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

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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.

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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;

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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.