mice (Fig. 7.3 A, B).

In Pax6-/- embryos, the ventral pallium progenitor zone is ventralized with a corresponding reduction in the ventral pallium mantle and an expansion of the LGE mantle (Yun et al., 2001). In Hoxa2 -/- embryos, ectopic expression of Pax6 into the subpallium, may be responsible for the thickening of the neuroepithelium most notable near the presumptive ventral pallium (arrow in Fig. 7.2 B,E). Ectopic expression of Pax6 in the MGE mantle may also be responsible for its abnormal enlargement and positional shift (Fig 7.2 C,F). Surprisingly, by E12.5, Pax6 expression was restored at the pallial/subpallial boundary of Hoxa2-/- embryos (arrow in Fig. 7.3 A), although coronal telencephalic sections at this stage did demonstrate an up-regulation of Pax6 within the pallium of Hoxa2 -/- mice. Similarly, profound early defects in the LGE of the Gsh2 -/- embryos (Szucsik et al., 1997; Yun et al., 2001) also exhibited dramatic compensation by E15.5 with a re-establishment of markers that define the cortical-striatal boundary (Corbin et al., 2000). It is unclear at present what are the molecular mechanisms that govern this recovery. Identification of genes involved in the development of MGE and globus pallidus may shed light on this phenomenon. These results suggest that there is a variance in the severity of the Hoxa2 -/- phenotype observed.
Also, we have shown for the first time expression of Islet1 at E10.5 in the lateral most region of the wild-type and heterozygote MGE which had been reported in the rat (Wang and Liu, 2001). In Hoxa2 -/- mice there was a marked increase in the level of Islet1 expression within the MGE. Similarly, Hoxa2 -/- mice also consistently displayed an up-regulation of Olig2, the marker of developing oligodendrocytes. Consequently, upregulation of Islet1 and Olig2 may have an impact on the generation of striatal projection neurons and oligodendrocytes, respectively. Taken together our findings have important implications since they provide insight into the molecular mechanisms involved in dorsoventral patterning of the pallium/subpallium as well as in the specification of striatal projections and oligodendrocytes within the developing murine forebrain.

7.3 Materials and Methods

7.3.1 Immunohistochemical and immunofluorescence analysis

Time pregnant C57BL/6J mice were considered embryonic day (E) 0.5 when the vaginal plug was found. Embryos were staged according to Kaufman (1992) via morphological examination of the embryos. Genotyping of C57BL/6J embryos was conducted using PCR analysis to identify Hoxa2 mutant mice (Gendron-Maguire et al., 1993). For Hoxa2 -/- mutant embryos, approximately an N=1 was analyzed for E10.5 embryos, N=6 for E11.5 embryos, N=5 for E12.5 embryos and N= 4 for E13.5 embryos. The embryos were fixed in 4% paraformaldehyde, cryoprotected in sucrose and embedded with OCT (Tissue-Tek, Miles Inc., Elkhart, IN). Transverse cryostat sections (8 µm) were collected and subjected to the immunohistochemical methodology as
outlined previously by Hao et al., (1999). Hoxa2, Hoxd1, and monoclonal Pax6 (BabCO, CA) primary antibodies were diluted 1:1000, 1:200, and 1:500 respectively, while a 1:500 concentration was used for the secondary antibody, biotinylated goat anti-rabbit IgG. Sections were stained with DAB (3,3′-diaminobenzidine) and/or with Vector® Nova Red. Similarly, immunofluorescence staining was conducted by diluting the primary antibodies anti-rabbit Mash1 (1:200; Chemicon), anti-mouse Islet1 (1:200; clone 34.4D5 ) and anti-rabbit Olig2 (1:2000; courtesy of Dr. Takebayashi) in PBS with 0.3 % Triton X-100 and incubating sections overnight at 4 °C. The secondary antibodies Cy3 goat anti-rabbit IgG (1:200 Jackson ImmunoResearch) and FITC donkey anti-mouse IgG (1:100 Jackson ImmunoResearch) were also diluted in PBS containing 0.3 % Triton X-100 and sections were incubated for 2 hours at room temperature. Sections were counterstained with Hoescht dye and mounted.

7.3.2 In situ hybridization histochemistry

Digoxigenin-labeled (Boehringer Mannheim, Indianapolis, IN) Hoxa2 and Hoxd1 anti-sense and sense RNA probes were transcribed with T7 and T3 RNA polymerase, respectively (Wolf, et al., 2001). In situ hybridization histochemistry was performed on corresponding consecutive adjacent sections as used for immunohistochemical analysis. Sections were treated with proteinase K, hybridized and stained with NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoly1-phosphate).
8.0  **Hoxa2 and the developing neural retina** *(Manuscript submitted)*

8.1  **Introduction**

The mammalian eye originates from coordinated interactions between various sources of primordial tissue. One such source, the wall of the diencephalon evaginates to form the optic vesicle of the developing eye. Subsequent contact between the optic vesicle and the overlying surface ectoderm is essential for lens placode development. Invagination of this lens placode results in the generation of the lens and the ensuing invagination of the optic vesicle to form the bi-layered optic cup. The optic cup gives rise to the inner layer of the neural retina and the outer pigmented epithelial layer characteristic of the vertebrate eye (reviewed in Graw, 1996).

The retina is a laminar structure consisting of three layers of neuronal and glial cell types. The outer nuclear layer contains the photoreceptor cells (rod and cones); the inner nuclear layer (INL) contains bipolar, horizontal, amacrine and Müller glia cells; and the ganglion cell layer (GCL) contains ganglion cells and displaced amacrine cells. During retinogenesis these various cell types differentiate in a conserved sequential manner from a dividing neuroblast layer of multipotent progenitor cells (Turner and Cepko, 1987). Although there is some overlap in retinal cell birth order, ganglion cell differentiation invariably is the first to occur followed by cone photoreceptors, amacrine, horizontal, rod photoreceptors, bipolar, and Müller glia cells (Cepko et al., 1996). Retinogenesis is not complete at birth but rather continues postnatally to develop rod, bipolar, Müller glia cells and a small number of ganglion and amacrine cells (Sidman, 1961; Young, 1985).
Several homeodomain transcription factors such as *Pax6*, *Chx10*, *Crx*, have been found to be required for normal development and cell type specification in the developing mammalian eye (Furukawa et al., 1999; Burmeister et al., 1996; Hill et al., 1991). One such gene, crucial to eye development, is *Pax6*. *Pax6* expression is conserved in several animal phyla including zebrafish, *Xenopus*, and mouse (Chow et al., 1999; Macdonald and Wilson, 1996; Puschel et al., 1992). Mutations in the *Pax6* gene results in the loss of eye formation in homozygous mice, as well as a reduction in the eye size and lens, with accompanying iris abnormalities in heterozygous mice (Hill et al., 1991). In humans, loss of this gene is well characterized by the conditions of aniridia (hypoplasia of the iris) (Glaser et al., 1992) and Peter’s anomaly (incomplete separation of the lens from the cornea) (Hanson et al., 1994). Furthermore, *Pax6* misexpression studies exhibit ectopic eye formation in *Drosophila* (Halder et al., 1995) and in *Xenopus* (Chow et al., 1999). Our previous analysis of the *Hoxa2* homeobox gene demonstrated co-localization of *Hoxa2* with *Pax6* in the developing diencephalon (Wolf et al., 2001). The co-localization of *Hoxa2* and *Pax6* in the diencephalon has led us to examine the spatial and temporal expression of *Hoxa2* protein and mRNA within the developing and murine retina.

### 8.2 Results and Discussion

*Hoxa2* expression is initially detected at E.9.5 in the proximal portion of the optic vesicle (Fig. 8.1 A-C), and within the optic stalk (Fig. 8.1 D-F). *Hoxa2* staining is also evident in the surface ectoderm of the developing murine eye (Fig. 8.1 C,E), which later invaginates to form the lens. By E10.5 the optic cup and the lens have developed, during
Figure 8.1 Immunohistochemical staining of transverse retinal sections from E9.5 (A-F), E10.5 (G,H) and E12.5 (I) mouse embryos. The antibody probes used are indicated on the bottom right hand corner of each figure. (B,C) are higher magnifications of the neural retina in (A); (E,F) are higher magnifications of (D); and (H) is higher magnification of (G). Hoxa2 expression is visible in the proximal optic vesicle (B,C), the optic stalk (E,F) and in the surface ectoderm (C,E). (G,H) were double-labeled with Hoxa2 and Pax6, where Hoxa2 expressing cells were stained with DAB (black) and Pax6 expressing cells stained (red-orange) with Nova Red substrate kit. The brownish-red stained cells exhibit co-localization of Hoxa2 and Pax6 in the optic stalk (G), lens and neural retina (arrows in H). Hoxa2 is visible throughout the neural retina by E10.5 (G) and expression is evident at E12.5 (I). Abbreviations: DOV, dorsal optic vesicle; L, lens; NR, neural retina; OS, optic stalk; POV, proximal optic vesicle; SE, surface ectoderm. Scale bars, 100µm.
which time Hoxa2 is co-localized with Pax6 in the neural retina and lens (arrows in Fig. 8.1 H) as well as in the optic stalk (Fig. 8.1G).

During retinogenesis, the ganglion cells are the first retinal cells to be generated from E11 to postnatal day 3 with a vast majority of cells being produced between E13 and E16 (Sidman, 1961; Young, 1985). At E12.5 we observed strong expression of Hoxa2 throughout the neural retina (Fig. 1I). Continued co-localization of Pax6 and Hoxa2 in the neural retina is also evident at E15.5 (Fig. 8.2 A-C; 8.3 E,F). Hoxa2 is co-localized with Islet-1, an amacrine and ganglion cell marker (Galli-Resta et al., 1997) in the neural retina at E15.5 (Fig. 8.2 D,E; 8.3 B,C), a time when amacrine and ganglion cell generation has reached its peak (Young 1985). Within the ventricular zone a few cells also exhibit Hoxa2/Islet-1 positive cells, which may be indicative of differentiated amacrine or ganglion cells en route to the inner region of the INL or GCL, respectively (arrow in Fig. 8.2 E). *In situ* hybridization of consecutive adjacent E15.5 tissue sections also revealed congruent expression of *Hoxa2* mRNA within the perspective GCL (Fig. 8.2 F). A large number of cells within the GCL in the retina are also displaced amacrine cells (Dräger and Olsen, 1981).

Further analyses using neurofilament 160 (NF-160), a marker for ganglion cells and horizontal neurons (Sechrist, 1969; Guillemot and Cepko, 1992; Brown et al., 2001) and syntaxin, a global amacrine cell marker (Inoue et al., 1992), were conducted in the retina of postnatal day 1 (PND 1) mice. Hoxa2 is co-expressed with both NF-160 (Fig. 8.3 H,I) and syntaxin (Fig. 8.3 K,L), indicating co-localization of Hoxa2 in the ganglion cells, horizontal neurons and amacrine cells. However, not all NF-160⁺ or syntaxin⁺ cells
Figure 8.2 Immunohistochemical staining of transverse retinal sections from E15.5 (A-E, H) and postnatal day 3 (I). Nuclei were visualized utilizing nuclear fluorescent HOESCHT counterstain (G). *Hoxa2* hybridized serial transverse retinal section from E15.5 (F). (B,C) are higher magnifications of the closed box in (A) and (E) is a higher magnification of the closed box in (D). The antibodies/molecular probes used are shown on the bottom right hand corner of each figure. (A-C) are double-labeled with *Hoxa2* (black) and Pax6 (red-orange) and co-localized cells in the neural retina are indicated by brownish-red staining (arrow in C). (D,E) are double-labeled with *Hoxa2* (black) and Islet-1 (red-orange) and numerous cells exhibit co-localization of *Hoxa2* and Islet-1 (arrow and arrowhead in E). Congruent *Hoxa2* transcripts are also visible in the GCL of the serial section (F). (H) illustrates expression of *Hoxa2* in the lens fiber cells.

Abbreviations: GCL, ganglion cell layer; pINL, presumptive inner nuclear layer; OS, optic stalk; V, ventricular zone. Scale bars, 100µm.
Figure 8.3  Figures (A-F) are immunofluorescent stained transverse retinal sections of E15.5 embryos and (G-O) of NB embryos. Nuclei were visualized utilizing nuclear fluorescent HOESCHT counterstain (A,D,G,J,M). *In situ* hybridized PND 3 sections probed with *Hoxa2* are shown in (P) and (Q), along with the corresponding *Hoxa2* immunohistochemical stained section in (R). At E15.5 *Hoxa2* protein is localized in the same nuclei as *Islet1* (arrows in B and C) in the GCL, which is also similarly shown with *Pax6* in figures (E) and (F) (arrows). *Hoxa2* (H) and NF-160 (I) along with *Hoxa2* (K) and syntaxin (L) are localized in the same cells in the GCL and within the pINL in the NB neural retina (arrows). *Hoxa2* also continues to be co-localized with *Islet1* in the GCL and pINL in the NB neural retina (N and O). *Hoxa2* protein and mRNA is visible in the inner region of the presumptive INL and GCL of PND 3 mice (P-R).

Abbreviations: GCL, ganglion cell layer; NB, newborn; pINL, presumptive inner nuclear layer; V, ventricular zone. Scale bars, 100 µm.
co-express Hoxa2, implying sub-type specificity of Hoxa2 in these cells. A large number of cells within the GCL in the retina are also displaced amacrine cells (Dräger and Olsen, 1981) which is evident by syntaxin staining in the GCL of PND 1 mice (Fig. 8.3 K,L arrow). However, in addition to labeling ganglion cells, NF-160 as mentioned above also labels horizontal cells (Brown et al., 2001). Hence, cells which demonstrate Hoxa2 as well as NF-160 labeling in the pINL of the PND 1 retina may either be horizontal cells or ganglion cells en route to the GCL (Fig. 8.3 H,I).

In situ hybridization and immunolabeling analyses revealed intense Hoxa2 mRNA (Fig. 8.3 P,Q) and protein (Fig. 8.3 R) expression throughout the GCL, and within a subset of cells in the inner region of the presumptive INL of the retina from postnatal day 3 (PND 3) mice. The INL consists of amacrine, bipolar, horizontal and Müller glia cells, with the amacrine cells residing in the inner region of INL (Young, 1985). Since we do not observe labeling in the middle portion of the presumptive INL of PND 3 mice (Fig. 8.3 P-R) the region where horizontal cells reside we postulate that the Hoxa2+ cells in the presumptive INL of the PND 1 retina which are double labeled with NF-160 (8.3 H,I) may likely be migrating ganglion cells. Hoxa2 mRNA expressing cells within the inner layer of the presumptive INL may be indicative of amacrine cells (Fig. 8.3 P,Q).

Hoxa2 expression in the lens is first evident at E10.5 (Fig. 8.1 H) when the lens is formed and expression continues at E15.5 (Fig. 8.2 H). Hoxa2 expression is absent from the lens in the PND-3 and significantly downregulated in the newborn lens (data not shown) primarily because the lens fiber cell nuclei have condensed and are no longer present. Hoxa2 expression is absent within the lens epithelium of PND-1 as well as PND-3 mice (Fig. 8.2 I).
Hoxa2 gene expression in the PND 1 neural retina was also identified using RT-PCR. Hoxa2 gene specific primers, from exon 1 and 2 (Tan et al., 1992), were designed to flank intronic sequences to distinguish between amplification of reverse transcribed Hoxa2 mRNA (yielding a band at 359 bp) from contaminating Hoxa2 genomic DNA. RT-PCR analyses of RNA extracted from E12.5 embryos, adult mouse spinal cord, neural retina of PND 1 Hoxa2+/− yielded a single band at 359 bp whereas a band of similar size was absent in neural retina from PND 1 Hoxa2−/− mice (Fig. 8.4). The RT-PCR amplified band from PND 1 Hoxa2+/− was further analyzed and the region determined to have identical sequence to the Hoxa2 mRNA.

Overall, Hoxa2 is initially expressed in the developing surface ectoderm, and proximal optic vesicle of the developing murine eye. As development progresses, Hoxa2 is expressed in the lens, the optic cup and later within the neural retina. The use of cell specific markers demonstrated Hoxa2 expression within ganglion and amacrine cells, however Hoxa2 is no longer apparent in the PND 3 lens as lens fiber nuclei condense. These findings are significant as this demonstrates novel Hoxa2 expression within the developing eye.
Table shows RT-PCR analysis of Hoxa2 and β-actin expression:

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<tr>
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<tr>
<td>2</td>
<td>Adult spinal cord</td>
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<tr>
<td>3</td>
<td>PND 1 Hoxa2 +/+ retina</td>
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<td>4</td>
<td>PND 1 Hoxa2 -/- retina</td>
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Figure 8.4 *Hoxa2* and β-actin RT-PCR analyses of RNA extracted from E12.5 embryos (lane 1), adult spinal cord (lane 2), the neural retina of PND 1 *Hoxa2* +/- (lane 3) and the neural retina of PND 1 *Hoxa2* -/- mice (lane 4). Amplification of Hoxa2 mRNA was not detected in the PND 1 *Hoxa2* -/- neural retina as compared to that seen with the control β-actin. The amplified fragment from the neural retina of PND 1 *Hoxa2* +/- was also subjected to sequence analysis confirming presence of Hoxa2 expression.
8.3 Materials and Methods

8.3.1 Tissue Preparation

The gestational age of the embryos was considered embryonic day (E) 0.5 at the time the vaginal plug was found and stage according to the morphology of the embryo. Harvested embryos were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in OCT (Tissue-Tek). Serial transverse sections (8 µm) were collected and subjected to immunohistochemical and in situ hybridization analyses.

8.3.2 Immunohistochemistry

Immunohistochemistry was performed as described previously in Hao et al., (1999). Sections were incubated with rabbit anti-Hoxa2 (1:1000), mouse anti-islet-1 (1:500, clone 39.4D5), mouse anti-Pax6 (1:500, Covance), mouse anti-HPC1/syntaxin (1:500, Sigma) and mouse anti-neurofilament 160 (NF-160; 1:1000, Sigma) antibodies. Biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories) and biotinylated donkey anti-mouse IgG (1:250, Vector Laboratories) were used as the secondary antibodies. DAB (3,3'-diaminobenzindine) and/or Nova Red substrate kit (Vector Laboratories) was used to visualize staining. Immunofluorescence staining was also similarly conducted by incubating sections with the aforementioned primary antibodies overnight at 4°C, followed by incubation with the secondary antibodies Cy3 goat anti-rabbit IgG (1:200, Jackson ImmunoResearch) and FITC donkey anti-mouse IgG (1:50, Jackson ImmunoResearch) for 2 hrs at room temperature. Cell nuclei were visualized utilizing nuclear fluorescent HOESCHT counter stain.
8.3.3 *In situ* hybridization histochemistry

Digoxigenin-labeled (Boehringer Mannheim) *Hoxa2* anti-sense and sense RNA probes were transcribed with T7 and T3 RNA polymerase, respectively (Wolf et al., 2001). *In situ* hybridization was performed on corresponding consecutive adjacent sections as used for immunohistochemical analysis. Sections were treated with proteinase K, hybridized and stained with NBT/BCIP (nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indolyl-phosphate).

8.3.4 RT-PCR

Total RNA from newborn neural retina was extracted using Tri Reagent ®. The *Hoxa2* cDNA was generated from RT-PCR reactions containing 1 µg of RNA, 0.5 mM dNTP, and 7.5 µg of random primer (Sigma). This mixture was incubated at 65 °C for 5 min, chilled on ice and a final concentration of 1X first strand buffer (50 mM Tris-HCL, 75 mM KCL and 3 mM MgCl₂), 10 mM DTT and 40 units of RNase and 200 units of Superscript RT II (Invitrogen) was added. These components were incubated at 25 °C for 10 min, followed by an additional incubation for 50 min at 42 °C and inactivated at 70 °C for 10 min. The RT-PCR product was then amplified with 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of either *Hoxa2* gene specific primer ((5'–TGACATCTTTTCCCCCTGTCG and 5'–GTGTTGGTGTACGCGGTTCTCAG) (Tan et al., 1992), or β-actin control primers (5'–GGCATCGGATGGACTCCG and 5'–GCTGGAAGGTGGACAGCGA) (Remacle et al., 2002), PCR buffer (10 mM Tris-HCL, 50 mM KCL, 0.1 % TritonX-100) and 2.5 units of Taq DNA polymerase. The reaction was incubated at 94 °C for 10 min followed by 30 cycles at 94 °C for 45 sec, 59 °C for 30
sec and 72 °C for 1.5 min. Samples were incubated for 10 min at 72 °C and the amplification products run on a 1% agarose gel and visualized with ethidium bromide staining.
9.0 General Discussion

Hox genes were believed to be not important in specifying the rostral regions of the brain early on in development and it had previously been assumed that this is also the case during the later embryonic stages of vertebrate development. Therefore, the expression profiles for the clustered Hox genes in older staged embryos (E10-NB) had not been studied. Although the anterior limits of most Hox genes is known along the early developing neural tube (E9.5), the anterior domain of Hoxd1 expression in the rhombomeres had not been reported. Also, little information had been available during the later stages of development in regards to the expression of Hoxd1. The purpose of this thesis was to conduct for the first time an expression analysis of Hoxa2 and Hoxd1 in the anterior regions of the brain with the primary focus being on the diencephalon, telencephalon and developing neural retina.

The research conducted has demonstrated for the first time novel expression of the homeobox genes Hoxa2 and Hoxd1 in the developing diencephalon and telencephalon. Furthermore, expression analysis has also revealed the presence of Hoxa2 within the developing retina and lens with expression becoming restricted to the ganglion and amacrine cells. The coordinated expression of Hoxa2, Hoxd1 and Pax6 within the three developmental stages of the diencephalon suggested that these genes may be involved in specifying the cytoarchitecture of the developing diencephalon. In addition, the temporal and spatial expression of these genes within the diencephalon suggests that they may be involved in the proliferation, specification and/or differentiation of the diencephalic neurons. However, further analysis in Hoxa2 -/- mice should be conducted to determine the role of Hoxa2 and Hoxd1 in diencephalic development.
Interestingly, the expression analysis in the diencephalon, telencephalon and developing eye consistently demonstrated co-localization of Hoxa2 with the divergent homeobox gene, Pax6. In addition, analysis of the telencephalon of Hoxa2-/- mice revealed a ventral expansion of Pax6 expression into the subpallium in some mutants at E11.5, while other mutants repeatedly demonstrated an up-regulation of Pax6 within the ventral, lateral, dorsal and medial pallium. As development continued, Pax6 continued to be up-regulated within the pallial telencephalic region in Hoxa2-/- mice. The co-localization of Pax6 and Hoxa2 as well as changes in Pax6 expression in Hoxa2 knock out mice suggests that Hoxa2 and Pax6 may potentially be involved in cross regulatory mechanisms. Further biochemical analysis need to be conducted to determine whether this is indeed the case.

One important observation that should be addressed is the fact that we did not consistently observe ectopic Pax6 expression within the ventral subpallium of the telencephalon in E11.5 Hoxa2-/- mice. As mentioned previously, some mutant embryos at this stage only demonstrated enhanced Pax6 expression within the pallium. The inconsistencies between the different mutant embryos at this stage could potentially be due to penetrance. Penetrance refers to “The proportion of individuals with a specific genotype who express that character in the phenotype” (http://cancerweb.ncl.ac.uk/omd/). Functional compensation by other transcription factors may also play a role in the phenotypical inconsistencies of Hoxa2-/- mice.

Apart from the appearance of some cells expressing Mash1 in the pallium of E10.5 Hoxa2-/- embryos, no apparent change in Mash1 expression was observed in either E11.5-E13.5 embryos. Hence, at this time it is difficult to assess whether there are
any regulatory interactions between Hoxa2 and Mash1 in the establishment of the
telencephalon and further experimental analysis in younger embryos (E10.5) must be
conducted to verify the significance of this change early on in development. In E10.5
HOXA2-/- embryos, we also noted a marked change in the expression levels of Islet1 in
comparison to that of the wildtype or heterozygote littermates and at E11.5 there also
appeared to be an up-regulation of Islet1 in HOXA2-/- mice. Although previous studies
have demonstrated that Islet1 marks the LGE and the differentiating striatal projection
neurons at E13.5, the onset of Islet1 expression has not been characterized in the
developing mouse. However, studies conducted in rats have determined Islet1 to be first
expressed at E13 (a time when the LGE has not yet formed) in the lattermost region of
the ventral telencephalon (Wang and Liu, 2001). This population of cells expressing
Islet1 in the rat at E13 is believed to consist of both postmitotic cholinergic and non-
cholinergic precursor cells (Wang and Liu, 2001). Hence, we can postulate from the
expression analysis of Islet1 conducted in the rat that the marked up-regulation of Islet1
in HOXA2-/- mutants could possibly be due to an increase in the number of postmitotic
cholinergic and non-cholinergic precursor cells. However, further analysis needs to be
carried out to determine if there are also changes in Islet1 expression in HOXA2-/-
knockout mice at later stages of development to determine if there is an effect on the
striatal projection neurons.

Changes in the expression of Olig2 (a marker of oligodendrocyte precursors) have
also been observed in the ventral telencephalon of HOXA2-/- mice. Increased levels of
Olig2 expression were observed in the MGE and LGE of the developing telencephalon
of E11.5, E12.5 and E13.5 HOXA2 mutant mice as compared to their wildtype or
heterozygote littermates. These results suggest that Hoxa2 may repress Olig2 in the wildtype embryos, ultimately limiting the number of oligodentrocytes generated.

Expression analysis conducted in the developing and newborn retina revealed novel Hoxa2 expression within the developing lens and neural retina. At later stages expression was specifically observed within the ganglion and amacrine cells of the postnatal retina. This expression data suggests Hoxa2 may be involved in the specification of the lens, differentiation of the lens fiber cells and may also play a role in ganglion and amacrine cell genesis. Expression analysis of Hoxa2 -/- and wildtype newborn mice using various markers specific to ganglion and amacrine cells will need to be conducted to determine whether there are any changes in the number of these cell types. If a change in cell number is observed for either ganglion or amacrine cells it would demonstrate that Hoxa2 is involved in ganglion or amacrine cell genesis. However, if there are no apparent changes in cell number, potentially the same scenario, that has been previously reported for Pax6, may exist. Pax6 is normally expressed in amacrine cells, yet analysis of Pax6 -/- mice did not display any changes in the amacrine cell number (Marquardt et al., 2001). While Pax6 alone was not sufficient for amacrine cell genesis it was demonstrated that the combination of homeobox genes and bHLH genes is required for cell type specification (Inoue et al., 2002). Hence, Hoxa2 together with various bHLH genes may be required for the specification of ganglion and amacrine cells.
9.1 Future Directions

Expression analyses of Hoxa2 in the developing embryonic murine diencephalon, telencephalon, and neural retina demonstrated co-localization of Hoxa2, along with the homeodomain containing transcription factor Pax6, in these regions. Indirectly, the co-localization of these two proteins suggests that these genes may be involved in the regulation of each other. In order to conclusively determine if this is indeed the case, future biochemical molecular analysis such as electromobility shift assays (EMSA) should be conducted. The utilization of transfection assays in cell culture may be additionally required to directly determine if Hoxa2 transactivates the Pax6 gene.

In regards to the diencephalon, further work may be conducted utilizing Hoxa2 -/- mice to examine if there are any morphological changes and/or changes in expression of other regulatory markers of the diencephalon such as Pax6. Furthermore, since we have demonstrated Hoxa2 expression in the diencephalon and telencephalon (pallio/subpallio border), analysis of thalamocortical and corticothalamic projections in Hoxa2 -/- mice by use of carbocyanine DiI (1,1'-dioctade-cyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) tracing would also be interesting to determine whether Hoxa2 is also involved in axonal pathfinding.

Furthermore, the analysis of Pax6 protein expression in the telencephalon of Hoxa2 -/- embryos revealed ectopic Pax6 expression in the ventral subpallium of the telencephalon. This ectopic expression indicates that Hoxa2 may play a role in maintaining this pallial/subpallial border and may be required to repress Pax6 expression. Further analysis would have to be conducted by analyzing Hoxa2 expression in Pax6 -/- knockout mice, as well as the analysis of homozygous double Pax6-/- / Hoxa2-/- mice to
determine whether Pax6 and Hoxa2 co-repress each other for maintenance of the pallial/subpallial border. Also, additional analysis of Hoxa2 −/− mice should be conducted to determine whether the loss of Hoxa2 affects the development of striatal projection neurons and oligodendrocytes at later stages of development.

Finally, in order to determine the function of Hoxa2 in the developing retina, misexpression of Hoxa2 could provide valuable information as to whether or not Hoxa2 alone is sufficient for ganglion and amacrine cell genesis. Also, the misexpression of Hoxa2 together with the bHLH genes that are found in amacrine and ganglion cells may also indicate whether a combination of genes is alternatively important for the specification of ganglion and amacrine cell fate.

9.2 Conclusion

The data presented in this thesis demonstrate for the first time novel Hoxa2, as well as Hoxd1 expression in the rostral diencephalic and telencephalic regions of the developing murine brain. In addition, Hoxa2 was also found to be expressed in the developing murine eye within the lens fiber cells as well as in the ganglion and amacrine cells of postnatal mice. These results suggest that Hoxa2 and Hoxd1 may be involved in the morphogenesis of these components of the forebrain. It is also of great interest that Hoxa2 consistently demonstrated co-localization with Pax6 within these regions. Further biochemical analysis as well as additional mutation analyses need to be conducted to determine the potential regulatory interaction between Hoxa2 and Pax6 within these various components of the developing forebrain.
10.0 REFERENCES


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APPENDICES

Appendix A. Western blot analysis to determine specificity of Hoxd1 antibody.

Appendix B. Materials and Methods

Appendix C. Differential expression of Hoxa2 protein along the dorsal-ventral axis of the developing and adult mouse spinal cord.
Appendix A. Western blot analysis to determine specificity of Hoxd1 antibody.

Western blot analysis of embryonic protein from E10-E18 day murine tissue, and recombinant Hoxd1 protein overexpressed in bacteria. The anti-F92 (Hoxd1) antibody recognized a single protein band in E10, E12, E14, E16 embryonic tissue which corresponds to the correct size of the Hoxd1 protein. Furthermore, this antibody also recognized a fragment of Hoxd1 protein (13 kDa), which was overexpressed in bacteria.
Appendix B. Materials and Methods

Protein Isolation

Embryonic protein was isolated from E9, E10, E12, E14, and E16 mouse embryos using Tri Reagent® (Molecular Research Centre, Inc.). Embryonic tissue (50-100 mg) was first homogenized with 1 ml of Tri Reagent® and stored at room temperature for 5 minutes. Chloroform (200 µl) was then added, samples shaken for 15 seconds, and incubated at room temperature for 10 minutes. The samples were centrifuged at 4 °C for 8 minutes at 12,000 g, the aqueous RNA layer removed and the DNA inter phase precipitated with 300 µl of 100 % ethanol. The samples were mixed by inversion, incubated at room temperature for 3 minutes and centrifuged at 4 °C for 5 minutes at 2000 g. The supernatant was removed and the protein was subsequently precipitated by the addition of 1.5 ml of isopropanol, incubated for 10 minutes at room temperature and the samples centrifuged at 12,000 g for 10 minutes at 4 °C. The protein pellets were then washed 3 times for 20 minutes at room temperature with 0.3 M guanidine hydrochloride in 95 % ethanol, and centrifuged for 5 minutes at 4 °C. A final wash in 100 % ethanol for 20 minutes was performed, the protein pellet was air dried and dissolved in 1% SDS at 50 °C. The protein for western blot analysis was quantified using the Bio-Rad protein assay according to manufacturers instructions.
SDS-PAGE and Western Blotting

The 12 % separating gel and 4 % stacking gel was prepared according to the manufacturers instructions (Invitrogen). Protein isolated from E9.0, E10, E12, E14, E16 embryos (20 µg) was resolved on the SDS-PAGE gel. The gel was then transferred to a PVDF membrane (NEN) with transfer buffer (12 mM Tris, 96 mM glycine, 20 % methanol) for 1.5 hours at 30 V using the Xcell II Blot Module ® (Invitrogen). The membrane was incubated overnight in 3 % skim milk (PBS) at 4 °C. The primary antibody, rabbit anti-F92 (Hoxd1) diluted 1:2000 in 3 % skim milk (PBS) was added to the membrane and incubated for 2 hours at room temperature. The membrane was then washed 5 times for 10 minutes each with 0.8 % Tween-20 in PBS and incubated with the horse radish peroxidase (HRP) conjugated secondary antibody, goat anti-rabbit diluted 1:3000 in 3 % skim milk (PBS) for 45 minutes at room temperature. The membrane was then washed with PBS containing 0.8 % Tween-20 and was incubated with a chemiluminescence reagent (PerkinElmer Life Sciences, Inc.) as per manufacturers instructions. The protein bands were subsequently visualized by exposure of the membrane to Kodak X-OMAT Blue XB-1 autoradiography film.

RNA Isolation

Total RNA from embryos and newborn (NB) mice was isolated using Tri-Reagent ® (Sigma). Tissue (50-100 mg) was homogenized in 1 ml of Tri-Reagent ® (Molecular Research Centre, Inc), and incubated at room temperature for 5 minutes. Bromochloropropane (100 µl) (Sigma) was added to each sample, shaken for 15 seconds and incubated at room temperature for 15 minutes. Samples were then centrifuged at
12,000 g for 15 minutes at 4 °C and the upper aqueous RNA layer removed. This aqueous layer of RNA was precipitated with 0.5 ml of isopropanol, incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 8 minutes at 4 °C. The RNA pellet was washed with 75 % ethanol and centrifuged at 12,000 g for 5 minutes at 4 °C. The pellet was air dried for 5 minutes and dissolved in diethyl pyrocarbonate (DEPC) water. Solubilization of the RNA was facilitated by incubation of samples at 60 °C for 15 minutes. The RNA concentration was quantified for subsequent use by spectrophotometry using the 260/280 nm ratio.

**Mouse Genotyping**

Genomic DNA from embryonic tissue and the newborn tail was isolated by digestion with 50 mM Tris (pH 8), 100 mM EDTA, 0.5 % SDS and 0.5 µg/µl of proteinase K (Invitrogen) at 55° C until tissue was completely digested. The DNA was cleaned twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) and the upper aqueous DNA layer was precipitated with 1/10 volume of 3M sodium acetate (pH 6) and an equal volume of 100 % ethanol. The genomic DNA was centrifuged at 12,000 g for 10 minutes at 4 °C and the pellet was washed with 70 % ethanol, air dried and reconstituted in autoclaved deionized water. Wild-type *Hoxa2* mice were identified by PCR amplification with the following *Hoxa2* primers, (5’ GTTGAACCTGACCTCTCTTG 3’) and (5’ GGGTCCGAGCAGGGTTATTCC 3’) yielding a 330 bp band. Conversely, a 380 bp Neomycin gene band was amplified in *Hoxa2* -/- mice utilizing the NEO-1 primer (5’ TCGCTTTCTATCGCCTTCTTG 3’) and the KO-1 primer (5’GTTGTACGCGTTTCTCAG3’). Briefly, the PCR
amplification was conducted with 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μM of either Hoxa2 primers (5' GTTGGAAACTGACCTCCTCTTG 3' and 5' GGGTCCGAGCAGGTATTC 3' ), or with the neomycin and KO-1 primers (5'-GGCATCGGATGGACTCCG and 5' GCTGGAAGGTGGACAGCGA), PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1 % TritonX-100) and 2.5 units of Taq DNA polymerase. The reaction was incubated at 94 °C for 10 min followed by 30 cycles at 94 °C for 45 sec, 59 °C for 30 sec and 72 °C for 1.5 min. Samples were extended for 10 min at 72 °C, cooled to 4 °C and the amplification products run on a 1% agarose gel and visualized with ethidium bromide staining.

**In Situ Hybridization**

Slides were initially pretreated with 0.1 % DEPC water overnight and autoclaved. Slides were then dipped in 0.25 M ammonium acetate (Sigma), dried at 60 °C and soaked for 30 minutes in a 50 μg/ml solution of poly-L-lysine (Sigma). They were then air-dried overnight and stored at 4 °C. All embryos used for in situ hybridization were fixed in 4 % paraformaldehyde (DEPC treated PBS) and cryoprotected in 30 % sucrose (DEPC treated PBS). Sections (8 μm) were cut and placed on the poly-L-lysine coated slides and incubated at 40 °C overnight. Sections were washed 2 times for 5 minutes each in DEPC-treated PBS (pH 7.4) and 2 times for 5 minutes each in DEPC-treated PBS containing 100 mM glycine. They were then incubated with DEPC-treated PBS with 0.3 % Triton X-100 and washed 2 times for five minutes each with DEPC-treated PBS. Sections were permeablized for 30 minutes with 1 μg/ml of RNase-free Proteinase K in TE buffer (100 mM Tris-Hcl, 50 mM EDTA, pH 8.0) at 37 °C and then fixed for 5
minutes at 4 °C in 4 % paraformaldehyde (DEPC-treated PBS). Following fixation slides were washed for two times five minutes each with DEPC-treated PBS and acetylated for 2 times for 5 minutes each with 0.1 M triethanolamine (TEA) containing 0.25 % acetic anhydride (Sigma). Sections were incubated for 20 minutes at 37 °C with 4X SSC (600 mM NaCl, 60 mM sodium citrate, pH 7.2) prehybridization buffer containing 50 % deionized formamide. Subsequently, the sections were hybridized with hybridization buffer (40 % deionized formamide, 10 % dextran sulfate, 1X Denhardt’s solution, 4X SSC, 10 mM DTT, 1 mg/ml yeast tRNA, 1 mg/ml denatured herring sperm) containing 10 ng of digoxigen labeled RNA probe, covered with hydrophobic plastic coverslips and incubated overnight at 42 °C in a hybridization oven. Following the removal of the plastic coverslips by immersing the slides in 2X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.2) sections were washed at 37 °C 2 times for 15 minutes each in 2X SSC and 2 times for 15 minutes each in 1X SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.2). Removal of unbound probe was performed by incubating sections with NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 μg/ml RNase A for 30 minutes at 37 °C and washing for 2 times 30 minutes at 37 °C with 0.1X SSC. Slides were then washed with buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 2 times for 10 minutes each and incubated with blocking solution (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 2 % sheep serum) for 30 minutes. The anti-DIG-alkaline phosphatase (Roche) was diluted 1:500 in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 1 % sheep serum, applied to the sections and incubated for 2 hours at room temperature. Sections were washed for 2 times for 10 minutes each with buffer 1 and incubated for 10 minutes with buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM
NaCl, 50 mM MgCl₂). The slides were then incubated with a staining solution containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 337.5 µg nitroblue tetrazolium (NBT), and 1750 µg 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

**Immunohistochemistry**

Embryos were fixed in 4 % paraformaldehyde in PBS, whereas NB mice were transcardially perfused with 4 % paraformaldehyde and cryoprotected with 30 % sucrose in PBS. Sections (8 µm) were collected on 0.5 % gelatin coated coverslips and dried at room temperature for 1.5 hours. Sections were washed 2 times for 10 minutes each in PBS and blocked with 3 % skim milk (0.1% Triton X-100, PBS) for 30 minutes at room temperature. The primary antibodies were diluted in 1 % skim milk (PBS) and sections were incubated overnight at 4 °C. Slides were then washed 2 times for 10 minutes in PBS and incubated for 30 minutes in 3 % skim milk. The sections were incubated for 30 minutes with the secondary antibody; biotinylated goat anti-rabbit IgG (1:500, Vector) in 1 % skim milk. Sections were washed 2 times for 10 minutes each and endogenous peroxidase activity was blocked by incubating coverslips for 15 minutes in methanol containing 0.3 % hydrogen peroxide. Slides were washed in PBS and blocked for 30 minutes in 3 % skim milk. A 1:125 dilution of avidin biotin complex (Vector Laboratories) in PBS was added to the coverslips and incubated at room temperature for 1 hour. Additional washes in PBS were conducted and sections were incubated for 10 minutes in 0.175 M sodium acetate. The staining of the sections was performed using a 3, 3'- diaminobenzidine (DAB) solution (0.66 mg/ml DAB, 62.5 mg/ml Ni₂SO₄, 0.175, 0.175 M sodium acetate) with the addition of 0.025 % hydrogen peroxide prior to use.
Sections were then washed in 0.175 M sodium acetate and PBS for 10 minutes. Coverslips were dehydrated in increasing concentration gradients of ethanol, followed by xylene and mounted with permount media. Double immunohistochemistry was similarly conducted by using the M.O.M® immunodetection kit (Vector Laboratories) according to manufacturers instruction and staining was conducted utilizing the Vector® NovaRed peroxidase substrate (Vector Laboratories).

**Immunofluorescence Staining**

The procedure for tissue fixation and sectioning was identical to that used for immunohistochemistry. Sections were washed 2 times for five minutes each in PBS and blocked for 30 minutes with 3 % skim milk in PBS containing 0.3 % Triton X-100. The primary antibodies (a polyclonal antibody and a monoclonal antibody) were diluted in PBS with 0.3 % Triton X-100 and sections were incubated overnight at 4 °C. The following day coverslips were washed 2 times for five minutes each in PBS. The secondary antibodies Cy3 goat anti-rabbit IgG (1:200 Jackson ImmunoResearch) and FITC donkey anti-mouse IgG (1:50 Jackson ImmunoResearch) were also diluted in PBS containing 0.3 % Triton X-100 and sections were incubated for 2 hours at room temperature. Coverslips were washed 2 times for 10 minutes each and counter stained with Hoescht dye for 15 minutes at room temperature. Sections were rinsed for 2 times five minutes in PBS and mounted with Citofluor mounting media (Marivac Limited).
Design of Probes for In Situ Hybridization

Primers designed for Dlx1 (accession number NM 010053), Gsh2 (accession number NM 133256), Ngn2 (accession number NM 009718) and Emx2 (Yoshida et al., 1997) contained Xho I and Hind III restriction enzyme sites. The cDNA was amplified from E12 and E14 RNA by conducting RT-PCR using random primers (Sigma). The RT-PCR product was then amplified via PCR with gene specific primers for Dlx2 (5' CTCGAGCCTAGCTCAACTCGGTCAGCAG 3' and 5' TTGCAATGGAACCATATCT TGACCTGCG 3'), Gsh2 (5' CTCGAGGCGAGCACCACCACGCACCTGTC 3' and 5' CTCGAGGCGAGCACCACCACGCACCTGTC 3'), Ngn2 (5' CTCGAGGCGAGCACCACCACGCACCTGTC 3' and 5' GCAGGGCAGTC 3' and 5' TTCGAACCGGCAGTGGTGTCGCCGAG 3') and Emx2 (5' CTCGAGGCCAGAGTTTCTTGTTCACAA 3' and 5' TTCGAAGCGCTGCTTGGTAGCAATTCTCC 3'). The cDNA for Hoxd1 was also amplified using the following gene specific primers 5' TCGAATTCGTGGCCTCGATTAAGCTT 3' and 5' TGCTCGAGTCTCTATACACAGCTCTCTCTCT 3' containing EcoRI and XhoI restriction enzyme sites respectively. The PCR was conducted similarly to that previously described for mouse genotyping with the exception of the use of an annealing temperature of 61 °C to amplify Gsh2 and Ngn2, 63 °C for Emx2 and Dlx2 and 58 °C for Hoxd1. DNA fragments of 322 bp, 350 bp, 348 bp, 301 and 273 bp respectively, were amplified and subsequently ligated into pBluescript SK + (Stratagene) plasmids.

Isolation of Plasmid DNA

Bacterial culture (1.5 ml) was pelleted by centrifuging at 12,000 g for 2 minutes. The supernatant was removed by aspiration and the bacterial pellet was resuspended in
ice cold buffer 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.). Freshly prepared solution 2 (0.2 NaOH, 1 % SDS) was added to the samples and incubated on ice for 5 minutes. The solution was neutralized by the addition of buffer 3 (3 M potassium acetate, pH 5.5) and incubated on ice for 5 minutes. Cellular debris was removed by centrifuging for 5 minutes at 4 °C at 12,000g and the supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous layer was removed and precipitated with 2 times volume of isopropanol. The plasmid DNA was then pelleted by centrifugation for 15 minutes at 12,000g and washed with ice cold 70 % ethanol. The DNA was air dried and resuspended in autoclaved deionized water.

**Plasmid Subcloning**

The pBluescript SKII + vector was prepared for subcloning by digesting 1 µg of the DNA plasmid with either Xho I (Invitrogen) and EcoR I (Invitrogen) or with Xho I and Hind III (Invitrogen) restriction enzymes. Briefly, the restriction digests was performed using 1 µg plasmid DNA, React 2 buffer (50 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl) (Invitrogen), and 1 unit of either Xho I and EcoR I or 1 unit of Xho I and Hind III. The reactions were incubated at 37 °C for 1 hour and run on an agarose gel. The digested DNA plasmid was eluted off the gel and spun through a microfuge tube containing siliconized glass wool. Yeast tRNA (10 µg/µl) was added to the DNA eluant and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The plasmid DNA was precipitated with 10 % volume of 3 M sodium acetate and 2 times volume of isopropanol. The DNA was pelleted by centrifugation at 12,000 g for 15 minutes at 4 °C. The pellet was washed with 70 % ethanol, air-dried and reconstituted in
autoclaved deionized water. Concentration of the plasmid DNA was determined by agarose gel electrophoresis.

Plasmid DNA Preparation for Sequencing

Plasmid DNA was treated with 10 mg/ml RNase A (Sigma) and incubated at 37 °C for 20 minutes. The samples were extracted 2 times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma), followed by an extraction of the aqueous phase with chloroform. The DNA was precipitated with an equal volume of isopropanol and centrifuged at 12,000 g for 10 minutes at 4 °C. The DNA pellet was washed with 70 % ethanol, air dried and reprecipitated with deionized water containing 0.4 M NaCl and 6.5 % PEG 8000. The sample was incubated on ice for 20 minutes and centrifuged for at 12,000 g for 15 minutes at 4 °C. The DNA pellet was washed with ice cold 70 % ethanol, air dried and resuspended in autoclaved deionized water. DNA was quantified by running on an agarose gel. DNA samples were sequenced via the Sanger sequencing kit (USP biochemicals) or at the Saskatoon Cancer Centre, University of Saskatchewan.

Ligations

T4 DNA ligations were performed as per manufacturers instructions (Invitrogen). Briefly, the reactions were conducted with 1X ligase reaction buffer [50 mM Tris-HCl, (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5 % (w/v) polyethylene glycol-800)], 0.3 µg DNA, 0.1 µg pBluescript SKII + (Stratagene), and 1 unit T4 DNA ligase (Invitrogen). The reactions were then incubated at 15 °C overnight.
Transformations

DH5α competent E. coli cells (100 µl) (Invitrogen) were added to 1 µl of ligation mixture and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 37 °C, and incubated on ice for 2 minutes. LB media (0.950 ml) was added to the cells and they were grown for 1 hour at 37 °C at 225 rpm in a shaking water bath. The E. coli cells were spread on LB agar plates containing 100 µg/ml ampicillin, X-gal (20 mg/ml) and isopropyl-β-D-thio-galactopyranoside (IPTG) (100 mM). Plates were then grown overnight at 37 °C.

RT-PCR

Total RNA from newborn neural retina was extracted using Tri Reagent ®. The Hoxa2 cDNA was generated from RT-PCR reactions containing 1 µg of RNA, 0.5 mM dNTP, and 7.5 µg of random primer (Sigma). This mixture was incubated at 65 °C for 5 min, chilled on ice and a final concentration of 1X first strand buffer (50 mM Tris-HCl, 75 mM KCl and 3 mM MgCl₂), 10 mM DTT and 40 units of RNase and 200 units of Superscript RT II (Invitrogen) was added. These components were incubated at 25 °C for 10 min, followed by an additional incubation for 50 min at 42 °C and inactivated at 70 °C for 10 min. The RT-PCR product was then amplified with 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of either Hoxa2 gene specific primer (5’- TGACATCTTTTCCCCCTGTGC and 5’ GTGTTGGTGACGCGGTTCTCAG) (Tan et al., 1992), or β-actin control primers (5’-GGCATCGGATGGACTCCG and 5’ GCTGGAAGGTGGACAGCGA) (Remacle et al., 2002), PCR buffer (10 mM Tris-HCl,
50 mM KCl, 0.1 % TritonX-100) and 2.5 units of Taq DNA polymerase. The reaction was incubated at 94 °C for 10 min followed by 30 cycles at 94 °C for 45 sec, 59 °C for 30 sec and 72 °C for 1.5 min. Samples were incubated for 10 min at 72 °C and the amplification products run on a 1% agarose gel and visualized with ethidium bromide staining.
Differential Expression of Hoxa-2 Protein Along the Dorsal-Ventral Axis of the Developing and Adult Mouse Spinal Cord

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ABSTRACT We have used synthetic oligopeptides derived from the coding sequence of the murine Hoxa-2 protein to produce polyclonal antibodies that specifically recognize the Hoxa-2 recombinant protein. Immunohistochemical studies reveal a distinct pattern of spatial and temporal expression of Hoxa-2 protein within the mouse spinal cord which is concomitant with the cytoarchitectural changes occurring in the developing cord. Hoxa-2 protein is predominantly detected in the nuclei of cells in the ventral mantle region of 10-day-old mouse embryos. Islet-1, a marker for motor neurons was also shown to be co-localized with Hoxa-2 in nuclei of cells in this region. As development progresses from 10-days to 14-days of gestation, Hoxa-2 protein expression gradually extends to the dorsal regions of the mantle layer. The Hoxa-2 protein expression pattern changes at 16-days of embryonic development with strong expression visible throughout the dorsal mantle layer. In 18-day-old and adult mouse spinal cords, Hoxa-2 protein was expressed predominantly by cells of the dorsal horn and only by a few cells of the ventral horn. Double labeling studies with an antibody against glial fibrillary acidic protein (GFAP, an astrocyte-specific intermediate filament protein) showed that within the adult spinal cord, astrocytes rarely expressed the Hoxa-2 protein. However, Hoxa-2 and GFAP double-labeled astrocytes were found in the neopallial cultures, although not all astrocytes expressed Hoxa-2. Hoxa-2 expressing oligodendrocyte progenitor cells were also identified after double-labeling with O4 and Hoxa-2 antibodies; although cells in this lineage that have begun to develop a more extensive array of cytoplasmic processes were less likely to be Hoxa-2 positive. The early pattern of Hoxa-2 protein expression across transverse sections of the neural tube is temporally and spatially modified as each major class of neuron is generated. This congruence in the expression of the Hoxa-2 protein and the generation of neurons in the cord suggests that the Hoxa-2 protein may contribute to dorsal-ventral patterning and/or to the specification of neuronal phenotype. Dev Dyn 1999;216:201-217.

Key words: Hoxa-2; spinal cord; central nervous system; immunohistochemistry; astrocytes; oligodendrocytes; neurons

INTRODUCTION

Homeobox genes are a family of genes that contain a 180 base pair sequence, known as the homeobox reviewed in McGinnis and Krumlauf, 1992; Manak and Scott, 1994; Mark et al., 1997). The homeobox encodes a homeodomain of 60 amino acid residues which forms a structure characteristic of the helix-turn-helix motif (Laughon and Scott, 1984) that can bind to specific nucleotide sequences and thereby regulate the transcription of downstream effector genes (Gehring et al., 1990; Kissinger et al., 1990; Li et al., 1990; Grebe et al., 1997; Akam, 1998). Many homeobox genes found in clusters at neighboring sites in the chromosome are structurally related to the Drosophila Antennapedia and Ubx/Abd-B group of homeobox genes (Kessel and Gniss, 1990; Simeone et al., 1991; Gellon and McGinnis, 1998). Genes in this class are collectively called the HOM complex (Akam, 1989; McGinnis and Krumlauf, 1992) and referred to as Hox/HOX genes in mice and humans, respectively. The Drosophila Antennapedia complex (ANT) and the Bithorax complex (BX) of homeobox genes exist on the same chromosome, whereas in the mammalian genome up to four clusters of Hox genes are present, each cluster residing on a different chromosome. The four mammalian chromosomal clusters of Hox genes, termed Hox A, Hox B, Hox C, and Hox D are believed to have been formed by two successive duplications of...
the ancestral AN1 and BX cluster of genes (Kappen et al., 1989; McGinnis and Krumlauf, 1992; Manak and Scott, 1994; Mark et al., 1997).

The order of Hox genes within each chromosomal cluster has been conserved during evolution. Interestingly, this order also corresponds to the order of anterior limit of expression along the anterior-posterior axis of the developing mouse (Flieberg et al., 1987; Holland and Hogan, 1988a; McGinnis and Krumlauf, 1992; Manak and Scott, 1994; Mark et al., 1997). Hence the Hox gene family can serve as a model system to study genetic control mechanisms of mouse development. Many Hox genes are expressed at high levels in the developing central nervous system (CNS) as well as in a variety of non-neural tissues and organ primordia during embryonic development (Holland and Hogan, 1988b; Awgulewitsch and Jacobs, 1990; Wilkinson, 1993).

The Hoxa-2 gene belongs to the Hox A chromosomal cluster of Hox genes and has mainly been studied in the mouse (Nazarali et al., 1992; Tan et al., 1992; Gendron-Maguire et al., 1993; Iijii et al., 1993; Mallo and Gridley, 1996; Nonechev et al., 1996; Mallo and Brandl, 1997; Gavalas et al., 1997; Kanzler et al., 1998) and the chick (Prince and Lumsden, 1994). The Hoxa-2 gene encodes a protein with a molecular mass of 41 kD (Tan et al., 1993). The transcriptional expression of the Hoxa-2 gene has been analyzed by in situ hybridization histochemistry and Northern blot analysis (Tan et al., 1992). During development, the highest level of poly(A)+ RNA was found in 12-day-old mouse embryos, with the levels progressively decreasing as development proceeded. An in situ hybridization study of 12.5- and 14-day-old mouse embryos showed that the Hoxa-2 gene was expressed in the hindbrain, VII and VIII cranial ganglia, spinal cord, dorsal root ganglia, larynx, lungs, vertebrae, sternum, and intestine (Tan et al., 1993). In the developing neural tube, Hoxa-2 expression was initially detected in the hindbrain in rhombomeres 3 and 5 (r3) at the 5- to 6-somite stage. As development progressed to the 10- to 20-somite stage, the Hoxa-2 expression was detected in rhombomeres r3 and r5. Later, at the 25-somite stage, Hoxa-2 expression extended anteriorly to the boundary of rhombomeres r1 and r2 (Krumlauf, 1993; Prince and Lumsden, 1994; Nonechev et al., 1996). Hence within the Hox gene family, Hoxa-2 is the most anteriorly expressed gene in the neural tube. Hoxa-2 gene like all other Hox genes has an overlapping domain of expression in the caudal spinal column of the embryo extending to a sharp anterior limit which is specific for each Hox gene.

An important step towards identifying the functions of vertebrate homeobox genes during development is a detailed analysis of their spatial and temporal patterns of expression. Although many homeobox genes have been isolated and their transcriptional expression patterns have been determined by Northern blot and in situ hybridization methods, little information existed on the precise localization of their protein products. We have developed murine Hoxa-2 specific polyvalent antibodies (anti-3 antibodies) to determine the expression pattern of the Hoxa-2 protein in the developing and adult spinal cord. Targeted mutations have been described for Hoxa-2 but a detailed analysis of the differentiation of the spinal cord neurons has not been reported (Gendron-Maguire et al., 1993; Rijli et al., 1993). Recent analysis of the Hoxa-2 gene revealed that the rhombomeres 2 (r2) and rhombomeres 3 (r3) were reduced in size resulting in an enlarged cerebellum and correspondingly reduced pontine structures (Gavalas et al., 1997). Additionally the trigeminal motor axons failed to exit the hindbrain at its proper location (Gavalas et al., 1997). We reveal differential patterns of Hoxa-2 protein expression in the spinal cord and in situ hybridization techniques on tissue obtained from embryos as young as 10 days after fertilization up to and including adult animals.

RESULTS

Specificity of Anti-J3 Antibodies

In order to test the specificity of the anti-J3 antibodies, the Hoxa-2 cDNA (1,232 bp) (Tan et al., 1992) was subcloned into the p-FLAG-2 expression vector (IBI, Kodak, New Haven, CT) and competent Escherichia coli cells transformed with this plasmid. The anti-J3 antibodies recognized a single protein band at 41 kD from the crude bacterial extract obtained from a culture transformed with the p-FLAG-2-Hoxa-2 cDNA plasmid (Fig. 2A, lane 5 and Fig. 2B, lane 1). To further test that the anti-J3 antibodies recognized the Hoxa-2 region that had formed the template for designing the J3 oligopeptide (Fig. 1), only the carboxy terminal portion of the Hoxa-2 cDNA, from nucleotide residues 1,681 to 2,199 (Tan et al., 1992), was subcloned into the p-FLAG-2 expression vector. As shown in Fig. 2B, lane 2, the anti-J3 antibodies specifically recognized a protein band of about 20 kD from the crude bacterial extract obtained from a culture transformed with pFLAG-2-carboxy term (1,681-2,199 bp) Hoxa-2 plasmid. These
HOXA-2 PROTEIN ALONG THE DEVELOPING SPINAL CORD

A

200-

18-

18-

97-

68-

43-

29-

68-

43-

29-

18-

B

200-

18-

18-

97-

68-

43-

29-

200-

97-

68-

43-

29-

18-

Fig 2. A. Western blot analysis of proteins extracted from mouse embryos at 10 days (E10) to 14 days (E14) after fertilization. Proteins (30 µg) were separated on a 10% polyacrylamide-SDS gel and after transfer to a PVDF membrane were probed with anti-J3-specific antibodies. Lane 1: E10; Lane 2: E12; Lane 3: E13; Lane 4: E14; Lane 5: crude bacterial extract of recombinant Hoxa-2 protein (144 kD). B. Western blot analysis of proteins extracted from 16-day-old mouse embryonic tissues (lanes 3 to 8) and of crude bacterial extracts of recombinant Hoxa-2 proteins (lanes 1 and 2). Proteins (30 µg) were separated on a 10% polyacrylamide-SDS gel and after transfer to a PVDF membrane were probed with anti-J3-specific antibodies. Lane 1: crude bacterial extract of a full-size recombinant Hoxa-2 protein (41 kD). Lane 2: crude bacterial extract of the carboxy-terminal fragment of recombinant Hoxa-2 protein (20 kD). The J3 peptide was derived from the carboxy-terminal region (see Fig. 1). Lane 3: brain; Lane 4: thymus; Lane 5: heart; Lane 6: kidney; Lane 7: liver; and Lane 8: lung.

findings confirm that the anti-J3 antibodies recognize the Hoxa-2 protein present in a crude bacterial extract. Furthermore, to test the specificity of anti-J3 antibodies in mice, protein extracts from whole embryos at different stages of development, 10 to 14 days after fertilization, as well as from several organs from 16-day-old mouse embryos, showed a single protein band at 41 kD (Fig. 2A and 2B). Results from western blot assays (Fig. 2A) also indicated decreasing Hoxa-2 protein levels as development progressed from 10- to 14-day-old mouse embryos, with the highest amounts detected in 12-day-old mouse embryos. This is similar to what was previously observed with highest poly(A)' RNA levels detected in 12-day-old mouse embryos (Tan et al., 1992; and our unpublished data). The strong correspondence between the levels of protein and poly(A)' RNA in the developing embryo suggests that Hoxa-2 expression is regulated at the transcriptional level. The tissue organs from the 16-day-old mouse embryos show highest level of Hoxa-2 protein in the heart and significantly lower levels in the brain (Fig. 2B).

In addition, anti-J3 antibodies stained cell nuclei in cryostat sections of the developing (Fig. 3b) and adult (Fig. 5b) mouse spinal cords. This finding is in agreement with the DNA binding and trans-activating functions known for the Drosophila and vertebrate homeodomain proteins (Gehring, 1987; Levine and Hoey, 1988; McGinnis and Krumlauf, 1992; Corsetti et al., 1995; Graba et al., 1997). The anterior limit of expression extending into the myelencephalon in a sagittal section of an E12 and an E14 embryo was the same for both the protein and RNA and omitting the anti-J3 antibodies from the immunostaining procedure resulted in only background staining (data not shown). Further evidence for antibody specificity has also been obtained by competition experiments where an excess amount of J3 peptide (200 µg/ml) was added to the incubation solution containing the anti-J3 antibodies; the resultant tissue sections showed only background staining (Fig. 3a).

The immunohistochemical and western blot results confirm the specificity of the anti-J3 antibodies in recognizing the Hoxa-2 protein in mouse tissue sections and in recognizing the Hoxa-2 recombinant protein from E. coli crude extracts. It is not known whether Hoxa-2 protein undergoes post-translational modification although many putative phosphorylation sites catalyzed by protein kinase C, cAMP-dependent protein kinase A or casein kinase II have been identified within the coding sequence (Tan et al., 1992). The Hoxa-2 protein from mouse embryo extracts had a similar molecular weight (41 kD) to the recombinant Hoxa-2 protein from crude bacterial extracts when analyzed by western blot assays (Fig. 2A and 2B) indicating that any post-translational modification does not alter the migration of the protein in an SDS-polyacrylamide gel.

Mid-Thoracic Spinal Cord of E10 to E14 Mouse Embryos

The anti-J3 antibodies were used to investigate the pattern of expression of the Hoxa-2 protein in the developing spinal cord of mice from five prenatal developmental ages including 10, 12, 14, 16, and 18-days
antibodies showed that the cytoarchitectural changes accompanied by a change in the expression pattern of the Hoxa-2 protein from ventral to dorsal parts of the developing grey matter (Fig. 4a). The dorsal-ventral pattern of Hoxa-2 protein expression from the ventral to dorsal regions is due to the movement of Hoxa-2 protein-expressing cells into the dorsal horn or as a consequence of de novo expression in the cells located in the dorsal horn of the spinal cord.

In an E10 mouse embryo, the neural tube consists mainly of a large inner layer of undifferentiated neuroepithelial cells, known as the ependymal layer (Kaufman, 1992). Immunohistochemical staining of spinal cord sections with anti-J3 antibodies showed that the cytoarchitectural changes that occur in the spinal cord during development is accompanied by a change in the expression pattern of the Hoxa-2 protein from ventral to dorsal parts of the developing grey matter (Fig. 4 and 5).

In an E10 mouse embryo, the neural tube consists mainly of a large inner layer of undifferentiated neuroepithelial cells, known as the ependymal layer (Kaufman, 1992). The Rat-401 staining (for nestin) shows an abundance of neuroepithelial stem cells in the E10 neural tube (Fig. 7c) and E14 neural tube (Fig. 8d). This antibody recognizes an epitope on nestin, an intermediate filament protein, expressed by progenitor cells of the developing CNS (Frederiksea and McKay, 1988; Friedman et al., 1990; Lendahl et al., 1990). As development progresses, most of the ependymal layer gets replaced by the growing mantle layer (the future grey matter of the cord) which consists of neural and glial progenitors cells. The distribution of the Hoxa-2 protein along the dorsal-ventral axis of the spinal cord at E10 mouse embryos showed intense staining in the two distinct ventrolateral regions (the basal plates) of the neural tube, corresponding to the presumptive ventral horns (Fig. 4a). No staining was detected in the more dorsal regions (the alar plates) of the mantle layer at this developmental age (Fig. 4a). The dorsal-ventral pattern of expression can also be clearly seen in sagittal sections (Fig. 4d). A higher magnification view of the basal plate (Fig. 1b) and the dorsal root ganglia (Fig. 4e) show intense staining in a majority of the cells in these two regions. Islet-1, a marker for motor neurons is also expressed in the basal plate in the E10 neural tube (Fig. 7b) exhibiting an overlapping domain of expression with that of Hoxa-2 positive cells (Fig. 4a, b, and Fig. 8d, e).

At 12 days after fertilization, a mid-thoracic section of the spinal cord (Fig. 4e) consists mainly of the ependymal and mantle layers and only a thin outer marginal layer (the future white matter of the cord) (Kaufman, 1992). At this stage, the mantle layer is enlarged in the region of the basal plate, whereas the alar plate (the future dorsal horn) is still relatively small, giving rise to a V-shaped appearance of the ependymal layer when viewed in cross-section. Accordingly, anti-J3 staining was visible mainly in the ventral and ventrolateral regions of the mantle layer (Fig. 4e, f). Both the sagittal and transverse sections revealed many more J3-positive cells in the basal plate (ventral region) (Fig. 4e, f, g).

By E14, the alar plates have encroached on the marginal zone such that the tip of each plate abuts upon the pial surface of the cord (Fig. 4h). As a consequence of development of the mantle layer, the number of ependymal cells is significantly reduced into a narrow layer surrounding the central canal. The marginal layer has increased substantially as a result of the appearance of ascending and descending pathways that traverse through this region of the cord. No anti-J3 staining was detectable in the marginal layer. At this developmental age, anti-J3-positive cells of the mantle layer displayed an intensity gradient from ventral to the dorsal region (Fig. 4h, i, and j). In the ventral horn, most of the larger cell bodies had intensely stained nuclei (Fig. 4j) whereas in the dorsal horn, in which there were a larger number of smaller, more closely packed cells, only a small percentage of the cells were J3-positive (Fig. 4k and l). Islet-1, positive motor neurons were also identified in the ventral horn in the E14 neural tube although the number of expressing cells had significantly decreased (Fig. 7g and Fig. 8h, i). The number of uncommitted neuroepithelial cells as indicated by Rat-401 nestin staining cells were mostly localized to the floor plate at this stage of development (Fig. 7j). It is not known whether the shift of Hoxa-2 protein expression from the ventral to dorsal is due to the movement of Hoxa-2 protein-expressing cells into the dorsal horn or as a consequence of de novo expression in the cells located in the dorsal horn of the spinal cord.

Mid-Thoracic Spinal Cord of E16 and E18 Mouse Embryos

At 16 and 18 days after fertilization, the mouse spinal cords had developed substantially such that the lumen of the central canal was barely visible and the central grey matter had assumed its characteristic butterfly shape. By E16 there were many more J3-positive nuclei in the dorsal horn (Fig. 5a, b). In the ventral horn, many cells were only weakly stained, especially in 18-day-old fetuses (Fig. 5d, f). In contrast, most nuclei in the dorsal horn were more intensely stained (Fig. 5d, f).

Based on cell size, the dorsal root ganglia of E16 and E18 mouse fetuses contained a heterogeneous population of cells. The nuclei of these cells were heterogeneously stained with the anti-J3 antibodies, with some cells showing intense nuclear staining while the nuclei
I NTRNAL ALONG THE DEVELOPING SPINAL CORD

Fig. 4. Immunohistochemical staining of sections of the mid-thoracic spinal cord from 10-day-old (a,b,c,d); 12-day-old (e,f,g) and 14-day-old (h,i,j,k) mouse embryos with anti-β specific antibodies. Sections in a,b,c,e,i,j,k are transverse sections of the mid-thoracic spinal cord and sections d,g, and k are sagittal sections of the mouse embryonic spinal cord. Hoxa-2 expressing cells were mainly located in the ventral spinal cord (basal plate) (a,b,d) and in the spinal ganglia (c) in 10-day-old mouse embryos. Section in (b) is a higher magnification of the ventral region (basal plate) in (a) and section in (c) is a higher magnification of the spinal ganglia in (a). As development progressed to 12-day-old mouse embryos, Hoxa-2 expressing cells were now also prominently visible in the dorsal region of the mantle layer (e and g). Section in (h) is a higher magnification of the ventral-lateral region of (a). The presence of Hoxa-2 protein remained and increased further in the dorsal horn in 14-day-old mouse embryos (i,j,k). At all three stages (10, 12, and 14 days) of development, Hoxa-2 expression remained in the ventral region of the mantle layer. Section in (i) is a higher magnification of the dorsal horn in (h) and section in (j) is a higher magnification of the ventral horn in (k). In g, e, and h dorsal is to the top, in d, g, and k dorsal is to the right. Abbreviations: c, central canal; ep, ependymal layer; ml, mantle layer; mg, marginal layer. Scale bar: 200 μm (a.b.h); 100 μm (b,c,e,i,j,k); 60 μm (d).
Fig. 5. Immunohistochemical staining of sections of the mid-thoracic spinal cord from 16-day-old (a,b,c) and 18-day-old mouse embryos (d,e) with anti-J3 specific antibodies. (f) is an in situ hybridization-stained section from the mid-thoracic spinal cord of an 18-day-old mouse embryo probed with Hoxa-2 digoxigenin-labeled antisense RNA (see Experimental Procedures). All sections except (g) are transverse sections of the mid-thoracic spinal cord. Section (d) is a sagittal section of the spinal cord from a 16-day-old mouse embryo. Hoxa-2 expressing cells were present in all of the mantle layer (dorsal and ventral regions) in 16-day-old mouse embryos (a,b, and c). Section in (f) is a higher magnification of the dorsal horn in (a). As development progressed to 18-day-old mouse embryos, Hoxa-2 expressing cells were more prominently visible in the dorsal region of the mantle layer (d and e). In the adult mouse spinal cord (g), expression of Hoxa-2 was more pronounced in the cells of the dorsal horn. Double-labeling of sections with an antibody against GFAP, indicated astrocytes present in the dorsal grey matter of the adult spinal cord (h) did not express Hoxa-2 (arrows point to astrocytes and their projections stained with GFAP (brown color) but no nuclear staining for Hoxa-2 was visible). In the same section other cells with a morphology that resembled neurons had clearly stained nuclei (black for Hoxa-2 staining). Section in (i) is a double-labeled section of the dorsal white matter from an adult mouse spinal cord. An occasional group of GFAP-stained cytoplasmic processes were closely associated with a Hoxa-2 positive cell (arrows). In a, d, and i dorsal is to the top; in c dorsal is to the right. Scale bar = 200 μm (a,c,d,h); 100 μm (b,g); 50 μm (i).
The expression patterns of the Hoxa-2 protein in the developing spinal cord, with its restricted expression in the basal plate in 10-day-old embryos followed by a gradual shift in expression toward the alar plate in E12 to E16 embryos (Figs. 4 and 5) parallels the spatial and temporal pattern of neuronal development in the spinal cord. The temporally related differences in expression between basal and alar portions of the mantle layer is intriguing since it is known that progenitor cells in these two regions give rise to different types of neurons. Neurepithelial cells in the ventral basal plate give rise to somatic motor neurons (both Aα and Aβ) to preganglionic autonomic neurons, and to a variety of interneurons (Tanabe and Jessell, 1986). The somatic and several motor neurons segment into columns and innervate their respective target muscle groups (Hoilet, 1980; Tsujiude et al., 1994). In the alar plate the progenitors differentiate into second order sensory projection neurons, which convey various types of sensory information to the brainstem and diencephalon, and to a large population of interneurons (e.g., substantia gelatiosa). Renshaw cells and 1a inhibitory interneurons which are located in the laminae VII and VIII of the cat spinal cord are two populations of ipsilaterally projecting interneurons (Scheibel and Scheibel, 1968; Jankowska and Lindstrom, 1972; Brown, 1981). Commisural interneurons are present throughout the dorsoventral axis of the spinal cord (Brown, 1981).

The motor neurons (basal plate derivatives) are the first major group of neurons to be generated in the developing spinal cord. These neurons are among the earliest cells of the spinal cord to differentiate into mature neurons with differentiation beginning prior to E10 in the mouse (Fig. 7b). In contrast, the interneurons of the dorsal horn (alar plate derivatives) are among the last neurons to be generated and to reach maturity (Altman and Bayer, 1984; Wentworth, 1984). The primordial pattern of Hoxa-2 protein expression along the dorsal-ventral axis of the thoracic portion of the neural tube is temporally and spatially modified, in congruence with the generation of each major class of neuron. This congruence would be consistent with a role of the Hoxa-2 protein in contributing to dorsal-ventral patterning in the spinal cord. A second possible role of Hoxa-2 is in contributing to the specification of neuronal phenotype in the grey matter of the developing spinal cord.

In the early developing spinal cord, dorsalizing signals from the surface ectoderm and roof plate along with ventralizing signals from the notochord and floor plate together specify cell types along the dorsal-ventral axis (Lumsden, 1991; Ericson et al., 1992; Yumada et al., 1992; Roelink et al., 1994; Marti et al., 1995; Dickinson et al., 1995; Lien et al., 1996; Chiang et al., 1996). The candidate dorsalizing signals are bone morphogenetic proteins (BMP), BMP-4 and BMP-7, both of which activate the expression of Pax3, Msh1, and Nog genes in the dorsal spinal cord (Lien et al., 1995). The candidate ventralizing signal is sonic hedgehog (Shh) (Roelink et al., 1994; Tanabe et al., 1995; Marti et al., 1995; Chiang et al., 1996; Ericson et al., 1996) which regulates the expression of transcription factors Pax3, Pax6, and Pax7 in the ventral spinal cord (Goulding et al., 1993, 1994). From these and other studies a general model has been put forward where signaling by Shh and BMP-4/7 regulate early patterning genes which in turn regulate downstream target genes in subsets of neurons along the dorsal-ventral axis to specify their phenotype and/or function (for review see Tanabe and Jessell, 1996). Hence combinations of transcription factors likely determine the identity of differentiating cells in the nervous system for

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**DISCUSSION**

The expression patterns of the Hoxa-2 protein in the developing cord, with its restricted expression in the basal plate in 10-day-old embryos followed by a gradual shift in expression toward the alar plate in E12 to E16 embryos (Figs. 4 and 5) parallels the spatial and temporal pattern of neuronal development in the spinal cord. The temporally related differences in expression between basal and alar portions of the mantle layer is intriguing since it is known that progenitor cells in these two regions give rise to different types of neurons. Neurepithelial cells in the ventral basal plate give rise to somatic motor neurons (both Aα and Aβ), to preganglionic autonomic neurons, and to a variety of interneurons (Tanabe and Jessell, 1986). The somatic and several motor neurons segment into columns and innervate their respective target muscle groups (Hoilet, 1980; Tsujiude et al., 1994). In the alar plate the progenitors differentiate into second order sensory projection neurons, which convey various types of sensory information to the brainstem and diencephalon, and to a large population of interneurons (e.g., substantia gelatiosa). Renshaw cells and 1a inhibitory interneurons which are located in the laminae VII and VIII of the cat spinal cord are two populations of ipsilaterally projecting interneurons (Scheibel and Scheibel, 1968; Jankowska and Lindstrom, 1972; Brown, 1981). Commisural interneurons are present throughout the dorsoventral axis of the spinal cord (Brown, 1981).

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**FIG. 6**. GFAP-positive astrocytes (brown color) in free-floating sections of the dorsal horn of adult mouse spinal cord (a,b) and in cell cultures isolated from the neopallia of newborn mice (c,d). The anti- β3 antibody, we find that oligodendrocyte progenitor cells were predominantly Hoxa-2 positive (Fig. 7a) but cells in this lineage did not stain for Hoxa-3. Astrocytes (brown color) in neopallial cultures, although not all astrocytes expressed Hoxa-2 (d), Cells exhibiting only Hoxa-2 positive nuclei (blank color) are seen in the vicinity of the dorsal horn. GFAP-positive astrocytes (brown color) in free-floating sections of the adult spinal cord (a,b) and in cell cultures also contained Hoxa-2 negative astrocytes (Fig. 6d). Astrocytes were also negative for Hoxa-1 (unpublished data. Fig. 8k) and Pax-2 expression (Fig. 8l). Cultures were also double-labeled with 0.1 monoclonal antibody to marker for oligodendrocyte progenitor cells, Sommer and Schachter, 1981), and the Hoxa-2 anti-β3 antibody. We find that oligodendrocyte progenitor cells were predominantly Hoxa-2 positive (Figs. 7b,h) but cells in this lineage have started to develop more extensive array of cytoplasmic processes were less likely to be Hoxa-2 positive (Fig. 7k).
Fig. 7. Cryostat sections of the neural tube in the thoracic region of a 10-day-old mouse embryo (a–c) and a 14-day-old mouse embryo (d–g). The cryostat sections were stained with cresyl violet acetoaldehyde (a,d) or with monoclonal antibodies against islet-1 (b,g) and nestin (c,i). The arrowhead in (c) denotes an area in the ventral region (basal plate) of the E10 neural tube in which there is a paucity of nestin-positive cells; note that this general region is also where there is the highest density of islet-1-positive cells. The remaining frames are photomicrographs of glioma cell cultures initiated from E15 mouse embryos. These cell cultures were double-labeled with the O4 monoclonal antibody (e,h,k) and with the anti-J3 specific Hoxa-2 polyclonal antibody (f,i,j) depicting the fluorescent and bright field images of the same fields. The arrowheads in (e),(f),(h),(i) point out oligodendrocyte progenitor cells that are identified by virtue of being O4 positive. Scale bars — 50 μm (a–c,d,g) and 25 μm (f,i,j); these bars apply also for e,h, and k respectively.
Fig. 8. Double-labeled immunohistochemical stained transverse sections of the spinal cord from 10-day-old (a–e) and 14-day-old mouse embryos (f–i). Figure 8a,b,c, and g, exhibit islet-1 expressing ventral motor neurons (black stained nuclei) and Pax-2 expressing intermediate neurons (reddish-orange stained nuclei). Section in (b) is a higher magnification of the basal plate in (a) and section in (c) is a higher magnification of (b). Two distinct groups of neurons express islet-1 and Pax-2 with no overlap in their expression domains. Figure 8d,e,h, and i, exhibit overlapping domains of expression of Hoxa-2 (black stained nuclei) and islet-1 (reddish-orange stained nuclei). Cells exhibiting co-localization of Hoxa-2 and islet-1 (arrowheads shown in Fig. 8e and h) are also visible. Section in (e) is a higher magnification of the basal plate in (d) and section in (i) is a higher magnification of the ventral horn in (h). Figure 8f is a double-labeled immunohistochemical section of the ventral horn exhibiting Hoxa-2 (black-stained nuclei) and nestin (reddish-orange neuroepithelial stem cells). Astrocytes from neopallial cultures were double-labeled with Hoxa-2 and GFAP (j), Hoxd-1 and GFAP (k), and Pax-2 and GFAP (l). Hoxd-1 and Pax-2 were not detected in astrocyte cultures whereas Hoxa-2 positive nuclei were visible (arrowhead in Fig. 8j). Scale bar = 100 μm (a–i) and 25 μm (j–l).

Shh signaling has been reported to cause ventral progenitors to give rise to motor neurons, while inhibition of Shh signaling leads instead to the generation of ventral interneurons (Ericson et al., 1996). One ventralizing inductive effect of Shh is in downregulating the expression of several transcriptional genes (i.e., Pax3, Pax7, Msx1, and Msx2) in the medial portion of the neural groove, prior to neural tube closure, in what will become the ventral portion of the mantle region of the developing spinal cord (Goulding et al., 1993; Ericson et al., 1996). The Hoxa-2 protein, however, is expressed in the ventral mantle region after the Pax3, Pax7, Msx1, and Msx2 genes have been downregulated by Shh. The continued expression of Hoxa-2 after Shh has exerted its ventralizing inductive effect raises the question as to whether Shh might induce progenitor cells in the
Hox genes exhibit early and dynamic domains of expression at the caudal levels of the developing spinal cord (Wilkinson et al., 1989; Aase and Robert, 1990; Kessel and Gruss, 1991; Sundin and Eichele, 1992; Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994; Krumlauf, 1994; Mark et al., 1997: Belting et al., 1998). Coordinated expression of Hox genes may augment and refine the pre-existing positional value established at the neural plate stages as well as determine neuronal phenotype. Several Hoxb cluster genes exhibit dynamic dorsal-ventral patterns of expression in the developing spinal cord (Graham et al., 1991; Krumlauf et al., 1991; Keynes and Krumlauf, 1994). At E10.5, the Hoxb1, b3, b4, b6, b7, b8 and b9 genes are all expressed uniformly across the spinal cord and this pattern is observed all along the axis within the anterior-posterior domain of expression for each gene (Graham et al., 1991). As development progresses to E11.5, Hoxb genes show a lateral expression pattern in the region where interneurons are born (Wentworth 1984). At this stage, none of the Hoxb genes were expressed in the presumptive ventral motor horn. At E12.5 stage of development, Hoxb genes exhibit a dorsally restricted expression pattern and this changes at E14.5 when expression reappears in the ventral region (Graham et al., 1991). The Hoxa4, a5, and c6 genes and Hoxb and c6 genes also exhibit dorsal-ventral pattern of expression in the neural tube of E12.5 embryos (Gaunt et al., 1990; Gaunt, 1991). The Hoxa genes in E12.5 spinal cords exhibited a ventral and medial domain of expression whereas the Hoxb genes show a ventral and central pattern of expression in the spinal cord at this stage.

The above findings are complementary to what we have observed with the Hoxa-2 protein but with some important differences. The uniform diffuse expression of Hoxb genes within the spinal cord at E10.5 does not necessarily imply its role in generating ventral motor neurons as was proposed by Graham et al. (1991). However, at this stage of development, Hoxa-2 protein is mainly restricted to the basal plate (Fig. 3a, b) and its expression matches closely with the motor neuron specific transcriptional factor, islet-1 (Erickson et al., 1992) (Fig. 7b, Fig. 8d, e, h, i). At E11.5, Hoxb genes exhibit expression in the lateral region where commissural neurons also develop and the remaining dorsolateral region of the spinal cord exhibits low but uniform expression (Graham et al., 1991). Interestingly, at about the same stage of development (E11.5–E12), cells in the lateral regions also exhibit Hoxa-2 protein expression but cells in the ventral motor horns also continue to exhibit Hoxa-2 expression (Fig. 6e, f, g). Between E12.5 and E14.5, the Hoxb genes exhibit dorsally-restricted expression with the exception that at E14.5, Hoxb genes show new ventral domains of expression. The Hoxa-2 protein expressing domain continues to gradually extend to the dorsal horn between E12 (Fig. 6e, f, g) and E14 (Fig. 4h, j, k) and by E16 stage of...
Hoxa-2 protein along the developing spinal cord. Schematic diagrams of the spinal cords at the mid-thoracic level indicate that in 10-day-old mouse embryos (E10) expression of Hoxa-2 protein is restricted to the basal plate (presumptive ventral horn) and expression extends to the alar plate (presumptive dorsal horn) in 12-day-old embryos (E12). Hoxa-2 expressing cells were found in both the dorsal and ventral horns in 14-day (E14) and 16-day-old (E16) mouse embryos. However the intensity of staining in 14-day-old embryos was greater in the ventral horn than in the dorsal horn. This pattern of expression was gradually reversed as development progressed where Hoxa-2 protein expressing cells in 18-day-old fetuses (E18) and in adult mice (A) were much more prominently expressed in the dorsal grey matter compared to the ventral grey matter. Abbreviations: a, alar plate; b, basal plate; c, central canal; dh, dorsal horn; vh, ventral horn.

Interestingly, Hoxa4, a5, and a6 genes which predominantly exhibit ventral and lateral expression at E12.5 (Gaunt et al., 1990; Gaunt, 1991) is similar to what we have observed with the Hoxa-2 protein at this stage of development (Fig. 4e, g). Hence our results confirm that Hox genes within each cluster have similar dorsal-ventral expression patterns but that this varies between Hox genes in different clusters (Gaunt, 1991). In addition, the coordinated temporal and spatial expression of Hox genes in a dorsal-ventral spinal cord is consistent with the generation of spinal neurons. Since the Hoxb genes also show new ventral domains of expression at E14.5, Graham et al. (1991) have proposed that this ventral re-expression reflects gliogenesis. This is interesting as we have observed Hoxa-2 expressing astrocytes (Fig. 6c, Fig. 8j) as well as Hoxa-2 expressing oligodendrocyte precursor cells in culture (Fig. 7a, b, c, d).

Ectopic expression or targeted deletion of Hox genes at the hindbrain levels induce changes in cranial motor neuron identity (Zhang et al., 1994; Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1998) hence it is possible that the identity of cells at more caudal levels of the neural tube may also be affected. Observations of Hox gene mutants suggests that Hox genes can contribute to the establishment of patterning within the spinal cord. For instance in the Hoxa-10 mutants, there is a shift in the segmental position of the lateral motor (LMC) neurons (Carpenter et al., 1997). Hoxa-10 mutants show alterations in the peripheral nerves (Rijli et al., 1995) reflecting circumambient alteration in the spinal cord. Hoxc-8 mutants possess defects in axonopod clenching due likely to defects in motor neurons at the brachial levels (LaMoureille et al., 1992). Recent evidence indicates that in the Hoxa-8 mutant mice, C7-T1 motor neurons exhibit enhanced apoptosis, subsequently leading to a decrease in functional motor neurons and an irreversible alteration of motor neuron pools (Tiret et al., 1998). Hence Hox genes are involved in patterning the nervous system, rostrally at the level of the developing hindbrain and caudally along the entire length of the emerging spinal cord.

Targeted mutations described for Hoxa-2 did not detail an analysis of the differentiation of spinal cord neurons (Gendron-Maguire et al., 1993; Rijli et al., 1995).
Multiple head and cranial abnormalities were identified in the homozygous *Hoxa-2* mutant mice (Gendron-Maguire et al., 1993; Rijli et al., 1993; Mallo and Gridley, 1995). Some of the abnormalities include a bilateral cleft of the secondary palate and defects in the formation of the middle and external ears. The stapes, the styloid process, and the lesser horn of the hyoid bone, all derived from the neural crest cells of the second branchial arch, were absent. This selective disruption of the development of second branchial arch derived structures corresponds to the rostral expression domain of *Hoxa-2* gene in the hindbrain (Krumlauf, 1993; Wilkinson, 1993; Nonchev et al., 1996). In contrast, the malleus, incus, tympanic, squamosal bones, and Meckel's cartilage derived from the neural crest cells of the first branchial arch were duplicated. This suggests that the loss-of-function of the *Hoxa-2* gene affects the specification of the neural crest cells such that derivatives of the second branchial arch undergo a homeotic transformation to derivatives of the first branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993). Recently Kanzler et al., (1998) have reported that *Hoxa-2* directs proper skeletal formation in the second branchial arch by inhibiting chondrogenesis and that in *Hoxa-2*-/- embryos, chondrogenesis is ectopically activated in the rostral expression domain of *Hoxa-2* resulting in mutant set of cartilages. *Hoxa-2* has also been implicated in regulating axonal guidance, in that rhombomere 2 (r2) and rhombomere 3 (r3) trigeminal motor axons failed to exit the hindbrain at the proper location from r2 but instead exit caudally from the r4 exit point in *Hoxa-2*-/- embryos (Gavalas et al., 1997). In addition, *Hoxa-2*-/- embryos exhibit a reduction in alar plates resulting in hindbrain defects. The cerebellum was found to be enlarged and pontine structures correspondingly reduced implying that fate changes in alar plates is likely responsible for these abnormalities (Gavalas et al., 1997). The differential expression of the *Hoxa-2* protein between the alar and basal plate derivatives (Fig. 3) of the developing spinal cord, as shown in the present study, could be contributing to dorsal-ventral patterning and specifying neuronal identity as well as influencing axonal growth by the projection neurons of this part of the neural tube. Further investigations are required to determine if a loss-of-function of the *Hoxa-2* gene affects the specification of the neural crest cells in the caudal rhombomeric regions of the developing neural tube or in the development of spinal cord neurons from the more caudal parts of the neural tube.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**

The J3 oligopeptide (SPLTSNEKNLKHFQHQS) derived from the coding sequence of the *Hoxa-2* protein (Tan et al., 1992) shown in Fig. 1 was synthesized on an Applied Biosystems (Foster City, CA) model 430A peptide synthesizer using t-Boc (tert. butyloxycarbonyl) amino acids (for review see Barry and Merrifield, 1980). The peptide was cleaved from Pam (3-(oxygen- methyl)phenylacetic acid amido methyl)-resin via the HF procedure (Tam and Merrifield, 1985). The cleaved peptide was purified by ion-exchange HPLC and purity of peptide determined by HPLC. Amino acid composition of the J3-peptide was determined by both amino acid analysis and mass spectrometry.

**Preparation of the Immunogen**

The J3 peptide was conjugated to bovine serum albumin (BSA) as described in Yeung et al., (1996). Briefly, 1 ml of ice-cold 0.05 M sodium phosphate (pH 7.0) and 100 mg of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to 30 mmol of the J3 peptide. After mixing, the reaction mixture was frozen at -20°C. Bovine serum albumin (28 mg), dissolved in the same phosphate buffer (1.5 ml), was added to the frozen peptide-peptide mixture. The reaction mixture was then placed on an orbital shaker for 18 hr at 4°C. The resultant conjugate was dialyzed against running water for 48 hr and stored frozen in 1 ml aliquots.

**Immunization Procedure**

Two groups of three male New Zealand White rabbits were used to obtain polyclonal antibodies against the J3 peptide hapten-bovine serum albumin conjugate. The immunogen was diluted in phosphate buffered saline (PBS) and emulsified in Freund's complete or incomplete adjuvant (Gibco, Grand Island, NY) to yield a concentration of 1 mg/ml. Rabbits were injected subcutaneously with 0.5 ml of the complete adjuvant emulsion at four sites. Booster injections were given at 4-week intervals, substituting incomplete adjuvant for complete adjuvant. Serum titres were monitored for the presence of peptide-specific IgGs by enzyme-linked immunoadsorption assays (ELISAs) as described in Yeung et al., (1996) using peptide conjugated to bovine serum albumin (BSA, pentax fraction V) via 1-ethyl-3-(dimethylaminopropyl) carbodiimide as antigen. Animals having the highest titres and most sensitive inhibition curves were exsanguinated under anesthesia from four to six months after the initial immunization. For long-term storage, the serum was kept frozen at -20°C in 10 ml aliquots. Once thawed, an equal volume of 50% glycerol was added and the solution was stored at -20°C. The glycerolated antibodies can be used for at least 6 months with no deleterious effect. The animals yielding high titre anti-J3 antibodies were chosen for use in our study.

**Western Blot Analysis**

Embryonic tissues were rinsed twice with ice-cold PBS and proteins extracted using TriReagent® (Molecular Research Center, Inc., Cincinnati, OH). Sample protein contents were measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Aliquots of 30 μg protein extracts from tissue samples were separated on a 10% polyacrylamide-SDS...
Additional corona sections of the adult spinal cord (8 μm thick, cut on a cryostat) were collected onto gelatin-coated coverslips.

A modification of the method of Zhang and Fedoroff (1997) was used for immunohistochemical analysis. All incubations and washes were performed at room temperature unless indicated otherwise. Briefly, tissue sections were rehydrated in PBS (two changes of 10 min each) and incubated in 3% solution of skim milk powder and 0.1% Triton X-100 in PBS (5% SM-PBS). The sections were then incubated overnight at 4°C with the anti-J3-specific antibodies at a dilution of 1:500 (cryostat sections) or 1:1000 (free-floating sections) in 1% solution of skim milk powder and 0.1% Triton X-100 in PBS (1% SM-PBS). The sections were then incubated at room temperature for 45 min with the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:500 (cryostat sections) or 1:200 (free-floating sections) in 1% SM-PBS, and then incubated in PBS for 20 min followed by an incubation in 0.3% hydrogen peroxide in methanol for 15 min to eliminate endogenous peroxidase activity. Sections were further washed in PBS (two washes for 10 min each) and then incubated in 3% SM-PBS for 30 min. This was followed by a one-hr incubation with avidin-biotin complex (Vectastain ABC; Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:125 in PBS. Sections were washed in PBS for 10 min followed by either washing in distilled water and direct staining using the Vector<sup>®</sup> Nova RED<sup>®</sup> kit or once again washed in PBS, for 10 min, followed by a 10 min incubation in 0.175 M sodium acetate solution for DAB staining. For DAB staining a 1 to 5 min incubation was performed in a horseradish peroxidase substrate solution (0.175 M sodium acetate solution containing 0.05% 3,3'-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO), 1.5% nickel sulfate and 0.03% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>),). The DAB treated sections were finally washed in 0.175 M sodium acetate solution for 10 min and in PBS for a further 10 min. At this point a majority of the cryostat sections were counterstained with nuclear fast red (unless they were to be further processed for double labeling, see below), dehydrated and mounted in Permount<sup>®</sup> (Fisher Scientific, Fair Lawn, NJ).

The free-floating or cryostat sections from the adult mouse spinal cord were then processed for immunohistochemical staining with a rabbit antiserum to glial

**Immunohistochemical Analysis**

Our study was based on an analysis of > 200 embryos which were staged according to Kaufman (1982). Mouse embryos dissected from timed-pregnant Swiss-CD-1 mice (the morning on which a vaginal plug was found was considered day zero of pregnancy) were fixed overnight in 4% paraformaldehyde in PBS. The following day, embryos were rinsed (three times in 20% sucrose solution containing 0.05% 3,3'-diaminobenzidine (DAB: Sigma Chemical Co., St. Louis, MO), 1.5% nickel sulfate and 0.03% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>),). The DAB treated sections were finally washed in 0.175 M sodium acetate solution for 10 min and in PBS for a further 10 min. At this point a majority of the cryostat sections were counterstained with nuclear fast red (unless they were to be further processed for double labeling, see below), dehydrated and mounted in Permount<sup>®</sup> (Fisher Scientific, Fair Lawn, NJ).
fibrillary acidic protein (GFAP; DAKO, Denmark), an astrocyte-specific intermediate filament protein, or with the islet-1 monoclonal antibody (clone 34.4D51) or with the Rat-401 monoclonal antibody to nestin. A 0.5 M solution of DAB (in the presence of 0.01% H2O2 in 0.05 M PBS) was used as the chromogen to localize the antibody binding sites, as described in Doucette (1993) producing a black reaction product or the Vector® Nova RED® kit was used to produce a red reaction product. For DAB-stained sections the anti-J3 staining was black in color and was confined to the nucleus of Hoxa-2 expressing cells, whereas the anti-GFAP staining was brown in color and was confined to the cytoplasm of astrocytes. The astrocyte cell cultures were also double-labeled with Hoxa-2 and GFAP or Hoxd-1 and GFAP or Pax-2 and GFAP antibodies, using the same protocol as described above (Fig. 8j,k, and l). In addition, cryostat sections of E10 and E14 neural tubes were also double-labeled with Hoxa-2 and islet-1 (Fig. 8d,e,h,i) or Pax-2 and islet-1 (Fig. 8a—g) or Hoxa-2 and Rat-401 (Fig. 8l) as described above.

The O4 mouse monoclonal antibody (O4 hybridoma; IgM; Sommer and Schachner, 1981) was used to identify oligodendrocyte progenitor cells in cultures isolated from the cerebral hemispheres of E18 mice. This antibody recognizes a cell surface epitope that is expressed by glial cells in the O-A cell lineage (Gard and Pfeiffer, 1989; Trotter and Schachner, 1989). For the O4 staining, the incubation was done at 4°C using live cells. The antibody was diluted 1:10 in MEM containing 10% horse serum and the living cells stained as described in Doucette and Devan (1994). After fixation and prior to being stained with an FITC-conjugated goat anti-mouse IgM (1:200), the cell cultures were processed for immunohistochemical staining with the rabbit polyclonal antibody to Hoxa-2 (anti-J3 antibody), as described above for the astrocyte cell cultures. Staining with the FITC-conjugated secondary antibody was done after the DAB/nickel sulfate/H2O2 incubation. The O4 staining was black in color and was confined to the nucleus of Hoxa-2 expressing cells, whereas the O4 staining was confined to the entire plasma membrane of oligodendrocyte progenitor cells.

In Situ Hybridization Histochemistry

Mouse embryos from Swiss-CD-1 mice were frozen and covered in embedding medium (OCT, Tissue-Tek, Miles Inc., Elkhart, IN) and serial cryostat sections, 8-μm thick were cut. We used a 518 bp Hoxa-2 gene fragment (nucleotide residues 1,681-2,199 from outside the homeobox region (Tan et al., 1992) that had been subcloned into pBluescript II SK (+) (Stratagene) as the template for the synthesis of RNA probes. The digoxigenin-labeled RNA probes were synthesized by incorporation of digoxigenin-UTP (Boehringer-Mannheim Biochemicals, Indianapolis, IN) catalyzed by T7 or T3 RNA polymerase. A modification of the method of Straehle et al., (1994) was used for in situ hybridization histochemistry. The tissue sections on slides were washed in PBS prepared with DEPC (diethylpyrocarbonate-treated water). Sections were then hybridized with digoxigenin-labeled antisense Hoxa-2 RNA probe (1:1000) diluted in hybridization solution, consisting of 50% formamide, 3 M sodium chloride, 10 mM phosphate buffer (pH 7.4), 10 mM EDTA, 10 mM Tris-HCl (pH 7.5), 10% dextran sulphate, 1 mg/ml tRNA, and 1× Denhardt's solution, at 55°C overnight in a humidified chamber. After hybridization, all slides were washed four times in 2× SSC (sodium chloride/sodium citrate) with 50% formamide at 65°C for 15 min each, followed with four more washes in 1× SSC and a final wash in 0.5× PBS at 65°C for 15 min each, respectively. The slides were then transferred to PBS at room temperature for 5 min and non-specific sites blocked by a further 30 min wash in PBS with 0.1% Tween 20 (PBT) containing 0.2% BSA. This was followed by an incubation at room temperature for 30 min in alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer-Mannheim), at a dilution of 1:400 in PBT. The anti-digoxigenin antibody had been pre-absorbed with mouse tissue powder prior to use (Harlow and Lane, 1988). The slides were then washed in PBT (four times for 15 min each) and incubated in a light-tight box in a staining solution to allow the color to develop. The staining solution consisted of 0.34 mg/ml nitroblue tetrazolium chloride (NBT; GIBCO BRL, Burlington, Ontario), 0.175 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; GIBCO BRL, Burlington, Ontario), 100 mM sodium chloride, 50 mM magnesium chloride, 100 mM Tris-HCl (pH 9.5), and 0.1% Tween 20.

Cell Cultures

To obtain cell cultures enriched in astrocytes, newborn (1–2 day old) mouse neopallia, after being cleaned of meninges, olfactory bulbs, basal ganglia and hippocampal formation, were diced into small pieces and passed through a 75 μm Nitex mesh (Doucette, 1993). The cells were planted onto glass coverslips inside 35 mm Falcon petri dishes at dilutions of 1 × 10^5 to 1 × 10^6 viable cells per ml. The cultures were fed with DMEM/F12 (1:1) containing 10% FBS (HyClone; Logan, UT) and were incubated in 95% air/5% CO2 humidified atmosphere at 37°C for 5–7 days. To obtain cell cultures of oligodendrocyte progenitor cells we used E15 mouse embryos, from which we dissected the entire cerebral hemisphere. The tissue was handled as described above for the neopallial tissue and the cell suspensions were plated down at 1 × 10^5 viable cells per ml. The cell cultures were harvested for immunohistochemical staining after 2–3 weeks incubation in vitro.

ACKNOWLEDGMENTS

Supported in part by research grants from the Natural Sciences and Engineering Research Council of Canada and the Health Services Utilization and Research Commission (to A.N.) and from the National Institute on Deafness and Other Communication Disorders (No. R01 DC 02370-04). National Institutes of
Health (to R.D.J.) We would like to thank Dr. Marshall Xiremburg, NIH, for reviewing the manuscript. We acknowledge the technical assistance of Ms. Cindy Farrar. The Rat-401 and Iisbol-1 monoclonal antibodies, which were developed by Drs. S. Hockfield and T. Jessell respectively, were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins’ University School of Medicine, and the Department of Biological Sciences, University of Iowa, under contract NO1-HD-8-3144 from the NICHD.

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NOTE ADDED IN PROOF

The following complementary articles on Hoxa-2 regulation were recently published:


