Studies on the development of the olfactory nerve pathway in the sea lamprey, Petromyzon marinus L.

Aliya Urooj. Zaidi

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Studies on the development of the olfactory nerve pathway in the sea lamprey, *Petromyzon marinus* L.

By

Aliya Urooj Zaidi

A Dissertation
Submitted to the Faculty of Graduate Studies and Research through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
1998

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ABSTRACT

These studies investigated in vivo regulation of olfactory receptor neuron (ORN) axon outgrowth and guidance by molecules of the extracellular matrix (ECM). The sea lamprey olfactory system provided the opportunity for spatial-temporal examination of axonal outgrowth during ontogeny, larval development and neurogenic replacement of ORNs following an olfactory nerve axotomy. During ontogeny, acetylated tubulin (AT)-immunocytochemistry, Dil tracing and transmission electron microscopy revealed short axons that spanned the short distance between the olfactory epithelium and the primordial olfactory bulb. In larvae, the entire olfactory nerve pathway could be viewed in single horizontal sections. Lectin and Dil labeling demonstrated small ORN fascicles that emerged from the olfactory epithelium and extended to the olfactory bulb as a single olfactory nerve. Expression of proteins associated with development (GAP-43, vimentin, acetylated tubulin and NCAM) confirmed that the larval olfactory system was still developing. The role of the ECM molecule, tenascin-C was investigated during ontogeny, in larvae and perturbation studies. Although tenascin-C immunoreactivity was present in the larval olfactory nerve pathway, it was absent during ontogeny and in the larval olfactory mucosa during initial ORN outgrowth following axotomy. In both circumstances, the presence of relatively fewer ORN axons, and the short axonal pathway infer that in the absence of tenascin-C, ORN axonal fibers do not undergo extensive growth or pathway guidance. Similarly, during antibody blocking of tenascin-C in larvae, AT-immunoreactivity was diffuse. On the other hand, when tenascin levels were increased by injections following axotomy, ORN axon outgrowth of irregular-looking fascicles was stimulated. These results support previous studies of tenascin-C that demonstrated boundary signaling during axonal outgrowth. The second ECM molecule, chondroitin sulfate proteoglycan (CSPG), was localized in the nasal cartilage, lateral to the olfactory nerve pathway. The fact that the pathway turned adjacent to the cartilage, suggested CSPG may influence ORN
axon outgrowth by repulsive cues. Following CSPG injections (0.33%) into olfactory-axotomized larvae, ORN axon outgrowth was deleteriously affected, suggesting that CSPG is a modulator of the olfactory nerve pathway. These studies demonstrate that the sea lamprey olfactory system is a valid in vivo model for investigating the regulation of axonal outgrowth.
To my wonderful parents, Urooj and Shabana Zaidi, for all their love, support and encouragement.
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# TABLE OF CONTENTS

**ABSTRACT** ........................................................................................................ III

**ACKNOWLEDGMENTS** .................................................................................. VI

**LIST OF FIGURE PLATES** ............................................................................... XI

**LIST OF TABLES** ........................................................................................ XI

**LIST OF ABBREVIATIONS** ............................................................................ XIV

## 1. INTRODUCTION ................................................................................................. 1

1.1 **OVERVIEW** ........................................................................................................ 1

1.2 **NEURONAL DEVELOPMENT** ............................................................................. 5

1.2.1 Axon Outgrowth and Pathfinding .................................................................... 6

1.2.2 Guidance Mechanisms .................................................................................. 7

1.2.3 Guidance Molecules ...................................................................................... 10

1.3 **THE VERTEBRATE Olfactory SYSTEM** .......................................................... 16

1.3.1 The Olfactory Nerve Pathway ......................................................................... 16

1.3.2 The Olfactory Mucosa .................................................................................... 17

1.3.3 The Olfactory Nerve ...................................................................................... 20

1.3.4 The Olfactory Bulb ......................................................................................... 22

   Molecules associated with neural activity ............................................................ 23

1.4 **THE Olfactory SYSTEM AS A MODEL FOR AXONAL OUTGROWTH** .............. 23

1.4.1 Neuronal proteins in the olfactory nerve pathway ........................................ 24

1.4.2 Guidance Molecules in the Olfactory Nerve Pathway ................................... 27

1.5 **THE SEA LAMPREY** ...................................................................................... 29

1.5.1 The Sea Lamprey Life Cycle .......................................................................... 29

1.5.2 Olfaction in Sea Lampreys ............................................................................ 33

1.5.3 The Sea Lamprey Olfactory Nerve Pathway .................................................. 34

1.5.4 The Larval Lamprey as a Model of Axon Outgrowth .................................... 36

1.6 **SUMMARY** .................................................................................................... 36

## 2. MATERIALS AND METHODS .......................................................................... 37

2.1 **MATERIALS** ..................................................................................................... 37

2.1.1 Equipment ..................................................................................................... 37

2.1.2 Chemicals ....................................................................................................... 38

2.1.3 Solutions ........................................................................................................ 41

2.1.4 Experimental Animals .................................................................................. 41

   Embryos and Prolarvae ...................................................................................... 41

   Larvae .................................................................................................................. 42

   Feeding and maintenance of larval sea lampreys ............................................. 42

   Adults .................................................................................................................... 43

2.2 **METHODS** .................................................................................................... 43

2.2.1 Tissue Preparation ......................................................................................... 43
2.2 METHODS .................................................................................................................................................. 43
  2.2.1 Tissue Preparation ................................................................................................................................. 43
    Wholmount embryonic and prolarval preparations ......................................................................................... 43
    Fixation of the olfactory organ ...................................................................................................................... 44
    Cryosections .................................................................................................................................................. 45
    Vibratome sections ....................................................................................................................................... 46
    Semi and ultrathin plastic sections .................................................................................................................. 46

EXPERIMENTS: PROLARVAE .............................................................................................................................. 47
  2.2.2 Dil labeling .............................................................................................................................................. 47
  2.2.3 Whole-mount immunofluorescence ......................................................................................................... 47
  2.2.4 Olfactory mucosal neural responses ........................................................................................................ 48

EXPERIMENTS: LARVAE .................................................................................................................................... 50
  2.2.5 Dil labeling of the olfactory nerve pathway ............................................................................................ 50
  2.2.6 GS-1-Isolectin B4 staining ...................................................................................................................... 51
  2.2.7 Immunocytochemical analyses of developmental and regulatory molecules within the larval olfactory nerve pathway ................................................................................................. 52
    Controls ......................................................................................................................................................... 53
  2.2.8 Western blotting for tenascin-C detection in larvae .................................................................................. 53
  2.2.9 Immunofluorescence within the olfactory nerve pathway by confocal imaging ...................................... 55
  2.2.10 Lesions of the olfactory nerve ............................................................................................................. 56
  2.2.11 Morphologic light microscope analyses of ORNs during retrograde degeneration ............................. 57
  2.2.12 Effects of axotomy-induced ORN retrograde degeneration on the localization of neuronal and developmental markers ....................................................................................................................... 57
  2.2.13 Perturbation studies ............................................................................................................................. 58
    Anti-tenascin-C .......................................................................................................................................... 59
    Tenascin-C .................................................................................................................................................. 60
    CSPG ......................................................................................................................................................... 60
  2.2.14 Golgi-Kopsch Silver Impregnation ......................................................................................................... 61
  2.2.15 Ultrastructural analyses ....................................................................................................................... 62

3. RESULTS .......................................................................................................................................................... 63
  3.1 ONTOGENY OF THE OLFATORY NERVE PATHWAY .............................................................................. 63
    3.1.1 Embryonic development ....................................................................................................................... 63
    3.1.2 Acetylated tubulin immunocytochemistry and Dil neuronal tracing ................................................. 65
    3.1.3 Olfactory Mucosal Neural Responses ................................................................................................. 68
  3.2 LARVAL OLFATORY NERVE PATHWAY .................................................................................................. 71
    3.2.1 Organization of the olfactory nerve pathway ....................................................................................... 71
    3.2.2 Topographic mapping of the olfactory nerve pathway ......................................................................... 74
    3.2.3 Distribution of acetylated tubulin and GAP-43-immunoreactivity .................................................... 77
    3.2.4 Distribution of tenascin-C immunoreactivity ...................................................................................... 79
    3.2.5 Specificity of tenascin-C antibodies .................................................................................................... 79
    3.2.6 Distribution of laminin and CSPG immunoreactivity ......................................................................... 82
    3.2.8 ORN axon outgrowth following axotomy-induced ORN retrograde degeneration ........................... 87
    3.2.9 Tenascin-C immunoreactivity following axotomy ................................................................................. 90
3.2.10 Ultrastructure of ORN fascicles during initial axon outgrowth .......... 91
3.2.11 Effects of anti-tenascin-C on ORN axons ........................................ 92
3.2.12 Effects of exogenous tenascin-C on ORN axon outgrowth ............... 95
3.2.13 Effects of exogenous chondroitin sulfate proteoglycan on ORN axon outgrowth ................................................................. 99
3.3 THE OLFACTORY BULB ..................................................................... 101
3.3.1 Distribution of nuclei .................................................................. 101
3.3.2 Golgi-Kopsch staining ............................................................... 103
3.3.3 Ultrastructure of ORN axons ...................................................... 105
3.3.4 Distribution of developmental markers ...................................... 105
3.3.5 Distribution of immunoreactivity associated with neuronal activity .. 108

4. DISCUSSION .................................................................................... 111
4.1 Properties of ORNs during ontogeny .............................................. 111
4.2 The larval lamprey as an in vivo model of axonal outgrowth .......... 113
4.3 Tenascin-C and ORN axonal outgrowth ......................................... 117
4.4 CSPG and ORN axonal outgrowth ............................................... 122
4.5 Conclusions .............................................................................. 123
4.6 Directions for future research ....................................................... 129

5. APPENDIX ...................................................................................... 131
   RECIPES ...................................................................................... 131
      Stock Solutions ........................................................................ 131
      Buffers .................................................................................. 131
      Western Immunoblot Solutions .............................................. 132
      Fixatives ............................................................................... 135
      Preparation of Semi and Ultra Thin Plastic Sections .................. 136
   Protocols ................................................................................... 137
      Preparation of Double-subbed Slides ...................................... 137
      Stains Utilized ....................................................................... 138
      Coverslipping of Slides ......................................................... 140
   Preliminary Results for the Effects of Exogenous Tenascin-C and CSPG Injections ................................................................. 141

6. REFERENCES .................................................................................. 143

VITA AUCTORIS ............................................................................. 158
LIST OF FIGURE PLATES

FIGURE 1: GUIDANCE MECHANISMS.................................................................8

FIGURE 2: A SCHEMATIC DIAGRAM OF ORNS WITHIN THE VERTEBRATE OLFACTORY NERVE PATHWAY.................................................................18

FIGURE 3: SEA LAMPREY LIFE CYCLE...............................................................31

FIGURE 4: SEA LAMPREY OLFACTORY ORGAN.............................................35

FIGURE 5: EXOGENOUS TENASCIN-C AND CSPG INJECTIONS INTO OLFACTORY AXOTOMIZED LARVAE.................................................................59

FIGURE 6: EMBRYONIC DEVELOPMENT.........................................................64

FIGURE 7: DEVELOPMENT OF AXONAL PATHWAYS IN PROLARVAE...............66

FIGURE 8: ONTOGENY OF THE SEA LAMPREY OLFACTORY ORGAN...............67

FIGURE 9: INNERVATION OF THE PROLARVAL NASAL CAVITY.......................69

FIGURE 10: OLFACTORY MUCOSAL NEURAL RESPONSES...............................70

FIGURE 11: OLFACTORY MUCOSAL NEURAL RESPONSES...............................72

FIGURE 12: ORGANIZATION OF THE LARVAL OLFACTORY NERVE PATHWAY.......73

FIGURE 13: TOPOGRAPHIC MAPPING OF THE LARVAL OLFACTORY NERVE PATHWAY...75

FIGURE 14: ACETYLATED TUBULIN, GAP-43 AND TENASCIN-C IMMUNOREACTIVITY WITHIN THE LARVAL OLFACTORY NERVE PATHWAY................................78

FIGURE 15: SPECIFICITY OF TENASCIN-C IMMUNOREACTIVITY..........................80

FIGURE 16: DISTRIBUTION OF LAMININ WITHIN THE LARVAL OLFACTORY NERVE PATHWAY.................................................................83

FIGURE 17: DISTRIBUTION OF CSPG WITHIN THE LARVAL OLFACTORY NERVE PATHWAY.................................................................84

FIGURE 18: INFLUENCE OF OLFACTORY AXOTOMY ON THE OLFACTORY EPITHELIUM....86

FIGURE 19: SPATIAL-TEMPORAL CHANGES IN IMMUNOLOCALIZATION OF ACETYLATED TUBULIN, GAP-43 AND TENASCIN-C FOLLOWING AXOTOMY OF THE OLFACTORY
LIST OF TABLES

TABLE I: Piavis’ Early Developmental Classification........................................32

TABLE II: Localization of acetylated tubulin, GAP-43 and tenascin-C
immunoreactivity during retrograde degeneration of the olfactory nerve
and subsequent axonal growth........................................................................91

TABLE III: Summary of experimental results and their possible
interpretations....................................................................................................124
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AT</td>
<td>acetylated tubulin</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>ORN</td>
<td>olfactory receptor neuron</td>
</tr>
<tr>
<td>ORNs</td>
<td>olfactory receptor neurons</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>TN-C</td>
<td>tenascin-C</td>
</tr>
</tbody>
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1. INTRODUCTION

1.1 Overview

The goal of these studies was to explore the feasibility of using the sea lamprey olfactory pathway as an in vivo model for examining the regulation of molecular processes during the development of the nervous system. Although the lamprey is a distant relative to the human model, basic developmental mechanisms of neurons can be tested directly and effectively in this vertebrate.

The development of axonal pathways involves many events (Purves et al., 1997). The guidance of axons is controlled by growth cones, the highly motile, specialized sensory structures, located at the tips of extending axons. These explore the extracellular environment by using finger-like projections, filopodia. Both positive and negative molecules, present in the local environment, are crucial in axon guidance. The primary focus of the present studies was to investigate the involvement of two ECM guidance molecules: tenascin-C and chondroitin sulfate proteoglycan (CSPG) in the modulation of the olfactory nerve pathway. Modulation by tenascin-C was particularly intriguing. Previous developmental studies of the nervous system have shown that this large glycoprotein has complex signaling properties, with several reports showing apparently conflicting functions for tenascin-C.

During ontogeny, a functional olfactory system develops shortly after hatching, when the young lampreys barely pass the neural tube stage. Acetylated tubulin immunocytochemistry and Dil tracings demonstrated that olfactory receptor neuron (ORN) axons spanned a very short distance between
the olfactory epithelium and the primordial olfactory bulb. These olfactory axons were organized in medial and lateral branches, an arrangement that persisted later in the larval stage. The medial axons, which predominated, were diffusely organized, rather than being hemmed in by a cord-like fascicle. During this period, when the ORN pathway was short and simple, tenascin expression was absent. Unfortunately, these very early life stages were available once annually and the use of this stage as an in vivo model for routine examination of the regulation of neuronal development was inappropriate.

The larval stage, which is widely available in the Great Lakes Basin, provided an opportune in vivo model for examining the mechanisms associated with axonal outgrowth and pathfinding. Study of ORNs was particularly suitable, as these cells undergo neurogenic replacement throughout the life of an organism (e.g. Monti-Graziadei and Graziadei, 1979). Sea lampreys share this basic plan of ORN turnover with other vertebrates (Thomhill, 1967). Following axotomy-induced retrograde degeneration of the olfactory nerve, synchronous neurogenesis and ORN axon outgrowth are induced within the olfactory epithelium. Axons extend from these newly generated ORNs and enter the olfactory nerve which extends to the olfactory bulb (Graziadei and Monti-Graziadei, 1978). In larval lampreys, examination of factors that regulate ORN axonal guidance is facilitated by the simple arrangement of the olfactory system and by the absence of a calcified cribiform plate between the olfactory epithelium and the olfactory bulb. Furthermore, the larval sea lampreys maintain characteristics of early development (Rubinson, 1990; Hardisty et al., 1986). As
the larval stage lasts several years, it provides a prolonged period to investigate
neuronal developmental processes. The larval olfactory system expressed both
molecules associated with neuronal development, GAP-43, vimentin and NCAM,
and molecules associated with neuronal activity, synapsin and tyrosine
hydroxylase. Expression of these molecules continued into the juvenile adult
stage, although the expression pattern differed slightly. The larval stage was
chosen to investigate for the experimental analyses, as these have been
maintained successfully in the lab for long periods of time.

Immunocytochemical techniques were used to localize both tenascin and
CSPG within the larval olfactory nerve pathway. The spatial distribution of
tenascin-C implied a regulatory function in this pathway. Tenascin-C
immunoreactivity was moderate and patchy in small fascicles emerging from the
olfactory epithelium, but more intense and widespread in the merged olfactory
nerve that extended to the olfactory bulb. This distribution, with tenascin-C most
concentrated at a location where the ORN axons spanned a considerable
distance, united into a single fascicle, suggested that tenascin-C may signal
events associated with outgrowth and with guiding growth within defined
boundaries. The function of tenascin-C was indirectly examined by observing
expression during ORN axon outgrowth following olfactory nerve axotomy.
Tenascin-C expression was absent during initial ORN outgrowth, and gradually
re-appeared with the growth of axons into nerve fascicles of the olfactory
pathway. When tenascin-C was blocked following the injection of the tenascin-C
antibody, acetylated tubulin expression decreased and became diffuse.
Therefore, the ORN axons appeared diffuse under three conditions with low or no tenascin-C levels: during ontogeny, during initial axon outgrowth following axotomy and during antibody blocking. These data support the hypothesis that tenascin-C modulates axon outgrowth along the defined boundaries of the olfactory nerve pathway.

The effect of tenascin-C on ORN outgrowth was directly tested by injecting tenascin-C during axon outgrowth induced by olfactory nerve axotomy. Here the tenascin-C injections promoted axon outgrowth and possibly fasciculation, with the axons following aberrant pathways.

In these studies, tenascin-C's activity as a signal of lateral boundaries that modulates axonal outgrowth and of the pathway taken by axons, is supported by the localization of tenascin-C in control and lesioned larvae, the effect of antibody blocking and of tenascin-C injections.

The localization of the second molecule of the ECM to be investigated, CSPG, inferred that it may modulate the development of the olfactory nerve pathway by functioning as an avoidance signal to ORN axons. CSPG was localized in nasal cartilage, lateral to the olfactory pathway. Injection of CSPG into the site of ORN outgrowth following olfactory nerve axotomy was followed by detrimental affects on ORN axon outgrowth. These studies demonstrated that CSPG influenced ORN axon outgrowth and may have prevented axons from entering inappropriate terrains.

These studies of the sea lamprey olfactory system have developed the larval sea lamprey as a suitable and applicable in vivo model for investigating the
function of ECM molecules on the growth and pathway selection by outgrowing axons. These results demonstrate that the complex and highly specific expression patterns of ECM molecules direct and guide axon outgrowth. Further development and refinement of this \textit{in vivo} model will yield a powerful tool for examining the regulation of axon outgrowth.

1.2 Neuronal Development

The initial generation of neurons from undifferentiated cells involves the processes of neural induction, axon outgrowth, axon pathfinding and synapse formation. Neural induction, the process whereby a population of neural precursor cells emerge from a subset of ectodermal cells results from spatial and temporal control of different sets of genes. After neurons have migrated to their intended destinations, they extend axons towards appropriate target cells and form synapses (Dodd and Jessell, 1988; Purves \textit{et al.}, 1997).

Of the many remarkable features of nervous system development, perhaps the most fascinating is the ability of growing axons to navigate through complex cellular terrains to find appropriate synaptic partners. Neural tissue differentiation and morphogenesis involves diverse interactions between neural cells and their extracellular environment. Many potentially important interactions occur with the extracellular matrix (ECM), a complex association of extracellular glycoproteins organized into aggregates and polymers (Purves \textit{et al.}, 1997; Letourneau \textit{et al.}, 1994).
1.2.1 Axon Outgrowth and Pathfinding

The mechanisms of axon outgrowth and guidance have been under investigation for over a century, since Cajal's pioneering observations first identified the growth cone (reviewed by Bixby and Harris, 1991). During embryonic development, each differentiating neuron sends out an axon which migrates through the embryonic environment to its synaptic target. In vivo observations of developing axonal projections have revealed that axons extend to the vicinity of their appropriate target regions in a highly stereotyped and directed manner, making few navigational errors (Dodd and Jessell, 1988; Tessier-Lavigne and Goodman, 1996). Growth cones, the growing tips of axons, accomplish this task by detecting molecular guidance cues that are presented to them by cells in their environment, integrating these external signals and transducing them into changes in the rate and direction of axon outgrowth. Considerable effort has been directed toward identifying molecular guidance and pathfinding cues both within the axon and in its environment (Wang and Denburg, 1992). In recent years, a detailed understanding of some of the cellular interactions that direct axonal pathfinding has been developed (reviewed by Tessier-Lavigne and Goodman, 1996). Of these investigations, many focused on interactions between growth cones and their surrounding environment; some have identified and characterized the cues that guide growing axons to their targets.
1.2.2 Guidance Mechanisms

During development, axons give the appearance of unerring navigation, remarkable given the large distances that many axons grow to reach their targets and the complexity of the molecular terrain that they cross. Axon outgrowth may be guided by axons that have already been directed and established contacts with their targets. The first axons that develop, pioneer axons, navigate through an axon-free environment when the embryo is relatively small. Pioneer axons or pre-existing axons provide a scaffold which is followed by newly arriving axons. Non-neuronal cues may also determine the route of axon outgrowth. Gilial scaffolds, as determined by their cell surface properties or surrounding ECM guide axons in this manner (Bartsch et al., 1989).

Axon outgrowth and guidance are also influenced both by promoting and inhibitory factors (Kapfhammer and Raper, 1987 a, b; Silver et al., 1987; Gong and Shipley, 1996). Positive factors promote neurite outgrowth and negative influences from surrounding tissues constrain axons to specific routes (Silver et al., 1987; Tosney and Oakley, 1990). Embryological, tissue culture and genetic experiments have demonstrated that axons respond to the actions of attractive and repulsive forces that guide growth cones (Fig. 1).
Figure 1 - Guidance forces. Four mechanisms contribute to guiding growth cones: contact attraction, chemoattraction, contact repulsion and chemorepulsion. Examples of ligands that are implicated in mediating these mechanisms are shown. Growth cones may be "pushed" from behind by a chemorepellent, "pulled" by a chemoattractant, "hemmed in" by attractive and repulsive local cues. Axons may also be guided by cues on pre-existing axons. These forces act in concert to ensure accurate guidance (modified from Tessier-Lavigne and Goodman, 1996).

- molecules with inhibitory effects on axon outgrowth
+ molecules with stimulatory effects on axon outgrowth
Attraction refers to a range of permissive and axon outgrowth promoting effects; repulsion refers to a range of inhibitory and repellent effects. Long range attractive and repulsive forces, termed chemoattraction and chemorepulsion, respectively, involve diffusible cues such as growth factors and netrins. Long range cues, secreted by target cells, guide axons to their targets by either attracting or repelling advancing axonal growth cones (Tessier-Lavigne and Goodman, 1996; Faissner and Schachner, 1995). Since this study focuses on short range cues, long range cues will not be described in detail. Short range attractive and repulsive mechanisms, termed contact attraction and contact repulsion, respectively, involve nondiffusible or ECM molecules. The process of contact attraction has been implicated in fasciculation where new growth cones follow the axon scaffold laid down by pre-existing axons, forming a nerve fascicle or bundle. Contact repulsion of axons is demonstrated when axon growth is channelled by a corridor of repulsive cues that serve to hem in the axons or by blocking the forward progression of axons. The guidance of axon outgrowth appears to involve the co-ordinate actions of both attractive and repulsive forces which are mediated by mechanistically and evolutionarily conserved ligand-receptor systems. Thus an axon might, simultaneously, be led by local attractive cues on the surface of a pre-existing axon trajectory but be laterally inhibited by local repulsive cues. Many molecules have been implicated in axonal pathfinding. Some have only recently been identified and undoubtedly, more remain to be discovered.
One source of axon guidance cues is the spectrum of molecules within the ECM, through which axons grow, neurons migrate and synapse (Caubit et al., 1994; Yip and Yip, 1992; Sanes, 1989; Bixby, 1991). Ablation of cells that produce ECM guidance molecules or functional blocking of ECM guidance molecules results in misdirected growth by many sensory axons (Cohen et al., 1998).

1.2.3 Guidance Molecules

The direction and extent of axonal outgrowth is influenced by molecules that can act as either diffusible or fixed attractants or repellents, as substrates and/or as guideposts for the growing axon (Doherty and Walsh, 1989; Hynes and Lander, 1992; Goodman, 1994; Tessier-Lavigne, 1994; Fig. 1).

Guidance molecules are local environmental cues that are: 1) present along developing and regenerating axonal pathways at times of active axon outgrowth and 2) are able to influence axon outgrowth. Both criteria must be met for a molecule to be categorized as a guidance molecule (Yip et al., 1995). Molecular cues can be provided by neighbouring cells, target cells and by cells the ECM. Consequently, two broad categories of signal molecules have a role in axon pathfinding: cell surface molecules (e.g., molecules of the immunoglobulin superfamily such as neural cell adhesion molecule) and ECM molecules (e.g., laminin, tenascin and proteoglycans). These are fixed (nondiffusible) molecular signals that guide axon outgrowth by creating gradients for growing axons. These equivalents of molecular highways provide either attractive or repellent forces (Sanes, 1989; Bixby, 1991).
The growth cone membrane contains specific receptors, collectively known as integrins, that bind to large ECM molecules. The binding of ECM molecules to integrins triggers a cascade of events. Within the growth cone, signaling includes changes in levels of intracellular messengers such as Ca\textsuperscript{2+} and inositol triphosphate. These generate signals that directly regulate locomotory activities: cytoskeletal components polymerize and bind to the plasma membrane and produce axon extension (Letourneau et al., 1994). In vitro assays of neurite extension have implicated several molecules in the guidance of axons including: neural cell adhesion molecule (NCAM; Rutishauser, 1985; Walsh and Doherty, 1997), laminin (Kafitz and Greer, 1997; Liesi, 1985), tenascin-C (Faissner and Steindler, 1995; Yip et al., 1995; Faissner, 1997; Kafitz and Greer, 1998) and chondroitin sulfate proteoglycan (CSPG, Snow et al., 1990a; Cole and McCabe, 1991; Margolis and Margolis, 1997).

**Laminin**

Laminin, an extracellular matrix glycoprotein (MW=900 kDa), is present in basement membranes where it provides an adhesive substrate for several cell types (Zhou, 1990; Timpl et al., 1979). In nervous tissue, laminin is found on the cell surface of several populations of cells. It is synthesized primarily by glia and secreted into the ECM. Within the developing nervous system, laminin is involved in axon elongation, axon guidance, and nerve pathway formation within the developing nervous system (Timpl et al., 1979; Cohen et al., 1986; Liesi and Silver, 1988). Liesi (1985) demonstrated the continuous expression of laminin in
astrocytes of the adult CNS that support axonal outgrowth and regeneration. He observed the presence of laminin in nerves that regenerate such as the frog and goldfish optic nerves and its absence from optic nerves in species that fail to support axonal growth, such as rat or chick optic nerves. Laminin's selective in vivo association with axons that undergo growth and regeneration, suggests that it provides the CNS with regenerative potential. When provided as a substrate, laminin promotes neurite outgrowth of cultured CNS and PNS neurons (Kafitz and Greer, 1997; Rogers et al., 1983; Liesi et al., 1984). In addition, laminin-containing gels significantly hastened axonal regeneration in the transected sciatic nerve (Madison et al., 1985).

**Tenascin**

Tenascin, is a highly conserved, large oligomeric glycoprotein (MW= 190-320 kDa) of the extracellular matrix, secreted by fibroblasts and glial cells in culture (Erickson and Bourdon, 1989). Tenascin glycoproteins are linked to multimers by disulfide bonds at the amino terminal end and appear as a six armed structure (hexabrachion) in rotary shadow electron microscopy preparations (Erickson and Iglesias, 1984). They are characterized by a serial arrangement of a cysteine-rich segment at the amino terminus, subsequent epidermal growth factor-type repeats followed by fibronectin type III repeats and homologies to fibrinogen-β and -γ at the carboxyl terminus. Chondroitin sulfate proteoglycan (CSPG) has been identified as a ligand of tenascin-C (Grumet et al., 1994; Maurel et al., 1994). The tenascin gene family is comprised of four
distinct genes, tenascin-C, tenascin-R, tenascin-X and tenascin-Y. Only
tenascins-C and -R have been reported in CNS tissues (Faissner, 1997). The
relatedness of tenascins-C and R can be seen from their structural similarity. In
mammals, tenascin-C is first expressed during gastrulation along the notochord
and subsequently, during neurulation along the neural tube, in pathways for
neural crest cell migration and along developing peripheral axonal pathways
(Erickson and Bourdon, 1989; Steindler, 1993; Yip et al., 1995; Faissner, 1997).
The transient quality of tenascin-C expression is seen in the mature CNS.
Expression of tenascin-C is undetectable in the majority of mature CNS
structures, with the exception of structures that exhibit plasticity, i.e. the olfactory
bulb (Gonzalez and Silver, 1994).

Tenascin-C’s importance is reflected by its conservation in many
vertebrates including: pigs, humans, chicks, fish, sharks, newts (Erickson, 1994)
and also in invertebrates like the moth, Manduca sexta (Kroll et al., 1994)

The tenascin proteins are multifunctional components of the ECM.
Neuronal processes respond by both extension and repulsion on tenascin-rich
areas (Schwab, 1996; Erickson and Bourdon, 1989). Tenascin-C may modulate
axonal outgrowth by repulsive molecular interactions that confine axonal
outgrowth within a specific boundary. For example: elongating axons are
retained by tenascin-C in the endoneurial tube (Martini, 1994; Mege et al.,
1992). The distribution of tenascin-C in boundary-like structures, which separate
anatomically functional processing units of the nervous system, also implicates
that tenascin-C serves to segregate forming axonal pathways (Faissner et al.,
Cultured CNS neurons exhibit avoidance reactions to tenascin-C-coated surfaces (Faissner and Kruse, 1990).

Tenascin-C's association with axonal growth extends to regenerating peripheral motoneurons. Following nerve injury, tenascin-C becomes expressed by proliferating fibroblasts and Schwann cells and accumulates in specific endoneurial, perineurial and perisynaptic locations (Sanes et al., 1986). Antibody perturbation studies that demonstrated delays in reinnervation have suggested that tenascin-C functionally contributes to the regeneration process (Fruttiger et al., 1995; Langenfeld-Oster et al., 1994). Likewise, following lesion of the newt caudal spinal cord, there is a sustained elevation of tenascin-C protein and mRNA expression (Caubet et al., 1994).

Tenascin-C's role in axon guidance is highly controversial. Some studies have shown it to be involved in the repulsion of CNS neuronal axon outgrowth (Faissner and Kruse, 1990) and in boundary signaling of functional units of the nervous system (Steindler, 1993; Krull et al., 1994). Others have shown it to promote neuronal outgrowth from embryonic chick dorsal root ganglion neurons (Crossin et al., 1990).

Chondroitin Sulfate Proteoglycan (CSPG)

Proteoglycans are large ECM and cell surface proteins (MW=325 kDa) with negatively charged, covalently linked glycosaminoglycans side chains (Krusius et al., 1987; Carney and Muir, 1988). Once thought of mainly as ECM molecules of cartilage and connective tissues, sulfated proteoglycans are now
known to be present in the CNS and PNS (Snow and Letourneau, 1992). Various proteoglycans exert different effects on growth cone behaviour. Some promote elongation of axons and dendrites (Shum and Chau, 1996; Faissner et al., 1994; Bovolenta et al., 1993) whereas others inhibit neurite elongation during development and following injury (Brittis et al., 1992; Cole and McCabe, 1991). A further contribution to the diversity of proteoglycan function is their ability to bind to a large number of molecules. These include tenascin, laminin, neural cell adhesion molecule, diffusible growth factors, enzymes and other proteoglycans (Schwab, 1996). Interactions of CSPG with ECM constituents and diffusible growth factors complicates the examination of its developmental function.

Responses of cells to CSPG in vivo may depend upon other molecules with which CSPG is complexed (Snow and Letourneau, 1992). Both in the CNS and PNS, CSPG occurs both as a membrane-associated protein and as a constituent of the ECM. The avoidance of future cartilage and bone CSPG-rich areas, by growing peripheral nerve fibers led to the postulation of inhibitory interactions (Tosney and Oakley, 1990). CSPG has been implicated as a negative modulator of neurite outgrowth both during development and after CNS injury (Snow et al., 1990a). The fact that CSPG is enriched in barrier regions in the developing CNS and in regions of glial scars suggests inhibitory or repulsive activity with respect to growing and regenerating axons. Importantly, there is a correlation between the presence of CSPG and the absence of neurite outgrowth, in vivo (i.e.) in the roof plate of the spinal cord (Snow et al., 1990a, b). Tissue culture experiments have confirmed this view. Growth cones turn at the border of chondroitin sulfate-
rich areas, and slow their growth; although they do not collapse (Snow et al., 1991; Snow and Letourneau, 1992).

Therefore, CSPG has been implicated in signaling axon outgrowth by repulsive cues (Cole and McCabe, 1991).

1.3 The Vertebrate Olfactory System

The olfactory system is a highly specialized sensory system which detects many odorant molecules with great accuracy and sensitivity. Olfaction plays an important role in food-finding, kin recognition, reproductive, homing behaviours and predator-prey relationships (Farbman, 1994). For many animal species, it may be one of the most important senses driving basic patterns of behaviour, since it develops well before visual and auditory abilities emerge (Brunjes and Frazier, 1986; Farbman, 1994).

1.3.1 The Olfactory Nerve Pathway

The olfactory nerve pathway which consists of: the olfactory mucosa (the olfactory epithelium and its underlying lamina propria), the olfactory nerve and the olfactory bulb (Fig. 2). Within the olfactory epithelium, ORN axons merge to form small aggregates. Within the underlying lamina propria, these become surrounded by Schwann cells and form olfactory nerve fascicles. These coalesce to form the olfactory nerve which extends to the olfactory bulb. Here the ORN axons arborize and terminate within discrete neuropil areas, the olfactory glomeruli (Farbman, 1992).
1.3.2 The Olfactory Mucosa

The olfactory mucosa, covered by a thick layer of mucus, lines the nasal cavity and is comprised of olfactory epithelium and lamina propria. A thin basement membrane separates these two layers (Farbman, 1992).
Figure 2- A Schematic diagram of ORNs within the vertebrate olfactory nerve pathway

Olfactory receptor neurons (ORNs), located within the olfactory epithelium (OE), extend axons that first converge within the OE, penetrate through the basement membrane, converge again within the lamina propria (LP) to form olfactory nerve fascicles (ONF). ONF merge to form the olfactory nerve (ON) which projects to the olfactory bulb (OB).
Olfactory epithelium

The olfactory epithelium is distributed along the surfaces of the nasal cavity. It is derived from the olfactory placode on the neural tube (Anholt, 1987 and 1993). It is a pseudostratified ciliated columnar-type of epithelium consisting of three principal cells: olfactory receptor neurons (ORNs), sustentacular and basal cells (Menco, 1977; Morrison and Costanzo, 1990; Morrison and Costanzo, 1992).

The ORNs are bipolar sensory neurons that extend an apical dendrite towards the mucosal surface and a proximal axon towards the olfactory bulb. The dendrite culminates in a knob, off of which a number of cilia extend and embed within a thick layer of mucus. Odourant receptors are located on the ciliary membrane, where olfactory signal transduction is initiated (reviewed by Getchell et al., 1984). Two additional aspects set the ORNs apart from other neurons. First, ORNs continually turn over during the lifespan of an organism, persisting into adult animals (Moulton et al., 1970; Thornhill, 1970; Graziadei and Monti-Graziadei, 1978). No other vertebrate neuron undergoes complete degeneration and reconstitution following an axotomy (Monti-Graziadei and Graziadei, 1979). Second, ORNs are the only neurons that are directly exposed to both the external environment and the CNS. The dendritic tip (the olfactory knob) is located in the nasal cavity, and the axon terminates in the olfactory bulb, an outgrowth of the forebrain.
**Sustentacular cells** are supporting cells that produce and secrete the mucus within which the ORN dendritic cilia are contained. Recent studies of sustentacular cells have localized detoxifying enzymes (e.g., cytochrome P450) which help neutralize toxic odourants (Adams *et al.*, 1991).

**Basal cells** constitute a neurogenic reservoir and are located immediately above the basement membrane (Costanzo and Graziadei, 1987). Loss of ORNs due to environmental damage (Harding *et al.*, 1978), senescence (Graziadei and Monti-Graziadei, 1978) or experimental lesions (Camara and Harding, 1984; Harding *et al.*, 1977) stimulates basal cells to differentiate into ORNs and re-establish functional connections within the olfactory bulb.

**The Lamina Propria**

The lamina propria is a much thicker area than the olfactory epithelium. It consists of loose connective tissue and is highly vascularized, allowing a substantial amount of blood to reach the olfactory epithelium. Many melanophores are present within the lamina propria of fish (Starcevic *et al.*, 1993). Small aggregates of ORN axons penetrate the basement membrane and form olfactory nerve fascicles within the lamina propria.

**1.3.3 The Olfactory Nerve**

In fish, olfactory nerve fascicles collect into a single olfactory nerve which projects to the olfactory bulb. In mammals, the olfactory bulb is relatively close to the olfactory mucosa and fascicles do not collect into a single nerve but project individually to the olfactory bulb (Farbmam, 1992).
Within the olfactory nerve, ORN axons are ensheathed by Schwann cells. The organization of the olfactory nerve is unique among peripheral nerves since ORN axons are organized in a way typical of axons in an early stage of embryonic development in the PNS. Embryonic axons are grouped together in a single bundle wrapped by Schwann cell cytoplasm. In unmyelinated adult nerves, axons are isolated within wrappings of Schwann cells. This is the case both in the embryonic and adult olfactory nerves. The olfactory nerve is the only nerve in the PNS that retains its embryonic relationship to Schwann cells. Schwann cells of the olfactory nerve also differ from other PNS non-myelinating Schwann cells in that some contain an intermediate filament protein, glial fibrillary acidic protein, typically seen in CNS glial cells (Barber and Lindsay, 1982). Unlike other peripheral nerves, which after injury by axotomy, simply regenerate axonal processes, it is ORN axons of newly differentiated ORNs that grow into the olfactory bulb after the olfactory nerve is injured, not the regenerating axons of mature ORNs (Doucette, 1990). Since ORNs are constantly turning over and extending their axons, the olfactory nerve is capable of regeneration. Olfactory nerves, unlike other nerves, retain the ability to grow from their peripheral location into the CNS. The fact that ORN axons are capable of growth into myelin containing regions of the CNS suggests that they may lack the receptors for CNS myelin inhibitory proteins (Farbman and Bucchoz, 1991; Farbman, 1992).
1.3.4 The Olfactory Bulb

ORNs receive information from the environment and transmit this information to the olfactory bulb, the first relay station for olfactory signaling in the brain, which develops from the region of the neural tube, just beneath the olfactory placode (Farbman, 1992). Information in the olfactory bulb is processed to provide an internal representation of the external world and is sent for further processing to higher centers. While the major constituents of the vertebrate olfactory bulb are recognized in lower vertebrates, lamination remains obscure (Iwahori et al., 1992). In fish and other lower vertebrates, the olfactory bulb is a diffusely organized structure with four discernible layers (laminae).

From the periphery inward, the following layers were distinguished: the olfactory nerve layer, glomerular layer, mitral cell layer and granule cell layer (Oka et al., 1982; Satou, 1990). In olfactory glomeruli of the glomerular layer, ORN axons form synaptic connections with mitral and tufted cells, the primary output cells. Glomeruli are not clearly visible because of the absence of juxtaglomerular or periglomerular cells (Byrd and Brunjes, 1995). In ultrastructure studies, ORNs were identified by their intense electron-density (Halasz, 1990). ORN axons, are instrumental in the specification of the entire forebrain, by determining the subsequent morphology of the tissue that they contact (reviewed in Farbman, 1992; Byrd and Burd, 1993). The inductive effect of ORN axons was confirmed by altered olfactory bulb formation following olfactory placode manipulation (Byrd and Burd, 1991 and 1993).
Molecules associated with neural activity

Specific proteins are expressed in functioning neurons. By using immunocytochemical techniques, the location of neurons and molecules contained within these neurons can be assessed. Therefore, by using immunocytochemistry, a functional profile of the brain (olfactory bulb) becomes available. Important molecules for immunocytochemistry for characterizing neural activity are enzymes in the pathway of neurotransmitter biosynthesis. These point to a particular neurotransmitter that is used at a specific location. Secondly, molecules associated with the synthesis and trafficking of neurotransmitter vesicles can be labeled. Tyrosine hydroxylase, the rate-limiting enzyme in the catecholamine synthesis pathway (Nagatsu et al., 1964) is expressed by dopaminergic juxtaglomerular neurons in the rodent olfactory bulb (Baker, 1986) and is a good marker for neural activity within the olfactory nerve pathway.

Synapsin I, a neuron-specific peripheral membrane protein, associated with synaptic vesicles, regulates the movement of synaptic vesicles to their sites of release within the nerve terminal (Bahler and Greengard, 1987; Baines, 1987). Therefore, this protein is a good marker for synaptic activity.

1.4 The Olfactory System as a Model for Axonal Outgrowth

The olfactory system is exquisitely suited to examine the mechanisms associated with axonal outgrowth and pathfinding because of persistent turnover of ORNs and the new ORN axonal outgrowth along the pre-existing olfactory
nerve pathway (Moulton, 1970; Monti-Graziadei and Graziadei, 1979). Studies in mammals and amphibians have shown that an olfactory nerve lesion or axotomy induces synchrony of the ORN renewal process. Following an olfactory nerve lesion, only mature ORNs degenerate, sparing immature ORN axons, that have not yet extended their axons to the site of the lesion (Monti-Graziadei and Graziadei, 1979; Morrison and Costanzo, 1989; Farbman, 1992). Mature ORNs then undergo retrograde degeneration: the axon degenerates followed by the soma (cell body) and finally the dendrite. Following retrograde degeneration of the olfactory nerve, increased neurogenesis occurs in the basal layer of the olfactory epithelium. Newly formed axons emerge from the developing olfactory receptor neurons and enter the olfactory nerve (Costanzo and Graziadei, 1983; Monti-Graziadei and Graziadei, 1979). Therefore, the olfactory system is a continually developing system and a suitable system to examine developmental outgrowth processes.

1.4.1 Neuronal proteins in the olfactory nerve pathway

Acetylated Tubulin

Acetylated tubulin is a component of microtubules, the first cytoskeletal elements to appear in axons (Wilson et al., 1990). Piperno and Fuller (1985) raised an antibody against it which labeled early axons in zebrafish (Chitnis and Kuwada, 1990). Wilson et al. (1990) used this antibody to demonstrate that the olfactory nerve forms part of the initial axon scaffold in the embryonic zebrafish brain. Acetylated tubulin has also been observed in early ORN axons in
embryonic mammals (Easter et al., 1993). Acetylated tubulin was previously observed in the retino-spinal axons of larval lampreys (Hall et al., 1991) and in the olfactory placode of embryonic lampreys (Kuratani et al., 1998). These results suggest that acetylated tubulin is a good marker to examine the ORNs within the olfactory nerve pathway.

**Growth Associated Protein-43**

Growth-associated protein (GAP-43) is an intraneuronal membrane-associated phosphoprotein (MW=46 kDa). Its expression is associated with neuronal development and synaptic plasticity (Pellier et al., 1994; Verhaagen et al., 1989). GAP-43 is a calmodulin-binding protein synthesized in large quantities by neurons when extending their axons, either during initial development of the nervous system or following axon injury (Benowitz and Perrone-Bizzozero, 1991). During neuroembryogenesis, GAP-43 first appears in differentiating neuronal precursors that have just begin to elaborate nerve fibers (Biffo et al., 1990; Dani et al., 1991). The levels of GAP-43 decrease in most neurons during postnatal development, although significant expression is observed in regions of the nervous system that maintain a high level of synaptic plasticity during adulthood (Benowitz et al., 1988; Neve et al., 1988). Non-neuronal cells transfected with the GAP-43 gene develop neuronal-like processes (Zuber et al., 1989; Kumagi-Tohda et al., 1993).

In the olfactory system of mature mammals, GAP-43 expression is restricted to only a subset of cells located in the basal region of the olfactory epithelium. These GAP-43 positive cells are new neurons derived from the basal
cells. GAP-43 is not expressed in basal cells or mature ORNs. Following olfactory nerve lesions, the mature pattern is lost and replaced with that seen in immature animals where most ORNs are GAP-43 positive (Gong and Shipley, 1995). When overexpressed in mature ORNs, gradual alterations in the morphology of olfactory synapses were observed. These experiments suggest that GAP-43 may serve an important role in ongoing structural synaptic plasticity in adult neurons and in neuronal membrane repair after injury to synaptic fields (Holtmaat et al., 1997).

**Neural Cell Adhesion Molecule**

Neural cell adhesion molecule (NCAM) is the best characterized cell surface molecule of the immunoglobulin superfamily, in the nervous system. It promotes cell-cell adhesion in a Ca$^{2+}$-independent manner (Paz et al., 1995). NCAM is expressed in multiple isoforms (180, 140 and 120 kDa) as a result of alternative splicing (Goridis et al., 1985; Rutishauser and Goridis, 1986). It has a temporally-regulated expression throughout nervous system development (Goridis and Brunet, 1992).

Although NCAM is usually expressed during early CNS development, it continues to be expressed in the olfactory system of amphibians and adult mammals (Gong and Shipley, 1996). This expression is consistent with the regenerative capability and continuous cell turnover within the olfactory system (Paz et al., 1995). Targeted deletions of NCAM demonstrated smaller olfactory bulbs and thicker olfactory nerve layers in mutant mice. This finding strongly supports NCAM’s role in the growth and fasciculation of primary ORN axons.
(Treolar et al., 1997).

Vimentin

Vimentin (MW=52-58 kDa), is an early developmental intermediate filament found in glial cells. It is expressed before neurofilaments in almost all dividing neuroepithelial cells and is gradually replaced by neurofilaments (Schwob et al., 1986).

The unusual continual expression of vimentin in ORNs may have implications for the unique plastic and regenerative capacities of ORNs (Gorham et al., 1991, Schwob et al., 1986). Immunocytochemical evidence shows that ORNs contain vimentin in dendrites, cell bodies, and axons. Immunoreactivity does not extend to ORN terminals in olfactory glomeruli in adult and juvenile mammals. Vimentin-antibody staining is uniform in both adult and juvenile ORN axon fascicles (Gorham et al., 1991).

1.4.2 Guidance Molecules in the Olfactory Nerve Pathway

In the nervous system, many glycoproteins have been implicated in developmental and regenerative processes (Nieke and Schachner, 1985; Miragall et al., 1989) and it is quite probable that these molecules are involved in sustaining the high plasticity of the olfactory system (Miragall, 1990). Gong and Shipley (1995) have shown that early ORN axons grow from the olfactory epithelium to the telencephalon along a highly specific pathway. Previous studies have demonstrated the expression of ECM and cell adhesion molecules within the olfactory nerve pathway during early development (Doucette, 1990;
Key and Akeson, 1990; Chung et al., 1991; Gong and Shipley, 1996; Kafitz and Greer, 1997) and it is believed that these could play a crucial role in mediating the guidance of ORNs to the olfactory bulb.

**Laminin**

Laminin may be one of the factors responsible for continuous ORN axon outgrowth and pathfinding within the olfactory system (Kafitz and Greer, 1997). In rats and frogs, laminin is continuously expressed by glial cells in the olfactory bulb and in regenerating olfactory nerves (Liesi, 1985). Interestingly, laminin is usually restricted to early developmental stages elsewhere in the brain (Chung et al., 1991). Spatio-temporal distributions of punctate laminin immunoreactivity observed in the embryonic olfactory system suggest a role in delineating the pathway for ORN axon elongation (Kafitz and Greer, 1997). Moreover, the continuous expression of laminin in the adult olfactory bulb probably relates to the regenerative activity and high plasticity of the olfactory system. (Julliard et al., 1998). The migration rate of ORN neuroblasts increased on laminin substrates (Calof and Lander, 1991). Recently, Kafitz and Greer (1997) have demonstrated that cultured embryonic ORNs show a growth preference for laminin substrates.

**Tenascin-C**

In mature rats, tenascin-C is undetectable by immunocytochemistry in the CNS, with the exception of the olfactory bulb (Gonzalez and Silver, 1994). During early development of the olfactory nerve pathway, specific tenascin-C staining was absent (Gong and Shipley, 1996). In the olfactory lobe of the moth,
*Manduca sexta,* tenascin-like molecules secreted from glial cells formed boundaries around developing glomerular units. The positioning of these tenascin-like molecules may constrain growing ORN axons to glomeruli (Krull et al., 1994). In this way tenascin may inhibit neuritic outgrowth.

Chondroitin Sulfate Proteoglycan

Gong and Shipley (1996) did not observe any specific CSPG staining pattern during early development of the rat embryonic olfactory nerve pathway. However, CSPG has been demonstrated around glomeruli, in association with tenascin, in the postnatal olfactory bulb (Gonzalez and Silver, 1994). Here it may act with tenascin to restrict ORN axons within their pathway.

1.5 The Sea Lamprey

Sea lampreys are modern-day representatives of the ancient vertebrate class, *Agnatha* that has survived well over 300 million years (Hubbs and Potter, 1971). Sea lampreys are particularly useful for neurobiological studies because of their large nerves (Rovainen, 1979), their ability to recover coordinated swimming following spinal transection (Rovainen, 1976; Selzer, 1978) and because of the ability of trans-sected spinal axons to regenerate (Swain et al., 1995).

1.5.1 The Sea Lamprey Life Cycle

The life cycle of the sea lamprey is divided in seven basic phases on the basis of behavioural, morphological and physiological differences. These are:
embryo, prolarva, larva (ammocoete) and transformer (metamorphosis), juvenile adult (parasite), upstream migrant and spawner (Fig. 3; Hardisty and Potter, 1971). Embryos are found within eggs in gravel or sand nests in streams or rivers. During the embryonic stage developmental changes include: gastrulation, neural tube and head formation. Piavis (1971) has subdivided the embryonic phase of the life cycle into 13 stages, each of which is distinguished by its own unique morphological characteristics (Table I).
Figure 3 - The sea lamprey life cycle

The sea lamprey life cycle is divided into the following stages: embryonic, prolarval, larval, transformer (metamorphosis), juvenile adult, upstream migrant and spawner. Embryos are encapsulated within a protective chorionic membrane. The prolarval stage begins with the loss of this membrane and terminates when lampreys exhibit burrowing behaviour. During the larval stage (duration of 3-17 years), lampreys remain burrowed within the sand and are filter feeders. Following metamorphosis, lampreys emerge from the sand and continue development to the juvenile adult (parasite) stage. Following the parasitic phase, lampreys prepare for spawning by migrating upstream. Shortly after spawning, lampreys die, completing their life cycle.
Sea Lamprey Life Cycle

- Spawner
- Embryo
- Prolarva
- Larva
- Upstream migrant
- Juvenile adult (Parasite)
- Metamorphosis
Table I: Piavis' Classification Scheme for Early Development (Water Temp-18-21°C)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Post-Fertilization Time</th>
<th>Stage</th>
<th>Morphological Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td>0</td>
<td>0</td>
<td>Ovulate, unfertilized egg</td>
</tr>
<tr>
<td>0-2 hours</td>
<td>1</td>
<td>Zygote</td>
<td></td>
</tr>
<tr>
<td>2-8 hours</td>
<td>2</td>
<td>Two cells</td>
<td></td>
</tr>
<tr>
<td>8-11 hours</td>
<td>3</td>
<td>Four cells</td>
<td></td>
</tr>
<tr>
<td>10-15 hours</td>
<td>4</td>
<td>Eight cells</td>
<td></td>
</tr>
<tr>
<td>13-15 hours</td>
<td>5</td>
<td>Sixteen cells</td>
<td></td>
</tr>
<tr>
<td>16-19 hours</td>
<td>6</td>
<td>Thirty-two cells</td>
<td></td>
</tr>
<tr>
<td>19-24 hours</td>
<td>7</td>
<td>Sixty-four cells</td>
<td></td>
</tr>
<tr>
<td>24-64 hours</td>
<td>8</td>
<td>Full blastula</td>
<td></td>
</tr>
<tr>
<td>64-104 hours</td>
<td>9</td>
<td>Gastrula</td>
<td></td>
</tr>
<tr>
<td>4-5 days</td>
<td>10</td>
<td>Neural plate and groove</td>
<td></td>
</tr>
<tr>
<td>5-6 days</td>
<td>11</td>
<td>Neural rod</td>
<td></td>
</tr>
<tr>
<td>6-8 days</td>
<td>12</td>
<td>Head</td>
<td></td>
</tr>
<tr>
<td>8-12 days</td>
<td>13</td>
<td>Pre-hatching</td>
<td></td>
</tr>
<tr>
<td>Prolarval</td>
<td>10-13 days</td>
<td>14</td>
<td>Hatching</td>
</tr>
<tr>
<td>13-16 days</td>
<td>15</td>
<td>Pigmentation</td>
<td></td>
</tr>
<tr>
<td>15-17 days</td>
<td>16</td>
<td>Gill-cleft</td>
<td></td>
</tr>
<tr>
<td>17-33 days</td>
<td>17</td>
<td>Burrowing</td>
<td></td>
</tr>
</tbody>
</table>

At stage 14 (hatching, 10-13 days post-fertilization), when the embryo forces itself out of its protective chorionic membrane, it is classified as a prolarva (body length ~6.5 mm). Prolarvae abandon nests to settlement sites (Applegate, 1950).

The onset of the larval phase is marked by the exhibition of burrowing behaviour into the sandy, slity mud. Larvae (ammocotes) are sedentary filter feeders that primarily feed on organic detritus, micro-organisms, ciliates and
diatoms from the water (Margolin and Moore, 1989). During the larval phase, lampreys maintain some characteristics of early development such as the lack of photoreceptors and gender (Rubinson, 1990; Hardisty et al., 1986). Lampreys have an extended larval stage which can last anywhere from three to seventeen years.

In response to some unknown signal, larvae undergo a programmed and highly synchronized process of metamorphosis. During metamorphosis, lampreys experience both internal and external changes; they stop feeding, internal organs either disappear or are replaced by new ones, gonads develop, gills are modified, they develop an oral disc, a suction-like mouth lined with teeth (Youson and Potter, 1979) and experience changes in the olfactory mucosa (Vandenbossche et al., 1995). Metamorphosed lampreys (parasites, juvenile adults) emerge from their gravel beds, migrate downstream towards the sea or lake to feed on fish for the next few years (Beamish, 1980; Youson, 1980). During the juvenile adult stage, parasitic lampreys experience a tremendous amount of growth. After this phase, sexually mature adult sea lampreys migrate upstream to build nests, spawn and die shortly thereafter (Hardisty and Potter, 1971).

1.5.2 Olfaction in Sea Lampreys

Olfaction is the dominant sense in lampreys (Braun, 1996), especially in larvae since their visual system is not functional (Rubinson, 1990). Olfaction is crucial to lampreys for feeding (Kleerekoper and Mogensen, 1963), reproductive and migratory behaviours (Teeter, 1980). Both physiological and behavioural
studies have shown that the larval and adult olfactory systems respond to
chemostimulation (Teeter, 1980; Li and Sorensen, 1995; Zielinski et. al., 1996)

1.5.3 The Sea Lamprey Olfactory Nerve Pathway

The sea lamprey is monorhinic with a single nostril located on the dorsal
surface of the head and connected to a naso-hypophyseal passage
(Kleerekoper, 1972) that leads to a heart-shaped nasal cavity at the level of the
olfactory mucosa (Vandenbossche et al., 1995) located on the dorsal surface of
the head. Lampreys have an easily accessible olfactory nerve, in comparison to
higher vertebrates, where the ethmoid bone and cribiform plate obstructs access
for direct observation of the entire olfactory pathway. The arrangement of the
olfactory pathway in the lamprey allows for visual examination of this region and
thus the study of the spatial-temporal characteristics of axonal outgrowth. The
olfactory nerve pathway, which connects the olfactory epithelium to the olfactory
bulb, can be viewed in its entirety in horizontal sections of lampreys (Fig. 4). In
addition, the lamprey olfactory system provides an evolutionary comparison to
that of higher vertebrates. The sea lamprey olfactory pathway has previously
been characterized by Tobet et al. (1996) using GS-1-B₄ lectin (glycoconjugate)
immunohistochemistry. Intense staining was observed in the olfactory epithelium
and bulb. Therefore, GS-1-B₄ lectin may be a good marker for ORN axons within
the olfactory nerve pathway.
Figure 4 - A model of the sea lamprey olfactory organ

The nostril, located on the dorsal surface of the larval head, leads into a heart-shaped nasal cavity, lined with olfactory epithelium. ORN axons merge to form olfactory nerve fascicles (not shown) which further converge forming the olfactory nerves which synapse within the olfactory bulbs. An accessory olfactory organ is located ventrally.
1.5.4 The Larval Lamprey as a Model of Axon Outgrowth

The larval stage of the sea lamprey maintains characteristics of early development, such as the absence of photoreceptors (Rubinson, 1990), gender (Hardisty et al., 1986) and a folded olfactory epithelium (VanDenbossche et al., 1997). These qualities suggest that larval lampreys are in a "suspended" early developmental stage. The larval lamprey seems to provide a prolonged period to investigate events that occur transiently during early development in other animals. Thus, the larva is an appropriate animal for investigating the early development of the olfactory system and processes that modulate neuronal development in a young animal.

1.6 Summary

The objectives of my research, on the development of the olfactory nerve pathway in the sea lamprey, were: i) to determine the timing of morphological and physiological differentiation of the sea lamprey olfactory organ during embryonic and prolarval development, ii) to investigate the expression of regulatory and guidance molecules that guide the development of the olfactory nerve pathway of sea lamprey, iii) to perturb the olfactory nerve pathway, inducing synchronous neurogenesis, to observe changes in the spatio-temporal distribution of guidance molecules during ORN degeneration and subsequent outgrowth processes and iv) to observe the regrowth of the olfactory nerve pathway following the application of exogenous putative guidance molecules.
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

Animal Holding
30 L Aquaria
Aqua-clear filters
Filter sponges
Ammonia and carbon-removing chips

Tissue preparation

Fischer Scientific microcentrifuge model 59A (Pittsburgh, PA)
Mettler BB244 Delta Range Balance (Switzerland)
Coming pH meter 240 (New York)
Fine Science Tools (Vancouver, BC)
  Micromanipulator
  iridectomy scissors
  #5 Dumont needle forceps
  serrated curved forceps
RMC 6000 XL ultramicrotome (EM Lab Equipment Inc., Whitby, ON)
Glass-Col Homogenizer (Terre Haute, IN)
Fischer Roto-Rack
Nikon SMZ-2B dissecting scope with NKII Fiber Optic Light (Nikon, Canada, Mississauga, ON)
Microm HM-500 OM cryostat (Zeiss; Heidelberg, Germany)
Vibratome-Series 100 (Technical Products International, St. Louis, MO)

Tissue analyses

Philips Transmission Electron Microscope 201
Seanix Computers with Pentium Processors (Ontario)
  Equipped with Windows NT, Adobe Photoshop 4.0, Confocal Assistant,
  JAVA software (Jandel Scientific)
BioRad MRC 600 equipped with a Krypton/Argon laser (Dr. Greer's
  Neurosurgery lab at Yale)
BioRad MRC 1024 Confocal Laser Scanning Microscope equipped with a
  Krypton/Argon laser (Univeristy of Windsor)
Nikon Eclipse E800
Nikon Optiphot microscope:
  FX-35 WA Camera
  Super high pressure mercury lamp power supply (HB 10101AT)
  UKX-II photomicrographic attachment
  XF 32 Interference Filter (Omega Optical, Brattleboro, VT)

Western Immunoblots

BioRad Minigel and transblot apparatus (Hercules, CA)
BioRad Power Pac 300
Hyperfilm ECL (High performance chemiluminescence film, Amersham Life Science, Buckinghamshire, England)

Electrophysiology

BMA 931 AC/DC Bioamplifier (CWT Incorporated, Ardmore, PA)
Tungsten micro-electrodes (Longreach Scientific, Maine)
Dataq data acquisition system (Akron, Ohio).

2.1.2 Chemicals

Amersham Life Science (Buckinghamshire, England)
ECL western blotting detection kit

Argent (Redmond, WA)
Finquel

BDH (Toronto, ON)
Acetone
Entellan (mounting medium for light microscopy)
Gelatin
Glycine
Nitric acid
Toluidine blue
Tris
Xylene

BioRad (Mississauga, ON)
Western immunoblot reagents
  30% acrylamide/bis solution
  Ammonium persulfate
  Non-fat dry milk
  Broad range molecular weight marker
  Gel dryer filter paper backing
  Glycine
N,N,N', N'-tetra-methylethlenediamine (TEMED)
Transblot medium (nitrocellulose membranes)

**Chemicon** (Temecula, CA)
Human tenascin-C purified protein
Polyclonal human anti-rabbit tenascin-C antibody
Polyclonal anti-rabbit laminin

**Dr. Andreas Faissner** (University of Heidelberg)
KAF-9 and EGF polyclonal teriascin antibody gifts

**Dr. Greengard** (Rockefeller Institute)
Synapsin-I antibody gift.

**Dr. Peter Sorensen** (University of Minnesota at St. Paul)
Petromyzonal sulfate gift

**Electron Microscopy Sciences** (Fort Washington, PA)
PAP Pen

**Fischer Scientific** (Unionville, ON)
Glacial acetic acid
Sodium citrate (granular)
Permound

**FMC Bioproducts** (Rockland, ME)
Agarose

**Inctar** (Stillwater, MN)
Monoclonal anti-tyrosine hydroxylase

**J.B. EM Services** (Dorval, PQ)
4% osmium tetroxide solution
Paraformaldehyde EM grade
Propylene oxide

**Kodak Canada** (Toronto, ON)
Kodafix
Polycontrast III RC paper
T developer
T-MAX 100 film
T-MAX 400 film
Developer and fixer for Hyperfilm
Lipshaw (Pittsburgh, PA)
M-1 embedding matrix

Molecular Probes (Eugene, OR)
1,1'-dioctodecyl-3, 3', 3'-tetramethylindocarbocyanine (Dil)

Pelco (Redding, CA)
2,4,6-tri(dimethylaminomethyl)phenol (DMP-30)
Copper grids (200-400 mesh)
Diamond scriber
Dodecenyl succinic sulphate (DDSA)
Glutaraldehyde, aqueous (25%)
Lead nitrate
Lettraset
Medcast resin
Nadic methyl anhydride (NMA)
Osmium tetroxide aqueous (4%)
Propylene oxide
Self closing forceps
Sodium cacodylate trihydrate

Sigma (St. Louis, MO)
3,3'-diaminobenzidine tetrahydrochloride tablets with metal enhancers
Chondroitin sulfate proteoglycan type A
Chromium potassium sulphate
D-arginine
Homoarginine
L-arginine
L-citrulline
L-histidine
L-serine
Methyl salicylate
Monoclonal anti-acetylated tubulin
Monoclonal anti-chondroitin sulfate proteoglycan
Monoclonal anti-growth associated protein-43
Monoclonal anti-neural cell adhesion molecule (NCAM)
Monoclonal anti-vimentin
N,N-dimethyl formamide
N<sup>6</sup>-methyl-L-arginine
N<sub>ω</sub>-nitro-L-arginine
Poly-L-lysine solution
Potassium chloride
Sodium bicarbonate
Sodium chloride
Sodium hydroxide (anhydrous pellets)
Sodium phosphate monobasic
Sodium phosphate dibasic
Sucrose
Taurocholic acid
Triton X-100
Tween-20

**Squibb Pharmaceutical** (Montreal, PQ)
Orabase

VWR (London, ON)
Picric acid

**Vector Laboratories** (Burlingame, CA)
Biotinylated anti-rabbit IgG
Biotinylated GS-1-B4 lectin
Fluorescein anti-mouse IgG
Fluorescein avidin D
Rabbit and mouse IgG vectastain ABC Elite Kits
Texas red anti-mouse IgG
Texas red avidin D
Vectashield

### 2.1.3 Solutions

Recipes of all solutions are given in the Appendix

### 2.1.4 Experimental Animals

All experimental procedures reported in this study were in compliance with guidelines established by the Canadian Council of Animal Care.

**Embryos and Prolarvae**

Embryos and prolarvae were procured by aquaculturing techniques. Sea lamprey *in vitro* fertilization was carried out at the instruction of Dr. Kim Fredricks, at the Lake Huron Biological Station in Millersburg, Michigan. Upon the observation of spawning behaviour, (males attaching onto females which were
anchored to large nest stones, and vibrating vigorously for a short period of time), the spawning pair was quickly removed and placed into separate holding cages. The female was taken out of the cage, massaged gently to release her eggs into a dish of Lake Huron water. The male's sperm were extruded similarly into the egg-containing dish to initiate the process of fertilization. The eggs were initially incubated in an aerated 17°C, UV sterilized water flow-through system and later, at the Department of Biological Sciences at the University of Windsor under similar conditions. Following fertilization, embryos were examined twice daily with a dissecting microscope and staged according to Piavis's staging scheme (Piavis, 1971).

Larvae
Larval sea lampreys (8-10cm in length) were obtained from the U.S. Department of the Interior (U.S. Fish and Wildlife Service; Lake Huron Biological Station, Millersburg, Michigan) and from the Department of Fisheries and Oceans Canada (Bronte Creek, Burlington, Ontario). Larvae were maintained at the Department of Biological Sciences at the University of Windsor at 10°C.

Feeding and maintenance of larval sea lampreys
Larval lampreys were maintained in 30 L aquaria, with sand to a depth of approximately 5 cm, in a 10°C cold room. Ammonia and carbon were removed through a filter containing carbon and ammonia-removing chips, as the tank water was recirculated. 1/3 of the water was changed daily. Once a week, lampreys were fed dry baker's yeast (0.5g yeast/250 mL of dechlorinated water).
Filters were unplugged, to allow the yeast to settle and about 7 hours later, all
the yeast-containing water was removed and replenished with fresh
dechlorinated water. Once a month, carbon and ammonia chips were replaced.

Adults

Juvenile adult lampreys (parasites) were acquired from the Lake Huron
Biological Station in the summers of 1995 and 1996.

2.2 Methods

2.2.1 Tissue Preparation

Wholemount embryonic and prolarval preparations

For whole mount immunofluorescence and Dil labeling, embryos and
prolarvae were anesthetized (0.2g Finquel/50 mL of dechlorinated H₂O). After
removing the chorionic membrane from embryos and making a mid-dorsal
incision in prolarvae, all extraneous tissue was removed to increase penetration
of the fixative and ensure proper fixation. Embryos and prolarvae were fixed in
4% paraformaldehyde (0.1M phosphate buffer, pH=7.4, Appendix), for 2-4 hours
and 1 hour at 4°C, respectively. Embryos were fixed every few hours, from
stages 0 to 13. During prolarval development, changes weren’t as rapid and
lampreys were fixed every 12 hours, stages 14 to 18. Fixed embryonic and
prolarval heads were transferred to 0.1M phosphate buffer (pH=7.4) for 10-15
minutes at room temperature. The whole-mount method was based on the
 technique previously used by Wilson et al., (1990) and Easter et al. (1993).
Ethanol and xylene clearing steps were used to remove yolk from cells up to
stage 14 (day 13 post-fertilization, hatching embryos) prolarvae. Dissected embryonic and prolarval heads were placed in distilled water, followed by 5 minutes in each step of an ascending series of ethanols (30, 50, 70, 90 and 100%) to xylene and then returned through the same series of solutions. The tissue was permeabilized by placing it in acetone at -20°C for 10 minutes and finally back to distilled water. The tissue was blocked for 1 hour at room temperature with an immunofluorescence blocker (Appendix). Immunocytochemistry against developmental and guidance markers followed.

Fixation of the olfactory organ

Larvae

Anesthetized larvae (0.4g Finquel/50 mL dechlorinated H₂O) were decapitated. The nasal cavity was immediately flushed with Zamboni’s fixative (Appendix). Fixative was injected, with a syringe, into the nostril and the cranial cavity to ensure proper fixation. The olfactory organ was dissected by cutting away extraneous tissue surrounding the nostril. A mid-dorsal incision was made into the skin, posterior to the nostril, to enhance penetration of the fixative into the olfactory organ. The dissected olfactory organ was immersion fixed at 4°C for 4 hours for immunocytochemical and GS-1-B₄ lectin studies.

Dil-labeled larval heads were fixed in 4% paraformaldehyde (in 0.1M phosphate buffer, pH=7.4), two days after the application of Dil, for 4 hours at 4°C.

For Golgi staining, larval heads were dissected and immersion fixed in
1.25% paraformaldehyde, 1.25% gluteraldehyde in 0.1 M phosphate buffer (pH=7.4) overnight at room temperature.

For transmission electron microscopy and light microscopy of 1 μm sections, the olfactory organ and the brains were dissected out from decapitated heads of anesthetized larvae and immersion fixed in modified Karnovsky's fixative (Appendix) for 2 hours at 4°C, for transmission electron microscopy.

Adults

For immunocytochemistry, anesthetized adults (parasites) were decapitated and fixed as larvae were.

Cryosections

To prevent the formation of ice crystals, Zamboni-fixed larval and adult (parasites) olfactory organs were cryoprotected through an ascending sucrose gradient: 10% sucrose for 1 hour, 20% sucrose for 2 hours and 30% sucrose overnight, in 0.1M phosphate buffer (pH=7.4, Appendix), at 4°C. Following sucrose infiltration, the olfactory organs were placed in M-1 cryo-embedding matrix and frozen at -40°C in a cryostat. 10-20 μm cryostat sections taken from larval and adult stages were collected on double subbed, poly-L-lysine coated slides (Appendix) to ensure adhesion of the sections to the slide, allowed to air dry for at least an hour at room temperature and stored at -20°C overnight or until used. Prior to any immunocytochemical processing, slides were warmed to room temperature for 10 minutes, rehydrated for 5 minutes with 0.1M PBS (pH=7.4), cold-fixed in acetone at -20°C for 10 minutes and rehydrated with 0.1M
PBS (pH=7.4) for 5 minutes. Tissue sections were further processed for immunocytochemistry.

Vibratome sections

Fixed Dil-labeled heads of larvae were embedded in 2% agarose and sectioned at a thickness of 50 μm with a vibratome. For Golgi-staining, larval heads were washed in 2 changes of 0.1M phosphate buffer (pH=7.4), 15 minutes each, embedded in 2% agarose and sectioned in 80% ethanol, at a thickness of 50 μm, on a vibratome. All vibratome sectioning was done in Dr. Charles Greer’s lab in the Departments of Neurosurgery and Neurobiology at Yale University School of Medicine.

Semi and ultrathin plastic sections

Karnovsky-fixed tissue was stained *en bloc* in 2% uranyl acetate, dehydrated through an ascending series of ice-cold ethanol, passed through propylene oxide and embedded into epoxy resin (Zielinski *et al*., 1996). Semi-thin sections (1μm) were taken on an ultramicrotome, stained with 1% toluidine blue, viewed by brightfield microscopy and photographed on a Nikon Optiphot Microscope.

For transmission electron microscopy, ultrathin sections (65-90 nm) were prepared with an ultramicrotome, collected onto copper electron microscope grids, stained further with 7% aqueous uranyl acetate and lead citrate (Reynolds, 1963), then viewed and photographed on a Philips 201 transmission electron microscope.
EXPERIMENTS: PROLARVAE

2.2.2 Dil labeling

To characterize the morphological development and structural organization of the prolarval olfactory nerve pathway, prolarval nasal cavities (n=10) were anterogradely labeled with the lipophilic dye, 1,1' dioctodecyl-3, 3', 3'-tetramethylindocarbocyanine (Dil). Dil crystals were dissolved in N,N-dimethyl formamide, spread on a microscope slide, allowed to crystallize, wrapped in aluminum foil and stored at -20°C. A few crystals were scraped off the slide, placed onto the tip of a microelectrode, which was mounted on a micromanipulator. Paraformaldehyde-fixed prolarvae were transferred to 0.1M phosphate buffer (pH=7.4). The nasal cavity of the prolarva was viewed with the aid of a dissecting microscope. The microelectrode was inserted into the nostril and withdrawn after the crystals were placed within the nostril. The tissue was then immersed into 0.1M phosphate buffer for 1 day at 37°C, to allow the dye to diffuse along the olfactory nerve pathway. Samples were analyzed as wholemounts on a Nikon Optiphot epifluorescence microscope equipped with a XF32 interference filter (excitation and emission wavelength of 535 and 590 nm, respectively).

2.2.3 Whole-mount immunofluorescence

For immunolocalization studies of the developmental neuronal marker, acetylated tubulin and the ECM molecule, tenascin-C, tissue from hatching embryos (n=10) and prolarvae (n=20) was prepared as whole mounts as described under section 2.2.1. Following tissue permeabilization and blocking,
embryonic and prolarval heads were incubated in either anti-mouse acetylated tubulin (1:1000) or anti-rabbit tenascin-C (1:500) overnight at 4°C, in a porcelain staining dish that was placed in a container lined with wet paper towels. Primary antibody incubation was followed by three 1 hour rinses with 0.1M PBS (pH=7.4) at room temperature, an overnight incubation in appropriate secondary antibodies (fluorescein anti-mouse IgG for anti-acetylated tubulin, fluorescein anti-rabbit IgG for anti-tenascin-C) at 4°C and another set of three 1 hour rinses with 0.1M PBS (pH=7.4). The tissue was dehydrated through an ascending series of alcohols, cleared in xylene and mounted between two coverslips with Entellan (Appendix). This enabled the preparation to be viewed from either side, after placing it on a microscope slide. A Nikon Optiphot epifluorescence microscope was used for photographic analyses (Kodak TMAX 400 film).

2.2.4 Olfactory mucosal neural responses

Multi-unit responses were recorded from prolarvae (n=33) to assess the function of the olfactory organ. Anesthetized stage 17 prolarvae (length of 7.5 to 9.0 mm) were placed onto a small ramp glued within a small Petri dish (6 cm) filled with 50 mM NaCl (in deionized Lake Huron water). This solution was slightly more dilute than lamprey Ringer's solution (91 mM NaCl; Wickelgren 1977). When prolarvae were in this solution, multi-unit recordings were recorded from the prolarval olfactory epithelium for up to an hour. Once the animal was placed onto the ramp, the nostril, a small depression on the dorsal surface of the head, was visible under a dissecting microscope. Each prolarva remained still throughout the experimental procedure (about 1 hour/animal).
Stimulus compounds included L-arginine, N-ω-nitro-L-arginine, taurocholic acid, D-arginine and 3α,7α,12α,24-tetrahydroxy-5α-cholan-24-sulfate (petromyzonol sulfate). In addition, water conditioned by conspecifics was tested. This included water conditioned by eggs, prolarvae and ammocoetes. Water was conditioned by ammocoetes by placing 20 ammocoetes into a bucket of lake water, and allowed to stand for 20 minutes before testing. To prevent mechanoreception from interfering with the olfactory responses, test solutions (30-100 μL) were applied gently from a syringe onto the water surface at the edge of the Petri dish. The effectiveness of this application was tested using a coloured dye, which spread into the dish and over the head of the prolarva. The application of 50 mM NaCl in deionized Lake Huron water failed to elicit a response. This solution was used as the negative control. To test for a change in the sensitivity of the preparation, L-arginine (10^-6 M) was chosen as the standard stimulus and was regularly applied to trace any changes in responsiveness in the electrophysiological preparation throughout the experiment.

The recording electrode, an epoxy-insulated tungsten microelectrode was positioned into the posterior surface of the nasal cavity since transmission electron microscopy revealed ORNs at this location (Zielinski, 1996 Technical Report to GLFC). A ground electrode was placed on the tail of the prolarva. AC recordings were fed into a AC/DC Bioamplifier with a Super Z impedance probe. Integrated neural activity was recorded using a Dataq data acquisition system. The integrated neural activity showed sustained tonic activity to the continuous
presentation of the stimulus. Between each response recording, the holding solution was removed and the prolarva was rinsed three times with dilute de-ionized lake water saline. To test receptor specificity between L-arginine, L-arginine analogues and bile compounds, L-arginine was first applied and the neural response was recorded. After one minute, the second stimulus was applied (taurocholic acid, N-ω-nitro-L-arginine). For data standardization, responses to stimuli were bracketed by responses to the standard stimulus L-arginine and to the control water (dilute saline). Stimulus compounds were applied at an interval of 3 minutes.

EXPERIMENTS: LARVAE

2.2.5 Dil labeling of the olfactory nerve pathway

The pathway of the olfactory nerves was characterized by anterograde labeling from the nasal cavity of anesthetized lampreys (n=5). Dil was applied in crystal form as previously described under section 2.2.2. As the lamprey is monorhinic (Kleerekoper, 1972), application of Dil into the single nasal cavity enabled access to both olfactory nerves. The Dil-labeled larvae were kept in holding tanks for two days (10°C), then fixed and sectioned as described in section 2.2.1. Vibratome sections were mounted with Vectashield and examined on a BioRad MRC 600 confocal laser scanning microscope. Images were processed through Adobe Photoshop 3.0 and printed on a Codonix NP1600M color printer.
2.2.6 GS-1-Isolectin B₄ staining

The organization of the olfactory nerve pathway was further analysed using lectin staining (n=8). Biotinylated GS-1-isoelectin-B₄ staining was used to map the olfactory nerve, its fascicles and arborizations within the olfactory bulb, from the dorsal aspect of the olfactory organ to the ventral. The GS-1-isoelectin B₄ staining protocol used was modified from the method used by Tobet et al., 1996. Olfactory organs were fixed and sectioned as described in section 2.2.1. Briefly, the tissue was incubated with biotinylated GS-1-B₄ lectin (diluted to 10 μg/mL) for 3 hours at room temperature. Slides were washed with 0.1M PBS (pH=7.4) for 10 minutes at room temperature. For fluorescence, this was followed with a 1 hour Texas Red-Avidin D (1:100) incubation at room temperature and three 10 minute rinses with 0.1M PBS (pH=7.4). Sections were coverslipped with Vectashield, sealed with nailpolish and examined on a BioRad MRC 1024 confocal laser scanning microscope; Nikon Eclipse E-800; 20-60X objectives; 0.5-1 μm Z step.

For 3,3'-diaminobenzidine (DAB) histochemistry, following the PBS wash sections were incubated with the ABC reagent (Vectastain ABC Elite Kit) for 30 minutes at room temperature, followed by three 10 minute rinses with 0.1M PBS (pH=7.4). DAB fast tablets were used to visualize the lectin distribution throughout the olfactory nerve pathway. Slides were dehydrated through an ascending series of alcohols, cleared in xylene and coverslipped with Entellan (Appendix).
2.2.7 Immunocytochemical analyses of developmental and regulatory molecules within the larval olfactory nerve pathway

Cryosections of the larval and adult olfactory pathway were stained using immunocytochemical procedures (n>100). For DAB immunocytochemistry, endogenous peroxidase was suppressed with 0.3% H₂O₂ for DAB immunocytochemistry (30 minutes at room temperature) prior to the primary antibody incubation. Non-specific sites were blocked with a 20 minute incubation with horse serum at room temperature. The tissue was incubated with an array of primary antibodies (1:1000 acetylated tubulin, 1:1000 GAP-43, 1:40 vimentin, 1:300 CSPG, 1:100 neural cell adhesion molecule, 1:10 000 tyrosine hydroxylase and 1:500 tenascin) overnight at 4°C. The antibody diluent was 0.1M PBS (pH=7.4) with 0.2% Triton X-100 for all antibodies except for neural cell adhesion molecule, CSPG and tenascin. For these, Triton X-100 was omitted from the antibody and wash diluents. The ABC elite kits (mouse and rabbit IgG) and standard ABC protocols were used. Briefly, the tissue was rinsed after overnight incubation with the primary antibodies, all rinses consisting of three 10 minute washes, in 0.1 M phosphate buffered saline (pH=7.4) at room temperature. Subsequently, biotinylated secondary antibodies were applied for 30 minutes at room temperature, followed by the PBS rinse. The ABC reagent was applied for an additional 30 minutes at room temperature, followed by the PBS rinse. DAB tablets were used to visualize immunoreactivity. The sections were dehydrated through an ascending series of alcohols, cleared in xylene and mounted with Entellan (Appendix), then viewed and photographed on a Nikon
Optiphot microscope.

**Controls**

Negative controls, with primary antibodies omitted from the staining procedure, were included with each immunocytochemical run.

A pre-adsorption control was performed to establish the specificity of the polyclonal anti-rabbit antibody against tenascin-C (Chemicon antibody). A 10 fold molar excess of purified human tenascin-C antigen \(10^{-5}\) to \(10^{-3}\) M) was added to a 1:500 antibody dilution. IgGs were assumed to be at a concentration of 1 mg/mL for serum antibodies. The antigen-antibody mixture was allowed to stand for 18 hours at 4°C, with mild agitation. The antigen-antibody mixture was centrifuged at 100,000g for 20-30 minutes. Any precipitating antigen-antibody complexes formed would be in the pellet. The supernatant or the preadsorbed serum, was pipetted into a clean vial and used in place of the anti-rabbit tenascin-C antibody. Preadsorbed samples were processed in parallel with tissue incubated in normal anti-rabbit tenascin-C antibody.

**2.2.8 Western blotting for tenascin-C detection in larvae**

The specificity and the molecular weight of the polyclonal anti-rabbit tenascin-C antibody (courtesy of Dr. Faissner) was determined by western immunoblot analysis. Anesthetized larvae \(n=15\) were decapitated. Two preparations were made. First, the skin, which contained tenasin immunoreactivity. Second, the olfactory organ and olfactory bulb. The dissected tissue was immediately immersed in liquid nitrogen to inactivate all enzymatic
processes, then stored at -80°C until the experiment was initiated. The tissue was weighed in a 10°C cold room, suspended in five times the volume of homogenizing buffer (Appendix). Sand was mixed into the tissue-buffer solution and homogenization was carried out on ice. The sample was centrifuged at 10,000 rpm for 25 minutes at 4°C. The supernatant was decanted and stored at -80°C until required. The collected supernatants were combined with an equal volume of sample buffer (Appendix). This mixture was boiled for 5 minutes. A 6% SDS-polyacrylamide gel was prepared and 5µL of a broad range molecular weight marker was applied to lane 1, 10 µL of bovine serum albumin (1 µg/mL; negative control) was applied to lane 2, 10 µL of the purified human tenasin-C protein (5µg/mL; positive control) was applied to lane 3, 10 µL of skin sample was applied to lane 4 and 10 µL of the olfactory sample was applied to lane 5. These samples were electrophoresed for 1 hour at room temperature at 130V. The resolved protein bands were transferred onto nitrocellulose membranes at 100 V for 60 minutes in transfer buffer (Appendix). The membranes were blocked overnight at 4°C in blocking buffer (Appendix). The blocking buffer was decanted, blots were incubated for 1 hour at room temperature with the polyclonal anti-rabbit tenasin-C antibody (courtesy of Dr. Faissner; diluted 1:1000 in blocking buffer). Blots were then washed with washing buffer (Appendix) for 30 minutes on a shaker at room temperature. The blots were subsequently incubated with an anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (diluted 1:2500 in blocking buffer) for 1 hour at room temperature, followed by another 30 minute washing on a shaker. ECL detection
reagents were used to visualize the reaction. The blots were exposed for 1 hour onto hyperfilm in a Kodak film cassette.

2.2.9 Immunofluorescence within the olfactory nerve pathway by confocal imaging

For acetylated tubulin (1:1000), tenascin (1:500), laminin (1:1000), GAP43 (1:1000) and CSPG (1:300) immunofluorescence (n=10), all the steps were similar to those followed for DAB immunocytochemistry in section 2.2.9 (except for the peroxidase blocking step, which was replaced with a 1 hour incubation with an immunofluorescence blocker (Easter et al., 1993) at room temperature, Appendix), up to the secondary antibody stage. Following the post-primary antibody wash, sections were incubated in fluorescein or texas red-conjugated secondary antibodies (fluorescein and texas red anti-rabbit and mouse IgG; diluted 1:100) for 1 hour at room temperature, followed by three rinses of 10 minutes with 0.1 M PBS (pH=7.4). For tenascin-C-immunofluorescence, a biotinylated anti-rabbit IgG (1:100) was applied for an hour, followed by three 10 minute rinses with 0.1M PBS. Sections were incubated with fluorescein avidin D (1:100) for 1 hour at room temperature, followed by three 10 minute rinses with 0.1M PBS.

Sections were mounted with Vectashield and examined on a BioRad 600 and 1024 MRC confocal laser scanning microscopes; Olympus IMT2 and Nikon Eclipse E800; 20-60X; objectives 0.5-1 μm Z step). Digitized images were processed through Adobe Photoshop 3.0 and printed with a Codonix NP1600M color printer.
In an attempt to determine the role of CSPG in the larval olfactory organ, a double label experiment (n=3) was performed to examine CSPG immunolocalization in relation to outgrowing ORN axons. CSPG immunofluorescence was performed as described above at a dilution of 1:300 with a secondary antibody conjugated to Texas Red. Following the post-secondary wash, tissue sections were blocked for 1 hour at room temperature (Appendix) and run up for acetylated tubulin immunofluorescence (as described above) using fluorescein anti-mouse IgG. Following the post-secondary wash, sections were coverslipped, viewed and analyzed as described above.

2.2.10 Lesions of the olfactory nerve

Anesthetized larvae (n=15) were placed in a water flow-through system to ensure that their gills were perfused with water. A scalpel was used to make an incision anterior to the pineal gland. The skin was folded back and adipose tissue was removed with forceps. A dissecting microscope was used to view the olfactory nerves. The nerves were cut with iridectomy scissors. A metal probe was passed through the lesioned region to ensure that the olfactory nerves were trans-sected. An antiseptic cream, Orabase, was applied to the wound to prevent infection. The skin was folded back over the incision. The incision site healed within a few days of the larvae’s return to holding tanks. For sham operations, the surgical procedure was carried out but the olfactory nerve was not lesioned. On days 3, 7, 9 and 14 following the lesions, olfactory axotomized and sham-operated larvae were anesthetized and sacrificed for morphological and immunohistochemical analyses.
2.2.11 Morphologic light microscope analyses of ORNs during retrograde degeneration

In 1μm epoxy sections of the larval olfactory epithelium, ORNs are identifiable by the rounded ciliated olfactory dendritic knobs protruding into mucociliary matrix, slight narrowing of the apical portion of the dendrite and by the non-vesicular cytoplasm, compared to neighbouring vesicular sustentacular cells (Vandenbosche et al., 1995). The results from preliminary experiments suggested that following trans-section of the olfactory nerve, ORNs were largely absent from the olfactory epithelium by the third day following the lesion. By the fourteenth day, the dendritic tips of newly generated ORNs were present. The effect of retrograde degeneration on the numerical density of ORN was investigated by light microscopy of 1μm epoxy sections in unlesioned controls (day 0), days 3 and 14. The olfactory organs from 9 larvae (3 larvae for each of days 0, 3 and 14) were prepared for electron microscopy as described in section 2.2.1. Semi-thin sections (1 μm) were made with an ultramicrotome, stained with 1% toluidine blue and viewed on a Nikon Optiphot light microscope. The number of ORN per 100 μm length of olfactory epithelium were counted using JAVA image analysis software, Vandenbossche et al., 1995). For each animal, ORNs from three lengths of olfactory epithelium on separate sections were counted and averaged. The average of these values was calculated from each time point.

2.2.12 Effects of axotomy-induced ORN retrograde degeneration on the localization of neuronal and developmental markers

To determine the effects of an olfactory nerve lesion on the expression of guidance molecules within the larval olfactory nerve pathway, axotomized larvae
were placed in holding tanks at 10°C. The animals sacrificed were 3 and 14
days post-lesion, since preliminary light microscopic analyses indicated that
ORNs are virtually absent from the olfactory epithelium, three days post-lesion
and by the fourteenth day post-lesion, ORNs repopulate the olfactory epithelium.
The olfactory organ from 15 larvae (n=3 at each time point) was prepared for
immunocytochemistry on days 0, 3 and 14 following olfactory nerve lesions, as
described in section 2.2.1. Larvae were also sacrificed at intermediate time
points (n=3 at 7 and 10 days post lesion, each) to monitor ORN axonal
outgrowth. For the expression of the putative guidance molecule, tenascin-C
(Chemicon) immunostaining patterns were examined. ORN axon outgrowth
patterns following olfactory axotomies were revealed by acetylated tubulin and or
GAP-43 immunostaining.

2.2.13 Perturbation studies

To examine the roles of tenascin-C and CSPG on the process of ORN
axon outgrowth and olfactory nerve regrowth, larvae were injected daily with
either CSPG or tenascin-C up to 14 days following olfactory nerve lesions. To
ensure that ORN axon outgrowth had begun, axotomized animals were injected
daily from day 3 post-lesion onwards since ORN axon outgrowth was seen at
that time point (Fig. 2.2.12). Shams were lesioned and injected with 0.1M PBS,
pH=7.4, daily from day 3 post-lesion.
Figure 5: Exogenous tenascin-C and CSPG injections into olfactory axotomized larvae

The injections were made in the nostril and subcutaneously, just below the olfactory organ. These sites were chosen because injections into the nostril bypassed the blood-brain barrier providing direct access to the olfactory nerve pathway. Subcutaneous injections were made in the vicinity of ORN axon outgrowth to ensure that the injected protein was present in areas of active ORN axon outgrowth, to exert its effect. Injected tissue was run up for tenascin-C and CSPG immunofluorescence to confirm that injected tenascin-C and CSPG was present along the olfactory nerve pathway. Results demonstrated that tenascin-C and CSPG were highly abundant in the olfactory organ up to the olfactory bulb.

Anti-tenascin-C

To determine the role tenascin plays in the outgrowth of ORN axons, tenascin-C antibody perturbation experiments were carried out. In these experiments, injection of anti-tenascin-C antibody inactivated the function of
tenascin-C based on the assumption that the antibody binds onto tenascin-C's active site. An anti-rabbit tenascin-C antibody (diluted 1:500; 1 μL tenascin-C protein in 499 μL of 0.1M phosphate buffer, pH=7.4, courtesy of Dr. Faissner Langenfeld-Oster et al., 1994;) was introduced into the olfactory nerve pathway of unlesioned larvae (n=3) by daily subcutaneous injections in the nostril and anterior to the pineal gland for 14 days. The animal was then sacrificed and run up for acetylated tubulin immunofluorescence (section 2.2.9) to examine the effects of blocking tenascin function on the ORN pathway.

**Tenascin-C**

Effects of the anti-tenascin-C antibody were evident at a 1:500 dilution of anti-tenascin-C antibody. For this reason, the 1:500 dilution was also used for exogenous tenascin-C injections into the lesioned larval olfactory nerve pathway (n=10). Lesioned larvae were injected daily with 0.2% tenascin-C, sacrificed on days 6, 10 and 14 and processed for acetylated tubulin immunofluorescence (see time line).

**CSPG**

To determine the dose of CSPG, unlesioned animals (n=9, 3 at each concentration) were injected with increasing concentrations of CSPG (0.165, 0.25 and 0.33% in 0.1M phosphate buffer, pH=7.4), until a significant effect was observed on ORN outgrowth. 0.33% was calculated from the anti-CSPG dilution used in immunocytochemical studies. After 14 days of daily injections, the animals were sacrificed and processed for acetylated tubulin
immunocytochemistry. In animals that were injected with 0.165 and 0.25% CSPG; there was no observed effect on the ORN pathway. In the animals that were injected with 0.33% CSPG, the pathway exhibited decreased ORN axon outgrowth and remaining axons appeared to be extending in misguided directions. Therefore, 0.33% was the concentration used to elucidate CSPG’s role in ORN axon outgrowth.

The tenascin-C, CSPG and PBS (n=1 at each time time point) injected animals were sacrificed, fixed (as described in section 2.2.1) at 6 (n=4 for each treatment), 10 (n=2 for each treatment) and 14 (n=4 for each treatment) days post-lesion. To visualize the effects on the olfactory nerve pathway, tenascin-C, CSPG and PBS injected tissue was processed for acetylated tubulin immunofluorescence (section 2.2.9). Sections were analyzed by confocal microscopy on a BioRad MRC 1024 confocal laser scanning microscope, processed using Confocal Assistant and Adobe Photoshop 4.0. Images were printed on a Hewlett Packard Colour jet printer.

2.2.14 Golgi-Kopsch Silver Impregnation

The rapid Golgi-Kopsch staining method used was slightly modified from that of Klenoff and Greer, 1998). All steps of the Golgi stain were carried out at room temperature. Fixed larval heads (n=5, section 2.2.1) were washed in 2 changes of 0.1 M phosphate buffer (pH=7.4), 15 minutes each, then immersed into a dichromate/osmium solution overnight (Appendix). The tissue was next rinsed in a silver solution and incubated in a 1% silver nitrate solution overnight (Appendix). The heads were embedded and sectioned as described in section
2.2.1. The sections were further dehydrated by placing them in 2 changes of 95% and 100% ethanol for 30 minutes each. The sections were cleared in a 1:1 mixture of methyl salicylate and 100% ethanol for 15 minutes followed by 2 rinses in 100% methyl salicylate, 15 minutes each. Finally, the sections were incubated in 2 changes of xylene, 15 minutes each and mounted in Permount.

2.2.15 Ultrastructural analyses

To examine the ultrastructural organization of the olfactory epithelium and nerve fascicles, anesthetized larvae (n>100) were decapitated, olfactory organs and brains were dissected out and prepared for electron microscopy as described in section 2.2.1.
3. RESULTS

3.1 Ontogeny of the olfactory nerve pathway

3.1.1 Embryonic development

Embryonic development occurred in the egg, within an encapsulating chorionic membrane. Initial development occurred rapidly with hourly morphological changes. Each stage was identified by its own unique characteristics. Cell divisions resulted in a decrease of cell size but an increase in cell number (not shown). By stage 7, there were a large number of cells. Cell boundaries were indistinct and the surface appeared smooth (Fig 6A). The appearance of the head, caused by a sudden increase in the length of the neural rod, marked the commencement of stage 12 (Fig 6B). Initially, the head protruded above the yolk mass as a bud but later took on the shape of a fully formed head (Fig. 6C). At stage 13, the head began to exhibit muscular activity and at stage 14, the embryo pierced the membrane, emerging as a free-swimming prolarva (Fig. 6D).

Observation of embryonic whole mounts, prepared for immunocytochemistry, failed to show acetylated tubulin and tenascin-C immunoreactivity prior to hatching. Once the embryos hatched (stage 14), acetylated tubulin-IR axonal fibers were observed throughout the head (Fig. 7).
Figure 6 - Embryonic development

Dissecting microscope images of live embryos.

Scale bars for A and B are 400\mu m.

A. Stage 7 embryos are seen within their chorionic membranes (arrowhead).
   Numerous cells are visible on the surface of the embryo.

B. Stage 12 embryos are identified by their larger size and the appearance of a
   head bud (arrow).

C. As stage 12 advances, the head bud develops into a complete head (arrow)
   Scale bar is 650\mu m.

D. At stage 14, the embryo pierces the chorionic membrane (arrowhead) and
   emerges as a free-swimming prolarva.
   Scale bar is 200\mu m.
3.1.2 Acetylated tubulin immunocytochemistry and Dil neuronal tracing

A single olfactory placode was located on the anterior ventral surface of the head on the day of hatching (stage 14, day 13). von Kupffer (1899) originally identified this site as the olfactory placode. The work of Gorbman and Tamarin (1985) supported this finding. At this early developmental stage, the olfactory placode contained acetylated tubulin-IR cells (Fig. 7A). Acetylated tubulin IR neural fibers extended from the olfactory placode toward the brain. Other labeled axonal fibers were seen scattered throughout the head. At higher magnification and after closer examination, the cells within the olfactory placode, appeared to be somewhat bipolar in shape (Fig. 7B). By stage 17, the intensely labeled nostril was located on the dorsal surface of the prolarval head (Fig. 7C). Acetylated tubulin-IR fibers were seen innervating the mouth and the primordial eye.

During prolarval development, a single nasal cavity invaginated at the site of the olfactory placode, with the olfactory epithelium located at the base of the cavity, and immediately adjacent to the brain (Figure 8A). Previous studies have demonstrated the presence of ORNs at stage 15 and the presence of axons from presumptive ORNs in the small space between the olfactory epithelium and the brain (Fig. 8B; Zielinski et al., 1996). Although axonal pathways were forming during early prolarval development, tenascin immunoreactivity was not observed (data not shown).

Dil, a neuronal tracing dye, was used to identify the prolarval olfactory nerve pathway. Following the application of Dil into the nasal cavity, diffuse Dil
Figure 7 - The development of axonal pathways in prolarvae

Whole-mount prolarval preparations stained for acetylated tubulin with immunofluorescence.

A. In this sagittal view of a stage 14 prolarva (just after hatching), the olfactory placode, containing intense acetylated tubulin immunoreactivity, was located on the rostro-ventral region of the head (arrow). Neural fibers extended from the placode; other axonal fibers, scattered throughout the head were also visible.

Scale bar is 100μm.

B. In this high power micrograph of the stage 14 prolarva shown in A, the intensely stained cells (arrow) within the olfactory placode appear to take on the characteristic bipolar shape of ORNs. Scale bar is 50 μm.

C. In the stage 17 prolarva, the nostril was located on the dorsal aspect of the head (arrow). Intensely stained axonal pathways were observed around the eye (e). The black processes are melanophores.

Scale bar is 100μm.
Figure 8 - Ontogeny of the olfactory organ in sea lampreys

A and B are stage 15 (day 15 post-fertilization) prolarvae, two days after hatching. A is a toluidine blue stained 1 μm epoxy section and B is a transmission electron micrograph.

A. The olfactory epithelium (OE) lies at the base of a shallow invagination on the anterior surface of the head. The base of the olfactory epithelium is adjacent to the brain.

Scale bar is 25 μm.

B. This uranyl acetate and lead citrate stained section shows that axons (ax) fill the small space between the olfactory epithelium (OE) and the brain (Br).

Scale bar is 1.5 μm.
labeling was observed projecting from the posterior end of the nasal cavity (Fig 9). This labeling extended a short distance (~140 μm). A second Dil-labeled pathway projected laterally. Both sets of fibers lay between the olfactory epithelium and the olfactory bulb and were identified as the developing olfactory nerves. Therefore, at stage 17, the ORNs and their axons appear to have developed.

3.1.3 Olfactory Mucosal Neural Responses

To determine whether the prolarval olfactory system was functional, physiological tests were performed. Multi-unit neural responses in the nasal cavity of stage 17 prolarvae demonstrated stereo-specific responses to arginine: strong responses following application of L-arginine (10^{-6}M) and very weak following D-arginine (10^{-5}M; Fig. 10A). Another basic amino acid, L-histidine evoked very low response magnitudes and the neutral amino acid, L-serine, failed to elicit responses (not shown). Application of N-ω-nitro-L-arginine elicited small responses (Fig. 10B). When N-ω-nitro-L-arginine application was followed by L-arginine, the neural response was also small, suggesting that the binding site for L-arginine was blocked. On the other hand, when application of the L-arginine analogues (N-ω-nitro-L-arginine) was followed by the bile acid, taurocholic acid, a strong response was observed (Fig. 10B). After several rinses, the L-arginine response was restored. This result suggests that L-arginine and taurocholic acid bind onto different receptor proteins or that they are modulated by different transduction pathways.

The response magnitude was concentration dependent to taurocholic acid
Figure 9 - Innervation of the prolarval nasal cavity

A horizontal view of a whole-mount preparation of an anterogradely Dil-labeled stage 17 prolarva. Two sets of fibers extend from the nasal cavity (NC). Diffusely labeled streaks extended caudally (arrows). Well-defined fibers emerged caudo-laterally (arrowheads).

Scale bar is 100μm.
Figure 10 - Olfactory mucosal neural responses

Olfactory mucosal neural responses of stage 17 prolarvae to L-arginine, D-arginine, N-ω-nitro-L-arginine and to taurocholic acid.

A. $10^{-6}$ M L-arginine; $10^{-6}$ M D-arginine

B. $10^{-6}$ M L-arginine; $10^{-6}$ N-ω-nitro-L-arginine; $10^{-6}$ M L-arginine; $10^{-8}$ M taurocholic acid. These were presented in a sequence.

C. $10^{-10}$ M taurocholic acid; $10^{-8}$ M taurocholic acid
A

B

L-arg  D-arg

1 mV  

100 msec

L-arg  Nω-nitro-L-arg

L-arg  Taurocholic acid

C

Taurocholic acid $10^{-10}$M  Taurocholic acid $10^{-8}$M
(Fig. 10C), and to other chemostimulatory compounds. Application of the lamprey pheromone, petromyzonol sulphate \((10^{12}M, \text{Li et al., 1995})\) elicited very large magnitude responses \((4 \text{ mV}; \text{Fig. 11A})\). The magnitude of olfactory mucosal neural responses to water conditioned by conspecifics varied with the developmental stage of lampreys that conditioned the water. The response magnitudes evoked by water conditioned by stage 17 prolarvae varied from 1 to 4 mV \((\text{Fig. 11A})\). Water conditioned by Piavis stage 12 embryos elicited 2 to 2.5 mv responses \((\text{Fig. 11B})\). Water conditioned by ammoeocetes elicited olfactory mucosal neural responses of a lower magnitude than water conditioned by stage 17 prolarvae or \(10^{-6}M\) L-arginine \((\text{Fig. 11B})\).

3.2 Larval olfactory nerve pathway

3.2.1 Organization of the olfactory nerve pathway

Previous studies have shown that the olfactory mucosa in larval lampreys line a simple nasal cavity and contained ORNs with olfactory knobs of approximately 1 μm in diameter \((\text{Vandenbossche et al., 1995})\). During metamorphosis, the mucosa folded and the size of ORNs increased. These facts suggest that the olfactory organ of in the larval lamprey would be an appropriate model for examining signaling in a developing olfactory pathway.

Anterograde Dil labeling clearly showed the olfactory epithelium and two olfactory nerves extending into the olfactory bulb \((\text{Fig. 12A})\). In low power views of these preparations, the terminals of ORNs within the olfactory bulb were not clearly visible. The arrangement of axons within the olfactory pathway was
Figure 11 - Olfactory Mucosal Neural Responses

Olfactory mucosal neural responses of stage 17 prolarvae to petromyzonol sulfate and to water conditioned by conspecifics.

A. Petromyzonol sulfate \((10^{-12} \text{ M})\); water conditioned by stage 17 prolarvae.

B. Water conditioned by stage 12 embryos; water conditioned by ammocoetes.
Figure 12 - The organization of the larval olfactory nerve pathway

A. A horizontal view of Dil-labeling in the larval sea lamprey olfactory organ.

The olfactory epithelium (OE) lines the posterior surface of the two shallow alcoves in the nasal cavity. Axonal projections were seen extending from the OE, merging to form the olfactory nerve (ON) and contacting the olfactory bulb (OB).

Scale bar is 50 μm

B and C are transmission electron micrographs with the same magnification.

Scale bar shown in C is 1μm.

B. The organization of axons at the interface between the olfactory epithelium (OE) and the lamina propria (LP). The OE is located above the basal lamina (dotted line), and the LP is located below the basal lamina. Small clusters of axons (arrows) are located in the OE, subjacent to the basal lamina and within the LP.

C. An olfactory nerve fascicle ensheathed by a Schwann cell (Sc) located in the lamina propria. Axons are marked with an asterisk (*).
viewed by transmission electron microscopy. Small groups of ORN axons merged within the epithelial portion of the olfactory mucosa and traversed the basal lamina into the lamina propria (Fig. 12B). Bundles of axons of relatively uniform diameter joined, forming large unmyelinated fascicles surrounded by Schwann cells (Fig. 12C).

3.2.2 Topographic mapping of the olfactory nerve pathway

GS-1-B₄ histochemistry was shown to label the olfactory epithelium and the olfactory bulb in larval lampreys (Tobet et al., 1996). In the present study, GS-1-B₄ histochemistry of cryosections, revealed the pathway followed by the olfactory nerve. The images shown in figure 13 represent the right portion of the nasal cavity. The same pattern is mirrored in the contralateral side. Intense labeling was localized on the olfactory epithelium, especially at the apical and basal surfaces (Fig. 13). ORN axons merged within the olfactory epithelium, penetrated the basement membrane forming olfactory nerve fascicles. Two branches of the olfactory nerve emerged from the olfactory mucosa. The most dorsal, was a lateral branch (Fig. 13B-G). The lateral branch was thick, extending a depth of 140 μm (distance of A-B to G), and appeared to contain ORN axons that extended from the olfactory epithelium lining the lateral portion of the olfactory mucosa. The lateral branch extended parallel to the lateral surface of the olfactory mucosa, then merged with a second branch of the olfactory nerve, the medial branch (Fig. 13 E, F) and extended towards the olfactory bulb. The medial branch of the olfactory nerve recruited ORN axons from the medial portion of the olfactory mucosa (Fig. 13E-H). The medial branch
Figure 13 - Topographic mapping of the olfactory nerve pathway

Confocal imaging of cryosections, from the right portion of the nasal cavity, processed with GS-1-B4 isolectin histochemistry.

Scale bar is 50 µm.

A. A dorsal section through the olfactory organ demonstrating intense staining in the apical and basal regions of the olfactory epithelium. Olfactory nerve fascicles project laterally and medially from the olfactory epithelium (OE) into the lamina propria (LP).

B. Lateral olfactory nerve fascicles have merged to form the lateral branch (L) of the olfactory nerve. Short axonal projections are visible subjacent to the olfactory epithelium.

C. In this section, similar to B, the lateral branch of the olfactory nerve is again visible.

D. In this medial section, the lateral branch of the olfactory nerve has recruited many ORN axons from the lateral olfactory organ, demonstrated by its increasing thickness.

E. In this section, the medial branch (M) of the olfactory nerve is observed, recruiting ORN axons from the medial part of the olfactory organ. The medial branch is seen to merge with the lateral branch of the olfactory nerve. Small axonal aggregates are visible below the olfactory epithelium.

F. In this section, the medial branch appears to be thicker. Small axon aggregates, subjacent to the olfactory epithelium, have become more prominent.
G. In this section, lateral and medial branches are visible with small axon aggregates, below the olfactory epithelium.

H. In this ventral section, radially projecting fascicles are prominent within the LP.
was shorter than the lateral branch: (depth of 80 μm). At the ventral edge of the olfactory mucosa, abundant, diffusely arranged fibers filled the lamina propria. Small axon aggregates were visible from the medial olfactory organ to the ventral olfactory organ. This pattern shows that in the ventral region, the olfactory nerve innervates the olfactory epithelium evenly. Therefore, the topographic mapping of the olfactory nerve pathway is comprised of a lateral portion extending from the lateral olfactory mucosa, a medial branch from the medial olfactory mucosa and radial fibers in the ventral olfactory organ. This spatial pattern was constant in all larvae examined in this study.

3.2.3 Distribution of acetylated tubulin and GAP-43-immunoreactivity

Acetylated tubulin and GAP-43 immunocytochemical staining of horizontal sections of the larval lamprey clearly showed the olfactory nerve pathway and indicated its state of development. Intense acetylated tubulin-immunoreactivity was localized within the olfactory epithelium, in cilia, dendrites and axons of ORNs; also in non-myelinated nerve fascicles below the olfactory epithelium and in the olfactory nerve (Fig. 14A). Intense acetylated tubulin immunoreactivity within nerve fascicles of the lamina propria indicated the abundance of axons within nerve fascicles (Fig. 14A, B). GAP-43-immunoreactivity was intense in the middle portion of the olfactory epithelium and in olfactory nerve fascicles and in the olfactory nerve (Fig. 14B).
Figure 14 - Acetylated tubulin, GAP-43 and tenascin-C immunoreactivity within the olfactory nerve pathway

A. In the olfactory epithelium (OE), intense acetylated tubulin immunoreactivity was present in cilia, ORN dendrites and axons. Small nerve fascicles, the olfactory nerve (ON) and bulb (OB) were labeled intensely.

Scale bar for A is 50 μm and 25 μm for B and C.

B. GAP-43 immunoreactivity was intense in differentiating cells of the olfactory epithelium and in the olfactory nerve (ON).

C. Tenascin-C immunoreactivity was strong in the olfactory epithelium (OE) and in the olfactory nerve (ON). Weak, diffuse tenascin-C immunoreactivity was present in the loose connective tissue surrounding the ON.
3.2.4 Distribution of tenascin-C immunoreactivity

Whereas acetylated tubulin and GAP-43-immunoreactivity appeared to mark ORN axons, tenascin-C distribution was slightly different. Tenascin-C immunoreactivity was strong in the olfactory epithelium, moderate in small nerve fascicles and strong in the olfactory nerve (Fig. 14C, 19H).

3.2.5 Specificity of tenascin-C antibodies

Two polyclonal tenascin-C antibodies were used in this research. As polyclonal antibodies usually bind to more than one epitope, experiments were performed to confirm the specificity and validity of the immunoreactivity observed with these antibodies. Incubation with the preadsorbed tenascin-C antibody (Chemicon) did not exhibit any immunoreactivity. These results confirmed that this polyclonal tenascin antibody was specific to tenascin (Fig. 15A). In contrast, immunocytochemical preparation with this antibody revealed tenascin distribution in the olfactory epithelium and nerve fascicles within the olfactory nerve pathway (Fig. 15B).

The specificity of the second tenascin-C antibody (courtesy of Dr. Faissner, University of Heidelberg, Dept. of Neurobiology, Heidelberg, Germany) was tested by Western immunoblot analysis (Fig. 15C). Tenascin-C glycoproteins display molecular weights ranging from 190-220 kDa (Erckson and Bourdon, 1989). In the immunoblot, the first lane contained the broad range molecular weight marker (lane 1), the second, bovine serum albumin (lane 2). These lanes showed no immunoreactive (IR) bands as the proteins in lanes did not contain
Figure 15 - Specificity of tenascin-C immunoreactivity

The specificity of the two polyclonal tenascin-C antibodies was tested with a pre-adsorption control and a Western immunoblot.

A. The tissue treated with the pre-adsorbed tenascin-C antibody (Chemicon) did not exhibit immunoreactivity in the olfactory epithelium (OE) or in olfactory nerve fascicles of the lamina propria (LP), confirming the specificity of the antibody.

Scale bar is 100 μm.

B. This tissue, processed for immunocytochemistry using a polyclonal anti-tenascin-C antibody (Chemicon) and DAB as the chromagen, demonstrated immunoreactivity. Intense staining was observed throughout the olfactory epithelium (OE), especially in the apical portion and in olfactory nerve fascicles (arrowheads) within the lamina propria (LP).

Scale bar is 100 μm.

C. Polyclonal tenascin-C antibody specificity (courtesy of Dr. Faissner) was demonstrated by western immunoblot analysis and ECL detection of the coloured product formed from the interaction of an anti-rabbit IgG secondary antibody conjugated to horse radish peroxidase with luminol. The arrow demarcates the 202 kDa myosin molecular weight marker. The broad range molecular weight marker (lane 1) and bovine serum albumin (negative control, lane 2) demonstrated no immunoreactivity. Human tenascin-C purified protein (positive control, lane 3) and larval skin homogenate (lane 4) exhibited immunoreactive bands of similar intensities at approximately 200 kDa, as
determined from the molecular weight marker on the nitrocellulose membrane.

Larval olfactory organ and bulb homogenate (lane 5) exhibited a weaker immunoreactive band at 200 kDa.
tenascin. Purified human tenascin-C protein, the positive control (lane 3), exhibited an intense band at approximately 200 kDa. Larval skin was tested because of the intense immunoreactivity it exhibited in immunocytochemical preparations (lane 4). This lane demonstrated a highly IR band at 200 kDa of similar intensity to the positive control. The olfactory organ (lane 5) displayed a 200 kDa weaker IR band. These results, using pre-adsorption immunocytochemistry and Western immunoblot analysis, confirmed that the immunoreactivity observed with both antibodies was specific to tenascin-C.

3.2.6 Distribution of laminin and CSPG immunoreactivity

Laminin and CSPG are ECM molecules that have previously been shown to modulate axon outgrowth (Kafitz and Greer, 1997; Margolis and Margolis, 1997). Their immunolocalization within the larval olfactory nerve pathway implicates them as putative modulators of the olfactory nerve pathway. Laminin immunofluorescence was intense in the apical region of the olfactory epithelium (Fig. 16A). Olfactory nerve fascicles, the olfactory nerve and the olfactory nerve layer of the olfactory bulb were also highly IR (Fig. 16A, B). This distribution supports previous studies of laminin's role as a guidance and an axon outgrowth promoting molecule (Kafitz and Greer, 1997).

Intense CSPG immunofluorescence was observed in the nasal cartilage adjacent to the olfactory organ (Fig. 17A). On the lateral olfactory mucosa, small ORN fascicles that were present in the lamina propria fasciculated and turned caudally within 43 μm of the CSPG-rich nasal cartilage (Fig. 17B). ORN axons, in the lateral branch of the olfactory nerve, maintained this distance along the
Figure 16 - Distribution of laminin within the larval olfactory nerve pathway

Confocal images of laminin immunofluorescence within the olfactory nerve pathway.

Scale bars are 50μm.

A. Intense laminin-immunoreactivity was present within the apical region of the olfactory epithelium (OE), in olfactory nerve fascicles (arrowhead), along the olfactory nerve (arrows) and within the olfactory nerve layer of the olfactory bulb (OB).

B. At higher magnification, small nerve fascicles (arrowhead) within the lamina propria, exhibiting intense immunoreactivity were evident.
Figure 17 - Distribution of CSPG within the larval olfactory nerve pathway

A. Cryosection processed for CSPG immunohistochemistry with DAB as the chromagen. Intense immunoreactivity, seen as dark blue staining, was present in the nasal cartilage lateral to the olfactory epithelium (OE).

Scale bar is 25μm.

B. Confocal image of CSPG-IR (processed with texas red) and acetylated tubulin-IR (processed with fluorescein) within the olfactory nerve pathway demonstrating CSPG's localization in relation to ORN axons. Similar to figure 17A, CSPG immunofluorescence was localized within the nasal cartilage (NC). The acetylated tubulin-IR ORN axons (green), deflected towards the olfactory bulb, avoiding the CSPG-rich cartilage (red). ORN axons appeared to maintain a constant distance from the cartilage.

Scale bar is 50μm.
length of the cartilage and merged to form the main olfactory nerve, caudal to the cartilage. These results may suggest that CSPG influences outgrowth and guidance of the lateral branch of the olfactory nerve pathway or that ORNs axons cannot grow through cartilage.

3.2.7 The larval lamprey olfactory system as a model for the development of axonal pathways

To investigate the feasibility of using the olfactory pathway of the larval lamprey for examining axonal outgrowth, synchronous neurogenesis of ORNs was induced by olfactory nerve axotomy. The retrograde degeneration of ORNs influenced the morphology of the olfactory epithelium. In the olfactory epithelium from untreated larvae, ORNs were identified by the rounded ciliated olfactory dendritic knobs protruding into mucociliary matrix, slight narrowing of the apical portion of the dendrite and by the non-vesicular cytoplasm. compared to neighbouring vesicular sustentacular cells (Fig. 18A). The numerical density of ORNs was $11.86 \pm 3.4$ ORNs /100 $\mu$m), and the thickness of the olfactory epithelium was 70 $\mu$m (Figure 18A). On the third day following trans-section of the olfactory nerves (day 3), ORN density decreased to 6.30 $\pm$ 1.6 ORNs /100 $\mu$m and the thickness of the olfactory epithelium was reduced to 50 $\mu$m (Figure 18B). By day 14 post-lesion, ORN density and the thickness of the olfactory epithelium was almost restored to control levels (ORN density: 10.00 $\pm$ 2.9 ORNs /100 $\mu$m; thickness of olfactory epithelium: 68 $\mu$m, Fig. 18C). The numerical decline of ORNs during retrograde degeneration of the olfactory nerve and
Figure 18 - The influence of olfactory nerve axotomy on the olfactory epithelium

Sections were stained with toluidine blue. The basement membrane of the olfactory epithelium is indicated by a dotted line.

A. Before trans-section (D0), the olfactory epithelium contained ciliated olfactory knobs of olfactory receptor neuron dendrites (arrows). The apical region of sustentacular cells (sc) was wider and more vesicular than the olfactory receptor neuron dendrites.

B. Three days following axotomy (D3), olfactory knobs were absent and the olfactory epithelium was shorter.

C. Fourteen days following axotomy (D14), the olfactory epithelium regained its original thickness. Olfactory knobs (arrow) protruded from olfactory receptor neuron dendrites.

Scale bar is 10 μm
subsequent return of these cells after 14 days, confirmed the occurrence of synchronous neurogenesis following olfactory nerve axotomy.

3.2.8 ORN axon outgrowth following axotomy-induced ORN retrograde degeneration

Following an olfactory nerve axotomy, short immature ORN axons converged into olfactory nerve fascicles within the lamina propria. The location of axonal processes was revealed by acetylated tubulin and GAP-43 immunoreactivity. The examination of ORN axon outgrowth focused on fibers within the lamina propria of the olfactory mucosa. The intense acetylated tubulin IR fascicular staining pattern observed in unlesioned animals (Fig. 19A, B) was absent on the third day following olfactory nerve trans-section (Fig. 19C). Instead, short, diffuse, acetylated tubulin-IR unfasciculated processes extended from the olfactory epithelium into the lamina propria. These may have been axons extending from the cell bodies of young ORNs within the olfactory epithelium. Within the olfactory epithelium, acetylated tubulin-immunoreactivity was concentrated in the apical region, presumably reacting with tubulin within the cilia and striated rootlets of sustentacular cells (Vandenbossche et al., 1995). By day 14, bundles of intensely stained acetylated tubulin- IR fibers in the lamina propria indicated the re-appearance of ORN axons within fascicular boundaries, although some fibers coursed independently (Fig. 19D). GAP-43- immunoreactivity marked the location of developing ORN axons during retrograde degeneration and axon outgrowth. GAP-43-IR nerve fibers within fascicles in unlesioned tissue (Fig.19E), were absent by the third day following
Figure 19 - Spatial-temporal changes in immunolocalization of acetylated tubulin, GAP-43 and tenascin-C following axotomy of the olfactory nerve

Upper row, before trans-section (D0); middle row, day 3 following axotomy (D3); lower row, day 14 following axotomy (D14). Left panel: acetylated tubulin – immunoreactivity (AT); middle panel: GAP 43- immunoreactivity (GAP); right panel: tenascin-C- immunoreactivity (TN).

The dotted line indicates the location of the basement membrane between the olfactory epithelium and the lamina propria.

A. Acetylated tubulin- immunoreactivity was intense within the apical and basal regions of the olfactory epithelium (OE). In the lamina propria (LP) acetylated tubulin - immunoreactivity was present in nerve fascicles.

B. In the lamina propria, nerve fascicles (NF) viewed in cross-section showed a patchy distribution of acetylated tubulin immunoreactivity.

C. Acetylated tubulin on the third day following axotomy. The olfactory epithelium is to left of the dotted line and lamina propria is to the right of the dotted line. The olfactory epithelium contains relatively weak acetylated tubulin -IR. Diffusely arranged short acetylated tubulin-IR processes extend from the olfactory epithelium into the lamina propria (arrows).

D. Acetylated tubulin on the fourteenth day following axotomy; intensely stained IR nerve fascicles (arrows) are seen in the lamina propria.

E. GAP-43 immunoreactivity in unlesioned tissue. The lamina propria, located to the right of the dotted line contained nerve fascicles (NF) with GAP-43- IR fibers.

F. GAP-43 immunoreactivity on the third day following olfactory nerve lesion.
The olfactory epithelium (left of the dotted line) contains moderate staining. Diffuse GAP 43-IR strands (arrows) extend into the lamina propria. Black granules within the lamina propria at the top of the micrograph (m) are melanin granules within a melanophore.

G. GAP-43 IR on day 14. The white asterisks (•) indicate the location of the basement membrane between the olfactory epithelium and the lamina propria. The olfactory epithelium contains intense staining and immunoreactive fibers are seen within the lamina propria (arrows) that converge with large fascicles (NF).

H. Tenascin staining in unlesioned control tissue. The lamina propria, located to the right of the dotted line, contains tenascin-C -IR (arrow) with an uneven distribution with nerve fascicles.

I. On day 3, tenascin-C IR is absent from the lamina propria. Migratory phase melanophores (m) with retracted processes are present.

J. In the lamina propria at day 14, nerve fascicles are tenascin-C-IR (arrows). The surrounding loose connective tissue is diffusely stained.

Scale bar is 10 µm.
axotomy (Fig. 19F). At this time of initial axon outgrowth, GAP-43 immunoreactivity was limited to short autonomous processes extending from the olfactory epithelium. Melanophores were frequently observed in the lamina propria. By day 14, GAP-43 staining was intense, abundant and GAP-43 IR fibers were organized into bundles (Fig. 19G). Small fascicles that extended from the olfactory epithelium merged, forming larger bundles deeper in the lamina propria.

3.2.9 Tenascin-C immunoreactivity following axotomy

The olfactory pathway of the larval lamprey was used to investigate spatial-temporal distribution of tenascin during synchronous neurogenesis and axon outgrowth following olfactory nerve axotomy. In unlesioned animals, tenascin-C immunoreactivity had a fibrillar pattern within nerve fascicles (Fig. 19H). In the lamina propria, tenascin-C immunoreactivity did not appear to be uniform throughout each fascicle; it appeared to be stronger in some parts of the olfactory nerve fascicles and absent from other parts. By the third day following axotomy, tenascin-C immunoreactivity was completely absent from the lamina propria (Fig. 19I). By the fourteenth day, when the ORN axon fibers were aggregated into characteristic fascicles, tenascin-C immunoreactivity was present in nerve fascicles (Fig. 19J). Diffuse tenascin-C immunoreactivity was prevalent throughout loose connective tissue within the lamina propria. In specimens that had undergone sham operations, there was no change in tenascin-C immunoreactivity at the experimental time points. The results, summarized in Table II, indicate that tenascin-C was absent during the period of
ORN retrograde degeneration and initial axon outgrowth but returned when axons were long, numerous and organized into fascicles.

Table II: Localization of acetylated tubulin, GAP-43 and tenascin-C immunoreactivity during retrograde degeneration of the olfactory nerve and subsequent axonal growth.

<table>
<thead>
<tr>
<th>Axonal pathway</th>
<th>Days following lesion</th>
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<tr>
<td>Axons within the olfactory nerve pathway</td>
<td>Acetylated tubulin, GAP-43, Tenascin-C</td>
</tr>
<tr>
<td>Autonomous axons extending from the olfactory epithelium into the lamina propria</td>
<td>Absent</td>
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</table>

* included scattered acetylated tubulin-IR axonal processes in the lamina propria that presumably correspond to fibers innervating blood vessels.

3.2.10 Ultrastructure of ORN fascicles during initial axon outgrowth

The fact that tenascin-C-IR was absent during initial ORN axon outgrowth suggests that morphological differences may accompany the loss of tenascin expression. Transmission electron microscopy was used to examine the ultrastructure of ORN fascicles during early outgrowth (day 3) following an
olfactory axotomy. In unlesioned animals, groups of axonal processes were present above and below the basal lamina (Fig. 20A, previously shown as Fig. 12B). On the third day following axotomy, small axons were seen aggregated in the basal region of the olfactory epithelium. The basal lamina was absent from the site with these axons (Fig. 20B). In unlesioned animals, olfactory nerve fascicles within the lamina propria were enveloped by Schwann cells. Fascicles that contained large numbers of axonal processes were common in the lamina propria (>50 axons, Fig. 20C). In day 3 post-lesion animals, these large olfactory nerve fascicles were absent; small fascicles with fewer, irregularly shaped axons were present (<15 axons, Fig. 20D)

3.2.11 Effects of anti-tenascin-C on ORN axons

The role of tenascin-C on ORN axons was tested by an antibody perturbation study. In larvae that received PBS injections for 14 days, intense acetylated tubulin immunoreactivity was observed within the olfactory epithelium, in short axonal processes subjacent to the olfactory epithelium and in olfactory nerve fascicles within the lamina propria (Fig. 21A). In larvae that were injected with anti-tenascin-C (1:500) for 14 days, acetylated tubulin immunoreactivity was faint within the olfactory epithelium (Fig. 21B) and in olfactory nerve fascicles within the lamina propria. Olfactory nerve fascicles appeared thinner and discontinuous compared to PBS injected controls, indicating a reduction in ORN axon outgrowth. Axonal projections that emerged from the olfactory epithelium in PBS injected controls, were absent in anti-tenascin-C injected animals. These results show that when endogenous tenascin reacted to the injected tenascin
Figure 20 - Ultrastructural effects of an olfactory nerve lesion on olfactory nerve fascicles

Transmission electron micrographs of uranyl acetate and lead citrate stained sections from control (A, C) and three day post-lesion larvae (B, D).

All micrographs are at the same magnification.

Scale bar shown in A is 1μm.

A. In unlesioned tissue, previously shown as Fig. 12B, axonal processes were present in the olfactory epithelium (OE, arrow) and in the lamina propria (LP, arrowheads).

B. In 3 day post-lesion tissue, axons were aggregated in the basal region of the olfactory epithelium (OE). The basal lamina was absent from areas containing these axon aggregates.

C. In the lamina propria of unlesioned tissue, relatively large olfactory nerve fascicles (arrow), within the lamina propria were enveloped by Schwann cells (Sc). This fascicle contained more than 50 ORN axons.

D. In 3 day post-lesion tissue, small olfactory nerve fascicles (arrow) were present within the lamina propria (LP). This fascicle contained no more than 15 ORN axonal processes. The axons appeared vacuolated, with electron-dense patches.
Figure 21 - Effects of anti-tenascin-C on ORN axon outgrowth

PBS and anti-tenascin-C injected, unlesioned tissue sections processed for acetylated tubulin (AT) immunoreactivity.

Scale bars are 25 µm.

A. 14 day PBS injected tissue exhibited moderate AT-immunoreactivity in the olfactory epithelium (OE) and in short axons projecting from the OE (arrow). The olfactory nerve (arrowhead) within the lamina propria (LP) was highly immunoreactive, extending deep within the LP.

B. 14 day anti-tenascin-C injected tissue demonstrated faint AT-immunoreactivity within the OE. Olfactory nerve fascicles (arrowheads) were fainter staining and shorter; since they did not extend deep into the LP.
antibody; the expression of acetylated tubulin was reduced. Furthermore, the function of tenascin-C on ORN axons may be inferred. The loss of acetylated tubulin immunoreactivity immediately adjacent to the olfactory epithelium and the reduction in acetylated tubulin immunoreactivity throughout the lamina propria may suggest that tenascin-C is needed to maintain the normal converging pathway of ORN axons into small fascicles, then merging into the larger branches of the olfactory nerve.

3.2.12 Effects of exogenous tenascin-C on ORN axon outgrowth

Previous data showed an absence of tenascin, three days following an olfactory nerve lesion and its gradual re-appearance by day 14 post lesion (Fig. 19I and J). In the absence of tenascin-C, short, diffuse, unfasciculated axon processes extended from the olfactory epithelium. Fourteen days following axotomy, both tenascin-C and olfactory nerve fascicles were present. Functional blocking of tenascin-C, in the tenascin-C antibody perturbation study, showed a decrease in the expression of acetylated tubulin implying a reduction in the number and size of olfactory nerve fascicles. These results propose that tenascin-C may be needed to maintain axons within the olfactory pathway.

To view the effect of tenascin-C on the process of ORN axon outgrowth, exogenous tenascin-C was injected during the period of axon outgrowth (days 3-14) following olfactory nerve axotomy. Tenascin-C was injected into two sites in axotomized larvae, into the nostril and subcutaneously along the pathway of the olfactory nerve (between the olfactory epithelium and the pineal gland). 0.25mL of 0.2% tenascin-C was injected into each injection site (tenascin-C dose was
determined by antibody perturbation studies and a previous study by Langenfeld-Oster et al., 1994). Injections into the nostril, bypassed the blood-brain barrier providing direct access to the olfactory nerve pathway. Subcutaneous injections were made in the vicinity of ORN axon outgrowth to ensure that injected tenascin-C was present in areas of active ORN axon outgrowth, to exert its effect. The injections commenced on the third day following axotomy since initial ORN axon outgrowth following the lesion was visible at this time point (Fig. 19C, F). Animals were sacrificed on days 6, 10 and 14 following axotomy and examined for the spatial pattern of acetylated tubulin immunofluorescence.

Although four animals were processed for each of the tenascin experimental time points, not all larvae showed the effects described below. Preliminary observations are provided in a table in the Appendix. Axotomized larvae receiving daily injections of the carrier solution (PBS) were also examined. On the sixth day following axotomy, tissue from daily PBS injected animals showed intense acetylated tubulin immunoreactivity within the olfactory epithelium. ORN axon outgrowth was evident from short ORN fascicles subjacent to the olfactory epithelium and from axons growing between the aggregates (Fig. 22A). Tissue from animals injected daily with 0.2% tenascin and fixed on the sixth day following axotomy, exhibited intense acetylated tubulin immunoreactivity within slender processes in the apical portion of the olfactory epithelium typical of ORN dendrites (n=2, Fig. 22B). Olfactory nerve fascicles were seen extending from the olfactory epithelium and were spaced apart, with few, if any axonal processes visible in between the fascicles. On the tenth day
Figure 22 - Effects of exogenous tenascin-C on ORN axon outgrowth

Confocal images of PBS and tenascin-C injected, lesioned animals at 6, 10 and 14 days post-lesion. A and D is lesioned tissue injected with PBS. B, C and E is lesioned tissue injected with tenascin. Lesioned tissue was processed for acetylated tubulin immunofluorescence.

Scale bars are 25 μm.

A. In six day post-lesion, PBS injected tissue, the olfactory epithelium (OE) exhibited intense AT-immunoreactivity at the apical surface. Olfactory nerve fascicles (●) were seen projecting from the OE into the lamina propria. Smaller, developing axonal projections were seen dispersed between the fascicles.

B. In six day post-lesion, tenascin-C injected tissue, ORN dendritic processes exhibited intense immunoreactivity within the OE. Olfactory nerve fascicles (●) projected from the OE into the LP. Fascicles appeared to be spaced apart with little or no visible axon outgrowth between the fascicles.

C. In ten day post-lesion, tenascin-C injected tissue, acetylated tubulin immunofluorescence was clumped and irregular within the OE. ORN axonal fasciculation (●) was prominent throughout the lamina propria (LP).

D. In fourteen day post-lesion, PBS injected tissue, the OE exhibited intense immunoreactivity within the ORN dendritic processes and the lateral branch of the olfactory nerve (●). Many AT-IR axonal processes projected from the OE to the lamina propria. Larger immunoreactive olfactory nerve fascicles were observed within the lamina propria.

E. In fourteen day post-lesion, tenascin-C injected tissue, apical and basal regions of the OE stained intensely. Short axonal processes emerged from the OE which appeared to be thicker than those seen in the PBS control. Prominent olfactory nerve fascicles (●) were seen in the lamina propria.
following an olfactory nerve lesion, in 0.2% tenascin injected animals, the acetylated tubulin immunoreactivity in the olfactory epithelium was clumped and irregular. Axonal fasciculation was prominent within the lamina propria. These large fascicles extended caudally, towards the olfactory bulb (n=2, Fig. 22C). On the fourteenth day post-lesion, tissue injected with PBS, demonstrated intense acetylated tubulin immunofluorescence in ORN dendrites and initial axonal segments within the olfactory epithelium (Fig. 22D). In the lamina propria, acetylated tubulin immunoreactivity was present in small axonal aggregates and in the olfactory nerve. In 0.2% tenascin-C injected, fourteen day post-lesion tissue, acetylated tubulin immunoreactivity was intense in the apical and basal portions of the olfactory epithelium (n=3, Fig. 22E). Likewise, ORN axons revealed irregular patterns of outgrowth and fasciculation in the lamina propria, immediately subjacent to the olfactory epithelium. The axon fibers stained intensely for acetylated tubulin and seemed to be grouped into fascicles at the base of the olfactory epithelium. Posteriorly, within the lamina propria, prominent acetylated tubulin-IR olfactory nerve fascicles were seen.

These preliminary results: widely spaced fascicles during initial axon outgrowth, prominent large fascicles by day 10 and massive ORN axon outgrowth at day 14 that failed to follow the usual fascicular pattern of convergence suggest that tenascin-C may signal events associated with the selection of the pathway taken by outgrowing axons.
3.2.13 Effects of exogenous chondroitin sulfate proteoglycan on ORN axon outgrowth

Immunolocalization studies have demonstrated 1) the presence of CSPG within cartilage surrounding the olfactory organ, 2) the pathway of ORN axons is parallel to this cartilage (section 3.2.6). Following olfactory axotomy, the pathway of outgrowing ORN axons repeats this pattern. Outgrowing axons grow caudally toward the olfactory bulb, they do not grow toward the cartilage. As previous studies have shown that CSPG is a potent inhibitor of neurite elongation during development and following injury (Brittis et al., 1992; Cole and McCabe, 1991); CSPG from the nasal cartilage may signal to ORN axons to inhibit axon elongation in the direction of the cartilage. To help establish a role for CSPG in the modulation of the larval olfactory nerve pathway, exogenous CSPG was injected daily during ORN axon outgrowth. CSPG dosage was determined by dose response experiments in which only 0.33% CSPG had an effect on the ORN pathway. Tissue from 0.33% CSPG injected animals, on the sixth day following axotomy was processed for acetylated tubulin immunofluorescence. The arrangement of acetylated tubulin immunoreactivity appeared to be irregular (n=3, Fig 23B) compared to PBS injected controls (Fig. 23A). Although some acetylated tubulin nuclear structures were visible at the apical surface of the epithelium, the olfactory epithelium was distorted. Intensely stained axon fibers were observed coursing through the olfactory epithelium. In axotomized larvae, injected daily with 0.33% CSPG for 14 days, then processed for acetylated tubulin immunofluorescence, ORN axonal processes were very short (n=4, Fig. 23D). No fascicles were visible within the lamina propria as seen in PBS.
Figure 23 - Effects of exogenous CSPG injections on ORN axon outgrowth following olfactory nerve lesion.

Confocal images of PBS and CSPG-injected, lesioned animals at 6 and 14 days post-lesion, immunostained with acetylated tubulin.

Scale bars for A, B and are 50 μm

A. In six day post-lesion, PBS injected tissue, ORN axon fascicles (arrows) projected from the olfactory epithelium (OE) into the lamina propria (LP). Short axons were observed between the OE and ORN fascicles. A few aberrant axons were visible within the OE.

B. In six day post-lesion, CSPG-injected tissue, the olfactory epithelium (OE) had degenerated. Cells of the olfactory epithelium (OE) were unidentifiable. ORN axonal fibers were seen coursing along the degenerating OE in unusual directions but there was no evidence of new ORN axon outgrowth. Inset, in the upper left corner, shows the orientation of the olfactory organ and the extent of ORN axon outgrowth.

C. In fourteen day post-lesion, PBS injected tissue, the OE was highly immunoreactive with short immunoreactive axon processes projecting into the LP. Intensely immunoreactive olfactory nerve fascicles were seen projecting caudad to the olfactory bulb.

Scale bar is 10 μm.

D. In fourteen day post-lesion, CSPG injected tissue, short ORN axonal projections were observed below the OE but no fascicles were visible within the lamina propria (LP). Scale bar is 50 μm.
injected, fourteen day post-lesion tissue (Fig. 23C). The apical region of the olfactory epithelium stained intensely but the acetylated tubulin immunoreactivity was clumped. Rather than appearing in dendritic processes, as in the PBS injected tissue (Fig. 23C); the acetylated tubulin immunoreactivity was clumped with small processes, presumably cilia, extending into the lumen. These preliminary results, with exogenous CSPG injections, demonstrating a reduction in ORN axon outgrowth and distortion of the olfactory epithelium clearly show the destructive effects of CSPG on ORN axon outgrowth. This interpretation may be supported by the absence of ORN axon outgrowth in the vicinity of the CSPG-rich nasal cartilage of unlesioned larvae (Fig. 17A, B).

3.3 The olfactory bulb

3.3.1 Distribution of nuclei

The larval olfactory bulb is located immediately anterior to the telencephalon. As previously seen in adult lampreys, the olfactory bulb is diffusely organized into four primary layers: the olfactory nerve, glomerular, granule cell and ependymal cell layers (Iwahori et al., 1987, Fig. 24). The olfactory nerve layer contains ORN axons that project over the bulbar surface. It is a relatively thin layer covered with melanophores. Spherical regions of neuropil (glomerular-like structures) are located in the glomerular layer. Here, ORN axons form synaptic junctions with central (mitral cell) processes. The glomerular layer, the first integrative center of the olfactory bulb, contains very
Figure 24 - Distribution of nuclei within the olfactory bulb

This cresyl violet-stained cryosection demonstrates the presence of four diffuse layers within the olfactory bulb. The olfactory nerve layer (ONL) is a thin layer consisting of ORN axons ensheathed by melanophores. The glomerular layer (GL) contains paler-staining, spherical glomerular-like structures, outlined by darker staining periglomerular cell nuclei. The granule cell layer (GR) is a cell rich region characterized by the presence of scattered nuclei. The ependymal cell layer (EL) is distinguished by stratified nuclei which line the lateral ventricle. Scale bar is 50 μm.
few cell bodies. The next layer, the granule cell layer is a cell rich region characterized by the presence of scattered nuclei. The ependymal cell layer is distinguished by stratified nuclei lining the lateral ventricle.

3.3.2 Golgi-Kopsch staining

Golgi-Kopsch silver impregnation demonstrated the cellular organization of the larval olfactory bulb. Diffusely organized glomerular-like structures, containing an intricate network of neural fibers were present throughout the glomerular layer (Fig. 25A, B, D). These putative glomeruli contained incoming ORN axons and putative mitral cell dendritic projections. Cell bodies of these putative mitral cells were located adjacent to the glomeruli. The dendrites were unbranched as they extended from the cell bodies radially toward glomeruli. Branching of mitral cell dendrites occurred proximal to olfactory glomeruli (Fig. 25A, B). The granule cell layer contained nuclei and thin processes of granule cells (Fig. 25C). Ependymal cells, with spiny projections, were seen extending radially toward the ventricle into glomerular-like structures (Fig. 25D).

Although the major constituents of the vertebrate olfactory bulb were recognized, lamination was obscure (as previously observed with cresyl violet staining, Fig. 24). Mitral cells were located in periglomerular areas. Granule and ependymal cells were also observed in the larval olfactory bulb, similar to the findings of Iwahori et al. (1987) in the adult lamprey.
Figure 25 - Golgi stain: Cellular organization of the olfactory bulb

A and B are mitral cells. Scale bars for A and B are 25μm

A. A mitral cell, within the granular layer extending its primary dendrites into a glomerular-like structure.

Inset: Low power view of the same mitral cell, with the periphery of the olfactory bulb visible.

Scale bar is 50 μm.

B. Another mitral cell with its dendritic processes innervating a glomerular-like structure

Inset: Low power view of the same mitral cell. Scale bar is 20 μm

C. Two granule cells with fine processes seen within the granular layer of the olfactory bulb.

Scale bar is 10 μm

D. An ependymal cell, with spiny processes, extending radially into glomerular-like structures within the glomerular layer. Scale bar is 10 μm.

Inset: Lower view of the same ependymal cell. Scale bar is 50 μm.
3.3.3 Ultrastructure of ORN axons

Many electron dense ORN axons, arranged in longitudinal bundles (Fig. 26A, B) were located in the olfactory nerve layer. The glomerular layer was paler staining than the olfactory nerve layer. ORN axonal processes (Fig. 26A, C) were clearly visible because of their intense staining. ORN axon terminals were packed with synaptic vesicles and mitochondria. Synaptic contacts were recognized by electron-dense areas on pre and post-synaptic membranes (Fig. 26C).

3.3.4 Distribution of developmental markers

GS-1-B4 lectin histochemistry was previously shown to label the olfactory epithelium and the olfactory bulb in larval lampreys (Tobet et al., 1996). In the present study, this technique was used to reveal the pathway followed by the ORN axons within the olfactory bulb. Fibers within the olfactory nerve layer and the glomerular layer stained intensely by GS-1-B4 lectin histochemistry (Fig. 27A). In the glomerular layer, these were grouped into clusters: the olfactory glomeruli. Vimentin, an intermediate filament, localized in developing neurons (Schwob et al., 1986), exhibited intense immunoreactivity in periglomerular locations and in cells in the granule cell layer (Fig. 27B) and in the ependymal cell layer. Immunoreactivity against GAP-43, an intraneuronal membrane-associated phosphoprotein (Pellier et al., 1994), was observed in the olfactory nerve layer, the glomerular layer and the granule cell layer. GAP-43 immunoreactivity was weak in the ependymal layer (Fig. 27C). Neural cell
Figure 26 - Ultrastructure of ORN axons in the olfactory bulb

Transmission electron micrographs of the olfactory nerve and glomerular layers of the olfactory bulb.

A. Many electron-dense ORN axons are seen on the surface of the olfactory bulb, within the olfactory nerve layer (ONL). The paler-staining glomerular layer (GL) contained many cell types including intensely stained ORN axons (arrowheads).

Scale bar is 1.5μm.

B and C are at the same magnification. Scale bar shown in B is 1μm

B. A high-power micrograph of the olfactory nerve layer (demarcated by arrowheads) showing intensely stained ORN axons arranged longitudinally.

C. In the glomerular layer, ORN axon terminals were recognized by electron-dense staining and densely-packed synaptic vesicles. Mitochondria were also seen within the ORN axon terminals. Synaptic contacts between ORN axons and dendritic processes of putative mitral cells were numerous (arrowheads).
Figure 27 - Distribution of markers associated with development and neuronal activity within the larval olfactory bulb

This plate demonstrates that the larval olfactory system is in an immature developmental stage and exhibits evidence of neuronal and synaptic activity.

A. GS-I-B₄ isolectin intensely stained ORN axons within the olfactory nerve layer and glomerular-like structures (⁎) in the glomerular layer.

B. Vimentin immunoreactivity was intense in periglomerular locations. Immunoreactive cells were scattered throughout the granule and ependymal cell layers.

C. GAP-43 immunoreactivity was periglomerular, scattered throughout the granule cell layer and moderate in the ependymal cell layer.

D. NCAM immunoreactivity was periglomerular and clustered within the granule cell layer.

E. Synapsin-I immunoreactivity was patchy throughout the glomerular and granule cell layers and absent from the ependymal cell layer.

F. An intense mat of tyrosine hydroxylase immunoreactivity was observed within the glomerular and granule cell layers. Paler glomerular-like structures were also present. No immunoreactivity was observed within the ependymal cell layer.

Scale bar is 100 μm
adhesion molecule (NCAM), a glycoprotein widely expressed in the developing nervous system (Edelman and Crossin, 1991), demonstrated periglomerular localization. In the granule cell layer, NCAM immunoreactivity was visible in granular clusters (Fig. 27D). This distribution of developmental markers within the larval olfactory bulb implies that it is in a developmentally immature stage.

3.3.5 Distribution of immunoreactivity associated with neuronal activity

Synapsin I, a neuron-specific regulatory protein, associated with exocytosis of synaptic vesicles (Bahler and Greengard, 1987), exhibited intense immunoreactivity in the olfactory nerve layer, formed a meshwork in the glomerular and granule layers and was absent from the ependymal layer (Fig. 27E). Tyrosine hydroxylase, a regulatory enzyme, involved in the catalysis of catecholamine biosynthesis (Nagatsu et al., 1964), exhibited intense immunoreactivity in the olfactory nerve layer and moderate-immunoreactivity in the glomerular and granule layers. In the glomerular layer, tyrosine hydroxylase immunoreactivity appeared to be periglomerular. Tyrosine hydroxylase was absent from the ependymal cell layer (Fig. 27F). This distribution of neuronal activity markers indicates that the developing larval olfactory bulb is a functionally active structure.

3.3.6 The distribution of developmental and neuronal activity markers within the juvenile adult olfactory nerve pathway

In the juvenile adult, intense GAP-43 IR ORN axons entered the olfactory bulb and terminated in heavily-stained glomerular-like structures (Fig. 28A). Within the granular layer, GAP-43 immunoreactivity was granular. The
Figure 28 - The distribution of developmental and neuronal activity markers within the juvenile adult (parasite) olfactory bulb

Juvenile adult cryosections were processed for DAB immunocytochemistry.

Arrowhead at the invagination marks the end of the olfactory bulb and the beginning of the telencephalon.

Scale bar is 200 μm

A. GAP-43 immunoreactivity was intense in the olfactory nerve layer, in glomerular-like structures within the glomerular layer and in the ependymal cell layer. Moderate immunoreactivity was observed in the granule cell layer.

B. Vimentin-immunoreactivity was absent from the olfactory nerve and glomerular layers, with the exception of some periglomerular immunolocalization. Vimentin-IR cells were scattered throughout the granule and ependymal cell layers.

C. Tyrosine hydroxylase immunoreactivity was absent from the olfactory nerve layer. The glomerular layer exhibited moderate immunoreactivity with the exception of an intensely stained periglomerular area. Cells of the granule layer were intensely stained.
ependymal cell layer was highly IR for GAP-43. Vimentin-immunoreactivity was absent from the olfactory nerve and glomerular layers, with the exception of punctate periglomerular immunolocalization (Fig. 28B). Vimentin-IR cells were scattered throughout the granular and ependymal cell layers. Tyrosine hydroxylase immunoreactivity was absent from the olfactory nerve layer (Fig. 28C). The glomerular layer exhibited moderate immunoreactivity with the exception of one intensely stained periglomerular area. Cells within the granule layer stained intensely for tyrosine hydroxylase.

This distribution shows that development is ongoing in the olfactory system of the juvenile adults and that the olfactory bulb is functionally active.

The olfactory bulb is a good model to examine developmental processes because it appears to remain in an immature state for an extended period of time.
4. DISCUSSION

These studies of the developing olfactory pathway in lampreys demonstrate 1) the ontogeny of ORN pathway and physiological responses during early development, 2) that the larval lamprey is an excellent in vivo model for examining the regulation of the developing olfactory pathway, 3) that tenascin-C modulates ORN axonal outgrowth and boundary signaling, and 4) that CSPG is a molecule that interferes with ORN axon growth and may be a repulsive signal for ORN axonal outgrowth.

4.1 Properties of ORNs during ontogeny.

In the hatching embryo, acetylated tubulin-immunoreactive axons were localized within the olfactory placode, as previously described by von Kupffer, 1880, Gorbman and Tamarin (1985). A very recent study of Lampetra japonica showed the same result (Kuratani et al., 1998). ORNs are among the first neurons in the head that express acetylated tubulin in the sea lamprey, a fact previously observed in embryonic mammals (Easter et al., 1993). As in other vertebrates (Wilson et al., 1990; Easter et al., 1993), lamprey ORNs were among the pioneering neurons during ontogenic development. The early appearance of ORNs parallels a previous study in which ORNs are developed in embryonic teleost fish (Zielinski and Hara 1988; Hansen and Zeiske 1993). Although the prolarval olfactory system consisted of short ORN axons, the olfactory system demonstrated the ability to elicit differential responses to a variety of odourants. As prolarvae and larvae lack functional photoreceptors (Rubinson, 1990),
olfaction is probably one of their most relied upon senses. L-arginine, which evoked responses in prolarvae, was also a potent chemostimulant to larvae (Zielinski et al., 1996) and adults (Li et al., 1995). Low magnitude prolarval responses elicited by D-arginine may indicate contamination of the D-arginine stock by L-arginine, or nonspecific responses, previously observed from developing ORNs in rats (Gesteland et al., 1982). However, receptor specificity was observed in the prolarval neural responses. Responses to taurocholic acid were independent of blockers of the L-arginine response. This suggests that the L-arginine receptors do not bind taurocholic acid and that the signal transduction pathways may be different for the two ligands.

The lamprey specific bile alcohol, petromyzonol sulfate (Hasselwood and Tokes, 1969) evoked strong summated action potentials in prolarvae, even when applied at extremely low concentrations (10^{-12} M). Previous physiological and behavioural studies have demonstrated that petromyzonol sulfate is a pheromone to the upstream migrant stage (Li et al., 1995). The pheromonal activity of this molecule may extend to prolarval stage 17 because they leave nests and move downstream to settlement sites. Multi-unit responses were evoked following the application of water conditioned by stage 17 prolarvae. As the gall bladder had acquired bile-green color by stage 17 (Piavis, 1971), these young lamprey may have been excreting traces of petromyzonol sulfate.

Surprisingly low responses were recorded following the application of water conditioned by ammocoetes. This low activity may be due to the fact that conditioned water contains a nonsulfated derivative of petromyzonol sulfate.
rather than petromyzonol sulfate itself (Li et al. 1995). The prolarval olfactory receptor neurons may have restricted olfactory neural responses to the sulfated form of petromyzonol.

Eggs with stage 12 embryos may contain pheromones, as prolarval olfactory neural responses were recorded following the application of water conditioned by stage 12 eggs. These eggs probably did not contain petromyzonol sulfate, as the embryonic liver forms after stage 12 (Piavis 1971). The water conditioned by the stage 12 eggs may have contained molecules with chemostimulatory properties of endogenous or parental origin. Alternatively, L-arginine present in the water conditioned by eggs may have been the stimulatory compound.

The results from this physiological study demonstrate that the olfactory system is functional and highly specific by stage 17. Sea lamprey prolarvae may imprint onto water conditioned by conspecifics in the nest and respond to these odours during downstream movement to settlement sites. Olfaction may be used by these early life stages to recognize appropriate habitat during settlement and recruitment to feeding sites.

4.2 The larval lamprey as an in vivo model of axonal outgrowth.

The evolutionary position of the lamprey, a vertebrate order that has survived on earth for over three hundred million years, makes this animal a fascinating subject for the examination of physiological and developmental regulatory processes. An obvious stage for examining the regulation of axonal outgrowth is the earliest stage in ontogeny, when pioneering axon tracts form. During the
course of these studies, it became apparent that the larval rather than the embryonic stage would be of greatest use as an *in vivo* model for axonal outgrowth. Several facts support the use of the larval stage rather than embryonic stages.

1. **Availability.** Sea lamprey eggs were available to us once, at most twice or three times during each summer. The eggs required fastidious daily maintenance, often with large numbers of mortalities. In comparison, larval lampreys are widely available in the Great Lakes Area and are maintained successfully in a wet lab.

2. **Very rapid initial development of the olfactory organ.** Within a few hours on the day of hatching, the initial ORN axon fibers appeared to extend from the olfactory placode to the brain. This very rapid development makes examination of the factors regulating the process of axonal outgrowth difficult during ontogeny. In comparison, the larval stage spans a period of several years, without major structural change to the olfactory organ (Vandenbossche *et al.*, 1995; 1996). The larval stage offers the researcher a long period for perturbation studies.

3. **The pathway of ORN axons is very short during ontogeny.** Since the olfactory epithelium and the primordial olfactory bulb are in close proximity, spatial examination of molecules governing the outgrowth of ORN axons becomes difficult. In larvae, the linear arrangement of converging olfactory nerve fascicles from the olfactory epithelium, through the lamina propria and towards the olfactory bulb and the absence of a calcified cribiform plate
permits examination of processes that regulate axonal elongation toward a target.

4. **Size.** The extremely small size of embryos and prolarvae, ranging from 1 mm to approximately 6.5 mm, makes them unattractive models for perturbation studies. The larvae that were used in this study were usually 7 - 10 cm in length. The olfactory nerve, clearly accessible for lesioning with the aid of a dissecting microscope, was located subcutaneously, between the nostril and the pineal gland.

5. **The larva is an Immature developmental stage.** The larval lamprey retains characteristics of early development such as the lack of eyes and of gender (Rubinson, 1990; Hardisty and Potter, 1986). The olfactory mucosa is unfolded with narrow ORN dendrites compared to the adult stage (Vandenbossche et al., 1995). The larval olfactory organ expresses neuronal developmental markers: GAP-43, vimentin and NCAM; as well as markers of physiological activity, synapsin and tyrosine hydroxylase. The spatial distribution of laminin immunoreactivity along the larval olfactory nerve is similar to early mammalian distribution (Liesi, 1985; Kafitz and Greer, 1997). This demonstrates the applicability of developmental data to processes in higher vertebrates. The olfactory bulb in the juvenile adult stage was also highly immunoreactive for GAP-43 and vimentin. Although this made the juvenile adult a good candidate for a developmental model, that stage was not used to investigate the processes of axon pathfinding because of its limited availability and difficult upkeep.
6. **ORN neurogenesis following olfactory nerve lesion.** Larval lampreys share the basic plan of ORN turnover observed in adult lampreys (Thornhill, 1967) and in other vertebrates. In larval lampreys, olfactory nerve axotomy was followed by ORN degeneration, then neurogenesis and axon outgrowth of synchronously differentiating ORNs. Although this process was consistent in most larvae examined, special attention was necessary to make a complete lesion of the olfactory nerve. If the olfactory nerve was partially lesioned, the scale of the degeneration was greatly diminished. Another source of variation was the exact location of the lesion site. Although the larvae were considerably larger than prolarvae, the axotomy was very difficult to perform, at times, without damaging the olfactory bulbs. Although every attempt was made to standardize the surgical procedure, sometimes differences and variations were inevitable and spatial evaluation of regrowth of the olfactory nerve was variable.

7. **Molecular markers for the olfactory nerve pathway.** GS-1-B4 isolectin, previously used by Tobet et al. (1996) was a useful marker for the topographic organization of the olfactory nerve and in identifying the specific location of a section within the olfactory organ. This approach proved to be a useful guide in perturbation studies. Acetylated tubulin was another useful marker for perturbation studies. As the acetylated tubulin antibody was previously observed in retino-spinal axons of larval sea lampreys (Hall et al., 1991) and in whole mounts of prolarvae (Kuratani et al., 1998) this study extends acetylated tubulin localization to the larval olfactory system. The
presence of GAP-43 immunoreactivity in developing ORNs and in locations with presumptive basal cells, corroborates previous observations of GAP-43-IR in ORNs from young stages of mammalian development (Verhaagen et al., 1988). Therefore, GAP-43 immunoreactivity is evolutionarily conserved in lampreys and is a useful marker for developing ORNs.

4.3 Tenascin-C and ORN axonal outgrowth

Data from lampreys during ontogenic and larval development, and from larval perturbation studies, support the hypothesis that tenascin-C modulates ORN axon outgrowth and boundary signaling. The absence of tenascin-C immunoreactivity in the lamprey olfactory organ during ontogeny parallels a previous study by Gong and Shipley (1996) that failed to localize tenascin-C in the embryonic rat olfactory organ. In embryonic lampreys, the olfactory epithelium and primordial olfactory bulb were essentially adjacent, enabling ORN axons to reach their target without extensive pathfinding and boundary guidance cues. Other ECM molecules such as laminin, L1 or NCAM maybe involved in the guidance of ORN axons during embryonic development (Gong and Shipley, 1996). During ontogeny, medial ORN axons were diffusely organized as shown with Dil labeling. Formation of olfactory nerve fascicles was not evident in the early lamprey olfactory system, in the absence of tenascin. This may indicate that tenascin-C is involved in the lateral inhibition ("hemming in") of ORN axons within the olfactory nerve, during the development of the olfactory nerve pathway. Another possibility may be that tenascin-C and olfactory nerve fascicle fascicles appear at the same time with no causal connection. This is highly
unlikely when this data and previous studies, that convincingly implicate
tenascin-C in the formation of boundaries, are taken into consideration (Krull et al., 1994)

The spatial distribution of tenascin-C within the larval olfactory nerve pathway suggested a role in the modulation of this pathway. Immunoreactivity was present in nerve bundles during the larval stage when a converging pathway of fascicles was evident. Following trans-section of the olfactory nerve, ORNs degenerated, with the olfactory epithelium becoming narrower during the loss of ORN nuclei. Within 14 days, newly formed ORNs repopulated the olfactory epithelium, which returned to its original thickness. Following the destruction of ORN axons by retrograde degeneration of the olfactory nerve, tenascin-C immunoreactivity ceased. During the absence of tenascin-C immunoreactivity, diffuse developing, unfasciculated axonal processes appeared subjacent to the olfactory epithelium as viewed with AT-immunoreactivity. At the ultrastructural level, the olfactory epithelium basement membrane appeared to be discontinuous in areas containing ORN axon aggregates. The few axons in the lamina propria were electron-dense and vacuolated. These data show that in the absence of tenascin; axon outgrowth was diffuse and abnormal.

Although the source of tenascin-C in the olfactory pathway is not known, it likely originates from Schwann cells, as glial cell expression of tenascin-C has been previously demonstrated (Faissner, 1997, Scholze et al., 1996). Loss of tenascin-C immunoreactivity during retrograde degeneration may have been caused by the downregulation of glial tenascin-C expression following the loss of
ORN axons. Another possibility is that tenascin, being such a large molecule MW=190-320 kDa) and linked by disulfide bonds, is prone to enzymatic breakdown by processes that are activated or upregulated following an olfactory nerve lesion (i.e. glutathione; Starcevic and Zielinski, 1997). The discontinuity of the basement membrane in three day post lesion tissue might also have had effects on the expression of laminin, a basement membrane glycoprotein implicated in axon outgrowth and pathfinding (Timpl et al., 1979; Kafitz and Greer, 1997). Changes in the expression of regulatory proteins following olfactory nerve axotomy have been previously observed. For example, in the rat there was an increase in the expression of low affinity nerve growth factor receptor (Gong et al., 1994).

Upon the return of tenascin-C immunoreactivity on day 14 post-lesion, axonal processes were constrained to fascicles that were "hemmed in" by specific boundaries. The relative intensity of tenascin-C in the fascicles and the surrounding loose connective tissue confirms previous observations of tenascin-C upregulation during wound healing (Mackie et al., 1988; Sakakura and Kusano, 1991) and nerve regeneration (Martini et al., 1990). This spatio-temporal pattern of tenascin-immunoreactivity upholds tenascin-C's function as a modulator of axonal pathfinding and boundary shaping (Bartsch et al., 1992; Becker et al., 1995; Brodkey et al., 1995; Caubit et al., 1994). Repulsive interactions between tenascin-C territory and outgrowing axons (Steindler, 1993), presumably defined the axonal path. This mechanism was implied from the fact that axons (although quite short) were arranged parallel and were somewhat
spread out in the absence of tenascin-C, then formed characteristic olfactory nerve fascicles upon the return of tenascin-C immunoreactivity. Tenascin-C's constraining positioning was previously observed in olfactory glomeruli of the moth, *Manduca sexta* (Krull et al., 1994) and in the boundaries of vibrissae-related barrel fields in the somatosensory cortex of mice (Crossin et al., 1989; Steindler et al., 1989). Tolbert and Oland (1990) demonstrated that the removal of tenascin-C immunoreactive glial cells in the moth, prevented the formation of olfactory glomeruli. Thus, tenascin-C seems to be pivotal in the formation of boundaries by contact repulsive mechanisms.

Functional blocking of tenascin-C, by exogenous application of anti-tenascin-C antibody caused a decrease in the expression of acetylated tubulin immunoreactivity and ORN axonal processes. In tenascin-C injected, 14 day post-lesion tissue, acetylated tubulin staining was diffuse implying that olfactory nerve fascicles were smaller and fewer. In a previous anti-tenascin-C study, Langenfeld-Oster et al. (1994) observed a significant delay in reinnervation of endplates, after daily applications of anti-tenascin antibodies, indicating tenascin's promoting effect on axon outgrowth. In this study, immunocytochemical analyses of tenascin immunoreactivity following lesions demonstrated tenascin's presence at the time of abundant ORN axon outgrowth (14 days post lesion) and olfactory nerve fascicle formation. This suggests that tenascin may function in a contact repulsive mechanism to constrain or laterally inhibit axons within the larval olfactory nerve. Tenascin antibody perturbation experiments should have removed the lateral constraint, causing aberrant axon
outgrowth. This anti-tenascin-C antibody may have interacted with a domain that promotes outgrowth, the fibronectin domain (Faissner, 1997). This suggests that tenascin may not only function by contact repulsion in the olfactory system but may also promote ORN axon outgrowth through its other domains (Langenfeld-Oster et al., 1994). Another possibility is that the antibody because of its large size might sterically bind to growth promoting molecules (i.e.) laminin, L1, growth factors, inhibiting their function and eventually producing the observed effects.

Preliminary results following the introduction of exogenous tenascin-C, into the axotomized larval olfactory nerve pathway revealed highly immunoreactive aberrant axonal processes, subjacent to the olfactory epithelium which appeared be thicker than PBS injected controls, indicating that they were fasciculated. Therefore, exogenous tenascin-C appeared to promote axon outgrowth and may have induced axonal fasciculation. One speculation is that exogenous tenascin-C may have formed complexes with CSPG (Maurel et al., 1994), neutralizing CSPG's inhibitory function thus causing enhanced ORN axon outgrowth in axotomized larvae.

Antibody perturbation and exogenous tenascin-C experiments imply dual functions for tenascin-C in the olfactory system; inhibiting ORN axon outgrowth by contact repulsion within the olfactory nerve, as well as promoting axon outgrowth and ORN axonal fasciculation. Another possibility is that tenascin-C promotes outgrowth which leads to fasciculation by other mechanisms (i.e.) NCAM, L1. These conflicting functions are reflected in previous studies by Faissner and Kruse (1990) that demonstrated CNS neuron repulsion to tenascin-
C coated surfaces and promotion of neuron outgrowth onto tenascin-C substrates from embryonic chicken spinal cord (Crossin et al., 1990). These controversial reports can be attributed to different domains of the tenascin-C protein; EGF-like repeats have been shown to repel neurite outgrowth whereas fibronectin repeats promote neurite outgrowth (Faissner, 1997).

4.4 CSPG and ORN axonal outgrowth

CSPG immunolocalization in the larval olfactory nerve pathway, was considerably different from that of tenascin and laminin. CSPG immunoreactivity was observed only in nasal cartilage lateral to the anterior portion of the olfactory nerve pathway. ORN axons turned beside the cartilage, thus completely avoiding the CSPG-rich cartilage. Possibly, a small amount of CSPG, undetectable by immunocytochemistry, was present in the area between the cartilage and ORN axons, as a result of diffusion, explaining why ORN axons turn at a distance from the cartilage. These results could be explained by the fact that CSPG is a repulsive signal to ORN axons that functions as a barrier to the outgrowth of ORN axons. Previous studies have demonstrated that CSPG generates barriers to the growth of axons from the dorsal root ganglion (Katoh-Semba et al., 1995). The avoidance of future cartilage and bone areas by growing peripheral nerve fibers have led to the postulation of inhibitory interactions between CSPG and axon interactions (Tosney and Oakley, 1990). Another possibility is that the nasal cartilage serves as a physical barrier.

Introduction of CSPG, by exogenous injections, into the larval olfactory nerve pathway of axotomized animals, demonstrated distortions within the
olfactory epithelium and deleterious effects on the outgrowth of ORN axons. Complete inhibition of ORN axon outgrowth was not obtained. This is not surprising since axon outgrowth is a complex process regulated by many molecules. This preliminary study demonstrated deleterious effects of CSPG on ORNs and their axon outgrowth following axotomy-induced ORN retrograde degeneration.

4.5 Conclusions

These studies of the lamprey olfactory nerve pathway, corroborate previous studies and support the hypothesis that laminin, tenasin-C and CSPG modulate ORN axon outgrowth. Tenasin-C appears to influence ORN axon outgrowth both by contact repulsion/boundary signaling and by promoting ORN axon outgrowth and possibly even fasciculation. CSPG localization in nasal cartilage, lateral to the olfactory nerve, ORN axon avoidance of the CSPG-rich cartilage and the detrimental effects of exogenous CSPG may indicate that it is a repulsive or toxic cue for ORN axons. More studies are required to make any conclusions on laminin but its spatial distribution suggests that it probably acts as an attractive signal for ORN axons, promoting their outgrowth (see Table III and Fig. 29). Furthermore, developmental, physiological, anatomical and phylogenetic characteristics of the larval sea lamprey olfactory pathway make it an excellent model for examining the regulation of axonal outgrowth.
<table>
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<tr>
<th>EXPERIMENT</th>
<th>RESULTS</th>
<th>POSSIBLE INTERPRETATIONS</th>
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| Morphologic assessment of embryonic development | **Stage 7** - large number of cells  
**Stage 12** - head appears  
**Stage 13** – head exhibits muscular activity  
**Stage 14** - embryo hatches and is a prolarva | Early development occurs rapidly with frequent morphological changes                      |
| Axonal pathways in prolarvae     | **Stage 14** – ventrally located olfactory placode contained bipolar AT-IR cells, AT-IR fibers extended from the placode to the brain  
**Stage 17** - dorsally located nostril, AT-IR fibers innervated the mouth and eye | ORNs are pioneering neurons and among the first neurons to express AT                      |
<p>| Ontogeny of olfactory organ      | Olfactory epithelium and brain are in close proximity, ORNs are located in small area between the olfactory epithelium and the brain | ORN axon guidance is not required because the olfactory epithelium and the brain are in close proximity; hence the lack of tenascin-C |
| Innervation of prolarval nasal cavity | Two sets of fibers labeled, one projected laterally and the other posteriorly | Structural framework for the olfactory system is in place very early in development. This may imply that olfaction is heavily relied upon during early development |
| Olfactory mucosal neural responses | Strong response to L-arginine but weak to D-arginine; N-ω-nitro-L-arginine blocked the L-arginine response but not the strong taurocholic acid response; petromyzonal sulfate also elicited large magnitude responses | Olfactory system is functional, different transduction pathways exist for L-arginine and taurocholic acid |
| Organization of larval olfactory nerve pathway | Entire olfactory nerve pathway visible in one horizontal section; ORN axon aggregates seen above and below the olfactory epithelium; many ORN axons are enveloped by one Schwann cell | Organization similar to mammals; retains embryonic characteristics therefore serves as a good developmental model |
| Topographic mapping of the olfactory nerve pathway | Number and distribution of GS-1-B1 stained olfactory nerve fascicles varies throughout olfactory organ. Prominent lateral and medial branches | The extent of ORN axon outgrowth varies from region to region within the olfactory organ and therefore it is probable to have sections that do not demonstrate staining for ECM guidance molecules. |</p>
<table>
<thead>
<tr>
<th>AT, GAP-43 and tenascin-C within the olfactory nerve pathway</th>
<th>Intense IR with growth-associated markers and tenascin-C within the olfactory epithelium and nerve.</th>
<th>The olfactory nerve pathway is in an immature state. TN-C's spatial distribution suggests that it is involved in the modulation of the olfactory nerve pathway.</th>
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<tr>
<td>Specificity of TN-C immunoreactivity</td>
<td>Incubation with pre-adsorbed antibody exhibited no immunoreactivity (Chemicon) Immunocytochemistry with tenascin-C antibody exhibited intense IR within the olfactory nerve pathway (Chemicon) Western immunoblotting displayed a 200 kDa band with Faissner's antibody</td>
<td>The immunoreactivity observed with the both polyclonal antibodies is specific to tenascin-C</td>
</tr>
<tr>
<td>Laminin within the larval olfactory nerve pathway</td>
<td>Intense immunofluorescence within the apical and basal regions of the olfactory epithelium and along the olfactory nerve</td>
<td>Laminin's spatial distribution suggests that it influences ORN axon outgrowth and is likely involved in promoting outgrowth</td>
</tr>
<tr>
<td>CSPG within the larval olfactory nerve pathway</td>
<td>Intense immunofluorescence within the nasal cartilage. ORN axons maintained a distance from the nasal cartilage as they traveled caudally towards the olfactory bulb.</td>
<td>CSPG influences ORN axon outgrowth; it may do so by repulsive cues since ORN axons remain a constant distance away from nasal cartilage.</td>
</tr>
<tr>
<td>Influence of an olfactory nerve axotomy on the olfactory epithelium</td>
<td>Control, unlesioned animals = 11.86 ± 3.4 ORNs Day 3 post lesion = 6.30 ± 1.6 ORNs Day 14 post lesion = 10.00 ± 2.9 ORNs</td>
<td>Day 3 is when maximal ORN degeneration occurs Day 14 is when maximal subsequent ORN differentiation occurs</td>
</tr>
<tr>
<td>Changes in AT, GAP-43 and TN-C immunoreactivity following an olfactory nerve lesion</td>
<td>Day 3 post lesion – no fascicular staining pattern observed with AT/GAP-43, instead short, diffuse, unfasciculated immunoreactive processes extended from the olfactory epithelium, no TN-C immunoreactivity Day 14 post lesion – intensely stained AT/GAP-43-IR olfactory epithelium and converging olfactory nerve fascicles present within the LP, TN-C-IR reappeared with the olfactory nerve fascicles</td>
<td>Tenascin-C is absent when ORN axons are unfasciculated but present when ORN axons converge into fascicles. Therefore, TN-C may be involved in the outgrowth and fasciculation of ORN axons</td>
</tr>
<tr>
<td>Ultrastructural effects following an olfactory nerve axotomy</td>
<td>Unlesioned animals – axonal aggregates were present above and below the basement membrane, olfactory nerve fascicles contained numerous axonal processes in the lamina propria</td>
<td>Olfactory nerve fascicles are smaller post lesion, don't have a Schwann cell nucleus surrounding them. This may be one reason for</td>
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<td><strong>Day 3 post lesion animals</strong> – axons were aggregated in the basal region of the olfactory epithelium where the basal lamina was absent, large olfactory nerve fascicles were absent from the lamina propria, smaller ones were present with fewer, irregularly shaped and vacuolated ORN axons</td>
<td>reason for the absence of TN-C, 3 days post lesion. It is also possible that my immunocytochemical method is not sensitive enough to detect a small amount of tenascin-C that may be present 3 days post-lesion. There may also be laminin downregulation effects from the discontinuity of the basement membrane affecting ORN axon outgrowth.</td>
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<td><strong>Effects of anti-tenascin-C on ORN axon outgrowth</strong></td>
<td><strong>Control</strong> – intense AT immunoreactivity was observed within the olfactory epithelium and nerve fascicles, short IR processes were seen subjacent to the olfactory epithelium. <strong>Anti-tenascin-C injected</strong> – faint AT immunoreactivity was observed in the olfactory epithelium and olfactory nerve fascicles, axons subjacent to the olfactory epithelium were not present.</td>
<td>Anti-TN-C caused a decrease in AT immunoreactivity implying olfactory nerve fascicles were smaller, thinner and fewer. Therefore, blocking TN-C appears to affect ORN axon outgrowth.</td>
</tr>
<tr>
<td><strong>Effects of exogenous tenascin-C on ORN axon outgrowth</strong></td>
<td><strong>Tenascin-C injected</strong> 6 Day Post Lesion-intense AT-immunoreactivity in ORN dendritic processes, olfactory nerve fascicles spaced apart with no axon outgrowth in between. 10 Day Post Lesion-AT-immunoreactivity in OE appears irregular, olfactory nerve fascicles are AT-IR and prominent within lamina propria. 14 Day Post Lesion-intense immunoreactivity in apical and basal portions of OE; short ORN axonal projections below OE which appeared to be fasciculated, prominent olfactory nerve fascicles in lamina propria.</td>
<td>Tenascin-C is involved in the promotion of ORN axon outgrowth and possibly fasciculation.</td>
</tr>
<tr>
<td><strong>Effects of exogenous CSPG on ORN axon outgrowth</strong></td>
<td><strong>CSPG injected</strong> 6 Day Post Lesion-OE appears to be degenerating, ORN axonal fibers were coursing in unusual directions, no evidence of new ORN differentiation. 14 Day Post Lesion-OE very distorted with only a few abnormal axonal projections seen, no fascicles observed.</td>
<td>CSPG appears to have deleterious effects on ORN axon outgrowth. This could be due to the fact that CSPG provides repulsive cues to ORN axons or that CSPG may be toxic at this...</td>
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126
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<thead>
<tr>
<th>Distribution of nuclei in larval olfactory bulb</th>
<th>Organized into four primary layers: the olfactory nerve, glomerular, granule and ependymal cell layers</th>
<th>Organization of larval lamprey olfactory bulb similar to mammals but lamination is somewhat obscure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golgi stain</td>
<td>Cellular organization of the larval olfactory bulb included mitral, granule and ependymal cells</td>
<td>Cellular organization of the olfactory bulb simpler than mammalian system but very similar</td>
</tr>
<tr>
<td>Ultrastructure of ORN axons within olfactory bulb</td>
<td>Electron dense ORN axons were arranged in parallel bundles in the olfactory nerve layer. The glomerular layer was paler staining with darker staining ORN axonal processes, packed with synaptic vesicles and mitochondria.</td>
<td>Similar ultrastructure to mammalian olfactory bulb, therefore could be used as a model to further comprehend the mammalian olfactory bulb</td>
</tr>
<tr>
<td>Distribution of markers associated with development and neuronal activity within the larval olfactory bulb</td>
<td><strong>GS-1-1.4 isolectin</strong> stained ORN axons within the olfactory nerve layer and glomerular structures. <strong>Vimentin</strong> and <strong>GAP-43</strong> immunoreactivity was periglomerular and scattered throughout the granules and ependymal cell layers. <strong>NCAM</strong> immunoreactivity was periglomerular and clustered in the granule cell layer. <strong>Synapsin-I</strong> immunoreactivity was patchy in the glomerular and granule cell layers, absent from the ependymal cell layer. <strong>Tyrosine hydroxylase</strong> immunoreactivity was present in the glomerular and granule cell layers, absent from the ependymal cell layer.</td>
<td>The larval lamprey olfactory nerve pathway is in an immature stage, retaining characteristics of embryonic development</td>
</tr>
<tr>
<td>Distribution of markers associated with development and neuronal activity within the juvenile olfactory bulb</td>
<td><strong>GAP-43</strong> immunoreactivity was intense in the olfactory nerve, glomerular and ependymal cell layers. Moderate immunoreactivity was present in the granule cell layer. <strong>Vimentin</strong> immunoreactivity was absent from the olfactory nerve and glomerular layers but scattered in the granule and ependymal cell layers. <strong>Tyrosine hydroxylase</strong> immunoreactivity was absent from the olfactory nerve layer. The glomerular layer exhibited moderate immunoreactivity. Cells of the granule layer stained intensely.</td>
<td>The juvenile olfactory bulb is also in a developmental stage and may be used to examine the regulation of developmental processes</td>
</tr>
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</table>
Figure 29 - Modulators of the lamprey olfactory nerve pathway

A. During ontogeny, the olfactory placode (OP) and the primordial olfactory bulb (OB) are in close proximity to each other. During this period, tenascin-C is absent implying that tenascin-C is not required during early development of the lamprey olfactory system. Other molecules like laminin or NCAM may be involved. Another possibility is that guidance is not required because the olfactory placode and brain are virtually adjacent to each other.

B. During the larval stage, immuncytochemical studies demonstrated the presence of laminin (circles) along ORN axons and in the olfactory bulb (OB) implying that it may be involved in ORN axon outgrowth and guidance. Tenascin-C (triangles) was present along the periphery of the olfactory nerve pathway which is suggestive of the fact that tenascin-C may be involved in constraining ORN axons within the olfactory nerve. CSPG was intensely localized within the nasal cartilage (NC). Since CSPG is soluble, it may have diffused out of the NC into the lamina propria repelling ORN axons.

C. During functional blocking of tenascin and the introduction of excessive amounts of tenascin-C and CSPG, ORN axon outgrowth was dramatically influenced. Anti-tenascin-C bound to available tenascin (triangles linked to trapezoids) greatly diminishing ORN axon outgrowth. Exogenous tenascin- (triangles) appeared to promote ORN outgrowth and fasciculation, to a certain extent, following an olfactory nerve lesion. CSPG (rectangles) appeared to have deleterious and almost toxic effects on ORN axon outgrowth.
4.6 Directions for future research

Although my research has provided evidence suggesting tenascin-C, laminin and CSPG influence ORN axon outgrowth and guidance, many questions remain unanswered.

An in vitro system would be an appropriate approach to take to answer some of these questions. If ORNs could be established in culture, they could be subjected to areas plated with either laminin, tenascin-C, CSPG or a combination of these molecules, to examine their effects on ORN axon growth cones. Retraction of growth cones to the presence of these molecules would indicate that the molecule functions as a repellent to ORN axon outgrowth. A faster rate of ORN axon outgrowth would indicate that the ECM molecule functions as an attractant, promoting ORN axon outgrowth. Transplanting larval lamprey nasal cartilage, which was demonstrated in my research be a source of CSPG, into an ORN culture dish and examining the subsequent effects on ORN axon outgrowth would be one way to test the influence of CSPG on the outgrowth of ORN axons.

More in depth studies are required to determine the roles of CSPG and laminin in ORN axon outgrowth and guidance. CSPG and laminin immunolocalization needs to be examined both in the embryonic and adult olfactory systems to observe its spatio-temporal distributions, which will provide more information on their roles. To directly investigate their roles in the development of the lamprey olfactory nerve pathway, exogenous injections with anti-laminin and anti-CSPG would yield valuable information on the function of CSPG and laminin within the olfactory nerve pathway. To exclude larval nasal
cartilage as a physical barrier, chondroitin sulfatase or an anti-CSPG antibody should be injected into the nasal cartilage to deplete available CSPG. Following CSPG depletion, ORN axon outgrowth patterns will be examined.

Another experiment which would reveal more about tenascin-C's role in the outgrowth of ORN axons, would be the introduction of tenascin-C (by exogenous injections) into a system where it is normally absent. (i.e. ) embryos, prolarvae. Effects of exogenous tenascin-C and tenascin-C's importance on ORN axon outgrowth, during development will subsequently be investigated by staining for GS-1-B4 lectin or acetylated tubulin. Also, injecting higher concentrations of tenascin-C, following an olfactory nerve lesion, would help demonstrate whether tenascin-C could possibly enhance or accelerate subsequent ORN axon outgrowth. Anti-tenascin-C injections in lesioned animals would also be helpful in examining effects of blocking tenascin expression on subsequent ORN axon outgrowth.

A more advanced and complete study would involve the injection of anti-sense tenascin-C, laminin or CSPG vectors into ORNs and basal cells, in vitro, to examine the effects of blocking their molecular expression on ORN axon outgrowth.
5. APPENDIX

RECIPES

STOCK SOLUTIONS

Stock A = 27.6 g sodium phosphate dibasic (Na₂HPO₄) in 1L of nanopure water
Stock B = 28.4 g of sodium phosphate monobasic (NaH₂PO₄•H₂O) in 1L of nanopure water

BUFFERS

0.1M Phosphate Buffer, pH=7.4

460 mL of 0.2 M diphosphate buffer (stock A)
1540 mL of 0.2 M monophosphate buffer (stock B)
2000 mL nanopure water

0.2M Phosphate Buffer, pH=7.4

90 mL of stock A
30 mL of stock B

0.1M Phosphate Buffered Saline (PBS), pH=7.4

8.76 g NaCl
0.2 g KCl
Add to 1L of 0.1 M Phosphate buffer

Immunofluorescence blocker

0.2% gelatin (0.02g)
0.1% sodium azide (0.01g)
0.1M L-lysine (0.146g)
0.25% Triton X-100 (25μL)

Dissolve these chemicals in 10 mL of 0.1M PBS (pH=7.4)
Antibody Diluent

5 mL of 0.1 M PBS
10μL of triton X-100 (omit for neural cell adhesion molecule, CSPG and tenascin)
Shake well

Sucrose solutions

10% sucrose - 1g of sucrose in 10 mL of 0.1M phosphate buffer (pH=7.4)
20% sucrose - 2g of sucrose in 10 mL of 0.1M phosphate buffer (pH=7.4)
30% sucrose - 3g of sucrose in 10 mL of 0.1M phosphate buffer (pH=7.4)

Western Immunoblot solutions

10% SDS

10g SDS
add autoclave water up to 100 mL

Homogenizing buffer

1% SDS
10 mM Tris HCl (pH=7.4)

Sample buffer

dH₂O 4.0 mL
0.5M Tris HCl, pH=6.8 1.0 mL
Glycerol 0.8 mL
10% (w/v) SDS 1.6 mL
2-β-mercaptoethanol 0.4 mL
0.05% (w/v) bromphenol blue 0.2 mL

Dilute the sample 1X with this buffer and heat to 95°C for 4 minutes.
Transfer buffer

25mM Tris base
192 mM glycine
20% (w/v) methanol
pH to 8.3

Store at 4°C

Running buffer (5X)

Tris base 9g
Glycine 43.2g
SDS 3g
to 600 mL of dH₂O

Store at 4°C, warm to 37°C before use, if precipitation occurs.
Dilute 60 mL 5X stock with 240 mL dH₂O for one electrophoretic run

Blocking buffer

10mM Tris
100 mM NaCl
pH this to 7.5
0.1% Tween 20
5% non-fat milk

Washing buffer

10 mM Tris
100 mM NaCl
pH this to 7.5
0.1% Tween 20

6% resolving gel

H₂O 10.6 mL
30% acrylamide mix 4.0 mL
1.5 M Tris (pH=8.8) 5.0 mL
10% SDS 0.2 mL
10% ammonium persulfate 0.2 mL
TEMED 0.016 mL
5% stacking gel

H₂O 2.7 mL
30% acrylamide mix 0.67 mL
1.0 M Tris (pH=6.8) 0.5 mL
10% SDS 0.04 mL
10% ammonium persulfate 0.04 mL
TEMED 0.004 mL

Broad Range Molecular Weight Standard

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>202 kDa</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>116 kDa</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>84 kDa</td>
</tr>
<tr>
<td>Ovalalbumin</td>
<td>50.1 kDa</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>35.7 kDa</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>29.3 kDa</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>21.2 kDa</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>7.5 kDa</td>
</tr>
</tbody>
</table>
Tenascin-C immunoreactivity in larval skin

Intense IR in larval skin exhibited by the anti-tenascin-C antibody (courtesy of Dr. Faissner)

FIXATIVES

4% paraformaldehyde

4 g of paraformaldehyde
50 mL of distilled water
50 mL of 0.2 M phosphate buffer, pH=7.4

1. Dissolve the paraformaldehyde in distilled water and heat to approximately 55°C.
2. Add a little NaOH to help dissolve paraformaldehyde.
3. Cool the solution on ice.
4. Add 0.2 M phosphate buffer.

Zamboni's Fixative

20 g of paraformaldehyde (2%)
850 mL of 0.1M phosphate buffer, pH=7.4
150 mL of saturated picric acid (15%), PICRIC ACID WILL EXPLODE, IF HEATED!!

1. Dissolve paraformaldehyde in buffer and heat to approximately 70°C.
2. Add a little NaOH to help dissolve the paraformaldehyde.
3. COOL the solution.
4. Add picric acid and adjust pH to 7.4 with either HCl or NaOH.
Karnovsky’s Fixative (modified)

1. Prepare 0.14M cacodylate buffer by dissolving 2.996 g sodium cacodylate in 100 mL of nanopure water. pH solution to 7.5 with HCl.
2. Prepare 1.2% paraformaldehyde by dissolving 0.6 g of paraformaldehyde in 50 mL of nanopure water. Heat to approximately 55°C and add a bit of NaOH to facilitate dissolving. Put on ice to cool.
3. Prepare 2% gluteraldehyde by diluting 2 mL of 25% gluteraldehyde into 23 mL of nanopure water.
4. Combine 25 mL of 0.14M cacodylate buffer with 50 mL of 1.2% paraformaldehyde and with 25 mL of 2% gluteraldehyde.
5. Add 0.02 g of CaCl₂ to fixative.

Preparation of Semi and Ultra Thin Plastic Sections

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medcast</td>
<td>80 mL</td>
</tr>
<tr>
<td>Dodecanyl succinic anhydride (DDSA)</td>
<td>50 mL</td>
</tr>
<tr>
<td>Nadic methyl anhydride (NMA)</td>
<td>45 mL</td>
</tr>
</tbody>
</table>

Combine all three chemicals and mix slowly, for approximately 1 hour. Add 1.75 mL of 2,4,6-tri(dimethylaminomethyl)phenol (DMP-30), drop by drop, while mixing. Mix for an additional 20 minutes but slowly to avoid air bubbles. Freeze for long term storage. Before using make sure that plastic has completely dissolved.

Plastic tissue preparation

1. Inject modified Karnovsky’s fixative directly into the nostril of the anesthetized larval lamprey. For larvae, dissect out the olfactory organ and the brain. Immersion fix olfactory organs in Karnovsky’s for approximately 2 hours.
2. Tissue was osmicated in 1% OsO₄ for 2 hours in an ice bucket covered with aluminum foil.
3. The tissue was stained en bloc with 2% uranyl acetate for 30 minutes in an ice bucket covered with aluminum foil.
4. The tissue was dehydrated through ice cold ethanols (alcohols are stored in the freezer before use).
   50% ethanol for 10 minutes on ice
   70% ethanol for 10 minutes on ice
   80% ethanol for 10 minutes on ice
   90% ethanol for 10 minutes on ice
   100% ethanol for 10 minutes on ice
   100% ethanol for 10 minutes on ice
   100% ethanol (absolute alcohol) for 10 minutes (3
changes at room temperature)

5. Infiltrate with propylene oxide for 10 minutes (3 changes at room temperature)
6. Immerse tissue into a 3:1 mixture of propylene oxide:epoxy for 3 hours. If the tissue is large, keep it in this solution overnight.
7. Incubate tissue in a 1:3 mixture of propylene oxide:epoxy overnight.
8. The next day, place tissue in 100% epoxy and rotate vials on a rotator for 4-5 hours.
9. Orient tissue in embedding molds, pour in fresh 100% epoxy and place in a 37°C oven for one hour to remove air bubbles.
10. Once all the air bubbles have been removed, increase the temperature to 70°C and bake tissue overnight or until plastic hardens.

Protocols

PREPARATION OF DOUBLE-SUBBED SLIDES

Part 1 - Acid Wash

1. Load frosted slides into stainless steel racks
2. Soak each rack in concentrated nitric acid for 5 minutes-work in the fume hood, wearing gloves, goggles and a lab coat
3. Rinse each rack in distilled water, then transfer to a dishpan containing water
4. Once all the racks have been acid treated, transfer dishpan into sink under running cold tap water for 2- hours
5. Sub slides-slides do not have to be dry

Part 2 - Subbing Protocol

1. Dissolve 6 g of gelatin in 288 mL of distilled water, stir and heat to 55°C
2. Cool gelatin solution slightly, add 120 mL of 95% ethanol
3. Dissolve 0.4 g chromium potassium sulfate in 20 mL of distilled water
4. Transfer the 20 mL chromium solution to the gelatin solution
5. Add 28 mL of glacial acetic acid, in the fume hood
6. Filter before use
7. Dip each rack of acid cleaned slides into subbing solution
8. Place the racks of subbed slides on paper towels, cover loosely to protect from dust.
9. Repeat this process (after the slides dry)
10. Poly-L-lysine coat slides
Part 3 - Poly-L-lysine coating

1. Dilute the poly-L-lysine solution, 1:10, with distilled water prior to coating slides.
2. Allow diluted poly-L-lysine solution to come to room temperature before use.
3. Place subbed slides in diluted poly-L-lysine solution for 5 minutes.
4. Dry slides at room temperature or for 1 hour at 60°C.

STAINS UTILIZED

Cresyl violet

1. Immerse slides in 0.1% cresyl violet (make 1% cresyl violet [0.5g cresyl violet acetate in 50 mL of nanopure], dilute it 10 fold in nanopure water) for 1-2 minutes
2. Immerse slides in distilled water for 1 minute
3. Dehydrate slides through an ascending alcohol series
   - 70% ethanol 1 minute
   - 95% ethanol 1 minute
   - 95% ethanol + 1% acetic acid - dip once to destain
   - 100% ethanol 1 minute
   - 100% ethanol 1 minute
   - Xylene 2 minutes
   - Xylene 2 minutes
1. Air dry and coverslip with Entellan or Permound

Toluidine Blue

1. Dissolve 1 g of sodium tetraborate in 100 mL of nanopure
2. Add 1 g of toluidine blue, stir with a stir bar
3. Filter before use with a syringe filter
4. Place slide on a hot plate on medium heat
5. Use syringe to apply stain on the section
6. Remove slide, once stain has just dried
7. Immerse slide in coplin jar containing either distilled water or 50% ethanol (differentiated stain)
8. Allow slide to dry, coverslip with either Entellan or Permound
Uranyl acetate

1. Prepare 7% uranyl acetate by dissolving 0.35 g of uranyl acetate into 5 mL of 70% ethanol and stir. Cover the container since uranyl acetate is light sensitive. This may take some time.
2. Once the uranyl acetate is completely dissolved (should not be foggy, if it is, discard immediately), filter with a 0.2 μm syringe filter.
3. Place filtered uranyl acetate into the wells of a porcelain staining dish, place a grid in each well.
4. Put the staining dish into a wet container and incubate at 37°C for 20-30 minutes.
5. Allow to cool at room temperature for 10 minutes.
6. Remove each grid and place it through several changes of distilled water.
7. Allow grids to air dry within self closing forceps

Lead citrate

1. Dissolve 1.33 g of lead nitrate [Pb(NO₃)₂] and 1.76 g sodium citrate in 30 mL of distilled water.
2. Place solution in a 50 mL flask for 20 minutes, solution will appear milky white.
3. Add 8 mL of 1N NaOH, solution should clear up. Do not use solution, if foggy.
4. Add 12 mL of distilled water.
5. In a covered, wax-filled, glass petri dish, cover 1/2 of the dish with NaOH pellets to absorb moisture.
6. On the other half, place a piece of parafilm and add a drop of filtered lead citrate to it.
7. Place the uranyl acetate stained grid (tissue side down) in lead citrate, anywhere from 1.5 to 2 seconds, in the covered petri dish.
8. Place the grid in 2 washes of distilled water (25 seconds each and shower the grids with a syringe extensively to remove any residual stain).
9. Blot off grids with filter paper and allow to air dry.

Golgi-Kopsch Solutions

Dichromate solution

8g of dichromate/192 mL of 0.1M phosphate buffer, pH=7.4
Combine 140 mL of this with 20 mL of 2% osmium

Silver solution

1.5g of silver in 198.75 mL of dH₂O
Preparation of Dil Crystals

Dissolve 5mg of Dil in 0.5 mL of N, N-dimethyl formamide and plate onto a microscope slide

COVERSLIPPING OF SLIDES

1. Dehydrate slides processed for immunocytochemistry through an ascending alcohol series.
   
<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>2 mins</td>
</tr>
<tr>
<td>Xylene</td>
<td>2 mins</td>
</tr>
</tbody>
</table>

2. Air dry and coverslip with Entellan or Permount
Preliminary results for the effects of exogenous tenasin-C and CSPG injections

Larvae following axotomy were injected with PBS, 0.2% tenasin or 0.33% CSPG starting at day 3 following axotomy. Animals were sacrificed and processed for acetylated tubulin immunofluorescence on days 6, 10 and 14 following axotomy.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>DAYS POST-LESION</th>
<th>ANIMAL</th>
<th>RESULTS FROM ACETYLATED TUBULIN IMMUNOFLUORESCENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>6</td>
<td>1</td>
<td>Intense IR localized within the olfactory epithelium, in short individual ORN axons and in olfactory nerve fascicles subjacent to the epithelium</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1</td>
<td>Intense IR in ORN dendrites and axons within the olfactory epithelium. IR was also observed in the many axons projecting off the epithelium and in the olfactory nerve</td>
</tr>
<tr>
<td>Tenasin-C</td>
<td>6</td>
<td>1</td>
<td>Intense IR in the apical region of the olfactory epithelium, widely spaced short olfactory nerve fascicles seen subjacent to the olfactory epithelium</td>
</tr>
<tr>
<td>(0.2%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>Complete degenerated olfactory organs with no visible ORN axon outgrowth, a few axonal fibers were IR</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>Olfactory epithelium was clumped and irregular, axonal fasciculation was prominent within the lamina propria</td>
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<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>Not entirely lesioned, same as unlesioned probably</td>
</tr>
<tr>
<td>CSPG</td>
<td>6</td>
<td>1</td>
<td>Irregular olfactory epithelium with acetylated tubulin-IR nuclear structures at the apical surface. Misguided ORN axons were seen coursing through the olfactory epithelium. No olfactory nerve fascicles were seen to extend within the lamina propria.</td>
</tr>
<tr>
<td>(0.33%)</td>
<td></td>
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<tr>
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<td>2</td>
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<td></td>
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<td></td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>Olfactory epithelium degenerated and almost unidentifiable</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>Olfactory epithelium highly immunoreactive. A few immunoreactive axonal fibers are visible but no fascicles observed in the lamina propria</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>Very short ORN axons extended from the olfactory epithelium, no fascicles were visible within the lamina propria. ORN dendrites stained intensely but IR appeared to be clumped at the apical surface</td>
<td></td>
</tr>
<tr>
<td>----</td>
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<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6. REFERENCES


VITA AUCTORIS

Aliya U. Zaidi was born in 1972 in Windsor, Ontario. She graduated from Vincent Massey Secondary School in 1990. From there she went on to the University of Windsor where she obtained her B.Sc. Honours in Biology in 1994. She is currently a candidate for the Doctor of Philosophy degree at the University of Windsor.