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A comparison of the effects of male pheromone priming and optogenetic inhibition of accessory olfactory bulb forebrain inputs on the sexual behavior of estrous female mice

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ABSTRACT

Previous research has shown that repeated testing with a stimulus male is required for ovariectomized, hormone-primed female mice to become sexually receptive (show maximal lordosis quotients; LQs) and that drug-induced, epigenetic enhancement of estradiol receptor function accelerated the improvement in LQs otherwise shown by estrous females with repeated testing. We asked whether pre-exposure to male pheromones ('pheromone priming') would also accelerate the improvement in LQs with repeated tests and whether optogenetic inhibition of accessory olfactory bulb (AOB) projection neurons could inhibit lordosis in sexually experienced estrous female mice. In Experiment 1, repeated priming with soiled male bedding failed to accelerate the progressive improvement in LQs shown by estrous female mice across 5 tests, although the duration of each lordosis response and females' investigation of male body parts during the first test was augmented by such priming. In Experiment 2, acute optogenetic inhibition of AOB inputs to the forebrain during freely moving behavioral tests significantly reduced LQs, suggesting that continued AOB signaling to the forebrain during mating is required for maximal lordotic responsiveness even in sexually experienced females. Our results also suggest that pheromonal stimulation, by itself, cannot substitute for the full complement of sensory stimulation received by estrous females from mounting males that normally leads to the progressive improvement in their LQs with repeated testing.

1. Introduction

Numerous studies have shown that sexually naïve ovariectomized female rats and hamsters show full-blown copulatory and paracopulatory behaviors when administered ovarian hormones and first tested with a sexually active male (Blaustein and Erskine, 2002). By contrast, sexually naïve female mice initially display very low levels of receptive sexual behavior following ovariectomy and the induction of estrus by administering estradiol followed two days later by progesterone. Repeated hormone priming and behavioral testing with a sexually experienced male is required for ovariectomized females to display maximal, high levels of sexual receptivity (Bakker et al., 2002; Kudwa and Rissman, 2003; Mani et al., 1997; Thompson and Edwards, 1971). Rissman and co-workers (Bonthuis et al., 2011) reported that systemic administration of a histone deacetylase inhibitor to ovariectomized female mice significantly enhanced the rate at which the incidence of lordosis (indexed by lordosis quotients; LQs) improved over the course of repeated hormone treatments and behavioral tests. These results point to an epigenetic mechanism whereby repeated testing with a stimulus male augments the actions of either estradiol or progesterone at respective cognate neural receptors for these two ovarian steroids. The expression of lordosis in female rodents is also sensitive to stress. Restraint stress reduces the expression of lordosis in ovariectomized female rats after treatment with ovarian hormones (White and Uphouse, 2004). Blaustein and co-workers (Laroche et al., 2009a, 2009b) reported that the progressive improvement in LQs observed in estrous female mice over several tests in adulthood was significantly decreased by prior exposure to environmental or immune stressors around the age of puberty (specifically at 6 weeks of age). These observed behavioral defects induced by pubertal stress were correlated with a significant, long-lasting reduction in the expression of estradiol receptor type alpha (ERα) in the ventromedial hypothalamic nucleus (Ismail et al., 2011), raising the possibility that the stress-induced reduction in females' receptivity resulted from an epigenetic down regulation of neural ERα expression.

In addition to the actions of ovarian hormones, male pheromones that are detected by the accessory olfactory system also play an essential role in the activation of courtship behaviors in estrous female mice

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(Haga et al., 2010; Keller et al., 2006b; Martel and Baum, 2009). High molecular weight pheromones are detected in the accessory olfactory system by sensory neurons in the murine vomeronasal organ (VNO), which send pheromonal information via projections to the accessory olfactory bulb (AOB) (Baum and Kelliher, 2009; Restrepo et al., 2004). The AOB in turn sends mitral cell projections to the medial amygdala, a major integration center for chemosensory cues (Samuelsen and Meredith, 2009), which then projects to hypothalamic regions that control reproductive behavior (Choi et al., 2005; Kevetter and Winans, 1981). Lesions placed at different points along the VNO-AOB pathway reduce the expression of lordosis as well as females' interest in the investigation of opposite sex odor cues (DiBenedictis et al., 2012; Keller et al., 2006b; Martel and Baum, 2009).

Estradiol has been shown to significantly upregulate the ability of pheromones emitted from soiled male bedding to stimulate immediate-early gene expression in sensory neurons of the VNO, with a similar non-significant trend being seen in subnuclei of the medial amygdala of female mice (Halem et al., 1999). Thus ovarian steroids and pheromonal inputs appear to play a synergistic role in forebrain sites to facilitate the expression of female courtship behaviors in mice. However, it is not known whether repeated exposure (priming) to male pheromones can duplicate the effects of drug-induced, up regulation of hypothalamic ERα expression in augmenting lordotic responses in estrous female mice. It is also not known whether inputs from the accessory olfactory system continue to be required for the expression of lordosis in sexually experienced, hormone-primed females.

In a previous study (Haga et al., 2010), 30 min pre-exposure of estrous female mice to the putative male tear pheromone, exocrine gland-secreting pepide-1 (ESP-1), significantly augmented LQs. In Experiment 1 we asked whether repeated pre-test exposure (30 min) to the pheromones emitted from soiled male bedding and urine would significantly accelerate the progressive improvement in the display of receptive (lordosis) as well as paracopulatory (investigative) behavior that occurs in ovariectomized female mice brought into estrus at 4-day intervals using estradiol followed by progesterone. In Experiment 2 we asked whether intermittent, optogenetic inhibition of activity in the AOB mitral cells would significantly inhibit the expression of lordosis otherwise seen in sexually experienced estrous females. We used a viral vector to insert the neuronal silencing opsin, ArchT (Han et al., 2011), into mitral cells of the AOB. This opsin, when activated by a narrow spectrum green laser, hyperpolarizes infected mitral cells thereby allowing us to create “reversible” lesions in the AOB to acutely block pheromone processing by the accessory olfactory system during freely moving behavioral tests.

2. Methods

2.1. Subjects

For Experiment 1, seventeen female C57BL/6J mice were purchased at 5–7 weeks of age (Charles River Laboratories, Wilmington, MA). Note that in previous studies by Blaustein et al. (reviewed in Blaustein and Ismail (2013)) mice shipped at 6 weeks of age and later tested after ovariectomy and priming with ovarian hormones showed average LQs of 30% on the last of 5 behavioral tests whereas mice shipped before 6 weeks or at 7 or more weeks of age showed average LQs of 60%. The mice used in Experiment 1 resembled this latter group from the Blaustein et al. work in so far as their maximal (test 5) LQs were 60% regardless of whether or not they were primed with male pheromones prior to each test. For Experiment 2, Protocadherin21-Cre (Pcdh21-Cre) mice were used in which expression of Cre-recombinase is restricted to mitral and tufted cells of both the main and accessory olfactory bulbs (Nagai et al., 2005). Subjects were generated in the Boston University vivarium by breeding heterozygous Pcdh21-Cre males with C57BL/6J females. Offspring were genotyped by PCR from genomic tail DNA and Cre-primers (Integrated DNA Technologies, Coralville, IA) constructed from sequences suggested by the Mutant Mouse Regional Resource Center (UC Davis, CA). Ten heterozygous female offspring and five female litter mate controls (age 8–14 weeks) were used in behavioral studies. Females were group housed (3–5 mice per cage) in both experiments. Stimulus C57BL/6J males for both experiments ($n = 14$ for Experiment 1 and $n = 10$ for Experiment 2 purchased at 5–7 weeks from Charles River Laboratories, Wilmington, MA) were individually housed after receiving sexual experience with an estrous female. Mice were maintained on a reversed 12:12 h light:dark cycle with food and water available *ad libitum*. All procedures were approved by the Boston University Charles River Campus Institutional Animal Care and Use Committee.

2.2. Surgery

Female mice from both experiments (sexually inexperienced, age 8–14 weeks) underwent bilateral ovariectomy under 2% isofluorane anesthesia and were allowed to recover for 1 week before behavioral testing (Experiment 1) or intra-cerebral virus injection (Experiment 2). Behavioral testing in Experiment 2 began three weeks after virus injection (a total of 4 weeks after bilateral ovariectomy). Subjects were given analgesic on the day of surgery and for two subsequent days (carprofen, 5 mg/kg, s.c.). During stereotaxic viral injections into the AOB (Experiment 2), mice were anaesthetized using 2% isofluorane whereupon the head was fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Injections were made using pulled glass micropipettes (Drummond Wiretrol-Precision Bores with plunger, Drummond Scientific Company, Broomall, PA,) with ~20 μm inner tip diameter using a Quintessential Stereotaxis Injector (Stoelting, Wood Dale, IL.) Adeno-associated viral (AAV) vectors were used to introduce the neuronal silencing opsin ArchT (Han et al., 2011) into the AOB. The ArchT vector, AAV8-CAG-Flex-ArchT-GFP (a gift from Dr. Ed Boyden, manufactured and distributed by the University of North Carolina Vector Core, Chapel Hill, NC), drives both ArchT and green fluorescent protein (GFP) reporter in cells that express Cre-recombinase. Pilot studies in our lab using Pcdh21-Cre mice that had previously been infected with a viral vector expressing Cre-dependent channelrhodopsin in AOB mitral cells showed that laser delivered via a single fiber implanted between the left and right AOBs induced bilateral c-fos expression in the medial amygdala (data not shown). These results indicate that laser from a single fiber is sufficient to activate opsins in infected mitral cells from both AOBs in our experimental set-up. Therefore, in the present study AOBs were injected bilaterally with 0.3 μL of the AAV-ArchT virus per site at a rate of 0.1–0.2 μL/min. Injections were made at a 40° angle off the horizontal plane at the following coordinates: 1.0 mm rostral to the inferior cerebral vein, 0.8 mm lateral to the midline and 1.8 mm below the dura. After each injection, the electrode remained in place for 10 min to allow for complete diffusion and stabilization of pressures before removal of the electrode. After virus injection, a single optical fiber (0.37 numerical aperture, 200 μm diameter, 2.0 mm in length; Doric Lenses, Quebec, Canada) was lowered through the inferior cerebral vein between the left and right AOBs. Bleeding was controlled by gentle pressure, and a ferrule attached to the optical fiber was secured to the skull using dental cement and two stainless steel screws. The surgical incision was closed with sutures, and post-operative analgesia was given as described previously. Females were group housed and behavioral experiments began three weeks after virus injection. Littermate control females were implanted with a single fiber optic ferrule similar to the experimental mice, but did not receive bilateral virus injections in the AOB.

2.3. Odor stimuli

Urine and soiled bedding were collected from sexually inexperienced stimulus males ($n = 14$) for use during olfactory priming (Experiment 1). Urine was collected using a metabolic chamber, pooled and stored in 1 mL aliquots at −80 °C. To collect soiled bedding, 2–4