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THE LEECH HOX GENE LOX6 PLAYS A ROLE IN NERVOUS SYSTEM DIFFERENTIATION AND PATTERNING

by

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A DISSERTATION

**Submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements
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ABSTRACT

THE LEECH HOX GENE LOX6 PLAYS A ROLE IN NERVOUS SYSTEM DIFFERENTIATION AND PATTERNING

By

Melania E. Mercado-Pimentel

Adviser: Dr. Gabriel O. Aisemberg

The main site of Hox gene expression in metazoans is the nervous system. These transcription factors have an important role in nervous system development and function. I describe here the expression pattern and function of the Hox gene *Lox6* in the embryonic nervous system of the leech *Hirudo medicinalis*. Using a new, improved approach, I purified two different glutathione S-transferase fusion proteins containing large fragments of the coding regions of the leech Hox genes *Lox2* and *Lox6*. These fusion proteins were used for raising and purifying antibodies against *Lox2* and *Lox6*. The *Lox6* antibody allowed me to identify neurons and muscle cells of the central nervous system, the peripheral nervous system and the body wall that express this gene in the embryonic leech. *Lox6* expression was detected in the bipolar neurons, the mechanosensory P_D neurons, the netrin expressing NE-C neurons, the serotonergic

Retzius, AMS, and PMS neurons, a FMRFamide excitatory motor neuron, and putative glial cells. Some of these cells have been shown or suggested to play a role in axon guidance. I also identified *Lox6* expression in putative photoreceptors of the eye primordia and in other sensory neurons of the body wall. The developmental expression of *Lox6* had a widespread early phase and a restricted late phase. The long anterior-posterior domain of early *Lox6* expression suggested that *Lox6* is involved in general processes of early nervous system development, probably regulating cell-autonomous and non-cell autonomous molecules necessary for axon growth and guidance, and target selection. The late, restricted expression of *Lox6* may be involved in specifying segment-specific connections to peripheral sensory organs that express *Lox6* in one rostral neuromere. I also describe here the effects of knocking down *Lox6* expression by RNAi. *Lox6* RNAi disrupted axon patterning in all major nerves, indicating that *Lox6* plays a significant role in the early patterning of the nervous system. My results also suggested that *Lox6* controlled pioneer axon generation or growth in the bipolar neurons and partially regulated their expression of the axon guidance molecule netrin.

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During these years of research I learned that patience, perseverance and determination are important qualities in an individual to pursue the Ph.D. degree. I have found out that I am working out those qualities in my personality and that the bases were founded many years ago during my childhood by my parents. Therefore, I start to say thanks to my parents Rev. Preciliano Mercado Nash and Nohemi Blandon de Mercado, who taught me important values to obtain my goals in life. The support of all my family, parents, sisters and brother played an important role in all these years in the Ph.D. program. I would like to especially thank my husband, Angel Pimentel, for his understanding support and help in all these critical years. To my daughter Bianca Pimentel a thousand thanks for her understanding and patience to wait for me in the computer room to finish my experiments before going home during the night. All my family was an important factor so I could acquire this degree.

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"Have I not commanded you? Be strong and courageous, do not be terrified; do not be discouraged, for the LORD your God will be with you wherever you go."

Joshua 1:9

Dedicatory

This dissertation is dedicated to my parents, Rev. Preciliano Mercado Nash and Nohemi Blandón de Mercado whom with their advices, support, and practical life gave me the right elements for the completion of graduate school, to my daughter Bianca Eileen Pimentel who has bring joy to my life, to my husband Angel C. Pimentel for his love, support, help, and patience, to all my sisters (Sara Edith, Sarli Edinia and Noemi) and to my brother (Gilton) for their support.

"He that brakes a thing to find out what it is has left the path of wisdom."

J. R. R. Tolkien (1892-1973)

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CHAPTER I

INTRODUCTION

A brief history of Hox gene evolution

The original research on Hox genes in *Drosophila* revealed that these genes specify the anterior-posterior axis of the organism by specifying the characteristic structures of each of the body segments after the segmental boundaries have been established. In *Drosophila*, the Hox genes are organized in two clusters, the Antennapedia complex (ANT-C) (Kaufman, 1980) and the Bithorax complex (BX-C) (Lewis, 1978). ANT-C contains the homeotic genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*). BX-C includes *Ultrabithorax* (*Ubx*), *abdominal A* (*abdA*), and *Abdominal B* (*AbdB*). During the early development of *Drosophila*, each of these genes is expressed in a specific region of the embryo and thus gives identity to the different segments of this organism. This feature of Hox gene function indicated that these genes have similar functions in different regions of the body (Lewis, 1978). Lewis proposed that these genes evolved from a single gene by duplication and divergence, leading in that way to the evolution of insects with complex structures from an annelid-like ancestor that had simpler, repeated body segments.

Later studies supported many aspects of Lewis' hypothesis: Hox genes are transcription factors that share a 180 bp sequence, which encodes the homeodomain, a well-conserved 60-amino acid DNA-binding motif called the homeobox (McGinnis et al., 1984; Scott and Weiner 1984; Gehring 1987; reviewed in Scott et al., 1989). In *Drosophila*, Hox genes are found on chromosome 3, arranged in a specific order that is colinear with the order of expression along the body axis of the embryo, as well as with the timing of activation during development (Lewis, 1978). This colinearity rule is conserved in mouse, human (Gaunt et al., 1988; Duboule and Dolle 1989), and many other animal species (Ruddle et al., 1994). In mammals, there are four Hox clusters (A, B, C and D) located on different chromosomes (Scott 1992; Gehring et al. 1994) and containing genes numbered from 1 to 13. The equivalent Hox genes with highly conserved sequences and expression patterns present in the same position in the different vertebrate complexes form sets of unlinked genes called the paralogous groups (Scott, 1992). Kondo and Duboule deleted a 50 kb fragment upstream of Hox D11, which is near the 5' end of the complex, causing premature activation of Hox genes even at the 3' end of the complex (Kondo and Duboule, 1999). These results show that sequences important for the early implementation of the colinear mechanism are positioned outside the Hox D complex and therefore confirm the idea that Hox complexes are integrated genetic entities.

Hox genes also share conserved sequences motifs outside of the homeodomain. One is the hexapeptide motif of consensus sequence IYPWMK located upstream of the homeodomain in all Hox genes with the exception of *Abd-B* (Gehring et al., 1994). Another example of a Hox gene conserved motif is the hexapeptide KLPNTK at the 3' end of the *Dfd*-family of homeodomains (Burglin, 1993). The conserved nature of the

homeodomain, together with the conserved hexapeptide motifs and the conserved colinearity rule, indicate that Hox genes originated during animal evolution from a single primordial Hox gene cluster. Many studies in different species have shown that Hox genes are arranged in highly conserved clusters on chromosomal DNA, but the number of Hox complexes varies between different animal groups. For example, the polychaete *Chaetopterus*, an annelid, has a single Hox cluster (Irvine and Martindale, 2000); the beetle *Tribolium* has a single cluster; *Drosophila* has a single Hox gene complex, split into two separated clusters; the acorn worm, a hemichordate, has one Hox cluster; *Amphioxus*, a cephalochordate has two clusters; the lamprey, a primitive vertebrate, has three (or possible four) clusters (Pendleton et al., 1993); mammals have four clusters (reviewed in Gehring et al., 1994). These findings lead to the significant conclusion that these very diverse animal groups must have inherited their Hox clusters from a common ancestor and that the first Hox cluster probably originated by tandem duplication and divergence from a single ancestral Hox gene, supporting Lewis' hypothesis at a scale greater than he originally proposed.

In trying to understand the role that duplication of Hox clusters plays in development and evolution, Gaunt made comparisons of Hox gene expression patterns between different Hox clusters of the mouse and came to the conclusion that these expression patterns suggest that duplication of Hox clusters has permitted the development of a more complex body plan (Gaunt, 1991). Paralogous vertebrate Hox genes have similar expression patterns in the developing neural tube, but they have dramatic differences in their expression patterns in mesodermal and neural crest derivatives. Gene targeting studies of Hox genes show that there is a greater disruption of development in

mesodermal and neural crest derivatives than in the neural tube, suggesting that there is partial overlapping and redundant function of paralogous genes in the neural tube (Holland, 1992). According to Holland, these results suggest that Hox genes preserved ancestral roles in neural patterning after Hox cluster duplication, but added to these roles new secondary expression sites and functions (Holland et al., 1994). These results and ideas supported Ohno's hypothesis that gene duplications are a major force in the generation of organismal complexity (Ohno, 1970). However, newer results show that the zebrafish (*Danio rerio*) has seven Hox clusters (Amores et al., 1998). The teleost fishes are the most diverse group of vertebrates; they have less complexity in the anterior-posterior axis than mammals, but they have more copies of Hox clusters than mammals, possibly acquired by genome duplication. This type of result challenges the concept of a tight relationship of Hox cluster number and morphological complexity along the body axis. In searching the consequences of genome duplication, McClintock and coworkers carried out comparisons of Hox genes of the paralogous group 1 between zebrafish and mouse, showing that there have been specific changes in the developmental roles of Hox genes during vertebrate evolution (McClintock et al., 2001). They found variations in amino acid sequence within functional domains and differences in expression pattern, including new expression patterns in the midbrain of zebrafish. Zebrafish *hoxc1a* has no ortholog in tetrapods and is expressed in midbrain and hindbrain neurons. Another novelty found in zebrafish is the co-localization in the midbrain of *hoxa1a* expression with the *hoxc1a* population of neurons. This expression pattern of *hoxa1a* is different from the one of its *hoxa1* ortholog in mouse; the zebrafish *hoxb1b* plays in its place a developmental role similar to mouse *hoxa1*. McClintock and coworkers proposed that the

type of midbrain expression observed could have an ancient origin within the vertebrates and that this expression may be a common feature of teleosts. This hypothesis is supported by the finding that in the medaka fish (a distantly related teleost) *hoxa1a* is expressed in the midbrain with the same pattern as in zebrafish. Concerning the function shuffling of paralogous group 1 zebrafish Hox genes in which *hoxb1b* performs a function similar to mouse *hoxa1*, McClintock proposed that this feature may be a consequence of a genome duplication event that occurred in a teleost ancestor. Therefore, the discovery of extra Hox gene clusters in zebrafish and other fishes like the medaka fish (Naruse et al., 2000) and the pufferfish (Taylor et al., 2001; and references therein) has led to the hypothesis that genome duplication provided the genetic raw material for the teleost radiation. Genome duplications may provide a mechanism where the Hox genes undergo radical modifications resulting in major alterations in the body plan.

Hox gene function in nervous system development

The major site of Hox gene expression in vertebrates and invertebrates is the central nervous system (CNS). A comparison of the expression of Hox genes in *Drosophila* and Hox B genes in the mouse indicates that the anterior-posterior order of Hox gene expression is more alike in the central nervous systems of the fly and the mouse than in the CNS and epidermis of the fly. This points out that the pattern of Hox gene expression has a greater evolutionary conservation in the CNS than in the epidermis (Kammermeier and Reichert, 2001). Although there are many studies that shed light on the molecular

mechanisms of Hox gene function during development, there are fewer studies on Hox gene function during the development of the nervous system. Most of the progress in understanding the role of Hox genes in nervous system development comes from studies on vertebrates (reviewed in Krumlauf 1994; Lumsden and Krumlauf 1996) and a few studies from invertebrates (see for example Hirth et al., 1998; Hirth et al., 2001; Aisemberg et al., 1995; Aisemberg et al., 1993).

Genetic studies in vertebrates indicate that a major site of Hox gene action is in the rhombomeres of the hindbrain and in the spinal cord. The hindbrain is subdivided into smaller and temporary compartments called rhombomeres where cells can mix freely, but not with cells from adjacent rhombomeres (Guthrie and Lumsden, 1991). The branchial arches are adjacent to the rhombomeres and receive motor axon input from a specific pair of rhombomeres (Lumsden and Keynes, 1989). Many studies using targeted mutations of Hox genes (Goddard et al., 1996, overexpression (Zhang et al., 1994), and manipulation by exogenous agents like retinoic acid (Marshall et al., 1994) support the conserved Hox code model stating that the regional expression of Hox genes in the hindbrain gives identity to rhombomeres. However, these studies did not show whether Hox genes act as segment identifiers in mediating connectivity between the CNS and the peripheral targets. The Lumsden lab showed that ectopic overexpression of mouse *Hoxb1* in chick rhombomere 2 that normally does not express *Hoxb1* causes cells of this rhombomere to express specific markers, migration patterns, and target connections of rhombomere 4 (Bell et al., 1999). *Hoxb1* specifies the facial motor neuron precursors of rhombomere 4 (Goddard et al., 1996) and the neural crest cells that migrate from dorsal rhombomere 4 into facial motor neuron targets of the second branchial arch (Sundin and Eichele, 1990).

In these experiments, overexpression of *Hoxb1* in rhombomere 2 caused its transformation into rhombomere 4 regarding the expression of molecular markers and the migration patterns of neurons, but the transformed motor neurons connected to their normal target, the second branchial arch. Complementary experiments showed that normal axon projections into the periphery were truncated when their target selectively expressed the improper Hox gene (Bell et al., 1999). These results demonstrated that Hox genes confer identity to neurons in terms of location in the nervous system, molecular markers, pathway migration, and axonal targeting, mediating connectivity between the CNS and the peripheral nervous system (PNS).

Hox gene function in neuronal identity and axon pathfinding

Insect neurogenesis studies pioneered the understanding of neuronal differentiation, showing that neuronal identity may be specified by the regional expression of specific transcription factors (see for example Doe and Technau 1993; Schmid et al., 1999). In insects, the neuronal progenitors give rise to a specific subset of neurons in the ganglion and are identifiable by its position and pattern of gene expression. The cells produced by the neuronal progenitors differ also in the expression of specific transcription factor that control other genes involved in selective migration, axon outgrowth, target cell recognition, and molecular phenotype. Many studies in vertebrates as well as invertebrates point out that Hox genes belong to this class of transcription factors that

lead to changes in the way neurons differentiate, indicating that they play an instructive role in neuron type determination.

Vertebrate studies of spinal motor neurons and interneurons provide a number of examples of the roles that Hox genes play in neuronal identification. Targeted mutations of *Hoxb9* show that spinal motor neurons are generated on time and in normal numbers, but after they leave the cell cycle they begin to transiently express transcription factors of V2 interneurons (Arber et al., 1999). In addition, the motor neurons show abnormal migratory patterns, errors in axon projection, and defects in innervation of some target muscles. By analyzing the spatial distribution of transcription factors normally expressed by neuronal progenitors and immature neurons in targeted mutations, Davenne and coworkers showed that mouse *Hoxa2* and *Hoxb2* synergize in controlling the neuronal cell fate of ventral interneurons in rhombomere 3 (Davenne et al., 1999). These studies also showed that facial motor neuron markers of rhombomere 4 were down regulated in *Hoxb2* mutant mice.

Studies done in invertebrates like the leech have shown that Hox genes are involved in determining electrophysiological properties as part of the identity of neurons (Aisemberg et al., 1997). Ectopic expression of the *Scr* homolog *Lox1* by mRNA injection produced much larger action potentials in the anterior Pagoda (AP) motor neuron and the nut neuron. These results suggested that *Lox1* ectopic expression modified voltage-dependent ion channels in those neurons. This is the first and only evidence so far that Hox genes are involved in giving electrical properties to neurons as part of their identity. In other invertebrates like *C. elegans*, *lin-39* and *mab-5* act in a combinatorial way to determine

cell identities (Salser et al., 1993; Clark et al., 1993), and *egl-5* has been shown to be involved in the specification of the fate of the neuroectoblast P12 (Jiang and Sternberg, 1998). In Drosophila, the lineage of the thoracic neuroblast NB1-1 differs from the abdominal one with respect to the presence or absence of specific glial and neuronal components (Udolph et al., 1993). Later studies showed that the activity of the homeotic genes *Ubx* or *abd-A* is required for the expression of the abdominal lineage of NB1-1 and that ectopic induction of *Ubx* or *abd-A* superseded the thoracic determination (Prokop and Technau, 1994).

The results discussed above show that Hox genes are involved in giving identities to neurons by regulating cell-autonomous factors. However, neurons also acquire their identity from factors in their environment. Some studies have pointed out that Hox gene expression provides positional information in the environment where axons can be guided to reach their targets. Such is the case of the virally mediated misexpression of chick *Hoxc-6* in the mesoderm of the first thoracic somite that dramatically affected the outgrowth of spinal nerve axons (Burke and Tabin, 1996). The sensory and motor cell bodies of the neurons had normal expression of *Hoxc-6*, but the mesoderm through which the axons normally find their way misexpressed *Hoxc-6*. Therefore, the truncated outgrowth of the spinal nerve axons seems to be due to the lack of instructive mesodermal signals that normally are present in their pathway.

In *Drosophila*, *lab* and *Dfd* mutants show regional defects in axonal patterning in the tritocerebrum, and in the mandibular and maxillary neuromeres, respectively (Hirth et al., 1998) (Hirth et al., 2001). Neurons of these regions did not extend axons and did not

express neuronal markers. Also, descending and ascending axons from other parts of the brain did not extend through the mutant domain. The fact that neurons of the mutant region did not extend axons and did not express neuronal markers shows that Hox genes are controlling cell-autonomous factors. In addition, the fact that axons from other parts of the brain did not project through the *lab* and *Dfd* regions as they normally do shows that Hox genes are also controlling non-cell autonomous factors.

Studies in mice have shown that *Hoxa-2* is necessary for normal axon pathfinding of motor neurons of rhombomeres 2 and 3 (Gavalas et al., 1997). In *Hoxa-2* mutants, a large number of these motor axons turn posteriorly and exit to the periphery through the rhombomere 4 exit points, innervating in this way the second branchial arch instead of the first branchial arch. Other studies have shown that *Hoxa3* has an important role in ensuring the correct axon projection pattern of the three components of the nerve (motor neurons, sensory neurons of the proximal ganglia, and sensory neurons of the distal ganglia) (Watari et al., 2001). *Hoxa3* knockout mice showed specific truncation of the ninth cranial nerve (Manley and Capecchi, 1997). Analysis of these mice showed that the truncation effect was due to migration defects of glial and neuronal precursors tested by specific markers proper of these cells. When these cells fail to migrate to their right location, they fail to send their axons to the CNS, and when they migrate to wrong locations, they project axons through wrong pathways. These results showed that Hoxa3 is necessary for the correct axonal patterning of the ninth cranial nerve. All these studies show that Hox genes are necessary for the proper development of the nervous system by conferring neurons many aspects of their identity. When Hox gene expression is altered, neurons lose their identity, affecting in that way the proper patterning of the nervous

system necessary for mature nervous system function. A number of other transcription factors not discussed here are expressed in combination with Hox genes to specify functional classes of neurons. For example, the LIM homeobox transcription factors have been shown to participate in conferring neuronal identity (reviewed in Jurata and Gill 1998).

Leech development

The following description of early leech development is based on the work of Fernandez and Stent (1982. In general terms, development in all leech species is similar. *Hirudo* eggs are about 0.1 mm in diameter and contain very little yolk. After fertilization, they are deposited in a sealed cocoon that contains an albuminous fluid. At the beginning of embryogenesis, a sac with a mouth is produced and this cryptolarva ingests the albumen that provides the source of nutrition for development to proceed. By day four, the embryo grows to about 5 mm. The germinal plate forms at the surface of the cryptolarva. During the first two days of development, cleavage produces a 30-40-cell embryo. As in typical spiral cleavage, the majority of the cells are small micromeres and lie on top of four big cells called macromeres. A group of micromeres forms the cryptolarval sac and mouth. Macromeres A, B, and C do not form germinal plate structures. The D macromere forms five pairs of teloblasts, which divide to give rise to the primary blast cells. The blast cells arrange in columns named bandlets, which converge to form two germinal bands. The left and right germinal bands pair together, starting at the future anterior region of the

embryo, forming the germinal plate. Thus, the embryo develops with a rostral-caudal gradient, with more mature segments at the anterior end. The blast cells from the pairs of teloblasts designated as N, O, P, and Q form the ectoderm. Blast cells from the two M teloblasts form the mesoderm. The central nervous system of the leech forms in the middle of the germinal plate, reflecting the segmented body plan.

The segmented body plan of the leech arises from the primary blast cells, which are the segmental founder cells (blast cells composing the bandlets). Each blast cell gives rise to 10^2 differentiated descendants (Shankland et al., 1991). The blast cells within the same bandlet produce clones that are identical in composition and positioned in consecutive segments along the body length (Weisblat and Shankland, 1985; Zackson, 1982). According to Shankland, there is a fixed relationship between the birth rank of the primary blast cell in the bandlet and the segmental location of its descendant clones (Shankland, 1994). The first-born blast cell contributes to the anterior-most body segment and the subsequent blast cells contribute to the next posterior body segment in its respective order (Shankland, 1994). As in any tripoblastic animal, the leech nerve cord is of ectodermal origin. Most neurons (260-320 in the adult ganglion) are derived from the N teloblast pair (Kramer and Weisblat, 1985). The CNS or ventral nerve cord of the leech is composed of 32 segmentally iterated ganglia (Mann, 1962). The four rostral neuromeres (RN1-RN4) and the seven caudal neuromeres (CN1-CN7) are fused, composing in that way the rostral and caudal ganglia, respectively. The rostral or subesophageal ganglion is linked to the supraesophageal ganglion through two circumesophageal connectives. The supraesophageal ganglion is not of segmental origin (reviewed in Stent et al., 1992). The other twenty-one midbody ganglia (MG) are linked

by two lateral connective nerves and a median connective called Faivre's nerve. Each segmental ganglion contains about 200 bilateral pairs of neurons (Macagno, 1980). These neurons acquire their segmental identity from the segmental founder cells or primary blast cells (Shankland et al., 1991). Individual blast cells get different identities in accordance with the rank order of their birth, indicating a lineage-based mode of determination (Shankland et al., 1991). However, this segmental identity is not the only source of final neuronal identity, because at later stages neuronal differentiation also takes place through cell interactions (Gao and Macagno 1987a; Gao and Macagno 1987b: Loer et al., 1987).

Hox Genes in the leech

Several Hox genes have been cloned from the leech and some of them have been partially characterized. Among them are *Lox7*, *Lox6*, *Lox18*, *Lox1*, *Lox20*, *Lox5*, *Lox4*, *Lox2*, and *Lox15*. *Lox7* is homologous to *Drosophila lab* (Kourakis et al., 1997), *Lox6* and *Lox18* to *Dfd* (Wong and Macagno 1998; Kourakis et al., 1997; Kourakis and Martindale 2001). *Lox1* and *Lox20* to *Scr* (Aisemberg and Macagno 1994; Kourakis et al., 1997), *Lox5* to *Antp* (Kourakis et al., 1997), and *Lox2*, *Lox4*, and *Lox15* to *Ubx* and *Abd-A* (Wysocka-Diller et al., 1989; Wong et al., 1995; Wong 1997). The expression patterns of the Hox genes *Lox7*, *Lox6*, *Lox18*, *Lox20*, and *Lox5* have been partially characterized in *Helobdella robusta* (Kourakis et al., 1997; Kourakis and Martindale 2001). In *Hirudo medicinalis*, *Lox6* (Wong and Macagno, 1998), *Lox1* (Aisemberg and Macagno, 1994),

Lox4 (Wong et al., 1995), *Lox2* (Wysocka-Diller et al., 1989), and *Lox15* (Wong, 1997) have been partially characterized. The domain of expression of *Lox7* extends from RN1 to CG7. *Lox6* expression starts at the posterior end of RN2 and ends in CN7. *Lox 18* is expressed in the ventral midline of the leech along the entire length of the germinal plate. *Lox1* expression begins at the posterior end of RN4 and ends at MG12. The *Lox20* domain is from RN3 to MG2. The expression domain of *Lox5* begins at RN4 and extends to the posterior-most neuromere. *Lox4* is expressed from the posterior end of MG3 to MG21and *Lox2* from the posterior end of MG6 to CG7. *In situ* hybridization experiments show that *Lox15* is expressed in *Hirudo medicinalis* in the CNS and in peripheral tissues. within an anterior-posterior domain of expression caudal to those of *Lox2* and *Lox4* (Wong, 1997). All these genes are expressed during developmental stages when the nervous system of the leech is developing. Therefore, the logical question to ask is: what is the role that Hox genes play during the development of the nervous system?

An advantage of using the leech as a model to study Hox gene function is that we can do studies at the level of well-characterized single neurons that can be identified according to several properties. These identifying properties include the size and location of the cell body within the ganglia, electrophysiological properties, biochemical properties like expression of neurotransmitters and surface molecules, guidance signals, and axon morphology. Studies of Hox genes in *Hirudo* have allowed us to identify a number of neurons that express different Hox genes. I report here that, among other cells, the mechanosensory P_D neurons, the serotonergic Retzius, AMS, and PMS neurons of RN3 and RN4, and the bipolar neurons express *Lox6* (Chapter III). In addition, *Lox1* is expressed in several neurons that have been previously characterized, like the

serotonergic AMS and PMS neurons of MG2 and MG3, the HA neurons, and the RPE neurons (R. Gharbaran, personal communication). *Lox4* is also expressed in the RPE neurons but with a different temporal pattern (Wong et al., 1995). Staining with *Lox2*, *Lox4*, and FMRFamide antibodies, showed that *Lox2* is expressed in the CV motor neurons and the RMV neurons of the MG7-MG21 (N. Jordan, personal communication). *Lox15* is expressed in the RMV neurons of MG15-MG21, which also express *Lox4* and *Lox2*.

Cell lineages studies revealed that *Lox2* transcripts are not present at the developmental stages when segment identities are first established (Nardelli-Haefliger, 1992; Nardelli-Haefliger et al., 1994). 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 within the same range of body segments. When selective teloblast lineages were frame-shifted along the anterior-posterior axis, 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 order of the neuronal precursors that originate from the posterior end of the embryo.

Lox4 is expressed transiently in the specialized RPE neurons of MG6 and in their segmental homologs, the 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 segmental homologs 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 MG15-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 MG4-MG5 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 MG1-MG3 are much shorter and less regular and produce a more extensive network of fine processes (Wong *et al.*, 1995). Together, the above results and observations indicate that the main function of the leech Hox genes may be to act in a combinatorial manner to specify neuronal identity during nervous system development. 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.

I studied the role that *Lox6*, a homologue of *Drosophila Dfd*, plays in the development of the nervous system in the leech *Hirudo medicinalis*. *Lox6* was cloned and partially characterized at Columbia University by Victoria Wong (Wong and Macagno, 1998). These early *in situ* hybridization studies showed that *Lox6* is expressed in the CNS and

PNS at stages when development of these two systems takes place. To identify *Lox6*-expressing cells and to study the function of *Lox6*, I developed a *Lox6* antibody. Based on my analysis of *Lox6* expression, I hypothesized that, among other cell types, *Lox6* is expressed in cells that extend pioneer axons that establish connections in the CNS and between CNS and PNS. Therefore, blocking *Lox6* expression during early stages of nervous system development should affect nervous system connectivity.

I report here an improved technique for the purification of glutathione S-transferase (GST) fusion proteins, necessary for the bacterial expression of *Lox6* to produce *Lox6* antibodies. Using these *Lox6* antibodies, I was able to identify CNS and PNS neurons, and cells of the body wall that express *Lox6* in the *Hirudo medicinalis* embryo. Finally, I was able to knock down *Lox6* expression using RNA interference (RNAi) and study *Lox6* function during nervous system development.

CHAPTER II

EXPRESSION AND PURIFICATION OF FUSION PROTEINS FOR IMMUNOHISTOCHEMICAL STUDIES OF HOX GENE EXPRESSION

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INTRODUCTION

The expression in *E. coli* of foreign proteins fused to glutathione S-transferase (GST) is a popular system that allows easy production and purification of proteins to be used as immunogens or biological reagents. This system makes use of plasmid vectors that contain a GST gene under the control of an inducible *tac* promoter, followed by restriction sites that allow the insertion in-frame of a cloned gene (Smith and Johnson 1988; Smith 1993). Occasionally, the resulting fusion protein has properties that are similar to those of the non-fused GST and is therefore highly expressed upon induction, soluble, and easy to purify by affinity chromatography (Smith and Johnson 1988; Smith 1993). However, the GST fusion proteins are often expressed at lower levels than the original, non-fused GST and are insoluble or difficult to purify by standard procedures. Because protein expression and purification are in most cases attempted before the behavior of the protein during standard affinity purification is known in detail, it is

important to design a general approach applicable to the purification of most proteins fused to GST.

As part of our lab's research on the role of Hox genes during the development of the nervous system, I expressed in *E. coli* the leech genes *Lox2* (Wysocka-Diller et al., 1989), and *Lox6* (Wong and Macagno, 1998) using the GST vector pGEX-2t. The proteins of these genes were needed to raise polyclonal antibodies that were used for staining embryonic tissues and determine expression patterns of Hox genes. Because these genes are usually expressed at low levels, I expected that antibodies of high titer, specificity, and purity would be required to detect the endogenous Hox proteins in the embryonic nervous system. Therefore, I predicted that about ten milligrams of each pure fusion protein were needed for this project.

Rather than relying on the automatic application of one standard purification recipe to any fusion protein, I developed an approach that quickly customizes a general purification protocol to each particular construct. I report here a simple but general procedure that can be used to optimize the purification of milligram quantities of GST fusion proteins (Mercado-Pimentel et al., 2002). For each new protein, this procedure determines the optimal conditions for solubilization in a bacterial lysate, binding to glutathione-agarose (GA) beads, and elution. The conditions for solubilization and binding are based on the procedure described by Frangioni and Neel (1993). I also describe new conditions for the efficient elution of GST fusion proteins from GA beads that are compatible with the subsequent use of these proteins as antigens to generate antibodies suitable for gene expression studies.

MATERIALS AND METHODS

Lox6 expression construct

A 2.5 Kb *Lox6* cDNA clone (Wong and Macagno 1998; GenBank accession number AF017253) was used as a template to amplify a 791 bp fragment between positions 25 and 815 by PCR. The PCR product contains a fragment of the *Lox6* coding region, starting at the 9th amino acid of the open reading frame found in the cDNA clone and including the hexapeptide IFPWMK, the homeodomain, the C-terminus, and a small fragment of the 3' untranslated region. The upstream primer lox6pr1 (CAC GGA TCC CTC CTG TTT CAG GCC AA) contained a BamHI restriction site while the downstream lox6.1pr2 primer (CAC GAA TTC TGT CCT TCA ATT CGT GA) contained an EcoRI restriction site. The following PCR recipe was used: 10 µl *Lox6* cDNA template (0.01 ng/µl), 1 µl of each primer (50 µM), 10 µl 10X Thermopol buffer (Promega), 0.8 µl dNTP mixture (25 mM), 5 µl DMSO, 0.5 µl Vent polymerase (2 U/µl) and water up to 100 µl. Samples were spun down and four drops of mineral oil were added to each tube. The PCR was done in a thermal cycler model number DB66925 from Barnstead Thermolyne. The parameters used were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 40 cycles. The PCR products were vortexed with 100 µl of chloroform and spun down for 1 min in the microcentrifuge. The DNA (top layer) was pipetted out into a new tube bringing together the 200 µl of each different PCR reaction. Five microliters of each PCR reaction were analyzed in a 3% agarose gel (2% NuSieve, 1% SeaKem agarose; FMC Bioproducts) in 1X TAE buffer (40 mM Tris-acetate pH 8.5, 2 mM Na_2EDTA) run at 80 volts for 45 min.

The PCR products were passed through 1 ml Sephadex G-50 columns and centrifuged at 1000 rpm for 1 min. Then, they were extracted two times in 1:1 phenol-chloroform (PC) and one time in chloroform. The DNA was precipitated by adding 1/10 of the volume of 3 M sodium acetate (NaAcO) pH 5.2 and 2 volumes of 100% ethanol. This mixture was incubated at –80°C for 30 min and centrifuged 10 min at 14,000 rpm. The DNA pellet was resuspended in 17 µl of H_2O.

The purified *Lox6* PCR product and the pGEX-2t expression vector were digested with EcoRI and BamHI for ligation. The digested PCR product and the vector were electrophoresed in a 0.8% agarose/TAE gel and purified from the gel with a Geneclean II kit (Bio101, Inc.). In a 10 µl reaction, 1 µl of digested pGEX-2t (50 ng/µl) was ligated to 3.75 µl of digested *Lox6* PCR product (40 ng/µl) with 1 µl 10X T4 ligation buffer (Promega), 1 µl T4 DNA ligase (3U/µl), and 1 µl 5 mM ATP. This ligation reaction was incubated at 14°C for 24 hrs. Five microliters of ligation product were analyzed in a 0.8% agarose/TAE gel.

Electrocompetent *E. coli* Top10F' cells were used for transformation with the ligated DNA. One microliter of ligated DNA was pipetted into an Eppendorf tube and 20 µl of competent bacteria were added. The components were mixed gently by pipetting, avoiding production of bubbles, and the mixture was pipetted in to a pre-chilled Gene Pulser/E.coli Pulser cuvette (0.1 cm electrode, gap 50) from Bio-Rad. The cuvette was placed in a pre-chilled carriage and slided to the end of the set of the Gene Pulser. The cells were blasted with the Gene Pulser setting of 1.7 KV and the Pulse Controller setting of 25 µF, 200 Ω. One ml of SOC medium (For 1 l: 2 g tryptone, 0.55 g yeast extract, 1 ml

of 1 M NaCl, 1 ml of 1 M KCl, and, after autoclaving, 1 ml 1 M $MgCl_2$/1 M $MgSO_4$ and 1 ml 2 M glucose) was added to each electroporation. The electroporated cells were transferred to Eppendorf tubes and incubated at 225 rpm and 37°C for 1hr. Aliquots of 1, 10, and 50 µl of cells were diluted up to 100 µl with LB medium (For 1 l: 4 g tryptone, 2 g yeast extract, 2 g NaCl, 0.4 µl 1 N NaOH in 80 ml ddH_2O), plated in LB-ampicillin plates, and incubated overnight at 37°C.

Single colonies were grown overnight at 37°C in 10 ml aliquots of LB-ampicillin liquid medium. Plasmid isolation from each culture was carried out using the Wizard Plus SV miniprep kit from Promega. Each purified plasmid was digested with BamHI and with EcoRV in two separated reactions. Plasmids that produced the right fragments indicating the *Lox6* insert were subjected to restriction analysis to confirm their identity (see Results).

The pGEX-2T/*Lox6* plasmids thus obtained were transformed into BL21(DE3) *E. coli*, a protease-deficient strain. BL21(DE3) cells were made competent with $CaCl_2$ following standard procedures (Ausubel, 1987). Transformed cells were plated onto LB-ampicillin plates and incubated overnight at 37°C. One colony from each plate, originally from an electroporation colony with the right restriction map, was grown overnight at 37°C in a small volume of liquid LB-ampicillin medium, mixed with 1/10 volume of 80% glycerol, and frozen at -80°C for long-term storage.

***Lox2* expression construct**

A 596 bp fragment was amplified by PCR from an XhoI-linearized *Lox2* cDNA cloned in the pBlueScript vector (G. Aisemberg, unpublished results). The primers used were SE5 (CAA GGA TCC TGC ATC GAT GAC ATG GTC) and T3 (TAA CCC TCA CTA AAG GGA). Four identical reactions of 100 µl each were carried out simultaneously. Each reaction contained 10 µl 10X Thermopol buffer (New England BioLabs), 10 µl of *Lox2* cDNA (0.15 ng/µl), 1 µl primer SE5 (50 µM), 1 µl primer T3 (50 µM), 0.8 µl 25 mM each dNTPs, 0.5 µl Vent polymerase (2 U/µl). Samples were spun down and four drops of mineral oil were added. The PCR parameters were 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and 72° C for 8 min at the end. A 5-µl aliquot of each reaction was analyzed in a 3% agarose/TAE gel. The PCR product was then purified following the same procedure as for the *Lox6* PCR products. To prepare for ligation with the pGEX-2t vector, the purified PCR product was digested with BamHI. The Bam HI enzyme was then inactivated by heating for 10 min at 70°C. To purify the digested PCR product, it was loaded a 0.8% agarose/TAE gel and electrophoresed. The *Lox2* band of about 700 bp was excised and the DNA was isolated with the Geneclean II kit.

The purified, digested *Lox2* PCR product was quantified and ligated with the pGEX-2t vector. A 10-µl ligation reaction containing 1 µl of BamHI-digested pGEX-2t (50 ng/µl), 3 µl of *Lox2* DNA (50 ng/µl), 1 µl 10X T4 buffer, 1 µl T4 ligase (3 U/µl.), and 1 µl ATP (5 mM) was incubated overnight at 14°C. A 5-µl aliquot of the ligation reaction was analyzed in a 0.8% agarose/TAE gel.

Electrocompetent DH5α cells were transformed with the pGEX-2t/*Lox2* construct as described above. Thirty-six colonies were screened by PCR, using *Lox2*-specific primers. Each colony was transferred to100 µl of H_2O and boiled for 5 min. A PCR premix containing 380 µl 10X PCR buffer (Promega), 38 µl primer SE5 (50 µM), 38 µl ASLox2 primer (50 µM) (CTG GTT CGA ATT CGG GC*)*, 30.4 µl dNTPs (25 mM each), 19 µl Taq polymerase, 304 µl 25 mM $MgCl_2$, and H_2O up to 3.762 ml. Aliquots of this PCR premix (99-µl) and four drops of mineral oil were added to Eppendorf tubes containing 1 µl of boiled bacteria from each colony. PCR parameters were the same as for the amplification of the *Lox2* insert. Five microliter aliquots of each reaction were analyzed in a 1% SeaKem/2% NuSieve agarose/TAE gel. Five colonies showing the 200 bp band expected were inoculated into 10 ml of liquid LB-ampicillin medium and grown overnight at 37°C. For long-term storage, 900 µl of each culture was brought to 8% glycerol and frozen at -80°C. The remaining volume of culture was used for plasmid isolation with the Wizard Plus SV Miniprep Kit (Promega). To determine presence and orientation of the *Lox2* insert, the isolated plasmids were digested with EcoRI and electrophoresed in a 1% SeaKem/2% NuSieve agarose/TAE gel.

Protein solubilization

The transformed cells containing the plasmids pGEX-2t/Lox6 and pGEX-2t/Lox2 were grown overnight in 100 ml of LB medium containing 100 µg/ml of ampicillin. The next day, a 20-ml aliquot of the overnight culture was added to 1 liter of fresh medium and

incubated in two 2-liter conical flasks at 37°C and 225 rpm. After 1.5 hr, the cultures were induced with 0.1 mM IPTG and incubated under the same conditions for 4 hr longer. The induced bacteria were collected by centrifugation, resuspended in 60 ml of ice-cold STE (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 100 μg/ml PMSF), centrifuged again, and frozen at -20°C until used.

The pellet of fusion protein-expressing bacteria was resuspended in 60 ml of ice-cold STE buffer and treated with 100 μg/ml lysozyme for 15 min on ice, followed by addition of DTT to 5 mM. The lysozyme-treated bacteria were then divided into six aliquots and different concentrations of Sarkosyl (0, 0.24, 0.39, 0.91, 1.7, and 2.65%) were added to GST/Lox6. These aliquots were sonicated at a low setting (duty cycle = 20%, output = 2) with a microtip probe in ice until the viscosity disappeared. After sonication, the samples were centrifuged at 10,000×g for 25 min. The supernatants were saved and the pellets resuspended in STE buffer. These samples were tested by PAGE (polyacrylamide gel electrophoresis)-SDS for solubility of the fusion protein. The lowest Sarkosyl concentration able to solubilize the fusion protein was selected and applied to the remainder of the bacteria after lysozyme treatment. In the case of GST/Lox2, the concentration of Sarkosyl that worked for GST/Lox6 (0.24%) was used.

Binding to GA beads

Triton X-100 was added at different concentrations (0, 0.5, 1, and 2%) to aliquots of the supernatant containing Sarkosyl. The fusion protein contained in these aliquots was tested for its ability to bind to GA beads, as described below. The lowest Triton X-100 concentration able to allow binding to the GA beads was selected and applied to the remainder of the supernatant.

Five-µl aliquots of the supernatant containing Sarkosyl and Triton X-100 were mixed with different volumes of a 50% (v/v) slurry of GA beads (Sigma) in PBS (150 mM NaCl, 8.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4). The samples were then incubated in ice for 15 min with continuous agitation and centrifuged for 2 min at 8,000 rpm in a microcentrifuge. The supernatants were removed and the GA bead pellets were washed eight times with 1 ml of ice-cold PBS, centrifuging each time as before.

For large-scale binding, 20 ml of a 50% (v/v) slurry of GA beads was centrifuged at 3,200×g and 4°C for 2 min, and the PBS was removed. One quarter of the cleared lysate containing Sarkosyl and Triton X-100 from a 1-l induced culture (about 15 ml) was then combined with the beads. The beads were incubated at 4°C with shaking for 15 min and washed eight times with 100 ml 1X PBS.

Elution

Twenty-µl aliquots of 50% (v/v) slurry of GA beads in PBS that had been previously bound to fusion protein and washed as described above were centrifuged at 5,000 rpm for 30 sec in a microcentrifuge, the supernatant removed, and then replaced with 10 µl of elution buffer. The following elution buffers were tested: buffer 1 contained the GST ligand glutathione (75 mM HEPES pH 7.4, 150 mM NaCl, 5 mM DTT, 50 mM glutathione); buffer 2 contained SDS (75 mM HEPES pH 7.4, 150 mM NaCl, 5 mM DTT, 0.1% SDS); buffer 3 was high pH (50 mM TEA pH 11.5, 0.1% Triton X-100, 150 mM NaCl); buffer 4 was low pH (50 mM glycine-HCl pH 2.5, 0.1% Triton X-100, 150 mM NaCl). To neutralize the pH after using buffer 3, the eluate was collected on 0.2 volume of 1 M Tris-HCl pH 6.7. The eluate of buffer 4 was collected on 0.2 volume of 1 M Tris-HCl pH 9.0. After 15 min at room temperature with agitation, the beads were sedimented by centrifugation, and the eluate collected. Another 10 µl of the same elution buffer was added to each tube, the elution procedure was thus repeated, and the two eluates of each aliquot were pooled together. After elution, the remaining GA beads were subsequently incubated in a boiling water bath with an equal volume of SDS/electrophoresis sample buffer to release the fusion protein still bound.

For large-scale purification, cleared lysate from 250 ml of culture containing 0.73 g (wet weight) of bacterial cells was bound to 10 ml (bed volume) of GA beads and washed as described above, and finally eluted with two incubations of 15 min each at room temperature with shaking in 10 ml of elution buffer 2 (after each step, the eluate was

separated by centrifugation). The GA beads were then regenerated (Frangioni and Neel, 1993) and reused with more cleared lysate.

Immunization and antibody purification

My immunization protocol was carried out by Strategic BioSolutions (Ramons, California). Three rats were immunized subcutaneously with the purified GST/Lox6 protein to produce polyclonal antibodies. The primary injection was 125 μg of GST/Lox6 per rat in complete Freund's adjuvant. Three boosters were given every 21 days in incomplete Freund's adjuvant. Sera samples 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 after the third booster, the rats were exsanguinated. For the *Lox2* immunization, two rabbits and two rats were injected with the purified GST/Lox2 protein using the same protocol, except that the rabbits were injected with 300 μg of protein.

The antibodies were purified by affinity chromatography. GST/Lox6 protein (about 5 mg) was covalently bound to 0.3 g CNBr-activated Sepharose 4 Fast Flow (Pharmacia Biotech) following the manufacturer's instructions. Serum (2 ml) was diluted 1:5 with PBS (140 mM NaCl, 30 mM KCl, 43 mM $Na_2HPO_4.7H_2O$, 15 mM KH2PO4, 0.025% NaN_3) and filtered through a 0.2-μm celluiose acetate syringe filter (Nalgene). The filtered serum was passed through a 10-ml liquid chromatography column containing

about 1 ml (bed volume) of the GST/Lox6-Sepharose. The flow-through was collected and reapplied to the column. This second flow-through was saved and stored at 4°C for testing on Western blots. The column was washed with 20 ml of PBS and the antibody was eluted with 4 ml of low pH elution buffer (100 mM glycine-HCl pH 3) and collected in a tube containing 0.8 ml 1M Tris-HCl pH 9. The column was then washed with 10 ml of PBS. Additional antibody was eluted with 4 ml of high pH elution buffer (100 mM Na_2CO_3 pH 11), collected in a tube containing 0.8 ml 1M Tris-HCl pH 6.7, and combined with the antibody eluted at low pH. The column was finally washed with 10 ml of PBS and stored at 4°C. The purified *Lox6* antibody was adsorbed with four different affinity columns, containing Sepharose covalently bound to GST, *Lox1* protein, *Lox4* protein, and a 14-day-old leech embryo homogenate. The columns used for adsorption were cleaned with elution buffers, washed with 10 ml of PBS, and stored at 4°C. The adsorption flow-through containing specific *Lox6* antibodies was concentrated with Vivaspin concentrators (Vivascience), down to 0.5 ml, and washed in the same device 3 times with 10 ml of 1X PBS.

The *Lox2* antibodies were purified following the same protocol, except that in these purifications GST/Lox2 was coupled to Sepharose for the purification step, and the GST/Lox6 column was added to the adsorption step.

Western blots

Antibody specificity was tested on Western blots against total protein from bacteria expressing Lox6/GST, Lox2/GST, or non-fused GST. Total protein from bacteria grown in the same conditions as for the protein purification was electrophoresed and blotted onto nitrocellulose membranes. Induced cultures (10 ml) were centrifuged at 4000 rpm for 30 min. The pellets were resuspended in 1 ml of 1X Laemmli's buffer (BioRad) and boiled for 5 min. Each sample was diluted 1:100 and loaded into a single well in a 12% SDS-PAGE gel and run at 100 V. The proteins were electro-transferred onto nitrocellulose filters and various different aliquots gathered at different steps of the purification of antibodies were tested on the blots. These aliquots were diluted 1:100, 1:200, 1:500, and sometimes 1:1000 and 1:2000. Western blots were blocked with TBST (0.1 M Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20, 0.025 % NaN_3) for 30 min at room temperature with shaking. The blots were then placed in a Mini-Protean II Multiscreen Apparatus (Bio-Rad) and 600 µl of every dilution of the different antibody aliquots were added to each well. The blots were incubated for 30 min at room temperature on a shaker. Each well was rinsed three times with TBST and the blots were placed on small trays. The blots were washed three times with TBST for 10 min at room temperature on a shaker and then incubated with 5 ml of 1:5000 alkaline phosphatase-coupled anti-rat or anti-rabbit (depending on the source of the primary antibody) antibodies in TBST inside a plastic bag for 30 min at room temperature with shaking. After the second antibody incubation, the blot was washed three times at room temperature with TBST. The blots were rinsed with alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 5 mM $MgCl_2$) and 5 ml of developing buffer (33 µl of 50 mg/ml nitroblue tetrazolium

(NBT), 16.5 µl of 50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate in 5 ml of alkaline phosphatase buffer) were added to the blot in a plastic bag. When bands were visible, the blots were rinsed with TBST and air-dried.

Immunohistochemistry

Leech embryos were obtained from our *Hirudo medicinalis* colony, dissected in 6% ethanol 1X PBS, and pinned on Sylgard (Dow Corning) in small Petri dishes. After 1hr of fixation in 4% paraformaldehyde 1X PBS at room temperature, they were rinsed three times with PBS. The embryos were blocked with blocking solution (4% goat serum in PBS-TX; PBS-TX is 1X PBS, 1% Triton X-100) for 2 hr at room temperature, changing the blocking solution every 30 min. The primary antibody was diluted in blocking solution, 25 µl of the diluted antibody were added to each embryo, and incubated overnight in a humid chamber at 4°C. The embryos were then washed with PBS-TX for 3hr at room temperature with shaking, changing the PBS-TX every 30 min. The fluorochrome-labeled second antibody was diluted 1:200 in blocking solution, 25 µl of antibody were added to each embryo, and incubated 3 hr at room temperature in the dark. The second antibody was rinsed three times with PBS-TX and the embryos were washed with PBS-TX for 3 hr with shaking, changing the PBS-TX every 30 min. The embryos were clarified in a series of 25%, 50%, and 80% glycerol in 1X PBS. The embryos were finally mounted in gelvatol (23 g of polyvinyl alcohol 2000 dissolved in 100 ml PBS plus 50 ml glycerol, and 0.21 M 1,4-diazobicyclo-[2.2.2]octane.

RESULTS

Lox6 and *Lox2* constructs

A *Lox6* cDNA clone (Wong and Macagno, 1997; Gene Bank accession number AF017253) was used to amplify by PCR a 791 bp fragment (including added restriction sites) from position 24 to 815. These PCR products were ligated to the pGEX2-t expression vector. Bacteria were transformed by electroporation. Restriction enzyme digestion with EcoRV showed that three colonies out of 40 analyzed had the right construct to produce GST/Lox6fusion protein. For *Lox2*, cDNA Lox2.12 (G. Aisemberg, unpublished results) was used as a template to amplify a 596 bp fragment (including added restriction sites) between positions 2662 and 3225. These products were inserted into the pGEX-2t vector and transformed into bacteria by electroporation. I screened for colonies containing pGEX-2t-Lox2 by PCR and five colonies out of 36 contained the right construct. To find out if the constructs of those five colonies had the *Lox2* insert in the right orientation, I digested their plasmids with EcoRI, expecting two fragments of 380 bp and 5,156 bp. Two colonies showed the *Lox2* insert in the right orientation.

Protein purification

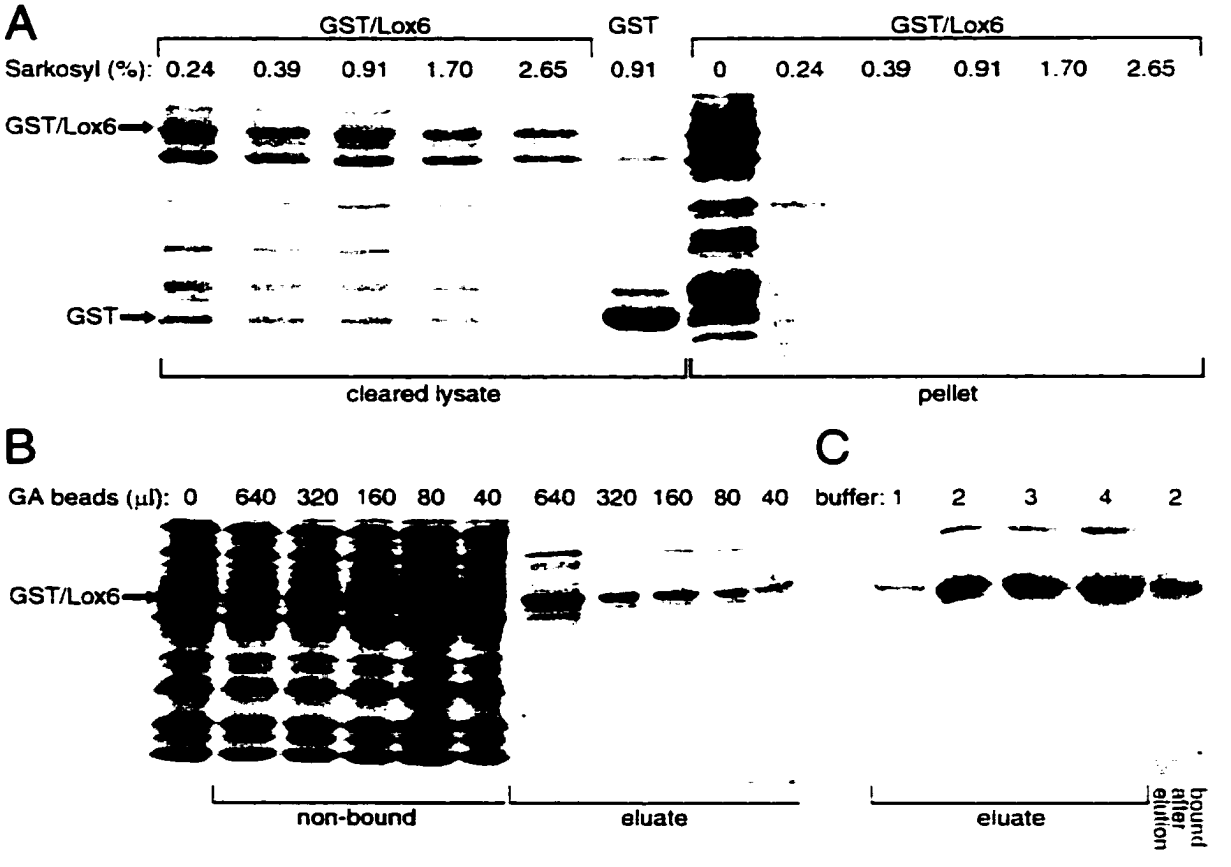
The early purification attempts using standard large-scale purification protocols (Smith and Johnson 1988; Smith 1993) had shown that the GST/Lox6 protein was insoluble in

the absence of denaturing detergents and was lost consequently at an early centrifugation step used to remove cell debris (see also Fig 1A). Therefore, I replaced the solution in which the cells were lysed with a buffer containing Sarkosyl and Triton X-100. This treatment has been shown to maintain fusion proteins in solution without affecting their ability to bind to affinity columns (Frangioni and Neel, 1993).

I also observed that excessive sonication produced fusion proteins that subsequently did not bind to GA beads (not shown), probably due to denaturation of the GST moiety of the fusion protein. Thus, I found it necessary to test the viscosity of the lysate with a Pasteur pipette after each pulse of ultrasound. Using this experimental setup, five pulses of 10 sec each reduced the viscosity to a level that allowed me to continue to the next step without inhibiting binding to GA beads at subsequent steps.

The optimal conditions for solubilization, binding to GA beads, and elution were determined for the fusion proteins (Fig. 1 shows representative results obtained for GST/Lox6; the optimization procedure was applied in the same way to GST/Lox2). In order to test for the optimal protein solubilization conditions, lysozyme-treated bacteria were sonicated in the presence of different concentrations of Sarkosyl. After lysate centrifugation, a fraction of each resulting supernatant and pellet was analyzed by SDS-PAGE (Fig. 1A). The fusion protein could be easily identified as a major band that was absent in bacteria that produced non-fused GST. These results showed that the lowest concentration of Sarkosyl, 0.24%, solubilized completely the GST/Lox6 protein. Therefore, I used 0.24% Sarkosyl for the following steps in the purification of this fusion protein.

Figure 1. *A. Solubilization of GST/Lox6 with Sarkosyl.* A bacterial pellet expressing GST/Lox6 was resuspended in STE buffer, divided into 10-ml aliquots and mixed with 10% Sarkosyl up to different concentrations. After sonication and centrifugation, equivalent samples of the resulting cleared lysate and pellet were electrophoresed. An arrow indicates the position of the GST/Lox6 protein, which can be identified by comparison with the cleared lysate containing non-fused GST, which is also indicated with an arrow. *B. Binding of GST/Lox6 to increasing amounts of GA beads.* Aliquots with 500 µl of GST/Lox6 cleared lysate containing 0.24% Sarkosyl and 0.5% Triton X-100 were incubated with different volumes of 50% (v/v) GA bead slurry. The GA beads were washed and eluted. Samples of the eluate and the fraction that did not bind to the beads were electrophoresed. *C. Elution of GST/Lox6.* Four different buffers (see Materials and Methods for buffer compositions) were tested for the elution of GST/Lox6 bound to GA beads. The protein still bound after elution with buffer 2 was released by boiling the remaining beads in SDS/electrophoresis sample buffer and is shown in the last lane.



Fusion proteins solubilized in Sarkosyl alone bind poorly to GA beads, while those in a mixture of Sarkosyl and Triton X-100 bind more efficiently to this medium (Frangioni and Neel, 1993). To improve the binding of the solubilized fusion protein, I divided a sample of the cleared lysate into aliquots and added different concentrations of Triton X-100. I found that Triton X-100 at 0.5% was sufficient to produce maximum binding of the fusion protein to the beads (not shown). Next, I tested the amount of GA beads required for affinity purification of the fusion protein. A sample of the cleared lysate with Triton X-100 containing solubilized fusion protein was divided into several aliquots and challenged with different amounts of GA bead slurry. The bound fusion protein was eluted from the beads and analyzed by SDS-PAGE, together with the corresponding non-bound fractions. The amount of bound GST/Lox6 protein increased with the volume of GA bead slurry added, without reaching a plateau at the highest amounts of GA beads tried (Fig. 1B). This test allowed me to determine that efficient binding of the fusion proteins requires an amount of beads several times larger than recommended in published protocols (Smith 1993; Smith and Johnson 1988). This is probably due to reduced access of the larger fusion proteins, compared to non-fused GST, to binding sites in the agarose gel.

The final step in this optimization procedure was to test different elution buffers and conditions. Elution following the protocol originally described for this expression system often fails to release most of the bound protein, probably because the fusion protein has low solubility in the elution buffer, which would thus cause fusion protein precipitation inside the affinity chromatography gel. Because my goal was to raise antibodies against the purified fusion proteins, I tested several elution buffers that were compatible with the

direct immunization of animals by injecting the resulting solution of fusion protein without any further purification steps. Consequently, I avoided at this step high concentrations of non-dialyzable detergents such as Sarkosyl or Triton X-100, which would be toxic at the concentrations required. I found that the fusion proteins were poorly eluted with glutathione (elution buffer 1). The other three buffers released a significantly higher amount of fusion protein. Although these buffers use transient denaturing conditions that may inactivate the biological activity of the fusion proteins, they produce protein that is suitable for immunization. This test allowed me to select the most efficient elution conditions (elution buffer 2) for the three fusion proteins (Fig. 1C). The concentration of SDS (0.1% in elution buffer 2) can be decreased by dialysis after elution. However, because the GST/Lox6 protein precipitated during dialysis, I eliminated this step from the purification procedure. As expected, the low concentration of SDS present in elution buffer 2 was not toxic to the immunized animals.

After the optimal conditions for solubilization, binding, and elution were determined, I easily scaled up this procedure. I obtained about 10 mg of GST/Lox6 per liter of culture. I also applied successfully this purification approach to the fusion protein GST/Lox2 (Fig. 2), and Nicole C. Jordan applied it to GST/Lox4 purification with very similar yields.

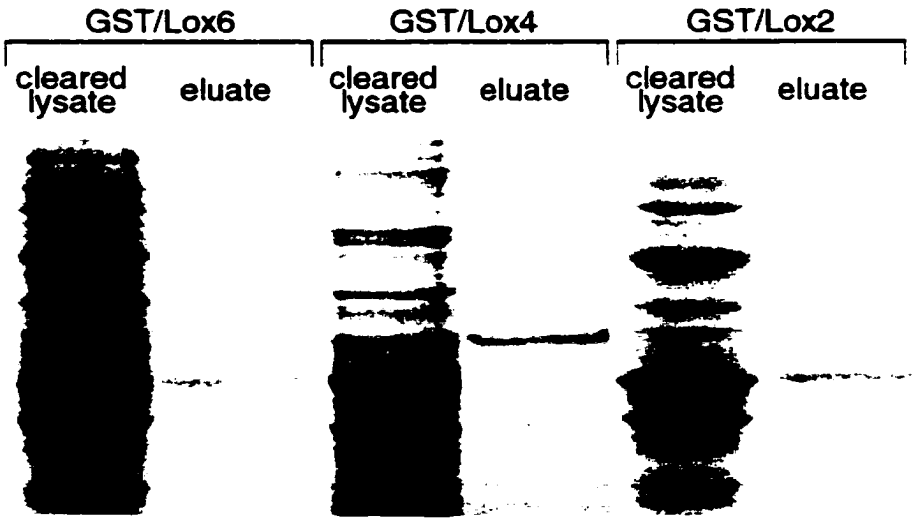


Figure 2. *Large-scale purification of GST fusion proteins.* The fusion proteins were purified from 1-liter cultures using optimized conditions for solubilization, binding to GA-beads, and elution. The mobility of each fusion protein matches its predicted molecular weight.

The fusion proteins were highly purified: They were the only bands detected with Coomassie Blue staining in the lanes containing purified GST/Lox2, though a minor contaminant was visible in the GST/Lox6 lane. In the absence of a specific assay for these proteins, the percentage of recovery of fusion protein from the lysate is difficult to measure and can be only roughly estimated to be over 50% from comparing the intensity of staining of the fusion protein band between equivalent aliquots of the cleared lysate, the eluate, and the various fractions that were discarded (see Fig. 1). These protein purification yields readily allowed us to prepare a sufficient amount of each fusion protein to immunize animals and make affinity chromatography columns containing fusion protein covalently linked to Sepharose for antibody purification. The specificity of these antibodies was tested in Western blots against total protein of bacteria transformed with the *Lox6* and *Lox2* constructs and against total protein from pGEX-2t transformed bacteria (Fig. 3A, B). Different aliquots acquired during the purification of the antibodies were tested. The purified *Lox6* antibody stained strongly a band of 56 kD that corresponds to GST/Lox6. The *Lox2* antibody stained strongly a band of 48 kD that corresponds to GST/Lox2 and very lightly a few smaller bands. Two of the *Lox2* antibodies (one from rat and one from rabbit) were successfully tested on embryonic tissue (Fig.4), producing the same pattern of expression as previously reported using *in situ* hybridization (Wysocka-Diller et al., 1989). Antibody staining of embryos with the *Lox6* antibody is described in the next Chapter.

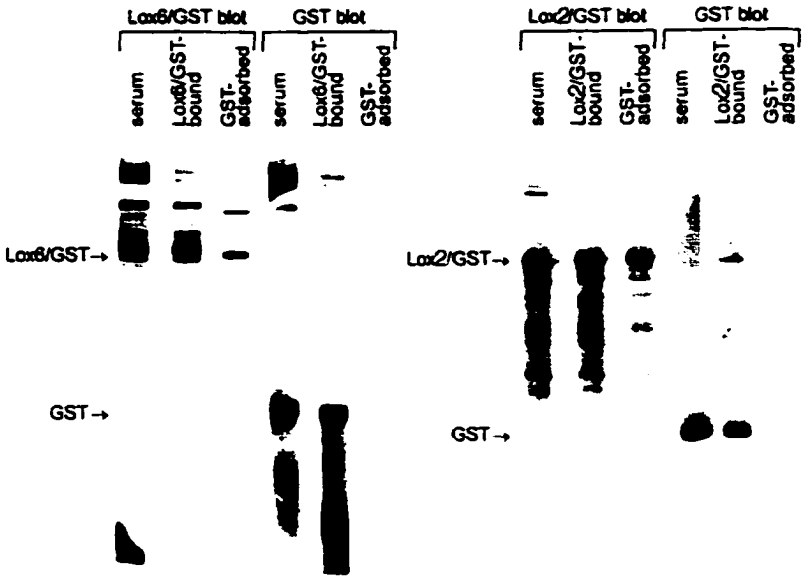


Figure 3. *A. Lox6 antibodies.* Aliquots from different steps of the antibody purification procedure were tested on Western blots. Purified *Lox6* antibodies showed the expected GST/Lox6 band of 56 kD and a band with higher molecular weight, bacterial chaperonin GroEL. *B. Lox2 antibodies.* Western blots showed the expected band of GST/Lox2 of 48 kD and a few minor bands stained by antibodies against *E. coli* proteins that contaminated the purified GST/Lox2 protein.

Figure 4. *Staining of leech embryos with antibodies raised against GST/Lox2.* Dorsal views of the central nervous system of early leech embryos were stained with antibodies against *Lox2*. As expected for these transcription factors, the staining for each Hox gene is restricted to the nuclei of a specific subpopulation of neurons. Scale bar 50 μm.

DISCUSSION

An optimization procedure for the purification of new GST fusion proteins is summarized in Figure 5. My results show that the application of the original isolation methods reported for these proteins (Smith and Johnson 1988; Smith 1993) often fails at either of three critical steps: protein solubilization, binding to GA beads, and elution. To overcome these problems, several simple tests can be carried out, allowing in most cases the efficient production of milligram quantities of purified fusion protein.

Eukaryotic proteins expressed in *E. coli* are often insoluble. This can be due, among other causes, to rapid overexpression of the protein under the control of strong bacterial promoters, improper folding of the nascent polypeptide in a more reducing intracellular environment, or to the absence of certain posttranslational modifications such as phosphorylation that may increase the overall polarity of the protein. The use of mixtures of ionic and nonionic detergents has been shown to solubilize these proteins without compromising their ability to bind glutathione, a GST ligand (Frangioni and Neel, 1993). The ionic detergent (Sarkosyl) is thought to help solubilize the protein by partially denaturing it; the subsequent addition of the nonionic detergent (Triton X-100) putatively sequesters the first detergent into its micelles (McNally et al., 1991) and allows the renaturation of at least the GST moiety of the fusion protein, thus enabling binding to GA beads. Refolding of transcription factors after solubilization with Sarkosyl from inclusion bodies has been achieved also by slowly diluting the detergent by dialysis (Nguyen, Jensen et al., 1993; Nguyen and Burgess 1996; Gentry and Burgess 1990; Ouhammouch et al., 1995).

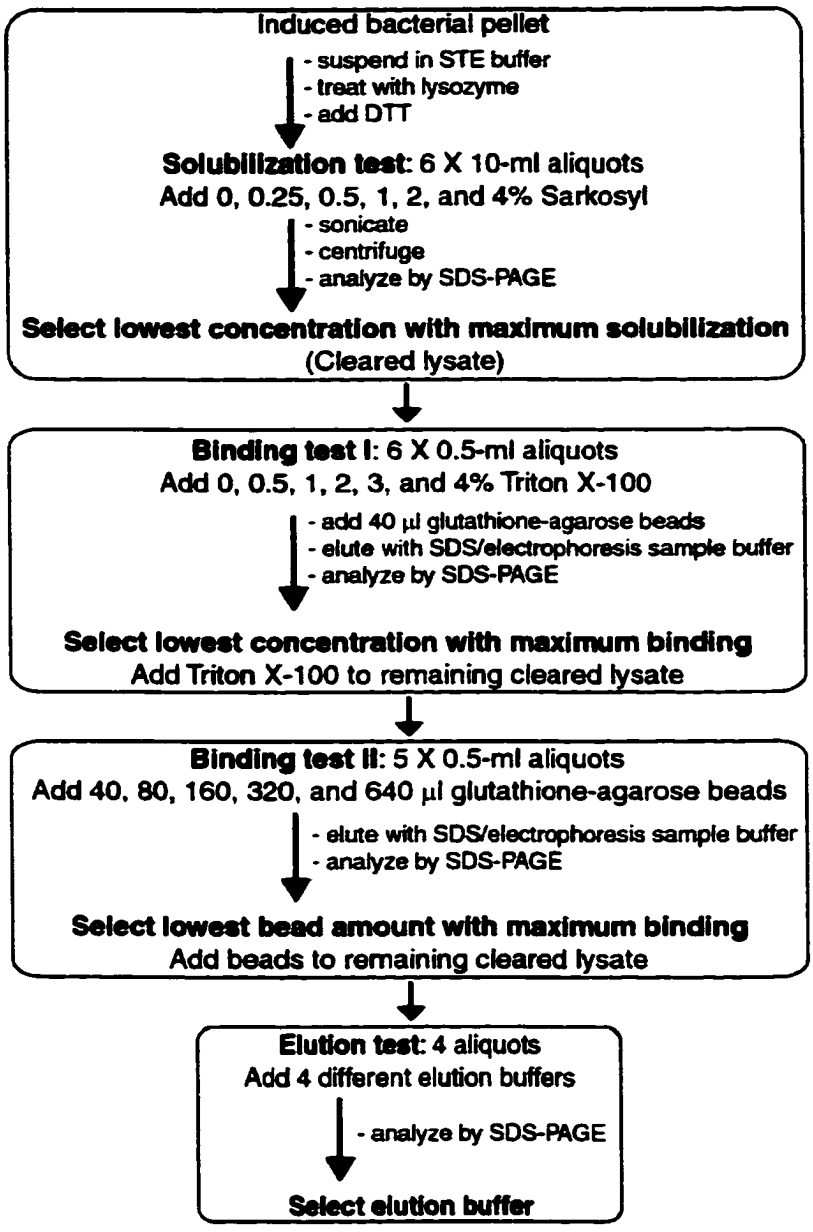


Figure 5. *Optimization procedure for GST fusion protein purification.* This protocol quickly tests different Sarkosyl and Triton X-100 concentrations for fusion protein solubilization, different amounts of GA beads for efficient binding, and different elution buffers.

The amount of GA beads used to bind GST fusion proteins is another critical factor influencing the yield of the purification. My results show that the binding capacity of the GA beads can be much lower with fusion proteins than with native GST. Consequently, failing to test for the amount of beads necessary for binding is likely to result in loss of a high percentage of the fusion protein at this step. This decrease in binding efficiency compared to the native GST protein is probably caused by reduced access of the bulkier fusion proteins to the binding sites on the GA beads.

Elution conditions are also crucial for high yield. Under conditions based on displacing the bound protein with excess glutathione, which efficiently elute non-fused GST, the GST/Lox6 fusion protein failed to be quantitatively recovered from the affinity medium. This could be due to either low solubility of the fusion protein or strong interaction with the affinity matrix. I have tested successfully three elution buffers that are compatible with the direct immunization of animals following elution and with the use of the purified protein for the affinity purification of the antibodies produced by the immunization. However, these elution conditions may not be compatible with the preservation of the activity of the protein for other studies.

When testing the specificity of the antibodies on Western blots, the *Lox6* antibody detected, in addition to the GST/Lox6 band of 56 kD, an additional, minor band of about 60 kD. A common problem in the purification of GST fusion proteins is the co-purification of *E. coli* GroEL (Thain et al., 1996). GroEL (chaperonin 60, Cpn60) is involved in protein folding (Xu and Sigler 1998; Sigler et al., 1998; Keskin et al., 2002) and makes a complex together with the GroES chaperonin that prevents the aggregation

of newly synthesized polypeptides, allowing their efficient folding in the bacterial cytosol (Sigler et al., 1998; Agashe and Hartl 2000; Gottesman and Hendrickson 2000). Co-purifying GroEL produced GroEL antibodies in rats. The GroEL antibodies could have been removed by adsorption with total protein from bacteria expressing non-fused GST. However, the specificity of the staining patterns observed in leech embryos (see next Chapter) suggested that this additional purification step was not necessary. In the case of *Lox2*, the Western blots do not show the GroEL band. This may be because the GST/Lox2 protein is more soluble than GST/Lox6. It is known that the GroEL/GroES complex associates with hydrophobic proteins (Lin et al., 1995; Ellis 2001). The *Lox2* blots also showed some minor, low molecular weight bands. These antibodies to contaminating *E. coli* proteins could have been removed by adsorption with a column containing total bacterial proteins, but this was considered unnecessary due to the high specificity that they showed on leech embryonic tissue. The low molecular weight bands detected in the Western blots stained with some of the *Lox2* antibodies could be due to minor contamination with bacterial proteins during purification of the *Lox2* fusion protein that was injected into the animals. These minor contaminants were probably more immunogenic in some animals. The antibodies raised against the two fusion Hox proteins were able to detect the endogenous, native protein in fixed tissue. The patterns of expression revealed by antibody staining were essentially the same as those previously reported using *in situ* hybridization (Wysocka-Diller et al., 1989; Wong and Macagno 1998).

CHAPTER III

LOX6 EXPRESSION DURING NERVOUS SYSTEM DEVELOPMENT

INTRODUCTION

Hox genes are a highly conserved family of transcription factors that control positional information during embryonic development. They specify regional identities along the anterior-posterior axis in all bilateral animals. Many studies carried out in vertebrates and invertebrates have shown that Hox genes also play an important role in the development of the nervous system. During neural development, many processes shape the structure and connectivity of the nervous system, including differential cell migration, axon outgrowth and navigation, and target recognition. The ability of neurons to make specific neuronal circuits, to migrate, and to have a pattern of axonal projections can be an effect of external molecular cues, or transcription factors that they acquire during the early stages of their differentiation, or both. The vertebrate nervous system is a perfect example in which migrations of neural crest cells take place to generate the peripheral nervous system (Douarin, 1980). Once these cells have arrived to their proper place, they begin to extend axons forming axonal pattern of connectivity necessary for the function of the nervous system (Goodman and Shatz, 1993). These axons navigate through complex environments to connect to their corresponding targets by responding to guidance cues supplied by the cells and the extra-cellular matrix. A major goal of developmental

neurobiology is to understand how these cues are provided and interpreted as well as how cells integrate information from multiple cues. Genetic studies in vertebrates indicate that Hox genes are involved in axial specification and patterning suggesting that a major site of their function is in the rhombomeres of the hindbrain and the spinal cord. For example, studies done in mice have shown that *Hoxa-2* is involved in the genetic control of motor neuron axon path finding in rhombomeres 2 and 3 (Gavalas et al., 1997). In the invertebrates, mutations of the *Drosophila lab* and *Dfd* Hox genes result in axonal patterning defects and lack of expression of neuronal markers in the neuromeres that these genes specify, showing that Hox genes are necessary for neuronal differentiation (Hirth et al., 1998).

Lox6 mRNA is expressed in the CNS and PNS at early stages of nervous system development, when gangliogenesis and axon pathfinding is taking place (Wong and Macagno, 1998). Based on the location and timing of *Lox6* expression, my hypothesis states that *Lox6*, a *Dfd* homolog, is expressed in neurons that extend pioneer axons, among other cell types. Therefore, blocking *Lox6* expression at early developmental stages should affect nervous system connectivity.

My results have revealed with more detail the pattern of expression of *Lox6* and allowed the identification of *Lox6* cells in the CNS, PNS, and body wall of the leech *Hirudo medicinalis*. *Lox6* is a leech ortholog of the *Drosophila* head gene *Dfd* that specifies the mandibulary and the anterior half of the maxillary neuromeres in the fly (Mahaffey et al., 1989). *Lox6* had been previously cloned and its pattern of expression had been determined by *in situ* hybridization (Wong and Macagno, 1998). These results revealed

the general pattern of *Lox6* expression during the development of the nervous system. To study the function of *Lox6* in nervous system development, it was necessary to identify cells that express *Lox6*. Thus, I could analyze the effects of blocking the expression of *Lox6* on single, identified cells. To find out the type of cells that express *Lox6*, I produced a polyclonal antibody (see Chapter II) to be used together with cell tracers and leech marker antibodies in double staining experiments. My studies showed that *Lox6* is expressed in two types of cells that are considered to be pioneers in axonal navigation. These cells are the bipolar neurons and the dorsal pressure sensory neuron (P_D). Other CNS and PNS cells were identified also as *Lox6*-expressing. Among the *Lox6* neurons are cells previously shown to interact, like the Retzius and P_D neurons (Fernandez-de-Miguel and Drapeau, 1995), neurons of sensillae 6-7 and the P_D neurons (Jellies et al., 1994), and bipolar neurons that are thought to establish the longitudinal connectives (Stewart et al., 1987). The expression of *Lox6* in these interacting partners at the time when the nervous system is developing suggests that *Lox6* may control molecules involved in axon path finding and synapse formation.

MATERIALS AND METHODS

Double antibody staining

Expression constructs, antibody production, and immunostaining were as described in Chapter II. The marker used for determining *Lox6* expression in the bipolar neurons was Laz1-1 (Stewart et al., 1987), diluted 1:4 from hybridoma culture medium. To find out if *Lox6* cells express netrin, I used an affinity purified polyclonal antibody to LNET-1 (Aisemberg et al., 2001), diluted 1:1000 from a 1 mg/ml stock. Lan3-2 monoclonal antibody was used to detect *Lox6* expression in sensillar cells. This antibody recognizes a surface glycoprotein involved in fascicle formation in a subgroup of peripheral neurons (McKay et al., 1983; Zipser et al., 1989; Peinado et al., 1987; Johansen et al., 1992). The dilution of this Lan3-2 monoclonal antibody was 1: 4 from hybridoma culture medium. The Laz1-1, Lan3-2, and Lan10-1 monoclonal antibodies were generous gifts of Dr. Birgit Zipser.

Intracellular injection

Cells were filled by ionthophoresis as described in Gao and Macagno (Gao and Macagno, 1987a). E9-E11 leech embryos were dissected and pinned down on Sylgard-lined Petri dishes containing Ringer solution with 6% ethanol. Before injecting the dye, the embryos

were changed to cold Wenning solution with 8% ethanol. P_D neurons were filled with 0.75 %, 1.5%, or 3% Lucifer Yellow dissolved in 0.1 M LiCl, or with 1.5% or 5% fluorescein-dextran (Molecular Probes) in 10mM Tris-HCl pH 8.0. The injections were carried out with a compound microscope using a 40X water immersion lens, and a 50 W mercury light source to visualize injected cells. The dye-filled electrode (glass filament, thin wall, 1.0 x 0.75 mm) was positioned over the embryo and grounded in the bath. The specific ganglion was found with a 10X lens and the electrode was positioned over the region of interest. The 40X lens was used to locate the P_D cell and the electrode was positioned on top of the cell. The resistance of the electrode was checked on the amplifier (a good resistance for this procedure is about 150 MΩ). The electrode was moved down, pressing the cell membrane. Then, the electrode was buzzed with the amplifier to penetrate the cell. Often, this was enough to deliver enough tracer into the cell. In those cases in which it was desirable to inject more dye, the electrode was moved deeper into the cell using the micromanipulator and pulses of 1-2 nA were applied. Embryos were fixed for 1hr in 4% paraformaldehyde in PBS at room temperature, rinsed 3-4 times with PBS, and left overnight at 4°C in PBS. The embryos were then processed for *Lox6* antibody staining using the method described in Chapter II.

RESULTS

Lox6 expression in the CNS

The expression of *Lox6* during embryonic development was studied using affinity-purified antibodies. *Lox6* is expressed in the CNS starting at E6, at the early stages of nervous system development. *Lox6* expression can be divided into early and late patterns. The early expression pattern begins at E6 in 5 or 6 pairs of cells at the posterior aspect of RN2 and in about 130 cells of RN3 (Fig. 1A). At this stage, *Lox6* is also expressed in the anterior midbody ganglia in about 80 cells. As ganglia form in more posterior segments, *Lox6* expression also extends caudally. The development of the leech proceeds in a rostral-caudal gradient and the anterior ganglia are more advanced than the posterior ones. At E8, *Lox6* expression reaches the most posterior neuromeres, including the last caudal neuromere (CN7). This early pattern ends at E9, when *Lox6* expression begins to decrease in the anterior midbody, while remaining high in RN2-RN4 (Fig.1B) and in fact increasing in caudal neuromeres. The late *Lox6* expression begins at E10, when *Lox6* is expressed in only about 4-5 pairs of cells in midbody and caudal neuromeres, at the same time as RN3 maintains a high number of *Lox6* cells (Fig.1C). As development continues, *Lox6* expression is stable in RN3 but diminishes in the other neuromeres, until it disappears from midbody and caudal neuromeres at about E17, the last stage examined.

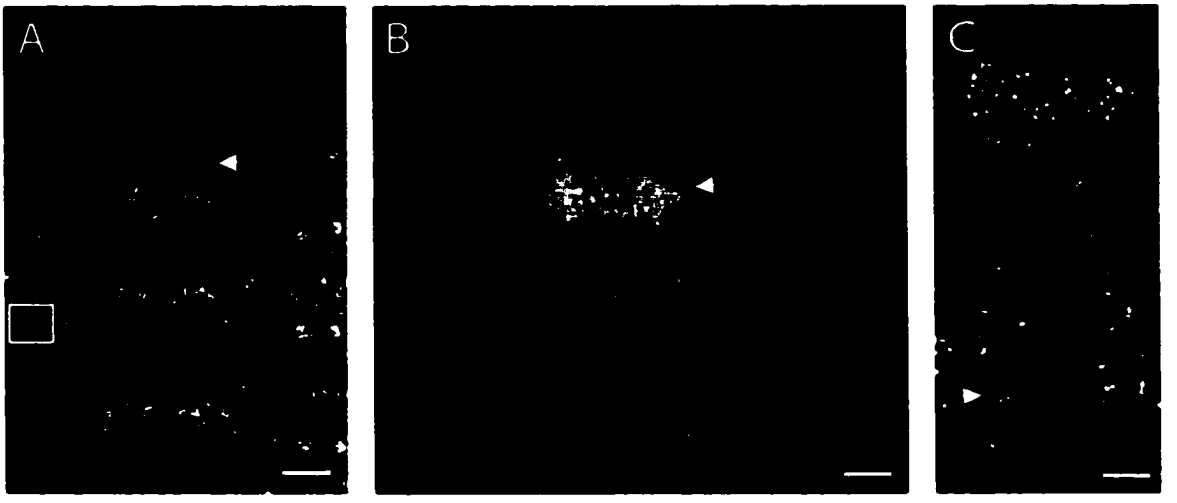


Figure 1. *A. Early pattern of* Lox6 *expression.* E7 embryo stained with *Lox6* antibody shows the anterior *Lox6* expression boundary at the posterior end of RN2 (arrowhead) in about 5 pairs of cells. *Lox6* expression is detected in the nuclei of the cells. Expression continues in the posterior segments and respective neuromeres and ganglia. *Lox6* is expressed in clusters of cells (square) and single cells in the body wall of the embryo. RN3 contains the highest number of *Lox6* cells. Scale bar 50 μm *B. End of early* Lox6 *expression.* The CNS and body wall of an E9 embryo show the end of early *Lox6* expression, when the highest number of *Lox6* expressing cells is at RN3 (arrowhead). *C. Late* Lox6 *expression.* An E12 embryo shows the late pattern of *Lox6* expression, when expression is restricted to RN3. RN4 contains few *Lox6* cells and the posterior ganglia have between four and eight pairs of *Lox6* cells (arrowhead). Scale bars for *B* and *C* 25 μm.

Identification of *Lox6*-expressing central neurons

The expression of *Lox6* in the developing CNS at a time when axonal growth is starting indicated that *Lox6* could be expressed in cells that have been suggested or shown to be pioneer neurons like the bipolar cells (Stewart et al., 1987) and the P_D cells (Kuwada 1985; Gan and Macagno 1995). Pioneer axons are the first axons that act in a searching manner by reading molecular cues in the environment, thus enabling follower axons that remain associated with them to grow and connect to their target (Lopresti et al., 1973) (Kuwada and Kramer, 1983). The Bipolar neurons are located at the posterior end of each ganglion and extend ipsilateral processes both anteriorly and posteriorly extending between three to five ganglia rostrally and between two to three ganglia caudally (Stewart et al., 1987). Their time of appearance in the central nervous system is between E6 and E9, and they go through apoptosis between E9 and E12. Due to their location and time of appearance in the nervous system, the bipolar neurons have been suggested to establish the longitudinal connective nerves and organize fascicles of axons within these nerves by providing cues for axons that grow after those of the bipolar neurons (Stewart et al., 1987). *In situ* hybridization and immunohistochemistry studies have shown that the bipolar neurons express netrin, a highly conserved axon guidance molecule (Gan et al., 1999; Aisemberg et al., 2001). Since *Lox6* is expressed during the same developmental stages as the bipolar neurons appear in the central nervous system, it was interesting to know whether they express *Lox6*. To determine whether bipolar neurons express *Lox6*, embryos of stages E7 to E9 were double stained with *Lox6* and the monoclonal antibody Laz1-1. Laz1-1 is a monoclonal antibody raised against adult nervous system extracts from the leech *Haemopis marmorata* that recognizes a cytoplasmic protein and is used

here as a marker for the bipolar neurons (Stewart et al. 1987). Double staining with *Lox6* and Laz1-1 antibodies confirmed that *Lox6* is expressed in the bipolar neurons (Fig. 2A). The anterior boundary of *Lox6* expression in this segmentally iterated pair of neurons is in RN3 (Fig. 2B). As development proceeds, *Lox6* expression in the bipolar neurons extends to about MG15 by early E8 and to the most posterior neuromeres thereafter. At E9, the bipolar neurons begin to die in the anterior ganglia, while they remain and express *Lox6* in the posterior ganglia (Fig. 2C). Before a bipolar neuron goes through programmed cell death, it stops expressing *Lox6*.

The dorsal pressure sensory neuron (P_D) is also a pioneer neuron that has been shown to be followed within its major field of innervation by axons of motor neurons like the annulus erector neurons (AE) and the anterior pagoda neurons (AP), and other sensory neurons like the touch sensory neurons (T_D) (Gan and Macagno 1995; reviewed in Wolszon 1995). The P_D is the first neuron to extend its axon to the periphery through the posterior nerve root (Kuwada, 1985), pioneering in this way the dorsal-posterior nerve (Jellies et al., 1996; Jellies et al., 1994). Previous studies done by *in situ* hybridization coupled with cell dye fills suggested that *Lox6* is expressed in the P_D neuron (Wong, 1997). To confirm that *Lox6* is expressed in the P_D neurons, embryos of stages E9-E12 were dissected and P_D neurons between MG1 and MG14 were injected with Lucifer yellow or fluorescein-dextran. These embryos were then fixed and processed for *Lox6* antibody staining. The results of these experiments corroborated that *Lox6* is expressed in the P_D neurons (Fig. 3A).

Figure 2. *A.* Lox6 *is expressed in the bipolar neurons.* Late E7 embryo stained with Laz1-1 (green) and *Lox6* (red) antibodies show expression of *Lox6* in the bipolar neurons, located at the posterior end of each ganglion. In this picture, bipolar neurons of RN3, RN4, and MG1 are visualized. Bipolar neurons have been suggested to pioneer other axons to establish the longitudinal connectives and extend their anterior axon through 3 to 5 ganglia and the posterior one through 2 to 3 ganglia. *B. Anterior boundary of* Lox6 *expression in the bipolar cells.* Double staining with *Lox6* (red) and netrin (green) antibodies in a late E7 embryo shows the RN3 anterior boundary of *Lox6* expression in the bipolar neurons (arrow). All the posterior bipolar neurons continue expressing *Lox6* and netrin during development until they begin programmed cell death. *C.* Lox6 *expression ends in bipolar neurons before apoptosis.* Double staining of *Lox6* and Laz1-1 (green) in an E9 embryo shows MG12 bipolar neurons going through programmed cell death after *Lox6* expression ended (arrow). Scale bars 25 μm

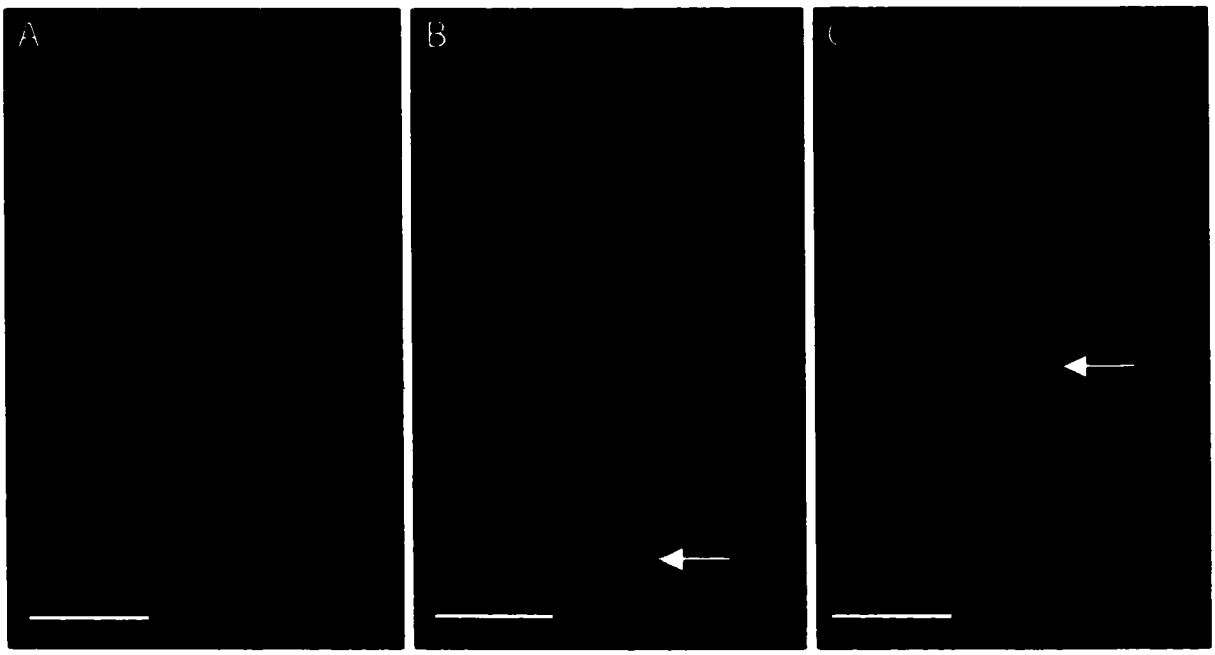
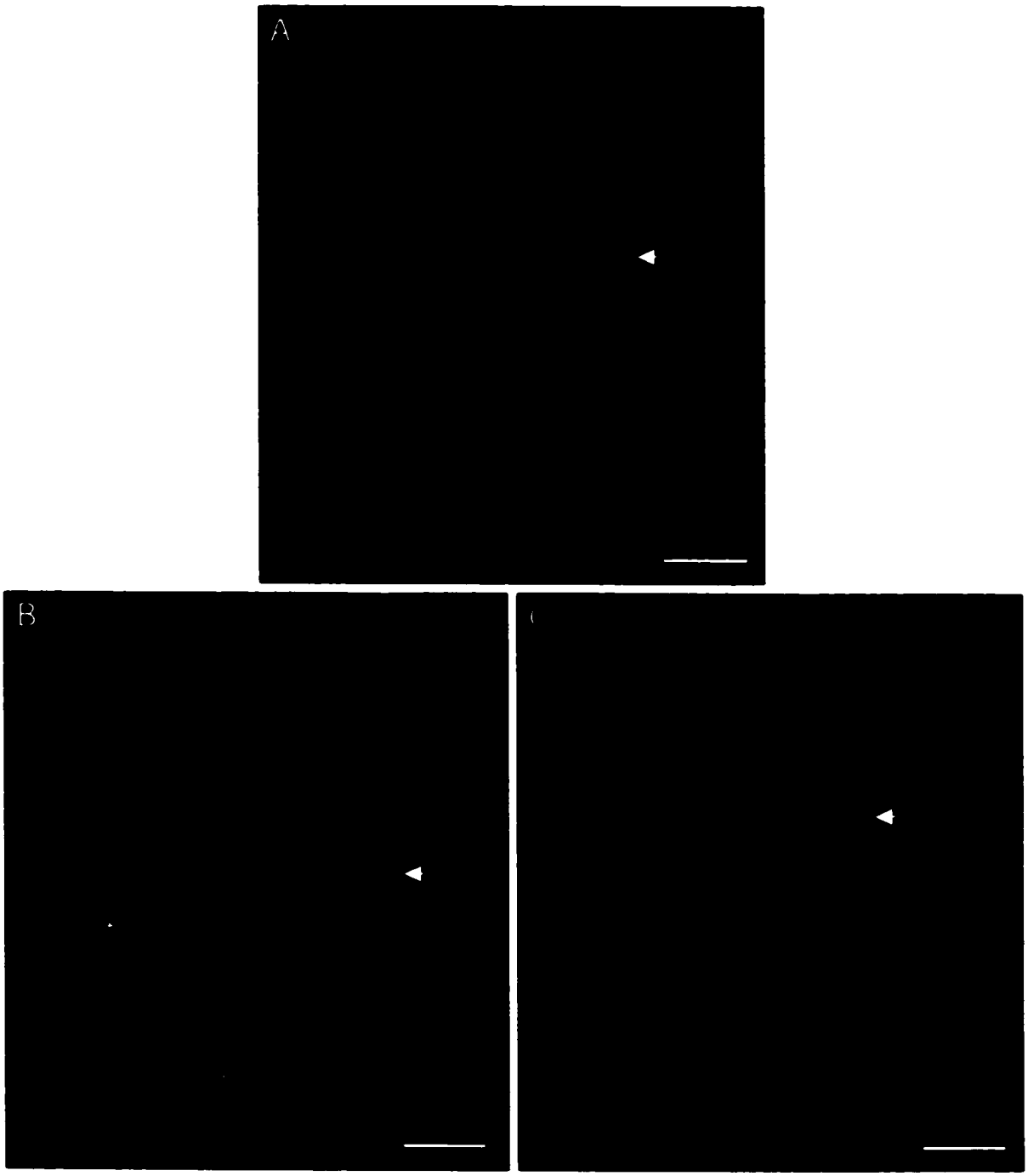


Figure 3. *A. The pioneer neuron of the posterior nerve root, P_D, expresses* Lox6. MG12 of an E9 embryo shows *Lox6* expression in one of the P_D neurons filled with fluorescein (green). The contralateral P_D neuron was not filled with the dye and also shows expression of *Lox6* (arrow). *B. Another netrin-producing cell, NE-C, expresses* Lox6. Double staining of a late E8 embryo with *Lox6* (red) and netrin (green) shows expression of *Lox6* in the left NE-C neuron in MG1 (arrow). The contralateral NE-C neuron did not express *Lox6*. Because of their location in the ganglia and their netrin expression, the NE-C neurons have been suggested to repel and attract central neuron axons to the periphery (Gan et al., 1999). In this embryo, the bipolar neurons of MG1 are going through apoptosis. *C. NE-C neurons of MG5.* MG5 shows expression of *Lox6* in the left NE-C neuron together with higher expression of netrin (arrow), while the right homolog has very low *Lox6* and netrin expression (same embryo as in panel *B*). Scale bars 25 μm.



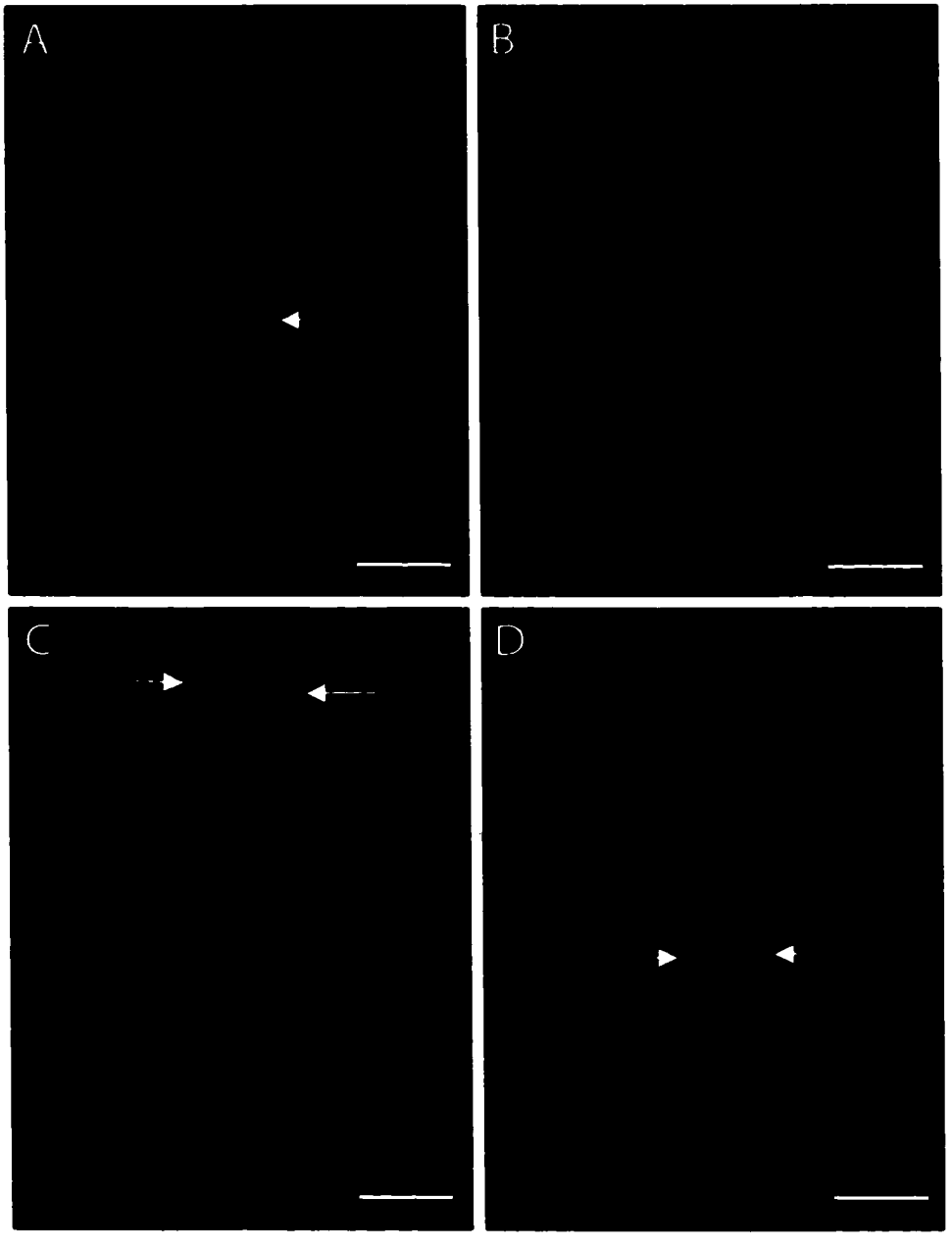
Netrins are guidance molecules that exist as diffusible (Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Placzek et al., 1990) and membrane-associated (Serafini et al., 1994) molecules. Netrins act as chemoattractants (Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996) or chemorepelents (Colamarino and Tessier-Lavigne, 1995) (Varela-Echavarria et al., 1997) for different types of axons, depending on the type of receptors with which they interact (Hedgecock et al., 1990; Chan et al., 1996; Leonardo et al., 1997). To identify other netrin sources (besides the bipolar neurons) that express *Lox6* during the time at which axons are navigating to connect to their targets, I used an antibody to leech netrin (Aisemberg et al., 2001) in conjunction with *Lox6* antibody to stain embryos of stages E7, E8, and E9. I found that at E9 *Lox6* is expressed in the segmentally iterated NE-C neuron. The NE-C neurons are located near the anterior nerve roots and they start to express netrin at E7. At later stages, these neurons become unpaired, alternating sides between segments (Gan et al., 1999; Aisemberg et al., 2001). Interestingly, the expression of *Lox6* in the NE-C neurons, observed at E9, was seen only in MG1-MG6 (Fig. 3B, C). *Lox6* expression was absent in one of the NE-C neurons in MG1 but netrin expression was sill present (Fig. 3C). Their location in the ganglia and their netrin expression indicate that they may be involved in attracting axons from the central neurons to extend to the periphery while preventing others from doing so.

The Retzius neurons and the P_D neurons are synaptic partners whose interactions in the leech CNS during development are well characterized. A specific, contact-dependent interaction of the Retzius neuron via synapse with the P_D neuron eliminates extrasynaptic response to serotonin on the P_D neuron (Merz and Drapeau 1992; Drapeau and Sanchez-Armass 1988; Drapeau et al., 1989). Cell surface glycoproteins of the Retzius neuron

mediate these specific interactions with the P_D neuron during synapse formation (Merz and Drapeau, 1994). These studies support the idea that appropriate synaptic connections during development of the nervous system require the recognition of specific molecules during selection of synaptic partners. Since the P_D neurons express *Lox6* at the time when these synapses are forming, I wanted to find out whether *Lox6* is also expressed in the Retzius neuron. E7 embryos stained with *Lox6* and serotonin antibodies showed that *Lox6* is expressed in Retzius neurons at E7 and at E8 (Fig. 4A, B), but only in RN3 and RN4 (not in midbody ganglia). At E9, *Lox6* ceased to be expressed in the Retzius neurons. These experiments allowed me to identify two other *Lox6*-expressing serotonergic neurons in RN3 and RN4: the anterior-medial serotonergic (AMS) and the posterior-medial serotonergic (PMS) neurons (Fig. 4C, D). The highest level of *Lox6* expression in these cells was found at E7-E8.

I tried to identify more neurons that express *Lox6* in the CNS using FMRFamide antibodies, which are known to stain a specific subpopulation of neurons in the adult leech (Evans and Calabrese, 1989; Norris and Calabrese, 1987). Norris and Calabrese (1987) reported excitatory and inhibitory motor neurons that express FMRFamide-like peptides on the dorsal side of the anterior and medial parts of midbody ganglia. Among the FMRFamide excitatory motor neurons in the anterior-lateral region of the ganglion are neurons 106, 107, 108, and 109. I observed that one of these neurons expresses *Lox6* only in RN3 at E8 and E9 (Fig. 5A), but because its location is too close to the other motor neurons, I was not able to identify which one of these was expressing *Lox6*.

Figure 4. *A.* Lox6 *is expressed in the Retzius neurons of RN3 and RN4.* E7 embryo stained with serotonin (green) and *Lox6* (red) shows expression of *Lox6* in the Retzius neurons of RN3 (arrow). *B. Axon morphology of Retzius cells at E7.* A different focal plane of the same Retzius neurons of panel *A* shows their axon morphology. The branches that leave through the segmental roots have not reached the periphery yet at E7. *C. AMS neurons express* Lox6. An E8 embryo shows expression of *Lox6* in the AMS neurons of RN3 (arrows). *D. PMS neurons express Lox6.* PMS neurons of RN3 show co-expression of *Lox6* and serotonin at E8. AMS and PMS express *Lox6* in RN3 and RN4 at E7 and E8 (arrows). Scale bars 25 μm.



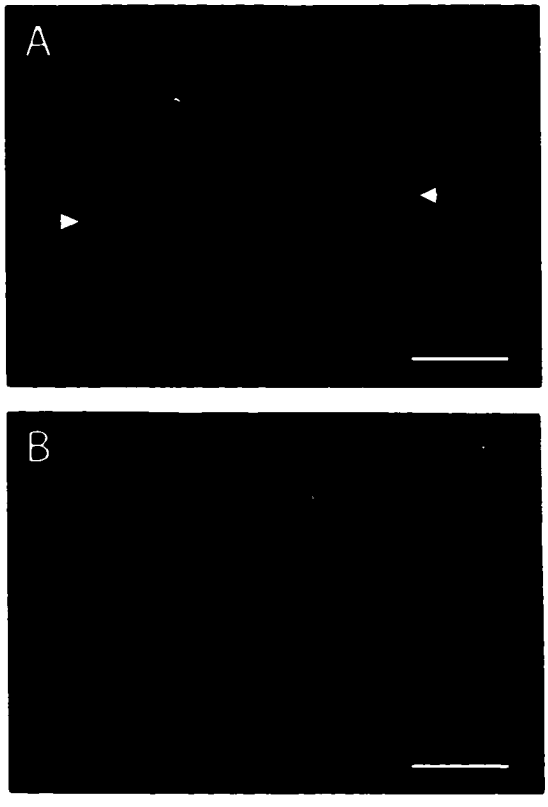


Figure 5. *A. A putative FMRFamide excitatory motor neuron expresses* Lox6 *only in RN3. A.* The RN3 of an E9 embryo stained with FMRFamide (green) and *Lox6* (red) shows expression of *Lox6* in a putative excitatory motor neuron (arrows). *B. RN3 contains the highest number of cells co-expressing* Lox6 *and FMRFamide.* A dorsal view of RN3 in an E9 embryo shows between several cells displaying double staining. Scale bars 25 μm.

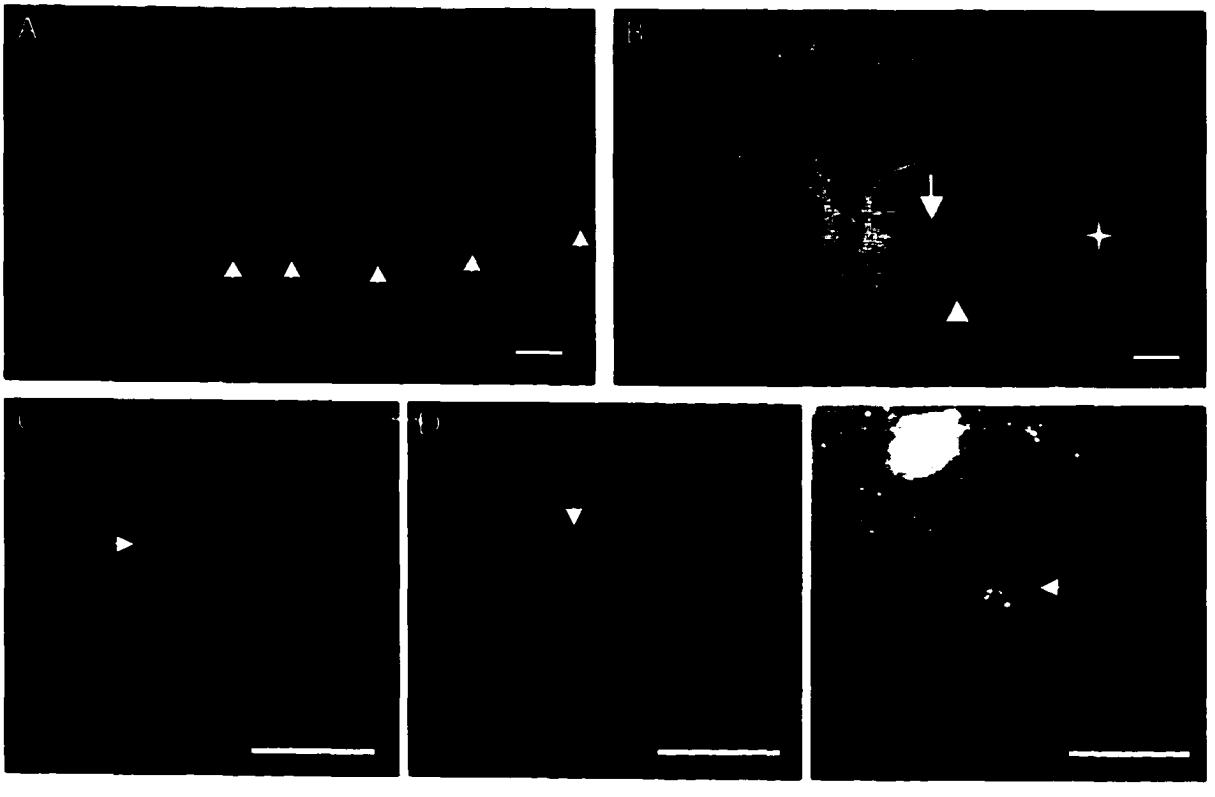
However, the expression of *Lox6* in this cell was not observed in other segments. The number of cells expressing both antigens, FMRFamide and *Lox6*, ranged between 12 and 16 in RN3 (Fig. 5B), and between 5 and 6 in RN4 and midbody ganglia.

Lox6 expression in sensillar cells

In the PNS, *Lox6* is expressed in cells of the peripheral sensory organs called sensillae (Fig. 6A). There are seven bilateral pairs of sensillae (named S1 to S7 from ventral to dorsal), located in the middle annulus of each midbody segment (Muller et al., 1981) (Johansen et al., 1992). The sensillae contain different types of sensory neurons, including photoreceptors, chemoreceptors, and mechanoreceptors (Phillips and Friesen, 1982). Sensillar neurons extend their axons to connect to the CNS through the anterior nerve root from S1-S5 and through the posterior nerve root from S6-S7 (Johansen et al., 1992). However, I observed in early leech embryos that this pattern of connections is different in the rostral segments. E9 embryos stained with Laz1-1 and *Lox6* antibodies showed that S7 connects to the CNS through the anterior nerve root in segment R3, together with eyes #3 and #4 (Fig. 6B). There was no evidence in R3 of S6 *per se* or of its connection through the anterior or posterior nerve roots. In addition, S1-S5 connected to the CNS in this segment through the posterior root. In segment R4, the pattern of connections is reversed: S1-S5 connected through the anterior nerve root, while S7 and eye #5 connected through the posterior nerve root. There was no S6 connecting to RN4; it

seems instead that eyes #4 and #5 in segments R3 and R4 replace S6, respectively. To determine *Lox6* expression in the sensillae, I stained E8 embryos with *Lox6* and Laz1-1 antibodies. My results show that *Lox6* is expressed in sensillar neurons, of which only one or two in each sensilla expressed both *Lox6* and the Laz1-1 antigen. At E14, *Lox6* expression is still detected in the sensillae. Staining of E12 embryos with Lan3-2 and *Lox6* antibodies revealed that S6 has the highest number of *Lox6*-expressing cells at that stage (Fig. 6C, D). *Lox6* was also expressed in the lip sensillae (Fig. 6E).

Figure 6. *A.* Lox6 *is expressed in sensillar neurons.* An E8 embryo stained with *Lox6* (red) and Laz1-1 (green) antibodies shows expression of *Lox6* in the sensillar neurons (arrows). Single cells expressing *Lox6* are detected in the body wall. Scale bar 25 μm. *B. Eye and sensillar pattern of connection to the CNS.* Normally, sensillae 1-5 (S1-S5) connect through the anterior root, while S6 and S7 connect through the posterior root. An E9 embryo stained with *Lox6* (red) and Laz1-1 (green) antibodies shows that in RN4, S1-S5 do not change the midbody pattern of connection through the anterior root (white arrowhead), but eye #5 (instead of S6, which is missing in this segment) connects through the posterior root (blue arrowhead), together with S7. RN3 has a different pattern of connections: S1-S5 connect through the posterior root and S7 connects through the anterior root, together with eyes #4 (white star) and #3 (blue star). The pattern of connection as well as the location, and number of sensillae is highly divergent for RN1 and RN2. Nerve DD (blue arrowhead) connects eye #5 to RN4; nerve DC (white arrow), which has two branches at its distal end, connects eye #4 (DCP branch) and eye #3 (DCA branch) to RN3; nerve DB (blue arrow) connects eye #2; nerve DA (green arrow) connects eye #1 (Kretz et al., 1976). Nerves DA and DB enter the CNS through the supraesophageal ganglion. Scale bar 50 μm. *C. Sensilla 6 has the highest number of* Lox6 *cells.* An E12 embryo stained with *Lox6* and Lan3-2 antibodies shows the highest number of *Lox6* expressing cells in S6 (arrow), compared with the other sensillae. Scale bar 25 μm. *D.* Lox6 *in sensilla 7.* S7 of the same embryo shown in panel *C* shows only 2-3 *Lox6* expressing cells (arrow). Scale bar 25 μm. *E. Lox6 is expressed in the lip sensillae.* An E14 embryo stained with *Lox6* shows expression of *Lox6* in the lip sensillae. A lip sensilla between eye #1 and eye # 2 shows 11 *Lox6* cells (arrow). Scale bar 25 μm.



Putative extra-sensillar sensory neurons express *Lox6*

I found additional *Lox6*-expressing cells in the four annuli of each segment that do not contain sensillae. The five annuli of each midbody segment are named, from anterior to posterior, a1, a2, m, p1, and p2 (Stewart et al., 1985). Several extrasensillar sensory neurons have been identified in a1, a2, p1, and p2 (Johansen et al., 1992; Briggs et al., 1993). To find out whether *Lox6* is expressed in these extrasensillar neurons, I stained E9-E12 embryos with Lan3-2 and *Lox6* antibodies. Lan3-2 is a monoclonal antibody that recognizes a surface glycoprotein involved in fascicle formation (McKay et al., 1983; Johansen et al., 1985; Peinado et al., 1987; Zipser et al., 1989). Lan3-2 labels all afferent axons, including those of extrasensillar neurons (Johansen et al., 1992; Briggs et al., 1993). E12 embryos stained with *Lox6* and Lan3-2 antibodies showed *Lox6* expression in one neuron of a cluster of three in the p1 annulus (Fig. 7A). At E14, the *Lox6* antibody stained a row of single cells in the p1 annulus and a cluster of two or three cells in the p2 annulus (Fig. 7B). These cells seemed to extend their afferent axons following the pathways of the sensillar axons. The late appearance of a similar group of extrasensillar neurons whose precise function is unknown has been described in *Macrobdella decora* (Briggs et al., 1993) and *Haemopis marmorata* (Stewart et al., 1985).

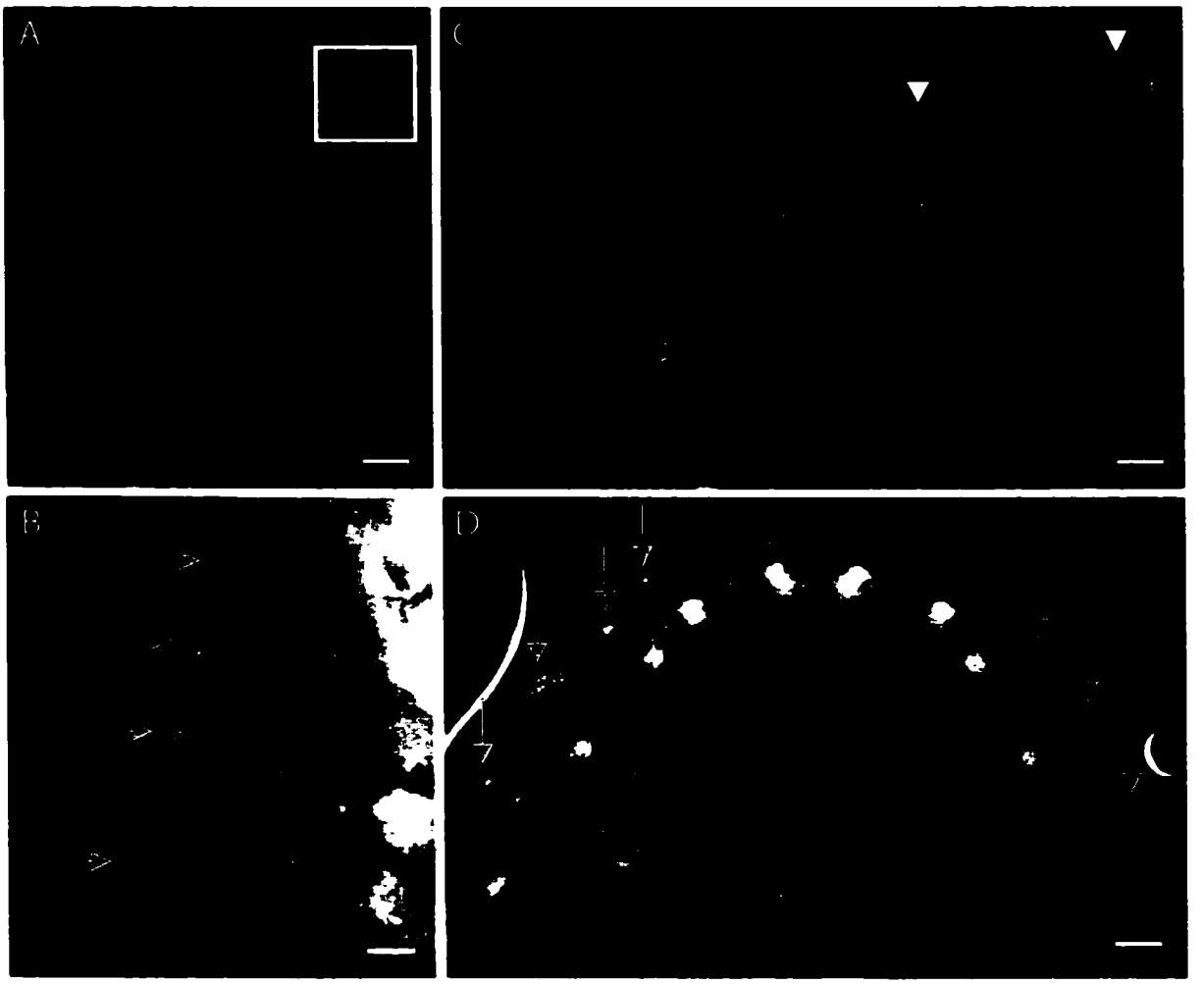
Figure 7. ***A. Extrasensillar neurons express*** **Lox6. High magnification image of an E12 embryo stained with Lan3-2 (green) and *Lox6* (red) antibodies shows expression in one of the extrasensillar clusters of sensory neurons located in M15. *B. Extrasensillar neurons of different annuli express* Lox6. E14 embryo shows *Lox6* expression in the p1 annulus in single cells (arrows) and in the p2 annulus in groups of three sensory neurons (arrowheads). The p1 and p2 annuli are posterior to the m annulus that contains the sensillae (anterior is down). Scale bars 25 μm.**

Lox6 expression in the five pairs of eyes

The leech *Hirudo medicinalis* has five pairs of eyes on the dorsal surface of the head, arranged on the anterior segments. They project their axons to the CNS via the four optic nerves. Each eye contains 30-50 photoreceptor cells that extend their axons into the center of the eye cup, where they fasciculate and leave the eye, forming the optic nerves (Kretz et al., 1976). Eye pair #1 connects through nerves DA, eye pair #2 through nerves DB, eye pairs #3 and #4 through nerves DC, and eye pair #5 through nerves DD. Nerves DA and DB enter the CNS through the supraesophageal ganglion; nerves DC and DD connect to the subesophageal neuromeres of segments R3 and R4 (Fig. 6B).

Lox6 is expressed in the five pairs of eyes. Eye expression started at E7, in a few cells of eyes #4 and #5 of R3 and R4 (Fig. 8A). At late E8, *Lox6* expression was detected in eyes #2-#5 (Fig. 8B). Eye development in the rostral segments seems to follow a temporal gradient that progresses from posterior to anterior (the opposite is true for general development and for *Lox6* expression in midbody and caudal regions). Only a few cells of eyes #4-#5 express *Lox6* at E7, later (at E8) a few cells in eyes #2-#3 and more cells in eyes #4-#5 express *Lox6*, and finally the five pairs of eyes express *Lox6* by E9. At E12, anterior eyes have more *Lox6* cells than the posterior ones (Fig. 8C). At E17, the higher number of *Lox6* cells in the anterior eyes is more noticeable (Fig.8D). The number of eye cells that express *Lox6* at E9 ranges between 23 and 40. This number, taken together with their location within the eye, suggests that these *Lox6* cells are photoreceptors.

Figure 8. *A. Early expression of* Lox6 *is first detected in a few cells of eyes #4 and #5.* Eye #4 (white square) and eye #5 (blue square) of a late E7 embryo stained with α-acetylated tubulin and *Lox6* antibodies shows four *Lox6* expressing cells, while the other eyes have not started to express *Lox6* yet. Scale bar 25 μm. *B. Development of the eyes and their* Lox6 *expression occur in a caudal-rostral order.* Late E8 embryo shows the order of eye development from posterior to anterior (arrows) and *Lox6* expression (anterior is up). Eyes #5, #4, #3, and #2 are visualized in this embryo. Eye #2 is beginning to express *Lox6* with two *Lox6* expressing cells, while the posterior eyes are ahead in number of *Lox6* expressing cells. An exception is eye #5, which has been reported to be the smallest eye in the adult leech (Kretz et al., 1976). Scale bar 50 μm. *C. The number of* Lox6 *expressing cell in the eyes increases at later stages.* An E12 embryo stained with Laz1-1 and *Lox6* antibodies shows an increased number of *Lox6* cells in eye pair #1 (arrowheads), left eye #2 and left eye #3. Scale bar 25 μm. *D. Eye* Lox6 *expression continues at E17.* An E17 embryo shows strong *Lox6* expression in the five pairs of eyes. The decreasing size of the eyes from anterior to posterior is clearly visible at this stage (anterior is up). Expression of *Lox6* in the lip sensillae is still present at E17 (arrows). Scale bar 50 μm.

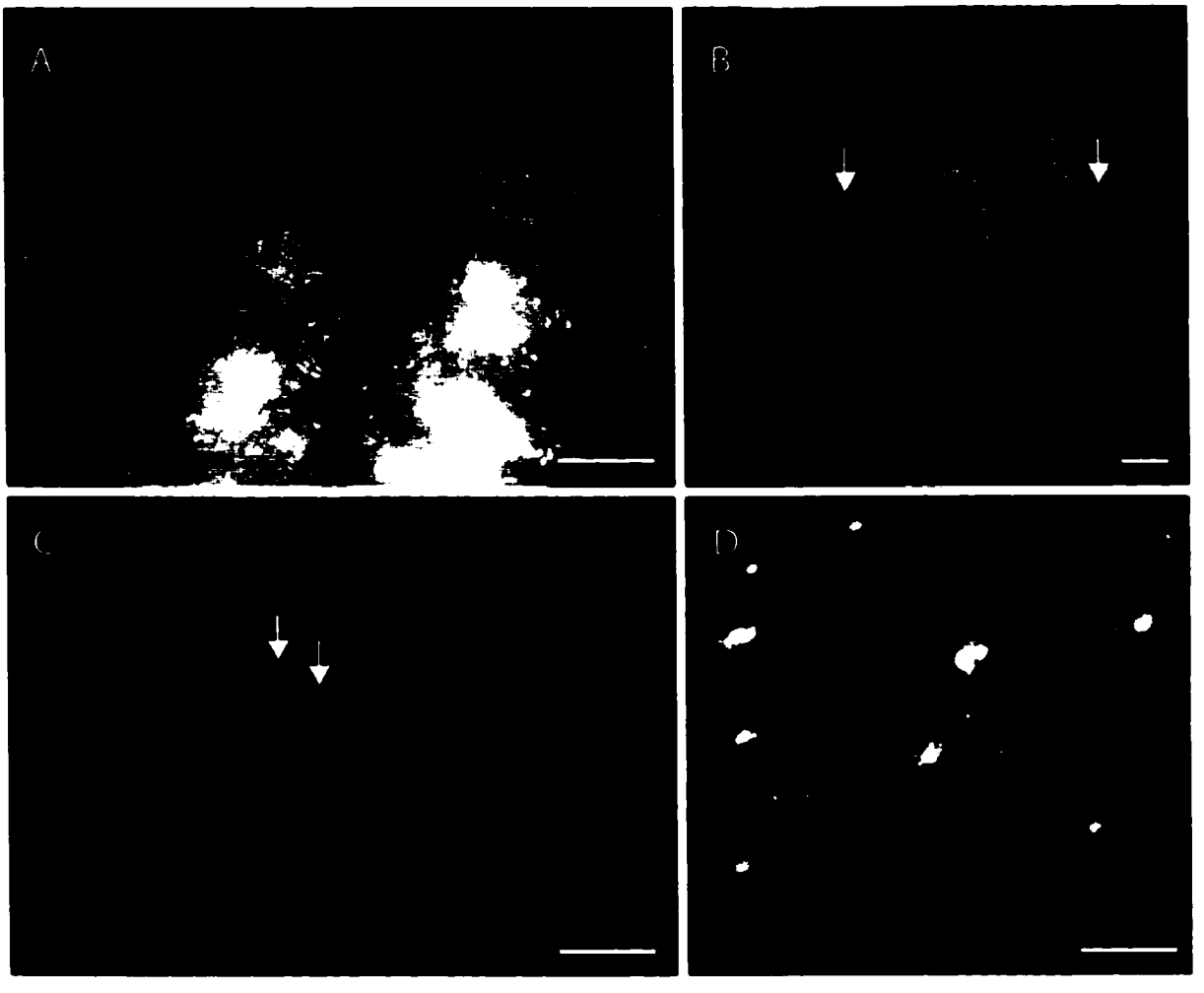


Lox6 is expressed in circular and longitudinal muscle

Development of muscles in the leech is accomplished during growth and expansion of the germinal plate (Stent et al., 1992). Three layers of muscle appear during development: first circular muscle (outer layer), second longitudinal muscle (inner layer), and third oblique muscle that form an intermediate layer between circular and longitudinal muscle (Stent et al., 1992). To find out if *Lox6* cells present in the body wall were muscle cells, embryos were double stained with *Lox6* and Laz10-1 antibodies. Laz10-1 is a marker monoclonal antibody that recognizes all muscle cells in the germinal plate of the leech embryo (Thorey and Zipser, 1991). My results showed that *Lox6* is expressed in longitudinal and circular muscle at early stages of development (E5-E9). At late E5, four longitudinal muscle cells located on the ventral midline of the embryo (where the CNS is starting to form) are the first ones to express *Lox6* (Fig. 9A). At this stage of development these muscles are the only structures connecting the ganglia. Later, pioneer axons of the longitudinal connective nerves follow the two lateral muscle fibers, and other axons follow the medial pair of muscle fibers, forming Faivre's nerve (reviewed by Stent et al., 1992). The Bipolar cells have been suggested to pioneer axons of the longitudinal connectives by reading guidance cues in these muscles. At E6-E7, *Lox6* expression is detected also in muscle cells beginning in the anterior end of R4 (Fig. 9B, C) and extending later to the posterior segments. *Lox6* expression in muscle cells diminishes at E9, remaining only in anterior segments. At E10, *Lox6* expression in muscle completely ceased. The *Lox6*-expressing longitudinal muscle cells located at the ventral surface of each ganglion also express netrin (Aisemberg et al., 2001); see also Fig. 9C). *Lox6* is also expressed in muscle cells of the cryptolarval membrane (Fig. 9D).

I also detected *Lox6* expression in cells located at the exit point of the anterior root of every ganglion (Fig.10). Based on their location, these may be root glial cells. There are four types of glial cells in the leech CNS (Elliot and Muller, 1981). These are the microglia of the ganglia, the neuropil and packet macroglia of the ganglia, the root glia, and the connective glia. A better characterization of these *Lox6*-expressing cells will be necessary to confirm their glial identity.

Figure 9. *A.* Lox6 *is expressed in the midline muscle cells.* An E5 embryo shows expression of *Lox6* in the first two pairs of longitudinal muscle cells located at the midline of the germinal plate (square), where the CNS develops. *B.* Lox6 *is present in longitudinal muscle of the body wall.* An E7 embryo stained with *Lox6* (red nuclei) and Laz10-1 (green vertical strips) antibodies reveals *Lox6* expression in the longitudinal muscle layer. The anterior boundary of *Lox6* expression in longitudinal muscle cells begins at the anterior end of R4 (arrow) and extends to the last posterior segment. Scale bar 50 μm. *C. Netrin-producing longitudinal muscle cells express* Lox6. An E7 embryo stained with *Lox6* (red nuclei) and netrin (green) antibodies shows that the ventral longitudinal muscle cells co-express *Lox6* and netrin (arrows). The anterior boundary of *Lox6* expression can be seen between RN3 and RN4. *D. Muscle cells of the cryptolarval membrane express* Lox6. Muscle cells (vertical and horizontal strips) of the cryptolarval membrane express *Lox6* (white spots) in an early E7 embryo. Scale bars for *A*, *C*, and *D* 25μm.



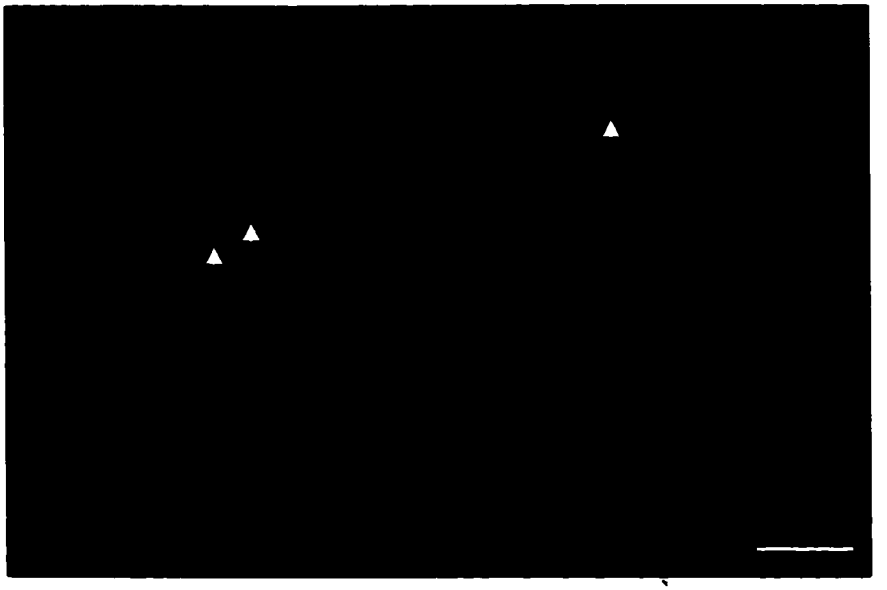


Figure 10. *Putative root glial cells express* Lox6. An E9 embryo stained with *Lox6* and serotonin (green) antibodies shows *Lox6* expression in the putative anterior root glial cells, located in a relatively straight line at the anterior root of MG11, at the position where axons leave the CNS to connect to the periphery. The anterior-lateral axonal branch of a Retzius neuron (pink arrow) appears to follow this string of glial cells. Scale bar 25 μm.

DISCUSSION

I have described in this chapter *Lox6* expression in the CNS, the PNS, and the body wall of the leech *Hirudo medicinalis*, at stages when important developmental processes are taking place in the nervous system. My results show an early *Lox6* expression pattern at E6-E9, during which time a long expression domain begins at the posterior aspect of RN2 and ends at CN7, followed by a late expression pattern at E10-E17 (last stage examined), which is restricted to RN3. The early *Lox6* expression pattern is dynamic, following the rostral-caudal developmental gradient of the leech embryo. During this time, the ganglia form, neurons differentiate, and axons start to grow. A probable role for early *Lox6* expression could be to control the neuronal expression of some of the cell-autonomous, cell surface, and extracellular factors required for axonal growth, guidance, or target recognition. It is possible that even those neurons that do not express *Lox6* need certain molecules in the environment that are normally present as a result of specific gene regulation by *Lox6* in other cells to extend axons into the right pathway and to select and connect to their targets. Thus, the entire pattern of connectivity in the nervous system of the leech embryo may be affected if *Lox6* is not present at the right time and place during nervous system development. In *Drosophila*, null mutants of *Dfd* have defects in the axonal patterning of the mandibular neuromere due to both cell-autonomous and non-autonomous effects (Hirth et al., 1998). In flies, *Dfd*$^-$ cells do not extend their axons and the axons of the wild type neurons do not enter the mutant domain. Since in *Drosophila* *Dfd* is expressed in a specific neuromere, the effect of the mutation is restricted to that neuromere. In the leech, the early *Lox6* expression takes place in the entire germinal

plate. Although the early expression of *Lox6* in the CNS begins at RN2, *Lox6* is expressed anterior to R2 in the PNS (in sensillae, extrasensillar neurons, lip sensillae, and eyes) and in muscle. Therefore, *Lox6* could affect patterning in the entire nervous system of the leech during early development.

One of the features of early patterning in the nervous system is that axons have to read molecular cues in the environment to find their pathways and form the right connections. There are several axon guidance mechanisms; one is guidance by pioneer axons that are the leaders of a group of fasciculated axons (Kolodkin et al., 1992). My results showed that *Lox6* is expressed in the mechanosensory P_D neurons, which have been shown to extend a pioneer axon into the periphery, thus forming the dorsal-posterior nerve (Kuwada 1985; Jellies et al., 1996; Jellies et al., 1994), and guide other axons with its peripheral branches, allowing them to connect to their targets (Gan and Macagno, 1995). In addition, *Lox6* is expressed during early development in the bipolar neurons, which have been suggested to be a pioneer for the formation of longitudinal connective nerves or for a group of axons in the longitudinal connectives. *Lox6* is also expressed during early embryonic development in muscle cells. In other organisms, muscle cells have been shown to provide molecular cues to motor neurons for axon pathfinding and target selection. They participate in early axon guidance by providing defasciculation signals and targeted growth, allowing motor axons to select their targets in the muscle fields (Landgraf et al., 1999). In other words, molecules expressed on muscle cells trigger the initial formation of entire nerve branches. *Lox6* expression at a critical time of development in pioneer CNS cells, in cell pairs known to interact early in development like the P_D and Retzius neurons, in peripheral sensory cells, and in differentiating muscle

cells suggests that an early *Lox6* function is to regulate the expression of molecules involved in axon pathfinding, target selection, or synapse formation.

Late *Lox6* expression becomes restricted to RN3, which is similar to the regionalized expression of *Dfd* in mandibulary and maxillary neuromeres of *Drosophila* during CNS development. A function of late *Lox6* expression may be to maintain a specialized identity of RN3 neurons, necessary for the connections to and from the peripheral organs that express *Lox6*. My results show that the connections of R3 sensillae are different from those of the sensillae of the other rostral and midbody segments. In the R3 segment, sensillae S1-S5 connect to RN3 through the posterior root, while S7 and eye pairs #3 and #4 connect through the anterior root and no S6 is present (Fig. 6b). These atypical connections to RN3 indicate that some *Lox6*-expressing neurons of RN3 may have a different axonal morphology or other specialized properties, compared to their segmental homologs in the other rostral and midbody neuromeres. It has been shown that the expression of the leech Hox gene *Lox4* correlates with three regionally divergent axonal morphologies of the segment-specific RPE motor neurons of MG6 and their segmental homologs of other segments, the RMV neurons (Wong et al., 1995). Hence, those RMV neurons that normally express *Lox4* in a continuous manner have an axonal morphology that is different from the one of the RPE neurons, which express *Lox4* transiently, and that is also different from a third axonal morphology found in the most anterior RMV neurons, which never express *Lox4*. The *Lox4* results support my hypothesis that late, stable *Lox6* expression in RN3 neurons may give rise to segment-specific identities. As a result, the segment-specific identities of RN3 neurons can be envisioned to maintain specialized connections to and from *Lox6*-expressing peripheral neurons of segment R3

through the segmental nerves or through the other cephalic nerves via the anterior longitudinal connective nerves. The late or stable expression of *Lox6* may be necessary for new cells (sensory neurons and muscle cells) produced in the periphery that need to extend axons to connect to the CNS or be contacted by efferent axons from central neurons.

In the leech, the primary blast cells that found the individual segments already possess segment identity at the time of their birth (Martindale and Shankland 1990; Gleizer and Stent 1993), before the Hox genes are expressed in the germinal plate (Nardelli-Haefliger and Shankland, 1992). This means that the initiation of segment identity in the leech does not require the Hox genes (Kourakis et al., 1997). It has been proposed that Hox genes would not play a role in early regional specification in the embryos of organisms like the leech, where cell lineage plays an important role in spatial organization, though they might exercise then other functions, and that only later in development would these genes be necessary for regional control functions (Davidson, 1991). All the Hox genes whose expression has been characterized in the leech have an early expression pattern and a late or extended expression pattern. The common feature of the early expression of these *Lox* genes is that it begins when the nervous system is starting its development (E5-E7) and ends around the time when gangliogenesis ends (E9-E10). Then there is a transition period in which the *Lox* expression declines. Finally, the extended or late expression continues, in some cases detected even in adult stages. For example, *Lox2* mRNA and protein is detected during gangliogenesis at E7; at E14, *Lox2* has a defined high expression in MG7-MG14 and is still active in adult animals (Wysocka-Diller et al. 1989; Nardelli-Haefliger and Shankland 1992; reviewed in Aisemberg et al., 1995; my

unpublished observations). *Lox1* expression was detected as early as E6 by antibody staining and as late as in adult stages by Northern blots (Aisemberg and Macagno 1994; R. Gharbaran, personal communication). Therefore, a probable role of early Hox gene expression in the leech and in other organisms in which cell lineage controls early anterior-posterior differentiation is to participate in general developmental processes of the nervous system. Late Hox gene expression may be necessary in these animals for the maintenance of the identity of functionally specialized neurons in specific regions of the nervous system.

CHAPTER IV

LOX6 PLAYS A ROLE IN NERVOUS SYSTEM PATTERNING

INTRODUCTION

The identification of neurons and other cells that express *Lox6* was a necessary step in these studies before studying the role that *Lox6* plays in the development of the nervous system. I used the RNA interference (RNAi) approach to knock down the expression of *Lox6* in embryos, followed by staining with neuronal markers to detect effects in axon patterning and in the properties of single cells. Introduction of double-stranded RNA (dsRNA) into cells can induce sequence-specific post-transcriptional silencing of genes in a variety of organisms (reviewed by Cogoni and Macino 2000; Vance and Vaucheret 2001; Waterhouse et al., 2001). The experimental application of dsRNA was termed RNAi and was first discovered by chance in the nematode *Caenorhabditis elegans*, in an attempt to knock down gene expression using antisense RNA molecules (Fire et al., 1998). It was noticed that during the *in vitro* transcription reactions small amounts of dsRNA molecules were produced and that they were very active in inducing sequence-specific gene silencing. RNAi involves mRNA degradation and it has been shown to work in a variety of organisms, including vertebrates and several groups of invertebrates (reviewed by Cogoni and Macino 2000). Studies carried out in the leech system have shown that RNAi is a viable approach. When dsRNA of the membrane receptor

phosphatase HmLAR2 was injected into the comb cells, it knocked down the expression of this gene and affected process outgrowth in the comb cells (Baker and Macagno, 2000). The mechanism of action of the dsRNA is not well understood. A proposed mechanism (reviewed by Matzke et al., 2001) involves dsRNA cleavage and processing into sense and antisense RNAs that are 21-25 nucleotides long by RNase III-type enzymes (named Dicer in *Drosophila*, and CAF in *Arabidopsis*). These small interfering RNAs (siRNAs) serve as guides for RNA-induced silencing complex (RISC) endonucleolytic cleavage of homologous mRNA. Recent work in *C. elegans* has elucidated the function of certain proteins in the upstream events of the mechanism of action of RNAi, showing that RDE-4 protein interacts with RDE-1, DCR-1, and a DexH-box helicase to direct RNAi (Tabara et al., 2002). The authors of these studies proposed a model in which RDE-4 and RDE-1 function together to detect and retain foreign dsRNA, and to present it to DCR-1 for processing.

RNAi has been used to knock down Hox gene expression in the milkweed bug *Oncopeltus fasciatus* (Hughes and Kaufman, 2000). To examine the role that *Lox6* plays in the development of the nervous system, I used RNAi to knock down its expression. My results show that *Lox6* RNAi interferes with normal axon patterning of the nervous system during the early embryonic stages of the leech. Several abnormal phenotypes were observed in the main nerves of the treated embryos; the one with the highest incidence was reduced nerve size in the segmental and longitudinal connective nerves. A more specific analysis of the effects of *Lox6* RNAi was carried out in the bipolar neurons and showed that their axons were shorter or absent, and that the expression of the guidance molecule netrin was down regulated. These results show that the Hox gene

Lox6 is necessary for proper axon patterning of the nervous system during early developmental stages.

MATERIALS AND METHODS

RNA synthesis

The template used for *Lox6* dsRNA synthesis was a PCR fragment produced with two primers designed to amplify a limited region of the coding sequence of the *Lox6* cDNA, between nucleotides 11 and 284 (GenBank accession number AF017253). Both PCR primers contained a T7 promoter sequence at their 5' end (ATT AAT ACG ACT CAC TAT AGG GAG). The T7 sequence was followed in the 5' primer (T7-5'Lox6) by the sequence ATG ACC CCT CCG GTC TC and in the 3' primer (T7-3'Lox6) by the sequence TTG TCG GCC GCC GAT AT. The template and the resulting dsRNA contained *Lox6* coding sequences directly upstream and including the first four nucleotides of the homeobox. A 5' primer without the T7 promoter sequence (Lox6pr1) was used with the T7-3'Lox6 primer to amplify a template used for single-stranded RNA (ssRNA) synthesis, as a control for dsRNA synthesis. As a negative control for RNAi, a non-coding dsRNA was made from the plasmid pBlueScript (BS dsRNA), using as a template a PCR fragment amplified with primers containing the T7 promoter sequence followed by plasmid sequences (CGT AAT CAT GGT CAT AGC in T7-BS800as, starting at position 800, and ACC GTA TTA CCG CCT TTG AG in T7-BS1119, ending at position 1119 of the pBlueScript sequence).

To produce the template for *Lox6* dsRNA, four 100-µl reactions were prepared. The following reagents were mixed in an Eppendorf tube: 40 µl of 10X PCR buffer, 4 µl of 50

μM T7-5'Lox6, 4 μl of 50 μM T7-3'Lox6, 40 μl of 0.01 ng/μl of linearized *Lox6* cDNA, 3.2 μl of 25 mM 4dNTPs, 2 μl of 5 U/μl Taq polymerase, 40 μl of 45 mM $MgCl_2$, and water up to 400 μl. The mixture was spun down and aliquoted into four PCR tubes. The parameters for the PCR reaction were: 1 min at 94°C, 1 min. at 55°C, and 2 min at 72°C, for 30 cycles, followed by 8 min at 72°C. The conditions and parameters of the synthesis of templates for *Lox6* ssRNA and BS dsRNA were the same, except that Lox6pr1 was used as a primer instead of T7-5'Lox6 for the Lox6 ssRNA template and T7-BS800as and T7-BS1119 were used as primers for the BS dsRNA template. For the PCR producing BS dsRNA template, the $MgCl_2$ concentration was reduced by using a 30 mM (instead of 45 mM) stock solution of $MgCl_2$. The PCR products were purified by 1:1 phenol:chloroform extraction and with Sepharose G-50 spin-columns, followed by ethanol/sodium acetate precipitation and resuspension in water. RNA synthesis was carried out with the RiboMax kit (Promega), according to the instructions of the manufacturer. A 100-μl reaction produced about 320 μg of RNA. A comparison of denaturing and non-denaturing agarose gel electrophoresis runs of small aliquots of the RNA showed that a very large fraction of the RNA was double stranded. This dsRNA was purified by 1:1 phenol:chloroform extraction and with Sepharose G-50 spin-columns, followed by ethanol/sodium acetate precipitation. The RNA was resuspended in 100 μl of H_2O and stored at -80°C.

Injections

E5-E12 embryos were anaesthetized with 3% ethanol in autoclaved, filtered leech water (0.5g of synthetic sea salts –Instant Ocean– per liter of tap water), and injected with 2.5μl

of dsRNA solution containing 2 μM or 5 μM dsRNA (*Lox6* or BS) in injection buffer (2% polyethylene glycol (MW 8000), 20 mM Hepes-NaOH pH 7.2, 0.1% Fast Green to help visualize the injectate, and 2.5% lipofectamine –Life Technologies–). The anaesthetized embryos were positioned with the ventral side up in a groove-shaped well carved in a Sylgard dish. The injections were performed with a dissecting microscope and glass microelectrodes, using a micromanipulator and positive air pressure provided by a manually operated 10-ml syringe connected by plastic tubing to the microelectrode holder. The microelectrodes were filled with dsRNA solutions using sequencing micropipette tips, before placing the microelectrodes in the holder attached to the micromanipulator. The dsRNA solutions were injected into the germinal cavity of the embryos through the anterior-lateral region of the germinal plate, avoiding the region where the CNS was developing.

After injection, each embryo was placed in a single well of a multiwell plate containing 2 ml of filtered, autoclaved leech water. The embryos were dissected after 1-4 days at 23°C and processed for antibody staining (see Chapter II).

RESULTS

Lox6 dsRNA knocked down *Lox6* expression

To find out the role that *Lox6* plays in the development of the nervous system, I used RNAi to block *Lox6* expression at early embryonic stages by injecting dsRNA into the germinal cavity. The dsRNA used was transcribed *in vitro* from PCR products obtained from the *Lox6* cDNA clone. Electrophoretic analysis showed that the RNA synthesized was predominantly double-stranded, since its mobility was shifted relative to a control ssRNA of the same size and corresponded to the one expected for *Lox6* dsRNA. Concentrations of dsRNA of 2 μM and 5 μM were tested (see Materials and Methods) and both produced knockdown of *Lox6*. To find out whether the *Lox6* dsRNA blocked gene expression, the injected embryos were dissected and processed for *Lox6* antibody staining, and compared to control embryos injected with BS dsRNA (Fig. 1). The developmental effects of *Lox6* knockdown were analyzed by simultaneously staining the same embryos with other antibodies that allowed me to visualize axonal morphology or expression of marker proteins (see next section). Since the leech embryo displays an anterior-posterior developmental gradient and *Lox6* expression is transient in the midbody, the regions affected by RNAi (regarding both *Lox6* expression and neuronal properties) depended on the stage injected. For example, embryos injected at E8 showed effects predominantly in segments posterior to the ones affected by injection at E7. In addition, the individual *Lox6* neurons that were affected by RNAi were observed to vary

from embryo to embryo, probably because the same cell in each embryo took up different amounts of dsRNA (Fig. 1).

Three groups of embryos were injected with dsRNA (Table 1). The first group was injected at E6, when *Lox6* expression is beginning in the CNS, and dissected between E9 and E10. The second group was injected at E7 and dissected between E9 and E10. The last group was injected at E8 and dissected at E12. The survival rate was low in the first group. Controls injected at E6 with injection buffer containing no dsRNA showed also the same low viability. In embryos injected at E6 with *Lox6* dsRNA, *Lox6* expression was knocked down in 78% of the surviving embryos (0% knockdown in the control group injected with BS dsRNA). In embryos injected at E7 with *Lox6* dsRNA, also 78% of the embryos showed *Lox6* knockdown (23% knockdown in the control group injected with BS dsRNA). *Lox6* knockdown could not be verified in embryos injected at E8 because these were dissected at E12, when *Lox6* expression in the CNS becomes restricted to RN3 during normal development.

Figure 1. ***RNAi knocks down Lox6 expression.*** A control embryo injected with BS dsRNA at E6 shows normal rate of development and normal *Lox6* expression in the rostral neuromeres (*A*). Experimental embryos injected with Lox6 dsRNA at E6 and dissected at E9 show intermediate (*B*) and strong (*C*) *Lox6* knockdown. Note the low number of *Lox6*-expressing cells in RN3 and RN4 compared to the control embryo. In the embryo with intermediate knockdown (*B*), development was retarded in the posterior neuromeres. Contralateral pairs of neurons and segmentally homologous neurons that would normally express *Lox6* at the same level show different levels of expression in the experimental embryos. The embryo with strong knockdown shows no *Lox6* expression in the germinal plate (*C*). The rostral ganglia of this embryo show characteristics of a 1-2 day younger embryo. A control embryo injected with BS dsRNA at E7 and dissected at E9 shows normal *Lox6* expression in the CNS, the five pairs of eyes, and in some of the anterior longitudinal muscle cells of the body wall (*D*). An experimental embryo injected with Lox6 dsRNA at E7 and dissected at E9 shows low *Lox6* expression in a few cells of RN3 and MG1 and no expression in the eyes and muscle cells (*E*). The rate of development of the anterior part of the germinal plate was not affected in this embryo. In all pictures anterior is up. Scale bars for *A*, *B*, and *C* are 25 μm and for D and E are 50 μm.

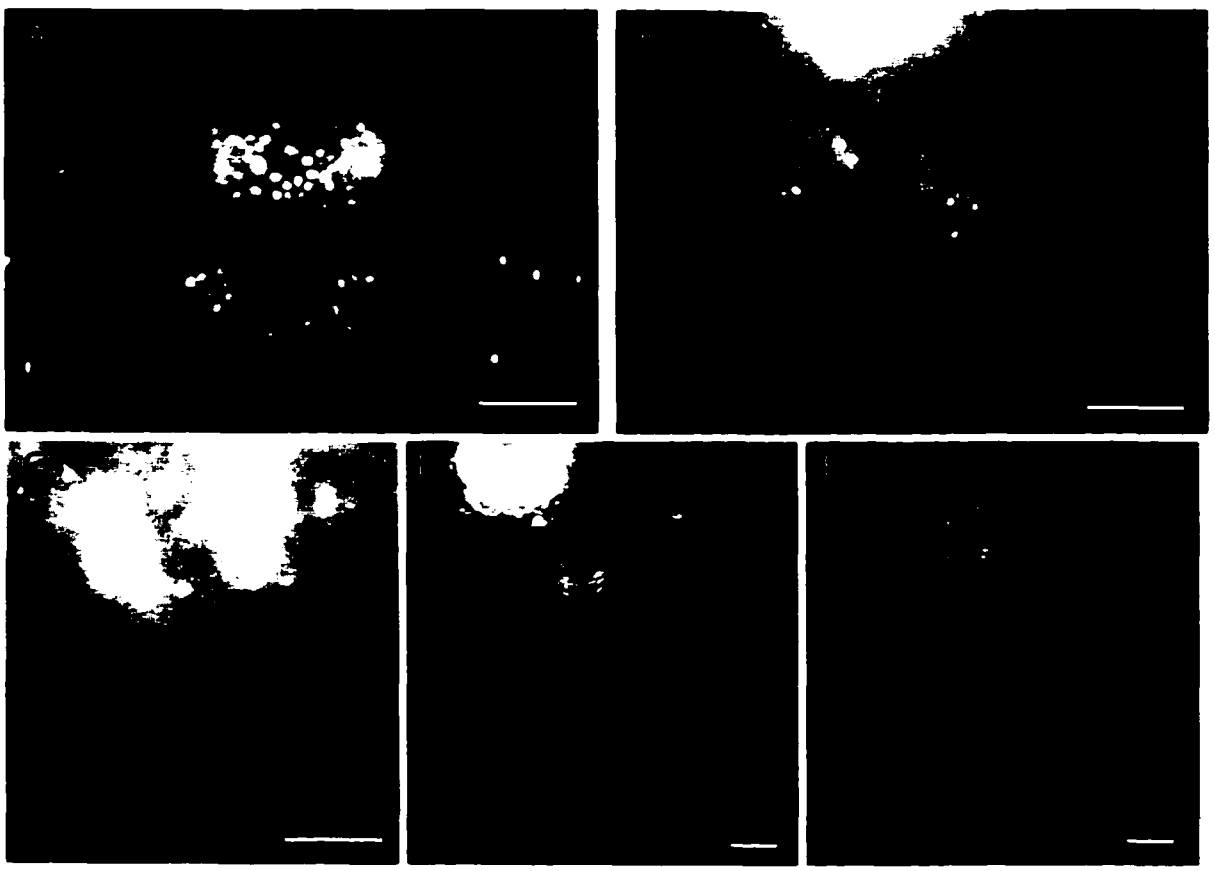


Table 1. ***dsRNA effects on embryo survival and Lox6 knockdown.***

Embryos injected with *Lox6* dsRNA at E7 and E8 had greater survival rate than those injected at E6. The rate of *Lox6* knockdown was the same between E6 and E7.

Stage injected – dissected	Lox6 *dsRNA*					BS dsRNA				
		Survival		Knockdown			Survival		Knockdown	
	N	N	%	N	%	N	N	%	N	%
E6 – E9/E10	26	9	35	7	78	19	4	21	0	0
E7 – E9/E10	33	27	82	21	78	24	22	92	5	23
E8 – E12	15	14	93	N/A	N/A	13	11	85	N/A	N/A

Lox6 RNAi had a general retarding effect on development in 100% of the embryos injected at E6 that were at least one day behind when dissected at E9-E10 (Fig. 1), while the corresponding controls maintained a normal developmental rate. At E7, development was retarded in 56% of the embryos injected with *Lox6* dsRNA. This retarding effect was not detected in embryos injected at E8.

Effect of *Lox6* RNAi on axon patterning

I looked at the effect of *Lox6* dsRNA on nerve patterning in the same embryos used to verify *Lox6* knockdown (see above), which were double-stained with *Lox6* and α-acetylated tubulin (ACTB) (Fig. 2). ACTB labels in the leech most central neurons and their axons, as well as a smaller population of peripheral, non-sensillar neurons (Jellies, Kopp et al. 1996; Jellies et al., 2000). Analysis of these embryos showed several abnormal phenotypes that affected the patterning of the segmental and connective nerves. The main phenotypes observed were abnormally reduced nerves that contain considerably fewer axons, defasciculation of axon bundles that would normally be packed together, visible axonal navigation defects, and short nerves that fail to extend fully (Table 2). The phenotype of reduced nerves refers to a marked decrease in the number of axons in all or some of the four segmental nerves (anterior-anterior, medial-anterior, dorsal-posterior, and posterior-posterior) and of the longitudinal connective nerves (two lateral connective nerves and the midline Faivre's nerve). Defasciculation

refers to axons being dispersed in the nerves instead of in tight bundles. Axonal navigation defects were recorded when it was evident that axons were traveling outside of nerves. Short nerves were nerves with axons that did not reach their targets and navigated short distances. Reduced nerves were found in 67% and short and reduced connective nerves were in 78% of the E6 embryos injected with *Lox6* dsRNA. Some of the embryos with short connective nerves showed completely fused ganglia (Fig. 2B). Among the embryos injected at E7, 81% had reduced nerves (Fig. 2C). The percentage for this phenotype was 33 % in the embryos injected at E8. In these embryos, nerve abnormalities were observed only in posterior segments (Fig. 2D).

Figure 2. *A. Embryo injected with BS dsRNA at E6 and dissected at E9.* The segmental roots of this E9 embryo in RN3 show normal size and normal patterning (in all the pictures anterior is up). *B. Embryo injected with* Lox6 *dsRNA at E6 and dissected at E9.* In this embryo only the nerves of the anterior root are present. They are very reduced and short. Nerves from the posterior root are not detectable. *C. Embryo injected with BS dsRNA at E7 and dissected at E9.* This embryo shows the normal size of the segmental nerves from RN1 to MG2. MG2 is located in the segment where the first pair of nephridia (N) is present. *D. Embryo injected with* Lox6 *dsRNA at E7 and dissected at E9. Lox6* RNAi seriously affected segmental nerve development in this embryo. Few axons left the ganglia and created thin nerves. *E. Embryo injected with* Lox6 *dsRNA at E6 and dissected at E10. Lox6* RNAi caused the fusion of MG2 and MG3. The connectives of MG3 to MG4 are very short. The correspondent nephridia are visible. *F. Embryo injected with* Lox6 *dsRNA at E8 and dissected at E12.* This embryo has moderately reduced segmental nerves in MG5 and MG6. Scale bars for *A*, *B*, and *E* 25 μm and for *C*, *D* and *F* 50 μm.

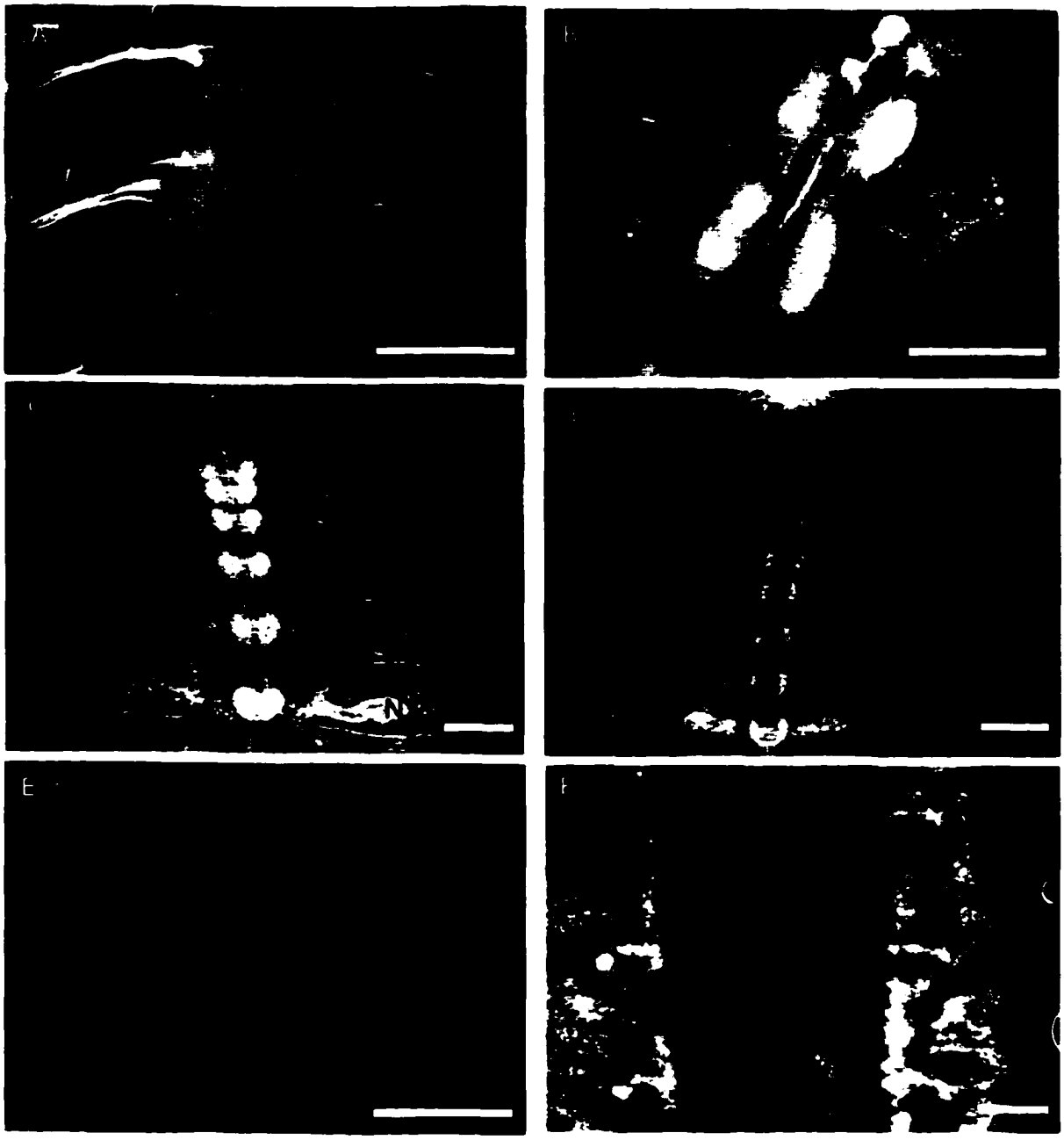


Table 2. Lox6 *RNAi affects the developmental rate and the patterning of axons.*

Lox6 dsRNA-injected embryos showed abnormalities in the segmental and connective nerves. The most frequent phenotype of *Lox6* RNAi embryos injected at E6 was reduced nerves and longitudinal connectives. The embryos injected at E7 were affected mostly in the segmental nerves and fewer embryos injected at E8 had more moderate effects in the segmental nerves of the posterior ganglia.

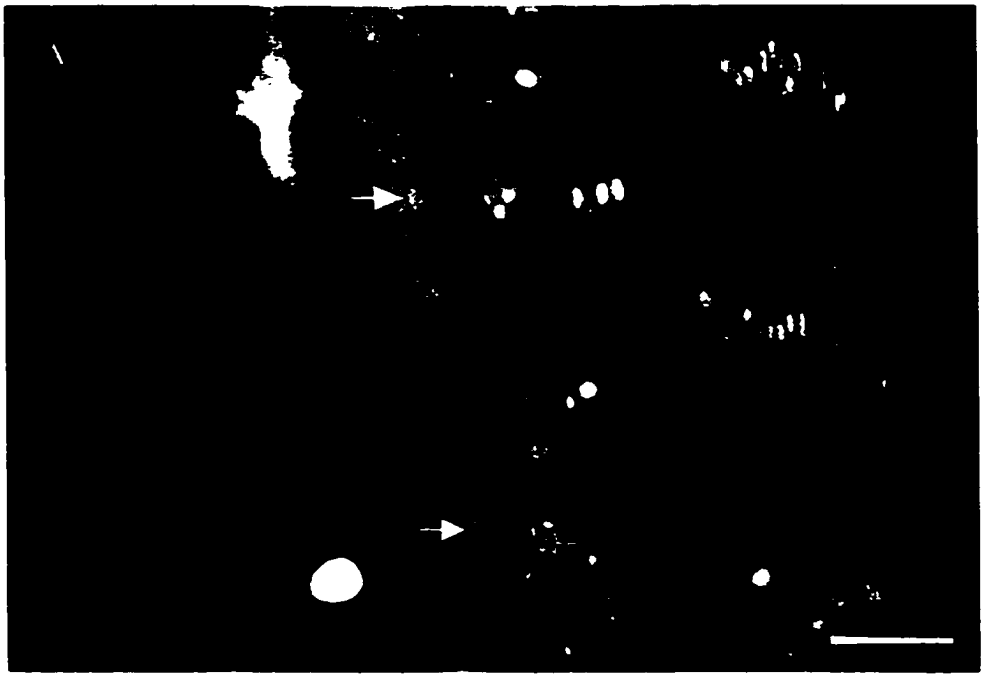
Stage	dsRNA	N	Phenocopies								
			Retarded development		Segmental root defects				Longitudinal nerve defects		
			N	%	Reduced nerves	Defascicu-lation	Navigation defects	Short nerves	Short/ reduced	Defascicu-lation	Navigation defects
E6	Lox6	9	9	100	6	2	1	2	7	4	1
E6	BS	4	0	0	0	0	0	0	0	0	0
E7	Lox6	27	15	56	22	3	6	8	6	1	0
E7	BS	22	4	18	1	1	1	1	0	0	0
E8	Lox6	14	0	0	6	1	0	0	0	0	0
E8	BS	11	0	0	0	1	0	0	0	0	0

***Lox6* RNAi specifically affects the bipolar neurons**

Since *Lox6* and netrin are expressed in the bipolar neurons at about the same time, I wanted to find out the effect of *Lox6* RNAi in two aspects of the identity of these neurons: axonal morphology and netrin expression. The fact that *Lox6* RNAi shortened the longitudinal connective nerves causing sometimes the fusion of ganglia suggested that *Lox6* may play a role in controlling axonal growth in the bipolar neurons. The bipolar neurons are the only early source of netrin signal in the leech CNS and seem to pioneer longitudinal connective nerves during early gangliogenesis before dying at E9-E12 (Stewart et al., 1987; Gan et al., 1999; Aisemberg et al., 2001) suggesting that they may play a role in the establishment of longitudinal tracts or in organizing fascicles of axons. To test the role that *Lox6* expression plays in both aspects of the bipolar cells, I injected embryos at E6 and at E7 with *Lox6* dsRNA, dissected them the next day, and stained them with *Lox6*, netrin, and Laz1-1 antibodies. Laz1-1 was used as a marker for the bipolar neurons. Eighteen embryos were injected with *Lox6* dsRNA and sixteen were injected with BS dsRNA. All the embryos survived except two that were injected with BS dsRNA. When analyzing these embryos I observed that netrin was present in the bipolar neurons before the *Lox6* protein became detectable and that netrin seemed to increase when *Lox6* expression was first evident in a bipolar neuron (Fig. 3). To find out whether knocking down the expression of *Lox6* had an effect on netrin expression in bipolar neurons, I counted the *Lox6*-positive bipolar neurons and the high-netrin bipolar neurons from RN3 (the most anterior expression of *Lox6* in the bipolar cells) to MG21. Both the number of bipolar neurons expressing *Lox6* and the number of these neurons

containing high netrin were significantly lower in the embryos injected with *Lox6* dsRNA when compared to the controls injected with BS dsRNA (Table 3). Netrin expression in the *Lox6*-negative bipolar neurons of RN1 and RN2 was not affected in embryos injected at E6 and E7. In addition, the bipolar neurons of the four embryos injected with *Lox6* dsRNA at E6 had shorter, stump-like, or missing axons not found in the controls (Fig. 4). Several bipolar neurons of embryos injected with *Lox6* dsRNA at E7 showed shorter axon that were not as affected as in the embryos injected at E6.

Figure 3. ***Netrin expression in the bipolar neurons is affected by*** Lox6 ***RNAi . A*** and ***B.*** ***Embryo injected with*** Lox6 ***dsRNA at E7 and dissected at E9.*** The two left bipolar cells of MG1 and MG2 show expression of *Lox6* (white arrows in *A*) together with an increased level of netrin (white arrows in *B*), while the right bipolar neurons do not express *Lox6* (yellow arrows in *A*) and their level of netrin expression is very low (yellow arrows in *B*). In this embryo, *Lox6* expression was almost normal in muscle cells, probably due to low uptake of dsRNA by body wall cells. Scale bars 25 μm.



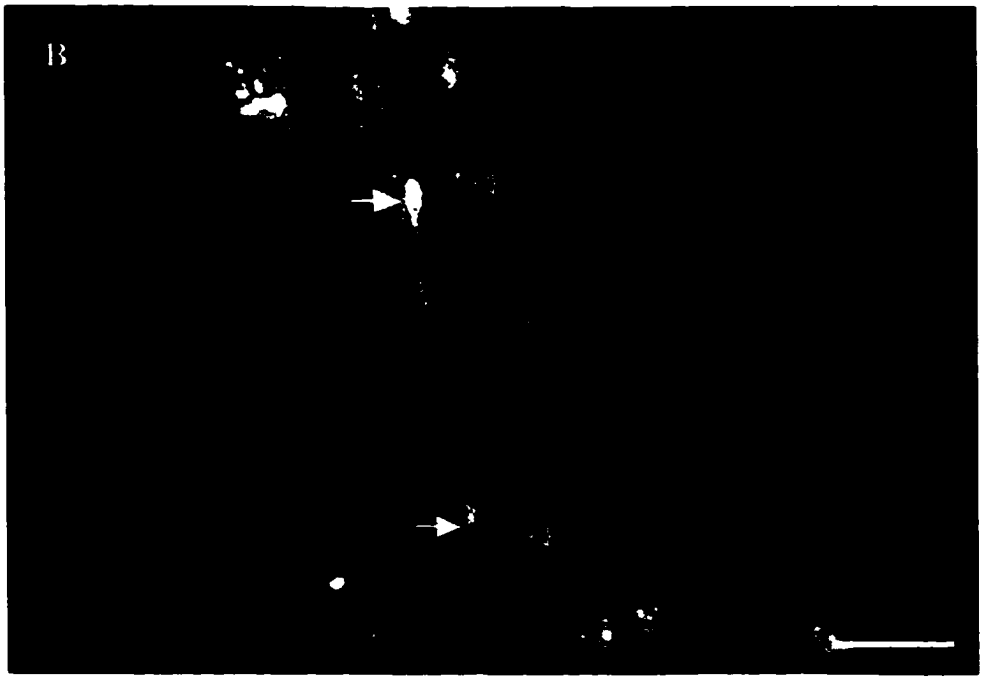


Table 3. Lox6 *RNAi knocked down* Lox6 *expression in the bipolar neurons and affected their expression of netrin.*

A significant number of bipolar cells did not express *Lox6* after *Lox6* dsRNA injections in E6 and E7 embryos compared to the BS dsRNA injected embryos (P= 0.0027, Student's t test). The knockdown of *Lox6* expression was parallel with repression of netrin in the bipolar cells (P= 0.0051, Student's t test). An outlying embryo with 46 high-netrin bipolar neurons (probably the result of an unexplained experimental error) was eliminated from the Lox6 dsRNA experimental sample.

Lox6 dsRNA	Embryo	Lox6 +	Netrin Hi
32. E6-E7	A2	4	0
	B5	3	3
	B2	16	1
	C2	1	0
33.E7-E8	A2	4	4
	A5	4	6
34. E7-E8	A4	2	0
	A6	0	0
35. E7-E8	A2	2	0
	A4	2	10
	A6	2	2
	B2	4	0
	B4	0	0
	C4	8	6
	C6	7	4
	D2	6	4
	D3	2	16
Mean		3.94118	3.2941176

BS dsRNA	Embryo	Lox6 +	Netrin Hi
32. E6-E7	C1	28	15
	A4	7	6
	B1	20	20
33. E7-E8	A3	13	4
34. E7-E8	A3	22	29
35. E7-E8	A1	2	0
	A3	21	4
	A5	0	8
	B1	21	14
	B3	3	14
	B5	8	4
	C3	5	3
	C5	7	11
	D1	2	2
Mean		11.35714	9.57142857

Figure 4. *Effect of RNAi on axon growth in bipolar neuron. A. Embryo injected with BS dsRNA at E6 and dissected at E7.* This control E7 embryo stained with netrin antibody showed the normal axon growth and pattern of the bipolar neurons in the rostral neuromeres. *B. Embryo injected with* Lox6 *dsRNA at E6 and dissected at E7. Lox6* expression was knocked down in the bipolar neurons, which were stained with Laz1-1 antibody and showed highly abnormal axon morphology. The axons grew in only two bipolar neurons and were small and thin (arrows). The cell bodies do not have their normal spindle-like shape. Scale bars 25 μm.

DISCUSSION

By combining immunohistochemistry and injections of dsRNA, I was able to knock down the expression of the Hox gene *Lox6*. Model organisms like *C. elegans*, *Drosophila*, mouse, and zebrafish are amenable to genetic analysis of Hox gene function. RNAi provides a promising tool in the leech system for the functional analysis of Hox genes at the level of single cells, complementing studies in other models. My data shows that knocking down *Lox6* expression affected nervous system patterning and development. The effect of *Lox6* RNAi on the rate of development and the anatomy of longitudinal connective nerves was most striking in embryos injected at E6. This was expected because *Lox6* expression begins at E6 in rostral neuromeres and perhaps because the bipolar neurons begin to appear at that stage. When I analyzed the results focusing only on the bipolar neurons, I found out that *Lox6* RNAi affected severely the bipolar neurons at E6. The cell bodies were small compared to the controls and some of the bipolar neurons generated very short axons, or did not form axons. These results suggest that the bipolar neurons play a role in establishing the longitudinal connective nerves and that *Lox6* may regulate molecules necessary for axonal growth. At E7, the main effect of *Lox6* RNAi was seen in the segmental nerves. More cells are sending their axons to the periphery at this stage and the longitudinal connectives of the posterior segments are being formed. In some embryos, the affected nerves appeared to have traveled a relatively short distance. These could be due to axons that had begun to navigate normally to the periphery before *Lox6* RNAi was introduced. Knocking down *Lox6* may have blocked the expression of molecules under the control of *Lox6* that are necessary for the axonal navigation. At E8, the effects of *Lox6* RNAi on axon patterning

were minimal, restricted to the segmental roots of posterior segments, and not present in all the embryos. An effect limited to the posterior midbody was expected at E8 because at this time of injection the anterior neuromeres were already formed. The effects observed in these experiments are similar to the phenotypes described for *Dfd* mutants in *Drosophila* (Hirth et al., 1998). In these mutants, the cells of the neuromeres specified by *Dfd* did not extend axons and ceased to express neuronal markers, while the axons of other segments did not enter the affected region. My results show that *Lox6*, a *Dfd* ortholog, may have a similar function in the nervous system of the leech. However, *Lox6* acts in a larger domain and consequently seems to affect more the development of the nervous system. It would be interesting to know if neuronal differentiation markers are lost or fail to appear in *Lox6* neurons when this gene is knocked down.

I observed that the expression of netrin seemed to precede by a short time the expression of *Lox6*. However, netrin expression was low in bipolar neurons before they expressed *Lox6* and high in those that had already started to express *Lox6*. This suggested that *Lox6* could play a role in regulating the expression of netrin. When E6-E7 embryos were injected with *Lox6* dsRNA, the number of bipolar neurons expressing *Lox6* as well as the number of these neurons with high levels of netrin was significantly lower than in control embryos, indicating that *Lox6* is likely to participate, either directly or indirectly, in regulating the levels of netrin in the bipolar neurons. If the level of expression of netrin were independent of *Lox6* control and it happened to increase with time, then the embryos injected with *Lox6* dsRNA should have had the same number of bipolar neurons with high levels of netrin as in the control embryos. It is worth noting that *Lox6* is also expressed in other netrin-producing cells like the NE-C neurons and longitudinal muscle

cells of the ventral body wall. Because other neurons that express netrin do not express *Lox6* and because bipolar neurons still show a low level of netrin when *Lox6* is knocked down, *Lox6* expression cannot be an absolute requirement for netrin expression.

An interesting point to consider is that not every cell in an embryo responded equally to RNAi. The *Lox6* knockdown was total in some embryos while a variable low number of cells continued to express *Lox6* after the treatment in other embryos. In the experimental embryos exhibiting residual *Lox6* expression, different cells were knocked down in different segments and in different embryos. It could be expected that the degree of knockdown would depend on the concentration of dsRNA, but all the concentrations I tried (1, 2, 5, 10, and 12 μM) produced variable degrees of knockdown, except that at the lowest concentration I never observed total knockdown. Therefore, I used concentrations of 2 and 5 μM for the experiments reported in this chapter. Under the complex conditions of these systemic RNAi experiments, it is not surprising that the phenotypes observed were pleiotropic and had partial penetrance. On the other hand, all the *Lox6* RNAi phenotypes observed were related to axonal growth and pathfinding in the major nerves of the leech, indicating a specific function for *Lox6* in the control of these important developmental processes.

CONCLUSIONS

The work presented in this dissertation focused on the role that the Hox gene *Lox6* plays during the development of the nervous system of the leech *Hirudo medicinalis*. The time of *Lox6* expression coincided with gangliogenesis and axon path finding. This suggested that *Lox6* plays a role in early nervous system patterning. Several *Lox6*-expressing cells were identified with the *Lox6* antibody, including two pioneer neurons (P_D and bipolar). It would be interesting to find out if other pioneer neurons express *Lox6*. P_D neurons and bipolar neurons seem to cover different tracks of axonal growth and different points of ganglion exit. Bipolar neurons are pioneers of the longitudinal connectives and the P_D neurons are pioneers of the posterior segmental roots. The P_V neuron has been suggested to pioneer the anterior root. Labeling the cell with dye and then staining the embryo with *Lox6* antibody could identify the P_V neuron.

The identification and characterization of other cells that guide axons to choose the right pathways can help to interpret the role that *Lox6* plays in axonal guidance. For example, the *Lox6*-expressing NE-C neurons, because they express netrin and are located at the proximal end of the anterior roots, have been suggested to attract or repel axons causing a specific group of axons to leave the ganglion through the anterior segmental root. Identification of the putative nerve glial cells that express *Lox6* could lead to studies of glial-neuronal cell interactions in the process of axon guidance. In addition, identification of specific sensory neurons in sensillae and among the extrasensillar peripheral neurons would help to understand better the connectivity between the CNS and the PNS when more functional experiments are carried out.

My results showed that *Lox6* expression plays an important role in early nervous system patterning. I suggested that the role of *Lox6* during its late expression may be to maintain the identity of segment-specific RN3 neurons, and of sensory neurons in eyes and sensillae, in addition to establishing and maintaining the reciprocal connections between RN3 and rostral peripheral organs (eyes and sensillae). To find out the role of the late *Lox6* expression in RN3 and the peripheral organs, embryos could be injected with *Lox6* dsRNA at early stages and dissected after E15. Then the embryos could be stained with marker antibodies to examine the effects on the afferent projections of eye and sensillar neurons.

It would be very interesting to find out the specific role of *Lox6* in the P_D neurons. Since these cells have been shown to pioneer the posterior dorsal nerve, blocking their expression of *Lox6* may interfere with its pioneering function, resulting in the absence or under-development of this nerve. This experiment can be done by injecting E6-E7 embryos with *Lox6* dsRNA and dissecting them at E9-E10. Then, the P_D neurons can be filled with fluorescent tracers and stained with *Lox6* and ACTB antibodies. The tracer will reveal P_D neuron morphology, the *Lox6* antibody will show if *Lox6* expression was knocked down, and the ACTB antibody will display the putative effect on the dorsal-posterior nerve.

Using *Lox6* RNAi, I showed that *Lox6* may partially regulate netrin in the bipolar cells. Another molecule that might be regulated by *Lox6* is the leech cell adhesion molecule (LeechCAM), which has been suggested to be homologous to N-CAM (Cunningham et al., 1987). *In vitro* studies have revealed that expression of Hox genes like HoxB9 and

HoxB8 activate the neural cell adhesion molecule (N-CAM) promoter (Edelman and Jones, 1998). LeechCAM is expressed in central and peripheral neurons but is differentially glycosylated with the Lan3-2 epitope (Huang et al., 1997). After knocking down the expression of *Lox6*, it should be possible to look at the expression of LeechCAM in the peripheral neurons. In other organisms, CAMs have been shown to mediate the correct patterning of neural connections by promoting selective outgrowth and fascicle formation of axons of specific neuronal types (Goodman and Shatz, 1993). Other molecules regulated by *Lox6* could be detected by studying the effects of *Lox6* RNAi in DNA microarrays. These techniques could allow us to examine differential gene expression specifically elicited by *Lox6* RNAi in the whole embryo or even in a single *Lox6*-expressing neuron.

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