HOXA2, HOXD1 AND PAX6 IN THE DEVELOPING MURINE BRAIN AND NEURAL RETINA

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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.

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LIST OF ABBREVIATIONS

AP	anterior-posterior
bHLH	basic helix-loop-helix
Bmps	bone morphogenic protein
CGE	caudal ganglionic eminence
CH	cortical hem
CNS	central nervous system
COR	cornea
CP	choroid plexus
CSB	cortical striatal boundary
CX	cortex
d	diencephalon
DiI	1,1'-dioctade-cyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
dLGE	dorsal lateral ganglionic eminence
DOV	dorsal optic vesicle
DP	dorsal pallium
dt	dorsal thalamus
DV	dorsal-ventral
E	embryonic day
ep	epithalamus
FgFs	fibroblast growth factors
FgF8	fibroblast growth factor 8
Gcl/GCL	ganglion cell layer
hb	hindbrain
hp	hypothalamus
INL	inner nuclear layer
L /LE	lens
LFC	lens fiber cells
lge/LGE	lateral ganglionic eminence
LP	lateral pallium
LV	lens vesicle
mb	midbrain
MZ	marginal zone
mge/MGE	medial ganglionic eminence
MP	medial pallium
NB	newborn
NR	neural retina
ON	optic nerve
ONL	outer nuclear layer
OS	optic stalk
OV	optic vesicle
p(1-6)	prosomeres 1-6
pINL	presumptive inner nuclear layer
PND	postnatal day
POV	proximal optic vesicle

RPE	retinal pigmented epithelium
RT-PCR	reverse transcriptase polymerase chain reaction
SE	surface ectoderm
Sey	*small eye*
Shh	sonic hedgehog
SVZ	subventricular zone
t	thalamus
v	third ventricle
vLGE	ventral lateral ganglionic eminence
VP	ventral pallium
vt	ventral thalamus
V/VZ	ventricular zone

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 likeliness 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 (Krumlauf, 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,

2004). Thirty-nine clustered vertebrate homeobox genes, which are referred to as the *Hox* genes, have been identified to date by virtue of their homology to the *Drosophila* HOM-C genes. Based on their sequence similarity with the HOM-C genes and their chromosomal position, the vertebrate *Hox* genes are organized into four separate clusters (*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 *et al.*, 1989) (see Fig. 1). These clusters of *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 *Pax* family of transcription factors (Mark *et al.*, 1997). Many of these genes also play a vital role in embryogenesis, especially in regards to the development of the eye.

2.2.1 Colinearity

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 *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 *et al.*, 1991; Dekker *et al.*, 1993) (Fig.1). *In-situ* hybridization illustrates the colinear manner in which *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 *et al.*, 1989; reviewed in Akin and Nazarali, 2004).

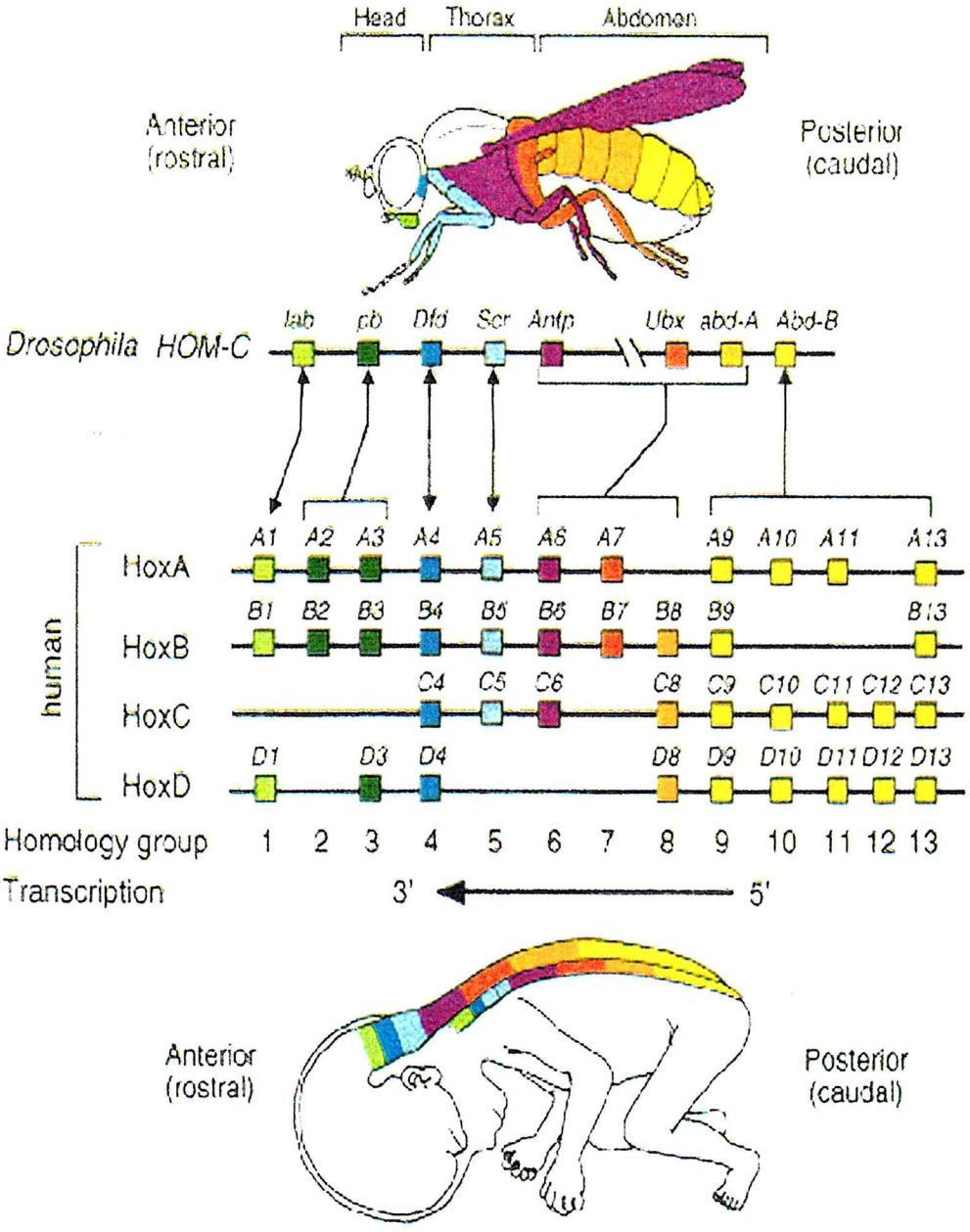

Figure 2.1 Schematic illustration of the colinear expression of the paralog cluster of *Drosophilia* homeotic genes *(HOM-C)* and the four mammalian *Hox* gene clusters. Each paralog gene represented in the different mammalian clusters and its related *Drosophila* HOM-C complex is denoted by the same 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 (*Hoxa1, 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).