The medial olfactory glomeruli in the sea lamprey, Petromyzon marinus: Peripheral input and central projections.

Xiang Ren

University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
https://scholar.uwindsor.ca/etd/7055

This online database contains the full-text of PhD dissertations and Masters’ theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000ext. 3208.
THE MEDIAL OLFACTORY GLOMERULI IN THE SEA LAMPREY, PETROMYZON MARINUS: PERIPHERAL INPUT AND CENTRAL PROJECTIONS

By: Xiang Ren

A Thesis
Submitted to the Faculty of Graduate Studies and Research through Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada
2006

© 2006 Xiang Ren
Abstract:

This thesis investigates the concept that the form of distinct sub-regions of the primary and secondary olfactory pathways, reveals function associated with locomotor responses to pheromones in the sea lamprey (*Petromyzon marinus*). The application of tract-tracing strategies has led to the discovery of spatially distinct peripheral and central candidate pathways that may participate in pheromone detection and subsequent neural channeling. Putative chemosensory neurons extended from the accessory olfactory organ, an acinar aggregate at the caudal end of the peripheral olfactory organ, to specific glomerular neuropil in the medial region of the olfactory bulb. Secondary olfactory neurons from medial glomerular territories projected through the telencephalon proper into locomotor coordinating nuclei in the hypothalamus and the ventral thalamus. This thesis is the first demonstration of a spatially and morphologically distinct peripheral olfactory sub-system in any fish species, and the first to link output neurons from specific olfactory glomeruli to exact diencephalic targets.
To my wife, Bing Wu, and to my daughter, Anqi Ren.
Acknowledgements:

I would like to express my gratitude to Dr. Barbara Zielinski for giving me the opportunity to have this experience to do graduate work in her lab. Thank you for introducing me to the field of neuroscience, for providing me with all the guidance, for your enthusiasm during my intellectual development. I learned from you not only how to design and perform scientific experiments; how to organize and analyze my results, but also how to enjoy the whole process.

I would like to thank my thesis committee members Dr. Doug Wahlsten and Dr. Jennifer Jakobi for their very useful suggestions and inspiration. I am thankful to the following individuals for their considerable assistance I received in conducting my experiments: Dr. Michael Crawford, Dr. Andrew Hubberstey and Dr. Lynda Corkum.

As friends, I want to thank Rachelle Belanger, thank you for everything. By everything, I mean your endless hours of conversation and deliberation, your pushes and pulls when I felt stuck, your encouragement on any small gains I got. Thank you. And, of course, my lab mates Steven Chang, Alyson Laframboise, Yolanta Kita, Dina Kokh and Dr. Shashi Jasra. Thank you for joining me on this journey and for your endurance and encouragement.

Several thanks to the support staff at the University of Windsor including Nancy Barkley, Ingrid Churchill, Usha Jacob, Candy Donaldson, Carolin Lekic and Pat Morrell.

Thanks everybody.
# Table of Contents

Abstract ........................................................................................................................................ iii  
Dedication .................................................................................................................................... iv  
Acknowledgements ..................................................................................................................... v  
List of Tables .............................................................................................................................. ix  
List of Figures .............................................................................................................................. x  
List of Abbreviations ............................................................................................................... xiii  

Chapter I General Introduction ................................................................................................. 1  
1.1 Sea lamprey control program ............................................................................................... 1  
1.2 Phylogenetic comparison of olfactory projection pattern ................................................... 2  
1.3 The olfactory system in fish ............................................................................................... 4  
1.4 The peripheral olfactory organ in the sea lamprey ............................................................ 5  
1.5 Structural organization of the olfactory bulb in the lampreys .......................................... 7  
1.6 The medial glomeruli in the sea lamprey ......................................................................... 8  
1.7 Thesis Hypothesis .............................................................................................................. 10  
1.8 Gross features and fiber connections of forebrain in the lampreys .................................. 10  
1.8.1 Telencephalon .............................................................................................................. 10  
1.8.2 Diencephalon ................................................................................................................ 11  
1.8.3 Neural Connectivity ..................................................................................................... 13  
1.9 Tract tracing techniques used in this project .................................................................... 14  
1.9.1 The general concept of tract tracing .......................................................................... 14  
1.9.2 Biocytin .......................................................................................................................... 15  
1.9.3 Biotinylated dextran amine ........................................................................................ 15  
1.9.4 Carbocyanine dyes ....................................................................................................... 15  
1.10 Specific Aims of this Thesis ............................................................................................ 16  

Chapter II Localization of the Medial Glomeruli in the Olfactory Bulb ................................. 18  
2.1 Material and Methods ......................................................................................................... 18  
2.1.1 Experimental animals ................................................................................................. 18  
2.1.2 *Griffonia simplicifolia* 1 isolectin histochemistry .................................................. 20  
2.1.3 *In-vivo* biocytin loading into the nasal cavity in the transformer lamprey .............. 20  
2.2 Results .................................................................................................................................. 22  
2.2.1 Localization of the medial glomeruli in the adult lamprey .......................................... 22  
2.2.2 Double labelling with GS1B4 histochemistry and biocytin in the transformer lamprey ................................................................................................................... 24  
2.3 Discussion ............................................................................................................................ 29  
2.3.1 An explanation of the absence of biocytin labelling from a subset of the medial glomeruli in the transformer lamprey .......................................................... 29  
2.3.2 Possible significance of GS1B4 labelling in the AOO .............................................. 29
4.3.1.2 Terminal fields in the dorsomedial telencephalic neuropil ........................................... 74
4.3.1.3 Projections to the medial pallium and habenula ..................................................... 74
4.3.2 Forebrain decussation of medial glomerular projections .............................................. 76
  4.3.2.1 Decussation through postoptic and posterior tubeculum commissures .................. 76
  4.3.2.2 Reciprocal bilateral connections between olfactory bulbs ..................................... 77
4.3.3 Homology between projections from medial glomeruli and the MOT in teleosts ........... 77

Chapter V Overall conclusions ................................................................................................. 79

Selected Bibliography ........................................................................................................... 80
Vita Auctoris ............................................................................................................................ 86
List of Tables

Table 1: summary of animal use in the thesis 18
List of Figures

Fig. 1: A drawing of the olfactory system in the sea lamprey .......................................................5
Fig. 2: Organization of the peripheral olfactory organ during metamorphosis ............................ 6
Fig. 3: Biochemical difference of the glomeruli in the olfactory bulb ..........................................9
Fig. 4: Schematic drawings of the transverse sections through the forebrain nuclei ................. 12
Fig. 5: Spatial pattern of olfactory glomeruli in the adult sea lamprey ........................................ 23
Fig. 6: Biocytin labelling in peripheral olfactory organ following nasal application in the transformer lamprey ..................................................................................................................................................................................25
Fig. 7: Double GS1B, and anterograde biocytin labelling in the peripheral olfactory organ in the transformer lamprey ..................................................................................................................................................................................26
Fig. 8: Double GS1B, and anterograde biocytin labelling in the AOO following dye application into the nasal cavity in the transformer lamprey ..................................................................................................................................................................................27
Fig. 9: Double GS1B, and anterograde biocytin labelling in the OB following dye application to the nasal cavity in the transformer lamprey ..................................................................................................................................................................................28
Fig. 10: Fluorescence spectra of Dil and DiA ................................................................................ 31
Fig. 11: Double Dil and DiA injection into different glomeruli in the olfactory bulb ............... 33
Fig. 12: Schematic drawings of tract tracing results in peripheral olfactory organ in the sea lamprey in the metamorphic stage ..................................................................................................................................................................................36
Fig. 13: Dil retrograde labelling in the OE following dye application to the lateral glomeruli in the transformer lamprey ..................................................................................................................................................................................37
Fig. 14: Ipsilateral neuronal projection of OSNs in the olfactory epithelium following dye application to the lateral glomeruli in the transformer lamprey ..................................................................................................................................................................................37
Fig. 15: Dil negative labelling in the AOO following dye application to the lateral glomeruli in the transformer lamprey ..................................................................................................................................................................................38
Fig. 16: Punctate distribution of OSNs in the OE following dye application to the lateral glomeruli in the transformer lamprey ..................................................................................................................................................................................39
Fig. 17: Morphological features of the Dil labelled OSNs in the OE in the transformer lamprey ..................................................................................................................................................................................39
Fig. 18: OSNs in the transformer lamprey ..................................................................................... 41
Fig. 19: DiA labelling in the AOO following dye application to the medial glomeruli in the transformer lamprey ..................................................................................................................................................................................41
Fig. 20: Double DiA and Dil labelling in the peripheral olfactory organ following Dil and DiA insertion into lateral and medial glomeruli respectively ............................................................... 42
Fig. 21: Carbocyanine dyes labelling at the lamellae fold following Dil and DiA insertion into lateral and medial glomeruli respectively ........................................................................................................... 42
Fig. 22: Biocytin retrograde labelling in the peripheral olfactory organ following biocytin insertion into medial glomeruli ........................................................................................................ 43
Fig. 23: Morphological features of the biocytin labelled OSNs in the OE in the transformer lamprey ......................................................................................................................... 43
Fig. 24: Morphological detail of the biocytin labelled AOO neurons ................................................................................................................................. 45
Fig. 25: Biocytin labelling in the AOO ........................................................................................................ 45
Fig. 26: Connectivity between the AOO vesicles with the nasal cavity in the ovulated adult sea lamprey ............................................................................................................................. 45
Fig. 27: Illustration of the surgical protocol for the anterograde labelling in the adult sea lamprey ........................................................................................................................................ 57
Fig. 28: BDA injection site in the medial glomeruli of unilateral OB of adult sea lamprey 59
Fig. 29: BDA labelling in the ipsilateral OB ........................................................................................................ 59
Fig. 30: Decussation of fibers from the medial glomeruli ............................................................................................................................. 61
Fig. 31: Trajectory of the fibers from the medial glomeruli in the ipsilateral pallium ......................................................................................... 61
Fig. 32: Reunion of the labelled axons at the end of telencephalon ......................................................................................................................... 62
Fig. 33: Diencephalic labelling from BDA injection in the medial glomeruli .............................................................................................................. 62
Fig. 34: BDA labelling in and beneath the dorsomedial neuropil (Dmtn) ............................................................................................................. 63
Fig. 35: Biocytin injection into the medial glomeruli in the unilateral OB .................................................................................................................. 63
Fig. 36: Morphological detail of the biocytin labelled neurons in the contralateral OB in the transformer lamprey .................................................................................................................... 65
Fig. 37: Diencephalic labelling from biocytin injection in the medial glomeruli ........................................................................................................... 66
Fig. 38: Dorsal pallial labelling from biocytin injection in the medial glomeruli .............................................................................................. 66
Fig. 39: Projection to the epithalamus from biocytin medial glomeruli injection ................................................................................................. 68
Fig. 40: Bilateral biocytin lateral glomerular injection .............................................................................................................................. 68
Fig. 41: Trajectory of the axons from mitral cells in the lateral glomeruli ........................................................................................................ 69
Fig. 42: Terminal fields found in the habenula from lateral glomeruli injection ........................................................................................................... 69
Fig. 43: Dil diffusion in the anterograde labelling experiment ............................................................................................................................. 71

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Fig. 44: Trajectory from the medial glomeruli revealed by Dil 71
Fig. 45: Decussations of the Dil labelled projections in forebrain commissures 71
Fig. 46: A schematic drawing, summarizing the secondary olfactory projection of the medial glomeruli in the sea lamprey 72
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOO</td>
<td>Accessory olfactory organ</td>
</tr>
<tr>
<td>BDA</td>
<td>Biotinylated dextran amine</td>
</tr>
<tr>
<td>CR</td>
<td>Calretinin</td>
</tr>
<tr>
<td>DiA</td>
<td>4-4-dihexadecylaminostyryl-N-methyl-pyridinium</td>
</tr>
<tr>
<td>DiI</td>
<td>1-1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>Dmtn</td>
<td>Dorsomedial telencephalic neuropil</td>
</tr>
<tr>
<td>Dp</td>
<td>Dorsal pallium</td>
</tr>
<tr>
<td>EC</td>
<td>Ependymal cells</td>
</tr>
<tr>
<td>FMRFamide</td>
<td>Molluscan cardio-excitative tetrapeptide</td>
</tr>
<tr>
<td>GC</td>
<td>Granule cells</td>
</tr>
<tr>
<td>GS1B4</td>
<td>Griffonia simplicifolia 1 isolectin</td>
</tr>
<tr>
<td>Hab</td>
<td>Habenula</td>
</tr>
<tr>
<td>Hyp:</td>
<td>Hypothalamus</td>
</tr>
<tr>
<td>LOT</td>
<td>Lateral olfactory tract</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>MOT</td>
<td>Medial olfactory tract</td>
</tr>
<tr>
<td>Mp</td>
<td>Medial pallium</td>
</tr>
<tr>
<td>MS-222</td>
<td>Tricaine methane sulfonate</td>
</tr>
<tr>
<td>NA</td>
<td>Nasal sacs</td>
</tr>
<tr>
<td>NOA</td>
<td>Nucleus olfactorius anterior</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
<tr>
<td>OE</td>
<td>Olfactory epithelium</td>
</tr>
<tr>
<td>OF</td>
<td>Olfactory fibers</td>
</tr>
<tr>
<td>OSNs</td>
<td>Olfactory sensory neurons</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffer saline with Triton X100</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Ppald</td>
<td>Lateral pallium dosalis</td>
</tr>
<tr>
<td>Ppir:</td>
<td>Lateral pallium</td>
</tr>
<tr>
<td>Pr</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>SM</td>
<td>Stria medullaris</td>
</tr>
<tr>
<td>Str</td>
<td>Striatum</td>
</tr>
<tr>
<td>Thd</td>
<td>Dorsal thalamus</td>
</tr>
<tr>
<td>Thv</td>
<td>Ventral thalamus</td>
</tr>
<tr>
<td>Tr. Ol. Th. Hy</td>
<td>Tractus olfacto-habenularis and tractus olfacto-thalamicus et hypothalamicus</td>
</tr>
<tr>
<td>Tr. Ol. Hab.</td>
<td>Tractus of olfactory habenula</td>
</tr>
<tr>
<td>Vimp</td>
<td>Lateral ventricle</td>
</tr>
<tr>
<td>VNO</td>
<td>Vomeronasal organ</td>
</tr>
</tbody>
</table>
Chapter I General Introduction

1.1 The sea Lamprey control program

The Great Lakes of North America are overpopulated with sea lamprey, which feed on economically important teleosts (salmon, trout, etc.) by sucking blood from these organisms. Thus, the predator lamprey is a threat to the normal ecology and to the fishing industry. The sea lamprey is an ancestral jawless fish species; it has a complex life cycle that consists of larval, parasitic and adult stages. Larval sea lampreys inhabit streams for nearly 17 years (Hardisty and Petter, 1971). After a radical metamorphosis, parasites migrate to lakes to predate on fish (Beamish, 1980). When approaching sexual maturation, adults migrate back to streams for reproduction and then die (Applegate, 1950).

The olfactory system is believed to be the key sensory modality that regulates and motivates basic behaviours in sea lampreys throughout the complex life history (Kleerekoper 1969). Each of the three development stages, larvac, parasite and adult, is regulated to some extent by odours detected in the environment. Sedentary larval sea lampreys have sensitive and mature olfactory sensory neurons (OSNs; VanDenbossche et al., 1995). The olfactory epithelium of the sea lamprey at all different life stages is highly sensitive to a unique repertoire of compounds (Li et al., 2002; Zielinski et al., 2005) that regulate prey search (Kleerekoper and Mogensen, 1963), migration and mating behaviours (Bjerselius et al., 2000).

Petromyzonol sulfates are bile alcohol derivatives that have been identified as the pheromonal substances serving as chemoattractants during the life cycle of sea lamprey (Li et al., 2002). The compound 5α-petromyzonol sulfate (5α-PZS) produced by the larvae, serves as a migratory pheromone that attracts both male and female adult to the breeding ground (Li et al., 1995). The oxidized form, 3-keto- 5α-PZS is produced by spermiating male lampreys, stimulates swimming movement and searching behaviours in the ovulating females, and attracts them to nests occupied by males, where spawning activity can proceed (Li et al., 2002).

The sea lamprey’s reliance on pheromones makes it possible to develop control strategies for exploiting these odorant induced reactions, and that are effective, efficient and environmentally benign. One of the mechanisms for controlling the overpopulation
of sea lampreys is to use 5α-PZS and its derivative as a bait to trap adults. The Great Lakes Fishery Commission is currently deploying trials for the use of pheromones as an alternative to lampricide based population control of lampreys (Great Lakes Fishery Commission, 2000).

The neurobiology of the olfactory system in the sea lamprey has been investigated during recent years. Electrophysiological experiments have shown the strong reactivity of the peripheral olfactory system to 5α-PZS (Li et al., 2002). However the functional organization of the lamprey peripheral olfactory organ and the neurons responsible for the 3-keto-5α-PZS detection are still elusive, and there is little information about the neural connections between central olfactory system and the locomotor regions in the lamprey brain. Consequently, increased understanding of how pheromones stimulate movement will address specific issues of sea lamprey control concern, and may lead to the establishment of effective application of the pheromones.

The purpose of this project is to investigate the olfactory pathways in the sea lamprey from the peripheral olfactory organ to the brain areas which are likely linked to the pheromone induced swimming.

1.2 Phylogenetic comparison of olfactory projection patterns:

The projection pattern of the axons of OSNs into the olfactory bulb (OB) is essential for understanding the ability of animals to discriminate between a vast number of odorants (Buck, 2004). The current knowledge of olfactory pathways in mammals and fish is summarized, followed by an introduction to the olfactory system in the sea lamprey.

In many vertebrate species, nasal chemosensory processes take place in two spatially distinct regions. General odour input is received by the main olfactory bulb from the ciliated OSNs located in the main olfactory epithelium. Each OSN expresses only one of the about 1000 odorant receptor genes (Buck and Axel, 1991) and the axons from all cells expressing that particular receptor converge onto one or few clusters of neuropil (glomeruli) in the main OB (Mombaerts et al., 1996). Responses to non-volatile pheromones are conducted to the accessory olfactory bulb via microvillar sensory neurons in the vomeronasal organ (VNO), a specialization of the peripheral olfactory...
system. The VNO is highly developed in snakes and rodents, but is absent in fish, birds and old world primates (Bhatnagar and Meisami, 1998; Eisthen, 1997).

The VNO is separated from the main olfactory organ and is enclosed within a capsule formed by the vomer-bone or vomer-cartilage. An elongated tube opens only at the anterior end and connects the nasal cavity with tubular lumen of VNO, which is partially lined with chemosensory vomeronasal epithelium. This epithelium contains bipolar sensory neurons similar to those of the main olfactory epithelium except that they generally bear microvilli on their exposed surface rather than cilia. The axons of the vomeronasal sensory neurons form the vomeronasal nerve and project into the accessory olfactory bulb, usually located posterior-dorsal to the main olfactory bulb.

The heterameric G proteins have an important role in transmembrane signalling events of odour recognition at the dendrites of olfactory neurons. The $G_\alpha$ subunit can mediate an adenylate cyclase cascade and induce responses to odours coupling with seven-transmembrane spanning domain odorant receptors. Following the binding of an odorant molecule to its receptor, the G protein is activated and in turn activates an adenylyl cyclase (Pace, 1986). The cyclase catalyzes the formation of cyclic AMP, a molecule that binds to the intracellular face of the an ion channel, enabling cations such as $\text{Na}^+$ and $\text{Ca}^{2+}$ to flow through the channel, causing the membrane potential to depolarize (Firestein and Shepherd, 1991). Separate distribution patterns of distinct G protein subtypes $G_{12}$ and $G_\alpha$ were observed in the afferent axons of olfactory neurons in mouse, suggesting their different functions in the odours detection in the main olfactory system and vomeronasal system (Wekesa and Anholt, 1999).

Electrophysiology and calcium imaging experiments have shown that vomeronasal sensory neurons in both mammals and reptiles respond sensitively and selectively to putative pheromone chemicals (Fadool et al., 2001; Leinders-Zufall et al., 2000). This intraspecific chemical communication, through pheromones, can produce dramatic effects on reproductive behaviours and physiology, and on aggressive behaviours, that depend on chemosensory input from the vomeronasal system (Keeverne, 1999).

The central neural connections of the vomeronasal system are consistent with its role in initiating social and reproductive responses since accessory olfactory bulb neurons receiving vomeronasal nerve input project to the medial nucleus of the amygdala and to
the medial part of the cortical nucleus. The neurons in the corticomedial amygdala project to more central preoptic and hypothalamic areas of the brain which are concerned with hormonal control, reproduction and regulatory functions in animals (Halpern, 1987). Access of stimulus molecules to the vomeronasal receptors is regulated by an autonomically controlled vascular pumping mechanism in most mammalian species. The pump consists of large blood vessels running alongside the VNO within the vomer capsule. The pump appears to operate in response to novel stimuli during situations that attract the animal’s attention. When the blood vessels are constricted by vasomotor action, the lumen of the organ expands, drawing potential stimuli in through the tube connecting lumen of VNO and the nasal cavity (Meredith and Fernandez-Fewell, 1994).

1.3 The olfactory system in fish

Fish have no vomeronasal organ or accessory olfactory bulb (Eisthen, 1997). A mixed population of ciliated, microvillar and crypt olfactory sensory neurons, located in the olfactory epithelium of a multi-lamellar olfactory organ, are capable of detecting both general odorants and pheromones (Finger et al., 2000).

Although the three types of OSNs are intermingled within the olfactory epithelium, they project axons to different regions in the OB. It was revealed that ciliated and microvillar sensory neurons in zebra fish terminated into different areas in the olfactory bulb in a mutually exclusive manner: the ciliated OSNs project axons mostly to the dorsal and medial regions of the OB, whereas the microvillous OSNs project axons to the lateral region of the OB (Sato et al., 2005). The segregation of the axonal projections was also characterized in catfish and carp (Hamdani and Døving, 2002; Hansen et al., 2003).

Odotopic representation in various teleost fish has been mapped in recent years. A calcium imaging strategy was used in zebra fish and showed that amino acids and nucleotides, typical odorants for food, activated the lateral chain of the OB, while the responses to reproductive pheromones were confined to two glomeruli located ventromedially near the olfactory nerve (Friedrich and Korsching, 1997). Electrophysiological data in the channel catfish showed that the excitatory responses to bile salts were observed primarily in a thin, medial strip in the OB; whereas responses to amino acids occurred more rostrally in the dorsolateral OB and the responses to
nucleotides were from dorsal, caudolateral OB (Nikonov and Caprio, 2001). In salmonids bile acid responsive neurons were found along the dorsal midline of the mid-section of the olfactory bulb while the amino acid responsive neurons were found mainly along the dorsoventral extent of the latero-posterior bulb (Hara and Zhang, 1998). These findings indicate that there might be two segregated neural pathways in teleost fish, which were responsible for coding and processing of different types of odor information.

1.4 The peripheral olfactory organ in the sea lamprey

The lampreys represent an ancient evolutionary line that diverged from most vertebrates 400 million years ago. The peripheral olfactory organ in the sea lamprey consists of three main parts: a nasal tube, a nasal sac, and the nasopharyngeal pouch (Kleerekoper, 1969) (Fig. 1). Only ciliated OSNs are found in the olfactory epithelium (Thornhill, 1967; Vandenbossche et al., 1995). This is in contrast to the presence of both microvillous and ciliated OSNs in teleost fish (Zielinski et al., 2006).

Fig. 1: A drawing of the olfactory system in the sea lamprey. This horizontal view of the dissected tissue shows the peripheral olfactory organ and rostral brain areas. The dorsal portion of the nasal sacs was cut off to better visualize the olfactory lamellae which are lined with the olfactory epithelium. A median septum partly separates the nasal cavity into halves. AOO is located within the connective tissue along the median line of the ventrocaudal end of the nasal sacs. The olfactory nerves in the sea lamprey are short and connect the peripheral olfactory organ with the OBs in the brain. Ppir: lateral pallium ventralis. Hyp: hypothalamus (Drawing by Yolanta Kita).

The ciliated pseudostratified olfactory epithelium in the larval sea lamprey is composed of olfactory sensory neurons, sustentacular cells and basal cells which include progenitor cells. The bipolar OSNs are tall and slender with an apical cylindrical dendrite
toward the surface of the epithelium. These primary sensory neurons end with an olfactory knob extending into the mucociliary complex at the free surface of the olfactory epithelium, where the odorant detection cascade takes place. Extensive metamorphic remodelling of the peripheral olfactory organ commences midway through metamorphosis and advances throughout the remaining periods. The simple sacs covered by olfactory epithelium become a complicated structure with 7-10 lamellae on each side of the head. These lamellar folds are raised from the floor of the sacs and form an olfactory rosette (Fig. 2). Moreover there are significant increases in the OSN numbers as metamorphosis progresses (VanDenbossche et al., 1995).

Fig. 2: Organization of the peripheral olfactory organ during metamorphosis. A: Low power scanning electron micrograph of the olfactory organ, showing the nasal tube (nt) and nasal sacs which are composed of 8 lamellae (L). B: In the olfactory epithelium the sustentacular cells (white arrows) separate the OSNs which project olfactory knobs (black arrows) into the mucociliary-complex at the free surface. C: Acinar structure of the AOO vesicles are surrounded by the huge supply of blood vessels (bv). D: The large cells (black asterisks) lining the AOO vesicle protrude cilia into lumen. (Adapted from Vandenbossche J, 1993)

Behind the nasal sac, an aggregation of epithelial vesicles constitutes the so-called accessory olfactory organ (AOO) (Hogben and De beer, 1925). The vesicle consist of an acinar-like arrangement of a single layer of low columnar cells: Some cells are large with a pronounced nucleus and cilia extending from the basal rootlets into the lumen. The
vesicles are surrounded by a supply of blood vesicles (Fig. 1.2) and in some cases vesicles are adjacent to non-myelinated nerve bundles (VanDenbossche, 1993).

Two separate groups of AOO vesicles develop during the life history. The AOO first occurs during the larval stage as paired shallow diverticuli at the caudal end of the nasal sacs (VanDenbossche et al., 1995). During metamorphosis AOO vesicles become prominent while individual vesicles continue to expand and forms gland-like structures during this period. In the adult stage the vesicles are clustered together and fill the connective tissue along the median line of the ventrocaudal end of the nasal sacs (Kleerekoper, 1969).

In addition to the AOO vesicles developed since the larval stage, some scattered vesicles appear at the late metamorphic stage: they are formed by the invagination of the base of lamellae folds and distributed along the cartilage surrounding the nasal sacs (Hogben et al., 1925). Hagelin (1955) suggested that the AOO is similar to the VNO of higher vertebrates due to the similarity of capsule and rich surrounding vascularization. However, the neuronal connections from the AOO to the brain, by which any cell-type in the lamprey AOO could convey sensory information, have not been demonstrated.

1.5 The structural organization of the olfactory bulb in the lampreys

The olfactory bulb in the lampreys is a sessile rostrodorsal protrusion of the rostral half of the telencephalon and is separated from the smaller caudally located cerebral hemispheres by a shallow circular fissure considered to represent the olfactory peduncle (Heier, 1948). A laminar structure was observed in the olfactory bulb of the arctic lamprey, *Lampetra japonica* and four layers were recognized cytoarchitecturally: from the periphery inward it consisted of 1) the layer of olfactory fibers, 2) the layer of olfactory glomeruli with mitral cells, 3) the layer of granule cells, and 4) the layer of ependymal cells.

The layer of the olfactory fibers was composed of axons of OSNs arranged in numerous bundles. They were the principal afferents to the OB and spread over the surface the whole bulb (Iwahori et al., 1987).

The olfactory glomerulus represents functional units in information processing in the OB (Shepherd, 1994). A glomerulus is composed of a dense agglomeration of the four components: the terminal portion of the OSN axons, the dendrites of the secondary
olfactory neurons, the terminal arborizations of the granule cells processes and the terminal portion of the processes of the ependymal cells. Dendrites of the mitral cell and processes from the granule cells converged in the olfactory glomeruli while the processes of the ependymal cells passed through the confines of the glomeruli and some collaterals seemed to end there (Iwahori et al., 1987).

Mitral cells are the secondary olfactory neurons and are distributed mainly around and between the glomeruli. The morphological features of the mitral cells had been investigated with silver staining method in the arctic lamprey (Iwahori et al., 1987). There were two or more coarse primary dendrites that extended toward the olfactory glomeruli. On arriving at the glomeruli, the dendrites arborized repeatedly to break up into large and dense branches, and terminated totally within the glomeruli. Usually primary dendrites of several mitral cells converged into a single glomerulus, and occasionally a single mitral cell extended dendrites to two or more glomeruli. Thus, the glomeruli usually had several mitral cell efferents. Sometimes, two or more different glomeruli had common mitral cell efferents.

The granule cell layer occupied approximately two-thirds of the total depth of the OB and was composed of densely packed granule cells which have small cell bodies and extend 2-3 thin long processes terminating in the olfactory glomeruli. The ependymal cell layer was seen lining the olfactory ventricle at the deepest portion of the bulb. The ependymal cells were small spindle cells with long processes perpendicular to the ventricular surface.

1.6 The medial glomeruli in the sea lamprey

Griffonia simplicifolia I-isolectin B4 (GS1B4) is a biochemical marker for the OSNs axons in the sea lamprey (Tobet et al., 1996; Zielinski et al., 2000). This plant lectin has an affinity for D-galactose residues (DeBray et al., 1981), binds to the carbohydrate components of membrane-associated glycoconjugates expressed by the axolemma and terminal buttons of small-diameter primary sensory neurons in various vertebrates (Gerke and Plenderleith 2002). The membrane-associated glycoconjugates to which GSI-B4 binds may play a pivotal role in cell-cell recognition or adhesion during development (Lidierth and Wall, 1998). Through GS1B4 histochemical labelling, a stereotyped
epitope map of 41 to 65 glomeruli was revealed in the OB in the larval sea lamprey (Frontini et al., 2003). A consistent pattern of glomerular organization was observed in which 5 glomerular groups were characterized: a dorsal ring, an anterior plexus, a lateral chain, the medial glomeruli and a ventral cluster.

The olfactory-specific guanosine triphosphate (GTP)-binding protein alpha subunit, termed as $G_{olf}$ was characterized in the OSNs as well as in the glomeruli in the larval sea lamprey (Frontini et al., 2003). The double labelling experiment with GS1B$_4$ histochemistry and $G_{olf}$ immunohistochemistry showed that medial glomeruli in the sea lamprey maintained an absence of $G_{olf}$ expression compared to the remaining glomerular units (Fig. 3).

In addition to the biochemical distinction, recent experiment in our collaborative lab has found that medial glomeruli were physiologically unique among all the glomeruli in the OB in the sea lamprey: when the medial glomeruli were electrically stimulated, the signal was recorded in the reticular-spinal cord neurons, which can initiate the
locomotion in the sea lamprey; while other bulbar area, whatever the lateral, ventral or dorsal glomeruli can not induce such responses towards the electrical stimulation (Derjean et al., 2006).

1.7 Thesis hypothesis

It is hypothesized that there are a subset of specialized primary sensory neurons in the peripheral olfactory organ in the sea lamprey and that these sensory neurons project and converge into the medial glomeruli in the olfactory bulb where the odour information is further transmitted through a special pathway among the secondary olfactory projections into the locomotor control area in the forebrain. This hypothesis is based on previous findings. 1) Both teleost fish and higher vertebrate animals have segregated neural pathways for coding & processing of different types of odour information. 2) Distinct G protein subtypes are coupled with different odour detection cascades in the vertebrates. 3) The medial glomeruli in the sea lamprey are distinct from other glomeruli in respect to the G protein expression. 4) Only the odor information transmitted through the medial glomeruli reaches the reticularspinal neurons in the sea lamprey (Derjean et al., 2006).

1.8 Gross features and fiber connections of the forebrain in the lampreys

The brain is a complex interacting network that integrates various kinds of sensory information and sends signals to the control bodily movement. There are candidate brain regions for secondary olfactory projections from the OB (Heier, 1948; Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993). The topography of the forebrain nuclei will be introduced as background for studies of central olfactory connectivity.

1.8.1 Telencephalon

The telencephalon in lamprey species consists of a rostrally located OB and caudally located telencephalon proper which is subdivided into two parts: the cerebral hemisphere and the telencephalon medium (Fig. 4). Unlike the telencephalon in teleosts, the neural wall of the telencephalon medium in lampreys does not participate in evagination during
on the ontogenesis but remains in its initial embryonic position as a rostral and medial continuation of the diencephalon (Nieuwenhuys et al., 1998).

The lateral pallium dorsalis and ventralis together constitute the fully evaginated portion of the cerebral hemisphere. In these areas the layer of central grey is particularly wide and can be divided into two zones, an inner zone consisting of diffusely arranged cells and an outer zone made up by smaller and larger cell clusters.

The medial pallium forms the most dorsal part of the hemisphere and lies entirely within the telencephalon medium. The majority of its neuronal perikarya are situated within a narrow, compact layer of periventricular grey, but some have migrated outward into the stratum album. The dorsal pallium is a strip like zone that separates the medial pallium from the evaginated part of the pallium. Its neurons are arranged in a wide and rather diffuse zone of central grey.

Three ventrally located nuclei can be recognized in the telencephalon medium: a striatum, a septal and a preoptic nucleus. Caudally the striatum is a distinct plate of closely packed perikarya. However the rostral extent of the striatum is difficult to delineate, as the cells of this aggregate become smaller and more scattered. The septal nucleus is an oblique wedge of smaller migrated neurons which replaces the striatum at the rostral end of striatum. The nucleus preopticus forms the most ventrally located telencephalic center. It consists of some compact laminae of cells, which are situated at some distance from the ventricular surface.

1.8.2 Diencephalon

The diencephalon in the lamprey is composed of four regions, which are, from dorsal to ventral, the epithalamus, the dorsal thalamus, the ventral thalamus and the hypothalamus (Fig. 4). The ganglion habenulae in the epithalamus is delimited from the dorsal thalamus by a distinct sulcus subhabenularis. Most neurons in the dorsal thalamus are located periventricularly, thus composing the dorsal thalamic nucleus. Tracing experiments indicate that the dorsal thalamus receives a strong projection from the retina as well as the pineal organ (Puzdrowski et al., 1989).

The ventral thalamus is a rather narrow strip of central grey situated between the dorsal thalamus and the hypothalamus. Its widened caudal part, which surrounds the
In the telencephalon, the lateral pallium dosalis (Ppald) and ventralis (Ppir) are separated by the wall of lateral ventricle (vimp). Medial pallium (Mp) forms the most dorsal part of the hemisphere while dorsal pallium (Dp) is wedged between the medial pallium and the cerebral hemisphere. The three nuclei in the subpallium-septum, striatum (Str) and preoptic area (Pr) are packed ventral to the above structures. In the diencephalon, the ganglion habenulae (Hab) is situated at the top of the diencephalons. Dorsal thalamus (Thd) located below the habenula is identified as the sensory coordinating center for sensory integrity except olfaction. Ventral thalamus (Thv) forms a rather narrow strip of central grey and hypothalamus (Hyp) forms the largest part of the diencephalon. Habenula commissure and postoptic commissure are present at this level. The inserts show the horizontal schematic of lamprey brain and where the two cross sections are located (Modified from Auclair, 2004).

rostrodorsal part of the tuberculum posterior has been designated the nucleus tuberculi posterioris. Based on the findings from silver impregnation study, the ventral thalamus
was regarded as a motor coordinating centre which receives impulses from the whole
telencephalon and from all sensory regions of the brain, and conveys the impulses to
lower coordinating centers, to motor centers and to the hypothalamus (Nieuwenhuys et al., 1998).

The hypothalamus forms the largest part of the diencephalon and surrounds the
ventral part of the third ventricle (Heier, 1948). Throughout the hypothalamus the
neuronal perikarya are concentrated in a compact layer of periventricular grey. The
activation of the hypothalamus in vertebrates is involved in the hormonal regulation of
reproductive functions. Immunohistochemical study has revealed the presence of
neuromediator-specific neuron groups e.g. gonadotropin-releasing hormone containing
neurons in the preoptico-hypothalamic continuum (King et al., 1988).

1.8.3 Neural connectivity

The first descriptive study of the efferent pathways from the olfactory bulbs in the
lamprey species appeared more than a half century ago (Heier, 1948). The topography of
the nuclei in the forebrain and its fiber connections were examined in silver impregnated
material. The secondary olfactory projections were distributed throughout the lateral
pallium and medial-ventral area of cerebral hemisphere. Although there was no obvious
formation of compact nerve bundles of lateral and medial olfactory tracts as seen in
teleost fish (Finger et al., 2000), the trajectory of labelled fibers were still distinguishable.
These coursed caudally in the lateral, dorsomedial and ventral directions. Heier described
the tractus olfactorius, the tractus olfacto-habenularis and the tractus olfacto-thalamicus
et hypothalamicus (Tr. Ol. Th. Hy.), and concluded that the tract, Tr. Ol. Th. Hy.
connected telencephalon nuclei with the ventral diencephalon. It consisted partly of thick
axons and partly of fibers with a finer calibre. The thick axons originated from the mitral
cells of the rostromedial part of the OB and projected over the ventral thalamus and
thence, the hypothalamus. From the silver impregnated preparations these thick axons of
the mitral cells were seen to synapse with coarse dendrites of neurons in the caudo-
ventral part of the ventral thalamus, in the nucleus tubercle posterior and in the dorsal
hypothalamus. Moreover some thick axons from the contralateral OB passed through the dorsal commissure and joined the ipsilateral tractus.

The first experimental studies tracing the secondary olfactory projections in lamprey species were carried out with horseradish peroxidase (HRP) in silver lamprey, Ichthyomyzon unicuspis (Northcutt and Puzdrowski, 1988) and in the river lamprey, Lampetra fluviatilis (Polenova and Vesselkin, 1993). Most labelled fibers from the OB terminated in the lateral pallium. They also observed the OB efferents to the ventral thalamus and hypothalamus in both species. However which specific glomeruli projected fibers into the Tr. Ol. Th. Hy in the diencephalons were not established. HRP labelling diffused within the whole OB when the large amount HRP was applied into the OB in these global injections.

Therefore the goals of my project include characterization of the trajectory originating from the medial glomeruli, the terminal fields, and the brain nuclei that these projections travel through. The projection from these medial glomerular territories will be compared to an equally focal lateral injection. This result will be helpful to determine if and how the olfactory input through the medial glomeruli channels locomotion.

1.9 Tract tracing techniques used in this project

Neural connectivity from the peripheral olfactory organ to the olfactory bulb and then to brain nuclei will be examined through anterograde and retrograde labelling strategies with a variety of neuro-tracers.

1.9.1 The general concept of tract tracing

Neuro-anatomical tracers are typically used to ascertain the location of cell bodies that innervate a certain brain structure and the anatomical targets of a particular population of projection neurons. Retrograde axonal transport allows identification of the cells of origin of afferent nerve fibres to a particular target zone, while anterograde axonal transport enables the projection target(s) of individual or groups of cells to be charted. For retrograde transport, the tracer material is applied to a fibre tract or a terminal field of innervation, becomes incorporated into the cell and is then carried back to the cell body. For anterograde transport the tracer material is moved from the perikarya
along the axons to the cell’s synaptic terminals. Biocytin, biotinylated dextran and carbocyanine dyes including DiA and DiI were used in this project.

1.9.2 Biocytin

Biocytin, a soluble complex of biotin and the amino acid lysine, was applied initially as an intracellular marker by iontophoresis (Horikawa et al., 1988). It has since been used comprehensively in neuro-anatomical studies (Izzo, 1991). For anatomical tracing, biocytin can be injected in-vivo by extracellular pressure injection and is applicable for both anterograde and retrograde tracing. Compared to other tracers, the biocytin molecule is relatively small with a molecular weight of 372 kDa. A major asset is biocytin’s unique selectivity and high affinity for the glycoprotein avidin, which provides the basis for their detection (Lachica et al., 1991).

1.9.3 Biotinylated dextran amine

Pressure injections of biotinylated dextran amine (BDA) to discrete locations within the OB had been previously used for the characterization of the secondary olfactory projections in teleost fish (Matz, 1995). Low molecular weight (3,000 mol. wt) BDA was reported to be more effective for anterograde labelling than larger BDA, e.g., 7,000 MW in the Lateral line nerves of Xenopus laevis tadpoles (Fritzsch 1993). The tracer can be detected via the high-affinity binding of avidin or streptavidin that has been labelled with a substrate of some kind (e.g., HRP or a fluorescent tag). It was initially claimed that BDA labelled broken fibers of passage and damage to the targeted nerves was necessitated for its uptake. However it has been found recently that BDA also label intact and functioning fibers, suggesting that other mechanisms, such as endocytosis or pinocytosis, could bring dextran into neurons (Vercelli et al., 2000).

1.9.4 Carbocyanine dyes

Carbocyanine dyes exhibit strong light-excitable fluorescence. Their chemical structure and the highly lipophilic character facilitate their insertion in the lipid fraction of the plasma membrane of intact cells to diffuse freely within the membrane. These dyes
were used initially for optical recordings of membrane voltage and for studies of membrane fluidity (Waggoner, 1976).

In addition to their original application, these carbocyanine dyes, most notably Dil (1-1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate) have been exploited for use to label neurons in tissue that has been previously fixed in aldehyde fixatives. Neuronal processes and somata can be labelled along considerable distances in both the retrograde and anterograde directions (Vanselow et al., 1989). The staining is smooth and the complete extension of the neuronal processes can be labelled clearly. The resolution of fine processes is similar to that seen using intracellular injections and appears better than that achieved by Golgi methods or by intra-axonal transport of other tracer substances such as HRP. The slow appearance and time course of progression of the dye along axons suggest that the staining in fixed tissues occurs due to a process of passive lateral diffusion of the dye along the plasma membrane of neurons. It had been shown experimentally that the diffusion coefficient of Dil has been estimated to be about 0.2–0.6 mm per day in paraformaldehyde fixed specimens (Vanselow et al., 1989).

Specific aims of this thesis

The overall aim of this thesis is to explore a candidate neural tract that detects pheromones and connects this sensory input to integrative locomotor centers. The studies will focus on the adult stage, with the transforming stage investigated for comparison. Particular attention will be paid to the olfactory epithelium, where OSNs are located, to the AOO because of its possible function in chemoreception, and to the medial glomeruli, because of the predominance of medial pathways for pheromone detection and integration in teleosts (Zielinski and Hara, 2006), and the distinctive biochemical properties of the medial glomeruli in the sea lamprey (Frontini et al., 2003). First, the location of OSN axonal endings in the olfactory bulb will be defined by GS1B4 histochemistry (Tobet et al., 1996) and by anterograde labelling following dye application to the nasal cavity (Frontini et al., 2003). The uptake of these tract-tracing dyes by the olfactory epithelium and the AOO will be tested, and compared to the glomerular projections. Differential labelling of glomeruli by GS1B4 and anterograde labelling will be investigated for differential access of dye molecules into nasal sensory...
neurons projecting to the glomerular territories. Whenever possible, the tract tracing experiments will be confirmed by more than one dye marker, using *post mortem*, *in vivo* or *ex vivo* strategies. Reciprocal retrograde injections into medial glomerular territories will indicate the specific peripheral location of these sensory neurons. If the AOO is labelled following retrograde labelling into medial glomeruli, a double labelling strategy will be undertaken to test for possible targets in the lateral region of the olfactory bulb. If the AOO is labelled only following medial injection, then possibly, the AOO is a morphologically and spatially distinct olfactory subsystem. The morphology of the retrograde labelled cells projecting into medial glomeruli will be checked for the presence of axons. Connectivity between the lumen of the nasal cavity and the AOO will be examined to determine if odorous molecules are able to gain access to these cells from the lumen of the nasal cavity.

The central projections (secondary olfactory projections) from the medial glomeruli will be examined following *post mortem* and *ex vivo* focal application of tracers into medial glomeruli. Comparison to tracts labelled following injections into lateral glomeruli will reveal the specificity of the medial projections. Neural connectivity to integrative motor centers, such as the ventral thalamus and the hypothalamus will suggest channelling of neural output from the medial glomeruli to locomotor centers. The projections may also pass through or terminate in the latter, but may pass through the dorsal-medial telencephalic neuropil, pallium and habenula. The presence of decussation will indicate a mechanism for bilateral integration of output from the medial glomeruli. These tract-tracing and morphological studies will lay establish and anatomical basis for a spatially distinct neural pathway for pheromone detection and linkage to locomotor responses.
Chapter II  The localization of the medial glomeruli in the olfactory bulb

Due to the unique G protein expression and lectin binding properties, the medial glomeruli in the sea lamprey were hypothesized to receive projections from specialized sensory neurons in the peripheral olfactory organ and transmit the odour information via a specialized pathway to the higher brain nuclei. Therefore all the projections related with medial glomeruli, first from the peripheral olfactory organ to the OB, then from the OB to the diencephalic brain nuclei, will be investigated with tract tracing techniques. The strategy is to trace the neural connectivity in the peripheral and central olfactory system after the neuro-anatomical dyes are loaded into the medial glomeruli. Therefore the spatial localization of medial glomeruli in the spherical OB is first characterized. The techniques of histochemistry and anterograde dye loading are used to localize glomeruli in the sea lamprey.

2.1 Materials and Methods

2.1.1 Experimental animals

In order to exploit the potential developmental changes in the olfactory pathways and odorant directed locomotion control, sea lampreys from metamorphic to adult stages were used in this project. Adult sea lampreys (50 cm in length, 210 grams in weight on average) were collected from tributaries of Lakes Huron and Michigan from June to July 2005. Transformer sea lampreys (130 mm in length, 2.3 gram in weight on average) in the metamorphosis VII stage were collected from Oshawa Creek and Bronte Creek, Ontario in October 2005. The lampreys were transported to the U.S. Geological Survey Lake Huron Biological Station in Millersburg, Michigan, then transported to and maintained in recirculated dechlorinated water aquarium facilities at 10°C in the Department of Biological Sciences, University of Windsor. All experimental protocols including animal care, anaesthesia and surgery in this study were in compliance with guidelines established by the Canadian Council of Animal Care.
Table 1 Summary of sea lamprey specimens used in the thesis. Each technique was based on preliminary experiments using experimental animals.

<table>
<thead>
<tr>
<th>Thesis Section Aim of Experiment</th>
<th>Experimental Technique</th>
<th>Number of specimens</th>
<th>Life stage of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section 2. The localization of the medial glomeruli in the olfactory bulb</td>
<td><em>Griffonia simplicifolia</em> 1 isolectin histochemistry</td>
<td>3</td>
<td>ovulated adult</td>
</tr>
<tr>
<td></td>
<td><em>In-vivo</em> biocytin loading into the nasal cavity</td>
<td>1</td>
<td>transformer</td>
</tr>
<tr>
<td>Section 3. Primary olfactory projections into the medial glomeruli</td>
<td>Double carbocyanine dyes loading into the glomeruli</td>
<td>8</td>
<td>transformer</td>
</tr>
<tr>
<td></td>
<td>Biocytin application into the glomeruli</td>
<td>2</td>
<td>parasite</td>
</tr>
<tr>
<td></td>
<td>Biocytin application into the medial glomeruli</td>
<td>6</td>
<td>transformer</td>
</tr>
<tr>
<td></td>
<td>Biocytin application into the lateral glomeruli</td>
<td>1</td>
<td>parasite</td>
</tr>
<tr>
<td></td>
<td>Dil loading into the medial glomeruli</td>
<td>4</td>
<td>ovulated adult</td>
</tr>
<tr>
<td>Section 4. Secondary olfactory projections from the medial glomeruli</td>
<td>Biotinylated dextran amine pressure injection into the medial glomeruli</td>
<td>7</td>
<td>ovulated adult</td>
</tr>
<tr>
<td></td>
<td>Biocytin application into the medial glomeruli</td>
<td>5</td>
<td>transformer</td>
</tr>
<tr>
<td></td>
<td>Biocytin application into the lateral glomeruli</td>
<td>10</td>
<td>transformer</td>
</tr>
<tr>
<td></td>
<td>Dil loading into the medial glomeruli</td>
<td>5</td>
<td>transformer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>ovulated adult</td>
</tr>
</tbody>
</table>
2.1.2 *Griffonia simplicifolia 1* isolectin histochemistry

In this experiment, 3 adult ovulated female lampreys and 1 transformer lamprey were used. The fish were deeply anesthetized with 0.5% tricaine methane sulfonate (MS-222, FINQUEL, Ayerst Laboratory, New York) and sacrificed by decapitation. The female adults were termed as ovulated when eggs could be squeezed out from the fish abdomen. The brains were dissected out under the Olympus stereomicroscope (SZX-9, Carsen Group Inc., Markham, Canada) and fixed in 4% paraformaldehyde (Sigma-Aldrich, Mississauga, Canada) in 0.1 M phosphate buffer (PB), pH 7.6 for 24 hours and then cryoprotected in 10% (2h), 20% (2h) and 30% (overnight) solution of sucrose in 0.1 M PB, frozen in M-1 embedding matrix (Cat. 52945, Thermo Shandon, Pittsburgh, PA) and sectioned on a cryostat (Microm, Heidelberg, Germany). Serial cross sections at 25 μm thicknesses were thaw-mounted onto glass slides (Superfrost-plus Fisher Scientific, Cat. #12-550-15, Pittsburgh, PA). The staining of olfactory glomeruli by GS1B, had been previously described (Tobet et al., 1996; Zielinski et al., 2000). The sections were incubated in biotinylated *Griffonia simplicifolia 1* isolectin (GS-1 isolectin B4, B-1205, Vector, Burlingame, CA; 1 μg/ml in 0.1 M PBS, pH 7.5) for 3 hours at room temperature. The sections were rinsed in 0.1 M PBS for 10 minutes and then incubated in Alexa-488 conjugated streptavidin (S11236 1: 200 Molecular Probes, Eugene, OR) containing 0.4% Triton X100 (Sigma Stock. No. X-100) in 0.1 M phosphate buffer saline (PBST) (PH 7.6) for 2 hours at room temperature. After rinsing thoroughly in 0.1 M PBS for 10 minutes, the slides were mounted with Vectashield (H-100 Vector, Burlingame, CA). Sections were inspected on a fluorescence microscope (Zeiss Axioskop 2 FS, Toronto, Canada) and photographed using a Q-imaging Retiga 1300 digital camera (Q-Imaging Corporation, Burnaby, Canada) and Northern Eclipse software (EMPIX Imaging Inc., Mississauga, Canada). Some preparations were also examined with confocal optics (Bio-Rad 1024, Hercules, CA) attached to an upright microscope (Nikon Eclipse 800, Mississauga, Canada).

2.1.3 *In-vivo* biocytin loading into the nasal cavity in the transformer

Biocytin anterograde tracing is a direct method for showing the glomerular pattern of the OB in the sea lamprey. Similar methods with which biocytin (Koza and Wirsig-
Wiechmann, 2000) and calcium green dextran (Friedrich and Korsching, 1997; Frontini et al., 2002) was injected into nasal cavity had been carried out in amphibian and fish species. The calcium green dextran and biocytin gained entry into olfactory sensory neurons by endocytosis from ciliary processes at the surface of the olfactory epithelium, and were transported anterogradely along axons towards the neuronal terminals in the glomeruli in the OB.

In this experiment, 2 transformer lampreys were used. Glass micropipettes (Cat. TW100F-4, World Precision Instruments, Sarasota, FL) were first pulled using KOPT micropipette puller (Model 720, David Kopf Instruments, Tujunga, CA) and broken to a tip diameter of 40–50 μm. Each micropipette was backfilled with 4% biocytin (B-1592, Molecular Probes, Eugene, OR) in 0.05M Tris-HCl buffer (pH=7.4) containing Methylene blue assembled with the end of a pipette holder (H-7, Narishige East Meadow, NY) which was connected with picospritzer II (Parker instrumentation, General valve division, Fairfield, NJ) and affixed to the stereotaxic micromanipulator (MMN-1 Narishige East Meadow, NY) for positioning before the dye pressure injection. The transformer lamprey was anesthetized by immersion in aerated water containing 0.03% MS-222 solution. The animal was then placed under the dissection scope until it was totally sedate. The water in the nasal cavity was sucked out with a 23 gauge needle connected to a vacuum. The glass pipette was guided into the nasal cavity through the nostril using micromanipulator system.

The biocytin was loaded into the nasal cavity through the picospritzer II by applying 10 PSI (Pounds per square inch) of air pressure and 10 msec pulse. Repeated pulses were performed if necessary until the blue-color dye filled the nostril. The nostril was then quickly sealed with Oralbase paste (Bristol-Mayers Squibb Canada Inc.) to prevent dye leakage and the animal was allowed to recover in the aerated fresh water. Two serial injections were performed at 12 hours interval. The repeated injection and overall 36 hours incubation were necessitated for enough biocytin to be taken up by the primary olfactory sensory neurons in the peripheral olfactory organ and to be transported along the axons to the terminal fields in the glomeruli in the olfactory bulbs. Thirty six hours after the first injection the animal was deeply anesthetized with 0.5% MS222 and sacrificed by decapitation. The head was fixed with 4% PFA in 0.1 M PB overnight at 4
°C. The olfactory organ and brain tissue were dissected out the next day under the stereoscope before being cryoprotected in 10% (2h), 20% (2h) and 30% (overnight) solution of sucrose in 0.1 M PB, frozen in Shandon M-1 embedding matrix and sectioned on a cryostat. Serial cross sections at 25 µm thickness were thaw-mounted onto glass slides.

The labelling of biocytin in the glomeruli of the OB in the transformer lamprey was visualized through biotin-streptavidin histochemistry: the slides were incubated with Alexa-568 conjugated streptavidin (S11226; 1: 200 Molecular Probes) in 0.1 M PBST, PH 7.4 for 2 hours at room temperature and rinsed in 0.1 M PBS for 30 minutes. In order to check the co-localization of biocytin and lectin in the axons and terminal fields of OSNs in the OB, the slides were incubated with fluorescein-conjugated GS-1 isolectin B4 (FL-1201, 1 µg/ml, Vector Labs, Burlingame, CA) in 0.1 M PBS, pH 7.5 for 2 hours at room temperature. The slides were washed in 0.1 M PBS for 10 minutes and mounted with Vectashield. The sections were viewed under epifluorescence microscope or laser scanning confocal microscopy.

2.2 Results

The result showed the spatial localization of the medial glomeruli in the OB in the sea lamprey. They were closely packed together along the medial edge of the OB and situated in a very limited area from 200 µm caudal to the olfactory nerve to the anterior commissure in the telencephalon in the adult fish. The measurements were consistent and will be used for the tract tracing experiments targeted at the medial glomeruli.

2.2.1 Localization of the medial glomeruli in the adult lamprey

The olfactory glomeruli were identified as spherical regions from 45 to 90 µm in diameter. They were closely packed in single and sometimes double layers. Medial glomeruli were situated along the medial edge of the OB, ranged from 200 µm caudal to the olfactory nerve (Fig. 5A) and ended before anterior commissure and lateral ventricle which was 600 µm in deep to the olfactory nerve (Fig. 5D).

Compared with the compactness of the medial glomeruli, the lateral glomeruli were loosely interspersed under the surface of the lateral OB, and extended more caudally than
Fig 5: The spatial pattern of olfactory glomeruli in the adult sea lamprey. The glomeruli in the OB were visualized with GSIB₄ histochemistry and representative OB cross sections were rostro-caudally arranged in this layout. The olfactory nerve was divided into several bundles of fibers at the rostral end of the OB before spread over surface of the bulb. The olfactory glomeruli were characterized as spherical areas from 45 to 90 μm in diameter which were packed in single and sometimes double layers (Fig. 1A). Medial glomeruli (red arrows) were situated in the medial edge of the OB, ranged from 200 μm from the rostral most end of the OB (Fig. 1B) and ended before dorsal commissure (Dc) and the wall of lateral ventricle which was 600 μm in deep (Fig. 1E). Lateral glomeruli were loosely interspersed under the surface of the lateral OB, and extended more caudally than medial and ventral glomeruli (blue circle) (Fig.1F-H). Anterior nucleus of olfactorious (NOA) was wedged between the OB and the septum and preoptic areas (Fig. 1E-H). Lateral is on the right and ventral is at the bottom. Scale bar= 500 μm.
the medial and ventral glomeruli (Fig. 5E-H). In addition to the axon terminals from the primary sensory neurons in the nasal cavity, GS1B4 also labelled fibers which entered the anterior nucleus of olfactorious (NOA) (Fig. 5G) and proceeded caudomedially into the septum and preoptic areas in the ventral telencephalon (Fig. 5H).

The spatial pattern of glomeruli in the OB in the transformer lamprey was similar to that in adult fish (data not shown). All the glomerular groups including the medial, lateral, dorsal and ventral glomeruli were present in the transformer stage. Moreover the lateral glomeruli in the transformer lamprey were shallower than adult and arranged in a single layer under the olfactory nerve layer.

2.2.2 Double labelling with GS1B4 histochemistry and biocytin in the transformer lamprey

In order to judge the effectiveness of the biocytin glomeruli labelling, the staining pattern in the peripheral olfactory organ was first checked. Following the biocytin application into the nasal cavity in the live animal, all the different epithelial components, including olfactory sensory neurons (OSNs) and supporting cells in the olfactory epithelium and low cuboidal cells covering the nasal sac were labelled: the biocytin molecules were localized not only in the cytoplasm of epithelial cells but also in the neuronal processes of OSNs. The labelled OSNs in the olfactory lamellae projected axons into the lamina propria while OSNs at the base of the lamellar fold sent axons directly into the deep connective tissue (Fig. 6A). These labelled nerve bundles converged at the caudal end of the olfactory organ, forming the olfactory nerve and passing through the cartilage into the olfactory bulb in the brain (Fig. 6B).

The nerve fascicles of axons of OSNs in the lamina propria and in the deep connective tissue were positively stained for GS1B4. Anterogradely loaded biocytin were co-localized with GS1B4 in the olfactory nerve fascicles in the olfactory lamellae. There was strong GS1B4 labelling in the vesicles of the accessory olfactory organ, situated at the caudal portion of the peripheral olfactory organ (Fig. 7). The lumen as well as the mucus layer of the epithelial cells surrounding the vesicles of the AOO was GS1B4 positive. Ovoid shaped cells in the lining of the AOO lumen were strongly stained and
might be the secretary source of high concentration of carbohydrate within the lumen (Fig. 7).

In spite of the high intensity of GS1B4 staining, no cell components in the AOO vesicles took up biocytin applied into the nasal cavity as OSNs did in the olfactory epithelium in the transformer lamprey and no biocytin labelled nerve fibers were observed to originate from these GS1B4 positive vesicles (Fig. 8).

Fig. 6: Biocytin labelling in peripheral olfactory organ following nasal application in the transformer lamprey. Top: numerous epithelial components in the nasal cavity were stained. OSNs in the lamellae projected axons into the lamina propria (blue arrows) while those at the base of the lamellar fold sent axons directly into the deep connective tissue (yellow arrows). Bottom: The nerve fascicles bundled together and formed the olfactory nerve (red circles) and the cuboidal cells (red arrows) along the nasal tubes were also labelled by the biocytin. Scale bar=500 μm.
Fig. 7: Double GS1B₄ and anterograde biocytin labelling in the peripheral olfactory organ in the transformer lamprey. A: Biocytin labelled olfactory nerve fascicles projecting from the OSNs in the OE was evident in the connective tissue. B: GS1B₄ histochemistry in the same section labelled the olfactory nerve fascicles as well as the AOO. C: Double labelling showed the colocalization of biocytin and GS1B₄ in the nerve fascicles and the absence of biocytin in the AOO structure. Scale bar=250 μm. E: Strong GS1B₄ staining within the lumen of an AOO vesicle at high magnification. Scale bar=50 μm. F: GS1B₄ staining in the mucus layer of the AOO vesicle and in the cytoplasm of some ovoid shaped vesicle cells. Scale bar=50 μm. G: high magnification of the insert in E, showing the morphological features of GS1B₄ positive cells. Scale bar=5 μm.
Fig. 8: Double GS1B$_4$ and anterograde biocytin labelling in the AOO following dye application into the nasal cavity in the transformer lamprey. A: biocytin labelled olfactory nerve fascicles traveled within the connective tissue of the caudal portion of the peripheral olfactory organ. B: GS1B$_4$ histochemistry in the same section showed the labelled nerve fascicle and strongly labelled AOO vesicle. C: merged picture from red channel in picture A and green channel in picture B. D: showed no biocytin signals were present inside AOO system. D: High magnification of the insert in C, showing the absence of biocytin labelling in the AOO vesicle and the no biocytin labelled fibers projecting from the AOO vesicle. Scale bar=10 μm.

In the OB in the transformer lamprey, the glomerular organization revealed by biocytin labelling showed the same pattern as GS1B$_4$ lectin showed. However it is striking that a subset of the medial glomeruli were not filled by biocytin in the transformer lamprey (Fig. 7). Compared with the diffusive GS1B$_4$ labelling on the surface of neuronal membranes, the biocytin signal situated within the cytoplasm of the neurons provided better visualization of individual axons (Fig. 8): the labelled axons from OSNs ran in bundles along the free surface of the bulb and formed the olfactory nerve layer. The bundles traveled in various directions and crossed in a complex fashion.
in this layer. Finally the fiber bundles swung inward and entered the olfactory glomeruli to terminate therein, forming a dense fiber plexus (Fig. 9).

Fig. 9: Double GS1B4 and anterograde biocytin labelling in the OB following dye application to the nasal cavity in the transformer lamprey. D, E and F are high magnification of A, B and C respectively. A: Biocytin stained axons of OSNs (blue arrows) ran in bundles along the bulb surface before they swung into the glomeruli. B: GS1B4 histochemistry labelled all the glomeruli in the OB (white circle). C: Double staining showed the biocytin absence in the medial glomeruli (blue circle). Scale bar=500 μm.
2.3 Discussion

2.3.1 An explanation of the absence of biocytin labelling from a subset of the medial glomeruli in the transformer lamprey

Although numerous OSNs in the olfactory epithelium were visually positive to the biocytin staining under the fluorescence microscope, a subset of the medial glomeruli were still not labelled by biocytin in the OB in the transformer lamprey. The N value (N=2) in this experiment is too small to draw any conclusion about the neural source of the medial glomeruli in the peripheral olfactory organ. However we noticed the biocytin absence in the vesicles of the AOO located in the caudal portion of the peripheral olfactory organ. Small ducts and pores connect AOO and the nasal cavity (Hagelin and Johnels, 1955), so theoretically biocytin in the nasal cavity could enter the AOO vesicles through these passageways and label the AOO epithelial cells. Our results suggested that AOO vesicles were not connected to the olfactory epithelium and could not fill with biocytin from the nasal cavity under our experimental conditions and at the transformer stage; the AOO neurons therefore did not absorb and transport biocytin molecules, resulting the negative labelling in their terminal field in the medial glomeruli in the olfactory bulb. More discussion about the potential connectivity between AOO and these medial glomeruli can be found in chapter III in this thesis.

2.3.2 Possible significance for the GS1B$_4$ labelling in the AOO

Strong GS1B$_4$ histochemical staining in the lumen of AOO vesicles in transformer lamprey parallels lectin-binding properties of the olfactory epithelium and the VNO in rat and newt (Mendoza and Kuhnel, 1991; Saito et al., 1994). Lectins had been used as the histochemical probes for glycoconjugates (Sharon and Lis, 1986). There was intense staining in the free border and the secretory supporting cells of the VNO, whereas negative or weak staining in the cells of the olfactory epithelium. The heterogeneity of mucous environments in the olfactory epithelium and in the VNO resulted from the differences in quality and/or quantity of secreted elements (e.g. supporting cells). The odoriferous molecules traverse the mucus of the epithelium prior to reaching the cilia or microvilli of the receptor neurons (Pelosi 1996). Therefore, the mucous environment of the epithelial surface seems to influence the access of odoriferous molecules to the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
olfactory receptors (Getchell and Getchell, 1992; Menco et al., 1994). Although there still has not been definite conclusion about the function of the AOO in lamprey species, the different localization of glycoconjugates in the olfactory epithelium and AOO vesicles provide evidence of functional differences between olfactory epithelium and the cells within AOO.

2.4 Conclusion

From this experiment, we conclude that GS1B4 histochemistry was a reliable method to address the glomerular organization in the OB in the sea lamprey. The measurement from medial glomeruli to the anatomical markers on the surface of the spherical OB (e.g. dorsal commissure) was constant and was used for the tract tracing experiments targeted at the medial glomeruli.
Chapter III  Primary Olfactory Projections into the Medial Glomeruli

The objective of the following set of experiments was to investigate the connectivity between the peripheral olfactory organ and the medial glomeruli in the OB in the sea lamprey in both the metamorphic and adult stages. Our objective was to find out which primary sensory neurons projected their axon terminals into the medial glomeruli, morphological features of these projections and their distribution in the peripheral olfactory organ. Retrograde tract tracing techniques were used and medial glomeruli were injected with neuro-anatomical dyes. Moreover lateral glomeruli were also tested in the experiments so as to compare the two targets of primary projections from the peripheral olfactory organ.

3.1 Materials and Methods

3.1.1 The double labelling of peripheral projections into the olfactory bulb

The goal of this experiment was to trace simultaneously two primary projections originating from the peripheral olfactory organ by applying two carbocyanine dyes to spatially distinct OB glomerular territories. The 488 nm excitable DiA (4-4-dihexadecylaminostyryl-N-methyl-pyridinium) and 568 nm excitable Dil were used together for double labelling in this experiment Their excitation and emission spectra are shown (Fig. 10). It had been reported that all the green carbocyanine dyes including DiA diffused in the cell membranes significantly slowly than their red or infrared counterparts.

![Fig 10: Fluorescence spectra of Dil and DiA. Fluorescence excitation and emission spectra of DiA bound to phospholipids bilayer membranes (green block) and absorption and fluorescence emission spectra of Dil bound to phospholipids bilayer membranes (blue block). (Adapted from webpage of Invitrogen, 2006)](image-url)
such as Dil (Snider et al., 1992). In the sea lamprey the olfactory nerve projects into the OB from the dorso-medial direction and there was shorter distance from rostral pole of OB to the medial glomeruli than to the lateral glomeruli (personal observation). Therefore the slow-diffusing DiA was injected into medial glomeruli in our experiment and the fast-diffusing Dil was injected into the lateral glomeruli in the ipsilateral OB. This arrangement provided the appropriate head start needed by the DiA to reach the target areas simultaneously with Dil molecules at the end of the tissue incubation.

In this experiment, 8 transformer phase lamprey and 2 parasite phase lamprey were used for double carbocyanine dye injection. The lampreys were deeply anaesthetized with 0.5% MS 222 and then sacrificed by decapitation. The brains of the parasite lamprey were exposed by cutting off the soft tissues and cartilages dorsal and lateral to the brain cavity and then were fixed in 4% PFA in 0.1 M PB for 24 hours. The transformer has a small and delicate olfactory system; therefore the whole head was first fixed in 4% PFA in 0.1 M PB overnight. On the following day the brain as well as the whole olfactory organ was dissected out as a single unit under the stereoscope by cutting off most of the soft tissue and the cartilage surrounding the nasal cavity and brain tissue. Extra attention was paid not to damage the central olfactory system and the olfactory nerves which were short and fragile; these were especially vulnerable to damaging of nerve fibers. To facilitate microinjection of carbocyanine dyes to the glomerular areas, the whole tissue including the brain and the peripheral olfactory organ was embedded into 5% agarose (A0169, Sigma-Aldrich) in 0.1 M PBS and the brain was sliced in a cross-sectioned plane from caudal to rostral with a vibratome (V100T, Leica), until the front wall of the lateral ventricle was exposed on the cutting surface. Therefore, the targeted glomeruli in the olfactory bulb were anatomically accessible under the surface of the preparation. The tissue-agarose block was then incubated in fluorescein-conjugated GS-1 isolectin B₄ solution in 0.1 M PBS at 4 °C overnight in darkness. After rinsing thoroughly in 0.1 M PBS for 1 hour, the block was positioned under the Leica fluorescence dissection scope (Leica Microsystems Inc., Richmond Hill, Canada), the fluorescein-GS1B₄ labelled glomeruli in the olfactory bulb were visualized using the GFP filter set (#41018, Chroma Technology Corp, Rockingham, VT). Dil (D282, Molecular Probes,) crystals were
picked up onto the tip of the micro-dissection pin (Cat 501316, World Precision Instrument, Sarasota, FL) and inserted into the lateral glomerular region. Immediately after an equal amount of DiA (D3883, Molecular Probes) crystals were inserted into medial glomerular region in the unilateral OB using another micro-dissection pin (Fig. 11). The tissue surface was then sealed with melted agarose for preventing the crystals from dislodging from the injection site. The tissue was returned to the fixative and placed in the darkness for incubation in frequently renewed fresh fixative at 37°C to allow the dyes diffusion retrogradely from nicked axon terminals in the olfactory bulb along the olfactory nerves to the OSNs in the olfactory epithelium.

Fig. 11: Double Dil and DiA injection into different glomeruli in the olfactory bulb. The glomeruli in the OB in the post-mortem tissue became accessible after vibratome sectioning. Under the fluorescence dissection scope GS1B4 labelled glomeruli were visualized and inserted with suitable amount of Dil and DiA crystal. Scale bar=500 μm.

After 10 days, the tissues was sectioned in a longitudinal or horizontal or cross plane with the vibratome at the thickness of 40 μm and the sections were thaw-mounted onto untreated microscope slides and covered with cover slips. Serial sections were immediately examined with a epifluorescence photomicroscope (Zeiss Axioskop) equipped with the following filter cubes: DiA filter set with 480/30 exciter, 505 dichroic and 535/40 emitter filters (#31001, Chroma Technology Corp) and Dil filter set with 540/25 exciter, 565 dichroic and 605/55 emitter filters (#31002a, Chroma Technology Corp).
Photographs were taken using Q-imaging Retiga 1300 digital camera and Northern Eclipse software.

Confocal imaging was performed on representative sections using a Bio-Rad MRC laser scanning confocal microscopy with Argon/Krypt lasers. 488 nm excitation and emission filter set at 522 nm were used for DiA signal while 568 nm excitation and emission filter set at 598 nm were performed to collect Dil signal.

3.1.2 Biocytin loading of glomeruli

In order to confirm the projection patterns revealed from the application of carbocyanine dyes in the post-mortem lamprey tissue, biocytin retrograde labelling method was performed in the in-vitro live tissue in this experiment. Moreover, biocytin application was advantageous over carbocyanine dyes in some respects: the morphological features of the labelled sensory neurons were better characterized due to the application of thin sectioning and fluorophore marker for the signal amplification. The stability of biocytin made it possible for the long-term retention of the labelled sections while carbocyanine signal was stable for only a couple hours after tissue sectioning.

The specimens for this experiment included 6 transformers, 1 parasite lamprey and 4 ovulated adult lampreys. 10 μl 4% biocytin solution in Tris-HCl buffer (pH=7.4) was first dropped onto a microscope slide; the water was allowed to evaporate to form viscous dye solution. Then under the dissection scope the tip of the micro-dissection pin was dipped into the dye solution so that its surface was coated with a thin film of dye which dried completely in the air before use. This dissection pin was installed onto a Narishige pipette holder (H-7) which was affixed to the Narishige stereotaxic micromanipulator system (MMN-1).

The lamprey was anesthetised with 0.5% MS 222 and sacrificed by decapitation. The fish head was immersed in oxygenated lamprey Ringer solution (110 mmol/L NaCl, 2.1 mmol/L KCl, 2.6 mmol/L CaCl2, 1.8 mmol/L MgCl2, and 10 mmol/L Tris buffer at pH 7.4) in a Petri dish. The peripheral olfactory organ and forebrain were dissected out from the head under the stereoscope at the magnification of 40X before the preparation was positioned stable in the Petri dish with the 000 insect pin (Fine Science Tools, Cat
26002-20, Vancouver, Canada). The diencephalon and then caudal part of telencephalon were cut apart gradually from the preparation with the super-fine forceps. Extra attention was paid not to stretch or twist the brain tissue during the dissection. Eventually the front wall of the lateral ventricle was exposed on the cutting surface. Ringer’s solution was removed from the Petri dish with the transfer pipette and the cutting surface of the preparation was kept dry by wicking away any Ringer solution with a fragment of twisted Kimwipe paper. The micro-dissection pin coated with a film of biocytin crystal was moved under the stereoscope towards the cutting surface of the preparation with the guide from micromanipulator, then the pin tip was inserted as quickly as possible into the medial or lateral glomeruli under the surface of the preparation and kept in position for about 2-3 minutes for the dye solving into the brain tissue. The pin tip was then retracted out from the injection site and the preparation was immersed again into oxygenated lamprey Ringer solution in a perfusion chamber. The in-vitro tissue incubation lasted 4-5 hours at 10 °C to allow the biocytin transport retrogradely from the axon terminals in the olfactory bulb along the olfactory nerves to the OSNs somata in the olfactory epithelium in the nasal cavity. The tissue was then fixed in 4% PFA in 0.1 M PB for 24 hours and cryoprotected in 10% (2h), 20% (2h) and 30% (overnight) solution of sucrose in 0.1 M PB, frozen in Tissue-Tek, OCT and sectioned on the cryostat. Serial cross sections at 25 μm thicknesses were thaw-mounted onto superfrost-plus slides. The biocytin labelling was visualized by incubating the slides with Alexa-568 conjugated streptavidin in 0.1 M PBST, PH 7.4 for 2 hours at room temperature. The slides were viewed in a Zeiss fluorescence microscope (Axioskop) or Bio-Rad 1024 confocal optics attached to a Nikon E800 upright microscope.

3.2 Results

For unknown the reasons, the tract tracing experiments carried out in all the parasitic phase lampreys failed. The results from the fish at transformer stage were constant and were summarized in the following schematic drawing (Fig. 12). From the double carbocyanine dyes loading experiment two separate primary olfactory projections were revealed: DiI inserted into the lateral glomeruli retrogradely labelled OSNs distributed throughout the olfactory epithelium, while DiA inserted into the medial glomeruli.
labelled the neurons in vesicles of the accessory olfactory organ (AOO) and some OSNs along the lamellae folds. There was no overlap of the Dil and DiA labelling in the peripheral olfactory organ. Biocytin loading experiment in the live tissue in the transformer lamprey supported our results from carbocyanine dyes and the morphological features of AOO neurons were well characterized. Moreover the connectivity between AOO vesicles and the nasal cavity was shown to be more prominent in the adult lamprey than in the transformer lamprey.

Fig. 12: Schematic drawings of tract tracing results in peripheral olfactory organ in the sea lamprey in the metamorphic stage. Left: medial glomerular injection labelled the AOO neurons and some OSNs along the olfactory lamellae. Right: Lateral glomerular injection labelled the OSNs distributed throughout the OE in the lamellae.

3.2.1 OSNs projections into the lateral glomeruli

Ten days after deposition of DiI into lateral glomerular regions, numerous OSNs in the olfactory epithelium were retrogradely labelled in lamellae in the ipsilateral half of the olfactory organ. Beneath the epithelial layer, the olfactory nerve fascicles within the lamina propria in each lamella, consisting of small bundles of axonal processes from OSNs, were also labelled by DiI (Fig. 13). Neither OSNs in the olfactory epithelium nor
the nerve fascicles in the lamellae propria were Dil labelled in the contralateral half of the olfactory organ, demonstrating the lack of decussation in the projection in the primary olfactory pathway (no OSNs in the olfactory epithelium projected to the contralateral OB). The strict ipsilateral projection was clearly seen in the Dil labelled median septum lamella of the olfactory organ. The ipsilateral side was positively stained with Dil while the contralateral side was entirely negative (Fig. 14).

Fig. 13 Dil retrograde labelling in the OE following dye application to the lateral glomeruli in the transformer lamprey. The retrogradely labelled OSNs were shown in the cross section of the olfactory rosette in the transformer lamprey, and the olfactory nerve fascicles extended within the lamina propria from the base of the each lamella to the tip end. Dorsal is at the top and lateral at the right. Scale bar= 500 μm.

Fig. 14 Ipsilateral neuronal projection of OSNs in the olfactory epithelium following dye application to the lateral glomeruli in the transformer lamprey. It is shown in the bright field micrograph that olfactory lamellae projected centrally and the median septum lamellae partly separated the nasal cavity into two halves. Dil injected into the lateral glomeruli retrogradely labelled the OSNs in the ipsilateral half of the nasal cavity in the red channel. Dorsal is at the top and lateral at the right. Scale bar= 500 μm.
Compared with the small size of the glomerular area in the olfactory bulb in the transformer lamprey, the size of Dil crystal applied to the lateral glomeruli in the first experiments was in fact too huge and caused the dye diffusion into the lateral half of the olfactory bulb. Many glomeruli except the medial ones were strongly stained in the OB sections (data not shown). In the peripheral olfactory organ, numerous OSNs were observed to be retrogradely labelled and clustered in the pseudo-stratified olfactory epithelium. However there were still Dil negative areas left in the peripheral olfactory organ: they were the AOO and epithelial structures at most lamellae folds. Although the AOO was juxtaposed tightly with Dil strongly labelled olfactory nerve fascicles within the connective tissue, Dil molecules did not diffuse into these structures. There was a clear boundary between Dil labelled olfactory epithelium and Dil negative AOO-lamellae folds areas (Fig 15).

Fig 15 Dil negative labelling in the AOO following dye application to the lateral glomeruli in the transformer lamprey. Left: AOO vesicles (green arrows) located at the caudal portion of the peripheral olfactory organ in the transformer lamprey were Dil negative in this horizontal sections after Dil was inserted into lateral glomeruli. Moreover the OSNs in the lamellae folds were also Dil negative. Right: The AOO vesicle beneath the lamellae fold seemed to have an opening towards the nasal cavity. Rostral is at the top and medial at the right. Scale bar= 500 μm.
Morphological features of the OSNs in the olfactory epithelium were characterized after smaller amount of Dil was applied in the experiment. The labelled OSNs were distributed sparsely in the olfactory epithelium in lamellae of the ipsilateral olfactory organ (Fig. 16). The labelled neurons showed the typical morphological characteristic of

![Image of OSNs distribution](image1)

**Fig. 16** Punctate distribution of OSNs in the OE following dye application to the lateral glomeruli in the transformer lamprey. Dil labelled OSNs were distributed throughout all the olfactory lamellae in this longitudinal section after suitable amount of Dil crystal was micro-injected into lateral glomeruli in the transformer lamprey. Olfactory nerve bundles at the caudal portion of the olfactory organ and the rostral end of the OB were shown in this section. Rostral is at the top and dorsal at the right. Scale bar = 150 μm.

![Image of OSNs morphology](image2)

**Fig. 17** Morphological features of the Dil labelled OSNs in the OE in the transformer lamprey. The typical morphological characteristic of OSNs was shown in this Z-series projections: the neuron was bipolar with the cell body at the deep region of the epithelium (OE). The slender neuron projected an apical dendrite toward the surface of the epithelium and ended with an olfactory knob (red arrow), the cell body tapered into an axon (green arrows) and traveled into the lamina propria (LP). Scale bar = 50 μm.

OSNs in the sea lamprey: they were tall and slender bipolar cells with the cell bodies at the deeper layer of the epithelium, the neuron projected an apical dendrite toward the surface of the epithelium and ended with an olfactory knob extending into the mucosal
surface. The cell body was located at the deep two-thirds of the epithelium and gradually tapered into the axon which could be traced into the nerve fascicles in the lamina propria (Fig. 17). The labelled OSNs were located at different depths in the olfactory epithelium in the transformer lamprey. Predominately labelled cells were of the tall type with long dendrites towards the surface and perikarya located near the bottom of the epithelial layer, while other neurons with intermediate height had their cell bodies in the middle part of the epithelium and possessed short dendrites at the top (Fig. 18).

3.2.2 AOO projections into the medial glomeruli

The DiI negative AOO areas from the lateral glomeruli injection were retrogradely labelled by DiA when the dye crystal was inserted into the medial glomeruli in this experiment. It was clearly shown that AOO was composed of a large number of small epithelial vesicles; they were located within the connective tissue and packed together along the median line of the ventrocaudal part of the peripheral olfactory organ. The cells lining the AOO vesicles were arranged into simple cuboidal or low columnar epithelium with an obvious lumen at the centre. Each vesicle was composed of 10-15 cells, some of which were labelled by DiA (Fig. 19). It was hard to characterize the morphological detail of the individual cells even at high magnification with laser scanning confocal microscopy because 488 nm from argon laser does not match the absorption maximum of DiA.

In addition to this vesicle cluster, scattered AOO vesicles beneath most lamellae folds were also labelled with DiA. Some are so closely associated with the olfactory epithelium that only the basement membrane separated them (Fig. 20). A small bundle of DiA labelled fibers was observed to be immediately adjacent to the vesicle, and Dil labelled olfactory nerve fibers nearby projected rostrally into the lamina propria within the olfactory lamellae. There was no co-localization of DiA and Dil signals within the nerve fascicles. Above the AOO vesicle a few OSNs along the lamellae folds were also labelled and there was no morphological variance between these DiA labelled OSNs and adjacent DiI labelled ones (Fig. 21).
Fig. 18 OSNs in the transformer lamprey. Left: The cell body of a neuron with short dendrite was situated in the middle region in the epithelium. Right: The perikarya of an OSN was located near the bottom of the epithelial region with long dendrites towards the surface. Scale bar =10 μm.

Fig. 19 DiA labelling in the AOO following dye application to the medial glomeruli in the transformer lamprey. Left: the bright field micrograph of this cross section shows that the aggregation of AOO vesicles are located along the medal line (white dotted line) within the connective tissue at the caudal end of the olfactory organ. They were labelled after DiA crystals were injected into the medial glomeruli in bilateral OBs. The picture was shot under epi-fluorescence scope with DiA filter set. Scale bar =500 μm. Right: High magnification of the insert area in Left picture, showing the morphological detail of AOO vesicle. In the cluster each AOO vesicle contained a lumen and was composed of 10-15 cells, some were labelled by DiA. Some DiA labelled fibers were observed surrounding the AOO vesicles. Scale bar = 25 μm.
Fig. 20 Double DiA and Dil labelling in the peripheral olfactory organ following Dil and DiA insertion into lateral and medial glomeruli respectively. A single AOO vesicle was located beneath the lamellar fold and labelled by DiA inserted into the medial glomeruli (green channel). A small nerve fascicle stained by DiA (white arrow) was observed beside the vesicle. Several nerve bundles and some OSNs in the OE were retrogradely labelled by Dil which was inserted into lateral glomeruli (red channel). Scale bar = 25 µm.

Fig. 21 Carbocyanine dyes labelling at the lamellae fold following Dil and DiA insertion into lateral and medial glomeruli respectively. Left: DiA crystal inserted into medial glomeruli not only labelled AOO neurons but also labelled a few OSNs at the lamellae fold situated above the AOO vesicle (green channel). Dil crystal in the lateral glomeruli only stained OSNs in the OE (red channel). Right: High magnification of the Dil and DiA labelled OSNs in the OE, there seemed no morphological variance between the DiA and Dil loaded neurons. Scale bar = 10 µm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
3.2.3 The morphology of backfilled OSNs and AOO neurons

Although the double labelling experiment can not be performed with biocytin, the segregation of primary olfactory projections was also revealed by combining the results from separate biocytin injections in different animals: AOO vesicles as well as some OSNs in the olfactory epithelium at lamellar folds were labelled after medial glomeruli injection (Fig. 22); only OSNs which were distributed throughout the whole olfactory

![Biocytin retrograde labelling in the peripheral olfactory organ following biocytin insertion into medial glomeruli. A few OSNs in the OE at the caudal most portions of the lamellae (upper blue circle) and AOO neurons (lower blue circle) were labelled in the ipsilateral OB. Neither OSNs nor AOO neurons (red circles) in the contralateral half of the olfactory organ was labelled. The strong labelling in the green circle was the biocytin injection site and the green arrows indicate olfactory commissure. Scale bar= 500 μm.](image)

![Morphological features of the biocytin labelled OSNs in the OE in the transformer lamprey. The axon (red arrows) originated from the cell body in the middle epithelial layer and projected along the basal membrane and then entered the lamina propria. The olfactory knob (green arrows) sent out 2-3 cilia and projected into the surface of the nasal cavity. Different from the labelling from carbocyanine dyes, the cytoplasm instead of the cell membrane was labelled by the biocytin molecule. Scale bar= 10 μm.](image)
lamellae were labelled when biocytin were inserted into lateral glomeruli, no positive labelling occurred in the AOO neurons (data not shown).

At high magnification the morphological features of OSNs were clearly shown by biocytin which was inserted into lateral glomeruli in the transformer lamprey. The axons originated from the perikarya located in the middle epithelial layer and projected first along the basal membrane and then entered the lamina propria (Fig. 23). The olfactory knob at the apical end sent out cilia and projected into the lumen of the nasal sacs. The whole cytoplasm was uniformly labelled while the nuclei were not stained by biocytin.

AOO neurons showed polymorphic shapes and were different from the OSNs in the olfactory epithelium. These labelled cuboidal neurons had no dendritic structures but possessed prominent cilia or ciliary appendages at the apical surface towards the lumen and sent out individual axons at the base of the vesicle (Fig. 24). Biocytin negative cells were interspersed among the positive labelled neurons. Among the thick olfactory nerve branches a small bundle of labelled nerve fibers was observed to innervate the vesicle and some of the fibers could be traced to the neuronal cell bodies in the lining of the vesicle lumen (Fig. 25).

Overall the retrograde tracing experiments showed that AOO neurons instead of OSNs projected axon terminals into the medial glomeruli in the OB in the sea lamprey; these neurons had characteristic morphology and distribution pattern in the peripheral olfactory organ.

3.2.4 Connectivity between the lumen of the nasal cavity and the AOO

These experiments demonstrated that the lumen of AOO vesicles was found to be connected with the nasal cavity in the sea lamprey. Developmental changes in the connection pattern were obvious from metamorphic to adult stages. In the transformer lamprey only single AOO vesicles scattered along the cartilage had small pores towards the nasal cavity at the point of the lamellae fold (Fig. 15). Most of the AOO vesicles were aggregated in the connective tissue at the most ventrocaudal end of the olfactory organ. The linear distance from these well developed AOO vesicles to the base of the median septum lamella was approximately 20-50 μm.
Fig. 24 Morphological detail of the biocytin labelled AOO neurons. A: Two AOO neurons were labelled in the vesicle, these neurons had no dendritic structures but possessed prominent ciliary-like appendages at the apical surface (yellow arrows) and sent out axons at the base of the vesicle (green arrows). Biocytin negative cells were interspersed among them. B: Two AOO vesicles were clustered together containing typical AOO neurons. Some fine fibers were labelled with the thick olfactory nerve bundles travelling alongside the AOO vesicles. Scale bar= 10 µm.

Fig. 25 Biocytin labelling in the AOO. The AOO vesicle (green circle) comprised of biocytin labelled neurons. These have different staining intensity and polymorphic shapes. A small bundle of labelled nerve fibers was observed to innervate the vesicle and some of fibers can be traced into the neuronal cell bodies in the lining of the vesicle lumen (green arrows). Scale bar= 10 µm.

Fig. 26 Connectivity between the AOO vesicles with the nasal cavity in the ovulated adult sea lamprey. In this horizontal section of the peripheral olfactory organ, the small duct (thick green arrow) connecting single AOO vesicle “C” with the nasal cavity at the base of lamellae fold “B” was observed. Tiny pores (yellow arrow) between the AOO vesicles “E” and “F”, which were located in the deep connective tissue, connected all the vesicles together. Scale bar= 500 µm.
The connectivity between the AOO and nasal cavity in the adult lamprey was more obvious than that in the transformer lamprey. Not only were the connecting ducts between the scattered AOO vesicles and the nasal cavity well established at this life stage, numerous tiny pores between individual vesicles integrated all the vesicles into a functional unit in the respect to the communication with nasal cavity since these ducts and pores were prominent throughout the all the AOO areas (Fig. 26).

3.3 Discussion

The neural nature of the AOO in the sea lamprey was identified in our experiment, for the first time since its discovery by Scott in 1887. The morphological features of AOO and its projection into the OB were characterized anatomically using tract tracing techniques. Moreover specific projections between the OB and OSNs in the olfactory epithelium and the AOO neurons were also revealed in this set of experiments. The AOO neurons and some OSNs along the lamellae folds projected into medial glomeruli, whereas lateral glomeruli received input from OSNs distributed throughout the olfactory epithelium in the ipsilateral olfactory lamellae.

3.3.1 Are there AOO neurons?

There have been conflicting opinions regarding the function of AOO in the lamprey species. Hogben (1925) suggested that AOO was a glandular structure because of its intimate relationship to the surrounding blood vesicles and its lack of obvious communication with nasal cavity. The opposite opinion has been proposed in that the AOO could be homologous with the vomeronasal organ of higher vertebrates (Scott, 1887). The neural nature of AOO in the sea lamprey was identified in our experiment after the cells in the AOO vesicles were retrogradely labelled by the neuro-anatomical tracers applied to the candidate OSN terminal fields in the OB. Although the morphology of these AOO neurons differed from OSNs in the olfactory epithelium, the cilia-like structures extended into central lumen and axons converged into medial glomeruli. These observations suggest that AOO may be a chemosensory organ in the olfactory system in the sea lamprey.
The morphology of the AOO cells has been previously characterized with conventional microscope in the river lamprey, *L. fluviatilis* (Hagelin and Johnels, 1955) and in the larval sea lamprey, *P. marinus* (VanDenbossche, 1993). Some AOO cells in both species possessed pronounced nuclei at the cellular base and the cilia protruding into the lumen of the vesicles. Moreover the basal part of the cells has been observed occasionally to proceed in a thin process which joined a bundle of similarly stained fibers that run towards the olfactory nerve (Hagelin and Johnels, 1955). The dye labelled AOO cells in our tracing experiment resembled the cells in the non-experimental studies and their neural nature was further identified by proving their output into the OB.

Other AOO cells in the river lamprey, *L. fluviatilis*, exhibited morphological similarities to the supporting cells in the olfactory epithelium and were suggested to form an interstitial system by separating the first type cells from each other (Hagelin and Johnels, 1955). In the future the biocytin negative cells in our tract tracing experiments should be investigated with biochemical markers specific for supporting cells in the olfactory epithelium e.g. calnexin (Czesnik *et al.*, 2006) in order to determine whether these neuro-tracer negative cells in the AOO in the sea lamprey were biochemically and functionally homologous to the sustentacular cells in the olfactory epithelium.

The biocytin labelled fibers innervating AOO neurons were in accordance with the description from Leach (1951) who noticed the innervation of the aggregation of AOO vesicles by fiber branches within the olfactory nerve in the river lamprey, *L. fluviatilis*. Previous silver impregnation studies have found that the nerve fibers of the olfactory nerve which supply the AOO and the rest of the olfactory nerve appeared to be histologically identical (Hagelin and Johnels, 1955). Overall we concluded that AOO neurons were clustered together in the ventrocaudal portion of the peripheral olfactory organ and separated from OSNs in the olfactory epithelium; however the projections from AOO neurons joined the olfactory nerve with axons from OSNs in the olfactory epithelium until running into the medial glomeruli in the OB.

3.3.2 Speculation on the chemosensory function of the AOO

Molecular evidence supported the hypothesis that AOO was another chemoreceptive structure in the peripheral olfactory system in lamprey species. Several genes of
presumed odorant receptors and vomeronasal receptors have been cloned out in sea
lamprey in Li lab in Michigan State University (personal communication). The
eexpression pattern of these genes products in the peripheral olfactory organ were assessed
in the \textit{in situ} hybridization experiment. It was found in their results that both gene
products of odorant receptors and vomeronasal receptors were localized in the olfactory
epithelium and more importantly the presumed vomeronasal receptors genes were
expressed within the neurons in the AOO vesicles. Although it was still unclear about the
function of this vomeronasal receptor -like gene in coding for a "pheromone" versus a
"general odour" receptor, this result indicated that odour repertoire difference between
OSNs and AOO neurons definitely exist.

In tetrapods, the main olfactory system and vomeronasal system have separate
sensory epithelia and morphologically distinct projection sites into the OBs. The
segregation of the AOO neurons from the nasal cavity in sea lamprey and their unique
projections towards the medial glomeruli in the OB suggest that the AOO in lamprey
species might be the phylogenetic prototype of vomeronasal organ and may be involved
in the recognition of a specific subset of social and sexual behaviour relevant stimuli.

In addition to the similarity of neuronal distribution between the VNO and AOO,
similar vascular plexus surrounding the neural structure has been found in both VNO and
in AOO (Hagelin and Johnels, 1955; VanDenbossche \textit{et al.}, 1995). In tetrapods the
connective tissue within the VNO is extremely vascular; the dense network of large blood
vessels were in close vicinity of the wall of the VN epithelium and were densely filled up
by blood cells (Meredith and Fernandez-Fewell, 1994). This vasculature within the VNO
functions as a pump which operates under novel stimulus conditions and delivers
chemicals into the VNO lumen: when the blood accumulates in the vascular plexus, the
VNO become constricted and partly emptied of its contents. On the other hand, the blood
was drained off and the mechanical tension inside the VNO was reduced when the pump
is activated; consequently the VNO lumen will expand and become refilled with contents
from the olfactory sacs.

Although the direct evidence is not currently available, we proposed that vasculature
in AOO in sea lamprey may serve as a pump mechanism and was capable of emptying or
filling the AOO vesicles with water from the nasal cavity. During this process the
diameter of the pores and ducts connecting AOO with the exterior may change. This hypothesis may explain the absence of biocytin labelling in the AOO neurons in the anterograde labelling experiment in transformer lamprey. The pump may operate under specific stimulus conditions that do not include anaesthesia MS222. Instead the pump may be activated by a specific stimulus such as sex pheromones. The AOO loading experiment could be tried in the future with biocytin solution containing proposed odour stimuli in unanaesthetized animals. Such an experiment may demonstrate the existence of a pump mechanism in the olfactory system and an effective stimulus for pump activation in this species. Coupling pump mechanism with anterograde labelling of the AOO neurons would provide direct anatomical evidence about the AOO projection pattern in the OB and whether or not there were direct connections between AOO and particular olfactory nuclei in the brain.

The localization of intense GS1B\(_4\) staining in the AOO vesicles, compared to the olfactory epithelium, may indicate a specific environment for odoriferous molecules to dissolve in before these chemoreceptive neurons as seen in the olfactory and vomeronasal mucociliary complex (Pelosi 1996). Therefore the heterogeneity of mucous environments at the epithelial surface in the olfactory epithelium and in the AOO resulted from the differences in quality and/or quantity of secreted elements necessitated for the different odour detection cascade.

### 3.3.3 Connectivity between the AOO and the Lumen of the Nasal Cavity

One of the important reasons why AOO was categorized into glandular structure at the beginning of its discovery was its lack of connectivity with the nasal sacs (Hogben et al., 1925). If the AOO is currently suggested to be a chemosensory structure, it should have a passageway to the lumen of the nasal cavity for detecting chemical stimuli dissolved in the nasal mucus. The lack of large openings of AOO into the nasal cavity at both metamorphic and adult stages raise the question as to how chemical stimuli in the nasal cavity would effectively reach the chemosensory neurons in the AOO vesicles. Although the connectivity increases in the adult stage, the narrow ducts connecting nasal cavity with AOO vesicles beneath the lamellae folds revealed in adult and transformer lampreys still seem inefficient for the odorants transport. However numerous tiny pores
existing between the adjacent vesicles integrate all the vesicles into a functional unit, therefore AOO neurons in the vesicles may be accessible to chemical stimuli in the nasal cavity through these openings at the base of lamellae folds in the adult stage. Moreover the possible pumping mechanism from the vascular plexus surrounding the AOO vesicles may change the diameters of the connecting pores and ducts in AOO and pump in effectively the odorants when it is activated.

Conspicuous connecting passageways were found in the AOO in the river lamprey, *L. fluviatilis* (Hagelin and Johnels, 1955): two thick ducts, originating from the AOO vesicles were clearly observed even with low-magnification under the microscope. These opened into the nasal cavity at the base of the median septum lamella, forming the connectivity between the bilateral AOO vesicles aggregations and the nasal sacs. Since potential ducts may be overlooked if tissue is not sectioned at a favourable angle, more tract tracing experiments need to be carried out with extra attention directed to search for the ducts arising from vesicle aggregations in the sea lamprey. The results will ascertain the exact communication pattern and exclude the possible species difference.

3.3.4 Projections from AOO cells and olfactory epithelial OSNs into medial glomeruli

There are different developmental processes between the clustered AOO vesicles along the median line of the nasal sacs and the scattered ones beneath the lamellae folds (Hagelin and Johnels, 1955; VanDenbossche, 1993). However both subgroups were found in our tracing experiments to have the same primary olfactory projection patterns and send the neuronal processes into the medial glomeruli in the OB. It is reasonable to speculate that both AOO vesicle subgroups might have the same odorants repreorior despite their different developmental origin.

Some OSNs along the lamellae folds were labelled with both the biocytin and carbocyanine dye experiments in which the dyes were injected into medial glomeruli. Since the scattered AOO vesicles originated from the invagination of lamellae folds (VanDenbossche, 1993), the labelling pattern and spatial closeness of the two dyes suggest that the OSNs along the lamellae folds and AOO neurons might be integral and have the same primary olfactory projections. To be complete, the clustered AOO vesicles
along the median line of the nasal sacs, the scattered AOO vesicles and OSNs along the lamellae folds comprise a chemosensory structure and project together into the medial glomeruli in the OB in the sea lamprey in metamorphic and adult stages.

However there is another possibility that the OSNs along the lamellae folds do not belong to this structure and actually do not project to the medial glomeruli, but to the adjacent glomeruli instead. The neuro-tracers may diffuse from the injection site in the medial glomeruli to the adjacent glomerular territories during the experiments. These glomeruli might have trajectories and neuronal sources closely aligned with those from the medial glomeruli. Therefore the actual relationship between the AOO neurons with the OSNs along the lamellae folds needs to be checked in the future with molecular markers such as odorant receptors specific for the OSNs and vomeronasal receptors for AOO neurons.

3.3.5 Do the AOO neurons terminate in the medial glomeruli?

Although we identified the neuronal nature of the AOO structure in the sea lamprey and speculate its chemosensory function, it is important to keep in mind that the results of medial glomerular injections should be interpreted with caution, because the potential coexistence of genuine primary olfactory axons with extrabulbar projecting olfactory nerve fibers or terminal nerve projections in the OB in lamprey species (Tobet et al., 1996; Eisthen and Northcutt, 1996; Von Bartheld, 2004). Our retrograde tracing experiments in this chapter revealed the projections of the AOO neurons to the medial glomeruli in the OB, but we can not determine whether the axons of AOO neurons terminated within the medial glomeruli or formed en-passant synapses therein before entering higher forebrain structures. Practically the neuro-tracers we used could be picked up from the injection site in the medial glomeruli which contained either the terminal processes of AOO neurons or the transacted extra-bulbar axons of AOO neurons.

BDA had been proved to retrogradely labels OSNs and or neurons in the AOO vesicles by injection into the glomerular territories in the OB in the transformer lamprey (unpublished data), as we observed in the biocytin insertion experiments. Since dextran conjugated calcium indicators have the same absorption and transport mechanism as BDA, calcium imaging of the slice of the peripheral olfactory organ will be carried out in
the future following the injection of olfactory glomeruli with dextran conjugated calcium indicators. This functional experiment will provide direct evidence whether or not the AOO is a chemosensory organ and characterize its odour repertoire.

3.3.6 Are AOO cells labelled through contamination?

In this set of experiments the projection of AOO neurons was revealed with two kinds of anatomical tracers: carbocyanine dyes applied in the post-mortem lamprey tissue and crystalline biocytin inserted into medial glomeruli in the live tissue. Theoretically there was a possible defect with biocytin technique: the potential leakage of the hydrophilic dye crystals from the injected sites in the medial glomerular areas to the Ringer's solution in the incubation chamber. Sensory neurons, either OSNs in the olfactory epithelium or in the AOO vesicles, therefore have the possibility to take up the biocytin molecules once dissolved in the solution and cause a false positive result in the neuronal labelling in the retrograde tracing experiment.

However, the nerve fascicles in the olfactory nerve were strongly labelled and the axon processes could be traced from the labelled AOO neurons back to the injection site in the OB. Second, the biocytin labelling was confined within the ipsilateral side of the AOO instead of any irregular distribution in the bilateral side of the peripheral olfactory organ. Lastly, despite the short distance between the injection site in the OB and aggregation of AOO vesicles at the caudal end of the olfactory organ, there was no contact between these tissues in-vitro except through the small pores connecting the AOO system with the nasal cavity. It was less likely that for the biocytin to diffuse through the Ringer solution to the deep AOO neurons without labelling any OSNs in the nasal cavity. All these observations support that there was no contamination in the AOO labelling in the biocytin tracing experiment.

We conclude that a unique primary olfactory pathway exists in the sea lamprey. It originates from the AOO neurons and projects into the medial glomeruli in the OB; OSNs in the olfactory epithelium seem to contribute little to this pathway. AOO in the sea lamprey is a potential chemosensory structure responsible for pheromone detection. Direct evidence will be collected in the functional experiments in the future.
Chapter IV Secondary Olfactory Projections from the Medial Glomeruli

In chapter III the unique primary olfactory projection from the AOO neurons to the medial glomeruli was revealed in the sea lamprey. This projection was suggested to be responsible for pheromone detection. Therefore it is hypothesized that the odor information from AOO neurons is further transmitted to the locomotor control regions in the forebrain after the synapse delay in the medial glomeruli. The objective of the following set of experiments was to investigate the trajectory of mitral cells from the medial glomeruli and to figure out the terminal fields and the nuclei receiving this olfactory projection. In order to address this issue, anterograde tract tracing techniques were used and medial glomeruli were injected with neuro-anatomical dyes. Moreover projections from the lateral glomeruli were also characterized in some experiments for comparing differences in the distribution patterns and contribution towards the locomotor areas.

4.1 Material and Methods

4.1.1 Biotinylated dextran amine pressure injection into the medial glomeruli in adult lamprey

In this experiment 7 ovulated adult lampreys were used for medial glomeruli injections. The fish were anaesthetized with 0.5% MS222 and sacrificed by decapitation. The head of the lamprey was immersed in oxygenated lamprey Ringer’s solution in a Petri dish. The forebrain was exposed from the brain cavity by cutting off the surrounding soft tissue and cartilage under the dissection scope.

A micropipette was pulled using KOPT micropipette puller and broken to a tip diameter of 40–50 μm. The Pipette was backfilled with 0.5 μl of 10% BDA 3,000 in distilled water (PH 7.4) (B-1592, Molecular Probes) and attached to the end of a Narishige H-7pipette holder which was connected with picospritzer II. The holder was then affixed to the Narishige stereotaxic micromanipulator (MMN-1) for positioning before the dye injection. The tip of the glass pipette was guided to the surface of the injection site, which is the medial part of the OB between its rostral end and the anterior commissure. Using picospritzer II with the setting of 10 PSI air pressure and 10 msec
pulses, approximately 20 nl BDA solution was pressure-injected through the tip of the pipette into the medial glomeruli in the unilateral OB under the stereoscope at 40X magnification. The glass pipette was kept in position for about 2-3 minutes for the dye diffusion into the brain tissue. Then the tip was retracted out from the injection site and the preparation was immersed again into oxygenated lamprey Ringer solution in the perfusion chamber. The in-vitro tissue incubation lasted 5 hours at 10°C to allow the BDA to be transported anterogradely from the medial glomeruli into the terminal fields in the forebrain nuclei. At last the tissue was fixed in 4% PFA in 0.1 M PB for 24 hours and then cryoprotected in 10% (2h), 20% (2h) and 30% (overnight) sucrose in 0.1 M PB, frozen in Shandon M-1 embedding matrix and sectioned on the cryostat. Serial cross sections at 25 μm thicknesses were thaw-mounted onto superfrost-plus slides.

Serial cross sections from the rostral end of the OB to the dorsal commissure were incubated with Alexa 568 streptavidin (1:200, Molecular Probes) in 0.1 M PBST containing 1 μg/ml fluorescein-conjugated GS-1 isoelectin B4 (FL-1201, Vector Labs, Burlingame, CA). The lectin was used as a marker to visualize all the glomeruli and determine the BDA loading scope from the injection site in the medial glomeruli.

Molluscan cardio-excitatory tetrapeptide (FMRFamide) is a potential neurotransmitter or neuro-modulator expressed in the periventricular nuclear region of hypothalamus in the diencephalon in lamprey species (Ohtomi et al., 1989). FMRFamide antiserum was used as a marker in our experiment to distinguish the diencephalons structure in which BDA labelled fibers traveled through. For the other sections from dorsal commissure to the end of diencephalons, BDA tract tracing was combined with immunohistochemistry to FMRFamide antiserum. First, the sections were incubated with polyclonal anti- FMRFamide antibody (1:1000, Cat. 20091 Immunostar Inc, Hudson, WI) in 0.1 M PBST for 48 hours. After rinsing in 0.1 M PBS, these slides were incubated in Alexa 488 anti-rabbit secondary antibody (1:200, Molecular Probes) containing Alexa 568 streptavidin (1:200). The specificity for FMRFamide immuno-labelling was tested through pre-absorption controls.

The labelled neuronal components from injection site in the OB to the terminal fields in the diencephalon were visualized using TRITC filter set (Cat# 41002, Chroma Technology Inc.) under the Zeiss epifluorescence scope, while the GS1B4 histochemical
results in the OB and FMRFamide-immuno-positive substances in forebrain nuclei were observed with FITC filter set (Cat# 41001, Chroma Technology Inc.). Representative sections were shot with Bio-Rad MRC laser scanning confocal microscope.

4.1.2 Biocytin application into transformer glomeruli

The objective of this experiment is to investigate the secondary olfactory projections originating from medial glomeruli in the transformer sea lamprey and compare this trajectories with that from lateral glomeruli. Different neuro-anatomical dye and loading methods were performed in this experiment. The reason why pressure injection was stopped was due to a technical difficulty: The size of the glomeruli in the transformer lamprey has been shown to be much smaller than those in adult fish in chapter II in my thesis; the micropipette with much smaller tip was therefore used to guarantee the pressure injection into the small glomerular areas. In the first experimental trials, the dura covering the surface of the OB prevented the smooth penetration of the micropipette, the tip of which bent into a curve at the surface of the brain. With sustained pressure, the micropipette was pushed into the deeper region of the OB than the glomerular layer and damaged the brain structure. Therefore the tungsten micro-dissection pin with the 1μm tip diameter (Cat. 501316, World Precision Instruments, Sarasota, FL) was chosen for the smooth bulbar insertion. A crystalline tracer was previously used in the calcium imaging experiment in the olfactory bulb in the frog (Delaney et al., 2001). Compared to pressure injection, the dye insertion method is advantageous in providing a maximal dye concentration at the site of deposition. The biocytin crystal preparation is less hydrophilic than BDA and would not melt much at the free surface of the brain before inserted into the targeted nuclei.

In this experiment, 15 transformer lampreys were used for biocytin glomerular injection. 5 fish were used for medial glomeruli injection and the other 10 for lateral glomeruli injection. 10 μl 4% biocytin in 0.05 M Tris-buffer solution (PH=7.4) was first dropped onto the glass slide and the water was allowed to evaporate to form a viscous dye solution. Under the dissection scope the tip of the micro-dissection pin was dipped into the dye solution so that its surface was coated with a thin film of dye which dried completely in the air before use. This dissection pin was installed onto a Narishige pipette.
holder (H-7) which was affixed to the Narishige stereotaxic micromanipulator system (MMN-1). The olfactory bulbs in the transformer lamprey were exposed using the same dissection method as mentioned above. Any solution on the surface of the telencephalon was wiped off with a fragment of twisted Kim wipe paper before the dye application to prevent the dye from melting on the brain surface during dye insertion process. The tip of the dissection pin coated with a film of dye crystal guided under the stereoscope to the surface of the injection site (either medial or lateral glomeruli) with the help of micromanipulator. After the quick insertion into the given targeted areas, the microdissection pin was kept in position for about 2-3 minutes for the dye solving into the brain tissue. After the tip retracted from inside the olfactory bulb, the tissue was rinsed with Ringer's solution before incubating into oxygenated lamprey Ringer solution in the perfusion chamber. The in-vitro tissue incubation lasted 5 hours at 10°C and the tissue was fixed in 4% PFA in 0.1 M PB for 24 hours and cryoprotected in 10% (2h), 20% (2h) and 30% (overnight) sucrose in 0.1 M PB, frozen in Tissue-Tek, OCT and sectioned on the cryostat.

4.1.3 Dil loading into medial glomeruli

In order to confirm the live tissue biocytin and BDA tracing experiments, we applied carbocyanine dye Dil into the medial glomeruli in the post-mortem lamprey brain tissue in both adult and transformer lamprey. Moreover an asset of this dye insertion technique is that fixed-tissue sectioning facilitated the application of Dil crystal to the most accurate targeted area inside the OB.

5 transformer lampreys and 4 ovulated adult lampreys were used for medial glomerular injection with Dil crystal in this experiment. The transformer lampreys were anesthetised with 0.5% MS 222 and sacrificed by decapitation. The brains were dissected out and fixed in 4% PFA in 0.1 M PB. To facilitate Dil insertion to the exact glomerular areas the fixed brain was first embedded into 5% agarose in 0.1 M PBS. Cross section of the brain-agarose block were sliced with a vibratome from the rostral end of the olfactory bulbs until medial glomeruli were accessible under the cutting surface. Then the block was immersed in fluorescein-conjugated GS-1 isolectin B4 solution at 4 °C overnight in
darkness. After rinsing thoroughly in 0.1 M PBS for 1 hour, the block was positioned under the Olympus fluorescence dissection scope, the fluorescein-GS1B4 labelled glomeruli in the olfactory bulb were visualized using the GFP filter set. A small cut was made into medial glomeruli with a clean micro-dissection pin and a suitable amount of Dil crystals were then picked up onto the tip of the micro-dissection pin and inserted into the targeted area under the fluorescence dissection scope (Fig. 27).

Fig. 27: Illustration of the surgical protocol for the anterograde labelling in the adult sea lamprey. Top: The schematic drawings of the tissue-agarose block: fixed brain tissue was embedded in the agarose and sectioned transversely from the olfactory nerve to the targeted glomeruli. (Modified from Nieuwenhuys, 1998) ON: olfactory nerve. OB: olfactory bulb. Ppir: lateral pallium. MP: medial pallium. Hyp: hypothalamus) Left bottom: The glomeruli in the tissue-agarose block was visible under the fluorescence dissection scope after the incubation in fluorescein GS1B4. DiA crystal was inserted into medial glomeruli (blue circle). Right bottom: The same tissue block with DiA crystal (black circle) shot under the bright field micrograph. Scale bar= 300 μm.

The injection site was then sealed with melted agarose after injection in order to prevent the crystals from dislodging, and the samples were returned to the fixative and placed in the darkness for incubation in frequently renewed fresh fixative at 37°C to
allow dye diffusion anterogradely from the medial glomeruli in the olfactory bulb to the terminal fields. After 10 days incubation, the brain-agarose blocks were sectioned in cross plane with the vibratome at the thickness of 40 μm and the sections were thaw-mounted onto microscope slides and covered with coverslips. Serial sections were examined with rhodamine filter set under Zeiss epifluorescence photomicroscope. Representative sections were photographed using Q-imaging Retiga 1300 digital camera and Northern Eclipse software. The pictures were transferred to a standardized series of brain drawings with the aid of software Corel Draw 12.

4.2 Results

This is the first description of the secondary olfactory projections from specific glomerular territories in the olfactory bulb in the sea lamprey. The analysis of these tract tracing results was based on the silver impregnation studies which comprehensively described the topography of individual brain territories and traced the connections between the different nuclei in the brain of lamprey species.

4.2.1 Tracts from the medial glomeruli in the adult lamprey revealed by BDA injection

4.2.1.1 Precise BDA loading into the medial glomeruli

In the project aimed to trace the trajectory from medial glomeruli, precise dye loading without diffusion into adjacent glomerular areas is necessary before the subsequent analysis of dye-labelled passageway in the forebrain area is deserved to be carried out.

The BDA injection site in the medial glomeruli was clearly shown in the cross sections of the OB (Fig. 28). The hole at the center of the injection site was generated by the insertion of the glass pipette and by the pressurized air injection using picospritzter. The GS1B4 histochemistry showed that glomeruli in the OB were injected by BDA that did not diffuse outside the limits of medial glomeruli. The BDA molecules may have been absorbed by the endocytosis from the perikarya or through the broken axons in the glomeruli before they were transported anterogradely along the processes towards the distal ends in the forebrain nuclei. Rostral to the injection site, several perikarya of the
Fig. 28: BDA injection site in the medial glomeruli of unilateral OB of adult sea lamprey. Glomeruli in the OB were visualized by GS1B4 histochemistry in the OB cross section (green channel). BDA was confined within the limits of medial glomeruli (red channel) and the hole at the center of injection site was generated by the insertion of the micropipette and pressurized air during injection. Scale bar= 500 µm.

Fig. 29: BDA labelling in the ipsilateral OB. Some perikarya located in the medial glomeruli were observed to be strongly labelled in these sections rostral to the injection site. All the glomeruli were marked in C and no perikarya outside the medial glomeruli were labelled with BDA. Some granule cells with the long processes were observed in the deeper layer of the OB. Scale bar= 500 µm.
mitral cells were strongly labelled and the fine fibers surrounding these perikarya might be their dendritic terminals (Fig. 29). The perikarya of granule cells and their long processes extending bi-directionally were retrogradely labelled at the deeper layer of the OB (Fig. 29 D).

4.2.1.2 Projections into the ventral thalamus and hypothalamus

Following BDA injection into the medial glomeruli of the OB, the coarse fibers were strongly labelled and observed to course ventrocaudally and pass diffusely through the lateral neuropil of the ipsilateral telencephalon and the diencephalon in the adult lamprey. These labelled fibers assembled in the vicinity of the dorsal commissure, through which some fibers decussated and projected into the contralateral OB (Fig. 30). Then the axons traveled from the dorsal commissure and nucleus olfactorius anterior (NOA) and to the posterior end of the corpus striatum in the telencephalon (Fig. 31). These labelled fibers comprised of two separate bundles, which passed dorsal and ventral to the wall of lateral ventricle through the telencephalon medium. The ventral division was adjacent to the striatum and the neuron-free zone of preoptical area. The dorsal division ran rostrocaudally and gathered as a thick bundle in a thin area between the dorsomedial telencephalic neuropil (Dmtn) and the roof of the lateral ventricle. These dorsal fibers passed medially from the lateral pallium to the telencephalon medium through the nuclei of dorsal pallium (Fig. 32). After the reunion of the dorsal and ventral division at the caudal end of the corpus striatum, the tract arched ventrally to enter the diencephalon. The fibers ran rostro-caudally beside the nuclei of the periventricular cell layer of the ventral thalamus and the lateral dorsal hypothalamus. These fibers became thinner gradually and terminated diffusively inside these areas (Fig. 33) since BDA labelled fibers were not visible any more in the adjacent caudal tissue sections. Overall, the trajectory running into the diencephalic structure was located along the median line of the forebrain, not extending into the lateral portion of the lateral pallium in the telencephalon.
Fig. 30: Decussation of fibers from the medial glomeruli. A: Thick axons (green arrows) were observed to decussate through the dorsal commissure to the contralateral hemisphere. Some fine fibers were also labelled in the commissure (yellow arrows). B: The thick axons traveled in the symmetric position in the contralateral hemisphere after the decussation (left circle). The inserts were the low magnification of the brain sections. Scale bar= 500 µm.

Fig. 31: Trajectory of the fibers from the medial glomeruli in the ipsilateral pallium. The thick axons comprised of two separate bundles, traveling dorsal and ventral to the lateral ventricle respectively. The dorsal division (green arrows) ran between the dorsomedial neuropil (Dmtn) and the roof of the lateral ventricle (VL). The ventral division (red arrows) traveled adjacent to the neuron-free zone of preoptic area. Some fine fibers were also labelled and travelled along the thick axons of mitral cells underneath the Dmtn. The insert was the low magnification of the cross section showing nuclei in the telencephalic proper. Scale bar= 500 µm.
Fig. 32: Reunion of the labelled axons at the end of telencephalon. The dorsal division turned medially and traveled through dorsal pallium (Dpal) (red circle in Fig. B), and eventually reunited at the caudal end of the striatum (colourful circle in Fig. D) with the ventral division. The fine fibers (green arrows) separated from the thick axons and turned dorsal into the medial pallium (Mpal). Scale bar= 500 μm.

Fig. 33: Diencephalic labelling from BDA injection in the medial glomeruli. Left: high magnification of the insert of cross section of diencephalons in right picture. The periventricular nuclei in the hypothalamus were labelled by the FMRFamide antisera (green channel) and the dotted terminal processes from mitral cells were distributed throughout the ventral thalamus (Vth) and the lateral dorsal hypothalamus (Hyp) (red channel). Scale bar= 500 μm.
4.2.1.3 Projections into the dorsal-medial telencephalic nuclei and the medial pallium

Compared with the thick mitral cell axons located beneath the dorsal-medial telencephalic nuclei (Dmtn), many fine labelled fibers were observed within the Dmtn and formed a dense neuronal network. These fine fibers filled the ipsilateral Dmtn and terminated there throughout the entire rostro-caudal extent of this formation (Fig. 34), and did not label the contralateral counterpart.

Fig. 34: BDA labelling in and beneath the dorsomedial neuropil (Dmtn). Thick fibers in the medial glomeruli traveled caudally underneath the Dmtn (red arrows). Many fine fibers formed a dense neuronal network within the Dmtn (red circle) and terminated throughout the entire rostro-caudal extent of this formation. Scale bar= 100 μm.

Fig. 35 Biocytin injection into the medial glomeruli in the unilateral OB. Strong labelling was observed in the injection site (red circle). Granule cells (green arrows) at the deeper layer of the ipsilateral OB and a few neuronal components (red arrow) in the medial glomeruli of the contralateral OB were also labelled. Scale bar= 500 μm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Bundles of thin fibers were also labelled from medial glomeruli BDA injection. Although the perikarya of these fine fibers were not visualized in the sections, the fibers seemed to begin from NOA. Some fibers ran dorsally of the lateral ventricle by accompanying the thick axons underneath the Dmtn (Fig. 31). When the thick fibers travel ventrally through the dorsal pallium, these fine fibers turned dorsally into the medial pallium. Other fine fibers ran ventral to the lateral ventricle before turning medially and entering the medial pallium. Upon entering the medial pallium, these fibers bend caudo-dorsal through the stratum nervosum where some may terminate (Fig. 32). Having passed through the vertical nucleus along the posterior border of the medial pallium, it turned dorsally and ran through the thalamic eminence in the epi-thalami and entered the ipsilateral habenula.

Our BDA tracing experiment revealed the trajectory of the secondary olfactory projection from the medial glomeruli. Combined with histochemistry and immunohistochemistry the results showed that thick axonal processes from the medial glomeruli projected through the telencephalon medium and terminated in the ventral thalamus and hypothalamus in the diencephalon; terminal fields were also observed in the Dmtn and medial pallium in the telencephalon.

4.2.2 Projections from the medial glomeruli in the transformer lamprey revealed by biocytin injection

The projection pattern revealed from this experiment is similar to the result in the BDA experiment: fibers bundles from the medial glomeruli to the diencephalon were labelled and the terminal fields in the ventral thalamus and hypothalamus were clearly shown in the transformer lamprey. Moreover the crystalline biocytin insertion method provided the advantage of a maximal dye concentration at the site of deposition (Fig. 35) and resulted in more comprehensive labelling in the forebrain nuclei.

A few neuronal components were clearly observed in the contralateral OB. These included anterogradely labelled beaded fibers and retrogradely labelled perikarya. Many neurons were interpreted as mitral cells because of their characteristic morphological feature e.g. two primary dendrites and a thin axon at the proximal end. Some neurons
with only one ramifying apical dendrite were also labelled in the glomerular layer; a few granule cells with tiny perikarya and long bi-directional processes were dispersed in the inner granular layer (Fig. 36).

Fig. 36: Morphological detail of the biocytin labelled neurons in the contralateral OB in the transformer lamprey. A: a typical mitral cell with two primary dendrites (yellow arrows) projecting from the large fusiform perikarya and a thin axon at the proximal end (green arrows). B: Two closely packed OB neurons with only one thick apical dendrite ramifying profusely toward the glomeruli. The proximal ends of the axons were also observed (green arrows). The neurons in C and D resembled the mitral cells in Fig. A while OB neurons in F resembled those in Fig. B. E: a granule cell with long bi-directional processes (green arrows). Scale bar= 10 μm.
The dorsal and ventral fiber bundles originating from the medial glomeruli were clearly labelled and can be traced into the ventral thalamus and hypothalamus (Fig. 37).

Fig. 37 Diencephalic labelling from biocytin injection in the medial glomeruli. Left: More fibers were labelled in the diencephalon and the terminal field covered the ventral thalamus and the whole dorsal hypothalamus. Some thick fibers were also observed in the symmetrical areas in the contralateral hypothalamus and fine fibers could be traced from the habenula commissure into ventral thalamus and hypothalamus (green arrows). Right: Nucleus tuberculi posteriors at the most caudal portion of the diencephalons contained biocytin positive fibers. Scale bar= 500 μm.

Fig. 38: Dorsal pallial labelling from biocytin injection in the medial glomeruli. Left: The dorsal bundle of axons from medial glomeruli was strongly labelled with biocytin. They ran medially from the lateral pallium and traveled through the dorsal pallium. Scale bar= 500 μm. Right: high magnification of the insert, showing the varicosities distributed irregularly along the axons of their whole length. Scale bar= 50 μm.
Within the diencephalon, the terminal field of the secondary olfactory projection was found to extend into a larger area and the fibers fanned out over the ventral thalamus and the entire hypothalamus and the most caudal portion of the terminal field was traced into the nucleus tuberculi posteriors. In the telencephalon, the labelled fibers in the dorsal bundle were compactly arranged under the Dmtn, turned medially from the lateral pallium and ran through the nuclei of dorsal pallium. These labelled axons possess irregularly distributed varicosities along their length (Fig. 38), indicating the existence of pre-synaptic elements of synapses en passage and potential synaptic contacts with the dorsal pallium nuclei.

Following biocytin application to the medial glomeruli, the fine fibers were prominent in the medial pallium (Fig. 39). These labelled fibers ascended to the epithalamic region and decussated in the habenular commissure. Terminal fields may be formed in the epithalamia or habenula, however some fibers ran through the habenular commissure and separated into two bundles and gave rise to diffusive terminal fields respectively: some fibers re-directed back into the pallial areas of the contralateral hemisphere while the others traveled ventrally into the ventral thalamus and hypothalamus in the diencephalons (Fig. 37). These decussating fibers did not redirect back to the OB, which appeared to receive secondary olfactory fibers solely via the dorsal commissure.

Biocytin stained axons were found to decussate through postoptic commissure and posterior tuberculum commissure (Fig. 39). Terminal fields were present in the contralateral hypothalamus, nucleus tuberculi posteriors and even the rostral most portion of mesencephalon (Fig. 37).

4.2.3 Projections from lateral glomeruli in transformer lampreys revealed by biocytin injection

The GS1B4 histochemistry labelling showed that the medial glomeruli were compressed along the medial edge of the OB compared to the lateral ones which were shallow and spread under the surface of the OB in the transformer lamprey (Fig. 40). Therefore it was impossible to label all the lateral glomeruli from one insertion point. In this experiment biocytin crystal was inserted into glomeruli directly opposite the medial
glomeruli in bilateral olfactory bulbs and therefore selected lateral glomeruli were labelled. Biocytin diffusion area was larger in the right OB than that in the contralateral side, and more lateral glomeruli in the glomerular layer and more granule cells in the deeper layer were labelled on the right side (Fig. 40).

Fig. 39: Projection to the epithalamus from biocytin medial glomeruli injection. Left: Some fine fibers ran dorsally in the medial pallium (Mpal) (green arrows), while the thick fibers projected ventrally in the hypothalamus, Right: These fine fibers ascended to the epithalamic region (green arrows) and decussate in the habenular commissure (green circle). Scale bar= 500 μm.

Fig. 40: Bilateral biocytin lateral glomerular injection. Glomeruli in the OBs (blue circles) in the transformer lamprey were revealed with GS1B4 histochemistry (green channel). Selected lateral glomeruli were labelled after biocytin was inserted the opposite the medial glomeruli. Scale bar= 500 μm.
The thick fibers from the lateral glomeruli were found to course caudally within the superficial half of the ipsilateral lateral pallium and formed a terminal field throughout the entire rostrocaudal extent of the lateral pallium, mainly in the superficial neuropil zone than in the central cellular area (Fig. 41). Moreover retrogradely labelled neuronal perikarya were also observed in this area, the projection of which formed the reciprocal connections with the anterogradely labelled fibers between the OB and lateral pallium (Fig. 41). Compared with the huge fiber bundles projecting into the diencephalons from the medial glomeruli, only a few labelled fibers from the lateral pallium turn medially and coursed though the dorsal pallium. These fibers could be traced into the neuropil of

![Fig. 41: Trajectory of the axons from mitral cells in the lateral glomeruli. Left: The thick axons travelled caudally within the superficial half of the lateral pallium (between the red dashed lines) and terminated throughout the entire rostro-caudal extent. Few fibers were traced in the central cellular zone. Some fibers passed through dorsal pallium (Dpal) before entering the medial pallium (red arrow). Right: huge amount of perikarya (green arrows) in central cellular zone in the lateral pallium were retrogradely labelled. Scale bar= 500 μm.](image)

![Fig. 42: Terminal fields found in the habenula from lateral glomeruli injection. A few fine fibers terminated inside the habenula and no labelled fibers were found in the ventral thalamus or hypothalamus. Scale bar= 500 μm.](image)
medial pallium before reaching the habenula in the diencephalon (Fig. 42). Since the bilateral OBs were injected with biocytin, we can not tell whether there existed decussation through the habenula commissure. Different from the terminal field from medial glomeruli, no obvious fibers in the ventral thalamus or hypothalamus were observed in this experiment.

4.2.4 Projections from medial glomeruli revealed by Dil

Despite the effort to reduce the dye application in the experiment, the DiI crystal usually diffused beyond the boundary of the medial glomeruli area and labelled adjacent neural compartments, especially in the ventral glomeruli and the caudally located NOA (Fig. 43). We can not exclude the possibility that some labelled fibers in this experiment actually originated from DiI diffused areas other than the medial glomeruli. Accordingly, the data from DiI injection experiment were listed for the supplement to the biocytin and BDA experiment.

The secondary olfactory projections revealed from BDA and biocytin injection experiments can be found in the DiI labelled fibers in the medial glomeruli injection experiment in the postmortem lamprey brain. Two separate DiI labelled fiber bundles were located dorsal and ventral to the lateral ventricle respectively, and some fine fibers turned from lateral pallium into medial pallium, and DiI signal was not found in the lateral portion of lateral pallium (Fig. 44). Decussating fibers were observed in the dorsal commissure, postoptic commissure and habenula commissure (Fig. 45), thus unilateral medial glomeruli established neuronal connections with bilateral forebrain nuclei in the forebrain through these commissures.

Overall, the secondary olfactory projections from the medial glomeruli in the sea lamprey were revealed from the above tracing experiments. These were distinct from the trajectory from lateral glomeruli since the major components of the former trajectory include the projections into the ventral thalamus and hypothalamus in the telencephalon. Moreover the decussated fibers through forebrain commissures were prominent in the projections from medial glomeruli and there was no evident developmental change in
Fig. 43: Dil diffusion in the anterograde labelling experiment. Despite the small size of the Dil crystal applied to the medial glomeruli in the *post-mortem* tissue, Dil molecule, as the animation showed, still diffused in all directions into the adjacent glomeruli, deeper OB layer and even NOA. However the lateral glomeruli were not labelled in this preparation. Scale bar= 500 μm.

Fig. 44: Trajectory from the medial glomeruli revealed by Dil. Same as the result in BDA and biocytin experiment, two separate bundles located dorsal and ventral to the lateral ventricle were Dil labelled. No Dil signal was found in the lateral portion of lateral pallium. Scale bar= 500 μm.

Fig. 45 Decussations of the Dil labelled projections in forebrain commissures. A: Decussation through dorsal commissure connecting bilateral OBs. B: The projection in the medial pallium ran through stria medullaris (SM), entered habenula and crossed the commissure there. C: The huge projections traveled through postoptic commissure and entered the contralateral hypothalamus. D: some decussated fibers re-directed back to the telencephalon and terminated into lateral pallium contralateral to the injection site. Scale bar= 500 μm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
this projection pattern. The projection pattern of the medial glomeruli in the sea lamprey is shown schematically (Fig. 46).

![Diagram of olfactory system](image)

**Fig. 46** A schematic drawing, summarizing the secondary olfactory projection of the medial glomeruli in the sea lamprey. The red line represented the pathway being determined from the BDA injection results, while blue lines represented the additional fibers we labelled from biocytin and DiI experiments. Drawn by M. Francois Auclair, U. de Montreal.

### 4.3 Discussion

The secondary olfactory projections in the sea lamprey were investigated in our tract tracing experiments. The results from different neuro-anatomical dyes were compared and trajectories revealed in these experiments confirmed previous results of silver impregnation (Heier, 1948) and tract tracing studies in other lamprey species (Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993). More importantly we differentiated the trajectories from the lateral glomeruli and the medial glomeruli whose terminal fields were functionally related to the locomotion modulation in the sea lamprey.

#### 4.3.1 Efferents from medial glomeruli into the forebrain

Despite the comprehensive investigations about the olfactory neural connections in the brain of lamprey species (Heier, 1948; Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993), the trajectory from the individual glomeruli was not carried out due
to the lack of understanding in odotopic representation in the OB. The projections into the locomotor control area in the sea lamprey were found to be ascribed to the medial glomeruli in our micro-injection experiment, and the terminal fields from medial glomeruli corresponded well to the fiber network revealed from Golgi staining (Heier, 1948).

4.3.1.1 Projections into the ventral thalamus and hypothalamus

Difference in the projection patterns between medial glomeruli and lateral glomeruli were characterized from our glomerular micro-injection experiments. The results showed the tractus to the ventral thalamus and hypothalamus from medial glomeruli, while the thick axons originating from lateral glomeruli mainly terminate in the neuropil area of the lateral pallium in the telencephalon. Moreover the trajectory of dorsal and ventral bundles from medial glomeruli revealed that our tracing results agreed with the description from Heier’s work: these two separate bundles passed dorsal and ventral to the lateral ventricle through the wall of the telencephalon medium. The ventral division has an intimate relation to the migrated cells of the corpus striatum and the neuron-free zone of preoptica area, while the dorsal fibers pass medially from the lateral pallium to the telencephalon medium through the nuclei of dorsal pallium. These two subdivisions re-unite at the caudal end of the corpus striatum and terminate into the ventral diencephalon.

The medial and lateral glomeruli injection were performed in our OB tract tracing experiment, and the projections in the ventral glomeruli had be proven to be confined within telencephalon and innervate the septal and preoptica area according to Heier’s silver staining work. We conclude the efferents to the ventral thalamus and hypothalamus mainly originate from medial glomeruli in the OB in the sea lamprey, while other glomeruli may also contribute in this tract.

Our conclusion can be consolidated from a retrograde tract tracing experiment performed in the larval sea lamprey (Pombal et al., 1996). When the dextran was loaded into the ventral thalamus in the diencephlon, some putative mitral cells with huge perikarya and dual dendrites were retrogradely labelled in the medial region of the OB; no mitral cells were traced in other parts of the OB. The result of this experiment was of
significance since the direct connection between the medial OB and the diencephalic nuclei were traced in lamprey species through the technique complement to ours.

4.3.1.2 Terminal fields in the dorsomedial telencephalic neuropil

The silver impregnation study showed that the dorsomedial telencephalic neuropil (Dmtn) contained small-sized neurons and was ventrally bound by a thin cell plate (Heier, 1948). Experimental studies found that HRP injection in the OB resulted in the labelling of this formation throughout its entire rostrocaudal extent in the silver lamprey, *Ichthyomyzon unicuspis* and the river lamprey, *L. fluviatilis* (Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993). Northcutt suggested (1988) that some secondary olfactory projection from mitral cells in the OB might terminate inside the Dmtn and formed synapses with 3rd order olfactory neurons, which project tertiary olfactory fibers caudally along with the thick axons of mitral cells located underneath the Dmtn.

The fiber network in the Dmtn was clearly observed in our experiments when the dyes were injected into the medial glomeruli in the OB, while there was no labelling from the dye lateral glomerular injection. We therefore agreed to the previous results that Dmtn was one of the terminal fields of secondary olfactory projections. Moreover we concluded that the neural termination in the Dmtn originate from the medial glomeruli than other glomeruli. However the physiological significance of this neuropil and any possible correlations with the major terminal fields from medial glomeruli e.g. ventral thalamus and hypothalamus still need to be elucidated.

4.3.1.3 Projections into the medial pallium and habenula

The neural projections to the medial pallium and habenula in lamprey species were first described in the silver staining study (Heier, 1948). The tractus of olfacto-habenularis (Tr. Ol. Hab.) was found to project into the medial pallium and habenula in the silver lamprey. After the thorough analysis of the fibers connection, Heier (1948) claimed that this tract actually originated from various part of telencephalon nuclei other than from the OB. The dorsal division originated from the lateral and dorsal portion of NOA and the periventricular layer in the lateral pallium. The ventral division came from
the medial portion of NOA, the ventral part of the lateral pallium and the nucleus of septi and preoptica. These fibers run dorsocaudally through the medial pallium and terminate into the habenula.

Heier's description about the origin of the Tr. Ol. Hab. match the results of our Dil anterograde tracing experiments in which the dye molecules diffused outside the limits of the medial glomeruli and labelled perikarya in NOA, and maybe the rostral portion of the lateral pallium. The clear labelling of the fine fibers in the medial pallium and habenula therefore originate from the telencephalon nuclei other than the medial glomeruli in the OB.

However the medial pallium and habenula labelling from our BDA injection can not be explained from Heier's description about Tr. Ol. Hab. A suitable amount of BDA solution was pressure injected into the medial glomeruli and no diffusion to either NOA or other telencephalon nuclei were found. Although the number of BDA labelled fibers and labelling intensity was reduced compared to those in the biocytin experiment, the fine fibers can still be traced from the injection site into medial pallium and habenula. Up to now the mitral cells are believed to be the only efferent component in the OB in lamprey species since the processes of granule cell, the interneuron in the olfactory bulb, were confined within the bilateral OBs (Iwahori et al., 1987). Therefore we conclude that there exist a direct connection between the medial glomeruli and medial pallium and habenula. The secondary olfactory projections from the medial glomeruli include trajectory into those diencephalon areas.

The direct connection between the medial glomeruli and medial pallium and habenula is now proven by the BDA retrograde tracing experiment. After the injection of BDA into habenula in the adult sea lamprey, mitral cell perikarya as well as their characteristic dendritic processes were well labelled retrogradely in the medial glomeruli (St-Pierre et al., 2006). Although this anatomical tracing technique can not determine whether habenula is one of the terminal fields of mitral cells in medial glomeruli, the labelling confirmed the existence of a direct connection from medial glomeruli to the habenula in the diencephalon.

In addition to the projection into the ventral thalamus and hypothalamus in the diencephalic areas, secondary olfactory neurons in the medial glomeruli in the sea
lamprey has now been proven from our tract tracing experiments to have other trajectory and terminate into other diencephalic nuclei. Despite the minor contribution to the whole projections from the medial glomeruli, this neural connectivity suggests that the odour information converged on the medial glomeruli is not only related to locomotion control but also is integrated for other physiological processes.

4.3.2 Forebrain decussation of medial glomerular projections

The strict ipsilateral projection in the primary olfactory pathway in the sea lamprey was revealed in our retrograde labelling experiments in chapter III: the primary sensory neurons including AOO neurons and OSNs in the olfactory epithelium sent out axons to the glomerular territories in the ipsilateral side of the OB. However, there is difference in the secondary olfactory pathways in the sea lamprey. The axons of mitral cells in the medial glomeruli were found to decussate to the contralateral hemisphere via different commissures in the forebrain. This result combined with the complex circuits within the OB structures in vertebrates (Shepherd, 1994) suggests that the integration and modulation of the olfactory output occur as early as the OB level.

4.3.2.1 Decussation through postoptic and posterior tubeculum commissures

Our Biocytin and DiI labelling experiments showed the trajectory from medial glomeruli to the ventral diencephalon. Some labelled fibers turned medially and decussated through all the major commissures in the forebrain: dorsal commissure, postoptic commissure and posterior tubeculum commissure. However our BDA labelling experiment revealed no decussation of labelled fibers except in the dorsal commissure. Up to now there are no consistent results about the decussation of secondary olfactory projections in lamprey species. HRP labelling experiment in the river lamprey, *L. fluviatilis* indicated that bulbar neurons took advantage of both dorsal commissure and postoptic commissure for projections to the contralateral pallial structures and ventral diencephalon areas (Polenova and Vesselkin, 1993); however the decussation through the postoptic commissure was not evident in another lamprey species --the silver lamprey, *I. unicuspis* (Northcutt and Puzdrowski, 1988).
Based on our data analysis in the sea lamprey, we disagree with the idea of species differences with the respect to decussation of secondary olfactory projections (Nieuwenhuys et al., 1998). The absence or presence of the decussated fibers seemed to depend on the amount of tracer at the injection site. The insertion of biocytin crystal provided the optimal tracer concentration at the deposition site and resulted in the strong labelling of all the mitral cells. Therefore their projection including the terminal fields and decussating commissures was clearly labelled. The absence of labelling in decussated fibers in the diencephalon in BDA experiment might be due to insufficient amount of tracer: all the mitral cells in the medial glomeruli were not labelled in that experiment or the BDA signal at the distal end of labelled axons in the diencephalon areas was too dim to be detected by means of fluorescence observation.

4.3.2.2 Reciprocal bilateral connections between olfactory bulbs

Different tracer loading amount can also explain the different neural components labelling in the contralateral OB. The thick axons of the mitral cells anterogradely labelled by BDA were observed in the dorsal commissure and were traced in the contralateral telencephalon proper. It seemed that the large tracer amount, provided from the biocytin insertion technique, was needed for uptake of the tracer from neuronal terminals and the retrograde labelling of perikarya of mitral cells in the contralateral OB. In addition to the mitral cells with typical morphological features (Iwahori et al., 1987), several neurons were also labelled in the symmetrical location of the injection site in our biocytin labelling experiment. They were closely packed and sole primary dendrites projected to the same glomerulus. We can not identify the nature of these neurons from morphological criteria determined from Golgi staining in the river lamprey, *L. fluviatilis*.

4.3.3 Homology between projections from medial glomeruli and the MOT in teleosts

Using different neuro-anatomical tracers, our tract tracing experiments showed that medial glomeruli in the sea lamprey mainly project to the ventral thalamus and hypothalamus. This direct connection between medial glomeruli and ventral diencephalon is distinct from the projection pattern of lateral glomeruli which terminated heavily in lateral pallium in the telencephalon. It was suggested before that the tract
running and terminating into lateral pallium in the river lamprey, *L. fluviatilis* was homologues to the lateral olfactory tract in teleost fish, while the medially directed bulbar projection traveling along the median line of the forebrain structures was recognized as the homology of the medial olfactory tract (Polenova and Vesselkin, 1993). The medial olfactory tract was generally believed to be responsible for the transmission and integration of the pheromones and functionally related to the fish social and reproductive responses e.g., aggression and mating.

The ventral thalamus in lamprey species had been proposed as a locomotor-coordinating center (Nieuwenhuys *et al.*, 1998; Heier, 1948), the huge compact fiber bundles from the medial glomeruli to this structure indicated the direct physiological connections between animal movement coordination and the chemoreceptive processes, which might be involved in the pheromone responses. Recent physiological experiments using semi-dissected lamprey has shown that movement can be induced when the medial OB was electronically stimulated, but not the lateral OB (Derjean *et al.*, 2006). Direct electrophysiological and behavioural evidence of movement triggering needs to be collected in the future by stimulating the peripheral olfactory organ with different odours, especially the identified sex pheromones in the sea lamprey. These experiments will be helpful to determine whether the pheromonal stimulation can induce the olfactory responses in the candidate glomerular territories in the OB and be transmitted forward into the locomotor control areas which modulate the animal movement.
Chapter V Overall conclusions

This thesis describes the discovery of sensory neurons located within the AOO, that project to the medial glomeruli, and output neurons from this neuropil in the medial glomeruli extending to specific diencephalic centers that are associated with locomotor control. These studies support the hypothesis of a spatially and morphologically distinct olfactory subsystem in the sea lamprey. Furthermore, this pathway may regulate locomotor responses to pheromones. This study also suggests that the tetrapod VNO olfactory subsystem may have originated as long as 400 million years ago, with the evolution of initial ancestral vertebrates.
Selected Bibliography


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Vita Auctoris

Xiang Ren was born in 1973 in Jinan, P. R. China. He graduated from the Shandong Medical School in China in 2000 with a M. D degree. He practiced as an otolaryngological specialist in the QiLu Hospital in China for four years. Xiang is currently a candidate for the Master’s degree in Biological Sciences at the University of Windsor.