2006

The neural connectivity of olfactory bulb regions in ovulated female lampreys (Petromyzon marinus).

Steven Chang
University of Windsor

THE NEURAL CONNECTIVITY OF OLFACTORY BULB REGIONS IN OVULATED FEMALE LAMPREYS (*PETROMYZON MARINUS*)

by

Steven Chang

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 of Science at the University of Windsor

Windsor, Ontario, Canada

2006

Your file Votre référence
ISBN: 978-0-494-17075-5
Our file Notre référence
ISBN: 978-0-494-17075-5

Abstract

This study aims to discover neural pathways stimulated by reproductive pheromones eliciting an attractive locomotor response in sea lampreys. Distinct glomerular territories in the olfactory bulb of ovulated female lampreys were injected with biocytin. Dorsoanterior injections revealed labeling in integrative areas (dorsal, medial, and lateral pallia, habenula, thalamus, hypothalamus) and a locomotor control area (striatum). Medial injections labeled the medial pallium, the thalamus and the striatum. Projections to the hypothalamus and the lateral pallium from the dorsoanterior region of the olfactory bulb, that were absent from medial injections, infer functional difference between these two regions. Lateral injections labeled the striatum, suggesting the lateral olfactory bulb is strongly associated with locomotion. The results demonstrate an anatomical difference in the neural connections of distinct regions of the olfactory bulb.

Co-authorship statement

I certify that this thesis and research are original products of my research, and that ideas and quotations from the work of others, published or otherwise are fully acknowledged in accordance with the referencing practices of the discipline. I acknowledge the valuable contributions of Dr. Barbara Zielinski, Dr. Réjean Dubuc and Mr. François Auclair.

Acknowledgements

I would like to thank Dr. Barbara Zielinski for her guidance and support as my advisor. Thanks to Michele Askew, Heather MacDonald and Jessica Vaisica and Dr. Shashi Jasra for their assistance. I acknowledge Dr. Réjean Dubuc and Mr. François Auclair at the University of Montreal for their knowledge and expertise on all things lamprey. Thanks go to my predecessors, Wes Arbuckle and Ashley Firby for training and orienting me to the lab. I acknowledge fellow students, Rachelle Belanger, Xiang Ren and Alyson Laframboise for their feedback and valued contributions.

I would like to thank all the staff, including Usha Jacob, Ingrid Churchill, Candy Donaldson, Elaine Rupke, Nancy Barkley, Carolin Lekic and Pat Miles, for their support in my tenure as a graduate student in Windsor.

Finally, I thank my family for pushing me back into research and supporting me throughout this experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

OE = olfactory epithelium

OB = olfactory bulb

OSN = olfactory sensory neuron

ON = olfactory nerve

TEL = telencephalon

Di = diencephalon

Mes = mesencephalon

Rhomb = rhombencephalon

D Pal = dorsal pallium

L Pal = lateral pallium

M Pal = medial pallium

Hab = habenula

Hyp = hypothalamus

Thal = thalamus

Str = striatum

PA = preoptic area

3-kpzs = 3-keto petromyzonol sulfate

EOG = electro-olfactogram

LOT = lateral olfactory tract

MOT = medial olfactory tract

HRP = horseradish peroxidase

WGA-HRP = wheat germ agglutinin

1.0 Introduction

Olfaction is an important sense as it allows for the collection of diverse information from the environment, such as food sources and the location and reproductive status of potential mates. The importance of olfaction to lampreys is demonstrated by its use to locate prey (Kleerekoper & Morgensen, 1963), spawning streams (Fine et al. 2003) and mates (Li et al. 2002).

When lampreys reach sexual maturity and are ready to spawn, the mature spermiated males of *P. marinus* release a pheromone, 3-keto petromyzonol sulfate (3-kpzs), that is detected by mature, ovulated females and elicits the females to swim towards the pheromone source (Li et al. 2002). In fact, lampreys at all stages of development can detect this pheromone peripherally, at the level of the olfactory sensory neurons, (Teeter et al. 2003), but it is only the ovulated females that demonstrate locomotor activation in response to this male pheromone (Li et al. 2002).

Based on these observations, we hypothesize the presence of a secondary olfactory pathway from the olfactory bulb, to locomotor control regions, that is stimulated by this pheromone. In channel catfish, distinct regions of the forebrain are stimulated by different classes of odourants (Nikonov et al. 2005). These regional differences in the forebrain may also be seen in lamprey. Distinct areas of the lamprey forebrain (olfactory bulb) may have different projection patterns. One region of the olfactory bulb may be physically linked with a locomotor control area. This thesis demonstrates the anatomy of a potential neural pathway in the brain of the lamprey that stimulates locomotor activation in response to a sex pheromone.

1.1 Organization of the Olfactory Pathway

In all vertebrates, olfactory sensory neurons (OSNs) are the primary sensory cells of the olfactory epithelium. Their dendrites project to the apical surface of the olfactory epithelium. The axons of these neurons exit the olfactory epithelium and form the olfactory nerve (cranial nerve I) which projects to the olfactory bulb, the most rostral portion of the forebrain (prosencephalon). Olfactory sensory neuron axons terminate in discrete, marginal areas of the olfactory bulb called glomeruli which are dense areas of neuropil. OSNs are termed primary olfactory projections as they are the first cells of the olfactory pathway to respond to odours before transmitting this information within the olfactory glomeruli. Mitral cells are olfactory bulb neurons associated with olfactory glomeruli that accept incoming olfactory information from olfactory sensory neuron axons and transmit information to other parts of the brain via their axons. Mitral cells are large, conspicuous cells whose somata are located close to the periphery of the olfactory bulb. Mitral cell axons that extend to more caudal targets in the brain are termed secondary olfactory projections, and are the focus of the current investigation.

1.2 Mapping Olfactory Glomeruli

Olfactory glomeruli are dense areas of neuropil in the olfactory bulbs that receive incoming information from the axons of olfactory sensory neurons, and relay that information via mitral cells, the output neurons. In zebrafish, individual glomeruli receive afferent input from either ciliated or microvillous olfactory sensory neurons, but not both, lending support to a hypothesis that different glomeruli process different odours (Sato et al. 2005). If glomeruli do process different odours, then a

map of where different odours are processed in the olfactory bulb can be constructed, called an odotopic map.

To visualize glomeruli under a microscope, they must be stained in some way to distinguish them from the surrounding tissue. Methods usually exploit some property of glomeruli that are unique or exist in greater abundance than the surrounding tissue. A glomerular map has been constructed for larvae of *P. marinus* (Frontini et al. 2003). The lectin *Griffonia simplicifolia* isolectin B_4 ($GS1B_4$) has a high affinity for galactose residues (Kirkeby and Moe 2001), and was used to stain olfactory glomeruli in larvae. The visualization of glomeruli is important for at least two reasons;

1) to understand where olfactory sensory neurons in the olfactory epithelium project

2) to visualize where secondary projections (mitral cells) originate.

This information can then be applied to linkage between chemotopic responses in the olfactory bulb to downstream integrative and motor output centers. For example, in the channel catfish, the two main types of olfactory sensory neurons (ciliated and microvillous), project to distinct regions of the olfactory bulb (Nikonov and Caprio 2001). Also in the channel catfish, different classes of odourants stimulate different regions of the forebrain. Amino acids and nucleotides stimulate the lateral and pallial forebrain and bile salts stimulate the medial forebrain (Nikonov et al. 2005).

For this study, our goal was to discover which regions of the brain are connected to specific glomerular territories.

1.3 Stimulation of Olfactory Sensory Neurons

An odour is any compound that is detected by the olfactory sensory neurons of the olfactory epithelium. Because fish live in an aqueous medium, odourants in the surrounding water stimulate odourant responses. Binding of an odour to G-protein coupled receptors located on the apical surface of olfactory sensory neurons enhances the opening or closing of membrane channels. Ion flux through the channels changes membrane conductance which spreads electrotonically and can generate action potentials that are then conducted along olfactory sensory neuron axon terminals to the olfactory bulbs. This electrical event is exploited by a technique known as the electro-olfactogram (EOG) which samples the generator potentials of a population of olfactory sensory neurons in response to application of a compound. An EOG recording indicates that the compound being tested can be classified as an odour (Scott and Scott-Johnson 2002).

A pheromone is "a chemical substance which carries a message about the physiological or behavioural state [of an organism] to members of its own species, resulting in 'a specific reaction, for example a definite behaviour or a developmental process that is species specific' " (Karlson & Luscher 1959). In fish, pheromones that are gonadal steroids or prostaglandins that influence the behaviour or physiology of a conspecific, often acting as an attractant, are considered sex pheromones (Dulka et al. 1987).

1.4 The Location and Function of the Lateral and Medial Olfactory Tracts

In vertebrates (goldfish - *Carassius auratus* – Dulka 1993 and Sorensen et al. 1991: brown ghost knifefish - *Apteronotus leptorhynchus* – Sas et al. 1993; Chinook salmon – *Oncorhyncus tshawytscha* – Matz 1995; salamander – *Plethodon shermani* – Laberge and Roth 2005; mouse - *Mus musculus* – Inaki et al. 2004; rat - *Rattus norvegicus* –Schwob and Price 1994), the axons of mitral cells form two distinct pathways; the medial olfactory tract (MOT) and the lateral olfactory tract (LOT). This anatomical division of the olfactory tract reflects a division of information transmission which is supported by lesion experiments. In goldfish (*C. auratus*- Stacey and Kyle 1983) and crucian carp (*Carassius carassius* – Hamdani et al. 2001, Weltzien et al. 2003) lesioning the MOT resulted in a reduction in sexual behaviour, while lesioning the LOT resulted in reduction in feeding behaviours

This functional and anatomical division of transmission of olfactory information indicates that different regions of the brain are stimulated by different classes of odours.

1.5 Tract Tracing is Used to Analyze Mitral Cell Outputs in Vertebrates

Our hypothesis posits that olfactory information is relayed from specific glomerular regions to integrative areas of the brain, including those that control locomotion. In fish, these areas include; the pallial regions of the telencephalon, the striatum, the habenula, the thalamus and the hypothalamus.

There are a number of studies that trace the secondary olfactory projections in vertebrates and employ different methods.

Carbocyanine tracers, such as DiI or DiO are fluorescent molecules that are used to trace fibers, usually in fixed tissue, but can also be used *in vivo*. After application, the lipophilic dye molecules insert themselves in the plasma membrane of cells. The signal provided is strong with great detail shown, however, transport of the dye takes weeks and the staining is ephemeral, as the dye eventually diffuses out of the target cells. Lipophilic tracers have been used to trace secondary olfactory connections in myxinoid fish - *Eptatretus stouti* (Wicht and Northcutt 1998); and teleost fish - *Acipenser baeri* (Huesa et al. 2000); *Gadhus morhua* (Rooney et al. 1992).

Horseradish peroxidase (HRP) has been used in goldfish (von Bartheld et al. – 1984, Levine and Dethier – 1985); lungfish (von Bartheld et al. 1990); rabbit (Ojima et al. 1984); winter flounder (Rao and Finger 1984); brown ghost knife fish (Sas et al. 1993). HRP was intracelluarly injected into cells *in vivo* and transported retrogradely and anterogradely along the length of the cell. Animals were then sacrificed and their brains sectioned. Sections containing HRP were then exposed to peroxidase that produced a chromogen that allowed fibers to be visualized.

Biotinylated molecules, such as biotinylated dextran amine (BDA) or biocytin, can also be used for tract tracing. These compounds are directly applied as crystals or in solution to lesioned axons *in vivo*, for intracellular transport in the retrograde and anterograde directions. Animals are then sacrificed and the brains sectioned. An avidin complex is then added (usually tagged to a fluorophore) to visualize the biotinylated molecule in the cells (King et al. 1989; Izzo 1991; Xue et al. 2004). The

use of biotinylated molecules and dextrans has often replaced techniques like HRP, as the methods are simpler, quicker and lend directly to live cell imaging.

BDA has been successfully used in teleost fish to trace primary and secondary olfactory projections (Chinook salmon – Matz 1995; sturgeon – Huesa et al. – 2000, cod – Rooney et al. 1992). BDA injection to the olfactory bulb of the Chinook salmon (*Oncorhyncus tshawytscha*) demonstrated two main output tracts of the bulb; lateral and medial, which project to parts of the telencephalon and diencephalon (Matz 1995). Projections were seen to terminate in multiple nuclei in the ventral telencephalon (ventral, dorsal, lateral and supracommisural nuclei) and the dorsal telencephalon (lateral-ventral and posterior zones). The posterior tuberal region of the ventral diencephalon was also labeled, as was the preoptic area. The nomenclature of brain regions, used by Matz (1995), is problematic, in that a direct comparison to lamprey brain regions is not possible. For the purpose of direct comparison of the Matz study with this thesis, the ventral telencephalon in Chinook salmon encompasses the dorsal pallium, striatum and preoptic area of the lamprey, the dorsal telencephalon encompasses the medial pallium and the lateral telencephalon encompasses the lateral pallium (see **Figure 1** for a dorsal view of the lamprey brain).

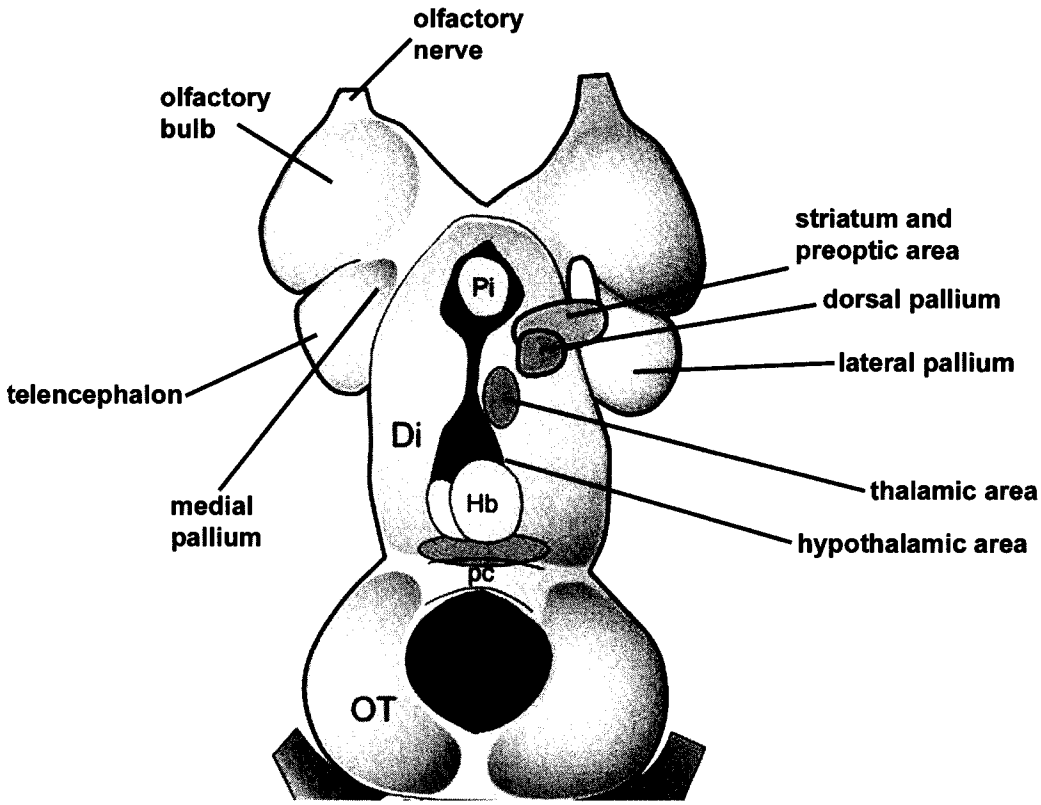

Figure 1: Dorsal view of the adult lamprey brain including the prosencephalon, diencephalon and mesencephalon. Brain regions of interest for this study are shown. Rhombencephalon is not shown. Di = diencephalon, Pi = pineal gland, Hb = habenula, pc = posterior tuberculum, OT = optic tectum.

In sturgeon (*Acipenser baeri*), secondary olfactory projections were traced using a variety of techniques (lipophilic tracers, biotinylated tracers and HRP). Secondary projections were seen in the dorsomedial telencephalon, anterior commissure, dorsocentral telencephalon, preoptic area, thalamus, habenula and the hypothalamus (Huesa et al. 2000).

In cod (*Gadhus morhua* L.), secondary projections were traced with HRP (Rooney et al. 1992). Areas labeled included the telencephalon, the anterior commissural and preoptic areas, habenula, dorsal thalamus, and the diencephalon.

Separate divisions of the olfactory tract (LOT, lateral bundlet of the medial olfactory tract [lMOT] and medial bundlet of the medial olfactory tract [mMOT]) each had unique targets with some overlap of targets. The LOT projected to the lateral, dorsal posterior regions of the dorsal telencephalon and the ventral region of the telencephalon. The lMOT projected to the lateral and central regions of the dorsal telencephalon. The mMOT projected to the ventral telencephalon and the posterior region of the dorsal telencephalon.

In goldfish, secondary olfactory projections were traced to the ventral and dorso-lateral areas of the telencephalon, the preoptic area and the posterior tubercle in the diencephalon. Fibers were seen crossing to the contralateral side in the anterior and habenular commissures as well (von Bartheld et al. 1984).

In summary, injection of tracers to the olfactory bulbs in teleost fish results in labeling in discrete areas of the dorsal and ventral telencephalon and labeling in discrete areas of the diencephalon. Rarely are cells labeled in the mesencephalon, and in those cases that do report mesencephalic labeling, the signal is faint. No reports exist that report labeling in the rhombencephalon. The lack of labeling in the mesencephalon and diencephalon suggests that secondary olfactory projections are limited in length and do not reach areas such as the reticulospinal neurons, which directly control locomotion.

In the salamander, *Plethodon shermani*, biocytin injection to the main olfactory bulbs resulted in labeling in the pallial areas of the telencephalon, which are integrative areas. One of the areas of interest labeled was the striato-pallial transition area which is homologous to the striatum in lampreys. The striatum is involved in

locomotor activity and coordination. The striato-pallial transition area is sub-divided into three parts; rostral striatum, intermediate striatum and caudal striatum. The caudal striatum has descending projections to the ventral hypothalamus, which is also an integrative region (Laberge & Roth 2005).

In an HRP study, rabbit (*Oryctolagus cuniculus*) mitral cells projected to the olfactory cortex (Ojima, Mori and Kishi 1984). The olfactory cortex is where olfactory information is received and integrated. Mitral cell somata located in the ventral half of the olfactory bulb projected axons to the LOT. Some LOT projections terminated into discrete areas of the olfactory cortex (i.e. anterior olfactory nucleus, anterior piriform cortex and the olfactory tubercule). It is in the olfactory cortex that incoming olfactory information is processed and co-ordinated with locomotor function in mammals.

In summary, secondary olfactory projections spread to a variety telencephalic and diencephalic targets, some of which integrate sensory information (thalamus, pallial areas). A subset of these target areas co-ordinate locomotor function (striatum).

1.6 The Striatum as a Gate for Olfactory Information

In the lamprey, the striatum has descending connections to the ventral thalamus, which in turn connects to reticulospinal neurons, which directly control swimming (Pombal, El Manira and Grillner, 1997). This pathway leads us to the expectation that there is a direct connection between the olfactory bulb and the striatum. Some of the somata in the striatum are immunoreactive to GABA indicating that the striatum inhibits or modulates incoming information from the

olfactory bulbs. The concept of the striatum as a gate or relay area of olfactory information is reviewed in Grillner et al. 1997.

1.7 Life Cycle of *Petromyzon marinus*

P. marinus has a well defined and complex life cycle. Larvae (ammocoetes) hatch from eggs in freshwater streams and live as sedentary filter feeders for 3-20 years. The ammocoetes then undergo metamorphosis and enter the transformer phase which lasts for about three months. Transformers then enter the parasitic stage which lasts 1-2 years. The parasites migrate to open waters to feed on the blood and other tissues of prey fish. The parasites (now upstream migrants) travel to freshwater streams, reach sexual maturity (ovulating females and spermiating males), spawn and die soon after.

The ammocoete and the adults possess markedly different physiologies. For instance, the gall bladder is absent in adults, but present in ammocoetes (Yamamoto et al. 1986). The peripheral olfactory organ is different as well. The ammocoete olfactory epithelium lines a simple sac as compared to the multi-lamellar epithelium of the adult olfactory epithelium. The differences in physiology and anatomy between the larval and adult stages may also be reflected in neural connections in the brain. Using exclusively ovulated females is ideal, in this study, as ovulated females exclusively demonstrate a locomotor response to the male pheromone.

1.8 Olfaction and *P. marinus*

The olfactory sense is an important component of how lampreys localize their prey. In behavioural assays, parasitic *Petromyzon marinus* showed a preference for conditioned water taken from trout (*Salvelinus foario fontinalis* and *Salmo foario trutta*) which are normal prey for this lamprey species (Kleerekoper & Morgensen, 1963). This preference was shown by changing their orientation to the odour source and increasing their locomotor (swimming) activity in response to introduction of these odours.

When lampreys reach sexual maturity, they migrate back to freshwater streams to spawn. Adults spawn in streams where there are established populations of larvae, indicating an appropriate place to spawn. Larvae release a pheromone that is detected by mature adults which acts as an attractant to the spawning streams. This pheromone is comprised of several elements, including petromyzonamine disulfate, petromyzosterol disulfate and petromyzonol sulfate and behavioural and electrophysiological testing has shown that petromyzonamine disulfate is the most potent component of this migratory pheromone (Sorensen et al. 2005).

Sexually mature (spermiated) males of the sea lamprey, *Petromyzon marinus*, release a sex pheromone, 3-keto petromyzonol sulfate (3-kpzs) (Li et al. 2002) which is chemically similar in structure to petromyzonol sulfate, a component of the migratory pheromone (Sorensen et al. 2005). Odour detection of 3-kpzs was determined electrophysiologically via electro-olfactogram. Behavioural assays have revealed that only ovulated females show a preference for this odour, when compared to non-ovulated females. (Li et al. 2002). The ovulated females increased search

around the odour source and swam towards the odour source (Li et al. 2002). These results suggest that 3-kpz is a sex pheromone used by males to attract females for mating, and so, examining the neural connectivity of ovulated females is the most logical starting point.

1.9 The Lamprey Brain

The brain of an adult lamprey in a dorsal view (**Figure 1**), shows the relative positions of different brain regions. A lateral view of the lamprey brain is shown in **Figure 2A.**

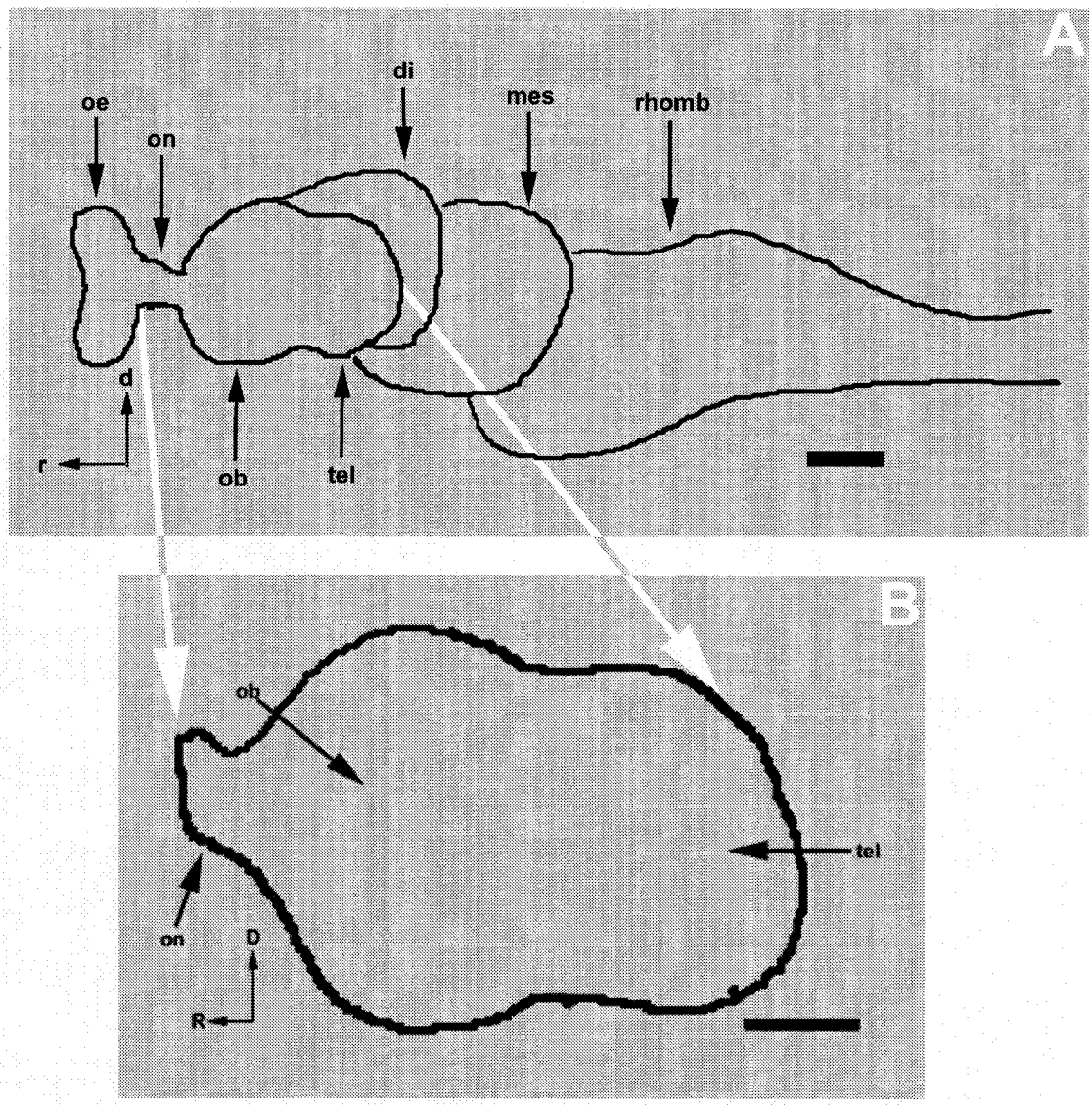

Figure 2: Cartoon sketch of an adult lamprey brain, lateral view. A – complete lamprey brain in lateral view; B – prosencephalon in lateral view.
oe - olfactory epithelium; on - olfactory nerve; ob – olfactory bulb; tel - telencephalon; di – diencephalon: mes – mesencephalon; rhomb – rhombencephalon. Scale bar = 500 μm.

The olfactory bulbs are the most rostral part of the lamprey brain and are the first regions to receive olfactory input from the olfactory epithelium. An enlarged lateral view of the olfactory bulb and the telencephalon is shown in **Figure 2B**. At the periphery of the bulbs are glomeruli; dense areas of neuropil that are the first level

of olfactory neurotransmission in the brain. Here, incoming olfactory information is accepted and relayed to other parts of the brain (the telencephalon and the diencephalon). The information is relayed by mitral cells which are the main output neurons of the olfactory bulbs. Directly attached to the olfactory bulbs is the telencephalon which contains three broad regions referred to as the dorsal, medial and lateral pallia. The pallial areas receive input from sensory systems (olfactory, visual, somatosensory – Northcutt and Puzdrowski 1988, Northcutt and Wicht 1997), indicating that there is multi-sensory integration taking place in these areas. Also located in the telencephalon are the striatum and the preoptic area. These two structures are almost continous and are medially located, bordering the central ventricle. The striatum (analogous to the basal ganglia in higher vertebrates) plays a role in execution of movement and is located lateral to the preoptic area (Grillner et al. 2005). The preoptic area is a periventricular region densely populated by somata. Some of these cells are immunoreactive for gonadotropin releasing hormone (GnRH), a hormone responsible for promoting reproductive status (Eisthen and Northcutt 1996). The striatum is known to have projections to the ventral lateral pallium region of the telencephalon. The ventral lateral pallium in turn projects to the ventral thalamus which contains descending connections to reticulospinal neurons which directly control locomotion in lampreys (Pombal et al. 1997). Directly posterior to the pallial areas, and dorsally situated, is the habenula which is an integrative center between the striatum and the limbic and motor systems (Yanez and Anadon 1994). At about the same level of the habenula, looking ventrally, are the thalamus and hypothalamus, which are also integrative centers. The hypothalamus is located

posterior to the thalamus. The four integrative centers in the lamprey brain mentioned (pallium, habenula, thalamus and hypothalamus) are all putative targets for olfactory input that may be associated with locomotor responses to odourant stimuli.

1.10 Olfactory Bulb Output Connections in Lampreys

There are many other species of lamprey, most of which have parasitic life stages, as *P. marinus*. The neural connections of two species of lamprey have previously been studied. The silver lamprey (*Icthyomyzon unicuspis*) is a parasitic species native to the Ohio region of Lake Erie. The neural connections of the olfactory bulb were studied following injection of horseradish peroxidase (HRP) and wheat germ agglutinin-horseradish peroxidase (WGA-HRP) to the olfactory bulb of adult lampreys (Northcutt and Puzdrowski 1988). Injections were large and along the dorso-ventral axis, but glomerular territories were not defined. This study reported connections from the olfactory bulb to the pallial areas (dorsal and lateral) and also to the septum and preoptic area. The pallium of the brain refers to broad areas in the medial, dorsal and lateral telencephalon and are also areas of sensory integration.

A similar tract tracing method was employed to explore the olfactory and non-olfactory projections in the river lamprey, *Lampetra fluviatilis* (Polenova and Vesselkin 1993). This species of lamprey, found in Western Europe, is also parasitic. The authors injected HRP and WGA-HRP to various areas (the olfactory bulb, pallial areas, primordial hippocamp and the dorsal thalamus) to explore the location and extent of secondary olfactory projections. Injections resulted in a general pattern of projections from the olfactory bulb to the pallial areas of the telencephalon, the septum, striatum, preoptic area, dorsal thalamus and the posterior tuberculum. The

telencephalon and the thalamus are both integration centers, and the striatum is involved in locomotor control.

The Northcutt and Puzdrowski (1988) and Polenova and Vesselkin (1993) investigations focused on the location and extent of all secondary olfactory projections and comparison of these projections with those found in more complex vertebrates led to the conclusion that the telencephalon developed as an olfactory center first and later acquired input from other sensory modalities. Both papers show a general view of projections from the olfactory bulb. Glomerular territories were not defined and the areas labeled cannot be linked to a glomerular region and so, cannot be linked to stimulation by a specific odourant.

The objective of this thesis was to link glomerular territories of the olfactory bulb to specific output regions in ovulated female lampreys.

1.11 Delineation of Developmental Stages

The Northcutt and Puzdrowski (1988) and Polenova and Vesselkin (1993) investigations did not distinguish sex or reproductive status in their experimental animals. In *P. marinus*, only the ovulated females demonstrate the locomotor response to the male reproductive pheromone (Li et al. 2002) and so, there may be sexually dimorphic characteristics in the brain that would explain this phenomenon. Also, the Northcutt and Puzdrowski diagrams show their injection sites in very caudal/posterior sites of the olfactory bulb/telencephalon which could mean that rostral glomerular territories may not have been contacted by their injections. Due to the nature of the investigation, specific glomerular territories and their respective projection patterns were not discussed.

The bulbar injections in the Polenova and Vesselkin study are more rostrally located than in Northcutt and Puzdrowski's work, but they are still very posterior and so could still be missing the projection pattern of very rostrally located glomerular territories.

This study exclusively uses ovulated female *P. marinus.* Adult status was determined by size of the animal (length and weight). Reproductive status (and hence, developmental status) was confirmed by the presence of eggs, after sacrifice and dissection of each animal. It was vital to our study that the animals used were only ovulated females, as it is at this stage of development that a locomotor response to the male sex pheromone, 3-kpzs, is seen (Li et al. 2002).

1.12 Experimental Strategy

Our goal was to inject glomerular territories in the olfactory bulb of an ovulated female lamprey and label the projections from these territories to their downstream targets in the brain. The hypothesis was that different glomerular territories would have unique patterns of projections and these secondary olfactory projections would project to integrative centers such as the pallial areas, habenula, thalamus or hypothalamus which would then control locomotion.

2. Materials and Methods

2.1 Materials

2.1.1 Experimental Animals

Ovulated female lampreys (*Petromyzon marinus*) were collected four times in 2005 (May 16, June 15, July 20, August 23) and were sourced from the Cheboygan River in Cheboygan, Michigan, the Ocqueoc River in Millersburg, Michigan and the St. Mary's River in Sault St. Marie, Ontario by the staff at the Department of Fisheries and Oceans and at the Hammond Bay Biological Station in Millersburg, Michigan. Forty-two (42) ovulated females [length 50.9 cm ± 0.9, weight 253.5g ± 2.7] were used for this study. Animals were housed at the University of Windsor in 250 L glass aquaria at 7°C. Aquaria were oxygenated and equipped with a carbon and UV filter (Aquatic Ecosystems, catalog # ALS10).

2.2 Methods

2.2.1 Tissue Preparation

Animals were anaesthetized using MS-222 (tricaine methanesulfonate) (0.05g/L) from Argent Laboratories (catalog # C-FINQ-UE-100G, Redmond, WA). Animals were sacrificed by decapitation and the brain was rapidly exposed by dissection. Ovulation was confirmed *post mortem* by the presence or absence of eggs in the body cavity. For cresyl violet or $GS1B_4$ lectin experiments, brains were immediately fixed in 4% paraformaldehyde in 0.1 M PBS and left overnight. For injection experiments, tissue was immersed in cold lamprey Ringer's solution (7.6g NaCl, 0.156g KCl, 0.382g $CaCl_2$, 0.366g $MgCl_2$, 0.953g HEPES, 0.721g dextrose, 0.084g $NaHCO_3$ in 1L distilled water, pH 7.4) and pinned into a Sylgard ® lined dish.

The dish was then placed on a cooled Peltier plate to keep the tissue cool during dissection and manipulation, which we termed an *ex vivo* preparation.

2.2.2 Cresyl Violet (Nissl) Stain of Ovulated Female Brain Sections

We first sought to familiarize ourselves with the organization of the adult lamprey brain in cross-sectional views because all of the literature on lamprey brain anatomy has been shown in the cross-sectional plane (Heier, 1948; Niewenhuys, 1977; Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993). Our goal was to duplicate the results obtained by Heier (1948) in identifying specific neural structures that are known to be densely populated with cell bodies, such as the striatum and the preoptic area. Heier's work is important as a reference in lamprey research, as it was the first complete atlas of the entire adult lamprey brain. Ovulated females (n=4) were anaesthetized with MS-222 [0.05g/L] and decapitated. Brains were exposed by dissection as described above and immediately fixed in 4% paraformaldehyde (in 0.1 M PBS, pH 7.4). Brains were allowed to fix at least overnight, then removed from the cranium and passed through a series of sucrose solutions (10%, 20%, 30% sucrose in 0.1 M PBS, pH 7.4) to cryoprotect the tissue. Tissue was cryosectioned in the transverse plane at 20 μm intervals and collected on Superfrost slides (Fisher Scientific, Nepean ON, catalog # 12-550-20). Slides were allowed to air dry before being processed for the Nissl stain.

A 0.1% solution of cresyl violet in distilled water was applied to the slides for 2 minutes and then washed in distilled water to remove the excess stain. Slides were dehydrated and cleared through as series of washes [70% ethanol, 1minute; 95% ethanol, 1 minute; 95% ethanol + 1% acetic acid in 95% ethanol, dip once; 100%

ethanol, 2 minutes (x2); 100% xylene, 2 minutes (x2)]. Slides were then dried and mounted in Permount (FisherChemicals, Fair Lawn, NJ, catalog # SP15-500). Tissue was analyzed on a Zeiss AX-2 Axioscope under bright field illumination and images were captured using a CCD camera and Northern Eclipse Software.

Morphometric data on the olfactory bulbs was required to determine the distance biocytin would need to travel in order to reach the telencephalon. The olfactory bulbs are about 1000 μm long and the telencephalon is about the same length, making the whole forebrain (or prosencephalon) about 2000 μm long. The anterior lateral ventricle appears at about 360 μm and continues for about 180 μm (**Figure 3**).

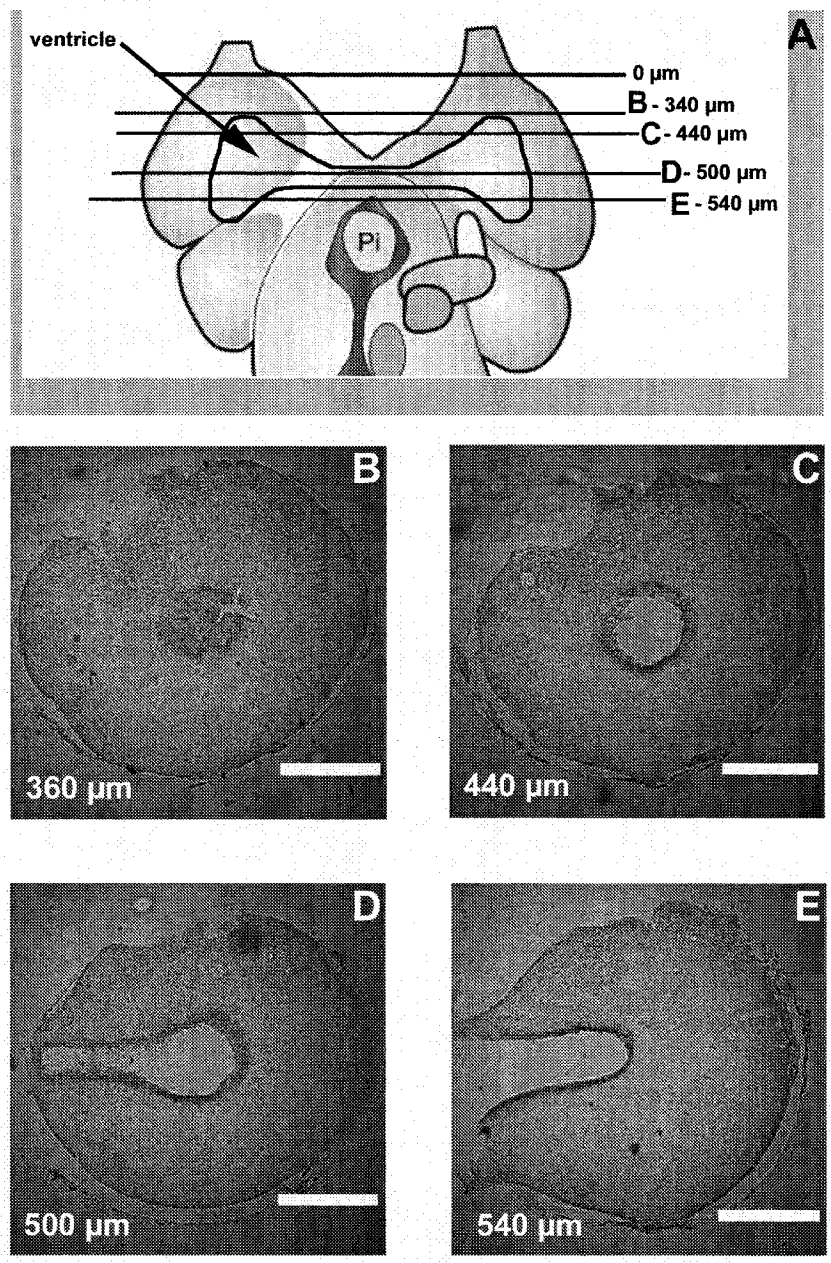

Figure 3: The location of the ventricle in the olfactory bulb in cresyl violet stained sections of the brain of an adult ovulated female lamprey.
The lateral ventricle begins in the rostral olfactory bulb and continues through the telencephalon, joining the main or central ventricle in the brain. A – cartoon diagram of a lamprey brain showing the level of sections taken; B – rostral edge of the lateral ventricle in the olfactory bulb; C – the lateral ventricle enlarges in volume; D – the lateral ventricle enlarges in the medial direction to join the main ventricle; E – the lateral ventricle now is part of the main ventricle. Scale bar = 500 µm.

The rostro-caudal distance to the ventricle is a vital piece of information because it is necessary for us to avoid injecting the biocytin into the ventricle, thereby causing the tracer to leak and penetrate unintended targets. With our global injections, we were not concerned with leakage of biocytin into the ventricle because our goal, at that point, was to label as many output neurons as possible to gain an overall understanding of what the general projection pattern looked like. As we refined our study to glomerular territory injections, it became more important to avoid penetrating the ventricle to ensure that our injections were restricted to the glomerular territory of interest and so, any labeling seen would arise from the restricted injection.

2.2.3 $GSIB_4$ Lectin Staining of Glomerular Territories in the Olfactory Bulbs of Ovulated Female Lampreys

Knowing which olfactory glomeruli are associated with our projection pattern is crucial to understanding where secondary olfactory projections originate. Previous work has identified that *Griffonia simplicifolia* isolectin B_4 labels olfactory sensory neuron axons in glomeruli of larval *P. marinus* (Frontini et al. 2003). For our own experiments, animals (N=4) were sacrificed, dissected, fixed and cryoprotected as described for cresyl violet staining. Brains were cryosectioned in the transverse plane at 20 μm. Biotinylated GSL I – isolectin B4 (Vector Laboratories, Burlington, ON, cat. # B-1205) (1:1000 in 0.1 M PBS, pH 7.4) was incubated overnight on slides. Slides were then washed three times in 0.1 M PBS and then incubated in fluorescein avidin DCS (Vector Laboratories, cat. # A-20111:100 in 0.1 M PBS, pH 7.4) for 2 hours at room temperature. Slides were then coverslipped with Vectashield (Vector Laboratories, Burlington, ON catalog # H-1000) and sealed with nail polish. Slides

were analyzed on a Zeiss Axioscope equipped for fluorescence and fitted with a CCD camera. Images were captured using Northern Eclipse software.

2.2.4 Lesion and Injection of Biocytin

The original intent of this thesis was to create large, global injections to the olfactory bulb to see the complete pattern of secondary olfactory projections in ovulated *P. marinus* females. Our guides were the studies of poorly defined life stages in silver lamprey (*I. unicuspis* - Northcutt and Puzdrowski, 1988) and in river lamprey (*L. fluviatilis* - Polenova and Vesselkin, 1997). The authors made large, global injections to the olfactory bulbs in an effort to contact as many olfactory glomeruli as possible. Our attempts, however, were unable to avoid the lateral ventricle present in each olfactory bulb. This resulted in dye leakage to posterior regions of the brain through the ventricle and labeling in multiple and inappropriate locations in the brain. Results could not be confidently linked to glomeruli and glomeruli could not be confidently linked to labeled areas.

In their illustrations of the lamprey brain and injection sites, Northcutt and Puzdrowski (1988) showed their injections of horseradish peroxidaseindeed touch olfactory glomeruli (as indicated in their diagrams) however, the diagrams show both lobes of the brain, implying that injections were not sufficiently rostral to include only one olfactory bulb and indicating that injections were posterior and therefore, could not have contacted rostral olfactory glomeruli.

In their 1997 paper, Polenova and Vesselkin used similar diagrams to illustrate their injection pattern. In this work, the authors clearly showed that their injection sites overlapped the lateral ventricle, showing that their indicator dye

(WGA-HRP) had leaked into the ventricle. Since the lateral ventricle connects to the main ventricle, the dye could have been transported to the telencephalon, diencephalon, mesencephalon or even the rhombencephalon, which could have resulted in labeling in these areas. Labeling due to inappropriate dye transport could have confounded results as labeling in the brain could not be exclusively attributed to the original injection sites in the olfactory bulb.

Our attempts at global olfactory bulb injections consistently resulted in leakage through the lateral ventricle and so, we began with dorsal anterior injections as this region contained abundant glomeruli (Frontini et al. 2003). We hypothesized that different olfactory glomeruli would have a unique projection patterns, so we attempted to injected different glomerular territories by injecting different regions of the olfactory bulb (lateral and medial regions).

2.2.5 Dye Loading into Glomerular Territories

The olfactory bulb was lesioned using an insect pin or the tip of a fine glass pipette. Glass micropipettes were prepared with a vertical pipette puller (KOPF™ model 720; 3 1/2" Drummond capillaries #3-000-203-G/X). The tips of the micropipettes were broken under a dissecting microscope to a diameter of approximately 50 μm and filled with the biocytin solution (2% in 0.1 M PBS, pH 7.4) by capillary action. Small eppendorfs were filled with 20 μl of the biocytin solution, and 4 micropipettes were filled from this solution, yielding 5 μl per micropipette. The micropipette was fitted to a holder that allowed the user to blow the dye out of the micropipette. In every case, the entire volume of one micropipette was injected to a single region of the olfactory bulb. The intracellular labeling of cells using biocytin

is dependent upon damaging axons for the biocytinto enter (MacDonald 1992). Once a cell has been lesioned and dye taken up by the cell, axoplasmic transport passively carries the biocytin along the cell in the retrograde and anterograde directions.

The *ex vivo* preparations were kept in cold, oxygenated lamprey Ringer's solution to keep the tissue viable. Tissue was sectioned and compared to tissue that was fixed immediately *post mortem*. If the *ex vivo* preparations were not viable, tissue necrosis would have created holes or cavities in the sections. No such necrosis was visible in the *ex vivo* preparations.

The following is a table of injections to the different regions of the olfactory bulbs in ovulated female *P. marinus*.

Table 1: Summary of Injections to Different Regions of the Olfactory Bulb of Ovulated Female Lampreys. Injection volume is about 5 µl per animal. Dorsally injected animals n=12 , medially injected animals (n=8) and laterally injected animals (n=2).

Location of Injection	Code	Date	Size of Lesion ($\mu m^3 x 10^{6)}$	Angle of Section	Length of Specimen (cm)	Weight of Specimen (g)
Dorsal Anterior	HHH	Jul-14	2.2	H	49	248
	KKK	Jul-15	3.45	H	46.8	241
	PPP	Jul-19	4.25	H	55	260
	YYY	Jul-22	9.00	H	58	269
	ZZZ	Jul-22	37.50	X	61	274
	HHHH	Jul-27	0.74	X	54	258
	AAAAA	Aug-30	0.56	X	57	272
	EEEEE	Sep-07	0.93	X	58	273
	FFFFF	Sep-07	0.30	X	56	270
	LLLLL	Oct-07	0.55	X	54	260
	MMMMM	Oct-07	0.30	X	55.9	263
	NNNNN	Nov-03	0.65	X	51	254
Medial	III	Jul-14	0.29	H	45	239
	LLL	Jul-15	0.48	H	45	238
	IIII	Jul-27	0.12	X	45	247
	NNNN	Jul-29	0.78	X	47	245
	OOOO	Jul-29	1.20	X	47.5	247
	PPPP	Jul-29	0.60	X	46.6	241
	QQQQ	Jul-29	1.30	X	44.8	235
	TTTT	Aug-03	1.20	X	49.7	255
Lateral	CCC	Jul-08	3.58	X	60.4	275
	EEE	Jul-08	4.50	X	56.6	257

2.2.6 Tissue Incubation and Biocytin Transport

After 20 minutes to allow for biocytin uptake, the tissue was then placed in a Sylgard ® lined chamber cooled to 7°C, equipped for flow through (78 mL/hour) of oxygenated lamprey Ringer's. Tissue was incubated for 2 hours (N=8), 4 hours (N=26) or overnight (N=6), to determine the optimal incubation period for maximal distance of biocytin transport.

2.2.7 Criteria for Analysis of Secondary Olfactory Projections

Slides were analyzed on a Zeiss A-2 Axioscope equipped for fluorescence and a CCD camera. Images were captured using Northern Eclipse software. Dr. Réjean Dubuc and Mr. François Auclair at the University of Montreal had previously created cross-sectional templates of the adult lamprey brain (Auclair et al. 2004). From these templates, another series of templates was created for this thesis. For each experimental animal, injections and subsequent labeling patterns were marked on separate templates. The new templates were then organized to match sections across animals to see if there was a pattern to labeling. The original slides were also analyzed at the University of Montreal in collaboration with Dr. Dubuc and Mr. Auclair. Each experimental animal was carefully analyzed to ensure there were no extraneous lesions and subsequent loading in inappropriate locations (i.e. lesion and injection in the wrong region of the olfactory bulb, multiple lesion and injection sites in the olfactory bulb or lesion and injection to areas other than the olfactory bulb). The Dubuc lab has many years of experience working with lamprey brains and their expertise in identifying brain regions was instrumental in compiling this thesis and organizing the results. Brain regions were identified as labeled or not labeled based

on visual inspection of the intensity of fluorescence in each section. In cases where fluorescence was faint or ambiguous, the section was rejected for analysis. Images were analyzed using a Nikon Eclipse E600 microscope equipped for fluorescence and a Nikon Digital Camera (DXM 1200) and captured using Act-1 software.

3.0 Results

The results show three distinct patterns of labeling arising from three separate injections of biocytin to discrete glomerular territories.

From injections into the dorsal hemisphere of the olfactory bulb, the following areas/structures were labeled; olfactory bulb, striatum, preoptic area, dorsal, medial and lateral pallia, habenula, and the hypothalamus.

From injections into the medial hemisphere of the olfactory bulb, the following

areas/structures were labeled; olfactory bulb, striatum, medial pallium and thalamus.

From injections into the lateral hemisphere of the olfactory bulb, the following areas/structures were labeled; olfactory bulb, striatum, preoptic area and dorsal, lateral and medial pallia.

3.1 The Lamprey Brain: The Prosencephalon to the Diencephalon.

A Nissl stain on untreated tissue was used to familiarize us with cross sections of the adult lamprey brain (n=4). Images were compared to those seen in Schober (1964), who used adults and juvenile lampreys [*Lampetra fluviatilis* and *Lampetra planeri*], in an effort to visualize broad neuroanatomical structures and nuclei such as the preoptic area and the pallial regions.

Figure 4A shows the striatum and the preoptic area as located in the telencephalon. The striatum is identified as an area in the telencephalon that is medial to the lateral pallium. Identification is based on presence of somata and proximity to the lateral ventricle (**Figure 4B**). Continuous and medioventral to the striatum is the

preoptic area. The preoptic area lines the main or central ventricle in a dorso-ventral line (**Figure 4C**), is densely populated by somata and is comprised of two lamina.

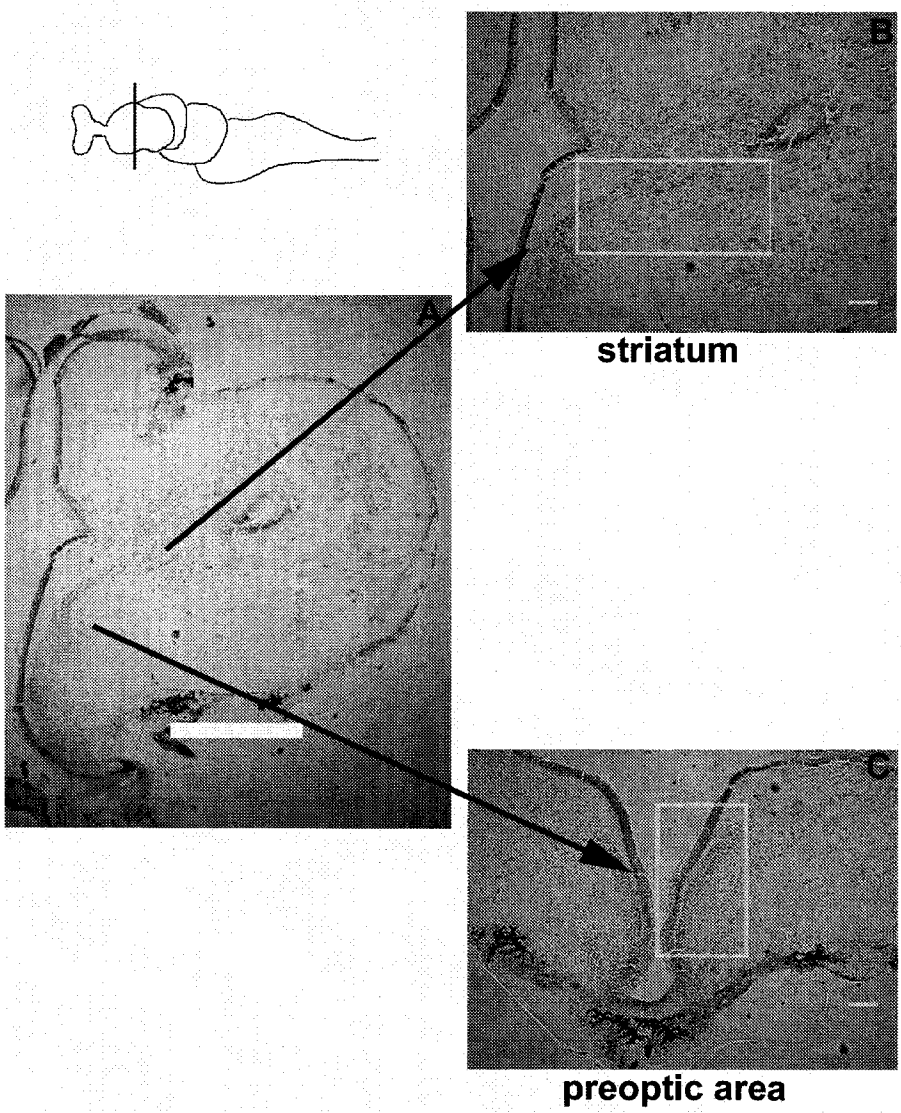

Figure 4: Cross sectional view of cresyl violet stained sections of adult, ovulated female lamprey telencephalon. The striatum and preoptic are nearly continuous structures located in the telencephalon. A – A low power micrograph of the lamprey telencephalon showing the striatum and telencephalon: B – the striatum is identified by a loose aggregation of cell bodies, located near the preoptic area; C – the preoptic area has two lamina that are periventricular, densely packed with somata. Scale bars = 500 μm. A-C are cresyl violet stained brain sections.

Figure 5 shows the broad regions of the telencephalon known as the pallial areas or pallial regions. The medial pallium possesses very few somata and is medially and dorsally located in the telencephalon (**Figure 5B**). The lateral pallium is broadly populated by somata and occupies the large evaginated portion of the telencephalon (**Figure 5C**). The dorsal pallium is located directly below the medial pallium and medial to the lateral pallium, and is identified by a loose aggregation of somata (**Figure 5D**).

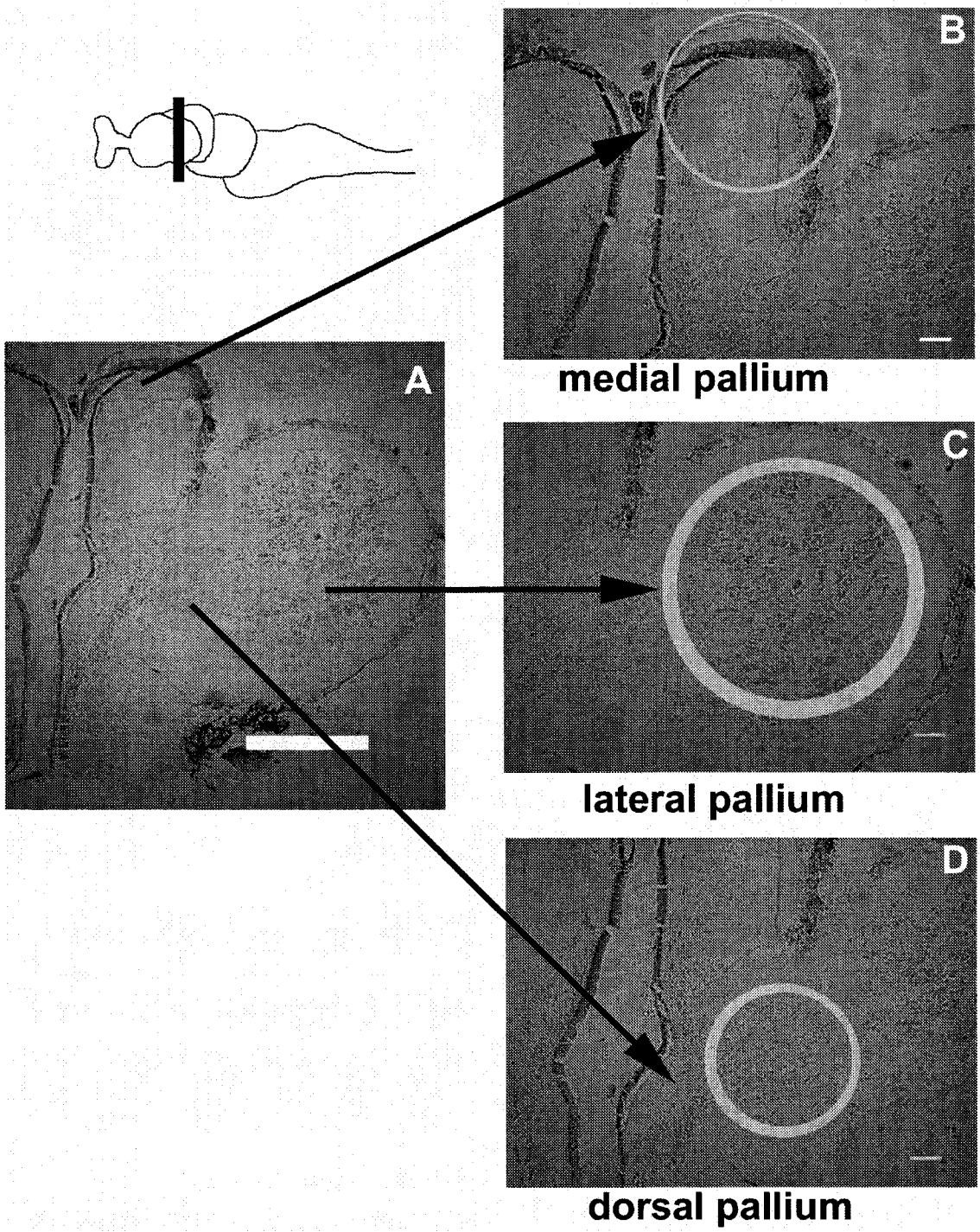

Figure 5: Cresyl violet stained sections showing the pallial areas of the lamprey telencephalon. A – all three pallial formations (dorsal, medial, lateral) are visible at low magnification and are identified by shape of brain slice and/or presence of cell bodies: B – medial pallium; C – lateral pallium; D – dorsal pallium. Scale bar in A = 500 μm. Scale bar in B – D = 100 μm.

Figure 6 shows the habenula which has a characteristic morphology in the lamprey. The right side of the habenula is larger than the left side.

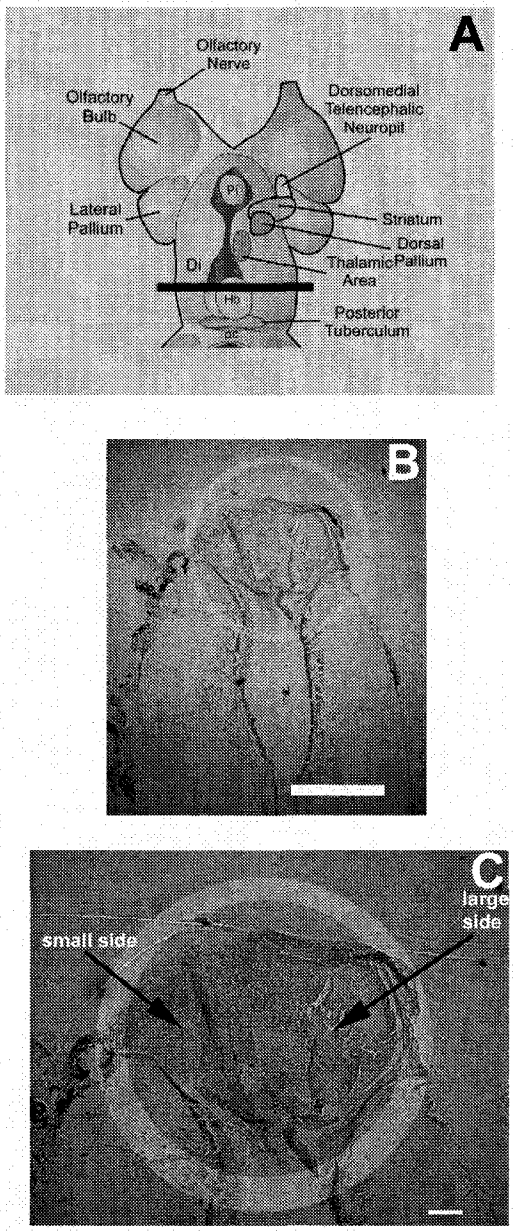

Figure 6: Cresyl violet stained brain sections showing the habenula. A - cartoon of lamprey brain indicating location; B – low power view of habenula; C – high power view of B. Scale bar in B = 500 μm. Scale bar in C = 100 μm.

Figure 7 shows the thalamic area and is identified by architectural elements, such as the absence of the telencephalon, the shape of the ventricle and the shape of the brain section. The thalamic area is located in the middle to bottom two thirds of the brain.

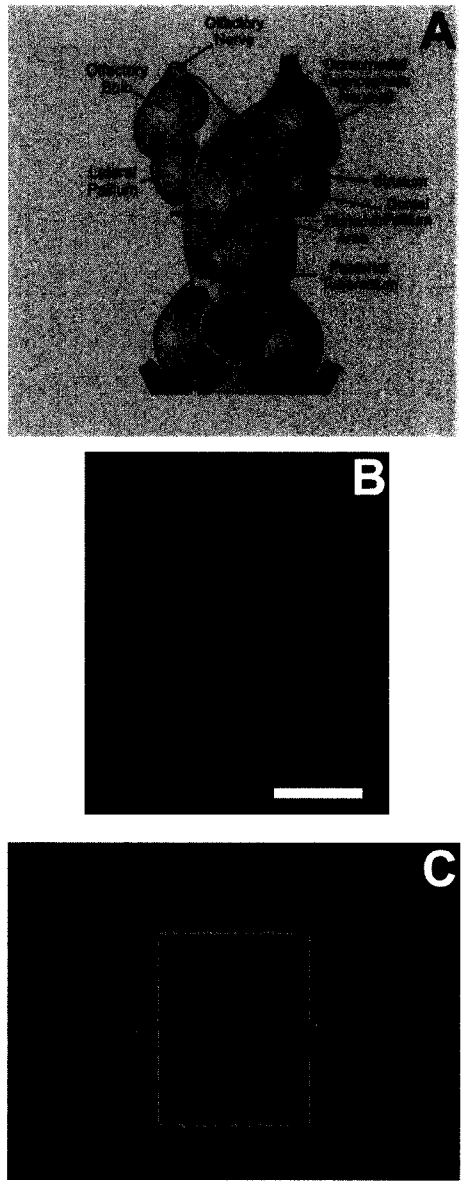

Figure 7: Cresyl violet stained brain sections showing the thalamus, A - cartoon of lamprey brain indicating location; B – low power view of thalamic area; C – high power view of B. Scale bar in B = 500 μm. Scale bar in C = 100 μm.

Figure 8 shows the hypothalamus at the level of the mammillary recess. The hypothalamus is a ventral structure and is also identified by architectural elements such as absence of the telencephalon, the shape of the ventricle, the shape of the brain section and a small invagination at the ventral end of the brain, which is the mammillary recess.

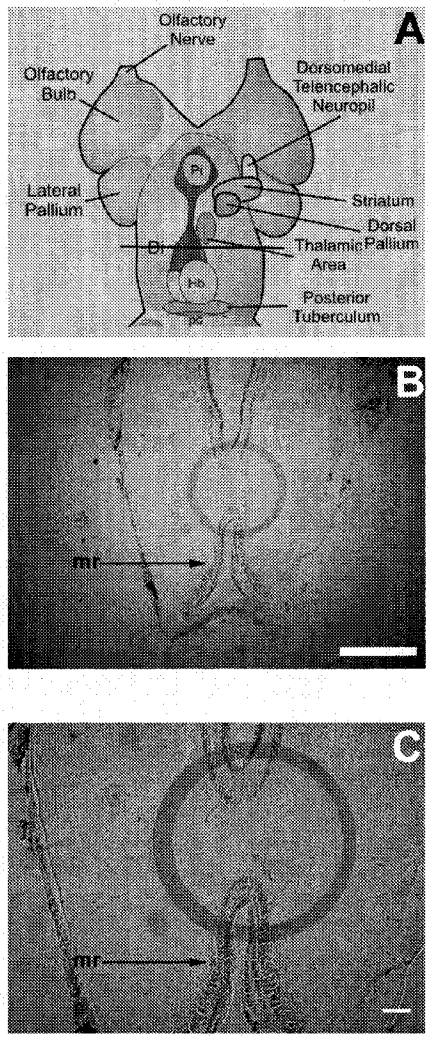

Figure 8: Cresyl violet stained brain sections showing the hypothalamus and the mammilary recess (mr). A – cartoon of lamprey brain indicating location; B – low power view of hypothalamus and mamillary recess; C – high power view of B. Scale bar in B = 500 μm. Scale bar in C = 100 μm.

3.2 $GSIB_4$ Lectin Staining of Glomerular Territories

Use of the biotinylated form of $GS1B_4$ lectin resulted in a consistent pattern of labeling of ovoid structures around the periphery of the olfactory bulb and telencephalon. Glomerular territories were easily distinguishable from the surrounding tissue as ovoid, fluorescent areas that were prominently visible with respect to the interiors of the olfactory bulb and telencephalon (**Figure 9**).

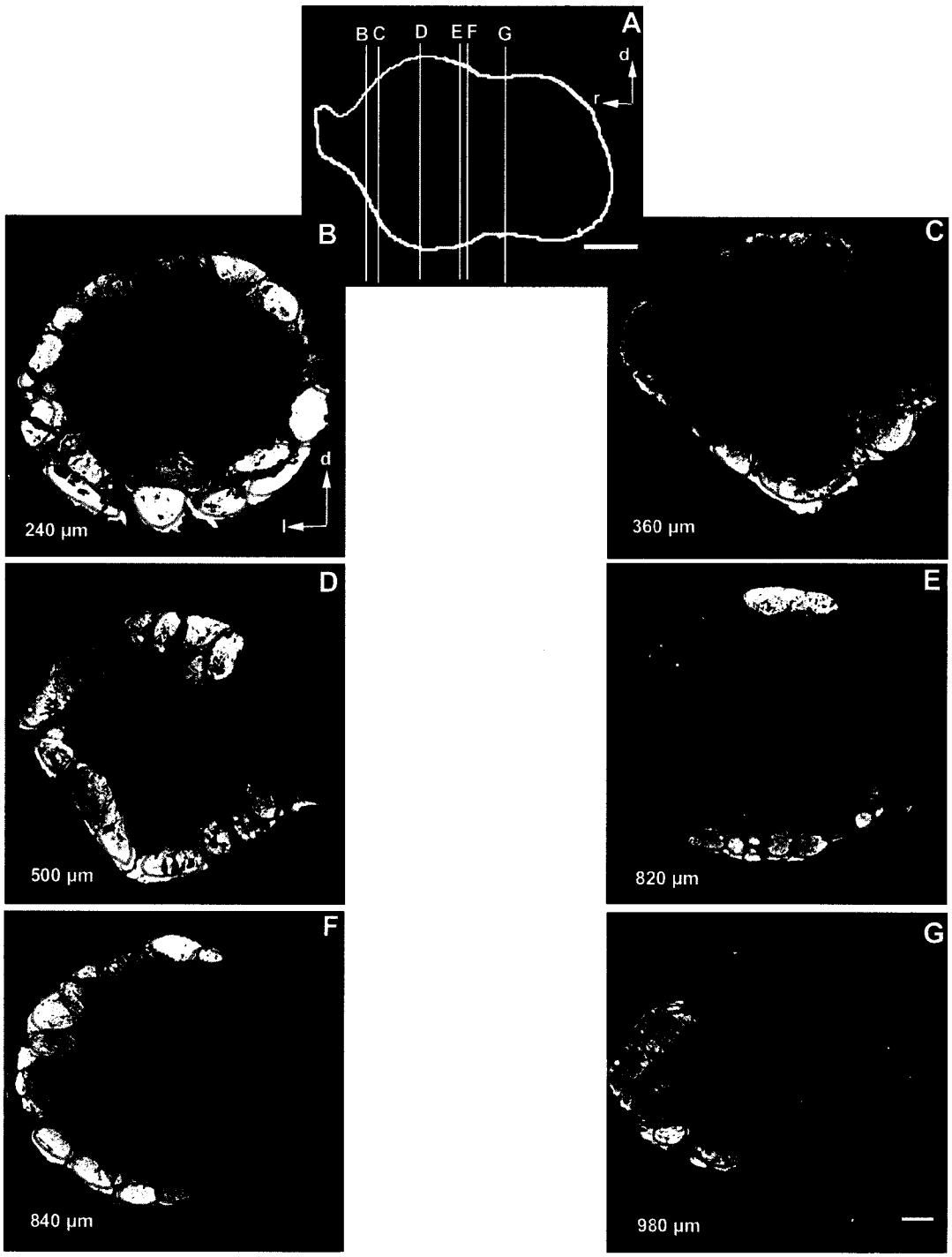

Figure 9: GS1B$_4$ lectin stained glomerular territories in the adult ovulated female lamprey olfactory bulb. A - cartoon representation of locations of olfactory bulb/telencephalon slices (scale bar = 500 µm); B – dorsal, lateral and medial glomerular territories are present rostrally; C – E the medial glomerular territory is restricted to the ventral hemisphere of the bulb; F – G – the medial glomerular territory is no longer visible and dorsal glomerular territories disappear with only the lateral glomerular territories persisting deep into the telencephalon. B-G = 20 µm sections. Scale bar for B-G in G = 500 µm

3.3 Anterograde Transport of Biocytin

The maximal distance of biocytin transport was determined by inspecting for fluorescence in serially sectioned tissue, following biocytin application and tissue incubation. The final section showing fluorescence was used to determine maximal distance of biocytin transport. Distances were averaged from all injected specimens. Comparison of different times for transport of biocytin revealed that four hours was the optimal time to allow for maximal distance of biocytin travel, with an average distance of about 2620 μm.

Table 2: Summary of Ovulated Female Lamprey and the Maximal Distance of Biocytin Travel Following Injection Of Bioctyinto the Olfactory Bulb.

Specimen Code	Date	Maximal Distance of Biocytin Travel (μm)
HHH	Jul-14	2760
KKK	Jul-15	2720
PPP	Jul-19	2710
YYY	Jul-22	2740
ZZZ	Jul-22	2690
HHHH	Jul-27	2840
AAAAA	Aug-30	2760
EEEEE	Sep-07	2700
FFFFF	Sep-07	2680
LLLLL	Oct-07	2760
MMMMM	Oct-07	2820
NNNNN	Nov-03	2740
III	Jul-14	2800
LLL	Jul-15	2810
IIII	Jul-27	2760
NNNN	Jul-29	2780
OOOO	Jul-29	2780
PPPP	Jul-29	3000
QQQQ	Jul-29	2830
CCC	Jul-08	2620
EEE	Jul-08	2520

3.4 Description of Biocytin Labeling

The three different injection sites and their respective labeling patterns follow in their own respective sections.

3.4.1 Unilateral Injections to the Dorsal Anterior Region of the Olfactory Bulb in Ovulated Female Lampreys

In total, twelve specimens had appropriately located injections to the dorsal anterior region of the olfactory bulb; four were sectioned horizontally and eight were cross sectioned. Every specimen examined displayed the diffuse organization of fibers in the olfactory bulb. Six of the eight cross sectioned specimens had labeling in the striatum. All of the cross sectioned specimens had labeling in the preoptic area. Six of the eight cross sectioned specimens had labeling in the dorsal pallium, and all eight had labeling in the lateral and medial pallia. Four specimens had labeling in the habenula and five had labeling in the hypothalamus. The thalamus was not labeled in any of the cross sectioned tissue.

Table 3: Summary of Labeling Pattern Resulting from Unilateral Injections to the Dorsal Anterior Region of the Olfactory Bulb in Ovulated Female Lamprey (N=12). OB = olfactory bulb, str = striatum, PA = preoptic area, D Pal = dorsal pallium, L Pal = lateral pallium, M Pal = medial pallium, Hab = habenula, Hyp = hypothalamus, Thal = thalamus. Note: analysis from horizontal sections is not complete as structures are not readily identifiable in horizontal sections and are shown as blank cells. Only a general pattern of fiber location and direction is possible to be identified. N indicates that labeling was not seen.

Code	Date	Size of Lesion ($\mu m^3 x 10^6$)	Angle of Sectioning	OB	Str	PA	D Pal	L Pal	M Pal	Hab	Hyp	Thal	Length of Specimen (cm)	Weight of Specimen (g)
ZZZ	Jul-22	37.5	X	✓	✓	✓	✓	✓	✓	✓	✓	N	61	274
HHHH	Jul-27	0.74	X	✓	✓	✓	✓	✓	✓	✓	✓	N	54	258
AAAAA	Aug-30	0.56	X	✓	✓	✓	✓	✓	✓	N	✓	N	57	272
EEEEE	Sep-07	0.93	X	✓	✓	✓	✓	✓	✓	N	✓	N	58	273
FFFFF	Sep-07	0.30	X	✓	N	✓	N	✓	✓	N	N	N	56	270
LLLLL	Oct-07	0.55	X	✓	N	✓	N	✓	✓	N	N	N	54	260
MMMMM	Oct-07	0.30	X	✓	✓	✓	✓	✓	✓	✓	✓	N	55.9	263
NNNNN	Nov-03	0.65	X	✓	✓	✓	✓	✓	✓	✓	N	N	51	254
HHH	Jul-14	2.2	H	✓						✓			49	248
KKK	Jul-15	3.45	H	✓						✓			46.8	241
PPP	Jul-19	4.25	H	✓						✓			55	260
YYY	Jul-22	9.0	H	✓						✓			58	269

Lesions and injections of biocytin were made to the dorsal anterior region of the olfactory bulb, and the biocytin solution was applied to this lesion (**Figures 10, 11, 12**).

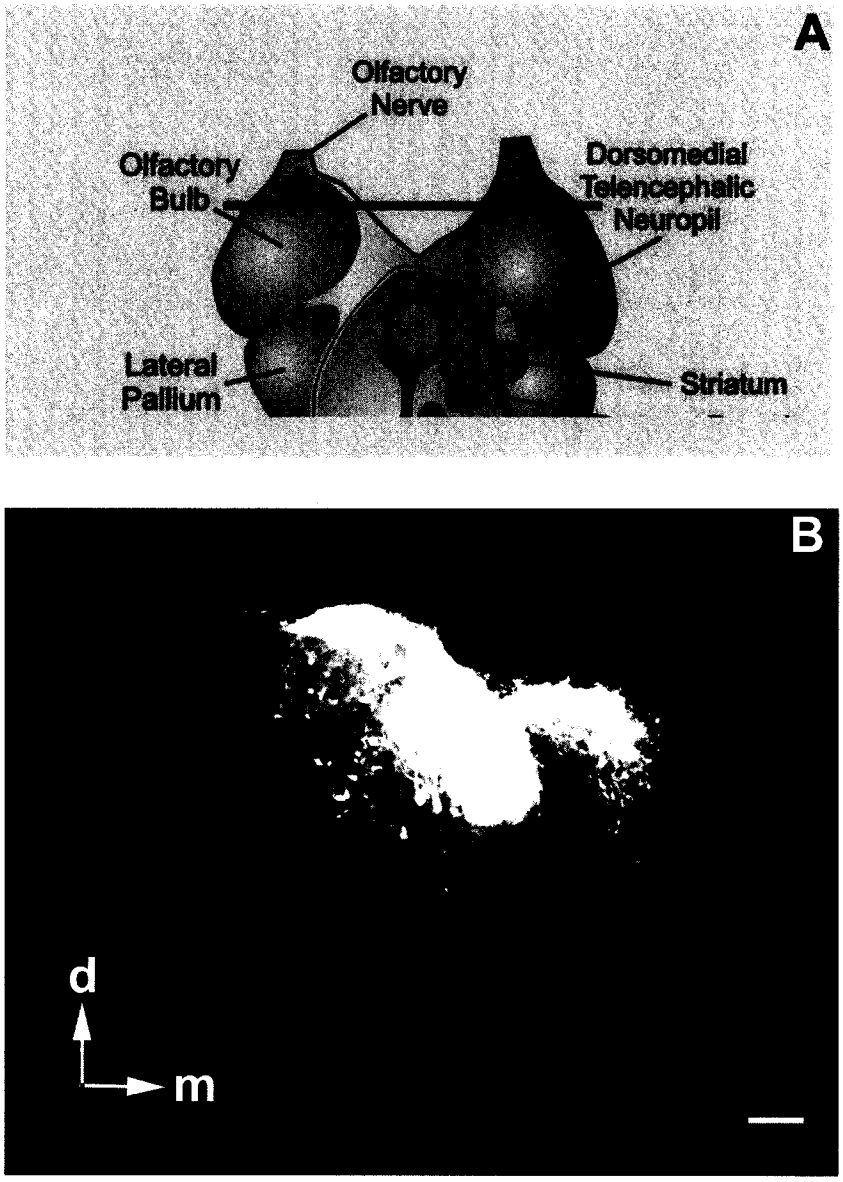

Figure 10: Injection biocytinto the dorsal anterior region of the olfactory bulb of an adult, ovulated female lamprey. A - Cartoon diagram of the lamprey brain showing the level of sectioning and injection site – asterisk shows injection; B – micrograph of the left olfactory bulb with a lesion and injection to the dorsal anterior region. Bar = 100 μm. [Animal AAAAA]

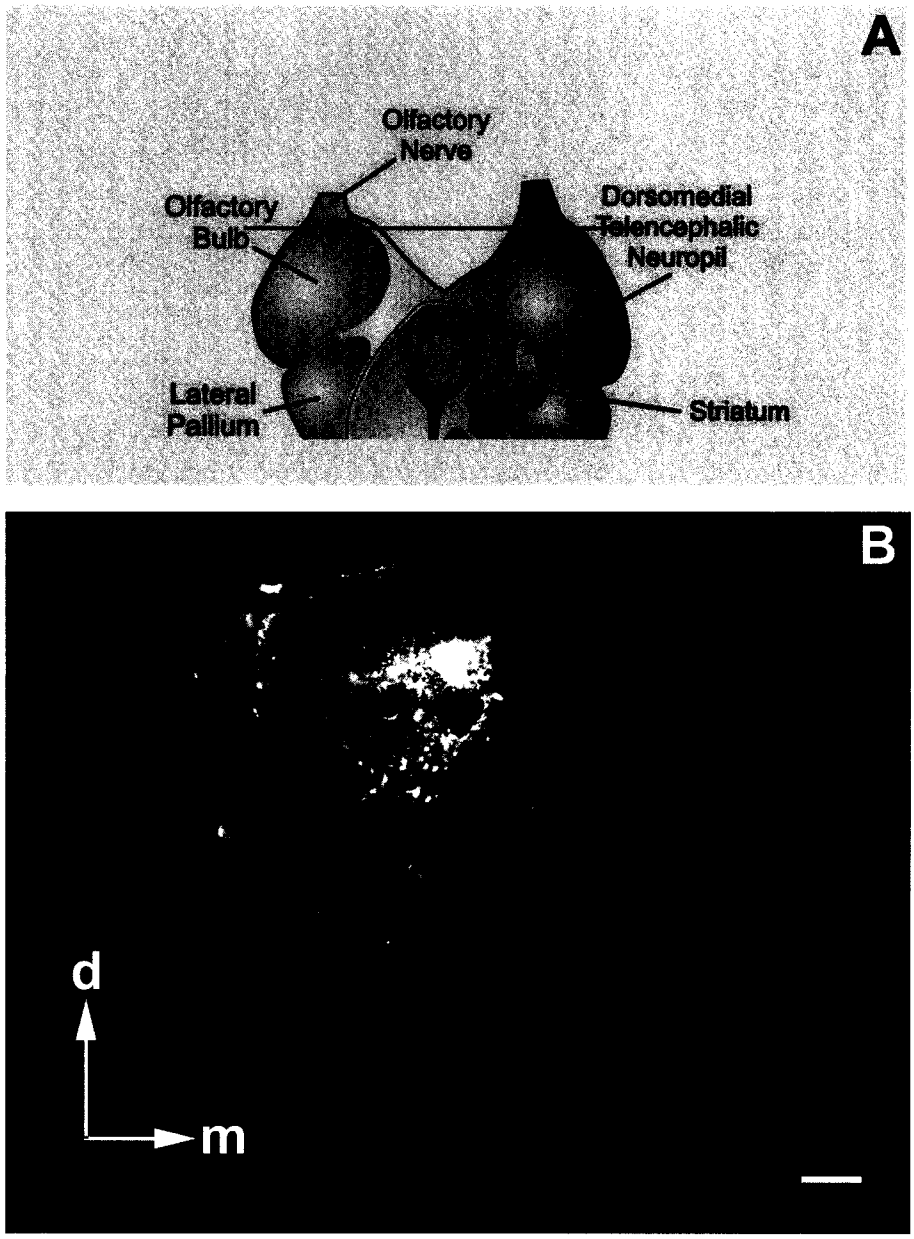

Figure 11: Injection of biocytinto the dorsal anterior region of olfactory bulb of an adult, ovulated female lamprey. A - Cartoon diagram of the lamprey brain showing the level of sectioning and injection site – asterisk shows injection; B – micrograph of the left olfactory bulb with a lesion and injection to the dorsal anterior region. Bar = 100 μm. [Animal MMMMM]

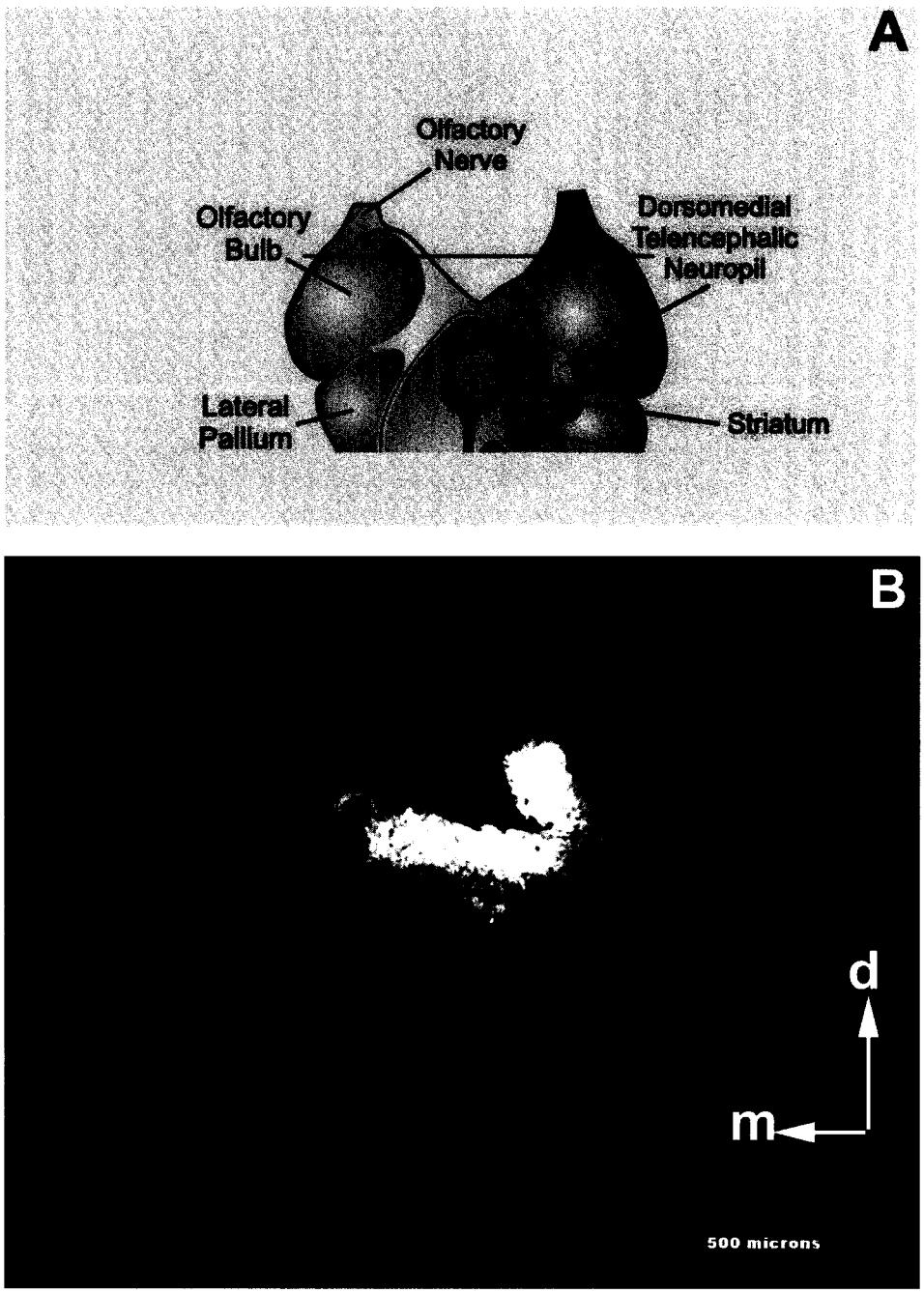

Figure 12: Injection to biocytinto the dorsal anterior region of olfactory bulb of an adult, ovulated female lamprey. A - Cartoon diagram of the lamprey brain showing the level of sectioning and injection site – asterisk shows injection; B – micrograph of the left olfactory bulb with a lesion and injection to the dorsal anterior region. Bar = 500 μm. [Animal ZZZ]

From the dorsal anterior injections, the following pattern of labeling arises. Visual inspection revealed preferential filling of fibers at the margins or periphery of the olfactory bulb (**Figure 13**). This preferential or marginal labeling was most prominent at the border of the olfactory bulb and telencephalon.

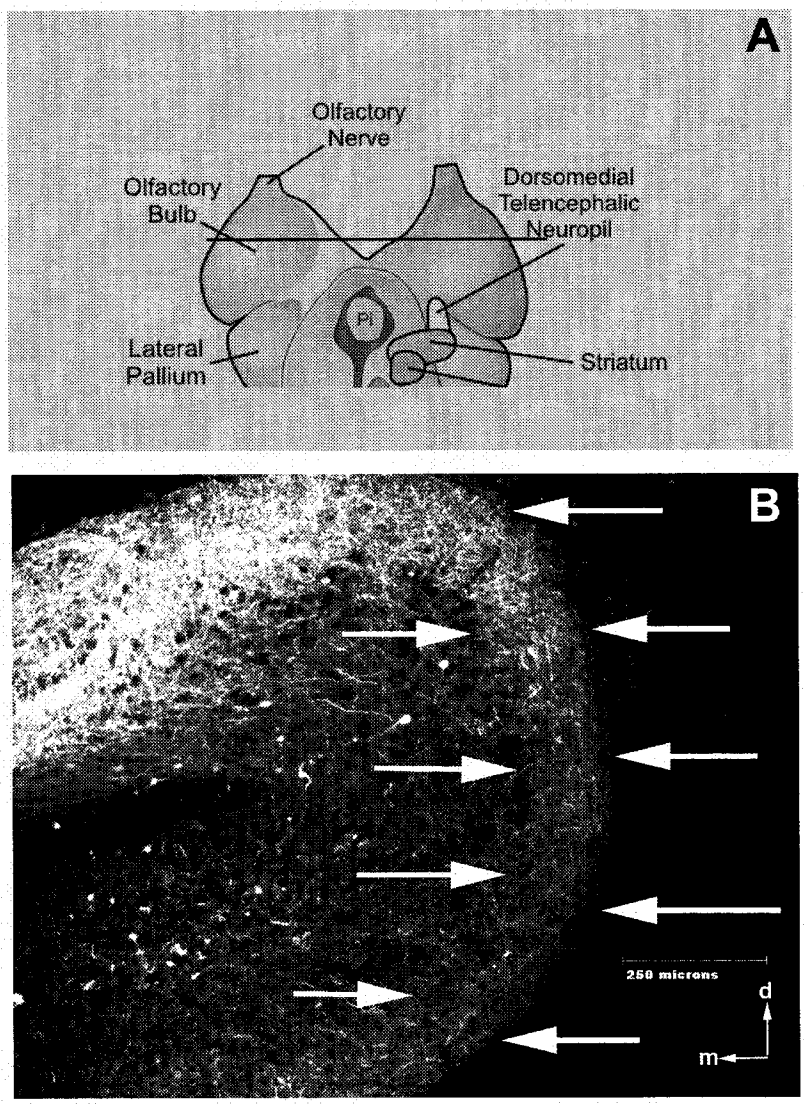

Figure 13: Peripheral labeling at the olfactory bulb/telencephalon border following injection of biocytinto the dorsal anterior region of the olfactory bulb in an adult, ovulated female lamprey. A – cartoon of lamprey brain showing the level of sectioning; B – intense labeling is seen in the dorsal hemisphere and the periphery of the olfactory bulb/telencephalon. [Animal HHHH]

Labeled fibers crossed to the contralateral olfactory bulb via the dorsal commissure (**Figure 14**). The fibers spread to the contralateral bulb where the labeling was not as intense as on the ipsilateral side.

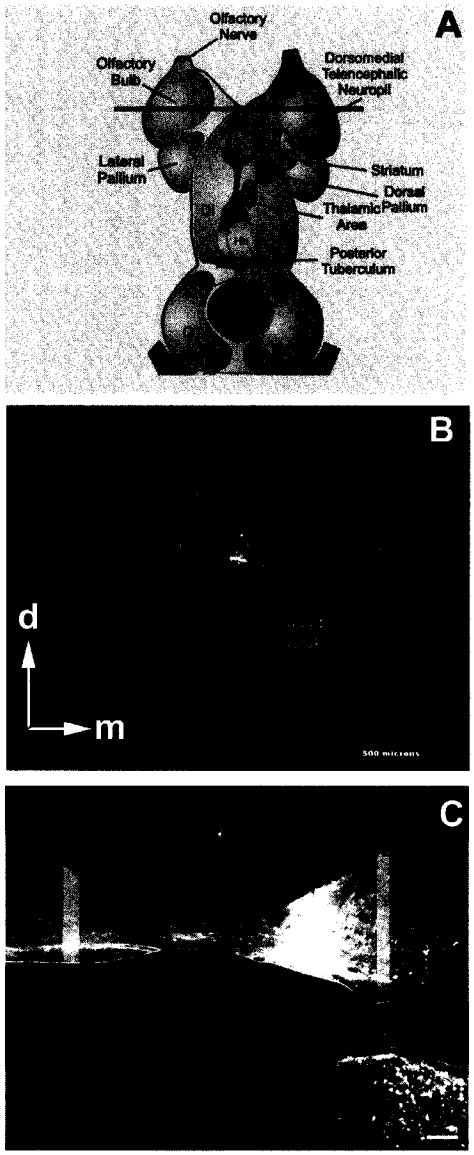

Figure 14: Fibers in the dorsal commissure are visible following injection of biocytinto the dorsal anterior region of the olfactory bulb of an adult ovulated female lamprey. The dorsal commissure links the olfactory bulbs. A – cartoon of the lamprey brain, showing the depth of section; B – low power view of fibers in the dorsal commissure labeled with biocytin; C – high powered view of fibers labeled in the dorsal commissure. Bar in C = 100 μm. [Animal AAAAA]

In the striatum, we saw labeled cell bodies and fibers (**Figure 15**).

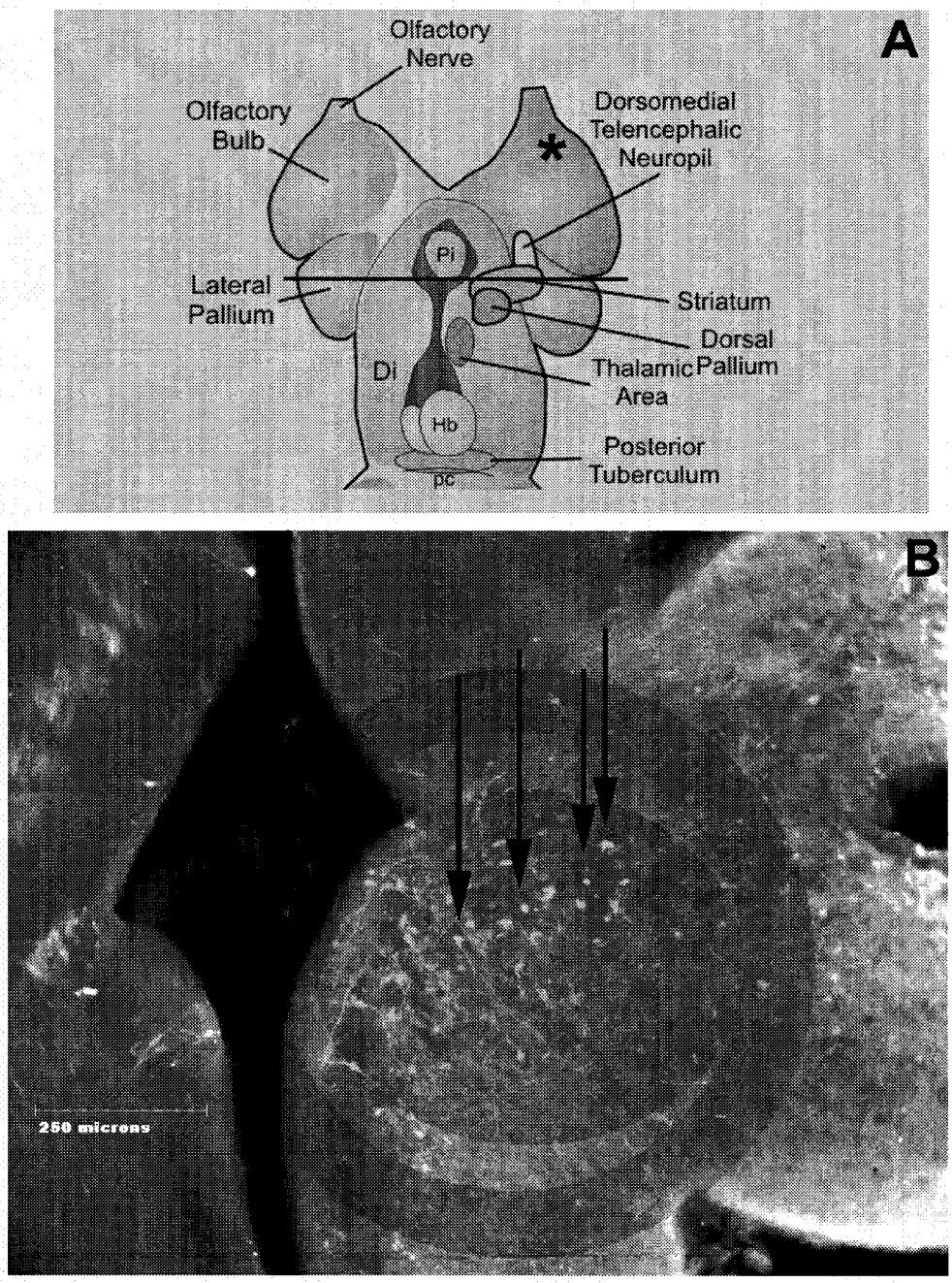

Figure 15: Labeling of cells and fibers in the rostral striatum following injection of biocytinto the dorsal anterior region of the olfactory bulb of an adult, ovulated female lamprey. A – cartoon of lamprey brain showing the level of sectioning; B – somata and fibers in the rostral striatum - labeling extends throughout the entire rostro-caudal extent of the striatum. [Animal HHHH]

Looking posterior, from this point, we saw a prominently labeled bundle of thick and thin fibers that connects the medial and lateral pallia that projected ventrally from the dorsal end of the medial pallium and turned laterally to the lateral pallium (**Figure 16**).

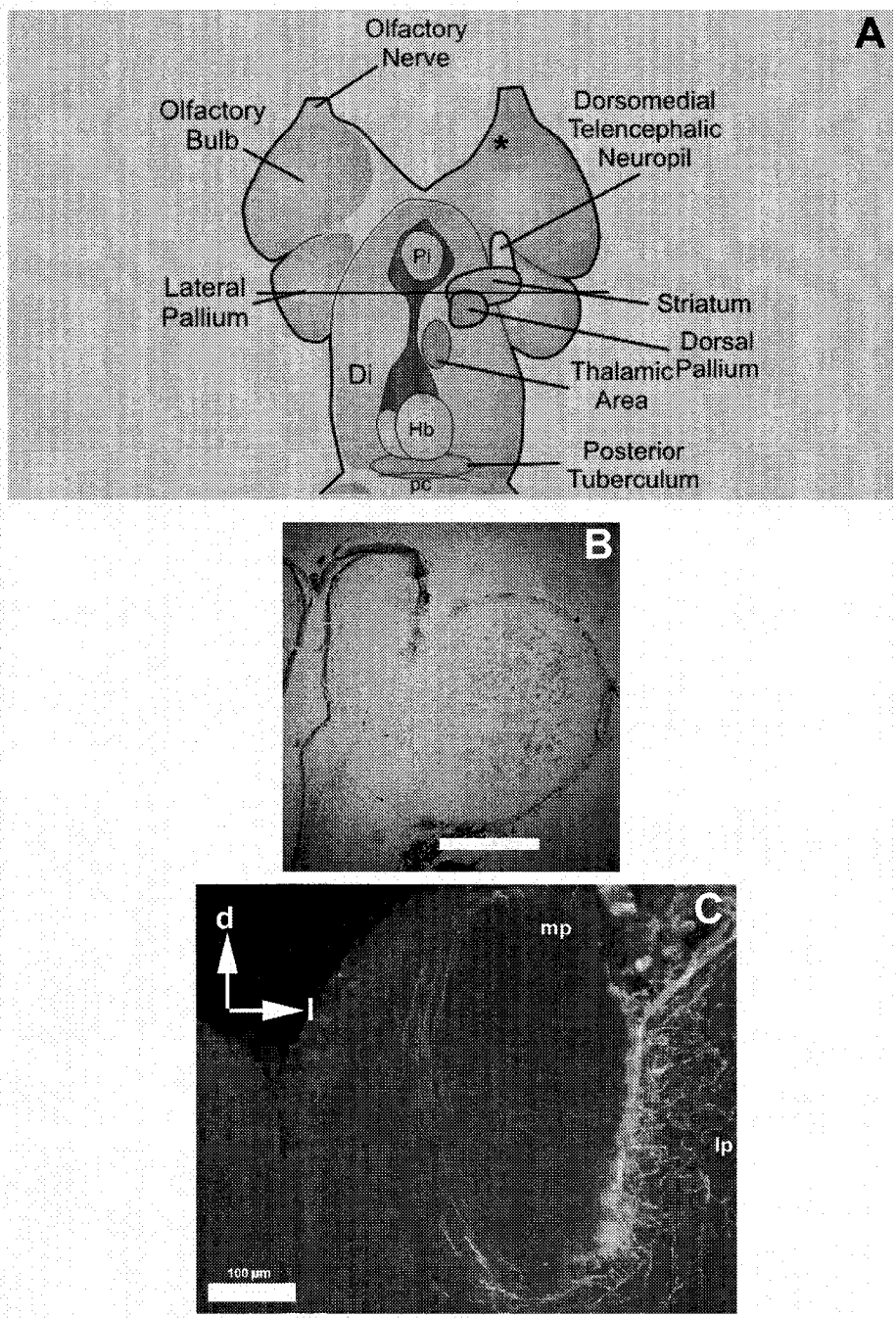

Figure 16: Fibers in the lateral and medial pallia are revealed after injection of bioctyinto the dorsal anterior region of the olfactory bulb of an adult ovulated female lamprey. The medial and lateral pallia are connected by a bundle of fibers. A – cartoon of lamprey brain showing the level of sectioning; B – low power view of pallial area in cresyl violet stained section; C - tract of fibers connecting the medial and lateral pallia in the right side of the brain following injection to the right olfactory bulb. mp = medial pallium, lp = lateral pallium. [Animal HHHH]

The large side of the habenula also displayed labeled fibers (**Figure 17**) that extended to the small habenula and crossed to the contralateral side.

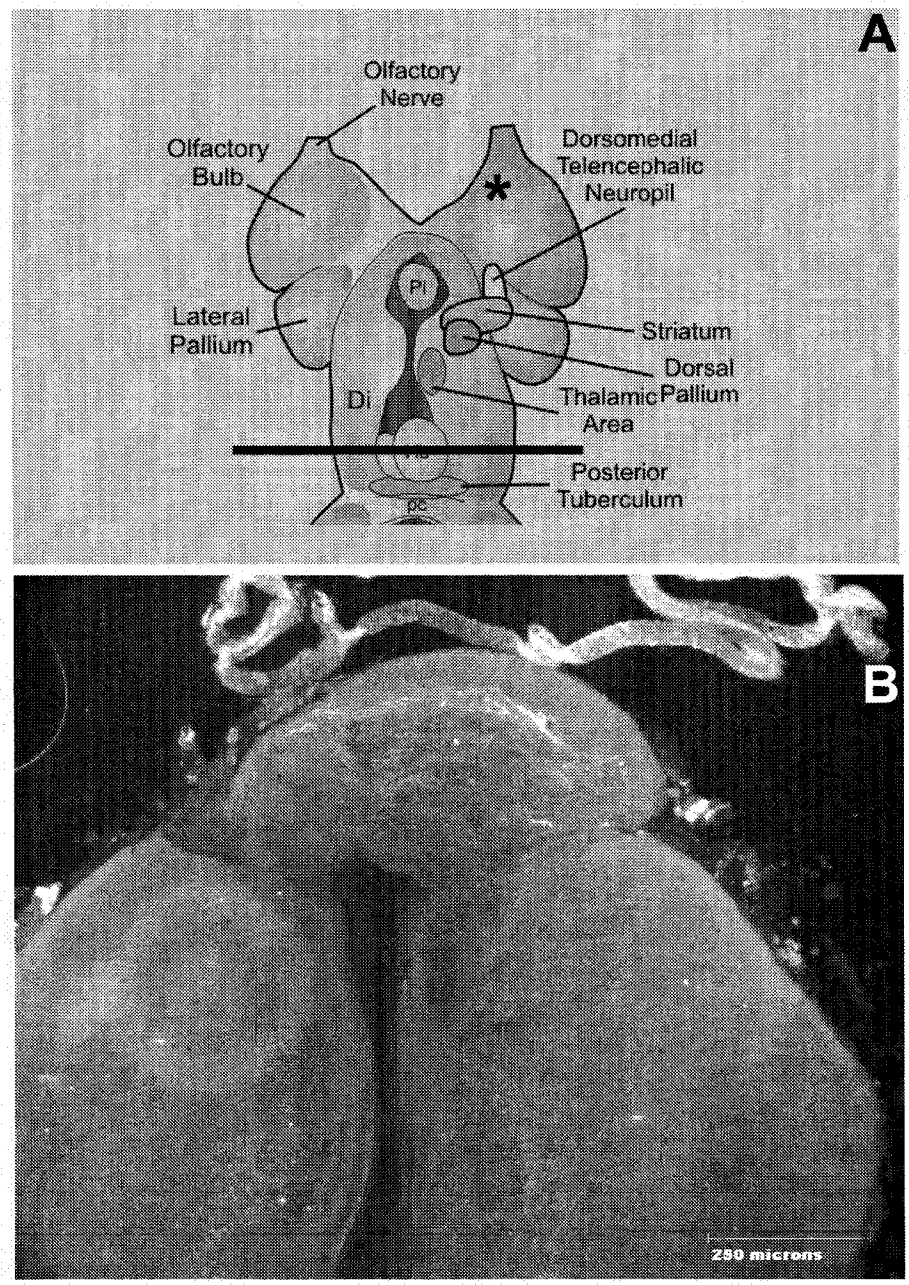

Figure 17: Fibers in the habenula revealed after injection of biocytinto the dorsal anterior region of the olfactory bulb of an adult, ovulated female lamprey. The habenula is a conduit for fibers to cross to the contralateral side. A – cartoon of lamprey brain showing level of sectioning; B - fibers in the right habenula, following injection to the right olfactory bulb. [Animal HHHH]

From the thick bundle connecting the medial and lateral pallia in Figure 17, a second contingent of fibers emerged, bifurcated from the main bundle, passed through the dorsal pallium and coursed ventrally to the hypothalamus, where the fibers then crossed to the contralateral side (**Figure 18**).

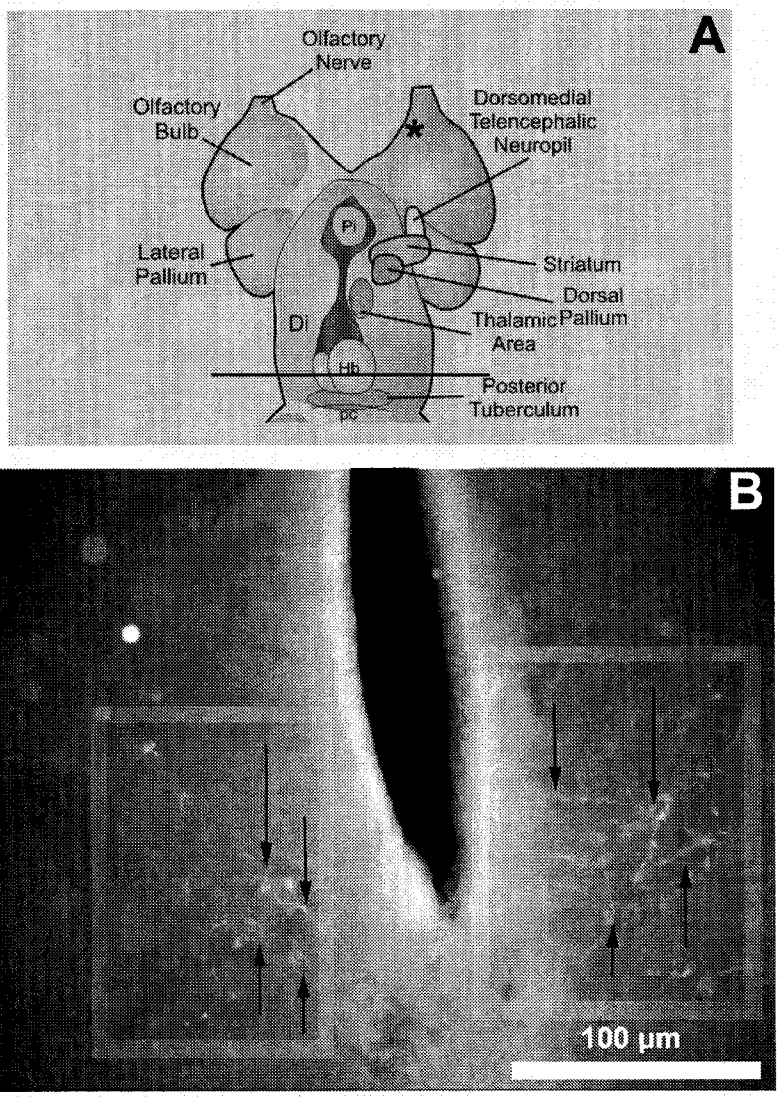

Figure 18: Coarse fibres crossing to the contralateral side at the level of the mammillary recess revealed after injection of bioctyinto the dorsal anterior region of the olfactory bulb of an adult ovulated female lamprey. A – cartoon of lamprey brain showing level of sectioning; B - coarse fibers, shown by arrows, in the hypothalamus seen in the ipsilateral and contralateral side, at the level of the mammilary recess. Bar = 100 µm.

The projection pattern following biocytin injections to the dorsal anterior region of the olfactory bulb is summarized in **Figure 19**. From the dorsal anterior injection site, fibers projected to the contralateral olfactory bulb via the dorsal commissure as well as through the telencephalon to reach the striatum, the dorsal, lateral and medial pallia, the habenula and the hypothalamus.

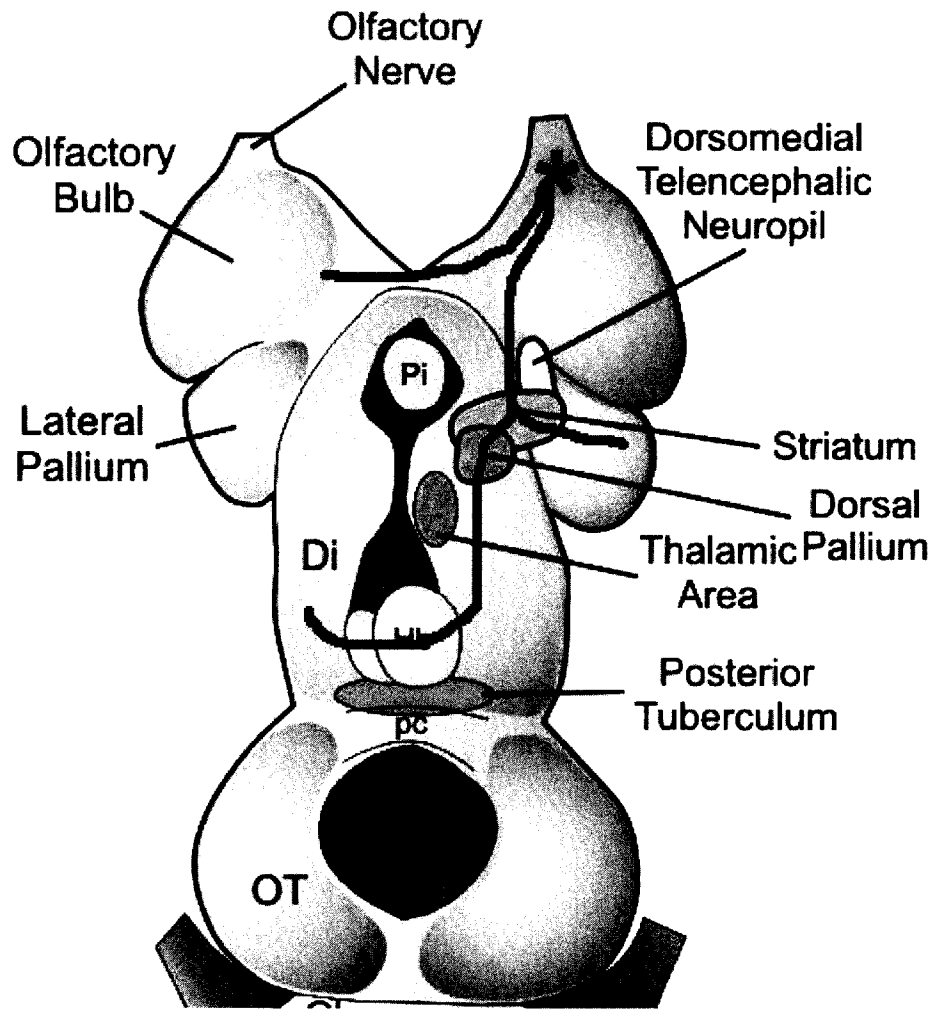

Figure 19: Summary of telencephalic and diencephalic observations following injections to the dorsal anterior region of the olfactory bulb. Labeled areas include the olfactory bulb, the striatum, the dorsal, lateral and medial pallia, the habenula and the hypothalamus.

3.4.2 Unilateral Injections to the Medial Region of the Olfactory Bulb in Ovulated Female Lampreys

In total, eight specimens had appropriately located injections to the medial region of the olfactory bulb; two were sectioned horizontally and six were cross sectioned. Every specimen examined displayed the diffuse organization of fibers in the olfactory bulb. Four of the six cross sectioned specimens had labeling in the striatum. None of the specimens had labeling in the preoptic area. All of the cross sectioned specimens had labeling in the preoptic area. All of the cross sectioned specimens had labeling in the dorsal and medial pallia. None of the specimens had labeling in the lateral pallium, the habenula or the hypothalamus. Three specimens had labeling in the thalamus.

Table 4: Summary of Labeling Pattern Resulting from Unilateral Injections to the Medial Region of the Olfactory Bulb in Ovulated Female Lamprey (N=8). OB = olfactory bulb, str = striatum, PA = preoptic area, D Pal = dorsal pallium, L Pal = lateral pallium, M Pal = medial pallium, Hab = habenula, Hyp = hypothalamus, Thal = thalamus. Note: analysis from horizontal sections is not complete as structures are not readily identifiable in horizontal sections and are shown as blank cells. Only a general pattern of fiber location and direction is possible to be identified. N indicates that labeling was not seen.

Code	Date	Size of Lesion ($\mu m^3 x 10^6$)	Angle of Sectioning	OB	Str	PA	D Pal	L Pal	M Pal	Hab	Hyp	Thal	Length of Specimen (cm)	Weight of Specimen (g)
IIII	Jul-27	0.12	X	✓	✓	N	✓	N	✓	N	N	✓	45	247
NNNN	Jul-29	0.78	X	✓	✓	N	✓	N	✓	N	N	✓	47	245
OOOO	Jul-29	1.20	X	✓	N	N	✓	N	✓	N	N	N	47.5	247
PPPP	Jul-29	0.60	X	✓	✓	N	✓	N	✓	N	N	✓	46.6	241
QQQQ	Jul-29	1.30	X	✓	N	N	✓	N	✓	N	N	N	44.8	235
TTTT	Aug-03	1.20	X	✓	✓	N	✓	N	✓	N	N	N	49.7	255
III	Jul-14	0.29	H	✓									45	239
LLL	Jul-15	0.48	H	✓									45	238

A vertical lesion was made to the medial region of the olfactory bulbs, and the biocytin solution was applied to this lesion (**Figure 20 & 21**).

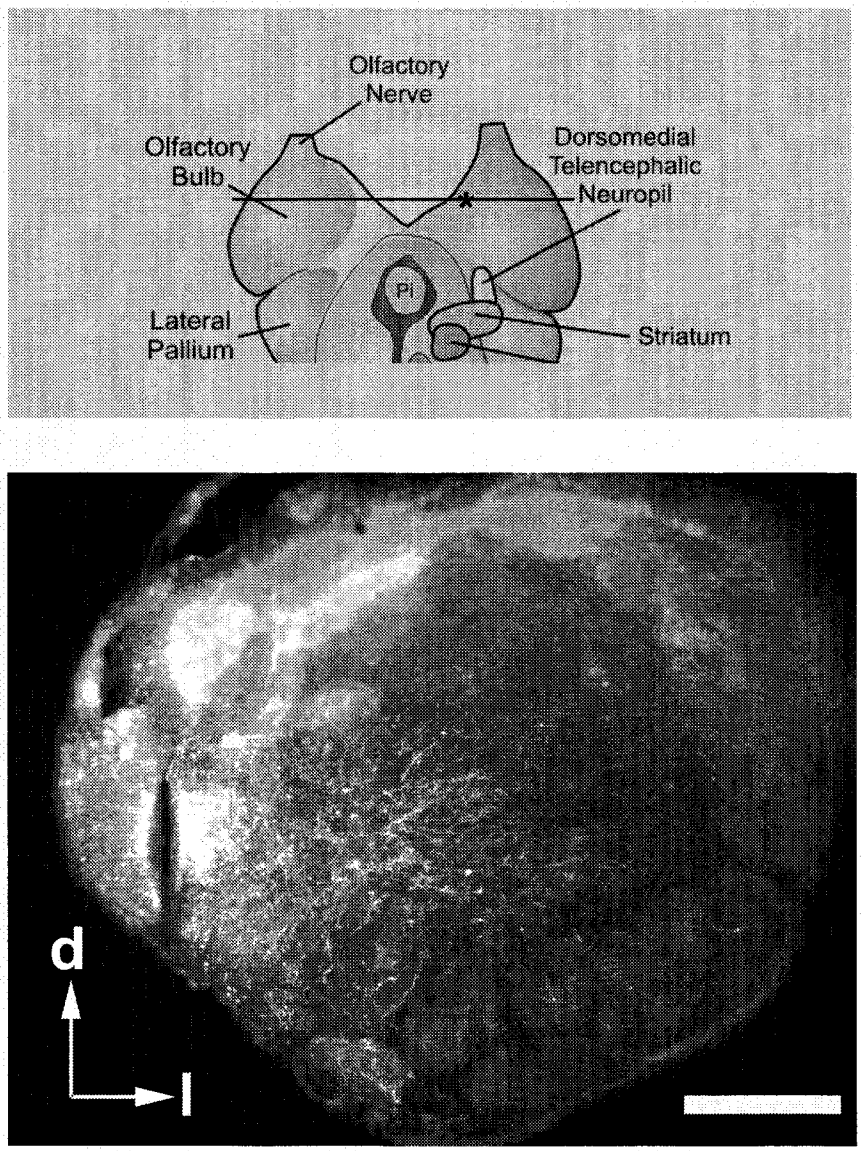

Figure 20: Injection of biocytinto the medial region of the olfactory bulb of an adult, ovulated female lamprey. A - cartoon diagram of lamprey brain showing the level of sectioning and injection site; B – micrograph of the right olfactory bulb with a lesion and injection to the medial region. Bar = 500 μm. [Animal PPPP]

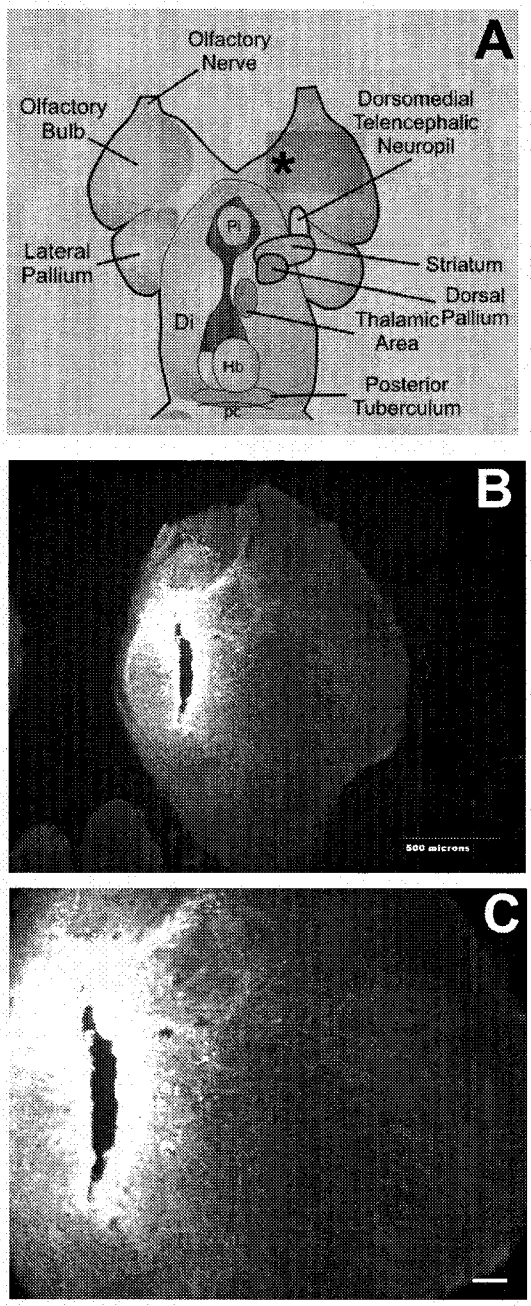

Figure 21: Injection of biocytinto the medial region of the olfactory bulb of an adult ovulated female lamprey – horizontal view. A - cartoon diagram of lamprey brain showing the injection site – shaded box is region shown in B; B – low power micrograph of the right olfactory bulb with a lesion and injection to the medial region; C – high power micrograph of B with fibers that course throughout the bulb from the injection site. Bar in C = 100 μm. [Animal LLL]

In the olfactory bulbs, intense labeling was seen in the area of the lesion, with fibers extending from the injection site in an unspecific manner throughout the bulb. The spread of fibers in the olfactory bulb was confirmed in horizontally sectioned tissue (**Figure 21**). There was evidence of the same pathway seen in the dorsally injected animals, connecting the medial and lateral pallia, however the pathway was not as definitively labeled (**Figure 22**, dorsal to encircled area).

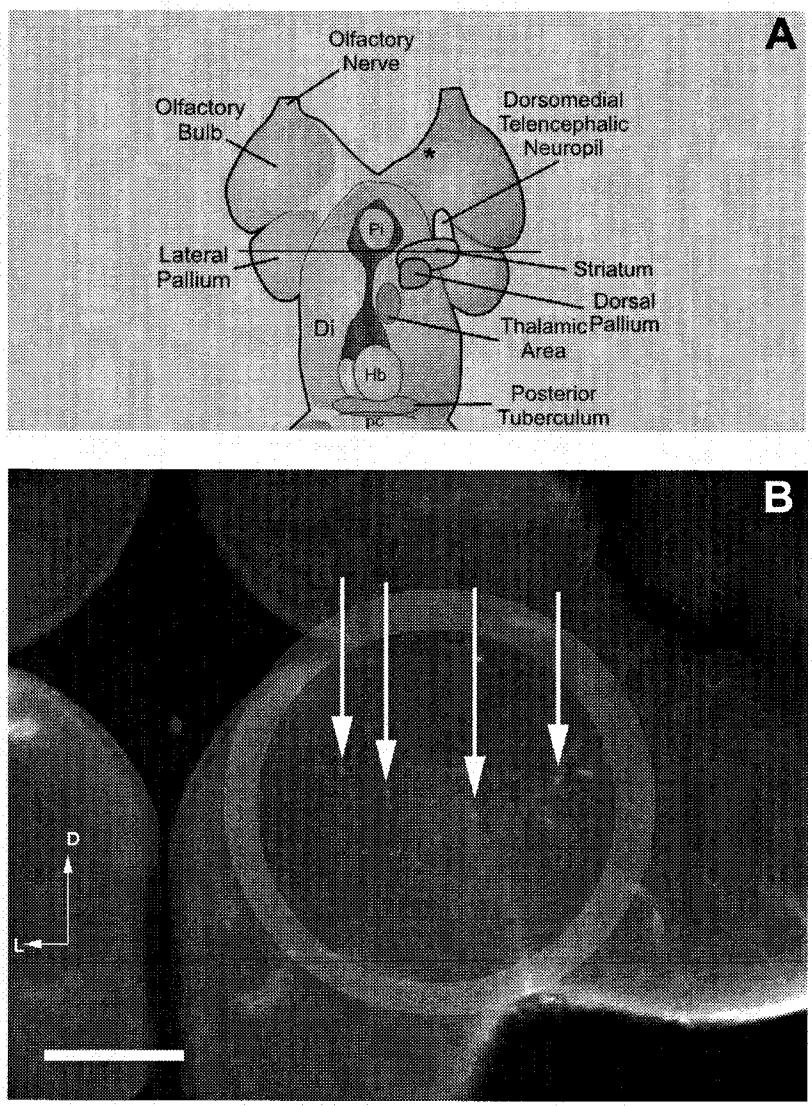

Figure 22: Labeling of cells and fibers in the rostral striatum. A – cartoon of lamprey brain showing the level of sectioning; B – somata and fibers in the rostral striatum. Bar = 500 μm. [Animal PPPP]

In the striatum, cell bodies and fibers were visible, similar to what was seen in the dorsally injected specimens, however, the preoptic area was not labeled (**Figure 22**). Lastly, there were long, thick fibers in the region of the diencephalon at the region where the medial and dorsal pallia transition to the thalamus (**Figure 23**).

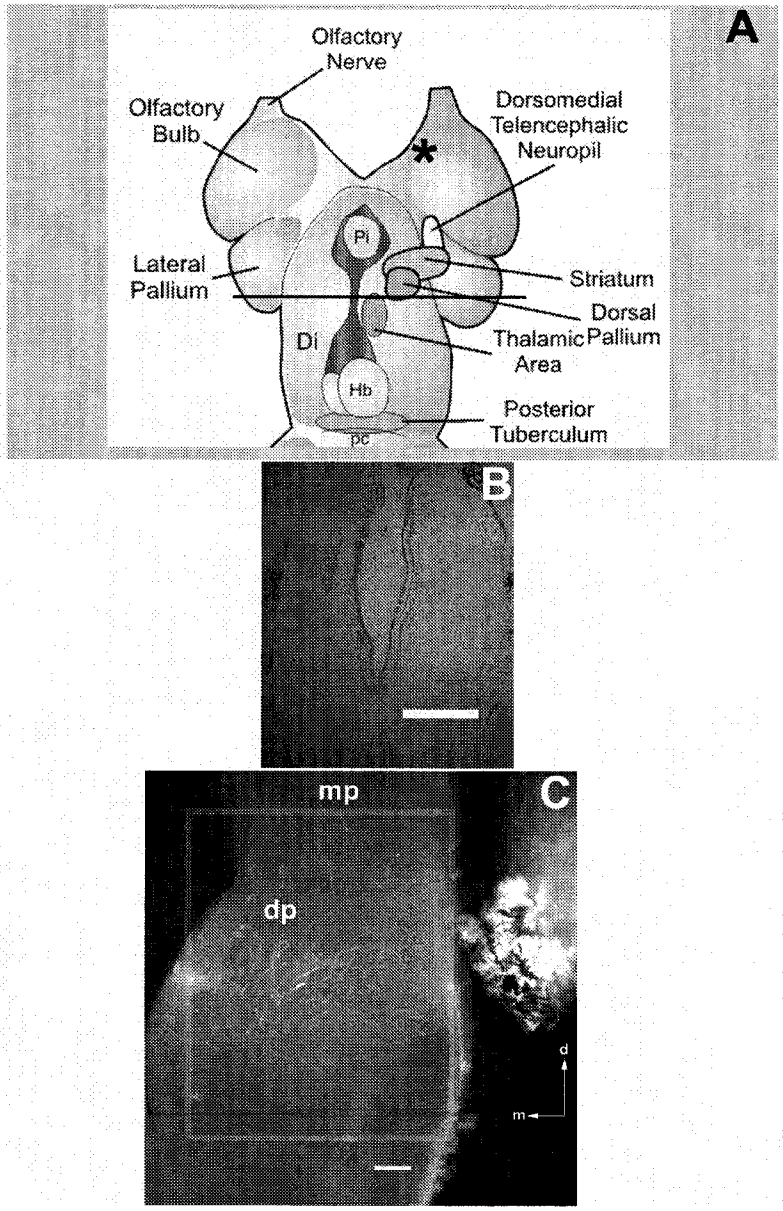

Figure 23: Labeling of fibers in the medial/dorsal pallium. A – cartoon diagram of lamprey brain showing level of sectioning; B – low power view of transition area, from caudal pallial area to thalamic area, in cresyl violet stained section - bar = 500 μm; C - long, coarse fibers in the medial/dorsal pallium. Scale bar = 100 μm. [Animal PPPP]

These fibers radiated dorsally and laterally from a single point, located medially at the margins of the central ventricle. In sections immediately posterior, thick coarse fibers were seen in the thalamic area at the same dorso-ventral distance as the fibers in the dorsal/medial pallium (**Figure 24**).

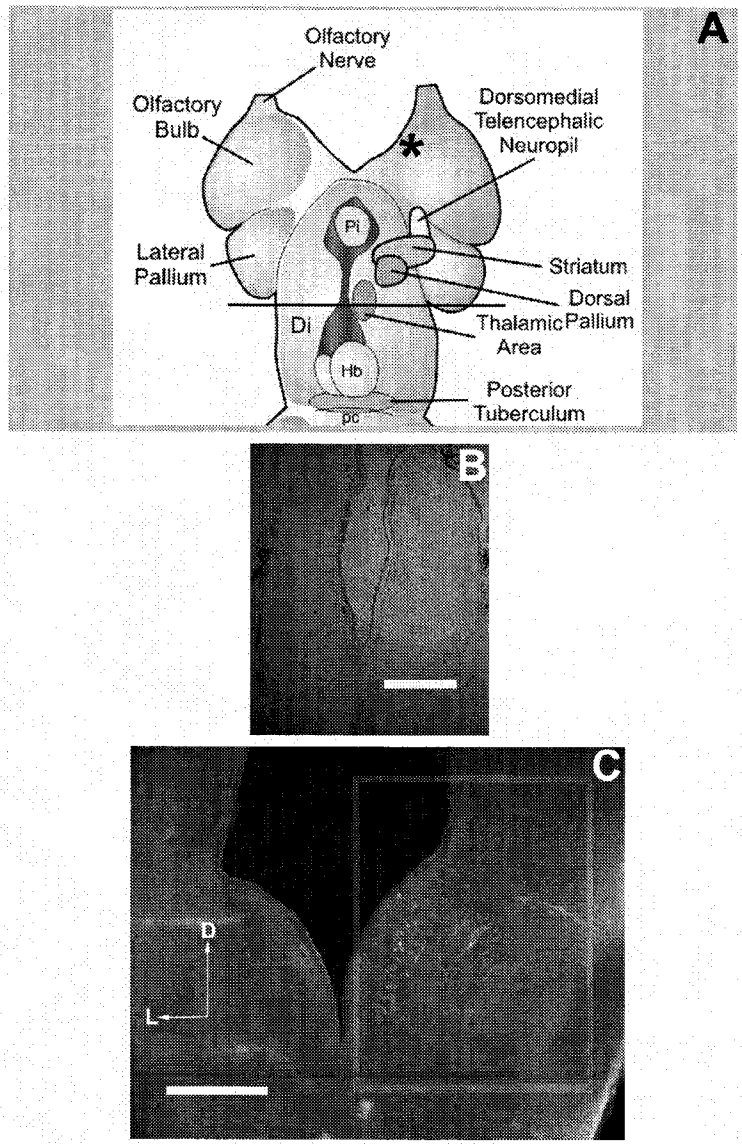

Figure 24: Labeled fibers in the thalamic area. A – cartoon of lamprey brain showing level of sectioning; B – low power view of transition area, from caudal pallial area to thalamic area, in cresyl violet stained section – bar = 500 μm; C - continuation of fibers from Figure 23, now seen in the thalamic area. Scale bar = 100 μm. [Animal PPPP]

The neural connectivity of output neurons is supported by looking at horizontal sections. From a medial injection, long fibers can be seen connecting the olfactory bulb with the telencephalon, as well as the diencephalon (**Figure 25**).

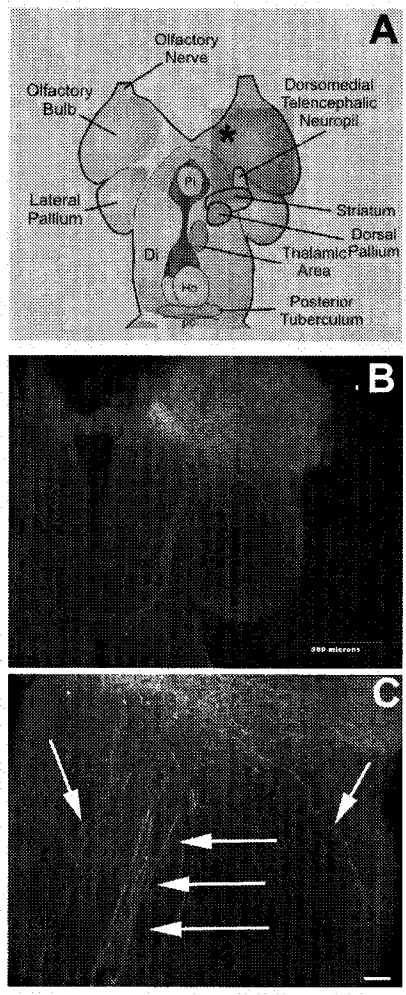

Figure 25: Coarse, thick fibers project from the olfactory bulb through the telencephalon towards the diencephalon following injection of biocytinto the medial region of the olfactory bulb of an adult ovulated female lamprey. A – cartoon of lamprey brain showing injection site; B– low power view of fibers leaving the olfactory bulb and passing through the telencephalon towards the diencephalon; C – high powered view of B showing that fibers leaving the olfactory bulb are thick, coarse fibers highlighted by arrows. Scale bar in C = 100 μm. [Animal LLL]

The projection pattern following biocytin injections to the medial region of the olfactory bulb is shown in **Figure 26**. From the medial injection site, fibers projected to the striatum and to the dorsal and medial pallia. Also, from this injection, there was labeling seen in the thalamus. Labeling was not seen in the hypothalamus.

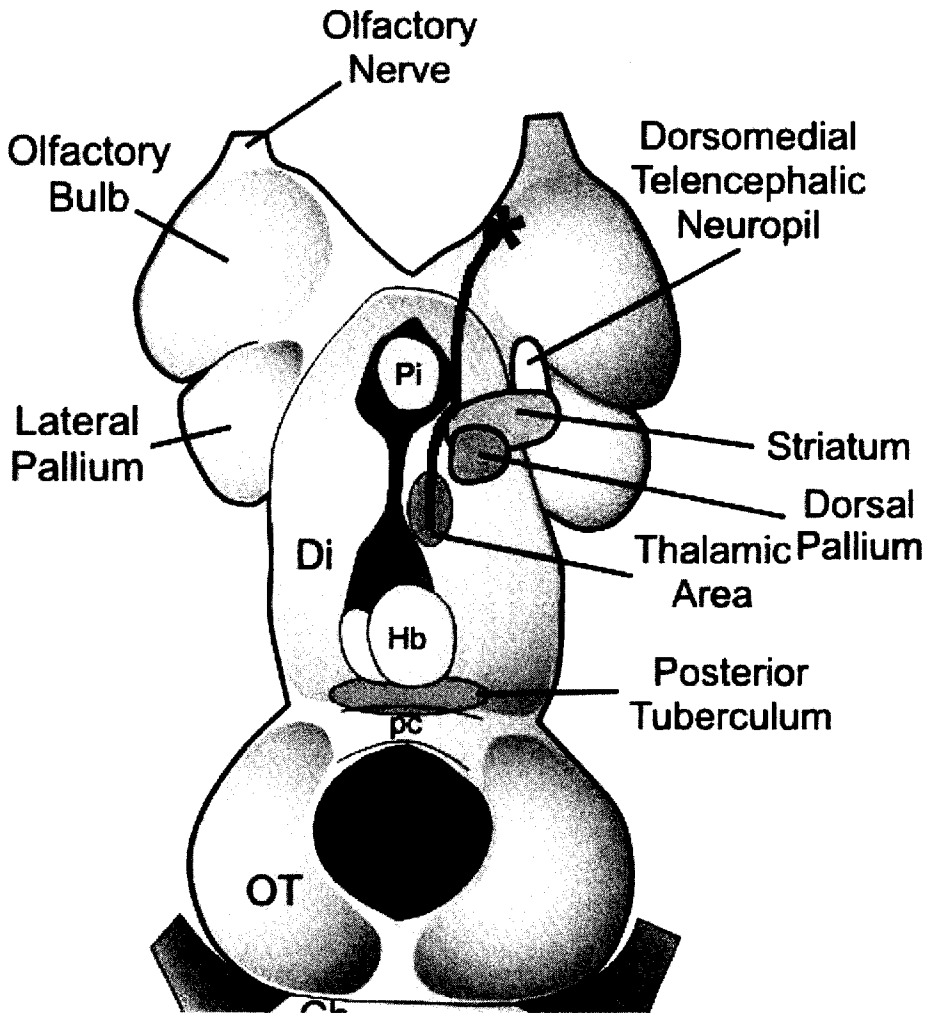

Figure 26: Summary of telencephalic and diencephalic targets following injections to the medial region of the olfactory bulb. Labeled areas include the olfactory bulb, the striatum, the dorsal and medial pallia and the thalamic area.

3.4.3 Unilateral Injections to the Lateral Region of the Olfactory Bulb in Ovulated Female Lampreys

In total, two specimens had appropriately located injections to the lateral region of the olfactory bulb; both were cross sectioned. Both specimens displayed the diffuse organization of fibers in the olfactory bulb, as well as labeling in the striatum and preoptic area, and in the dorsal and lateral pallia. Labeling was not seen in the medial pallia, habenula, hypothalamus or thalamus.

Table 5: Summary of Labeling Pattern Resulting from Unilateral Injections to the Lateral Region of the Olfactory Bulb in Ovulated Female Lamprey. OB = olfactory bulb, str = striatum, PA = preoptic area, D Pal = dorsal pallium, L Pal = lateral pallium, M Pal = medial pallium, Hab = habenula, Hyp = hypothalamus, Thal = thalamus. N indicates that labeling was not seen.

Code	Date	Size of Lesion ($\mu m^3 x 10^6$)	Angle of Sectioning	OB	Str	PA	D Pal	L Pal	M Pal	Hab	HYP	Thal	Length of Specimen (cm)	Weight of Specimen (g)
CCC	Jul-08	3.58	X	✓	✓	✓	N	✓	✓	N	N	N	60.4	275
EEE	Jul-08	4.50	X	✓	✓	✓	N	✓	✓	N	N	N	56.6	257

A vertical lesion was made to the lateral region of the olfactory bulbs, and the biocytin solution was applied to this lesion (**Figure 27**).

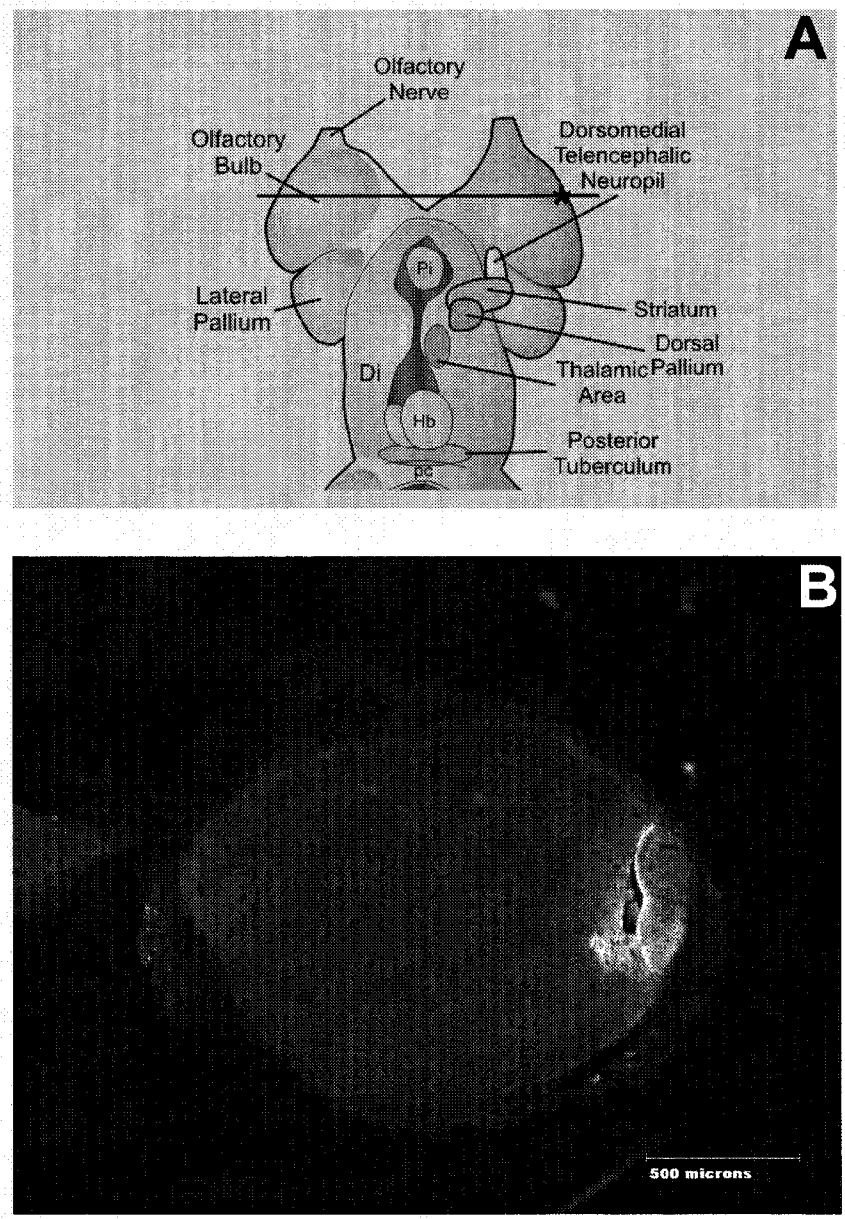

Figure 27: Injection to lateral region of the olfactory bulb. A - cartoon diagram of lamprey brain showing the level of sectioning and injection site; B - micrograph of the right olfactory bulb with a lesion and injection to the lateral region, B. [Animal EEE]

What remained the same was the broad, non-specific labeling of somata and fibers in the olfactory bulb. The striatum and preoptic area displayed positively labeled somata and fibers (**Figure 28**).

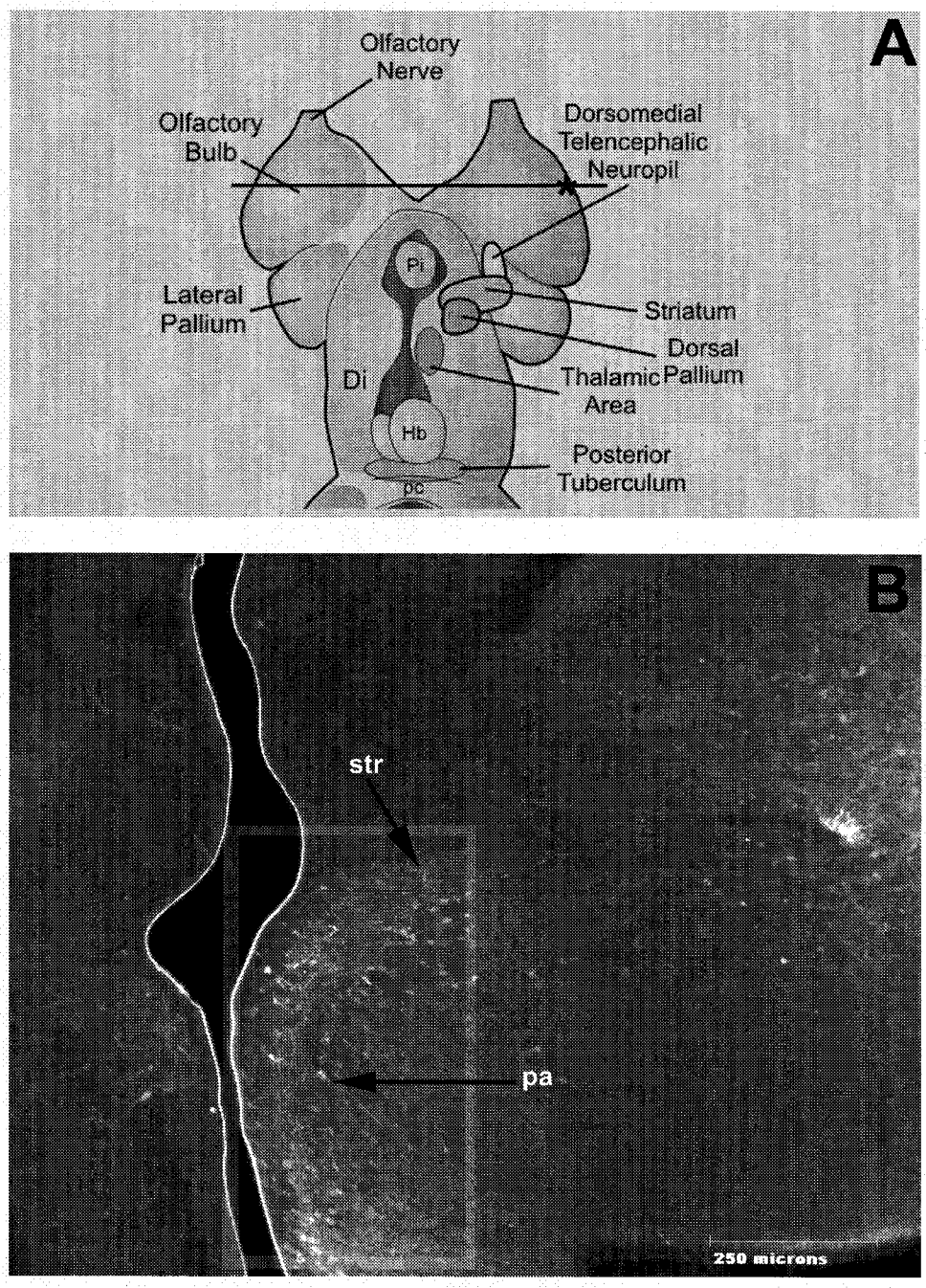

Figure 28: Labeling of cells and fibers in the rostral striatum following injection of biocytinto the lateral region of the olfactory bulb of an adult ovulated female lamprey. A – cartoon of lamprey brain showing the level of sectioning; B – somata and fibers in the rostral striatum - labeling extends throughout the entire rostro-caudal extent of the striatum. Striatum and pre-optic area are virtually indistinguishable in this section. [Animal EEE]

The dorsal pallium contained many labeled fibers and somata (**Figure 29**).

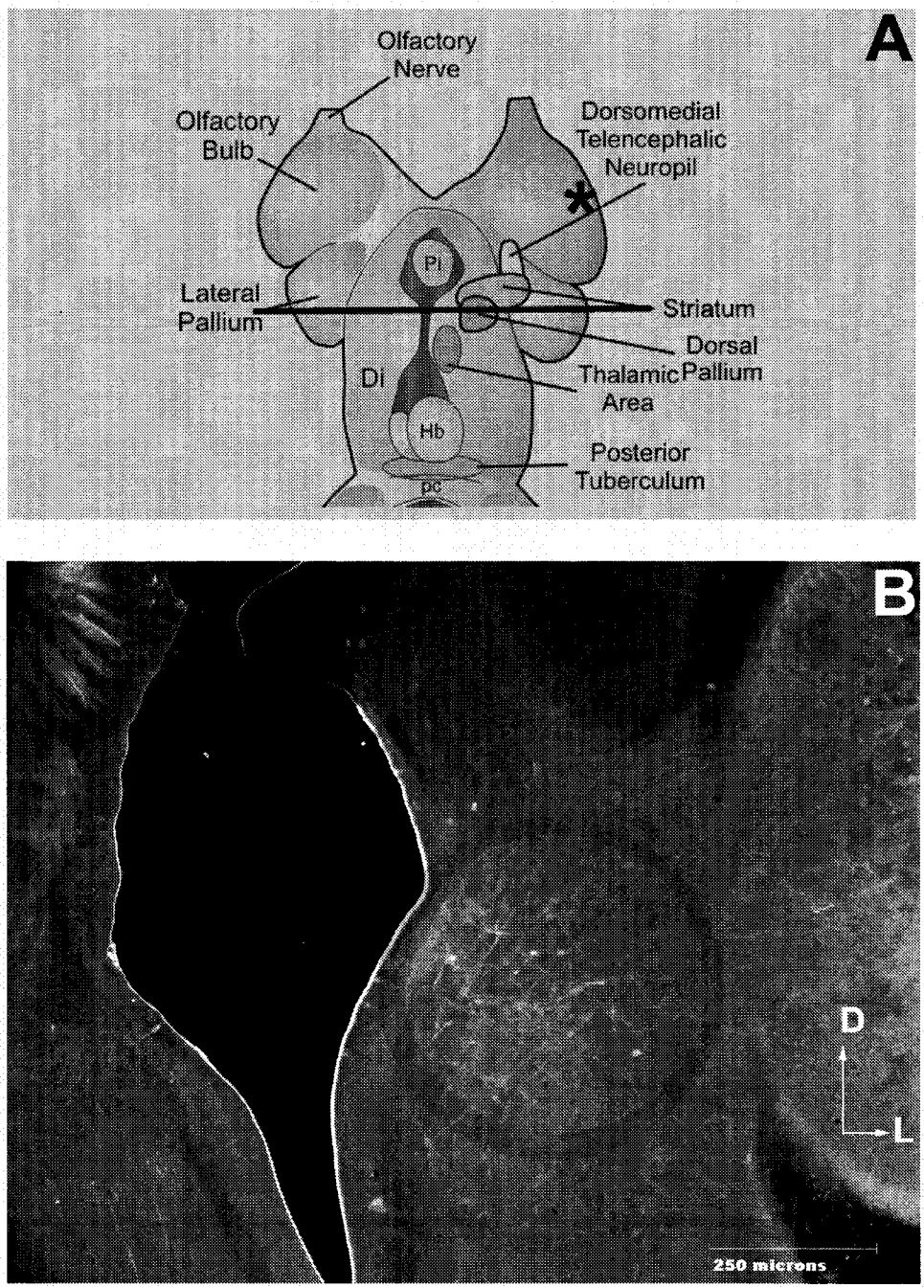

Figure 29: The dorsal pallium is densely populated by coarse fibers revealed after injection of biocytinto the lateral region of the olfactory bulb of an adult ovulated female lamprey. A – cartoon of lamprey brain showing level of sectioning; B – dorsal pallium with numerous coarse fibers. [Animal EEE]

The projection pattern following biocytin injections to the lateral region of the olfactory bulb is shown in **Figure 30**. From the lateral injection site, the striatum, preoptic area and dorsal thalamus were positively labeled.

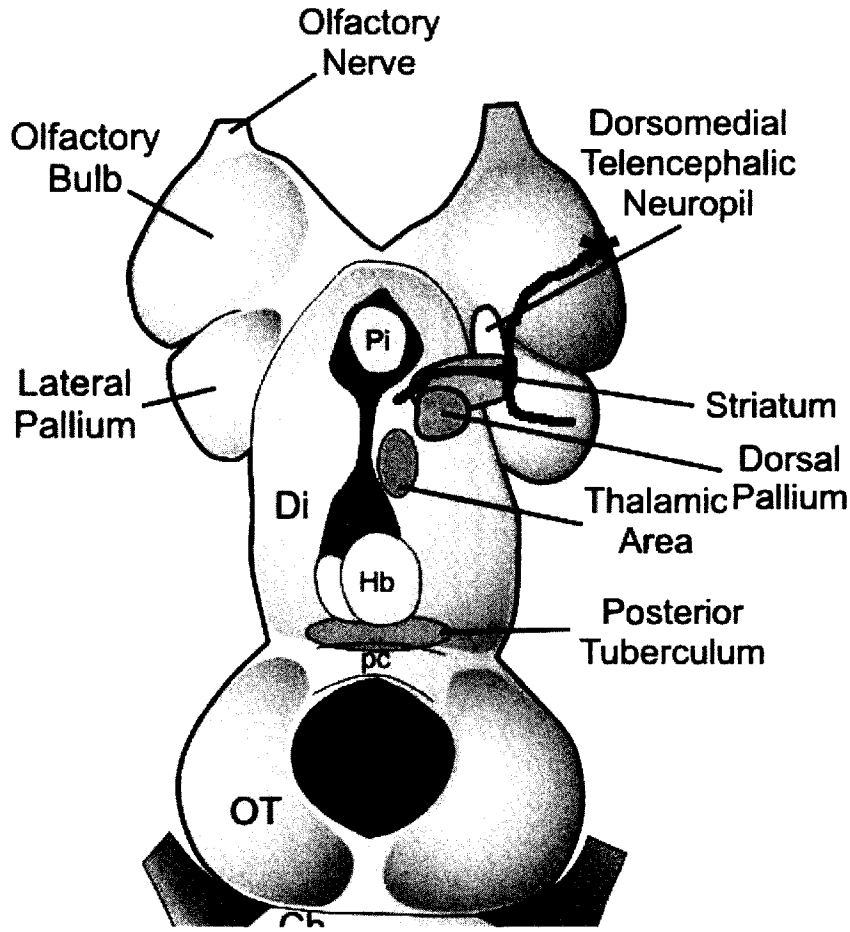

Figure 30: Summary of telencephalic and diencephalic targets following injections to the lateral region of the olfactory bulb. Labeled areas include the olfactory bulb, the striatum and the dorsal pallium.

Table 6: Summary of Biocytin Injections and Brain Regions Labeled. OB = olfactory bulb, str = striatum, PA = preoptic area, D Pal = dorsal pallium, L Pal = lateral pallium, M Pal = medial pallium, Hab = habenula, Hyp = hypothalamus, Thal = thalamus. N indicates that labeling was not seen.

Code	Date	Size of Lesion ($\mu m^3 x 10^6$)	Angle of Sectioning	OB	Str	PA	D Pal	L Pal	M Pal	Hab	Hyp	Thal	L (cm)	W(g)
HHH	Jul-14	2.2	H	✓						✓			49	248
KKK	Jul-15	3.45	H	✓						✓			46.8	241
PPP	Jul-19	4.25	H	✓						✓			55	260
YYY	Jul-22	9.00	H	✓						✓			58	269
ZZZ	Jul-22	37.50	X	✓	✓	✓	✓	✓	✓	✓	✓	N	61	274
HHHH	Jul-27	0.74	X	✓	✓	✓	✓	✓	✓	✓	✓	N	54	258
AAAAA	Aug-30	0.56	X	✓	✓	✓	✓	✓	✓	N	✓	N	57	272
EEEEE	Sep-07	0.93	X	✓	✓	✓	✓	✓	✓	N	✓	N	58	273
FFFFF	Sep-07	0.30	X	✓	N	✓	N	✓	✓	N	N	N	56	270
LLLLL	Oct-07	0.55	X	✓	N	✓	N	✓	✓	N	N	N	54	260
MMMMM	Oct-07	0.30	X	✓	✓	✓	✓	✓	✓	✓	✓	N	55.9	263
NNNNN	Nov-03	0.65	X	✓	✓	✓	✓	✓	✓	✓	N	N	51	254
III	Jul-14	0.29	H	✓									45	239
LLL	Jul-15	0.48	H	✓									45	238
IIII	Jul-27	0.12	X	✓	✓	N	✓	N	✓	N	N	✓	45	247
NNNN	Jul-29	0.78	X	✓	✓	N	✓	N	✓	N	N	✓	47	245
OOOO	Jul-29	1.20	X	✓	N	N	✓	N	✓	N	N	N	47.5	247
PPPP	Jul-29	0.60	X	✓	✓	N	✓	N	✓	N	N	✓	46.6	241
QQQQ	Jul-29	1.30	X	✓	N	N	✓	N	✓	N	N	N	44.8	235
TTTT	Aug-03	1.20	X	✓	✓	N	✓	N	✓	N	N	N	49.7	255
CCC	Jul-08	3.58	X	✓	✓	✓	N	✓	✓	N	N	N	60.4	275
EEE	Jul-08	4.50	X	✓	✓	✓	N	✓	✓	N	N	N	56.6	257

The projection patterns from the different injection sites are summarized in **Figure 31**.

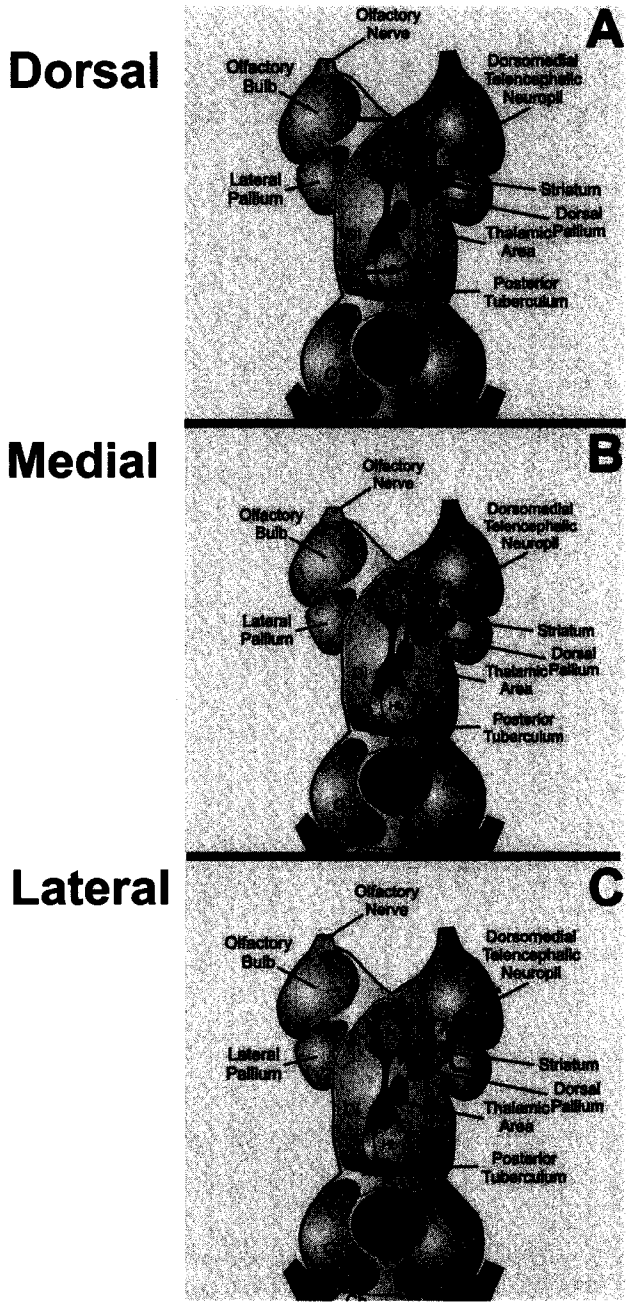

Figure 31: Injections to the dorsal anterior, medial and lateral regions of the olfactory bulb result in distinct projection patterns with some overlap in downstream targets. Each cartoon represents a summary of the projection pattern following injection to a unique zone of the lamprey olfactory bulb. A – dorsal anterior injection; B – medial injection; C – lateral injection.

From the dorsal anterior injection site, fibers projected to the contralateral olfactory bulb via the dorsal commissure as well as through the telencephalon to reach the striatum, the dorsal, lateral and medial pallia, the habenula and the hypothalamus.

From the medial injection site, fibers projected to the striatum and to the dorsal

and medial pallia. Also, from this injection, there was labeling seen in the thalamus. Labeling was not seen in the hypothalamus.

From the lateral injection site, the striatum, preoptic area and dorsal thalamus were positively labeled.

4.0 Discussion

Injections of biocytinto different glomerular territories resulted in unique patterns of labeling with respect to each glomerular territory investigated. Following each injection a number of diencephalic and mesencephalic targets were labeled, including sensory integration centers (pallial areas, habenula, thalamus and hypothalamus) and locomotor control areas (striatum).

4.1 Injections to the Dorsal Anterior Region of the Olfactory Bulb

With respect to areas being positively labeled in some animals and not labeled in others, variability in results may be accounted for by the rigorous criteria used to identify areas with positive labeling. Because positive labeling was seen in the preoptic area and the lateral and medial pallia of every specimen, it can be concluded that these areas are linked to dorsal anterior glomerular territories. Conversely, because the thalamus was not labeled in any specimens, the thalamus is not linked to dorsal anterior glomerular territories. The pallial areas are broad integrative areas and so may be an area that integrates olfactory information to relay to locomotor areas. From the injection site, the broad distribution of labeled fibers in the olfactory bulb is in agreement with findings in other teleosts (salmon – Matz 1995, sturgeon – Huesa et al. 2000, cod – Rooney et al. 1992, goldfish – von Bartheld et al. 1984). Each of these studies showed a diffuse organization of fibers in the olfactory bulb, following tracer application to various regions in the olfactory bulb, including rostral locations.

Some of the areas labeled included the striatum and preoptic area, and the pallial formations (dorsal, medial, lateral). The same structures are labeled in teleosts

such as salmon (Matz 1995), sturgeon (Huesa et al. 2000), cod (Rooney et al. 1992), goldfish (von Bartheld et al. 1984), following tracer application to multiple regions of the olfactory bulbs, including rostral locations. Direct comparison of these studies to our investigation in the lamprey is complicated by the fact that each study uses a different nomenclature for these areas of the telencephalon. None of the teleost studies link their projection pattern to a specific glomerular site in the olfactory bulb, but are valuable in noting that fish have similar projection patterns from the olfactory bulb to the telencephalon.

The hypothalamus has many functions, including sensory integration, and is labeled following dorsal anterior injections, suggesting that it is linked to dorsal anterior glomerular territories.

4.2 Injections to the Medial Region of the Olfactory Bulb

The broad distribution of labeled fibers extending from the injection site supports the idea of a diffuse pattern of mitral cell output in the olfactory bulb from this medial territory. The well defined, coarse axons that extended medially from the olfactory bulb to *and* through the pallial areas of the telencephalon, in horizontal sections (**Figure 25**), suggest direct connections to telencephalic structures like the striatum. The striatum displayed labeled somata and fibers. The same labeling of fibers and somata was seen following injection to the dorsal anterior region of the olfactory bulb, indicating that the striatum is connected to dorsal anterior glomerular territories as well as medially located glomerular territories. Labeled somata and fibers following injections to the bulb, suggest reciprocal connections between the striatum and the olfactory bulb. This result has the same implications for gating of

information from the olfactory bulb, namely, inhibitory cells in the striatum may modulate incoming olfactory signals, to the striatum. The coarse and fine fibers in the thalamus may originate from the ventral portion of the thalamus and radiate dorsally and laterally. The thalamus is known to accept input from various stimulus modalities (proprioceptive, vision, olfactory), which suggests this region is an integrative center (Polenova and Vesselkin 1993).

Because positive labeling was seen in the dorsal and medial pallia of every specimen, it can be concluded that these areas are linked to medially located glomerular territories. Conversely, because none of the samples displayed labeling in the preoptic area, the lateral pallium, the habenula or the hypothalamus, these areas are not linked to medial glomerular territories.

4.3 Injections to the Lateral Region of the Olfactory Bulb

The broad distribution of labeled fibers extending from the injection site supports the idea of a diffuse pattern of mitral cell output in the olfactory bulb from this lateral territory. The dense labeling of fibers and somata in the striatum and preoptic area made the delineation of these two structures almost impossible. This labeling suggests that lateral glomerular territories are linked to the striatum. Absence of labeling in the thalamus and hypothalamus, suggests no direct integration of information from lateral glomeruli to behaviours or functions controlled by these caudal structures.

Because positive labeling was seen in the striatum, preoptic area, and the lateral and medial pallia, it can be concluded that these areas are linked to laterally located glomerular territories. Conversely, because none of the specimens had labeling in the

dorsal pallium, habenula, hypothalamus or the thalamus, these areas are not linked to lateral glomerular territories.

4.4 The Striatum May Act as a Gate for Olfactory Information

In the lamprey, the entire rostro-caudal extent of the striatum displayed positively labeled somata and fibers following injections to the dorsal anterior region of the olfactory bulb. Injections to the medial or dorsal regions of the olfactory bulb also resulted in labeling in the striatum; however, labeling was restricted to the rostral and medial striatum. These positively labeled somata indicate that these cells are being filled with biocytin along projections that reach rostrally, toward the olfactory bulb and the injection site. These projections from the striatum to the olfactory bulb are termed centrifugal or bulbofugal fibers. The striatum projects to the ventral lateral pallium of the telencephalon of the brain which in turn, projects to the ventral thalamus (Pombal, El Manira and Grillner 1997). The ventral thalamus has descending connections to reticulospinal cells which directly control locomotion (Pombal, El Manira and Grillner 1997), demonstrating an indirect pathway from striatum to the reticulospinal neurons. The striatum is rich in somata that are immuno-reactive towards GABA and fibers immuno-reactive towards serotonin (Pombal, El Manira and Grillner 1997). These inhibitory and modulatory neurotransmitters may be used to inhibit or modulate incoming olfactory information at this level of the brain.

Our result of labeling from the olfactory bulb to the striatum, considered with the indirect pathway from the striatum to reticulospinal neurons and coupled with the inhibitory and modulatory neurotransmitters lends further support to the hypothesis

that the striatum acts as a gate for olfactory information. This pathway may be stimulated by the male lamprey sex pheromone 3-kpzs to elicit the swimming response seen in ovulated female lampreys.

4.5 Conclusions

The pattern of labeling in the ovulated female lamprey brain following injections of biocytin to different regions of the olfactory bulb support the idea that there is a functional separation in the olfactory bulb, as previously seen in teleost fish (Nikonov and Caprio 2001; Nikonov et al. 2005; Sato et al. 2005). In our study, distinct regions of the olfactory bulb projected to different areas of the brain. The dorsal anterior region of the olfactory bulb has projections to integrative areas (dorsal, lateral and medial pallia, habenula, thalamus and hypothalamus) and the striatum. The medial region of the olfactory bulb has projections to integrative areas (dorsal and medial pallia, the thalamus) and the striatum. The lateral region of the olfactory bulb has projections to the dorsal pallium and the striatum.

All three regions of the olfactory bulb project to the striatum, suggesting that olfactory information from all three regions can elicit a locomotor response. The dorsal anterior and medial regions of the olfactory bulb have considerable overlap, with respect to their outputs; however, the medial region did not project to the hypothalamus or the lateral pallia. The absence of labeling in these integrative areas, following medial injections but not dorsal anterior injections, demonstrates a possible functional difference between these two areas of the olfactory bulb. The lateral region of the olfactory bulb projected to one integrative area, the dorsal pallium. The striatum, however, was strongly labeled, following injection to the lateral region of

the olfactory bulb, suggesting that the lateral region of the olfactory bulb is strongly associated with locomotor responses.

This study is the first to link glomerular territories in olfactory bulb of the ovulated female lamprey to specific areas in the brain. Injections of biocytin to the olfactory bulb revealed connections to integrative centers (pallial areas, habenula, thalamus and hypothalamus) and a locomotor control area (the striatum). Only ovulated females show a locomotor response to the male sex pheromone and the peripheral detection of this pheromone must be transmitted to integrative or locomotor control areas to initiate the response. The connections between glomerular territories and specific brain regions, described in this study, are candidates for the neural pathway that is stimulated by the male lamprey sex pheromone that elicits the locomotor response in ovulated female lampreys.

4.6 Future Directions

This thesis has anatomically identified candidate areas to investigate for activity in ovulated female lampreys in response to the male reproductive pheromone, 3kpzs. The same *ex vivo* preparation could be used to confirm which areas are actually responsive. Odourants, in this case, the pheromone, would be introduced to the exposed olfactory epithelium and responses would be recorded via electrophysiology. Using the same preparation, and possibly simultaneously, a calcium indicator dye (such as Fluo-4, AM or calcium green-1 dextran) could be used to measure calcium dynamics as an indicator of activity in candidate regions.

References

Auclair et al. Immunohistochemical distribution of tachykinins in the CNS of the lamprey *Petromyzon marinus*. Journal of Comparative Neurology. 479 (2004) 328-346.

Duchamp-Viret P and Duchamp A. Odor Processing in the frog olfactory system. Progress in Neurobiology. 53 (1997) 561-602.

Dulka JG. Sex pheromone systems in goldfish: Comparisons to vomeronasal systems in tetrapods. Brain Behavior and Evolution. 42 (1993) 265-280.

Dulka JG, Stacey NE, Sorensen PE and Van der Kraak GJ. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. Nature. 325 (1987) 251-253.

Eisthen HL and Northcutt, RG. Silver lampreys (*Icthyomyzon unicuspis*) lack a gonadotropin-releasing hormone- and FMRFamide- immunoreactive terminal nerve. The Journal of Comparative Neurology. 370 (1996) 159-172.

Fine JM and Sorensen PW. Biologically relevant concentrations of petromyzonol sulfate, a component of the sea lamprey migratory pheromone, measured in stream water. Journal of Chemical Ecology. 31 (2005). 2205-2210.

Fine JM, Vrieze LA and Sorensen PW. Evidence that Petromyzontid lampreys employ a common migratory pheromone that is partially comprised of bile acids. Journal of Chemical Ecology. 30 (2004), 2091-2110.

Frontini et al. Glomerular territories in the olfactory bulb from the larval stage of the sea lamprey *Petromyzon marinus*. Journal of comparative neurology. 465 (2003) 27-37.

Gonzalez MJ, Yanez J and Anadon R. Afferent and efferent connections of the torus semicircularis in the sea lamprey: an experimental study. Brain Research. 826 (1999) 83-94.

Hamdani EH, Kasumyan A, Døving KB. Is feeding behaviour in crucian carp mediated by the lateral olfactory tract? Chemical Senses. 26 (2001) 1133-1138.

Heier P. Fundamental principles in the structure of the brain. A study of the brain of Petromyzon fluviatilis. Acta Anatomica Supplementum VIII. 1948. 1-213.

Inaki K, Nishimura S, Nakashiba T, Itohara S, Yoshihara Y. Laminar organization of the developing lateral olfactory tract revealed by differential expression of cell recognition molecules. Journal of Comparative Neurology. 479 (2004) 243-256.

Iwahori N, Kiyota E and Nakamura K. A Golgi study on the olfactory bulb in the lamprey, *Lampetra japonica*. Neuroscience Research. 5 (1987) 126-139.

Karlson P and Luscher M. Pheromones: a new term for a class of biologically relevant compounds. Nature. 183(4653) (1959) 55-56.
Kirkeby S and Moe D. Binding of *Griffonia simplicifolia* 1 isolectin B4 (GS1 B4) to α-galactose antigens. Immunology and Cell Biology. 79 (2001). 121-127.

Kleerekoper H. and Mogensen J. Role of olfaction in the orientation of *Petromyzon marinus* I. Response to a single amine in prey's body odor. Physiol. Zool. 36 (1973) 347-360.

Kotrschal, K. Taste(s) and olfaction(s) in fish: a review of spezialized sub-systems and central integration. Pflugers Arch – Eur J Physiol. 439[Suppl] (2000) R178-R180.

Laberge F. and Roth G. Connectivity and cytoarchitecture of the ventral telencephalon in the salamander, *Plethodon shermani*. Journal of Comparative Neurology. 482 (2005) 176-200.

Li W et al. Bile acid secreted by male sea lamprey that acts as a sex pheromone. Science. 296 (2002) 138-141.

Lindsay SM and Vogt RG. Behavioral responses of newly hatched zebrafish (*Danio rerio*) to amino acid chemostimulants. Chemical Senses. 29 (2004) 93-100.

Matz SP. Connections of the olfactory bulb in the Chinook salmon (Oncorhynchus tshawytscha). Brain Behav. Evol. 46 (1995) 108-120.

McDonald AJ. Neuroanatomical labeling with biocytin: a review. Neuroreport. 3(10) (1992) 821-7.

Niewenhuys R. The brain of the lamprey in a comparative perspective. Annals of the New York Academy of Sciences. 299 (1977) 97-145.

Nikonov AA and Caprio J. Electrophysiological evidence for a chemotopy of biologically relevant odors in the olfactory bulb of the channel catfish. Journal of Neurophysiology. 86 (2001) 1869-1876.

Nikonov AA, Finger TE and Caprio J. Beyond the olfactory bulb: An odotopic map in the forebrain. Proceedings of the National Academy of Sciences. 51 (2005) 18688-18693.

Northcutt RG and Puzdrowski RL. Projections of the olfactory bulb and nervus terminalis in the silver lamprey. Brain Behavior and Evolution. 32 (1988) 96-107.

Northcutt RG and Wicht H. Afferent and efferent connections of lateral and medial pallia of the silver lamprey. Brain Behavior and Evolution. 49 (1997) 1-19.

Ojima H,. Mori K. and Kishi K. The trajectory of mitral cell axons in the rabbit olfactory cortex revealed by intracellular HRP injection. Journal of Comparative Neurology. 230 (1984) 77-87.

Polenova, OA and Vesselkin NP. Olfactory and nonolfactory projections in the river lamprey (*Lampetra fluviatilis*) telencephalon. Journal für Hirnsforschung. 34 (2) (1993) 261-279.

Pombal MA, El Manira A, Grillner, S. Organization of the lamprey striatum – transmitters and projections. Brain Research. 766 (1997) 249-254.

Rooney D, Doving KB, Ravaille-Veron M and Szabo T. The central connections of the olfactory bulbs in cod, Gadhus morhua L. J. Hirnforsch. 33 (1992). 63-75.

Rossler W, Kuduz J, Schurmann FW, Schild D. Aggregation of F-Actin in olfactory glomeruli; a common feature of glomeruli across phyla. Chemical Senses. 27 (2002) 803-810.

Sato Y, Miyasaka N, Yoshihara Y. Mutually exlusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. Journal of Neuroscience. 25(20) 2005 4889-4897.

Schmidt A. and Roth G. Central olfactory and vomeronasal pathways in salamanders. Journal fur Hirnforschung. 31 (1990) 543-553.

Schober W. Vergleichend-anatomische untersuchungen am Gehirn der larven und adulten tiere von *Lampetra fluviatilis* (Linné, 1758) und *Lampetra planeri* (Bloch, 1784). Journal fur Hirnforschung. (1964) 107-209.

Sorensen PW, Fine JM, Dvornikovs V, Jeffrey CS, Shao F, Wang J, Vrieze LA, Anderson KR and Hoye TR. Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. Nature Chemical Biology. 1 (2005) 324-328.

Sorensen PW, Vrieze LA and Fine JM. A multi-component migratory pheromone in the sea lamprey. Fish Physiology and Biochemistry. 28 (2003) 253-257.

Stacey NE and Kyle AL. Effects olfactory tract lesions on sexual and feeding behavior in the goldfish. Physiology and Behavior. 30 (1983) 621-628.

Stein PSG, Grillner S, Selverston AI and Douglas DG. Neurons, Networks and Motor Behavior. 1997

Tabor R, Yaksi E, Weislogel JM, Friedrich RW. Processing of odor mixtures in the zebrafish olfactory bulb. Journal of Neuroscience. 24(29) (2004) 6611-6620.

Teeter J, Lischka F, and Li W. Pheromone responses in olfactory receptor neurons of sea lampreys (*Petromyzon marinus*). Chemical Senses. 28: A49 (Abstract 195, pg 49).

Tobet SA, Chickering TW and Sower SA. Relationship of gonatotropin-releasing hormone (GnRH) neurons to the olfactory system in developing lamprey (Petromyzon marinus). Journal of Comparative Neurology. 376 (1996) 97-111.

Valentincic T. et al. Expression of a reflex biting/snapping response to amino acids prior to first exogenous feeding in salmonid alevins. Physiology and Behavior. 67 (4) (1999) 567-572.

Wiltzien F, Höglund E, Hamdani EH, Døving KB. Does the lateral bundle of the medial olfactory tract mediate reproductive behavior in male crucian carp? Chemical Senses. 28 (2003). 293-300.

Von Bartheld CS, Meyer DL, Fiebig E and Ebbesson SO. Central connections in the goldfish Carassius auratus. Cell Tissue Research. 238 (1984). 475-487.

Xue H et al. The anterograde and retrograde axonal transport of biotinylated dextran amine and biocytin in the nervous system of teleosts. Brain Research Protocols. 13 (2004) 106-114.

Yamamota K, Sargent PA, Fisher MM, Youson JH. Periductal fibrosis and lipocytes (fat-storing cells or ito cells) during biliary atresia in the lamprey. Hepatology. 6 (1986) 54-59.

VITA AUCTORIS

Steven Chang was born in 1977 in Oakville, Ontario. He graduated from the University of Windsor in 1999 with a B.A. in Psychology. He continued his studies at the University of Windsor, and in 2001, he graduated with an Honours B. Sc. in Biology and Psychology with the distinction of being the first graduate of what would eventually become the Behaviour, Cognition and Neuroscience (B.C.N.) program. Steven is currently a candidate for the Master's degree in Biological Sciences at the University of Windsor and hopes to enter a Ph. D. program in September 2006.