HOX GENES AND NEURONAL DIFFERENTIATION DURING LEECH DEVELOPMENT

by

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ABSTRACT

HOX GENES AND NEURONAL DIFFERENTIATION DURING LEECH DEVELOPMENT

By

Rajendra Gharbaran

Adviser: Dr. Gabriel O. Aisemberg

One of the most fascinating and important biological findings over the past two decades was the discovery of the Hox gene family. These genes code for transcription factors that regulate the expression of other genes to bring about the complex organization of the nervous system, their main site of expression. Using an improved technique for antibody purification, I carried out a detailed characterization of the expression of two leech Hox genes (*Lox1* and *Lox2*) and studied the role of *Lox1* in the differentiation of a pair of specialized motor neurons (RPE neurons) that innervate the male sex organ.

Lox1 expression was detected in segmental ganglia R4 to M13. *Lox2* expression was detected in segmental ganglia M6 to M21. From M8 to M13, the number of $Lox1^+$ nuclei was found to decrease while the number of $Lox2^+$ nuclei remained large. These observations suggest that *Lox2* may down-regulate *Lox1* expression.

In addition, double-staining experiments led to the identification of several central neurons that express *Lox1* or *Lox2*. These experiments used antibodies that recognize

neuronal markers (FMRFamide, serotonin, enkephalin, and Laz1-1 antigen) in combination with *LOX1* and *LOX2* antibodies. The *Lox1*-positive (*Lox1*$^{+}$) neurons identified in this manner included the AMS and PMS neurons of M2 and M3, the RPE neurons of M6, the cell 261 of M7-M12, and the HAL neurons of M4. Among the identified neurons that expressed *Lox2* were the Rz neurons of M8-M21, the PMS neurons of M7-M21, the RMV and CV neurons of M7-M21, and the AP, NUT, and HAL neurons of M7-M17.

Lox1 expression was also detected in the primordia of the male and female sex organs. Because the sex organs are known to provide guidance cues to the axons of neurons in the nearby sex ganglia (M5 and M6), this observation suggested that *Lox1* may control the expression of signaling molecules (by the sex organs) that would be required for axon guidance, target recognition and neuronal differentiation.

RNA interference (RNAi) experiments suggested a role for *Lox1* in controlling the differentiation of the RPE neurons, segment-specific motor neurons of ganglion M6 that innervate the male sex organ. Knockdown of *Lox1* expression caused a decrease in FMRFamide staining in the RPE neurons, as well as axonal defects.

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CONTENTS

LISTS OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

One of the most fascinating and important findings in biology over the last two decades or more was the discovery of the Hox gene family. These genes code for transcription factors that regulate the expression of other genes to bring about the otherwise complex organization of metazoans. Initially discovered in *Drosophila*, where their mutations cause serious and widespread alterations in segmental identities, these genes are master architects of the body plan of all vertebrates and invertebrates studied thus far (Lemons and McGinnis, 2006). They are recognized by a characteristic, highly-conserved 180-base pair (bp) sequence termed the homeobox, which encodes a 60-amino acid DNA-binding domain called the Homeodomain (HD), making them part of the larger class of HD-containing proteins. A number of remarkable and distinctive features have emerged from the study of Hox genes in many diverse animals. Firstly, they are always arranged or organized in gene clusters or complexes. Secondly, they are expressed in specific regions along the main or anterior-posterior (A-P) body axis, in the same relative order as they are arranged in the chromosome. And thirdly, these genes are activated in a temporal sequence that mirrors their locations in the clusters.

In this chapter, current knowledge about Hox genes will be explored, including information about their biochemistry, evolution, expression patterns, cluster organization, and role in the development of the nervous system. In addition, the organization of the

leech body plan, the anatomy of the leech CNS, and the embryonic development of the leech will be discussed.

Homeobox, Homeodomain, and Homeodomain-DNA Interaction

HOX proteins belong to a large class or superfamily of transcription factors that contain a highly conserved DNA-binding domain. The DNA-binding domain, called the homeodomain (HD) is encoded by a 180 bp nucleotide sequence, the homeobox. The DNA-binding motif of HD-proteins is crucial for regulating the activities of other genes (downstream target genes) by either inhibiting or activating their expression, a property that makes the Hox genes major developmental regulators of the body plan of all metazoans examined thus far (Gehring, 1987). HD-containing proteins are responsible for sequence-specific DNA recognition (Affolter *et al*., 1990; Hayashi and Scott, 1990), forming an HD-DNA complex. Studies using nuclear magnetic resonance -NMR- (Otting *et al*., 1990) and X-ray crystallography (Wolberger *et al*., 1991), which included HD proteins from Hox and non-Hox genes, suggest that, despite striking differences in the primary structure, the HD has a conserved tertiary structure. The three-dimensional structure of the HD of the *Drosophila* Hox gene *Antp* has been well studied using both NMR and X-ray crystallography. Qian *et al*. (1989) have shown that the HD of *Antp* consists of four alpha helices (Fig. 1-1), of which helices 2 and 3 form a variation of a helix-turn-helix (HTH) motif analogous to the repressor HTH of prokaryotes, followed by an N-terminal arm. Helix 3, termed the recognition helix, is inserted into the major groove of the DNA, where it binds to specific nucleotide sequences; additional base contacts are made by the N-terminal arm (Gehring *et al*., 1994), which reaches into the adjacent minor groove (Fig. 1-1 B). More recent data from at least two HOX proteins

(*Dfd* and *Ubx*) suggest a more precise role of the N-terminal arm, whose position determines the binding mode and the interaction of the recognition helix with the nucleotides in the major groove (Frazee *et al*., 2002). The other two helices serve to help stabilize the position of the third helix by orienting it into the major groove.

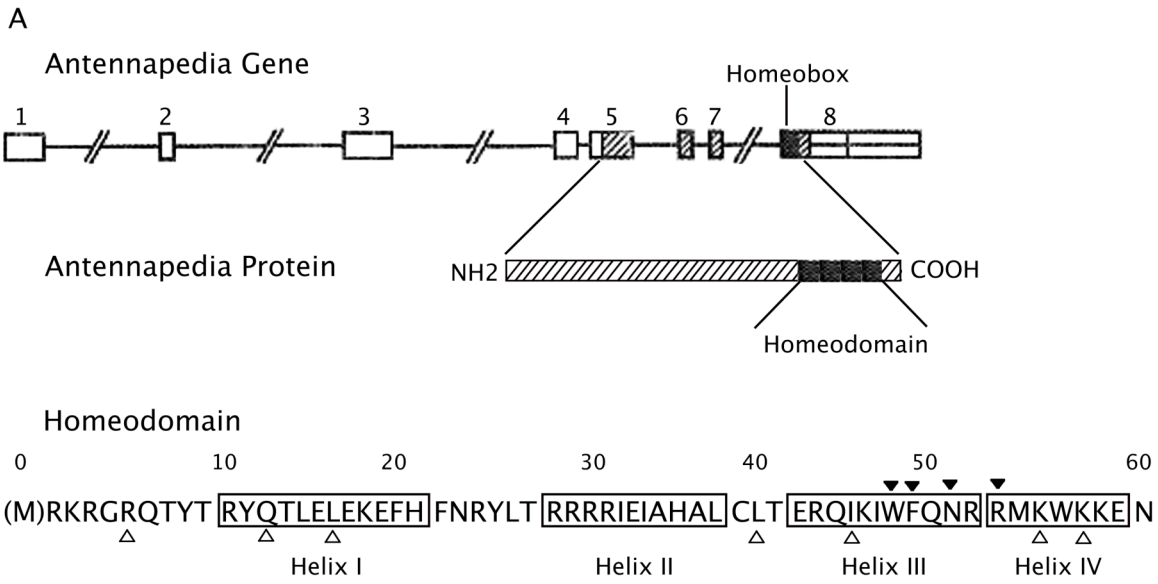
The interaction between the HD and DNA occurs in the regulatory regions of the target genes, the promoter and enhancer regions. In these regions of several Hox target genes, core consensus binding sites for the HD have been identified and defined as a 4-bp element, typically TAAT, TTAT, or TTAC (Shen *et al*., 1997; Ekker *et al*., 1994; Hayashi and Scott, 1990).

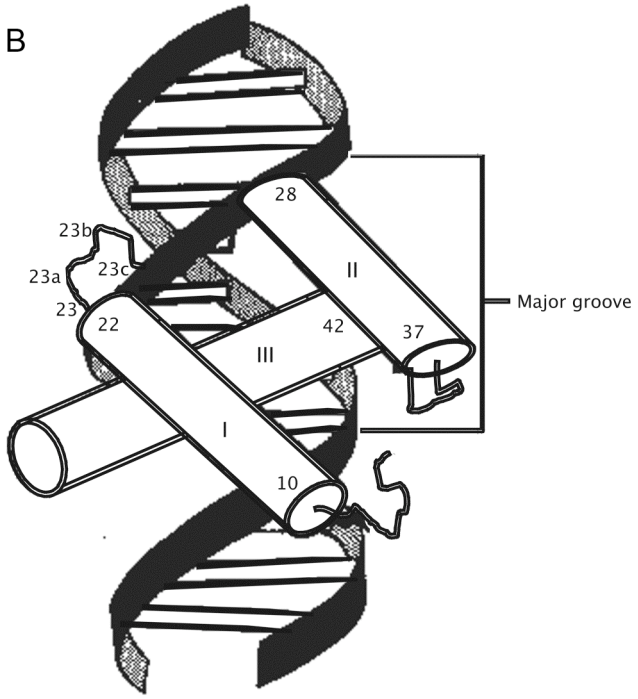
Figure 1-1 ***Structural representation of the Antennapedia gene and protein*****.**

A. Primary structure of the Antennapedia gene and protein. Boxes represent the exons at the DNA level, shaded or dark parts of exons are translated, and the homeobox is black. The black region of the primary protein structure represents the homeodomain, and the shaded area to the left is the N flanking terminal, to the right is the C flanking terminal.

B. Three dimensional structure of DNA-homeodomain interaction. The tertiary structure of the HD, as first determined by solution NMR studies of the Drosophila Antennapedia HD, consists of three alpha-helices (1, 2, 3) and a flexible N-terminal arm.

Adapted and modified from Gehring et al., *(1994)*.


A
Antennapedia Gene
Homeobox
1
2
3
4
5
6
7
8
Antennapedia Protein
NH2
COOH
Homeodomain
Homeodomain
0
10
20
30
40
50
60
(M)RKRGRQTYT
RYQTLELEKEFH
FNRYLT
RRRRIEIAHAL
CLT
ERQIKIWFQNR
RMKWKKE
N
Helix I
Helix II
Helix III
Helix IV


B
28
23b
23a
23c
23
22
II
III
I
42
37
10
Major groove

In addition to the HD, Hox genes also have other highly conserved sequences. The hexapeptide motif, IYPWMK is located upstream of the HD in all Hox genes with the exception of *Abd-B* (Gehring *et al*., 1994) and it has been implicated in the recruitment of *PBX* proteins (another class of HD proteins) to modulate the activity of Hox genes (Chang *et al*., 1995). KLPNTK is another conserved hexapeptide located downstream of the HD in *Dfd*-related Hox genes. To date, it is not known what may be the function of the KPLNTK motif.

Colinearity, Structure, and Evolution of the Hox Cluster

The Hox genes of all metazoans examined to date have clustered arrangements into complexes on chromosomes. In some animals (humans and mice, for example) the genes are arranged in a tandem of tightly organized genomic clusters. In other animals (e.g., *Drosophila*) the Hox cluster has been invaded by non-Hox genes and split into two complexes. In a few others yet (e.g., tunicates), the Hox cluster seems to have undergone a drastic disintegration, in which large intergenic distances may separate the Hox genes. In most cases, despite these differences in the structural organization of the cluster, the location of the Hox genes along the chromosome mirrors their expression pattern along the A-P axis of the animals. Spatially, the most 3' genes in the cluster are expressed anteriorly and the 5' genes in posterior segments. Thus, fly *labial* (*lab*, a 3' gene) is expressed in an anterior domain where it is required for the for the specification of head segments and *Abdominal B* (*AbdB*, a 5' gene) confers identity to posterior abdominal segments. The timing of expression of Hox genes during embryonic development is also well coupled to their relative order along the chromosome. The 3' genes are expressed early and those that are 5', late in development. These two remarkable phenomena are

referred to as spatio-temporal colinearity (Kammermeier and Reichert, 2001; Manak and Scott, 1994; Duboule and Morata, 1994). These properties of Hox genes are observed in most animals examined thus far.

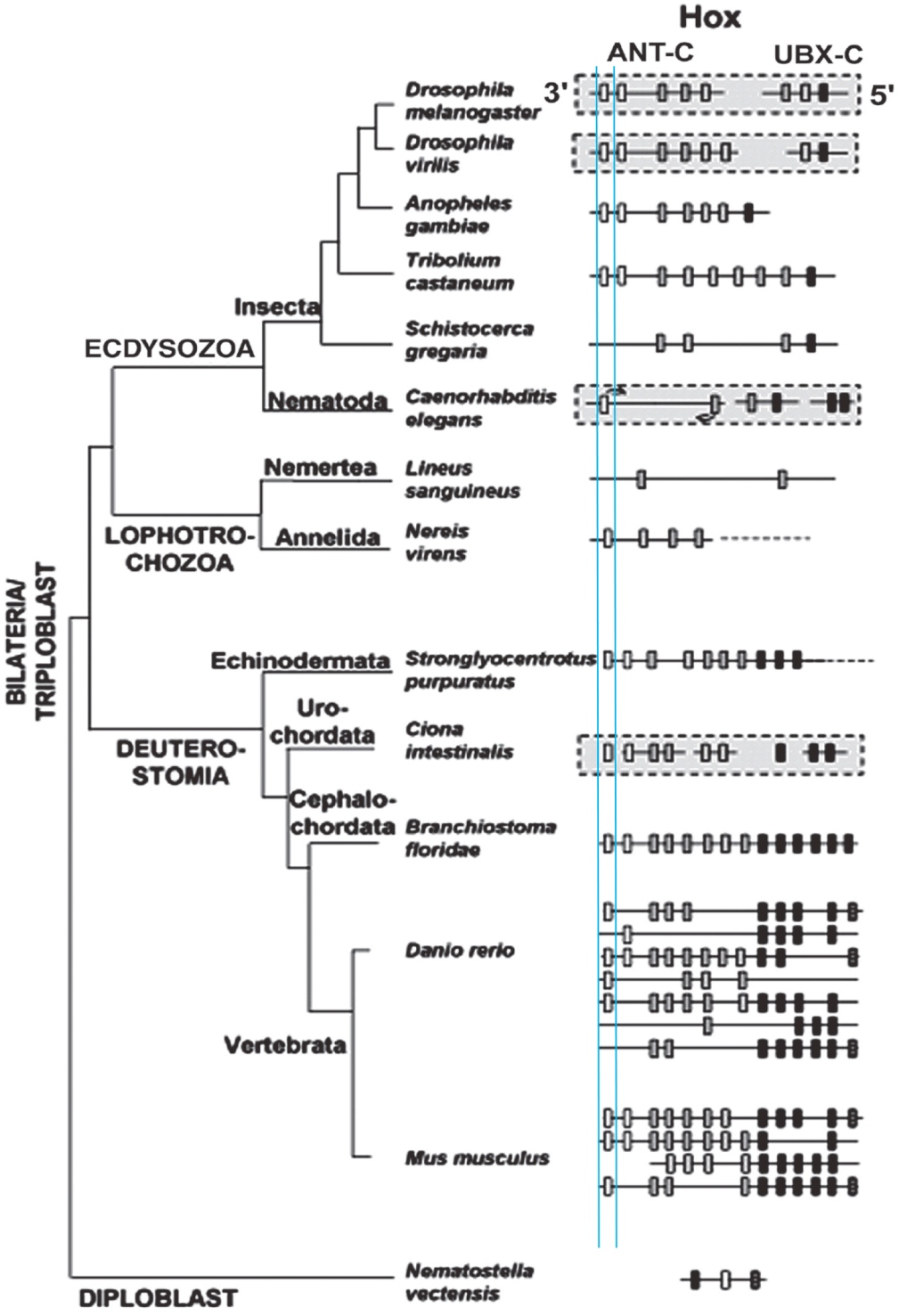
The Hox genes have a wide phylogenetic distribution across all animal phyla (Fig. 1-2) in which the vast majority of animals are bilaterally symmetric or bilaterians. Bilaterians include both diploblasts, animals whose adult forms arise from two embryonic germ layers, and triploblasts, animals that develop from three germ layers (Fig. 1-2). Most bilaterians are triploblasts. The triploblast clade is subdivided into two superphyla: deuterostomes (including echinoderms and chordates) and protostomes. Protostomes consist of Ecdysozoa (including arthropods and nematodes) and Lophotrochozoa (including mollusks, nemertea, and annelids). A single Hox cluster appears in those representatives of protostomes that have been studied (Kaufman *et al*., 1990; Burglin and Ruvkun, 1993; Martinez *et al*., 1999). The Hox cluster of *Drosophila melanogaster* consists of eight Hox genes located on two complexes: the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C) -Lewis, 1978-, along a 650,000 bp stretch of DNA of chromosome number 3. The homeotic genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*) are located on ANT-C; *Ultrabithorax* (*Ubx*), *abdominal A* (*abdA*), and *Abdominal B* (*AbdB*) are members of the BX-C. The complexes are separated by 9.5 Mbp of DNA. Whereas the split occurs between *Antp* and *Ubx* in *D. melanogaster*, in *Drosophila virilis*, which contains the same number of Hox genes, the split is located between *Ubx* and *Abd-A* (Von Allmen *et al*., 1996). Mutation analysis suggests that further splitting of the BX-C can still produce viable *Drosophila melanogaster.* The significance of this split is not quite clear. The split

is missing in other insects such as the mosquito *Anopheles gambiae* and the flour beetle *Tribolium castaneum* which contain seven (Powers *et al*., 2000; Devenport *et al*., 2000) and nine (Beeman *et al*., 1989) Hox genes, respectively, in a single cluster with no splits and no separations. These observations suggest that the split in *Drosophila* species is a derived character, which occurred after the divergence of *Anopheles* and *Drosophila.*

Figure 1-2 ***Phylogenetic distribution, organization, and evolution of Hox clusters.***

The Hox clusters that have broken up are highlighted in grey boxes. The gene shading shows Anterior Hox and *Gsx* genes in white; group 3 central Hox and Xlox genes in gray; and posterior Hox and Cdx genes in black. Striped boxes (not quite visible but shown in blue outline) are *Evx* genes linked to the Hox clusters of vertebrates and cnidarians. X represents loss of a ParaHox gene. The gaps in the Hox clusters of *S. gregaria* and *L. sanguineus* represent lack of information on the intervening genes and not gene absence. The two complexes (ANT-C and UBX-C) of *Drosophila melanogaster* are shown. The actual names for all the genes are not shown.

Adapted and modified from Ferrier and Minguillon (2003).


Hox
ANT-C
UBX-C
3'
5'
Drosophila melanogaster
Drosophila virilis
Anopheles gambiae
Tribolium castaneum
Schistocerca gregaria
Caenorhabditis elegans
Lineus sanguineus
Nereis virens
Stronglyocentrotus purpuratus
Ciona intestinalis
Branchiostoma floridae
Danio rerio
Mus musculus
Nematostella vectensis
Insecta
Nematoda
Nemertea
Annelida
Echinodermata
Uro-chordata
Cephalo-chordata
Vertebrata
ECDYSOZOA
LOPHOTRO-CHOZOA
DEUTERO-STOMIA
BILATERIA/ TRIPLOBLAST
DIPLOBLAST

Another interesting feature of the Hox cluster in insects is its invasion by other genes. Hox genes in *Drosophila* do not form a tightly continuous, integrated cluster. Instead, non-Hox HD genes such as bicoid (*bcd*) and fushi tarazu (*ftz*) are located along the same segment of DNA as the Hox genes (not shown in Fig. 1-2). It is not quite clear what are the functional or evolutionary implications of this phenomenon in the development of *Drosophila*.

As in insects, *Caenorhabitis elegans* (a nematode, which is a sister taxon of the insects) contains a single Hox cluster that has only six genes. It was initially thought that this animal contained only four Hox genes, which resemble some of those of *Drosophila*: *ceh-13* (*lab*), *ceh-15* (*Dfd*), *mab-5* (*Scr*/*Antp*/*Ubx*) and *egl-5* (*Antp/Abd-B*). However, a more recent analysis found that there are two additional genes (*nob-1* and *php-3*) at the 5' end of the *C. elegans* Hox cluster (Van Auken *et al*., 2000). Thus, the present inventory of the Hox cluster consists of an anterior gene (*ceh-13*), two central genes (*lin-39* and *mab-5*), and three posterior genes (*egl-5*, *php-3* and *nob-1*), spread widely across 5 Mbp on chromosome III (reviewed in Aboobaker and Blaxter, 2003; Van Auken *et al*., 2000).

The difference in the number of Hox genes between *C. elegans* and insects has a number of evolutionary implications. Some of the Hox genes present in insects may have arisen via tandem duplication after insects diverged from nematodes, thus accounting for their larger number of Hox genes. Alternatively, the last common ancestor between arthropods and nematodes (*C. elegans*) could have had a larger number of Hox genes on a single cluster, some of which were later lost during evolution after these two groups diverged. In addition, the more complex organization of the fly's body plan (e.g antenna, legs,

wings etc) seems to reflect the number of Hox genes, suggesting that the development of greater complexity requires a larger number of Hox genes.

In the context of evolutionary biology and molecular systematics, where relationships of gene families are recognized on the basis of sequence similarities (nucleotides sequences in the DNA or amino acid sequence in the protein), two types of homology can be distinguished: paralogy and orthology. Paralogy is used to refer to genes showing the highest degrees of sequence similarity to other genes of the same species. Among mammals for example, mouse *Hoxa-13, Hoxb-13* and *Hoxc-13* are paralogues because they have a higher sequence similarity to each other than, say, to mouse *Hoxb-1*. Thus, *Hoxa-13, Hoxb-13* and *Hoxc-13* are members of paralogous group 13. Paralogous groups of genes may have arisen from the entire or partial duplication of an ancestral prototypical cluster. Further, the several member genes of this ancestral prototypical cluster are likely to have appeared by tandem duplication in a very primitive animal genome containing a single Hox gene. Orthology defines a set of genes that have the highest sequence similarity between two different animal species. For instance, *Scr* of *Drosophila melanogaster* has a higher sequence similarity to *Hoxc-5* of mammals and *Lox1* of annelids such as *Hirudo medicinalis*, than to other Hox genes within the fly cluster.

Hox clusters have also been extensively studied in members of the deuterostome clade, including chordates (vertebrates, cephalochordates, and urochordates), hemichordates, and echinoderms. Despite the lack of comprehensive knowledge on leech genome, whereas all studies thus far suggest that most invertebrate protostomes have only a single Hox cluster, there is some indication that annelids (leeches) have more than one cluster.

Evidence of this are derived from more than one instance in the context of some leech Hox genes that bear more striking similarities to each other than with their equivalent in arthropods. The number of identified Hox genes in annelids to date is about nine, compared to insects that have only eight. For example *Lox2, Lox4* and *Lox15* are more similar to each other than they are to their homologs *Ubx* and *AbdA* in insects (Wong *et al.,* 1995)- *Lox2* and *Lox15* are closest. With this in mind, it could be that *Lox2* may be on the same cluster with *Lox4*, and *Lox15* on another. Also, several line of evidence suggests that *Lox6* and *Lox18* belong to the *Dfd* class of Hox genes. *Lox6* and a fragment of *Lox18* was isolated from *Hirudo* (Wong, 1997). *Lox6* alone has been reported in *H. robusta* (Koroukis *et al.,* 1997). Also, Kourakis and Martindale (2001) reported both *Lox6* and *Lox18* in *H. triserialis*. These data suggest a duplication of this locus in some species of leeches. However, there are considerable variations between *Lox6* and *Lox18* (Kourakis and Martindale, 2001). This has led to the idea that *Lox6* and *Lox18* may be parlogs, suggesting that may be located on different Hox clusters.

The number of Hox genes may be limited in invertebrates but the number varies greatly among vertebrates. The genomes of vertebrates are more Hox-rich than those of invertebrates. Initially, it was thought that all vertebrates (including human and mouse) have 39 Hox genes organized into four clusters (HoxA, HoxB, HoxC, and HoxD) whose members can be aligned to each other and to their *Drosophila* orthologs (reviewed by Carrol, 1995; Ruddle *et al*., 1994). To date, 13 paralogous groups have been identified in vertebrates. However, not all paralogs are represented in all mammalian clusters or in the *Drosophila* cluster. The presence of this large number of Hox genes suggests that duplication of an ancestral Hox cluster gave rise to the diversity of highly complex

organization of vertebrate animals. This idea supports the hypothesis of gene duplications as major forces in the generation of organismal complexity (Ohno, 1970). Contrary to this hypothesis, a greater and varied number of Hox genes are located on a larger number of clusters in a many teleost fishes, including the zebrafish (*Danio rerio*), which is generally assumed not to be as complex as a mammal. Fish in general are perhaps the most successful and species-rich vertebrates, accounting for the greatest vertebrate diversity. To date, 49 Hox genes on 7 clusters located on different chromosomes have been identified in zebrafish (Amores *et al*., 1998). These clusters which are designated Aa, Ab, Ba, Bb, Ca, Cb, and Da and are homologous to the four mammalian Hox clusters and may have arisen by means of one duplication from the four-cluster state, quite possibly after tetrapods and teleost fish diverged, giving fish 8 clusters, one of which (*HoxDb*) was subsequently lost. However, the pufferfish also has seven clusters, but only one HoxC cluster, suggesting that the independent loss of a different Hox cluster occurred after pufferfish and zebrafish diverged (Amores *et al*., 2004).

As with protostome invertebrates, a deuterosome cephalochordate (the sister group of the vertebrates), the amphioxus *Brachiostoma floridae*, has a single Hox cluster with 14 contiguous Hox genes, (Ferrier *et al*., 2000), the most gene-rich Hox cluster reported thus far. Each of the first thirteen (*AmphiHox1* to *AmphiHox13*) has a ortholog in the vertebrate paralogous groups (Ferrier *et al*., 2000; Sharman, 1999), suggesting that the multiple Hox clusters seen in vertebrates may have arisen by genome-wide duplications that occurred during vertebrate evolution. *AmphiHox14* has no vertebrate equivalent (Ferrier *et al*., 2000), suggesting it may have arisen independently by tandem duplication of a prior *AmphiHox* gene, possibly *AmphiHox13*, after the divergence of amphioxus and

vertebrates. Alternatively, vertebrates with multiple clusters may have initially acquired 14 paralogous groups of Hox genes but later lost the *Hox14* group.

A single Hox cluster has also been reported for animals that do not display a distinct anterior-posterior axis. Such animals include the echinoderms. Echinoderms are pentameral, radially symmetric invertebrates that display little or no cephalization; they are quite unlike other animals, having diverged radically from the basic A-P organization of bilaterians. Examples are the starfishes, sea urchins, and sea cucumbers. The sea urchins *Stronglyocentrotus purpuratus* has a single Hox cluster (Popodi *et al*., 1996) containing 10 genes (Martinez *et al*., 1999), including orthologs of most of the *Drosophila* Hox genes. However, the sea urchin member of the *Hox4* group (e.g., *Drosophila Dfd*) may have been lost, although orthologs of this group are present in the chordates and the protostomes. As PCR analysis has identified more than three posterior Hox genes other echinoderms, it is possible that they have more than 10 Hox genes (Mito and Endo, 2000).

A single Hox cluster has also been reported for ascidians. These urochordates are considered the most primitive chordates (Di Gregorio and Levine, 1998; Satoh and Jeffrey, 1995; Wada and Satoh, 1994). During larval development, ascidians show typical chordate features such as a caudal notochord, dorsal neural tube and segmental muscles, all of which disappear during metamorphosis. A typical urochordate, the sea squirt *Ciona intestinalis*, contains 9 Hox genes on a single cluster that seems to have undergone disintegration (Spagnuolo *et al*., 2003; Dehal *et al*., 2002).

There is some evidence that Hox genes may have originated well before the emergence of bilaterally and radially symmetric metazoans. PCR fragments of at least two *Antp*-like

homeobox genes have been isolated from sponges (Ohno, 1996; Degnan *et al*., 1995), animals that belong to the Poriferan phylum, which is a sister taxon to Bilateria and Radiata (Cnidaria, Ctenophora, and Placozoa). Poriferans are the most basal metazoans. The occurrence of Hox genes in sponges suggests that these genes may have emerged at least 800 million years ago. However, it has not been determined whether these are indeed true Hox genes. Though it may be argued that the Hox-like genes of sponges bear some resemblance to *Antp*-class genes but do not fully qualify as true Hox genes, this observation has nevertheless important implications for the evolution of Hox genes. It is possible that Hox-like genes in sponges represent an ancestral Hox prototype.

The foregoing discussion is relative to current data forming a rapidly changing picture. Additional Hox genes have been recently found in at least three instances (*C. elegans*, amphioxus, and echinoderms). These recently found genes are all posterior Hox genes. The discovery of more than four clusters in teleost fishes challenges the older paradigm of body complexity and number of Hox genes. As research on the function and evolution of Hox genes continues, new theories and ideas will replace the older ones. However, it is generally accepted that initially there was a single Hox gene, which underwent tandem duplication, giving rise to other Hox genes and forming a cluster. In some animals, the number of Hox genes increased, while in others it decreased. Later genome or chromosome duplication would then account for the larger number of clusters seen in vertebrates.

Hox Genes as Master Architects of Nervous System Development

The Hox genes have a main site of expression in the central nervous system (CNS), a developmentally dynamic and complex system that contains a larger variety of cell types

than any other organ system. Studies in *Drosophila* and mice have provided the most extensive details. In fly, these genes are expressed in the developing brain and the ventral nerve cord. Their anterior-posterior order of the expression follows the principle of temporal and spatial colinearity (Maconochie *et al*., 1996; Manak and Scott, 1994; Dubule and Morata, 1994): The Hox genes located at the 3' end of the Hox cluster are expressed earlier and more anteriorly during development than those at the 5' end. The only exception is the *labial* gene, whose anterior expression boundary is posterior to that of *proboscipedia -pb-* (Kammermeier and Reichert 2001). Hirth *et al*. (1998) have shown that a loss-of-function mutation of *lab* results in profound defects in the establishment of the tritocerebral neuromere in the embryonic brain of *Drosophila.* Such effects include reduced longitudinal connectives, frontal connectives which project ectopically, and reduced or missing tritocerebral commisure. In *Dfd* null mutants, defects associated with the mandibular neuromere were observed: reduced longitudinal connective, missing mandibular commisure. Some of these phenotype alterations for both types of mutants are similar, but occur in different neuromeres. In the tritocerebrum of these mutants, the commissural and longitudinal connectives were significantly reduced or completely missing.

Hox genes are also seen master molecular architect in patterning the nervous system of vertebrates. The mouse hindbrain develops from embryonic segmental units called rhombomeres whose identity and morphology has been compellingly shown to be determined by a unique combination of Hox genes. For example, targeted inactivation of *Hoxa1* results in partial loss of rhombomeres 4 (r4) and 5, in defects of the development of facial motor neurons, and in malformations of several cranial nerves (Gavalas *et al*.,

1998; Carpenter *et al*., 1993; Mark *et al*., 1993). In addition, loss of *Hoxb1* leads to early changes in the identity of r4 and to the impairment of facial motor neuron development. Several studies have also shown that *Hoxa2* specifies the identity of r4-derived neural crest cells, the patterning of r2 and r3, and axonal pathfinding of a subset of trigeminal motor neurons (Gavalas *et al*., 1997; Gendron-Maguire *et al*., 1993; Rijli *et al*., 1993). These and other studies suggest that the expression and functions of Hox genes in the CNS are conserved across all animal phyla, though in other tissues they are not.

In more caudal regions of the CNS, some regional and segmental differences in the developing spinal cord are also under the influence of Hox instructions. For example, Carpenter *et al*. (1997) have shown that targeted disruption of *Hoxd-10* results in a decrease in the number of motor nerve fibers projecting through the sacral plexus to innervate the hindlimb musculature. In addition, Tiret *et al* (1998) have shown that inactivation of *Hoxc-8* results in the loss of an advantage in selective growth cone pathfinding and target selection in motor neurons of the seventh and eighth caudal neuromeres.

Hox Genes as Determinants of Neuronal Fate and Axonal Pathfinding

The Hox genes function as master architects shaping the nervous system of animals because they specify some unique properties of neurons. As a result, Hox genes participate in the differentiation of the wide diversity of neuronal subtypes found in the nervous system of animals. This diversity of neuronal subtypes is displayed as differences in cell body morphology, arborization pattern, neurotransmitter and receptor type, innervation target, synaptic partner, axonal pathfinding, etc. Understanding the

molecular mechanisms by which this cellular diversity is accomplished is a crucial step in understanding the development and pathology of the nervous system.

Details about the role of Hox genes in specifying the properties of neurons in the CNS have emerged from studies mostly done in *Drosophila* and mouse and several other vertebrates. In *Drosophila,* though most of the Hox genes are expressed in the developing CNS, only two of them have been shown to be important for neuronal cell fate. A loss-of-function mutation of *lab* results in the loss of neuronal markers and absence of axonal projections in the *lab*-expression domain of the brain, suggesting a loss of neuronal identity (Hirth *et al*., 1998). In addition, axons originating from outside the *lab* domain stop at this region or are projected ectopically, suggesting that *lab* is also required for the development of a permissive environment for axonal navigation.

The vertebrate orthologs of fly *lab* are *Hoxa1*, *Hoxb1*, and *Hoxd1*. These Hox genes are temporally the earliest genes to be expressed, beginning at gastrulation, and eventually establishing their anterior boundary in the hindbrain at the level of r3/r4 (Kolm and Sive, 1995; Godsave *et al*., 1994; Frohman and Martin, 1992; Murphy and Hill, 1991; Frohman *et al*., 1990; Sundin *et al*., 1990; Wilkinson *et al*., 1989; Wilkinson, 1989). Triple morpholino-knockdown of *Hoxa1*, *Hoxb1*, and *Hoxd1* has resulted in lack of segmentation and reduced hindbrain of *Xenopus* frog embryos (McNulty *et al.,* 2005). At the neuronal level, Studer *et al* (1996) have shown that facial brachiomotor neurons and contralateral vestibuloacoustic efferents (CVA) neurons are incorrectly specified in the fourth rhombomere (r4) of the developing hindbrain of *Hoxb1* mutant mice. In these mutants, motor neurons differentiate but fail to migrate into their proper positions (from r4 to r5), resulting in the subsequent loss of the facial nerve. In addition, inactivation of

Hoxa1 results in partial loss of facial motor neurons in the r4 and r5 regions of the developing hindbrain (Gavalas *et al*., 1998; Carpenter *et al*., 1993; Mark *et al*., 1993).

In other parts of the CNS, other Hox genes play important role in determining neuronal cell fate. Members of all four mammalian Hox clusters are expressed in broad A-P domains along the spinal cord. The expression of HoxC genes has been demonstrated in motor neurons (Liu *et al*., 2001; Tiret *et al*., 1998) and the regional expression of different HoxC genes may provide positional identity for motor neurons at different spinal cord levels (Liu *et al*., 2001). Inactivation of *Hoxc8* or *Hoxd10* alters the production and projection of motor neurons: Carpenter *et al.* (1997) showed that a deletion of *Hoxd-10* resulted in decrease in the number of spinal segments that project motor nerve fibers through the sacral plexus to innervate the musculature of the hindlimb; Tiret *et al.* (1998) showed drastic reduction of motor neurons that innervate the forepaw in *Hoxc8* mutant mice, suggesting that these neurons in part lost their target selection and pathfinding ability, important steps in establishing a functional nervous system.

Other aspects of neuronal differentiation by Hox genes have been explored by various authors. A mutational analysis of the *Abdominal-B* class *C. elegans* gene *egl-5* by Lints and Emmons (1999) has shown that dopaminergic male ray sensory neurons. These neurons normally innervate the nine pairs of sensilla (rays) located in the tail of the animal and express EGL-5 protein, but fail to adopt a dopaminergic cell fate, indicating that Hox genes may be involved in the specification of some important pharmacological and biochemical properties of neurons. In addition, del Toro *et al*. (2001) have shown that novel functional neuronal circuits are generated in the hindbrain of *Hoxa1* mutant mice, suggesting a role of this Hox gene in specifying the synaptic partners of neurons.

Hox Genes in the Leech

Hox genes are present in all multicellular animals. In *Hirudo medicinalis*, nine of these genes, called *Lox* genes, have been cloned and partially characterized (Kourakis *et al*., 1997; Aisemberg *et al*., 1995). Among these genes are *Lox1*, *Lox2*, *Lox4*, *Lox5*, *Lox6*, *Lox7*, *Lox15*, *Lox18*, and *Lox20*. *Lox1* and *Lox20* are putative orthologs of the *Drosophila Scr* gene (Aisemberg and Macagno, 1994); *Lox2*, *Lox4*, and *Lox15* are orthologs of fly *Ubx* and *abd-A* (Wong, 1995; Aisemberg *et al*., 1995; Wysocka-Diller *et al*., 1989); *Lox5* is a likely ortholog of *Antp* (Kourakis *et al*., 1997); *Lox6* and *Lox18* are orthologs of *Dfd* (Wong and Macagno, 1998); *Lox7* is a ortholog of *lab* (Kourakis *et al*., 1997).

The expression patterns of the leech Hox genes have been established and partially characterized in a number of studies. The expression patterns of *Lox5*, *Lox6, Lox7*, *Lox18*, and *Lox20* have been characterized in *Helobdella robusta* (Kourakis *et al*., 1997; Kourakis and Martindale, 2001). In *Hirudo medicinalis*, the expression patterns of *Lox1* (Chapter II), *Lox2* (Wysocka-Diller *et al*., 1989), *Lox4* (Wong *et al*., 1995), *Lox6* (Wong and Macagno, 1998), and *Lox15* (Aisemberg *et al.,* 1995) have been also characterized. Immunohistochemistry analysis showed overlapping A-P expression domains that extend from the posterior end of rostral neuromere 2 (R2) to caudal neuromere 7 (C7) for *Lox6*, from the posterior region of R4 to midbody ganglion 13 (M13) for *Lox1* (current unpublished data, see Chapter II), from the posterior region of M3 to M21 for *Lox4*, and from the posterior region of M6 to M21 for *Lox2* (Chapter II). *In situ* hybridization studies also showed that expression extends from R1 to C7 for *Lox7* (Kourakis *et al*., 1997), from R4 to C7 for *Lox5* (Kourakis *et al*., 1997) both in *Helobdella robusta,* and from about M15 to C7 for *Lox15* (Wong, 1997) in *Hirudo*. The overlapping expression

domains of leech Hox genes suggest that a combinatorial Hox code may determine A-P regional identity in the leech CNS in a manner that is similar to the one observed in vertebrates.

Hirudo medicinalis presents several advantages for the study of Hox gene function at the single cell level. It has a relatively simple, well-characterized nervous system. There are relatively few neurons per ganglion (about 400 for most midbody ganglia and about 700 for each sex ganglion). The neurons are relatively large and hardy. Many of the central neurons of the leech can be identified by their relative size and position in the ganglion. In addition, a number of these neurons have been well characterized in terms of their biochemical properties (surface markers, neurotransmitters, signaling molecules, etc.), target organs or synaptic partners, electrophysiological properties, the type of antibody marker they express, and their morphological properties. In our lab, we take advantage of the simplicity of the leech nervous system to identify central neurons that express Hox genes, some of which are presented in this study. *Lox1* is expressed in several neurons that have been previously characterized, like the anterior-medial serotonergic (AMS) and posterior-medial serotonergic (PMS) neurons of M2 and M3, the heart accessory (HA) neurons of M6, the RPE neurons (located only in M6), the Laz 1-1 positive HA-like cells of M4 (Chapter III). Previous work by Mercado-Pimentel (2003) showed that, among other cells, the mechanosensory P_D neurons, the serotonergic Retzius (Rz), AMS, and PMS neurons of RN3 and RN4, and the bipolar neurons express *Lox6*. *Lox4* is also expressed in the rostral penile evertor (RPE) neurons but with a different temporal pattern (Wong *et al*., 1995). In addition, among other cells, *Lox2* is expressed in the CV motor neurons and the RMV neurons of M7-M21 (N. Jordan, personal communication), in the

giant Rz cells from M8-M21, in the PMS of M7-M21 (current unpublished data, see Chapter III). *Lox15* is expressed in the RMV neurons of M15-M21, which also express *Lox4* and *Lox2*.

Cell lineage studies revealed that *Lox2* transcripts are not present at the developmental stages when segment identities are first established (Kourakis *et al*., 1997). Nardelli-Haefliger *et al* (1992) labeled individual stem cell lineages by intracellular injection of fluorescent tracers and found co-localization of the lineage tracer with the *Lox2* signal in the progeny of the five different pairs of teloblasts within the same range of body segments. When selective teloblast lineages were frame-shifted along the anterior-posterior axis by ablation, the expression of *Lox2* in the shifted lineage maintained its own anterior-posterior pattern. These results suggest that *Lox2* expression is not regulated by positional information, but only by the birth rank of the neuronal precursors that originate from the posterior end of the embryo.

Lox4 is expressed transiently in the specialized RPE neurons of M6 and in their segmental homologs, the rostral most ventral (RMV) neurons of other midbody ganglia. The RPE motor neurons innervate the male organ, but the RMV neurons do not. In addition, *Lox2* and *Lox15* are also expressed in RMV neurons, although in different anterior-posterior domains. Finally, *Lox1* is expressed in a stable manner in the RPE neurons, but not in the RMV neurons. Therefore, the expression pattern of Hox genes within this group of segmentally homologous neurons can be classified into at least five different classes: (1) the RMV neurons of MG1-MG3 never express these genes; (2) the RMV neurons of MG4-MG5 express *Lox4*, starting at E6; (3) the RPE neurons of MG6 express *Lox4* only from E6 to E8 but turn it off afterwards, while always expressing

Lox1; (4) the RMV neurons of MG7-MG14 express both *Lox4* and *Lox2* continuously throughout development, but not *Lox15*; and (5) the RMV neurons of M15-M21 express *Lox4*, *Lox2*, and *Lox15*. The RPE neurons, which express *Lox4* transiently and *Lox1* constantly, have two major projections that travel through specific sex nerves to the male organ. In contrast, the *Lox4*-positive RMV neurons of M4-M5 have primary projections that grow laterally and produce two pairs of major longitudinal branches that extend for approximately two body segments, while the main projections of the *Lox4*-negative RMV neurons of M1-M3 are much shorter and less regular and produce a more extensive network of fine processes (Wong *et al.*, 1995). Together, the foregoing observations suggest that leech Hox genes may act in a combinatorial way to bring about diverse neuronal cell fates. The use of the leech as a model to study the mechanisms of action of Hox genes in a well-characterized and accessible nervous system will complement studies carried out in systems where a genetic approach is possible.

Embryonic Development of the Leech

The early embryonic development of the leech has been described by Fernandez and Stent (1982) and Stent *et al* (1992). The eggs of *Hirudo* are about 0.1 mm in diameter and contain very little yolk. Fertilized eggs are deposited in a sealed cocoon that contains an albuminous fluid. At the start of embryogenesis, a sac-with-a-mouth cryptolarva ingests the albumen, which provides nutrients for development. Even though the very early development of *Hirudo* is not well documented, greater details about leech development ranging from cleavage all the way to the formation of the germinal plate have been obtained from studies done in *Helobdella*, another related species of leech (Stent *et al.,* 1992). During the first two days of development, spiral cleavage produces a 30-40-cell

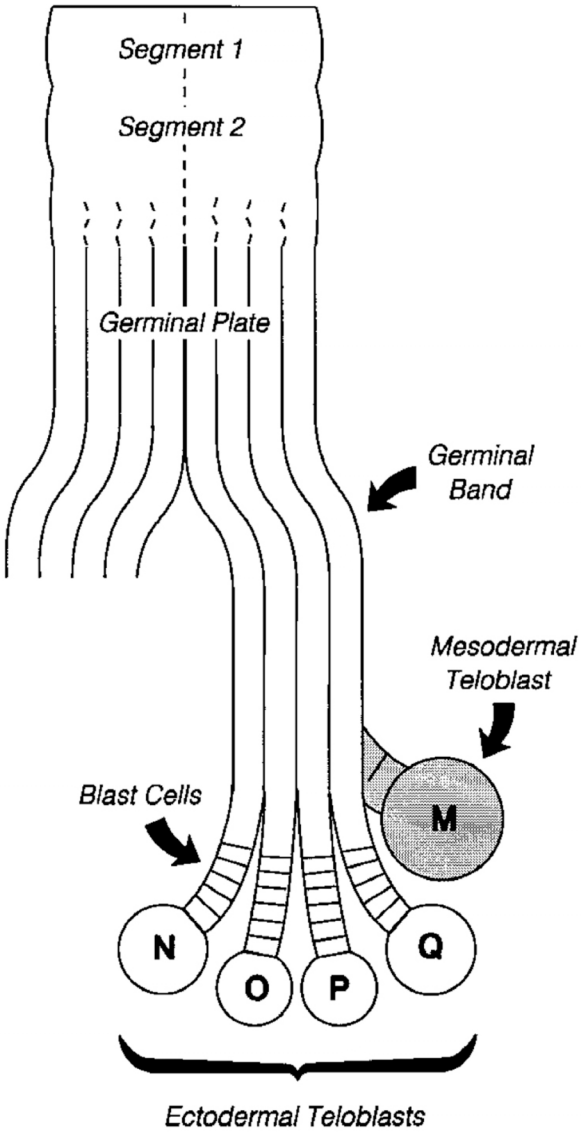
embryo. The majority of the cells are small micromeres and lie on top of four relatively large cells called macromeres. A group of micromeres forms the cryptolarval sac and mouth. By embryonic day four (E4), the embryo ingests enough albumen to grow to about 5 mm. The germinal plate, which gives rise to the animal body proper, forms at the ventral surface of the cryptolarva. Macromeres A, B, and C do not form germinal plate structures, but D originates five bilateral pairs of teloblasts (M, N, O, P, and Q), which divide to give rise to A-P bandlets of primary blast cells (two per segment) that join side by side to form the germinal plate.

Adult leeches have a metameric body organization with a constant number of segments. All metameric structures arise from the five pairs of teloblasts, (Weisblat, 1983; Fernandez *et al*., 1982; Stent *et al*., 1992), which are found at the posterior region of the early embryo and divide asymmetrically to produce two primary blast cells per segment (Fig. 1-3). The primary blast cells undergo a stereotypic pattern of cell divisions (Zackson, 1982) to form the germinal plate from which the segmented structures of the leech develop. The rostral segments are more advanced in development than the posterior segments. During development, cells derived from the N, O, P, and Q teloblasts form the ectoderm; cells derived from the M teloblast form the mesoderm. The germinal plate contains 32 embryonic metameres. The first four rostral segments (R1 to R4) form the head, which also contains non-segmental structures. The tail forms from the fusion of the seven most caudal segments (C1-C7). The 21 intervening segments are the midbody segments (M1-M21).

Figure 1-3 ***Embryonic development of the leech*****.**

Four stages are shown: (1) five bilateral pairs of stem cells (teloblasts) give rise to bandlets of primary blast cells; (2) primary blast cells undergo stereotypic cell division to produce two germinal bands; (3) germinal bands fuse to form the germinal plate; (4) segments separate, anterior segments develop before posterior segments.

Adapted and modified from Nardellli-Haefliger et al. *(1994)*.


Segment 1
Segment 2
Germinal Plate
Germinal Band
Mesodermal Teloblast
Blast Cells
M
N
O
P
Q
Ectodermal Teloblasts

Neuroanatomy of Hirudo medicinalis

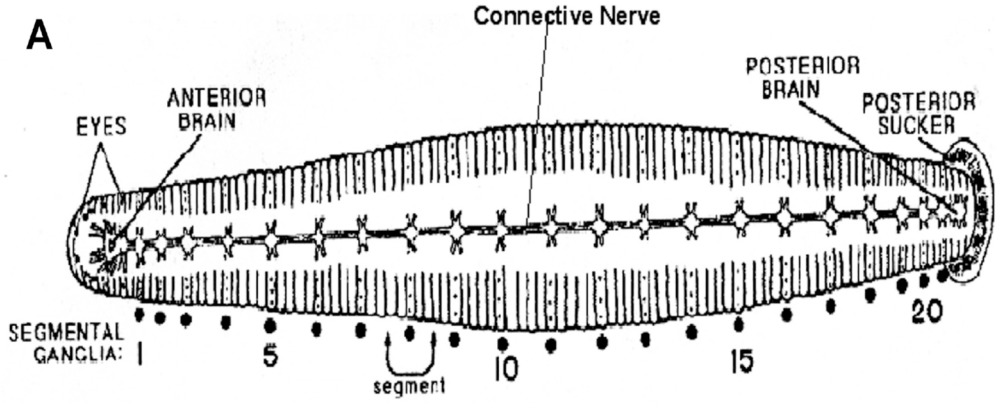
Hirudo medicinalis has a relatively primitive and simple CNS that consists of a segmented ventral nerve cord and an anterior, non-segmented supraesophageal ganglion or dorsal brain. The segmented CNS develops from a fixed set of ganglionic primordia called neuromeres (Fernandez and Stent, 1982; reviewed in Stent *et al*., 1992). Similar CNS metameric units are found also in insects and vertebrates (Wilkinson *et al*., 1989). As the primordia develop, the four anterior neuromeres fuse to form the subesophageal ganglion, and the seven posterior neuromeres join to form the tail ganglion (Fig. 1-4 A). The twenty-one intervening midbody neuromeres develop into separated ganglia but are connected by longitudinal nerves (Fig. 1-4 A). Although the midbody ganglia are very similar to one another, they show segment-specific differences (reviewed in Levine and Macagno, 1990). A midbody ganglion of an adult leech has about 400 neurons (Fig. 1-4 B), except for the sex ganglia (midbody ganglia 5 and 6), which have about 700 neurons each (Macagno, 1980).

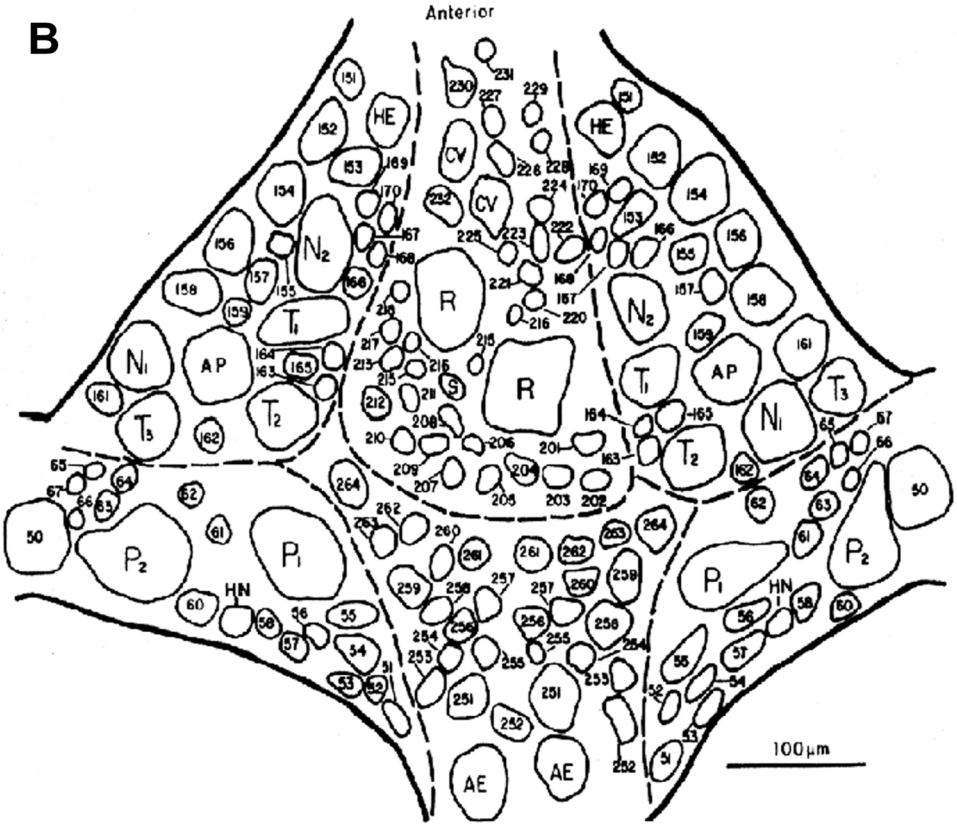
Figure 1-4 ***Neuroanatomy of the leech.***

A. The anterior brain of the leech is formed by a non-segmental supraesophageal ganglion and four fused rostral neuromeres (subesophageal or rostral ganglion). The posterior brain (caudal ganglion) is formed by seven fused caudal neuromeres. The 21 intervening segmental ganglia are called midbody ganglia and are linked by longitudinal connective nerves. The segmented CNS of the leech is ventral to the gut (ventral nerve cord).

B. Ventral view of a representative leech midbody ganglion. Only those neurons that are located near the ventral surface are shown in this drawing. Many neurons are large and easy to identify by their relative position in the ganglion. The midbody ganglia of the leech contain relatively few neurons (about 400).

Adapted and modified from Stent et al., *1992*.


A
Connective Nerve
POSTERIOR BRAIN
POSTERIOR SUCKER
ANTERIOR BRAIN
EYES
SEGMENTAL GANGLIA:
1
5
10
15
20
segment


B
Anterior
HE
CV
R
AP
AE
HN
100 µm

CHAPTER 2

EXPRESSION OF HOX GENES DURING EARLY EMBRYONIC DEVELOPMENT

INTRODUCTION

Hox genes code for a family of well studied transcription factors that contain a highly conserved DNA-binding motif, the homeodomain. The homeodomain is a 60-amino acid helix-turn-helix structure. The Hox transcription factors regulate the expression of downstream target genes and, in so doing, they assign positional values along the anterior-posterior axis and confer segmental or regional identities. The importance of these genes has been demonstrated by studies of their expression, and complementary functional analyses. Hox genes have been shown to have long expression domains with a certain degree of overlap. In the regions of overlap, unique combinations of Hox genes play important roles in the morphological and functional diversifications of body segments or regions.

Mutations of in Hox genes often lead to drastic changes in the identity of entire body segments or larger regions. For example, mutation in the *S*ex *c*omb *r*educed (*Scr*) gene of *Drosophila* transforms the first thoracic segment (T1) into the second thoracic segment (T2), and the labial into maxillary segment (Pattatucci *et al.,* 1991; Sato *et al.,* 1985; Struhl, 1983; Wakimoto andKaufman, 1981).

A main site of expression of Hox genes is the central nervous system (CNS), a developmentally dynamic system with a huge mosaic of different cell types. The cellular

diversity of the CNS manifests itself in a wide display of different cell body morphologies, patterns of arborization, neurotransmitters, receptor types, ion channels, adhesion molecules, etc. Understanding the molecular mechanisms by which this cellular diversity is accomplished constitutes a crucial step in understanding the development and pathology of the nervous system. Differential cell migration, axonal outgrowth and navigation, target recognition and synaptic formation, establishing neuronal circuitry, are among the numerous processes required to bring about the organization of the nervous system. The ability of neurons to accomplish these feats involves interplay between the neurons themselves, the environment, and their synaptic partners. The neurons may require transcription factors to determine their early stages of differentiation. The environment may contain guidance cues that may either repel or attract outgrowth of the neurons, as a way of guiding them toward the correct target. The synaptic partners may be required to express the appropriate surface receptor to bring about the correct target recognition. Studying the expression and function of Hox genes is an important tool for understanding CNS development and evolution.

The leech *Hirudo medicinalis* provides several unique advantages for the study of Hox gene function, at the single cell level. This animal has a relatively simple nervous system that has been well studied. The leech nervous system consists of a ventral nerve cord with 32 segmental neuromeres that form the segmental ganglia during embryogenesis and a supraesophageal ganglion of non-segmental origin. The four rostral neuromeres fuse during embryonic development to form the rostral ganglia. The seven caudal neuromeres also fuse, forming the tail ganglion. The remaining neuromeres form the 21 midbody ganglia. Each midbody ganglia contains about 400 neurons (Macagno, 1980), except the

sex ganglia (M5-M6), which contain about 500-600 neurons that are generated later in embryogenesis.

Many neurons can be reliably identified by their morphology and location in the ganglion. Though most are identical from segment to segment, some display segment-specific phenotypes and morphologies (reviewed by French and Kristan, 1994; Jellies *et al*., 1992; Stewart *et al*., 1991; Glover and Mason, 1986; Gao and Macagno, 1987a, b; Jellies *et al*., 1987). The functions of many leech neurons have been well studied, and their synaptic targets have been determined. Also, a number of the best known neurons have relatively large cell bodies, making it possible record their electrical activity, inject dyes or manipulate gene expression of in a single cell.

There are ten leech Hox genes (*Lox* genes). *Lox* genes have long expression domains along the rostral-caudal axis of the developing embryos. For example, *Lox4* is expressed M3 to M21, with most of the $Lox4^{+}$ neurons located in M13-21, and *Lox6* from R3 to M21, *Lox1* from posterior regions of R4-M13, *Lox2* in posterior M6-M21. Within the embryonic CNS, Hox expression overlaps extensively. Therefore, the *Lox* genes may specify the identity of central neurons in a combinatorial manner. One goal of our lab is to study the overlap of Hox gene expression in the CNS. First, we have to obtain detailed information about the expression of the individual Hox genes. After obtaining this information, we plan to study the expression of multiple Hox genes at the single cell level. This approach should yield insight into the unique combinations of Hox genes that participate in the specification of neuronal cell fate.

The leech Hox gene *Lox1* is a putative homolog of *Drosophila Scr* (Aisemberg *et al*., 1994) and vertebrate *Hoxc6.* In the fruit fly, it has been shown that *Scr* is expressed at

high levels in a small number of anterior segments, and at lower levels in posterior neuromeres (Riley *et al*., 1987). Within the CNS of the third instar larval stage, *Scr* expression becomes confined to a narrow band of cells in the subesophageal region of the ventral ganglion (Glicksman and Brower, 1988).

The leech Hox gene *Lox2*, is homologous to *Drosophila* Hox *Ultrabithorax* (*Ubx*) and *abdominalA* (*AbdA*). In a monoclonal antibody study, White and Wilcox (1985) showed that *Ubx* expression extends from the T1 to A8 in midline cells, from T2-A8 in the ventral nervous system and the epidermis, and from A1-A8 in the somatic mesoderm of fly embryos. *Abdominal-A* gene is expressed from the posterior half of the A1 neuromere to the posterior half of the A7 neuromere (Karch *et al*., 1990).

Lox7, another leech Hox gene is a homolog of *Drosophila labial* (*lab*). Independent studies showed that the patterns of expression of these genes (*Lox7* and *lab*) did not remain conserve. In the fruit fly, *lab* is expressed in the procephalon. It is a gene that is expressed in more anterior segments of the fly embryo. In the leech *Helobdella triserialis, in situ* hybridization studies showed that *Lox7* is expressed in all body segments (Kourakis *et al*., 1997). It is likely that the patterns of *Lox7* expression will be more conserved among leeches, than between leeches and fly.

Because several important experimental resources were already available for *Lox1* and *Lox2* (sequence and expression information, expression vectors, antibodies, etc.), my research focused mostly on *Lox1* and *Lox2*. In this Chapter, I report detailed expression patterns for these two genes that were obtained using immunostaining of leech embryos. I also report the sequencing of a *Lox7* cDNA fragment and its expression in bacteria. The earliest expression of *Lox1* in the CNS starts E6 in a few nuclei of R4-M6. As

development continues, *Lox1* expression extends to more posterior ganglia, stopping at M13. Peak *Lox1* expression was detected at late E9 to E10, with the largest number of $Lox1^+$ nuclei detected in M2 and M3. Posterior to M6, the number of $Lox1^+$ nuclei decreased progressively. In these observations, the *Lox1* expression domain is seen to extend from posterior R4 to M13, in contrast to earlier published work that reported *Lox1* expression in all the midbody ganglia of the leech embryo. *Lox1* expression was also detected in the putative primordium of the female sex organ, starting at late E4-E5, the earliest expression. By E8, both male and female sex organs expressed *Lox1*, hence remaining stable. These observations suggest that *Lox1* may be involved in the early regional patterning of the CNS and in the differentiation and innervation of the sex organs. The earliest expression of *Lox2* was detected at E4, in a region corresponding to the bandlets of primary blast cells produced by the teloblasts. In the CNS, *Lox2* expression was first observed at early E7 and continued at least until E18, from the posterior region of M6 to M21. Though the *Lox2* anterior-posterior domain was established by late E9, the number of nuclei of central neurons that expressed *Lox2* within this domain continued to increase at later stages of development. By E14, the largest number of *Lox2* positive ($Lox2^+$) cells was observed in M8 and M9, and a slightly smaller number was observed in M11-M15. Posterior to M15, the number of $Lox2^+$ neurons decreased progressively up to M21. No expression of *Lox2* was observed in the caudal ganglia, in contrast to earlier published studies (Wysocka-Diller *et al.*, 1989). A cDNA fragment of *Lox7* was sequence, cloned and expressed in bacteria, as a way of raising proteins for generating antibodies to study the expression of *Lox7*.

Polyacrylamide-SDS gel electrophoresis established that the cloned fragment yielded a 57 kDa band.

MATERIALS AND METHODS

Expression and Purification of LOX1 *Protein*

E. coli BL21(DE3), carrying a plasmid most of the *Lox1* coding region (amino acids 3 to 369) inserted into the pET3a vector (Studier *et al.,* 1990), was induced to produce the L-*LOX1* protein polypeptide (Aisemberg and Macagno, 1994). The bacteria were grown overnight at room temperature with gentle shaking in NZCYM 10 g NZ amine, 5 g sodium chloride, 5 g yeast extract, 1 g casamino acids) medium containing 1X M9 salts (consists of Na_2HPO_4 12 H_2O, KH_2PO_4, NaCl, NH_4Cl), 0.4 % glucose, and 200 μm/ml ampicillin, the transferred to 37^{o}C with strong shaking for continued growth up to OD_{600} = 0.3. Expression of *LOX1* protein was then induced with 0.4 mM IPTG (Isopropyl beta-D-1-thiogalactopyranoside) for 3 hr at 37^{o}C.

After centrifugation, bacteria from one liter of culture were incubated at room temperature for 20 min in 40 ml of SET buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-Hcl pH 8) containing 100 μg/ml PMSF (phenylmethylsulphonyl fluoride) and 1 mg/ml lysozyme, and lysed by adding sodium deoxycholate to 0.1% . After incubation at room temperature for 30 min with 10 μg/ml DNase I to reduce viscosity, the homogenate was centrifuged for 10 min at 12, 0000 x g at 4^{o}C. The supernatant was discarded and the pellet containing the inclusion bodies was then washed, first with 25 ml 1% Nonidet P-40 in SET buffer and later with 25 ml SET buffer (after each wash, the suspension was centrifuged under the same conditions as before). The washed pellet was suspended in 4

ml SET buffer with a Dounce homogenizer and then solublilized by adding an equal volume 2X sample buffer (for 10 ml stock: 0.420ml of 1.5M Tris, pH6.8, 5 ml of 50% glycerol, 0.2 g SDS, 0.54 g DTT, 0.001 g Bromophenol blue) and then heating the sample at 95^{o}C for 5 min. The L-*LOX1* protein was easily identified in an aliquot of this sample by electrophoresis in a 12% polyacrylamide –SDS gel stained with Coomassie blue (Shimoni and Reuveni, 1988; Wu and Welsh, 1996), present as a major band of about 50 kDa that was not present in a non-induced sample.

The L-*LOX1* protein was further purified by electrophoresing the solubilized pellet in preparative, 12% polyacrylamide-SDS gel, run overnight at 16^{o}C. The L-*LOX1* band was identified as a broad band of about 50 kDa after incubation with 0.3 M $CuCl_2$ for 5-10 min at room temperature. The band was cut out, rinsed with electrophoresis buffer (1 L 5X stock consists of 15.1 g Tris base, 72 g glycine, 5 g SDS) and loaded into a Bio-Rad Electro-Eluter (Model 422). The L-*LOX1* protein was eluted overnight at room temperature in electrophoresis buffer at constant current of 10 mA/well. The eluate was dialyzed overnight against PBS (140 mM NaCl, 30 mM KCl, 43 mM $Na_2HPO_4.7H_2O$, 15 mM KH_2PO_4, 0.025% NaN_3), using dialysis tubing (molecular weight cutoff 12, 000- 14, 000). The purified L-*LOX1* protein produced a single Coomassie blue band of approximately 50 kDa after an aliquot of the eluate was electrophoresed on a 12% polyacrylamide –SDS minigel. The L-*LOX1* protein was then quantified using the Bradford technique (Bradford, 1976) and subsequently used to generate and purify antibodies.

Immunization

To generate antibodies against the L-*LOX1* protein, three Guinea pig (maintained, injected, and bled at the facilities of Strategic BioSolutions) were injected with 100 μg each of L-*LOX1* protein suspended in complete Freund's adjuvant, followed by three boosters in incomplete Freund's adjuvant every two weeks. Preimmune serum preceding the primary immunization and test bleed following each booster were collected and tested on immunoblots.

Immunoblot Assay of L-LOX1 Antibodies

An induced *E.coli* homogenate containing L-*LOX1* protein was diluted at 1:1000, electrophoresed as described before, and then transferred to a 0.45 μm nitrocellulose membrane using a semi-dry blotting apparatus (Bio-Rad, Model: Trans-Blot), stained reversibly with Ponceau S to check that the transfer was successful, and rinsed briefly with electrophoresis buffer. The membrane was then blocked with TTBS (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 30 min at room temperature with gentle shaking. The blocked membrane was next sandwiched between the plates of a Bio-Rad Mini-Protean II Multi Screen apparatus (Model 170-4017) and probed with different serum samples (600 μl of 1:100 to 1:2000 serum, diluted in PBS) for 30 min at room temperature with gentle shaking. The serum samples were aspirated out of the wells, followed by three 5 min washes, each with 600 μl of TTBS. The membrane was next removed from the multiscreen apparatus, rinsed briefly with TTBS, and place in a sealed plastic bag containing 5 ml of a 1:5000 dilution of alkaline phosphatase-linked anti-Guinea pig antibody (Rockland) in TTBS and incubated for 30 min at room temperature with gentle shaking. The membrane was washed 3 times with 20 ml TTBS for 5 min with

gentle shaking. The antibody was finally visualized as a black-purple band by staining with nitro-blue tetrazolium (NBT, diluted 1:150 from a 50 mg/ml stock solution in 70% dimethylformamide) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, diluted 1:300 from a 50 mg/ml stock solution in 100% dimethylformamide) in AP buffer. The presence of a strongly stained band of about 50 kDa identified those serum samples that contained *LOX1* antibodies.

Antibody Purification

Antibodies against the L-*LOX1* antigen were purified by affinity chromatography. L-*LOX1* protein (about 5 mg in PBS) was covalently linked to 0.3 g CNBr-activated Sepharose 4 Fast Flow (Pharmacia Biotech), following the manufacturer's instructions. The L-*LOX1*-Sepharose was then packed in a small chromatography column, producing a bed volume of about 1ml, and subsequently washed with 20 ml of PBS. Immune serum (2 ml) was diluted 1:5 in PBS and flitered through a 0.2-um cellulose acetate syringe filter (Nalgene). The diluted serum was first passed through a column containing a leech embryonic homogenate as a way of eliminating antibodies with non-specific cross-reactivity to abundant leech antigens. The flow-through was next passed through the L-*LOX1*-Sepaharose column. The column was then washed with 20 ml PBS. The bound antibodies were eluted with 4 ml low pH elution buffer (100 mM glycine-HCl pH3) and collected in a tube containing 0.8 ml 1 M Tris-HCl pH 9. The column was then washed again with 10 ml PBS. Additional bound antibodies were eluted from the column with 4 ml of high pH elution buffer (100 mM Na_2CO_3 pH 11) and collected in a tube containing 0.8 ml 1 M Tris-HCl pH 6.7. The two eluates (from the high pH and low pH elutions) were combined and then adsorbed with three different affinity columns containing *LOX2,*

LOX4 and *LOX6* proteins as a way of eliminating cross-reactivity of *LOX1* antibodies to other leech HOX proteins. These columns were obtained from Melania Mercado-Pimentel (Mercado-Pimentel, 2003) and from Nicole Jordan (unpublished results). The adsorbed flow-through containing specific L-*LOX1* antibodies was concentrated to a volume of about 0.5 ml with a Vivaspin concentrator (Vivascience) and washed in the same device 3 times with 10 ml of PBS; each concentration step was attained by centrifugation at 1,699 g and 4^{o}C. The *LOX1* antibodies were finally concentrated to a volume of 50 μl. Antibodies independently purified from the three immunized animals were assayed against an immunoblot as described above. However, only one of the three Guinea pigs yielded antibodies that recognized the endogenous *LOX1* protein in the leech embryonic tissue.

Immunohistochemistry

Leech embryos were dissected in PBS containing 6% ethanol and pinned (ventral side down) with 3-5 mm fragments of 1-mil tungsten wire on Sylgard (Dow Corning), in 35-mm Petri dishes. After fixing for 1 hr at room temperature in 4% formaldehyde (EMS) in PBS, embryos were briefly rinsed three times with PBS and blocked-permeabilized with four changes of a blocking solution consisting of 4% goat serum in PBS-TX (1% Triton X-100 in PBS) for 2 hr at room temperature with gentle shaking. Embryos were next incubated in a moist chamber, each embryo covered with 25-μl drop of primary antibodies diluted in blocking solution (a 1:1,000 dilution worked best for the *LOX1* antibody and a 1:200 dilution was optimal for the *LOX2* antibody), either for 3 hr at room temperature or overnight at 4^{o}C. The embryos were next washed with 6 changes of PBS-TX for 3 hr at room temperature and with gentle shaking. The embryos were then

incubated with fluorochrome-labeled secondary antibodies diluted 1:200 in blocking solution (Jackson ImmunoReseach Laboratories) for 3 hr at room temperature in a dark, moist chamber. Finally, the embryos were washed as before with PBS-TX, passed through a graded glycerol series (25%, 50%, and 80% glycerol in PBS, 5 min each), and mounted in gelvatol 923 g polyvinyl alcohol 30,000 dissolved in 100 ml PBS plus 50 ml glycerol, containing 0.21 M 1,4-diazobicyclo-[2.2.2]octane- DABCO- as an anti-fade reagent), on microscope slides covered with number 1 cover slips. For double staining experiments, the dilutions used for marker antibodies were 1:5 for Laz1-1 (a mouse monoclonal antibody generously donated by Dr. Birgit Zipser), and 1:200-1:2,000 or three different rabbit polyclonal antibodies: anti-FMRFamide (Peninsula Laboratories, Inc.), anti-serotonin (Sigma), anti-leu-enkephalin (Sigma), anti-netrin (developed by our laboratory). Other monoclonal antibodies used include mouse anti-glutamate (Sigma) used at 1:200 to 1:800, mouse anti-small cardioactive peptide (anti-SCPb, generously provided by Dr. Dennis Willows of the University of Washington) used at 1:200-1:800, mouse anti-gama-aminobutyric acid (GABA)- Sigma- used at 1:20-1:100.

Purification of* LOX2 *Proteins and Generation of* LOX2 *Antibodies

The cloning and expression, and the subsequent raising and purification of antibodies for *Lox2* studies were initially completed by Melania-Mercado Pimentel *et al.* (2002) in our lab. In brief, a 596 bp fragment between positions 2662 and 3225 of the *Lox2* cDNA Lox2.12 (G. Aisemberg, unpublished results) was amplified to include additional restriction sites and inserted into the pGEX-2t vector. The construct was used to transform BL21 (DE3) *E. coli* bacteria by electroporation. The *LOX2* protein was large-

scale produced from cultures of transformed *E. coli* following conditions described by Mercado-Pimentel *et al*. (2002).

Lox2 ***Immunization and Antibody Purification***

Raising of antibodies followed the protocol described in Mercado-Pimentel (2002). In summary, two rats and two rabbits were immunized. Rats were immunized subcutaneously with the purified *GST/LOX2* protein to produce polyclonal antibodies. For the immunization, each rat was injected with 125 μg of *GST/LOX2* protein in complete Freund's adjuvant, followed by three boosters every 21 days in incomplete Freund's adjuvant. The immunization protocol for the rabbits was the same, except that 300 μg of *GST/LOX2* protein was used for each injection. Samples of sera taken before primary injection and before second booster were diluted and tested on Western blots containing total protein from bacteria expressing the corresponding fusion protein, to confirm that the animals responded. Ten days following the third booster, the animals were exsanguinated and the immunized sera harvested.

The purification of the *LOX2* antibodies followed the same steps as *LOX1* antibodies. The semipurified antibody was adsorbed with other *LOX* affinity columns as a way of minimizing cross-reaction of the *LOX2* antibody with epitopes of other leech HOX proteins.

Animals

The embryos used in this study were obtained from our leech colony. Adult leeches (*Hirudo medicinalis*) were nested at 23^{o}C in sphagnum moss soaked with artificial pond water (0.5 g synthetic sea salts- Instant Ocean- per liter of tap water). Cocoons were

harvested on a daily basis and stored in moist chambers at 23°C. Embryos were staged according to Fernandez and Stent (1982).

Imaging

Images were generated using either an Olympus epifluoroscent microscope (Model AX70) coupled to a Spot camera (Model R2023- Diagnostic Instruments, Inc.) or Nikon microscope (Model E800) coupled to a Bio-Rad MRC1024 laser-scanning confocal system. For descriptive clarity, the contrast and brightness of the images were edited. Images generated with the Spot camera were first edited with the SpotAdvance software, and further processed with Adobe Photoshop. Similarly, images and stacks of images obtained with the confocal microscope were first edited with the LaserSharp 2000 software, and then with Adobe Photoshop when necessary.

Cloning and Expression of Lox7 *cDNA*

Lox7 has been cloned and expressed according to Mercado-Pimentel (2002; 2003) into the pGEX-2t expression vector which was used to transformed BL21 (DE3) strain of *E. coli*. The protein obtained from the supernatant was recognized as a 57 kDA band when compared against uninduced samples on polyacrylamide-SDS gel electrophoresis.

RESULTS

Characterization of the LOX1 *Antibody*

A rabbit antiserum against the *LOX1* protein was already available (Aisemberg *et al*., 1994). Two expression constructs containing either the full-length *Lox1* open reading frame (L-*Lox1*) or a C-terminal fragment including the homeodomain (S-*Lox1*) inserted

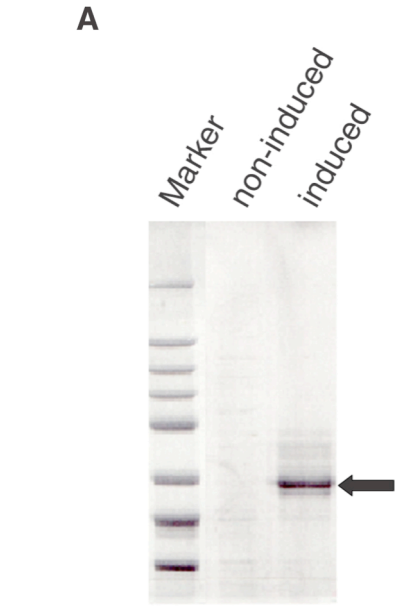
into the vector pET3a (Studier *et al*, 1990) were also available (Aisemberg and Macagno, 1994). The rabbit anti-*LOX1* polyclonal antibody was affinity-purified, using procedures that were not possible in 1993 because other *LOX1* proteins were not available (see below). A new antiserum against the *LOX1* protein was raised in Guinea pigs, to be used in double-staining experiments involving other antibodies that were raised in rabbits. In order to raise the new *LOX1* antibody, milligram quantities of S-*LOX1* protein was expressed and purified. This protein was identified as a 28-30 kDa band on polyacrylamide-SDS gel electrophoresis (data not shown). However, antibodies obtained from three Guinea pigs immunized with this protein did not recognize the *LOX1* antigen in leech embryonic tissue, though the antiserum recognized both the L-*LOX1* and S-*LOX1* proteins on Western blot (data not shown). This suggested that either the S-*LOX1* antigen was not immunogenic enough, or that it contained antigenic determinants not exposed in the native protein present in embryonic tissue. As a consequence, more protein was purified as described above, but from L-*Lox1*-transformed *E. coli* cultures. This protein was detected as a 50-55 kDa band in polyacrylamide-SDS gel electrophoresis (Fig. 2-1A). The protein thus produced was injected into three Guinea pigs to raise a polyclonal antibody. The antisera were tested on a Western blot of total protein from an induced bacterial culture containing the *Lox1* expression vector. Antisera from the three Guinea pigs detected the *LOX1* protein on this blot (Fig. 2-1B). The antibody was affinity-purified with a column containing covalently-bound L-*LOX1* protein. To eliminate antibody molecules that cross-react with other *LOX* proteins via the conserved motifs in their homeodomains, the *LOX1* antibody was then adsorbed to three other affinity columns containing the *LOX2, LOX4,* and *LOX6* proteins. Next, the purified

antibodies were tested on another Western blot containing the *LOX1* polypeptide. The purified antibodies from the three Guinea pigs detected again the *LOX1* protein on this blot (data not shown), but only one of these antibodies was later found to detect the *LOX1* protein in fixed leech embryos. The pattern of expression detected by this antibody was identical to that detected by the newly purified *LOX1* antibody made in rabbits. However, all the results reported in this thesis were obtained using only the Guinea pig antibody, which was available in much greater quantities and produced somewhat cleaner staining.

Figure 2-1 ***Detection of*** **LOX1** ***protein and*** **LOX1** ***antibody***

A. L-*LOX1* protein from induced cultures was identified as a 50-55 kDa band (arrow) as revealed by polyacrylamide-SDS gel electrophoresis. Non-induced cultures produced no detectable *LOX1* protein. The band was visualized by Coommassie blue staining.

B. Western blot analysis detected *LOX1* immunoreactivity in the serum obtained from immunized Guinea pigs (arrow). Preimmune and immune sera samples were tested at 1:1000.


A
Marker
non-induced
induced

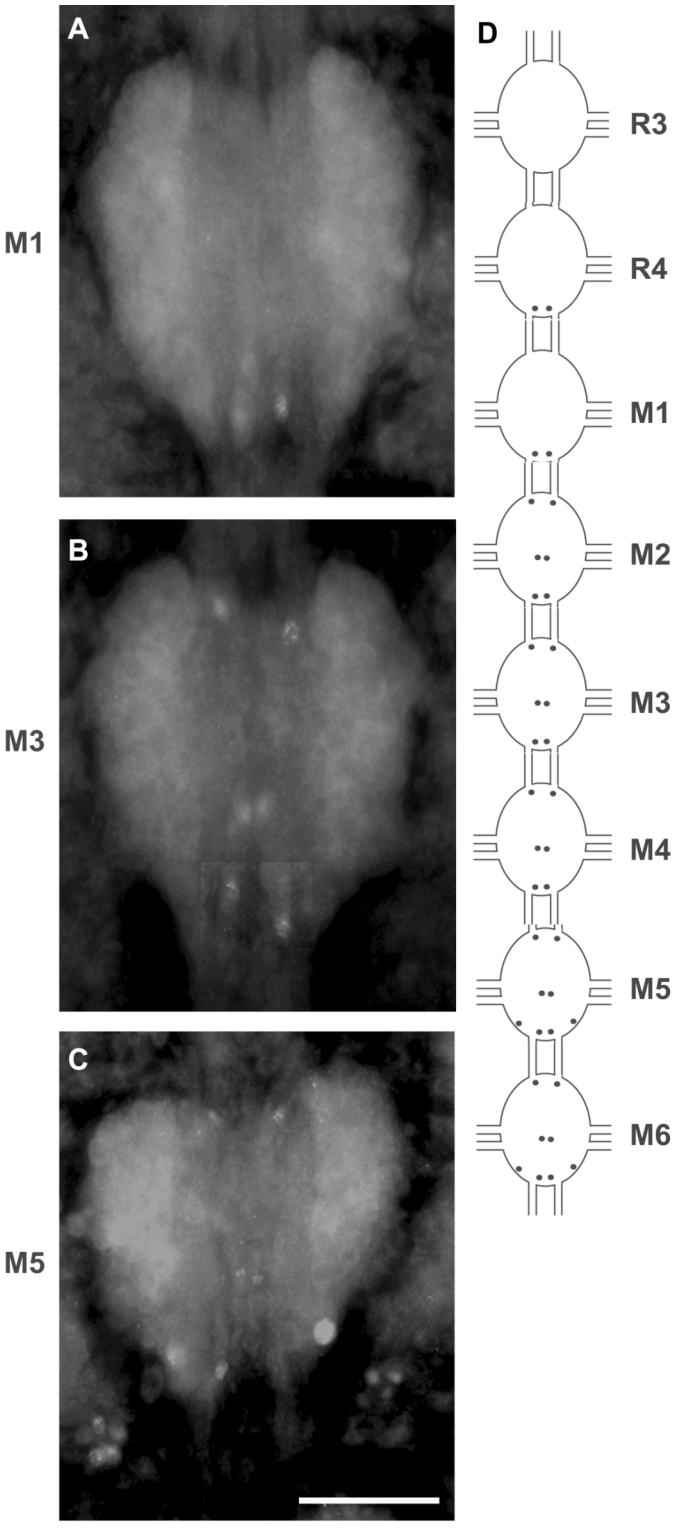

B
Serum samples
immune
preimmune
immune
preimmune
immune
preimmune

Temporal and Spatial Expression of Lox1 in Early Development of CNS

Formaldehyde-fixed embryos were incubated in Guinea pig *Lox1* antibody at a dilution of 1:1000, followed by incubation in TRITC (Tetramethyl Rhodamine Iso-Thiocyanate) - labeled anti-Guinea pig secondary antibody. The earliest expression of *Lox1* in the CNS was detected in whole-mounted E6 embryos. The expression pattern was restricted to segmentally repeated and segment-specific neurons of midbody neuromeres R4-M6 (Fig. 2-2). Only one pair of posterior-medial neurons expressed *Lox1* in R4 and M1 (Fig. 2-2A-B). In M2-6 (Fig. 2-2B-C), segmentally-repeated $Lox1^{+}$ neurons included a pair of anterior-medial neurons, a pair of central-medial neurons, and the posterior medial neurons seen in R4-M1. A pair of posterior-lateral, segment-specific $Lox1^{+}$ neurons was detected only in M5-M6 (Fig. 2-2C). This pattern was consistent in eight embryos examined.

Figure 2-2 *Early* Lox1 *expression in the CNS*.

Lox1 expression was detected in neuromeres R4-M6 at E6 by immuno-staining with Guinea pig anti-*Lox1* antibody and TRITC-labeled secondary antibodies. The schematic drawing on the right summarizes the total *Lox1* expression in these anterior ganglia, as observed in the individual embryos. In this and all subsequent figures, anterior is to the top. The scale bar is 50 μm.


A
M1
B
M3
C
M5
D
R3
R4
M1
M2
M3
M4
M5
M6

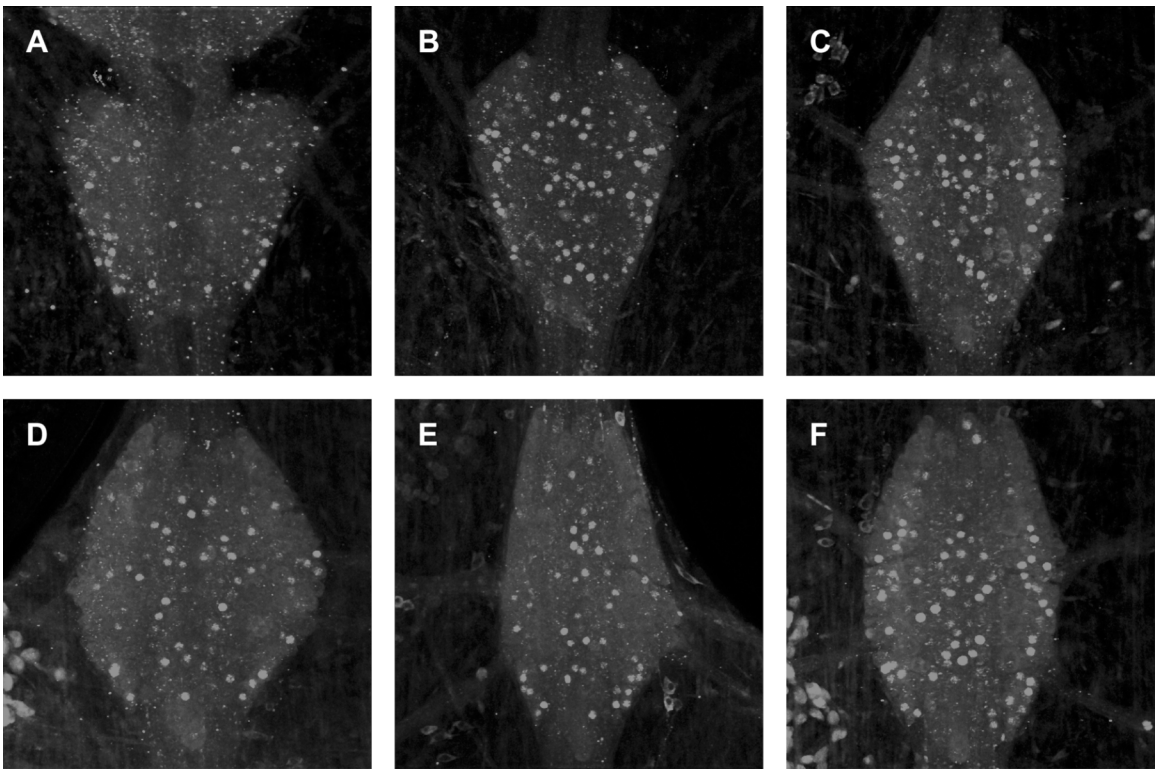
The number of *Lox1*$^{+}$ neurons increased dramatically from E6 to E10 (compare Fig. 2-2 with Fig. 2-3). By E8, the expression domain extended from R4 to M8-9. By E14, the domain of *Lox1* expression in the CNS became fully established, a stage when *Lox1* expression was restricted to a subset of segmentally repeated and segment-specific central neurons between the posterior limits of R4 and M13 (Fig. 2-3). A total of 10 embryos were used to generate the average number of nuclei that expressed *Lox1* in each ganglion. The largest number of *Lox1*$^{+}$ neurons appeared in M2 (average of 56) and M3 (average 60), decreased in M4 and M5 (average of 40) and increased in the male sex ganglia of M6 (average of 55). *Lox1* expression decreased again in more posterior ganglia: about 37 neurons in M7, 21 in M8 20-22 neurons in M9-11, average of 14 neurons in M12, and 2 in M13. No expression was detected posterior to M13. Though these numbers represent an average value, all the *Lox1*$^{+}$ nuclei did not exhibit the same level of fluoroscent signal; some are brighter than others. This difference could be partially explained on the basis of the levels of the *LOX1* protein expressed by individual neuron.

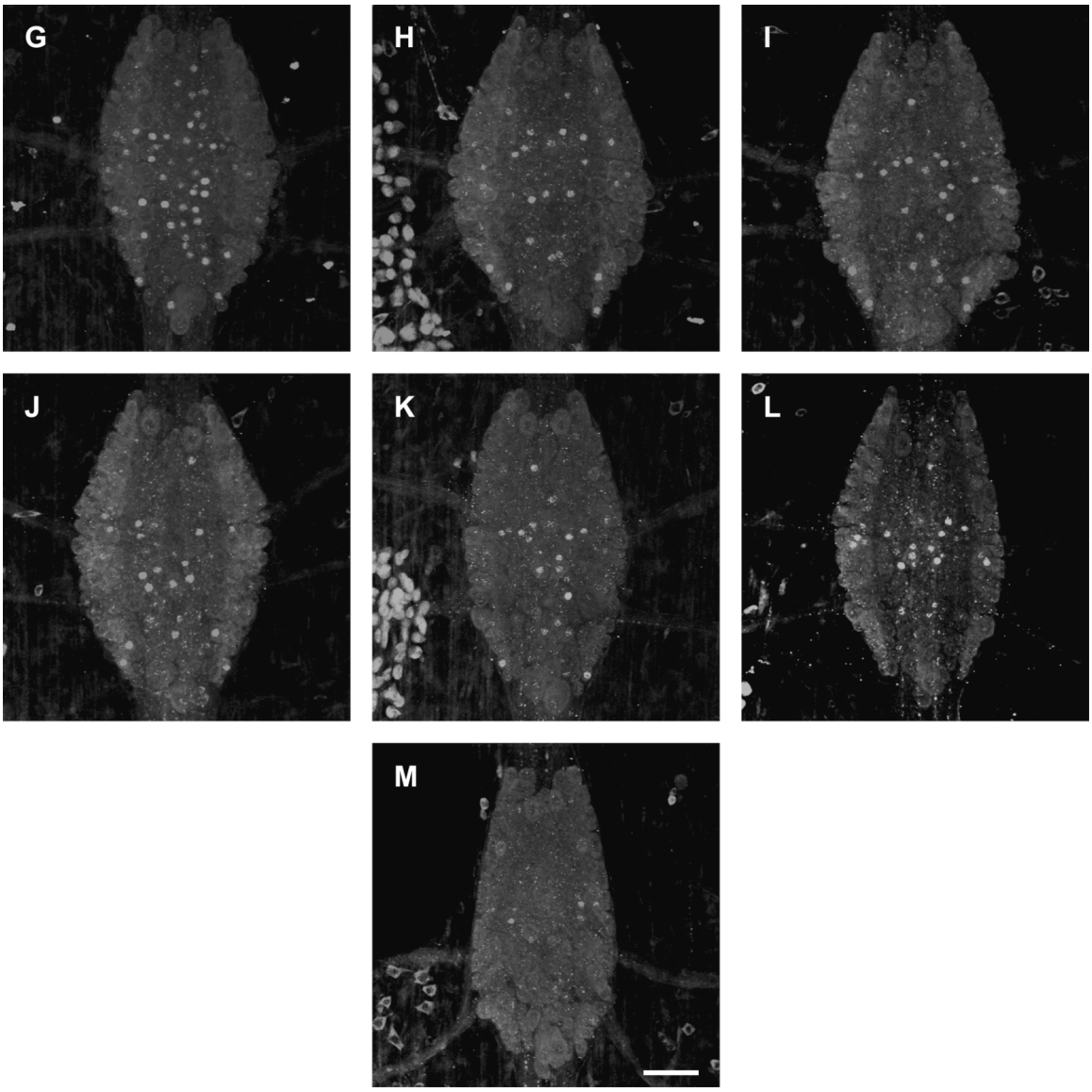
Between E9 and E16, the expression pattern of *Lox1* was dynamic. This dynamism was evident in doubled staining experiments (Chapter III). Some cells may die or stop expressing *Lox1* while others may start *Lox1* expression at later embryonic stages. The PMS neurons of M3 expressed *Lox1* at E9-E11 but later these cells became unpaired as one member of the pair continued to express *Lox1* while the other one dies. In addition, an anterior-lateral pair of *Lox1*-expressing neurons appeared in M3 at E14 but was not detected in embryos at E9-E11 (data not shown**).** However, most *Lox1*$^{+}$ neurons were found at all embryonic stages observed (E9-E21). The temporal expression pattern of

Lox1 can be partially explained with developmental gradient of leech embryos; at early stages a larger number of *Lox1*$^{+}$ neurons were present in the more developed anterior neuromeres.

Figure 2-3 Lox1 *domain at the peak of expression*.

Confocal projections of the CNS of an E14 embryo stained with Guinea pig anti-*LOX1* antibody, showing expression from posterior R4 (not show) to M13. The greatest number of *Lox1*$^{+}$ neurons appears in anterior ganglia within this domain (M2-M7). *Lox1* expression decreases progressively in more posterior ganglia (M8-13). Not all the *Lox1*$^{+}$ nuclei are shown in these images. ***A***, M1; ***B***, M2; ***C***, M3; ***D***, M4; ***E***, M5; ***F***, M6; ***G***, M7; ***H***, M8; ***I***, M12; ***J***, M13. The scale bar is 50 μm.


A
B
C
D
E
F

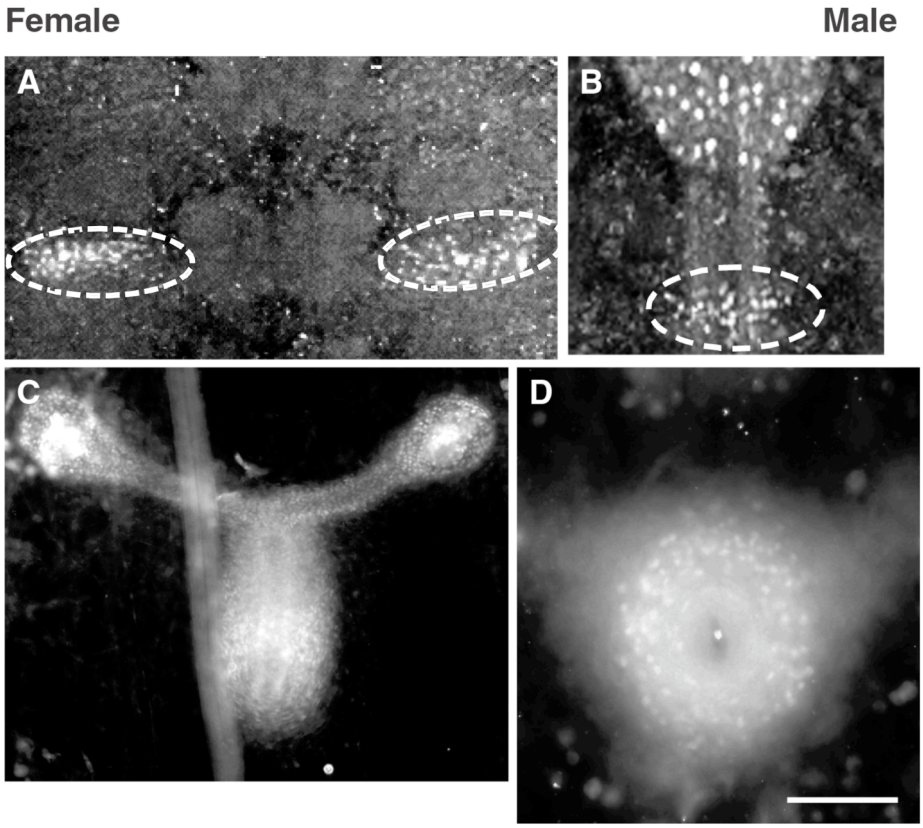

G
H
I
J
K
L
M

Expression of Lox1 *in the Primordia of the Genitalia*

Lox1 expression was also detected in the sex primordia, organs that later acquire innervations from central neurons located in the specialized sex ganglia of M5 and M6. Expression in the genital primordia was first observed in a region that corresponds to the position of the female sex primordium, starting at E4 (data not shown). In fact, this marks the earliest expression of *Lox1*. At this stage of development, there is no overt sign of the formation of the specialized sex ganglia. By E6, *Lox1* expression was observed in a position corresponding to that of the male sex organ primordium (Fig. 2-4 B) By E9, the intensity of *Lox1* expression increased in both structures, and remained stable through all subsequent stages examined, at least up to E21, (Fig. 2-4 C and D).

Figure 2-4 Lox1 *expression in the genital primordia*.

At E6 high *Lox1* was detected in the female organ primordium as ganglia started to form (***A***, dashed ovals). *Lox1* expression was also evident in the male sex organ primordium at E9 (***B***, dashed oval). This expression pattern continued at E17 (***C*** and ***D***), at a time when these anatomical features of these organs are well developed and defined. The scale bar is 50 μm.

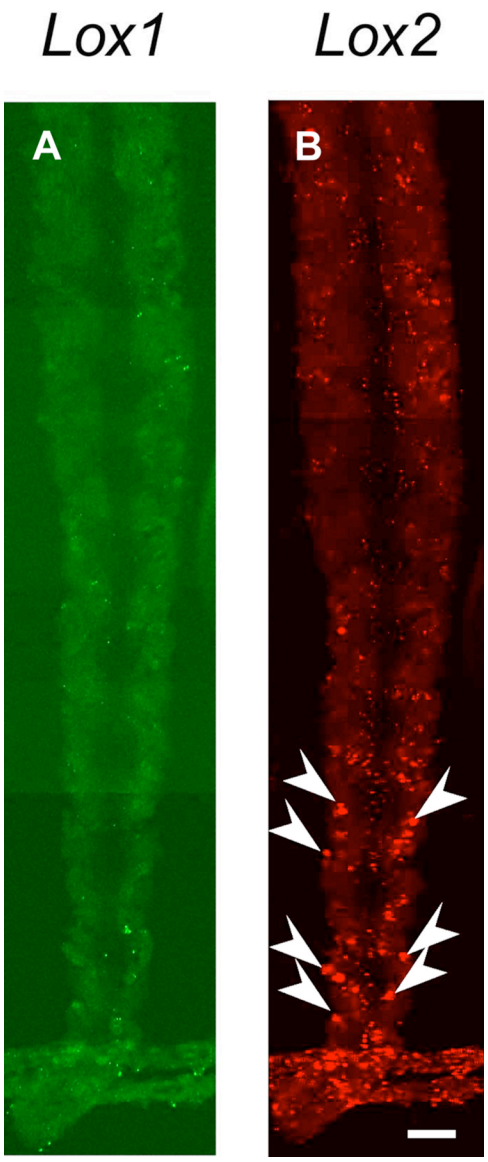

Female
Male
A
B
C
D

Early Lox2 *Expression*

A rat polyclonal antibody was used to study the temporal and spatial expression of *Lox2.* The earliest *Lox2* expression was detected in bands of relatively large nuclei in the posterior region of the developing embryo at E4 (Fig 2-5B). At this stage, there is no overt appearance of the developing nervous system, nor were there any sign of segmentation. This made it difficult pinpoint in which ganglia *Lox2* expression started at this stage. However, these cells appeared posterior to the level of the putative female sex primordium, which expressed *Lox1* (the earliest expression of *Lox1*) when the same animals were costained with the Guinea pig *LOX1* antibody. Also, the position of these nuclei at this stage corresponds to those of the primary bandlet of cells that form the germinal plate of the leech. No *Lox1* expression was observed in this region at this stage (Fig. 2-5A).

Figure 2-5 *Early* Lox2 *expression*.

E4 embryos stained with *LOX1* antibodies did not show any expression in the posterior region (***A***). The earliest expression of *Lox2* was detected at this stage (***B***, arrowheads). These are composite images projected from stacks of optical confocal sections. The *Lox1* staining was visualized with TRITC-labeled anti-Guinea pig antibody; *Lox2* was detected with Cy2-labeled anti-rat antibody. The scale bar is 50 μm.

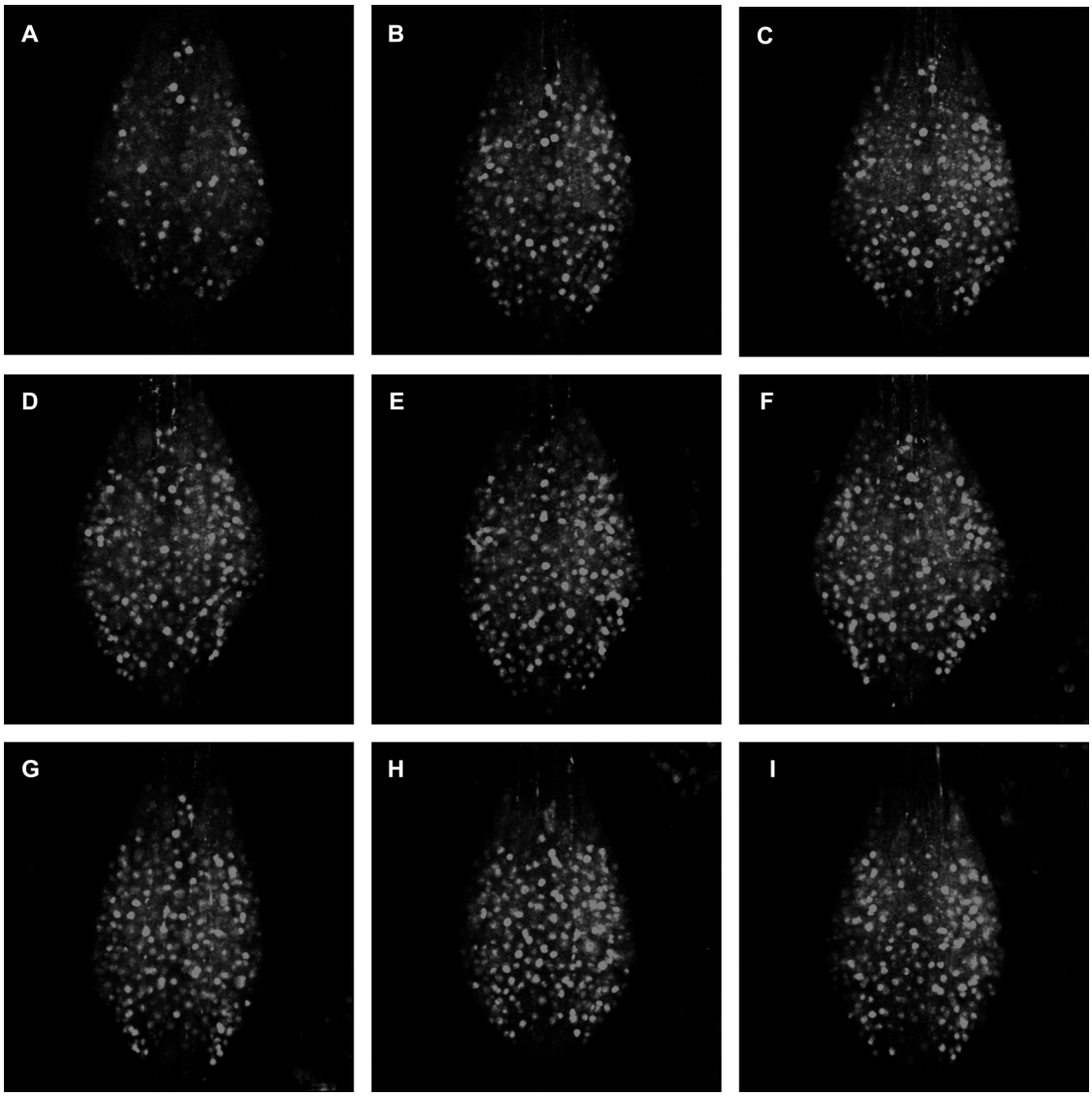

Lox1
Lox2
A
B

Temporal and Spatial Lox2 *Expression in the CNS*

The earliest *Lox2* expression in the CNS was detected at early E7 in a few neurons of M7-M10. The average number of *Lox2*$^+$ neurons per ganglion at each stage was obtained from 12 embryos. However, the nuclei did not all stain equally. By E9, M6 and ganglia posterior to M10 started to express *Lox2*, while the number of *Lox2*$^+$ neurons in M7-10 increased dramatically (25 in M7, 50 in M8, 55 in M9, 38 M10). By E10 a few (6) neurons expressed *Lox2* at the posterior edge of M6, the precise anterior boundary of *Lox2* expression domain. Between E10 and E14, the number of central neurons expressing *Lox2* increased further in more posterior ganglia, but the population of such neurons did not surpass those of the more anterior ganglia (M8-M10): 55 in M7, 121 in M8, 115 in M9, 88 in M10, 103 in M11, 98 in M12, 96 in M13, 88 in M14, 84 in M15, 72 in M16, 74 in M17, 60 in M18, 55 in M19, 40 in M20, and 32 in M21. During this period of embryonic development, the *Lox2* expression domain became stable (Fig. 2-6). The largest population of *Lox2*$^+$ neurons appeared in M8 and M9, decreased slightly in M10, and then increased in M11-M14 though not at the same level as M8 and M9. Posterior to M15, the number of *Lox2*-expressing neurons decreased progressively to M21 (where it was first detected late E10). No expression was detected in C1-C7.

Figure 2-6 ***Expression of*** **Lox2** ***in posterior ganglia.***

These images are confocal projections showing the *Lox2* domain within the CNS of an E14 embryo. The greatest number of *Lox2*$^{+}$ neurons was detected in M8 (***B***) and M9 (***C***), decreased slightly in M10 (***D***), and then increased in M11-15. (***E-I***). Some *Lox2*$^{+}$ nuclei are not visible these projections but were detected in individual optical sections. The *Lox2* staining was visualized with Cy3 anti-rat secondary antibody. ***A,*** M7; ***B,*** M8; ***C***, M9; ***D***, M10; ***E***, M11; ***F***, M12; ***G***, M13; ***H***, M14; ***I***, M15; ***J***, M16; ***K***, M17; ***L***, M18; ***M***, M19. The scale bar is 50 μm.


A
B
C
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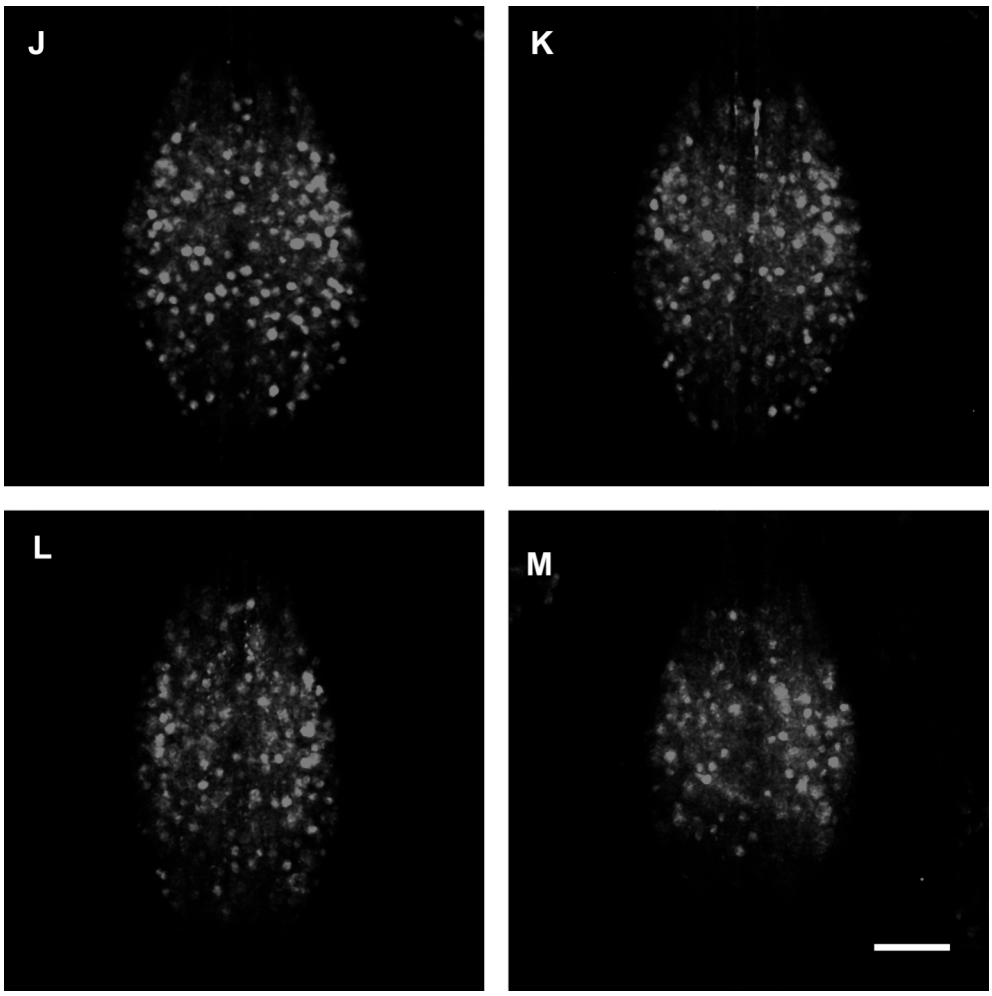
I


J
K
L
M

Structure of the Lox7 *cDNA*

The initial clone of the *Lox7* cDNA was obtained by low stringency to a *Lab* Homeobox probe by Victoria Wong (1997) of Columbia University. The longest continuous, non-overlapping fragment of the *Lox7* cDNA sequence produced an inferred translation product of about 368 amino acids (Fig. 2-7A). The upstream sequences obtained in this current work, aligned well into the open reading frame (ORF) of the already available sequences. There was no indication of the start codon, suggesting that this length does not represent the entire upstream of the *Lox7* gene. This current work also generated 136 amino acids sequence upstream of the available ELEKEF sequence of the Homeobox, suggesting that an additional region of the homeobox became available. In addition, the polyT segment was also present, but this region of the gene was reportedly difficult to sequence. The sequencing along this region of the cDNA produced lots of chatters, and ambiguity. Also in the available sequence, there was no indication of any conserved hexapeptides common to Hox genes.

Homologs of Lox7

The inferred *LOX7* amino acid sequence was compared to other related Hox genes using the protein alignment and comparison program, BLAST for possibility of conserve domains. *Lox7* homeobox shared a 90 % similarity with its homolog in another species of leech, *Helobdella triserialis* (Kourakis *et al.,* 1997), and 85 % with the *labial* gene of *Drosophila melanogaster* (Fig. 2-7 B). It has however, been well established that *Lox7* is indeed the homolog of *lab*.

Lox7 *Expression Construct*

A *Lox7* cDNA clone (Wong, 1997) was used to PCR-amplified a 822-bp fragment (Fig. 2-7, shaded sequence). This fragment started about 96 amino acids of the upstream of the homeodomain. The PCR products were ligated in-frame to the pGEX-2t expression plasmid. The ligated products were used to transformed BL21 (DE3) strain of *E. coli* by electroporation. PCR screening showed all colonies (18) tested contained the right insert, suggesting that the transformation was highly efficient; a single discrete band of (approximately 800 bp) appeared on a 1 % agarose gel electrophoresis, for each colony screened. In addition, restriction enzyme digestion with *Pst* I followed by agarose gel electrophoresis showed that the linearized construct of these colonies was of the correct size and electrophoretic mobility. To ascertain whether the construct of these colonies was inserted in the right orientation, the primers used in the PCR screening were used to sequence the insert. The sequences aligned correctly with the linearized pGEX-2t plasmid, using the DNA-alignment program, BioEdit.

The GST-*LOX7* fusion protein was purified according to Mercado-Pimentel *et al* (2002). SDS-PAGE showed that the protein was soluble, thus eliminating the need to solubilize it. The protein was detected as a 57 kDa band (data not shown).

Figure 2-7 *Structure of* Lox7 *cDNA*

A. Nucleotide sequence with the inferred translation of the longest open reading frame (ORF) of the *Lox7* cDNA. The homeodomain is underlined. There was no indication of the start codon, suggesting the sequence does not represent the entire *Lox7* coding region. The shaded region represents the fragment of the *Lox7* cDNA that was subcloned and expressed in bacteria.

B. Sequence alignment and comparison of the homeodomain of *Hirudo Lox7* with its homolog in another related species of leeches, *Helobdella triserialis*, and in *Drosophila melanogaster.* The result produced by the protein alignment program BLAST, shows that *Hirudo Lox7* is more closely related to its homolog in *H. triserialis* than to *lab* in the fruit fly. The supersripts indicate the amino acid position. The superscript at the beginning of *Hirudo Lox7* homeodomain represent the amino acid position based on translated the cDNA sequence available (not the complete gene). Dashes (-) indicate amino acid identities.

A

```
1     ACC AAA AGC TGG AGC TCC ACC GCG GTG GCG GCC GCT CTA GAA CTA   45

1      T   K   S   W   S   S   T   A   V   A   A   A   L   E   L    15

46    GTG GAT CCC CCG GGC TGC AGG AAT TCG GCA CGA GAG ACA CTC CGT   90
16     V   D   P   P   G   C   R   N   S   A   R   E   T   L   R    30

91    CAA AAG CTT TCC ATT TCT CCT GGA TAC CAC GAA CTC CAA TCC ACG   135
31     Q   K   L   S   I   S   P   G   Y   H   E   L   Q   S   T    45

136   CCG TCC GTT CCG GCG TTC GCC CAG CAC TTT GCC GGC AAC TCC GGC   180
46     P   S   V   P   A   F   A   Q   H   F   A   G   N   S   G    60

181   CAA TTC TAC AAC AAC AAT GGC TAC CGG CGA GGC CAG CAG TAT CCA   225
61     Q   F   Y   N   N   N   G   Y   R   R   G   Q   Q   Y   P    75

226   GCC TTC CAG CAG TTC GGC AGC GGC TTG GAC TCG CAA GCT CTT CAA   270
76     A   F   Q   Q   F   G   S   G   L   D   S   Q   A   L   Q    90

271   CAG CAA CTA CAA CTT GGC AAA AGC GCG AAT GGC TTT GAC TTG TCC   315
91     Q   Q   L   Q   L   G   K   S   A   N   G   F   D   L   S   105

316   TTG GAG TTG AAC CAC AAG CCT TCG CAA CAA CAG CAA CTC AGC AAC   360
106    L   E   L   N   H   K   P   S   Q   Q   Q   Q   L   S   N   120

361   AAC GCC ATC AAC CTC GGC CGA ACC AAC TTC ACT AAC AAG CAA CTG   405
121    N   A   I   N   L   G   R   T   N   F   T   N   K   Q   L   135

406   ACG GAG CTG GAA AAG GAG TTT CAC TTC AAC AAG TAC CTG ACA AGG   450
136    T   E   L   E   K   E   F   H   F   N   K   Y   L   T   R   150

451   GCT CGC AGG ATC GAG ATT GCC TCG GTC TTG GGT CTG AAC GAG ACA   495
151    A   R   R   I   E   I   A   S   V   L   G   L   N   E   T   165

496   CAA GTC AAG ATC TGG TTC CAG AAC CGA CGG ATG AAG CAG AAG AAA   540
166    Q   V   K   I   W   F   Q   N   R   R   M   K   Q   K   K   180

541   CGA CTT AGA GAG ACC GGA TCC AAG ACC GTC CTT CCC CAC TCC AGT   585
181    R   L   R   E   T   G   S   K   T   V   L   P   H   S   S   195

586   GAC GTC ACG GAC GGA GAG TCC ATC CAG ACG CCA TCC GGC CAA TCG   630
196    D   V   T   D   G   E   S   I   Q   T   P   S   G   Q   S   210

631   CCG ACC GGC CAG CAT CGA CCC GGC AAC AAC AAC TCA AAT CAT TCG   675
211    P   T   G   Q   H   R   P   G   N   N   N   S   N   H   S   225

676   TTA AGC CCA GGA AGC GAG AAT TCC AAA ATG GCT GAG AGT TCC CTC   720
226    L   S   P   G   S   E   N   S   K   M   A   E   S   S   L   240

721   CAT TGT GAC ACG ATG ACG TCA TGC GAC CAC GTG ATT ATC AAC TTC   765
241    H   C   D   T   M   T   S   C   D   H   V   I   I   N   F   255

766   CGG TCC GGT GAT GGC GGC GCT GAC GAT AAT AAG AGC GCC AGG ATT   810
256    R   S   G   D   G   G   A   D   D   N   K   S   A   R   I   270

811   TGC AAC GAT GGA TGC CAC TTG ATG AGC GAT GAT TCC CGG GCC GAT   855
271    C   N   D   G   C   H   L   M   S   D   D   S   R   A   D   285

856   TGT TTC TTC TCG TGA TTC GTC TGC TCT CAA AAT GTC CGT TCT AAT   900
286    C   F   F   S   *   F   V   C   S   Q   N   V   R   S   N   300

901   TTT TCT TTT TCT TTT TTA TTT TTA TGT TTT ATA TAT ATA TAT TTA   945
301    F   S   F   S   F   L   F   L   C   F   I   Y   I   Y   L   315

946   TTT ATT TAT TTA TAT GTT TAA ATG TTC GTT TAT TCG TTG AAT CTG   990
316    F   I   Y   L   Y   V   *   M   F   V   Y   S   L   N   L   330

991   AAC ATT TTG TGC AAT ATT TAT ATA TTT ATT CTC AAA TAT CTT AAG   1035
331    N   I   L   C   N   I   Y   I   F   I   L   K   Y   L   K   345

1036  CTT AAA CAG GGT CTT TTC TGT GGC TTT TGG TCA CTT TTT ATA ACA   1080
346    L   K   Q   G   L   F   C   G   F   W   S   L   F   I   T   360

1081  ATA AAC ATG TTT CAA AGT GAC AGC                              1104
       I   N   M   F   Q   S   D   S
```

B

Species	Homeodomain	Identities
H. medicinalis	^{124}LGRTNFTNKQLTELEKEFHFNKYLTRARRIEIASVLGLNETQVKIWFQNRRMKQKKRLRE^{184}	
H. triserialis	^{43}----------T----------H----K-^{102}	57/63 (90%)
D.melanogaster	^{508}NNF----------R----------NT-Q----------VK-^{568}	51/60 (85%)

DISCUSSION

This chapter reports the expression patterns of two leech Hox genes, *Lox1* and *Lox2*. *Lox1* was expressed as early as E4 (not show) in the sex primordia and maintained at all subsequent stages examined. In the CNS, the earliest *Lox1* expression was detected at early E6, beginning at the posterior margin of R4 and extending more posteriorly (to M13) at later stages, as the number of *Lox1*$^{+}$ neurons increased. The earliest expression of *Lox2* was detected in primary blast cells at E4. In the CNS, though a few *Lox2*-expressing neurons were detected at the posterior margin of M6 as early as E9-E10, the earliest expression of *Lox2* was detected at E7, beginning at M7, extended more posteriorly to M21 at later stages, as the number *Lox2*-containing neurons increased and continue to express *Lox2*. In essence, *Lox1* expression domain extends from posterior limits of R4 to M13, and that of *Lox2* from posterior M6 to M21. Thus there is a region of overlap between these two rostral--caudal expression domains: M6 to M13. There exists the possibility that *Lox1* and *Lox2* are both required to specify the fate of a subset of central neurons within those regions of overlap. These Hox genes begin their expression at a time when the main functional and structural properties of the nervous system, as well as the innervation and differentiation of the sex organs are developing in *Hirudo medicinalis*.

Dynamics of Early Lox1 *Expression: A New Insight into* Lox1 *Expression Pattern*

The expression patterns for both *Lox1* and *Lox2* reported here are considered more reliable and specific. For each gene, two different sets of polyclonal antibodies (anti *LOX1* made in guinea pig and rabbit; anti *LOX2* made in rat and rabbit) revealed identical

expression patterns. The difference between the early studies and the present observations could be attributed to the improvement in the antibody purification method. The antibodies were purified in such a way to avoid cross-reaction with the homeodomain of other leech HOX proteins. In this way, it is expected that the expression pattern observed here are more specific and reliable. These observations suggest that *Lox2* may play a role in the identity of central neurons in the posterior segmental ganglia. In addition, it is likely that both *Lox1* and *Lox2* play a role in the differentiation of central neurons in the region of the CNS where the expression domains of these genes overlap (posterior M6 to M13). In addition, *Lox2* expression may be imposing strict, tight regulation on *Lox1* expression, thus inhibiting and limiting its expression to more posterior ganglia, a type of control typical of posterior Hox genes, a control called posterior dominance (also poster prevalence or functional dominance).

Despite the relatively stable expression of *Lox1* at later embryonic stages, the results presented here suggest that *Lox1* expression is spatially and temporally dynamic during early development of the leech CNS. *Lox1* expression can be described in three phases: early phase (E5-6), mid phase (8-9), and late phase (E10-14). At the early phase, *Lox1* expression CNS was detected in a few segmentally repeated and segment-specific neurons from posterior R4 to M6 at around E6. This is a time when the anterior ganglia are in their earliest stages of development, suggesting that *Lox1* may play a role in neurogenesis or in the early differentiation of central neurons.

The mid phase of *Lox1* expression occurs between E8 and E9, a time when the number of *Lox1*$^{+}$ neurons increased dramatically. The *Lox1* expression domain at this time extended from R4 to M12. At this crucial period of CNS development, many neurons acquire their

specific identity and generate axons that grow toward their targets. Therefore, *Lox1* may play a role in fating a specific population in this domain.

More neurons start to express *Lox1* at its late phase of expression, after E10, and the domain of *Lox1* expression becomes fully established in a population of segmentally-repeated and segment-specific central neurons between R4 and M13. *Lox1* continues to be expressed at later stages of embryonic development, at least up to E21, the last stage examined. *Lox1* stable expression through later developmental stages of the CNS suggests that this Hox gene may not only play a role in determining neuronal fate, but also in maintaining the identity of the neurons.

Lox1 may be involved in controlling the neuronal expression of cell-autonomous, cell surface, and extracellular factors required for axonal axonal growth, guidance and target recognition. It is quite possible that the *Lox1*$^{+}$ neurons provide certain environmental factors required by non-*Lox1*-expressing neurons to differentiate, project their axons through the correct pathways and establish connections with the right synaptic partners. Moreover, non-*Lox1* expressing neurons not located in the *Lox1* expression domain, but having to extend axons for several ganglia through its domain may depend on *Lox1*-specified guidance molecules to do so. Through all these potential mechanisms, *Lox1* may play an important role establishing neuronal circuitry of the CNS

Aisemberg and Macagno (1994) reported that *Lox1* is expressed in more ganglia than reported here. *Lox1* was the very first leech Hox gene cloned and then used to generate antibodies in order to study its expression pattern. Thus, it was not possible to adsorb the *LOX1* antiserum to other *LOX* proteins as a way of avoiding cross-reactivity via their highly conserved homeodomain. As a consequence, some of the *Lox1*$^{+}$ neurons reported

by Aisemberg and Macagno (1994) may be the result of cross-reaction with other HOX proteins. The new pattern of *Lox1* expression reported here is reliable because two different preparations of *LOX1* antibodies, which were raised in two different animal species (rabbit and Guinea pig) and adsorbed to other *LOX* proteins to eliminate cross-reactivity, reveal identical expression patterns. In addition, this expression pattern matches *in situ* hybridization results showing *Lox1* expression in M1-M6 at E7 (Joanna Wysocka-Diller, personal communication).

The Lox1 *Expression Pattern May Reflect Simplicity of Body Plan*

Hox genes are not only required for the construction of the nervous system, but they are also important for conferring identity other tissues and organs. However, it may be argued that Hox expression in any part of an animal is directly or indirectly related to nervous system development. Nerves emanating from the CNS innervate most organs or tissues. Thus, their Hox-specified identity is essential for providing the guidance cues to attract axons of central neurons, and establish the right connections.

In *Drosophila, Scr*, the putative homolog of *Lox1*, displays a more restricted pattern of expression during embryonic development of the fly, than *Lox1* shows in *Hirudo. Scr* is an anterior Hox gene. Gorman and Kaufman (1995) showed that its expression is initiated early (stage 5) in a jagged dorsolateral stripe around the border of the maxillary and labial segment primordia which, as judged by stripes of engrailed expression, is neither segmental nor parasegmental in register. Subsequently, it resolves into a parasegmental register (PS2) ventrally (primarily progenitors of the central nervous system) and what is largely a segmental register in its dorsolateral domain - the labial epidermis- (Mahaffey *et al*., 1989). This early expression includes a small population of cells in the lateral

epidermis of the posterior maxillary compartment (Carroll *et al*., 1988: Riley *et al*., 1987). Later, the expression of *Scr* expands into the prothorax. This expansion begins in the anterior prothorax and eventually fills the entire prothoracic epidermis but does not expand into the most ventral region of the ectoderm (Gorman and Kaufman, 1995; Carroll *et al*., 1988; Riley *et al*., 1987).

Scr mutation causes the first thoracic segment (T1) to become transformed into the second thoracic segment (T2), and the labial segment is reportedly transformed to maxillary identity in the fly embryos (Pattatucci *et al.,* 1991; Sato *et al.,* 1985; Struhl, 1983; Wakimoto and Kaufman, 1981). However, not much data is available on how is the nervous system of the fly is affected by these mutations. In mouse, the vertebrate homolog of *Lox1* is expressed in the kidneys, testis, stomach, mesodermal lung components and parts of the intestine, as well as in the spinal cord with an anterior boundary at the posterior hindbrain (Sharpe *et al.,* 1988).

Lox1 expression has been detected in the CNS during the embryonic development of *Hirudo* and in the sex organ primordia. However, it is not known if *Lox1* is contained in tissues (e.g the gut) that have been removed during dissection of the animal. Despite this, the expression along the rostral-caudal axis of *Scr* and *Lox1* may be considered similar between fly and *Hirudo*, in the sense that they are both anterior and they both have a rostral boundary in a posterior head segment. However, the complexity of insect body plan suggests a more elaborate set of functions for *Scr* than *Lox1* has in *Hirudo* with a simpler body organization. Leeches do not have specialized appendages (such wings, legs, sex combs, etc.) known to require *Scr* in flies. In addition, flies do not have the expression of *Scr* in the sex primordia. Also, a longer *Lox1* domain reflects less A-P

specialization. So with high expression in the CNS, a conserved feature of Hox genes throughout the animal kingdom, suggest that the CNS function of *Scr* in fly and *Lox1* are conserved, but other functions are not.

Lox1 ***Expression Suggests a Role in the Differentiation and Normal Innervation of the Sex Organ Primordia***

Lox1 expression was also detected in the genital primordia. This observation does not only suggest a role for *Lox1* in the differentiation of these organs, but also implies that *Lox1* may be important for the provision of target-derived information for developing central neurons that innervate the sex organs. The pathfinding capability of neurons involves interplay between the neurons themselves and their target organs. Developing neurons are often not required to carry at the growing axonal tips pathfinding information about where their every branch will go. Instead, this information is distributed throughout the tissues of the embryo in the form of extracellular signaling molecules. Thus, each individual developing neurite has to selectively recognize the environmental guidance cues and respond to them appropriately. The directional guidance cues provided to the developing neurons include a wide variety of molecules ranging from diffusible proteins such as netrin (Colamarino and Tessier-Lavigne, 1995; Serafini *et al*., 1994), small soluble molecules such as the neurotransmitter acetylcholine (Zheng *et al.*, 1994), substrate-bound proteins such as cell adhesion molecule-like proteins (Chiba and Keshishian, 1995) and *Eph* receptor ligands (Tessier-Lavigne, 1995). Some of these molecules serve as attractants while others serve as repellents of the growing tip of the axon.

In *Hirudo*, it has been shown that the sex organs may play an important role in providing signals for central neurons in the specialized sex ganglia (M5 and M6). A number of the neurons that innervate the male sex organ have been identified. It was not fully established how many of these cells express *Lox1*, but the RPEs and Rz neurons could be identified. Both can be identified by E7: the Rz neurons contain serotonin; the RPE neurons express *Lox1* and contain FMRFamide-like peptides. Baptista and Macagno (1988) have shown that the RPE neuorns can find and contact the male sex organ even after ectopic transplantation of this organ to other segment, suggesting that the male sex organ provides guidance cues for the RPE neurons. In a similar manner, Loer *et al*. (1987) have shown that, in the absence of reproductive tissue, the Rz neurons of M5 and M6 adopt a morphology similar to that found in other ganglia, suggesting that the Rz cells of these specialized ganglia also interact with their unique targets. It is therefore likely that *Lox1* plays an important role in regulating the expression of guidance cues produced in the sex organs that attract the sex nerves toward their targets. Thus, *Lox1* may play a role in controlling the innervation of the male and female sex organs.

Regional Expression of* Lox2 *in Posterior Segmental Ganglia of the CNS

While *Lox1* is expressed in an anterior midbody domain, where it may play a role in the specification of neuronal cell fate in the CNS, *Lox2* is expressed in segmental ganglia of the posterior two-thirds of the CNS (but not in the caudal segments). The number of $Lox2^{+}$ nuclei increased in a stepwise manner from M6 to M7 and M8. The highest number of $Lox2^{+}$ neurons was located in M8 and M9. From M11 to M15, there is a large number $Lox2^{+}$ nuclei, but slightly lower than that of M8-M9. Posterior to M15, the $Lox2^{+}$ population progressively decreased. As a consequence, *Lox2* may be expected to play a

more important role in specifying the identity of neurons in the anterior region of its expression domain (M7-M15). The decrease in the number of *Lox2*$^{+}$ neurons in posterior ganglia could be attributed to expression of other genes that may down-regulate *Lox2* expression. For example, *Lox4* and *Lox15* which are genes expressed at high levels in posterior ganglia, and at low levels in anterior ganglia of their domain, could be suppressing or down-regulating *Lox2* in M16-M21.

The results presented here show *Lox2* expression up to late stages of embryogenesis (E20). In addition, mRNA of a number of *Lox* genes, including *Lox1* and *Lox2*, has been isolated from adult leeches, indicating *Lox* expression well beyond embryogenesis. The presence of *Lox2* gene products at later developmental stages suggests that this Hox gene, like *Lox1*, plays an important role not only in the differentiation, but also in the maintenance of the identity of central neurons in midbody ganglia located in the posterior two-thirds of the animal trunk. This idea is supported in part by the observation of Hox expression in adult tissues of other animals (reviewed in Morgan, 2006; Nicolas *et al.,* 2003).

Though the expression pattern of *Lox2* detected in this study is similar to those reported earlier, including the expression in a distantly related leech species (Wysocka-Diller *et al*., 1989), there are some differences. Wysocka-Diller *et al*. (1989) reported low levels of *Lox2* expression in the caudal neuromeres (C1-C7) of *Hirudo* during early stages of embryogenesis, using *in situ* hybridization. Caudal expression was not observed in this study using antibody staining. However, the expression pattern of *Lox2* reported for the distantly related leech species, *Helobdella robusta* in another *in situ* hybridization (Nardelli-Haefliger and Shankland, 1992) is very much similar to the one reported here;

not caudal expression. These differences could be attributed to differences in the techniques used to study the expression pattern. Though *in situ* hybridization has proven to be useful and reliable, there is a possibility that the hydrolyzed fragments of the *Lox2* riboprobe hybridized to conserved regions of the endogenous transcripts of other leech *Lox* genes in the caudal ganglia of *Hirudo*, especially with *Lox15* which is believed to be expressed in the tail and has a high sequence homology with *Lox2*, despite the fact that the homeodomains of Hox genes are more divergent at the nucleotide level, than at the amino acid. A less likely explanation would be that the riboprobe detected in C1-C7 was an alternative splicing form of the *Lox2* mRNA, containing the same 3'-utr as in M6-M21, but a divergent coding region that was not detected by the antibody. The polyclonal antibodies used to study *Lox2* expression were adsorbed to affinity columns containing other leech HOX proteins. A main advantage of this step is that it minimized cross-reaction of the *LOX2* antibody with other *LOX* proteins through their homeodomains. In addition, two different preparations of *LOX2* antibodies, raised in two different species (rat and rabbit), revealed the same pattern. Thus, the *Lox2* expression pattern reported here may be considered more reliable and specific.

Early Expression of Lox2 *Precedes Overt Segmentation*

At E4, a time when there is no overt manifestation of the neuromeres or any other segmented structure, *Lox2* was expressed in the posterior region of the embryo. There was no detectable expression for any other *Lox* gene examined in more anterior segments. However, *Lox1* expression at this stage was also detected in the primordia of the female genital, which is located between M6 and M7 (older animals) in the same embryos when costained with the *LOX1* antibodies. The *Lox2* expression started posterior to *Lox1*

expression. The *Lox2*$^{+}$ nuclei appeared as a column of cells along the longitudinal axis of the embryo. This is similar to the expression pattern in *Helobdella robusta*, whose earliest embryonic stages are easier to examine and more amenable to cell lineage studies. These longitudinal columns of cells are called bandlets and occur as five bilateral pairs, each descending directly from the five pairs of stems cells called teloblasts. The five pairs of bandlets of cells fuse to form the germinal plate of the embryo. In *Helobdella*, Nardelli-Haefliger and coworkers labeled individual stem cell lineages by intracellular injection of fluorescent tracers and found colocalization of the lineage tracer with the *Lox2* signal in the progeny of the five different pairs of teloblasts (Nardelli-Haefliger and Shankland, 1992). It is not clear whether early *Lox2* expression occurs in neuronal precursors in *Hirudo*. Nevertheless, this early pattern of *Lox2* expression suggests that this gene may play a role in the specification of some cellular precursors (both neuronal and non-neuronal) that will eventually give rise to specific tissues in the germinal plate, or some of the descendant cells of it.

Overlapping Expression of Lox1 *and* Lox2 *Suggests That* Lox2 *May Impose Posterior Dominance*

The expression domains of *Lox1* and *Lox2* overlap from posterior M6 to M13 (Fig. 2-8). This overlap suggests that both genes may play a role in assigning identity to neurons in a cooperative manner (the "Hox Code" hyopothesis). The overlap and coexpression of Hox genes is important for establishing morphological diversity along the rostral-caudal axis.

This overlapping expression also suggests that *Lox2* may be down-regulating *Lox1* expression, either directly or indirectly. This implication stems from the fact that the number of *Lox1*$^{+}$ neurons decreased progressively from M7 to M13, while *Lox2*-

containing nuclei progressively increased in these ganglia. In fact, it has been suggested that in regions of coexpression of Hox genes, these genes compete functionally for assigning positional values and specifying cell fate. Most often, in such competition, the posterior Hox gene prevails, and controls segment identity. This competitive interaction between Hox genes has been called functional dominance, phenotypic suppression or posterior prevalence Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al*., 1990; Duboule, 1991). This controlling effect of posterior Hox genes has been demonstrated in ectopic expression mutants of fruit fly and mouse, where segmental transformations have been observed anterior but not posterior to the normal expression domain of the misexpressed Hox gene (Duboule and Morata, 1994; reviewed by Botas, 1993; Lufkin *et al*., 1992; Gonzalez-Reyes and Morata, 1990; Mann and Hogness, 1990; Struhl, 1983). For example, Luftkin *et al*. (1992) have shown that the generalized overexpression of the mouse Hox gene *Hoxd-4* causes homeotic transformations of segments anterior but not posterior to its normal expression domain. In a like manner, the ubiquitous expression of *Ubx* in *Drosophila* transforms parasegments anterior but not posterior to its normal expression boundary (Gonzalez-Reyes and Morata, 1990; Mann and Hogness, 1990). These observations indicate that the posterior Hox genes may act as repressors of anterior Hox genes. This may be the case for *Lox1* and *Lox2*, with *Lox2* functioning as the repressor. At the molecular level, it has been shown that the *Drosophila* Hox gene *Abd-A* carried out its repressive regulatory activity via its downstream target gene, suggesting that the Hox genes may not repress other Hox genes through direct interaction with their regulatory elements (Capovilla and Botas, 1998).

It is possible that other posterior *Lox* genes also may be turning off or down-regulating *Lox1* expression in M7-M13. For instance, *Lox4* which is expressed at high levels from M7 may be repressing *Lox1* expression. In addition, it is possible that both *Lox2* and *Lox4* cooperate in suppressing *Lox1* expression.

In another example, *Lox4* may be turning off *Lox2*. *Lox4* is expressed at high levels from M13 to M21, to a magnitude of 100 neurons per ganglion. *Lox2* expression tapers off progressively to fewer neurons from M16 to M21. Therefore, *Lox4* may be turning off *Lox2* expression from M16 to M21, in a progressive manner that reflects lesser and lesser $Lox2^{+}$ neurons.

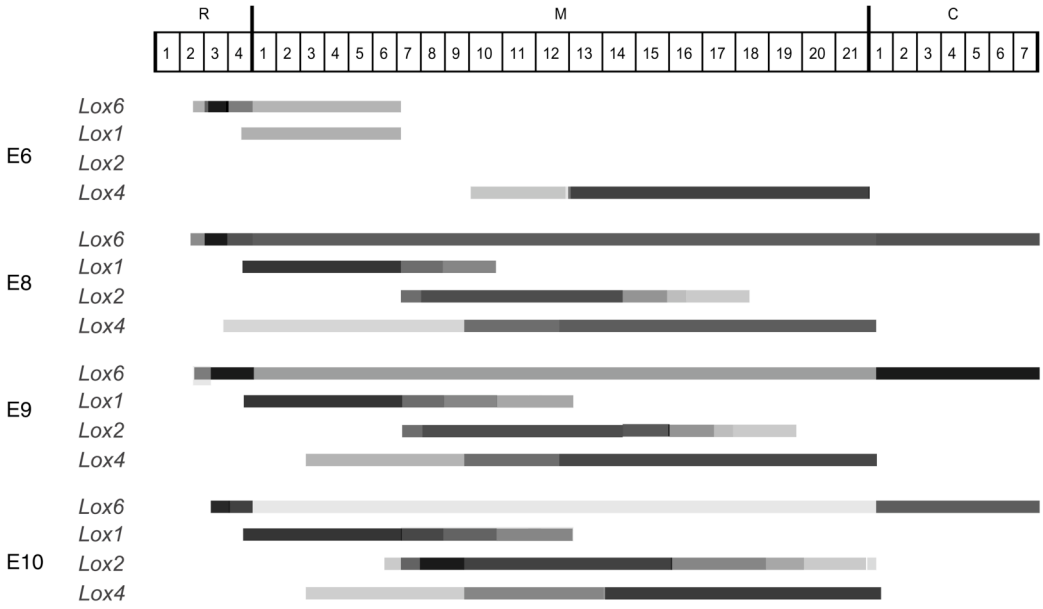
The concept of posterior dominance raises the question of whether anterior Hox genes with strong expression can repress posterior genes that have weak expression in their anterior boundary. Though there is not a large body of evidence to support this idea, the overlapping in *Lox1* and *Lox4* in the CNS of Hirudo may imply such a relationship in annelids. As reported in this study, *Lox1* has a stable, well-defined anterior expression domain in which its expression is highest from M1 to M7. From M3 to M9, *Lox4* is expressed in just a few nuclei per ganglion. The high expression of *Lox1* and the low expression *Lox4* suggest that *Lox1* may be the more dominant partner in specifying the identity of anterior ganglia. However, the role of other anterior *Lox* genes e.g. *Lox20*, should be taken into consideration, in their role in suppressing the posterior *Lox* genes in anterior segments.

In a similar scenario, *Lox2* may also suppress *Lox4* (M7-M12). *Lox4* is expressed at moderate levels from M6-M12 (Wong 1997), ganglia which contained a large number of

Lox2-containing nuclei. It could be that *Lox2* is repressing the expression of *Lox4* in these ganglia.

Figure 2-8 ***Expression domains of well-studied Hox genes during early development of the CNS of*** **Hirudo medicinalis.**

Lox1 is expressed in a relatively stable domain stretching from the posterior edge of R4 to M13 through later stages (21), with the anterior ganglia (R4 to M7) containing more *Lox1*$^{+}$ neurons. *Lox2* is expressed from the posterior region of M6 to M21, with most of the *Lox2*-containing neurons located in M8 to M15. *Lox4* is expressed from M3 to M21, however the anterior ganglia (M3-M9) of its expression domain contain few *Lox4*$^{+}$ neurons. By E13, *Lox4* expression reaches its peak (100 neurons/ganglion from M13-M21), but by E15 decreased to very few cells (Wong 1997). *Lox6* expression starts in R2 then (later it shifts to R3). At later stages, *Lox6* expression decreased drastically in midbody ganglia, but remained high in the R3-R4 and C1-C7. The widely overlapping expression of these genes supports the idea of the "Hox code", in which unique combination of Hox genes specify diversity of cell fates in the CNS. In the figure, shadowing represent high *Lox* expression in terms of number of neurons in the segmental ganglia and light shadowing represent lower levels of expression. Anterior is to the left.


R
M
C
1 2 3 4 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 1 2 3 4 5 6 7
E6
Lox6
Lox1
Lox2
Lox4
E8
Lox6
Lox1
Lox2
Lox4
E9
Lox6
Lox1
Lox2
Lox4
E10
Lox6
Lox1
Lox2
Lox4

A-P expression of Hox Genes in the CNS of Hirudo medicinalis

Whereas *Lox1* expression in the CNS and genitalia remains relatively simple and stable, other *Lox* genes display more elaborate and complex spatial and temporal expression, patterns that may reflect on the relative important functions of each Hox gene in constructing the nervous system of the leech. The manner of *Lox1* expression, which can be described in three phases: early phase (E5-6), mid phase (8-9), and late phase (E10-14), considered stable when compared with the expression *Lox* genes.

Lox6 displays an early phase and a late phase expression pattern. The early phase of *Lox6* expression (around E7), shows an anterior boundary in R2 within the CNS and extended to the last caudal neuromere by E8. In the peripheral nervous system, *Lox6* is expressed in the sensillae, extrasensillar neurons, lip sensillae, and eyes, and in muscle of all segments including those anterior to R2, suggesting that this gene may play a role in patterning the entire nervous system of the leech during early development. These observations are further bolstered by the expression of *Lox6* in highly specialized pioneer neurons: the mechanosensory P_D neurons and the bipolar neurons (Pimentel-Mercado, 2003). The P_D cells have been shown to generate a pioneer axon into the periphery, thus forming the dorsal-posterior nerve (Jellies *et al.,* 1994; Kuwada, 1985) and guide other axons with their peripheral branches, allowing them to connect to their targets. It has been suggested that the bipolar neurons pioneer the formation of the longitudinal connective nerve. These observations suggest that *Lox6* may be involved in a more elaborate set of functions in establishing the nervous system in the leech. Later on in development (after E9), the anterior boundary of *Lox6* expression shifts more posteriorly to R3, while its posterior expression becomes greatly restricted to very few neurons per

ganglion from M1-C7. This late pattern is stable throughout the remaining stages of embryonic development.

Lox4, a homolog of the *Drosophila* genes *Ultrabithorax* (*Ubx*) and *abdominal-A* (*Abd-A*) also displays a more complex and dynamic pattern expression than *Lox1*, from a regional perspective. Wong *et al* (1995) have shown that the earliest *Lox4* expression starts at E6, at about midbody segment 10, in a region known to contain precursors of central neurons. Late *Lox4* expression in the CNS, at about E9, shows a majority of $Lox4^{+}$ neurons in M10-M21. However, the anterior boundary of *Lox4* expression extends to few neurons per ganglion M3-M9. *Lox4* is also expressed in peripheral tissues of segments M13 to M21.

In summary, the expression of *Lox* genes occurs at a crucial time of development. The early phase of *Lox* expression in the CNS (E5-7) suggests that these genes may be involved in neurogenesis and in conferring the very early properties of neuronal identity. The later phases of expression suggest that *Lox* genes may also be involved in maintaining the identity of central neurons.

Expression Domains of Well-Studied Hox Genes in CNS of Hirudo

The new insight into the expression pattern of *Lox1* and *Lox2* obtained in the present study warrants a revision of Hox gene expression during the development of the CNS of *Hirudo*. The A-P expression pattern of several of the best-studied leech Hox genes (including those in this study) are summarized in Fig. 2-8. *Lox1* expression started at about E5, with an anterior boundary of posterior edge of R4. As development continues, the expression domain extends to more posterior ganglia, up to M13 by E14.

Lox2 expression develops in a similar manner to that of *Lox1*, with a few minor differences. *Lox2* expression in the CNS was first detected at a later stage (early E7) than *Lox1* expression. This difference could mean that early *Lox1* expression may be required for the early of *Lox2* expression. Subsequently, the onset of *Lox2* expression may inhibit posterior *Lox1* expression. On the other hand, this could be simply a consequence of the developmental gradient of the embryo, with R4 about a day ahead of M6 in its development. As development continues, more posterior ganglia started to express *Lox2*, as the number $Lox2^{+}$ neurons in the anterior ganglia increased. By 14, the expression domain of *Lox2* becomes stable and spans from the posterior edge of M6 to M21.

The temporal and spatial expression of *Lox6* in the developing CNS displays greater dynamism than either *Lox1* or *Lox2*, both of which, in comparison to *Lox6*, have relatively stable expression domains in time and space. The earliest *Lox6* expression in the CNS is detected at E7, with an anterior boundary in R2 (Mercado-Pimentel, 2003). Later in development, the domain of *Lox6* expression shifts more posteriorly, with R3 as its anterior boundary. It extends gradually to C7, with many neurons per ganglions. Most of these neurons in M1-C7 stop expressing *Lox6*. This could be a consequence of other *Lox* genes being turned on in the posterior ganglion and thus down-regulation *Lox6*. Such genes include *Lox1*, *Lox2*, *Lox4* and *Lox15*, in that order. The expression of *Lox6* at this stage is more stable than at earlier stages and overlaps with that of *Lox1* from M1-M13, and *Lox2* in M6-M21, although *Lox6* is expressed in very few cells in this region of overlap.

The earliest expression of *Lox4* within the CNS occurs at late E6 at M10. Later in development *Lox4* expression from E9 extends to a few neurons per segment from M3-

M9 and to many neurons in M10-M21. At this time, *Lox1* expression progressively decreased from M7-M13. It is likely that *Lox4* may play a role in down-regulating *Lox1* from M10-M13, even though *Lox2* may play a similar role from M7-M13. In addition, both *Lox2* and *Lox4* may cooperate to restrict *Lox1* expression from M7-M13. By E15 *Lox4* expression decreased in posterior ganglia (Wong, 1997), suggesting that its expression may be restricted by the expression of *Lox15* which is expressed from M15 to C7. However, it is not clear if *Lox15* expression remains high at E15.

Of particular interest in Fig. 2-8 is the extent of overlap of the leech Hox genes. The overlap partially supports the idea of the Hox code, in which unique combination of Hox genes specify the variety of cell fates in the CNS (Hunt *et al*., 1991).

Cloning and expression of Lox7 *cDNA*

The *Lox7* insert was correctly cloned inframe with the pGEX-2t expression vector. Protein alignment analysis and comparison confirmed that the fragment of *Lox7* is a *labial* class Hox gene. The GST-*LOX7* fusion protein is a soluble protein as it was detected in the supernatant during the purification steps. The fusion protein was recognized as a 57 kDa band on SDS-PAGE.

CHAPTER 3

IDENTIFICATION OF *LOX1*- AND *LOX2*-EXPRESSING CENTRAL NEURONS IN LEECH EMBRYOS

INTRODUCTION

A large number of studies have shown that Hox genes play an important role in the diversification of segments in animals with a metameric body organization. These genes specify the identity of body segments along that anterior-posterior axis. A main site of expression of Hox genes is the nervous system. The nervous system is highly complex and has the largest diversity of cell types in an animal. The cellular diversity of the nervous system is evident in cell body morphologies, patterns of arborization, action potential firing patterns, synaptic partners and targets, etc. Understanding the molecular mechanisms by which this cellular diversity develops is a very important step in understanding the development and pathology of the nervous system. Hox genes, as master molecular architects of development, have been shown to direct many processes that are involved in the genesis and correct specification of the nervous system. Differential cell migration, axonal outgrowth and navigation, target recognition, and synapse formation establish neuronal circuitry and are among the numerous processes required to bring about the organization of the nervous system. The ability of neurons to accomplish these feats involves interplay between the neurons themselves, the

environment, and their synaptic partners. The neurons may require transcription factors to determine their early stages of differentiation. The environment may contain guidance cues that may either repel or attract outgrowth of the neurons, as a way of guiding them toward the correct target. The synaptic partners may be required to express the appropriate surface receptor to bring about the correct target recognition.

Although many questions of Hox gene function in the development of the CNS have been addressed by studies in other animals ranging from fly to mouse, these animal models are not very amenable to provide great insight into the role of Hox genes at the single cell level. The medicinal leech *Hirudo medicinalis* provides several unique advantages for such studies. It has a relatively simple nervous system that has been well studied. The nervous system consists of a ventral nerve cord with which 32 segmental neuromeres and a supraesophageal ganglion of non-segmented origin. The four rostral neuromeres fuse to form the rostral ganglion and the seven most caudal neuromeres fuse to form the caudal ganglion. The remaining neuromeres form the 21 midbody ganglia. Each of these ganglia contains about 400 neurons (Macagno, 1980), except the sex ganglia (M5-M6), which contain about 500-600 neurons that are generated later in embryogenesis. Many neurons can be reliably identified by their morphology and their location in the segmental ganglia. Many neurons that are segmental homologs display segment-specific phenotypes. In addition, the functions of many neurons have been well studied and their synaptic targets have been determined. Also, a number of neurons have relatively large cell bodies, making it potentially possible to manipulate the expression of genes in a single cell, in an otherwise native or wild type environment.

The present chapter describes the identification of neurons that express *Lox1* or *Lox2*. The identification of neurons that express Hox genes provides the background for studying the function of these genes. *Lox1* expression was detected in the serotonergic AMS and PMS neurons of M2 and M3 (Macagno and Steward 1987). *Lox1* expression was also detected in the RPE neurons, specialized motors neurons of M6 that innervate the male sex organ (Becker *et al.*, 1996; Baptista and Macagno, 1988; Zipser, 1979), but not in the segmental homologs of the RPEs neurons, the RMV neurons. Double staining experiments using *LOX1* and Laz1-1 antibodies also showed that *Lox1* is expressed in the paired heart accessory-like (HAL) neurons of M4, the putative segmental homologs of the heart accessory (HA) neurons located exclusively in M5 and M6. On the other hand, double staining experiments using *LOX2* and Laz1-1 antibodies showed that *Lox2* is expressed in the HAL neurons of M7-M17. Double staining experiments using serotonin and *LOX2* antibodies showed that the Retzius (Rz) cells (the "giant neurons" of the leech; Kerkut and Walker, 1967) expressed *Lox2* from M8 to M21. *Lox2* is also expressed in the PMS neurons of M7-M21 and in cell 251- also called the NUT neuron (Becker *et al.*, 1996; Sargent, 1975). *Lox2* expression was also detected in the RMV and CV neurons of M7-M19. Though the CV neurons have been described by Stuart (1970), dye-filling and confocal microscopy studies showed that these neurons have contra-lateral projections that exit the ganglion through the anterior lateral root (Rela and Szczupak, 2007). Another interesting finding in this research is the expression of *Lox1* and *Lox2* in segmentally repeated homologous neurons that contain the Laz1-1 antigen, including unpaired neurons located in the anterior region of the ventral-medial glial packet that express *Lox1* in M4-M8 and their paired segmental homologs of M9-M16 that express

Lox2. This information provides a good background for studying the function of *Lox1* or *Lox2* in RNAi-knockdown experiments.

MATERIALS AND METHODS

Fixing and staining of embryos were as described in Chapter II. For double staining experiments, the monoclonal antibody Laz1-1 (a gift generously provided by Dr. Zipser) was used at a dilution 1:5 to 1:10, the rabbit antibodies against FMRFamide (Peninsula Laboratories, Inc.), serotonin (Sigma), and enkephalin (Sigma) worked well at 1:1000 to 1:2000 dilutions. Images were generated as described in Chapter II. The *LOX1* antibody was diluted 1:1000 and the *LOX2* antibody 1:200. *Lox1* expression was detected with an anti-Guinea pig TRITC-labeled antibody; *Lox2* expression was detected with an anti-rat Cy3-labeled antibody. An anti-mouse Cy2-labeled antibody was used to visualize cells that contain Laz1-1 antigen. An anti-rabbit Cy2-labeled antibody was used to detect serotonin-, FMRFamide-, and enkephalin-containing neurons.

RESULTS

Lox1 *Expression in Serotonergic Neurons*

A number of *Lox1*$^{+}$ neurons were identified using different marker antibodies and the *LOX1* antibody in double-staining experiments. Neurons were identified on the basis of the markers they expressed, their relative positions within the ganglia, and the relative sizes of their cell bodies.

For the *Lox1*-serotonin double staining, I studied eight embryos at E9 and six at E13. All the embryos showed identical *Lox1* and serotonin staining for each stage. Among the

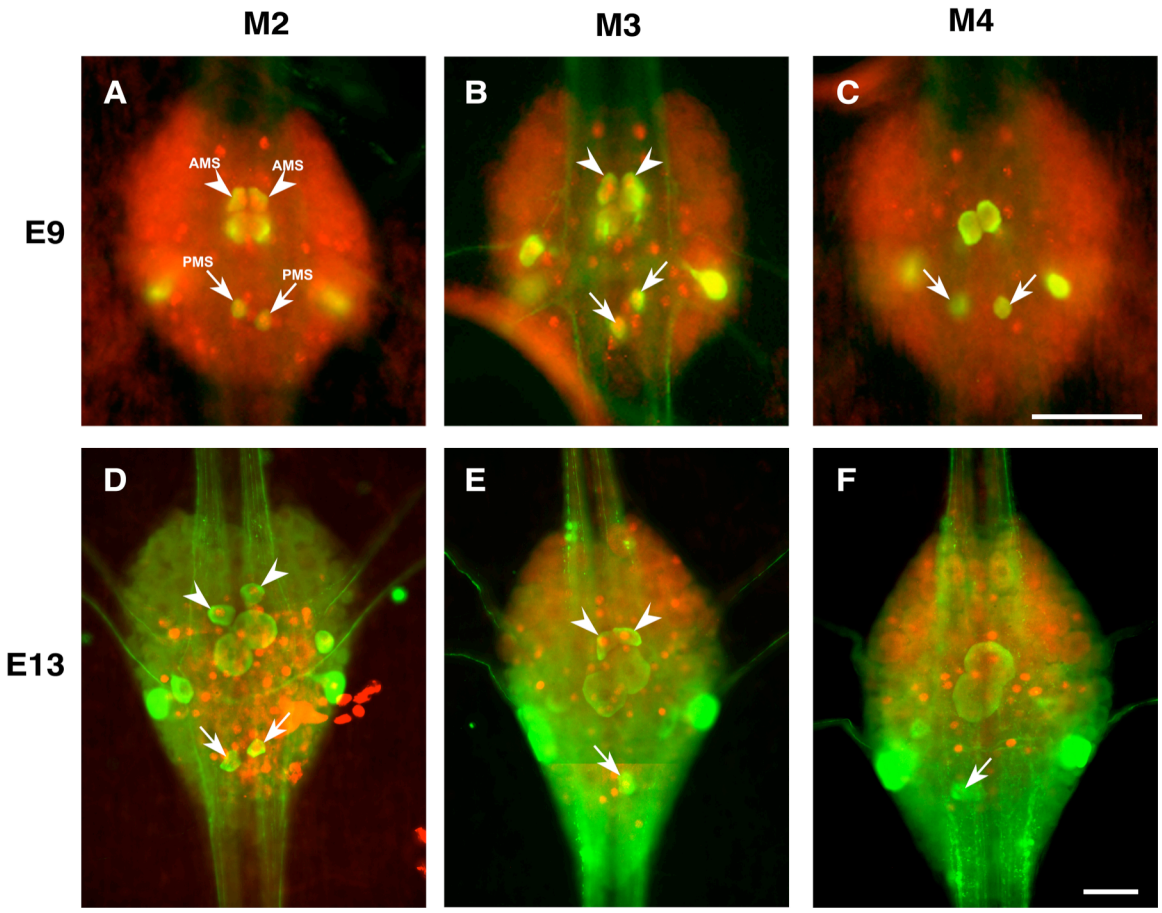
neurons identified using this approach were the anterior-medial serotonergic (AMS) and posterior-medial serotonergic (PMS) neurons of M2 and M3. *Lox1* and serotonin were detected in these neurons as early as E9 and through later embryonic stages (Fig. 3-1, see also Table 3-1). The AMS neurons are segment-specific interneurons that appear only in R1-M3, where they are located just next to the large, paired Rz cells. In M2 and M3, these neurons expressed *Lox1*. The PMS neurons are found in the ventral aspect of all midbody ganglia (Lent, 1982; Rude, 1969). These cells are interneurons that remain paired in R1-M2, but become unpaired posterior to M2. In M2, both PMS neurons expressed *Lox1* (Fig. 3-1 A, D). The unpaired PMS neuron of M3 continued to express *Lox1* at later embryonic stages (Fig. 3-1 E). These observations suggest that *Lox1* may be involved in the regional differentiation of these neurons. It is possible that expression of *Lox1* in the AMS and PMS neurons of M2 and M3 is related to two phenotypic transitions happening at this location: (a) the absence of AMS neurons posterior to M3 and (b) the disappearance of a single PMS neuron in every ganglion, starting at M3.

Table 3-1 ***Serotonergic neurons that express*** **Lox1** ***and/or*** **Lox2.**

Neurons	**Present**	***Lox1*$^{+}$**	***Lox2*$^{+}$**	**Fig.**	**Description**
AMS	R1-M3	M2-M3	--	3-1	paired interneurons relatively large somata intense serotonin and *Lox1* staining adjacent and anterior to the Rz cells
PMS	R1-M2 (paired) M3-M21 (unpaired)	M2-M3	M7-M21	3-1 3-7	paired or unpaired interneurons medium-size somata intense serotonin and *Lox1* staining moderate *Lox2* staining at later stages posterior-medial location
Rz	R1-M21	--	M8-M21	3-7	paired ipsilateral projections to periphery largest somata in the leech CNS moderate serotonin and *Lox2* staining central-medial location

Figure 3-1 Lox1 *expression in serotonergic interneurons.*

Lox1 expression was detected at E9 in the AMS neurons (arrowheads) and in the PMS neurons (arrows) of only M2 (***A***) and M3 (***B***), but not in M4 (***C***). This expression pattern was maintained at E14, but one of the two PMS neurons of M3 underwent programmed cell death (***D***: M2, ***E***: M3, ***F***: M4). *Lox1* staining is out of focus in the right PMS neuron of ***D***. Red fluorescence (TRITC) shows *Lox1* expression; green fluorescence (Cy2), serotonin. The scale bars are 50 μm.


M2
M3
M4
A
B
C
AMS
AMS
PMS
PMS
E9
D
E
F
E13

Lox1 ***Expression in FMRFamide-Like Immunoreactive Neurons***

Lox1 expression was detected in specific FMRFamide-like immunoreactive (FLI) central neurons from E9 to E14 (Figs. 3-2, 3-3, and 3-4; see also Table 3-2 for detailed descriptions of these neurons). For the *Lox1*-FMRFamide double staining, I observed 11 embryos at E14. All the embryos showed identical *Lox1* and FMRFamide staining. In each E9 embryo, just two to four FLI neurons express *Lox1* in mid body ganglia M2-M7 (data not shown). By E14, the number of $Lox1^+$ FLI neurons increased to about 4 cells in M1, 16 in M2, 11 in M3, 10 in M4, 8 in M5, 15 in M6, 11 in M7, 9 in M8, 10 in M9, 9 in M10, 7 in M11, and 9 in M12. A number of these cells were segmentally repeated (*i. e.*, they appeared in several consecutive ganglia –arrowheads in Figs. 3-2, 3-3 and 3-4–), while others were segment-specific (*i. e.*, they appeared only in some ganglia, in a non-consecutive manner –arrows in Figs. 3-2, 3-3 and 3-4).

Segment-specific $Lox1^+$ neurons included the VFLI-14 neurons of M3 (Fig. 3-2 F). The putative VFLI-14 homologs in other ganglia (e.g., M2 and M4, see Fig. 3-2 F, H) did not express *Lox1*. In addition, the $Lox1^+$ DFLI-3 neurons were detected only in M4 (Fig. 3-2 G) and M6 (Fig. 3-3 C). Other $Lox1^+$ FLI neurons that appeared to be segment-specific included the VFLI-1 neurons of M1 (Fig. 3-2 B), the DLFI-2 neurons of M2 (Fig. 3-2 C), the VFLI-5 neurons of M2 (Fig. 3-2 D; these neurons may be present in all ganglia, but with weak FMRFamide staining and no *Lox1* expression), the VFLI-11 neuron of M9 (Fig. 3-4 F), the VFLI-12 neurons of M12 (Fig. 3-4 L), and the RPE neurons of M6 (Fig. 3-3 D).

Some *Lox1*$^{+}$ FLIs neurons were segmentally repeated, but only within a specific region. For instance, the DFLI-1 neurons expressed *Lox1* in M1-M10 (Fig. 3-2 A, C, E and G, Fig. 3-3 A and C, and Fig 3-4 A, C, E and F) but not in other segments, where the DFLI-1 neurons appeared to be present and stained with the FMRFamide antibody. In a different case, the previously characterized cell 261 expressed *Lox1* in M7-M12 (Fig. 3-4 B, D, F, H, J and L) and was either absent or did not express FMRFamide-like peptides in other ganglia. In addition, the unpaired VFLI-9 neurons expressed *Lox1* in M6-M12 (Fig. 3-3 D; Fig. 3-4 B, D, F, H, J and L), but not in more anterior ganglia. Other *Lox1*$^{+}$ FLI neurons are segmentally repeated from M2 to M12, along the entire *Lox1* domain, including the VFLI-6 neurons, the unpaired VFLI-7 neurons, and the unpaired VFLI-13 neurons. Finally, some *Lox1*$^{+}$ FLI neurons only express *Lox1* in two to three consecutive segments (see Table 3-2), VFLI-3 (M2-M3), VFLI-4 (M2-M3), and VFLI-8 (M5-M6). VFLI-2 appeared M2-M4, but showed up again in M7.

Of particular interest are the RPE neurons of M6, which have been extensively studied in the past (Fig. 3-3 D). The RPE neurons contain FMRF-amide-like peptides (Kuhlman et al., 1985) and are unique to M6. These specialized motor neurons innervate the male sex organ (Zipser, 1979). The RPE neurons express *Lox1* and *Lox4* at early stages (E7-E8), but they express only *Lox1* at later stages. Their segmental homologs (the RMV neurons) do not express *Lox1*, but many express *Lox2* (M7-M21) and *Lox4* (M4-M5 and M7-M21).

Table 3-2 ***FLI neurons that express*** **Lox1**

Neurons	*Lox1*$^{+}$	Fig.	Description
DFLI-1	M1-M10	3-2 ACEG 3-3 AC 3-4 ACEG	paired small somata moderate FMRFamide staining intense *Lox1* staining posterior-lateral location
DFLI-2	M2	3-2 C	paired mid-sized somata anterior-medial location
DFLI-3	M4 M6	3-2 G 3-3 C	paired small somata moderate FMRFamide and *Lox1* staining location close to anterior nerve root
DFLI-7	M6	3-3 C	paired mid-sized somata intense FMRFamide and *Lox1* staining location close to anterior nerve root
VFLI-1	M1	3-2 B	paired small somata intense FMRFamide and *Lox1* staining anterior-medial location
VFLI-2	M2-M4 M7	3-2 DFH 3-4 B	paired mid-sized somata anterior-medial location
VFLI-3	M2-M3	3-2 DF	paired large somata anterior-medial location
VFLI-4	M2-M3	3-2 DF	paired small somata posterior-medial location
VFLI-5	M2	3-2 D	paired small somata posterior-medial location
VFLI-6	M2-M12	3-2 DFH 3-3 BD 3-4 BDFHJL	paired small somata posterior-medial location

Neurons	***Lox1*$^{+}$**	**Fig.**	**Description**
VFLI-7	M2-M12	3-2 DFH 3-3 BD 3-4 BDFHJL	unpaired small somata posterior-medial location
VFLI-8	M5-M6	3-3 BD	paired very small somata posterior-lateral location
VFLI-9	M6-M12	3-3 D 3-4 BDFHJL	unpaired mid-sized somata expressed *Lox2* (M9-M16) anterior-medial location
cell 261	M7-M12	3-4 BDFHJL	paired large somata central-medial location
VFLI-11	M9	3- 4 F	unpaired small soma location posterior to anterior nerve root
VFLI-12	M12	3-4 L	paired mid-sized somata location posterior to anterior nerve root
VFLI-13	M2-M12	3-2 DFH 3-3 BD 3-4 BDFHJL	unpaired large somata intense FMRFamide staining weak *Lox1* staining in posterior ganglia anterior-medial location
VFLI-14	M3	3-2 F	paired large somata moderate FMRFamide and *Lox1* staining anterior-medial location
RPE	M6	3-3 D	paired motor neurons large somata anterior-medial location

Figure 3-2 Lox1$^+$ *FLI neurons of ganglia M1-M4.*

In this and subsequent figures, arrows indicate *Lox1*$^+$ FLI neurons that are repeated in most ganglia of the *Lox1* domain and arrowheads indicate *Lox1*$^+$ FLI neurons that appear either in few ganglia (consecutive or non-consecutive) or in a single ganglion (segment-specific neurons). The left panels (***A***, ***C***, ***E***) show dorsal views and the middle panels (***B***, ***D***, ***F***) show ventral views. The *Lox1*$^+$ FLI neurons are indicated on the diagrams at the right side of the figure (in this and other similar figures, only one member of each pair is labeled). Red fluorescence (TRITC) shows *Lox1* expression; green fluorescence (Cy2), FMRFamide. The scale bar is 50 μm.

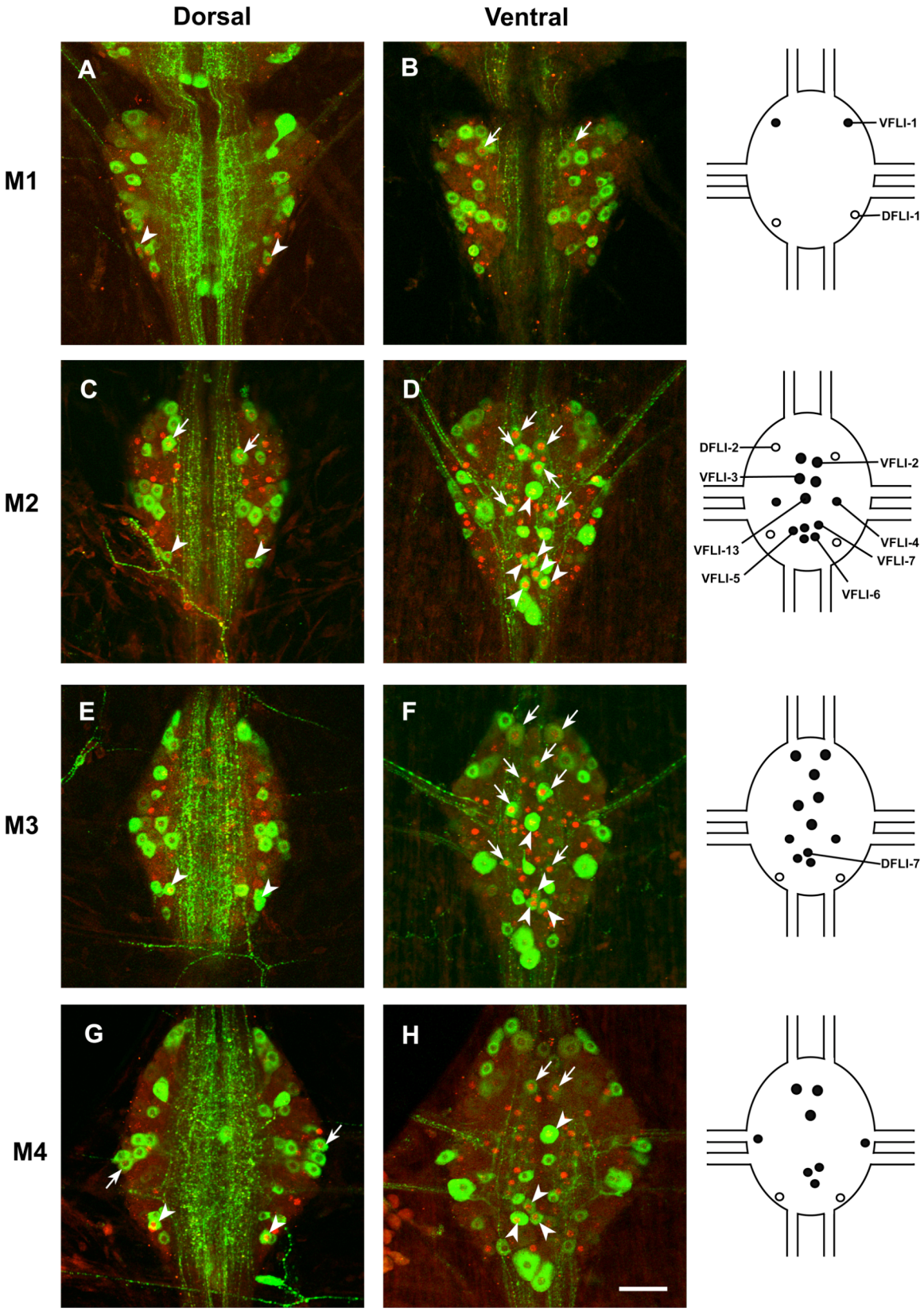

Dorsal
Ventral
M1
M2
M3
M4
A
B
C
D
E
F
G
H
VFLI-1
DFLI-1
DFLI-2
VFLI-2
VFLI-3
VFLI-13
VFLI-4
VFLI-7
VFLI-5
VFLI-6
DFLI-7

Figure 3-3. Lox1$^+$ *FLI neurons of the sex ganglia.*

Arrows and arrowheads are as in Fig. 3-2. Among the *Lox1*$^+$ FLI neurons were the segment-specific RPE motor neurons of M6 (***D***). The left panels (***A***, ***C***) show dorsal views and the middle panels (***B***, ***D***) show ventral views. The *Lox1*$^+$ FLI neurons are indicated on the diagrams at the right side of the figure. Red fluorescence (TRITC) shows *Lox1* expression; green fluorescence (Cy2), FMRFamide. The scale bar is 50 μm.

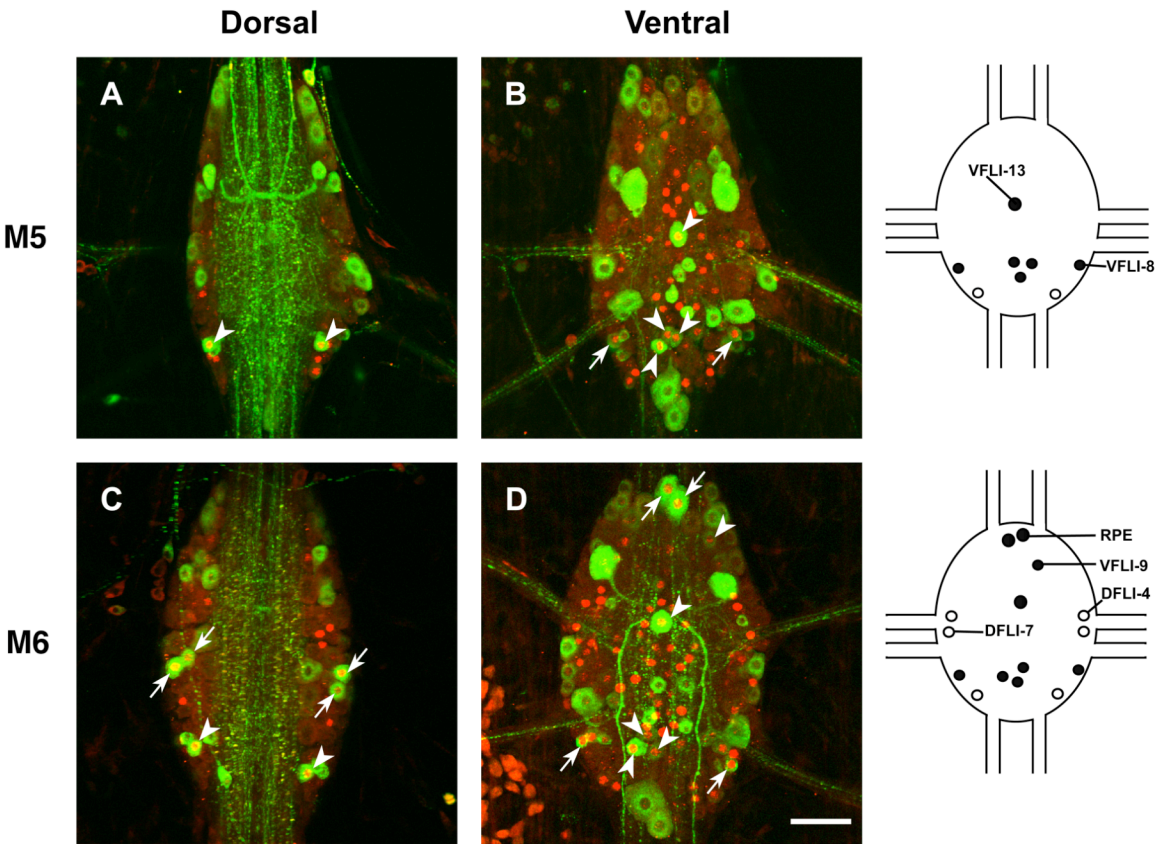
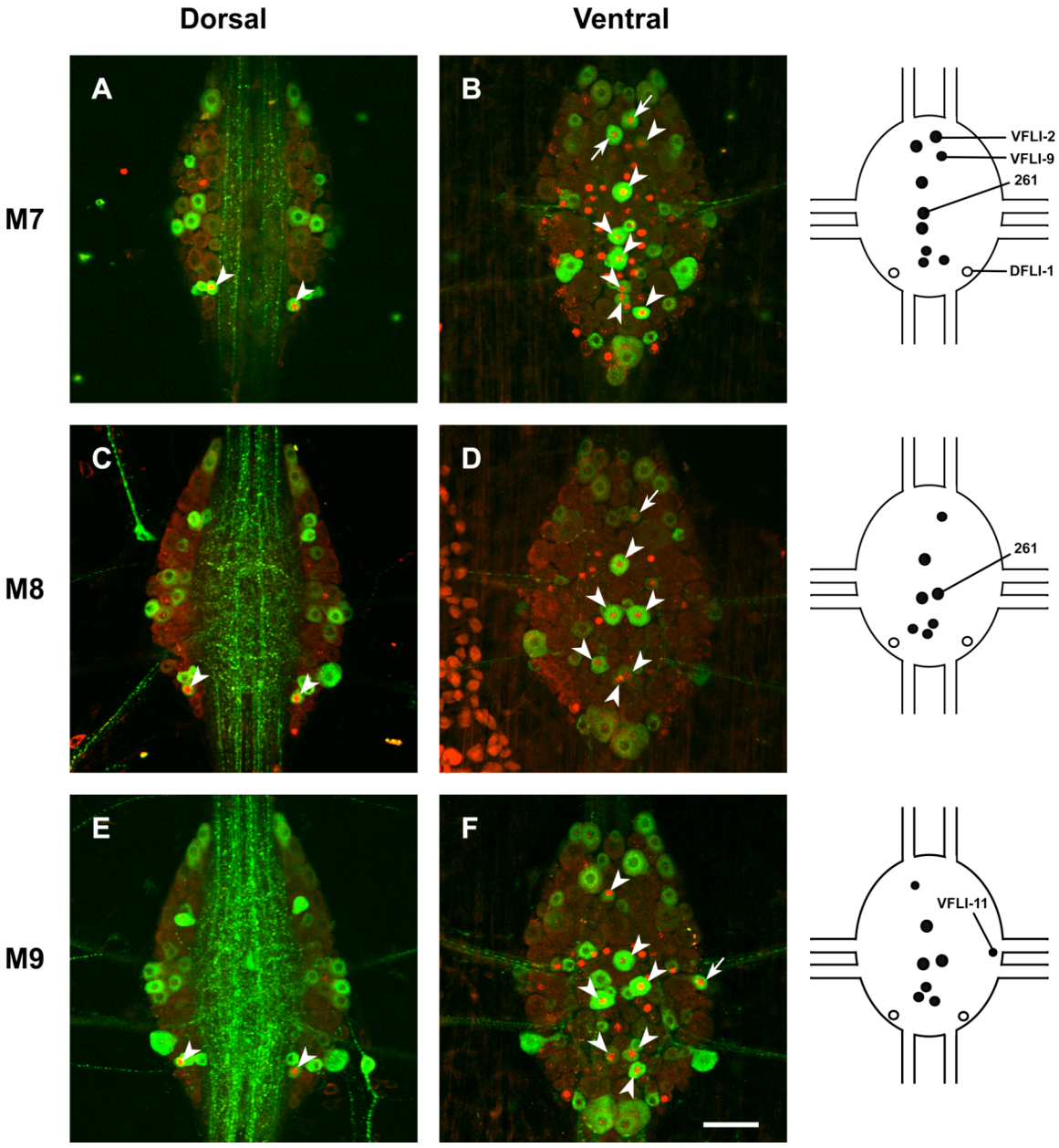
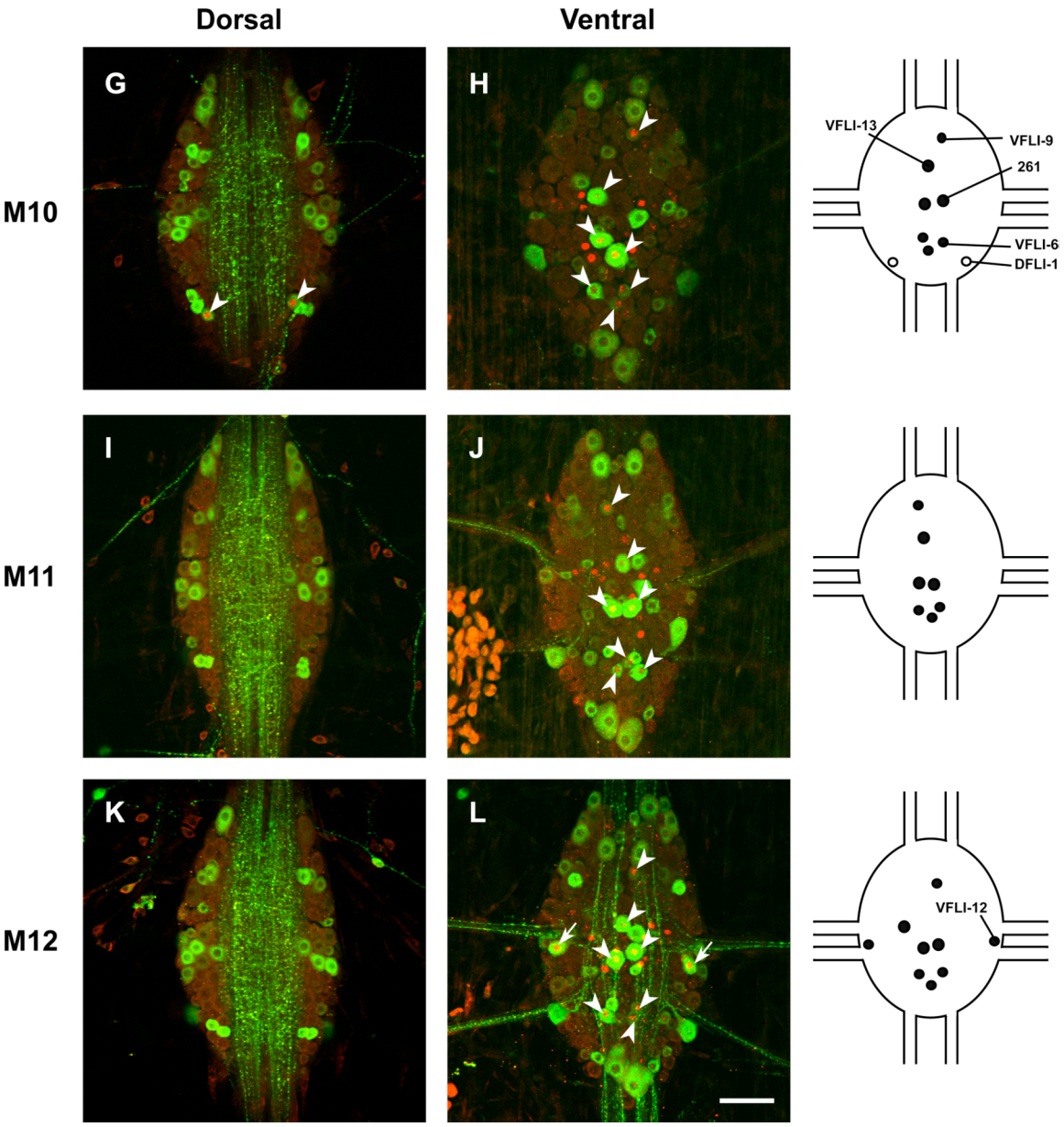

Dorsal
Ventral
A
B
C
D
M5
M6
VFLI-13
VFLI-8
RPE
VFLI-9
DFLI-4
DFLI-7

Figure 3-4 Lox1^{+} *FLI neurons of M7-M12.*

Arrows and arrowheads are as in Fig. 3-2. Note the *Lox1*$^{+}$ cell 261 of M7-M12 (***B***, ***D***, ***F***, ***H***, ***J*** and ***L***). These neurons were not detected in more anterior ganglia. The left panels (***A***, ***C***, ***E***, ***G***, ***I***, ***K***) show dorsal views and the middle panels (***B***, ***D***, ***F***, ***H***, ***J*** and ***L***) show ventral views. The *Lox1*$^{+}$ FLI neurons are indicated on the diagrams at the right side of the figure. Red fluorescence (TRITC) shows *Lox1* expression; green fluorescence (Cy2), FMRFamide. The scale bar is 50 μm.


Dorsal
Ventral
M7
M8
M9
A
B
C
D
E
F
VFLI-2
VFLI-9
261
DFLI-1
261
VFLI-11


Dorsal
Ventral
M10
M11
M12
G
H
I
J
K
L
VFLI-13
VFLI-9
261
VFLI-6
DFLI-1
VFLI-12

Lox1 ***Expression in Neurons that Contain Laz1-1Antigen***

Additional central neurons that expressed *Lox1* were identified by double-staining using *LOX1* and Laz1-1 antibodies. At E9, only a few neurons expressed Laz1-1, none of which expressed the *LOX1* protein. Between E13 and E15, the number of neurons that expressed Laz1-1 increased. At these stages, several central neurons co-expressed the *LOX1* and Laz1-1 proteins, in all the nine embryos observed at E14 (all the embryos showed identical staining with the *LOX1* and Laz1-1 antibodies). All *Lox1*$^{+}$ Laz1-1^{+} neurons were observed in the ventral aspect of the ganglion. Some of these neurons were present in several ganglia of the *Lox1* domain, some were detected in 2-3 consecutive ganglia, and some appeared in only one ganglion (Fig. 3-5, see also Table 3-3 for detailed descriptions of these neurons).

Lox1^{+} Laz1-1^{+} neurons repeated in several consecutive ganglia included the Lz3 and Lz5 neurons. The unpaired Lz3 neurons expressed *Lox1* in M4-M8 (Fig. 3-5 D-F; Fig. 3-6 A-B). These neurons project their axons contralaterally, with secondary branches extending into the anterior and posterior longitudinal connectives. Interestingly, the Lz3 neurons of M9-M16 were paired and expressed *Lox2* (Figs. 3-11 F and H 3-12 B, D, F and H; and 3-13 B and D). Unlike the trend with other unpaired neurons, the Lz3 neurons did not necessarily alternate sides between consecutive ganglia. The Lz5 neurons expressed *Lox1* in M4-M10 (Fig. 3-5 D-F and Fig. 3-6 A-D).

Lox1^{+} Laz1-1^{+} neurons that appeared in 2-3 consecutive ganglia included the Lz1 and Lz4 neurons. The Lz1 neurons expressed *Lox1* only in M2-M3 (Fig. 3-5 B-C). The

unpaired Lz4 neurons expressed *Lox1* in M4-M5 (Fig. 3-5 D-E). In different embryos, these neurons alternated randomly from one side to the next, in their respective ganglia.

Lox1^{+} Laz1-1^{+} neurons present in isolated ganglia included the heart accessory-like (HAL) neurons of M4 (Fig. 3-5 D), Lz6 neurons of and M6 (Fig. 3-5 F), and Lz7 neurons of M11 (Fig. 3-6 E). The HAL cells send their axon contralaterally, with one of the secondary branches extending into the anterior root, and the other into the anterior longitudinal connective (Stewart *et al*., 1991). The HAL neurons are homologs of the heart accessory (HA) neurons, which are unique to the sex ganglia (M5-M6) and bind to FMRF-amide antibodies (Kuhlman *et al*., 1985). The HA neurons did not express *Lox1*. Laz1-1-containing HAL neurons in more posterior ganglia did not express *Lox1* but expressed instead *Lox2* (Figs. 3-11, 3-12, and 3-13).

Table 3-3 ***Laz1-1^{+} neurons that express*** **Lox1.**

Neurons	*Lox1*$^{+}$	Fig.	Description
Lz1	M2-M3	3-5 BC	paired very small somata location behind posterior nerve roots
HAL (cell 158)	M4	3-5 D	paired expressed *Lox2*$^{+}$ in M7-M17 large somata moderate Laz1staining anterior-lateral location
Lz3	M4-M8	3-5 DEF 3-6 AB	unpaired in M4-M8 paired and *Lox2*$^{+}$ in M9-M16 mid-sized somata intense Laz1-1staining anterior-medial location
Lz4	M4-M5	3-5 DE	unpaired mid-sized somata moderate Laz1-1 staining; intense *Lox1* staining location behind anterior nerve root
Lz5	M4-M10	3-5 DF 3-6 ABCD	paired *Lox2*$^{+}$ in M12-M17 small somata intense Laz1-1 staining posterior-lateral location
Lz6	M6	3-5 F	paired mid-size somata intense Laz1-1 staining location anterior to the posterior nerve roots
Lz7	M11	3-6 E	paired small somata intense staining with Laz1-1 and *Lox1* location laterally, posterior to anterior lateral root

Figure 3-5 Lox1$^+$ *Laz1-1$^+$ neurons of M1-M6.*

A is M1, ***B*** is M2, ***C*** is M3, ***D*** is M4, ***E*** is M5, and ***F*** is M6. Arrowheads indicate *Lox1*$^+$ Laz1-1$^+$ neurons that were detected in several consecutively ganglia. Arrows indicate *Lox1*$^+$ Laz1-1$^+$ neurons that appeared in no more than two consecutive ganglia. Red fluorescence (TRITC) shows *Lox1* expression; green fluorescence (Cy2) shows Laz1-1 antigen. The scale bar is 50 μm.

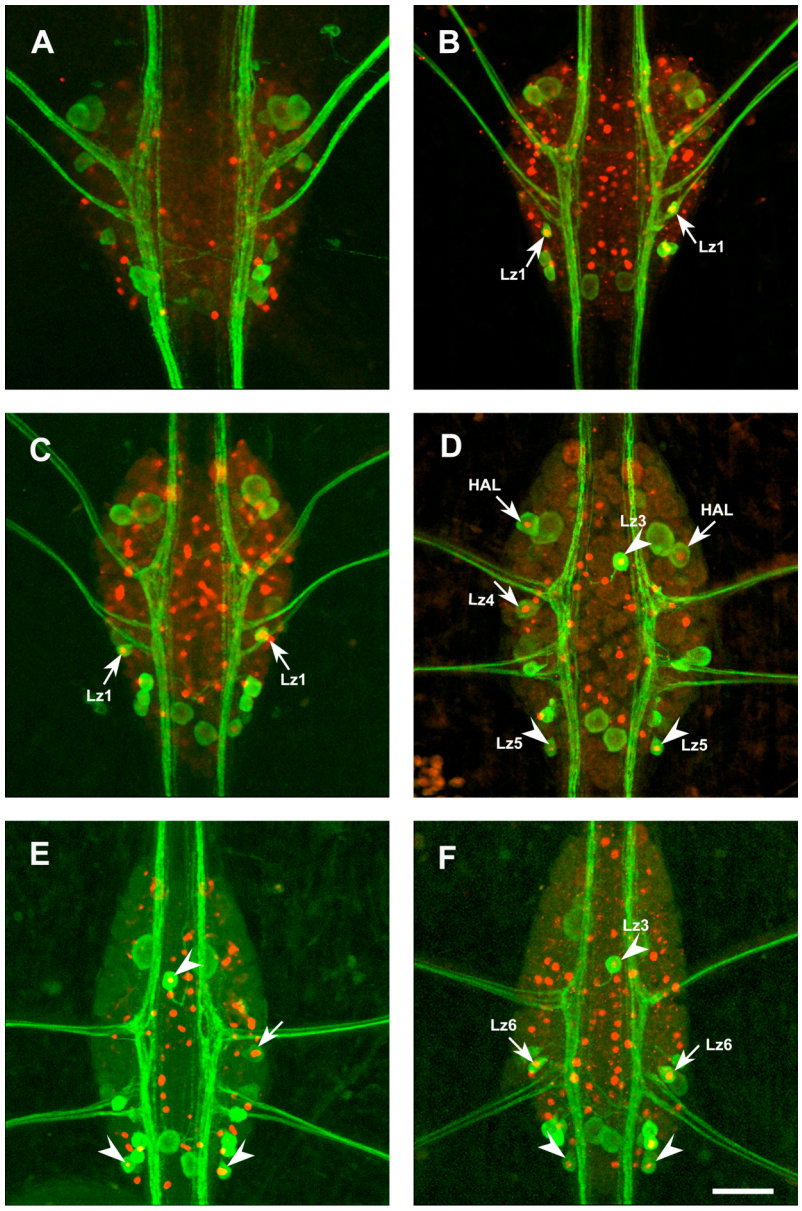
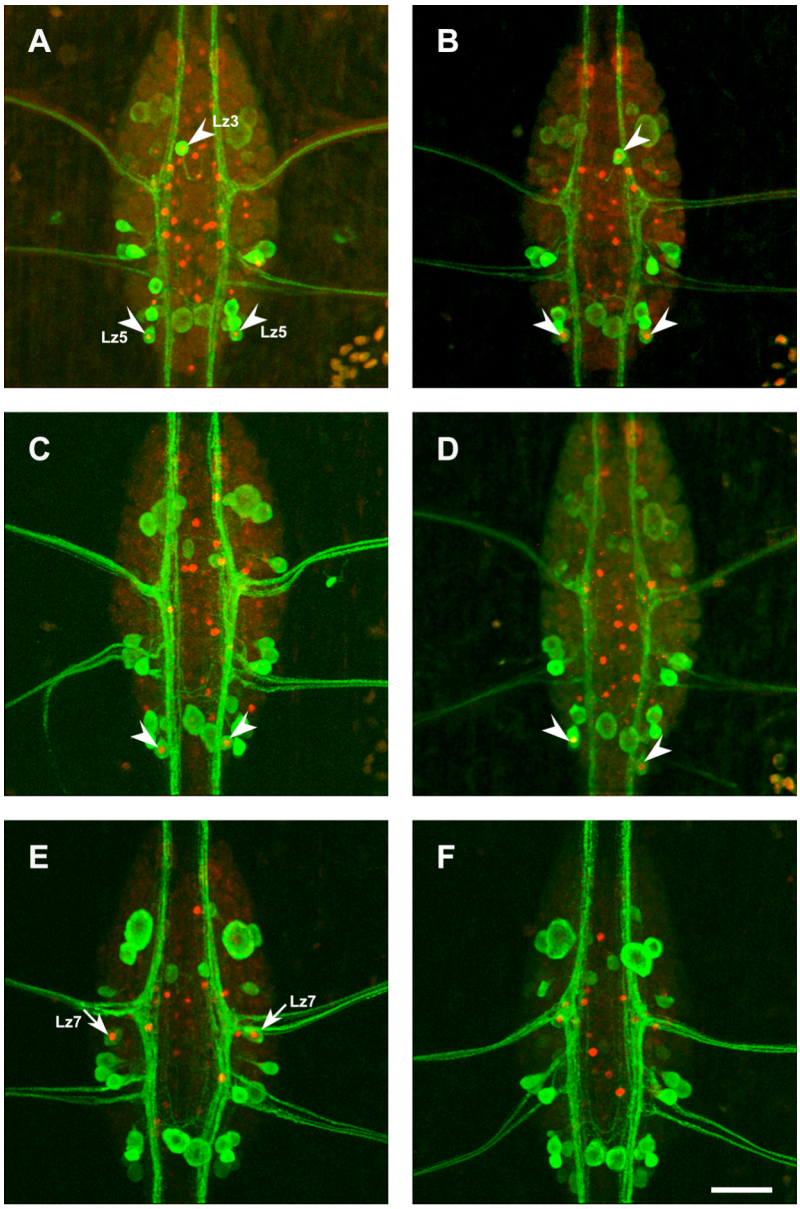

A
B
Lz1
Lz1
C
Lz1
Lz1
D
HAL
Lz3
HAL
Lz4
Lz5
Lz5
E
F
Lz3
Lz6
Lz6

Figure 3-6 Lox1$^+$ *Laz1-1*$^+$ *neurons of M7-M12*.

A is M7, ***B*** is M8, ***C*** is M9, ***D*** is M10, ***E*** is M11, and F is M12. Arrowheads and arrows are as in Fig. 3-5. Red fluorescence (TRITC) shows *Lox1* expression; green fluorescence (Cy2) shows Laz1-1 antigen. The scale bar is 50 μm.


A
Lz3
Lz5
Lz5
B
C
D
E
Lz7
Lz7
F

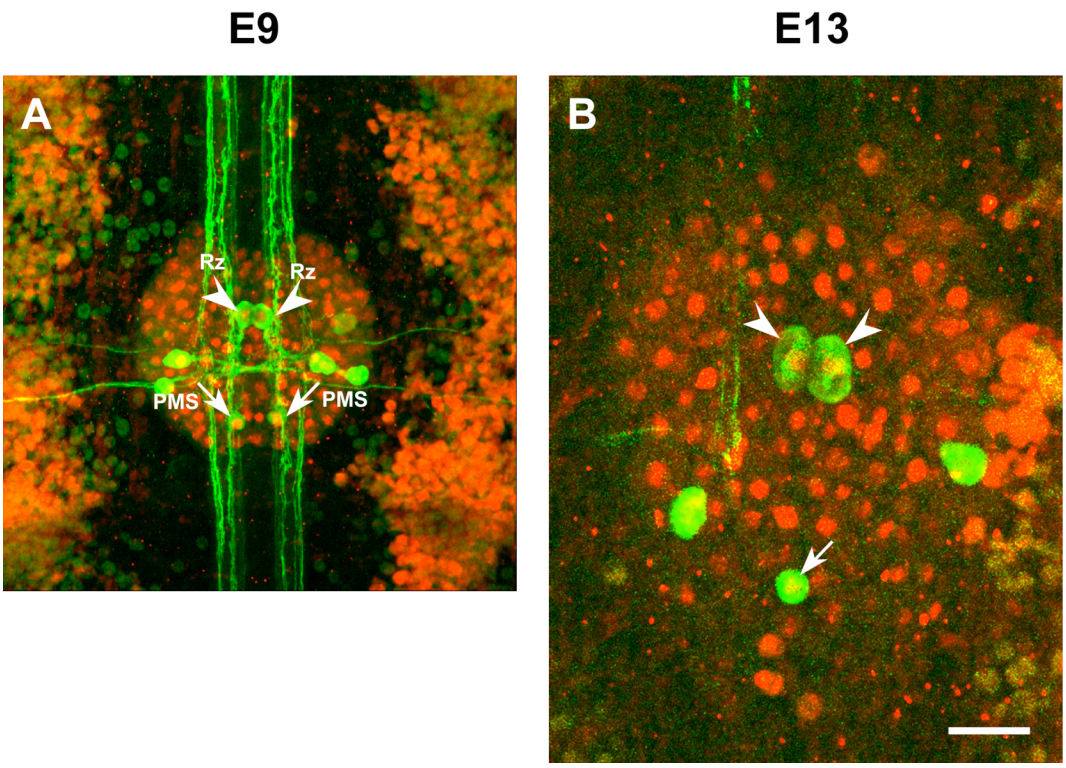
Lox2 *Expression in Serotonergic Neurons*

Starting at E9, *Lox2* was found to be expressed in two serotonin-containing, segmentally repeated neurons. These neurons were the Rz and PMS neurons (Fig. 3-7; see also Table 3-1). These results were obtained from observations of nine E9 embryos, which showed identical *Lox2* and serotonin staining. This pattern continued at later stages (eight E13 embryos showed identical *Lox2*-serotonin costaining). The Rz cells are among the best-studied neurons in the leech. They appear in all segments, including the head and tail neuromeres. They have the largest neuronal cell body, located in the anterior ventral-medial glial packet. Their functions include the regulation of mucus release, swimming, and relaxation of longitudinal muscles. The Rz neurons of the more mature, anterior ganglia started to express serotonin at E7-E8. By E9, these cells showed moderate levels of *Lox2* expression from M8 to M21.

The PMS neurons also expressed *Lox2.* These neurons are located in the posterior ventral-medial glial packet. Early in development, they start as bilateral pairs in all segments, but become later unpaired in M3-M21, as the other member of the pair undergoes programmed cell death (anterior to M3, the PMS neurons remain paired at all stages). The surviving member of the pair continues to express *Lox2*.

Figure 3-7 Lox2 *expression in serotonergic cells*.

Lox2 is expressed in the Rz cells (arrowheads) at E9 (***A***) starting in M8. At E13 (***B***), all the Rz cells of M8-M21 expressed *Lox2*. The PMS neurons (arrows) also expressed *Lox2* in M8-M21, starting as bilateral pairs (***A***), but becoming later unpaired (***B***). These confocal images show an M12 ganglion stained for *Lox2* (Cy3, red) and serotonin (Cy2, green). The scale bar is 50 μm.


E9
E13
A
B
Rz
Rz
PMS
PMS

Lox2 *Expression in FLI Neurons*

Some *Lox2*$^{+}$ FLI neurons appeared in several consecutive ganglia, while other neurons were restricted to only one ganglion (Figs. 3-8, 3-9, and 3-10; see also Table 3-4 for detailed descriptions of these neurons). In 12 embryos studied at E14, I observed the same pattern of *Lox2* and FMRFamide staining. Few *Lox2*$^{+}$ FLI neurons were present at E9 (data not shown); most *Lox2*$^{+}$ FLI neurons were detected at E14, from M7 to M19. There may be *Lox2*$^{+}$ FLI neurons in M20-21, but the thickness of the posterior sucker blocks this region of the animal. Most of the *Lox2*$^{+}$ FLI neurons in several consecutive ganglia were found in ventral sections. Among these cells were the RMV (rostral-most ventral) neurons and the CV motor neurons, which innervate circular ventral muscle (Fig. 3-8 B, D, F, H; Fig. 3-9 B, D, F, H; and Fig. 3-10 B, D, F, H, J). The RMV neurons expressed *Lox2* in M7-M19 (the *Lox2* staining is weak in M12-M19). The RMV neurons have been reported to be morphologically diverse along the midbody (Wong, 1997). The CV neurons express *Lox2* in M7-M19 (the *Lox2* staining is weak in M12-M19).

Neurons that expressed both *Lox1* and *Lox2* are of particular interest. The *Lox1*$^{+}$ cell 261 neurons of M7-M12 (Fig. 3-4) were found to also express *Lox2* in M7-M12 (Fig. 3-8 B, D, F, H; Fig. 3-9 B, D). The cell 261 was either absent or did not express FMRFamide in segments posterior to M12. The unpaired VFLI-9 neuron of M9-12 expressed both *Lox1* and *Lox2*.

Table 3-4 ***Summary of FLI neurons that express*** **Lox2** ***in the CNS***

Neurons	***Lox2*$^{+}$**	**Fig.**	**Description**
DFLI-4	M8-M14	3-8 CEG 3-9 ACEG 3-10 ACEG	paired very small somata intense FMRFamide staining anterior-lateral location
DFLI-5	M8-M9	3-8 CE	unpaired (either left or right side) small soma location behind posterior nerve root
DFLI-6	M15	3-10 A	paired small somata intense FMRFamide staining posterior-medial location
VFLI-9	M9-M16	3-8 FH 3-9 BDFH 3-10 BD	unpaired mid-sized somata very weak FMRFamide staining very intense *Lox2* staining anterior-medial location
cell 261	M7-M12	3-8 BDFH 3-9 BD	paired large somata intense FMRFamide staining central-medial location
VFLI-13	M8-M12	3-8 DFH 3-9 BD	unpaired large somata intense FMRFamide staining moderate to low *Lox2* staining central-medial location
VFLI-17	M7-M17	3-8 BDFH 3-9 BDFH 3-10 BDF	paired very small somata posterior-lateral location
VFLI-18	M8-M15	3-8 DFH 3-9 BDFH 3-10 B	unpaired, not alternating sides mid-size somata posterior-medial location
VFLI-19	M8-15	3-8 DFH 3-9 BDFH 3-10 B	paired small somata very weak FMRFamide and *Lox2* staining posterior-medial location

Neurons	***Lox2*$^{+}$**	**Fig.**	**Description**
VFLI-21	M7-M17	3-8 BDFH 3-9 BDFH 3-10 BDF	paired small somata intense FMRFamide and *Lox2* staining posterior-medial location
VFLI-23	M17	3-10 F	paired mid-sized somata moderate FMRFamide staining intense *Lox2* staining central-medial location
VFLI-24	M18	3-10 H	unpaired mid-sized soma moderate FMRFamide staining intense *Lox2*stainin central-medial location
RMV	M7-M19	3-8 BDFH 3-9 BDFH 3-10 BDFHJ	paired mid-sized somata intense FMRFamide staining in M7-M9 lower FMRFamide staining in M12-M19 intense *Lox2*staining anterior-medial location
CV	M7-M19	3-8 BDFH 3-9 BDFH 3-10 BDFHJ	paired mid-sized somata very low FMRFamide staining intense *Lox2* staining anterior-medial location

Figure 3-8 Lox2 *expression in FLI neurons of M7-M10.*

Arrowheads and arrows are as in Fig. 3-5. *Lox2*$^{+}$ FLI neurons include the CV motor neurons and the RMV neurons, which are segmental homologs of the RPE motor neurons (***B***, ***D***, ***F***, ***H***). Red fluorescence (TRITC) shows *Lox2* expression; green fluorescence (Cy2) shows FMRFamide staining. The scale bar is 50 μm.

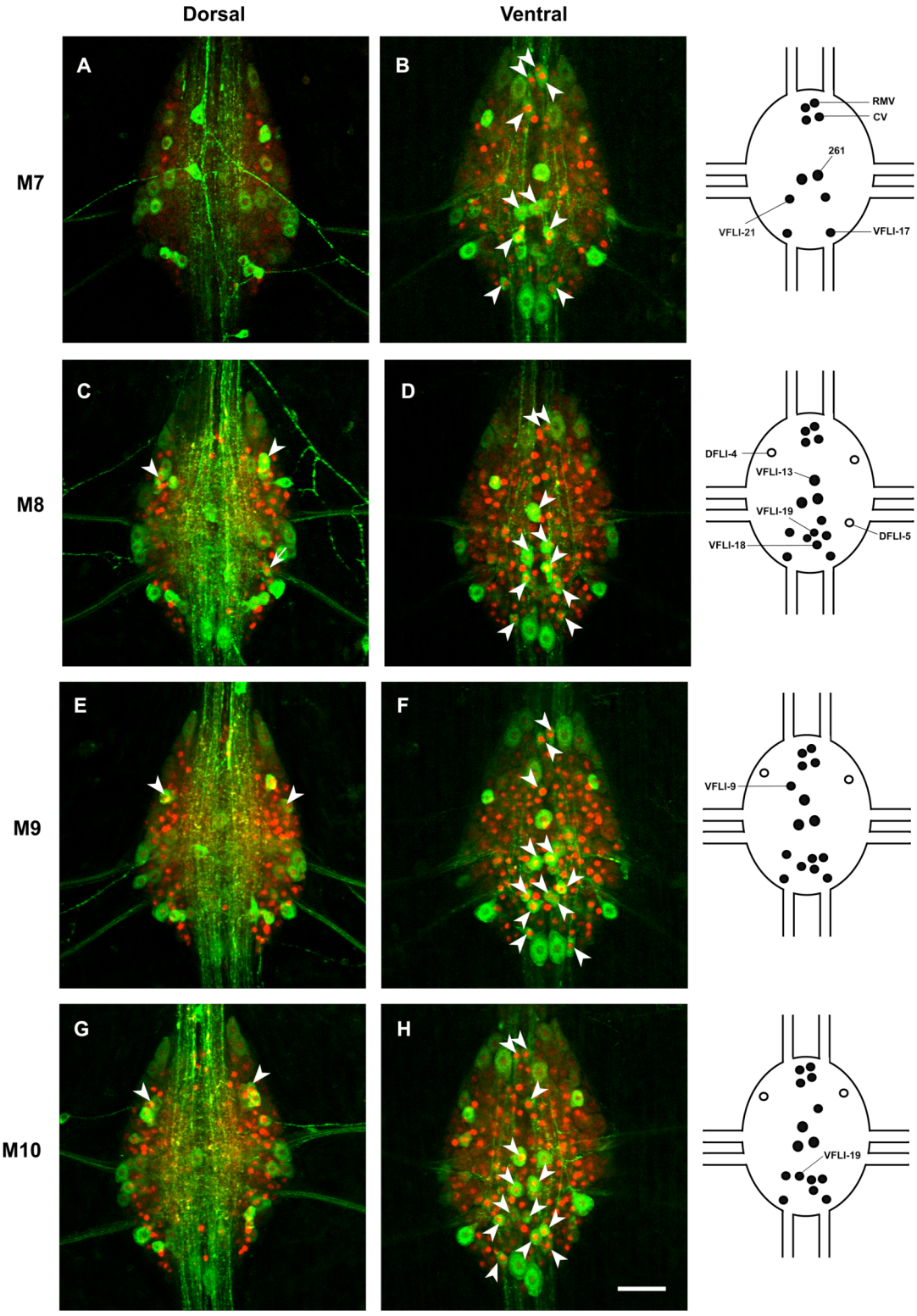

Dorsal
Ventral
A
B
M7
RMV
CV
261
VFLI-21
VFLI-17
C
D
M8
DFLI-4
VFLI-13
VFLI-19
VFLI-18
DFLI-5
E
F
M9
VFLI-9
G
H
M10
VFLI-19

Figure 3-9 Lox2 ***expression in FLI neurons of M11- M14.***

Arrowheads and arrows are as in Fig. 3-5. Note the continued presence of the RMV and CV neurons (***B***, ***D***, ***F***, ***H***). Red fluorescence (TRITC) shows *Lox2* expression; green fluorescence (Cy2) shows FMRFamide staining. The scale bar is 50 μm.

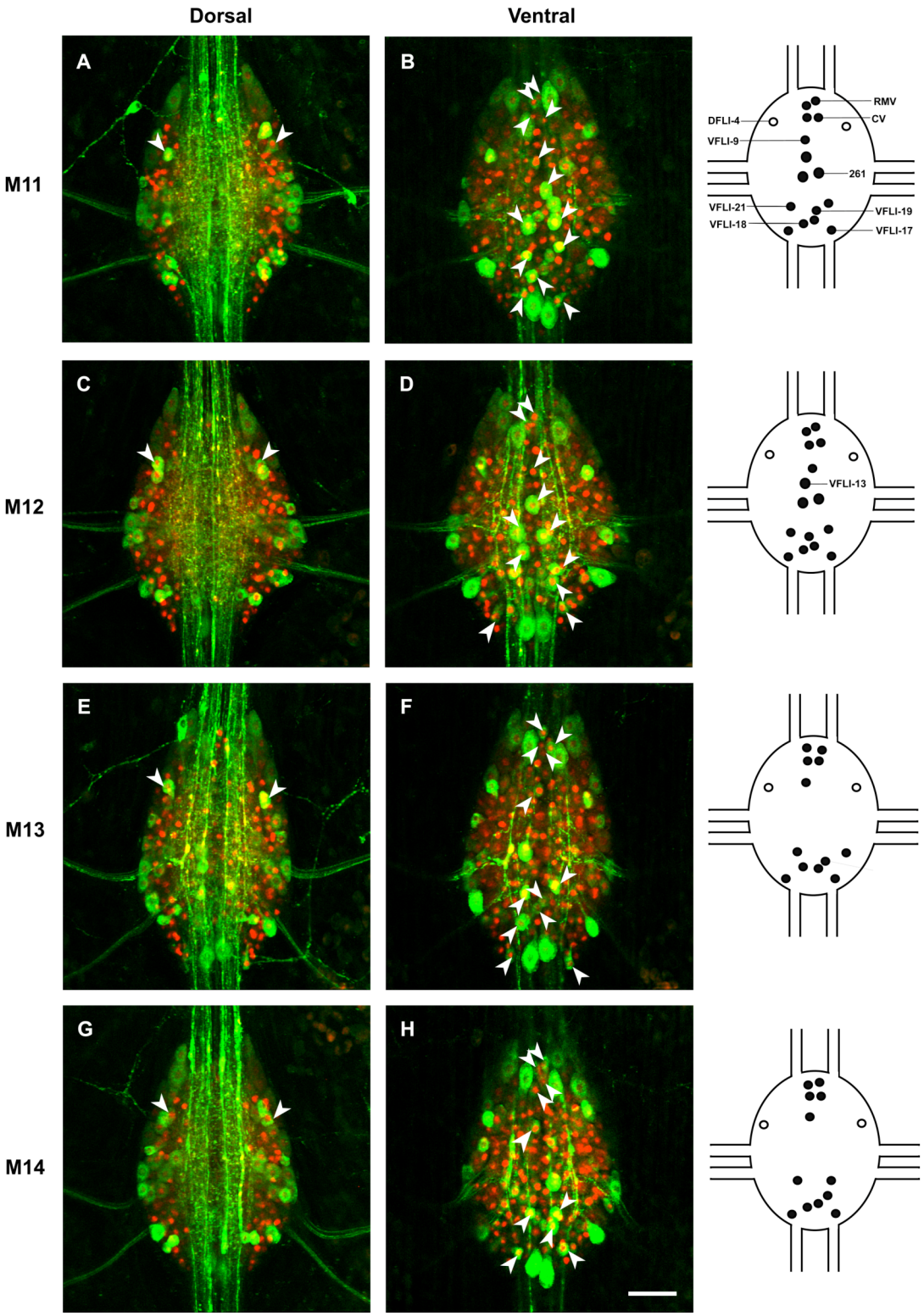
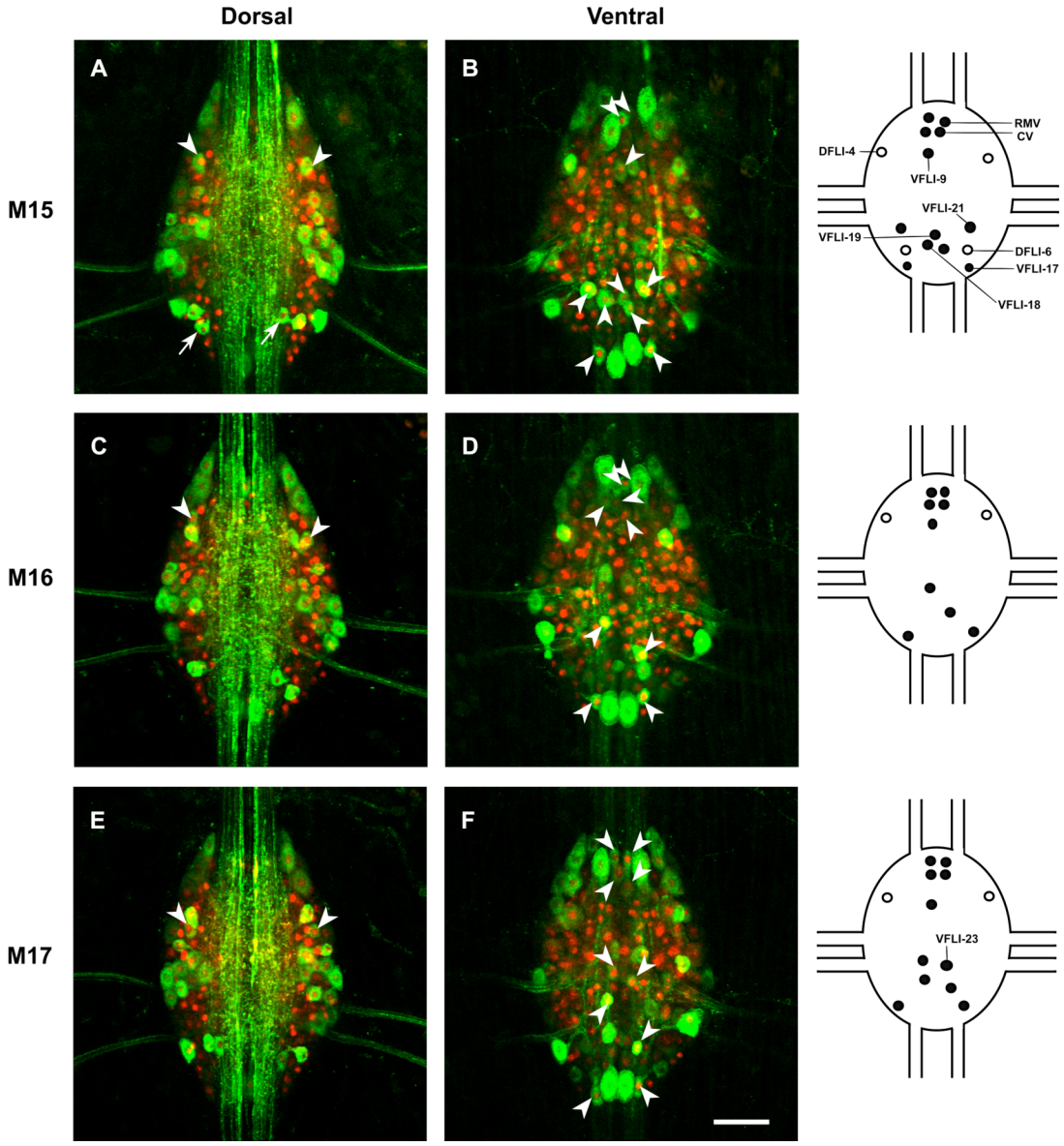

Dorsal
Ventral
A
B
C
D
E
F
G
H
M11
M12
M13
M14
RMV
CV
DFLI-4
VFLI-9
261
VFLI-21
VFLI-19
VFLI-18
VFLI-17
VFLI-13

Figure 3-10 Lox2 *expression in FLI neurons of M15-M19*.

Arrowheads and arrows are as in Fig. 3-5. There could be FLI neurons that express *Lox2* in M20-M21, but the posterior sucker was blocking them. Red fluorescence (TRITC) shows *Lox2* expression; green fluorescence (Cy2) shows FMRFamide staining. The scale bar is 50 μm.


Dorsal
Ventral
A
B
C
D
E
F
M15
M16
M17
RMV
CV
DFLI-4
VFLI-9
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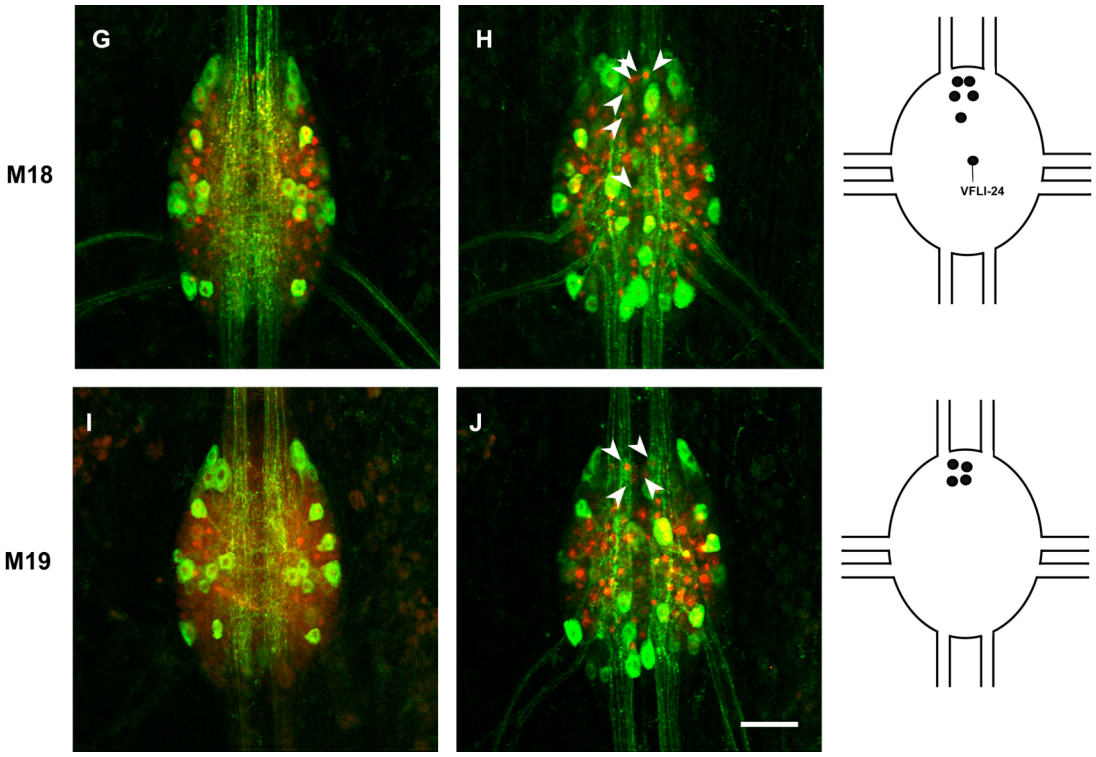
VFLI-18
VFLI-23


G
H
M18
VFLI-24
I
J
M19

Lox2 *Expression in Neurons Containing Laz1-1 Antigen*

Lox2 expression was also detected in central neurons containing the marker Laz1-1. For these experiments, 10 embryos were observed at E14. All the embryos showed the same pattern of *Lox2* and Laz1-1 staining. Most of the *Lox2*$^+$ Laz1-1$^+$ neurons were repeated in several consecutive segments, but some appeared in more restricted patterns (Fig. 3-11-Fig. 3-13, see also Table 3-5 for detailed descriptions of these neurons). For example, the DLz1 neurons expressed *Lox2* in M7-M11 and M13, but not in M12 (Fig. 3-11 A, C, E and G; Fig. 3-12 A and E). The segmental homologs of these cells were not detected posterior to M13 either because they do not express Laz1-1, or because they were absent. *Lox2*$^+$ Laz1-1$^+$ neurons that appeared in several consecutive ganglia along the *Lox2* domain include the AP, the HAL, and the NUT neurons as well as DLz6, all of which expressed *Lox2* in M7-M17 (Figs. 3-11 B, D, F and H, 3-12 B, D, F and H, and 3-13 B, D and F), and DLz4 which expressed *Lox2* in M8-M17 (Figs. 3-11 C, E and G; 3-12 A, C, E and G; 3-13 A and C). The AP neurons have the largest somata in the anterior-medial glial packets and send contralateral projections to the anterior and posterior roots. These neurons do not express *Lox1* and have been shown to alter their electrophysiological properties when *Lox1* mRNA is injected into them (Aisemberg *et al.*, 1997). Interestingly, the HAL neurons were found to express *Lox1*, but only in M4 (see above). The NUT neurons, which have ipsilateral axons, have been previously characterized (Sargent, 1975) and may be one of the synaptic partners of the P (pressure mechanosensory) neurons (Yang *et al.,* 1992). The NUT neuron of M6, which is known to innervate the male sex organ (Becker *et al.,* 1996), did not express *Lox2*. DLz6 neurons stained very weakly with Laz1-1, and moderately with *Lox2*. These paired cells

have large cell body located posteriorly and medially, in line with axons that formed the longitudinal connective. DLz4 is an unpaired neuron, and it did not alternate sides like other unpaired neurons. The Lz3 and Lz5 neurons expressed *Lox2* in fewer consecutive ganglia: the Lz3 cells in M9-M16 (Figs. 3-11 F and H, 3-12 B, D, F, H, and 3-13 B and D) and the Lz5 cells in M12-M17 (Figs. 3-12 D, F and H, and 3-13 B, D and F). The Lz3 cells were unpaired in M4-M8 and expressed *Lox1* (but not those of more posterior segments, see above). In M9- M16, the Lz3 neurons were paired and expressed *Lox2*, suggesting that *Lox1* may be involved in the unpairing of these neurons in M4-M8, whereas *Lox2* may maintain a pair of these cells in M9-M16. Other neurons that contained Laz1-1 and expressed *Lox2* were segment-specific: the VLz4 and VLz5 cells appeared only in M7 (Fig. 3-11 B) and M9 (Fig. 3-11 F), respectively.

Table 3-5 *Laz1-1*$^{+}$ *neurons that express* Lox2

Neurons	*Lox2*$^{+}$	Fig.	Description
DLz1	M7-M11 M13	3-11 ACEG 3-12 AE	paired very small somata intense Laz1-1 staining anterior-lateral location
DLz2	M7-M9	3-11 ACE	unpaired large somata intense Laz1-1 staining anterior-lateral location
DLz3	M7-M12	3-11ACEG 3-12 AC	paired mid-sized somata intense Laz1-1 staining posterior-lateral location
DLz4	M8-M17	3-11 CEG 3-12 ACEG 3-13 AC	unpaired mid-sized somata moderate Laz1-1 staining posterior-lateral location, (near posterior lateral root)
DLz5	M12-M16	3-12 CEG 3-13 AC	paired small somata intense Laz1-1 staining posterior-lateral location
DLz6	M7-M17	3-11 ACEG 3-12 ACEG 3-13 ACE	paired large somata very weak Laz1-1 staining moderate *Lox2* staining posterior medial location (next to longitudinal connective)
AP	M7-M17	3-11 BDFH 3-12 BDFH 3-13 BDF	paired large somata intense Laz1-1 staining intense *Lox2* staining in anterior region anterior-lateral location
HAL	M7-M17	3-11 BDFH 3-12 BDFH 3-13 BDF	paired *Lox1*$^{+}$ in M4 large somata, smaller in posterior region intense Laz1-1staining in all ganglia weaker *Lox2* staining in posterior region anterior-lateral location

Neurons	***Lox2*$^{+}$**	**Fig.**	**Description**
Cell 251 NUT	M7-M17	3-11 BDFH 3-12 BDFH 3-13 BDF	paired large somata intense Laz1-1 staining intense *Lox2* staining in anterior region posterior-medial location
VLz4	M7	3-11 B	paired very small somata intense Laz1-1 and *Lox2* staining posterior-lateral location
VLz5	M9	3-11 F	paired mid-sized somata intense Laz1-1 staining moderate *Lox2* staining posterior-medial location
Lz3	M9-M16	3-11 FH 3-12 BDFH 3-13 BD	paired in M9-M16 unpaired and *Lox1*$^{+}$ in M4-M8 mid-sized somata intense Laz1-1 and *Lox2* staining anterior-medial location
Lz5	M12-M17	3-12 DFH 3-13 BDF	paired *Lox1*$^{+}$ in M4-M10 small somata intense Laz1-1 staining moderate *Lox2* staining posterior-lateral location

Figure 3-11 Lox2$^+$ *Laz1-1*$^+$ *neurons of M7-M10*.

In this and the following two figures, note the paired *Lox2*$^+$ Lz3 neurons of M9-M16, whose segmental homologs were unpaired and expressed *Lox1* in M4-M8, and the HAL neurons and the cell 251 of M7-M17. *Lox2* expression was visualized with Cy3 (red) and the Laz1-1 antigen with Cy2 (green). The scale bar is 50 μm.

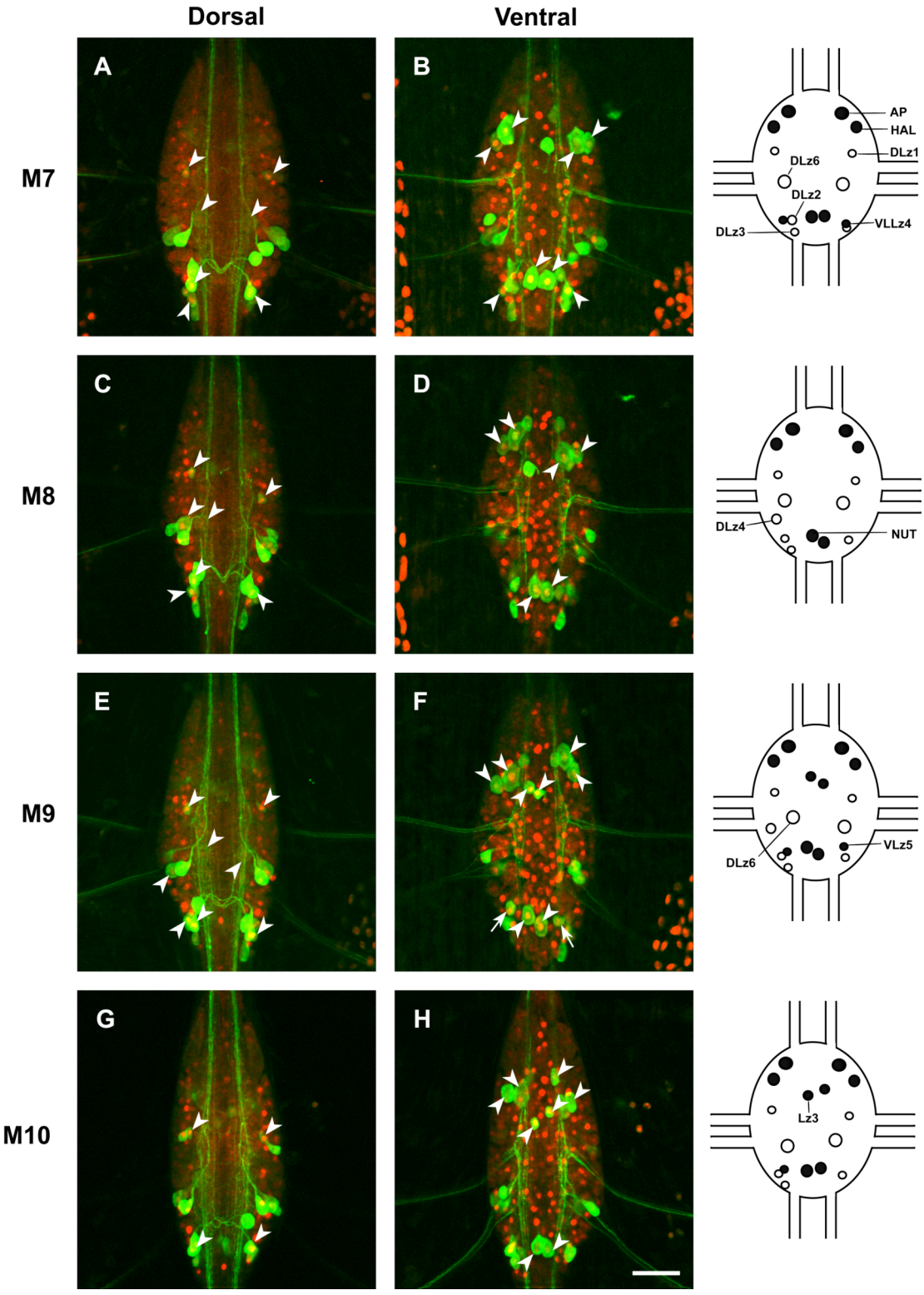

Dorsal
Ventral
A
B
C
D
E
F
G
H
M7
M8
M9
M10
AP
HAL
DLz1
DLz6
DLz2
VLLz4
DLz3
DLz4
NUT
VLz5
DLz6
Lz3

Figure 3-12 Lox2$^+$ *Laz1-1*$^+$ *neurons of M11-M14*.

The scale bar is 50 μm.

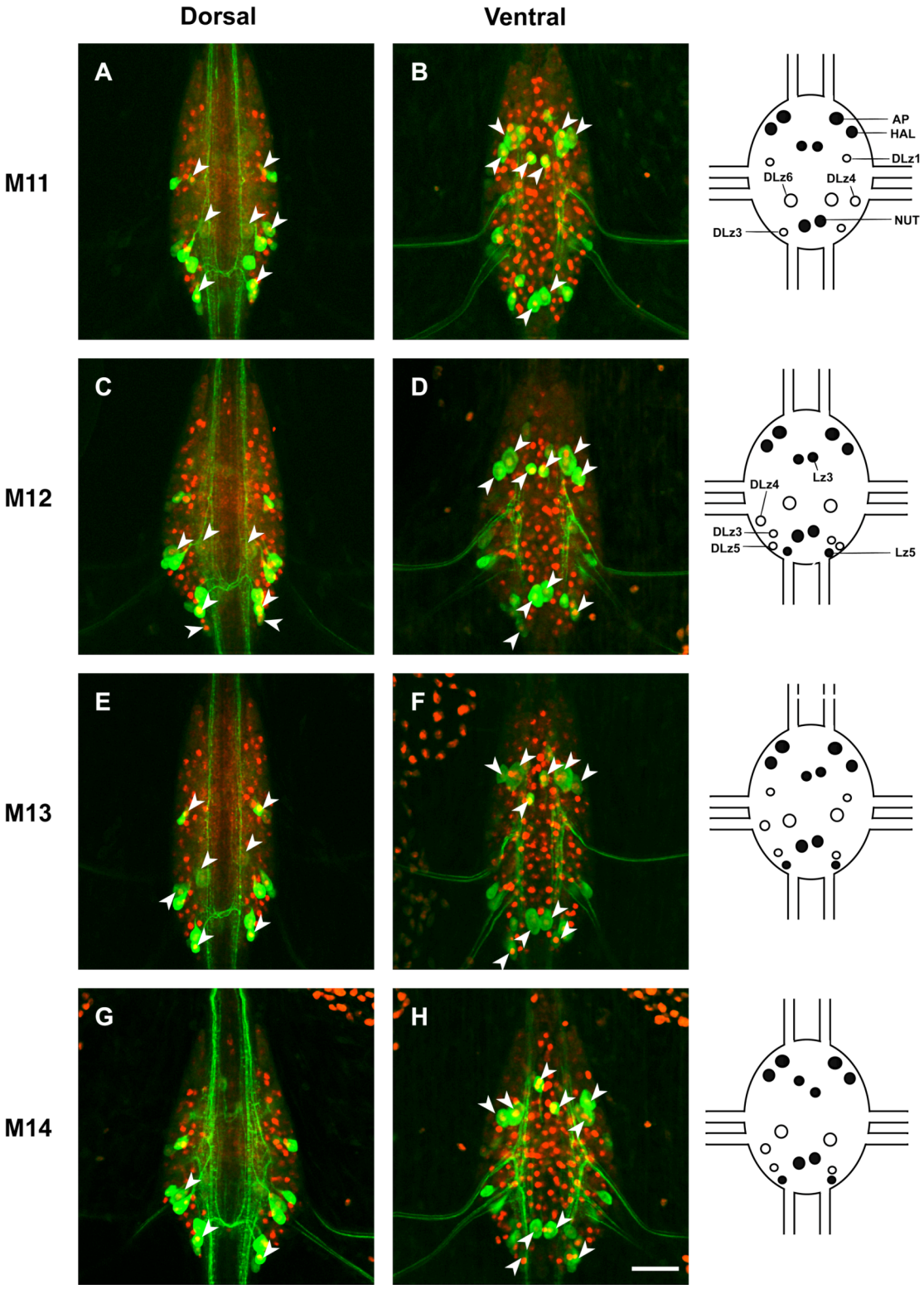
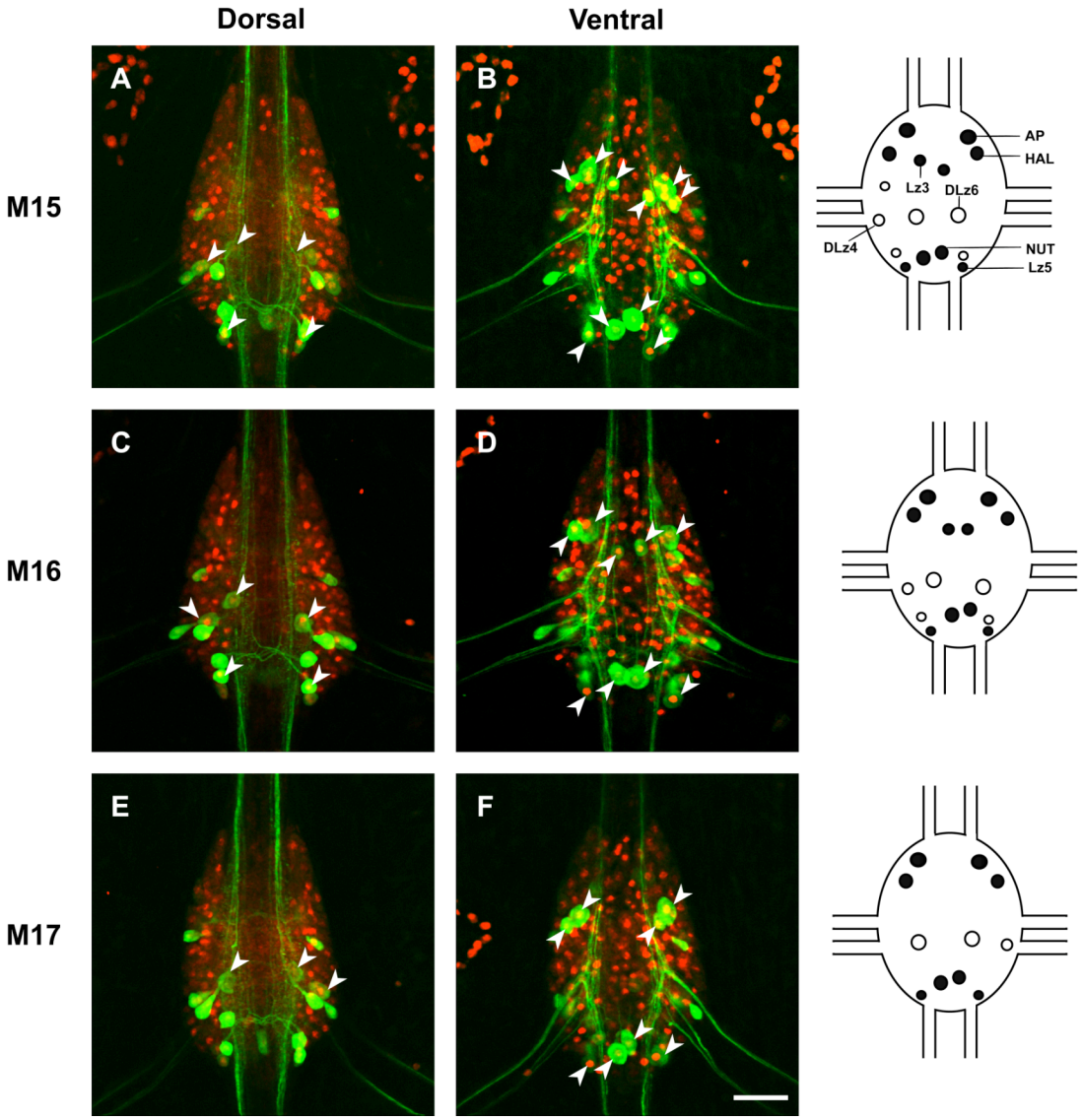

Dorsal
Ventral
M11
M12
M13
M14
A
B
C
D
E
F
G
H
AP
HAL
DLz1
DLz6
DLz4
NUT
DLz3
Lz3
DLz5
Lz5

Figure 3-13 Lox2$^+$ *Laz1-1*$^+$ *neurons of M15-M17*.

The scale bar is 50 μm.


Dorsal
Ventral
M15
M16
M17
A
B
C
D
E
F
AP
HAL
Lz3
DLz6
DLz4
NUT
Lz5

Lox2 *Expression in Enkephalin-Like Immunoreactive Neurons*

Lox2 expression was observed in a single, unpaired neuron that stained with a Leu-enkephalin antibody in M7-M21 (data not shown). This pattern was the same in seven embryos stained at E14. Enkephalins are small endogenous oligopeptides that bind to the same receptors as opiate analgesics and appear in two forms (Met- and Leu-enkephalins). Both enkephalins and their larger endorphin relatives are widely distributed among animals. One of the first invertebrates in which enkephalin expression was observed was the leech *Haemopis marmorata*, in which an unpaired neuron stains from M7 to M21 with a *Leu*-enkephalin antibody (Zipser, 1980). In *Hirudo medicinalis*, I observed that the enkephalin antibody stained not only the cell bodies, but also the axons of these neurons.

DISCUSSION

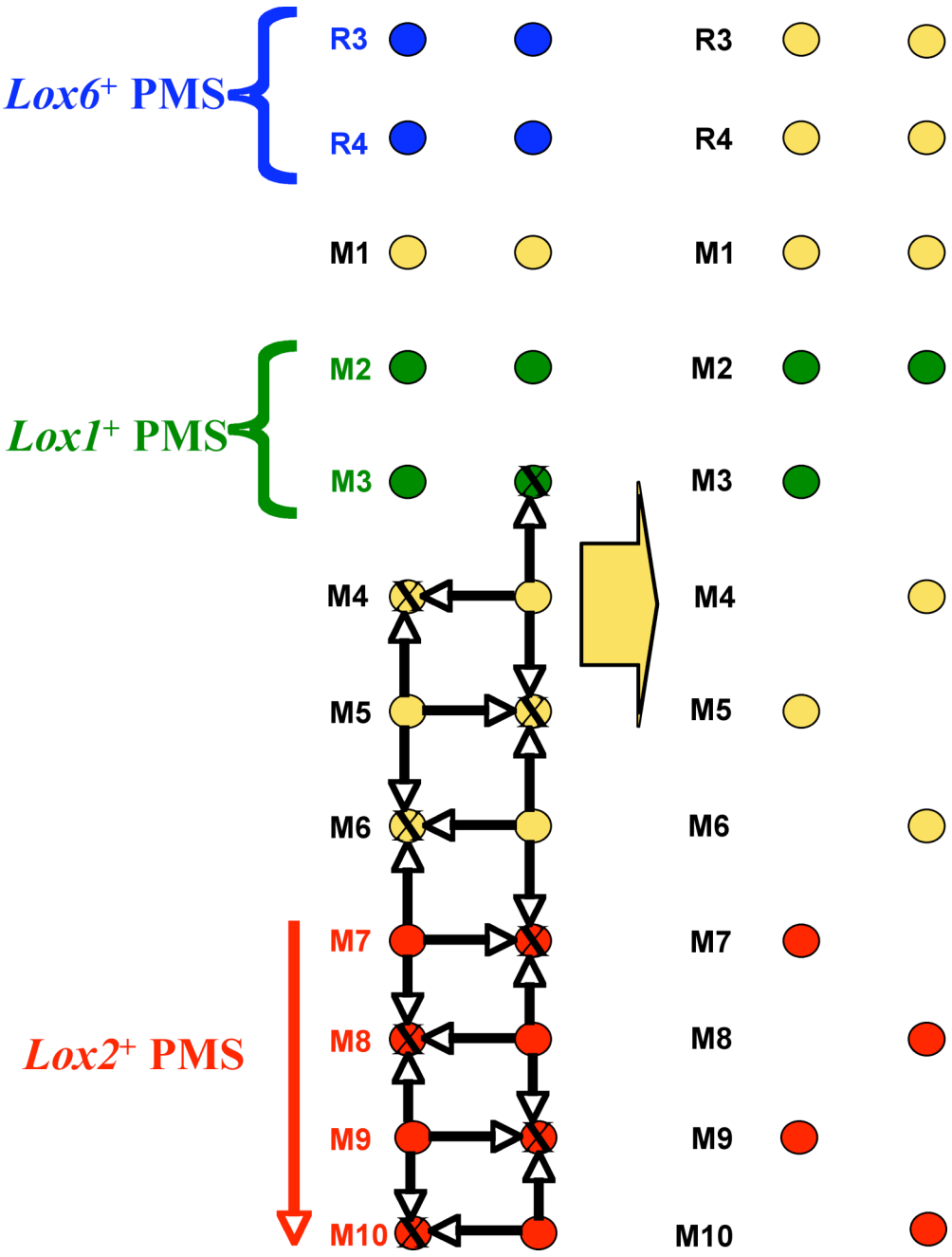
Region-Specific Transitions in Serotonergic Neurons (AMS and PMS)

I showed that previously identified serotonergic neurons express *Lox1* in a region-specific manner. The AMS interneurons appear only in R1-M3. It is not known whether there are homologs of the AMS neurons in segments posterior to M3 that do not produce serotonin. At early stages of CNS development, starting at E7, the PMS interneurons are paired and appear in every segment. At later stages, starting at E9, they remain paired in R1-M2, but become unpaired posterior to M2 (the other member of the pair undergoes apoptosis -Macagno and Stewart, 1987-). *Lox1* expression was detected in the AMS and PMS neurons in M2 and M3, starting at E9. In addition, these neurons express *Lox6* in R3 and R4 (Mercado-Pimentel, 2003). Starting at E8, the two PMS neurons of each segment between M7 and M21 express *Lox2*, but later one neuron dies in each segment,

while the surviving neuron continues to express *Lox2*. These observations suggest that *Lox1* may be involved in the regional differentiation of AMS and PMS neurons. The expression of *Lox1* in the AMS and PMS neurons of M2 and M3 may be related to the two phenotypic transitions happening at this location: (a) the absence of AMS neurons posterior to M3 and (b) the disappearance of a single PMS neuron in every ganglion, starting at M3. The absence of AMS cells posterior to M3 could be a consequence of *Lox1* repression by a more posterior Hox gene. These changes in Hox gene expression for posterior AMS neurons would either induce apoptosis or block serotonin synthesis. The surviving PMS neurons of M3-M21 alternate sides between consecutive segments. I propose that at early stages each PMS neuron of M4-M21 sends apoptosis-inducing signals through its axonal projections to the three neighboring PMS neurons: the contralateral PMS neuron of the same segment and the two ipsilateral PMS neurons of adjacent segments (Fig. 3-14). After a period of competition between the PMS neurons, the random first death of a PMS neuron decreases the amount of apoptosis-inducing signals received by its neighbors, allowing them to survive and thus causing their neighbors to die. This would produce a wave of cell death resulting in the alternating, unpaired PMS neurons observed at later stages in M3-M21. I propose that *Lox1* expression blocks the production or the release of apoptosis-inducing signals in the PMS neurons of M2-M3, resulting in the death of one PMS neuron in M3, which receives these signals from M4, and in no deaths anterior to M3. Thus, *Lox1* expression in M2-M3 would break the anterior propagation of the wave of PMS apoptosis initiated in more posterior ganglia. *Lox2* may play a role in inducing apoptosis in the PMS neurons of more posterior ganglia.

Figure 3-14 ***A model for the role of*** **Lox1** ***during the programmed cell death of PMS neurons.***

The left side of the drawing depicts early stages, when each paired PMS neuron of M4-M21 sends apoptosis-inducing signals through its axonal projections (arrows) to the three neighboring PMS neurons: the contralateral PMS neuron of the same segment and the two ipsilateral PMS neurons of adjacent segments. A wave of cell death leaves the alternating, unpaired PMS neurons observed at later stages in M3-M21 (right side of the drawing). *Lox1* expression would block the production or the release of apoptosis-inducing signals in the PMS neurons of M2-M3. *Lox2* would induce apoptosis in the PMS neurons of more posterior ganglia.


Lox6+ PMS
Lox1+ PMS
Lox2+ PMS
R3
R4
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
R3
R4
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10

Segment-Specific Motor Neurons (RPE) That Innervate the Male Sex Organ

Lox1 expression was detected in the RPE neurons. Zipser (1979) showed that these neurons control penile eversion in adult *Hirudinid* leeches. These cells also innervate the male organ selectively during embryogenesis (Baptista and Macagno, 1988). Each RPE neuron extends axons that cross the midline and form two branches. One branch extends through the longitudinal connective nerve to M5 and exits through the posterior lateral root from where it projects to the male sex organ; the other branch exits the anterior lateral root of M6 and projects to the male sex organ. The expression of *Lox1* in these cells suggests that *Lox1* may be important for enabling these neurons to acquire a segment-specific identity and to correctly innervate the male sex organ during early development, as well as for maintaining their identity at later stages.

The expression pattern of *Lox1* and *Lox4* in the RPE neurons and their segmental homologs, the RMV neurons, is very interesting. These cells have different morphologies. They express *Lox4* in M4-M21 at E7, but the RPE cells turn off *Lox4* by E9 (Wong *et al*., 1995). On the other hand, the RPE neurons continue to express *Lox1* as *Lox4* expression goes down. In addition, *Lox1* is never expressed in the RMV neurons. These observations suggest that *Lox1* down-regulates *Lox4* in the RPE neurons. I propose that the switch from *Lox4* to *Lox1* expression in the RPE neurons is necessary for determining their segment-specific identity.

Wong *et al.* (1995) showed that the RMV neurons of M4-M5 and M7-M21 express *Lox4* after early neurogenesis, resulting in different arborization patterns in M1-M3 and M4-M5. I also detected *Lox2* expression in the RMV neurons of M7-M19. The axonal

morphology of these posterior RMV neurons has not been examined yet, but it is likely that it will diverge from the one seen in M4-M5.

Segment-Specific Motor Neurons (HA and HAL) That Innervate the Heart Tubes

Lox1 and *Lox2* were also expressed in the HAL neurons, *Lox1* in M4 and *Lox2* in M7-M17. The HAL neurons are segmental homologs of the heart accessory (HA) neurons, which are found only in M5 and M6. Stewart *et al.* (1991) provide several lines of evidence indicating that the HAL neurons may be the segmental homologs of the HA neurons. Both HA and HAL neurons have identical electrophysiological spiking patterns, and seem to have a common source of inhibitory synaptic input, the HN interneurons, suggesting that the HAL cells are also part of the circuit that regulates the heart tubes. However, there are two features by which the HA neurons differ from the HAL neurons: HA neurons have a larger soma and no Laz1-1 antigen, which is present in the HAL neurons. It is likely that the repression of both *Lox1* and *Lox2* in the HA neurons, perhaps a consequence of the expression of an unidentified third Hox gene, plays a role in the differentiation of the HA neurons.

Giant Neurons (Rz)

Lox2 expression was detected in the well-studied giant Rz cells. The Rz neurons of the sex ganglia ("sex Rz cells" of M5 and M6) do not express *Lox2* and innervate the male sex organ; the Rz neurons of M8-M21 ("standard Rz cells") express *Lox2* and project axons ipsilaterally to innervate extensively the body wall of their segment. The firing of the standard Rz cells elicits a number of responses, including an increased rate of contraction and relaxation of the body wall muscle (Mason *et al*., 1979), a release of mucus from mucus glands in the skin (Lent, 1973), and an increased tendency of the

nerve cord to initiate the swimming program (Willard, 1981). These responses are mediated by the release of serotonin, the main neurotransmitter used by the Rz cells. I propose that the presence of the *Lox2* protein in standard Rz cells is responsible for the differentiation of these cells from the sex Rz cells, a hypothesis that can be tested by RNA interference.

Region-Specific Transitions in Laz1-1$^+$ Neurons (Lz3)

Another interesting finding was a correlation between the expression of *Lox1* and *Lox2* and a phenotypic transition in Lz3, a neuron that contains the Laz1-1 antigen. I found that Lz3 is unpaired and expresses *Lox1* in M4-M8, but the same cell is paired and expresses *Lox2* in M9-M16. These observations suggest that *Lox1* may be involved in unpairing these cells in anterior ganglia, while *Lox2* may be involved in maintaining a pair of Lz3 cells in more posterior segments. These roles would be different from the ones these Hox genes may play in the PMS neurons, which are paired and *Lox1*$^+$ in an anterior ganglion, but unpaired and *Lox2*$^+$ in more posterior ganglia. Not surprisingly, relationships between Hox gene expression and specific phenotypes seem to be complex and dependent on additional, yet unidentified factors.

CHAPTER 4

RNAi STUDIES ON HOX GENE FUNCTION IN RPE AND RETZIUS NEURONS

INTRODUCTION

The description of detailed expression patterns for *Lox1* and *Lox2*, followed by the identification of neurons that express these genes (Chapters II and III) led to the study of Hox gene function during CNS development at the single cell level. I made use of RNA interference (RNAi) and immunostaining to block Hox gene expression, detect loss of Hox protein, and assess phenotypic alterations of central neurons.

RNAi has become a very powerful tool for dissecting gene function. RNAi involves the introduction of double-stranded RNA (dsRNA) containing gene-specific sequences into the target cells, resulting in null or hypomorphic phenotypes (*i.e.*, phenotypes similar to those of loss-of-function mutants). The dsRNA accomplishes this goal by targeting its endogenous, homologous single-stranded mRNA counterpart for degradation (Zamore *et al.*, 2000; Bosher and Labousse, 2000; reviewed in Fire, 1999). This sequence-specific post-transcriptional silencing of genes has been demonstrated in a number of different organisms (reviewed by Vance and Vaucheret, 2001; Waterhouse *et al.*, 2001; Cogoni and Macino, 2000).

RNAi was discovered by chance. Before RNAi became available, antisense RNA and oligonucleotides were frequently used for gene-silencing. Single-stranded, antisense

RNA is not stable; most of the antisense RNA may be degraded before entering the cell. Fire *et al* (1998) discovered that a "negative control" of antisense RNA, which contained dsRNA, had the highest interfering activity. RNAi has been shown to produce specific phenocopies of null mutations in phylogenetically diverse animals such as, for example, *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), trypanosomes (Ngo *et al*., 1998), planaria (Sanchez and Newmark, 1999), and mouse (Wianny and Zernicka-Goetz, 2000). RNAi also has potential disadvantages. For example, some studies showed that RNAi phenotypes can be non-specific, probably due to dsRNA toxicity (Jackson *et. al.,* 2003). However, the use of dsRNA in gene silencing is in most cases gene-specific and more effective, because dsRNA is more stable than antisense RNA.

A mechanism has been proposed by which dsRNA brings about the disruption of the intracellular message (reviewed by: Milhavet *et. al.,* 2003*;* Matzke, *et al*. 2001). RNAi is initiated by ATP-dependent cleavage of dsRNA into small, 21-25 nucleotide-long double-stranded fragments called short interfering RNAs (siRNAs). This cleavage is catalyzed by a type III RNase (called DICER in fly and CAF in *Arabidopsis*). The siRNAs then form a complex with several proteins, the RNA-induced silencing complex (RISC). Subsequently, the siRNAs are unwound (another ATP-dependent process), separating the short sense and antisense RNA strands. The antisense strands are needed to activate RISC and act as guides that enable RISC to recognize and cleave the complementary, homologous mRNA. Thus, the mRNA becomes no longer available for translation. A single molecule of activated RISC can degrade multiple molecules of

mRNA, acting as a true catalyst. It has also been demonstrated that the generation of siRNA by DICER can be bypassed by directly introducing synthetic siRNAs into cells.

RNAi has been used with success in the leech system. Injection of dsRNA of the receptor tyrosine phosphatase gene HmLAR2 into either a single comb cell or into the body cavity of leech embryos causes inhibition of HmLar2 expression, followed by the collapse of growth cones (Baker and Macagno, 2000). In our lab, *Lox6* RNAi induced defects in the early patterning of the leech nervous system (Mercado-Pimentel, 2003). Also, netrin RNAi produced morphological alterations in the longitudinal connective nerves of leech embryos (Aloysius Phillips, personal communication).

RNAi studies on Hox gene function during early nervous system development will be described in this chapter. One of my initially proposed goals was to study the role of *Lox1* in the normal innervation of the male sex organ by the RPE motor neurons. My results showed that a *Lox1* dsRNA induced loss of *Lox1* and FMRFamide expression, as well as axonal defects in the RPE neurons. Several additional attempts to increase the efficacy of *Lox1* RNAi failed to produce similar effects. Finally, an RNAi analysis of the function of *Lox2* in the Rz cells of M8 –Rz(8) showed a trend to axonal defects but was inconclusive.

MATERIALS AND METHODS

dsRNA Synthesis

The first RNAi used (*Lox1* dsRNA) was transcribed from *Lox1* cDNA sequences upstream of the homeobox of this gene. The template for in vitro transcription was generated from the *Lox1* cDNA by PCR, using the primers T7-5'HLox1 (AGC ATG

TCA ACT GCA TCA) and T7-3'HLox1b (AGT GGC ACT CGC CGA ATT). Both primers included a T7 promoter sequence (ATT AAT ACG ACT CAC TAT AGG GAG) at their 5' ends. A template for the production of single stranded RNA (ssRNA, a control to compare against the dsRNA in gel electrophoresis) was also amplified under the same conditions, except that the primer T7-5'HLox1 was replaced with ME1Lox1, which contained the same *Lox1* sequence but not the T7 promoter. A template for a dsRNA to be used as a negative control was also amplified from a non-coding region of the pBlueScript plasmid (*pBS*), using the primers T7-BS800as (CGT AAT CAT GGT CAT AGC, starting at position 800 of the pBlueScript sequence) and T7-BS1119 (ACC GTA TTA CCG CCT TTG AG, ending at position 1119 of the pBlueScript sequence). The PCR reaction conditions were standard for Taq DNA polymerase, except that different $MgCl_2$ concentrations were found to be optimal for each reaction (15 mM for the *Lox1* dsRNA template, 15 mM for the *Lox1* ssRNA template, and 30 mM for the *pBS* dsRNA template). The program used was 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by 8 min at 72°C.

The PCR products were extracted with chloroform, digested with 0.5 µg/µl of proteinase K in the presence of 0.1% SDS for 10 minutes at 37°C, extracted with 1:1 phenol-chloroform, further purified through Sephadex G-50 spin-columns, precipitated with ethanol/sodium acetate, and resuspended in RNase-free water. The DNA concentration was estimated by serial dilution and staining after electrophoresis on a 2% agarose gel.

RNA synthesis was carried out with the RiboMax kit (Promega) according to the manufacturer's instructions. A typical in vitro transcription reaction used 4-10 µg of PCR product (DNA template) in a 100 µl reaction incubated for 4 hours at 37°C. The

DNA template was then removed by digestion with 1 μg/μl DNase I for 15 min at 37°C and the RNA extracted with 1:1 phenol-chloroform, passed through a Sepharose-G50 spin column, precipitated with ethanol/sodium acetate, resuspended in RNase-free PBS, and stored at -80°C. Samples of RNA were tested and compared using agarose gel electrophoresis under native and denaturing (formaldehyde) conditions, showing that very large proportions of the *Lox1* and *pBS* dsRNAs were indeed double-stranded. Other *Lox1* dsRNAs (generated from other regions of the *Lox1* cDNA) and a *Lox2* dsRNA were synthesized and purified following the same procedure (Table 4-1).

Table 4-1 ***Double-stranded RNAs***

All primers included a T7 promoter sequence (ATT) at their 5' ends, followed by the sequences included in this table.

dsRNA (length)	**Primers**	**Descriptions**
Lox1 (800)	T7-5'Hlox1 (AGCATGTCAACTGCATCA) T7-3'Hlox1 (AGGTCCATTATCGTCAG)	hexapeptide no homeobox
Lox1 1 (292)	T7-5'Hlox1short (GAGATTTCGAGCTGCGAT) T7-3'Hlox1c (TTGACCTGTTTCTCAGAG)	no hexapeptide no homeobox
Lox1 2 (769)	T7-5'Hlox1 T7-3'Hlox1c	no hexapeptide no homeobox
Lox1 3 (407)	T7-5'Hlox1c (CTCTGAGAAACACGTCAA) T7-3'Hlox1d (ATTTCGTCGACGTCATAG)	hexapeptide homeobox (part)
Lox1 6 (407)	T7-3'Hlox1_utr1 (GTGCATTAGCGAGATATG) T7-3'Hlox1_utr2 (CACGAAGAGTGTTGTCTG)	3'UTR
Lox1 7 (1,104)	T7-5'Hlox1c T7-3'Hlox1_3'utr2	homebox hexapeptide 3'UTR
Lox1 9 (1,419)	T7-5'Hlox1 T7-3'Hlox1_3'utr1 (CATATCTCGCTAATGCAC)	hexapeptide homeobox 3'UTR
Lox2 (255)	T7-Lox2pr1 (GGATTGAACTCAGTCACAACGC) T7-Lox2utr5 (CCTCTTCATCCATCTCCATCACTC)	5'UTR no homeobox no hexapeptide
pBS (270)	T7-BS1119 (ACCGTATTACCGCCTTTGAG) T7-BS800as (CGTAATTCATGGTCATAGC)	negative control

Injections

Embryos were placed in filtered, autoclaved leech water (0.5g of synthetic sea salts -Instant Ocean- per liter of tap water) within a 35-mm Petri dish with a bottom layer of Silgard with a wedged well carved in it. The well had a size that matched an average E6-E7 embryo. The embryo was positioned ventral side up and held with a narrow strip of Kimwipe. The injectate contained dsRNA, 2.5% Lipofectamine (Invitrogen), 2% polyethylene glycol MW 8000, 20 mM Hepes-NaOH pH7 7.2, and 0.1% Fast Green (a dye that helps to see the site and the progress of the injection).

The injections were carried out under a dissecting microscope. The injectate was loaded into glass microelectrodes that were then attached to a holder in a micromanipulator. The dsRNA was finally injected using positive air pressure into the germinal cavity through the anterior-lateral region of the germinal plate. Following injection, each embryo was placed in a different well of a 24-well plate, containing 2 ml/well of filtered, autoclaved leech water, and kept in an incubator for 2-3 days at 23°C. The embryos were then dissected stained with antibodies as described in Chapters II and III.

Data Analysis

The results were collected in spreadsheets and analyzed using the statistical package JMP Version 4.0 (SAS).

RESULTS

Role of Lox1 *during RPE Neuron Differentiation*

In order to test the hypothesis that *Lox1* plays an important role in the differentiation of the RPE neurons and their segment-specific innervation of the male sex organ, I used

RNAi to block the expression of this Hox gene. Purified dsRNA was injected into the germinal cavity of embryos at E7, before the RPE neurons showed any detectable signs of differentiation from their segmental homologs, the RMV neurons (*i. e.*, before the RPE neurons turn off *Lox4* expression, start to express *Lox1* and FMRFamide-like peptides, and extend processes that innervate the male organ). This is also the earliest embryonic age at which over 50% of the embryos survive systemic injections (Mercado-Pimentel, 2003). I tested concentrations of dsRNA in the injectate between 2.5 μM and 40 μM. Survival rates did not significantly change between 0 and 10 μM (data not shown). Thus, a dsRNA concentration of 10 μM was used in these experiments.

Embryos from the same cocoon were divided into two groups, injected at E7 with either *Lox1* or *pBS* dsRNA, and dissected at E9-E10. To test the effect of *Lox1* RNAi on the RPE neurons, the embryos were double-stained with *Lox1* and FMRFamide antibodies. The FMRFamide antibody stains the cell body and the axonal processes of the RPE neurons, which can be easily traced as the only stained axonal branches that enter the sex nerves. Developmental defects were determined by comparing *Lox1* dsRNA-injected embryos with their siblings from the same cocoon that were injected in the same experiment with *pBS* dsRNA.

The RPE neurons of embryos injected with *Lox1* dsRNA showed loss of *Lox1* expression (17 of 53 surviving embryos -32.1%-) more frequently than in the control group injected with *pBS* dsRNA (3 of 52 surviving embryos -5.8%-). The embryos injected with *Lox1* dsRNA displayed defects that suggest a failure of the RPE neurons to differentiate from their precursors, including loss of FMRFamide staining and missing axonal branches (50.9% of the embryos). Among the control embryos, significantly fewer (11.5%) had

undifferentiated RPE neurons (Table 4-2). The results also showed a strong association between the loss of *Lox1* staining and the observed lack of RPE differentiation in embryos injected with *Lox1* dsRNA (15 of 53 surviving embryos -28.3%-); this association was absent in embryos injected with the control dsRNA, in which loss of *Lox1* staining and RPE differentiation were distributed in a manner that closely matched that predicted if these were independent, random events (Table 4-3). These results indicate that *Lox1* controls aspects of the identity of the RPE neurons.

A great deal of time was spent trying to improve the effectiveness of *Lox1* knockdowns. To this end, I synthesized other dsRNAs from different regions of the *Lox1* cDNA (Table 4-1) and tested them on embryos under the same conditions as above. These dsRNAs failed to produce a significant frequency of *Lox1* knockdown.

Figure 4-1 ***RPE neuron defects due to*** **Lox1** ***RNAi.***

An embryo injected with *Lox1* dsRNA at E7 and examined at E10 showed loss of *Lox1* and FMRFamide staining and axonal defects in the RPE neurons (***B***) when compared to a control embryo injected with *pBS* dsRNA (***A***). Large arrowheads mark the RPE cell bodies; small arrowheads their axonal branches innervating the male organ (absent in ***B***). The scale bar is 100 μm.

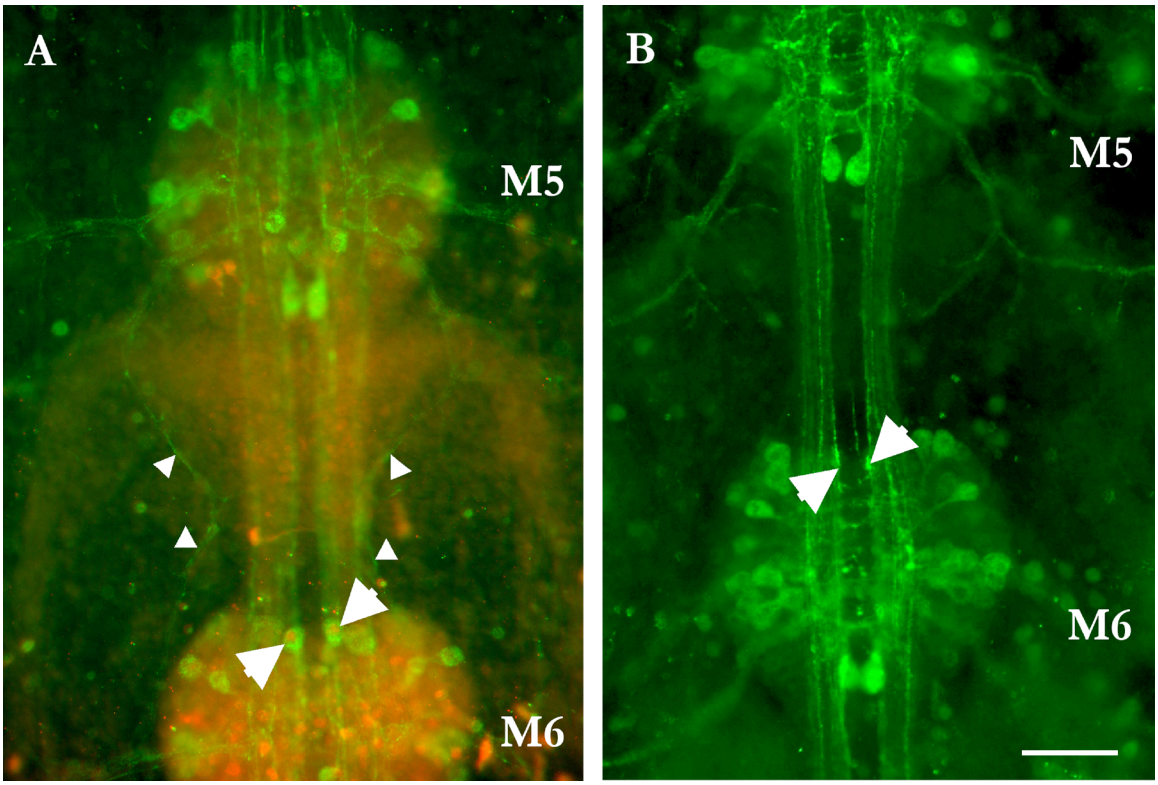

A
M5
M6
B
M5
M6

Table 4-2 ***Effects of*** **Lox1** ***dsRNA injection on RPE neuron development.***

The number of embryos in each category is reported.

dsRNA	**Injected**	**Surviving**	***Lox1*** **RPE neurons**	**Undifferentiated RPE neurons**
Lox1	74	53	17	27
pBS	74	52	3	6
Probability (Chi-square)			0.0003	<0.0001

Table 4-3 ***Frequency distribution of RPE neuron phenotypes after dsRNA treatment***

The number of embryos in each category is reported. RPE^+ means normally differentiated RPE neurons; RPE^- means undifferentiated RPE neurons (*i. e.*, loss of FMRFamide staining or missing axonal branches). Frequencies were predicted from the results of Table 4-2, assuming independence between *Lox1* expression and RPE neuron differentiation.

Phenotype	***Lox1 dsRNA***			***pBS* dsRNA**		
	Number	**Frequency**		**Number**	**Frequency**	
		Predicted	**Observed**		**Predicted**	**Observed**
***Lox1*$^+$/RPE$^+$**	24	0.33322	0.45283	43	0.83359	0.82692
***Lox1*$^-$/RPE$^+$**	2	0.15735	0.03774	3	0.05103	0.05769
***Lox1*$^+$/RPE$^-$**	12	0.34603	0.22642	6	0.10872	0.11538
***Lox1*$^-$/RPE$^-$**	15	0.16340	0.28302	0	0.00666	0.00000
Total	53	1.00000	1.00000	52	1.00000	1.00000

Effects of Lox2 *RNAi on Retzius neuron development*

I turned my attention to the very well-studied Retzius cells. These serotonergic neurons express *Lox2* in all segmental ganglia posterior to M7. I focused my attention on the Rz neurons of M8, which express *Lox2* at E9. The experiments were similar to those carried out for knockdown of *Lox1* (see above). The *Lox2* dsRNA was prepared, diluted with injection buffer to 20 μM, and injected in the same way as the *Lox1* dsRNA. The embryos were injected at E7 and examined two days later. The same *pBS* dsRNA used in the *Lox1* knockdown experiments was used as a negative control for *Lox2* knockdown.

Sixty-nine embryos were injected with *Lox2* dsRNA. Among the 60 surviving embryos, none of them showed any detectable loss of *Lox2* or serotonin staining in Rz(8) neurons. Seven Rz(8) neurons of embryos injected with *Lox2* dsRNA displayed reduced terminal branches, but similar defects were observed in four Rz(8) neurons of control animals (65 embryos were injected with *pBS* dsRNA, of which 55 survived). The difference between the experimental and control groups may indicate a trend, but was too small to be significant.

DISCUSSION

Using RNAi and immunostaining, I was able to show that *Lox1* controls important aspects of RPE neuron differentiation. The results showed highly significant differences between the *Lox1* dsRNA-treated embryos and their *pBS* dsRNA-injected counterparts. The RPE neurons of embryos injected with *Lox1* dsRNA displayed at a higher rate loss of *Lox1* staining, loss of FMRFamide staining, and axonal defects. This experimental population also showed a correlation between *Lox1* expression and RPE neuron

differentiation. Presence of *Lox1* staining was associated with normal RPE neurons in the embryos injected with *Lox1* dsRNA (45% of the embryos displayed these two traits together, well above the percentage that was expected if they were independent -33%-) but not in those injected with *pBS* dsRNA (83% observed, 83% expected). Absence of *Lox1* staining was linked to undifferentiated RPE neurons in the *Lox1* dsRNA-injected embryos (28% of the embryos showed both traits -16% expected-) but not in those that were *pBS* dsRNA-injected (0% observed -none out of 52-, 0.7% expected -predicting 0.35 embryos out of 52-). These differences indicate that the higher rate of abnormal RPE neurons after *Lox1* dsRNA injection is caused by *Lox1* knockdown, while those few embryos that have undifferentiated RPE neurons after *pBS* dsRNA injection are more likely to be either less developed than the rest, harmed by the experimental procedures, or poorly stained.

Only 32% of the embryos injected with *Lox1* dsRNA showed *Lox1* knockdown in the RPE neurons. The injections were not intracellular, but systemic: the dsRNA was introduced into the germinal cavity. Therefore, it is very likely that the amount of dsRNA taken by the RPE neurons in different embryos was highly variable. In addition, some embryos were noticed to leak and lose a fraction of the dsRNA solution after the injection, or to have an unevenly distributed injectate inside the germinal cavity. Another factor that must be considered is that there is a narrow window of time when RNAi is most effective under the conditions used. Embryos younger than E7 die at high rates after the injection; older embryos are knocked down at lower rates, probably because the ganglia become surrounded by connective tissue making the neurons less accessible to the dsRNA and because Hox expression may be difficult to knock down after it is

established (The *LOX1* protein already synthesized may be stable during the duration of the experiment and may also upregulate the expression of its own gene, counteracting the effect of the dsRNA). Finally, it is likely that the rate of *Lox1* knockdown was underestimated when observing the immunostaining of RPE neurons (a semi-quantitative method at best). In order to avoid bias, only those embryos in which the *Lox1* staining was very low or absent were scored as knocked down. It is then very probable that some embryos were partially knocked down by the *Lox1* dsRNA and still counted as having normal *Lox1* expression. This would explain why the percentage of experimental embryos with undifferentiated RPE neurons (51%) is actually higher than that with loss of *Lox1* staining (32%). In order to detect levels of Hox gene expression more accurately and test these assumptions, a real-time PCR assay may be needed in the future.

CONCLUSIONS

In this dissertation, I described in detail the early spatial and temporal expression of *Lox1* and *Lox2*, and identified several neurons that express either of these genes by immunohistochemistry. I also studied the role of *Lox1* in the differentiation of one pair of motor neurons using RNAi and immunohistochemistry. The stable *Lox1* expression domain extends from the posterior aspect of R4 to M13. The *Lox2* expression domain runs from the posterior region of M6 to M21. Between M6 and M13, the number of $Lox1^{+}$ nuclei decreases, while the number of $Lox2^{+}$ nuclei remains large. This suggests that *Lox2* (a posterior Hox gene) down-regulates *Lox1*, a process known as posterior dominance.

Also using immunohistochemistry techniques, I was able to identify several neurons that express either *Lox1* or *Lox2*. Neurons that express *Lox1* include the AMS and PMS neurons of M2 and M3, the RPE motor neurons on M6, and the HAL neurons of M4. Neurons that express *Lox2* include the PMS neurons of M7 to M21, the Rz neurons of M8-M21, and, between M7 and M21, cell 261, the CV neurons, the NUT neurons, and the AP neurons. Some of these neurons showed interesting patterns of either *Lox1* or *Lox2* expression. For example, the PMS neurons of M2 and M3 expressed *Lox1* whereas those of M8-M21 expressed *Lox2*. Later in deveopment, the PMS neurons become unpaired due to apoptosis of one member of each segmental pair from M3 to M21, whereas those of R1-M2 remained paired through all stages. It is likely that *Lox1* plays an important role in suppressing the wave of apoptosis in M2-M3. In addition, the AMS

neurons are only detected in R1-M3 and they express *Lox1* in M2 and M3, suggesting that *Lox1* also plays a role in the establishment of this boundary. A different behavior was observed in the Lz3 neurons, which express *Lox1* in M4-M8, where they are unpaired, but express *Lox2* in M9-M16, where they are paired.

Finally, I used RNAi to study the role of *Lox1* in neuronal differentiation. I observed significant differences in the RPE neurons between control and experimental embryos. The experimental embryos had RPE neurons with low levels of FMRFamide expression and axonal defects, suggesting that *Lox1* is needed for the differentiation of the RPE neurons of M6 from the RMV neurons of other segments.

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