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The neuroanatomical organization and chemosensory response characteristics of the olfactory bulb in the sea lamprey (Petromyzon marinus)

Warren W. Green

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By

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Declaration of Co-Authorship / Previous Publication

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

Chapter 2 is the outcome of joint research undertaken in collaboration with Alfred Basilious under the supervision of Dr. Barbara Zielinski. The key ideas, primary contributions, experimental design, data analysis and interpretation were performed by the author. The contribution of the co-author was through experimental assistance in retrograde labeling and examination of olfactory bulb projection neurons.

Chapter 3 is the outcome of research undertaken by the author under the supervision of Dr. Zielinski and Dr. Huiming Zhang. In all cases, the key ideas, primary contributions, experimental design, data analysis, and interpretation, were performed by the author. The contributions of Dr. Zhang and Dr. Zielinski were towards experimental design and data interpretation.

Appendix A is the outcome of joint research in which I am a co-author. I contributed to this published manuscript through conducting retrograde labeling experiments in the olfactory bulb, collecting and analyzing the resulting data, and contributing to the production of Figure 5A and Table 1.

Appendix B is the outcome of joint research in which I am a co-author. I contributed to this published manuscript through conducting a full literature review of olfactory-driven behaviours in fish. In addition, I conducted retrograde labeling experiments for olfactory sensory neurons in the peripheral olfactory organ and immunolabeled cilia against acetylated tubulin. I subsequently collected the confocal micrographs and produced Figure 2 for the publication.

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This thesis includes 2 original papers that have been previously published in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Appendix A	*Projections from the Accessory Olfactory Organ into the Medial Region of the Olfactory Bulb in the Sea Lamprey (Petromyzon marinus) - a Novel Vertebrate Sensory Structure?*	*Published 2009*
Appendix B	*Chemosensory-induced motor behaviors in fish*	*Published 2012*

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Abstract

Olfactory information is utilized for a variety of behaviours including feeding, migration and spawning. The olfactory bulb is the initial site of odour information processing in the central nervous system. The OB organizes this information along parallel processing pathways using topographic and temporal coding. In general, little is known about the neural substrate involved in transforming olfactory information into behavioural responses. In the sea lamprey (*Petromyzon marinus*) the responses of olfactory sensory neurons in the peripheral olfactory organ are transmitted along two distinct pathways to the medial and non-medial regions of the OB. The neural substrate for olfactory-locomotor transformation was recently identified in the sea lamprey, and demonstrated that projection neurons (PNs) in the medial OB region initiated movements by connecting to locomotor control centers. In contrast, PNs in the non-medial OB region projected to forebrain regions including the pallium. This thesis examines previously unknown anatomical and physiological characteristics of PNs in these medial and non-medial OB regions and relates the findings to these functionally distinct parallel output pathways. The medial PNs were anatomically isolated within the glomerular neuropil, exhibited varied somal shape, and had larger somata than non-medial PNs. Furthermore, the bulbar region containing these medial PNs responded to several different classes of odours with local field potentials (LFPs) being a mixture of transient and sustained responses and relatively short multiunit responses. The somata of non-medial PNs were below the glomerular neuropil and also exhibited varied shapes, but were smaller than the somata of the medial PNs. The LFP and multiunit recordings suggested that within the non-medial

OB region, the dorsal territory responded differentially to lamprey sex pheromones and migratory pheromones while lateral recordings exhibited sustained LFP responses and long multiunit responses largely to basic amino acids. These findings suggest that neural organization in the medial OB may be optimized for the initiation of olfactory-locomotor movements in response to diverse odours, while the nonmedial regions exhibit odour specificity and may be optimized for other functional processes such as odour information integration.

Dedicated to my wife Natalie, daughter Emilie and my parents Wayne and Trish Green.
Thank you for inspiring me each and every day.

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List of Abbreviations

3kACA	3-keto allocholic acid
3kPZS	3-keto petromyzonol sulphate
AA	Amino acid
ACA	Allocholic acid
AL	Antennal lobe
ANOVA	Analysis of variance
AOB	Accessory olfactory bulb
AON_{pE}	Anterior olfactory nucleus pars externa
AON_{pV}	Posteroventral part of the anterior olfactory nucleus
AOO	Accessory olfactory organ
APC_{VR}	Ventrorostral part of the anterior piriform cortex
cAMP	cyclic adenosine monophosphate
DAG	Diacylglycerol
EPL	External plexiform layer
GABA	γ-aminobutyric acid
GL	Glomerular layer
GLMC	Glomerular layer with mitral cells
GPCR	G protein coupled receptor
GrC	Granule cell layer
$GS1B_4$	*Griffonia simplicifolia* lectin 1, isolectin B4
IP_3	Inositol-1,4,5-triphosphate
IPL	Internal plexiform layer
LFP	Local field potential
LOT	Lateral olfactory tract
MC	Mitral cell
MCL	Mitral cell layer
MLR	Mesencephalic locomotor region
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
MOT	Medial olfactory tract
OB	Olfactory bulb
OE	Olfactory epithelium
OR	Odour receptor
OSN	Olfactory sensory neuron
OT_{CAP}	Anterolateral part of the olfactory tubercle

PADS	Petromyzonamine disulphate
PN	Projection neuron
PSDS	Petromyzonolsterol disulphate
PSTH	Post-stimulus time histogram
PT	Posterior tuberculum
PZS	Petromyzonol sulphate
RS	Taurocholic acid
V1R	Vomeronasal type 1 receptor
V2R	Vomeronasal type 2 receptor
VNO	Vomeronasal organ

CHAPTER 1

A review of olfaction and the olfactory system of the sea lamprey

1.1 General Introduction

The surrounding environment of an organism provides a vast array of sensory information that requires neural processing in order to produce the necessary behavioural responses to survive and reproduce. To adequately perceive and decode this information, sensory systems including the somatosensory, visual, auditory, and the olfactory systems utilize several levels of organization that can include topographic and temporal organization, as well as parallel and serial information processing (Strausfeld and Lee, 1991; Shepherd and Greer, 1998; Rauschecker and Tian, 2000; Ahissar and Arieli, 2001; Konishi, 2003). Through parallel processing, different functional aspects of a sensory stimulus are conveyed along different pathways in that system. How odour information is encoded, processed and how this leads to a behavioural response, are questions that have been under investigation in rodents, fish, and insects. Significant progress has been made in our understanding of peripheral and central neural activity associated with the olfactory system, and related behavioural responses. Examples of parallel processing and neural subsystems have been documented in the olfactory system of invertebrate and vertebrate organisms (Hildebrand and Shepherd, 1997); yet, the detailed neural substrates involved in odour driven behaviours are still poorly understood. The neural pathway from olfaction to locomotion has been elucidated in an invertebrate (*C. elegans*) (Gray et al., 2005) and also in the sea lamprey (*Petromyzon marinus*). Recently, Derjean et al. (2010) described a novel olfactory-locomotor neural substrate in the sea lamprey, involving parallel processing pathways in the medial and non-medial regions of the olfactory bulb (OB). Although the input and output pathways of the sea lamprey OB are

well characterized, almost nothing was known about the anatomical organization of bulbar projection neurons (PNs) and their odour-induced physiological response characteristics. Thus, using the sea lamprey as a model species, this thesis contributes to further understanding of odour information processing in the olfactory bulb, organization of odour information based on the function of OB output pathways, and their direct relevance to olfactory-locomotor output.

This thesis presents background on the neurobiology of the olfactory system, defines goals for experimental studies of the neural anatomy and chemosensory properties in the olfactory bulb of the sea lamprey's olfactory system, then describes and discusses these studies.

The purpose of this chapter is to review what is generally known regarding the olfactory system and its subsystems, with an emphasis of what is known in fish. Knowledge regarding the lamprey olfactory system is also presented. The details of olfactory information processing in the peripheral olfactory organ, the olfactory bulb, and pathways to higher brain structures are examined within the context of parallel olfactory processing pathways and olfactory driven motor behaviours.

1.2 Olfactory subsystems

Sensory input occurs via olfactory sensory neurons located in the peripheral olfactory organ. These afferent neurons form synaptic contacts within the central nervous system – the olfactory bulb (OB) for vertebrates, and the antennal lobe (AL) for insects. The mammalian olfactory system has two primary subdivisions, known as the main and accessory olfactory systems. Each has a distinct peripheral olfactory location (main olfactory epithelium; MOE and vomeronasal organ; VNO), distinct and

overlapping central targets (main olfactory bulb; MOB), accessory olfactory bulb; AOB) and parallel pathways to higher brain centers (Shepherd and Greer, 1998; Ma, 2007; Baum, 2012).

Early studies suggested that the VNO and MOE in mammals process different classes of odours, the main olfactory system responding to general olfactory cues and the accessory olfactory system to pheromones and other social cues. However, during the past 10 years researchers have seen overlap in the classes of odours that stimulate the two olfactory subsystems (Restrepo et al., 2004; Xu et al., 2005; Baxi et al., 2006; Brennan and Zufall, 2006; Spehr et al., 2006; Jakupovic et al., 2008; Slotnick et al., 2010).

While invertebrates do not possess an accessory olfactory organ, they also have parallel olfactory processing pathways. Olfactory receptor cells reside on the antennae of invertebrates and project their axons to the antennal lobes, where they synapse with projection neurons in aggregations of neuropil known as glomeruli (Tolbert and Hildebrand, 1981; Christensen et al., 1995). Within the ALs of the male cockroach (*Periplaneta americana*) and the male moth (*Manduca sexta*) there are so-called ordinary glomeruli as well as a macroglomerulus (*P. americana*) or macroglomerular complex (*M. sexta*) that solely process female species-specific sex pheromones (Matsumoto and Hildebrand, 1981; Christensen and Hildebrand, 1987; Christensen et al., 1989; Kanzaki et al., 1989; Kanzaki and Shibuya, 1992; Boeckh and Tolbert, 1993; Christensen et al., 1995; Hildebrand and Shepherd, 1997). Moreover, parallel medial and lateral antenno-protocerebral tracts have been identified in the honeybee (*Apis mellifera*) (Kirschner et al., 2006; Carcaud et al., 2012) where PNs extend their axons from the antennal lobe to distinct and separate regions of the mushroom bodies and lateral horn. Moreover, the

lateral and medial antenno-protocerebral tracts respond to different odours that vary in carbon chain length and functional group (Carcaud et al., 2012).

In teleost fish, the olfactory sensory neurons (OSNs) are intermixed in the peripheral olfactory organ, however in the brain, olfactory processing pathways are delineated as medial and lateral subdivisions of the olfactory system. The ciliated and microvillar OSN morphotypes are distributed stochastically throughout the olfactory epithelium. The axons of the ciliated type extend to the medial region of the olfactory bulb and the microvillar type project to the lateral region (Sato et al., 2005). The primary projection neurons of the medial and lateral OB project their axons along the medial and lateral olfactory tracts, respectively (Sheldon, 1912; Doving and Selset, 1980; Satou, 1990; Hamdani et al., 2000; Hamdani et al., 2001b; Hamdani and Doving, 2007), to the telencephalon, where these subdivisions are maintained (Nikonov et al., 2005). Generally, the medial olfactory pathway (ciliated olfactory sensory neurons) processes bile salt and pheromone odour information, while the lateral olfactory pathway (microvillar olfactory sensory neurons) processes amino acid and nucleotide odour information (Satou, 1990; Friedrich and Korsching, 1998; Hara and Zhang, 1998; Nikonov and Caprio, 2001; Hansen et al., 2003). Furthermore, within the medial and lateral subdivisions of the olfactory bulb (OB), specific subsets of projection neurons process specific subclasses of odours (eg. acidic, basic, or neutral amino acids in the lateral OB)(Nikonov and Caprio, 2004; Rolen and Caprio, 2007). It is clear that parallel processing pathways in the olfactory system are present across phyla, are important for processing different classes of odours, and can be organized by their functional output.

1.3 The olfactory sensory neurons – receptors, signal transduction and ion channels

In order to process specific odour information, the peripheral olfactory organ of invertebrate and vertebrate organisms utilize OSNs. Considerable diversity is seen, with respect to the morphological characteristics, odour receptor proteins, signal transduction mechanisms and ion conductance channels of OSNs (Hildebrand and Shepherd, 1997; Kay and Stopfer, 2006). These characteristics have many similarities across phyla. In many vertebrates, there are the two principal olfactory sensory neuron types - ciliated and microvillous. A third type known as crypt cells (Hansen and Finger, 2000) have been observed in the olfactory epithelium of some Actinopterygian fish including Acanthopterygian and Ostariophysian fishes (Hansen and Finger, 2000; Belanger et al., 2003; Hansen et al., 2003). In the lamprey, only ciliated OSNs are present in the olfactory epithelium (Thornhil, 1967; Vandenbossche et al., 1995), but in teleost fish, microvillous and ciliated OSNs intermingle in the peripheral olfactory organ (Zielinski and Hara, 2006). In mammals, ciliated OSNs predominate in the main olfactory epithelium and microvillous OSNs are located in the VNO (Ma, 2007). Ciliated cells are present on the antennae of insects (Schneider, 1969; Stengl et al., 1992), where there are generalist OSNs that bind and respond to a variety of compounds, as well as specialist OSNs that express only pheromone specific types of receptors, which bind components of the female sex pheromone (Christensen and Hildebrand, 1987; Hildebrand and Shepherd, 1997; Sato and Touhara, 2009).

In the MOE of mammals, each OSN expresses a single odour receptor (OR)(Ressler et al., 1994; Vassar et al., 1994; Buck, 1996; Mombaerts et al., 1996).

Unfortunately, the odor-ligands have been identified for only a handful of ORs. Zones of receptor expression overlap in the MOE, and a given odour receptor (OR) is expressed in several zones across the surface of the epithelium (Ressler et al., 1993; Buck, 1996; Iwema et al., 2004; Miyamichi et al., 2005; Ma, 2007). The ORs in different zones may be associated with the physicochemical properties of the odours (Kent et al., 1996; Schoenfeld and Cleland, 2006; Ma, 2007) and are functionally organized whereby a given odour will elicit activity in a specific cluster of OSNs as well as OSNs scattered across different zones of the olfactory epithelium (Ma and Shepherd, 2000).

In fish, each OSN also expresses only a single type of OR, and these are distributed throughout the olfactory epithelium (Speca et al., 1999; Laberge and Hara, 2001) The OSNs extend their dendritic ending into the olfactory mucus covering the olfactory epithelium where receptors on the surface of cilia, or microvilli, bind odours. This is similar in insects, except the odours pass through the porous waxy coating and bind to receptors on the surface of the cilia via olfactory binding proteins (Hildebrand and Shepherd, 1997).

The odour receptor proteins of OSNs are members of the seven-transmembrane G-protein coupled receptor (GPCR) superfamily. In rodents, OR-type receptors are in the main olfactory epithelium and the vomeronasal type one (V1R) and type two (V2R) receptors found in the VNO (Buck and Axel, 1991; Dulac and Axel, 1995; Berghard and Buck, 1996; Buck, 1996; Ryba and Tirindelli, 1997; Zhao and Firestein, 1999; Firestein, 2001). Once an odour binds to the GPCR, a signal transduction cascade involving second messengers opens cyclic nucleotide gated channels as well as other ion channels on the surface of the sensory neuron, leading to depolarization of the OSN and propagation of

the signal along the OSN axon to its terminus in the OB (Restrepo et al., 1996; Schild and Restrepo, 1998). Ciliated OSNs possess the G-protein $G_{\alpha olf}$, which uses the cyclic adenosine monophosphate (cAMP) second messenger cascade (Schild and Restrepo, 1998; Firestein, 2001) (Figure 1.1). Microvillous OSNs possess G-proteins such as $G_{\alpha i}/G_{\alpha o}/G_{\alpha q}$, which use the phosphatidylinositol-3-phosphate (IP_3) and diacylglycerol (DAG) second messenger cascade (Restrepo et al., 1996; Schild and Restrepo, 1998) (Figure 1.2).

In rodents, the ciliated and microvillous OSN types and their associated GPCRs and second messengers are the starting points for parallel olfactory processing pathways. Ciliated OSNs found in the main olfactory epithelium primarily use the canonical cAMP signaling pathway. Some ciliated OSNs express receptors or channels that differ from those typically involved in the cAMP pathway. These include trace amine associated receptors (TAARS), transient receptor potential channels (e.g. TRPM5), or the second messenger cGMP (GC-D) (Ma, 2007; Munger et al., 2009). The cGMP olfactory sensory neurons respond to components of urine and express guanylyl cyclase type D, as well as cyclic nucleotide gated channels gated by cGMP. The axons of these GC-D OSNs project to the caudal region of the main olfactory bulb, to a target region named the necklace glomeruli (Ma, 2007; Munger et al., 2009), and create one of several parallel processing pathways in the rodent olfactory system. Other further subdivisions of the main olfactory epithelium include the septal organ of Mesara and Grueneberg ganglion. For these, sensory neurons project to distinct regions of the main olfactory bulb.

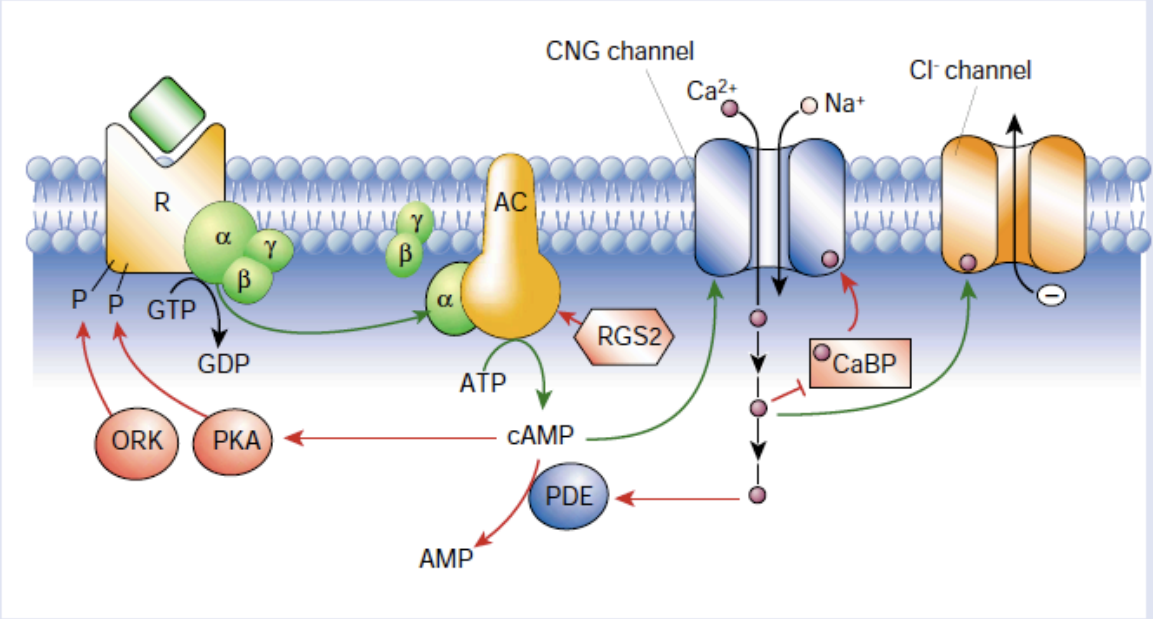

Figure 1.1. The olfactory signal transduction cascade involving the second messenger cyclic adenosine monophosphate (cAMP). The binding of an odour to a G-protein coupled receptor (R) releases the α subunit from the receptor, which binds to, and activates adenylyl cyclase (AC). The activated AC uses adenosine triphosphate (ATP) to produce cAMP, which in-turn binds to cyclic nucleotide-gated channels (CNG). The CNG channels open and Ca^{2+} and Na^{+} ions enter the intracellular environment. The increase in intracellular Ca^{2+} and Na^{+} ions causes a chloride channel to open, allowing Cl^{-} ions to flow into the extracellular environment. The movement of Ca^{2+} and Na^{+} ions inward and Cl^{-} ions outward depolarizes the olfactory sensory neuron generating an axon potential. Illustration from Firestein, 2001. Nature Vol 413: 211-218.

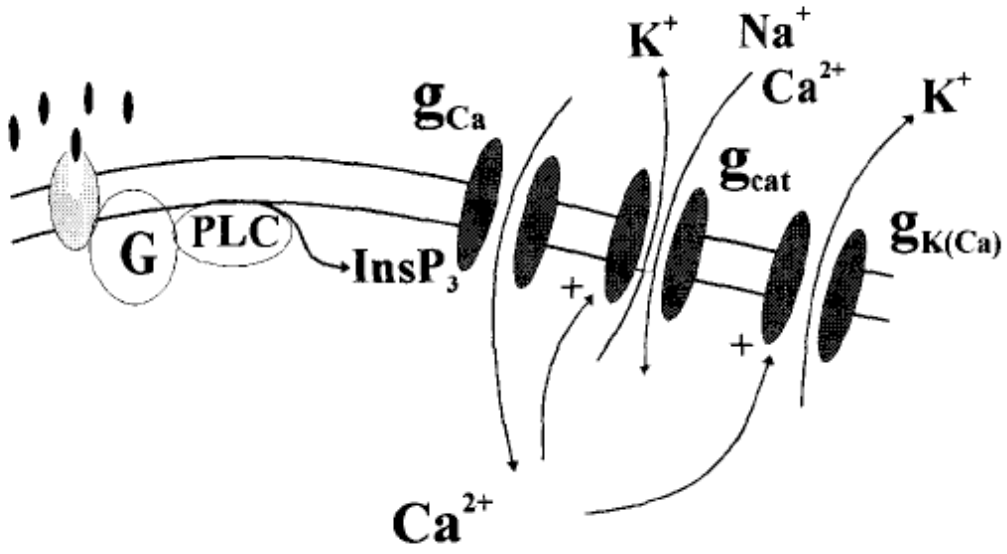

Figure 1.2. The olfactory signal transduction cascade involving the second messenger Inositol trisphosphate (IP_3). The binding of an odour to the G-protein coupled receptor activates phospholipase C (PLC). PLC in-turn produces $InsP_3/IP_3$, which binds to calcium channels, allowing Ca^{2+} to enter the intracellular environment. The increases Ca^{2+} concentration activates K^+ channels, allowing K^+ to move into the extracellular environment. The combined inward movement of Ca^{2+} and Na^+ ions inward and K^+ ions outward depolarizes the olfactory sensory neurons, generating an action potential. Illustration from Restrepo et al, 1996. Journal of Neurobiology. Vol 30: 37-48.

The rodent VNO is a primary example of a parallel pathway in the olfactory system. The VNO is located in a cavernous structure within the rostral part of the nasal cavity. The OSNs are microvillous, utilize the IP_3/DAG second messenger cascade and project their axons to the accessory olfactory bulb (AOB) (Ma, 2007). The OSNs in the dorsal portion of the VNO epithelium express V1Rs and the G-protein $G_{\alpha i}$, while OSNs in the ventral portion of the VNO express V2Rs and the G-protein $G_{\alpha o}$. The V1R expressing OSNs project their axons to the anterior portion of the AOB, while the V2R expressing OSNs project their axons to the posterior AOB (Dulac and Torello, 2003; Halpern and Martinez-Marcos, 2003).

In cyprinid fish, OSNs lack the peripheral compartmentalization seen in the rodent olfactory organ. The ciliated, microvillous, and crypt type OSNs are distributed throughout the epithelium. Ciliated OSNs project axons to the medial region of the OB, while the axons of microvillous OSNs project to the lateral region of the OB (Morita and Finger, 1998; Hamdani et al., 2001a; Hamdani and Doving, 2002; Hansen et al., 2003; Sato et al., 2005; Hamdani and Doving, 2007). The axons of crypt cells project into two small regions of the ventral portion of the OB (Hansen et al., 2003). From these examples, it is clear that diversity in location of sensory neurons, as well as OSN morphology, receptors, signal transduction mechanisms and channels are important contributors not only to olfactory sensory function, but also to the function of parallel pathways of information flow into the brain.

1.4 The Olfactory Bulb

1.4.1 What is the Olfactory Bulb?

The OB is a vertebrate forebrain structure that contains olfactory sensory input from cranial nerve I (the olfactory nerve). It is also a location of neural integration, output to higher brain centers, as well as input from centrifugal fibers projecting from higher brain centers (Shepherd and Greer, 1998). The OB contributes to vital olfactory functions, including odor coding, the sorting of sensory input towards appropriate locations in the higher brain centers, and of regulation by descending projections from higher brain centers. An example of OB function is seen in the channel catfish. The lateral region of the catfish OB receives the axons of microvillous OSNs that respond to amino acid odours, and the mitral cells (the bulbar projection neurons in teleost fish) propagate neural activity along the lateral olfactory tract, to the lateral region of telencephalon (Nikonov and Caprio, 2004; Nikonov et al., 2005). Although OB function is far from being completely understood, previous investigations in fish and in mammals have lead to some understanding regarding olfactory sensory and integrative function of this brain region. Neurophysiological studies of the analogous structure in insects, the antennal lobe, has contributed to an understanding of the processing of neural information during olfaction (Hansson et al., 1992; Kanzaki and Shibuya, 1992; Christensen et al., 1996; Laurent et al., 1996; Laurent, 2002).

1.4.2 Afferent input and the olfactory glomeruli

The OSN axons extend from the olfactory epithelium, fasciculate in the lamina propria, project into the olfactory nerve, and enter the rostral portion of the OB. These axons spread over the surface of the OB, forming the peripheral layer of this structure

(the olfactory nerve layer); then proceed deeper into the OB, and enter spherical regions of neuropil known as glomeruli. The OSN axons terminate in the olfactory glomeruli and form synaptic contacts onto post-synaptic partners, the dendritic endings of projection neurons (mitral and tufted cells) (Shepherd and Greer, 1998). Interneurons and descending projections from higher brain regions are also located in the glomerular region and participate in synaptic activity (Hildebrand and Shepherd, 1997; Gire and Schoppa, 2009).

1.4.3 Olfactory glomeruli and odour coding

Chemotopy, the spatial patterning of bulbar activity related to odorant chemical features is evident from studies of zebrafish (Friedrich and Korsching, 1997, 1998) channel catfish (Nikonov and Caprio, 2001) and mammals (Johnson et al., 1998; Mori et al., 2006; Johnson and Leon, 2007; Soucy et al., 2009). Specificity regarding OR expression on OSNs extending to the bulbar glomeruli is vital for chemotopy to occur. This is clearly illustrated in the mammalian OB, where each glomerulus receives peripheral axonal input only from OSNs expressing a single odour receptor gene (Wachowiak et al., 2004). Consequently, a specific odour ligand activates synaptic activity within the glomerulus that receives the OSNs that express the receptor for that odour ligand. The OSNs that respond to structurally similar compounds project to glomeruli that are grouped together and those that are less similar are further apart, so that the glomeruli produce a chemotopic map arranged around the surface of the OB (Johnson and Leon, 2007; Soucy et al., 2009). Imaging and electrophysiological experiments have shown that any given odour will activate a distinct group of glomeruli, with similar odours producing distinct but overlapping glomerular activity maps, and less

similar odours producing activity maps that overlap less or not at all (Sharp et al., 1975, 1977; Stewart et al., 1979; Imamura et al., 1992; Mori et al., 1992; Hara and Zhang, 1996; Friedrich and Korsching, 1997, 1998; Hara and Zhang, 1998; Johnson et al., 1998; Galizia and Menzel, 2000; Xu et al., 2000a; Xu et al., 2000b; Nikonov and Caprio, 2001; Wachowiak and Cohen, 2001, 2003; Xu et al., 2003; Nikonov et al., 2005; Xu et al., 2005; Johnson and Leon, 2007). In this way, odours are spatially encoded on the surface of the OB and within the glomeruli.

Chemotopic odour coding occurs by activating a combination of glomeruli (combinatorial coding), and also by stimulation of a single glomerular region (non-combinatorial coding). The activation of many glomeruli in the OB in response to a particular odour is known as combinatorial odour coding, due to the unique combination of glomeruli activated by a given odour. This has been observed in the zebrafish OB where amino acid odours activate a number of glomeruli in the lateral OB region (Friedrich and Korsching, 1998). There are also examples for the activation of only a single glomerulus by a given odour. This non-combinatorial spatial odour coding has been observed in the OB of zebrafish for sex pheromones (Friedrich and Korsching, 1998; Korsching, 2001) and in the antennal lobe of insects (Christensen and Hildebrand, 1987; Kanzaki et al., 1989; Kanzaki and Shibuya, 1992; Christensen et al., 1995; Hildebrand and Shepherd, 1997; Friedrich and Korsching, 1998; Korsching, 2001), where a single glomerulus (or isolated glomerular complex) is responsive to pheromones (Christensen and Hildebrand, 1987; Hansson et al., 1992; Boeckh and Tolbert, 1993; Christensen et al., 1995; Hildebrand and Shepherd, 1997; Friedrich and Korsching, 1998).

In some terrestrial vertebrates, parallel processing of olfactory information occurs in the glomeruli of two bulbar regions, the main and accessory olfactory bulbs. The MOB receives afferents from the MOE, and the axons from the VNO project to the accessory olfactory bulb. While there is some overlap in the odours that are processed in the main and accessory OB (Xu et al., 2005; Baxi et al., 2006; Spehr et al., 2006), generally the main OB processes a wide variety of volatile general odours and the accessory OB processes non-volatile pheromones and other social odour cues, which are commonly found in urine (Nodari et al., 2008; Meeks and Holy, 2009; Meeks et al., 2010; Turaga and Holy, 2012).

Generally, in fish, bile salt odours and pheromones (steroids or prostaglandins) activate glomeruli in the medial portion of the OB, except in zebrafish where pheromones activate a central portion of the ventral OB (Sato et al., 2005). In addition, amino acids activate glomeruli in the rostral lateral region of the OB and nucleotides activate glomeruli in the caudal lateral region of the OB. This pattern of glomerular activation has been observed in catfish (Nikonov and Caprio, 2001; Hansen et al., 2003), zebrafish (Friedrich and Korsching, 1997, 1998), and salmonids (Hara and Zhang, 1996; Hara and Zhang, 1998), but can vary in other fish species (See review by Zielinski and Hara, 2006).

1.4.4 The organization of neurons within the OB

The OB consists of cellular layers that are conserved across phyla, even though there are some differences with respect to cell types, stratification and organization. For example, there are six layers in the mammalian OB, and four layers in the teleost OB. The mammalian OB contains the olfactory nerve layer, glomerular layer, external

plexiform layer, mitral cell layer, internal plexiform layer, and the granule/internal cell layer (Shepherd and Greer, 1998), but the less laminar teleost OB does not contain two regions with concentrated neuropil, the external or internal plexiform layers (Satou, 1990).

In the mammalian OB, the glomeruli are very distinct due to the presence of periglomerular cells, interneurons that are in between and surround the glomeruli. Periglomerular cells innervate glomeruli and synapse with OSN axons and dendrites of the projection neurons, known as mitral cells and tufted cells. Synaptic neurotransmission within the glomeruli includes contact onto the dendrites of the primary projection neurons of the OB, the mitral/tufted cells (Shepherd and Greer, 1998). The dendrites of mitral cells extend into the glomerular layer, and the nuclei are located in the mitral cell layer. The dendrites of tufted cells are also located in the glomerular layer, but the cell bodies are located in the external plexiform layer (Shepherd and Greer, 1998). In addition, external tufted cells are present in the glomerular layer. Recent studies have shown that both external tufted cells and periglomerular cells synapse with OSN axons and form an excitatory/inhibitory microcircuit with one another in a glomerulus (Gire and Schoppa, 2009). This microcircuit acts as an intermediary between OSN axons and dendrites of mitral/tufted cells (internal and middle tufted) to process odour input information (Gire and Schoppa, 2009).

In the mammalian OB, the external plexiform layer (EPL) is located beneath the glomerular layer. It contains the dendrites of mitral cells as well as the cell bodies and dendrites of middle tufted and internal tufted cells, with middle tufted cell bodies located in the more dorsal portion of the EPL and internal tufted cell bodies located in the more

ventral portion of the EPL (Shepherd, 1972; Mori, 1987; Shepherd and Greer, 1998). The axons of both mitral cells and tufted cells project along the lateral olfactory tract. Mitral cell bodies are found within the narrow mitral cell layer, which is located between the EPL and the internal plexiform layer (IPL) (Shepherd, 1972; Mori, 1987; Shepherd and Greer, 1998). The IPL contains local interneurons as well as the axons of external tufted cells that project to the contralateral OB (Mori, 1987; Shepherd and Greer, 1998; Shepherd et al., 2007). The most internal cell layer, the granule cell layer, contains local interneurons known as granule cells which form dendrodendritic synapses with mitral cells (Mori, 1987; Shepherd and Greer, 1998; Shepherd et al., 2007) (Figure 1.3).

The OB of teleost fish has four layers: the olfactory nerve layer, glomerular layer, mitral cell layer, and granule cell layer. Fish do not possess periglomerular or external tufted cells and therefore have less-defined glomeruli/glomerular layer than that of mammals (Dryer and Graziadei, 1994). Examination by transmission electron microscopy has shown that in fish, OSN axons synapse onto the dendrites of mitral cells within a glomerulus (Satou, 1990). The primary projection neurons of the teleost OB are mitral cells. The somata are in the mitral cell layer and dendrites extend into the glomerular layer where they synapse with the axon terminals of OSNs. Interestingly, while a single mitral cell only innervates one glomerulus in mammals, they innervate more than one glomerulus in fish, as has also been observed in the mammalian accessory OB (Meisami and Bhatnagar, 1998). Mitral cells also receive inhibitory dendrodendritic synapses from granule cells (Satou, 1990; Laberge and Hara, 2001).

Some species of fish including the goldfish, catfish, sea eel, and zebrafish have another form of projection neuron known as ruffed cells, which have a distinctive ruffle

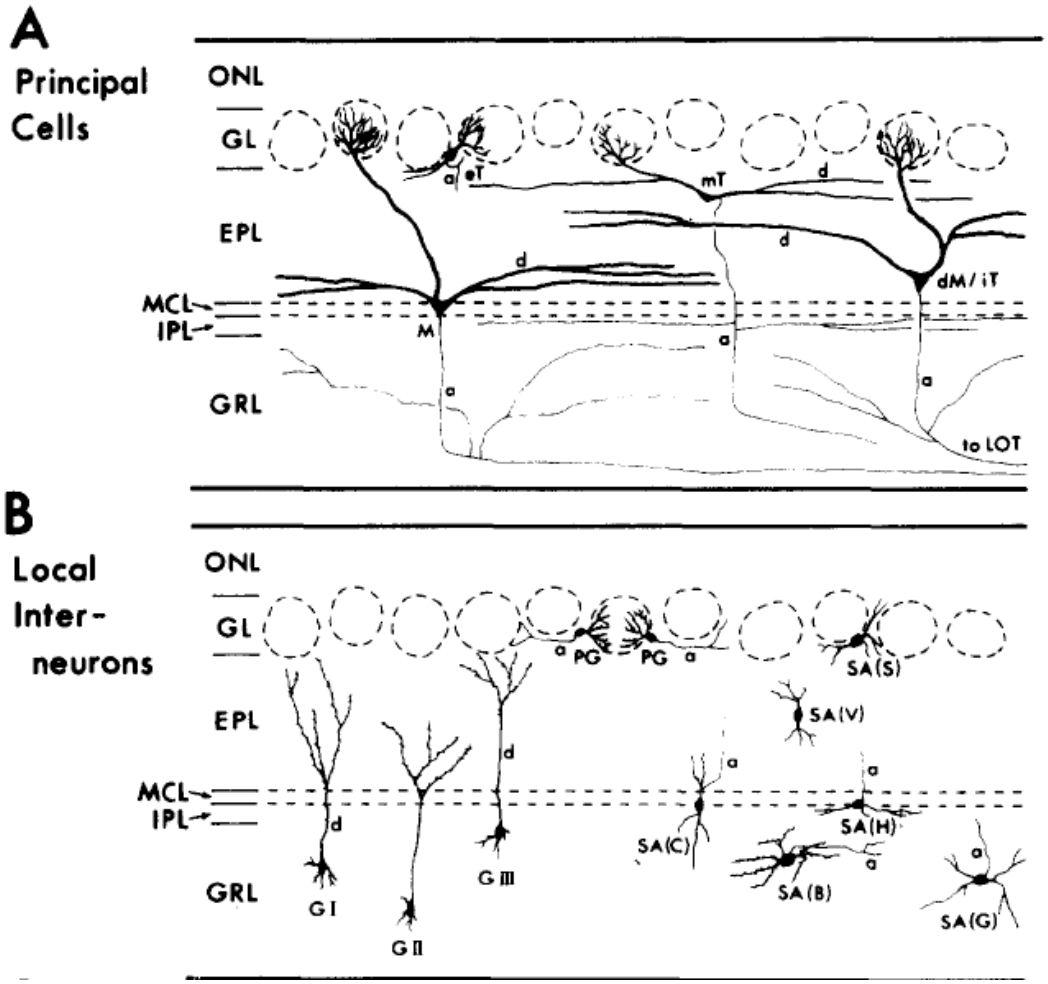

Figure 1.3. The layers and neurons of the olfactory bulb (OB) in mammals. The layers of the olfactory bulb are the olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GCL). The glomeruli are denoted by spherical structures of dashed lines in the GL. A: The principle neurons of the OB are the mitral cells (m), external tufted (eT), middle tufted (mT), and internal tufted (iT). Mitral and tufted cells possess primary and lateral dendrites (d) as well as axons (a) that project along the lateral olfactory tract (LOT). B: The local interneurons of the olfactory bulb include the periglomerular cells (PG) and granule cells (G), as well as short axon cells (SA). Modified from Mori, 1987. Progress in Neurobiology. Vol 29: 275-320.

on the initial segment of the axon (Kosaka and Hama, 1979; Kosaka, 1980; Kosaka and Hama, 1980, 1981; Satou, 1990; Fuller and Byrd, 2005). Ruffed cells are situated in the mitral cell layer and do not directly synapse with OSN axons or mitral cells, but instead receive input by axodendritic connections with granule cells, which in turn synapse with mitral cells (Kosaka and Hama, 1982). Ruffed cells project axons along the olfactory tract and may play a role in oscillatory activity observed in the OB of fish (Zippel et al., 1999; Zippel et al., 2000). Moreover, ruffed cells respond to a variety of odours in theopposite fashion as mitral cells (i.e. a given odour may elicit an excitatory response in a mitral cell and a suppressed response in an associated ruffed cell)(Zippel et al., 1999; Zippel et al., 2000; Laberge and Hara, 2001). The role of ruffed cells in odour information processing is still relatively unknown.

1.4.5 Temporal coding in the OB

Olfactory information is not only spatially encoded in the OB, but temporally encoded as well, by the firing pattern of primary projection neurons (mitral/tufted cells in mammals and fish; projection neurons in insects) (Laurent et al., 1996; Wehr and Laurent, 1996; Friedrich and Laurent, 2001; Laurent, 2002; Friedrich and Laurent, 2004; Kay and Stopfer, 2006). Primary projection neurons make dendrodendritic synapses with inhibitory interneurons, which shape the temporal structure of the PN response patterns through lateral inhibition (Laurent et al., 1996; MacLeod and Laurent, 1996; Shepherd and Greer, 1998; Urban and Sakmann, 2002; Wiechert et al., 2010). The temporal coding of odour responses along parallel processing pathways in the OB can be informative of odour identity in higher brain structures (Laurent, 2002; Wick et al., 2010). Odours

activate several mitral cells at the same time but each individual mitral cell has its own slow temporal firing pattern with increasing and decreasing firing rates over the course of the response. In addition, any given mitral cell may respond to several odours of similar structure (i.e. two different amino acids). Activity of the responding mitral cells get redistributed over the group of responding mitral cells and becomes more unique to that particular odour over the course of the response by decorrelation (Friedrich and Laurent, 2001). This process of decorrelation makes the temporal response pattern of a group of mitral cells unique to a specific odour over time, and therefore different from similar odours (Friedrich and Laurent, 2001; Laurent, 2002). This allows for higher order structures to interpret the overall slow temporal firing pattern of the collection of mitral cells to decode the stimulus identity (Friedrich and Laurent, 2001; Laurent, 2002; Wick et al., 2010). Fast oscillatory responses are also present in the OB and are generated in mitral cells by the dendrodendritic inhibitory interneurons and their circuit connections with primary projection neurons. These fast oscillatory waveforms are believed to play a role in making odour representations more unique from one another and aiding in odour identity in olfactory cortical regions for mammals (Laurent and Davidowitz, 1994; Laurent, 2002; Kay and Stopfer, 2006; Kay et al., 2009).

1.4.6 OB projection sites

The bulbar projection neurons transmit the spatially and temporally coded odour information from the OB to higher brain regions where it is integrated and ultimately affects behavioural responses. In the main OB, mitral/tufted cell axons project along the lateral olfactory tract to the anterior olfactory nucleus, olfactory tubercle, piriform cortex, amygdala, and entorhinal area. Projections from these areas go to the hypothalamus and

higher cortical regions (See review by Ma, 2007). Recent evidence suggests mitral and tufted cells in the main OB make up two parallel processing pathways that project their axons to different regions of the olfactory cortex from one another (Igarashi et al., 2012). Furthermore, mitral cells in the accessory OB have direct axonal projections to various nuclei of the limbic system. From limbic system nuclei, the accessory olfactory pathway continues to hypothalamic nuclei (Meredith, 1998; Dulac and Wagner, 2006; Ma, 2007).

In fish, mitral cells project their axons along the lateral or medial olfactory tracts to the telencephalon (Doving and Selset, 1980; Kosaka and Hama, 1982; Satou, 1990; Hamdani and Doving, 2007), where a chemotopic map similar to that observed in the OBs persists (Nikonov et al., 2005). Moreover, in sturgeon, cod, and goldfish, mitral cells also project to midbrain structures such as the preoptic area, thalamus, habenula, and hypothalamus (Vonbartheld et al., 1984; Rooney et al., 1992; Huesa et al., 2000, 2003, 2006; Zielinski and Hara, 2006). Studies by Hamdani and collaborators (Hamdani et al., 2000; Hamdani et al., 2001a; Hamdani et al., 2001b; Hamdani and Doving, 2007) in crucian carp have utilized retrograde degeneration to investigate the relationship between fish behaviour, and the lateral and medial olfactory tracts. They found that lateral olfactory tract (containing mitral cell axons from the lateral portion of the OB) controlled feeding behaviours; the medial portion of the medial olfactory tract (containing mitral cell axons from the medial region of the OB) controlled alarm reaction, and the lateral portion of the medial olfactory tract controlled reproductive behaviours. These studies have begun to explore the role of bulbar output on the organization of the olfactory system. There is clearly much more to be explored with regards to the role of targets of bulbar projection neurons on the function and organization of the olfactory system.

1.5 Olfaction in the sea lamprey

1.5.1 Olfactory signals during the life cycle

The life cycle of the sea lamprey (*Petromyzon marinus*), an extant species of ancient vertebrate lineage, consists of distinct life history phases including a larval stage, transformer stage (metamorphosis), parasitic juveniles, migrating adults, and spawning adults. Larval stage lamprey remain buried in stream sediments for approximately 3-18 years until they reach a size of 150 mm, at which time they undergo a radical metamorphosis and begin migrating downstream (Youson and Potter, 1979; Purvis, 1980; Potter et al., 1982). Once metamorphosis is complete the now parasitic lamprey move into open water and actively feed on other fish for 12-15 months (Potter et al., 1982; Bird et al., 1994). During the parasitic feeding stage, lamprey are thought to locate their prey using amino acids and amines released by the fish (Kleerekoper and Mogensen, 1963). In the spring, migrating adult lamprey move upstream and encounter a mixture of compounds that are released from larval sea lamprey in the streambed. This mixture includes petromyzonol sulphate (PZS), allocholic acid (ACA), and two disulphated aminosterol derivatives known as petromyzonamine disulphate (PADS) and petromyzonolsterol disulphate (PSDS). This mixture acts as a pheromone that migrating adult lamprey detect and use as an indicator of suitable spawning habitat (Li et al., 1995; Bjerselius et al., 2000; Sorensen et al., 2005). Moreover, once in the spawning stream it is known that spermiated male sea lamprey release the pheromones 3-keto petromyzonol sulphate (3kPZS) and 3-keto allocholic acid (3kACA), which induces movement/searching behaviours and spawning behaviours, respectively in ovulating female sea lamprey (Li et al., 2002; Siefkes et al., 2003; Siefkes and Li, 2004; Johnson et

al., 2005; Johnson et al., 2009). After spawning, adult lampreys quickly deteriorate and die.

1.5.2 The peripheral olfactory organ

The peripheral olfactory organ in lampreys, contains both a main olfactory epithelium (MOE) as well as tubular diverticula known as the accessory olfactory organ (AOO) (Hagelin and Johnels, 1955) (Figure 1.4). The MOE of sea lamprey consists of columnar cells that contain three morphotypes of olfactory sensory neurons (OSN). The three morphotypes have been termed tall, intermediate, and short OSN with the tall morphotype being most prominent and the intermediate and short morphotype less prominent (Laframboise et al., 2007). The 3 morphotypes were also found to be present in both metamorphic and reproductive adult sea lamprey indicating that these cells develop early and remain through adulthood (Laframboise et al., 2007). Interestingly, the density of OSN present in the MOE is greatest during the larval life stage when the cells are narrow and steadily decreases throughout maturation/development as the cells increase in width (Vandenbossche et al., 1995). Furthermore, a detailed examination of the peripheral olfactory organ (MOE and AOO) revealed that drastic remodelling occurs during metamorphosis. Specifically, the entire olfactory organ enlarges in size and weight as it changes from an epithelial lined tube to a nasal sac containing lamellar folds with a partial midline septum that separates the nasal sac into two regions (VanDenBossche et al., 1997). Moreover, during metamorphosis the AOO also becomes more prominent as two distinct cell types begin to form diverticula that are surrounded by blood vessels and nonmyelinated nerve bundles (Hagelin and Johnels, 1955; VanDenBossche et al., 1997).

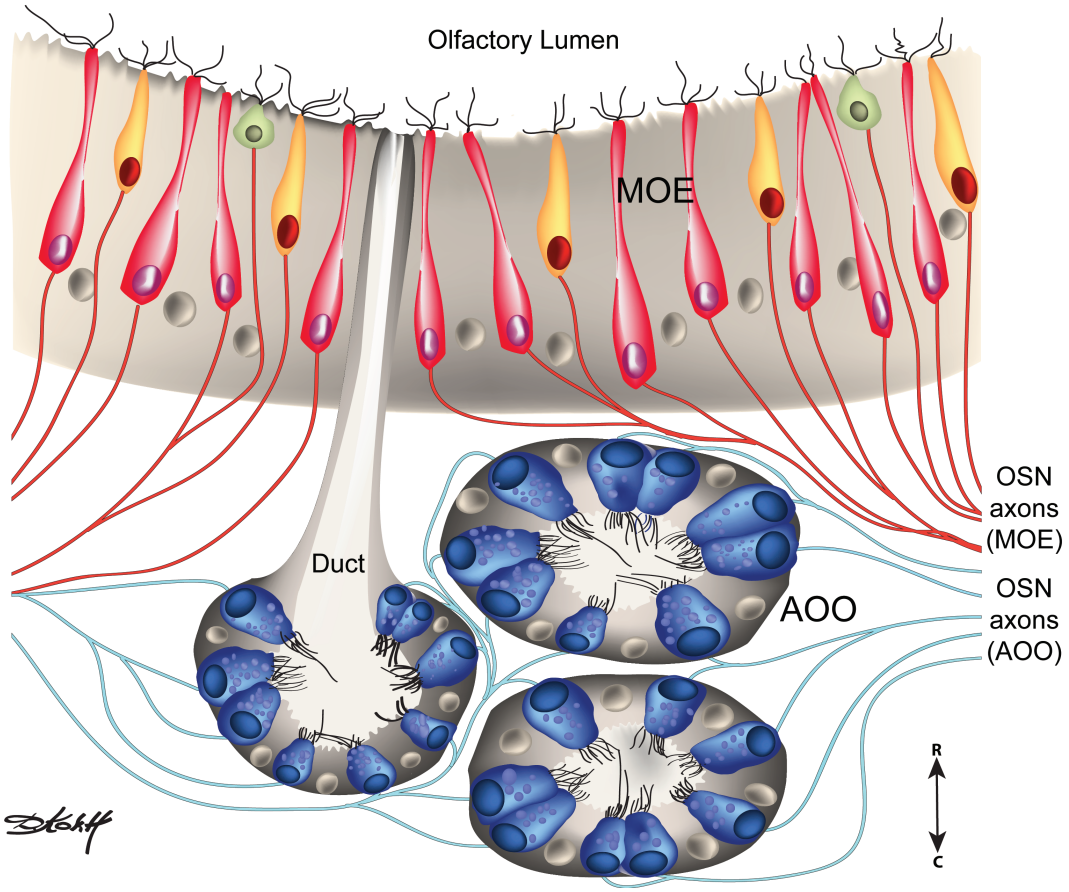

Figure 1.4. The Main and accessory olfactory organs of the sea lamprey. The main olfactory epithelium (MOE) consists of tall (red), intermediate (yellow) and short (green) olfactory sensory neurons (OSNs). The OSNs extend their cilia into the lumen of the olfactory organ and project their axons (red) to primarily the non-medial regions of the olfactory bulb, and sparsely to the medial region of the olfactory bulb. The OSNs of the accessory olfactory organ (blue) are ovoid shaped and extend their cilia into the lumen of the accessory olfactory organ (AOO), which is connected to the MOE via ducts. The OSNs of the AOO project their axons exclusively to the medial region of the olfactory bulb in the sea lamprey.

Several electrophysiological studies have demonstrated that the MOE physiologically responds to amino acids as well as a variety of biologically important bile acids. Li et al (1995) recorded extracellular field potentials from the MOE of migratory adult sea lamprey in response to the bile acids PZS and ACA of larval sea lamprey. Interestingly, of 38 bile acids tested, migratory adult sea lamprey showed very high olfactory sensitivity, with threshold responses near 10^{-12} M, for PZS and ACA (Li et al., 1995). Furthermore, a subsequent cross-adaptation (inhibition) study suggested that there are at least four independent receptors within the MOE for bile acids including PZS and ACA (Li and Sorensen, 1997). An examination of the genome revealed that lampreys possess the canonical olfactory receptors (ORs), as well as trace amine associated receptors (TAARs), and vomeronasal type 1 receptors (V1Rs) (Libants et al., 2009). Due to the relatively inaccessible location and small size there is presently no information regarding the physiological responses of the AOO to odourants.

1.5.3 The lamprey olfactory bulb and bulbar projections

Iwahori et al (1987) described the OB of the lamprey, *Lampetra japonica*, as having four layers that include: the layer of the olfactory fibers, layer of the olfactory glomeruli with mitral cells, the granule cell layer, and the layer of ependymal cells. The layer of olfactory fibers consists of fiber bundles that cover the entire surface of the OB and travel in many different directions. These fiber bundles eventually terminate in olfactory glomeruli within the glomerular layer of the OB (Iwahori et al., 1987). The glomerular layer of the OB contains numerous aggregations of spherical or oval

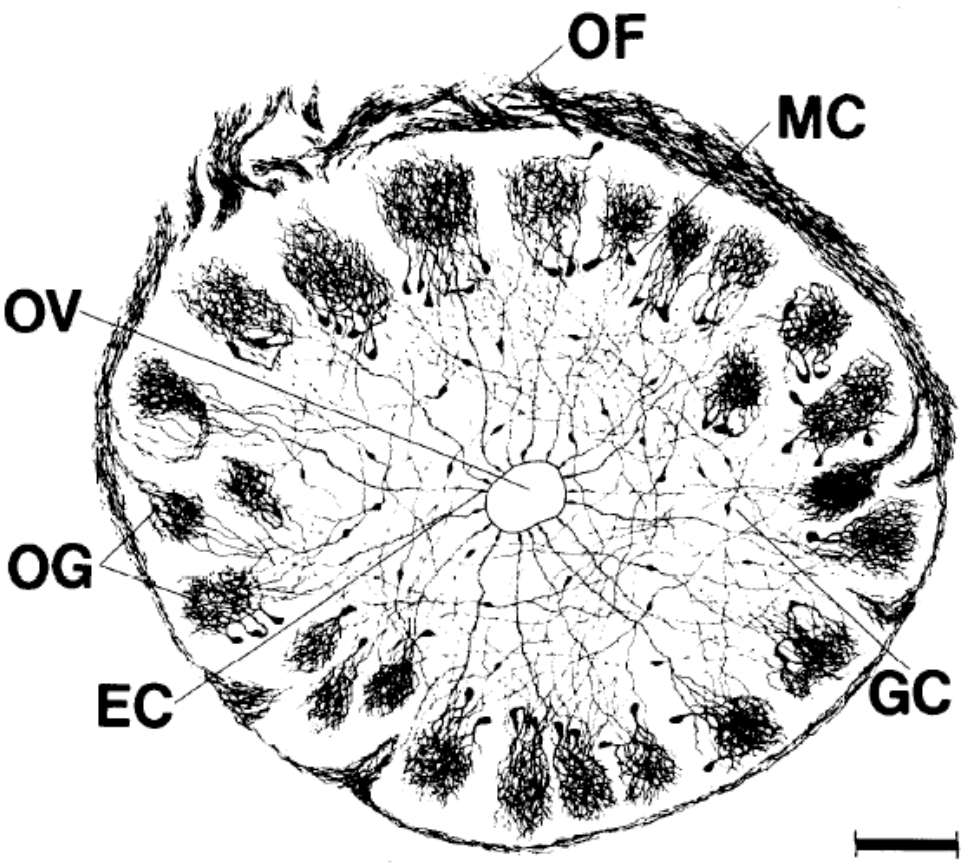

Figure 1.5. The layers of the olfactory bulb (OB) of the lamprey (*Lampetra japonica*). The lamprey OB consists of four layers including the layer of the olfactory fibers (OF), layer of the olfactory glomeruli (OG) with mitral cells (MC), the granule cell layer (GC), and the layer of ependymal cells (EC). The olfactory ventricle (OV) is also shown. Mitral cells (MC) are located near the glomeruli as well as deeper in the OB near the granule cell layer. The layers of the lamprey OB are not as laminar as seen in the OB of mammals or teleost fish. Scale bar is 200 μm. Illustration from Iwahori et al, 1987. Neuroscience research. Vol 5: 126-139.

glomeruli that contain olfactory nerve fibers, mitral cell bodies and dendrites, as well as projections from both the granule and ependymal cells (Figure 1.5). Two morphologically distinct types of mitral cells were observed in the lamprey OB based on soma size and shape and both were found in close proximity to OB glomeruli. Interestingly, Iwahori et al (1987) also found that some mitral cell dendrites terminated in two or more glomeruli but that a majority of mitral cells terminate into a single glomerulus. The granule and ependymal cell layers comprise a large portion of the lamprey OB and processes from these cells extend into the glomerular cell layer as well (Iwahori et al., 1987).

Currently, in sea lamprey it is unknown how olfactory information is organized in the OB and whether a chemotopic map, similar to that observed in teleost fish, contributes to odourant discrimination. Frontini et al (2003) demonstrated signs of glomerular territories by reporting that OSNs projecting to dorsal, lateral and ventral glomeruli were immunoreactive for the olfactory regulator G protein G_{aolf}, the OSNs projecting to the medial region lacked this immunoreactivity. This indicates that the medial region of the OB is biochemically different from the other regions of the OB and lends evidence towards the existence of spatial organization within the sea lamprey OB. Tract tracing experiments have shown that OSNs from the MOE extend to the medial and lateral regions of the ipsilateral OB and that the AOO contains sensory neurons that project axons solely to the medial region of the OB (Ren et al., 2009). Moreover, PNs in the medial region of the OB project their axons to the posterior tuberculum (PT), which relays to the mesencephalic locomotor region (MLR); neurons in the MLR project to the reticulospinal neurons in the hindbrain responsible for locomotion in the sea lamprey

(Derjean et al., 2010). This pathway from the medial OB to the hindbrain constitutes the neural substrate for olfactory-driven locomotion in the sea lamprey (Derjean et al., 2010) (Figure 1.6). Neural projections of primary projection neurons from the lateral OB of sea lamprey project to the lateral pallium as well as other forebrain regions and do not seem to connect to reticulospinal neurons (Derjean et al., 2010) (Figure 1.6). Therefore, this other olfactory pathway is likely not directly involved in locomotion but may be involved in further integration of odour information (Derjean et al., 2010). This division of neural projections from the peripheral olfactory organ to the OB also indicates that there is a possibility of spatial organization within the OB of the sea lamprey. This further implies that the OBs may be organized according to functional output rather than olfactory sensory neuron input.

1.6 Overview of the thesis

This thesis examines olfactory subsystems at the base of vertebrate evolution in the OB of the sea lamprey. Previous work by Ren et al. (2009) showed that peripheral input from the accessory olfactory organ into the lamprey OB was limited to the medial region of the OB. In addition, Derjean et al. (2010) showed that olfactory-locomotor control is exerted by projection neurons in the medial region of the OB. These studies provide a foundation for experiments that probe the following two predictions: 1) that there are neuroanatomical differences between the projection neurons located in the medial region of the OB that extend to the posterior tuberculum and those bulbar projection neurons in the nonmedial OB regions that project to the lateral pallium (Chapter 2); and 2) that chemosensory responses display regional differences in the OB, which correlate with the output organization of medial and non-medial projection

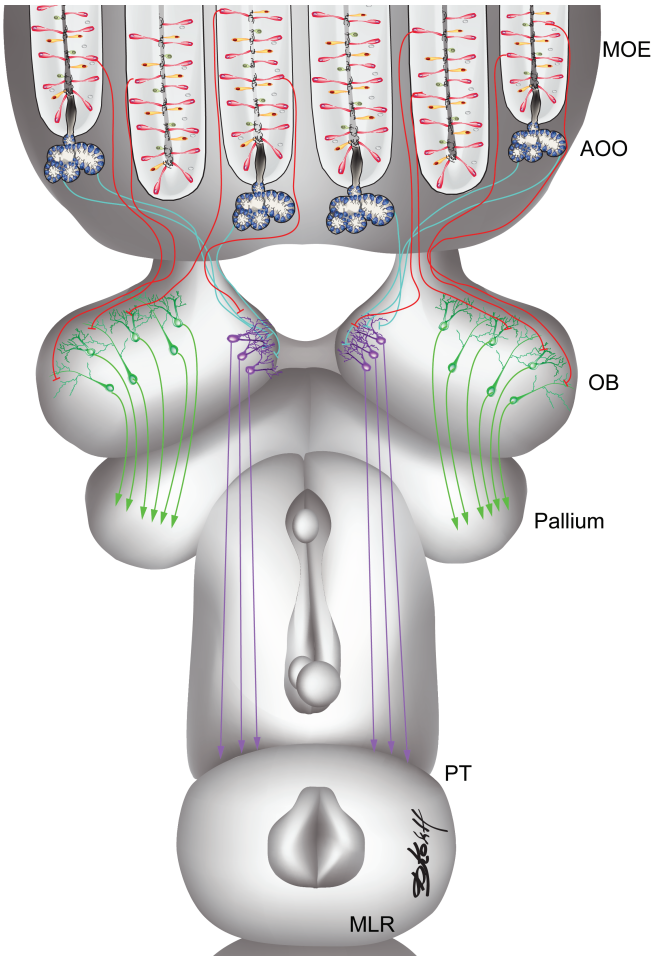

Figure 1.6. Parallel olfactory processing pathways of the olfactory bulb in the sea lamprey. The medial region of the olfactory bulb receives inputs primarily from the OSNs (blue) of the accessory olfactory organ (AOO) (blue) and sparsely from the OSNs (red) of main olfactory epithelium (MOE) (red). Primary projection neurons of the medial olfactory bulb (purple) project their axons (purple) to the posterior tuberculum (PT). Neurons of the PT project to the mesencephalic locomotor region (MLR), which connects to the reticulospinal cells in the hindbrain that are responsible for initiating locomotion. This medial OB pathway constitutes the neural substrate for olfactory-driven locomotion in the sea lamprey. Conversely, the non-medial regions of the olfactory bulb receive inputs exclusively from the MOE (red). Primary projection neurons of the non-medial OB regions (green) project their axons (green) to pallial regions of the forebrain. This pathway is proposed to be involved with further integration of olfactory information in the sea lamprey (Derjean et al, 2010).

neurons (Chapter 3).

In particular, I examined the distribution, location, size, and shape of projection neuron soma in the parallel processing pathways (medial and non-medial) of the sea lamprey OB. In addition, I used two different electrophysiological methods known as multi-unit and local field potential recordings to examine the odour-induced response of primary projection neurons to four different groups of odours in three distinct regions of the OB (Figure 1.7). These three regions were the medial, dorsal, and lateral regions of the OB, which relate to the medial and non-medial parallel olfactory pathways previously described in the sea lamprey. Two recording techniques were utilized in order to examine odour-induced OB responses at both the individual/few neuron response scale and population level response scale. Moreover, in both types of recordings the spatial and temporal characteristics of the odour-induced responses were examined across all four odour groups and all three OB regions.

Finally, over the duration of my thesis I co-authored two manuscripts not presented in the main body of this thesis, but still relevant to my studies in the OB of the sea lamprey. This work examined the organization of olfactory sensory neuron input to the OB from both the main and accessory olfactory organs of the sea lamprey, which contributed to a published manuscript I co-authored entitled "Projections from the accessory olfactory organ into the medial region of the OB in the sea lamprey (*Petromyzon marinus*): A novel vertebrate sensory structure?" by Ren et al. (2009) in the *Journal of Comparative Neurology* (Appendix A). In addition, I co-authored a review paper entitled "Chemosensory-induced motor behaviours in fish" by Daghfous et al. (2012) in the journal *Current Opinion in Neurobiology* (Appendix B). This manuscript

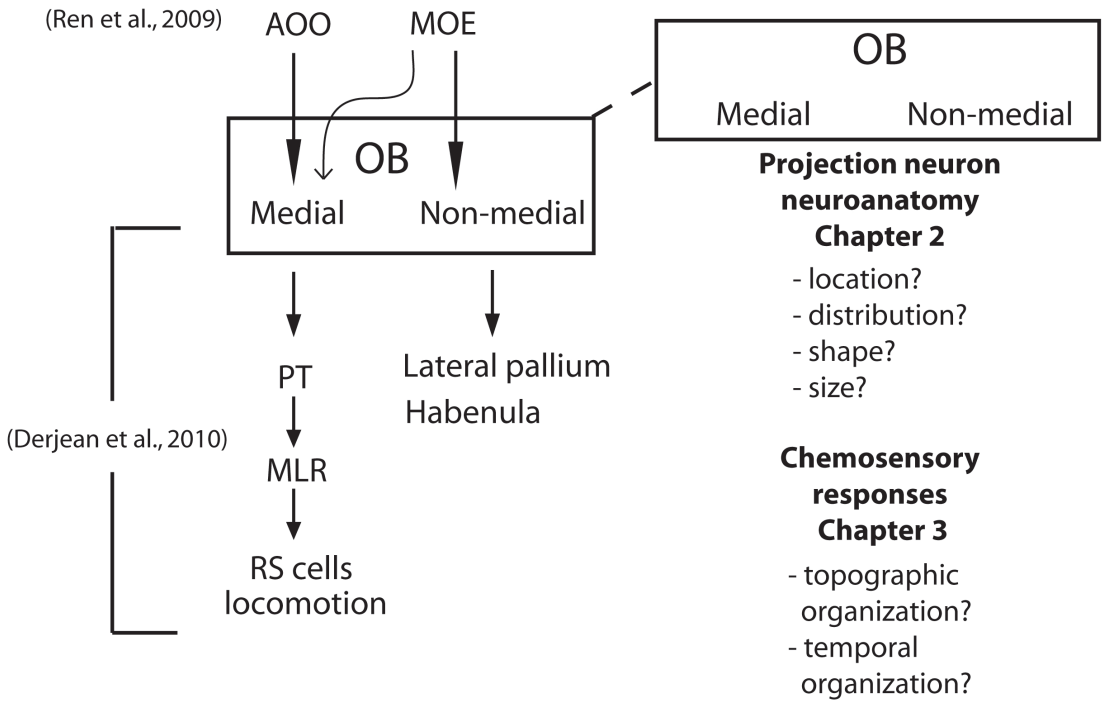

Figure 1.7. Flow diagram of the knowledge gaps and questions addressed in this thesis. Previous work by Ren et al. (2009) and Derjean et al. (2010) illustrate the parallel olfactory pathways in the lamprey. The 'flyout' of the olfactory bulb (OB) shows the questions that this thesis addresses with respect to the neuroanantomy and physiology of projection neurons in the OB. Accessory olfactory organ (AOO), main olfactory epithelium (MOE), posterior tuberculum (PT), mesencephalic locomotor region (MLR), reticulospinal cells (RS cells).

examines the role of chemosensory systems, especially the olfactory system, in driving behaviours in fish and highlights the olfactory-locomotor neural substrate of the sea lamprey described in part by Ren et al (2009) and in its entirety by Derjean et al (2010).

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CHAPTER 2

The neuroanatomy of projections neurons in two parallel pathways of the olfactory bulb

2.1 Introduction

An animal's environment contains a variety of chemosensory cues with the potential of eliciting context-specific movements (Gray et al. 2005; Kimchi and Dulac 2007; Johnson et al. 2009). Recently, the entire neural substrate underlying olfactory-based locomotor transformation was described in a vertebrate, the sea lamprey (*Petromyzon marinus;* Derjean et al. 2010). Olfactory sensory input is conducted by projection neurons (PNs) in the medial region of the olfactory bulb (OB) to the posterior tuberculum and stimulate neurons projecting to the mesencephalic locomotor region, where projecting axons activate reticulospinal cells - the motor command neurons of the hindbrain. The sensory inputs to this medial region of the OB are predominately from the accessory olfactory organ (Ren et al. 2009) and are biochemically distinct from the rest of the OB (Frontini et al. 2003). The axons of olfactory sensory neurons projecting into the glomeruli of the OB (both medial and nonmedial) can be visualized by labeling with GS1B4 lectin (Tobet et al. 1996; Frontini et al 2003). The axonal projections of projection neurons from non-medial regions of the lamprey OB are located in various forebrain structures, but not in the posterior tuberculum (Northcutt and Puzdrowski 1988, Polenova and Vesselkin 1993) and stimulation of these non-medial bulbar regions does not stimulate locomotor activity (Derjean et al., 2010). However, it is unknown if the arborization of the dendrites and localization of the cell bodies of the medial second order neurons differs from the PNs in rest of the OB. Moreover, it is not known if these PNs differ during the migratory or spawning adult stages, when locomotor responses to pheromones are of primary importance (Sorensen et al. 2005; Johnson et al. 2009).

Dendritic morphology is an important indicator of synaptic interactions. For invertebrate olfactory antennal PNs, (*Bombyx mori*), dendrites are heterogenous and depend on the innervating glomeruli (Namiki and Kanzaki 2011). For the PNs in the mammalian OB (mitral/tufted cells), a single primary dendrite extends radially from the cell body into a single glomerulus and lateral branches (secondary dendrites) extend horizontally into the external plexiform layer (Shepherd 1972). Mitral cells possess multiple primary dendrites that extend radially into multiple glomeruli in the mammalian accessory olfactory bulb (e.g. Shepherd and Greer 1998; Meisami and Bhatnagar 1998; Del Punta et al. 2002) and in the OB of teleost fish (e.g. Dryer and Graziadei 1994; Alonso et al. 1988; Satou 1990; Fuller et al. 2006).

J.B. Johnson (1902) observed that mitral cells were not well developed in lamprey, and Nieuwenhuys (1977) pointed out that the position and shape of mitral cells in this basal vertebrate differed from the typical mitral cells of teleost fish or terrestrial vertebrates. The cell bodies did not form a distinct layer, but were dispersed between as well as beneath glomeruli (Heier 1948; Iwahori et al. 1987; Viller-Cervino et al. 2011); multiple primary dendrites extended into glomerular neuropil and lateral branches were absent (Iwahori et al. 1987). These descriptions of mitral cells relied on Golgi impregnation (Johnson 1902; Heier 1948; Iwahori et al. 1987), Bodian staining (e.g. Heier 1948), immunocytochemistry and *in situ* hybridization (Viller-Cervino et al. 2011). While effective at staining neurons, these techniques do not allow for precise localization of PNs that target specific regions of the brain. Both neural connectivity and dendritic morphology are evident by retrograde tract tracing.

The objective of this study was to investigate the distribution and morphology of

the dendrites and the somal position of PNs that transmit olfactory inputs to locomotor centers (the medial projections to the posterior tuberculum; Derjean et al. 2010), to compare these with the PNs that connect to the lateral pallium; and to look for clues of spatial boundaries imposed on the medial PNs compared to the non-medial counterparts. Since retrograde neuronal labelling was utilized, we have designated the labelled cells as PNs, rather than mitral cells. These studies show that medial PNs exhibit a confined pattern of dendritic localization and morphometric parameters that likely affect the transmission of synaptic input to locomotor output in newly transformed as well as spawning adult stages.

2.2 Materials and Methods

2.2.1 Experimental Animals

We compared metamorphic stage seven or downstream migrants (transformers) and spawning-phase adult sea lamprey (Potter et al. 1982) (n=60) to test for possible morphological or morphometric differences in the PNs that extended from the OB during these two life stages. Transformers and spawning-phase adults were caught from Michigan streams and held at the U.S. Geological Survey Lake Huron Biological Station in Millersburg, Michigan, or were caught in Ontario streams and housed at either the Department of Fisheries and Oceans Canada Sea Lamprey Control center in Sault Ste. Marie, Ontario, or at the Boyd Field Centre, Toronto Region Conservation Authority, Downsview, Ontario. All animals were transported to the University of Windsor and housed in aquaria at 7 °C ± 1 °C in dechlorinated water under static renewal conditions. Transformer and adult sea lamprey weighed 4.2g ± 0.4 and 259.5g ± 6.9 (mean ± SEM) respectively, and measured 12.8 cm ± 0.8 and 47.2 cm ± 0.8 (mean ± SEM), respectively.

All housing conditions and experimental procedures were in compliance with the Canadian Council on Animal Care. Special care was taken to minimize the number of animals used in this study.

2.2.2 Cresyl violet staining of the olfactory bulb

Sea lampreys were euthanized in a 1 g/L solution of tricaine methane sulfonate (MS222, FINQUEL, Ayerst Laboratory, New York) and decapitated. The brain was isolated within the cartilaginous skull and the olfactory bulbs, pallia, and midbrain were exposed. The entire dissection was conducted in cold, oxygenated Ringer's solution (130 mM NaCl, 2.1 mM KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4.0 mM HEPES, 4.0 mM dextrose, and 1.0 mM $NaHCO_3$, pH 7.4). The excised tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4) for at least 24 hours. The tissue was then cryoprotected using a sucrose gradient (10%, 20%, then 30% sucrose in 0.1M phosphate buffered saline; PBS) and cryosectioned (Leica CM3050 S, Leica Microsystems, Germany) in serial 14 μm coronal sections. Tissue sections were dehydrated using an ethanol gradient (70%, 95%, 100%), cleared (Histosol™, National Diagnostics, Georgia, USA. Cat # HS-100), rehydrated (100% - 70% ethanol), and stained with cresyl violet. Tissue sections were subsequently differentiated (in acetic acid solution), dehydrated (70 – 100 % ethanol), cleared (Histosol™) and coverslipped with permount (Fisher Scientific, cat # SP15-100).

2.2.3 Retrograde tracing of olfactory bulb PNs

Biocytin (B-1592, Invitrogen, Burlington, ON) was utilized here to retrograde label the cell bodies and dendrites of PNs extending from the OB. Biocytin's relatively small molecular weight (372 kDa), makes it useful when labelling fine processes. It was

previously used as an anterograde and retrograde tracer (Kobbert et al. 2000), and as a retrograde label in the sea lamprey (Ren et al. 2009). For this study, biocytin was dissolved in deionized water, and then allowed to dry on a slide. The remaining biocytin crystals were applied onto the tip of tungsten probes (tip diameter = 1μm; 501316, World Precision Instruments, Sarasota, FL) for loading into brain tissue. Sea lampreys were anaesthetized in a 100 mg/L solution of tricaine methane sulfonate (MS222, FINQUEL, Ayerst Laboratory, New York) and decapitated. The brain was isolated within the cartilaginous skull and the olfactory bulbs, pallia, and midbrain were exposed. The entire dissection was conducted in cold, oxygenated Ringer's solution (130 mM NaCl, 2.1 mM KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4.0 mM HEPES, 4.0 mM dextrose, and 1.0 mM $NaHCO_3$, pH 7.4). Derjean et al. (2010) showed that the axons of PNs in the medial region of the OB terminate in the ventral diencephalon, specifically, the posterior tuberculum. Accordingly, these PNs were labeled by inserting biocytin crystals into the ventral diencephalon including the posterior tuberculum. Since axons of non-medial PNs project to and terminate in the lateral pallium (Derjean et al. 2010), these PNs were labeled by cutting the pallium and inserting biocytin crystals into the entire lateral pallium. The brain was then incubated in cold oxygenated Ringer's solution for 8 hours to allow biocytin to move retrogradely in the axon and label PN cell bodies and dendrites. Following incubation, the tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4) for at least 24 hours. The tissue was then cryoprotected using a sucrose gradient (10%, 20%, then 30% sucrose in 0.1M phosphate buffered saline; PBS) and cryosectioned (Leica CM3050 S, Leica Microsystems, Germany) in serial 35 μm coronal sections. The cryosections were rehydrated in 0.1M PBS and then placed in

Alexa Fluor 568 streptavidin (S11226: 1:200 Molecular Probes, Eugene, OR) in 0.1 M PBS overnight at 4 °C to visualize the biotinylated PNs. Slides were rinsed with 0.1M PBS 3 times for 10 minutes and coverslipped using fluoromount-G (cat. # 0100-01, Southern Biotech, Birmingham, AL). The dura was removed from all preparations, since endogenous expression of avidin binding sites was located in the dura (data not shown) in negative controls that were labelled with streptavidin, but did not receive an insertion of biocytin. The region between adjacent olfactory bulbs was inaccessible for removing dura; therefore a small amount of dura remained on the medial surface of the OB for these experiments.

2.2.4 $GS1B_4$ labelling of olfactory glomeruli

The *Griffonia simplicifolia* lectin I, isolectin B4 ($GS1B_4$) binds to galactosyl residues present on the axons of lamprey olfactory sensory neurons (Tobet et al. 1996; Zielinski et al. 2000). It was utilized to show the spatial relationship between retrogradely labeled PNs and the axons of olfactory sensory neurons. After the biocytin filled PNs had been labelled with Alexa Fluor Streptavidin 568, these slides were placed into Fluorescein 488 *Griffonia simplicifolia* Lectin I, isolectin B4 (10 μg/ml, FL-1201, Vector Laboratories, Burlington, ON) and incubated overnight at 4°C. Slides were then rinsed in 0.1 M PBS (3 rinses, 10 minutes each) and coverslipped.

2.2.5 Microscopy

Tissue sections were observed on a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) and images were archived with a Q-Imaging Retiga 1300 digital camera (Q-Imaging Corporation, Burnaby, B.C., Canada) using Northern Eclipse software (EMPIX Imaging Inc., Mississauga, Ontario, Canada). Confocal microscopy

(Olympus FluoView FV1000 FV-10 ASW, Olympus, Tokyo, Japan) was used to investigate the morphology of PNs, including the dendritic arborizations.

2.2.6 Morphometry

The location and size of the bulbar region containing the medial PNs was determined in transformer (n=4) and migratory adult (n=3) lampreys. The rostral-caudal location of the medial PNs as well as the length of the OB was determined by counting the number of sections from the rostral surface to the caudal-most section containing glomeruli – as indicated by $GS1B_4$ lectin labelling, and multiplying the number of sections by the section thickness (35 μm). The distance occupied by the medial PNs was determined by multiplying the number of sections that contained labelled PNs somata by the thickness of each section (35 μm).

The diameter of PN cell bodies on their major (longest) axis was measured using Northern Eclipse software (EMPIX Imaging Inc., Mississauga, Ontario, Canada). We measured only PN cell bodies that were visible in their entirety by focusing through sections with a 40X oil immersion objective (NA 1.3). The length of the major axis (diameter along the longest axis) was measured in the plane of focus where the cell was largest. A total of 50 PNs were selected in medial (n=5) and non-medial labelled (n=5) OB tissue in both transformer and adult sea lamprey. Values reported for PNs cell body diameters are mean ± SD. Post acquisition analysis of differences in cell body diameter between the medial and non-medial regions of the OB passed the statistical test for normality but not for equal variance, thus a Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey *post hoc* pairwise-comparisons was used (Sigmastat, Systat software Inc).

The primary dendrites were directed outwardly towards a glomerulus (Shepherd 1972) and were differentiated from an axon based on size (dendrites have a thicker diameter than axons (Iwahori et al. 1987)). Generally, PN cell dendrites project towards the surface of the OB, while axons project towards the centre of the OB (Iwahori et al. 1987). We utilized nomenclature defined by Fuller et al. (2006) for designating the dendritic composition of the PNs as unidendritic (possessing a single primary dendrite) or multidendritic (with two or more primary dendrites). Tallies of PNs (unidendritic and multidendritic) were performed utilizing two transformer and two adult lamprey for both the medial (10 PNs/tissue; n=4; 40 PNs total) and non-medial (20 PNs/tissue; n=4; 80 PNs total) regions of the OB. Only PNs that included the entire cell body and at least the proximal portion of these neuronal processes within a tissue section were assessed as unidendritic or multidendritic. Moreover, the classification of PNs as unidendritic or multidendritic was established by through-focus examination and confocal microscope z-stack reconstruction of these cells. In the confocal z-stacks, each section was examined from the proximal portion of the dendrite at the cell body to the distal end of the dendrite to ensure the continuity of the dendrites.

2.3 Results

In accordance with Iwahori et al. (1987), two strata - the layer with glomeruli and mitral cells and the granule cell layer (Figure 2.1) - were prominent in coronal views of the lamprey OB.

2.3.1 Localization of Olfactory Bulb PNs

Biocytin, inserted into the ventral diencephalon, retrogradely labeled medial PNs along the rostral-caudal extent of the medial glomerular neuropil, without labeling cells

in other regions of the OB (Figure 2.2 A-C). Non-medial PNs were labeled following biocytin application to the lateral pallium (Figure 2.2 D-F); and the dendrites of these non-medial PNs did not extend into the medial glomerulus (Figure 2.3). Axonal fibers extended caudally and were traced back to the biocytin injection site. In both post metamorphic and spawning adult stages, the medial PNs were clustered within glomerular neuropil that spanned the middle third of the length of the OB. Although the OB was approximately 1.5 X longer in the spawning adult compared to the post metamorphic stage, the position and relative length occupied by dendrites and somata of the medial PNs remained unchanged (Table 2.1). These measurements indicate the bulbar length occupied by the medial PNs increased proportionately with the total length of the OB, from the transformer to the spawning adult stage.

The double labelling of PNs (with biocytin) and of the extracellular surface of the axons of olfactory sensory neurons (with $GS1B_4$ lectin) revealed that the cell bodies and dendrites of the medial PNs were confined to the glomerulus (Figure 2.4 A, B). For non-medial PNs, the somal position was outside (often proximal) to glomeruli, and dendrites extended into glomerular neuropil (Figure 2.4 C, D).

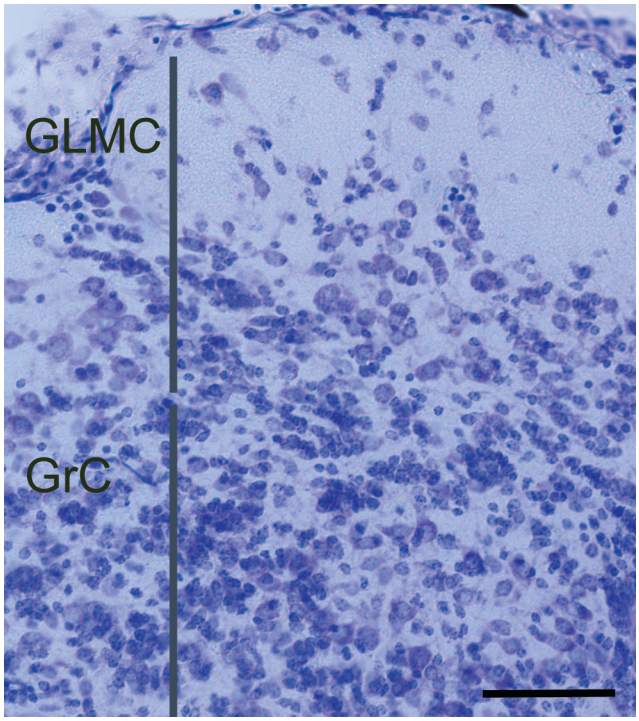

Figure 2.1. A cresyl violet stained cross section of the olfactory bulb from a transformer stage sea lamprey shows the layer with mitral cells and glomeruli (GLMC) and the granule cell layer (GrC). The scale bar is 100 μm.

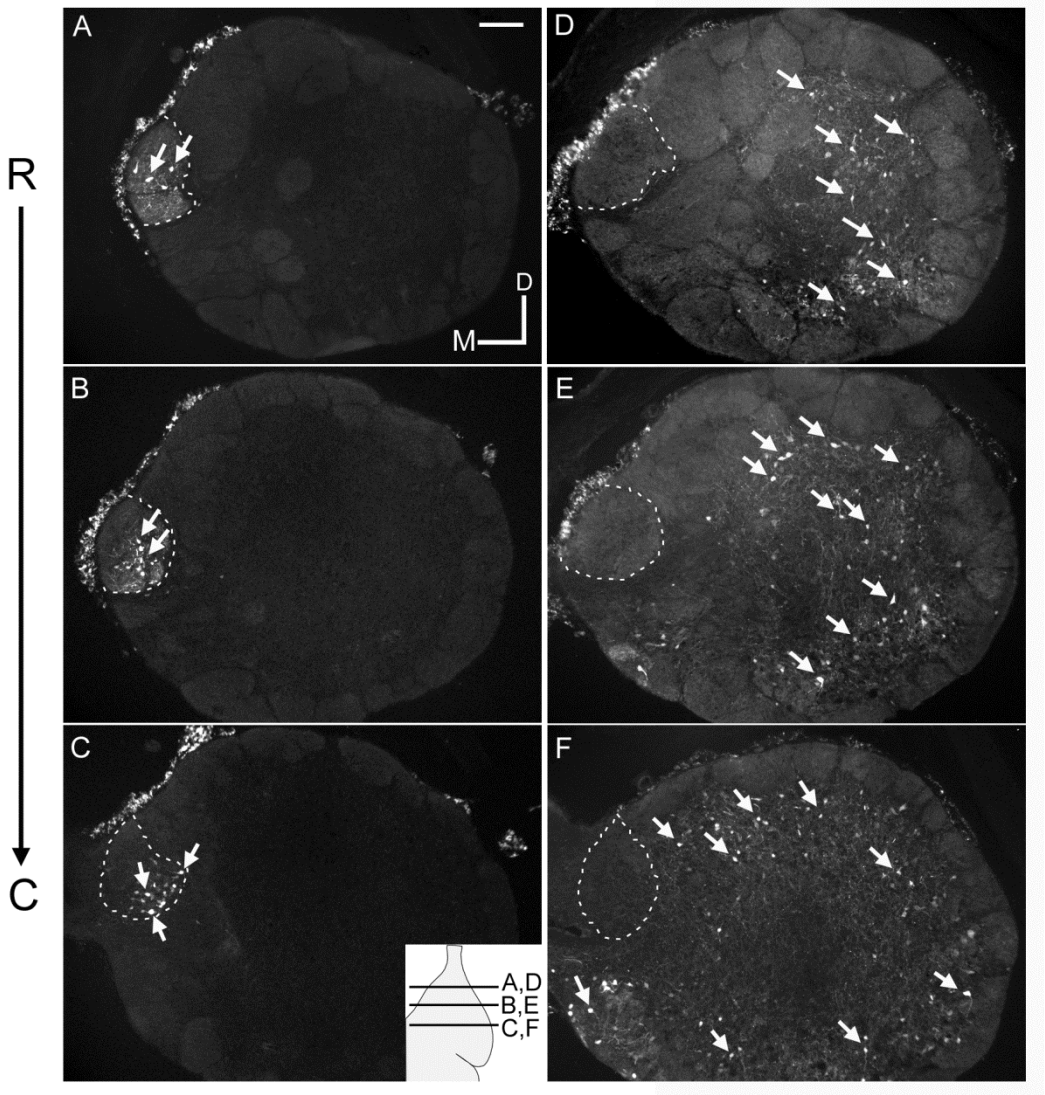

Figure 2.2. Rostral-caudal distribution of retrograde labeled PNs in coronal sections of the transformer olfactory bulb following insertion of biocytin into the posterior tuberculum (A-C) or the lateral pallium (D-F). Biocytin insertion into the posterior tuberculum exclusively labelled medial PNs (A-C) while biocytin insertion into the lateral pallium labelled non-medial PNs (D-F). Scale bar, shown in A (125 μm) is the same for all images. Inset in C is a diagram of the olfactory bulb in the horizontal plane with lines representing the location of the coronal sections. Dashed lines in A-F outline the location of the medial glomerular neuropil. Arrows point to retrograde labeled cell bodies.

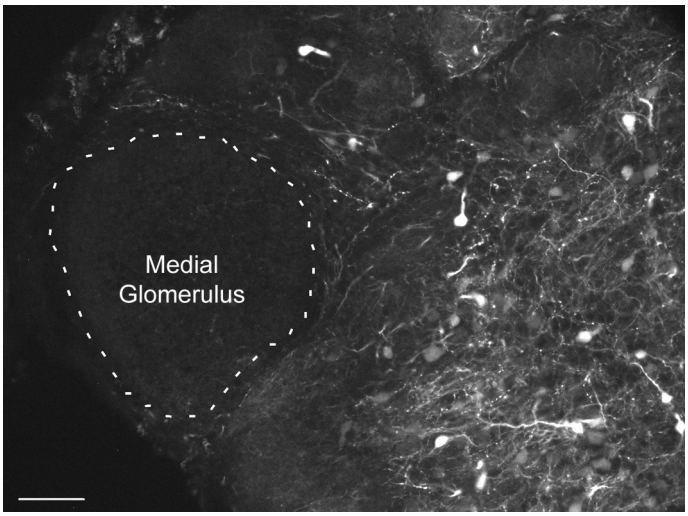

Figure 2.3. Non-medial projection neurons (PNs) retrograde labeled from the lateral pallium did not enter the medial glomerulus. Dashed line denotes the boundary of the medial glomerulus. Scale bar is 60 μm.

2.3.2 Size of PN Somata

In adult lamprey, the cell body diameter of medial PNs was 19.56 ± 1.82 μm (mean ± SD) compared to 15.32 ± 1.93 μm (mean ± SD) in non-medially located PNs (Kruskal-Wallis, H_3=137.5, P<0.001). A difference in cell body size was also observed in transformer stage lamprey, as medial PN cell body diameter was 14.05 ± 1.50 μm (mean ± SD) compared to 12.56 ± 0.90 μm (mean ± SD) in non-medial PNs (Kruskal-Wallis, H_3=137.5, P<0.001). These findings indicate that PN cell body size was always greater in spawning adult lamprey compared to transformer lamprey regardless of whether cell bodies were medial or non-medial (Table 2.1). However, medial PNs were larger than non-medial PN cell bodies in both post metamorphic (transformer) and spawning adult lampreys.

2.3.3 Projection neuron morphology

The morphology of medial (Figure 2.5 A-D) and non-medial (Figure 2.5 E-H) retrogradely labelled PNs was similar in many ways. In both locations, the shape of the labelled cell bodies was mitre, ovoid, or elongate. The dendrites were classified as primary dendrites (Shepherd et al. 1972) since they extended towards glomeruli, into glomeruli or were situated entirely within glomeruli (in the case of the medial PNs). Secondary dendrites (extending laterally into the granular layer) were absent. Dendrites of medial PNs were oriented radially. Dendrites of non-medial PNs were also radial, although some tangentially oriented dendrites were also observed (Figure 2.5 G). Unidendritic and multidendritic PNs were located in both the medial and non-medial

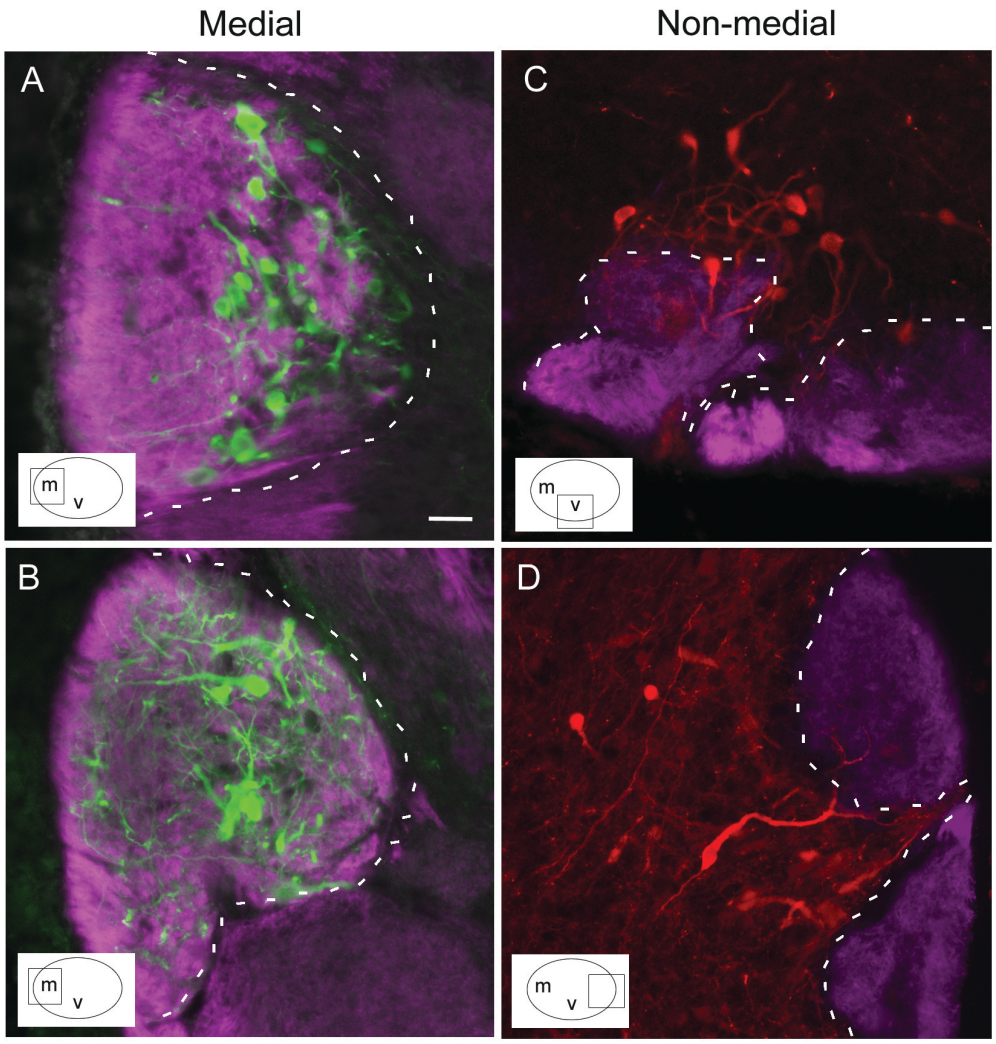

Figure 2.4. The spatial relationship between olfactory glomeruli and retrograde labeled PNs in transformers. The axons of olfactory sensory neurons were labeled with *Griffonia simplicifolia* Lectin I, isolectin B4; (purple). The cell bodies (A) and dendrites (B) of retrograde labeled medial PNs (green) are located within the glomerular neuropil. For non-medial retrograde labeled PNs (red), the cell bodies were observed proximal (deep) to the glomerular layer (C), and dendrites projected into the non-medial glomeruli (D). Scale bar shown in A is 30 μm, and is the same for all images. The location of each micrograph is indicated by a box within a diagram in the bottom left corner of the images indicating the medial (m) and ventral (v) aspects of the olfactory bulb.

Table 2.1. Length of the olfactory bulb and medial glomerulus containing projection neurons (PNs) and the soma size of PNs in the medial and non-medial olfactory bulb of post metamorphic and spawning adult sea lamprey. All values are mean ± SD. Sample size is indicated in brackets for each category. Asterisk denotes a significant difference ($p<0.001$) between medial and non-medial PN soma size within a given life stage.

	Transformer	Spawning Adult
Length of the olfactory bulb (μm)	645 ± 34.2 (4)	962.5 ± 24.7 (3)
Percent length occupied by medial PNs	43 ± 0.032 (3)	41 ± 0.04 (3)
Soma Size of medial PNs (μm)	14.05 ± 1.5 (50)*	19.56 ± 1.8 (50)*
Soma Size of nonmedial PNs (μm)	12.56 ± 0.9 (50)	15.32 ± 1.9 (50)

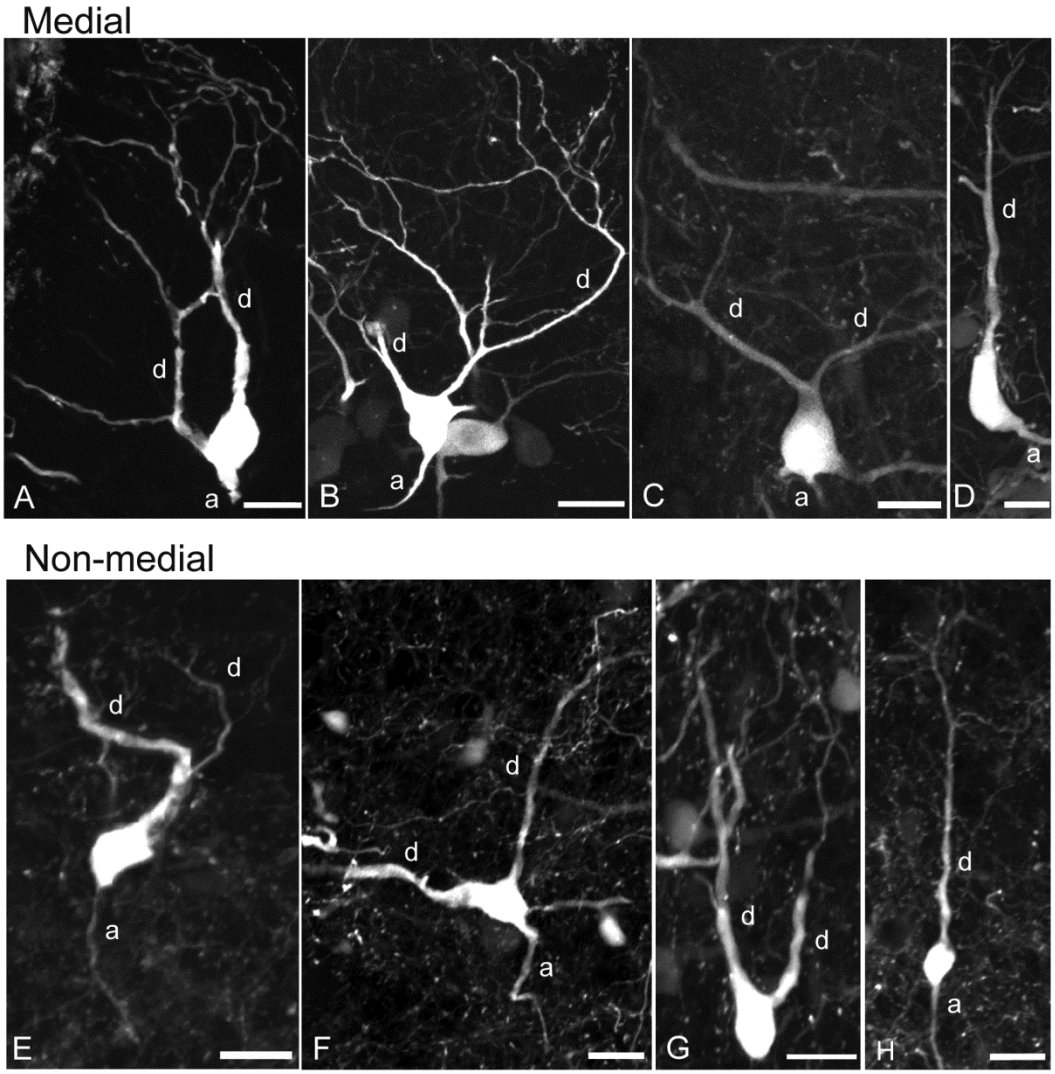

Figure 2.5. The morphology of medial and non-medial PNs retrograde labelled with biocytin in the olfactory bulb of transformers and adults. Multi-dendritic PNs are shown in the medial (A-C) and non-medial (E-G) region of the olfactory bulb. Unidendritic PNs were observed in the medial (D) and non-medial (H) olfactory bulb. Axons (a) and dendrites (d) of PNs are indicated on each image. Each of the PNs shown were radially oriented, except G, which was tangential. Scale bar is 20 μm.

regions (Figure 2.5). In the non-medial region, 68.75% of the PNs were unidendritic (55/80; n=4; $\chi^2 = 11.25$; $p<0.001$; df=1); and in the medial region, 62.5% were unidendritic (25/40; n=4; $\chi^2 = 2.5$; $p>0.10$; df=1). Of the multidendritic PNs observed, a larger proportion was located in the medial region (37.5%; 15/40, n=4) compared to the non-medial region (31.25%; 25/80, n=4). These findings show that the medial and non-medial retrograde labelled PNs shared a common somal shape and dendritic morphology.

2.4 Discussion

The location of somata and dendrites of medial PNs entirely within the medial glomerulus suggests that these second order neurons recruit synaptic information solely from this region. Conversely, the cell bodies of nonmedial PNs were widespread, and predominantly outside glomerular neuropil. Moreover, the dendrites of non-medial PNs did not extend into the medial glomerulus.

The anatomical isolation of PNs inside the medial OB glomerulus represents a unique condition with potentially profound functional consequences. The PNs of the medial glomerulus obtain synaptic information exclusively from dendritic and somatal surfaces within glomerular neuropil, and not from deeper bulbar regions. This suggests that neural signals processed in the medial glomerulus are not influenced directly by the processing of information by PNs in other regions of the OB; however, they may be subject to lateral inhibition by granule cells associated with PNs of the non-medial glomerulus. Moreover, because the somata and proximal dendrites of the medial PNs are able to come into contact with synaptic terminals entirely within glomerular neuropil, the space constant will be considerably small for these inputs, and there will be a large input

effect on these medial PNs.

The organisation that we observed for medial PNs in the sea lamprey is quite different from that of the mammalian main olfactory bulb; and suggests a potential difference in the functional PN circuit compared to the mammalian main olfactory bulb where inhibition of the lateral dendritic branches of mitral cells is widespread (Yokoi et al. 1995; Johnson et al. 1998; Aungst et al. 2003; review by Shepherd et al. 2007).

Three observations support a distinct functional role compared to that of the rest of the olfactory bulb; 1) these medial PNs are the origin of an olfactory pathway that leads to locomotion (Derjean et al. 2010); 2) the isolated nature of the medial PNs (dendrites are entirely within the glomerulus); and 3) the location of these medial PNs is the sole projection site of the sensory neurons of the accessory olfactory organ (Ren et al. 2009).

The relative length of the region occupied by the cell bodies and dendrites of the medial PNs was constant in both post metamorphic and adult stages. The post metamorphic stage leads directly to the parasitic feeding stage that moves in response to the odours of prey (Kleerekoper and Mogensen 1963); whereas the migratory adult and spawning adult lamprey show movement responses to pheromones (Li et al. 2002; Sorensen et al. 2005; Johnson et al. 2009). Possibly, this olfactory-locomotor substrate is important for both life stages.

The arrangement of cell bodies beneath and around glomeruli for non-medial PNs confirmed the arrangement of mitral cells previously seen in lamprey (Johnson 1902; Heier 1948; Nieuwenhuys 1977; Iwarhori et al. 1987; Villar-Cervino 2011). The olfactory bulbs of elasmobranchs and teleosts also demonstrate compartmentalization and

specificity of the medial region. Dryer and Graziadei (1993) demonstrated a medial "sub-bulb" that has different inputs and outputs compared to the lateral "sub-bulb" in sharks. The medial region of the OB in teleost fish is also distinct. Mitral cells in the medial region predominantly display responses to bile acids and project axons along the medial olfactory tract, whereas mitral cells in the lateral OB predominately respond to amino acids and project axons along the lateral olfactory tract (e.g. Hamdani and Doving 2003; Fuller et al. 2006; Hara and Zhang 1998; Nikonov and Caprio 2001; Hansen et al. 2003). Although sea lampreys display anatomical compartmentalization of the medial OB, odourant specificity of the medial OB (and other bulbar regions) has yet to be fully elucidated. In the moth antennal lobe, PNs innervating the macroglomerular complex (responding to pheromones) did not have extraglomerular processes (Namike and Kanzaki, 2011). Del Punta et al. (2002) have shown that the multi-dendritic second order neurons in the accessory olfactory bulb of mice project dendrites to glomeruli innervated by axons of sensory neurons expressing the same V1R or the same V2R gene.

The somal shape of the PNs investigated in the present study matched the morphology of mitral cells in the Arctic lamprey seen by Iwahori et al. (1987). However, we also observed unidendendritic PNs, previously seen by retrograde labelling in the OB of zebrafish (Fuller et al. 2006). Also in accordance with PNs (mitral cells) in zebrafish (Fuller et al. 2006); a greater proportion of multidendritic PNs were located in the medial region and soma size for medial PNs was greater than for non-medial PNs. In elasmobranchs, two types of mitral cells based on dendritic branching are also distributed throughout the OB (Dryer and Graziadei 1994). Furthermore, in the tench (*Tinca tinca*), a teleost, two types of mitral cells (based on soma) were differentially distributed in the

OB (Alonso et al. 1988).

The results of the present study demonstrate that the medial region of the OB in the sea lamprey is an anatomically defined and morphologically distinct region that seems to be isolated from the other regions of the OB. Previous work has demonstrated that the medial OB of sea lamprey has a unique output pathway to locomotor command centres (Derjean et al. 2010) and is the only site of sensory input from the accessory olfactory organ (Ren et al. 2009). The unique neuroanatomical features described here suggest that the medial region may be not only anatomically different, but also functionally different from other OB regions. Furthermore, this study supports the hypothesis of Derjean et al. (2010) that there are two olfactory pathways originating in the sea lamprey OB, one for odour discrimination and one for generating olfactory-mediated motor responses. Future work in our lab is focused on determining the mitral cell odour response profiles in the medial OB and how these responses are modulated.

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CHAPTER 3

Chemosensory responses of the olfactory bulb

3.1 Introduction

Parallel processing pathways serve to process different features of the sensory stimulus and have been observed in the visual, auditory, somatosensory, and olfactory systems. The processing of olfactory information occurs in the olfactory bulb (OB) and is projected along output pathways to specific targets in the central nervous system, ultimately driving odour-mediated behaviours. The olfactory systems of invertebrate and vertebrate organisms possess parallel processing pathways whereby different types of odourants are processed in different parallel olfactory subsystems. In the male moth and cockroach general odours are processed in glomeruli of the antennal lobe (AL) while sex pheromones are processed in the macroglomerulus/macroglomerular complex (Christensen and Hildebrand, 1987; Hansson et al., 1992; Christensen et al., 1995a; Hildebrand and Shepherd, 1997; Kay and Stopfer, 2006). Moreover, in rodents, general volatile odours are processed by the main olfactory system while non-volatile social odours are processed by the accessory olfactory system, although some overlap does occur (Restrepo et al., 2004; Xu et al., 2005; Spehr et al., 2006; Slotnick et al., 2010). In the honeybee, distinct medial and lateral parallel olfactory subsystems exist that are anatomically distinct (Kirschner et al., 2006) and differentially respond to odourant features such as carbon chain length and functional groups (Carcaud et al., 2012). Studies in cod (*Gadus morhua*) and crucian carp (*Carassius carassius*), where the different bundles of the medial and lateral olfactory tracts (MOT and LOT) were selectively cut one at a time, and the behaviour to feeding, reproductive, or alarm odours were examined, have shown that different bundles of the lateral and medial olfactory

tracts are functionally distinct (Doving and Selset, 1980; see review Hamdani and Doving, 2007). In particular, the LOT propagates feeding information; the lateral bundle of the MOT propagates reproductive information; and the medial bundle of the MOT propagated alarm reaction information from the OB to the telencephalon (Hamdani et al., 2000; Hamdani et al., 2001; Weltzien et al., 2003). In addition, the chemotopic organization of odour activity seen in the olfactory bulbs of the channel catfish (*Ictalurus punctatus*) is maintained in the telencephalon (Nikonov et al., 2005). Bile salts are spatially represented in the medial region of both the OB and telencephalon, while amino acids and nucleotides are spatially represented in the rostral lateral and caudal lateral regions, respectively, in both structures (Nikonov et al., 2005). Interestingly, a recent study by Igarashi et al (2012) has shown parallel output pathways from the main olfactory bulb to the olfactory cortex in mice. The mammalian olfactory bulb possesses two types of primary projection neurons, mitral and tufted cells, which are located in different layers of the OB (Shepherd and Greer, 1998). Mitral cells have a longer onset latency (slower response) and project to many regions of the olfactory cortex, while tufted cells have shorter onset latencies (faster response) and project to four small specific regions of the olfactory cortex including the anterior olfactory nucleus pars externa (AON_{pE}), the posteroventral part of the AON (AON_{pV}), the ventrorostral part of the anterior piriform cortex (APC_{VR}), and the anterolateral part of the olfactory tubercle (OT_{CAP})(Igarashi et al., 2012), suggesting a functional difference between these two OB output pathways within the main olfactory subsystem of rodents

In the OB, odour-evoked activity patterns contain both spatial and temporal aspects, which encode olfactory information (Laurent, 2002; Wachowiak and Shipley,

2006; Johnson and Leon, 2007). In the main olfactory bulb (MOB), afferents (i.e. olfactory sensory neurons, OSNs) expressing the same receptor converge onto one or a few circular aggregations of neuropil known as glomeruli (Mombaerts et al., 1996), where they synapse with primary projection neurons (PNs) (Pinching and Powell, 1971; Shepherd, 1972). Different odours activate different combinations of glomeruli and therefore each odour is spatially represented in the OB by a combinatorial glomerular activity pattern (Friedrich and Korsching, 1997, 1998; Johnson et al., 1998). Moreover, odours of similar chemical structure display similar glomerular activity patterns (Zhao et al., 1998; Malnic et al., 1999) and activate regionally localized glomeruli creating a chemotopically organized map on the surface of the OB (Friedrich and Korsching, 1997; Bozza and Kauer, 1998; Friedrich and Korsching, 1998; Hara and Zhang, 1998; Johnson et al., 1998; Nikonov and Caprio, 2001; Johnson and Leon, 2007; Soucy et al., 2009). Additionally, in the antennal lobe (AL) of insects, the spatial representation of general odours by combinatorial glomerular activation patterns has been observed (Galizia et al., 2000; Hansson et al., 2003; Sandoz, 2006; Zube et al., 2008) while sex pheromones elicit activity exclusively in a male-specific macroglomerulus/macroglomerular complex of the AL (Christensen and Hildebrand, 1987; Christensen et al., 1995b; Christensen et al., 1995a; Hildebrand and Shepherd, 1997). The spatial coding of odour information by a single glomerulus is known as non-combinatorial odour coding, and while considered to be rare in the OB, has been observed in the AL of cockroaches and moths, as well as in the OB of zebrafish (Hansson et al., 1992; Friedrich and Korsching, 1998). Non-combinatorial odour coding involves information of a single odour, usually a pheromone,

being encoded in the OB by a single glomerulus and can be considered a functionally distinct subsystem of the OB (or AL).

Within a glomerulus, the axons of OSNs synapse with the PNs known as mitral (and tufted) cells (known as PNs in the AL of insects) (Pinching and Powell, 1971; Shepherd, 1972; Boeckh and Tolbert, 1993; Shepherd and Greer, 1998). These PNs also interact with local inhibitory interneurons, which help shape the temporal aspect of odour-evoked signals prior to being propagated to other brain regions (Satou, 1990; Laurent, 1996, 2002; Sachse and Galizia, 2002; Urban and Sakmann, 2002; Aungst et al., 2003; Friedrich and Laurent, 2004; Tan et al., 2010). Individual PNs display different temporal firing patterns to different odours (Friedrich and Laurent, 2001) and a given odour can simultaneously activate multiple projection neurons, each with their own unique temporal firing pattern to the same odour (Macrides and Chorover, 1972; Kauer and Moulton, 1974; Meredith, 1986; Buonviso et al., 1992; Friedrich and Laurent, 2001). Hence, when recording local field potentials (LFPs; population level responses) in the OB (or AL) the temporal component of the LFP changes over the time course of the odour response due to periodic synchronized firing of multiple projection neurons with different temporal firing patterns (Wehr and Laurent, 1996; Friedrich and Laurent, 2001; Laurent, 2002; Kay et al., 2009). The synchronized firing of projection neurons and the dendrodendritic local circuit interactions of excitatory projection neurons and inhibitory interneurons generates the oscillatory activity generally observed in LFPs of the OB (Shepherd and Greer, 1998). The LFPs convey general odour information to higher brain regions but do not provide information about the precise odour identity (Friedrich et al., 2004). The temporal firing pattern of a group of non-synchronized primary projection

neurons in the OB and AL to chemically similar odours has been shown to initially be very similar to one another, but quickly diverge over the course of the odour-evoked response, so that the temporal response pattern of the ensemble of mitral cells (MC) become very different (decorrelate) from one another to the different odours (Friedrich and Laurent, 2001; Laurent, 2002; Stopfer et al., 2003; Daly et al., 2004; Friedrich and Laurent, 2004). This process of decorrelation allows for temporal response patterns of ensembles of neurons to become more informative about the precise identity of chemically similar odours and allows for odour discrimination (Friedrich et al., 2004; Friedrich and Laurent, 2004; Blumhagen et al., 2011). Interestingly, the temporal aspects of MC responses have been shown to vary in the main olfactory and accessory olfactory subsystems of rodents. Mitral cells in the accessory olfactory bulb (AOB) exhibit sustained responses to natural odours that last up to 30 seconds after odour sampling has stopped (Luo et al., 2003). Moreover, recent work by Shpak et al (2012) demonstrated that MC responses to brief electrical stimulation in the main olfactory bulb (MOB) were transient while those responses of MCs in the AOB were sustained, lasting up to minutes after the stimulus was removed. The sustained MC responses in the AOB emphasize the functional differences of the main and accessory olfactory subsystems of the rodent and are proposed to have functional consequences for social interactions (Luo et al., 2003; Shpak et al., 2012). The aforementioned studies demonstrate that there are parallel pathways of the OB that are anatomically, physiologically, and functionally distinct.

The detailed neural substrate through which olfactory information generates locomotor responses is not well understood, except in the sea lamprey (*Petromyzon marinus*) (Derjean et al., 2010). The sea lamprey, a model species for studies of

locomotion (Grillner et al., 1981; Grillner et al., 1983a; Grillner et al., 1983b; Grillner and Wallen, 1985; Dubuc et al., 2008), provides an ideal preparation for examining OB projection neuron response characteristics and their functional consequences. This preparation is ideal due to the ease by which an ex-vivo preparation of the entire intact nervous system can be maintained, easy access to large olfactory bulbs relative to the rest of the brain, and the benefit of a nervous system that has two well-defined parallel OB output pathways with connections to a well characterized locomotor system. The medial region of the OB receives inputs primarily from an accessory olfactory organ (Ren et al., 2009) and medial OB projection neurons project solely to the posterior tuberculum (Derjean et al., 2010). From the posterior tuberculum a multisynaptic pathway through the mesencephalic locomotor region ends at the reticulospinal cells (motor command neurons) in the hindbrain, forming an olfactory –locomotor neural substrate (Derjean et al., 2010). In contrast, the non-medial regions of the OB receive inputs only from the main olfactory epithelium (Ren et al., 2009) and non-medial OB projection neurons project to several forebrain structures including the contralateral OB, ipsilateral and contralateral pallia, the striatum, as well as other forebrain regions (Derjean et al., 2010). In addition, recent work by Green et al. (Chapter 2) showed that medial OB projection neurons are spatially isolated and anatomically unique compared to those projection neurons in the non-medial OB regions. While these studies suggest a functional difference in the two OB output pathways, very little is known about the odour response characteristics of the projection neurons or their functional consequences for either pathway.

To examine whether the response characteristics of projection neurons have temporal and spatial differences in the medial and non-medial OB pathways, extracellular local field potential and multiunit responses of these projection neurons were recorded in the medial, dorsal, and lateral OB regions in response to known amino acid, bile salt, and pheromone odours (Kleerekoper, 1963; Li, 1994; Li et al., 1995; Li and Sorensen, 1997; Bjerselius et al., 2000; Li et al., 2002; Fine et al., 2004; Sorensen et al., 2005; Fine and Sorensen, 2008). Overall, medial PNs showed responses to all of the odours tested and exhibited transient temporal response characteristics that differed from the dorsal and lateral (non-medial) OB projection neurons. In contrast, PNs in the lateral OB responded only to amino acid odours and exhibited sustained temporal response characteristics. These results provide further evidence that the parallel pathways of the OB are organized based on their functional output and support the hypothesis that the medial OB pathway functions to initiate olfactory-mediated motor behaviours.

3.2 Materials and Methods

3.2.1 Animal collection

All animals were captured from the wild and all experiments were performed in accordance with the guidelines of the University of Windsor Animal Care Committee and the Canadian Council on Animal Care. Metamorphic stage-seven sea lamprey were caught via electroshock techniques from the Black mallard river (2009 & 2010), Millersburg MI and the Little carp river (2011), Baraga MI in cooperation with the United States Geological Survey Hammond Bay Biological Station, Millersburg MI. Spawning stage adult sea lamprey were caught in traps on the Humber River, Toronto ON, and the St. Mary's River, Sault Saint Marie, ON (2008 & 2009) by the Toronto

Region Conservation Authority, Toronto, Ontario and the Department of Fisheries and Oceans, Sault Saint Marie, Ontario, respectively. All animals were transported to the Department of Biological Sciences at the University of Windsor and held in dechlorinated water under static renewal conditions at 7 °C for 1 week prior to use.

3.2.2 Animal preparation

Lamprey were anesthetized in a solution of MS222 (150 mg/L) in dechlorinated water (pH adjusted to 7 using 2M NaOH) and decapitated at the 3rd brachial pore. Tissue was transferred to a solution of ice-cold lamprey Ringer's (NaCl 130 mM, KCl 2.1 mM, $CaCl_2$ 2.6 mM, $MgCl_2$ 1.8 mM, HEPES 4 mM, dextrose 4 mM, $NaHCO_3$ 1 mM) for the remainder of the dissection. The ventral surface of the remaining tissue, including the oral disk, was removed down to the level of the lumen of the nasopharyngeal pouch and the buccal cavity. The tissue was then placed dorsal side up and the skin, musculature, and cartilage were removed to expose the entire cranial and nasal cavities. Every effort was taken to keep the maximal amount of olfactory epithelium intact. The *dura mater* was then removed from the OBs and pallia. The explant preparation included the intact olfactory epithelium and entire brain up to the rostral-most portion of the spinal cord.

Once the dissection was complete the explant preparation was transferred to a chilled recording chamber and submersed in a chilled (10 °C) solution of lamprey Ringer's. The explant preparation was continuously perfused with chilled Ringers at a flow rate of 1 ml/min. Ringers solution entered the chamber near the caudal region of the brain and flowed towards the rostral region (the olfactory epithelium) via suction (Figure 3.1). In addition, chilled Ringer's solution continuously perfused the olfactory

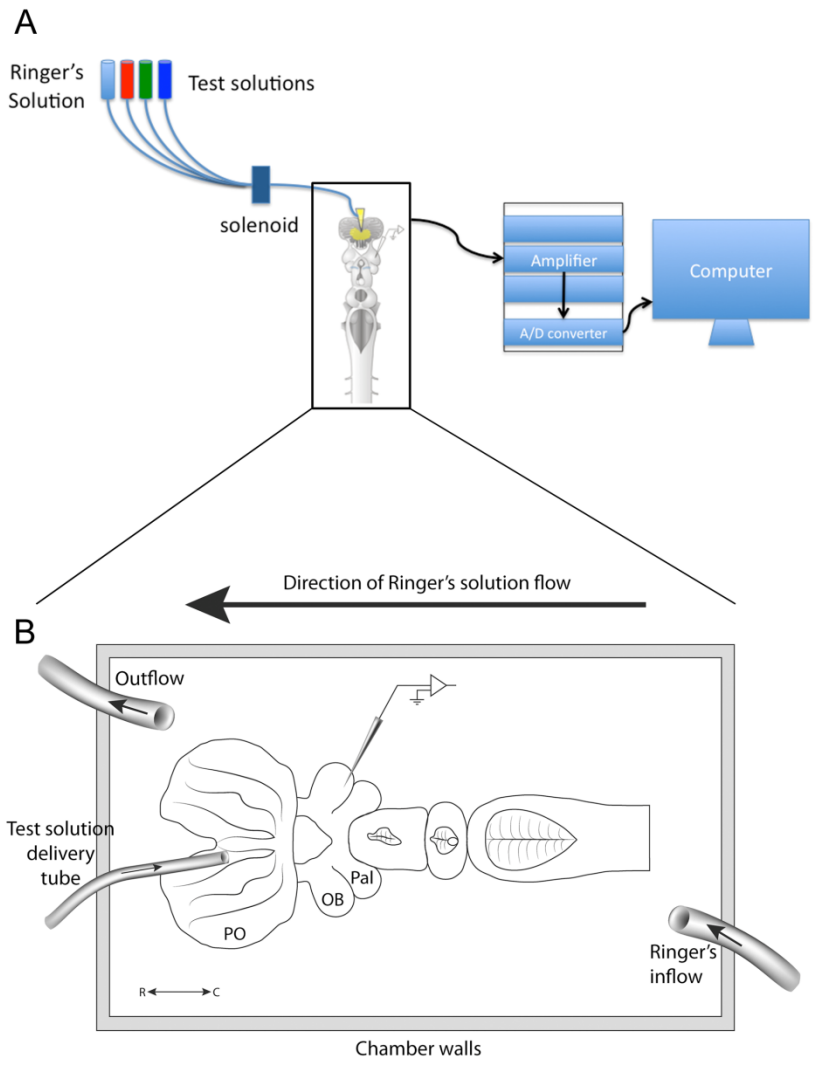

Figure 3.1. Illustration of the test solution delivery apparatus (A), recording chamber, and *ex-vivo* preparation of the sea lamprey peripheral olfactory organ (PO) and brain (B). Extracellular local field potential and multiunit responses to odorant test solutions were recorded from the olfactory bulb (OB). The test solutions flowed into the PO via a test solution delivery tube. Cooled Ringer's solution continuously flowed through this tube, except during the delivery of odour test solutions, which entered into this tube through a solenoid apparatus. The olfactory organ/brain preparation was perfused by cooled Ringer's solution flowing into the rear of the chamber and out via suction at the front of the chamber at a rate of 1 ml/min. The olfactory test solution was removed from the PO through this outflow tube as well. The walls of the recording chamber contained cooled re-circulating antifreeze in order to help maintain the Ringer's solution at a consistent temperature of 10 °C. Rostral (R), caudal (C), pallium (Pal).

epithelium via an odour delivery system (Figure 3.1) (see *odour delivery* below). The preparation was allowed to recover for approximately 1 hour prior to the beginning of experimentation. Ringer's solution was made fresh daily, chilled, oxygenated (95% oxygen, 5% carbon dioxide), and the pH was adjusted to 7.4 using 5 M NaOH prior to use. The lamprey Ringer's solution was oxygenated continuously throughout the experiment.

3.2.3 Preparation and delivery of odourant test solutions

Stock solutions of L-arginine (A5006, Sigma-Aldrich, Oakville, ON, Canada) and L-histidine (H-8000, Sigma-Aldrich) were made daily at a concentration of 10^{-2} M in chilled lamprey Ringer's. Just prior to experimentation a mixture of 10^{-3} M L-arginine and L-histidine was made in Ringer's solution and serial dilutions of this mixture were made down to 10^{-5} M. A stock solution of taurocholic acid (T4009, Sigma-Aldrich) was made daily at a concentration of 10^{-2} M. Just prior to experimentation the stock solution was diluted in Ringer's solution to working concentrations of 10^{-4} M and 10^{-5} M. The following five putative pheromones (Li et al., 2002; Sorensen et al., 2005) were utilized throughout all electrophysiological experiments: 3-keto petromyzonol sulfate (3KPZS), 3-keto allocholic acid (3KACA), petromyzonol sulfate (PZS), Petromyzonamine disulfate (PADS), Petromyzonsterol disulfate (PSDS). All pheromones were acquired from Dr. Weiming Li (Michigan State University) in powder form (pheromones received May 29, 2008; July 21, 2009; May 20, 2010)(Batch IDs: 3KPZS – 06 08 2006; 3KACA – 152-EJH-158-2; PZS - 152-EJH-282-3; PADS – 155-TNN-287D; PSDS – 158-IDE-223-1). Stock solutions of these pheromones were made by reconstituting each pheromone in a

1:1 solution of high purity methonal (cat no. CABDH6048-4, VWR, Mississauga, ON, Canada) : ultra-pure water (18 MΩ.cm, Milli-Q reference system, Millipore, Billerica, MA, USA) at a concentration of 1 mg/mL. Once reconstituted, aliquots were stored in 2 mL screw top glass vials (cat no. 224881, Wheaton, Millville, NJ, USA) at -80°C. Substocks of 10^{-5} M pheromones in Ringer's solution were made from stock solutions on the day of testing. Working solutions of pheromone mixtures were made each day just prior to experimentation. The sex pheromone mixture contained 10^{-6} M 3KPZS and 3KACA while the migratory pheromone mixture contained 10^{-6} M PZS, PADS and PSDS. Serial dilutions were made down to a concentration of 10^{-9} M of these mixtures.

A custom-made 9-chamber odour delivery/cooling system was utilized to chill and deliver background medium (Ringer's solution) and odours over the olfactory epithelium at the same temperature as the chilled preparation. Briefly, background Ringer's solution was continuously perfused over the olfactory epithelium via a gravity fed, valve-controlled odour delivery system and an electronically triggered, computer controlled, three-way solenoid valve (adapted from a design kindly provided by Dr. D. Restrepo, U. of Colorado) allowed fast switching from background Ringer's to a desired odour and back to background Ringer's with no interruption in flow or changes in pressure to the naris (Figure 3.1 and 3.2). Odour deliveries were 5s (LFP) or 30s (multiunit) in duration and were at least 3 minutes apart to prevent adaptation to odourants (Figure 3.2). Prior to experimentation, adaptation of odour responses in the OB were examined, and 3 minutes was found to be sufficient time to allow for consistent olfactory responses to be observed to repeated deliveries of odour test solutions. Each odour was delivered between 3-6 times and a negative control (either Ringer's solution or

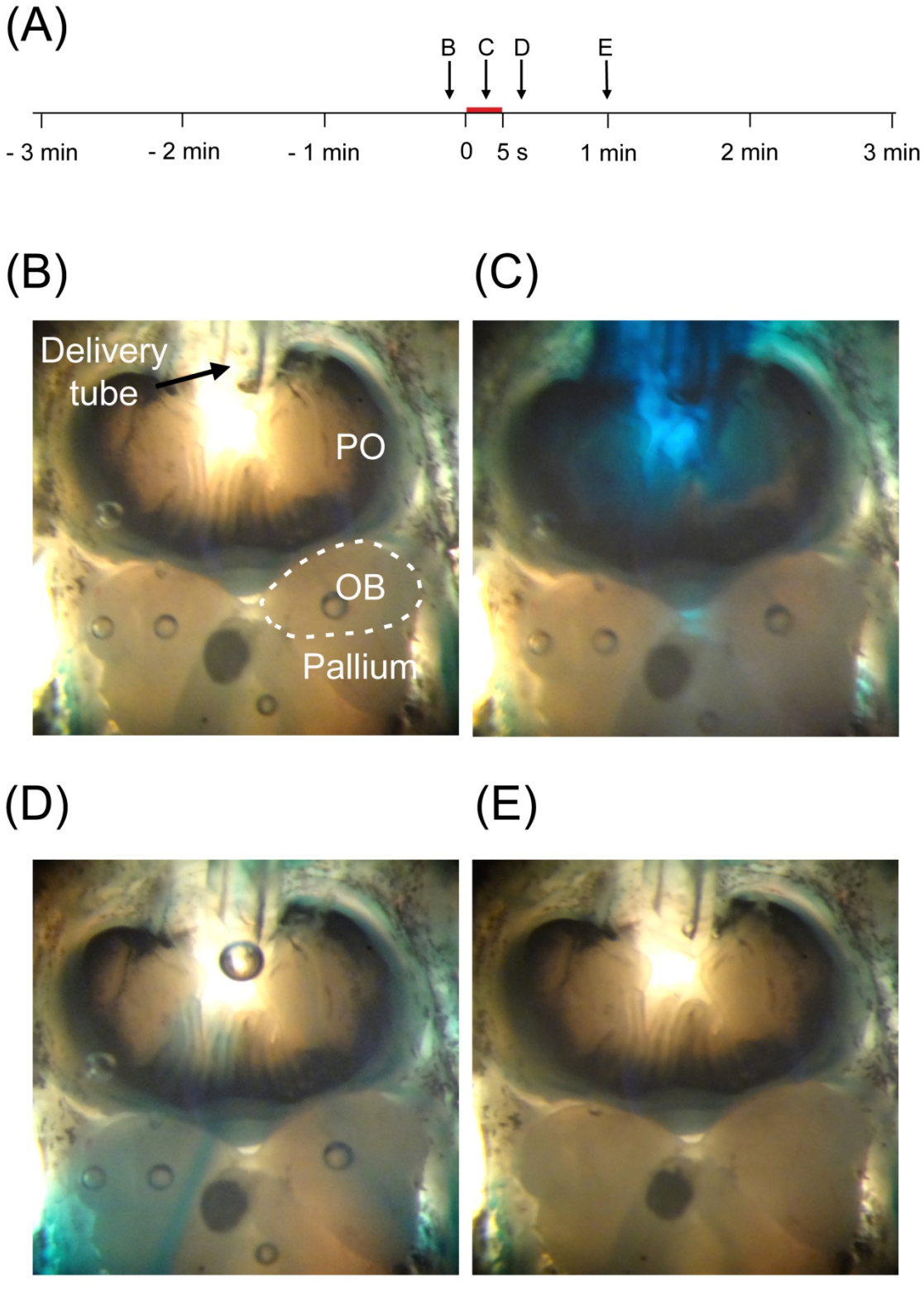
(A)
B
C
D
E
- 3 min
- 2 min
- 1 min
0
5 s
1 min
2 min
3 min
(B)
(C)
Delivery tube
PO
OB
Pallium
(D)
(E)

Figure 3.2. The time course and delivery of test solutions into the peripheral olfactory organ (PO) during the LFP experiments. A blue solution (5% Fast Blue) was utilized to demonstrate the passage of an odour test solution. **A.** The timing of odour test solution delivery during an LFP experiment. Background Ringers was perfused for 3 minutes (t= - 3 to 0 minutes); the test solution was applied for 5 seconds (LFP) or 30 seconds (multiunit) (t= 0; red bar), then the Ringers solution was switched back for 3 minutes (t=5 sec to 3 minutes). **B.** Prior to the delivery of the test solution, lamprey Ringer's solution flowed into the PO via the test solution delivery tube. **C.** The fast blue solution filled the PO. **D.** Ringer's solution returned to flowing into the PO. Some pale blue was seen surrounding the PO. **E.** The Fast Blue was not seen in the peripheral olfactory organ.

methanol: ultra pure water diluted in Ringer's solution down to the same dilution as the odours being tested, depending on the vehicle for the odours) was delivered at various intervals throughout each recording. There was no olfactory response to the negative controls containing the methanol : ultra pure water mixture (0.01 % for 10^{-6} M pheromones).

3.2.4 Extracellular electrophysiology recordings

Extracellular local field potentials (LFPs) in the olfactory bulb (N=29) were recorded from either the medial (N=8), dorsal (N=6), or lateral (N=15) surface of OB (Figure 3) using glass micropipettes (~ 8-10 μm tip diameter) filled with 2M NaCl (0.1 Mohm impedance). Micropipettes were connected to a headstage via a silver wire and the headstage-amplifier circuit was grounded and referenced via silver wires connected to the headstage, placed in the recording bath. Signals were filtered between 1 Hz and 1 kHz, amplified 10,000 times (model P511L amplifier, Grass Technologies Inc., West Warwick, RI, USA) and subsequently digitized at 10 kHz (Powerlab 4/30, model ML866, ADInstruments, Colorado Springs, CO, USA). LFPs were viewed online during

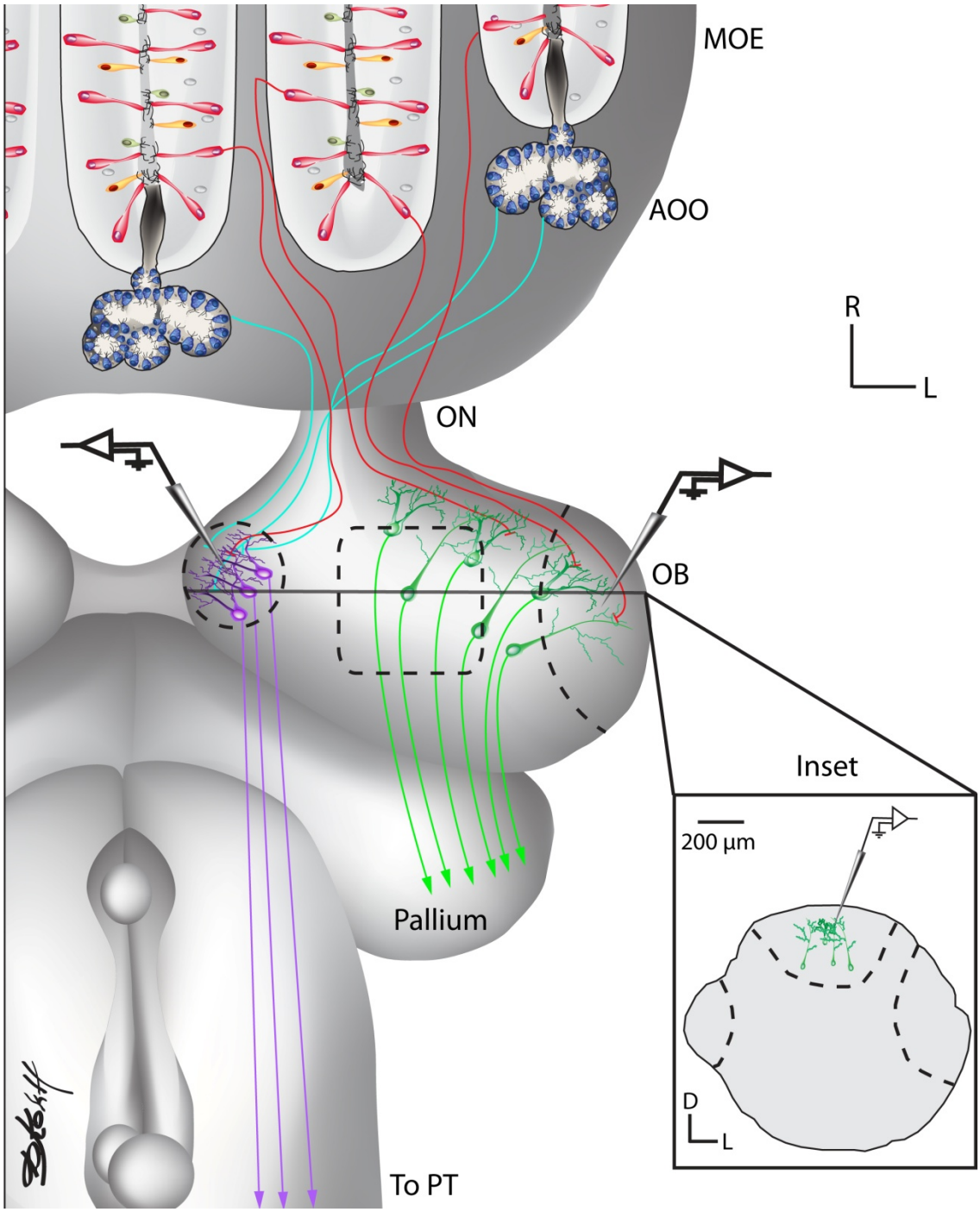

Figure 3.3. Illustration of the local field potential and multiunit recording locations in the medial, dorsal, and lateral regions of the olfactory bulb (OB). Hatched lines denote the medial, dorsal, and lateral recording regions in both the dorsal and cross-section views. Olfactory sensory neuron inputs via the olfactory nerve (ON) to the medial olfactory bulb from the accessory olfactory organ (AOO) (teal) and main olfactory epithelium (MOE) (red) are shown. The medial OB projection neuron projections to the posterior tuberculum (PT) are purple. Olfactory sensory neuron inputs to the dorsal and lateral

olfactory bulb from the main olfactory epithelium (red) are shown. The OB projection neuron projections to the lateral pallium are green. The inset shows a cross-section view of the olfactory bulb and the position of the recording electrode in the dorsal region. Scale bar is 200 μm. The innervation of the olfactory bulb by sensory input from the peripheral olfactory organ is from Ren et al. (2009) and the location of the targets for the OB projection neurons is summarized from Derjean et al. (2010).

recording to ensure good signal to noise ratio and digitally filtered and analyzed offline using LabChart software (version 6.1.3, ADInstruments).

Extracellular multi/few unit recordings (N=25) were recorded from either the medial (N = 11), dorsal (N = 5), or lateral (N = 9) OB (Figure 3.3) using glass micropipettes (2-3 μm tip diameter) filled with lamprey Ringers solution (2-5 Mohm impedance). Micropipettes were connected to a headstage via a silver wire and the headstage-amplifier circuit was grounded and referenced via silver wires connected to the headstage, placed in the recording bath. The recording electrode was slowly inserted into the desired region of the OB using a manual hydraulic micro-drive (Narishige Inc.) to a depth of ~ 150 μm for metamorphic stage seven lamprey and to a depth of ~ 300 μm for adult lamprey. Previous histological examination of the OB had shown that the somata of PNs were located at these depths (Figure 3.4) (Also see Green et al, Chapter 2). Signals were filtered between 10 Hz and 10 kHz, amplified 10,000 times (model 1800, A-M systems, Sequim, WA, USA) and subsequently digitized at 20 kHz (powerlab 4/30, model ML866, ADInstruments). Spikes were viewed online during recording to ensure adequate signal to noise ratio and analyzed offline for changes in spike frequency during odour delivery using LabChart software (version 6.1.3, ADInstruments).

3.2.5 Data analysis

Each case (a single recording location within an animal) was examined for odour responses and was only included in the analysis if a consistent response (across three repetitive deliveries) was observed to at least one test odour and the negative control (blank) exhibited no response. Both local field potential and multiunit recordings were examined for spatial and temporal response patterns across the medial, dorsal, and lateral OB regions.

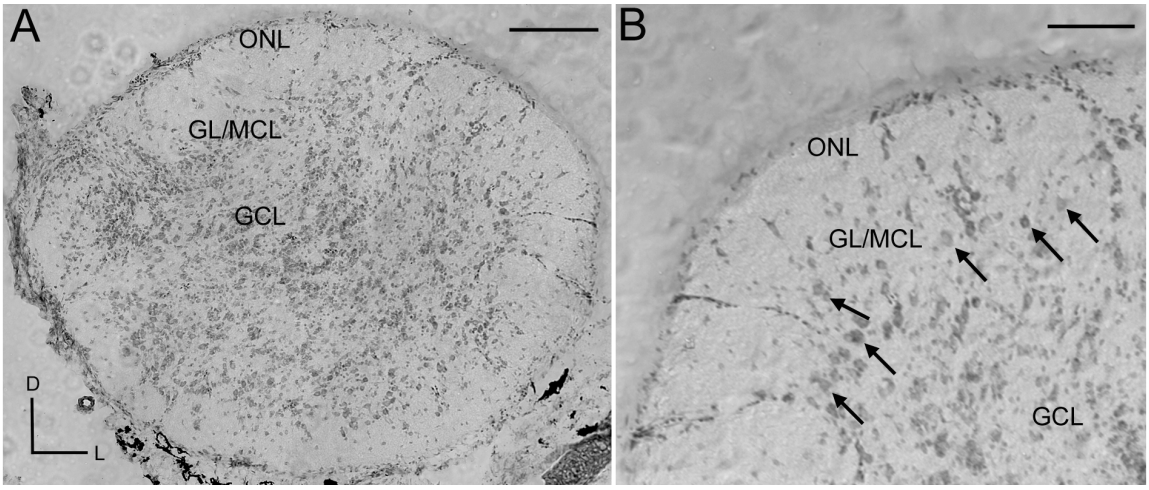

Figure 3.4. Cross sectional view of the sea lamprey olfactory bulb stained with cresyl violet. **A.** Three layers of the olfactory bulb as classified by Iwahori et al, (1987). The olfactory nerve layer (ONL) is thin, while the layer of glomeruli with mitral cells (GL/MCL), and the granule cell layer (GCL) are prominent. Beneath the ONL, glomeruli are located around the periphery as ovoid regions of neuropil with scarce nuclei. Scale bar is 200 μm. **B.** Arrows show mitral cells beneath the glomeruli. The mitral cells are recognized by the large nuclear size. Scale bar is 100 μm.

3.2.6 Local field potentials

Prior to analysis all local field potentials were digitally filtered post-acquisition with a 100 Hz low-pass filter using LabChart software (version 6.1.3, ADInstruments). Within each case, individual odour deliveries were examined and for each odour delivery a pre-odour baseline mean and standard deviation was calculated for the 5-second time period just prior to the onset of odour delivery. The response threshold for each odour delivery was then calculated as 3-times the baseline standard deviation. This threshold was utilized to distinguish odour evoked LFP responses from baseline deviations and spontaneous projection neuron activity. In addition, odour-evoked LFPs were distinguished from background spontaneous activity by comparing odour-evoked responses to spontaneous activity rates and ensuring that odour-evoked responses were consistent and repeatable across multiple deliveries of a given odour. Only those responses that were consistent and repeatable were considered an odour-evoked response. Local field potential peaks within an odour response period that were greater than the threshold were counted as part of the response and the total number of LFP peaks in each response were recorded. In addition to the number of peaks in an LPF response, the amplitude of each peak, time of each peak, interpeak interval, and change in peak amplitude over the time course of each sustained response were determined. Peak amplitude was measured as the voltage change between the pre peak baseline mean and the peak maximum. To further characterize the temporal aspects of each peak within an LFP response, the waveform data (time and amplitude values every 0.0001 seconds) for each peak was examined using custom written routines in R (Version 2.14.0, R Development Core Team 2011). These routines were used to determine the time at which

10%, 50% and 90% of the pre and post peak maximum amplitude occurred. The time values were then used to calculate the peak duration (10% pre-max to 10% post-max), halfwidth (50% pre-max to 50% post-max), falling phase (10% pre-max to 90% pre-max), and rising phase (90% post-max to 10% post-max) of each peak (Figure 3.5). The 10% and 90% of peak-maximum time points were used in order to account for changes in the LFP due to any signal noise at the beginning, maximum, and end of each peak. In this way, more representative temporal values were determined for each peak, regardless of signal noise. The differences among treatments for a given LFP peak characteristic was analyzed using a two-way ANOVA on ranks (Scheirer-Ray-Hare extension of the Kruskal-Wallis test) testing for the main effects of OB region, odour, and the OB region x odour interactions followed by a Holm-Sidak post-hoc analysis. Differences among groups were considered statistically significant when $p \leq 0.05$. In order to examine the spatial relationship of odour responses in the medial, dorsal and lateral regions of the olfactory bulb; the percentage of cases showing responses to a particular odour(s) in each OB region was also calculated.

3.2.7 Multiunit recordings

The change in spike frequency (spikes/s) was examined for the time course (180 s) of each odour delivery over the 30 seconds pre odour delivery, 30 second odour delivery period, and 120 seconds post odour delivery period using the spike histogram module (version 2.4.1) within LabChart software. Post-stimulus time histograms (PSTHs) were made for the time course (180 s) of each odour response period with spikes being

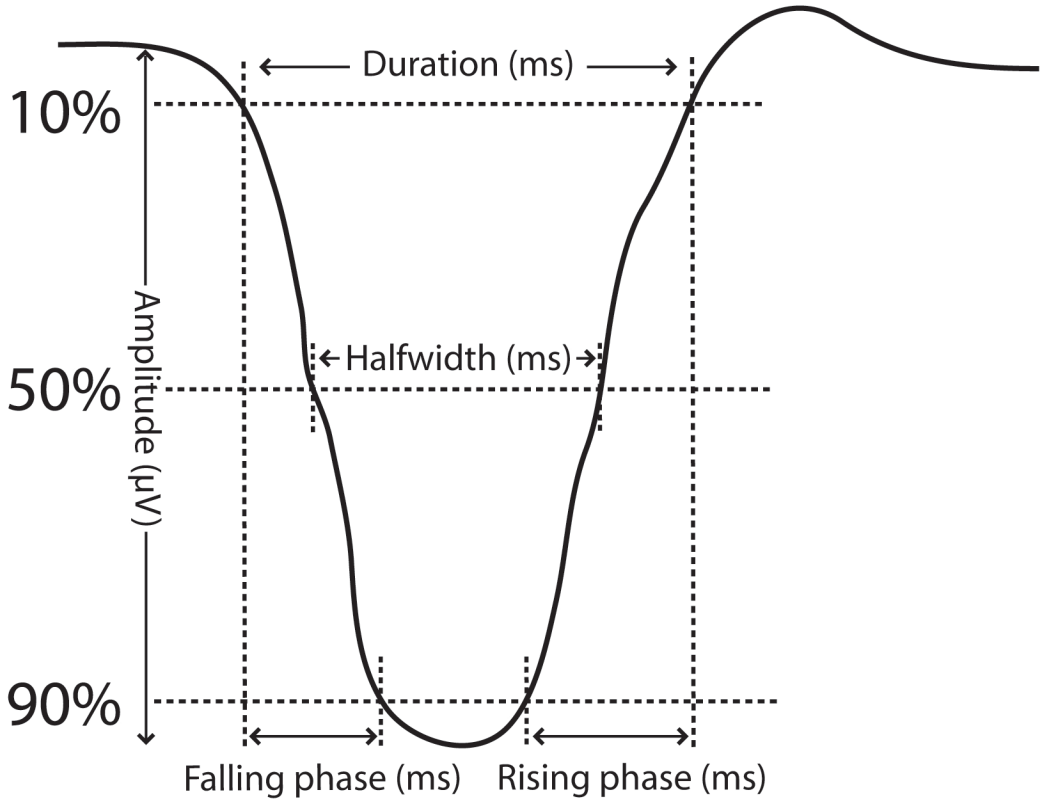

Figure 3.5. Local field potential characteristics. Five measurements were made for each individual peak of an odour response: peak amplitude, duration, halfwidth, falling phase, and rising phase. Peak amplitude was measured as the voltage change between the pre peak baseline mean and the peak maximum; peak duration was measured as the time between 10% pre and 10% post maximal peak; halfwidth was measured as the time between 50% pre and 50% post maximal peak, falling phase was measured as the time between 10% and 90% pre maximal peak; and rising phase was measured as the time between 90% and 10% post maximal peak.

sorted into 1-second time bins. PSTHs were used to examine the change in spike frequency over the 180 second time period and classify responses as excitatory, inhibitory, or null. An odour response was considered excitatory if spike frequency at least doubled in rate above background spontaneous activity during the odour delivery period. An odour response was considered inhibitory if spike frequency at least decreased by half (50%) in rate below background spontaneous activity during the odour delivery period and was considered null if there was no change in spontaneous firing rate. Odour responses were also examined for consistency and repeatability across multiple odour deliveries. Once each odour delivery was classified as excitatory, inhibitory, or null, the percentage of cases exhibiting an excitatory response was calculated for each odour in each OB region that was tested. The response duration of each odour delivery was determined by examining the time of an odour response onset (spike frequency doubling in rate or halving in rate compared to average spontaneous background rate) and an odour response offset (spike frequency returns to average spontaneous background rate) using PSTHs in LabChart spike histogram module (version 2.4.1). The odour response duration data for all odours were pooled for each OB region and analyzed offline using a one-way Kruskal-Wallis ANOVA on ranks using MATLAB (Version 7.9, The MathWorks Inc., Natick, MA 2009). Differences among groups were considered statistically significant when $p \leq 0.05$.

3.3 Results

Local field potential and multiunit neural responses were recorded from the OB in response to stimulation with odours previously shown to elicit electro-olfactogram

responses from the olfactory epithelium (Li, 1994; Li et al., 1995; Li and Sorensen, 1997; Li et al., 2002; Sorensen et al., 2005). These odours included the basic amino acids L-arginine and L-histidine; the bile acid taurocholic acid; the sex pheromones 3-keto petromyzonol sulfate and 3-keto allocholic acid; and the migratory pheromones petromyzonol sulfate, petromyzonamine disulfate, and petromyzosterol disulfate. Within a given recording location each test odour was delivered 3-6 times and the resulting response patterns were consistent > 90% of the time. A minimum of three consistent odour responses was required in order to include data for analysis. In addition, no responses were observed to the negative control (Ringer's solution or a methanol/Ringer's solution, depending on the vehicle in which the odour was dissolved/diluted).

3.3.1 Local field potential recordings exhibit transient and sustained odour-evoked responses

The LFP recordings exhibited transient and sustained responses to odour test solutions. Transient odour-induced LFP responses consisted of a single peak that occurred within the five-second odour delivery period, followed by a brief period of hyperpolarization and return to baseline (Figure 3.6). Sustained odour-induced LFP responses consisted of two or more peaks occurring within or lasting longer than the odour delivery period (Figure 3.6). Sustained responses were observed to be complete when neural activity subsided to pre-odour delivery baseline spontaneous firing rates. Local field potential responses were observed in all 29 cases (a case is a single recording location in an animal).

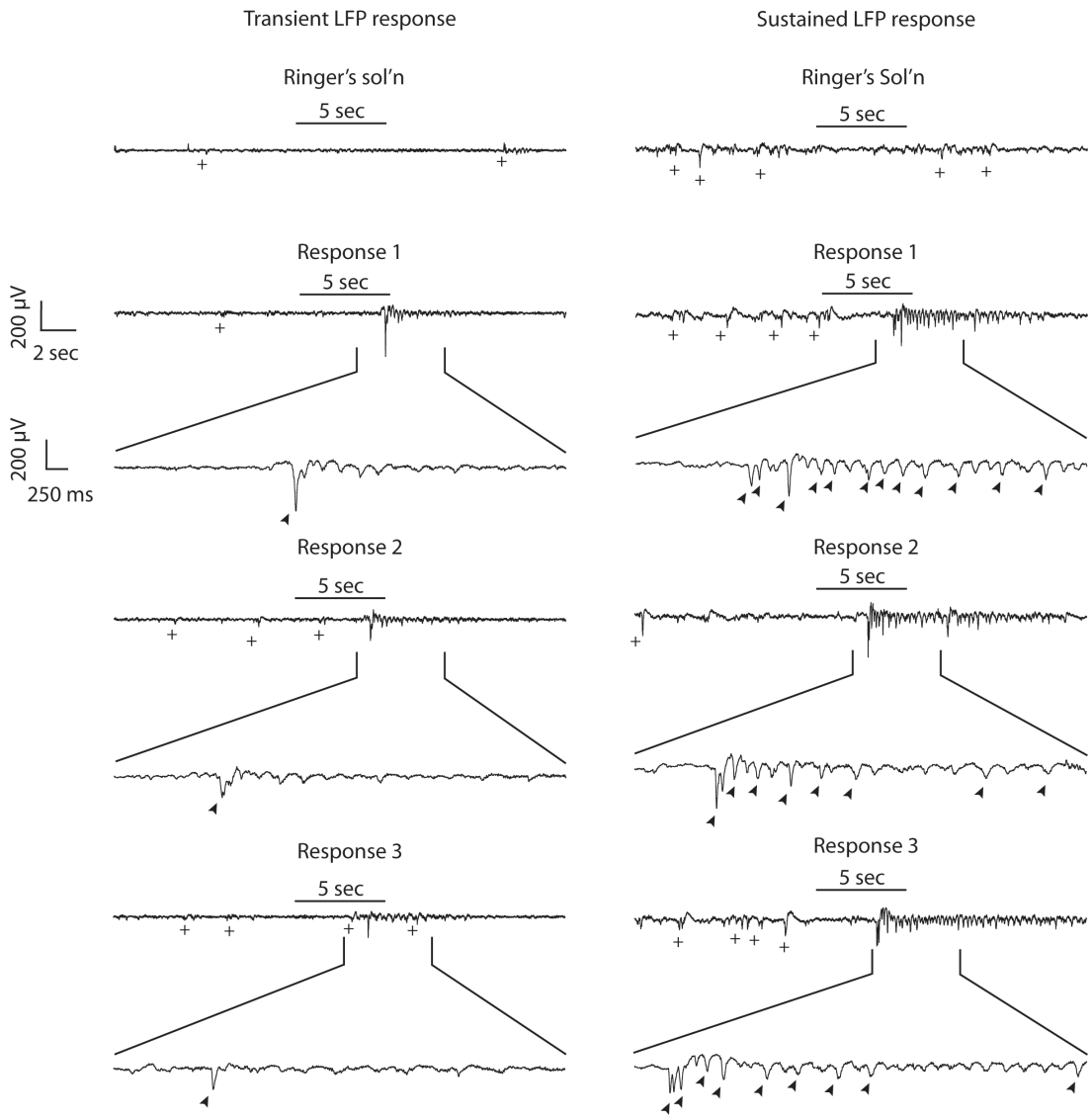

Figure 3.6. Examples of transient and sustained local field potential (LFP) responses to odour test solutions. The Ringer's solution was applied as a negative control. The transient responses include a prominent peak, as shown by the arrowhead, during the five second odour delivery period. Several prominent peaks, shown by arrowheads, illustrate a sustained response over the five second odour delivery period (solid bar above each response). Local field potential responses 1, 2, and 3 are repetitive deliveries of an odour test solution. Each delivery is shown in two views. The upper trace is 25 seconds in duration and a lower trace that is 5 seconds in duration. The latter shows a detailed view. The solid line above the upper trace denotes the 5 second test solution delivery period in which the 3-way solenoid valve was activated. The 'plus' symbols indicate spontaneous activity and the arrowheads indicate peaks recorded during the delivery of the test solution.

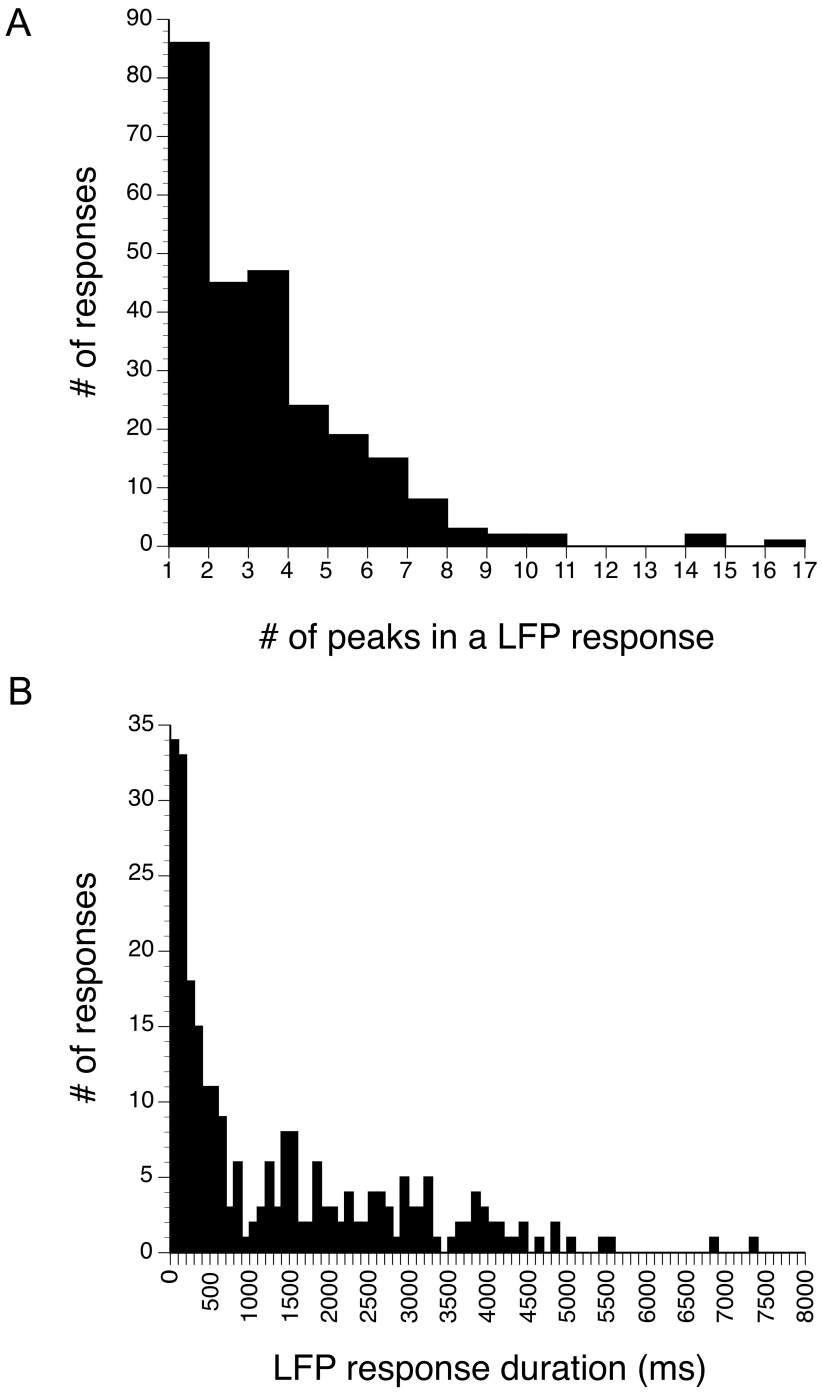

Figure 3.7. The distribution of all odourant test solution response durations for the local field potential (LFP) recordings in the olfactory bulb. **(A)** Histogram of the number of peaks in a local field potential (LFP) response. **(B)** Histogram showing the response duration of transient and sustained LFPs. The duration of transient responses was measured from the beginning to the end of the single peak. The duration of sustained responses was measured from the beginning of the first peak to the end of the last peak.

In 5.3 % of LFP cases, responses were entirely transient, 36.8% of cases had entirely sustained responses, and 57.9% of cases had a mixture of transient and sustained LFP responses. The transient or sustained response pattern to a given odour was consistent within a recording location. In 33.8% of LFP responses, a transient (single peak) response was observed (Figure 3.7A). In addition, LFP responses exhibited a range of durations, some lasting longer than the five-second odour delivery period (Figure 3.7B). The first peak of sustained local field potentials were typically the largest and each subsequent peak got smaller, although there were exceptions to this response pattern (Appendix C Figure 3).

3.3.2 Multiunit recordings exhibit excitatory and inhibitory odour-evoked responses of varying durations

Multiunit recordings exhibited spontaneous activity that was variable within and across recording locations (Figure 3.8A). Excitatory multiunit responses were observed in the medial, dorsal, and lateral OB regions. Excitatory responses varied in duration and typically exhibited a rapid increase in spike frequency at the odour onset (Figure 3.8 B-E). Responses were considered excitatory if they had at least a doubling in spike frequency during the odour delivery period. In addition, inhibitory responses were also observed during multiunit recordings in the OB (Figure 3.9). Odour-evoked responses were considered inhibitory if spike frequency was reduced by at least half during the odour delivery period. Inhibitory responses were also consistent in duration and repeatable across odour deliveries (Figure 3.9). The duration of 64 (38 %) multiunit responses lasted ≤ 30 seconds, the duration of the odour delivery period (Figure 3.8 B) (Figure 3.10). In addition, the duration of 50 (29.5 %) multiunit responses was between

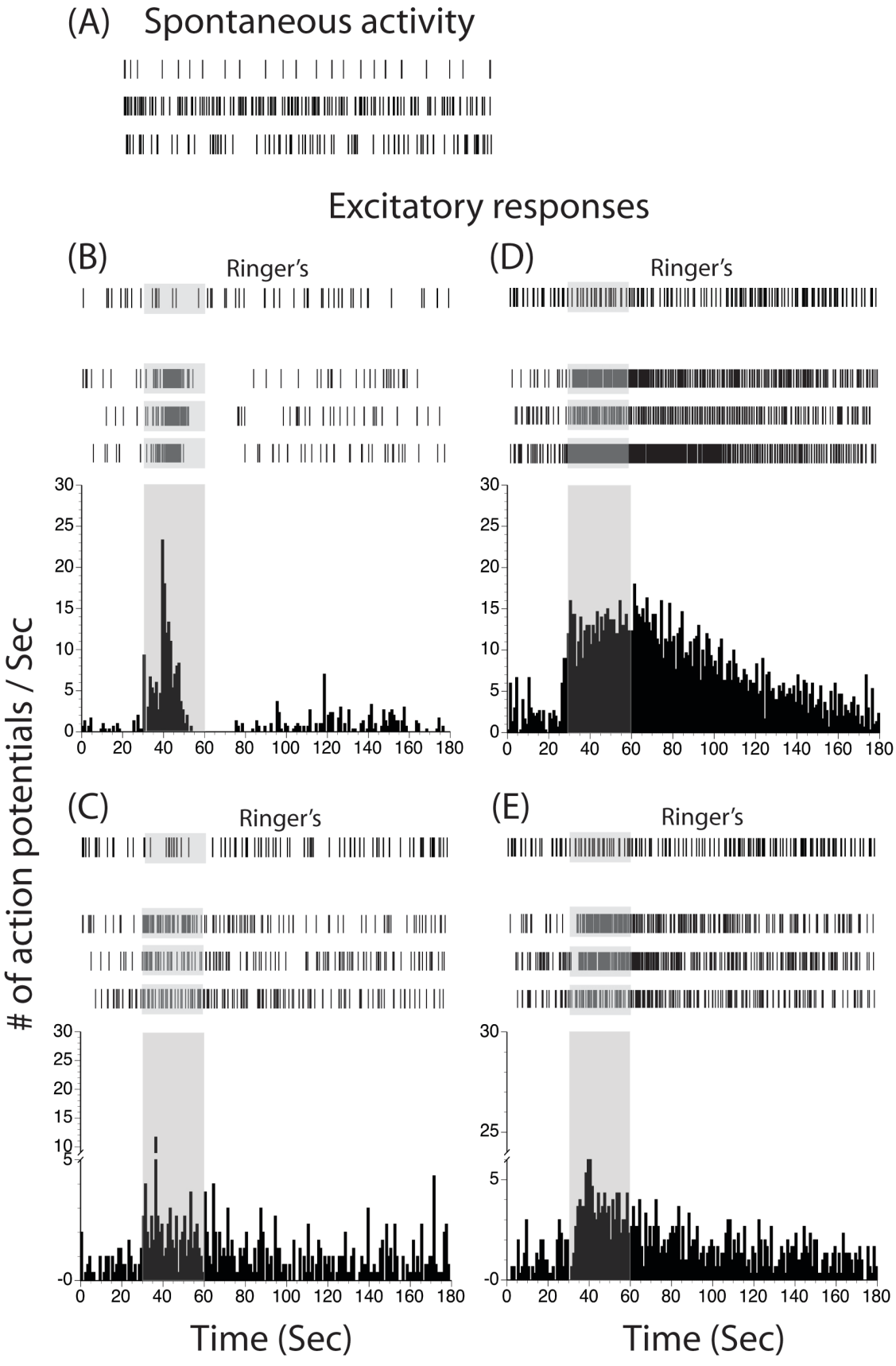
(A) Spontaneous activity
Excitatory responses
(B)
Ringer's
(D)
Ringer's
(C)
Ringer's
(E)
Ringer's
# of action potentials / Sec
Time (Sec)
Time (Sec)

Figure 3.8. Examples of excitatory multiunit recordings from the olfactory bulb during the delivery of odour test solutions to the peripheral olfactory organ. Each test solution was delivered for 30 seconds (gray overlay). The three rasters above each post-stimulus time histogram show three repetitive recordings from a preparation during the delivery of the same test solution. The 3 responses were averaged, and the corresponding average post-stimulus time histogram is shown below the rasters. **A.** Three example raster plots of spontaneous activity from three different recording sites in the olfactory bulb. Each raster plot is 180 seconds in duration. **B - E** are examples of excitatory multiunit responses of various durations. Delivery of a negative control odour test solution (Ringer's) is shown for each example.

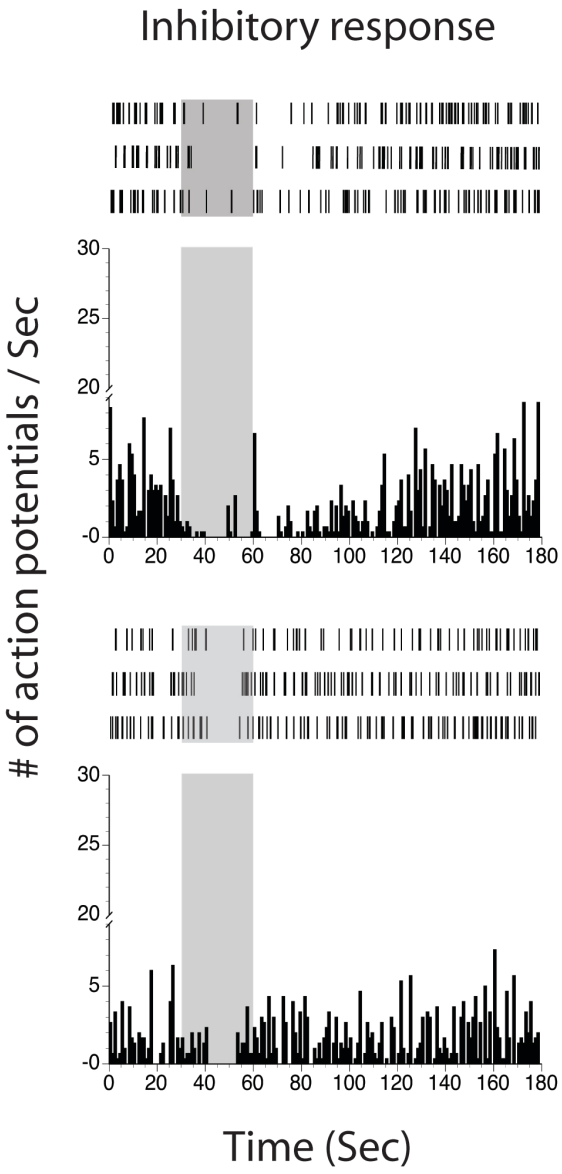

Figure 3.9. Examples of inhibitory multi-unit responses observed in the olfactory bulb. The rasters above each plot show three sequential recordings from the same location to the same stimulus. The corresponding post-stimulus time histogram (below) is averaged from the three multi-unit rasters. The grey shaded area denotes the 30 second delivery period for the olfactory test solution.

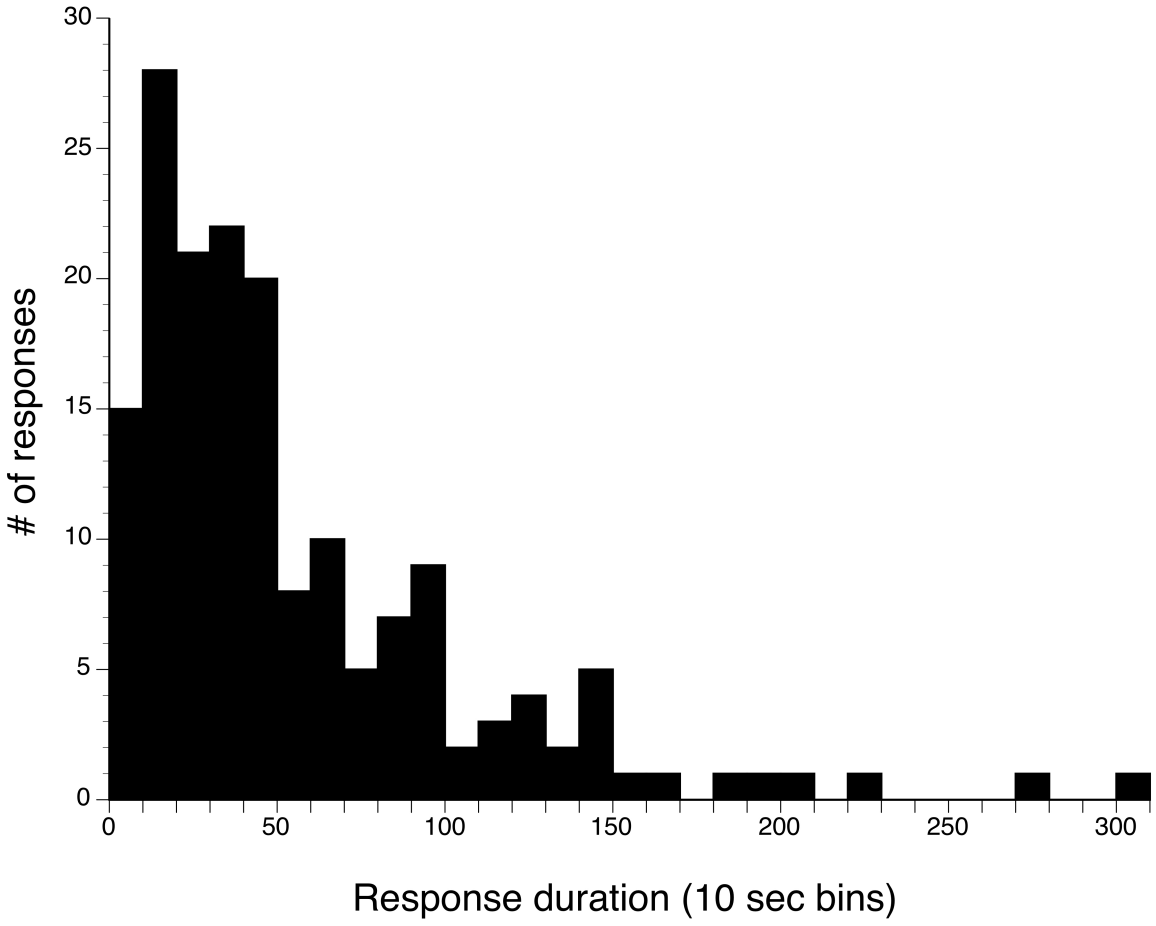

Figure 3.10. The distribution of all odourant test solution response durations for the multiunit recordings in the olfactory bulb. Each bin is 10 seconds.

30 and 60 seconds (Figure 3.8 C) (Figure 3.10) and 55 (32.5 %) multiunit recordings had response durations longer than 60 seconds (Figure 3.8 D, E) (Figure 3.10). Overall, odour-evoked responses of varying durations were observed using LFP and multi-unit recordings in the OB with excitatory responses being more prominent than inhibitory responses in the multiunit recordings.

3.3.3 Transient and sustained odour-evoked responses exhibit regional variation

Local field potential recordings (Figure 3.11) in the medial OB had 54.5% transient responses and 45.5% sustained responses. The lateral region had 23 % transient and 77 % sustained responses while the dorsal region had 35.8% transient and 64.2% sustained responses (Figure 3.12). In addition, sustained LFP responses in the medial OB have fewer peaks per response (Appendix C Figure 1) (Figure 3.11) and shorter interpeak intervals (Appendix C Figure 2) compared to sustained LFP in the dorsal and lateral OB regions. Interestingly, the proportions of each odour contributing to the transient and sustained responses in each region varied. In the medial OB approximately 46% of the transient odour responses were from the migratory pheromone mix while approximately 55 % of sustained responses in the medial OB were from the sex pheromone mix (Figure 3.12). In contrast, the transient and sustained LFPs in the lateral OB were all from amino acids while the dorsal region showed that roughly equal proportions of each odour contributed to the sustained LFPs (Figure 3.12).

The response duration of multiunit recordings to odour test solutions varied across the medial, dorsal, and lateral regions of the OB (Figure 3.13). The duration of odour-evoked multi-unit responses in the medial, dorsal, and lateral OB regions were not

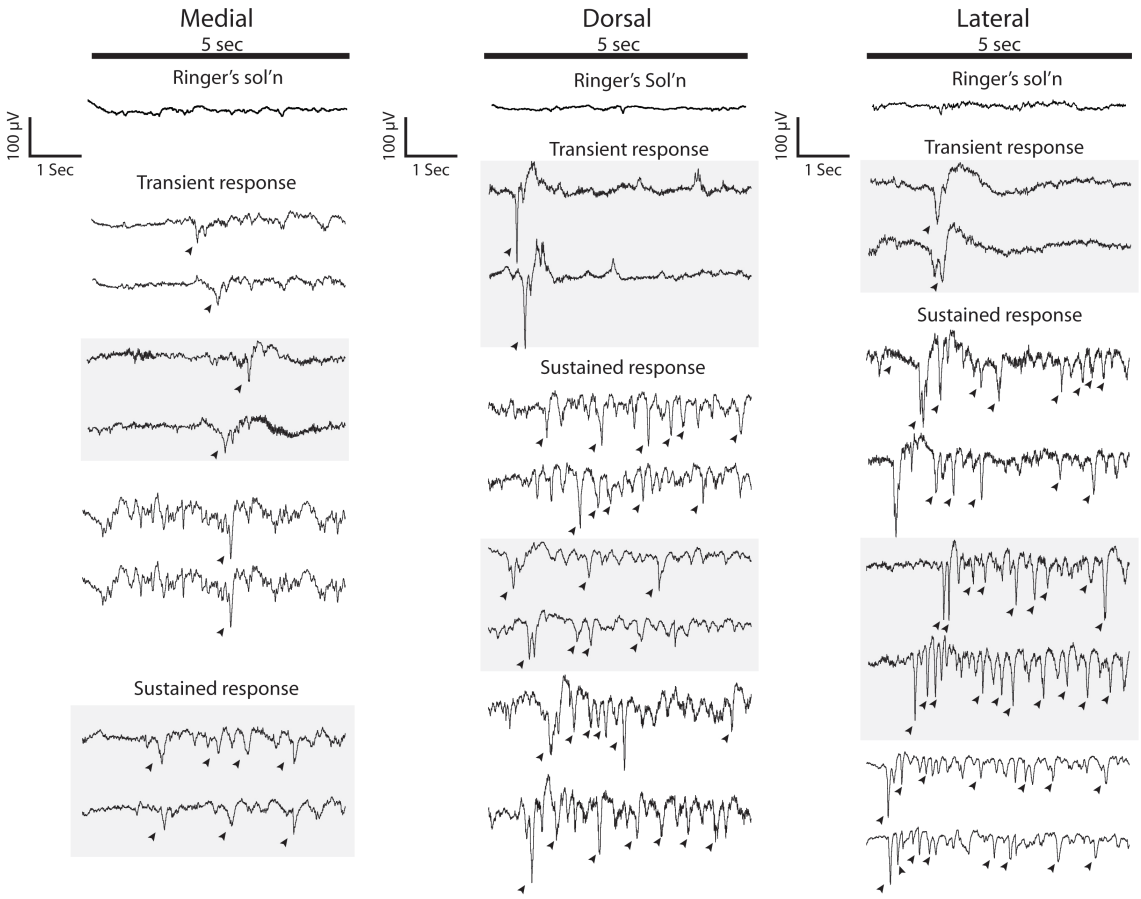

Figure 3.11. Examples of local field potential (LFP) recordings from the medial, dorsal and lateral OB regions during a 5 second application of odour test solutions (black bar above traces) to the peripheral olfactory organ. In all regions, no peaks were observed when Ringers solution was applied. The transient responses include a prominent peak, as shown by the arrowhead, during the odour delivery period. Several prominent peaks illustrate a sustained response over the odour delivery period. In the medial OB, transient responses were more common than in the dorsal and lateral regions. The shaded and unshaded blocks are example pairs of LFP traces, with each pair of LFP traces being from the same recording site in response to the same odour test solution.

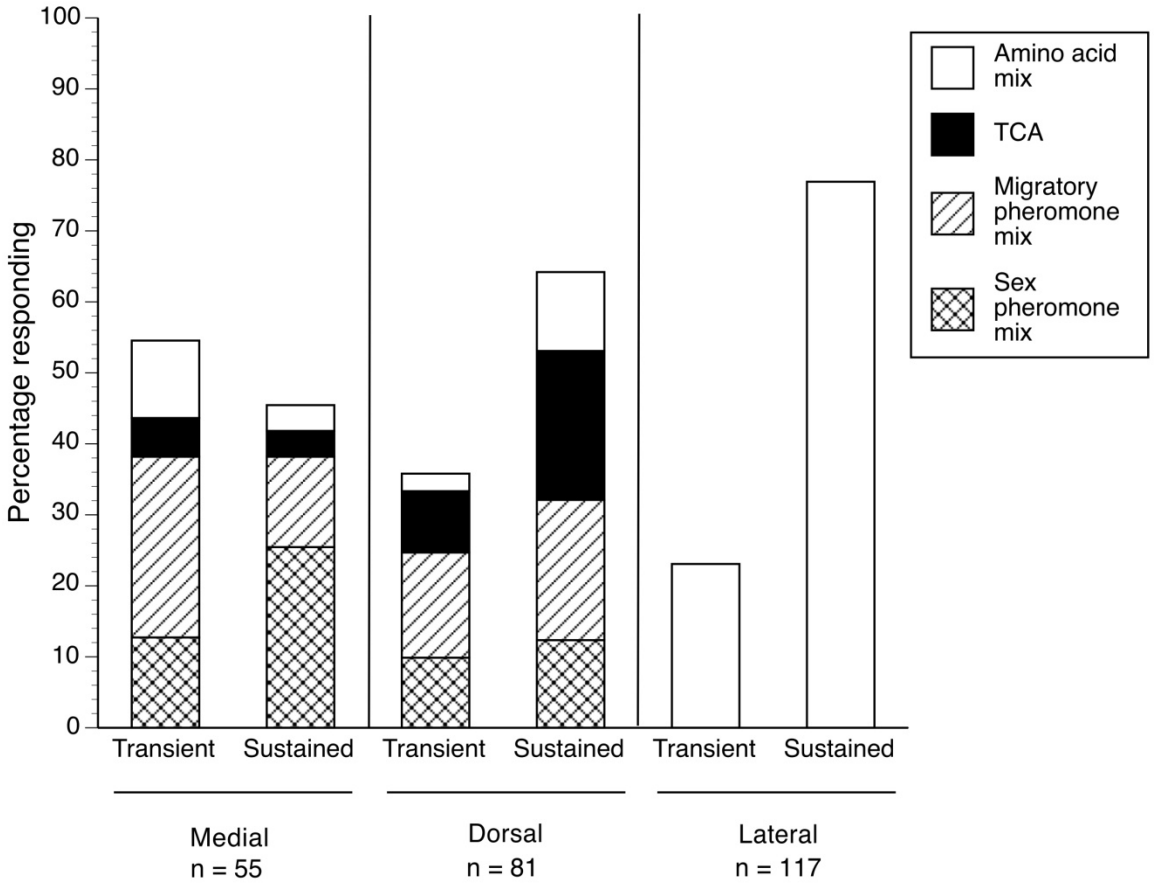

Figure 3.12. The percentage of local field potential responses to odourant test solutions applied to the peripheral olfactory organ that exhibited transient or sustained response types in the medial, dorsal, and lateral regions of the olfactory bulb. The sample size for each region represents the total number of responses across all odours for that region.

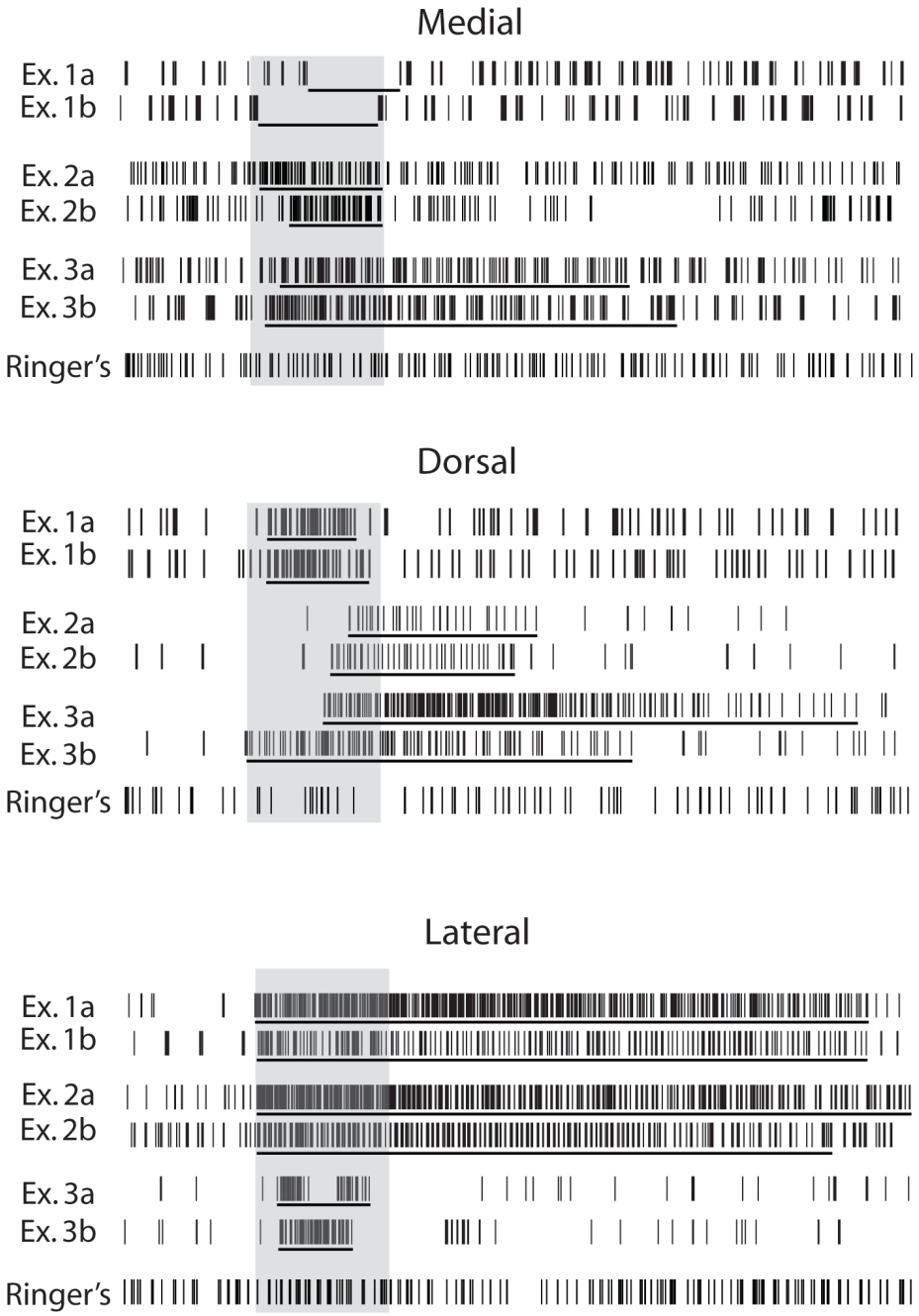

Figure 3.13. Examples of multiunit recordings of varying durations from the medial, dorsal and lateral regions of the olfactory bulb during the application of test solutions to the peripheral olfactory organ. Paired raster plots show responses to two deliveries of the same odour during a given recording (i.e. Ex 1a/1b). Three example recordings are shown for each region of the olfactory bulb (i.e. Ex 1, 2, 3). The bars underneath each raster mark the odour response period. The shaded area denotes the 30 second odour delivery period. All rasters are 180 seconds in duration.

significantly different (Kruskal-Wallis, $H_{(2)} = 4.56$, P = 0.1022) (Figure 3.14), however, response durations in the lateral OB region did show a trend towards longer durations than those in the medial and dorsal OB regions (Figure 3.14). Interestingly, the mean response duration of multiunit recordings to both the migratory pheromone and sex pheromone mixtures was consistent across the medial, dorsal, and lateral OB regions (Figure 3.15). In contrast, the mean response duration to both the amino acid mixture and the taurocholic acid varied across all three OB regions (Figure 3.15). Overall, both the LFP and multiunit recordings demonstrate that the response duration to odourant test solutions varies across all three regions of the OB and that different odours exhibit different response durations in each of the three regions.

3.3.4 The medial OB responds to all odours while the dorsal and lateral OB regions show odour specificity

Previous experiments (Ren et al., 2009; Derjean et al., 2010) demonstrated that the OB has two olfactory pathways with each pathway having different inputs from the peripheral olfactory organ to distinct regions of the OB. These two distinct regions, known as the medial and non-medial regions, also have outputs to different areas of the brain. Therefore we wanted to test whether the medial and two non-medial regions (dorsal and lateral) of the OB respond to different odours.

The medial OB region elicited LFP responses to each of the odour categories tested (Figure 3.16) with 60% of trials exhibiting responses to the amino acid mix, 50% of trials responding to TCA, 85.7% of trials responding to the migratory pheromone mix,

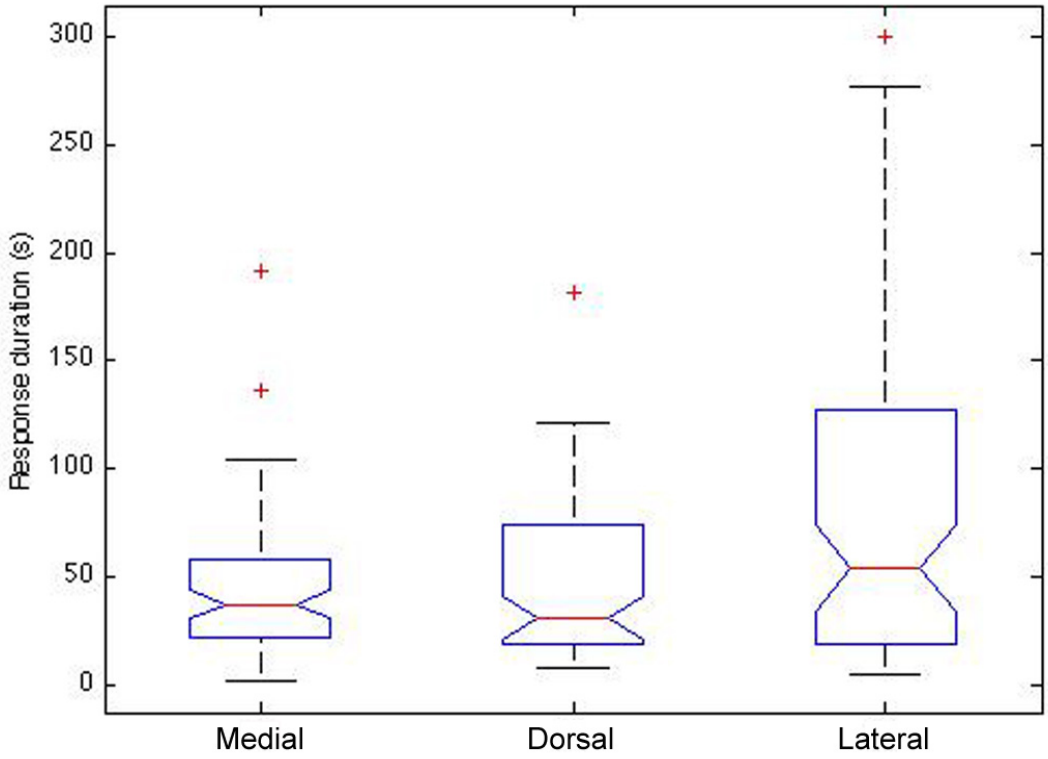

Figure 3.14. Distribution of multiunit response durations in the medial, dorsal and lateral olfactory bulb regions. The upper and lower bounds of each box represent the 75th and 25th percentile respectively, and the centre line in each box represents the median of each data set. The 'plus' symbols denote outliers in the data.

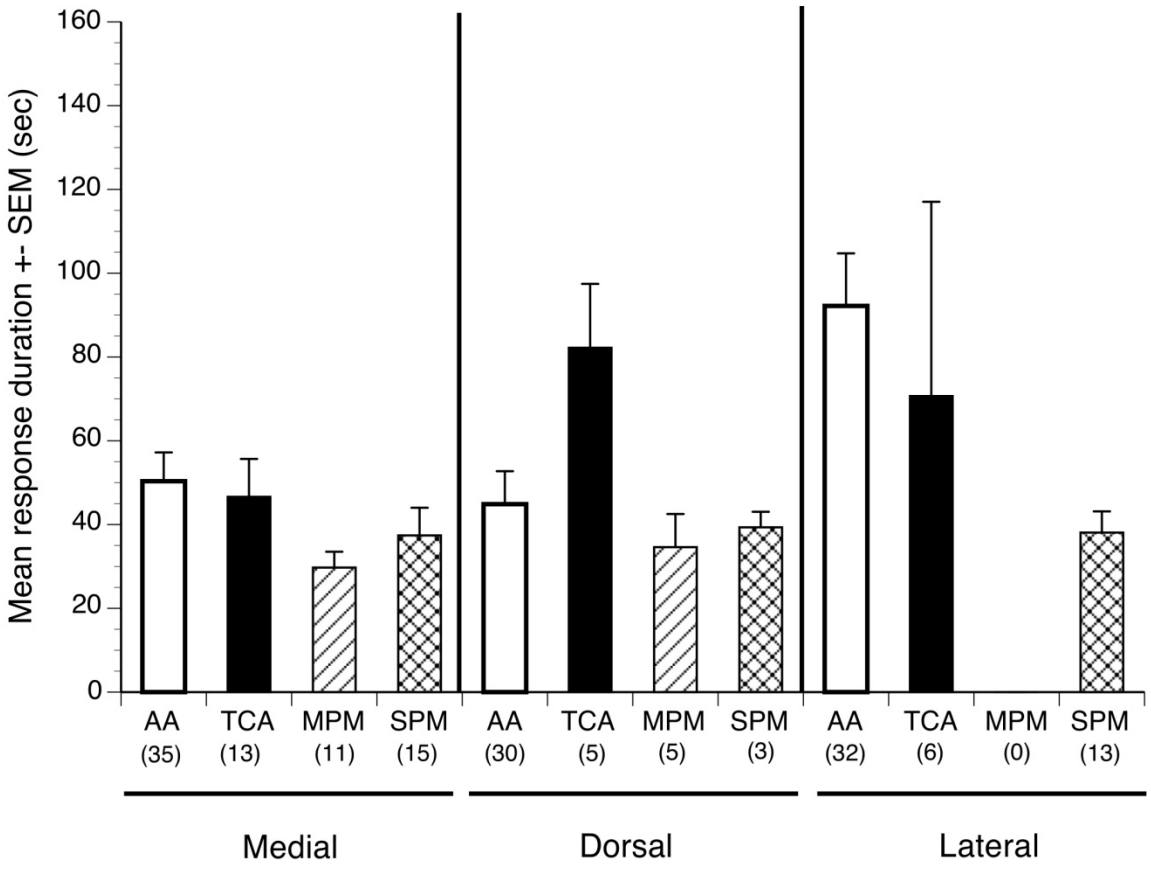

Figure 3.15. The duration of multiunit responses (Mean ± SEM) to each of four odour test solutions within the medial, dorsal, and lateral olfactory bulb. Odourant test solutions included an amino acid mixture (AA: L-arginine, L-histidine), taurocholic acid (TCA), sex pheromone mixture (SPM: 3KPZS, 3KACA), and a migratory pheromone mixture (MPM: PZS, PADS, PSDS). The number of multiunit responses (sample size) is indicated in brackets below each bar.

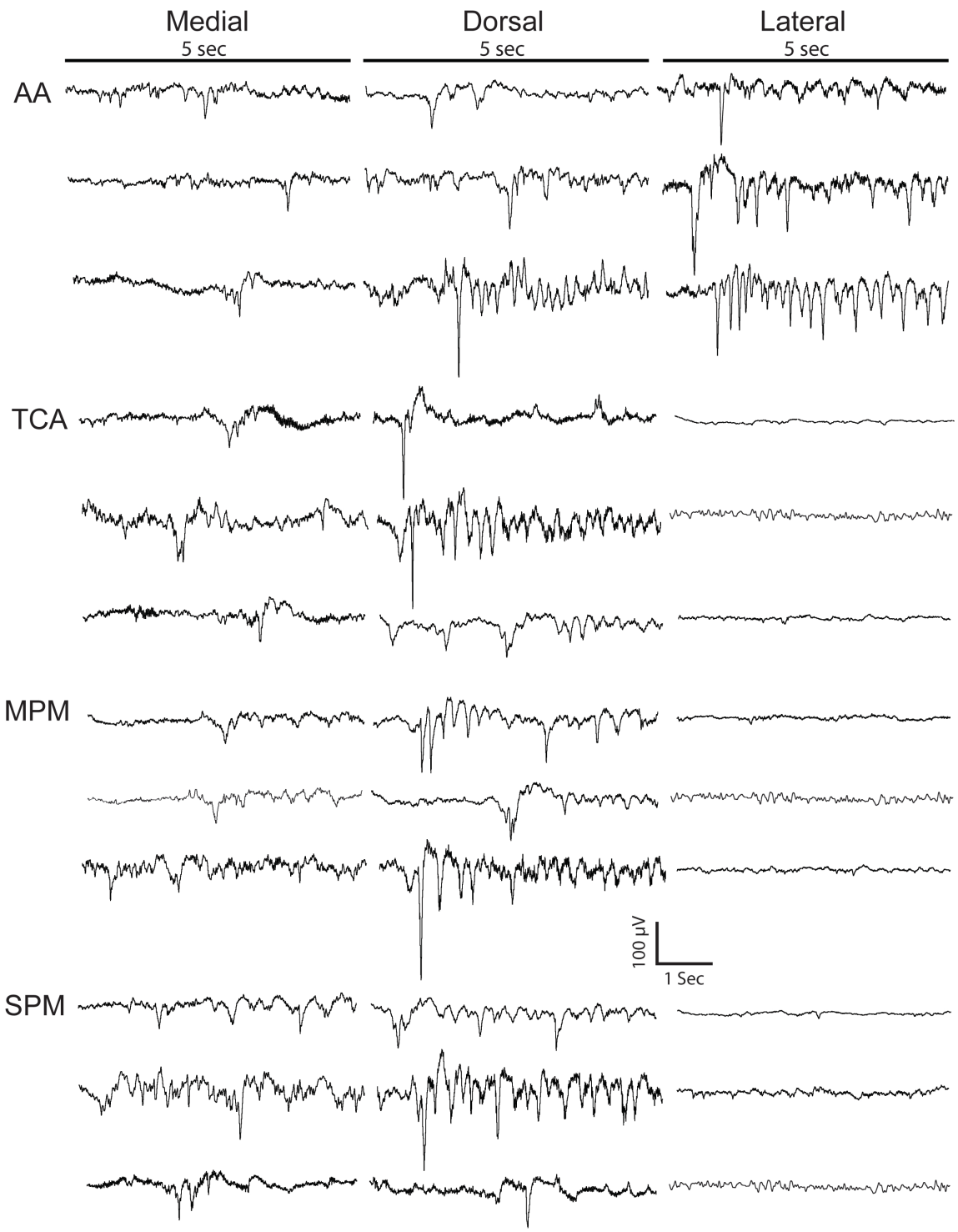

Figure 3.16. Sample recordings of local field potential (LFP) responses to test solutions delivered to the peripheral olfactory organ recorded from the medial, dorsal, and lateral regions of the olfactory bulb (OB). Odourant test solutions included an amino acid

mixture (AA: L-arginine, L-histidine), taurocholic acid (TCA), sex pheromone mixture (SPM: 3KPZS, 3KACA), and a migratory pheromone mixture (MPM: PZS, PADS, PSDS). Example LFP responses from three different recording sites are shown for each odour in each OB region. Each LFP trace is 5 seconds in duration and the bar at the top of each column denotes the 5 second odour delivery period.

and 87.5% of trials responding to the sex pheromone mix (Table 3.1). In addition, multiunit recordings in the medial OB exhibited responses to each of the odour categories tested (Figure 3.17). Medial multiunit responses were observed to the amino acid mix and TCA in 72.7% and 63.6% of trials, respectively, while responses to the migratory pheromone and sex pheromone mixtures were observed in 33.3% and 18.2% of trials, respectively (Table 3.2). In the dorsal OB region both the LFP (Figure 3.16) and multiunit recordings (Figure 3.17) also showed responses to each of the odour categories tested. The TCA, migratory pheromone mix and sex pheromone mix elicited LFP responses in 100% of the trials while LFP responses to the amino acid mixture were observed in 75% of trials (Table 3.1). A high percentage of responses to the amino acid mixture (80%) were also observed in the multiunit recordings (Table 3.2), however, the response to TCA (60%), the migratory pheromone mix (50%) and sex pheromone mix (50%) (Table 3.2) was lower than those observed in the LFP recordings. Finally, in the lateral OB local field potential responses were only observed to the amino acid mixture (100%)(Figure 3.16, Table 3.1) while multiunit recordings in the lateral OB exhibited excitatory responses to the amino acid mixture (80%) (Figure 3.17, 3.18; Table 3.2) as well as a small percentage of excitatory responses to TCA (11.1%) and the sex pheromone mixture (11.1%) (Table 3.2; Figure 3.18). Taken together, both LFP and multiunit recordings in the medial and dorsal OB

Table 3.1. Percentage of local field potential recording responses to test solutions applied to the peripheral olfactory organ in the medial, dorsal and lateral regions of the olfactory bulb. The test solutions were the amino acid mixture (AA: L-arginine, L-histidine), taurocholic acid (TCA), sex pheromone mixture (SPM: 3KPZS, 3KACA), and a migratory pheromone mixture (MPM: PZS, PADS, PSDS).

Region	Odour	Sample size	% responding
Medial	AA	5	60
	TCA	4	50
	MPM	7	85.7
	SPM	8	87.5
Dorsal	AA	4	75
	TCA	6	100
	MPM	3	100
	SPM	3	100
Lateral	AA	15	100
	TCA	7	0
	MPM	7	0
	SPM	6	0

Table 3.2. Percentage of excitatory multiunit recording responses to test solutions applied to the peripheral olfactory organ in the medial, dorsal and lateral regions of the olfactory bulb. The test solutions were the amino acid mixture (AA: L-arginine, L-histidine), taurocholic acid (TCA), sex pheromone mixture (SPM: 3KPZS, 3KACA), and a migratory pheromone mixture (MPM: PZS, PADS, PSDS).

Region	Odour	Sample size	% responding
Medial	AA	11	72.7
	TCA	11	63.6
	MPM	3	33.3
	SPM	11	18.2
Dorsal	AA	5	80
	TCA	5	60
	MPM	2	50
	SPM	4	50
Lateral	AA	9	77.8
	TCA	9	11.1
	MPM	2	0
	SPM	9	11.1

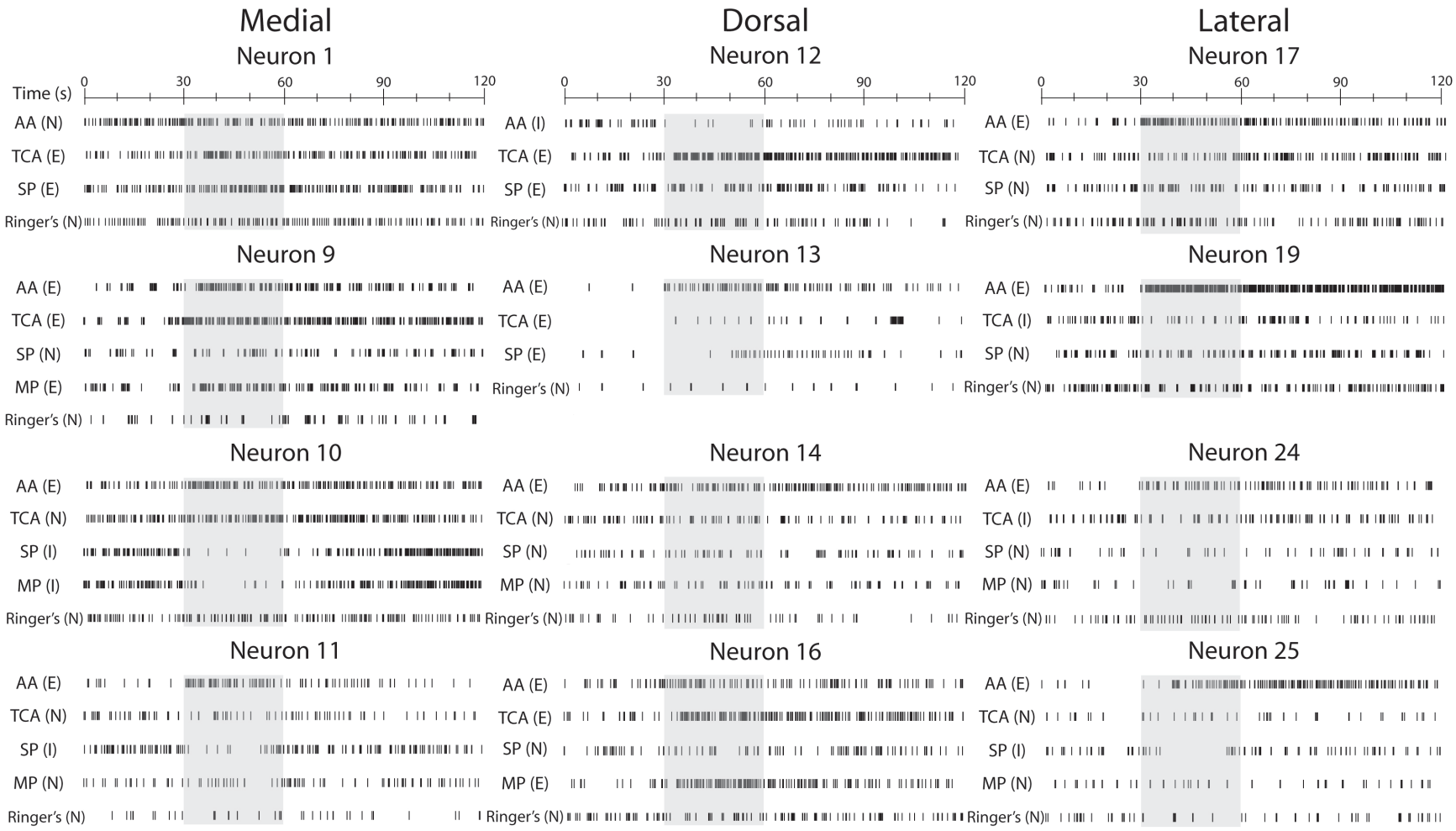

Figure 3.17 (caption on following page)

Figure 3.17. Examples of multiunit rasters during the delivery of test solutions to the peripheral olfactory organ, recorded from the medial, dorsal and lateral regions of the olfactory bulb (OB). The test solutions were an amino acids mixture (AA), taurocholic acid (TCA), sex pheromone mixture (SP) or migratory pheromone mixture (MP). Responses were classified as excitatory (E), inhibitory (I) or null (N). The time course of each raster plot is shown at the top of each column with the shaded region representing the 30 second odour delivery period.

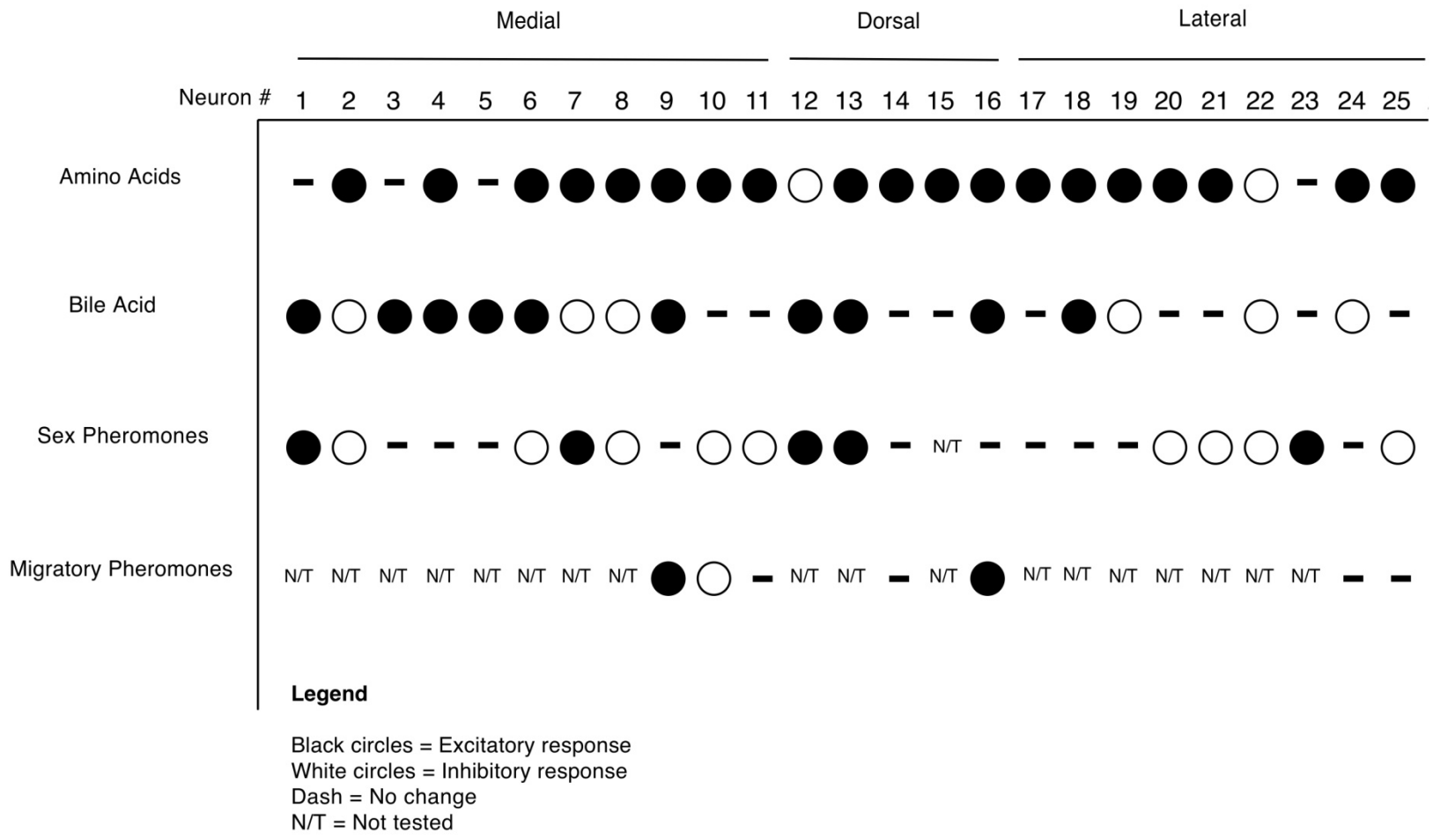

Figure 3.18. Summary of the multiunit recordings in the olfactory bulb during the application of odourant test solutions to the peripheral olfactory organ. Black circles denote an excitatory response, white circles an inhibitory response, dashes denote no change in spike firing rate, and N/T denotes that an odour was not tested.

regions exhibited responses to all of the tested odors while the lateral OB region exhibited responses solely (LFP) or primarily (multiunit) to the amino acid mixture and not to the TCA, sex pheromone mix or migratory pheromone mix. A more detailed examination of the LFP response peak characteristics demonstrates further regional differences in odour-evoked response patterns (see below).

3.3.5 Medial OB local field potential peak characteristics are the same for all odours

The temporal characteristics of the first peak of all the LFP responses from each region of the OB were examined for peak amplitude, duration, half-width, fall time, and rise time as described in the methods. We examined only the first peak of the LFP response in order to standardize the analysis across transient and sustained response patterns. A two-way ANOVA on ranks was conducted for each of the measured LFP peak characteristics and significant main effects of OB region ($p<0.001$) and the OB region x odour interaction ($p<0.001$) were determined for each of peak amplitude, duration, half-width, fall time, and rise time (See Table 3.3 for statistical details).

In the medial OB region peak amplitudes for all odours were similar in size, with mean values ranging from approximately 35 to 70 μV. The peak amplitudes for both TCA and the migratory pheromone mix in the medial region were smaller than their respective peak amplitudes in the dorsal region ($p<0.05$), which were typically larger than 100 μV (Figure 3.19A). Although the peak amplitude for all test odours in the medial region were small, the temporal features (peak duration, halfwidth, falling phase and rising phase) were as long as those in the other regions of the OB ($p>0.05$). Thus, LFP responses to all odours in the medial OB were on average between 110-170 ms in

Table 3.3 Statistical values for the two-way ANOVA on ranks conducted for local field potential peak amplitude, duration, halfwidth, falling phase, and rising phase (as shown in Figures 3.19 A-E).

LFP peak measure	F	df	P
Amplitude			
OB region	27.880	2, 79	<0.001
Odour	0.901	3	0.446
Region x odour interaction	8.215	6, 79	<0.001
Duration			
OB region	19.898	2, 79	<0.001
Odour	1.484	3	0.227
Region x odour interaction	6.509	6, 79	<0.001
Halfwidth			
OB region	18.109	2, 79	<0.001
Odour	1.613	3	0.194
Region x odour interaction	6.415	6, 79	<0.001
Falling phase			
OB region	19.226	2, 79	<0.001
Odour	1.256	3	0.297
Region x odour interaction	5.429	6, 79	<0.001
Rising phase			
OB region	17.839	2, 79	<0.001
Odour	1.657	3	0.184
Region x odour interaction	7.047	6, 79	<0.001

duration, producing small amplitude long duration LFP peaks (Figure 3.19 B, C, D, E). Interestingly, the medial region was the only region of the OB, where for any given peak characteristic, the amplitude and temporal values were approximately the same for all of the odours ($p>0.05$) (Figure 3.19 A-E).

3.3.6 Dorsal and lateral OB local field potential peak characteristics are odour dependent

The peak characteristics of LFPs in the dorsal region of the OB demonstrate that although LFP responses are observed to all odours in this region, two particular types of odours are shorter. All odours exhibited similar peak amplitudes with mean values greater than 100 μV (Figure 3.19A). However, the LFP peaks for the amino acid mixture and taurocholic acid exhibited a non-significant trend ($p = 0.06 – 0.09$ depending on the odour) towards having shorter average peak durations between 60 and 80 ms respectively, while those peak durations in response to the sex pheromone and migratory pheromone mixture averaged between 170 and 180 ms, respectively (Figure 3.19B). These non-significant trends ($p = 0.06 – 0.09$ depending on the odour) towards differences in the dorsal temporal peak characteristics for the amino acid mixture and taurocholic acid odours were also observed for the peak halfwidth, falling phase, and rising phase as well (Figure 3.19 C, D, E). Overall, local field potential responses in the dorsal OB region exhibit large amplitude, long duration responses for both the sex pheromones and migratory pheromones and large amplitude, shorter duration responses to amino acids and taurocholic acid.

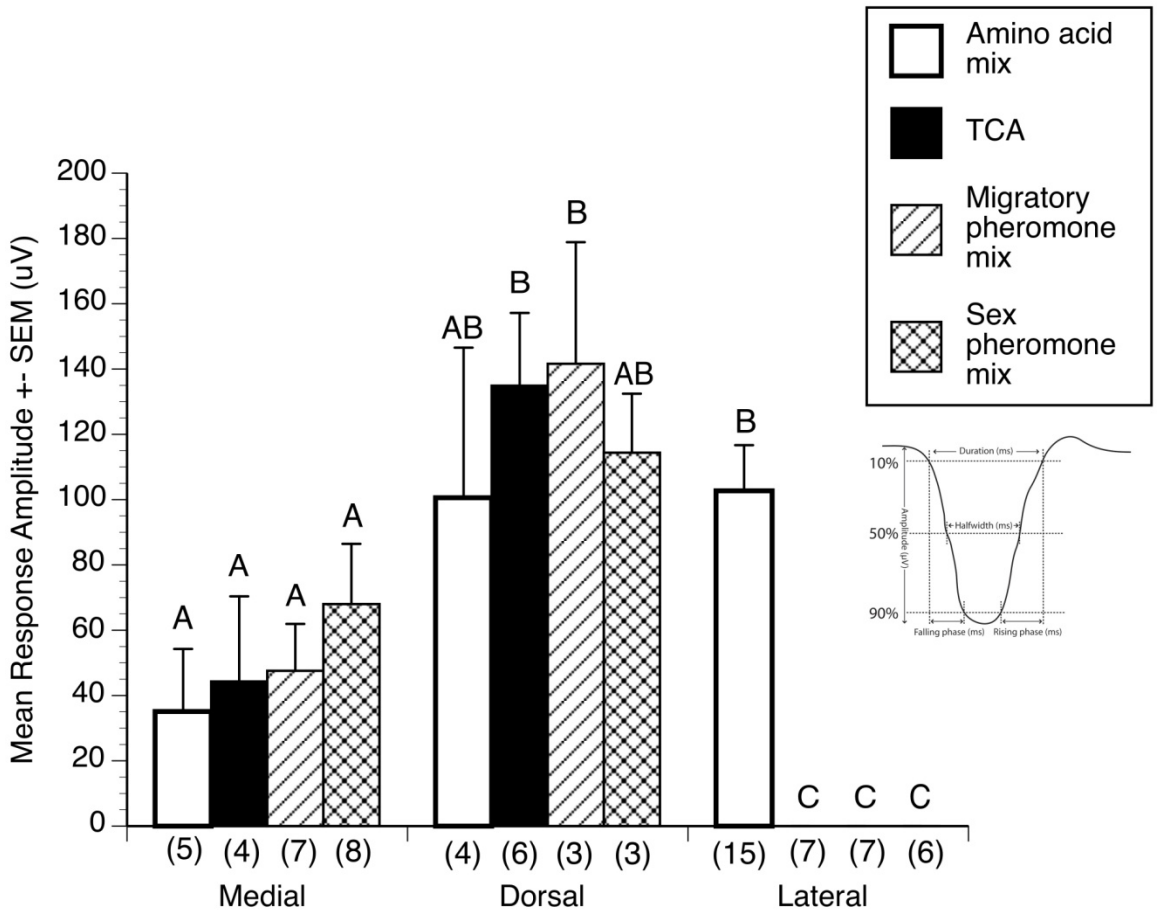

Figure 3.19A. Amplitude and temporal measurements of local field potential (LFP) recordings from the olfactory bulb during the application of four odour classes to the peripheral olfactory organ. (A) peak amplitude (mean ± SEM, μV), (B) peak duration (mean ± SEM, ms), (C) peak halfwidth (mean ± SEM, ms), (D) peak falling phase (mean ± SEM, ms), and (E) peak rising phase (mean ± SEM, ms). The LFP peak characteristics were determined using only the first peak of the LFPs in order to standardize values across transient and sustained LFP response types. The number below each bar is the sample size. **A.** Bars that do not share the same letter within an OB region or with a corresponding odourant are significantly different ($p<0.05$). **B-E**. Asterisk indicates a significant difference ($p<0.05$). See text for statistical details.

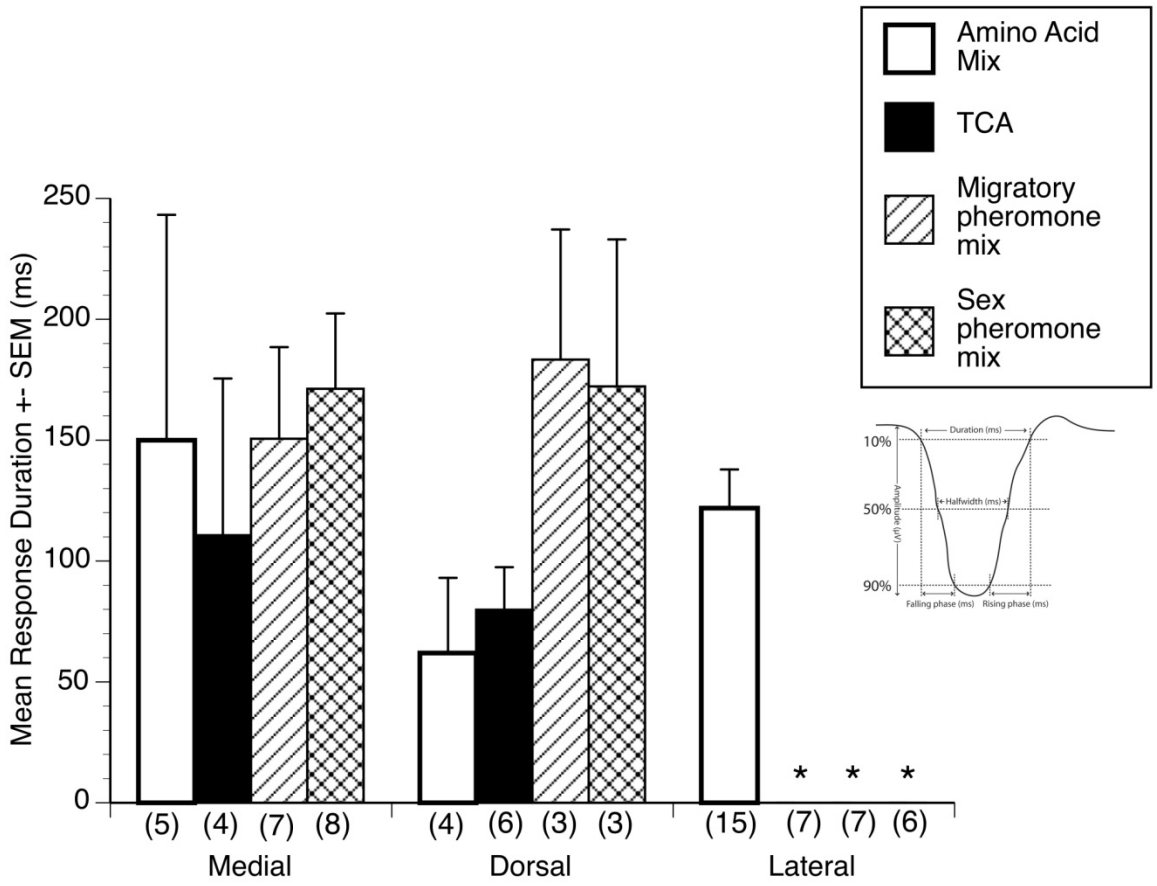
Amino Acid Mix
TCA
Migratory pheromone mix
Sex pheromone mix
Mean Response Duration +- SEM (ms)
250
200
150
100
50
0
(5) (4) (7) (8)
(4) (6) (3) (3)
(15) (7) (7) (6)
Medial
Dorsal
Lateral
Duration (ms)
10%
50%
90%
Halfwidth (ms)
Amplitude (μV)
Falling phase (ms)
Rising phase (ms)

Figure 3.19B

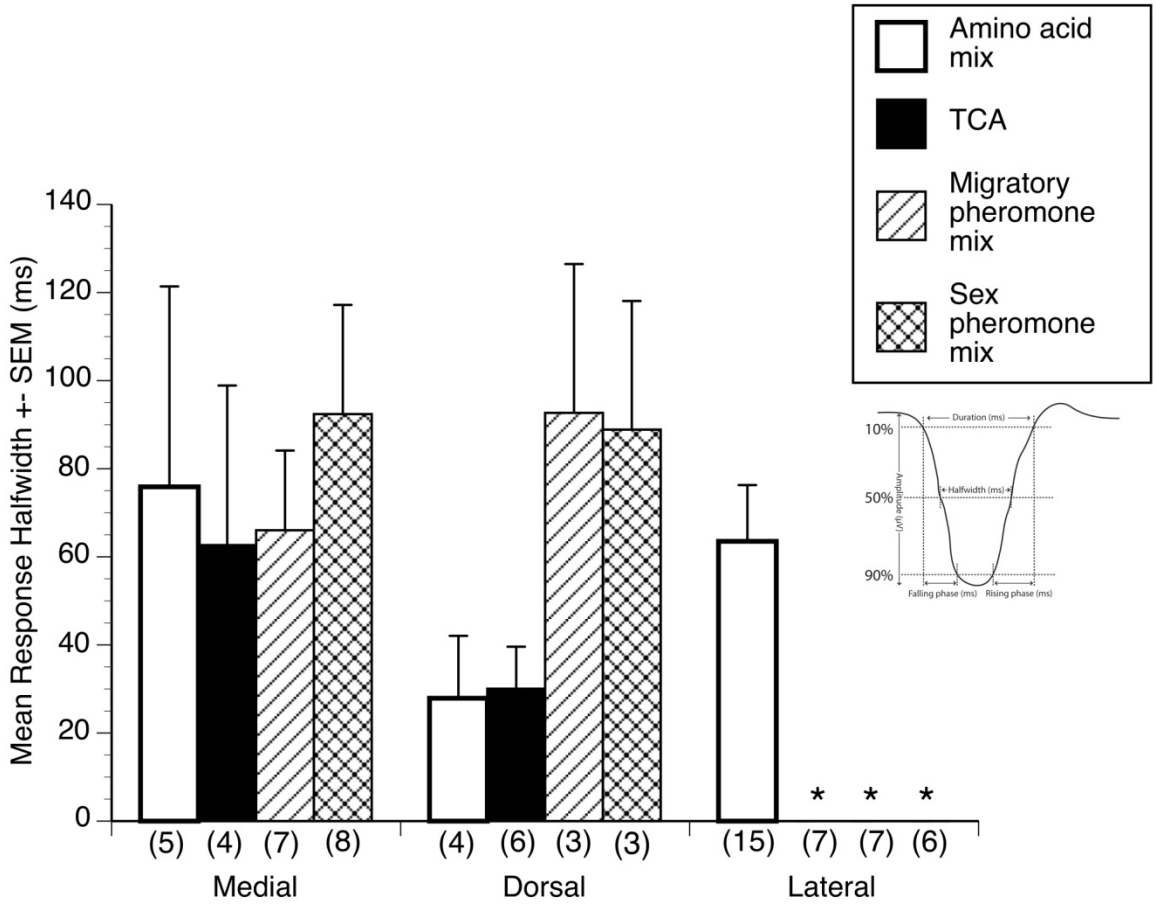
Amino acid mix
TCA
Migratory pheromone mix
Sex pheromone mix
Mean Response Halfwidth +- SEM (ms)
140
120
100
80
60
40
20
0
(5) (4) (7) (8)
(4) (6) (3) (3)
(15) (7) (7) (6)
Medial
Dorsal
Lateral
Duration (ms)
10%
50%
90%
Amplitude (uV)
Halfwidth (ms)
Falling phase (ms)
Rising phase (ms)

Figure 3.19C

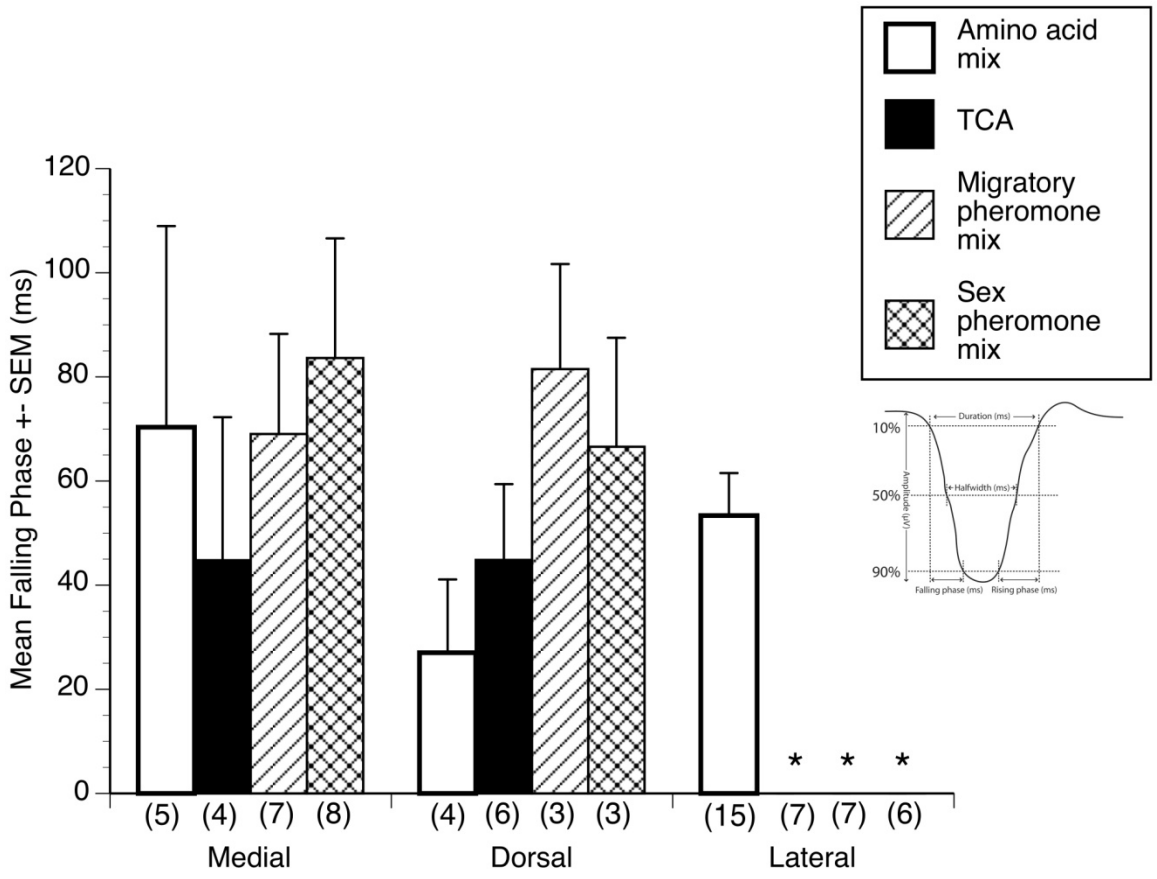
Amino acid mix
TCA
Migratory pheromone mix
Sex pheromone mix
Mean Falling Phase +- SEM (ms)
120
100
80
60
40
20
0
(5) (4) (7) (8)
(4) (6) (3) (3)
(15) (7) (7) (6)
Medial
Dorsal
Lateral
Duration (ms)
10%
50%
90%
Halfwidth (ms)
Amplitude (μV)
Falling phase (ms)
Rising phase (ms)

Figure 3.19D

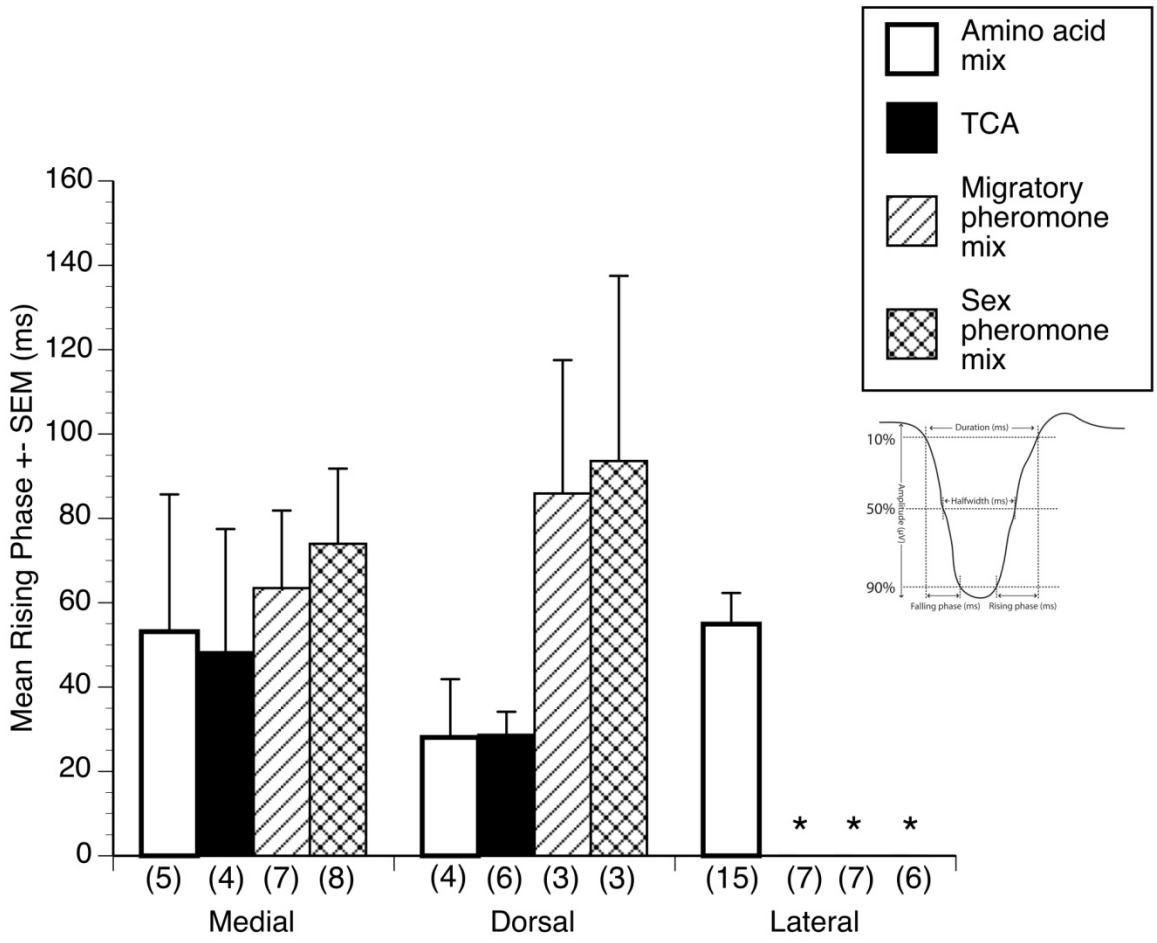
Amino acid mix
TCA
Migratory pheromone mix
Sex pheromone mix
Mean Rising Phase +- SEM (ms)
160
140
120
100
80
60
40
20
0
(5) (4) (7) (8)
(4) (6) (3) (3)
(15) (7) (7) (6)
Medial
Dorsal
Lateral
Duration (ms)
10%
50%
90%
Halfwidth (ms)
Amplitude (μV)
Falling phase (ms)
Rising phase (ms)

Figure 3.19E

The lateral OB region exhibited local field potential responses to only the amino acid mixture. The amplitude of these LFP peaks was large (typically greater than 100 μV) and similar to peak amplitudes in the dorsal region ($p>0.05$) but larger than peak amplitudes in the medial region ($p<0.05$) (Figure 3.19A). The temporal characteristics of the lateral responses to amino acids (peak duration, halfwidth, falling phase and rising phase) were similar to both medial and dorsal OB region temporal peak characteristics ($p>0.05$)(Figure 3.19 B, C, D, E). Overall, lateral local field potential responses exhibit large peak amplitudes and peak durations. The detailed examination of LFP peak characteristics clearly demonstrates that the medial region of the OB responds to amino acids, TCA, migratory and sex pheromones with similar amplitude and temporal characteristics while LFP responses in the dorsal region are odour specific and more prominent to both migratory and sex pheromones. In addition, the lateral OB responds only to amino acid odours.

3. 4 Discussion

The present study investigated odour coding in the OB as it relates to parallel processing pathways in a model olfactory-locomotor species, the sea lamprey. In the medial OB, a known olfactory-locomotor transformation region (Derjean et al., 2010), spatial as well as temporal differences in odour-evoked activity patterns were observed compared to the dorsal and lateral regions of the OB. Odour-evoked responses in the medial OB were generally transient and single LFP peaks in this region had smaller amplitudes with long durations. Responses in the medial OB were observed to amino acids, bile salts, and bile derived pheromones. In comparison, odour-evoked responses in

the lateral OB were generally sustained and LFP peaks had large amplitudes and long durations in response only to amino acid odours. In the dorsal region of the OB odour responses in both the multiunit and LFP recordings were observed to each of the odour types tested. Interestingly, the LFP peaks elicited larger amplitudes and durations specifically to the sex pheromones and migratory pheromones. Collectively these results suggest that odour information in the OB is temporally coded in relation to medial and non-medial OB output pathways, that the OB is chemotopically organized, and utilizes non-combinatorial odour coding in the single medial glomerulus responsive to all odours (Figure 3.20). Furthermore, our results demonstrate that the medial OB is functionally distinct from other regions of the OB and supports the conclusion that olfactory driven motor behaviours are initiated in this medial region as suggested by (Derjean et al., 2010).

3.4.1 Temporal pattern of odour-evoked responses

The medial region of the OB exhibited transient or shorter duration odour-evoked responses while the lateral region exhibited mainly sustained responses to odours. In mice, natural odours have been shown to induce transient and sustained responses in the MOB and AOB, respectively (Luo et al, 2003). Interestingly, Shpak et al (2012) also showed that transient and sustained mitral cell responses exist in the MOB and AOB, respectively, using electrical stimulation. Luo et al (2003) suggested that sustained responses in the AOB might be due to the additional time necessary to clear the

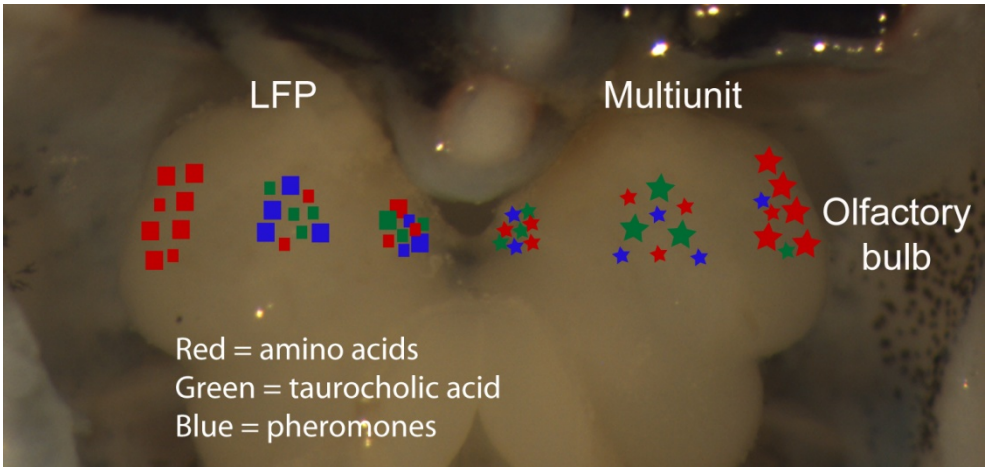

Figure 3.20. Photograph depicting the temporal, chemotopic and non-combinatorial organization of olfactory bulb regions within parallel olfactory pathways of the sea lamprey. Projection neurons elicited a mixture of transient (small symbols) and sustained (large symbols) responses to amino acids (Red: L-arginine and L-histidine), a bile salt (Green: taurocholic acid), putative sex pheromones (Blue: 3-keto petromyzonal sulfate and 3-keto allocholic acid), and putative migratory pheromones (Blue: petromyzonal sulfate, petromyzonamine disulfate, petromyzonsterol disulfate). The red, green, and blue symbols are representative recording locations to odour test solutions.

vomeronasal organ (VNO) of odours. Although this may be a factor in the sustained responses, Shpak et al (2012) provide evidence of a physiological basis for the sustained responses as being mediated by a calcium activated non-selective cationic current that utilizes TRPM4 channels (Shpak et al., 2012). The AOB processes non-volatile social odour cues and both Luo et al (2003) and Shpak et al (2012) suggests that the sustained firing of AOB neurons functions to integrate and perceive these social cues. In the current study, PNs of the lateral OB region also had primarily sustained LFP or longer duration multiunit responses and, based on the known projections of these neurons to the lateral pallium, suggests that neurons in this region are integrating and perceiving the odour cues associated with this region. Interestingly, sustained responses were observed in the lateral OB, which receives inputs only from the MOE. Therefore, the time required to clear the odour from the nasal cavity would be less of a factor due to the quick rinse out of odours from the MOE, and suggests a physiological basis for the sustained responses observed. In contrast, transient responses were observed in the medial OB, which receives olfactory inputs mainly from the AOO. If the medial OB is an olfactory centre responsible for fast initiation of locomotion and not integration as suggested by Derjean et al (2010) then transient responses may facilitate a quick olfactory-locomotor response.

Local field potentials in the OB and AL are typically oscillatory waveforms that can have varying frequencies depending on the species being studied and the neural circuitry involved (Kay and Stopfer, 2006; Rojas-Libano and Kay, 2008; Kay et al., 2009). Generally there are three frequency bands of oscillations in the OB, the theta (~1-12 Hz), beta (~15-30 Hz), and gamma (~40-100 Hz) frequency bands, although fish, frogs, and invertebrates show some deviation from these categories with odour-evoked

gamma-like oscillations ranging from 10-30 Hz (See review by Kay et al 2009). Oscillatory activity in the OB is linked to the respiration cycle of the animal and serves to make odour representations sparser, and therefore odour identification simpler. The lamprey OB did not exhibit any clear oscillatory activity in response to odour stimulation; however, a few responses did exhibit dampened oscillation-like activity. A possible explanation for the apparent lack of oscillatory activity in the OB is related to the lack of active sniffing and respiration in the ex-vivo preparation. Since OB oscillations are tightly linked to the respiratory cycle it is possible that a lack of respiratory activity, due to the removal of all respiratory tissue (gills), would cause the removal of oscillatory activity. However, extracellular recordings from the respiratory nuclei in the hindbrain still elicited strong stereotypical activity of 1 Hz throughout the recordings (data not shown). Regardless of the few oscillation-like responses, a majority of odour-evoked activity elicited slow temporal patterns that were either transient (single peak) responses or non-oscillatory sustained (multiple peak) responses. Interestingly, in the AL of locusts and honeybees, blocking of $GABA_A$ receptors removed the oscillatory component of the PN response but left the slow temporal pattern intact (Stopfer et al., 1997). The locusts and honeybees were still able to discriminate structurally different odours but not similar ones (Stopfer et al., 1997). The slow temporal patterns observed by Stopfer et al (1997) look similar to the LFPs observed in the current study of the sea lamprey OB. However, it is possible that the ex-vivo preparation used in the current study somehow causes the blocking of $GABA_A$ receptors; although similar preparations have been used by Friedrich and Korsching (1997) and many subsequent studies by Friedrich with no such problem reported. In addition, this preparation has been used in previous

lamprey studies (Derjean et al., 2010) and the blocking of $GABA_A$ leading to a loss of oscillatory waveforms has not been reported.

The medial region of the OB had several response characteristics that differed from the dorsal and lateral OB regions. This includes multiunit response duration, the number of peaks in an LFP response, the LFP response amplitude, and LFP peak duration. The medial OB is anatomically different from the other OB regions with medial PN cell bodies located inside the glomerulus and thus closely associated with OSN axons; while dorsal and lateral PN cell bodies are located deeper in the OB and project their dendrites into the glomerular layer (Green et al, chapter 2). This anatomical difference may be driving the physiological differences observed in the current study. There are fewer PNs in the medial OB compared to the rest of the OB, and since the medial OB is responsive to all odours tested, it may be that only a few medial PNs are driving the odour-evoked response to any given class of odour. This may account for the smaller amplitude of the medial LFPs observed. The lack of oscillatory activity suggests that the lamprey OB, and in particular the medial region of the OB, has a different basic circuitry than what is typically observed in a vertebrate OB. Oscillatory LFP activity is indicative of a high level of synchrony by projection neurons and is generated by the excitatory-inhibitory dendrodendritic interactions between primary projection neurons and granule cells (Shepherd and Greer, 1998). A lack of oscillatory LFP activity suggests a lack of synchronization of projection neurons in the lamprey OB and a possible difference in the projection neuron – interneuron circuitry or differences in innervation from higher brain centers. In the medial region where transient responses were prominent, it may be that granule cells have a different role or are not present at all.

Therefore another type of interneuron, known as periglomerular cells, may play an important role in modulating responses in the medial OB and have been shown to presynaptically modulate mitral/tufted cells in the mammalian OB (Gire and Schoppa, 2009) by releasing dopamine, thereby decreasing the amount of glutamate released onto the dendrites of mitral/tufted cells (Kay and Stopfer, 2006). The possible difference in the type and/or role of interneurons may be the reason for the small amplitude, but long duration, odour-evoked responses in the medial region of the lamprey OB. However, another explanation for the temporal differences observed in the odour responses in the medial OB may be the type of G-protein used in signal transduction. Previous work by (Frontini et al., 2003) indicated that olfactory sensory neurons projecting to the medial glomerulus do not contain the cAMP-dependent G protein G_{olf} while olfactory sensory neurons projecting to all other regions of the lamprey OB contain G_{olf}. Further studies are needed to focus on the transduction mechanisms related to the medial region of the OB.

3.4.2 Spatial pattern of odour-evoked responses

The topographic organization of odour information in the olfactory bulbs based on odourant chemical structure (chemotopy) has been extensively studied in mammals (Johnson and Leon, 2007). Similar findings have been observed in the olfactory bulbs of fishes, which are in general, chemotopically organized into medial and lateral regions based on odourant type and known medial and lateral output pathways of the OB (reviewed by Zielinski and Hara, 2006). Our findings in the sea lamprey, an extant agnathan vertebrate fish, demonstrate that the OB is divided into medial and non-medial (dorsal and lateral) regions, which are chemotopically organized as well as functionally organized according to their output pathways. Our results show that the medial OB

responds to amino acids, bile acids, and bile derived lamprey pheromones. Previous work in zebrafish (Friedrich and Korsching, 1998), catfish (Nikonov and Caprio, 2001; Hansen et al., 2003), and salmonids (Hara and Zhang, 1998) have shown that bile acids activate the medial OB region, while sex pheromones activate the medial OB in goldfish (Fujita et al., 1991). In addition, amino acids elicited weak responses in the medial region as well as most of the posterior half of the OB in brown trout (Hara and Zhang, 1998; Laberge and Hara, 2001, 2004). Taken together, these studies in conjunction with our findings suggest that depending on the species, the medial OB may be responsive to several types of odourants. Interestingly, bile acids have been shown to activate the dorsal OB in salmonids (Hara and Zhang, 1998), which is consistent with our results in the sea lamprey, that bile acids and bile derived pheromones elicit the strongest responses in the dorsal OB. Amino acids have been shown to activate the lateral region of the OB in zebrafish, catfish, and salmonids (Friedrich and Korsching, 1998; Hara and Zhang, 1998; reviewed in Laberge and Hara, 2001; Nikonov and Caprio, 2001; Hansen et al., 2003; Zielinski and Hara, 2006) and was also observed in the present study in sea lamprey. Nucleotides (which were not tested in the current study) have been shown to activate the caudal lateral region of the dorsal surface of OB in catfish (Nikonov and Caprio, 2001; Hansen et al., 2003). The peripheral olfactory organ of sea lamprey has been shown to be most sensitive to basic amino acids (Li, 1994) as well as a variety of bile acids including bile acid derived pheromones (Li et al., 1995; Li et al., 2002). The current study only focused on basic amino acids, a common bile acid (TCA) in fish and the known/putative sea lamprey pheromones. Future studies should examine the OB responses to nucleotides and a putative alarm substance (Wagner et al., 2011) and also examine whether

individual neurons in the medial and non-medial OB regions of the lamprey are responsive to particular chemical features (e.g. carbon chain length or functional group) of a specific odour class.

Although the canonical view of spatial encoding of olfactory information is that a given odour activates multiple glomeruli in a combinatorial fashion, there is growing evidence that particular odours, specifically pheromones, activate only a single glomerulus and therefore odour information is encoded in a noncombinatorial glomerular activity pattern (Friedrich and Korsching, 1998; Suh et al., 2004; Semmelhack and Wang, 2009). Previous work in the sea lamprey has shown that the medial region of the OB is comprised of a single glomerulus (Green et al, chapter 2) and that PNs associated with this glomerulus are part of an olfactory-locomotor pathway (Derjean et al., 2010). The current study demonstrates that the sex pheromones 3KPZS and 3KACA, as well as the migratory pheromones PZS, PADS, AND PSDS all activate PNs within the medial glomerulus. In addition, the general fish bile salt TCA and two amino acids L-arginine and L-histidine also activate the medial glomerulus PNs, suggesting that several classes of odours, not just pheromones, are represented in a noncombinatorial pattern in the medial OB. Interestingly, each of these odour classes were also represented in different non-medial OB regions in presumably a combinatorial activity pattern, although glomerular activation patterns were not examined using imaging techniques in the present study. Friedrich and Korsching (1998) proposed that noncombinatorial glomerular activation patterns of odours in a single glomerulus might require less processing by higher brain structures than combinatorial glomerular activation patterns across many glomeruli. The transient nature of responses observed in the single glomerulus of the

medial OB would presumably require less processing and interpretation by higher brain structures than the sustained responses observed in the non-medial OB regions. Considering that the medial OB output constitutes a direct olfactory-locomotor pathway (Derjean et al., 2010) it may be beneficial to have such a transient response that requires less processing in order quickly induce olfactory driven motor responses to biologically relevant odours.

The medial glomerulus receives inputs primarily from olfactory sensory neurons (OSNs) in the accessory olfactory organ of the lamprey. The ligand-receptor specificity of OSNs in the accessory olfactory organ are unknown, but our current data suggests that they may be broadly tuned because the medial PNs are activated by a variety of odours. However, electro-olfactogram recordings have shown that there are at least four independent receptor sites for bile acid derived odours, including pheromones, in the main olfactory organ of the sea lamprey (Li and Sorensen, 1997). Moreover, the medial glomerulus has sparse OSN inputs from the main olfactory organ and these OSNs may be specifically tuned to a particular class of odourant, in-turn activating a specific subset of PNs in the medial glomerulus to that odourant. Further experimentation is required to understand the ligand-receptor specificity of OSNs in the accessory olfactory organ as well as that of the sparse OSNs in the main olfactory organ that project into the medial glomerulus.

3.4.3 Functional organization of projection neurons driving behaviour

The activity of primary projection neurons from the OB of vertebrates, or the antennal lobe of invertebrates, ultimately initiates and modifies the behaviours of an organism. Odours are first processed in the OB and there is growing evidence that the OB

is organized by its functional output rather then by its odour inputs. Recently, the OB of the sea lamprey has been shown to have two olfactory output pathways, one of which (the medial pathway) functions to initiate olfactory-locomotor behaviours (Derjean et al., 2010). Evidence of the OB being functionally organized based on output pathways related to specific behaviours has been observed in other vertebrates as well as invertebrates. In mice the OB is functionally divided into dorsal and ventral regions, with the dorsal regions having two subdomains, one initiates fear responses to a predator odour while the other initiates aversive responses to spoiled food (Kobayakawa et al., 2007; Matsumoto et al., 2010; Mori and Sakano, 2011). In addition, Igarashi et al (2012) recently demonstrated that mitral and tufted cells project to spatially segregated regions of the olfactory cortex. Tufted cells in particular transmit fast signals to cortical regions that potentially have a role in olfactory behaviour, although the functional role of these cortical areas is still largely undetermined (Igarashi et al., 2012). Attraction and aversion behaviours in *Drosophila* are mediated by individual glomeruli in the AL. Suh et al (2004) demonstrated that a single glomerulus in the AL was involved in avoidance behaviours to CO_2 while Semmelhack and Wang (2009) showed that two glomeruli, either one of which, when activated by a food odour was capable of eliciting attraction responses. Interestingly, when the food odour concentration was increased, an additional single glomerulus was activated that elicited aversive behaviours (Semmelhack and Wang, 2009). Tract-tracing studies in the ALs of the honeybee have shown two olfactory pathways whereby PNs innervating the ventral-rostral AL project along the lateral antenno-cerebral tract and PNs innervating the dorsal-caudal AL project along the medial antenno-cerebral tract (Kirschner et al., 2006). The medial and lateral antenno-cerebral

tracts project to the mushroom bodies and lateral horn where they innervate distinctly separate regions of these higher brain centres (Kirschner et al., 2006). These findings are similar to those in the sea lamprey where PNs of the medial OB innervate a single medial glomerulus (Green et al., chapter 2) and project along an isolated olfactory-locomotor pathway (Derjean et al., 2010). In addition to attraction and aversion behaviours to odours related to predators and food, olfactory pathways in mice have been associated with mating behaviours (Boehm et al., 2005; Yoon et al., 2005).

The results of the current study demonstrate that the PNs in the medial OB of the sea lamprey exhibit generally transient responses to each of the odours examined; while those PNs in the dorsal and lateral regions of the lamprey OB are generally more sustained and exhibit regionally specific odour responses suggesting chemotopic organization. Furthermore, our results support the hypothesis of two functionally distinct parallel olfactory pathways in the lamprey, a medial pathway for locomotion and a non-medial (dorsal and lateral) pathway(s) that may play a role in integration and perception of odours (Figure 19). Further studies are needed to examine whether the functional organization of the OB based on its output pathways is a widespread phenomenon and whether distinct fast-acting olfactory-locomotor pathways exist in other species.

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CHAPTER 4

Concluding Remarks

4.1 Concluding Remarks

The olfactory bulb (OB) is a vital component of the olfactory system. Information regarding odours that activate OSNs in the periphery, may be associated with a variety of biological functions including foraging, reproduction, and predator avoidance, are processed by neurons located in the OB and ultimately drive behaviour. To perceive and decode this information the OB utilizes parallel processing pathways that are topographically and temporally organized (Friedrich and Korsching, 1997; Laurent, 2002; Kay and Stopfer, 2006; Johnson and Leon, 2007). How odour information is encoded, processed and how this leads to a behavioural response are questions that have been under investigation in rodents, fish, and insects. While much progress has been made into understanding how odours are detected in the peripheral olfactory organ(s) and processed in the OB, the detailed neural substrates involved in odour driven behaviours are still poorly understood. However, significant progress in determining the olfactory-locomotor neural substrate has been made in the sea lamprey.

The neural circuits that control locomotion in the sea lamprey are well characterized, as this basal vertebrate has been utilized as a model species for the study of central regulation of motor systems, for several decades (Grillner and Wallen, 1985; Dubuc et al., 2008). Recently, a growing body of literature has examined odour responses in the peripheral olfactory organ and odour-induced behaviours of the sea lamprey (Li and Sorensen, 1997; Li et al., 2002; Fine et al., 2004; Sorensen et al., 2005; Johnson et al., 2009). This background knowledge regarding the neurobiology of olfaction and locomotor control makes the sea lamprey an ideal model species for studying the

olfactory-locomotor neural substrate. Previous work by (Ren et al., 2009) and (Derjean et al., 2010) described the neural input pathways and output pathways of the OB, respectively. These two papers provided evidence of parallel olfactory pathways in the sea lamprey and describe a neural pathway from the medial region of the OB to the locomotor command neurons (reticulospinal cells) in the hindbrain. However, very little to nothing was known about the anatomical organization of bulbar PNs and their odour-induced physiological response characteristics. Thus, using the sea lamprey as a model species, this thesis contributes to further understanding of odour information processing in the OB, organization of odour information based on the function of OB output pathways, and their direct relevance to olfactory-locomotor output.

Chapter 2 examined whether projection neurons in the medial and non-medial OB exhibited anatomical differences including distribution, location, size and shape of the cell bodies in these two parallel OB pathways (Figure 4.1). Using retrograde labeling the findings show that the cell bodies (soma) and dendrites of PNs in the medial OB are clustered inside the glomerular neuropil, compared to the cell bodies of non-medial projection neurons that were predominantly located outside the glomeruli (frequently deeper in the OB). In addition, the somal size was greater in medial than in non-medial PNs and in both locations projection neurons contained single or multiple primary dendrites (Figure 4.1). This is the first study to show that there are anatomical differences in projection neuron location and size in the parallel medial and non-medial pathways of the sea lamprey OB. Furthermore, these findings provide more evidence for the anatomical basis of parallel olfactory pathways in the sea lamprey.

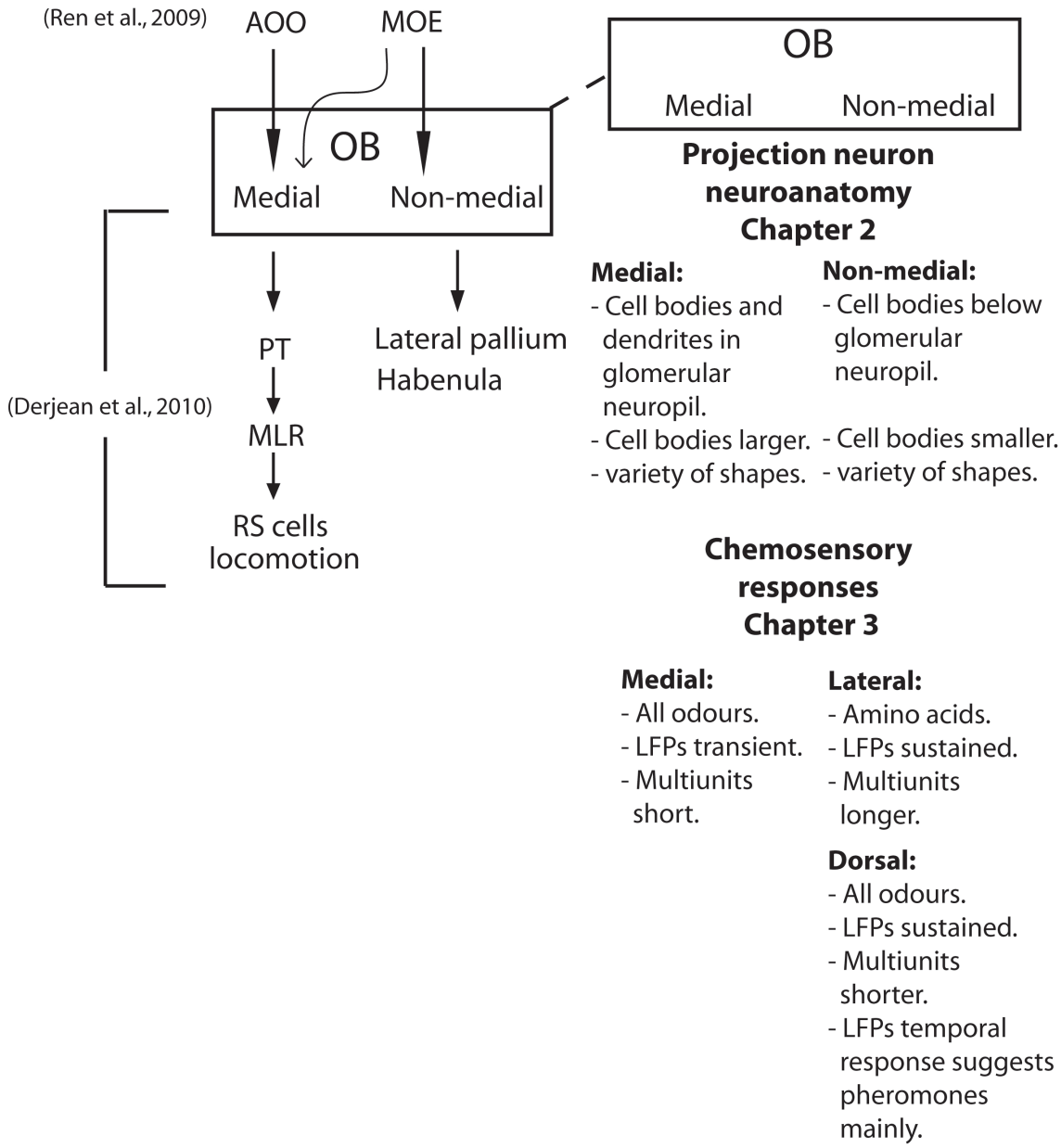

Figure 4.1. Flow diagram of the knowledge gaps and questions addressed in this thesis. Previous work by Ren et al. (2009) and Derjean et al. (2010) illustrate the parallel olfactory pathways in the lamprey. The 'flyout' of the olfactory bulb (OB) illustrates a summary of the results of the thesis. Accessory olfactory organ (AOO), main olfactory epithelium (MOE), posterior tuberculum (PT), mesencephalic locomotor region (MLR), reticulospinal cells (RS cells).

Chapter 3 examined the spatial and temporal coding of olfactory information in the medial and non-medial (dorsal and lateral) regions of the OB as they relate to the known odour processing pathways using a novel *ex-vivo* preparation and two electrophysiological recording techniques, LFPs and multiunit recordings (Figure 4.1). These findings supported the prediction that the chemosensory responses in the lamprey OB displays regional differences that correlate to the output organization of the medial and non-medial PNs. The data from LFPs and multiunit recordings were analyzed for topographic and temporal coding differences for four different classes of odours across the medial, dorsal, and lateral regions of the OB. Local field potentials revealed that projection neurons in the lamprey OB exhibit two types of responses to odours, transient and sustained. Both types of responses were observed in all three OB regions, however, the medial region showed a slightly higher proportion of transient responses, while the dorsal and lateral OB regions both showed much higher proportions of sustained LFP responses (in particular the lateral region) (Figure 4.1). From the multiunit recordings, the medial and dorsal OB regions exhibited an overall, trend towards shorter duration multiunit responses compared to the lateral region. Finally, both the LFP and multiunit recordings to the odour test solutions demonstrate that the medial OB is responsive to all four odour classes tested including basic amino acids, taurocholic acid, migratory pheromones, and sex pheromones. In contrast, LFP recordings showed that the lateral OB was only responsive to the basic amino acids, while the multiunit recordings showed that a few lateral projection neurons were responsive to taurocholic acid and sex pheromones, although these were the minority. The LFP and multiunit datasets show that the dorsal OB is responsive to amino acids, taurocholic acid, sex pheromones and migratory

pheromones. However, there were temporal differences in the LFP recordings between responses to pheromones compared to the amino acids and TCA, suggesting that the dorsal OB is more responsive to lamprey sex and migratory pheromones (Figure 4.1). Overall, the electrophysiological recordings demonstrate that the medial OB has different topographic and temporal odour coding compared to the dorsal and lateral OB regions, and that the lateral region is more responsive to basic amino acids than to taurocholic acid and pheromones. Odour information is topographically and temporally coded in the sea lamprey OB. Furthermore, when considering this dataset in the context of the known medial and non-medial output pathways of the OB it demonstrates that these two pathways are not only anatomically separate but are also physiologically different and that a wide variety of odours can activate the medial olfactory-locomotor pathway.

Overall, the results presented in Chapters 2 and 3 are the first studies to date on projection neuron anatomy and physiology in the sea lamprey OB. Furthermore, these results demonstrate the anatomical and physiological basis for odour information coding and processing in the sea lamprey OB. When considered in the context of the known medial olfactory-locomotor pathway, the unique findings that the medial OB responds to several different odour classes, each of which have an important biological function, support the concept of the OB being organized based on function. Taken together, the results from Chapters 2 and 3 as well as the previously published co-authored manuscripts presented in Appendix A and B contribute to the overall knowledge of parallel olfactory processing pathways and their relevance to understanding the neural substrate for olfactory-locomotor output.

Throughout the collection of data presented in this thesis many interesting research questions have been generated. Some are currently being examined, and others that have the potential to be examined in future studies. The medial OB region of the sea lamprey receives sensory neuron inputs primarily from the accessory olfactory organ (AOO) (Ren et al., 2009). At present, the odour ligands of the AOO sensory neurons are unknown. Since the AOO send input projections to the medial OB and it is involved in olfactory-locomotor transformation, it is imperative that the odour ligands of the AOO sensory neurons are characterized. In addition to the regional variation of odour responses observed in the OB it would be interesting to determine if single neurons in each of the regions respond to different chemical structures of a given class of odours. For example, do individual amino acid responsive projection neurons in the lateral OB respond to particular functional groups or side chain lengths of amino acid odours as seen by Nikonov and Caprio (2004)? This may provide further insight into the odour coding and processing mechanisms in the sea lamprey OB. The results of chapter 2 raise another interesting question as to the synaptic organization of projection neurons and interneurons in the medial and non-medial parallel olfactory pathways. Due to the projection neuron somata and dendrites being located entirely within the medial glomerular neuropil, it may be that the synaptic organization of these projection neurons and associated interneurons is different from the rest of the OB, which exhibits a more typical projection neuron arrangement to other fish. The differences in response duration observed in chapter 3 suggest that there may be an anatomical difference driving the physiological characteristics observed. Finally, previous anatomical studies have shown the presence of neurotransmitters such as glycine, glutamate, dopamine, γ-aminobutyric

acid, and serotonin in the OB (Zielinski et al., 2000; Villar-Cervino et al., 2008; Barreiro-Iglesias et al., 2009; Villar-Cervino et al., 2011). Their role in modulating the observed projection neuron responses is currently unknown and is important to fully understand the odour processing mechanisms present in the parallel olfactory processing pathways of the sea lamprey.

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APPENDIX A
Projections from the Accessory Olfactory Organ into the Medial Region of the Olfactory Bulb in the Sea Lamprey (*Petromyzon marinus*)– a Novel Vertebrate Sensory Structure?

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Abbreviated Title: The accessory olfactory organ in the sea lamprey

Key words: olfactory sensory neurons, tract tracing, olfactory bulb, fish

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ABSTRACT

While four different primary olfactory pathways have been described in tetrapod vertebrates, polymorphic olfactory sensory neurons comingle in the olfactory epithelium and project axons into separate bulbar regions in teleost fish. However, spatially segregated neurons may exist in the peripheral olfactory organ of lampreys, extant representatives of ancestral jawless vertebrates. In lampreys, the caudoventral portion of the peripheral olfactory organ contains tubular diverticula, named the accessory olfactory organ (AOO). Short ciliated AOO cells retrogradely labelled following application of biocytin or carbocyanine dyes to the medial region of the olfactory bulb. Tracer application to eight radial locations within the layer of glomeruli with mitral cells, of the olfactory bulb, showed that AOO projections were restricted to the medial region of the olfactory bulb. The outer boundary of the AOO projection extended to the ventromedial region of glomerular neuropil in 43% of the specimens. The olfactory sensory neurons in the main olfactory epithelium projected to glomerular neuropil throughout the olfactory bulb, including sparse projections to the medial region of the olfactory bulb. This study shows that these AOO neurons and their projections in the medial region of the olfactory bulb are anatomically distinct regions of the primary olfactory pathway in the sea lamprey.

INTRODUCTION

A wide variety of receptor cell types is seen within the olfactory epithelium of various vertebrates. These range from olfactory sensory neurons located in separate nasal epithelial regions, to different morphotypes within a single stretch of olfactory epithelium. Four spatially distinct olfactory subsystems, the main olfactory epithelium

(MOE), the vomeronasal organ, the septal organ of Masara and the Grüneberg ganglion have been observed in terrestrial vertebrates. In rodents, the axons of ciliated sensory neurons in the MOE terminate in the main olfactory bulb; microvillous primary sensory neurons located in the vomeronasal organ (at the rostral base of the nasal septum) extend into the accessory olfactory bulb (Meredith et al., 1980; Meredith, 1991); the septal organ of Masara (an island of MOE) is located within respiratory epithelium near the base of the nasal septum rostral to the nasopharyngeal canal (Levai and Strotmann, 2003), and the Grüneberg ganglion comprises of a cluster of rounded cells located in the submucosa of the MOE lining the rostral tip of the nasal septum, and with axons terminating in the caudal region of the olfactory bulb (Storan and Key, 2006). Diverticula lined with chemosensory epithelium extending off the main olfactory regions of the nasal cavity are termed Jacobson's organ or the vomeronasal organ in amphibians and reptiles (Negus, 1956; Freitag et al., 1995; Zuri and Halpern, 2003). Three olfactory sensory neuron morphotypes – ciliated, microvillous and crypt - intermix in the olfactory epithelium of teleost fish (Morita and Finger, 1998; reviewed by Hansen and Zielinski, 2005). The ciliated olfactory sensory neurons project to the lateral region of the olfactory bulb; the microvillous forms terminate in the medial region, and crypt cells extend to the ventrocaudal and ventrocentral region of the olfactory bulb (Morita and Finger, 1998; Hansen *et al.,* 2003; Sato *et al*., 2005; Hamdani and Doving, 2005). Although separate peripheral locations for olfactory subsystems are absent from teleost fish, an accessory sensory olfactory structure may exist in lampreys, extant representatives of ancient vertebrates that existed 530 million years ago (Shu et al., 1999). In 1887, W.B. Scott discovered the accessory olfactory organ (AOO) - diverticula of the MOE concentrated in

the caudoventral region of the peripheral olfactory organ of lampreys. In histological sections of *Lampetra fluviatilis*, *Lampetra planeri* and *Petromyzon marinus*, the AOO appeared as epithelial pockets surrounded by nerve fibers and blood vessels, (Scott, 1887; Beer, 1925; Leach, 1951; Hagelin and Johnels,1955, Vandenbossche et al., 1995) and sparse minute ducts linked the AOO to the lumen of the peripheral olfactory organ (Hagelin and Johnels, 1955). The presence of ciliated putative chemosensory neurons within this tubular structure lead Scott (1887) and Hagelin and Johnels (1955) to propose that the AOO is homologous to the vomeronasal organ. Exocrine and endocrine functions have also been postulated (e.g. Beer, 1925; Leach, 1951), and Lubosch (1905) considered the AOO follicles to be vestigial olfactory sacs in modern lampreys, which had been more extensively developed in earlier stages of evolution.

The cytoarchitecture of the olfactory bulb includes a circular layer of glomeruli and mitral cell bodies beneath the olfactory nerve layer in the lampreys *L. fluviatilis (*Heier, 1948) and *L. japonica* (Iwahori *et al.* 1987). The medial location of the layer of glomeruli and mitral cells contains biochemically distinct afferent projections in the sea lamprey *P. marinus* (Frontini et al., 2003), medial projection neurons project to the ventral thalamus (El Manira *et al*., 1997) and the posterior tuberculum (St. Pierre *et al.,* 2007), and locomotion responses were activated following the activation of this medial region of the olfactory bulb (Derjean et al., 2008). Consequently, in the sea lamprey, the medial region of the olfactory bulb is able to link olfaction sensory input to locomotor neural activity.

In this study of *P. marinus*, retrograde neuroanatomical tract tracing techniques reveal the presence of olfactory sensory neurons within the AOO and the projection of

these axons into the medial region of the olfactory bulb; while the axons of olfactory sensory neurons in the MOE extend broadly into the layer of glomeruli and mitral cells, including the medial area. Further, this study demonstrates structural complexity in the olfactory system in aganathan vertebrates.

MATERIALS AND METHODS

Experimental Animals

All experimental procedures used in this study were in compliance with protocols approved by the Canada Council for Animal Care. For this study, wild caught metamorphic stage 7 (transformers, Youson and Potter, 1979), parasitic phase and spawning phase (reproductively mature) lampreys were used. These were maintained at 6°C in recirculated dechlorinated water in the Department of Biological Sciences at the University of Windsor. The transformers and parasitic phase lampreys were captured during the autumn; and the spawning phase, during the spring and summer. The transformers and spawning phase lampreys were collected from Michigan streams, housed in the U.S. Geological Survey Lake Huron Biological Station in Millersburg, Michigan, then transported to the University of Windsor; or collected in Ontario streams and held at the Canada Department of Fisheries and Oceans Sea Lamprey Control Center in Sault Ste Marie, Ontario, before transport to the University of Windsor. The parasitic phase lampreys were captured by commercial fishers in Lake Huron, and held in Millersburg or Sault Ste Marie, then transported to the University of Windsor. For the transformers, the body length ranged from 11 – 17 cm and the weight, between 1.1 and 3. 4 grams. For the spawning-phase adults, the weight ranged from 106 - 324 grams and the body length from 34 to 52 cm. Specimens were deeply anesthetised with 0.5% MS

222 and decapitated prior to the processing of tissues for histology or neuroanatomical tracing experiments.

Semi-thin Sections

The cellular composition of the AOO follicles and the surrounding tissues was examined following toluidine blue staining of semi-thin sections. The peripheral olfactory organ was removed and fixed in a modified Karnovky's fixative containing 2.5% glutaraldehyde at 4°C overnight, then post-fixed in 1% OsO_4. The samples were dehydrated in ethanol and embedded into epoxy resin as previously described (Vandenbossche et al. 1995). Semi-thin sections (1 μm thick) were made on an RMC ultramicrotome, and stained with 1% toluidine blue in 1% aqueous sodium tetraborate.

Neuronal tract tracing

The peripheral origin of neurons projecting into specific regions of the olfactory bulb and the location of bulbar glomerular territories was investigated by *ex vivo* and *post mortem* tract tracing.

The central connectivity of the AOO cells and neurons in the MOE was examined by biocytin backfilling (Laframboise et al., 2007) from various regions of the olfactory bulb. The diencephalon and the caudal part of telencephalon were cut gently from the dissected peripheral olfactory organ/brain preparation and the targeted olfactory bulb region was exposed, then briefly lifted from the solution for biocytin application (Invitrogen, B1592). A micro-dissection pin (mounted onto a micromanipulator (Narishige, Tokyo, Japan)) was coated with flakes of biocytin then inserted into the olfactory bulb. The pin was kept within the olfactory bulb loading location for about 2 minutes to allow for the biocytin to dissolve into the tissue, before incubating in the cold

oxygenated Ringers solution (130 mM NaCl, 2.1 mM KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4 mM HEPES, 4 mM dextrose, 1 mM $NaHCO_3$, pH 7.4) for 4 to 18 hours, then fixed, cryoprotected and cryosectioned as previously described. Some slides with sections of the retrogradely labeled peripheral olfactory organ were then immunostained with anti-acetylated tubulin for viewing cilia and microtubules, using a monoclonal anti-acetylated antibody (known as 6-11B-1) in ascities fluid generated against acetylated a-tubulin from the outer arm of sea urchin sperm axonemes, (T6793; Sigma, St. Louis, MO, USA). The 6-11B-1 antibody has a single binding site located on the a3 isoform axonemal a-tubulin of *Clamydomonas* (Piperno and Fuller, 1985) within four residues of Ly-40 when this amino acid is acetylated (LeDizet and Piperno, 1987). Western immunoblotting demonstrated the binding (to native and denatured a-tubulin); 2-dimensional isoelectric focusing confirmed binding specificity; and solid-phase antibody-binding competition assay showed that this antibody bound to axonemal tubulin proteins but did not appreciably recognize cytoplasmic tubulin (Piperno and Fuller, 1985). The 6-11B-1 antibody has been used to detect a-tubulins acetylated on Lys-40 from a variety of organisms (LeDizet and Piperno, 1991), and binding to acetylated a-tubulin in axonemes was evident from previous applications that localized neurons: in *L. japonica* during cranial nerve development (Kuratani et al., 1997); in *P. marinus* during axon tract scaffolding (Barreiro-Iglesias et al., 2008) and in afferent gustatory fibers (Barreiro-Iglesias, 2008). This antibody also localized cilia in *P. marinus* olfactory sensory neurons (Frontini *et al*., 2003) and the round goby, *Neogobius melanostomus,* a teleost fish (Belanger *et al*., 2003). The slides were incubated in a blocking solution containing 4% normal horse serum in 0.1 M PBS for 20 minutes, then in the acetylated tubulin antibody

(1:1000 in 0.1 M PBS pH 7.4 containing 0.1% Triton X-100) overnight at 4°C, washed 3 times for 10 min in 0.1 M PBS and incubated with Alexa 488 conjugated anti-mouse secondary antibody (1:200; A21151 Invitrogen) for 2 hours at room temperature before viewing by fluorescence or confocal microscopy

The olfactory nerve was anterogradely labelled *ex vivo* with biotinylated dextran amine (3000 MW, Molecular Probes D-7135) for examining the bulbar distribution of olfactory sensory neuron fibers and terminals. The peripheral olfactory organ and brain were removed as a single piece and immersed into a Petri dish containing cold oxygenated Ringers solution. The olfactory nerve was exposed by gently removing the olfactory lamellae, then 3 – 4 flakes of biotinylated dextran amine were picked up on the tips of fine forceps, placed directly onto the olfactory nerve and allowed to absorb into the nerve for a few minutes before incubating in cooled oxygenated Ringers for 4 to 18 hours, depending on the length of the olfactory nerve (shortest in the transformer stage and longest in the spawning adult stage). Following this incubation, the tissue was fixed by immersion into 4% paraformaldehyde (Sigma-Aldrich, Mississauga, Canada) in 0.1 M phosphate buffer (PB), pH 7.6 at 4 °C for 24 hours. The fixed tissue was then cryoprotected in 10% (2h), 20% (2h) and 30% (overnight) sucrose solution in 0.1 M PB and transverse sections were prepared on a cryostat (Microm, Heidelberg, Germany). Serial cross-sections were thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), then incubated in Alexa-568 conjugated streptavidin (1:100 in PBS; Molecular Probes, Eugene, OR), washed and coverslipped.

Bulbar topography of AOO projections

The bulbar location of the AOO cell projections was investigated by *post mortem* retrograde labelling with the lipophilic carbocyanine compound DiI (1,1 -dioctadecyl 3,3,3,3 -tetramethylindocarbocyanine perchlorate; D-282, Invitrogen Inc.). The specific bulbar location of these axons was probed by applying DiI crystals to specific foci in the layer of glomeruli and mitral cells (Heier, 1948; Iwahori et al. 1987). This layer forms a circular pattern (similar to a clock face) beneath the more superficial olfactory nerve layer. The radial distribution of olfactory glomeruli (within the layer of glomeruli and mitral cells) was visualized in cross-sectional views of the olfactory bulb of *P. marinus* that were anterogradely labelled from the olfactory nerve (described above), or histochemically stained with the α–D-galactose binding lectin *Griffonia simplicifolia* I-isolectin B4 (GS1B4), which labels olfactory sensory neuron axons the sea lamprey (Tobet et al., 1996; Zielinski et al., 2000; Frontini et al., 2003). For the GS1B4 staining, the head was removed, then immersed into fixative (4% paraformaldehyde in 0.1 M PB) overnight. Transverse cryosections were prepared, then incubated with biotin conjugated GS-1 isolectin B4 (1:100, B-1105; Vector Labs, Burlington, ON) in 0.1 M PBS pH 7.4 , rinsed in 0.1 M PBS, then incubated in Alexa-488 streptavidin (1:100, Molecular Probes) in 0.1 M PBS for 2 hours at room temperature before rinsing and mounting, then viewed by fluorescence microscopy.

Eight radial DiI loading regions (dorsal, dorsolateral, lateral, ventrolateral, ventral, ventromedial, medial and dorsomedial) were selected, representing 45 degree arcs in the layer of glomeruli with mitral cells (Iwahori *et al*., 1987) seen in cross sectional views of the olfactory bulb. For the DiI loading, the olfactory pathway, including the peripheral

olfactory organ and forebrain, were carefully removed from a fixed lamprey head (4% paraformaldehyde in PB), embedded into 5% agarose (Sigma, A0169) then mounted onto a vibratome (Leica VT 1000S) in the transverse plane. The olfactory bulb was exposed for dye loading by shaving off caudal regions with the vibratome. The rostral edge of the lateral ventricle of the olfactory bulb served as an indicator of the location for DiI loading within the olfactory bulb for this study. Crystals of DiI were transferred onto the tip of a micro-dissection pin and inserted into one of these eight foci. The DiI-loaded preparation was then sealed with melted agarose, placed into fresh fixative and stored in darkness at 37°C for 13 to 30 days, to allow for retrograde diffusion from the olfactory bulb to the peripheral olfactory organ. Vibratome cross-sections of the olfactory bulb and peripheral olfactory organ were prepared and examined immediately for labelling by fluorescence microscopy. Possible contribution of extra-bulbar fibers was tested by examining AOO labelling following injection into the pre-optic area (N=3) and the area of the dorsal and ventral hypothalamic nuclei (N=3), both regions known to contain extrabulbar projections (Northcutt and Puzdrowski, 1988). Both application sites labelled fibers extending out of olfactory sensory neurons in the main olfactory epithelium, but did not label AOO cells or medial glomeruli.

The peripheral origin of fibers extending to medial and lateral regions of the layer of glomeruli and mitral cells, was directly compared by adapting a double labelling strategy with two spectrally distinct carbocyanine fluorophores, DiI (excited at 488 nm) and DiA, (excited at 568 nm; 4-4-dihexadecylaminostyryl-N-methyl-pyridinium iodide, D-3883 Invitrogen Inc.), a strategy previously used by Sullivan and Beltz (2001), Dunlop (2003) and Hu *et al.* (2004). The tissue was fixed and loaded with the carbocyanine

tracers under a stereomicroscope as previously described. The DiI was applied to the lateral region of the olfactory bulb, the DiA was applied into medial region; the tissue was incubated in fixative, and sectioned as previously described before examining by confocal microscopy.

Microscopy

The brightfield and fluorescence sections were photographed with a Q-imaging Retiga 1300 digital camera (Q-Imaging Corporation, Burnaby, Canada) on a Zeiss Axioskop 2, using Northern Eclipse software (EMPIX Imaging Inc., Mississauga, Canada). The confocal images were taken on an Olympus Fluoview FV1000 (FV10-ASW)) or a Biorad MRC 1024 equipped with Lasersharp software. Images were cropped, arranged and modified for contrast and brightness using Adobe Photoshop.

RESULTS

The AOO was located in the lamina propria beneath olfactory epithelium situated in the troughs between adjacent olfactory lamellae, in the caudoventral region of the peripheral olfactory organ of transformed and adult phase sea lampreys (Fig. 1A, B). These AOO pockets commenced midway along the rostral-caudal axis of the peripheral olfactory organ (Fig. 1A) and extended caudally beyond the olfactory lamellae. The simple cuboidal epithelium lining the AOO was noticeably shorter than the pseudostratified columnar MOE (Fig. 1C), and included ciliated cells (Fig. 1D, E), previously noted as putative sensory neurons by Scott (1887) and by Hagelin and Johnels (1955). Biocytin, applied throughout the olfactory bulb layer of glomeruli with mitral cells (Iwahori et al., 1987), retrogradely labelled olfactory sensory neurons in the MOE and in the AOO, as well as olfactory nerve fascicles in the lamina propria (Fig. 2A).

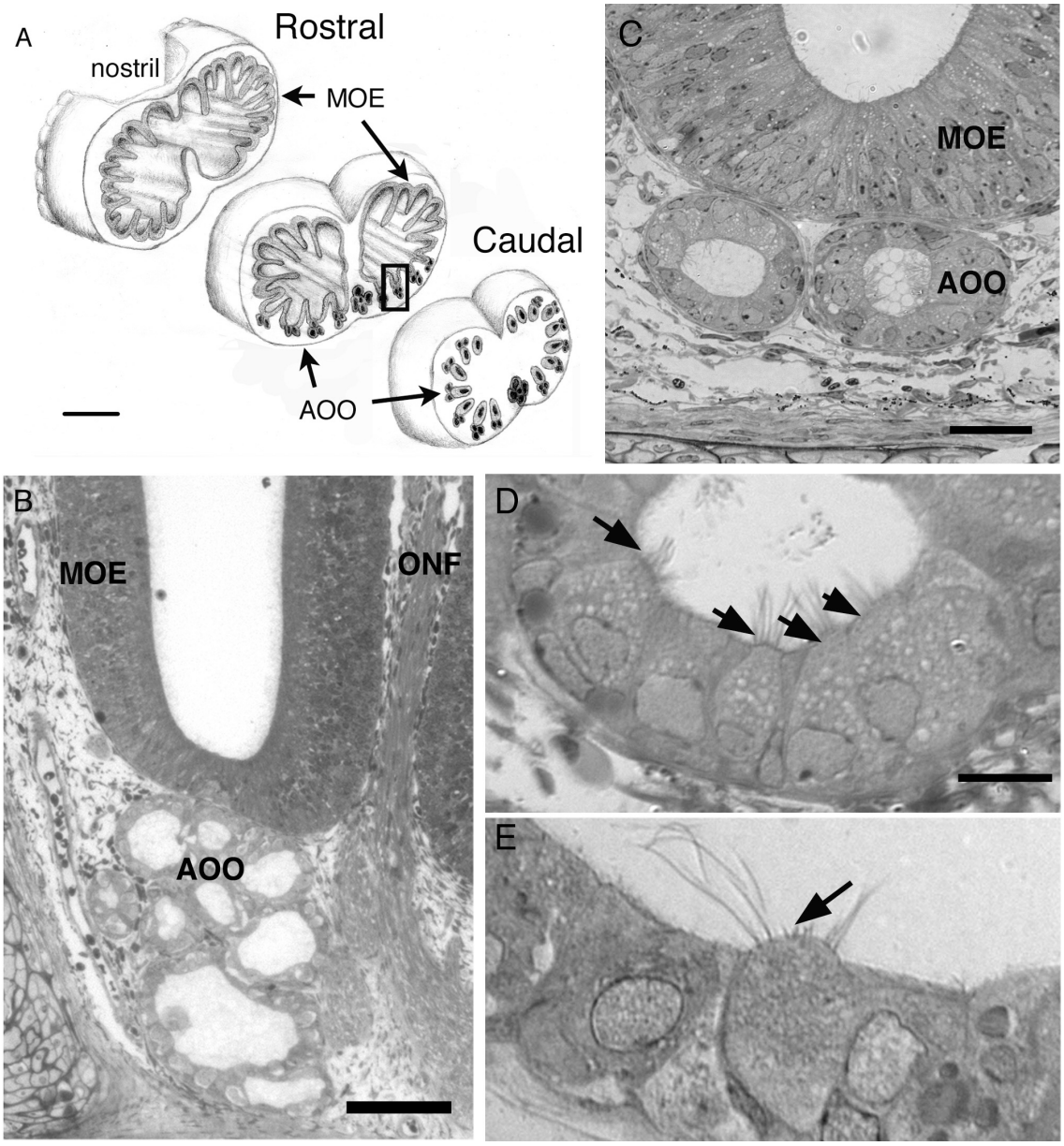

Figure 1. The accessory olfactory organ in *Petromyzon marinus*. **A:** Diagram illustrating cross-sectional views of the rostral, mid and caudal regions of the peripheral olfactory organ. The single peripheral organ contains bilaterally symmetrical cavities, lined by lamellae that are covered by the main olfactory epithelium (MOE). The rostral-most AOO follicles extend from the ventral region of the peripheral olfactory organ, from the base of the olfactory lamellae. The AOO follicles are also prominent in the caudal region of the peripheral olfactory organ. The area enclosed by a rectangle shows the region for sections shown in B to E. Bar is 1 mm. **B to E:** are epoxy semi-thin sections stained with toluidine blue. **B:** The AOO follicles are located in the lamina propria in a trough between adjacent olfactory lamellae that are covered by MOE. Fascicles containing olfactory sensory neuron axons (ONF) are located in the lamina propria of the olfactory

lamella. Bar is 100 μm. **C:** The MOE is a pseudostratified columnar epithelium, and the AOO epithelium is simple cuboidal epithelium. Bar is 25 μm. **D and E** are high power views of the AOO epithelium. The AOO contains rounded ciliated cells (arrows). Unciliated cells are adjacent to the ciliated cells. Bar is 10 μm.

Labeled fibers traversed the loose connective tissue in the lamina propria and entered AOO follicles (Fig. 2B) and into individual AOO cells (Fig. 2C). These retrogradely labelled AOO cells were located in all AOO sacs, including prominent clusters caudal to the MOE and rostral to the olfactory bulb (Fig. 2 D,E), where the retrogradely labelled follicles were arranged in semi-circular pattern, surrounding olfactory nerve fascicles, with the largest AOO clusters positioned ventrally (Fig. 2E). High power views of the retrogradely labelled cells in the MOE and the AOO revealed the shape and composition of these olfactory sensory neurons. While the MOE neurons were predominately elongate (Fig. 3A), the AOO neurons were much shorter and rounder (Fig. 3B). A rounded olfactory knob was situated on the luminal surface of the MOE neurons, compared to the flatter and less bulbous apical region of the AOO cells (Fig. 3). A single axon extended from the basal region of the cell body of sensory neurons both in the MOE and the AOO (Fig. 3, 4) and abundant hair-like apical processes on the apical surface of the AOO cells were identified as cilia by acetylated tubulin-immunofluorescence (Fig. 4). The localization of acetylated tubulin immunoreactivty in the cell body, dendritic and axonal compartments of the AOO cells further substantiated their neuronal identity (Fig. 4B). Previous studies have shown that olfactory sensory neurons located in the MOE of the sea lamprey are ciliated (Vandenbossche et al., 1995; Laframboise et al., 2007), and that the cilia, cell bodies and axons of these cells are acetylated tubulin-immunoreactive (Frontini *et al.*, 2003). The AOO neurons were generally bipolar, with a short dendritic

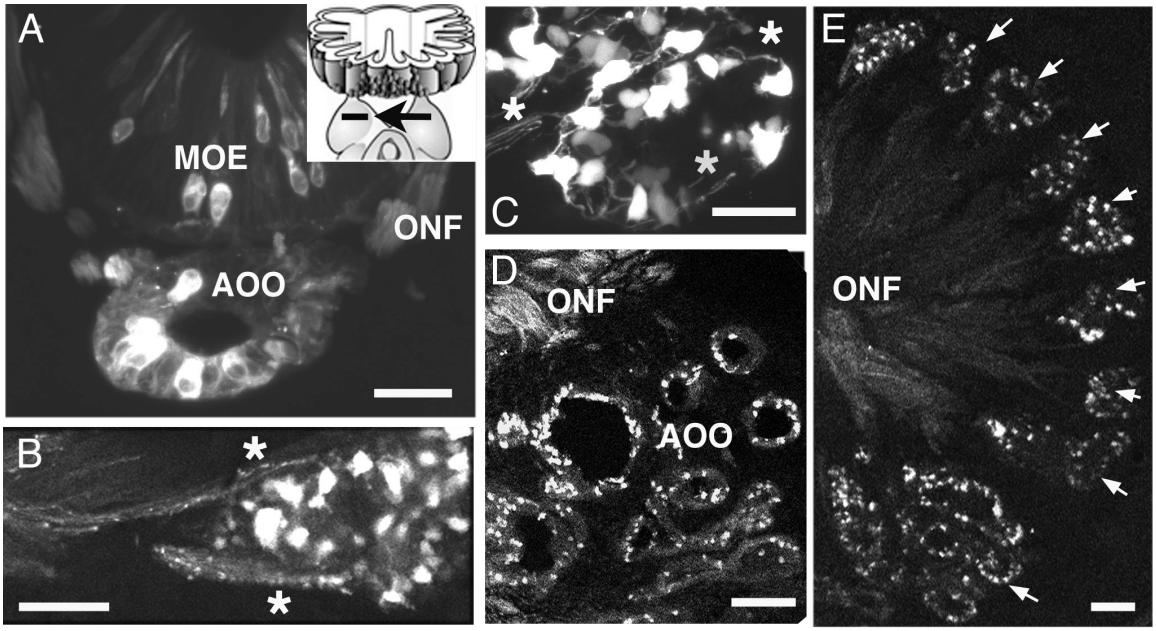

Figure 2. Retrograde labelling of AOO cells from the olfactory bulb following biocytin application to the olfactory bulb. **A:** The retrogradely labelled AOO cells are short compared to the olfactory sensory neurons in the MOE. Olfactory nerve fibers (ONF) are located in the lamina propria beneath the MOE. Bar is 25 µm. The inset illustrates the location of biocytin application in the olfactory bulb. **B:** Retrogradely labelled nerve fibers (asterisk) extend toward and enter the AOO follicles, and fibers extend from the retrogradely labelled AOO cells. Bar is 50 µm. **C:** Retrogradely labelled nerve fibers (asterisk) are adjacent to AOO cells. Bar is 24 µm **D:** Abundant AOO follicles are located in the submucosa caudal to the olfactory lamellae, and close to the olfactory nerve (ONF). Bar is 50 µm. **E:** This cross-sectional view of the region between the peripheral olfactory organ and the olfactory bulb shows a radial arrangement of labelled olfactory nerve fibers (ONF) and surrounding AOO follicles (arrows). A high concentration of AOO follicles is seen in the ventromedial corner, shown at the bottom of the image. Bar is 50 µm.

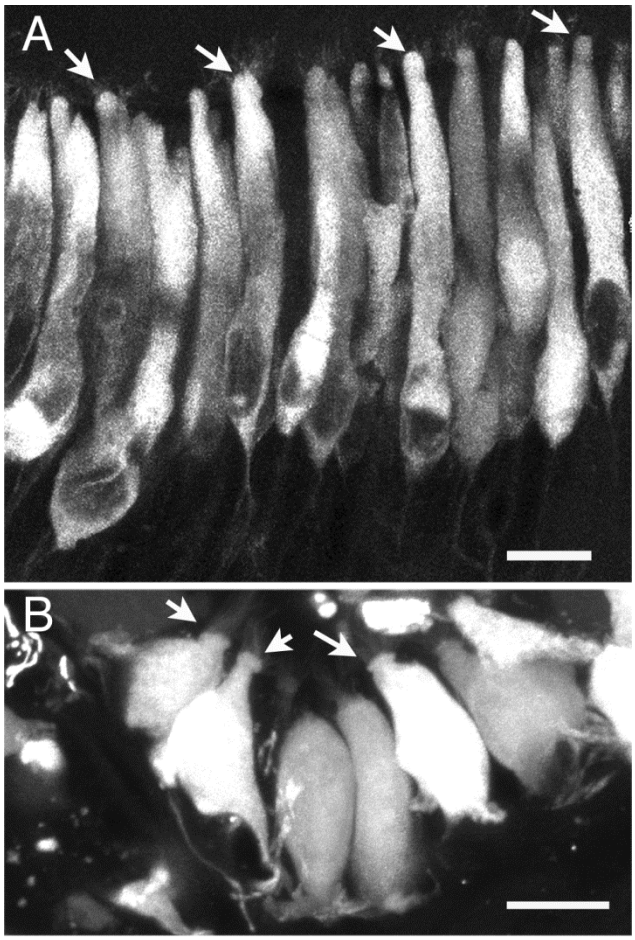

Figure 3. Confocal Z-stack images of backfilled olfactory sensory neurons in the MOE and the AOO. **A:** The olfactory sensory neurons in the MOE (backfilled with biocytin) are tall and narrow, with ciliated bulbous olfactory knobs (arrows). Bar is 10 µm. **B:** The AOO retrogradely labelled with biotinylated dextran amine contains ciliated rounded cells, some with a flattened, narrow olfactory knob (arrows). Bar is 5 µm.

apical portion, an ovoid cell body and a basal axon (Fig. 4). Some variation was observed in the relative size of the dendritic portion, with some cells appearing more elongate than others (Fig. 4A). The slight difference in neuronal shape constituted a continuum rather than specific neuronal subtypes.

The bulbar location of the AOO projections was identified by anterograde labelling from the olfactory bulb. The olfactory glomeruli formed a circular pattern, when viewed in transversely sectioned preparations of the olfactory bulb (Fig. 5A) and previously described in *Lampetra japonica* by Ichikawa *et al.* (1987). Anterograde labelling from the olfactory nerve revealed a tangle of very fine axons in the glomerular neuropil (Fig. 5B), including the medial region of the olfactory bulb. The bulbar projections of the AOO olfactory sensory neurons were systematically examined by retrograde DiI labelling of these bulbar glomerular regions in fixed *post mortem* specimens. The circular distribution of afferent input into the olfactory bulb allowed for systematic DiI application into eight radial regions of the layer of glomeruli with mitral cells in transversely sectioned *post mortem* olfactory bulbs (Fig. 5), for revealing specific topography of AOO projections. Global loads to all bulbar regions labelled olfactory sensory neurons in both the MOE and the rostral AOO follicles (directly abutting onto the MOE; Fig. 6A). These AOO follicles labelled only when the medial region of the olfactory bulb was included in the dye load (Fig. 6), with olfactory sensory neurons in both the MOE and the AOO labelling, even when a minute quantity of DiI was applied medially (Fig. 6C). These medial DiI loads labelled individual rounded AOO neurons, as

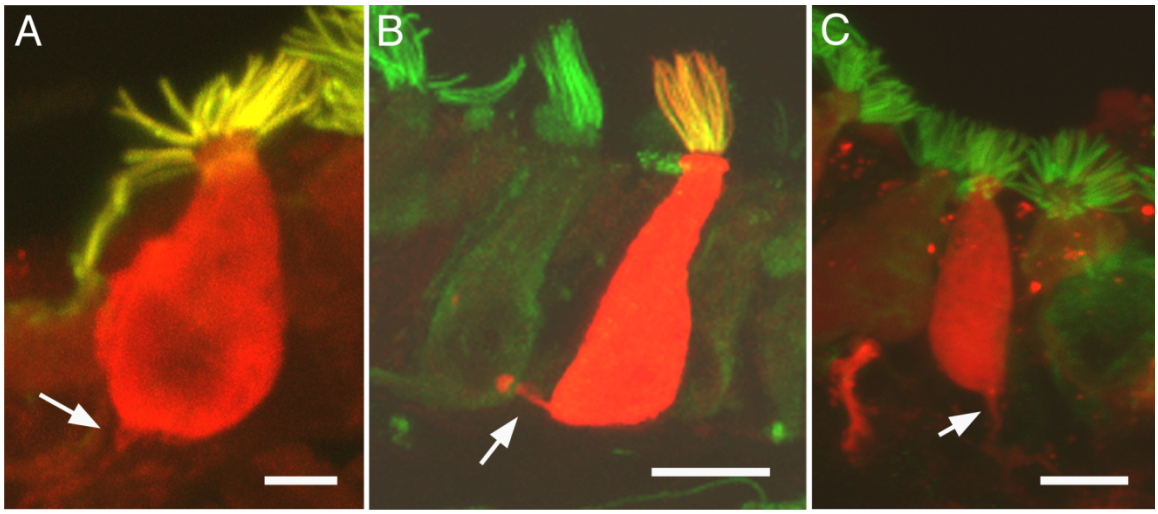

Figure 4. High power Z-stack confocal views of biocytin backfilled AOO olfactory sensory neurons (magenta), immunolabeled with acetylated tubulin (green). A to C) The backfilled AOO cells include a basal axonal process (arrows) and apical cilia. B and C) Microtubules (green) are seen within the cilia, cytoplasm and axons of AOO neurons that were not retrogradely labelled with biocytin (magenta). Bar is 10 µm.

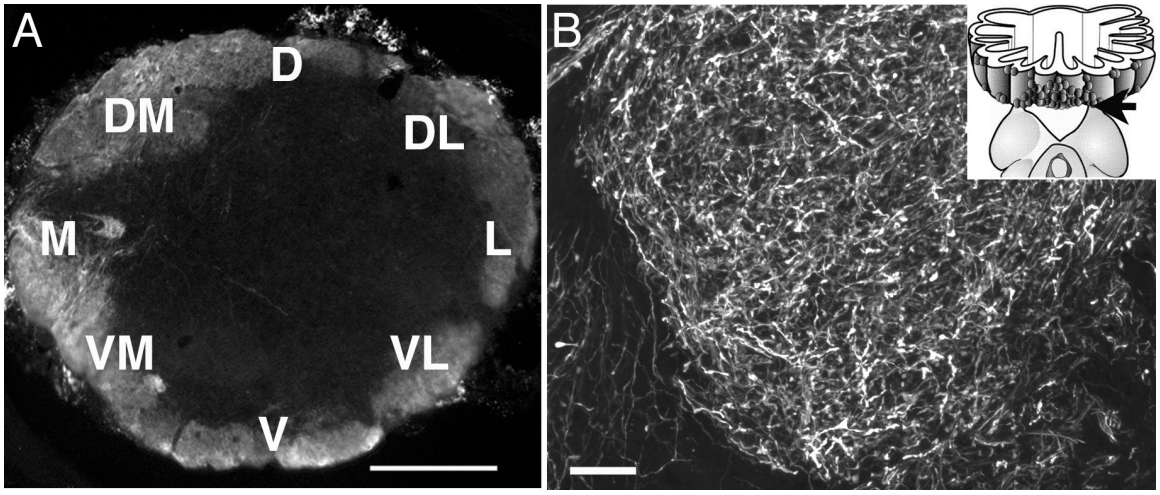

Figure 5. The glomeruli in the olfactory bulb in the parasitic phase lamprey. **A:** This cross-sectional view of the olfactory bulb was labeled with GS1B$_4$ lectin. The eight regions of the layer of glomerui with mitral cells are indicated: M, medial; DM, dorsomedial; D, dorsal; DL, dorsolateral; L, lateral; VL, ventrolateral; V, ventral; VM, ventromedial. Bar is 250 µm. **B:** A high power confocal Z stack showing olfactory nerve fibers in the layer of glomeruli with mitral cells, anterogradely labelled from the olfactory nerve with biotinylated dextran amine. The axons of olfactory sensory neurons are a meshwork of fine fibers. Bar is 20 µm. The arrow in the inset shows the olfactory nerve site of the biocytin application.

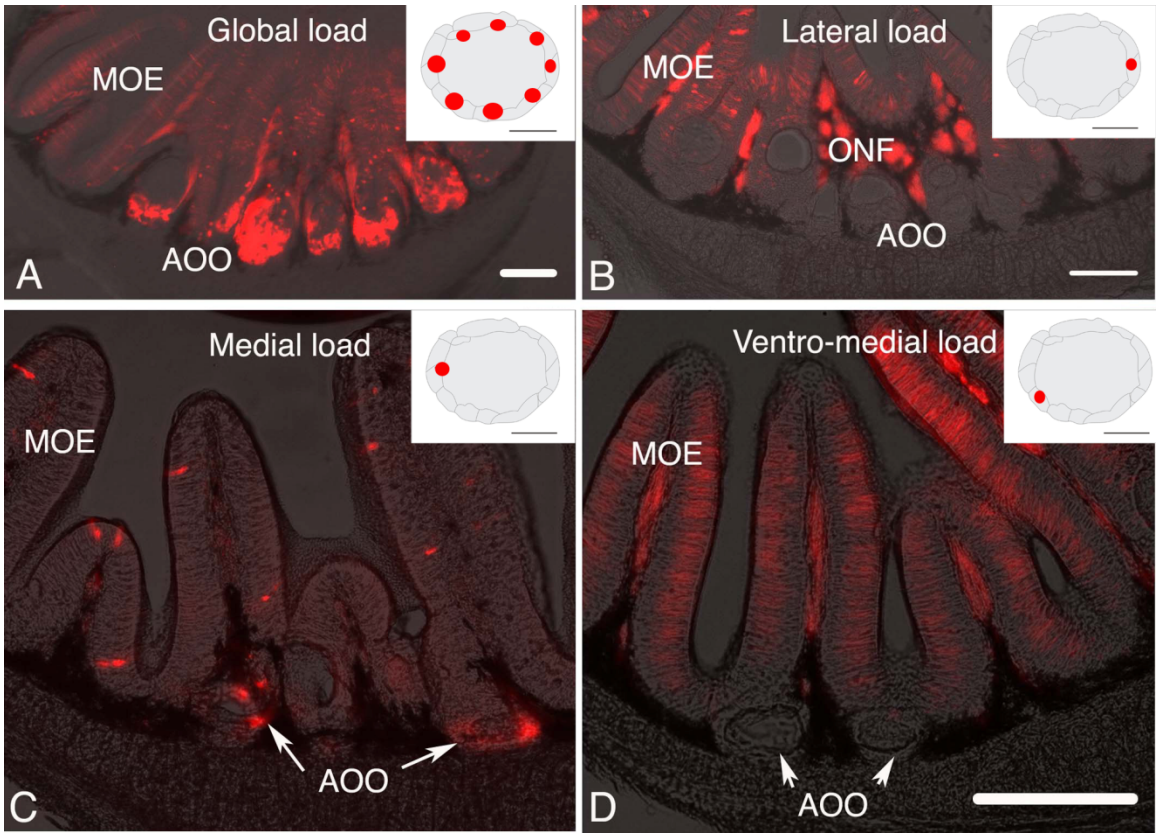

Figure 6. Rostral AOO follicles label with DiI following global and medial olfactory bulb loads. A and B are low power views; C and D are high power views. Bar is 200 µm for all. C and D are the same magnification. The bar is 500 µm in the insets shown in A - D. **A:** The labeling is located in AOO follicles and in the MOE following diffuse (global) dye application to the olfactory bulb. **B:** Following application to the lateral region of the olfactory bulb (illustrated in the inset), labelling was observed in the MOE and in olfactory nerve fascicles (ONF), but absent from AOO follicles. **C:** Labelled cells were seen in the AOO follicles and in the MOE following application to the medial region of the olfactory bulb. **D:** Labelling was present in the MOE and in olfactory nerve fascicles, but absent from AOO follicles following application to the ventromedial region of the olfactory bulb.

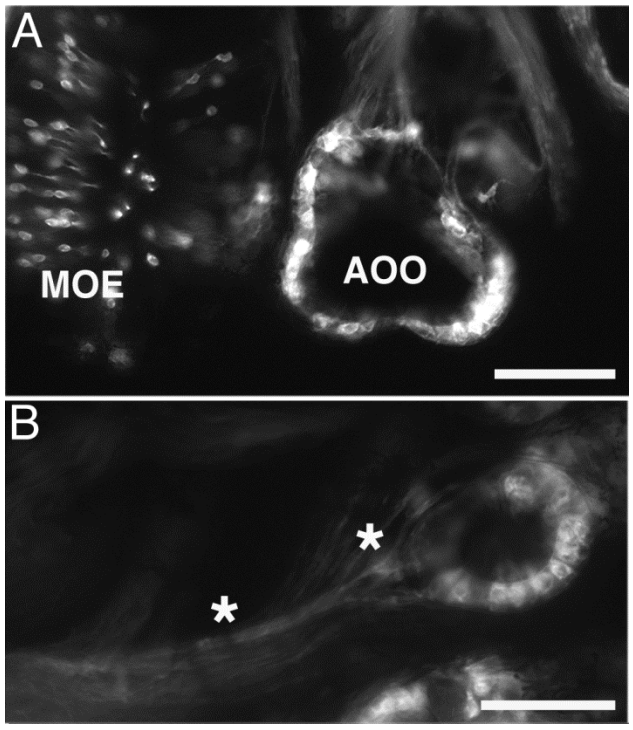

Figure 7. High power fluorescence views of rostral AOO follicles retrogradely labelled with DiI from the medial region of the olfactory bulb. Bar is 100 µm. **A:** Labelled olfactory sensory neurons are located in the MOE, as well as in the AOO. **B:** Axons (asterisks) extend into the AOO follicle. Labelled AOO cells line the AOO epithelium.

well as the axonal processes extending into the AOO follicles (Fig. 7). These labelled AOO neurons were bipolar, with small round cell bodies and a slightly constricted supranuclear region. The caudal AOO follicles (located ventral to the olfactory nerve) also labelled following DiI tracer application to the medial glomeruli, and failed to label following DiI application to the remaining glomerular regions (Fig. 8). From these experiments, it became clear that AOO neurons (from the rostral to the caudal follicles), project to the medial region of the olfactory bulb. Medial application of DiI consistently labelled olfactory sensory neurons in the AOO, as well as in the MOE (Table 1), indicating that the medial region of the olfactory bulb contained projections from olfactory sensory neurons in the MOE as the AOO. Abundant olfactory sensory neurons labelled in the MOE following application to the non-medial territories (Table 1). Although most ventromedial loads failed to label AOO neurons, the boundary of the AOO bulbar projection may extend to the ventromedial region of the olfactory bulb, since 3 out of 7 ventromedial bulbar applications labelled very sparse AOO neurons (Table 1). Alternatively, the DiI may have diffused to the medial region of the olfactory bulb during these ventromedial tracer application experiments.

The medial-only AOO axonal projection site was confirmed by a double labelling strategy with the carbocyanine dyes DiA and DiI (Fig. 9A). Following application of DiA to the medial region of the olfactory bulb, and DiI to the lateral region, only DiA was seen in AOO sensory neurons (Fig. 9B), and the neurons in the MOE were labelled with DiI or with DiA (Fig. 9C).

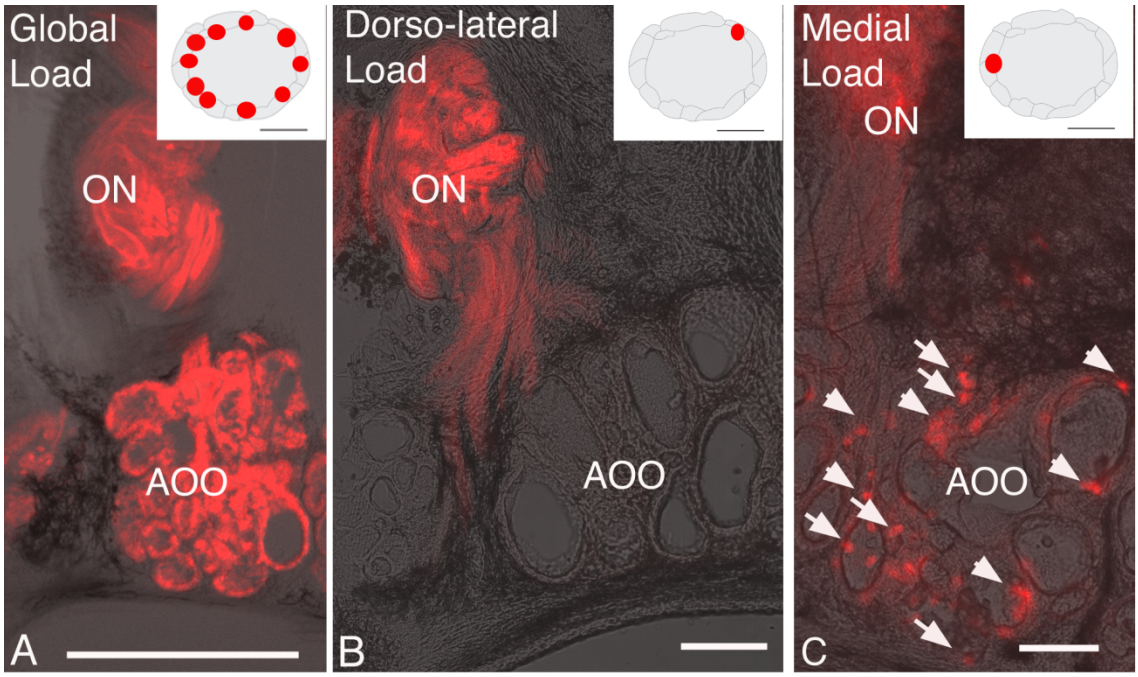

Figure 8. The medial region of the olfactory bulb contains projections from AOO cells caudal to olfactory lamellae. Bars are 500 µm in inserts shown in A – C. **A:** Following DiI application to all glomerular territories, labelling was observed in AOO follicles and the olfactory nerve (ON). Bar is 500 µm. **B:** Fluorescence was present in the olfactory nerve, and absent from AOO cells following DiI application to the lateral region of the olfactory bulb. Bar is 100 µm. **C:** Following a minute DiI application to the medial region of the olfactory bulb, labelling was present in ipsilateral AOO cells (arrows) and the olfactory nerve (ON). Bar is 100 µm.

TABLE 1. Labelling of Olfactory Sensory Neurons in the AOO and the MOE Following DiI Tracer Application Into Specific Glomerular Territories[1]

Olfactory bulb region with tracer	AOO	MOE	Number of specimens examined
Dorsal	– (6)	+++ (6)	6
Dorsolateral	– (4)	+++ (4)	4
Lateral	– (6)	+++ (6)	6
Ventrolateral	– (4)	+++ (4)	4
Ventral	– (5)	+++ (5)	5
Ventromedial	– (4) + (3)	+++ (7)	7
Medial	+++ (9)	+ (9)	9
Dorsomedial	– (8)	+++ (8)	8

[1]+++, abundant labelled cells; +, rare labelled cells; –, absence of labelled cells. Number in parentheses indicates the number of animals for each observation (N value).

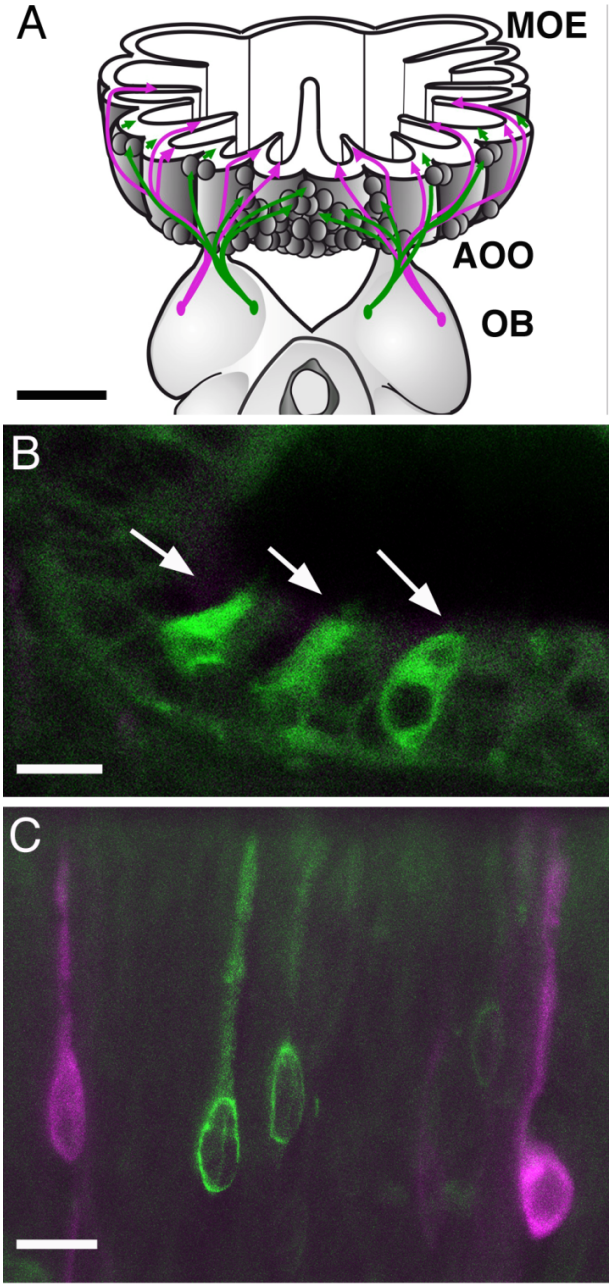

Figure 9. Retrograde double labelling of the peripheral olfactory organ from the medial and lateral region of the olfactory bulb. **A:** A diagrammatic representation of the retrograde carbocyanine double label exeperiment. The green dye (DiA) was applied to the medial region of the olfactory bulb (OB), and the megneta dye (DiI) was placed onto the lateral region of the olfactory bulb. Bar is 1 mm. **B:** Cells located in AOO epithelium follicles labelled with DiA (arrows). These AOO cells included flask shaped cells with a narrow apex, and the MOE labelled with DiI . Bar is 10 µm. **C:** Olfactory sensory neurons labelled with laterally applied DiI or medially applied DiA were seen in the MOE located adjacent to the AOO follicles. Bar is 10 µm.

DISCUSSION

These experiments show that olfactory sensory neurons in the AOO specifically target the medial region of the olfactory bulb, and those in the MOE project broadly into the layer of glomeruli and mitral cells. The presence of this accessory olfactory pathway in the sea lamprey, an agnathan vertebrate, may be significant in an evolutionary context.

Although previous investigators had noticed these small ciliated cells located in the crypt-like invaginations of the olfactory epithelium of lampreys, and suggested olfactory sensory function for these cells (e.g. Scott, 1887; Hagelin and Johnels, 1955; Pombal et al., 2002), the use of tract tracing strategies has confirmed that axons project from AOO cells to the olfactory bulb. The short stature of the AOO neurons reflects their cuboidal epithelial morphology, compared to taller pseudostratified MOE (e.g. Thornhill, 1967; Vandenbossche, 1997; Laframboise et al., 2006). In the lamprey, both the AOO and MOE sensory neurons cells are ciliated. The apical surface of the AOO cells is flatter than the more protruding olfactory knobs seen in the ciliated sensory neurons of the MOE in lampreys (Thornhill, 1967; Vandenbossche et al., 1992; Zielinski et al., 2000) and other vertebrates (Hansen and Zielinski, 2005; Menco et al., 1997). The flattened AOO cell apical surface, is reminiscent of the low olfactory knob seen in microvillous olfactory sensory neurons in teleost fish (Hansen and Zielinski, 2005). The more rounded shape of the AOO sensory neurons resembles the short cells in the MOE of the sea lamprey (Laframboise et al., 2007), but the abundant cilia covering the apical surface of the AOO sensory neurons differ from the single submerged cilium seen in the short olfactory sensory neurons (Laframboise et al., 2007) and in teleost crypt cells (Hansen and Finger,

2000). These morphological characteristics may indicate molecular differences between the olfactory sensory neurons in the AOO and the MOE.

The anterograde labelling of the olfactory afferents confirmed the circular arrangement of these projections into glomeruli in the lamprey olfactory bulb, previously illustrated by silver impregnation techniques (Heier, 1948; Iwahori *et al*., 1987). The neuropil in this layer of glomeruli and mitral cells contained finely meshed afferent fibers, characteristic of olfactory sensory neuron terminals (e.g. Au *et al*., 2002). The location of the medial glomerular neuropil containing the AOO projections differed from the more ventral location of extrabulbar fibers passing into the telencephalon and diencephalon from the olfactory epithelium (Northcutt and Puzdrowski, 1988; Eisthen and Northcutt 1996; Tobet *et al*., 1996; Von Bartheld, 2004), and confirmed in our studies as well, by retrograde labelling from the pre-optic area and the ventral and dorsal hypothalamic nuclear regions.

The retrograde tract tracing experiments support the previous observation of biochemically distinct afferent neurons in the medial glomeruli of sea lampreys (Frontini et al., 2003), and demonstrate that the AOO olfactory sensory neurons terminate exclusively in the medial region of the olfactory bulb. This medial AOO region of the olfactory bulb links olfactory sensory input to locomotor output. Strong depolarizations of reticulo-spinal cells were evoked following stimulation of this medial bulbar region, but not the other bulbar regions (Derjean et al., 2008), and tract-tracing studies showed input from mitral cells located in the medial region of the olfactory bulb to the ventral thalamus (El Manira *et al*., 1997) and the posterior tuberculum (St. Pierre, 2007) – both regions with strong links to locomotor control (Derjean et al., 2008). On the other hand,

tracers applied broadly to the olfactory bulb extended largely to the pallial regions and the dorsal thalamus (Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993). According to our neuroanatomical studies, there are spatially distinct projections from the medial vs lateral regions of the olfactory bulb (Chang, 2006; Ren, 2006; St-Pierre, 2007). Consequently, the synaptic pathway through the medial region of the olfactory bulb which receives the axons from the AOO sensory neurons, may also be functionally separate from the remaining region of the glomeruli and mitral cells with projection neurons extending broadly into the forebrain and midbrain. Behavioural implications of the medial pathway that connects locomotor centers may be represented by the sea lamprey's strong upstream movement response to pheromones in natural spawning streams (Johnson *et al*., 2009).

The AOO is an accessory olfactory structure.

Both similarities and differences are seen when comparing the lamprey AOO to the vomeronasal organ, the Grüneberg ganglion and the septal organ of Masara - secondary nasal chemosensory structures in terrestrial vertebrates. 1) While the vomeronasal epithelium is located in a fairly wide sac in amphibians and reptiles (Negus, 1956; Freitag et al., 1995; Taniguchi et al., 2008), both the mammalian vomeronasal organ and the lamprey AOO are tubular structures, linked to the nasal cavity by narrow ducts. The AOO ducts are located within the MOE (Lubosch, 1905) and the vomeronasal ducts empty into nasal respiratory epithelium at the base of the nasal septum (Meredith et al., 1980). Consequently, the apical surface of the sensory neurons in the AOO and in the vomeronasal organ of terrestrial vertebrates face a lumen, compared to the cells of the Grüneberg ganglion, a ductless cell cluster located in the submucosa inferior to olfactory

epithelium (Storan and Key, 2006). 2) Both the neurons in the AOO and septal organ of Grüneberg have a rounded shape and are considerably shorter than the sensory neurons seen in the vomeronasal organ. 3) Abundant blood vessels surround both the AOO and the mammalian vomeronasal organ. The constriction and dilation of these blood vessels regulates the passage of fluids to the vomeronasal organ (Meredith, 1980), and may also have a similar function in the lamprey AOO. 4) The cells located within all three - AOO, vomeronasal organ and septal organ of Grüneberg - project axons to discrete glomerular locations in the olfactory bulb. The AOO olfactory sensory neurons project to the medial glomeruli in the sea lamprey; vomeronasal axons extend medial to the olfactory bulb and enter the accessory olfactory bulb located caudal to the main olfactory bulb (Halpern and Martinez-Marcos, 2003; Taniguchi *et al.,* 2008); the nerve fibers from the septal organ extend to the caudal, ventromedial bulb region (Levai and Strottman, 2003); and the cells in the Grüneberg ganglion project to the caudal main olfactory bulb (Storan and Key, 2006). 5) The output of the mammalian accessory olfactory bulb has long been considered to be separate from the efferent connections of the main olfactory bulb, however recent studies have revealed convergence between these two pathways (Pro-Sistiaga *et al.,* 2007). In the sea lamprey, the posterior tuberculum predominates as the target of the secondary neurons from the medial glomeruli, compared to more diffuse projections from the remaining glomeruli to many forebrain structures, but excluding the posterior tuberculum (Derjean *et al*., 2008). Clearly, the lamprey's AOO differs from accessory olfactory structures in tetrapods, yet the location, cellular components and projection site, point to some similarities between the AOO and the vomeronasal organ, the septal organ of Masera and the Grüneberg ganglion.

Spatial segregation within the olfactory bulb, with respect to afferent input, is also seen in the teleost olfactory bulb - which splits up afferent input that is intermixed in the olfactory epithelium. In the olfactory bulb of zebrafish, channel catfish and carp, ciliated olfactory sensory neurons project medially, microvillous olfactory sensory neurons extend laterally, and crypt cells, ventrally (Morita and Finger, 1998; Sato et al., 2005; Hansen et al., 2003; Hamdani and Doving, 2006). Although the separate peripheral location of the lamprey AOO cells differs from the blended teleost polymorphic olfactory sensory neurons, the feature of a separate bulbar projection site for a olfactory neuron sub-type is conserved in both lampreys and teleost fish.

We conclude that the axons of AOO olfactory sensory neurons project into the medial region of the layer of glomeruli and mitral cells in the olfactory bulb. These observations affirm that in sea lamprey, AOO cells constitute a separate primary olfactory pathway, and signify the existence of an anatomically and biochemically distinct olfactory subsystem in the superclass Agnatha (jawless fish), positioned at the base of vertebrate evolution.

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APPENDIX B
Chemosensory-induced motor behaviors in fish

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Abstract

Chemical sensory signals play a crucial role in eliciting motor behaviors. We now review the different motor behaviors induced by chemosensory stimuli in fish as well as their neural substrate. A great deal of research has focused on reproductive, foraging, and escape behaviors but it is only recently that the molecules mediating these chemotactic responses have become well-characterized. Chemotactic responses are mediated by three sensory systems: olfactory, gustatory, and diffuse chemosensory. The olfactory sensory neuron responses to chemicals are now better understood. In addition, the olfactory projections to the central nervous system were recently shown to display an odotopic organization in the forebrain. Moreover, a specific downward projection underlying motor responses to olfactory inputs was recently described.

Abbreviations

AOO accessory olfactory organ
LOT lateral olfactory tract
MLR mesencephalic locomotor region
MOT medial olfactory tract
MOE main olfactory epithelium
OSN olfactory sensory neuron

Introduction

Most animals display orientation responses to chemical stimuli. In fish, chemosensory-mediated locomotion is associated with several essential behaviors such as homing, predator avoidance, reproduction, foraging and feeding. These locomotor responses require the activation of specialized chemical senses. The sensory signals are then transformed into a coherent motor behavior through a series of processes in the central nervous system. Although the link between chemosensory inputs and the motor control areas in the nervous system has long eluded scientists, recent discoveries in this area of research have provided some interesting clues as to the neural substrate involved.

Chemotactic responses in the environment

Homing behavior and reproduction

Among the most spectacular examples of chemosensory-guided behaviors are migratory behaviors of fish such as salmons and lampreys. For instance, sea lampreys, begin their lives upstream as freshwater larvae before metamorphosing into parasitic phase, and then migrate to the sea or a lake to feed. Once sexually mature, adults travel long distances to locate a freshwater spawning site in a stream or a river [1]. Adults are attracted to these sites, by migratory pheromones released by larvae [2-4]. Spermiating males, in turn, release sex pheromones that attract ovulating females to their nests for mating [5, 6, 7**; Fig. 1]. A major advance in this field of research in the last years has been the characterization of the pheromone blends implicated in these migratory [4] and sexual responses [5].

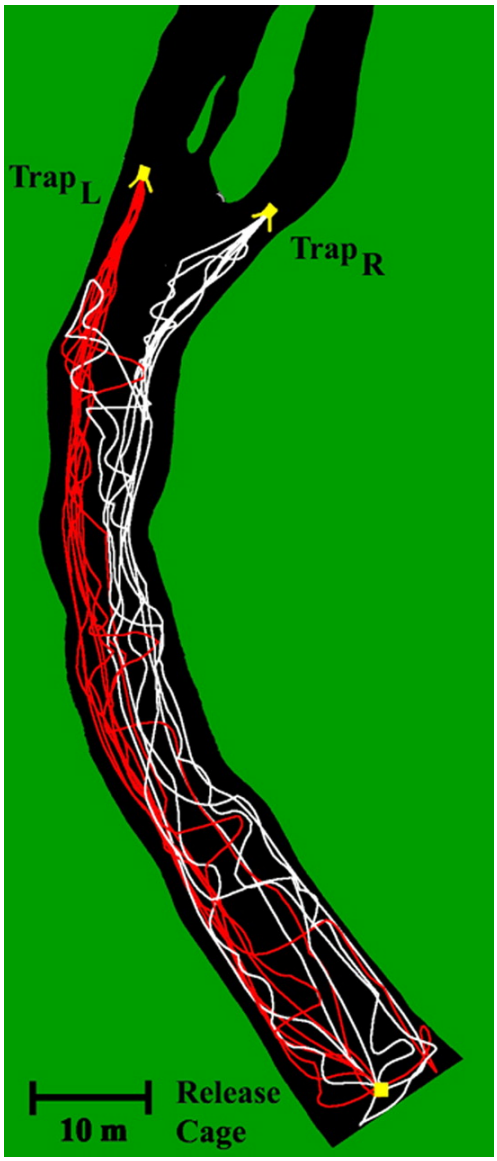

Figure 1: Observed movements of ovulated female sea lampreys in a natural stream segment. Behavioral responses to a synthetized component of the male mating pheromone: 7, 12, 24-trihydroxy-5-cholan-3-one 24-sulfate (3kPZS) were measured. A randomly selected trap ($Trap_L$ or $Trap_R$) was baited with 3kPZS, whereas a control solvent was applied in the other trap ($Trap_L$ or $Trap_R$). Red lines illustrate the path followed by lampreys entering the left trap when the latter was baited with 3kPZS. White lines illustrate the path followed by lampreys entering the right trap when the latter was baited with 3kPZS. The river is illustrated in black and the surrounds in green.
Credits: Reproduced with permission from from Ref. [7**]: Proceedings of the National Academy of Sciences of the United States of America, vol. 106 no. 4, Johnson et al., "A synthesized pheromone induces upstream movement in female sea lamprey and summons them into traps", 1021-1026. Copyright (2009) National Academy of Sciences, U.S.A.

Salmons are well known for returning to their home (natal) stream during their spawning migration. During the juvenile stage, salmons migrate downstream and imprint to (learn) chemical cues from their natal river. Once sexually mature, this olfactory imprinting is critical for successful completion of the salmon upstream spawning migration to their home-stream [8, 9]. The chemical nature of the imprinting odorant substance and the neural mechanisms underlying olfactory imprinting are still largely unknown. However, Yamamoto and colleagues [10*] have recently shown that dissolved free amino acids may be part of the imprinting odorant substance in lacustrine sockeye salmon. In another species, the masu salmon, spermiating males are attracted to an amino acid in the urine of ovulated females once they arrive to spawning beds [11]. While masu salmon have provided the only example of an amino acid sex pheromone, the prevalence of released hormonal compounds (steroids and prostaglandins) as sex pheromones that stimulate behavioral responses (attraction, nuzzling, quivering) in ciprinids has been demonstrated by Norm Stacey and his colleagues [e.g. 12, 13]. Conspecific attraction through sex pheromones has been observed in perciform fish, as well. Pheromones released by reproductive males stimulate olfactory sensory responses and attraction from females in the peacock blenny [14], and the round goby [15-17].

While released substances are important for stimulating behavioral responses during reproduction, the chemical signature of the home reef may be important for recruiting larval coral reef fish. During larval settlement, spiny damselfish prefer the water collected from their home reef over foreign reefs, and spend more time in home reef water under experimental conditions [18]. Anthropomorphic changes may affect recruitment to home reefs. The ability of larval clownfish to locate suitable settlement

sites is impaired in a chemical environment with elevated atmospheric carbon dioxide [19**].

Feeding behavior

For fish that do not rely on vision for hunting or foraging, the chemical senses are considered as the sensory modalities that are primarily responsible for initiating food search, localizing food sources, triggering the capture and ingestion of prey and the rejection of unpalatable food. It is generally accepted that amino acid constituents of prey provide cues for many aspects of feeding behavior. Fish respond to amino acids by initiating species-specific food search patterns. In zebrafish, L-alanine and L-valine induce an appetitive swimming behavior characterized by an increased number of turning behaviors [20*], whereas L-cysteine, L-alanine, L-lysine, L-proline induce bottom searching in rainbow trout, and L-arginine, L-glutamate, L-proline induce a "pecking" behavior in goldfish. Both chemically induced food search patterns are preceded by a period of arousal (increased locomotor activity) [21]. Amino acids are detected with threshold concentrations ranging from micromolar to nanomolar (same orders of magnitude for amino acid concentrations in natural waters) [22]. Importantly, environmental toxins also act on olfactory-mediated behaviors elicited by amino acids, including the attraction of fish to contaminated and potentially harmful areas [23,24]. Interestingly, the alarm substance of Ostariophysan fishes, which elicits a stereotyped predator avoidance behavior in prey species, has been shown to mediate feeding responses in predatory fish. Wisenden and Thiel [25] demonstrated that the skin extracts of the fathead minnow, which contain an alarm substance, elicited strong attractive strike responses from predatory fish. On the other hand, predatory fish are repelled by chemical

defenses released by prey. Recently, Nusnbaum and Derby [26*] described food rejection behavior, (decreased feeding strikes, food avoidance, food rejection) by predatory fish (bluenose wrasse), to ink secreted by the prey, *Aplysia califonica*, with olfactory sensory input mediating response related to food capture, and non-olfactory chemical senses contributing to the food acceptance phase.

Alarm reaction

Alarm reaction is a stereotyped predator avoidance behavior displayed by fish exposed to injured congeners. This reaction was originally described in ostariophysan species [27], which possess specialized epidermal cells called club cells that produce and store a chemical alarm cue released upon injury [for a review, see 28**]. Besides ostariophysans, damage-released alarm substances have been found in other fish taxa including gobies, salmonids, cottids, cichlids and percids [29]. Interestingly, cross-species reactions (i.e. response of one species to alarm cue from another species) between closely related species have been reported in percids, ostariophysans, and salmonids [29-31]. The fright reactions induced by the chemical detection of the alarm substances are, on the other hand, species-specific. In minnows, for example, the fright reaction is characterized by increased school cohesion, dashing and freezing behaviors [32], whereas in other cyprinids like crucian carp it involves a fast swimming behavior against to the bottom that disturbs the mud and debris, potentially hiding the fish [28].While the motor responses to alarm substances differ, in all these examples, fish avoid an area containing the alarm substance [33].

Neural substrate

Chemotactic responses, whether directed toward the source (i.e. positive chemotaxis) or away from the source (i.e. negative chemotaxis), require that the organisms locate the source by detecting the chemical stimuli. Three sensory systems (olfactory, gustatory, and diffuse chemosensory) are primarily responsible for chemical detection and thus potentially mediate chemotactic behaviors in fish. However, the neural mechanisms underlying chemotaxis remains unknown. Behavioral studies have provided interesting clues concerning the underlying principles. In sharks, olfactory orientation is achieved using internarial time differences and not internarial concentration differences, as previously believed [34**]. Sensory deprivation experiments in ictalurid catfish have shown that source localization of chemical stimuli is not mediated by olfaction, but by an extremely well developed gustatory system comprised of thousands of taste buds covering the body surface and the barbels, whisker-like appendages located near the mouth [35, 36]. However, a unilateral impairment of taste by surgically eliminating the barbels and the flank taste buds resulted in circling toward the intact side, and suggested that a bilateral comparison of stimuli is normally processed [35]. In this case, the bilateral comparison relies on differences in concentration and not on differences in the time of arrival of chemosensory signal [37]. Few studies have investigated the role of solitary chemosensory cells (of the diffuse chemosensory system) in the chemo-orientation behavior of fish. In rocklings, solitary chemosensory cells situated on the anterior fin respond to non-conspecific-conditioned water [38], whereas in the sea robin pectoral fin, solitary chemosensory cells are activated by food-related stimuli [39]. Taken together,

these findings suggest a possible involvement of the diffuse chemosensory system in at least two chemotactic behaviors: predator avoidance and food seeking.

Peripheral chemosensory systems

The peripheral olfactory system of most fish is composed of paired nasal cavities located near the dorsal surface of the head, in front of the eyes. The chambers are opened to the exterior medium by an anterior and a posterior nostril that allows the water to flow through. Lampreys and hagfish constitute an exception to this general pattern by having a single nostril and nasal cavity [40, 41*]. Typically, the floor of the olfactory chambers forms folds or lamellae lined with a sensory epithelium containing olfactory sensory neurons (OSNs) of distinct morphologies [42-44, Fig. 2]. Fish display a considerable variety in the number, surface area, and arrangement of olfactory lamellae. Consequently, it has been hypothesized that "size" and/or lamellar folding of the peripheral olfactory organs and olfactory acuity might be correlated [45, 46]. This was recently refuted in bony fish [42] and elasmobranchs [47]. Flow regulatory mechanisms and other factors that affect fluid dynamics and thus odorant transport were proposed to play a role in olfactory acuity [48]. For example, in the round goby, the nasal chamber is tube-shaped and unilamellar; but the enhanced flow of water passing through the nasal cavity due to the pumping action of accessory nasal sacs, may contribute to the good overall olfactory abilities of this fish [48]. In addition to the olfactory system, fish possess chemosensory cells located on the surface of their body, pharynx and branchial cavity. Taste buds, barrel-shaped aggregates of approximately one hundred chemosensory cells, constitute the peripheral receptors of the gustatory system. Taste buds are found in all craniates except hagfish, which have similar but not homologous sensory organs known as

Schreiner organs [49]. Taste receptor cells are tuned to respond to food-related substances and allow evaluation of food items and regulation of feeding [50, 51]. Isolated chemosensory cells (i.e. not gathered into taste buds) are referred to as solitary chemosensory cells and constitute a diffuse chemosensory system [52]. This chemosensory system, present in fish [50, 53] amphibians [54], and mammals [52] is poorly understood and its function has not been clearly defined yet. In fish, it has been suggested that it could constitute a parallel chemosensory system used to detect predators and prey [50].

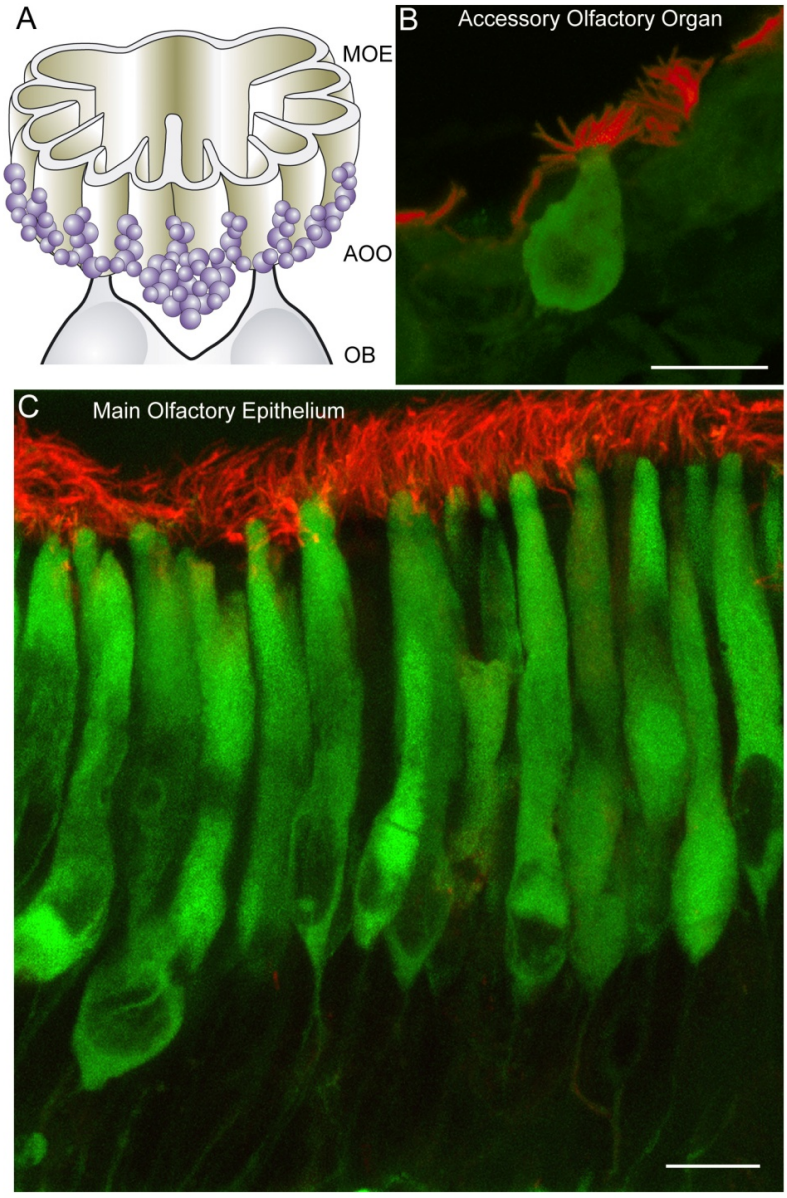

Figure 2: Olfactory sensory neurons in the sea lamprey. (A) The peripheral olfactory organ consists of a multi-lamellar main olfactory epithelium (MOE) and aggregations of spherical diverticula (purple) known as the accessory olfactory organ (AOO).The AOO is linked to the MOE via minute ducts. Both the MOE and AOO have distinct projections to the olfactory bulbs [44, 76**]. (B) and (C) are confocal Z-stacks of olfactory sensory neurons retrogradely labeled with biocytin (green); and cilia immunolabelled against acetylated tubulin (red) [44]. (B) An olfactory sensory neuron in the AOO. (C) Tall MOE olfactory sensory neurons. In the sea lamprey, the MOE contains three OSN morphotypes: tall, intermediate (not shown), and short (not shown) [43].Scale bars in B and C = 10 μm.

Central projections of chemosensory systems

The OSNs, located in the olfactory epithelium, project their axon to the glomerular layer of the olfactory bulbs, where synapses are made with the dendrites of the second order neurons, the mitral cells. In turn, the axons of mitral cells project via olfactory tracts to olfactory centers in the brain [reviewed in 55, 56]. In fish, olfactory tracts can be divided in medial olfactory tract (MOT) and lateral olfactory tract (LOT), each one being subsequently divided into a lateral and a medial part. The targets of secondary olfactory projections include several forebrain structures and can reach as far caudally as the midbrain.

Unlike OSNs, taste receptor cells gathered in taste buds are not neurons but specialized epithelial cells forming chemical synapses with afferent taste neurons [57]. The neurotransmitter of this synapse has not been formerly identified but a recent study by Finger *et al.* [58**] shows that ATP fulfills all criteria for linking taste buds to afferent nerve fibers. Taste buds located over the body surface, lips and anterior part of the mouth are innervated by the facial nerve (VII), taste buds located on the anterior most gill arches and associated oral tissues are innervated by glossopharyngeal nerve (IX). Finally, taste buds situated on the posterior part of the mouth and gill arches are innervated by the vagal nerve (X) [36, 59]. Typically, the primary afferent fibers terminate in a single nucleus (homologous to the solitary nucleus of tetrapods) in the visceral sensory column of the medulla oblongata. However, this primary taste center is subdivided in facial and vagal lobes in catfish and certain cyprinids with a highly developed sense of taste [59, 60]. The main target of second order neurons of the taste pathway is a dorsal brainstem nucleus, the secondary gustatory nucleus [50]. This nucleus

projects to gustatory areas mainly located in the diencephalon [61, 62]. Like taste receptor cells, solitary chemosensory cells are specialized epithelial cells in close association with a nerve fiber. Depending on the nerve providing somatosensory innervation of the body region where they are located, solitary chemosensory cells can be innervated by either cranial or spinal nerves [50].

The peripheral anatomy and central projections of olfactory and gustatory systems (and diffuse chemosensory system a lesser extent) have received significant attention over the years and are thus relatively well known. However, the connections of these chemosensory systems with motor control systems are still poorly understood.

Sensory-motor transformations

The early work of Grimm showed that electrical stimulation of the olfactory tract induced feeding behavior in goldfish [63]. Following this result, Døving and Selset [64] demonstrated that specific electrical stimulation of one the four olfactory bundles constituting the olfactory tracts elicited distinct stereotypical behaviors in cods. Alarm reaction and reproductive behavior were respectively evoked by stimulation of the medial and lateral part of the MOT, whereas feeding behaviors were evoked by stimulation of the LOT. Since then, these results were reproduced in crucian carps [65-67]. The latter group found that ciliated OSNs, crypt cells, and microvillous OSNS respectively synapse with secondary order neurons projecting through the medial part of the MOT, the lateral part of the MOT, and the LOT [68-70]. Because each bundle of the olfactory tracts mediates a specific behavior believed to be associated with a specific class of chemical compounds, a correlation between morphological types of OSNs and response to specific classes of chemical compounds was expected. Interestingly, each morphological type of

OSN was found to express different classes of membrane receptors: microvillous OSNs express V2R-type membrane receptors, whereas ciliated OSNs express OR-type membrane receptors [71]. The membrane receptors on crypt cells are currently unknown. However, as crypt cells are putatively associated with reproductive behavior, they are expected to express membrane receptors for sex pheromones [69], but physiological studies have shown responses to amino acids [72]. The anatomic and functional studies discussed above show a remarkable parallel segregation of the olfactory pathways from the sensory epithelium to the telencephalon in fish. In an elegant study, it was shown that an odotopic map is maintained up to telencephalic levels in channel catfish [73**; Fig. 3]. This telencephalic odotopic map does not simply mirror the odotopy of the olfactory bulb but displays convergence of responses to odors subserving the same biological function. Aside from pallial areas, an olfactory projection to the posterior tubercle, a ventral diencephalic area, has been reported in several fish species [74, 75, 76**]. This projection is of special interest because it was recently shown to be part of an olfactory-motor pathway in lampreys [76**]. The overall pathway consists of olfactory inputs from the OSNs to the medial part of the olfactory bulb, then on to the posterior tubercle, reaching the mesencephalic locomotor region and finally the reticulospinal cells, which are command neurons responsible for the activation of spinal locomotor networks. Activation of this pathway by odor or electrical stimulation generates large excitatory responses in reticulospinal cells and swimming movements (Fig. 4E). These findings constitute the first demonstration of a specific subsystem of the central nervous system dedicated to produce locomotor responses to olfactory inputs in vertebrates.

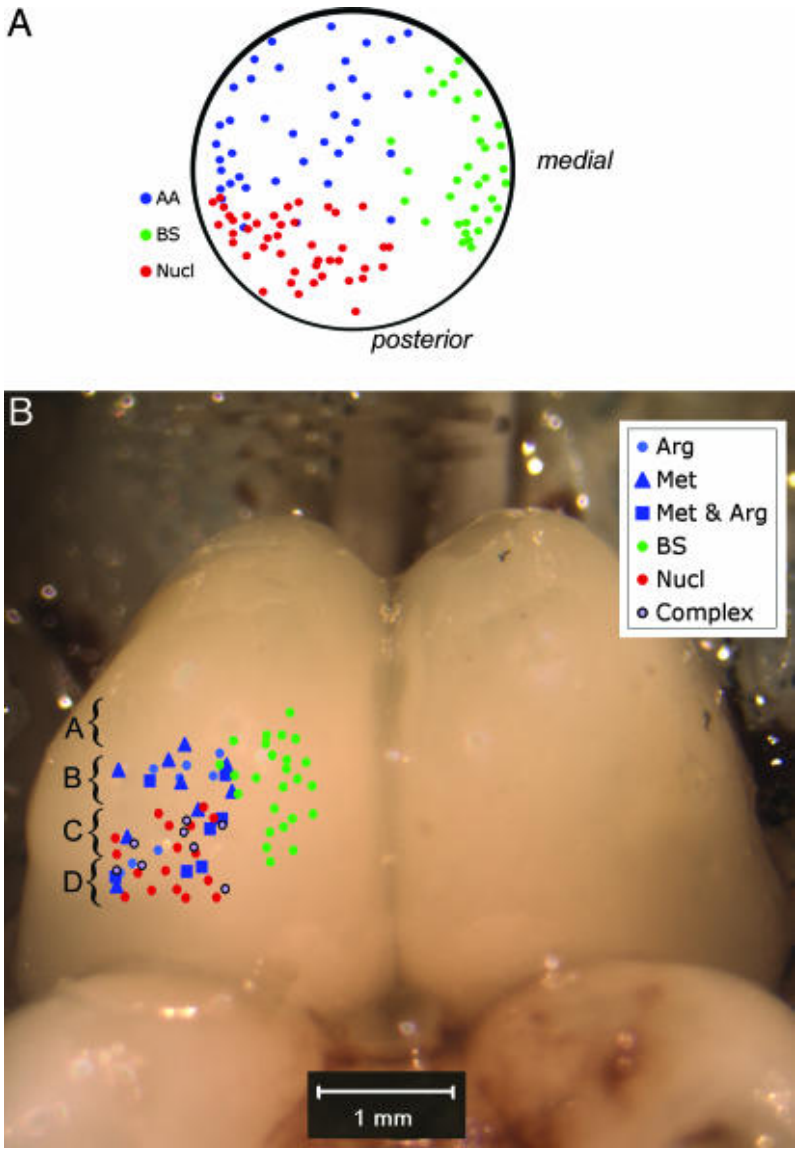

Figure 3: Odotopic organisation of amino acids, nucleotides, and bile-salts responsive units in the forebrain of the channel catfish. Interestingly, the odotopy (i.e. spatial mapping of different odors) is maintained from olfactory bulbs (A) to telencephalic levels (B). However, distinctive response properties emerge at this level, with odors subserving the same behavioral function showing convergence. Credits: Reproduced with permission from Ref. [73**]: Proceedings of the National Academy of Sciences of the United States of America, vol. 102 no. 51, Nikonov et al., "Beyond the olfactory bulb: an odotopic map in the forebrain", 18688-18693. Copyright (2005) National Academy of Sciences, U.S.A.

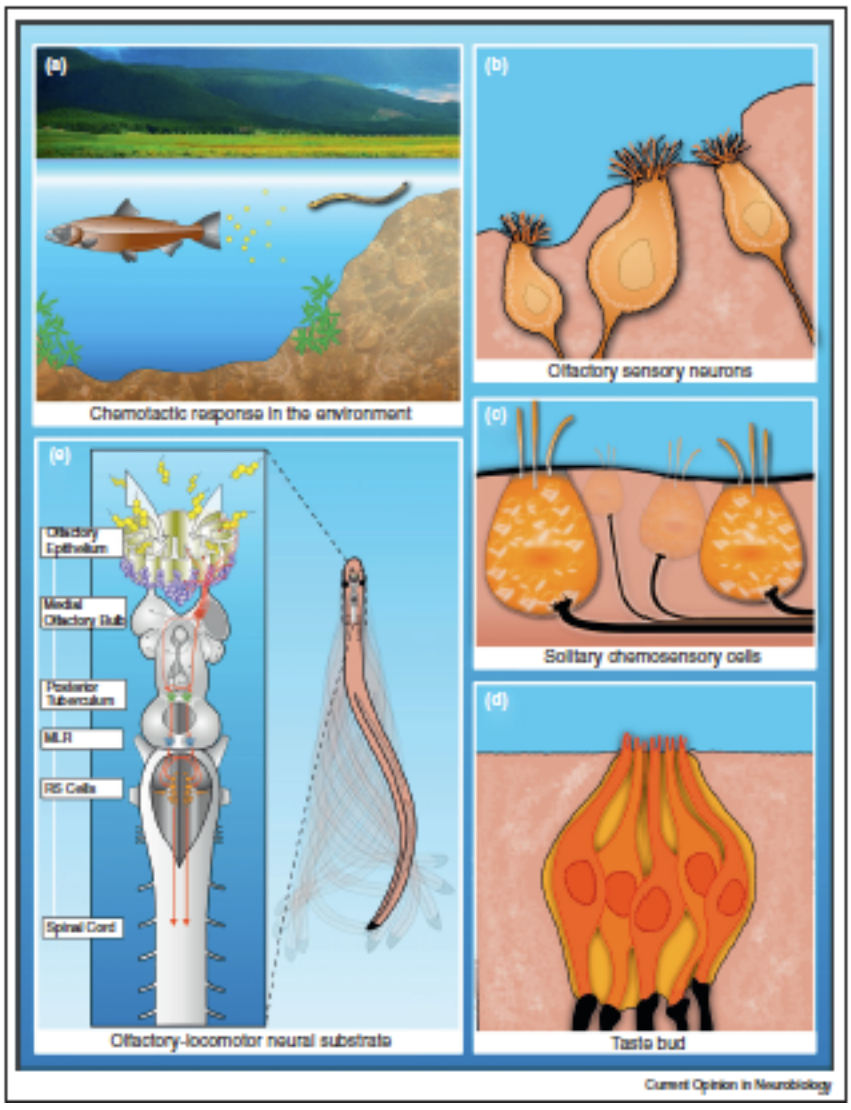

Figure 4: Summary figure illustrating chemically induced motor behaviors in fish and an underlying neural substrate in lampreys. (A) The olfactory system is presumed important for prey recognition. Chemical cues released by a prey will induce goal directed locomotion in a predator such as the sea lamprey. (B, C, D) Schematic representation of sensory cells in three chemosensory systems present in fish: Olfactory sensory neurons (B); Solitary chemosensory cells; (C) Taste sensory cells gathered in a taste bud (D). The illustrated sensory cells are from the lamprey model. (E) Schematic representation of the olfactory-locomotor circuitry in lampreys. Olfactory sensory neurons in the periphery activate neurons in the olfactory bulbs. There is a direct projection from the medial part of the olfactory bulb to the posterior tuberculum in the ventral diencephalon. From the posterior tuberculum, there is a projection to the mesencephalic locomotor region (MLR), known to play a crucial role in controlling locomotion in all vertebrate species. The MLR

projects in turn to brainstem reticulospinal neurons, which act as command cells for locomotion. Reticulospinal cells project directly to spinal cord neurons that are responsible for generating locomotion. Credits: (C) Adapted with permission from Ref. [77]: Journal of Zoology, vol. 199 no. 3, Whitear and Lane, “Oligovillous cells of the epidermis: sensory elements of lamprey skin”, 359–384. Copyright (1983) John Wiley and Sons. (D) Adapted with permission from Ref. [50]: Brain, Behavior and Evolution, vol. 50 no. 4, Finger, “Evolution of Taste and Solitary Chemoreceptor Cell Systems”, 234-243. Copyright (1997) S. Karger AG, Basel. (E) Reproduced from Ref. [76**]: PLoS Biology, 8(12): e1000567. doi:10.1371/journal.pbio.1000567, Derjean et al., "A Novel Neural Substrate for the Transformation of Olfactory Inputs into Motor Output". Creative Commons Attribution License (2010).

Conclusion

Chemotactic responses (Fig. 4 A) are mediated by similar chemosensory systems in different fish species. They consist of three systems: olfactory, diffuse chemosensory, and gustatory (respectively Fig.4 B, C, and D). In lampreys, a connection between the olfactory centers and the locomotor control system was recently described (Fig. 4 E). Direct projections from the olfactory bulb to the diencephalon have also been described in amphibians [77] and mammals [78, 79]. It is not unlikely that they could also be part of olfactory-motor pathways allowing for the production of motor behaviors in response to odors in theses vertebrates. The evolutionary conservation of such a neural pathway could underlie the presence of motor responses to olfactory cues seen in all vertebrate species. Future research will tell whether these pathways seen in other vertebrates underlie motor responses as seen in lampreys.

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APPENDIX C

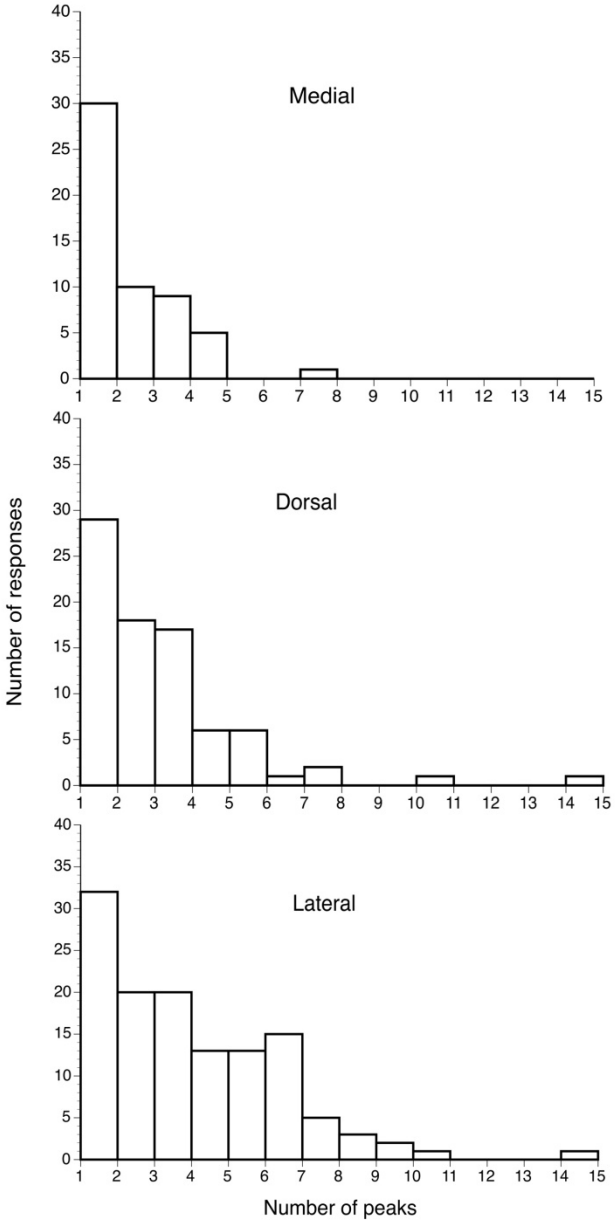

Figure C-1. Histograms of the number of peaks per odour-evoked local field potential (LFP) response in the medial, dorsal, and lateral olfactory bulb regions. Local field potentials in the medial OB exhibit the fewest number of peaks per response and the dorsal and lateral regions exhibit increasing numbers of peaks per LFP response.

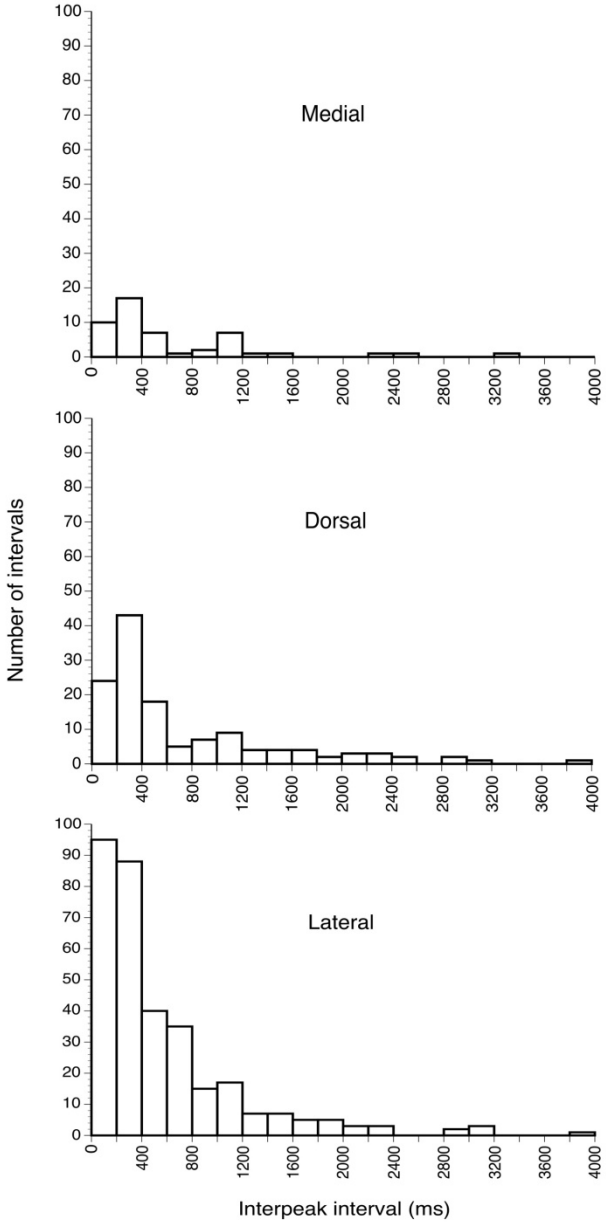

Figure C-2. Histograms of the interpeak intervals of sustained (multiple peak) odour-evoked local field potentials (LFPs) in the medial, dorsal, and lateral olfactory bulb. The medial region has relatively few; short, interpeak intervals while the dorsal and lateral regions have increasing numbers of interpeak intervals with increasing interpeak durations.

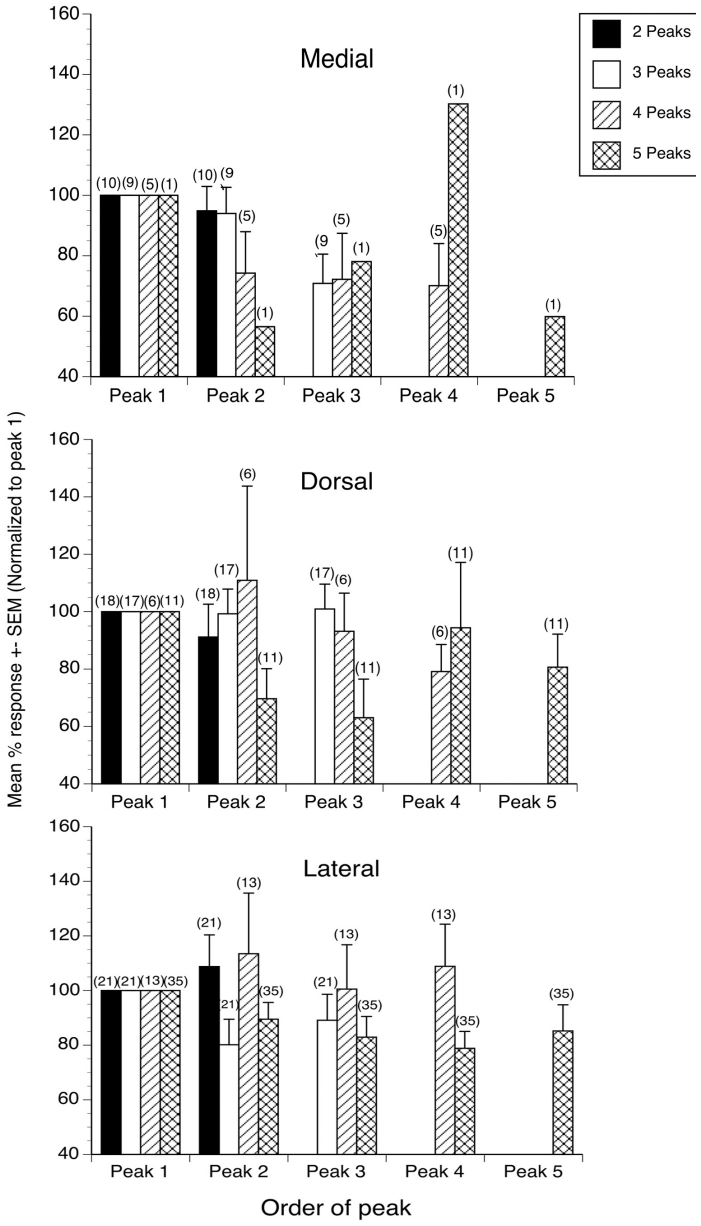

Figure C-3. The change in response amplitude of each peak subsequent to the first peak in sustained (multiple peak) local field potential responses. Responses were normalized to the amplitude of the first peak in a multiple peak response and are expressed as a percentage (Mean ± SEM) of the first peak. The change in peak amplitude over a multiple peak response was examined for local field potentials consisting of 2, 3, 4, or 5 peaks in the medial (A), dorsal (B), and lateral (C) olfactory bulb regions. The numbers above each bar are the number of responses in each region with a given number of peaks.

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