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Warren W. Green
University of Windsor

Karl Boyes
University of Windsor

Charrie McFadden
University of Windsor

Gheylen Daghfous

François Auclair

See next page for additional authors

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RESEARCH ARTICLE

Odorant organization in the olfactory bulb of the sea lamprey

Warren W. Green[1], Karl Boyes[1], Charrie McFadden[1], Gheylen Daghfous[2,3], François Auclair[2,3], Huiming Zhang[1], Weiming Li[4], Réjean Dubuc[2,3] and Barbara S. Zielinski[1,5,*]

ABSTRACT

Olfactory sensory neurons innervate the olfactory bulb, where responses to different odorants generate a chemotopic map of increased neural activity within different bulbar regions. In this study, insight into the basal pattern of neural organization of the vertebrate olfactory bulb was gained by investigating the lamprey. Retrograde labelling established that lateral and dorsal bulbar territories receive the axons of sensory neurons broadly distributed in the main olfactory epithelium and that the medial region receives sensory neuron input only from neurons projecting from the accessory olfactory organ. The response duration for local field potential recordings was similar in the lateral and dorsal regions, and both were longer than medial responses. All three regions responded to amino acid odorants. The dorsal and medial regions, but not the lateral region, responded to steroids. These findings show evidence for olfactory streams in the sea lamprey olfactory bulb: the lateral region responds to amino acids from sensory input in the main olfactory epithelium, the dorsal region responds to steroids (taurocholic acid and pheromones) and to amino acids from sensory input in the main olfactory epithelium, and the medial bulbar region responds to amino acids and steroids stimulating the accessory olfactory organ. These findings indicate that olfactory subsystems are present at the base of vertebrate evolution and that regionality in the lamprey olfactory bulb has some aspects previously seen in other vertebrate species.

KEY WORDS: Chemotopy, Neurophysiology, Odour responses, Tract tracing

INTRODUCTION

By detecting and discriminating specific stimuli, the olfactory system enables an organism to respond to its chemical surroundings. The olfactory system plays a crucial role in the survival and reproduction of the sea lamprey. Amino acids are associated with feeding (Kleerekoper and Mogensen, 1963; Kleerekoper, 1969). During spawning migration, sea lampreys encounter a mixture of bile acid steroids released from larval sea lamprey in the streambed, and these larval odours have an important role in identifying a suitable river for spawning and larval rearing (Li et al., 1995; Sorensen and Vrieze, 2003). Once a spawning location is found, spermiated male sea lampreys release steroid pheromones, which induce movement/searching and spawning behaviours in ovulated female sea lamprey (Li et al., 2002; Siefkes et al., 2003; Siefkes and Li, 2004; Johnson et al., 2005, 2009). Physiological studies have also investigated olfactory function in the sea lamprey. Basic amino acids and a variety of steroid bile acids (including the lamprey pheromones) stimulate olfactory sensory responses from the main olfactory epithelium (e.g. Li et al., 1995, 2002; Li and Sorensen, 1997; Siefkes and Li, 2004; Sorensen et al., 2005; Johnson et al., 2009). However, the peripheral olfactory organ also houses abundant tubular diverticula known as the accessory olfactory organ (Hagelin and Johnels, 1955). While sensory neurons are located in the accessory olfactory organ (Ren et al., 2009), there are presently no physiological recordings of odorant responses in the sea lamprey accessory olfactory organ, because of the relatively inaccessible location and small size of the many recesses of this structure.

As in other vertebrates, lamprey olfactory sensory neurons propagate odorant responses along the olfactory nerve into the olfactory bulb, where the axons terminate in regions of synaptic interactions called glomeruli. In the sea lamprey, these glomeruli include prominent dorsal, lateral and medial clusters (Frontini et al., 2003). In the olfactory bulb, the glomeruli receiving sensory inputs from the main olfactory epithelium are spatially segregated from that receiving accessory olfactory organ input. Olfactory sensory neurons in the main olfactory epithelium project to non-medial regions of the olfactory bulb (Ren et al., 2009) where output connects to the lateral pallium and extra pallial forebrain targets (Derjean et al., 2010; Green et al., 2013). A single glomerulus in the medial region of the olfactory bulb receives axons from olfactory sensory neurons located in the accessory olfactory organ (Ren et al., 2009) and medial projection neurons extend to the posterior tuberculum (Derjean et al., 2010; Green et al., 2013). The neurochemistry and cyto-architecture also differs between the non-medial and medial olfactory bulb regions. Olfactory sensory neurons projecting to the non-medial glomeruli are immunoreactive for the olfactory regulatory G protein $G_{\alpha olf}$ and those projecting to the medial region lack this immunoreactivity (Frontini et al., 2003). The dendrites and somata in the medial projection neurons are confined to the medial glomerular neuropil and dendrites of non-medial projection neurons do not enter this territory, suggesting that neural signals processed in the medial glomerulus are not influenced directly by neural activity in the non-medial regions (Green et al., 2013). While the medial and non-medial bulbar regions differ neuroanatomically, the odotopy – the spatial organization of odour responses in the lamprey olfactory bulb – has not been investigated.

Odour-processing streams within the olfactory bulb encode and discriminate odours and relay information to higher brain centres (Munger et al., 2009). In teleost fish, olfactory responses to amino acids or to amino acid derivatives have been linked to feeding (Braubach et al., 2009), as well as to home stream imprinting (Shoji et al., 2003; Yamamoto et al., 2010; Bandoh et al., 2011),

[1]Department of Biological Sciences, University of Windsor, Windsor, ON, Canada N9B3P4. [2]Groupe de Recherche en Activité Physique Adaptée, Département des sciences de l'activité physique, Université du Québec à Montréal, Montréal, QC, Canada H3C3P8. [3]Groupe de Recherche sur le Système Nerveux Central, Département de neurosciences, Université de Montréal, Montréal, QC, Canada H3C3J7. [4]Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824, USA. [5]Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON, Canada N9B3P4.

*Author for correspondence (zielin1@uwindsor.ca)

B.S.Z., 0000-0002-5666-4477

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Journal of Experimental Biology

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pheromone communication (Yambe et al., 2006) and kin recognition (Hinz et al., 2013). Amino acids activate olfactory sensory neurons that project to the rostral lateral olfactory bulb region, and sensory neurons projecting to the more medial bulbar regions respond to bile salts and pheromones (steroids or prostaglandins), except in zebrafish where a reproductive pheromone activates a central portion in the ventral olfactory bulb region (catfish – Nikonov and Caprio, 2001; Hansen et al., 2003; zebrafish – Friedrich and Korsching, 1997, 1998; and salmonids – Hara and Zhang, 1996, 1998). While odour responses take place in a single olfactory system in teleosts, many amphibians and terrestrial vertebrates process chemosensory information in the main and accessory (vomeronasal) olfactory subsystems (Eisthen and Polese, 2006). Within the main olfactory bulb of the larval stage of the amphibian *Xenopus laevis*, a lateral processing stream responds to amino acids, an intermediate stream responds to bile acids, amines and occasionally to amino acids, and the medial stream responds to amines and alcohols and infrequently to bile acids (Gliem et al., 2013). In the mammalian main olfactory bulb, glomeruli that respond to structurally similar compounds are congregated within a bulbar region, yet this grouping is rather coarse (Johnson and Leon, 2007; Soucy et al., 2009).

In this study, we investigated the spatial organization of odorant responses in three locations (lateral, dorsal and medial) in the sea lamprey olfactory bulb. First, we examined the peripheral location of sensory neurons that project to these territories; then, we recorded electrophysiological responses during the application of odorants to the peripheral olfactory organ. We found evidence for three streams in the olfactory bulb. The lateral region received axons from sensory neurons throughout the main olfactory epithelium and responded only to amino acids; the dorsal region also contained input from sensory neurons that were widespread in the main olfactory epithelium, but responded to steroid (pheromones and taurocholic acid) as well as to amino acids. These same odorants stimulated short responses from the medial bulbar region, the location of axons projecting from the accessory olfactory organ.

MATERIALS AND METHODS

Animal collection

All sea lampreys were captured from streams in Ontario, Canada, and Michigan, USA, in cooperation with the Toronto Region Conservation Authority, the Department of Fisheries and Oceans, and the United States Geological Survey Hammond Bay Biological Station. They 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. All experiments were performed in accordance with the guidelines of the University of Windsor Animal Care Committee and the Canadian Council on Animal Care. For this study, both transformer and spawning stage lampreys were used.

Tissue preparation

Lamprey were anaesthetized in a solution of tricaine methanesulphonate (MS222, 150 mg l^{-1}) in dechlorinated water (pH adjusted to 7 using 2 mol l^{-1} NaOH) and decapitated at the third brachial pore. Tissue was transferred to ice-cold lamprey Ringer's solution (130 mmol l^{-1} NaCl, 2.1 mmol l^{-1} KCl, 2.6 mmol l^{-1} $CaCl_2$, 1.8 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} Hepes, 4 mmol l^{-1} dextrose, 1 mmol l^{-1} $NaHCO_3$). The olfactory epithelium and brain as well as the rostral-most portion of the spinal cord were isolated. The explant preparation was transferred to a chilled (8°C) recording chamber and continuously perfused with chilled lamprey Ringer's solution at a flow rate of 1 ml min^{-1}. In addition, the olfactory epithelium was continuously perfused with chilled Ringer's solution via an odour delivery system (described below). Ringer's solution was made fresh daily, chilled, oxygenated (95% oxygen, 5% carbon dioxide), and pH adjusted (7.4) with 5 mol l^{-1} NaOH.

Neuroanatomy

Sea lampreys which had recently transformed from the larval to the feeding adult stage (metamorphic stage seven; Youson and Potter, 1979) (N=9) were anaesthetized and an explant preparation was prepared as described above. Fluorescent dextran powder (Alexa Fluor 488, Life Technologies, catalogue no. D-22910) was applied to the tip of a tungsten probe (tip diameter 1 µm) and inserted into the medial (N=3), dorsal (N=3) or lateral region (N=3) of the olfactory bulb. The explant preparation was then submerged and continuously perfused with fresh, oxygenated (95% oxygen, 5% carbon dioxide; pH adjusted to 7.4) and chilled (8°C) lamprey Ringer's solution for 12–16 h. Once the incubation was complete, the brain was placed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer for 24 h and subsequently cryoprotected in a sucrose gradient (10%, 20% and 30% sucrose in 0.1 mol l^{-1} phosphate-buffered saline). Tissue was embedded in Cryomatrix and cryosectioned (Leica CM3050 S, Leica Microsystems, Germany) in serial 18 µm coronal sections. Slides were coverslipped using Vectashield hardset mounting medium. Tissue sections were observed with a Leica DM2500 microscope and micrographs were captured using a Leica FX360 camera and Leica LAS 4.5 software.

Neurophysiology

Electrophysiological recordings from lateral, dorsal and medial regions of the olfactory bulb were made during the application of odorants to the peripheral olfactory organ. The *ex vivo* preparation described in the 'Tissue preparation' section (above) was utilized. Amino acids, a bile acid (taurocholic acid) and bile acid steroid pheromones previously shown to be stimulatory to the main olfactory epithelium (Li et al., 1995, 2002; Bjerselius et al., 2000; Siefkes et al., 2003; Siefkes and Li, 2004; Sorensen et al., 2005; Johnson et al., 2005, 2009) were tested. The pheromone test solution included the migratory pheromones petromyzonol sulphate (PZS), and two disulphated aminosterol derivatives known as petromyzonamine disulphate (PADS) and petromyzonolsterol disulphate (PSDS) (Li et al., 1995; Bjerselius et al., 2000; Sorensen et al., 2005), as well as the reproductive pheromones 3-keto petromyzonol sulphate (3KPZS) and 3-keto allocholic acid (3KACA). Stock solutions (10^{-2} mol l^{-1}) of L-arginine, L-histidine and taurocholic acid were made daily and diluted to working concentrations (10^{-3} mol l^{-1} to 10^{-6} mol l^{-1}) in chilled lamprey Ringer's solution. For the lamprey pheromones (3KPZS, 3KACA, PZS, PADS, PSDS), stock solutions were made by reconstituting each pheromone in a 1:1 solution of high purity methanol:ultra-pure water at a concentration of 1 mg ml^{-1} and were stored at −80°C. Micromolar solutions of the pheromones were combined and this mixture was serially diluted prior to experimentation. For both the local field potential and multiunit recordings, three stimulatory solutions were tested: (1) a mixture of amino acids (10^{-3} mol l^{-1} L-arginine and 10^{-3} mol l^{-1} L-histidine), (2) a mixture of steroid pheromones (10^{-7} mol l^{-1} 3KPZS, 3KACA, PZS, PADS and PSDS) and (3) an individual bile acid (10^{-4} mol l^{-1} taurocholic acid). These concentrations were empirically determined by examining electrophysiological responses to a range of odorant

Journal of Experimental Biology (2017) 220, 1350-1359 doi:10.1242/jeb.150466

concentrations. The concentrations utilized were above threshold and elicited reproducible responses in the olfactory bulb.

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. This allowed for fast switching between background Ringer's solution and a desired odorant with no interruption in flow to the naris. Each odorant delivery was 5 s in duration. Consecutive deliveries were at least 3 min apart to prevent adaptation. Each odorant was delivered at least 3 times to elicit three individual responses. Dye trials were used to determine the test solution dwell-time in the peripheral olfactory organ. The dye appeared very dilute 3 s after a dye application was terminated, and completely cleared this region by 1 min.

Local field potentials were recorded from three locations (lateral, dorsal, medial) in the olfactory bulb of an *ex vivo* preparation of the sea lamprey. The fairly large olfactory bulbs were fully exposed, making the placement of the recording electrodes (midway between rostral and caudal olfactory bulb boundaries) reproducible between experiments (location shown in the cartoon drawing in Fig. 1). Earlier work (Green et al., 2013) provided in-depth knowledge regarding the structural organization of the sea lamprey olfactory bulbs. This knowledge, along with the relatively large size of the olfactory bulbs, allowed reproducible electrode placement in the lateral, dorsal or medial region to be readily achieved using a stereo-microscope. A glass micropipette filled with 2 mol l^{-1} NaCl (tip diameter 8–10 µm, impedance 0.1 MΩ) was placed on the surface of the olfactory bulb. Signals were band-pass filtered (1 Hz to 1 kHz) and amplified (×10,000) using a P511Ll amplifier (Grass Technologies Inc., West Warwick, RI, USA) and digitized at 10 kHz using ML866 Powerlab 4/30 (ADInstruments, Colorado Springs, CO, USA).

Extracellular multiunit recordings were made from the olfactory bulb in a total of 25 cases using a glass micropipette filled with lamprey Ringer's solution (tip diameter 2–3 µm, impedance 2–5 MΩ). Recording electrodes were inserted into the olfactory bulb (medial N=11, dorsal N=5, lateral N=9) using a manual hydraulic micro-drive (Narishige Inc., Tokyo, Japan) to a depth of ∼150 µm for metamorphic stage seven lamprey and ∼300 µm for adult lamprey, where somata of projection neurons are located (Green et al., 2013). Signals were band-pass filtered (10 Hz to 10 kHz) and amplified 10,000 times using a model 1800 amplifier (A-M Systems, Sequim, WA, USA) and digitized at 10 kHz using ML866 Powerlab 4/30 (ADInstruments). For both local field potential and

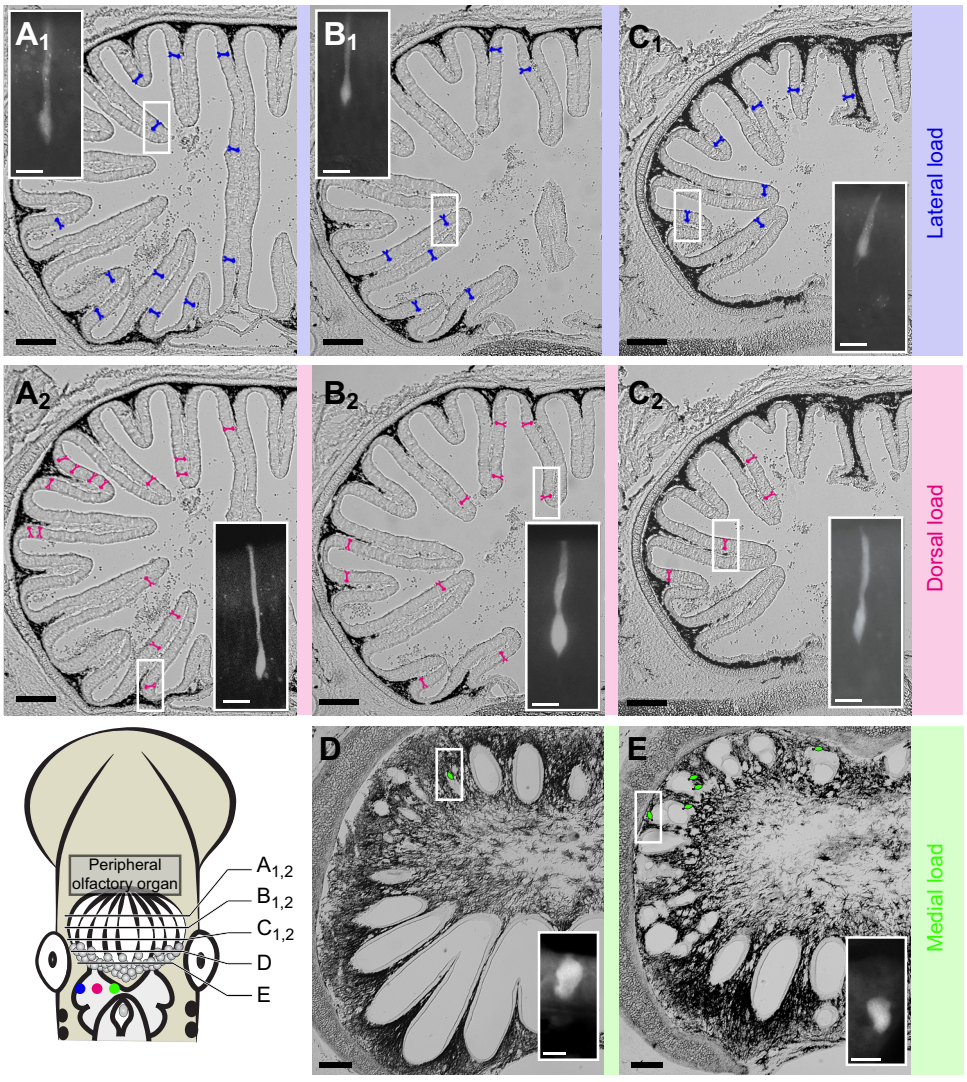

Fig. 1. Retrograde labelling of olfactory sensory neurons following the application of fluorescent dextran into the lateral, dorsal and medial regions of the olfactory bulb. Low power, greyscale, cross-sectional views of rostral (A) to caudal (E) regions of the peripheral olfactory organ. The coloured lines on each micrograph (blue for A_1, B_1, C_1; pink for A_2, B_2, C_2; and green for D and E) were drawn to show the ipsilateral rostro-caudal distribution of retrogradely labelled olfactory sensory neurons that project to lateral (blue), dorsal (pink) and medial (green) regions of the olfactory bulb. A_1 and A_2 show the rostral portion of the main olfactory epithelium, B_1 and B_2 show the middle portion, C_1 and C_2 represent the caudal portion, D shows the transition from the caudal region of the main olfactory epithelium to the accessory olfactory organ and E shows the accessory olfactory organ. The blue lines in A_1, B_1 and C_1 show the distribution of olfactory sensory neurons retrogradely labelled following dextran application to the lateral region of the olfactory bulb. The rectangles in A_1, B_1 and C_1 show the location of high-power views of retrogradely labelled olfactory sensory neurons that are shown as an inset of each micrograph. The pink lines in A_2, B_2 and C_2 show the location of labelled olfactory sensory neurons following dextran application to the dorsal bulbar region, and the green lines in D and E show the labelling following dextran application to the medial bulbar region. The drawing on the lower left of the figure shows the location of the lateral, dorsal and medial dextran injections into the olfactory bulb and the location of the peripheral olfactory organ sections shown in A–E. The scale bars are 200 µm in A–E and 10 µm for the insets in A–E.

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 1350-1359 doi:10.1242/jeb.150466

multiunit recordings, spikes were monitored online and analysed offline using LabChart software (version 6.1.3, ADInstruments).

Data analysis and statistics

Prior to analysis, all local field potentials were 100 Hz low-pass digitally filtered (LabChart version 6.1.3, ADInstruments). Within each recording, a pre-odour baseline mean and standard deviation were calculated for the 5 s time period immediately prior to the onset of each odorant delivery. A threshold was set as 3 times the baseline standard deviation and was utilized to distinguish odour-evoked responses from baseline deviations in neural activity. Odorant-induced local field potentials were exhibited with either a single deflection or multiple deflections from the baseline. For a response with a single deflection, the duration of the response was defined as the time lapse between the two points when a response reached 10% of its peak value during the rising and falling phases. For a local field potential response with multiple deflections, the duration was defined as the time lapse between the point when the response reached 10% of the peak value of the first deflection during a rising phase and that when the response reached 10% of the peak value of the last deflection during a falling phase. The mean odour-evoked response duration was calculated for each odour within each animal.

Multi-unit recordings

Multi-unit recordings were completed in order to confirm results from long field potential recordings regarding the spatial distribution of odour-evoked responses in the olfactory bulb. The change in the rate of action potential discharge was examined over the 30 s pre-odorant delivery period, 30 s odorant delivery period and 120 s post-odorant delivery period (LabChart version 2.4.1). Peri-stimulus time histograms were made for the entire time course of a recording (180 s), with spikes being sorted into 1 s time bins. Odorant-evoked responses were classified as excitatory, inhibitory or null. A response was considered excitatory if the rate of spiking was increased by at least 100% during odorant delivery in reference to the rate of spontaneous spiking. A response was considered inhibitory or excitatory if the rate was changed by at least 50% during odour delivery in reference to spontaneous spiking.

Statistics

The duration of odorant-elicited local field potential responses was compared between the lateral, dorsal and medial olfactory bulb regions using one-way analysis of variance (ANOVA) followed by *post hoc* Tukey analysis using Graphpad Prism (version 6). Differences in odorant response between bulbar regions were tested using Fisher's Exact Test on a subset of the local field potential data in which all three odorants were tested in a single case (lateral n=9; dorsal n=10; medial n=8). This subset of data is presented as Euler diagrams illustrating, for each bulbar region and each class of chemicals, the number of trials in which a response was observed.

RESULTS

The peripheral distribution of olfactory sensory neurons projecting to the lateral, dorsal and medial olfactory bulb regions

Tract-tracing experiments were conducted to establish whether odorants entering the lumen of the peripheral olfactory organ have the potential to stimulate responses in the dorsal, lateral and medial olfactory bulb regions. Earlier tract-tracing studies had shown that the lateral and dorsal bulbar regions receive axonal projections from olfactory sensory neurons in the main olfactory epithelium (Ren et al., 2009); however, it was not known whether olfactory sensory neurons projecting to lateral and dorsal territories were located in a specific region in the main olfactory epithelium. This question was addressed by labelling olfactory sensory neurons using Alexa 488 dextran, a retrograde tract tracer, separately applied to the lateral or dorsal region of the olfactory bulb. Olfactory sensory neurons with axons extending into either the lateral or dorsal bulbar regions were widespread (rostro-caudal and dorso-lateral) in the ipsilateral main olfactory epithelium, but were not seen in the accessory olfactory organ (Fig. 1). Labelled olfactory sensory neurons were not observed in the contralateral portion of the main or accessory olfactory epithelium. These olfactory sensory neurons were predominantly tall, with a narrow elongated dendrite and a cell body in the lower portion of the olfactory epithelium, and some were of an intermediate morphotype, with the cell body in the mid-epithelial region (Fig. 1A–C). The dendrites and cell bodies of

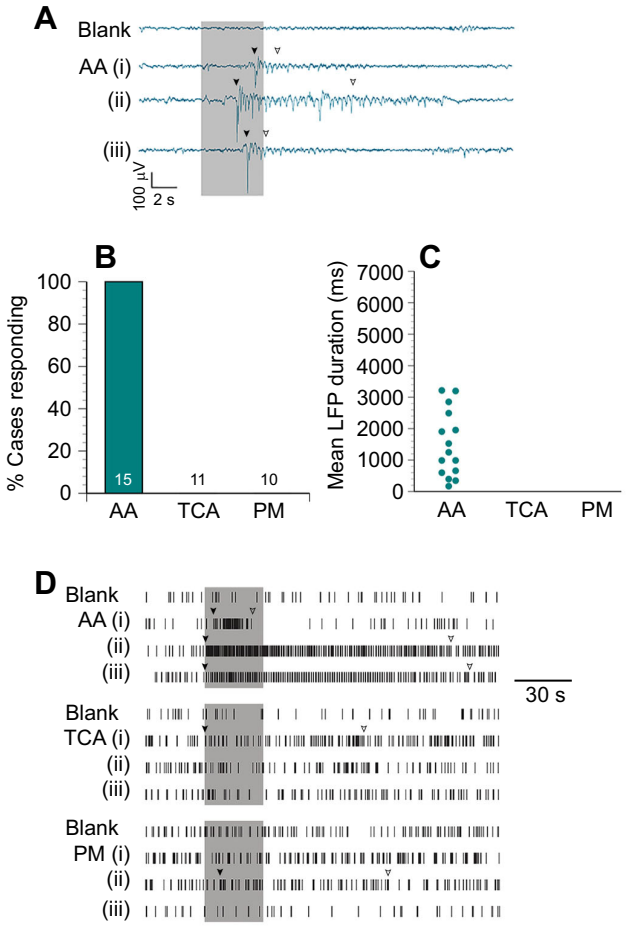

Fig. 2. The lateral olfactory bulb region exhibits responses primarily to amino acids. (A) Example local field potential recordings in response to amino acids (AA). Shaded area indicates the 5 s odour delivery period. Three representative responses from three different animals are shown (i–iii). The black triangles indicate onset and the white triangles indicate offset, calculated as a return of signal to less than 10% deviation from the baseline mean value. (B) The percentage of local field potential cases showing responses to each of the odours tested (AA, amino acids; TCA, taurocholic acid; PM, pheromone mixture). (C) Mean duration of local field potential (LFP) responses. (D) Example multi-unit recordings in response to AA, TCA and PM. Shaded area indicates the 30 s odour delivery period. A representative response from three different animals is shown for each odour (i–iii). The black triangles denote response onset and the white triangles show offset, when the rate returned to below 3 times the baseline standard deviation.

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 1350-1359 doi:10.1242/jeb.150466

olfactory sensory neurons that extend their axons to the medial glomerular region were widespread throughout the ipsilateral accessory olfactory organ but were absent from the main olfactory epithelium (Fig. 1C,D). These cells were rounded with a narrow apical region, as previously described by Ren et al. (2009). Therefore, tall and intermediate olfactory sensory neurons (Laframboise et al., 2007) projecting to a lateral or dorsal olfactory bulb territory are broadly distributed within the ipsilateral main olfactory epithelium, and would be activated by odour application into the peripheral olfactory organ. However, only sensory neurons located in the ipsilateral accessory olfactory organ project to the medial glomerular region.

Odorant selectivity in the lateral, dorsal and medial olfactory bulb regions

Local field potentials recorded from the lateral bulbar region (Fig. 2A) exhibited responses to a mixture of amino acids, and not to the steroids (pheromones or taurocholic acid; Fig. 2B,C). The response duration ranged from 170 to 3200 ms, with an average response duration of 1500±270 ms (mean±s.e.m., n=15; Fig. 2C). A similar odorant selectivity in the lateral region was seen in multi-unit recordings, in which 80% of recordings exhibited excitatory responses to the amino acid mixture (Fig. 2D). A smaller percentage of multiunit recordings exhibited weakly excitatory responses to taurocholic acid (11%) and to the pheromone mixture (11%). In some cases, the multi-unit responses did not return to baseline after the offset of odorant stimulation, although other responses were substantially shorter (Fig. 2D). Thus, both local field potential and multiunit recordings suggest that the lateral olfactory bulb region responds primarily to amino acids.

The dorsal olfactory bulb recordings (Fig. 3A) displayed different odour selectivity from the lateral recordings. Local field potential recordings indicated that 50% (7/14) of the cases generated responses to the amino acid mixture, while 100% (18/18) and 80% (12/15) of cases generated responses to taurocholic acid and to the pheromone mixture, respectively (Fig. 3B). The response duration recorded from the dorsal region ranged from 94 to 6600 ms across all odorants tested and the average duration did not differ among responses elicited by the amino acid mixture (mean±s.e.m. 1570±400 ms, n=7), taurocholic acid (1530±400 ms, n=18) and the pheromone mixture (1360±300 ms, n=12) ($F_{2,34}$=0.0713; P=0.9313; Fig. 3C). Multiunit recordings from the dorsal region (Fig. 3D), showed excitatory responses to the amino acid mixture (80%), taurocholic acid (60%) and pheromone mixture (75%). Some of these multi-unit responses persisted beyond the offset of odorant stimulation and others terminated shortly after the offset (Fig. 3D). These data show that amino acid and steroid bile-derived odorants activate the dorsal region of the olfactory bulb.

In the medial region, which receives sensory input exclusively from olfactory sensory neurons located in the accessory olfactory organ, local field potential responses (Fig. 4A) to the pheromone mixture were elicited in 100% of the lampreys examined (n=12/12),

Table 1. Contingency table for local field potential responses in the lateral, dorsal and medial olfactory bulb regions to odorants (shown in Fig. 5)

	AA	AA+PM	AA+PM+TCA	PM	PM+TCA	TCA	Total
Lateral	9^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	9
Dorsal	0^{a}	$0^{a,b}$	$5^{a,b}$	$0^{a,b}$	3^{b}	2^{b}	10
Medial	0^{a}	1^{b}	$5^{a,b}$	1^{b}	$1^{a,b}$	$0^{a,b}$	8

AA, amino acids; PM, pheromone mixture; TCA, taurocholic acid. Each superscript letter denotes a subset of odorant response categories with column proportions that do not differ significantly from each other at the 0.05 level.

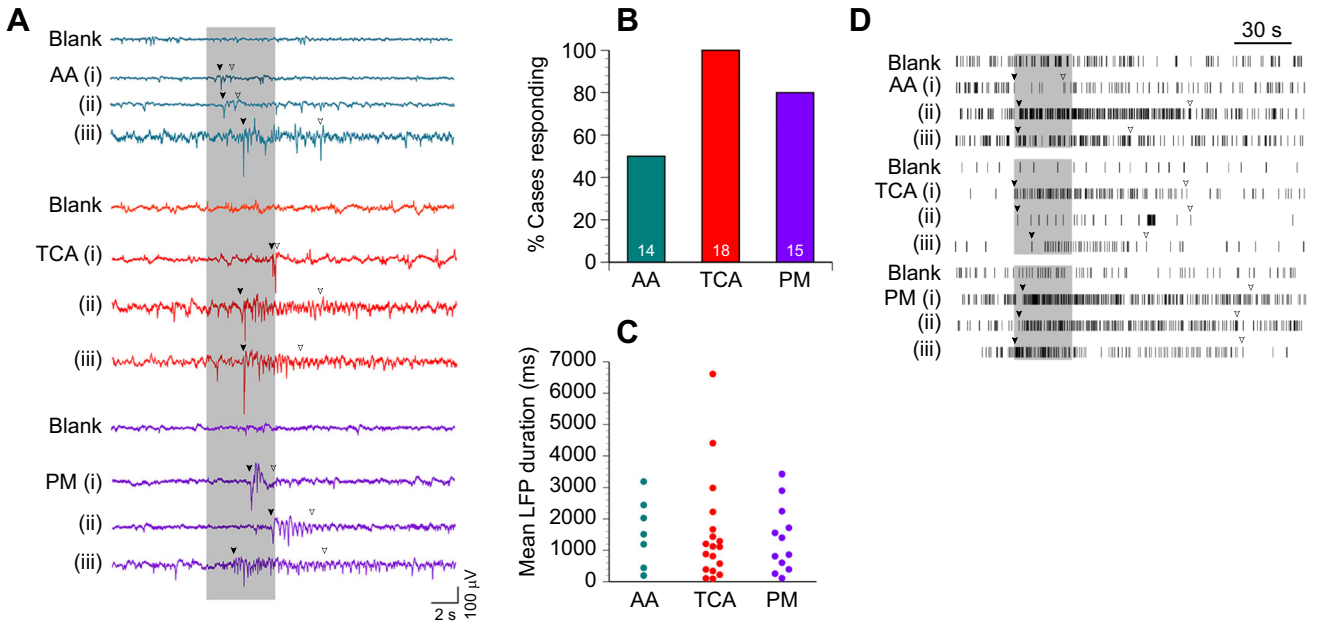

Fig. 3. The dorsal olfactory bulb region exhibits a greater proportion of responses to bile acids and pheromones. (A) Examples of local field potential recordings in response to amino acids (AA), taurocholic acid (TCA), and a pheromone mixture (PM). Shaded area indicates the 5 s odour delivery period. Representative responses from three animals (i–iii) to each odour are shown. The black triangles indicate response onset and the white triangles indicate offset, calculated as a return of signal to less than 10% deviation from the baseline mean value. (B) The percentage of local field potential cases showing responses to each of the odours tested (sample size given in the bars). (C) Mean duration of local field potential (LFP) recordings. (D) Example multi-unit recordings in response to amino acids, taurocholic acid and pheromone mixture. Shaded area indicates the 30 s odour delivery period. A representative response from three different animals is shown for each odour (i–iii). The black triangles denote response onset and the white triangles indicate the offset, when the rate returned to below 3 times the baseline standard deviation.

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 1350-1359 doi:10.1242/jeb.150466

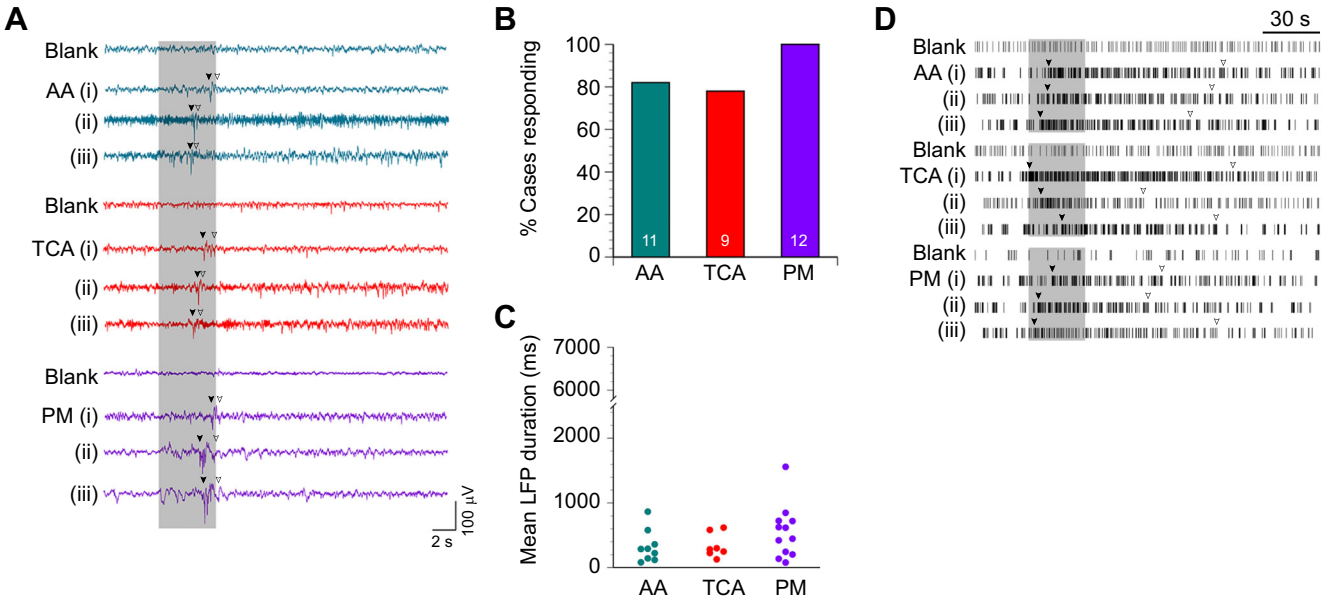

Fig. 4. The medial olfactory bulb region exhibits shorter duration responses to amino acids, bile acids and pheromones. (A) Example local field potential recordings in the medial region of the olfactory bulb in response to amino acids (AA), taurocholic acid (TCA) and a pheromone mixture (PM). The shaded area indicates the 5 s odour delivery period. Representative responses from three animals (i–iii) to each odour are shown. The black triangles indicate onset and the white triangles indicate offset, calculated as a return of signal to less than 10% deviation from the baseline mean value. (B) The percentage of cases in the medial region showing responses to each of the odours tested. (C) Mean local field potential (LFP) duration. (D) Example multi-unit recordings in response to amino acids, taurocholic acid and pheromone mixture. Shaded area indicates the 30 s odour delivery period. A representative response from three different animals is shown for each odour (i–iii). The black triangles denote response onset and the white triangles indicate offset, when the rate returned to below 3 times the baseline standard deviation.

responses to taurocholic acid were elicited in 78% of the cases examined (n=7/9) and those to the amino acid mixture were elicited in 82% of the cases (n=9/11) (Fig. 4B). The duration of local field potential recordings in the medial region ranged from 80 ms to 1550 ms across all odours tested. The average duration did not differ among responses to the amino acid mixture (mean±s.e.m. 325±80 ms, n=9), taurocholic acid (340±70 ms, n=7), and the pheromone mixture (550±120 ms, n=12) ($F_{2,25}$=1.626; P=0.2169) (Fig. 4C). Multiunit recordings from the medial region also showed excitatory responses to the amino acid mixture (74%), taurocholic acid (55%), and the pheromone mixture (27%), and the response returned to the baseline firing rate shortly after the offset of the odorant stimulus in each trace (Fig. 4D).

The duration of the local field potential responses in the medial region was significantly shorter than that in the dorsal and lateral regions, while the duration of odorant responses was similar in the dorsal and lateral regions (mean±s.e.m. medial: 425±60 ms, n=28; dorsal: 1480±225 ms, n=37; lateral: 1500±270 ms, n=15; one-way ANOVA, $F_{2,77}$=9.222, P=0.0003; data not shown). For the multi-unit recordings, the longest response was shorter in medial (103 s) than in lateral (139 s) or dorsal (120 s) recordings (Figs 2D, 3D, 4D).

The contingency table for local field potential responses in the lateral, dorsal and medial regions showed that the three regions responded to amino acids and that dorsal and medial regions both responded to pheromones and to taurocholic acid, but the lateral region did not (Table 1). Fisher's Exact Test indicated a significant difference (P<0.01) in odour response among olfactory bulb regions. *Post hoc* multiple comparisons (z-tests) showed significant differences for odorant response profiles for each olfactory bulb region (Table 1, P<0.05; Fig. 5). The lateral region responded to amino acids in 100% of cases and is the only region to respond to amino acids only. The dorsal region responded to taurocholic acid

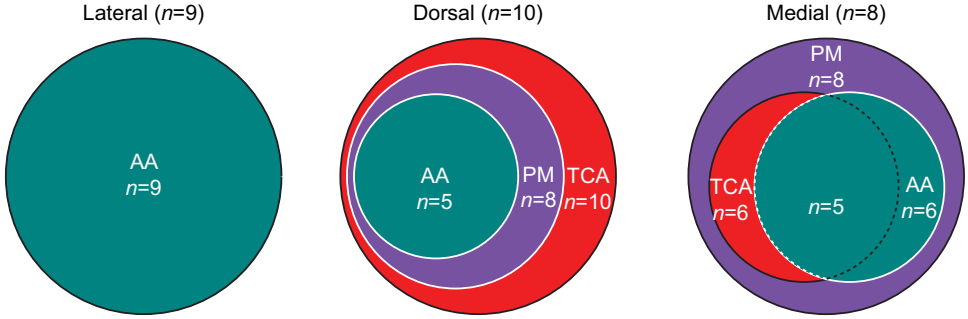

Fig. 5. Euler diagrams showing the olfactory local field potential responses to amino acids, taurocholic acid and pheromones in the lateral, dorsal and medial region of the olfactory bulb. In the lateral bulbar region, all responses were to amino acid odours (AA; green). In the dorsal region, there were 10 out of 10 (red) responses to taurocholic acid (TCA). Of these, 8 also responded to pheromones (PM; purple), and 5 responded to amino acids, taurocholic acid and pheromones (green). In the medial region, all 8 (purple) responded to pheromones; of these, 5 responded to amino acids, pheromones and taurocholic acid (green), 6 responded to pheromones and taurocholic acid (red) and 6 responded to amino acids and pheromones (green).

in 100% of cases. Of these, 80% also responded to pheromones and 50% of cases responded to taurocholic acid, pheromones and amino acids (Fig. 5). The medial region responded to pheromones in 100% of cases. Of these cases, 62.5% responded to pheromones as well as taurocholic acid and amino acids. In addition, 75% of medial cases that responded to pheromones also responded to amino acids or taurocholic acid (Fig. 5). *Post hoc* multiple comparisons (*z*-tests) on the transposed contingency table showed that there is a significant difference in olfactory bulb region for responses to amino acid only and for responses to all three odorants (amino acids, taurocholic acid, pheromones; $P<0.05$). These findings indicate that the lateral, dorsal and medial regions did not respond in the same way.

DISCUSSION

The present study investigated peripheral input and functional characteristics for three regions of the olfactory bulb in the sea lamprey. The lateral and dorsal regions received the axons of olfactory sensory neurons that were broadly distributed in the ipsilateral main olfactory epithelium and the time course of odour responses varied from short to prolonged, though the odorant tests had rapidly cleared the peripheral olfactory organ. A spatial pattern of bulbar responses to amino acid and bile acid odorants was observed. However, it is important to note that the full spectrum of odorants that stimulate the lamprey olfactory system may extend beyond amino acids and bile acids. The lateral bulbar region responded overwhelmingly to amino acid odours. From local field potential recordings, the dorsal region predominantly responded to taurocholic acid (and less often to pheromones and amino acid odours). In the medial bulbar region, the olfactory sensory input was exclusively from neurons in the accessory olfactory organ; the odour responses were short and local field potential responses were always seen to pheromones. Multi-unit recordings showed that these odour responses occurred in a small spatial scale equivalent to the action potentials of individual neurons.

We saw that olfactory sensory neurons projecting to the lateral and dorsal bulbar regions were widespread in the main olfactory epithelium. This broad distribution of specific sensory neurons within the main olfactory epithelium maximizes the likelihood of odour ligands encountering specific odorant receptors (zebrafish: Ngai et al., 1993; mammals: Ressler et al., 1993). The tall and intermediate morphology of these olfactory sensory neurons is consistent with the findings of a previous study (Laframboise et al., 2007).

The delivery of odorants in Ringer's solution may affect olfactory sensitivity, and ultimately the observed responses in the olfactory bulb. However, studies in zebrafish and mice have previously utilized similar *ex vivo* preparations in Ringer's solution in order to examine olfactory responses in the olfactory epithelium, main and accessory olfactory bulbs, and higher brain structures, with spatial organization of odour information similar to that reported *in vivo* (Friedrich and Korsching, 1997, 1998; Meeks and Holy, 2009). In this *ex vivo* preparation, the lateral region of the lamprey olfactory bulb was a locus for amino acid responses and the dorsal region of the sea lamprey olfactory bulb responded to the steroid bile acid taurocholic acid in all cases, to both taurocholic acid and the steroid pheromones in 30% of cases, and to amino acids, taurocholic acid and pheromones in 50% of cases, and was the only region with cases that responded to taurocholic only (20%). Amino acids activate the lateral olfactory bulb region in teleost fish (Hansen et al., 2003; Hara and Zhang, 1998; Fujita et al., 1991; Laberge and Hara, 2001, 2004; Friedrich and Korsching, 1998; Nikonov and Caprio, 2001) and in the larval stage of the amphibian *Xenopus laevis* (Gliem et al., 2013). However, while the olfactory system in teleosts responds to a wide variety of basic, acidic and aromatic amino acids (Hansen et al., 2003; Laberge and Hara, 2001), the lamprey olfactory system responds only to basic amino acids (Li et al., 1995). In teleosts, primarily bile acids and sex pheromones activate glomeruli in the medial region (Hansen et al., 2003; Hara et al., 1998; Fujita et al., 1991; Laberge and Hara, 2001, 2004; Friedrich and Korsching, 1998; Nikonov and Caprio, 2001). In *Xenopus*, sulphated steroids stimulate the dorsal and medial region of the main olfactory system, rather than the lateral region (Sansone et al., 2015). Amino acid responses predominated in the lateral region and a low percentage was seen in a region known as the intermediate cluster (located between the medial and lateral glomeruli), which also responded to bile acids, amines and alcohols (Gliem et al., 2013). This topography, with lateral responses to amino acids and more medial responses to bile acids and steroids, seen in teleost fish and in amphibians may have originated during agnathostome evolution and conserved during gnathostome evolution of fishes and amphibians. The longer local field potential responses to odorants in the lateral and dorsal regions indicate prolonged periods of graded potentials (probably synaptic events) compared with the medial region. Synaptic receptors, such as NMDA receptors, known to be associated with prolonged synaptic potentials are present in the olfactory bulb of lamprey (Villar-Cervino et al., 2010) and may have a role in the response duration.

As the lateral pallium and extra-pallial structures are the destinations of lateral and dorsal (non-medial) olfactory bulb projection neurons (Northcutt and Puzdrowski, 1988; Derjean et al., 2010; Green et al., 2013), odour processing in these bulbar regions may be involved in the integration and/or perception of odour cues. In mammals, vomeronasal and main olfactory epithelia also respond to similar odorants (Brennan and Zufall, 2006), but sustained firing of neurons in the accessory olfactory bulb compared with the main olfactory bulb is due to the activity of specific ion channels and has been proposed to function in the integration and perception of conspecific odours (Luo et al., 2003; Shpak et al., 2012).

In this study, we saw that the medial glomerular region received the axons of sensory neurons located exclusively in the alveolar-like recesses of the olfactory epithelium, named the accessory olfactory organ. Ren et al. (2009) showed that the medial region of the olfactory bulb received axons predominantly from olfactory sensory neurons in the accessory olfactory organ. In the current study, label application was confined to the medial glomerular region containing the dendrites and cell bodies on the medial projection neurons that extend to the posterior tuberculum (Green et al., 2013) and the retrogradely labelled sensory neurons were restricted to the accessory olfactory organ. The short, rounded shape of these sensory neurons matched the morphology reported by Ren et al. (2009), and is similar to crypt cells that project to the lateral margin of the olfactory bulb in lungfish (Gonzalez et al., 2010) and to crypt cells distributed throughout the olfactory epithelium and projecting to dorso-medial glomeruli in the zebrafish olfactory bulb (Gayoso et al., 2012; Ahuja et al., 2013; Biechl et al., 2016).

In sea lamprey, all local field potential recordings from the medial region of the olfactory bulb responded to pheromones and approximately 90% of these responded to two odorants (to pheromones and taurocholic acid or to pheromones and amino acids). Consequently, there were more cases responding to both steroids (pheromones and taurocholic acid) and to amino acids in the medial region than in the dorsal or lateral regions. While it is unknown at this time whether the accessory olfactory organ sensory neurons respond to more than one odour, this is the case for other olfactory sensory neurons. Salmon olfactory crypt cells respond

Journal of Experimental Biology

to amino acids, bile salts or gonadal extracts (Bazaes and Schmachtenberg, 2012) and mammalian olfactory sensory neurons projecting to 'necklace' glomeruli in the main olfactory bulb (Baker et al., 1999) respond to multiple odorants and may signal odorant detection rather than odorant discrimination (Greer et al., 2016).

There are neuroanatomical and physiological comparisons between the sea lamprey accessory olfactory system, the accessory olfactory system in lungfish (Gonzalez et al., 2010) and the mammalian necklace olfactory subsystem which responds to multiple odorants and may signal odour detection rather than odour discrimination (Baker et al., 1999; Munger et al., 2009; Greer et al., 2016). All three types of sensory neurons are associated with 'cul-de-sac' regions of the main olfactory epithelium. The sensory neurons of the lamprey accessory olfactory organ are located in diverticuli extending from the main olfactory epithelium (Ren et al., 2009), and the lungfish crypt cells and mammalian necklace olfactory sensory neurons are located in a similar cul-de-sac region (Gonzalez et al., 2010; Greer et al., 2016). While signal transduction via G proteins is a hallmark of olfactory sensory transduction (Ma, 2007), and is seen in the lungfish crypt cells (Gonzalez et al., 2010), mammalian necklace sensory neurons express MS4A four-pass transmembrane proteins rather than G-coupled receptor proteins (Greer et al., 2016). In the sea lamprey, immunoreactivity for the G protein G_{olf} is absent from the lamprey accessory olfactory organ, yet localizes to olfactory sensory neurons in the main olfactory epithelium (Frontini et al., 2003). The accessory sensory neurons may express other G proteins, as specific olfactory sensory morphotypes express different G proteins in teleosts (e.g. Belanger et al., 2003; Hansen et al., 2003; Ahuja et al., 2014), lungfish (Gonzalez et al., 2010) and mammals (Ma, 2007; Munger et al., 2009). It must be noted that while it is still unknown whether necklace receptor MS4A proteins are found in the lamprey olfactory system, according to Chang et al. (2013) the same odour receptors are expressed in main and accessory olfactory epithelia.

The location of soma and dendrites of medial projection neurons entirely within the medial glomerular region (Green et al., 2013) suggests neural signals processed in the medial glomerulus are not influenced directly by neural activity in the non-medial regions which contain axon terminals from the olfactory sensory neurons from the main olfactory epithelium. Moreover, projection neurons in the medial region have short dendrites and are spatially confined in a small region (Green et al., 2013). These morphological features might facilitate the synchronization of synaptic events elicited by inputs from the accessory olfactory organ. Such synchronization could lead to the short medial local field potentials that were observed in this study. Other mechanisms contributing to these short responses may be briefly activated ion channels and reduced temporal summation.

The coding of odorant information in the olfactory bulb of vertebrates is best understood in rodents and teleost fish. Briefly, in response to binding of odour receptors by a given odorant, increased activity of differing intensities can be observed across ensembles of glomeruli within a localized region of the olfactory bulb, generating a chemotopic map of combinatorial glomerular activity (Friedrich and Korsching, 1998; Johnson and Leon, 2007; Soucy et al., 2009; Kermen et al., 2013). Likewise, the main olfactory system of larval *X. laevis* is organized into medial and lateral streams. Each stream possesses groups of olfactory sensory neurons that utilize different G proteins for signal transduction, are spatially segregated within the main olfactory epithelium, and innervate different glomerular clusters (medial, intermediate, lateral) of the main olfactory bulb (Gliem et al., 2013). Together, these findings suggest that chemotopically organized, parallel odour-processing streams are conserved among vertebrates.

It appears that the lateral, dorsal and medial regions of the lamprey olfactory bulb are not redundant, but signal different information. The activity of projection neurons in functionally distinct glomeruli of the olfactory bulb in vertebrates, and the antennal lobe in insects, has been shown to initiate and/or modify specific odour-driven behaviours. In *Drosophila*, specific individual glomeruli have been shown to mediate avoidance of CO_2 and attraction to food odours (Suh et al., 2004; Semmelhack and Wang, 2009), while in zebrafish, pheromones activate a single glomerulus that may drive mating behaviours (Friedrich and Korsching, 1998; Kermen et al., 2013). In the sea lamprey, taurocholic acid (which stimulated medial and dorsal bulbar regions) may have multiple olfactory roles. In their natural environment, lampreys are likely to encounter taurocholic acid that has been excreted in teleost bile. As teleosts predate on larval and metamorphic sea lampreys (Dawson et al., 2015), taurocholic acid may signal escape behaviour during these stages. As parasitic adult sea lampreys predate on teleosts (Smith and Tibbles, 1980), taurocholic acid may stimulate behaviour associated with predation during this life stage. This phenomenon may explain why an odorant may activate multiple bulbar regions.

We saw that in the sea lamprey, the lateral region responded to amino acids and the medial and dorsal regions responded to steroids as well as to amino acids. Responses to similar odorants in different olfactory subsystems are also seen in gnathostomes. For example, in *X. laevis*, sulphated steroids are processed by sensory neurons projecting to the main olfactory system as well as by those in the accessory olfactory system (Sansone et al., 2015). In mice, the vomeronasal system (Hammen et al., 2014) and the necklace glomeruli of the main olfactory system respond to sulphated steroids (Greer et al., 2016).

The results of the current study demonstrate that the olfactory system of the sea lamprey has three olfactory pathways, and that two of these (lateral and dorsal) possess similarities to the olfactory systems of teleost fish and amphibians and may play a role in the integration of odour information with respect to responses to odour quality. Furthermore, our results support the hypothesis of a spatially distinct medial olfactory pathway that is activated through a variety of odorants. These findings indicate that olfactory subsystems are present at the base of vertebrate evolution and that the regionality in the lamprey olfactory bulb has some aspects in common with those previously described in other vertebrate and invertebrate species.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

W.W.G., H.Z., R.D. and B.S.Z. conceived and designed experiments. W.W.G. and K.B. performed experiments and analyzed data for electrophysiology studies. C.M., G.D. and F.A. performed experiments and analyzed data for anatomical tracing studies. W.L. assisted with the experimental design, provided the custom synthesized lamprey pheromones and assisted with the data analysis. All authors contributed to writing and editing the manuscript.

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