Journal of Neuroscience Methods 177 (2009) 440–447

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

# An *ex vivo* preparation of the intact mouse vomeronasal organ and accessory olfactory bulb

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ARTICLE INFO

*Article history:*
Received 17 August 2008
Received in revised form 31 October 2008
Accepted 4 November 2008

*Keywords:*
Vomeronasal
Accessory olfactory bulb
*Ex vivo*
Mitral cell
Pheromone
Mouse
Inhibition
Electrophysiology

ABSTRACT

The accessory olfactory system (AOS) in mammals detects and processes information from liquid-phase environmental odorants, including pheromones. The AOS carries out tasks such as individual recognition, learning, and decision-making with relatively few stages of neural processing; it thus represents an attractive system for investigating the neural circuits that carry out these functions. Progress in understanding the AOS has long been impeded by its relative inaccessibility to standard physiological approaches. In this report, we detail a novel dissection and tissue perfusion strategy that improves access to the accessory olfactory bulb (AOB) while maintaining afferent connections from sensory neurons in the vomeronasal organ (VNO). Mitral cells demonstrated spontaneous and evoked firing patterns consistent with recent *in vivo* reports. We assayed cell degradation in the AOB tissue using Fluoro-Jade C and found that the VNO and AOB glomerular, external plexiform, and mitral cell layers showed minimal signs of degeneration for up to 6 h. Whereas histology indicated some degeneration in the deep inhibitory granule cell layer over time, electrophysiological assays demonstrated intact inhibitory function on mitral cells. Pharmacological blockade of $GABA_A$ receptors with 3 μM SR95531 (gabazine) resulted in increased evoked mitral cell activity. Furthermore, mitral cells displayed suppression of responses to preferred urine stimuli when preferred and non-preferred stimuli were mixed, an effect thought to involve functional laterally connected inhibition. These results demonstrate the utility of whole mount *ex vivo* preparations for studying sensory processing in the AOS, and suggest that similar strategies may improve experimental access to other difficult-to-study neural circuits.



## 1. Introduction

Pheromone signalling through the accessory olfactory system (AOS) in mammals guides several behaviors, including territorial aggression, mating, estrous cycling, individual recognition, and pregnancy maintenance. Despite its strong role in guiding behavior, the AOS remains one of the most poorly understood sensory modalities in mammals. The obstacles to progress in the mammalian AOS are many, key among them the anatomical inaccessibility of the vomeronasal organ (VNO) and its long, fragile connections to the first brain region in the AOS, the accessory olfactory bulb (AOB). The ability to record activity across connected AOS brain regions will be important for increasing our understanding of pheromonal sensory processing.

The study of central sensory areas separated in space from upstream neurons has typically occurred *in vivo* or in slice preparations that maintain portions of the axonal connectivity. Slice preparations have been an invaluable tool for increasing our understanding of elementary neuronal networks, but are clearly restricted by the complexity and non-planar nature of axonal and dendritic projections. Awake, behaving *in vivo* electrophysiological recordings have advantages when stimuli can be adequately controlled during self-guided behavior, but are limited when self-guided behavior interferes with stimulus control. Anesthetized *in vivo* methods can increase experimental control by removing active locomotion and behavioral variance, but introduce the caveats of systemically altered synaptic and intrinsic activity due to anesthetic actions (Richards, 1983; Krnjevic, 1992; Ishizawa, 2007). Both slice (Hayashi et al., 1993; Dudley and Moss, 1995; Jia et al., 1999) and *in vivo* (Meredith and O'Connell, 1979; Reinhardt et al., 1983; Luo et al., 2003; Zhang et al., 2007; Hendrickson et al., 2008) approaches have been used to study the AOS previously. We envisioned that an intermediate approach, one that attempts to maintain the connectivity benefits of *in vivo* studies while improving optical and electrophysiological access, would improve our capacity to study AOS processing in the AOB.

*Ex vivo* strategies have allowed neurophysiological and optical studies of mammalian circuit function to progress substantially over the past decades. Among the most well-known *ex vivo* preparations are retinal explants (Ames and Gurian, 1960). Retinal explants

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doi:10.1016/j.jneumeth.2008.11.013

and Organ of Corti preparations (Flock and Strelioff, 1984) maintain circuits housed within a contained physical structure. In the AOS, maintenance of the circuit homologous to the retina requires dissection of two neural structures connected via ∼1 cm long axonal projections that pass through the cribriform plate. *Ex vivo* preparations of somatosensory neurons and the spinal cord have maintained the primary afferents and targets in the spinal cord in the face of similar obstacles (Ritter et al., 2000; Woodbury et al., 2001). The potential for maintaining even very large structures *ex vivo* exists via perfusion of oxygenated aCSF through the vasculature (Llinás and Mühlethaler, 1988; Mühlethaler et al., 1993; Babalian et al., 1997).

In this report, we present a novel *ex vivo* strategy for studying the AOS that maintains functional VNO–AOB connectivity. We describe an acute dissection method that quickly isolates the connected VNO and AOB within a single hemisphere of the mouse skull. We constructed a tissue chamber that allows fast superfusion and local oxygenation of the perfusate to extend the electrophysiological viability of the AOB. We found that AOB mitral cell responses to ectopic and natural stimuli are similar to those observed *in vivo*. We found histological evidence, using Fluoro-Jade C staining for dead and degenerating neurons, that the VNO and the AOB glomerular and mitral cell layers were protected from degeneration up to 6 h post-dissection. We observed an increase in Fluoro-Jade C staining in the deeper AOB granule cell layer, but subsequent experiments indicated that $GABA_A$-mediated inhibition onto AOB mitral cells remained intact. We conclude that this *ex vivo* strategy maintains functional VNO–AOB connectivity, allows for precise stimulus control, and improves optical and electrophysiological access to the AOB.

## 2. Materials and methods

### 2.1. Ex vivo dissection and VNO cannulation

Male B6D2F1 mice (F1 hybrid between C57BL/6 and DBA/2 strains, Jackson Laboratories, Bar Harbor, ME) aged 8–12 weeks postnatal were anesthetized using isofluorane and decapitated. The dissection procedure is diagrammed in Fig. 1. Throughout the initial dissection, the tissue is submerged in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 125, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 25, $NaH_2PO_4$ 1.25, Na-ascorbate 0.4, Na-pyruvate 2, myo-inositol 3, glucose 25. During initial dissection an additional 7 mM $MgCl_2$ is added to the ice-cold aCSF to limit potential excitotoxicity. Following decapitation, the lower jaw is removed, and the scalp, facial skin, and orbital cavities are stripped (Fig. 1B). The brain and skull caudal to the frontal cortex is removed, leaving a small portion of the frontal cortical lobes attached to the olfactory bulbs (brain tissue shown in tan color in diagram; Fig. 1 C and D1). The soft palate is removed from the ventral surface, allowing direct visualization of the vomeronasal organs (Fig. 1 D2, red ovals on schematic diagram). Following soft palate removal, bones attached to the vomeronasal capsule are broken rostral and caudal to the contralateral VNO (gray lines in diagram, Fig. 1 D2). Subsequently, the skull is divided at the midline by advancing a straight-edge razor from the caudal surface (i.e. first through the frontal cortical lobe) just contralateral to the midline (Fig. 1 E1 and E2). At this point, the maintained hemisphere contains both VNOs (Fig. 1 E2, red ovals), bilateral septal tissue, the septal cartilage and bone, the ipsilateral olfactory bulb including the AOB (Fig. 1 E2, pink oval at right) and a small portion of the frontal cortical lobe. Depending on the angle of the straight edge razor, a small section of contralateral olfactory bulb and frontal cortical lobe may overlie the ipsilateral tissue.

After one intact hemisphere has been removed, the lateral surface of the snout is adhered to a small, flat delrin plastic platform with tissue glue (Fig. 1F). The platform is then immediately adhered inside a custom delrin plastic tissue chamber (see Fig. 2) using a small volume of silicone grease. Once inside the tissue chamber, oxygenated, room-temperature (23–25 °C) aCSF is actively perfused around the tissue at 7–8 mL/min. The final dissection procedures are especially delicate, and the additional support afforded by placement within the tissue chamber improves experimental outcome greatly. In the tissue chamber, remaining contralateral olfactory bulb and cortex is removed, followed by careful separation of the ipsilateral frontal cortex from the AOB (Fig. 1 G1 and G2). Then, the contralateral septal tissue, septal cartilage, septal bone, and contralateral VNO capsule are removed (Fig. 1 G1 and G3). At this stage, a small-diameter (0.0057 in. internal diameter) polyimide cannula attached to a pressure-driven solution-switching device (Automate Scientific, Berkeley, CA) is threaded into the VNO capsule, and pH-buffered, oxygenated Ringer's solution is perfused into the VNO lumen at a rate of 0.2–0.4 mL/min. Our superfusion strategy (see Section 2 and Fig. 2) ensured little chance of spillover of stimuli from the VNO cannula to the AOB. At average flow rates, stimulus solution is diluted at least 20-fold in the superfusing aCSF. Additionally, the high-flow rate superfusion solution enters the recording chamber very near the AOB surface and the exit channel is near the VNO, meaning any VNO stimulus dilution happens against prevailing superfusion currents. To visualize the pattern of fluid flow, we injected Chicago Sky Blue diluted in Ringer's solution into the VNO through our stimulus cannula and observed dye diffusion via the dissection microscope. This verified that the majority of injected dye was immediately flushed into the exit channel by the bulk solution flow (data not shown). Some dye became caught in eddy currents, but did not visibly accumulate near the AOB over 5 min of observation. VNO Ringer's solution contained (in mM): NaCl 115, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 25, HEPES 10, glucose 10. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. Animal protocols were approved by the Washington University Animal Studies Committee.

### 2.2. Physiology chamber construction

A custom tissue chamber for electrophysiology was constructed from black delrin polymer (McMaster-Carr, Santa Fe Springs, CA). A schematic for this chamber is shown in Fig. 2. Inlet solution was pre-heated to 35–40 ° C and pre-oxygenated (not shown). The pre-warming and pre-oxygenation step served to reduce the burden on the in-line temperature controllers and prevent outgassing inside the perfusion tubing. Perfusion solution temperature was then controlled very near the chamber inlet via parallel in-line heater elements under control of a digital temperature controller (Multichannel Systems, Reutlingen, Germany). The temperature in the chamber was monitored every hour via a digital thermometer (Omega Engineering, Stamford, CT) and adjustments made via the temperature controller as necessary. The tissue chamber is built with an initial 95% $O_2$/5% $CO_2$ bubbling chamber to maximize solution oxygenation and maintain proper pH (Fig. 2). Following a series of channels to limit transmission of fluid waves, the solution enters the main chamber containing the VNO–AOB *ex vivo* preparation. The volume of this main chamber (∼4 mL) was minimized to increase the rate of total solution exchange directly around the tissue. At 7–8 mL/min flow rates, this allows for 1–2 complete exchanges of fluid volume per minute. A suction tube is placed at the fluid surface within a suction well separated from the main chamber by a series of channels to remove spent aCSF.

In order to encourage flow of oxygenated aCSF through deep layers of the AOB, in a subset of experiments we applied suction to the lateral face of the MOB using a microsuction device (Fig. 2B). This device was constructed of multi-wall polyimide tubing (0.039–0.042 in. in diameter). The tube end was beveled and a series of nylon meshes (10–50 μm pore size, with the finest mesh