Neuroscience 177 (2011) 148–158

HIGH CONVERGENCE OF OLFACTORY AND VOMERONASAL INFLUENCE IN THE TELENCEPHALON OF THE TERRESTRIAL SALAMANDER *PLETHODON SHERMANI*

F. C. ROTH AND F. LABERGE*

Brain Research Institute, University of Bremen, D-28334 Bremen, Germany

Abstract—Previous work suggested that the telencephalic pathways of the main olfactory and vomeronasal systems of vertebrates are mostly isolated from each other, with the possible exception of convergence of the two systems into a small part of the olfactory amygdala. We tested the hypothesis of convergence between the main olfactory and vomeronasal systems by investigating the physiology of telencephalic olfactory responses in an *in vitro* brain preparation of the salamander *Plethodon shermani*. This animal was chosen because its olfactory and vomeronasal nerves can be separated and stimulated independently. The nerves were stimulated by short current pulses delivered through suction electrodes. Evoked field potentials and intracellular responses were systematically recorded in the telencephalon. The results showed an abundant overlap of olfactory and vomeronasal nerve-evoked field potentials in the ipsilateral lateral telencephalon and the amygdala. Single neurons receiving bimodal main olfactory and vomeronasal input were found in the dorsolateral telencephalon and amygdala. A classification of response latencies suggested that a subset of these neurons received direct input from both the main and accessory olfactory bulbs. Unimodal excitatory main olfactory responses were mostly found in neurons of the caudal telencephalic pole, but were also present in the striato-pallial transition area/lateral pallium region and striatum. Unimodal excitatory vomeronasal responses were found in neurons of the striato-pallial transition area, vomeronasal amygdala, and caudal amygdala. We conclude that the main olfactory and vomeronasal systems are extensively integrated within the salamander telencephalon and probably act in concert to modulate behavior.

Key words: amphibian, olfaction, vomeronasal, evoked potential, intracellular recording, *in vitro* brain preparation.

*Correspondence to: F. Laberge, Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, ON, Canada N1G 2W1. Tel: +1-519-824-4120, ext. 56238; fax: +1-519-767-1656.
E-mail address: flaberge@uoguelph.ca (F. Laberge).

Abbreviations: AC, anterior commissure; AMY, amygdala; AOB, accessory olfactory bulb; cAMY, caudal amygdala; cPole, caudal pole of the telencephalon; di, diencephalon; DP, dorsal pallium; DPAL, dorsal pallidum; DT, dorsal thalamus; H, habenula; HYP, hypothalamus; LP, lateral pallium; MOB, main olfactory bulb; MP, medial pallium; NA, nucleus accumbens; OC, optic chiasm; ON, olfactory nerve; OT, optic tectum; POA, preoptic area; rP, rostral pallium; S, septum; SCN, suprachiasmatic nucleus; SPTA, striato-pallial transition area; STR, striatum; VCP, ventral cellular prominence; VN, vomeronasal nerve; vomAMY, vomeronasal amygdala; VT, ventral thalamus; I, cranial nerve 1 (olfactory); II, cranial nerve 2 (optic).

Thanks to recent research in rodents, it has become evident that the main and accessory olfactory (or vomeronasal) systems display overlapping functional properties. For example, the sensory neurons of both olfactory systems express odorant receptors once thought restricted to the main olfactory epithelium (Lévai et al., 2006) and both systems can detect volatile chemicals (Trinh and Storm, 2003; Xu et al., 2005; Muroi et al., 2006), a function once conceived as the exclusive domain of the main olfactory system. Further, the main olfactory system is also involved in the detection of reproductive pheromones (Hudson and Distel, 1986; Dorries et al., 1997; Kelliher et al., 1998; Swann et al., 2001; Xu et al., 2005; Wang et al., 2006), a typical function of the accessory olfactory system. The vomeronasal system has also been involved in the detection of social cues in mammals (Bean, 1982; Wysocki and Lepri, 1991; Del Punta et al., 2002; Leypold et al., 2002; Stowers et al., 2002; Chamero et al., 2007; Kimchi et al., 2007). The increasing variety and complexity of signals considered as pheromones in mammals has produced great uncertainty regarding the working definition of a pheromone (see Johnston, 1998; Restrepo et al., 2004; Stowers and Marton, 2005; Baxi et al., 2006; Brennan and Zufall, 2006; Martínez-García et al., 2009). Besides its role in social and reproductive behaviors, abundant evidence implicates the accessory olfactory system in the detection of prey and predator chemosensory cues (Kirschenbaum et al., 1986; Burghardt, 1993; Alving and Kardong, 1996; Miller and Gutzke, 1999; Placyk and Graves, 2002; Ben-Shaul et al., 2010; Papes et al., 2010).

Another possibility for functional overlap between the two olfactory systems is convergence in the central nervous system. When first discovered, the projection of the accessory olfactory bulb to the medial amygdala was thought to represent a parallel route of chemosensory influence to the hypothalamus separate from the main olfactory pathway (Winans and Scalia, 1970; Scalia and Winans, 1975). Licht and Meredith (1987) demonstrated functional convergence between the two olfactory systems onto a small proportion of neurons in the hamster posteromedial cortical amygdala, but they concluded that main olfactory input in this region was mediated through secondary connections between the main olfactory and vomeronasal amygdala. Recent reports of olfactory projections using modern tracer substances in mammals suggested that axons of projection neurons of both the main and accessory olfactory bulbs target the vomeronasal amygdala directly, and possibly additional regions in the basal telencephalon (Martinez-Marcos and Halpern, 2006;

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2010.12.013

Pro-Sistiaga et al., 2007; Kang et al., 2009). A report in the leopard frog also suggested that projections of the main and accessory olfactory bulbs contact directly neurons in the cortical amygdaloid nucleus (Scalia et al., 1991). Note that the cortical amygdaloid nucleus of Scalia and collaborators corresponds to the main olfactory amygdala of Laberge and collaborators (2006) or the lateral amygdala of Moreno and González (2004).

Previous work showed widespread distribution of telencephalic olfactory responses using an *in vitro* brain preparation of the fire-bellied toad (Laberge and Roth, 2007). However, in the latter study, the olfactory and vomeronasal nerves could not be separated and were, thus, stimulated simultaneously. The present study takes advantage of the fact that the olfactory and vomeronasal nerves can be easily separated in the salamander *Plethodon shermani* and that the efferents of the main olfactory and accessory olfactory bulbs are well described in this animal (Laberge and Roth, 2005). Further, courtship pheromones applied to freely-behaving females of this species activate the vomeronasal organ and central brain regions involved in reproduction (Wirsig-Wiechmann et al., 2002a; Laberge et al., 2008). Here, we used an *in vitro* brain preparation of the latter species to test for convergence between main olfactory and vomeronasal influence in the telencephalon, and evaluated whether convergence of olfactory bulb outputs was direct or polysynaptic.

EXPERIMENTAL PROCEDURES

Animals

A total of 26 female red-legged salamanders *Plethodon shermani* were used in the present study. The animals were collected from a single locality in Macon Co., NC, USA (35°10′48″ north, 83°33′38″ west; collecting permit Dr. Lynne Houck). The animals were held by groups of 10 in 80 l terraria provided with soil bedding, several hiding covers and water. They were fed once a week with crickets. All experiments were approved by the veterinary office of the Ministry of Health of the state of Bremen, Germany. All efforts were made to minimize the number of animals used and their suffering.

Recording procedures

The experiments were carried out *in vitro* in isolated brain preparations, as described in Laberge and Roth (2007). Briefly, the animals were deeply anaesthetized with 0.5% tricaine methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA), quickly decapitated and the brain was dissected out with the intact olfactory/vomeronasal nerve bundles cut as far as possible from the brain. Using fine scissors, the proximal part of the vomeronasal nerve was separated from the brain surface and cut just before it merges alongside the early portion of the olfactory nerve. Artificial stimulation of the nerve bundles was performed using custom-built glass suction electrodes and stimulators. A 700 μs square current pulse of 0.2 mA was used for stimulation. This current value was determined by reliable production of evoked potential responses of maximal amplitude. It was chosen to insure that all fibers within the nerve bundles would be stimulated; a necessary condition to assess convergence effectively. In other words, absence of response to a nerve stimulus had to result from lack of input to a neuron not lack of activation of the sensory pathway. In order to test for the presence of bimodal olfactory responses, two suction electrodes were used simultaneously on the olfactory and vomeronasal nerve bundles on one side of the brain.

For recordings, the brain was pinned down at the bottom of a recording chamber equipped with an overlooking dissecting microscope and continuously perfused with Ringer's solution (Na^+ 129 mM, K^+ 4 mM, Ca^{2+} 2.4 mM, Mg^{2+} 1.4 mM, Cl^- 115 mM, HCO_3^- 25 mM, glucose 10 mM, bubbled with 95% O_2/5% CO_2, pH 7.3) at a flow rate of 6 ml/min and a temperature of 14–18 °C. Evoked potentials were measured using glass micropipettes filled with a solution of 2 M NaCl with the tip cut at a diameter of approximately 10 μm. Bilateral responses were systematically recorded at 15 ventral and 12 dorsal telencephalic sites that could be reliably identified across animals. Efforts were made to randomize the recording site sequences. Intracellular potentials were measured using the sharp electrode technique. Recording electrodes were made with glass micropipettes filled with a solution of 3 M potassium acetate or 2% biocytin (Sigma-Aldrich) dissolved in 0.3 M potassium chloride. The impedance of the intracellular electrodes ranged from 80–250 MΩ. A silver wire pinned on the floor of the recording chamber served as reference electrode. Electrical potential was measured with a differential electrometer (Duo 773, World Precision Instruments, Sarasota, FL, USA) connected to an A/D interface (micro 1401mkII, Cambridge Electronic Design, Cambridge, UK) and operated from a computer using the Signal 2.16 data acquisition program (CED). When searching for cells, a hyperpolarizing current of 0.2 nA was applied for 200 ms every second, while the electrode was moved dorsoventrally in small steps with the help of a hydraulic three-axis micromanipulator (model ONO-131, Narishige, Tokyo, Japan). Cell membranes were penetrated by application of a slight overcompensating current (tickling). The criteria for a valid intracellular recording included a drop of the membrane potential to at least −25 mV, which had to remain stable. Further, before nerve stimulation began, the baseline activity had to be silent following cessation of the hyperpolarizing current used to search for cells.

Biocytin labelling

Following nerve stimulation, biocytin injection was performed by iontophoresis (1 nA pulsed current for 4 min) in a subset of neurons. After injection, the brains were stored in Ringer's solution at room temperature for 4 h and at 4 °C overnight. Brains were then fixed in a solution of 2% paraformaldehyde–2% glutaraldehyde, embedded in 4.4% gelatin, and 50-μm thick transverse sections were cut using a vibrating microtome (VT1000S, Leica, Nussloch, Germany). Biocytin was visualized by means of an avidin–biotin–peroxidase complex (Vectastain standard kit, Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine (Sigma) as chromogen with heavy-metal intensification. Sections were lightly counterstained with 0.1% Cresyl Violet, dehydrated in ascending ethanol concentrations, cleared in xylene, and coverslipped with Eukitt (Kindler O. & Co., Freiburg, Germany). The photomicrographs presented were scanned with a digital camera (AxioCam HR, Carl Zeiss, Inc., Jena, Germany) and optimized for brightness and contrast using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Data analysis

For the evoked potentials experiment, five responses were obtained in each animal at each brain site at an interval of 1 min between stimulations. These evoked potentials were averaged using the software Signal 2.16 (CED) and the response latency, latency to response peak, and response amplitude were measured. Intracellular recordings were analyzed by measuring the latency to response and latency to response peak on the original recording traces using Signal. Because basal activity was absent, identification of the responses to nerve stimulation was unambiguous. Each neuron received at least two stimuli with each nerve separated by at least 10 s. Response type never changed across stimulations of the same nerve.

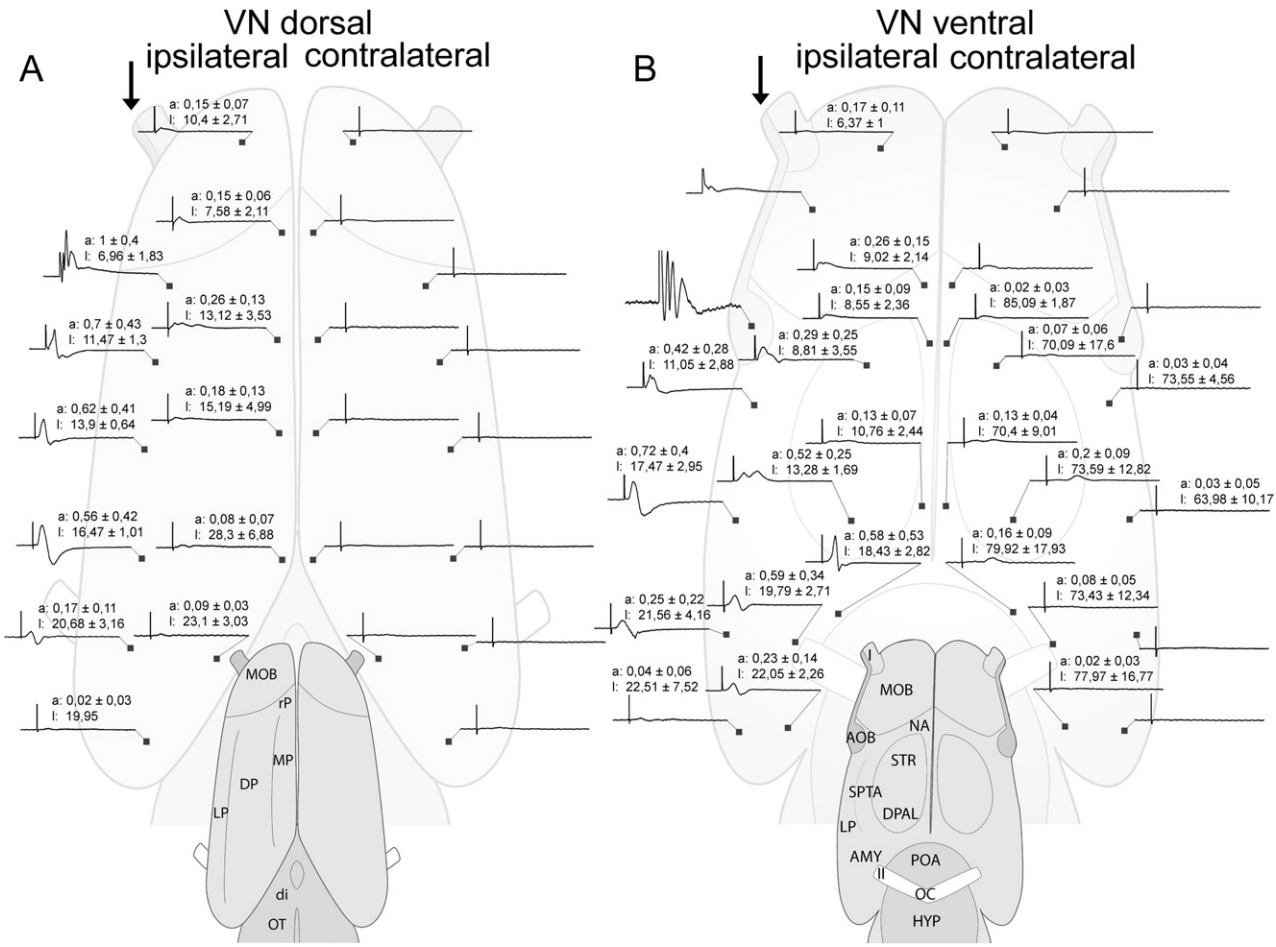

Fig. 1. Evoked field potentials in the salamander telencephalon after electrical stimulation of the vomeronasal nerve. Evoked responses are illustrated on the dorsal (A) and ventral (B) surfaces of the telencephalon. Arrows indicate the side of stimulation. Recording sites are shown as squares with a line connecting to the corresponding traces. Example traces were obtained from one preparation, while the values listed above each trace are averages of all preparations (a: amplitude in mV; l: latency to onset of response in ms; values are mean±SD; sample size=5). Time is 50 ms until the stimulation artifact, the size of which corresponds to an amplitude of 1.5 mV, except for the response in the accessory olfactory bulb where it is 3 mV. The latter trace was enlarged for clarity. This fast oscillatory response in the accessory olfactory bulb could not be measured with precision. The lower part of panels (A) and (B) have corresponding schematic drawings of the salamander telencephalon in dorsal and ventral views, respectively, that are used to define brain regions. Rostral is to the top, caudal to the bottom. Brain regions are indicated on the left and also apply to Fig. 2. See list for abbreviations.

RESULTS

Evoked potentials

The 54 recording sites surveyed in each animal are represented by squares in Figs. 1 and 2. As can be seen in Fig. 1, responses evoked by vomeronasal nerve stimulation were generally found in the lateral telencephalon on the side of stimulation and in the amygdala region in both hemispheres, although in the latter case responses were larger on the side of stimulation. Additional small responses were observed along the dorsal striato-pallidum where the ventral branch of the accessory olfactory tract courses in *P. shermani* (see Laberge and Roth, 2005). Latency of response was shortest in the accessory olfactory bulb (AOB) and increased caudally. The onset of the AOB response could not be measured because of an overlap with the stimulus artifact. Oscillatory responses were seen in the AOB, whereas simpler biphasic or single-peak responses were observed elsewhere.

Fig. 2 shows that the most important responses evoked by olfactory nerve stimulation were distributed over nearly the entire bilateral telencephalon, with the exception of the AOB and some sites contralateral to the stimulation side. Latency of response was shortest in the main olfactory bulb (MOB) and increased in the more caudal parts of the telencephalon. Overlap between the evoked response and the stimulus artifact prevented measurement of latencies in the ventral part of the MOB on the side of stimulation. On the contralateral side, latency of response increased with distance away from the habenular commissure. Many telencephalic responses that followed olfactory nerve stimulation displayed multiple peaks and long duration, as opposed to the simpler response patterns seen following stimulation of the vomeronasal nerve. Overlap in

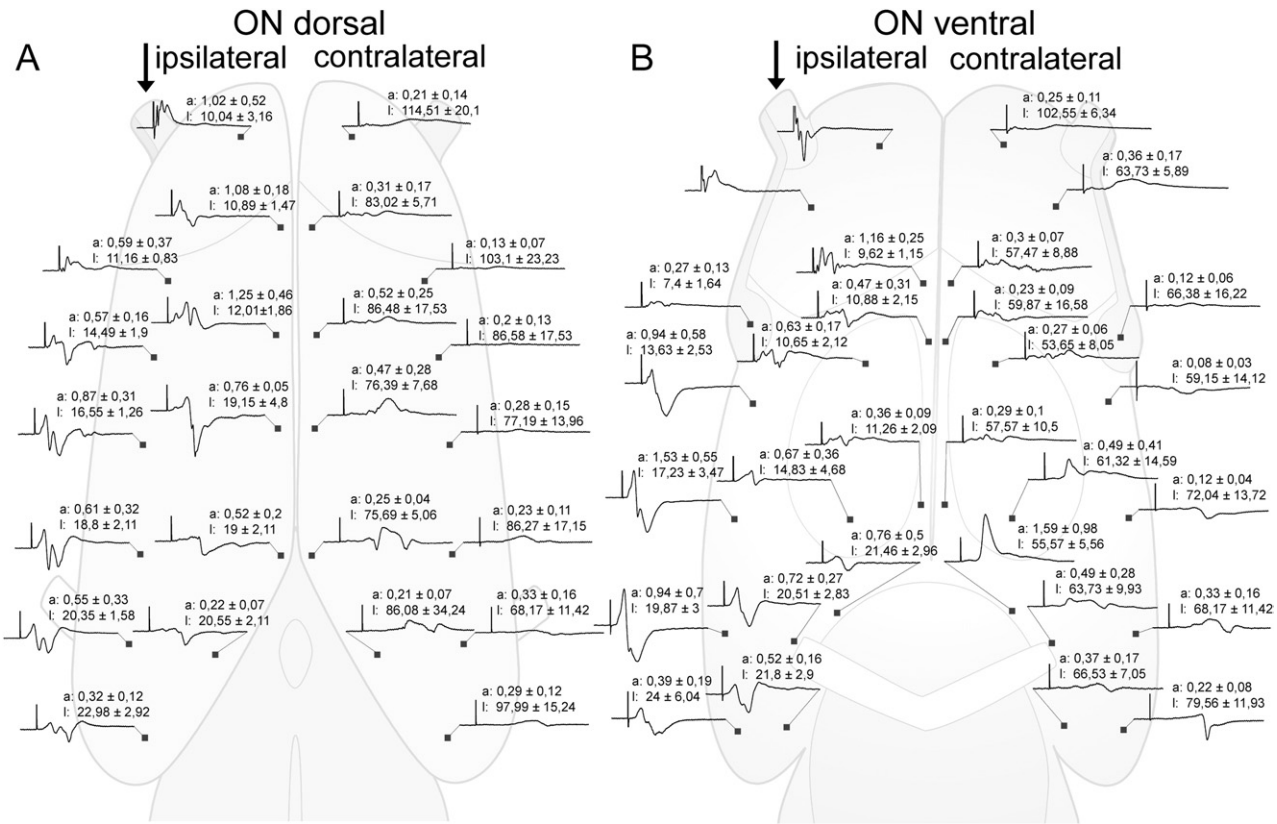

Fig. 2. Evoked field potentials in the salamander telencephalon after electrical stimulation of the olfactory nerve. Evoked responses are illustrated on the dorsal (A) and ventral (B) surfaces of the telencephalon. Arrows indicate the side of stimulation. Recording sites are shown as squares with a line connecting to the corresponding traces. Example traces were obtained from one preparation, while the values listed above each trace are averages of all preparations (a: amplitude in mV; l: latency to onset of response in ms; values are mean±SD; sample size =5). Time is 50 ms until the stimulation artifact, the size of which corresponds to an amplitude of 1.5 mV. The fast oscillatory responses in the olfactory bulb could not be measured at ventral sites. See Fig. 1 for detail of the brain regions.

responses evoked by stimulation of the two different nerves occurred along the lateral telencephalon and the caudal ventral hemisphere on the side of stimulation, where the olfactory divisions of the amygdala are found (see Laberge et al., 2006). Those regions of potential functional overlap between the two olfactory systems were investigated in more detail using intracellular recording.

Intracellular responses

Intracellular responses were investigated only on the side of stimulation using sequential stimulation of the main olfactory (ON) and vomeronasal (VN) nerves. Fig. 3 shows examples of the different response types that were obtained. Responses displaying excitation only or excitation followed by inhibition were classified as excitatory. Inhibition followed by rebound excitation was not observed. Unimodal neurons were defined as showing excitation in response to stimulation of one nerve, and inhibition or no response following stimulation of the other nerve (Fig. 3A, B). Bimodal neurons were those displaying excitatory responses following stimulation of both nerves (Fig. 3C–E).

A total of 254 neurons were analyzed for their response patterns. They are listed in Table 1 according to general localization determined by the position of the recording electrode. Table 1 also refers to an analysis of response latencies that was conducted in order to tentatively distinguish neurons that received direct input from the olfactory bulbs from those that received polysynaptic input. Fig. 4 highlights the latter analysis of response latencies. It shows the frequency of observed response latencies organized into 3 ms time bins following the time of stimulation for both VN and ON stimulation at four different rostrocaudal levels of the salamander telencephalon. VN response latency distribution showed one peak, whereas ON latency distribution appeared to show two peaks at the most rostral sites (Fig. 4A, B). Response latencies in the caudal pole of the telencephalon were generally longer than expected from conduction distance alone, possibly because of slower conduction speed of axons innervating that region. The choice of the boundary between a “short” (one synapse between MOB/AOB and neuron) and “long” (two or more synapses between MOB/AOB and neuron) latency took into account the latency of onset of the evoked field potentials shown in Figs. 1 and 2. We arbitrarily determined that a delay of 3 ms following the onset of evoked potentials in the lateral telencephalon, at the sites closest to the olfactory tracts, would comprise monosynaptic “short” intracellular responses, while latencies be-

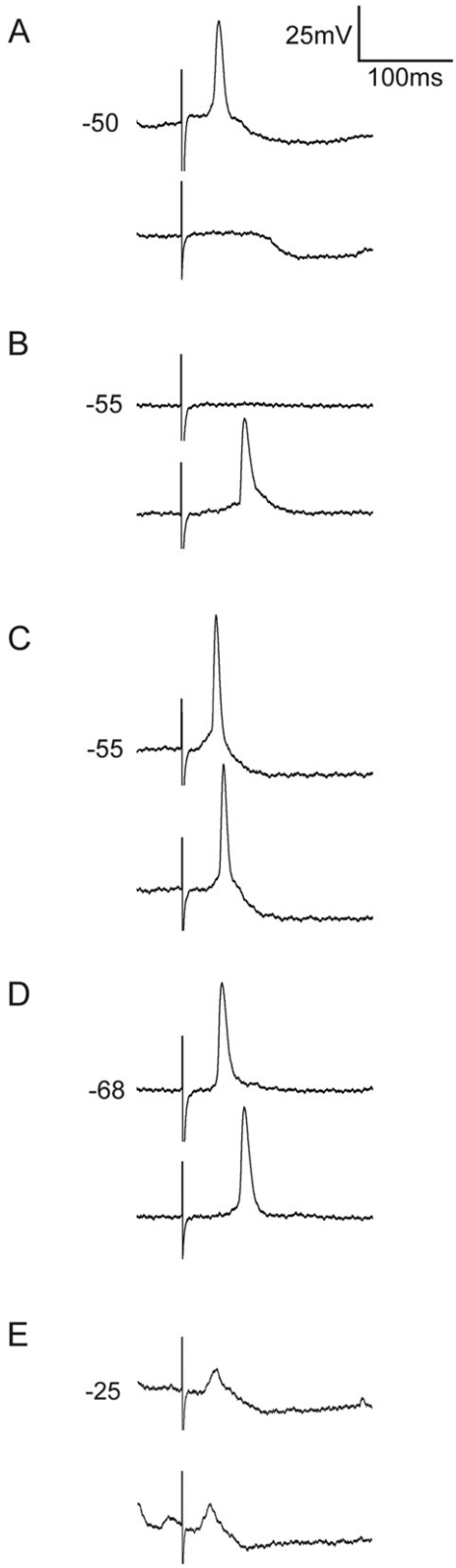

Fig. 3. Five examples of neuron responses recorded intracellularly with electrical stimulation of the vomeronasal (VN, top traces) and main olfactory (ON, lower traces) nerves. (A) Neuron in the vomeronasal amygdala displaying an excitatory VN response (latency 25.8 ms) and an inhibitory ON response (latency 83.2 ms). (B) Neuron in the caudal pole of the telencephalon displaying a unimodal excitatory ON response (latency 34.6 ms). (C) Neuron in the vomeronasal amygdala displaying bimodal excitatory responses with short latency (19.3 ms) to VN and long latency (27.0 ms) to ON stimulation. (D) Neuron in the caudal pole of the telencephalon displaying long latency excitatory responses with both VN (31.4 ms) and ON (43.4 ms) stimulation. (E) Neuron in the caudal amygdala displaying bimodal excitatory responses with long latency (27.8 ms) to VN and short latency (22.0 ms) to ON stimulation. Resting membrane potential (in mV) is shown on the left. Scale bars for amplitude and time are shown on top right.

yond that boundary would represent polysynaptic "long" responses. As can be seen in Fig. 4, the boundary between "short" and "long" responses increased with distance away from the olfactory bulbs at a speed of approximately 0.08 mm/ms. Accordingly, 29 neurons displayed "short," presumably monosynaptic responses following stimulation of both olfactory and vomeronasal nerves. There were overall 152 neurons that displayed bimodal excitatory responses, 32 that displayed unimodal vomeronasal excitatory responses, and 55 that displayed unimodal olfactory excitatory responses.

In order to refine neuron localization within the brain, intracellular injections of biocytin were performed in a subset of the recorded neurons listed in Table 1. A total of 41 neurons (or cluster of neurons) were successfully labeled using this method and a further five neurons could be successfully localized by being in the same recording track as a successfully labeled neuron. Fig. 5 illustrates the localization and response types of these neurons. Note that neurons displaying only inhibitory responses or those found outside of the brain regions listed in Table 1 were not included in the figure. Responses of the latter are described below. Biocytin-labeled bimodal neurons with short latency responses were localized in the lateral pallium, unimodal VN neurons were found in the striato-pallial transition area (SPTA) and vomeronasal amygdala, and unimodal ON neurons were concentrated in the caudal pole of the telencephalon, but some were also found in the striatum (see below). Fig. 6 shows examples of neurons labeled by intracellular injection of biocytin. Single injections often labeled clusters of neurons. The dendrites of labeled neurons typically fanned outwardly into the white matter near the cell body. However, some dendritic branches clearly reached above neighboring brain regions in most cases. There were no marked differences in dendritic morphology when compared to the previous study of Laberge and Roth (2005), and the reader is referred to the latter work for a more complete description of cytoarchitecture of the telencephalon in *P. shermani*.

Some recordings were also made in the dorsal pallium and underlying regions. However, these recordings suffered from poor localization accuracy when biocytin labeling was unsuccessful and produced a sample of neurons outside of the targeted regions identified for potential functional overlap between the two olfactory systems. Of these neurons, 10 could be successfully localized by biocytin labeling. They are not included in Table 1, but are charted in Fig. 5. Two neurons in the dorsal pallium displayed

Table 1. Types of intracellular responses in the salamander telencephalon following sequential stimulation of the vomeronasal and olfactory nerves

Response type	Brain region			
	SPTA/LP (n=89)	vomAMY/LP (n=86)	cAMY/LP (n=34)	Caudal pole (n=45)
VN(+ short)[a] − ON(+ short)	15 (−/3)[b]	8 (2/−)	5 (1/−)	1
VN(+ short) − ON(+ long)	23 (4/1)	35 (2/−)	10	—
VN(+ short) − ON(−)	11 (1/−)	4	1	—
VN(+ short)	1 (1/−)	2 (1/−)	—	—
VN(+ long) − ON(+ long)	17 (4/1)	25 (1/−)	6	2
VN(+ long) − ON(−)	4 (1/−)	4 (3/−)	1	—
VN(+ long)	—	2	2	—
VN(−) − ON(−)	6 (−/1)	—	2	2 (1)
VN(−)	1 (1/−)	—	—	—
ON(+ short) − VN(+ long)	—	1	4 (1/−)	—
ON(+ short) − VN(−)	1	—	—	3
ON(+ short)	3	—	—	1
ON(+ long) − VN(−)	5	5	—	22 (2)
ON(+ long)	1	—	—	14 (4)
ON(−)	1	—	3	—

[a] (+), excitatory response; (−), inhibitory response; "short" and "long" refer to the latencies established in Fig. 4.
[b] Numbers in parentheses refer to the number of neurons localized by biocytin labeling (n=31 in this table) or by penetration in the same recording track as a successfully labeled neuron (n=5). The first and second numbers represent the first and second brain regions listed above. Abbreviations: cAMY, caudal amygdala; caudal pole, caudal pole of the telencephalon; LP, lateral pallium; ON, olfactory nerve; SPTA, striatopallial transition area; VN, vomeronasal nerve; vomAMY, vomeronasal amygdala.

bimodal responses of long latencies; one neuron in the medial pallium displayed a unimodal main olfactory response; two neurons in the striatum displayed unimodal ON responses and one neuron in that region displayed a bimodal response with long latency; two neurons in the nucleus accumbens displayed bimodal responses with long latency and one displayed a unimodal main olfactory response; finally, a neuron localized just below the AOB displayed a bimodal response with long latency.

DISCUSSION

Electrophysiological measurements in an *in vitro* preparation of the salamander brain were used to investigate convergence between the two olfactory subsystems in the telencephalon. The results confirmed the observation of Licht and Meredith (1987) in the hamster and showed an unexpectedly high amount of convergence between the main olfactory and vomeronasal systems onto single telencephalic neurons in the salamander. Further, the present report is the first to demonstrate that the outputs of the two olfactory bulbs directly converge onto a subset of neurons in the telencephalon.

Methodological considerations

One important point to consider is whether the observed responses represent distinct influences of the olfactory and vomeronasal nerves. Evidence that the two nerves were independently stimulated can be seen by the absence of evoked potential response in the MOB upon vomeronasal nerve stimulation, and conversely, in the relatively minor AOB response seen after olfactory nerve stimulation. Responses evoked by nerve stimulation could have been influenced by concurrent stimulation of the terminal nerve, which projects widely into the telencephalon and is involved in neuromodulation (Oka and Matsushima, 1993; Mousley et al., 2006). The terminal nerve principally runs inside the ventromedial part of the olfactory nerve bundles in salamanders and thus should have been absent from the region of the vomeronasal nerve that was used for stimulation in the present study (Wirsig-Wiechmann et al., 2002b). It was not possible to separate the olfactory nerve from the terminal nerve. The latter might explain the complex field potential responses observed with stimulation of the olfactory nerve, an aspect that cannot be conclusively elucidated with the present study.

Working with an *in vitro* brain preparation proved useful because it eliminated basal activity, enabling precise measurement of the onset of telencephalic responses, as was previously observed in the toad (Laberge and Roth, 2007). The choice of a 3 ms post-evoked potential boundary for the latency analysis was arbitrary. However, this value should be considered conservative because it is based on the earliest evoked potential response at a brain site, likely reflecting the first synaptic afferents to that region. When using the present preparation, the delay between the onset of an evoked potential and its peak is typically several milliseconds. If one adds synaptic delay and the action potential refractory period, it is clear that some monosynaptic bulbar responses should have occurred beyond the 3 ms boundary. The stringent cutout latency used in this analysis was primarily aimed at demonstrating the existence of neurons that received bimodal monosynaptic input from both nerves, not to ascertain their true proportion in the telencephalon. The fact that a significant proportion of these neurons could still be detected using this criterion is strong evidence that primary convergence has an important role to play in the salamander telencephalon.

The general rostrocaudal positions used in Table 1 were easy to assess visually during the recording proce-

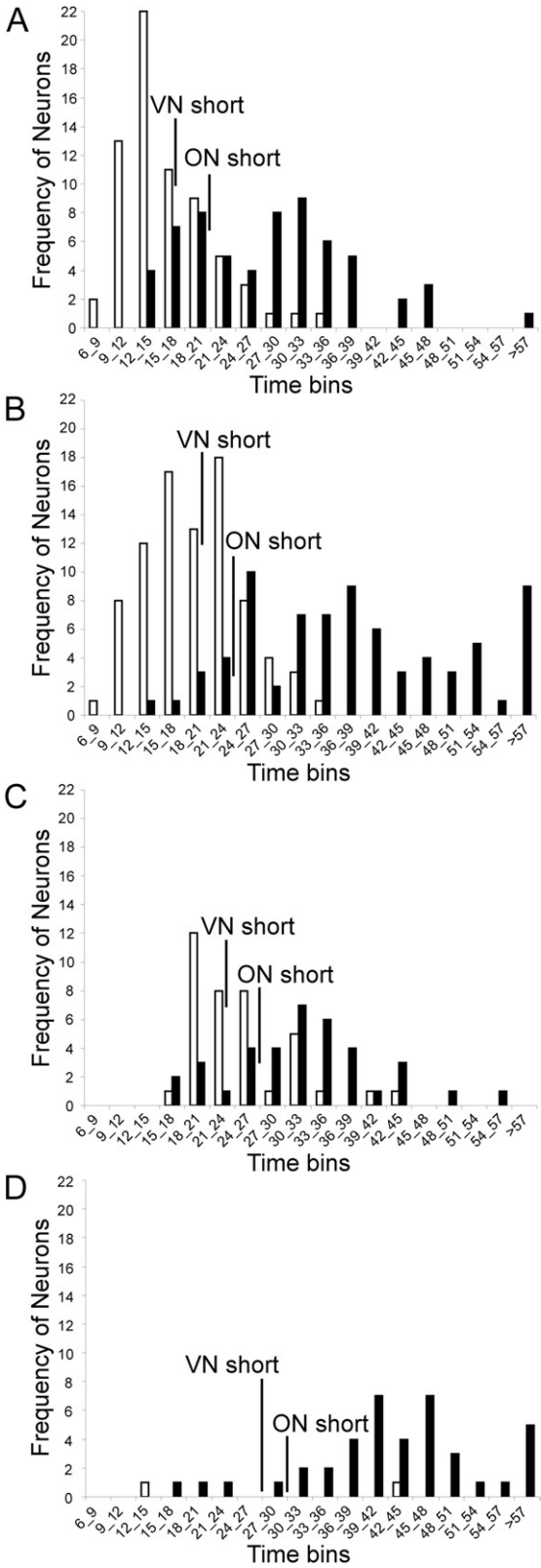

Fig. 4. Distribution of intracellular response latencies at four different rostrocaudal levels of the salamander brain. (A) Striato-pallial transition area level; (B) vomeronasal amygdala level; (C) caudal amygdala level; (D) level of the caudal pole of the telencephalon. Histograms represent the number of recorded neurons displaying latencies within the specified 3 ms time bins. All recorded neurons were included. White bars are vomeronasal nerve responses, whereas black bars are olfactory nerve responses. The chosen demarcations between short and long latencies are shown in each panel for each nerve.

dure. Despite the fact that only a small proportion of neurons were precisely localized by biocytin labeling, the position of these neurons supported the general anatomical divisions used in Table 1. In addition, it provided precise information about the localization of a subset of neurons, enabling the interpretation presented below.

Functional organization of the olfactory telencephalon in amphibians

The distribution of olfactory and vomeronasal responses matches the anatomical findings of Laberge and Roth (2005) on the telencephalic projections of the olfactory bulbs in *P. shermani*. The measured potentials correspond well to the course of the olfactory and accessory olfactory tracts on both sides of the telencephalon. Short latency responses correlate with either the presence of terminal fields or passing fibers which could have “en passant” synaptic contacts. In contrast to the widespread distribution of main olfactory responses, vomeronasal telencephalic responses are more restricted. This could point toward a more direct relay of information involved in initiation of behavioral/neuroendocrine responses in the vomeronasal pathway.

Neurons responding exclusively to vomeronasal stimulation were mainly found in the SPTA and vomeronasal amygdala. This finding supports the existence of an extended vomeronasal amygdala, as proposed by Laberge and collaborators (2006). However, as seen above, that region is not devoid of main olfactory influence, as it comprises many bimodal neurons. The caudolateral part of the amygdala was previously proposed as a possible site of the main olfactory division of the salamander amygdala (Laberge and Roth, 2005; Laberge et al., 2006). The bimodal neuron with a short latency response to stimulation of the olfactory nerve shown in Fig. 5E could represent that part of the amygdala. Because few neurons were labeled there, the identity of that region will require confirmation by additional study.

Neurons responding exclusively to main olfactory stimulation were predominantly localized in the caudal pole of the telencephalon. This is in line with the description of MOB input to the most caudal poles of the telencephalon in amphibians (Scalia et al., 1991; Laberge and Roth, 2005). Interestingly, conduction speed of projections to that region appeared slower, possibly representing a different axon type projecting to that region. The present data warrant revision of the previous notion that the lateral pallium near the lateral olfactory tract represents the main olfactory pallial region in amphibians (see Northcutt and Kicliter, 1980; Scalia et al., 1991; Bruce and Neary, 1995). The lateral pallium in *P. shermani* is not strictly a main olfactory pallium, as it displayed abundant bimodal responses. In fact, no unimodal main olfactory neuron could be precisely

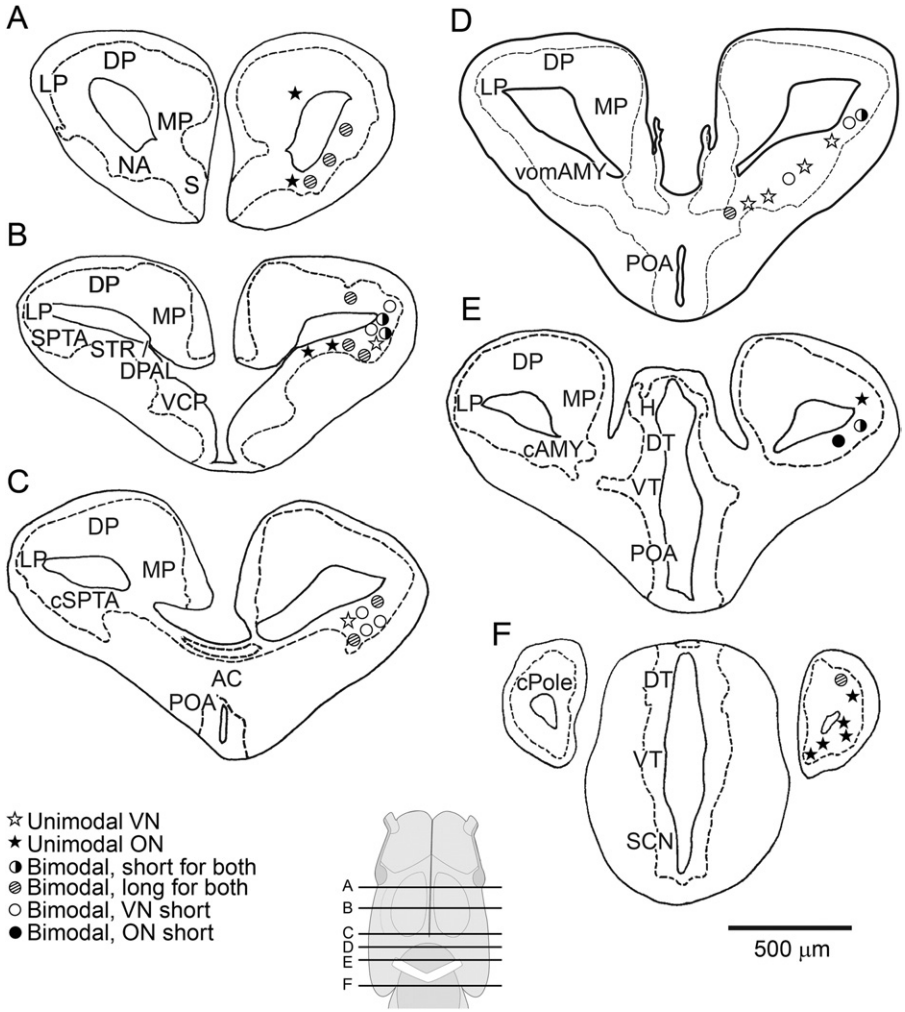

Fig. 5. Location and response type of the neurons labeled with biocytin. (A–F) Location and response type are pictured on the right side of the schematic sections, while brain regions are indicated on the left side. The symbols representing different response types are described at the bottom of the figure on the lower left, while the level of sections are illustrated on a schematic ventral view of the salamander telencephalon displayed in the middle at the bottom of the figure. In the latter, distance between sections is 250 μm, except between (C-D) and (D-E) where it is 125 μm. The position of labeled neurons was assigned to the closest rostrocaudal section pictured in this figure.

localized in that region. However, this does not mean that it is devoid of any of these neurons because a small number of unimodal main olfactory neurons were sampled nearby the lateral pallium in the present study, but none were precisely localized by biocytin labeling. The caudal poles of the telencephalon should be considered as the main olfactory pallium in amphibians, with MOB input similar to the mammalian piriform and lateral entorhinal cortices (Scalia and Winans, 1975; Martinez-Marcos and Halpern, 2006; Pro-Sistiaga et al., 2007). Further investigation of the characteristics of neurons in the caudal poles of the telencephalon is needed to evaluate its possible homologues in other vertebrates.

Convergence between the two olfactory systems

Bimodal neurons with excitatory responses to sequential stimulation of the two separate nerves represented the majority of all recorded neurons and were found in the salamander SPTA, lateral pallium and amygdala. Some of these neurons received short latency input from both nerves, which likely represented direct synaptic input from the olfactory bulbs. The dual olfactory hypothesis of Winans and Scalia (1970) and Scalia and Winans (1975) was based on the assumption that the main olfactory and vomeronasal systems provide parallel, separate routes of chemosensory influence into the hypothalamus via distinct territories in the amygdala. This assumption depends critically on the existence of separate pathways between the two olfactory subsystems in the telencephalon prior to output to the hypothalamus.

As mentioned in the Introduction, Licht and Meredith (1987) have previously demonstrated functional convergence between the two olfactory systems onto a small proportion of neurons in the hamster posteromedial cortical amygdala. The convergence they observed was thought to represent secondary (polysynaptic) influence of

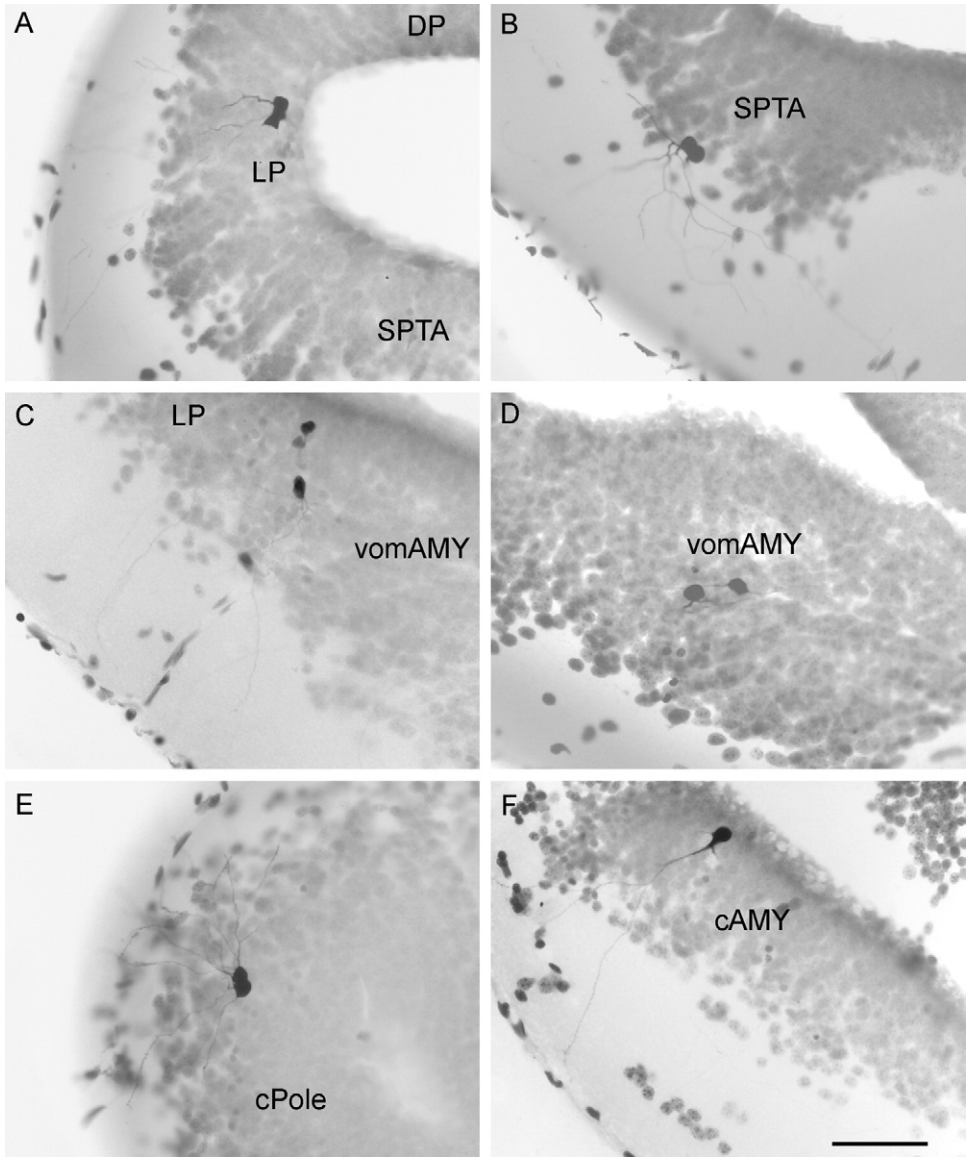

Fig. 6. Examples of neurons labeled by intracellular injection of biocytin. Labeled clusters of neurons in the lateral pallium (A), the striato-pallial transition area (B), the lateral vomeronasal amygdala (C), the central vomeronasal amygdala (D), the lateral part of the caudal pole of the telencephalon (E), and the caudal amygdala (F). Scale bar in panel (F) is 50 μm, and it applies to all micrographs in this figure.

the main olfactory system on this "vomeronasal" part of the amygdala and was proposed as the substrate of observed chemosensory influences on the circuits involved in male hamster mating behavior (Meredith, 1998). Compared to their work, a much bigger proportion of bimodal neurons could be identified in the present study. Four percent of recorded neurons displayed bimodal responses in the hamster and the proportion of non-responsive neurons was high (62%). In the present study, bimodal excitatory responses were observed in the majority of neurons in most regions (62% in SPTA/LP; 80% in vomAMY/LP; 74% in cAMY/LP). This could represent a species difference or be due to the fact that Licht and Meredith (1987) did not stimulate all olfactory fibers when using restricted MOB stimulation sites. Despite the latter concerns, the question whether high convergence between the two olfactory systems is common to all vertebrates is a valid one, especially in light of recent anatomical results showing that the telencephalic projections of mammalian olfactory bulbs are more extensive than previously thought (Martinez-Marcos and Halpern, 2006; Pro-Sistiaga et al., 2007; Kang et al., 2009).

The dual olfactory hypothesis cannot explain organization of most of the olfactory telencephalon, at least in salamander. The observation of such a high amount of convergence in the salamander telencephalon suggests a complementary role for the two olfactory subsystems. This was already proposed by Martínez-García and colleagues (2009) based on observations of reproductive behavior in rodents. However, since some unimodal neurons were

observed, one cannot exclude the possibility that labeled line processing streams are found within a small subset of vomeronasal and/or main olfactory neurons. Other hypotheses on the function of the vomeronasal and main olfactory systems have been proposed. The vomeronasal organ is present in some amphibians, reptiles and mammals. There is convincing evidence that it arose in aquatic tetrapods and therefore is not an adaptation to terrestrial life (Eisthen, 2000). Two hypotheses on the dual roles of the olfactory subsystems have been reviewed by Baxi and colleagues (2006). First, the learning hypothesis states that the vomeronasal system mediates unlearned responses to odorants, which can be associated with the main olfactory system through experience. Second, the volatility hypothesis (attributed to Halpern and Kubie, 1980 and Wysocki et al., 1980) states that the vomeronasal system mediates responses to molecules of low volatility, whereas the main olfactory system mediates responses to more volatile molecules. The latter would represent a new function in terrestrial animals because of evolution of the vomeronasal organ in the aquatic environment (Eisthen, 2000). These two hypotheses are not mutually exclusive as shown by the proposal of Martínez-García and colleagues (2009) that the vomeronasal system is involved in innate responses requiring contact between individuals and the main olfactory system is involved in learned responses toward stimuli emitted at a distance from their source, eliciting investigation. Another hypothesis presented by Dulac and Wagner (2006) proposed that the vomeronasal system mediates detection of biologically important molecules often present as blends, whereas the main olfactory system is of a more generalist nature, involved in appraising changes in the environment through fine discrimination of single molecules. The present results showing abundant convergence between the two olfactory subsystems in the salamander telencephalon evidently point toward complementary roles of the two organs, which only offer support for the learning hypothesis by presenting abundant opportunity for associations to take place between input from both systems.

CONCLUSION

The present results show abundant overlap between main olfactory and vomeronasal input onto single neurons in the salamander telencephalon. Unimodal input is however present, especially as regards main olfactory input to the caudal pole of the telencephalon. The results suggest a great variety of olfactory cell types in the SPTA, lateral pallium and amygdala region. Despite the fact that natural stimulation of the olfactory organs will almost certainly produce different neural activity patterns compared to those induced by artificial nerve stimulation, the excitatory nature of projection neurons in both olfactory bulbs (Jung et al., 1990; Mulligan et al., 2001) should result in information convergence in natural situations. Investigations of convergence using natural odors are now needed. Abundant integration of main olfactory and vomeronasal information in the brain would confirm the complementary roles of the two vertebrate olfactory systems in behavior and neuroendocrine control.

Acknowledgments—This study was supported by Deutsche Forschungsgemeinschaft LA 2383/1-1, NSF IOS 0818554, and a NSERC Discovery Grant to F. Laberge. We thank Dr. Lynne Houck for organizing the capture and shipment of salamanders.

REFERENCES

Alving WR, Kardong KV (1996) The role of the vomeronasal organ in rattlesnake (*Crotalus viridis oreganus*) predatory behavior. Brain Behav Evol 48:165–172.

Baxi KN, Dorries KM, Eisthen HL (2006) Is the vomeronasal system really specialized for detecting pheromones? Trends Neurosci 29:1–7.

Bean NJ (1982) Modulation of agonistic behavior by the dual olfactory system in male mice. Physiol Behav 29:433–437.

Ben-Shaul Y, Katz LC, Mooney R, Dulac C (2010) *In vivo* vomeronasal stimulation reveals sensory encoding of conspecific and allospecific cues by the mouse accessory olfactory bulb. Proc Natl Acad Sci U S A 107:5172–5177.

Brennan PA, Zufall F (2006) Pheromonal communication in vertebrates. Nature 444:308–315.

Bruce LL, Neary TJ (1995) The limbic system of tetrapods: a comparative analysis of cortical and amygdalar populations. Brain Behav Evol 46:224–234.

Burghardt GM (1993) The comparative imperative: genetics and ontogeny of chemoreceptive prey responses in natricine snakes. Brain Behav Evol 41:138–146.

Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, Cravatt BF, Stowers L (2007) Identification of protein pheromones that promote aggressive behaviour. Nature 450:899–902.

Del Punta K, Leinders-Zufall T, Rodriguez I, Jukam D, Wysocki CJ, Ogawa S, Zufall F, Mombaerts P (2002) Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. Nature 419:70–74.

Dorries KM, Adkins-Regan E, Halpern BP (1997) Sensitivity and behavioral responses to the pheromone androstenone are not mediated by the vomeronasal organ in domestic pigs. Brain Behav Evol 49:53–62.

Dulac C, Wagner S (2006) Genetic analysis of brain circuits underlying pheromone signaling. Annu Rev Genet 40:449–467.

Eisthen HL (2000) Presence of the vomeronasal system in aquatic salamanders. Philo Trans R Soc Lond B 355:1209–1213.

Halpern M, Kubie JL (1980) Chemical access to the vomeronasal organs of garter snakes. Physiol Behav 24:367–371.

Hudson R, Distel H (1986) Pheromonal release of suckling in rabbits does not depend on the vomeronasal organ. Physiol Behav 37: 123–128.

Johnston RE (1998) Pheromones, the vomeronasal system, and communication. From hormonal responses to individual recognition. Ann N Y Acad Sci 855:333–348.

Jung MW, Larson J, Lynch G (1990) Role of NMDA and non-NMDA receptors in synaptic transmission in rat piriform cortex. Exp Brain Res 82:451–455.

Kang N, Baum MJ, Cherry JA (2009) A direct main olfactory bulb projection to the "vomeronasal" amygdala in female mice selectively responds to volatile pheromones from males. Eur J Neurosci 29:624–634.

Kelliher KR, Chang YM, Wersinger SR, Baum MJ (1998) Sex difference and testosterone modulation of pheromone-induced neuronal Fos in the ferret's main olfactory bulb and hypothalamus. Biol Reprod 59:1454–1463.

Kimchi T, Xu J, Dulac C (2007) A functional circuit underlying male sexual behaviour in the female mouse brain. Nature 448: 1009–1014.

Kirschenbaum DM, Schulman N, Halpern M (1986) Earthworms produce a collagen-like substance detected by the garter snake vomeronasal system. Proc Natl Acad Sci U S A 83:1213–1216.

Laberge F, Roth G (2005) Connectivity and cytoarchitecture of the ventral telencephalon in the salamander *Plethodon shermani*. J Comp Neurol 482:176–200.

Laberge F, Roth G (2007) Organization of the sensory input to the telencephalon in the fire-bellied toad, *Bombina orientalis*. J Comp Neurol 502:55–74.

Laberge F, Mühlenbrock-Lenter S, Grunwald W, Roth G (2006) Evolution of the amygdala: new insights from studies in amphibians. Brain Behav Evol 67:177–187.

Laberge F, Feldhoff RC, Feldhoff PW, Houck LD (2008) Courtship pheromone-induced c-Fos-like immunolabeling in the female salamander brain. Neuroscience 151:329–339.

Lévai O, Feistel T, Breer H, Strotmann J (2006) Cells in the vomeronasal organ express odorant receptors but project to the accessory olfactory bulb. J Comp Neurol 498:476–490.

Leypold BG, Yu CR, Leinders-Zufall T, Kim MM, Zufall F, Axel R (2002) Altered sexual and social behaviors in trp2 mutant mice. Proc Natl Acad Sci U S A 99:6376–6381.

Licht G, Meredith M (1987) Convergence of main and accessory olfactory pathways onto single neurons in the hamster amygdala. Exp Brain Res 69:7–18.

Martínez-García F, Martínez-Ricós J, Augustín-Pavón C, Martínez-Hernández J, Novejarque A, Lanuza E (2009) Refining the dual olfactory hypothesis: pheromone reward and odour experience. Behav Brain Res 200:277–286.

Martinez-Marcos A, Halpern M (2006) Efferent connections of the main olfactory bulb in the opossum (*Monodelphis domestica*): a characterization of the olfactory entorhinal cortex in a marsupial. Neurosci Lett 395:51–56.

Meredith M (1998) Vomeronasal, olfactory, hormonal convergence in the brain: cooperation or coincidence? Ann N Y Acad Sci 855: 349–361.

Miller LR, Gutzke WH (1999) The role of the vomeronasal organ of crotalines (Reptilia: Serpentes: Viperidae) in predator detection. Anim Behav 58:53–57.

Moreno N, González A (2004) Localization and connectivity of the lateral amygdala in anuran amphibians. J Comp Neurol 479:130–148.

Mousley A, Polese G, Marks NJ, Eisthen HL (2006) Terminal nerve-derived neuropeptide Y modulates physiological responses in the olfactory epithelium of hungry axolotls (Ambystoma mexicanum). J Neurosci 26:7707–7717.

Mulligan SJ, Davison I, Delaney KR (2001) Mitral cell presynaptic Ca^{2+} influx and synaptic transmission in frog amygdala. Neuroscience 104:137–151.

Muroi Y, Ishii T, Komori S, Kitamura N, Nishimura M (2006) Volatile female odors activate the accessory olfactory system of male mice without physical contact. Neuroscience 141:551–558.

Northcutt RG, Kicliter E (1980) Organization of the amphibian telencephalon. In: Comparative neurology of the telencephalon (Ebbesson SOE, ed), pp 203–255. New York: Plenum.

Oka Y, Matsushima T (1993) Gonadotropin-releasing hormone (gnrh)-immunoreactive terminal nerve cells have intrinsic rhythmicity and project widely in the brain. J Neurosci 13:2161–2176.

Papes F, Logan DW, Stowers L (2010) The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. Cell 141:692–703.

Placyk JS Jr., Graves BM (2002) Prey detection by vomeronasal chemoreception in a plethodontid salamander. J Chem Ecol 28: 1017–1036.

Pro-Sistiaga P, Mohedano-Moriano A, Ubeda-Bañon I, Del Mar Arroyo-Jimenez M, Marcos P, Artacho-Pérula E, Crespo C, Insausti R, Martinez-Marcos A (2007) Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J Comp Neurol 504:346–362.

Restrepo D, Arellano J, Oliva AM, Schaefer ML, Lin W (2004) Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice. Horm Behav 46:247–256.

Scalia F, Winans SS (1975) The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. J Comp Neurol 161:31–55.

Scalia F, Gallousis G, Roca S (1991) Differential projections of the main and accessory olfactory bulb in the frog. J Comp Neurol 305:443–461.

Stowers L, Marton TF (2005) What is a pheromone? Mammalian pheromones reconsidered. Neuron 46:699–702.

Stowers L, Holy TE, Meister M, Dulac C, Koentges G (2002) Loss of sex discrimination and male-male aggression in mice deficient for TRP2. Science 295:1493–1500.

Swann J, Rahaman F, Bijak T, Fiber J (2001) The main olfactory system mediates pheromone-induced fos expression in the extended amygdala and preoptic area of the male Syrian hamster. Neuroscience 105:695–706.

Trinh K, Storm DR (2003) Vomeronasal organ detects odorants in absence of signaling through main olfactory epithelium. Nat Neurosci 6:519–525.

Wang Z, Balet Sindreu C, Li V, Nudelman A, Chan GC, Storm DR (2006) Pheromone detection in male mice depends on signaling through the type 3 adenylyl cyclase in the main olfactory epithelium. J Neurosci 26:7375–7379.

Winans SS, Scalia F (1970) Amygdaloid nucleus: new afferent input from the vomeronasal organ. Science 170:330–332.

Wirsig-Wiechmann CR, Houck LD, Feldhoff PW, Feldhoff RC (2002a) Pheromonal activation of vomeronasal neurons in plethodontid salamanders. Brain Res 952:335–344.

Wirsig-Wiechmann CR, Wiechmann AF, Eisthen HL (2002b) What defines the nervus terminalis? Neurochemical, developmental, and anatomical criteria. Prog Brain Res 141:45–58.

Wysocki CJ, Lepri JJ (1991) Consequences of removing the vomeronasal organ. J Steroid Biochem Mol Biol 39:661–669.

Wysocki CJ, Wellington JL, Beauchamp GK (1980) Access of urinary nonvolatiles to the mammalian vomeronasal organ. Science 207:781–783.

Xu F, Schaefer M, Kida I, Schafer J, Liu N, Rothman DL, Hyder F, Restrepo D, Shepherd GM (2005) Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. J Comp Neurol 489:491–500.

(Accepted 9 December 2010)
(Available online 20 December 2010)