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Research report

Sex differences in olfactory-induced neural activation of the amygdala

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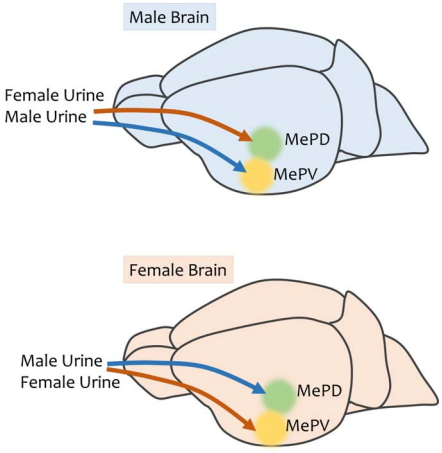
GRAPHICAL ABSTRACT



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ABSTRACT

Olfactory signals, including the scent of urine, are thought to be processed by specific brain regions, such as the medial amygdala (Me), and regulate sexual behavior in a sex-dependent manner. We aimed to reveal the sex-specific neural circuit from the accessory olfactory bulb (AOB) to Me by using a transgenic mouse. We quantified the long-lasting green fluorescent protein (GFP) expression profile, which was controlled by the c-fos promotor in a sex-dependent manner by the scent of urine. Female urine predominantly activated neurons of the posterodorsal medial amygdala (MePD) in male mice and the posteroventral medial amygdala (MePV) in female mice. Male urine, in contrast, generated the opposite pattern of activation in the Me. Secondary, the selective artificial activation of these circuits was used to examine their specific behavioral function, by using a dual Cre-loxP viral infection. AAV-hSyn-FLEX-hM3Dq-EGFP–the designer receptor exclusively activated by a designer

Abbreviations: AOB, accessory olfactory bulb; MePD, posterodorsal medial amygdala; MePV, posteroventral medial amygdala; Me, medial amygdala; VNO, vomeronasal organ; VNS, vomeronasal neural system; MeA, anterior amygdala; MOB, main olfactory bulb; AAV, adeno-associated virus; DREADD, designer receptor exclusively activated by designer drug; TTC, tetanus toxin C fragment; CNO, Clozapine-N-oxide; aCSF, artificial cerebrospinal fluid; PB, phosphate buffer; DMSO, dimethylsulfoxide; VMH, ventromedial hypothalamic nucleus; BNST, bed nucleus of the stria terminalis; MOS, main olfactory system

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drug–was infused into the AOB after infection with trans-synaptic AAV(DJ)-CMV-mCherry-2A-Cre-TTC into either the MePD or the MePV. Double virus-transfected mice were injected with hM_3Dq activator and their sexual behavior was monitored. However, selective activation of sex-dependent circuits, i.e., the AOB-MePD or AOB-MePV, did not significantly alter mounting or attack behavior in male mice. There were clear sex differences in the pheromone conveying circuits in the AOB-Me of mice. The sex-dependent functional activation of the Me, however, no effect on behavior. This suggests that a diverse number of nuclei and brain areas are likely to function in concert to successfully facilitate sexual and aggressive behaviors.

1. Introduction

Pheromones are chemical cues essential for intra-species communication, particularly with regard to sex and aggression. In mice, the vomeronasal organ (VNO), which is located beneath the nasal septum, detects chemicals that convey biologically relevant information, including sex and reproductive status. The coordination of sex-specific behaviors is highly dependent on the vomeronasal neural system (VNS) [1–3]. For example, the ablation or functional loss of the VNO impairs sexual behavior [4,5], even when sex-related odor discrimination is intact [6,7], suggesting that the VNO mediates pheromonal signals that trigger sex-specific patterns of behavioral plasticity. The accessory olfactory bulb (AOB) is the primary target of the VNO, and efferents from the AOB extend to the medial amygdala (Me) [8,9]. Therefore, some of the pheromonal signals responsible for the induction of sex behavior are conveyed to the Me via the VNS.

The Me is one of the sexually dimorphic nuclei in the brain and consists of the anterior (MeA), posterodorsal (MePD), and posteroventral (MePV) components. Neurons located in the MePD are larger and more abundant in male mice, compared to female mice [10,11]. Recent neurochemical analyses of the MePD demonstrated that expression profiles of genes encoding the GPCRs *Brs3* and *Cckar* were sexually dimorphic. These sex differences are steroid hormone dependent and control the expression of sex-specific behaviors [12,13]. In addition, neurons that express aromatase are critical for the conversion of testosterone to estradiol in the MePD, which is a process that promotes territorial and maternal aggression, in male and female mice, respectively [14]. Neural fibers in the Me project to discrete nuclei in the hypothalamus that are known to regulate sex-specific behaviors, including aggression and sex [15]. It remains to be determined if olfactory-related sensory information is uniquely decoded in the Me by each sex to facilitate adaptive behavioral responses.

The Me shares connectivity with other brain areas involved in the regulation of complex social behaviors. Retrograde labeling studies have demonstrated that the MeA, MePV, and MePD all receive afferents from the AOB and piriform cortex [9]. In addition, the MeA and MePD are directly targeted by the main olfactory bulb (MOB) [16], and the MePV/MePD receives a prominent projection from the AOB [16]. The MePV and MePD can, however, be distinguished by their respective patterns of innervation. The MePD, unlike the MePV, receives axons from the rostral zone of the AOB [16]. Different patterns of neuronal activation in the MePV and MePD, in response to exposure to male or ovariectomized female mice, have been identified using whole brain analyses in males. Signals in response to female mice were most dominant in the MePV, while those in response to male mice equally activated the MePV and MePD [17]. In addition, the differences in neural activation between the male and female brain in response to male urine was examined; male urine increased c-fos expression in the MePD in female mice but not in male mice [18]. However, the temporal dynamics of the cellular, molecular, and functional contributions of these distinct neural circuits in the Me remains unclear, particularly with regard to the expression of sexually dimorphic behaviors.

In the current study, we examined sex-dependent neural activation across subregions of the Me by a prominent sex signal, i.e., urine. Using a transgenic mouse model, we observed the long-lasting green fluorescent protein (GFP) expression in these neurons via c-fos activation, which was temporally specific and doxycycline suppressible [19]. Importantly, neurons that were selectively activated by male or female urine were measured within the same individual. The expression of a different immediate early gene of Zif268 was used to label neurons activated by the second stimulus during the suppression of additional GFP expression due to doxycycline, thus, preserving the label from the first stimulus. Using this system, we observed the separate neural activations by male and female urine, separately in the same mouse. Further, we compared male and female recipients to visualize sex-specific cells that were activated in response to urine.

In the second experiment, we tried to examine the role the sex-dependent functional connection from the AOB to Me plays in sexual/aggressive behavior in male mice. To evaluate this, we utilized adeno-associated viral vectors (AAV) carrying transgenes of Cre and lox-p-dependent designer receptor exclusively activated by a designer drug (DREADD). Using this system, we observed any behavioral effects that were increased by the activation of the connection between the AOB and Me [20]. Here, AAV-Cre was infused into the Me and allowed sufficient time to infect the presynaptic nerve ending in the MePD or MePV and be incorporated in retrograde projection neurons from the AOB. We fused tetanus toxin C fragment (TTC) to the C terminus of Cre recombinase. TTC is necessary and sufficient for retrograde transport of tetanus toxin [21] and can be utilized to transport fused proteins retrogradely [22,23]. The second virus carrying DIO-hM_3Dq-EGFP allowed the expression of this receptor under Cre. Therefore, the neurons located in the AOB, which were projecting fibers to the MePD/MePV, expressed hM3Dq. These neurons in particular were activated by the systemic administration of clozapine-N-oxide (CNO), an agonist of hM3Dq, which allowed us to examine the behavioral function of AOB neurons innervating the MePD or MePV.

2. Methods

2.1. Animals

All mice were housed under a standard 12-h light/dark cycle (lights on from 6 a.m. to 6 p.m.). The vivarium was maintained at a constant temperature (24 ± 1 °C) and level of humidity (50 ± 5%). Food and water were provided ad libitum.

The double transgenic mouse line *c-fos*-tTA, Fos-EGFP was generated by crossing *c-fos*-tTA and tetO-EGFP transgenic mice [19]. They were bred at The HAKUBI Center of Kyoto University (Sakyo-ku, Kyoto, Japan). After being transferred to the Azabu University, these male mice were crossed with wild-type C57BL/6 (B6) female mice. The genotypes of the offspring were confirmed using a polymerase chain reaction-based genotyping protocol, with DNA purified from tail clips. All pups were weaned at 28 days of age and housed in groups of the same sex until eight weeks of age. For viral infection studies, experimental B6 mice (purchased from CLEA Co. Ltd, Hamamatsu Japan, and bred in the lab) were group-housed according to their sex and age in larger cages (175 × 245 × 125 mm). Immediately after viral infections, mice were singly housed for two weeks until measurements of behavior.