
Andrea. Frontini

University of Windsor

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

Recommended Citation

https://scholar.uwindsor.ca/etd/2783

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.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeib Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

By
Andrea Frontini

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

Windsor, Ontario, Canada
2002

© 2002 Andrea Frontini
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.
Abstract.
Olfaction is an extremely important communication modality for most organisms, yet it is probably the sensory system least understood. Recent findings have shed light on some of the basic principles that control olfactory detection and transmission of signals into the central nervous system, but the mechanisms for spatial resolution of odorants within the brain are still poorly understood. In this study we have used histological, immunocytochemical and \textit{in vivo} optical imaging techniques to investigate the spatial and functional organization of olfactory bulb glomeruli of the developing Agnathan, the sea lamprey, \textit{Petromyzon marinus}. We characterized the glomerular pattern of olfactory sensory neuron terminals in the brain, and found discrete subsets of olfactory glomeruli. The 45 kD protein \(G_{\text{olf}}\) a cAMP dependent GTP binding protein, was localized in the dorsal, anterior, lateral and ventral glomeruli, but was absent from medial glomeruli. This result shows that \(G_{\text{olf}}\) expression was present during early vertebrate evolution and is fundamental to the function of a subpopulation of vertebrate olfactory sensory neurons. We pursued the goal of combining physiological and morphological analysis of defined axonal projections by developing a preparation of the pathway of olfactory sensory axons for dynamic optical imaging. The larval stage of the lamprey provides a very powerful model, allowing for view of the entire olfactory pathway since olfactory epithelium, nerves and olfactory bulb are located in the same horizontal plane. Using a calcium indicator (\(Ca^{2+}\)-green 1-dextran), we anterogradely labeled the glomeruli, and observed pre-synaptic activity following potassium induced synchronous depolarization and olfactory stimulation with the basic amino acid L-arginine. This study established the lamprey as an appropriate model for examining spatial and temporal events during olfactory coding.
Acknowledgments.

I like to thank Dr. Barbara Zielinski for giving me the opportunity to have this experience as graduate student in her lab. It was extremely enjoyable and informative to work with you and I appreciate your total understanding during my initial period here in Canada, which made it easy to overcome the language barrier and any other trouble that an experience in a new country brings with it. Thank you very much Barb.

I would like to thank the Committee members Dr. Paul Taylor (also for his technical advices) and Dr. Stephen Loeb.

I also like to thank Dr. David Cotter to chair my defence.

Thanks of course to my many lab mates. The two graduate students Rachelle Belanger and Andrea Belanger; thank also goes to Christina Dakhil and Cortney Smith for their extremely helpful work during the last years, thank all of you guys also for you friendship and the funny time during work.

I also like to thank Dr. Weiming Li and Dr. Yun Sang at Michigan State University and Dr. Fritz Lischka at Monell Chemical Senses Center in Philadelphia for their technical expertise.

I like to thank also the faculty and the staff (especially Nancy, Usha and Ingrid) for their kindness.

Finally, I would like to thank all fellow graduate students and all the persons that made my stay in Canada very similar to be at home and they made this experience, not just a work experience but mainly a life experience.

Thank you everybody.
TABLE OF CONTENTS

Abstract .................................................................................................................. III
Acknowledgments ................................................................................................... IV
List of figure plates ................................................................................................. VIII
List of Abbreviations ............................................................................................... XI

Chapter 1. General Introduction ........................................................................... 1
1.1 The primary olfactory pathway ......................................................................... 1
1.2 Rhinotopy ........................................................................................................ 7
1.3 Odour signaling ................................................................................................ 10
1.4 The occurrence of G-protein coupled receptors in vertebrate evolution .......... 12
1.5 The lamprey as a model in neurobiology ......................................................... 14

Chapter 2. Morphological characterization of lamprey olfactory bulb .............. 19

2.1 Material and Methods ..................................................................................... 19
  2.1.1 Experimental animals ............................................................................... 19
  2.1.2 Tissue fixation ........................................................................................ 21
  2.1.3 Characterization of olfactory sensory neurons through GSL I-isolecind B₄ histochemistry and Gₐₛ/olf & Gₛ₀ immunoreactivity .............................................................. 22
  2.1.4 Anterograde labeling of olfactory sensory neurons through Calcium green-1 dextran loading ................................................................. 23
  2.1.5 Molecular weight determination of Gₐₛ/olf through Western immunoblotting ................................................................. 24
  2.1.6 Microscopic imaging acquisition ............................................................... 25

2.2 Results .............................................................................................................. 26
  2.2.1 Organization of glomerular units within the olfactory bulb ....................... 26
    • GSL I-isolecind B₄ staining ........................................................................ 26
    • Calcium green-1 dextran loading .............................................................. 27
  2.2.2 Gₐₛ/olf expression in the olfactory pathway and in the olfactory bulb ......... 29

2.3 Discussion ........................................................................................................ 37
  2.3.1 The spatial pattern of OSN axon terminals ............................................... 37
  2.3.2 Discrete subsets of olfactory glomeruli with Gₐₜ localization .................... 38
2.4 Conclusions........................................................................................................40

Chapter 3. The advantages and limitations of current imaging methods for spatial analysis of activity in the olfactory pathway..................................................................................42
3.1 A preparation of the primary olfactory pathway for dynamic optical imaging. Objectives ..................................................................................................................44
3.2 The Calcium indicator: Calcium green 1-dextran........................................................................................................47

3.3 Material and Methods..........................................................................................49
3.3.1 Dye loading.........................................................................................................49
3.3.2 Assessment of OSN dye loading through Hu-immunocytochemistry..........................50
3.3.3 Assessment of the effect of dye loading on olfactory cilia through acetylated tubulin immunocytochemistry..........................51
3.3.4 Preparation of the primary olfactory pathway for dynamic microscopy..........................53
3.3.5 Perfusion chamber..............................................................................................54
3.3.6 Presentation of odor stimuli..................................................................................55
3.3.7 Image acquisition.................................................................................................57

3.4 Results..................................................................................................................58
3.4.1 Calcium green-1 dextran loading of glomerular units...............................................................................................................59
3.4.2 Structural integrity of OSNs following dye loading.................................................62
3.4.3 Calcium imaging responses to 43mM K⁺ ................................................................65
3.4.4 Calcium imaging responses following amino acid stimulation..............................66

3.5 Discussion..............................................................................................................67
3.5.1 Calcium green-1 dextran as a tool for study of neuronal activity in the primary olfactory pathway of lamprey..........................68
3.5.2 Glomeruli are physiologically active units in the larval lamprey olfactory bulb..........................................................................................69
3.5.3 Complications associated with olfactory bulb imaging...........................................70

3.6 Conclusions.........................................................................................................72
Chapter 4. Overall conclusion...............................................................73
Appendix..............................................................................................75
Selected Bibliography........................................................................81
Vita Auctoris.........................................................................................91
List of figure plates.

**Figure 1:** A schematic diagram of the pathway of OSNs in vertebrates ........ 2

**Figure 2:** A view of the larval lamprey head ............................................. 3

**Figure 3:** Horizontal section of the larval lamprey head ............................. 4

**Figure 4:** Glomerular units in the lamprey olfactory bulb ............................ 6

**Figure 5:** Schematic model for the mechanism of odorant signal transduction .................................................................................. 10

**Figure 6:** Schematic diagram of an odorant receptor ..................................... 13

**Figure 7:** A photograph of larval lampreys .................................................... 14

**Figure 8:** The olfactory pathway in a larval lamprey ................................. 16

**Figure 9:** A photograph of an adult sea lamprey .......................... 17

**Figure 10:** Lamprey holding facility ............................................................. 20

**Figure 11:** The organization of olfactory glomeruli in the olfactory bulb of the larval lamprey ............................................................. 28

**Figure 12:** $G_{a s/ol}$ expression in lamprey olfactory epithelium .................. 31

**Figure 13:** Double labeled images with $G_{a s/ol}$ and AT-immunoreactivity in larval olfactory epithelium ..................................................... 32

**Figure 14:** Double labeled preparations of $G_{a s/ol}$-immunoreactivity and GSL I-isoelectin B4 in larval olfactory bulb ................................. 33

**Figure 15:** Double labeled preparations of
$G_{\alpha s/olf}$-immunoreactivity and GSL I-isolectin B$_4$

in the medial olfactory bulb.................................................. 34

**Figure 16:** Double labeled preparations with

$G_{\alpha s/olf}$-immunoreactivity and anterograde

transport of CGD in the larval olfactory bulb......................... 35

**Figure 17:** Double labeled preparations of $G_{\alpha s/olf}$ immunoreactivity

and GSL I-isolectin B$_4$ expression in the adult olfactory bulb........36

**Figure 18:** Western immunoblotting for $G_{\alpha s/olf}$ in

olfactory epithelium preparation............................................37

**Figure 19:** Diagram of pre-synaptic calcium influx..................45

**Figure 20:** Emission spectrum of Calcium green-1 dextran.............47

**Figure 21:** Preparation of the olfactory pathway for

dynamic optical imaging.......................................................53

**Figure 22:** Diagram of the perfusion system.......................... 54

**Figure 23:** Time course of presentation of odour stimuli............... 57

**Figure 24:** An *in vivo* image of CGD loading in the OSN

Pathway................................................................. 59

**Figure 25:** Diagram of time course loading in OSNs...................60

**Figure 26:** An *in vivo* through focus series of glomeruli

units loaded with CGD.....................................................61

**Figure 27:** Acetylated-tubulin immunocytochemistry

IX
following CGD loading

Figure 28: Hu immunoreactivity following CGD loading

Figure 29: The effect of 43mM K⁺ on Calcium green

1-dextran fluorescence emission in two specific glomeruli-I

Figure 30: Effect of 10⁻⁶ M L-Arginine on calcium green

1-dextran fluorescence in olfactory glomeruli

Figure 31: The effect of 43mM K⁺ upon Calcium green

1-dextran fluorescence emission in two specific glomeruli-II
List of Abbreviations.

AT  Acetylated Tubulin
CAMP  cyclic Adenosin Mono Phosphate
CNS  Central nervous system
CGD  Calcium green-1 dextran
mRNA  messenger Ribo Nucleic Acid
MS-222  Tricaine methane sulfonate
GSL IB₄  Griffonia simplicifolia lectin I-isolectin B₄
IP₃  Inositol Trisphosphate
-IR  Immunoreactivity
K⁺  Potassium cation
KCl  Potassium chloride
OB  Olfactory bulb
OE  Olfactory epithelium
ON  Olfactory nerve
OSN  Olfactory sensory neuron
OSNs  Olfactory sensory neurons
OR  Olfactory receptor
PBS  Phosphate buffer saline
PFA  Paraformaldehyde
TME  Tris buffer

(10 mM Tris, 3 mM MgCl₂, 2 mM EGTA, pH 8.2)
Chapter 1. General Introduction.

1.1 The primary olfactory pathway.

For many animals, olfaction is the primal sensory modality allowing communication with their environment. Chemosensitivity is probably the most ancient sense in the animal kingdom and it has been critical for the survival of organisms since it is involved in a host of behaviors including predation, reproduction and various social interactions.

It is common to refer to the human olfaction “as the sense of luxury”. This may explain why many researchers, until the past few decades, avoided the topic of olfaction and spent most of their research effort in the understanding other senses. However, the trend has completely changed and lately, this chemical-detecting system has received considerable attention. One of the breakthroughs that shed light on the complex sense of smell, was the discovery of a novel gene family encoding seven trans-membrane proteins, that were considered as candidate odorant receptors with about four percent of the genomes of many higher eukaryotes devoted to encoding these proteins (Buck & Axel 1991). The opening of this research line, together with the advent of new techniques offer a fresh prospective to the study of the olfactory system, making it one of the hottest topics in neurobiology.

The basic organization of the primary olfactory pathway is conserved in all vertebrates, from agnathans to primates. Central players in olfactory perception are the olfactory sensory neurons (OSNs) (Fig. 1). These are the primary sensing cells and are organized in a neuroepithelial sheet. This epithelial layer lines a series of cartilaginous outcroppings, called turbinates, in the upper reaches of the nasal cavity in mammals. Other vertebrates have similar specialized structures containing OSNs. In teleost fish, the paired olfactory organs are usually located on the dorsal side of the head.
Fig. 1: The olfactory epithelium is a relatively simple tissue consisting of only three cell types: olfactory sensory neurons (OSNs), supporting or sustentacular cells and a stem-cells population, known as basal cells, from which new OSNs are generated. Each OSN expresses only one of the about 1000 OR genes (green and blue) and the axons from all cells expressing that particular receptor converge onto one or few glomeruli in the olfactory bulb (OB).

The OSNs are bipolar neurons with unique features. An apical dendrite extends towards the mucosal surface and a proximal axon towards the olfactory bulb (Fig. 1). The dendrite culminates in a knob that contains a number of cilia, which extend into a thick layer of mucus. In all vertebrates, odorant receptor proteins are located on the ciliary membrane, where olfactory signal transduction is initiated. There are two additional aspects that set the OSNs apart from other neurons. First OSNs continually turn over during the lifespan of an organism, persisting into adult animals (Thornhill, 1967). Second, OSNs are the only neurons that are directly exposed to both the external environment and the CNS since the dendritic tip (the olfactory knob) is located in the nasal cavity, and the axon terminates in the olfactory bulb. The dendrites and cell bodies of OSNs are located within the olfactory epithelium, and axons project
onto distinct neuropil olfactory bulb glomeruli within the olfactory bulb, an outgrowth of the forebrain (Fig. 1).

The lamprey is a vertebrate with a cartilaginous skeleton; it is a jawless member of the Agnathan family and its olfactory pathway is even simpler than in teleosts. The lamprey is monorhinic with a single nostril located on the dorsal surface of the head (see Fig. 2) and connected to a naso-hypophyseal passage (Kleerekoper, 1972).

![Fig. 2: close view of larval lamprey head. The arrow shows the single nostril on the top of the head. The eyes in the ammocoete (larval stage) are located beneath the skin and are not fully developed. However the olfactory epithelium is functional and most likely it is the primary sense modality in this stage. The larvae are filter feeders and live burrowing into benthic substrate. Scale bar is 5mm.](image_url)

The olfactory mucosa is located in heart-shaped outcroppings in the posterior surface of this passage (Vandenbossche et al., 1995). Lampreys have an olfactory nerve easily accessible through surgery, in comparison to higher vertebrates, where the ethmoid bone and cribiform plate obstruct access for direct observation of the entire olfactory pathway. The OSN pathway, which connects the olfactory epithelium to the olfactory bulb, can be viewed in its entirety in horizontal section of the lamprey's head (Zaidi et al., 1998)(Fig. 3).
Fig. 3: Horizontal section of the larval lamprey head. The olfactory epithelium (OE) the olfactory nerve (ON) and the olfactory bulb (OB) are located in the same plane. Scale bar is 500 μm

In the teleost olfactory bulb, there are four concentric laminae. The most superficial layer is the olfactory nerve layer followed by the glomerular layer, the mitral cell layer and the internal cell layer (Oka et al. 1982).

The organization of the olfactory bulb was studied in the adult lamprey by Iwahori et al. (1987) and although, not as discrete as in many vertebrates, a laminar organization was recognized. From the periphery inward, four different layers were identified: 1) the layer of the olfactory fibers, 2) the olfactory glomeruli with the mitral cells, 3) the granule cells, and 4) the ependymal cells. The olfactory glomerulus is one of the clearest anatomical modules in the entire nervous system. In mammals, a glomerulus is a spheroidal neuropil structure
comprising preterminal axons and terminal boutons of OSN axons and distal dendritic tufts of mitral, tufted, and periglomerular cells. The border of the glomerulus is demarcated by several layers of glial processes and by a ring of cell bodies of the periglomerular cells (Harison and Scott, 1986; Mori, 1987; Shepherd, 1990; Kauer and Cinelli, 1993; Scott et al., 1993; Shepherd, 1993, Shipley et al., 1995). The glomeruli are the sites of the pre-synaptic calcium influx into OSN axons and of asymmetric synapses that generate excitatory postsynaptic responses onto mitral cells. OSN axons synapse onto mitral cells and local interneurons in the olfactory glomeruli (Halasz and Greer, 1993; Klenoff and Greer, 1998; Lin and Ngai, 1999). These secondary neurons project through the olfactory tract into higher brain areas, most of which are telencephalic areas. The olfactory glomeruli in lamprey (Fig. 4) are composed of a dense agglomeration of four components: the terminal portion of the olfactory fibers, the primary dendrites of the mitral cells, the terminal arborisation of the granule cell processes and the terminal portion of the processes of the ependymal cells (Iwahori et al., 1987). The principles afferent to the glomeruli are the olfactory fibers that derive from OSNs, on the other hand the main efferent of the glomeruli are the primary dendrites of the mitral cells. Usually in high vertebrates primary dendrites of several mitral cells converge into a single glomerulus, and occasionally a single mitral cell extends dendrites to two or more glomeruli. The morphology of the mitral cells of the lamprey is different from that found in mammals (Johnston, 1902). In the mitral cells of the lamprey, usually two or more primary dendrites were seen, whereas no secondary dendrites were observed. The absence of secondary dendrites in the mitral cells seemed to be one of the main causes for the lack of a clear laminar organization in the lamprey bulb (Iwahori et al., 1987). In mammals glomeruli are 50-200 μm in diameter (Leise, 1990), whereas in
amphibia and fish the glomeruli are smaller (20-50 µm diameter) and less distinct, owing partly to a paucity of surrounding periglomerular cells (Pinching and Powell, 1971).

![Glomeruli](image)

**Fig. 4:** Glomerular unit in larval lamprey olfactory bulb highlighted through anterograde transport with CGD. Periglomerular cells are not present in this ancestral organism and the glomerular units are not clear as in higher vertebrates. Top is anterior and right is medial. Scale bar is 25 µm.

The glomerulus is a tremendous convergence area and is the functional unit in odour processing. In the input side, the numbers of OSN projections, far exceed the numbers of glomeruli. For example the ratio of convergence in rabbit is 25,000:1 (with 50⁶ the number of OSNs and 2000 the number of glomeruli). On the output side, there are about 50,000 mitral cells (about 25 in each glomerular) and 100,000 tufted cells in rabbit (Allison, 1952), yielding modest divergence of 1:25 and 1:50, respectively. Fish and amphibia characteristically have of the order of 1 million olfactory sensory cells (Allison, 1953). The olfactory bulb in the zebrafish, a teleost, contains about 80 glomeruli (Baier and Korshing, 1994). The ratio of convergence on the input side is thus 12,500:1. No studies have been done on the glomeruli organization in lamprey and this project attempted to search for glomerular characteristics
that are fundamental to the function of the developing vertebrate olfactory bulb.

1.2 Rhinotopy.

In the auditory, visual, and somatosensory system, receptor cells encode specificity about the sensory stimulus (e.g. auditory frequency, visual or somatosensory spatial pattern) by virtue of their exact placement in the sensory sheet. In the olfactory system of mammals and teleosts, the sensory sheet does not form a map of spatial information about the environment. The connections between the olfactory epithelium and the OB lack a strict point-to-point topography. Evidence from several studies now show that the neighbour relations between OSNs in the olfactory epithelium are not preserved in their projection to the OB (Mombaerts et al., 1996) and are, therefore, unlike the connections between the periphery and central targets in the visual, auditory and somatosensory systems.

A few decades ago, multi-electrode recordings provided support for the idea that odour information is represented by activity occurring in parallel in different locations within the olfactory system. Lately with the advent of functional imaging methods, it was possible to observe these events with spatial resolution that gives global views of distributed processes. Four themes for organizing principles have emerged from these studies on the mammalian olfactory bulb. 1) There is a roughly quadrant to quadrant patterning. Olfactory sensory neurons in the dorso-medial olfactory epithelium projecting to glomeruli in the dorso-medial main olfactory bulb, lateral to lateral main olfactory bulb etc. This zone-to-zone mapping has been termed rhinotopy (eg. Schoenfeld et al., 1994). 2) Neurons are widely dispersed within a zone converge on a single glomerulus or cluster of glomeruli. Different laboratories
have demonstrated that the epithelium can be divided into sharply bounded zones on the basis of the differential expression of odorant receptor genes and cell surface molecules that are concentrated on olfactory axons (Mori et al., 1995; Yoshihara et al. 1997). This has been done using monoclonal antibodies, which differentially label the dorso-medial versus ventro-lateral olfactory epithelium and the axonal projection onto the bulb. The differential pattern of gene expression in the epithelium translates to differential levels of protein on olfactory axons that project from the periphery onto the bulb, including their termination in the glomerular layer (Schwob and Gottlieb, 1986). 3) Axons of OSNs that express seven transmembrane domain putative odorant receptors, no matter where they are found on the epithelial sheet, project to spatially conserved glomeruli (eg. Mombaerts 1996). It has also been shown, in rat, that OSNs expressing a particular molecular receptor in the left or right nasal cavity, distributed within one of the olfactory epithelium receptor distribution zones, project their axons in a convergent fashion onto (usually) two glomeruli symmetrically situated on either side of the ipsilateral olfactory bulb (Buck, 1996). It is not known what determines which subset of OSNs from this population projects to one or the other of the two glomeruli. The presence of paired glomeruli is interesting because there are anatomical pathways connecting homologous lateral and medial OB regions (Schoenfeld et al.; 1985) that might use the information coming to the two glomerular target to enhance detection or discrimination. It is generally thought that all the OSNs projecting to the two glomeruli share the same response profile, because they express the same receptor. Finally 4) there are two anatomical and functionally distinct components, termed “generalist” and “specialist” by reference to their sensitivity to odorants (Hildebrand and Shepherd, 1997). Candidates for the specialist OSNs are located within “necklace olfactory glomeruli” projecting
adjacent to the accessory olfactory bulb, and with elements of a cyclic guanosine monophosphate (cGMP) signal transduction pathway (Juilfs et al., 1997). Single-unit (Duchamp et al., 1974; Getchell, 1974; Revial et al., 1978, 1982), field potential (Scott et al., 1996, 1997), and optical imaging (Youngentob et al., 1995) studies in amphibians and rodents have demonstrated that individual odorants differentially activate broad regions of the olfactory epithelium and that OSNs are “broadly tuned”, responding to many, often structurally dissimilar, molecules.

In summary, these results suggests that OSN afferents can segregate by odorant responsiveness and that local regions of the OB may receive inputs from OSNs with similar response properties (Kauer, 1980, 1987; Shepherd, 1994; Mori, 1995). This idea is supported by physiological data suggesting that individual odorants are represented in part by the spatiotemporal activation of ensembles of glomeruli (Kauer et al., 1993; Friedrich and Korsching, 1997). In mammals, the scale of this organization is phenomenal. The olfactory system responds to approximately 2000 odors, expresses about 1000 putative olfactory G protein linked receptors (Mobaerts, 1999) and contains about 2400 glomeruli (Meisami and Sendera, 1993). In teleost fish, the number of putative olfactory receptor genes drops to 300, there are approximately 80 olfactory glomeruli, and the spatial organization of glomerular groupings conform to a specific configuration (Baier and Kosching, 1994). The medial glomeruli respond to stimulation by bile acids; the anterior and lateral glomeruli, to amino acids; and a medial ventral glomerulus responds to a reproductive prostaglandin pheromone and may contain projections of specialist OSNs (Friedrich and Korsching, 1997). In lampreys, the family of olfactory receptor genes is even smaller (Berghard and Dryer, 1998; Freitag et al., 1999). Not surprisingly, lampreys respond to few odorants: basic amino acids, such as L-arginine, and
bile acids (Li and Sorensen, 1995). Pheromonal communication for spawning migration is guided by the bile alcohol petromyzonol sulfate (Bjerselius et al, 2000) and reproductive behaviour, by its ketone metabolite (Li, 2002). These characteristics of the lamprey olfactory system predict a simple organization of glomeruli in the olfactory bulb, with the possibility of specialist OSNs projecting to specific olfactory glomeruli.

1.3 Odour signaling.

Following the binding of an odorant molecule to a receptor protein within the compact layer of cilia, chemical energy is transformed into an electrical signal that can be transmitted to the brain. Once the receptor has bound an odor molecule, a cascade of events is initiated (Fig. 5).

![Diagram of odorant signal transduction]

Fig. 5: Schematic model for the mechanism of odorant signal transduction. The chemical energy following the binding of an odorant molecule to receptor coupled to a G-protein ($G_{alb}$), is transformed to an electrical stimulus by a cascade of enzymatic reactions. The pathway using the second messenger cAMP is represented in this diagram.

The ligand-bound receptor activates a G-protein (an olfactory specific sub-type, $G_{alb}$) (Jones and Reed, 1989), which in turn activates an adenylyl cyclase (ACIII) (Pace, 1986). The cyclase converts the abundant intracellular molecule ATP into cyclic AMP, a molecule that has numerous signaling roles in cells. In the case of OSNs, the cAMP binds to the intracellular face of an ion channel, enabling it to conduct cations such as $\text{Na}^+$ and $\text{Ca}^{2+}$ (Firestein and Shepherd,
Inactive OSNs normally maintain a resting voltage across their plasma membrane of about $-65 \text{ mV}$ (inside with respect to outside). When the cyclic nucleotide-gated channel opens, the influx of $\text{Na}^+$ and $\text{Ca}^{2+}$ ions causes the inside of the cell to become less negative (Nakamura and Gold, 1987). If enough channels are open for long enough, causing the membrane potential to become depolarize, about $20 \text{ mV}$ less negative, the cell reaches threshold and generates an action potential. The action potential is then propagated along the axon, to the axon terminal and synaptic junction onto second-order neurons (mitral cells) in the olfactory bulb. A survey of numerous odorous compounds indicated that all odorants, which have previously been shown to activate adenylyl cyclase (Sklar et al., 1986), induced a rapid cAMP response. Odorants that failed to elicit cyclic nucleotide accumulation, produced another second messenger response; those odorants induced an increase in inositol 1,4,5-trisphosphate ($\text{IP}_3$), instead (Boekhoff, 1990). $\text{IP}_3$-gated ion channels have been discovered in the plasma membrane of olfactory neurons from various species (Restrepo, 1990); Assaying a whole range of odorants on isolated cilia indicated that they activate either the cAMP or the $\text{IP}_3$ pathway and suggest that odorants may be categorized into one of two classes, each class activating a different transduction pathway (Breer and Boekhoff, 1991; Schild, 1998). However, a study on catfish cilia, which contain cAMP-activated channels and $\text{IP}_3$-activated channels, showed that individual amino acids elicit responses through both pathways (Bruch and Teeter, 1990). Coexistence of both pathways within the same OSN raises the possibility of an efficient interaction between the two systems, thus, providing the potential for considerable positive and negative interactions, which would affect the generation of electric responses. The existence of two alternative transduction cascades would be of particular
relevance if they could be related to the observed alternative excitatory or inhibitory response of olfactory neurons to odor stimulation (Dionne, 1992). Biochemical experiments confirm that $G_{olf}$ can mediate ligand-activated adenyl cyclase activation in a heterologous system (Jones et al., 1990). In the screen for $G$ protein $\alpha$ subunits expressed in the olfactory system, no additional, novel subunits that might transduce signals through the IP$_3$ pathway were identified. However, one study reported that IP$_3$ stimulation in rat olfactory cilia could be blocked by pretreatment with pertussin toxin (Boekhoff et al., 1990). The mRNA encoding the pertussin toxin substrate $G_{o}$ is enriched in neurons of the olfactory epithelium (Jones et al., 1990). The presence of the $G_{o}$ protein at high levels in the cilia (Jones et al., 1990) suggests that the $G_{o}$ protein may be responsible for IP$_3$-mediated odorant detection (Reed, 1992).

1.4 The occurrence of G-protein coupled receptors in vertebrate evolution.

Olfactory signal transduction starts by the binding of odorant molecules to receptor proteins located on the cilia of olfactory receptor neurons. Biological and biochemical evidence in several vertebrate species (Jones and Reed, 1989; Abogadie and Farbman, 1995) have indicated that the olfactory signaling process is mediated by heterotrimeric ($\alpha\beta\gamma$) GTP-binding protein (G-protein). As such, odorant receptors are transmembrane proteins that belong to the large superfamily of G protein-coupled receptors. All members of this superfamily have seven transmembrane alpha helices that form pockets for binding of ligands and that allow for interactions with G proteins (Fig. 6). Thus, they form the first element in a transduction cascade.
Fig. 6: Schematic diagram of an odorant receptor. In this scheme, three extracellular loops alternate with three intracellular loops to link the seven transmembrane domains characteristic of G-protein coupled receptors. The high degree of variability, shown by olfactory receptors, in transmembrane domains III, IV and V (shown by black balls) is associated with the capability of these receptors to bind a large number of odorants of diverse molecular structures. (Adapted from Buck and Axel, 1991).

The expression of G_{olf} by mammalian OSNs is necessary for olfactory sensory transduction of odors (Belluscio et al., 1998). The G_{olf} protein may have appeared quite early in vertebrate evolution. In the amphibian *Xenopus laevis*, a nonmammalian G_{olf} subtype is expressed by OSNs that signal airborne odorants (Mezler et al., 2001), and in teleost fishes, G_{olf} immunoreactivity is localized to ciliated OSNs (Hansen et al., 2001; Belanger et al, 2002 submitted). In catfish, the projection of these ciliated OSNs is spatially conserved to the ventral region of the olfactory bulb (Morita and Finger, 1998). In the mammalian vomeronasal organ, receptor neurons expressing the G proteins G_{m2} and G_{b3} project to segregated glomeruli in the accessory olfactory bulb (Jia and Halpern, 1996). In the amphibian *Xenopus laevis*, clearly defined subpopulations of OSNs that express G_{olf} respond to aqueous odorant molecules (Mezler et al., 2001). In the catfish, microvillous OSNs express G_{olf} (Hansen et al., 2001) and project to the dorsal region of the olfactory bulb.
(Morita and Finger, 1998). Therefore, two principles appear to govern the expression of vertebrate G proteins. First, \(G_{olf}\) is essential for the function of some OSNs, and second, some chemoreceptive neurons have evolved alternate G proteins for sensory transduction. When the agnathan olfactory organ is taken into consideration, \(G_{olf}\) expression is expected to occur if this G protein is a vital component of the function of vertebrate OSNs with adenylyl cyclase activity. If olfactory sensory transduction by alternate G proteins is also fundamental to OSN function, clearly defined subpopulations of OSNs that do not express \(G_{olf}\) may be represented in the lamprey olfactory bulb.

1.5 The lamprey as a model in neurobiology.

The animal used for this study is the lamprey, *Petromyzon marinus*. The lamprey was chosen as the model for these experiments since it offers many advantages in order to study neurobiology. It also has an interesting evolutionary history and is an environmentally important species in the Great Lakes region. This organism goes through seven different stages during its life cycle; these stages are divided on the basis of behavioural, morphological and physiological differences. They are: embryo, prolarva, larva (ammocoete), transformer (metamorphosis), juvenile adult (parasite), upstream migrant and spawner. The larval stage is a fairly small, eel shaped animal with dark green/brown colour. The year 1 to year 3 class larvae range from 80 to 120 mm in length (see Fig. 7).

**Fig. 7:** Larval Lamprey. The year 1 to year 3 class larvae range from 80 to 120 mm in length and 0.6 to 2.7 grams of weight. Their color is green/brown and they are filter feeders.
The larval stage burrows within benthic substrate and is a filter feeder. The larvae don’t require tedious animal care. They thrive in a flow-through tank with water at 10-12 °C and 5-8 cm of sand at the bottom. These are practical advantages for using the lamprey as a model organism for laboratory based studies. This jawless vertebrate represents over 400 million years of evolution separate from the main vertebrate line. Lampreys and hagfish, the living jawless fish, are now considered as the only vestiges of a large and diverse group of early craniates which flourished nearly half a billion years ago (Stensio, 1968; Forey and Janvier, 1993, 1994). The nature of the earliest ancestor of craniates and the major evolutionary transitions between craniates and vertebrates can only be inferred by studying the living descendants of the earliest craniates, which retained essentially the same form over several hundred million years. Although many neurological traits are very different between Agnathans and other vertebrates, others have an ancient origin, and have been conserved through lamprey and gnathostome evolution. For example, the ultrastructure of ciliated OSNs is similar in all vertebrates, from lamprey to humans (Takagi, 1989). Some facets of the lamprey olfactory system, the number of olfactory receptors genes, for example, is lower than in gnathostome vertebrates. Three olfactory receptors intronless open reading frames corresponding to three different olfactory receptors types have been sequenced from the lamprey, *Lampetra fluviatilis* (Berghard and Dryer, 1998).
Fig. 8: The olfactory pathway in larval lamprey. In this micrograph the pathway was highlighted through anterograde transport of Calcium green-1 dextran injected into the nasal cavity, giving direct evidence of the wiring from the periphery to the central nervous system. Scale bar is 500 μm.

These genes are expressed in a punctate pattern in the olfactory epithelium (Berghard and Dryer, 1998). They share little sequence similarity with one other and with other vertebrate ORs. The lamprey is the most ancient vertebrate in which OR genes have been identified. Therefore neurobiological processes that are common to both lampeys and other vertebrate species are likely to be conserved characteristics, and fundamental to the function of the vertebrate nervous system. A very useful feature of lamprey’s anatomy, in relation with olfactory system studies, is that the entire pathway of OSNs is located in the same horizontal plane (Zaidi et al., 1998). A horizontal section of the animal’s head allows for the dendrites and cell bodies in the olfactory epithelium to be displayed, as well as the axons in the olfactory nerve and the axon terminals in the olfactory bulb (Fig. 8).

This allows for spatial analysis of intracellular events at these locations. The lamination of the olfactory bulb in the adult stage of the lamprey Lampetra japonica has been characterized (Iwahori, 1987), and connectivity in adult Ichthyomyzon has been demonstrated (Northcutt and Puzdrowski, 1988).
Moreover, during the larval stage the visual system is at an early developmental stage (Rubinson, 1990), however, the olfactory mucosa responds physiologically to chemostimulation by basic amino acids, bile acids and petromyzonol sulfate (Li, 1995; Zielinski, 1996). The larval stage provides an extended period for examining the regulation of developmental processes, as this life stage lasts for at least three years. Finally, lampreys are important to the Great Lakes ecosystem, making it an even more interesting subject. In the past years the joint effort of Canada and USA, brought to establish a Fishery Commission for the control of the sea lamprey. Sea lamprey is indeed, a Great Lakes invader; these organisms are native to the Atlantic Ocean. They show a migratory behaviour and they can live in both salt and fresh water. In its juvenile stage it is a parasite (see Fig. 9) that feeds on body fluids, often scarring and killing host fish.

**Fig. 9:** Adult sea lamprey. This sexually mature lamprey was held in the U.S. Geological Survey Lake Huron Biological Station, Millersburg, Michigan USA. At this stage the animal doesn’t feed. The animals were sent from Michigan and quickly delivered to the University of Windsor to avoid any unneeded stress; the animals were then sacrificed and the tissue of interest (olfactory organ and olfactory rain) was quickly removed and fixed.

Sea lamprey have had an enormous negative impact on the Great Lakes fishery. Because they did not evolve with naturally occurring Great Lakes fish species, their aggressive, predaceous behaviour gave them a strong advantage over their native fish prey. Sea lamprey prey on all species of large Great Lakes fish such
as lake trout, salmon, rainbow trout, whitefish, chubs, burbot, walleye and catfish.

In precedent studies of sea lamprey olfactory biology, both electrophysiological and behavioural evidence, have demonstrated that adult migratory lampreys rely on waterborne chemical cues (pheromones) released by larval conspecifics to locate spawning streams (Li and Sorensen, 1995). In the field, migratory adult lampreys are found to select stream with high larval densities but fail to find any stream if their olfactory system is occluded (Bjerselius and Sorensen, 2000; Li et. al., 2002). Further characterizations of the olfactory system in this aquatic vertebrate will assist in developing and employing pheromone based strategies and techniques for sea lamprey population management.
Chapter 2.
Morphological characterization of lamprey olfactory bulb.

The objective of the following set of experiments was to characterize the olfactory bulb of the larval lamprey. We wanted to find evidence for a spatial organization of the glomerular units within the olfactory bulb that was comparable with the one found in other aquatic vertebrates, such as the zebrafish, a teleost fish (Baier and Kosching, 1994). In order to address this issue we labelled OSNs through histochemical staining and anterograde labeling with the purpose to have further direct evidence. We investigated the expression of $G_{olf}$ protein in the olfactory pathway of lamprey, in different stages of its life cycle: larvae, transformer and adult. The rationale was to check if this G-protein is conserved through out vertebrate evolution, with the lamprey representing the base of this evolutionary scale. The overall goal was to have a mapping of the glomerular units. These results will bring new insight in the morphological characterization of the lamprey’s brain and they will be extremely useful for spatial analysis of olfactory bulb.

2.1 Materials and Methods.
All experimental protocols reported in this study were in compliance with guidelines established by the Canadian Council of Animal Care.

2.1.1 Experimental animals.
During this study, animals from different locations were used. During the first year of the project (2000), the animals were obtained from Bronte Creek, Burlington, Ontario. About 50 lampreys were used for the morphological studies. The larval lampreys from this site were 80 – 100 mm in length and 0.6 – 1.8 gm in weight. The lampreys were maintained in 30 L
aquaria, with sand to a depth of 5 cm, in a 10°C cold room in the Department of Biological Sciences at the University of Windsor. Ammonia and carbon were removed through a filter containing carbon and ammonia-removing chips, as the tank water was recirculated. Once a week, the lampreys were fed dry baker’s yeast and the filters were unplugged, to allow the yeast to settle. About 8 hours later, 1/3 of the water was removed and replenished with fresh dechlorinated water and the filters were turned back on. Once a month, carbon and ammonia chips were replaced. In September 2001, approximately 400 larvae were sent from Sault Ste Marie by Canada Department of Fisheries and Oceans. These lampreys were bigger in size, ranging from 100 – 120 mm in length and 1.7 – 2.7 gm in weight. In 2001, lamprey holding facility in Department of Biological Sciences at the University of Windsor, expanded. There are 3 flow-through tanks (215x63x22 cm) with 3-5 cm of sand at the bottom (see Fig. 10).

![Image](image.jpg)

**Fig. 10:** Lamprey holding facility. The flow-through tanks (215 x 63 x 22 cm) are kept in the animal quarters at the University of Windsor. A light cycle was also set-up in order to keep the animals with at least 8 hrs of dark. The water's temperature ranges from 12 to 14 °C.

The animals were fed once a week and the water flow was shut down to allow the yeast to set at the bottom. The sand was replaced every 6 months. Larval
stage lampreys were primarily used, however transformers and a few adult organisms were used throughout the project. In July and August 2001, sexually mature adult sea lampreys (Fig. 9) were examined in order to obtain a comparative analysis to larval animals. The adults were trapped or collected by hand from tributaries to lakes Huron and Michigan, and then transported to the U. S. Geological Survey Lake Huron Biological Station, Millersburg, Michigan USA. The adults were held in flow-through tanks (1000 L) with Lake Huron water (7°C to 20°C) before being euthanized for collection of olfactory organs. Prior to experimentation, all lampreys were deeply anesthetized with tricaine methane sulfonate (MS-222 0.05%) and killed by decapitation.

2.1.2 Tissue fixation.

The steps described below for fixing and sectioning tissue, are common to all the histochemical and immunocytochemical procedures used in this study. Following decapitation, tissue surrounding the nostril and the forebrain of the larval head was trimmed away on the ventral and lateral sides. This extraneous tissue was removed to increase penetration of the fixative and ensure proper fixation. The larval heads were immersed into Zamboni’s fixative (see Appendix pp. 77) for 4 to 20 hours at 4°C. To prevent formation of ice crystals during cryostat sectioning, fixed larval olfactory organs were cryoprotected by passage through a sucrose gradient (10-20-30% in phosphate buffer, see Appendix). Olfactory organs were then infiltrated with embedding matrix (Shandon, M-1 embedding matrix, Pittsburgh, PA, USA) by placing the samples and the matrix under the vacuum for 5 minutes at a pressure of about 15 psi. Horizontal sections (20-30 μm) were cut on a cryostat (Microm, Heidelberg) at a temperature of -21°C. The cryostat sections are collected on Superfrost/Plus slides (Fisher Scientific, USA) to ensure adhesion during the
many steps of the immunohistochemical procedure. Sections were air-dried for at least 1 hour, and then placed in a glass container and stored at -20 °C. The sections were washed in 0.1M PBS (see Appendix) for 10 minutes to rehydrate; drained and washed in acetone for 10 minutes to permeabilize the tissue; drained again, and washed in 0.1M PBS (see Appendix) for 10 min.

Each slide was then carefully wiped without touching the sections. One drop of diluted goat normal serum was added to each section and left for 15 min. The blocker was drained, and the slides were carefully wiped.

2.1.3 Characterization of olfactory sensory neurons through GSL I-isoelectin B₄ histochemistry and $G_{\alpha s/olf} \& G_{\alpha olf}$ immunoreactivity.

For histochemical labeling of lamprey OSNs with GSL I-isoelectin B₄ (Tobet et al., 1996) biotin-conjugated, (Griffonia simplicifolia lectin I-isoelectin B₄, Vector, Burlingame, CA, catalog. No. B-1205; 10 µg/ml in 0.1M PBS, pH 7.5) was prepared and applied onto sections for 3hr or overnight. Slides were kept in a box with dampened paper towels at the bottom to ensure a light level of moisture and were incubated at 4 °C. The slides were then rinsed in PBS three times for 10 minutes. The PBS was drained and air-dried for a few minutes. The slides were incubated with a secondary antibody, DCS avidin-fluorescein (1:100, see Appendix) for 1 hr, washed in PBS three times in the dark, and mounted with Vectashield™ (Vector, Burlingame, CA). The edges of the coverslips were sealed with nailpolish. Usually, an hour was needed to allow the nail polish surrounding the coverslip to dry, before observing by fluorescence microscopy. Negative controls were also performed omitting the GSL I-isoelectin B₄.

The immuno-labeling of the two heterotrimeric G proteins ($G_{\alpha s/olf} \& G_{\alpha olf}$) was performed similarly to the procedure previously described. All the
steps concerning washing and wiping were the same. For labeling, we tried different dilution series for $G_\alpha$ s/olf (1:500; 1:1000; 1:2000, Santa Cruz Biotechnology, Inc; catalog. No. sc-383). We found that the 1:1000 labeled OSNs optimally. We continued the labeling using $G_\alpha$ s/olf antibody at the concentration of 1:1000 in PBS overnight. The following day sections were washed three times in PBS and incubated in Alexa 568 goat anti-rabbit IgG (Molecular Probes, 1:100 in PBS, pH 7.4) for 1 hr. The tissue was then washed in PBS three times, wiped and mounted with Vectashield\textsuperscript{TM}. Negative controls were also performed omitting the primary antibody. We did not observe any staining with the $G_\alpha$ (Santa Cruz Biotechnology, Inc; catalog. No. sc-387), however positive control tissue from the round goby labeled positively. Therefore we hypothesized that $G_\alpha$ antibody binding epitopes in the mammalian $G_\alpha$ antibody were not present during lamprey evolution.

For double labeling for GSL I-isolectin B$_4$ histochemistry and $G_\alpha$ s/olf immunocytochemistry, slides were first immunolabeled for $G_\alpha$ s/olf using Alexa 568. After this slides were washed three times with PBS and then incubated with GSL I-isolectin B$_4$ for 3hr. These were rinsed in PBS, then incubated in DCS avidin-fluorescein for 1 hr, washed, and mounted with Vectashield\textsuperscript{TM}.

2.1.4 Anterograde labeling of olfactory sensory neurons through Calcium green-1 dextran loading.

The glomerular units containing OSN projections in the olfactory bulb were anterogradely labelled following the application of calcium green dextran into the nasal cavity. Prior to experimentation, all lampreys were individually anesthetized, by immersion in a 0.05 % solution of tricane methanolsulfate (MS222). After the animal was sedate, it was wrapped in a wet tissue paper. A solution of 2.5 % Calcium green-1 dextran (potassium salt, 3000 MW, anionic
Molecular probes, Eugene, OR), 0.2% Triton X-100, and 1 mM NaCl, 0.1 M Na bicarbonate in water, was injected into the nasal cavities using a Hamilton syringe with a 23 gauge needle. The lampreys were allowed to recover, and then another injection was performed the following day. On the fifth day following this injection, the lamprey was immersed into a solution of MS222 (tricaine methane sulfonate), deeply anesthetized and killed by decapitation. The larval heads were immersed into 4% paraformaldehyde fixative overnight, at 4 °C, then cryoprotected by passage through a sucrose gradient (see Appendix). Cryosections (20 µm) were cut in horizontal planes on a cryostat (Microm). Coverslips were mounted with Vectashield™.

2.1.5 Molecular weight determination of G_{s/s} through Western immunoblotting.

This experiment was conducted in Dr. Li’s laboratories at Michigan State University, Department of Fisheries and Wildlife.

The cilia were dissociated from OSN by calcium shock (Schandar et al., 1998). Sea lamprey olfactory epithelia were dissected out and agitated with a high calcium buffer (10 mM CaCl, 20 mM ACES, 0.3 M sucrose, 10 ug/ml leupeptin, 76.8 nM aprotinin, 0.7 µM pepstatin, 0.83 mM benzamidine, 0.23 mM PMSF (Phenyl-Methyl-Sulfonil-Fluoride), 1 mM iodoacetamide) in an end-over-shaker at 4 C for 20 min. The solution was spun for 15 min at 6000 g and the supernatant collected. The pellet was resuspended in the same buffer and spun down again for collection of supernatant. The combined supernatant was spun for 15 min at 18,000 g to precipitate cilia. The ciliary pellet was washed with TME buffer (10 mM Tris, 3 mM MgCl, 2 mM EGTA, pH 8.2) and resuspended. Aliquots were store at -80 C until use. The deciliated olfactory mucosa was prepared from the pellet of the first centrifugation according to
DellaCorte et al., (1996). The deciliated tissue was homogenized using a glass pestel and mortar and centrifuged at 30,000g for 90 min at 0 C. The supernatant was removed and stored at -80 C. The protein concentration was determined with a DCA protein analysis kit (Pierce). 10 and 20ug protein of cilia and deciliated mucosa were loaded on to a 10 % SDS PAGE gel and 5% stacking gel, which were subjected to 150 V for 1 h in a standard running buffer. The protein bands in the gel were transferred to a PVDF (polyvinylidene fluoride) membrane. The membrane was then blocked with a blocking solution containing 5% non-fat dry milk in TBST (Tris buffer), incubated with the primary antibody (anti-G_{q} \text{a/}\beta; 1:200 or 1:500) in the blocking solution, washed 3 times with TBST, and incubated with horseradish peroxidase conjugated secondary antibody (1:5000) in the blocking solution. Finally, the membrane was washed three times and incubated in chemoluminescence substrate for 10-15 minutes. A film was exposed and developed.

2.1.6 Microscopic image acquisition.

The sections with fluorescently labeled probes were viewed on a BioRad MRC 1024 laser scanning confocal microscope (Hercules, CA) equipped with argon-krypton laser and attached to an up-right Nikon eclipse E-800 scope. Serial optical sections were imaged using a 20X dry objective or 60X oil immersion objective; at axial intervals of 0.2-0.3 \text{\textmu}m and compiled in a Z-series with Lasersharp 3.0 processing. Individual frames (512 X 512 pixels/frame) were averaged together using the Kalman filter function of the confocal system. For double-labeling, two separate images, one for each fluorochrome, were scanned in sequence to build the Z-series. The two images were then merged using pseudocolors from Confocal Assistant. An epifluorescence Zeiss
Axioskop FS was used in order to analyze slides at lower magnification. A 5X dry objective and 20X water immersion objectives were used. This microscope is equipped with a fluorescein-green filter and a rhodamine-red filter. Empix, Northern Eclipse acquisition software was used. Adobe Photoshop 6.0 was used to convert merged color transmission and fluorescence images to grayscale, to crop images, to build collages and to label figures.

2.2 Results.

Our results characterized the glomerular organization in the olfactory bulb of the developing Agnatha, the larval sea lamprey. The glomerular units were organized into a consistent spatial pattern, when viewed by microscopy following histochemical and immunocytochemical treatment.

2.2.1 Organization of glomerular units within the olfactory bulb.

- **GSL I-isolectin B₄.** A consistent pattern of glomerular organization was seen in serial horizontal sections of the olfactory bulb stained with GSL I-isolectin B₄ (Fig. 11 A-D) and later confirmed by anterograde labeling with fluorescent dextran. The glomerular groupings included: a dorsal ring, an anterior plexus, a lateral chain, medial glomeruli and a ventral cluster. The glomerular units of the dorsal ring contained closely spaced modules located in the rostral caudal edge (Fig. 11A). The anterior plexus, located ventral to these dorsal glomeruli, comprised of a cohesive mesh of OSN axon terminals (Fig. 11B), a characteristic previously observed in the anterior plexus in the zebrafish olfactory bulb (Baier and Korsching, 1994). A space, about 50μm wide separated the anterior plexus from the lateral chain (see arrow in Fig. 11B).
Sections taken from the plane containing the ventral portion of olfactory nerve interface with the olfactory bulb, contained a circular merged anterior plexus/lateral chain, a small glomerulus caudal to this ring, and three medial glomeruli (Fig. 11C). The most medial of these glomeruli was adjacent to the anterior commissure, a second medial glomerulus was posterior to the olfactory nerve, and a third medial glomerulus was positioned radially within the granular layer (the radiomedial glomerulus). In some specimens, two radiomedial glomeruli were found. The ventral region of the olfactory bulb contained a cluster of closed spaced glomeruli (Fig. 11D). Frequently, extrabulbar axons of olfactory receptor neurons were seen extending in an extrabulbar pathway to the ventral diencephalon, as previously reported (Tobet et al., 1996).

- **Calcium green-1 dextran loading.**

The anterograde labeling of the OSN axons terminals, confirms the spatial pattern of glomeruli organization within the lamprey’s olfactory bulb. The staining was not as intense as the GSL I-isolectin B₄ because we did not use a fixable dextran in order to perform physiological studies. However it is possible to clearly localize a dorsal glomerular ring, an anterior plexus, a lateral chain, medial glomeruli and a ventral cluster (Fig. 11 E-H).
Fig. 11: The organization of olfactory glomeruli in the olfactory bulb of the larval lamprey. In each micrograph, the lateral edge is on the left, medial is on the right and rostral is on top. The axons of the olfactory receptor neurons were labeled with GSL I-isoelectin B4. A. The dorsal ring of olfactory glomeruli. B. The anterior plexus (AP) and lateral chain (LC). C. The medial glomeruli (arrows). D. The ventral cluster. The same pattern was demonstrated through anterograde label with Calcium green-1 dextran (E, F, G and H). Scale bar in A is 100μm.

2.2.2 $G_{\text{olf}}$ expression in the olfactory pathway and in the olfactory bulb.

$G_{\text{olf}}$ immunoreactivity was localized on the base of cilia that extend from the apical portion of the OSNs into the mucociliary complex (Fig. 12B). Staining was also found on the cell body as well as in the axonal process of the OSNs (Fig. 12A-C). $G_{\text{olf}}$ immunoreactivity was also evident in subcellular components of OSNs located in the olfactory epithelium of all life stages (Fig. 12B-C). The boundary between $G_{\text{olf}}$ and non-$G_{\text{olf}}$ expressing nasal epithelium is sharp and clear. The abundance of $G_{\text{olf}}$ immunoreactivity in cilia of the olfactory epithelium was evident from double labelling experiments with antibodies against $G_{\text{olf}}$ and acetylated tubulin (Fig. 13), and from high power views of OSN cilia (Fig. 12A).

Double labeling with acetylated tubulin and $G_{\text{olf}}$-immunocytochemistry, allowed for visualization of all cilia, those originating from ciliated non-sensory cells as well as OSNs. This technique showed that $G_{\text{olf}}$ was expressed exclusively in the olfactory epithelium sheet lining the nasal cavity, recognizable by its thickened pseudostratified appearance (Fig. 13A-B). $G_{\text{olf}}$ expression in cilia, dendrites, cell bodies and axons persisted after metamorphosis (Fig. 12A), when OSNs were considerably larger than during larval development (Vandenbossche et al., 1995; 1996).

In the olfactory bulb of the larval lamprey, glomerular units of the dorsal ring, anterior plexus, lateral chain and ventral cluster contained $G_{\text{olf}}$ - immunoreactivity (Fig. 14B-D-F). This staining was most intense in dorsal and ventral glomeruli, slightly weaker in the anterior plexus and lateral chain (Fig.
14D) and absent from the medial glomeruli when viewed in double labelled preparations with both GSL I-isoelectin B₄/Gₐlf immunocytochemistry (Fig. 15) and fluorescent dextran/ Gₐlf immunocytochemistry (Fig. 16). The glomerular subsets with differing Gₐlf expression persisted in the adult stage; with the medial glomeruli maintaining an absence of Gₐlf –IR compared to the remaining glomerular units (Fig. 17A-B-C).

These results were consistent in larvae and adults animals coming from different locations and in different seasons through out the year.
Fig. 12: $G_{af}$-IR in transformier olfactory epithelium (A & B) and larval olfactory epithelium (C). $G_{af}$-IR is present throughout the plasma membrane of OSNs, at the base of ciliary processes and in the axonal projection. Scale bar in A is 100μm; OE = olfactory epithelium; ON = olfactory nerve; OB = olfactory bulb. In B and C scale bar is 50 μm.
Fig. 13: Double label with AT (red) and $G_{s/{\alpha}_{f}}$ (green) in the olfactory epithelium. In A and B red shows nasal epithelium without $G_{s/{\alpha}_{f}}$-IR. The yellow region with $G_{s/{\alpha}_{f}}$ and AT double labeling indicates olfactory epithelium. C was taken from the apical region of the olfactory epithelium. Cilia from non-sensory cells are in red and cilia with $G_{s/{\alpha}_{f}}$-IR from OSNs are in yellow. Scale bar in A is 100μm, in B scale bar is 50μm and in C is 25 μm.
Fig. 14: GSL I-B₄ (red) vs. Gₜ (green) in the larval OB. Gₜ is present in the dorsal glomeruli (B), in the anterior plexus and lateral chain (D) and in the ventral cluster (E). In Fig. E the arrows point to a glomerulus, with medial location, not expressing Gₜ-IR. Scale bar in A is 100μm.
Fig. 15: GSL I-B₄ isolectin (red) vs. Gₛₑ (green) in the medial glomeruli of larvae. Fig. A is the result of the merging of B-D and C-E, shows that the medial glomeruli (arrows in A) do not express Gₛₑ. Scale bar in A is 100 μm.
Fig. 16: Medial olfactory glomeruli in larvae double labeled with CGD and G_{olf} immunocytochemistry. A and C show serial sections from the same preparation in which the glomeruli were anterogradely labeled with Calcium green-1 dextran. In B and D the same sections were viewed under the red channel of the confocal microscope in order to compare the staining against G_{olf}. The yellow arrows in A and C shows that there are medial glomeruli not immunoreactive to G_{olf}. Scale bar in D is 100 μm.
Fig. 17: GSL I- B₄ vs. Gₐlf in adult lamprey OB.
In Fig. A GSL I-B₄ staining is present in the anterior, lateral and medial glomeruli. B. Gₐlf-IR is located in anterior and lateral glomeruli but is absent in the medial (arrows). Scale bar in A is 500μm.
Western blotting demonstrated the specificity of the $G_{olf}$ antibody. The molecular weight of $G_{olf}$ in the cilia of lamprey OSNs was 45 KDa with a slightly higher molecular weight component likely from the phosphorylated form of this G protein (Fig. 18).

![Fig. 18: Western Immunoblot of $G_{olf}$ in a preparation from the olfactory epithelium of an adult sea lamprey. On the left is the molecular weight marker. Lane A: 20μg deciliated lamprey olfactory epithelium. Lane B: 10μg deciliated lamprey olfactory epithelium. Lane C: 20μg ciliated lamprey prep. Lane D: 10μg ciliated lamprey prep. The antibody concentration tested was 500 in A and C and 1:200 in B and D.]

2.3 Discussion.

The results of this study show that the olfactory bulb in the larval lamprey contains olfactory glomeruli organized in a consistent precise manner. The olfactory receptor linked protein $G_{olf}$ was expressed by OSNs projecting to dorsal, anterior, lateral and ventral glomerular subsets but was absent in the medial glomeruli subsets. Although these lampreys were from naturally occurring populations that represented diverse gene pools, there was remarkable consistency in these morphological, and spatial characteristics. The results have been confirmed in adult organisms.

2.3.1 The spatial pattern of OSN axon terminals.

The pattern of glomerular organization in the larval lamprey was similar yet considerably reduced from the pattern in the olfactory bulb of zebrafish teleost, *Danio rerio* (Baier and Korsching, 1994). There were five groups of glomeruli in the larval lamprey, compared to eighteen groups in the zebrafish. In both species, the dorsal and ventral regions of the olfactory bulb contained a
glomerular ring, with glomeruli clustered in the very top part. The anterior plexus stretched from the dorsal to the ventral olfactory bulb, and the lateral group appeared as a chain of glomeruli. The overall simplicity of the lamprey's glomerular arrangement can be seen from the ring of glomeruli encircling the dorsal and ventral regions. Images from previous studies of the olfactory bulb of the adult silver lamprey, *Ichthyomyzon unicuspis*, (Northcutt and Puzdrowski, 1988) and sea lamprey (Tobet, 1996) have displayed examples of this ring-like pattern, as well as medially located glomeruli. The medial glomeruli were quite reduced compared to the teleost pattern, with three medial glomeruli compared to *D. rerio*, where several glomeruli are located along the medial edge and occupy radial positions (Baier and Korsching, 1994). The absence of glomeruli at the dorsal interface between the olfactory bulb and the olfactory nerve is likely due to the dorsomedial neuropil that contains contralateral secondary olfactory projections (Northcutt and Puzdrowski, 1988), and may account for the sudden appearance of medial glomerular units in the ventral hemisphere of the olfactory bulb.

2.3.2 Discrete subsets of olfactory glomeruli with $G_{olf}$ localization.

$G_{olf}$ labeling was readily demonstrated in the glomerular units of the dorsal ring, anterior plexus, lateral chain and ventral cluster. This is somewhat in contrast with prior reports in the rodent where $G_{olf}$ was not detected in the olfactory bulb, although other molecules including olfactory marker protein and odor receptor mRNA are present in OSN axons (Wensley, 1995). This difference may underscore a species-specific phenotype but nevertheless emphasizes that subsets of lamprey OSNs are using $G_{olf}$ in transduction cascades. Detection in the axons may reflect further on the importance of $G_{olf}$ in not only odor transduction, but also modulation of growth cone behavior.
through cascades that involve $G_{olf}$ and cyclic nucelotide-gated channels, as suggested by Kafitz et al., (2000). Of equal importance, the absence of $G_{olf}$ immunoreactivity in the lamprey medial glomeruli points to a differing signal transduction mechanism for these compared to the other glomerular groupings. In various teleosts, the medial olfactory path is associated with bile acid and steroid perception (Doving et al., 1980; Satou, 1992), and also in zebrafish, medial glomeruli respond to chemostimulation by these compounds (Friedrich and Korschning, 1998). In mammals, the posterior/medial region of the olfactory bulb contains the putative specialist neurons with cGMP mediated signal transduction. Therefore, the OSN medial glomeruli in the lamprey may also constitute a subtype of OSN specialist expression. The molecular weight of the lamprey $G_{olf}$, 45 kDa, is identical to that of $G_{olf}$ that regulates adenyl cyclase activity in mammals (Jones and Reed, 1989) and teleosts (Abogadie et al., 1995; DellaCorte et al., 1996). The ciliary localization of lamprey $G_{olf}$ shown both immunocytochemically and through Western immunoblotting, supports the involvement of $G_{olf}$ in olfactory sensory transduction. Therefore this G protein appeared in vertebrate evolution over 400 million years ago, and is fundamental to the function of OSNs in both gnathostomes (Belluscio et al., 1998) and agnathostomes. However, it is not surprising to find a subpopulation of lamprey OSNs without $G_{olf}$ expression. The expression of various G proteins in OSN subtypes appears to be a principle of the vertebrate olfactory system (eg. Hansen et al., 2001; Jia & Halpern, 1996; Mezler et al., Shinohara et al., 1992; Wekesa and Anholt, 1999). The medial location of the glomeruli that do not express $G_{olf}$, implies that the OSNs that project to these glomeruli use an alternate G protein during olfactory sensory transduction. In teleosts bile acid and putative steroid phermones stimulate the medial glomeruli and the medial olfactory tract (Friedrich and Korschning, 1998). In tetrapods,
chemoreceptive neurons of the vomerosal system rely on the G proteins $G_{a2}$ and $G_\alpha$ for sensory transduction (Jia and Halpern, 1996), and in the mammalian olfactory system, "necklace olfactory glomeruli" projecting adjacent to the accessory olfactory bulb, and contain elements of a cyclic guanosine monophosphate (cGMP) signal transduction pathway (Julfs et al., 1997). Therefore, the use of G protein subtypes arose during gnathostome evolution, and may be paralleled in agnathostome vertebrates.

2.4 Conclusions.
Summarizing the results of this first set of experiments we achieved two main goals: 1) We characterized the spatial organization of glomeruli units within the olfactory bulbs of larval lamprey; 2) We characterized the expression of $G_{\text{olf}}$ in the glomerular units grouped in the dordal cluster, anterior plexus and lateral chain and 3) We identified a medial glomeruli subset lacking $G_{\text{olf}}$ expression.

We found a consistent pattern of organization within the larval lamprey olfactory bulbs. This pattern was comparable, although at a different dimensional scale, with the one found in a teleost fish (Baier and Korshing, 1994). We also explored the expression of $G_{\alpha_{s/olf}}$ in the primary olfactory pathway of this ancestral vertebrate. We found a few common traits compare with higher vertebrates, but also some interesting divergences.

In the olfactory epithelium the expression of the protein occurs in the sensory part of the epithelium, through out the plasma membrane of OSNs and at the base of the cilia. Through Western blotting analysis performed on a ciliary preparation, from adult organisms, we verified the presence of a 45 kD protein immunoreactive to $G_{as/olf}$. The molecular weight was consistent with other species, such as rat and catfish (Jones and Reed, 1989; Abogadie, 1995).
The expression of this protein on the axonal projections and in the glomerular units throughout the life cycle of the sea lamprey was peculiar. We detected expression in most of the glomerular grouping we previously identified except for the medial subsets. This is somewhat in contrast with prior reports in the rodent where $G_{olf}$ was not detected in the olfactory bulb (Wekesa, 1999).

The absence of $G_{olf}$ immunoreactivity in the lamprey medial glomeruli points to a differing signal transduction mechanism for these compared to the other glomerular groupings. Bile acid and putative steroid pheromones stimulate the medial glomeruli and the medial olfactory tract in teleosts (Friedrich and Korschning, 1998) but we only speculate, at the moment, about the same detection pattern in lamprey olfactory bulbs.

These results give us a very useful tool in linking neurobiological processes that are common to both lampreys and other vertebrate species. These characteristics are likely to be conserved and fundamental to the function of the vertebrate nervous system. The characterization of the glomerular pattern, within the lamprey olfactory bulbs, will greatly help in the attempt to further characterize the lamprey olfactory bulb through physiological studies.

These data will be fundamental for linking different stimuli, with foci of activity in different glomerular groupings and hopefully achieve a spatial mapping of glomerular activation in the olfactory bulbs following the application of a given stimulus.
Chapter 3.

The advantages and limitations of current imaging methods for spatial analysis of activity in the olfactory pathway.

In the past decade, advances in microscopy have been coupled with new methods of labeling cells to generate the new science of functional in vivo imaging. Imaging technologies allow investigators to look directly inside living cells and neuronal circuits and probe their form and function in unprecedented detail. This approach is revolutionizing many aspects of biomedical research, particularly neuroscience, in which visual techniques have traditionally been so important. Functional imaging methods permit analysis of neuronal systems in which activity is broadly distributed in time and space (Kauer and White, 2001). The main advantage that this technique gives is the possibility to resolve the spatial (and in some fortuitous cases, temporal) aspects of many neuronal events occurring simultaneously.

Imaging methods potentially provide advantages for examining a number of important functional attributes of nervous system as well as a) changes in real time activity in response to perturbations by stimulation, b) plastic responses to long term external or internal environmental changes and c) changes due to injury, senescence, and normal (e.g. apoptotic) and abnormal (disease-associated) degeneration. In this prospective a few limitations must be keep in mind. To characterize each of these processes completely, one would need to obtain information with a) spatial resolution that ranges from nanometers (molecular size) to meters (organism size); b) temporal resolution extending from microseconds (for molecular events such as enzymatic reactions) to years (for developmental, plastic and degenerative changes); c) the ability to observe in three, as well as two, dimensions (using a tool like confocal
or multiple photon microscopy); d) the ability to access the signal in tissue without invasive surgery; and e) the ability to assess function without perturbation by the observation process. Methods presently available can approach some of these goals, but as well as other techniques they fail in other issues.

In the olfactory system the dimension that describes odorant stimuli in “odorant space” are still poorly defined. The distributed nature of olfactory information processing was first observed 50 years ago by Adrian (1953). Subsequent studies using serial single electrode recordings (Levetea and MacLeod, 1966) and, in some cases, multi-electrode recordings (Moulton, 1976) provided additional support for the idea that odor information is represented by activity occurring in parallel in different locations within the olfactory pathway. Until the advent of functional imaging methods, it was not possible to observe these events with spatial resolution that gives a global view of distributed processes.

Fundamental for the successful application of this technique is the optical accessibility to the structure to be observed. Imaging methods that have been applied to analysis of olfactory function include 1) examination of neuronal activity by measuring glucose uptake with radiolabeled 2-deoxyglucose (2DG) (Kennedy et al., 1975); 2) observation of changes in activity-related gene expression, such as e-fos (Sagar et al., 1988); 3) measurement of intracellular calcium concentration using fluorescent markers such as fura-2 (Gryniewicz et al., 1985) or calcium green (Friedrich and Korshing, 1997); d) measurement of transmembrane voltage using fluorescent voltage-sensitive dyes (Cohen and Lesher, 1986; Blasdel and Salama, 1986).
3.1 A preparation of the pathway of olfactory receptor axons for dynamic optical imaging. Objectives.

Electrical signaling in nerve cells is accompanied by changes in membrane potential and the generation of currents in the extracellular space, which can be recorded with various types of electrodes. Intracellular recordings with micropipettes can monitor the membrane potentials of single neurons, while extracellular electrodes are usually used to record activity in populations of neurons. Both types of recording techniques have their advantages and disadvantages. Intracellular electrodes can record action potentials as well as subthreshold type activity (e.g. synaptic potentials), but this method is not well suited to monitor the activity patterns in groups of cells since only one or few neurons can be recorded at a time (McClellan, 1994). Extracellular electrodes can monitor the activity patterns in many neurons at the same time, but it is often difficult to separate the patterns of activity in individual neurons and to know which neurons are contributing to the activity. In addition, extracellular electrodes are usually used to record action potentials, while subthreshold activity may go undetected. Because of the limitations in the above electrophysiological recording techniques, we chose a different approach for the issue of resolving the spatial pattern of neuronal activation in the olfactory system. Indeed, the dynamic optical recording potentially allows monitoring of intracellular activity in as many neurons as can be visualized simultaneously in the field of view of the microscope (e.g., Cohen, 1986; O'Donovan et al., 1993; Tsau et al., 1996).

Since calcium influx often accompanies neuronal depolarization and action potentials (Fig. 19), calcium sensitive dyes offer an alternative approach

Fig. 19: The sensory cells, the presence of Na⁺/K⁺ ATPase pumps and of open K⁺ channels on cell membranes allows K⁺ to accumulate in the cell cytoplasm, keeping the ratio between intracellular and extracellular side equal to 140mM to 5mM. Thus in the normal condition, a continual efflux of K⁺ is responsible for the resting membrane potential to be in electrochemical equilibrium at about -65mV. Following the odorant-receptor binding in the
cilia membrane, a cascade of events initiates and this leads to the opening of cyclic nucleotide gated channels with inward current of cations such as Ca\(^{2+}\) and Na\(^{+}\). The consequence is a membrane depolarization and firing of actions potential. The depolarization propagates along the neuronal cell body and the axons, where voltage-dependent Na\(^{+}\) channels open, allowing an inward current of Na\(^{+}\) and further depolarization. At pre-synaptic level, the change in membrane potential determines the opening of voltage-dependent Ca\(^{2+}\) channels with a consequent inward current of Ca\(^{2+}\). The use of calcium sensitive dyes such as Calcium green-1 dextran is therefore a useful tool in order to monitor calcium influx at pre-synaptic level.

A calcium indicator fluo-3, has been used to show that odorant stimulation leads to uniform Ca\(^{2+}\) changes within OSNs cilia and then an increase in Ca\(^{2+}\) spreads to the knob, dendrite, and soma, decaying in the different compartments with different time courses (Leinders-Zufall and Zufall, 1998). This imaging method extended patch clamp recordings from single ciliary membranes (Nakamura and Gold, 1987) by clearly showing the degree of involvement and the dynamic spread of the initial response.

Presynaptic activity in OSN terminals of OB glomeruli labeled by anterograde transport of a Ca\(^{2+}\) indicator dye delivered to the olfactory epithelium was initially shown in zebrafish by Friedrich and Korshing (1997). This pioneering work showed that stimulation with various amino acids clearly generated different patterns of activity that are shared by certain glomeruli for a number of the compounds. The organization of the olfactory system in the larval sea lamprey allows for the spatial analysis of the pathway of olfactory receptor neurons, as the olfactory epithelium, olfactory nerve and olfactory bulb are located in the same horizontal plane. The objective of this project was to monitor temporal and spatial changes in Ca\(^{2+}\) levels upon stimulation, following loading of a fluorescent calcium indicator, Calcium green-1 dextran, into the olfactory pathway. The axons of OSNs projected into distinct glomerular modules located in specific dorsal, anterior, lateral, medial, and
ventral positions. An important asset of this preparation is the possibility to view the entire pathway, from the peripheral to the central nervous system. The larval lamprey provides a very stable preparation for live cell imaging. First, the brain and surrounding tissue is relatively thin, which allows optical signals to be recorded from neurons at different depths within the tissue. Second, previous works have shown that the nervous system can be maintained in vitro for hours or days, which provides a reasonable window of time to collect optical signal data (McClellan, 1994).

3.2 The Calcium indicator: Calcium green-1 dextran.

The long-wavelength calcium indicator Calcium green-1 dextran (CGD; Excitation wavelength = 488nm), is a visible light-excitable probe derived from fluorescein. Upon binding to calcium, this indicator exhibits an increase in fluorescence emission intensity with little shift in wavelength.

![Emission spectra of Calcium green-1 dextran](image)

The spectral characteristics of the long-wavelength indicators have three major advantages: 1) Their emissions are in regions of the spectrum where cellular autofluorescence and scattering background are minimal; 2) The energy of the excitation light is low, reducing the potential for cellular photodamage; 3) The wavelength required for optimal excitation are compatible with those produced by fluorescent filter systems.
CGD is more fluorescent, compared with other calcium indicators, at low calcium concentration. This facilitates the determination of baseline Ca\(^{2+}\) levels and increases the visibility of resting cells. In addition, CGD has proven to be less phototoxic than fluo-3 in confocal studies (Stricker, 1992).

One limitation in the use of Ca\(^{2+}\) sensitive dyes is that the activity-related changes in Ca\(^{2+}\) fluorescence occur much more slowly than the electrical events of neuronal action potentials. As consequence, the technique may be most useful for recording the activity of neurons in a qualitative manner, rather then attempting to quantify the number of action potentials produced.

Another problem related to the degree of fluorescence change may depend upon how well the cell takes up the dye. Different sized cells or cell at different distance from the application site may differ in the degree of fluorescence change. It should be possible to realize a rough calibration of the CGD response at the end of the experiment, by adding a calcium ionophore (e.g. ionomycin) to make the cells selectively permeable to Ca\(^{2+}\). Subsequent exposure of the cells to known Ca\(^{2+}\) concentrations should provide a crude estimate of the range of Ca\(^{2+}\) concentrations that arose during the experiment, but note that this method assumes that no fading or other dissipation of the dye has occurred. Others limitations must keep in mind when attempting to record neuronal activity through this calcium indicator.

All fluorescent dyes, including CGD, are subject to fading when exposed to ambient light, so it may be helpful to dim the room lights during dye application, and to cover the dye against unnecessary light. Similar precautions would apply during dissection of the tissue and any handling prior to the recording session. Dye bleaching is a much greater problem during the recording period, due to intense illumination. The use of a neutral density filter to keep the light as low as possible is a good precaution. By adjusting the gain
on the camera it is possible to record from cells too dimly fluorescent for
unaided eyes to see. Also the shutter controlling tissue illumination should be
kept closed except during the recording periods of the experiment. We need to
keep in mind the features of the product we are going to use and its advantages
and limitations in order to use it in the best way and with better results.
The capability of Calcium green-1 dextran in detecting pre-synaptic activity will
assist in the issue of resolving spatial activity in the olfactory bulb following
stimulation with odor molecules.

3.3 Materials and Methods.
3.3.1 Dye loading.

The loading of the dye in the olfactory pathway of larval lamprey was
obtained through anterograde transport by performing two sequential
injections into the nasal cavity. The calcium indicator dye CGD (10,000 MW,
Molecular Probes, Eugene, OR USA) was dissolved in a solution made by
0.1mM NaCl plus 0.1% Triton X-100 (modified from Friedrich and Korschning,
1997). About fifty larval lampreys (average length 12 cm) were used in this
study. Each first anesthetized with 0.05% tricaine methanosulphate (MS-222)
and wrapped in a wet paper towel. A Hamilton syringe loaded with 2.5%
Calcium green was positioned into the nostril under a dissecting microscope
and the solution was injected into the nasal cavity of the lamprey. The animals
were allowed to recover in a separate tank, and 4-5 hours later they were re-
anesthetized and the procedure was repeated a second time.

For reasons not known, during winter periods throughout the two years
of the project we had major problems to obtain a good loading of the dye into
OSNs, compared to largely successful loading from Spring to Autumn.
To improve labeling, a second, more accurate method of dye loading was used. A solution of 0.1% Triton X-100 was injected directly into the nasal cavity prior to dye loading. Triton X-100 serves to permeabilize the cells but has been proven to knock off cilia from the olfactory and respiratory epithelium allowing a better uptake of the dye. Ultrastructural studies in zebrafish have shown that cilia grow back within 72 hours after the treatment (Friedrich and Korsching, 1997). Lamprey were anesthetized with MS-222, wrapped in a wet paper towel and placed under a light source. A Hamilton syringe was loaded with 0.1% Triton X-100 solution, its tip was inserted into the nostril and the solution was injected. The animal, well wrapped in wet paper towels, was left to absorb the Triton X-100 for 2 minutes before dye loading. A 0.15 mm Ø capillary (World Precision Instruments Inc cat. # PG52151-4, Sarasota, FL USA), was pulled in order to reach an appropriate tip shape using a KOPF vertical pipette puller set to heater = 7.9 and solenoid = 5. The capillary was loaded with 2.5% CGD (2-3μl), and assembled with the pipette holder connected to a Picospritzer II (Parker instrumentation, General valve division, Fairfield, NJ). The tip was inserted into the nostril and the dye was injected at a pressure of 5 psi, with brief and repetitive pulses. All lamprey loaded with dye were kept in separate tanks until they were used for experiments. Animals were tested at different times following the application of the dye (6, 12, 24, 48 and 72hrs).

3.3.2 Assessment of OSN dye loading through Hu-immunocytochemistry.

This antibody (full name anti-human neuronal protein HuC/HuD anti-Hu) binds specifically to antigens present exclusively in neuronal cells and are thus useful as markers of neuronal cells in tissue. The Hu antigen is an RNA-
binding protein. The antibody specifically labels neuronal cells in zebrafish, chick, canaries and humans and is likely to label neuronal cells in most vertebrate species (Fisher, 2001; Formaro, 2001). Labeling is visible early in development, at about the time that the neurons leave the mitotic cycle. We used this antibody to highlight the OSN cell bodies within the epithelial sheet, to determine if CGD extend structurally coherent OSNs.

During the labeling procedure, the sections were washed in 0.1M PBS for 10 minutes to rehydrate; were drained and washed in acetone for 10 minutes to permeabilize the tissue; were drained again, and washed in 0.1M PBS for 10 min. Each slide was then carefully wiped without touching the sections. One drop of goat normal serum was added to each section and left for 15 min. The blocker was tapped off and the slides wiped carefully.

A stock solution of 10 µg/ml (anti-human neuronal protein HuC/HuD anti-Hu, Molecular Probes, Eugene, OR) was prepared and stored at −20 °C and aliquoted in 50 µl volume. The performance of a dilution series gave optimal staining results at 1:2000 (in PBS 0.1 M, Triton X-100 0.2%).

After incubation overnight with the primary antibody, followed by three washes in PBS and then incubation with the secondary antibody (FITC, Vector lab. 1:100 in PBS) for 1 hr, as with the other staining procedure, the tissue received a final wash and was Vectashield mounted.

3.3.3 Assessment of the effect of dye loading on olfactory cilia through acetylated tubulin immunocytochemistry.

In order to assess the structural integrity of the olfactory pathway following the dye loading, we performed a labeling with acetylated tubulin histochemistry (monoclonal anti-acetylated tubulin, mouse Ascites Fluid, SIGMA, Sant Louis, Missouri USA). This antibody recognizes an epitope
located on α-tubulin. The tubulin is the major building block of microtubules, thus following immunohistochemistry assay we could clearly visualize cilia and many microtubular structures.

Following CGD loading, several animals at several different times were tested with this antibody. Animals were individually anesthetized, decapitated and fixed with PFA 4% (see Appendix) for 1 day, 3 days and 8 days after the CGD injection. After three hours, the tissue was trimmed and the olfactory organ and olfactory bulb were further fixed overnight. The following day to prevent formation of ice crystals during cryostat sectioning, PFA-fixed larval olfactory organs were cryoprotected by passage through a sucrose gradient (see Appendix). Olfactory organs were then infiltrated with embedding matrix by placing the samples and the matrix in the vacuum for 5 minutes at a pressure of about 15 psi. Horizontal sections (20-30 μm) were cut on a cryostat (Microm) at a temperature of -21°C. The cryostat sections were collected on Superfrost/Plus slides (Fisher Scientific, USA) and then stained.

During the labeling procedure, the sections were washed in 0.1M PBS for 10 minutes to rehydrate; were drained and washed in acetone for 10 minutes to permeabilize the tissue; were drained again, and washed in 0.1M PBS for 10 min. Each slide was then carefully wiped without touching the sections. One drop of goat normal serum was added to each section and left for 15 min. The blocker was tapped off and the slides wiped carefully. A stock solution of acetylated tubulin antibody was prepared, aliquoted in 5μl volume and stored at -20°C. The stock solution was made by adding 95μl of PBS 0.1 M, Triton X-100 0.2% it reached a final concentration of 1:1000. After incubation overnight with the primary antibody, followed by three washes in PBS and then incubation with Alexa 568 goat anti-mouse IgG (Molecular
Probes, 1:100 in PBS, pH 7.4, see Appendix) for 1 hr; as with the other staining procedure, the tissue received a final wash and was Vectashield mounted.

3.3.4 Preparation of the primary olfactory pathway for dynamic microscopy.

Following injection with Calcium green Dextran, the lampreys were left for 3-5 days. The animals were re-anesthetized with 0.01% MS-222 and then decapitated. The heads were pinned down to a Sylgard base, leading within a dissecting plate and dissected dorsally creating a window to expose the olfactory pathway (Fig. 21).

Fig. 21: Preparation of the olfactory pathway for dynamic optical imaging, in larval lamprey. On the top it is possible to see the nostril flap, just underneath there is the olfactory organ that leads to the nasal cavity. In the preparation shown in the above picture, the organ was left intact but in other preparations the top of the organ was cut open for a direct view. Below the organ are the olfactory nerves that connect to the frontal part of the brain, the olfactory bulbs. A thin layer of melanophores is covering the brain. In the right part of the brain the melanophores layer was peeled off to improve the acquisition of imagines under fluorescence light. The bright white spot between the two sides of the brain is the pined gland. On the right corner is a forceps n° 5.

The skin overlying the nostril was removed with care to not damage essential olfactory receptors in the nasal cavity as well as the axons connecting it to the brain. All excess surrounding connective tissue and melanophores on the bulbs were carefully removed to clearly expose the bulbs. Cold Ringer’s solution was applied to the tissue during the dissection and also the dissecting plate was kept
in ice most of the time. Sterile dissecting utensils were used to prevent contamination of the tissue.

3.3.5 Perfusion Chamber.

**Fig. 22a.** A preparation for imaging olfactory bulb responses in live lamprey tissue. On the left side a wider view of the set up showing the gravity fed perfusion system. We designed two separate influx lines; the blue one is connected with a 4 liters tank of Ringer’s solution. A thumb-wheel flow regulator at about 7 to 10 ml/min controls the rate of influx. The solution is aerated with 95% O₂ / 5% CO₂ and is maintained at 15-17 °C. The red line is connected to a 60 cc. syringe and merged with the Ringer’s line by a V connector. This allows us to deliver pulses of odorant test solutions to the preparation. The green line represents the out flux from the perfusion system and is connected with a vacuum. The perfusion chamber is mounted on a Zeiss Axioscope 2 FS The microscope equipped with a monochrome high performance digital CCD camera at the top (QImaging Retiga 1300). The camera is connected to a computer and the data is processed by Northern Eclipse software (version 6.0) to display changes in fluorescence intensity in selected areas of interest over time. **Fig. 22b.** Closer view of the perfusion chamber mounted on the stage of the
microscope. The specimen chamber was made by drilling out an elongated hole through a piece of Plexiglas and the floor lined with Sylgard. The shaded area collects the overflow, which is vacuum aspirated. The out pipe is located within this level to avoid noise in the specimen chamber during recording. The pipe is held in place with modeling clay and the end of the tip is beveled. The specimen is held in place by needles that pierce down into the Sylgard. The image acquisitions are taken by a 20X water immersion objective.

The preparation was maintained in this chamber of our design (Fig. 22; see also in Appendix pp. 78 for details) containing a continuous flow of Ringer's solution, which was circulated through an ice bath to maintain the temperature near 15 °C. A second line was planed in order to deliver stimuli. In the dual line system, the Ringer solution and odorant containers were placed at a higher level to allow steady flow of the solutions into the chamber. The tubing was coiled into an ice bath in order to maintain the solutions at a temperature of about 15°C before reaching the chamber. Adjusting a thumbwheel flow regulator at about 7 to 10 ml/min regulated the flow rates of the solution through the lines. The Ringer's solution was oxygenated with 95% oxygen balance with 5% CO₂.

In experiments using the Picospritzer for odorant delivery, the set-up for the Ringer solution flow was the same, yet the second line is not required. Rather, a stand holding the capillary loaded with the odorant was positioned next to the microscope and it was adjusted to reach the base of the chamber where the lamprey brain will be located. To dispose of the solution in the chamber and make room for fresh solution to flow in, another tubing apparatus was placed on the upper level of the chamber and held in place with plasticine. This tube was connected to a sink, and when the water was running it acts as a vacuum for aspirating the fluid from the chamber.
3.3.6 Presentation of odor stimuli

Experiments were performed using two different methods of stimulant application. The first method made use of the dual line system described in the previous section. In order to test that the CGD within neurons was still functioning as an intracellular calcium sensitive dye we performed a KCl treatment, where 43mM potassium in Ringer's solution (see Appendix pp.69) was placed in the second line/container. To present the stimulant to the olfactory system, the flow of Ringer's solution into the perfusion chamber was stopped and the flow from the second line containing the stimulant was opened. This allowed the solution to enter the chamber at one end, flow over the nostril and be aspirated by vacuum on the other end of the chamber. This bath application method directly acted upon the exposed olfactory bulbs rather than solely through the olfactory sensory neurons. To improve the accuracy of odorant application directly into the nasal cavity, a second method was established using the Picospritzer (see Appendix pp. 79 for details). A capillary tube loaded with the test solution was assembled to the picospritzer device. The animal was pinned to the Sylgard at the base of the chamber and completely immersed in Ringer's solution whose flow is continuous into the chamber. Under low magnification (5x), the fine tip of the capillary tube is guided into the nostril by adjusting the knobs on the stand. Once the tip was positioned, the olfactory bulb was focused under high magnification (20X immersion objective) and the stimulant is applied at a pressure of 50psi, for a duration of 5sec (Fig. 23). We tried different pressures and different duration, in relation with the opening of the tip of the capillary, in order to fill the nasal cavity quickly and also have a fairly quick wash out of the stimuli (see Fig. 23 for details on time course of odor presentation). The stimuli were applied
against the current of the Ringer's solution to avoid direct effects of the test solution on the olfactory bulbs (Fig. 23B).

**Fig. 23:** Time course of the application of the food dye. The graph shows two different regions where the signal was recorded. Region 1 (Fig. A) is located within the nasal cavity; region two is on the OB. The graph shows intensity of light vs. time (sec). It's possible to see how the intensity of the light drastically decreases within 3 sec after the application of the dye and slowly (about 20 sec) returned to the start level, in the nasal cavity.

Region 2 located on the OB (Fig. A) was not affected from the presentation of the food dye. We also verified visually (under 5X objective) that the plum was completely filling the nasal cavity and was not moving upon the OB (Fig. B). We hypothesized that the same dynamic was occurring with the presentation of the real stimulus.

### 3.3.7 Image acquisition.

In order to acquire dynamic optical images, we used an epifluorescence Zeiss Axioscope FS connected with a CCD camera (Qimaging, Retiga 1300) hooked up with a PC running Northern Eclipse 5.0 software (Fig. 22A).
This camera was a high-resolution camera (1280 x 1024 pixel) with high-speed low noise electronics (up to 100 fps; up to 40 fps binning and 12 fps full resolution) and 10 or 12-bit digitization with 1024 or 4096 gray levels for precise gray level discrimination. The tissue was first viewed under a 5X dry objective for checking the success of dye load procedure in the entire pathway. The objective was then switched to a higher magnification. Usually (depending on the size of the larval head) with the 20X objective, it was possible to view the entire (left or right) side of the brain. The record was made under a 20X, water-immersion objective, with the Ringer’s solution (see Appendix pp.69) flowing on the tissue during the experiment. The dorsal surface of one olfactory bulb was illuminated with 480±25 nm (fluorescein filter block) light; the illumination was provided through a mercury short arc photo optic lamp (OSRAM, HBO 103 W/2). A neutral density filter was used to decrease the light intensity and avoid problems due to quenching the dye and photo bleaching of the tissue. Once the area of interest was in the right plane of focus the experiment was started and the changes of fluorescence against time were recorded using the bright fast configuration. With this configuration several parameters are under the direct control of the user in real time. Images were acquired and digitalized with a 1280 x 1024 pixel and with different exposure time (depending on the amount of the dye load within individual glomeruli) and with 2 X 2 time binned. Single data points were recorded each 100-300 msec and directly saved in Excel for further processing.

3.4 Results.

The main goal we achieved was to anterogradly label the entire olfactory pathway without major damages into the epithelium and to assess the physiological activity of the dye within glomerular units following the load. The
recording of activity in some glomeruli after odor stimulation was encouraging for further use of this technique for observing bulbar activity and odor coding.

3.4.1 Calcium green-1 dextran loading of glomerular units.

Following loading of the dye by nasal injection, the animals were kept for varying lengths of time; the pathway was exposed and viewed under epifluorescent light with a fluorescein filter. We found that at least three days (about 70 hrs) are needed for the dye to reach the OB (Fig. 24).

![Image](image.png)

**Fig. 24:** Olfactory pathway in vivo preparation, 3 days after loading with the calcium indicator Calcium green-1 dextran. The olfactory epithelium shows a high concentration of dye. The dye is present in the olfactory nerves and OB. The nasal cartilage obstructs the view of the olfactory nerves in this preparation. Scale bar is 500µm.

Animals tested before three days, did not show the presence of dye within the OB, however the dye was clearly visible within the olfactory epithelium and in some cases along the nerves (Fig. 25).

CGD diffuse from OSN dendrites to the axon terminals. Thus, the fluorescent intensity was much higher in the olfactory epithelium (Fig. 24), which is the application site and gradually diminished along the olfactory nerve (Fig. 24). The larger surface are of the olfactory epithelium compared to the

59
olfactory nerves and OB, may also have contributed to the greater fluorescent signal emitted by the olfactory epithelium.

![Diagram showing time course of Calcium green-1 dextran loading in larval OSNs](image)

**Fig. 25:** Time course of Calcium green-1 dextran loading in larval OSNs. The calcium sensitive dye was injected in the nasal cavity in association with Triton X-100, to remove cilia from the epithelium, greatly improving dye uptake (A). In preparations observed within 24 hrs from the application (B), the dye was found in the soma of the OSNs and in some cases along the axonal projections. The cilia were floating in the nasal cavity detached from the epithelium. The dye needed at least 3 days to reach the olfactory bulbs (C). In preparations observed after this period of time the dye was found within the glomeruli. The cilia grew back more or less at the same time.

In animals viewed 70 hrs following the application of the dye, it was possible to observe the distribution of the dye within a single glomeruli (Fig. 26).

It was possible to clearly observe single fibers within the neuropil of glomeruli at higher magnification (Fig. 26).
Fig. 26: An in vivo through focus series of glomeruli within the larval OB loaded with CGD. A is taken with the focal plane just above the labeled glomerular units. B is focused 100 μm deeper than A and the glomeruli are visible; the glomerulus indicated in C by an asterix in C was the last visible, 200 μm deeper.
Scale bar in B is 100 μm. In all the pictures, left is medial and top is anterior.
3.4.2 Structural integrity of OSNs following dye loading.

Together with the goal of loading the dye into the primary pathway of larval lamprey, we needed to confirm the integrity of the pathway following the load. First we checked the structural integrity of subcellular components of signal transduction. This included the olfactory cilia, the location of G-protein that is the starting point of the cascade of events for the transmission of the signals into the brain. In order to verify the preservation of cilia, we performed immunocytochemistry against acetylated tubulin, in animals previously treated with injection of CGD. The animals tested within 24 hrs of the injection showed a loss of cilia from the epithelium due, most likely, to the effect of Triton X-100 in removing cilia (Friedrich and Korshing, 1997). The cilia were visible as thin filaments detached from the epithelium and floating in the lumen of the nasal cavity (Fig. 27A). In animals tested after a longer period of time, the simultaneous presence of the dye within the pathway and the integrity of the pathway itself confirmed the efficiency of the technique (Fig. 27B-D). The cilia were visible on the epithelium (arrow in Fig. 27B) and OSNs were well loaded with the dye.

The results obtained through Hu-immunoreactivity, also confirmed the integrity of the OSNs after dye loading. As expected, immunoreactivity with Hu-antibody, gave staining exclusively in the OSNs of the olfactory epithelium (Fig. 28A). Staining was not observed in the respiratory epithelium. Higher magnification images give a closer view to the integrity and density of the OSN’s within the olfactory epithelium after loading confirming the presence of structurally intact OSNs (Fig. 28B-C). The OSNs were recognizable by a bottle like shape with the nucleus located in the bottom part of the epithelium (Fig. 28B).
Fig. 27: Acetylated-tubulin immunocytochemistry (red) in the olfactory epithelium following CGD loading (green). Fig. A and A-control show olfactory epithelium stained with AT. In A the staining was performed 12 hrs following the TritonX-100 treatment and load of the dye. The cilia are floating in the nasal cavity confirming the deciliating TritonX-100 effect, compared to the untreated preparation (A-control). B shows an olfactory epithelium 4 days after the injection of TritonX-100 and load of the dye. Staining with AT showed the structural integrity of OSNs. OSNs are loaded with CGD and the cilia grew back (arrows in B). C and D are higher magnification of Fig. B. Scale bar in A, A-control and B is 50μm; scale bar in C and D is 10μm.
Fig. 28: Hu-immunoreactivity in larval olfactory epithelium shows OSNs following TritonX-100 treatment. A shows the presence of OSNsexclusively in the posterior part of the nasal cavity where the olfactory epithelium is located. B and C show the shape and density of OSNs within the olfactory epithelium 80 hrs after the treatment. Hu is expressed in the perikaryon and dendritic compartements. Scale bar in A is 100µm; scale bar in B and C is 25µm.
3.4.3 Calcium imaging responses to 43mM KCl.

The following step was the assessment of the physiological activity of CGD after the injection. We decided to test the responsiveness of the excitable cells (neurons) through K⁺ treatment. Ringer’s solution with high concentration of K⁺ (see Appendix pp.69) was applied on the olfactory pathway through the second line that was designed on the perfusion chamber. In the membrane of the sensory cells, the presence of Na⁺/K⁺ ATPase pumps and of open K⁺ channels allows cells to K⁺ to accumulate in the cell cytoplasm, keeping the ratio between intracellular and extracellular sides equal to 140mM to 5mM. Thus in normal conditions, a continual efflux of K⁺ is therefore responsible for the resting membrane potential to be in electrochemical equilibrium at about -65mV. If the [K⁺]ₘₐₓ is altered the resting membrane potential is also altered because K⁺ moves inward. When [K⁺]ₘₐₓ raises high enough, the membrane potential reaches threshold with consequent firing of action potential (Purves, 1997). The depolarization travels along the neuronal cell body and the axons, where Na⁺ voltage sensitive channels open, allowing an inward current of Na⁺ and a further depolarization. At the pre-synaptic level, the change in membrane potential determines the opening of voltage sensitive Ca²⁺ channels with a consequent inward current of Ca²⁺. As a result the enhancement of [K⁺]ₘₐₓ leads to a calcium influx at a pre-synaptic level and consequently to an increase of fluorescence emission by the calcium indicator in these location.
Fig. 29: The effect of 43mM KCl upon Calcium green-1 dextran fluorescence emission in two specific glomeruli (1 and 2 on the right). Both glomeruli showed an increase of fluorescence (measured in arbitrary units), with consistent pattern, upon stimulation with high potassium ion solution. In the picture right is medial and top is anterior. Scale bar is 100μm.

The results show that the OSNs are physiological active 5 days after the application of CGD in the perfusion chamber (Fig. 29). At the same time we also showed that CGD did not lose its property, and upon Ca$^{2+}$ binding it was possible to observe an increase in fluorescence emission in two selected areas (Fig. 29) corresponding with dorso-medial glomeruli. Other preparations showed evidence of physiological activity of the dye (Fig. 31 in Appendix pp. 80) in different dorsal glomeruli units.

3.4.4 Calcium imaging responses following amino acid stimulation.

In order to further pursue the goal of physiologically characterizing the pattern of neuronal activity within the larval brain, we tested the preparation with L-Arginine stimulation. The application of the amino acid directly onto the nasal cavity was done through the Picospritzer (see Appendix pp. 79 for details). The stimulation with 10^{-6} M L-Arg resulted in an increase in fluorescent emission in a specific glomerulus, labeled in Fig. 30.

During the recording phase the base line seemed to decrease probably due to photo-bleach of the dye.
Fig. 30: Effect of $10^{-6}$ M L-Arginine on OSNs. The signal was recorded from a single glomerulus (labeled in the right picture). The arrows in the graph show the application of stimuli pulses. Each pulse was 500 msec in duration. The increase of fluorescence is measured in arbitrary units. Scale bar in the right picture is 100μm. Right is medial and top is anterior.

We tested about fifteen preparations and two of them gave a response following (10^{-6} M) L-Arginine stimulation. In the preparations where it was possible to record physiological activity, the dorsal glomeruli were tested. We estimated an increase in fluorescence of about 4%. Medial glomeruli tested in different trials, did not respond to L-Arginine stimulation.

3.5 Discussion.

The ability to load Calcium green-1 dextran into the primary olfactory pathway of larval lamprey provides a very useful tool for studies concerning the pattern of neuronal activation within the olfactory bulbs. The assessment of the physiological activity of the dye following the load and some preliminary results with amino acid stimulation confirmed the activity of the glomeruli units within the larval lamprey olfactory bulbs. However the preparation still needs some improvements, in order to achieve most persuasive results. Some limitations are intrinsic to the technique, but several improvements may be possible in the
preparation set up and in the images acquisition in order to consistently obtain better results.

3.5.1 Calcium green-1 dextran as a tool for study of neuronal activity in the primary olfactory pathway of lamprey.

The presence of Calcium green-1 dextran within the olfactory bulbs of lamprey, 3 days following the injection into the nasal cavity, was confirmed both in fixed postmortem tissue and \textit{in vivo} preparations. The dye appeared to be concentrated in the application site. In some preparations it was possible to observe small crystals embedded into the lumen of the nasal cavity and in several parts into the epithelium sheet. There is a substantial difference in the anatomy of the model we used in this study compare with the zebrafish model used by Frederich and Korsching. Lamprey is a monorinic organism and so after the injection of the dye the nostril cannot be flushed efficiently; this may caused the accumulation of the dye in small crystal clusters scattering in the olfactory epithelium. However we used lower concentrations of Calcium green-1 dextran with respect to the zebrafish model used by Friedrich and Korsching (1997). We injected dye at 2.5\% whereas they used 12\%. The fluorescence intensity of the dye gradually decreased by moving far away from the epithelium and toward the brain. Less intense staining was detectable within the glomeruli units, with optimal results occurring between 4 and 5 days following the dye application.

Immunocytochemistry with acetylated tubulin and Hu antibodies confirmed the integrity of the pathway following the load of the dye. The dye was injected in association with a detergent, Triton X-100. This compound knocks off cilia from the epithelium with a consequent improvement of dye uptake.
In zebrafish ultra-structural analysis and electrophysiological recording showed that cilia grew back within 72 hrs after the Triton X-100 treatment (Friedrich and Korshing, 1997). In lamprey the time is slightly longer due probably to the lower temperature (10-12 °C) at which the animals are kept. Regenerative processes are strongly affected by temperature and the zebrafish model used in Friedrich and Korshing study was kept at higher water temperature (between 25-28 °C). However we were able to visualize loading of the dye into OSNs and the presence of cilia in the epithelial sheet approximately 4 days following the injection.

K+ treatment confirmed the excitability of OSNs following loading of Calcium green-1 dextran into the olfactory bulbs and also confirmed the presence of presynaptic Ca2+ channels in dorsal and medial glomeruli of the lamprey.

Taken together, these results show that Calcium green-1 dextran can be employed as useful tool in the study of neuronal activity in larval lamprey olfactory bulb.

3.5.2 Glomeruli are physiologically active in the larval lamprey olfactory bulb.

Previous works of the olfactory mucosa, in the larval stage, showed physiological responses to chemostimulation by basic amino acids, bile acids and petromyzonol sulfate (Li, 1995; Zielinski, 1996). The results achieved in this study, show that the glomerular units within the larval brain are physiologically active following stimulation by the basic amino acid L-Arginine.

It is currently unknown which glomerular units respond to stimulation by amino acids. Functional imaging performed on teleost fish have suggested signal induction by amino acid is exclusively in the lateral chain while bile acids
elicited signals in medial and posterior regions of the olfactory bulb (Friedrich and Korshing, 1997; Fuss, 2001). These findings are consistent with field potential recordings made also in teleost fish, which showed the same pattern of activation, with amino acid activating lateral parts of the olfactory bulbs and activity elicited by bile acids highest in the medial olfactory bulbs (Døving et al, 1980). We tested two different amino acids, L-arginine and taurocolic acid.

We were able to record activity only after L-arg stimulation and in a few preparations; in both cases the glomeruli imaged where located in the dorsal part of the olfactory bulb. Due to the low number of functional experiments we cannot accurately speculate on a pattern of glomeruli activation within the olfactory bulbs of the larval lamprey following amino acid stimulation. However these preliminary results showed the possible application of dynamic imaging in association with calcium sensitive dye in the study of glomeruli activity in larval lamprey brain.

3.5.3 Complications associated with olfactory bulb imaging.

The results from the physiological section of this project are still in a preliminary phase and further improvement in the preparation is required before spatial analysis of neuronal activity in the olfactory bulb can be completed. Dynamic optical imaging is a relative new technique and its use in neurobiological studies, although extremely powerful, is in a developing phase. As in many laboratory techniques, there are limitations. It is very useful to analyze these limitations in relationship with the preparation we used in this study, in order to target potential sources of problems and consequently improve the results and the actual recording phase. The first issue comes with the sample preparation. First of all the loading of the dye occurred by a mechanical altering the olfactory epithelium. The injection of Triton X-100
knocked off cilia and permeabilized the plasma membrane. Through immunocytochemistry we showed that the tissue recovered after the injection of the dye in association with the Triton X-100. The cilia grew back and the OSNs looked healthy, with a characteristic bottle like shape. Following the dye injection, in order to view the primary olfactory pathway, we must surgically expose the olfactory organ and the brain. This, of course, is quite an invasive procedure and some physiological changes are expected within the tissue. During the dissection we took all the precautions possible to make the procedure less damaging for the tissue. The samples were kept in ice to avoid major damage due to enzymatic reactions. The dissection is a relative fast procedure (about 10 min) and Ringer's solution was applied on the tissue through out the duration of the surgery. During the image acquisition phase, fresh Ringer's, bubbled with 95% O₂/5% CO₂, was continuously perfused on the preparation and the temperature was ranging between the optimal values (12-15 °C). Because of these precautions and because of the physiological conditions in which the tissue was kept during the dissection and the image-recording phase, we believe loading of the dye and sample preparation did not affect the responsiveness of the olfactory system. Other limitations are related to the recording phase. We were able to record activity in just a few experiments and in a few glomeruli. An issue we must keep in mind is the optical accessibility. During the stimulation we visualized just a small area of the olfactory bulb, usually composed by few glomeruli. In all the experiments the location of the glomeruli that were imaged was the dorsal part, the only part accessible when the image acquisition occurs through an epifluorescent microscope. It seems obvious, that other foci of activity, located in different parts of the olfactory bulb, might go undetected. In their latest work Wachowiack and Cohen (2001), imaged odorant-evoked input to dorsal
glomeruli (which are the only visually accessible) in olfactory bulb from mice, using odorants known to activate dorsal glomeruli in rodents. However, it is unknown which odorant activates different foci of activity in specific glomeruli in the lamprey.

3.6 Conclusions

In the present study, we have combined anterograde tracing of OSN axons with a Ca$^{2+}$ sensitive fluorescent dye and optical imaging the OB. This technique allowed us to selectively monitor the dynamic patterns of presynaptic activity in glomeruli, induced by a given stimulus.

The results are still preliminary but the technique has been greatly improved in the last year and hopefully we will be able to address some interesting issues related with pattern of neuronal activation within the olfactory bulbs, in fairly short time. It is important to point out the power of dynamic optical imaging to neurobiological studies. The technique seems very appropriate to resolve some of the open questions concerned olfactory discrimination.

Together with skills and experience, also a good model and some luck appeared to be fundamental to achieve positive results. The lamprey offers an extremely interesting model because of its advantages for in vivo studies and because its relative simplicity in the olfactory wiring. The design of its olfactory pathway also gives the unique feature to visualize the entire OSNs from the periphery to the central nervous system.

Continual development and use of this stable preparation will lead to successful harvesting of important information broadly applicable to the function of the vertebrate olfactory system.
Chapter 4. Overall conclusions.

The overall goal of this project was to combine morphological evidence found in the olfactory bulbs of the developing Agnathan, the sea lamprey, *Petromyzon marinus* with physiological studies concerning spatial analysis of neuronal activation within the olfactory bulb.

The first set of experiments consisted in histological, immunocytochemical and anterograde labeling of the primary olfactory pathway with particular attention on the olfactory bulb. We found a consistent pattern of glomerular organization within the larval lamprey olfactory bulb. This pattern was comparable, although at a different dimension and scale, with the one found in a teleost fish (Baier and Korshing, 1994). We also explored the expression of $G_{\alpha_s/olf}$ in the primary olfactory pathway of this ancestral vertebrate. We found common traits compared with higher vertebrates, but also some interesting divergences.

The presence of $G_{olf}$-IR in the primary olfactory pathway of lamprey highlights the importance of this protein in the olfactory system during evolution. The molecular weight of $G_{olf}$ (45 kD), determinated through Western blotting analysis in ciliary preparation from adult lamprey olfactory epithelium, confirmed the homology with the protein found in other vertebrates. However the expression of $G_{olf}$ within glomerular units of the dorsal cluster, anterior plexus, lateral chain and ventral cluster is a peculiar characteristic, but at the moment we cannot make any speculation upon the function of this protein in the olfactory bulb.

The absence of $G_{olf}$ immunoreactivity in the lamprey medial glomeruli points to a differing signal transduction mechanism for these compared to the other glomerular groupings.
In the second part of the project, we combined anterograde tracing of OSN axons with a Ca\textsuperscript{2+} sensitive fluorescent dye and optical imaging the OB. This technique allowed us to selectively monitor the dynamic patterns of presynaptic activity in given glomeruli, induced by different stimuli.

The results are still preliminary but the technique has been greatly improved in the last year and hopefully we will be able to address some interesting issues related with pattern of neuronal activation within the olfactory bulbs, in fairly short time. It is important to point out the power of dynamic optical images applied in neurobiological studies. The technique seems very appropriate to resolve some of the open questions concerned olfactory discrimination and odor coding.

Furthermore, this study shows that the larval lamprey offers a useful tool for investigating the organization and modulation of spatial mapping of odors onto the vertebrate olfactory bulb.

Further characterization of the olfactory system in this aquatic vertebrate will assist in developing and employing pheromone based strategies and techniques for sea lamprey population management.
APPENDIX

RECIPES

STOCK SOLUTIONS
Stock A = 27.6 g sodium phosphate monobasic (NaH₂PO₄) in 1L of distilled water
Stock B = 53.7 g sodium phosphate dibasic (Na₂HPO₄) in 1L of distilled water

BUFFERS

0.2M Phosphate Buffer (PB), pH=7.4
190 mL of stock A
810 mL of stock B

0.1M Phosphate Buffer, pH=7.4
1L of 0.2M Phosphate buffer
1L of distilled water

0.1M Phosphate Buffered Saline (PBS), pH=7.4
8 g Sodium Chloride (NaCl)
0.2 g Potassium Chloride (KCl)
1L of 0.1M Phosphate buffer

1° Antibody Diluent for GSL I-isolectin B₄, pH=7.5
10 mM HEPES
0.15 M NaCl
0.2% Triton-X
2° Antibody Diluent for GSL I-isolectin B₄, pH=7.5

(Fluorescein Avidin DCS)

0.1 M Sodium Bicarbonate
0.15 M NaCl

Dilution Buffer for Calcium green-1 dextran, pH=8.0

1 mM NaCl
0.1% Triton-X

SUCROSE SOLUTIONS

10% sucrose: 10 g sucrose in 100 mL of PB 0.1 M
20% sucrose: 20 g sucrose in 100 mL of PB 0.1 M
30% sucrose: 30 g sucrose in 100 mL of PB 0.1 M

FIXATIVES

4% Paraformaldehyde

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

1. Dissolve the paraformaldehyde in distilled water and heat to approximately 55°C.
2. Add a small amount of NaOH to help dissolve the paraformaldehyde.
3. Cool the solution on ice.
4. Add 0.2 M phosphate buffer.
Zamboni's Fixative

20 g of paraformaldehyde (2%)
850 mL of 0.1 M phosphate buffer, pH=7.4
150 mL 1.2% saturated picric acid

PICRIC ACID WILL EXPLODE IF HEATED!!

1. Dissolve paraformaldehyde in buffer and heat to approximately 70°C.
2. Add a small amount of NaOH to help dissolve the paraformaldehyde.
3. Cool the solution on ice.
4. Add picric acid and adjust pH to 7.4 with either NaOH or HCl.

RINGER’S SOLUTIONS

For Lamprey 1L

130mM NaCl  (7.60g)
3mM KCl  (0.22377g)
2mM MgCl₂  (0.365g)
2mM CaCl₂  (0.294g)
5mM glucose  (0.901g)
10mM HEPES  (2.283g)

RINGER’S SOLUTIONS with high K⁺.

90mM NaCl  (5.26g)
43mM KCl  (3.206g)
2mM MgCl₂  (0.365g)
2mM CaCl₂  (0.294g)
5mM glucose  (0.901g)
10mM HEPES  (2.383g)
Perfusion chamber.

The chamber was constructed using a block of plastic with outer dimensions of 10cm x 5.75cm that was carved to create two inner levels. The upper level (8cm x 4.5cm) consists of a horizontally drilled hole that connects the Ringer's solution container to the chamber via a tube, and the lower level (4.5cm x 2cm) is lined with a layer of Sylgard to which the specimen will be stabilized. The custom built chamber sits at the center of a plastic platform, on one side of which two holes are drilled 7cm apart. This platform fits perfectly onto the base of the Zeiss Axioskop 2 fluorescent microscope and is held in place by two screws.
Presentation of odor stimuli through Picospritzer.

In order to localize the odor stimuli into the nasal cavity and thus directly on the olfactory epithelium, we used the Picospritzer (Parker instrumentation, General valve division, Fairfield, NJ). The capillary (World Precision Instruments Inc cat. # PG52151-4, Sarasota, FL USA) micropipettes was prepared on an electrode puller, with a given set-up of the puller, the shape and the wideness of the tip opening was checked under 4X objective on a monocular microscope. The capillary was then assembled with the pipette holder connected with the picospritzer. Different trials were performed, in which different pressures were use, in order to obtain a fairly wide plum that was able to completely fill the nasal cavity shortly and as well, wash out in a relative short time. We obtained these results with a 50msec pulse at the pressure of 15psi (see Fig 23). Through a food color test we also confirmed that the design of the chamber was appropriate. Puffing the odors against the current and sucking on the same side of the application site, we avoided the odors to spill out of the nasal cavity and move on the brain, thus being sure of not direct effect of odors on the olfactory bulb.
Further evidence of physiological activity of the dye achieved by $K^+$ treatment.

Fig. 31: Further evidence of physiological activity of two glomerular units, following $K^+$ treatment. The two glomeruli imaged are labeled in the micrograph beside. Scale bar is 100$\mu$m. Top is anterior and right is medial.
Selected Bibliography.


Belanger RM, Smith C, Corkum LD and Zielinski B. “Morphology and histochemistry of the peripheral olfactory organ in the round goby, Neogobius melanostomus”. Submitted to Journal of Morphology.


Juilfs et al. (1997). “A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway.” Proc Natl Acad Sci U S A. 1; 94(7):3388-95.


Vita Auctoris

Andrea Frontini was born in 1972 in Reggio-Emilia, Italy. He graduated from L. Vanvitelli Secondary School in 1991. From there he went on to the University of Ancona, Italy, where he obtained his B.Sc. Honours in Marine Biology in 1998. He started his M.Sc. degree at the University of Windsor, Ontario, in January 2000.