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Inhibitory Neurons Respond to Social Odors in the Medial Amygdala in Male Syrian Hamsters

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ABSTRACT

Behavioral responses to social odors in Syrian hamsters are regulated by brain structures including the medial amygdala (Me) and bed nucleus of the stria terminalis (BNST). The role of these areas in regulating social behaviors has been previously studied in detail. However, the chemical phenotypes of neurons in these areas have not been well defined. Based on previous literature, we hypothesize that there is an increase in Me GABAergic signaling in response to social odors in male hamsters. To test this, we quantified colocalization of Calbindin and Fos in the Me of male hamsters after odor exposure. There were no significant differences in the percentage of Fos+ cells that were CB+ between odor conditions, but the percentage of CB+ cells that were Fos+ was higher in subjects exposed to conspecific odors versus clean odors. This implies that CB+ neurons transmit social information in the Me of male hamsters.

INDEX WORDS: Medial amygdala, Social behavior, Syrian hamsters, Fos, Calbindin, Odors, GABA, Calbindin

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INTRODUCTION

Most mammals as well as other vertebrates rely heavily on processing odor information to express appropriate social behavior. Syrian hamsters have been used as a model to study social behavior because they use conspecific odor cues to facilitate readily observable social behaviors such as sexual behavior. Their neuroanatomy has also been well studied and documented (Petrulis, 2009). In the wild, Syrian hamsters live in isolation and males rely on the perception of female odors to locate a receptive female for copulation. This behavior is regulated by a network of brain structures including the medial amygdala (Me) and bed nucleus of the stria terminalis (BNST). Olfactory sensory neurons in the nasal cavity project to the main and accessory olfactory bulbs in the brain, which then directly project onto the medial amygdala (Me) (Davis et al., 1978). The anterior (MeA) and posterior medial amygdala (MeP) are heavily interconnected, process chemosensory information and are necessary for opposite sex odor preference (P.M. Maras and Petrulis, 2010) (Maras and Petrulis, 2006) (Lehman et al., 1980). The MeA receives the majority of olfactory system projections while the steroid-sensitive MeP relays sex specific odor information to further downstream brain regions including the BNST (Been and Petrulis, 2011). Therefore, the Me is the critical initial processing area in the neural circuitry responsible for regulating appetitive behavior in the Syrian hamster.

Although the role of the Me in odor preference has been previously investigated, the neurochemical organization of the neurons in these areas has not been clearly defined. In male hamsters, neurons in the Me that project to the BNST are preferentially activated in response to opposite sex odors (Been and Petrulis, 2011). Furthermore, lesions that functionally disconnect the Me from the BNST result in a loss of volatile odor preference in male hamsters (Been and Petrulis, 2012), suggesting that these neurons are necessary for normal odor preference. However, the neurotransmitters these neurons release is unknown. Neurons communicate primarily through chemical synaptic transmission, and their communication properties are largely determined by the neurotransmitters they

release (Bear, 2006). To understand the neurobiology behind social information processing, it is essential to identify the types of neurotransmitter systems involved in neuron-to-neuron communication within the Me as well as between the Me and BNST. The current study aims to address this topic by investigating the role of GABA, the major inhibitory neurotransmitter in the brain, in the processing of sexually specific odor stimuli.

There is evidence that GABAergic neurons are preferentially activated within the Me in response to appetitive social stimuli. Neurons that express GABA have been found to be heavily distributed in the Me in rodents (Simmons and Yahr, 2003), (Stefanova, 1998), (Nitecka and Ben-Ari, 1987). GABA A type receptors are also densely distributed throughout the BNST (Araki et al., 1992), (Egli and Winder, 2003). In addition, more GABAergic neurons were activated in the Me of male rats exposed to female odors than in the Me of male rats exposed to clean odors (Pereno et al., 2010). Recently, our laboratory has found that volatile odor preference is disrupted when the GABA A type receptor antagonist, bicuculline, is injected into the BNST of male hamsters (Petrulis et al., 2012). Taken together, these data suggest that sex specific investigatory behavior in male hamsters may be facilitated by GABA signaling from the Me to the BNST. Consequently, the current study aims to investigate the role of GABA in the Me in response to relevant odors in male Syrian hamsters.

Male subjects were exposed to female odors, male odors or clean odors and the MeA and MeP were double labeled for the proteins Fos, to quantify activated neurons, and Calbindin-D28k (CB), to quantify GABAergic neurons. Fos is a protein encoded by the immediate early gene *fos* and is expressed in response to extracellular stimuli such as odors (Pfaus and Heeb, 1997), (Kovács, 2008). CB is a calcium binding protein and while its full function is unknown, studies suggest it acts as a calcium buffer and calcium transporter in neurons (Schmidt, 2012). Previous studies have shown that virtually all neurons that express CB (CB+) in the amygdala are GABAergic and that over 50% of GABAergic neurons are CB+ in the lateral nucleus, basal nucleus and accessory basal nucleus in rats,

(Kemppainen and Pitkänen, 2000), as well as in the rat basolateral amygdala (McDonald and Mascagni, 2001), (Muller et al., 2006).

Considering the discussed evidence, we hypothesize that there is a preferential increase in GABAergic activation in the MeP in response to opposite sex odors versus same sex odors and clean odors. We hypothesize that only the MeP will show this increase because previous studies have shown that the MeP transmits sex specific odor information (Been and Petrulis, 2011) while the MeA receives and processes the majority of chemosensory information (P.M. Maras and Petrulis, 2010). We predict that a higher number of neurons labeled for both Fos and CB (Fos+/CB+) are expressed in the MeP of male hamsters after exposure to female odors over male or clean odors. The results from our experiment will contribute in identifying the chemical organization and nature of neurons within the Me and will add to an increased knowledge of the neurochemical properties in this social information processing region.

RESEARCH METHODS

Animals

Adult male Syrian hamsters were purchased from Harlan laboratories (Prattville, AL, USA) and served as either experimental subjects (n=12) or odor stimulus donors (n=24). Experimental hamsters were individually housed while stimulus donors were group housed with 3 animals of the same sex per cage. All animals had access to food and water ad libitum. All hamsters were maintained in a reversed 14 light, 10 hour dark cycle with lights going out at 10:00am.

Gonadectomy and hormone implant

The Me, and BNST are known to be hormone sensitive regions that play a critical role in

mediating social and sexual behavior (Wood, 1997). Hormones have also been shown to have an effect on GABA synthesis with steroids increasing GABA synthesis in the Me of male rats (Grimes et al., 2003) and may also influence neuronal activity in these areas (Wood and Newman, 1999). Further, male hamsters experience a surge in testosterone in response to opposite sex odors but not same sex or clean odors (Petrulis, 2013). To prevent this confound effecting experimental results, the amount of testosterone in all experimental subjects was maintained at the same level. This was accomplished by bilateral gonad removal via a midline abdominal incision followed by cauterization of the ductus deferens and blood vessels. Hamsters were anesthetized during surgery under 2% isoflurane gas vaporized in 100% oxygen, which was done in a sterile environment to prevent infection. Testosterone levels were maintained by a hormone capsule subcutaneously implanted between the scapulaes immediately after gonad removal. Hormone capsules consisted of Silastic tubing (i.d. 1.57 mm, o.d. 2.41 mm; Dow Corning, Midland, MI, USA) 20mm in length and packed with powdered testosterone (Sigma, St. Louis, MO, USA). Incisions were immediately stitched with vicryl suture (size 4-0; Ethicon, Somerville, NJ, USA) and skin was closed with wound clips following stitching. Hamsters were given injections of ketoprofen (5 mg/kg Henry Schein, Melville, NY, USA) subcutaneously and 1 ml of saline to minimize post-operative pain and dehydration.

Fos Induction

Subjects were allowed to recover for at least one week after surgery before testing. On the day of testing, all experimental subjects were brought into the testing room and allowed to sit undisturbed for at least one hour. Experimental subjects were randomly assigned before testing to male, female, or clean odor stimulus exposure. Odor exposure was accomplished by placing each subject into a cage previously housing either male hamsters, female hamsters or clean bedding. Odor stimulus cages were prepared by removing group housed hamsters from their home cage as well as removal of food and

water. All stimulus cages were prepared directly before odor exposure to ensure the freshness of the stimuli. For clean odor exposures, experimental subjects were placed into a clean cage with clean bedding. During Fos induction, experimental subjects were allowed to freely explore the odor stimulus cage for 70 minutes. At the end of this time, subjects were immediately anesthetized and perfused (see below).

Histology

Animals were injected with a large dose of sodium pentobarbital (0.2 ml, 100mg/kg; Sleep Away, Ft. Dodge, IA, USA) until a deep anesthetic state had been reached, which was determined by lack of response to multiple toe pinches. Animals were then transcardially perfused with 200ml of 0.1M PBS followed by 200ml of a 4% paraformaldehyde fixative. Brains were extracted promptly and post fixed overnight in 4% paraformaldehyde at 4˚C. Brains were blocked and then cryoprotected in a 30% sucrose solution for approximately 48 hours at 4˚C. Coronal slices (35µm thick) through the Me were cut on a cryostat and stored in cryoprotectant at 20º C until immunohistochemistry procedures.

Immunohistochemistry

The MeA and MeP were identified from free floating tissue sections with the assistance of a published Syrian hamster brain atlas (Morin and Wood, 2001) and rinsed in a series of 10 five minute PBS washes. Tissue was then incubated in 0.3% hydrogen peroxide solution to reduce endogenous peroxidase activity and then washed again in 10 five minute PBS washes followed by an overnight incubation at room temperature in a polyclonal primary antibody developed against Fos, rabbit anti-Fos (Santa Cruz Biotechnology, (4): sc-52, Santa Cruz, CA, USA), at a 1:20,000 concentration diluted in 0.4% Triton-X 100 and 0.1M PBS. Primary antibody was then washed out in a series of 10 five minute PBS washes followed by an hour incubation in a goat anti-rabbit secondary antibody (Jackson

ImmunoResarch, West Grove, PA, USA) at a 1:600 concentration diluted in 0.1M PBS and 0.4% Triton-X 100. Sections were next rinsed in a series of 10 five minute PBS washes, incubated for one hour at room temperature in an avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) followed by 5 five minute PBS washes and 3 five minute washes in 0.175M sodium acetate. To initiate a color change, sections were incubated for 15 minutes in a Ni-DAB solution containing 1ml of 30% hydrogen peroxide, followed by 3 five minute washes in sodium acetate to stop the chromagen reaction. Sodium acetate was washed out with 5 five minute PBS washes. To prevent nonspecific staining of the calbindin antibody, tissue was then incubated for 1 hour in a 2% normal horse serum solution diluted in 0.1M PBS and 0.4% Triton-X 100. Tissue was incubated in the monoclonal primary mouse anti-calbindin antibody (Sigma-Aldrich, C9848, St. Louis, MO, USA) at 1:75,000 concentration in 1% normal horse serum, 0.1M PBS and 0.4% PBS for 48 hours at 4˚C. The primary antibody was rinsed out with PBS and tissue was incubated in a secondary horse anti-mouse antibody at a concentration of 1:200 in PBS and 0.4% Triton-X 100 for 2 hours followed by rinsing with PBS. Tissue was then incubated for 1 hour in an avidin-biotin complex in PBS and 0.4% Triton-X 100 for 1 hour, rinsed with PBS and then rinsed with a tris buffer 3 times for five minutes. To stain for CB, tissue was incubated in a DAB solution consisting of 1 ml 30% hydrogen peroxide for 10 minutes followed by 3 five minutes washes with tris buffer and 5 five minute washes with PBS. Tissue was finally mounted on labeled slides. After slides had dried, sections were dehydrated in a series of alcohol and xylene washes, coverslipped in Permount and stored at room temperature until further analysis.

Data Analysis

Cells that were labeled for CB (CB+), Fos (Fos+), and both CB and Fos (CB+/Fos+) were quantified within the MeA and MeP. These areas were identified with the use of a brain atlas and a

Nikon Eclipse E800 Microscope set at a 4X magnification. A QImaging digital camera was attached to the microscope and projected the slide image from the microscope onto an iMac computer screen using iVision software. Each brain section was identified and matched to the corresponding figure in the brain atlas. The camera was then positioned at 10X magnification so as to align the counting domains of the MeA and MeP (Fig 1).

CB+ cells were identified as having a brown staining in the shape and size of a neuron. Fos+ cells were identified as having a black central nuclear staining. Cells that were identified as CB+/Fos+ had a black central staining with a brown outer ring. All cells were manually counted with the researcher blind to the stimulus exposure of all animals. Densities of cells were determined by calculating the total number of CB+, Fos+, or CB+/Fos+ separately in each counting domain and dividing by the total area of the counting domain ($0.649mm^2$).

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses and significance was determined at $P<0.05$. To identify differences in the number of single or double labeled cells in the MeA and MeP across stimulus conditions (male, female, clean odors), separate one-way ANOVAs were ran followed by Tukey's-B post hoc tests.

RESULTS

Fos expression in the MeA and MeP

Fos expression differed across the three odor groups in both the MeA ($F(2,11) = 41.9$, $p<.01$) and MeP ($F(2,11) = 27.7$, $p<.01$) (Figure 6). Tukey-B's post hoc test revealed that male hamsters exposed to male or female odors had significantly more Fos+ cells in the MeA and MeP compared to males exposed to clean odors (Figure 2). However, Fos expression did not differ between males exposed to male versus female odors.

Calbindin distribution in the MeA and MeP

As expected, the densities of CB+ cells did not differ across the three odor conditions (Figure 6). The average densities of CB+ cells across the MeA and MeP are shown in figure 3. There are no differences in the distribution of CB+ cells across the MeA or the MeP.

Colocalization of Fos and CB in the MeA and MeP

Overall, there was a low number of Fos+/CB+ neurons regardless of odor exposure (Figure 6). The percentage of Fos+ cells that were also CB+ did not differ between the three odor groups in either the MeA or the MeP (Figure 4). However, the percentage of CB+ cells that were also Fos+ was greater in male hamsters exposed to male or female odors than in males exposed to clean odors in both the MeA ($F(2,11)=8.5$, $p<.01$) and MeP ($F(2,11)=48.9$, $p<.01$) (Figure 5).

DISCUSSION

There were no differences in the percentage of Fos+ cells in the MeA and MeP that were also CB+ in response to any odor condition. Our hypothesis was not supported. There are many possibilities why this could have occurred. Firstly, CB does not label all of the GABAergic neurons in the Me. Previous studies have shown that CB colocalizes with over 50% of GABAergic neurons in various sub regions in the amygdala of rodents (Kemppainen and Pitkänen, 2000), (McDonald and Mascagni, 2001). Since all GABAergic neurons were unable to be identified, it is possible that we are missing a much higher percentage of Fos+ cells that colocalize with GABA in response to conspecific odor exposure. Future studies can look to electrophysiology or in situ hybridization to quantify inhibitory neuronal activity in the MeA and MeP of male hamsters in response to socially relevant odor information.

Previous studies have quantified Fos expression in the MeA and MeP of male Syrian hamsters in response to odors (Been and Petrulis, 2011), (P.M. Maras and Petrulis, 2010), (Maras and Petrulis, 2010), (Fiber et al., 1993). Conspecific odor exposure increased Fos expression in the MeA and MeP compared to neutral odors in all these studies and we observed similar results in the current study. Also similar to our results, previous studies have found no differences in Fos expression in the MeA and

MeP in male hamsters in response to female versus male odor exposure (Been and Petrulis, 2011), (Maras and Petrulis, 2010). However, unlike our results, Fos expression has been significantly higher in response to female conspecific odor exposure than male odor exposure in the MeP of male hamsters (Pamela M Maras and Petrulis, 2010), and male mice (Samuelsen and Meredith, 2009) as well as male rats (Donato Jr. et al., 2010). The cause of these inconsistencies is not clear but may be due to variations in the counting domain positioning between researchers or the quality of the odorants used in experiments. These differences may also be attributable to early experience of the experimental subjects with female siblings. Since the subjects were sexually naïve, perhaps some male subjects, who had more experience with female odors from their sisters perinatally, respond differently to female odors than those who may have had little to no sisters.

Fos is an indirect marker of neuronal activation and may not portray all neuronal activity. There are other immediate early genes that may perhaps better quantify neuronal activation in response to odors. However, a previous study looked at both Fos and the immediate early gene EGR1 in the Me of male Syrian hamsters in response to odor exposure and found similar levels of both Fos and EGR1 suggesting immediate early genes are consistent in marking neuronal activation in response to odor stimuli (Been and Petrulis, 2011). Perhaps immediate early gene products are not characterizing the full extent of neuronal activation in the medial amygdala in response to odor stimuli (Kovács, 2008).

The percentage of CB+ neurons that were activated in response to conspecific odors increased in the MeA and MeP of male hamsters compared to male hamsters exposed to clean odors. These results suggest that CB+ neurons transmit socially relevant information. CB is implicated in short term synaptic plasticity as well as modulating neurotransmitter release due to its calcium binding properties and buffering characteristics (Schmidt, 2012). It is interesting that more inhibitory neurons of this nature are recruited and suggests an increase in inhibitory control in the Me of male hamsters in response to social information. Future studies can aim at characterizing to where these neurons project.

In summary, this study demonstrates that at least some socially relevant odor information is transmitted by CB+ neurons in the MeA and MeP. Neurons in the MeA and MeP were activated more in response to socially relevant odors versus a clean odor. Neurons in the MeA and MeP were not preferentially activated in response to opposite sex versus same sex odors in male Syrian hamsters. The percentage of activated neurons in response to socially relevant odors versus clean odors did not differ in the MeA and MeP of male hamsters. Taken together, these data suggest that subpopulations of GABAergic neurons that have the characteristic of colocalizing with the calcium binding protein, calbindin, are not preferentially activated in response to social odors. However, more CB+ neurons are recruited in response to socially relevant odors than neutral clean odors. Future studies can aim at identifying the response of the entire population of GABAergic neurons in response to sexually relevant odor information.

FIGURES

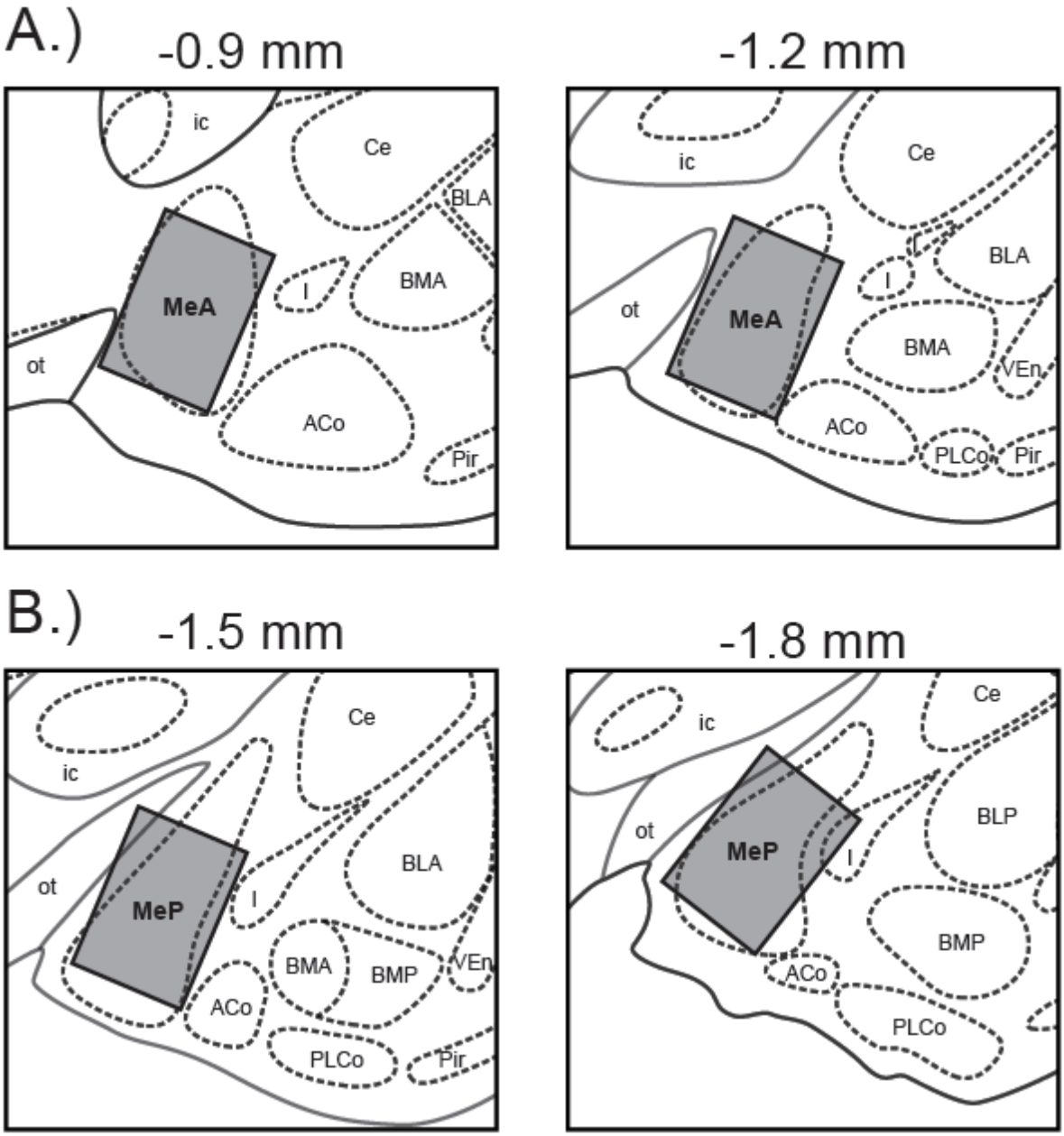

Figure 1: Gray rectangles represent the counting domains used for analysis of CB+, Fos+ and CB+/Fos+ neurons in the medial amygdala. **A.)** Cells were counted and summed within the domains of two representative sections of MeA. **B**.) Cells were counted within the domains represented by grey rectangles of two representative sections of MeP. Illustrations modified from hamster brain atlas (Morin and Wood, 2001).

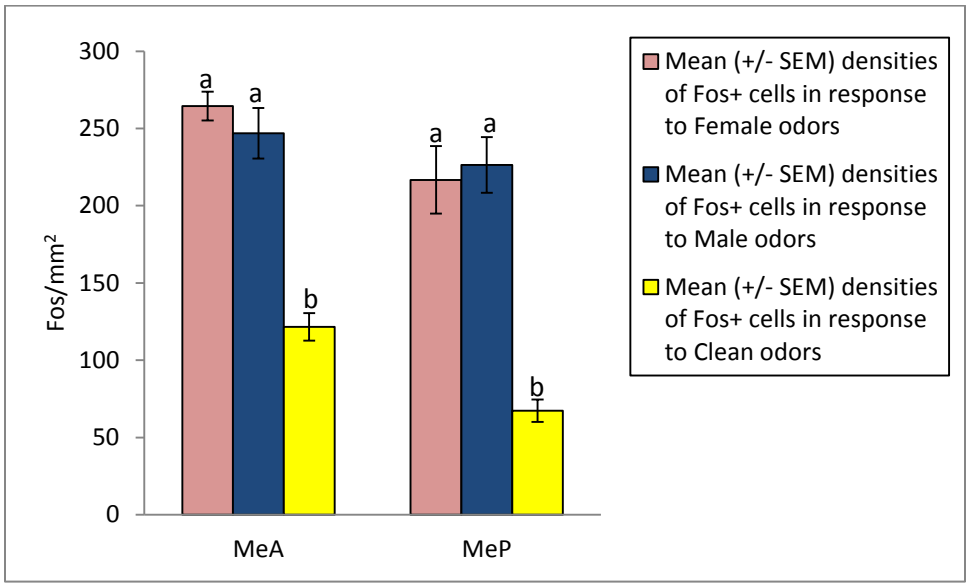

Figure 2: Average densities of Fos+ cells in the anterior (MeA) and posterior medial amygdala (MeP) in response to female (pink), male (blue), or clean (yellow) odor exposure. Different letters reflect significant differences between odor stimulus conditions within the MeA and MeP separately (Tukey's post-hoc comparisons, P<.01).

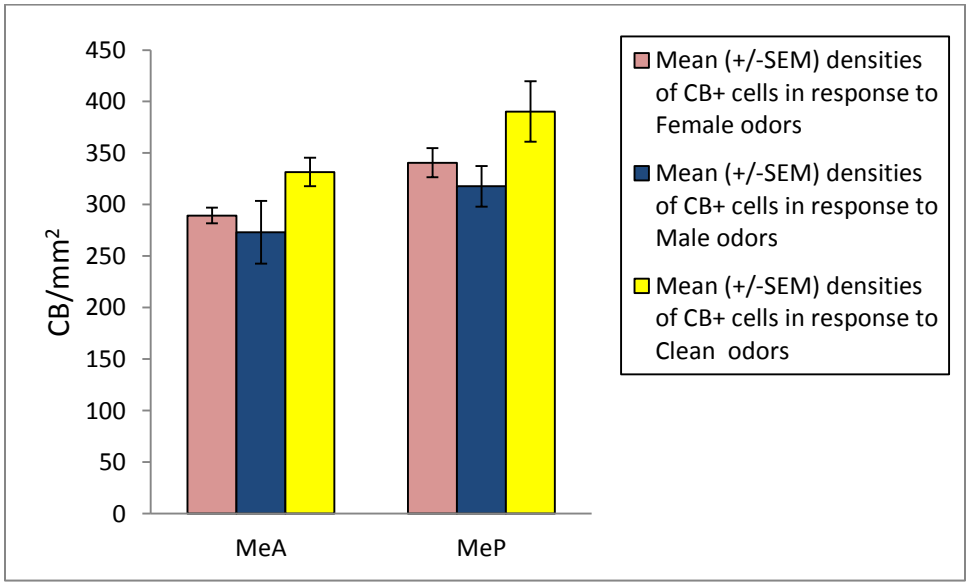

Figure 3: Average densities of CB+ cells in the anterior (MeA) and posterior medial amygdala (MeP) in response to female (pink), male (blue), or clean (yellow) odor exposure.

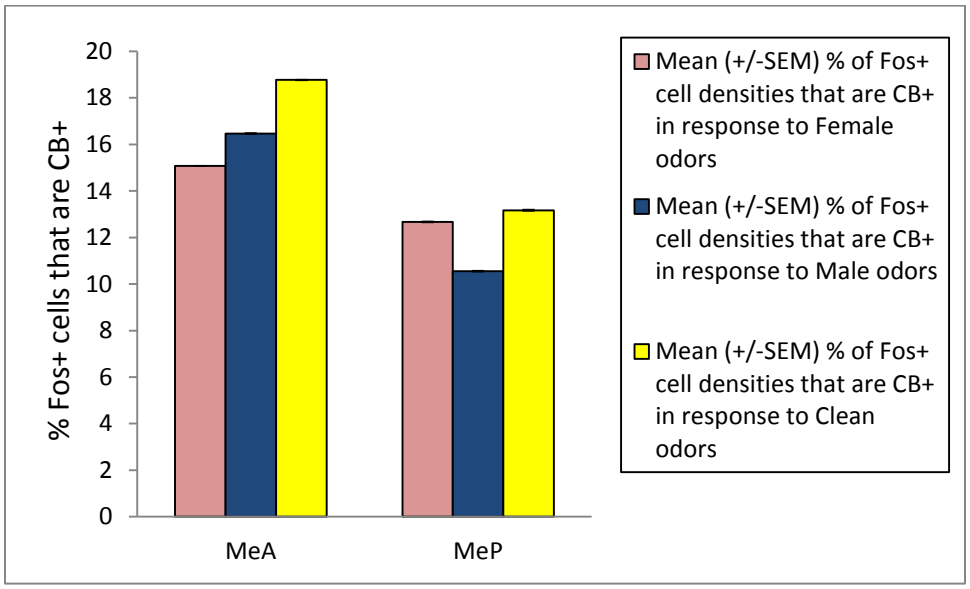

Figure 4: Average percentage of Fos+ cells that were also CB+ across odor conditions in the anterior and posterior medial amygdala.

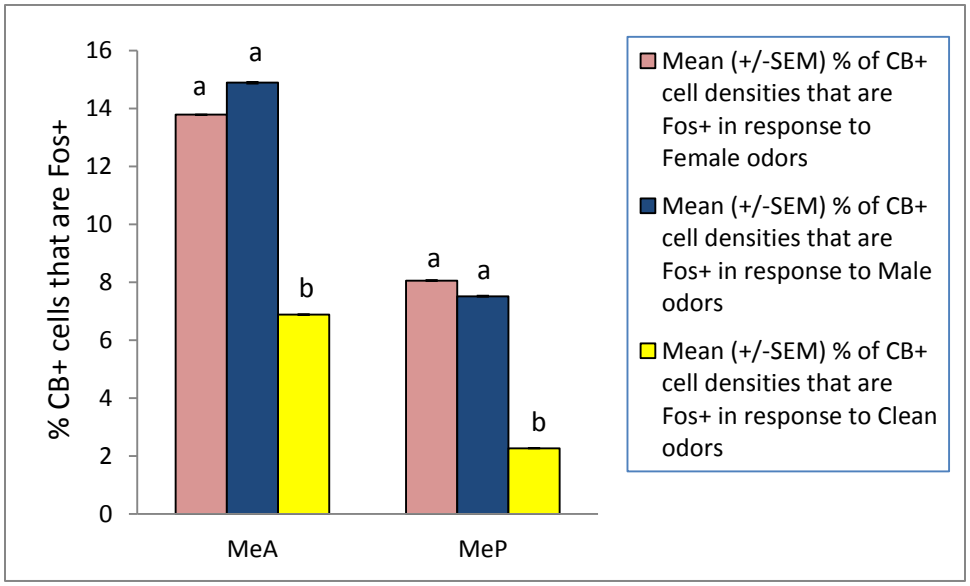

Figure 5: Average percentages of CB+ cells that are also Fos+ across odor conditions in the anterior and posterior medial amygdala. Different letters reflect significant differences between odor stimulus conditions within the MeA and MeP separately (Tukey's post-hoc comparisons, $P<.01$).

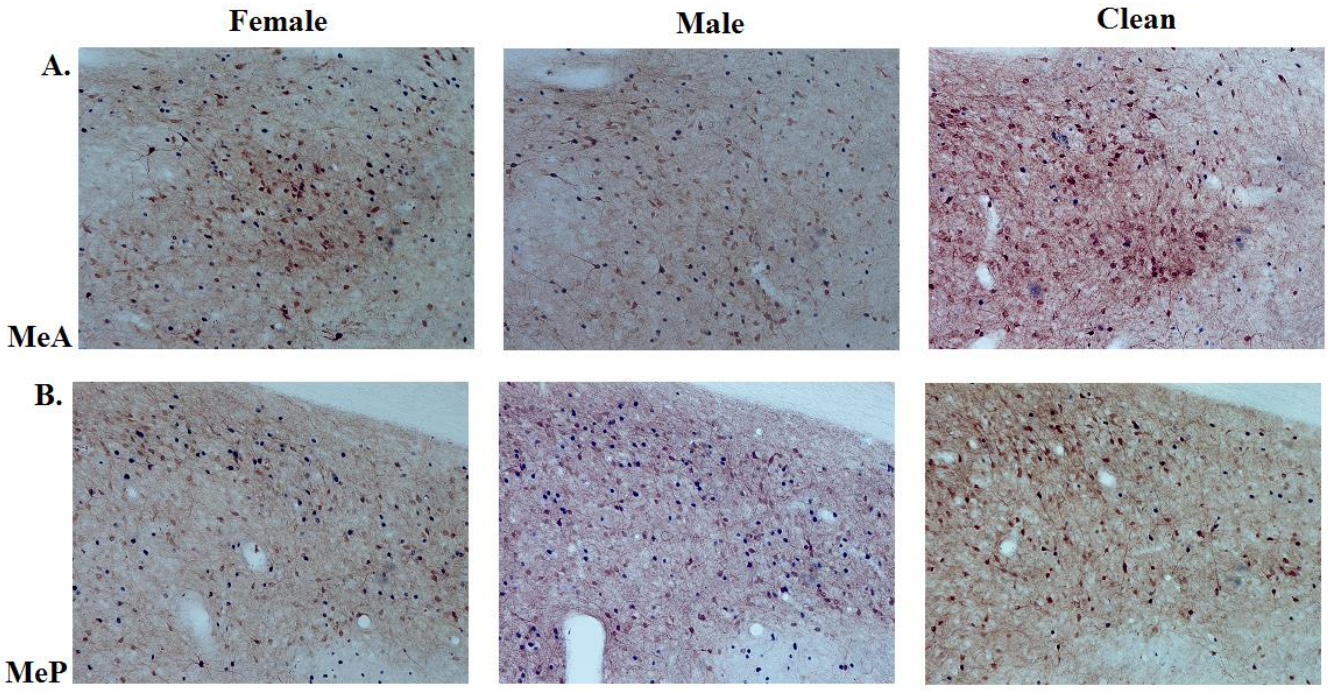

Figure 6: Photomicrographs from representative sections labeled for calbindin (CB) and Fos in the anterior medial amygdala (A.) and posterior medial amygdala (B.) of male hamsters exposed to either female, male or clean odors.

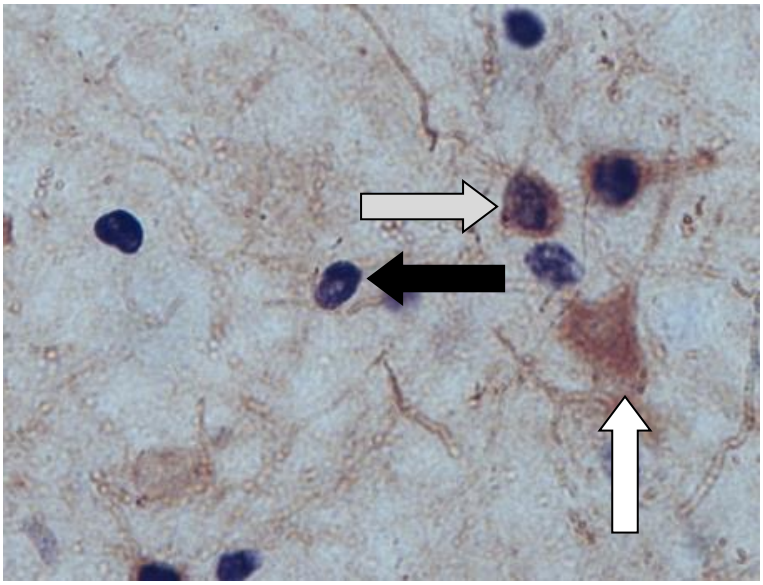

Figure 7: Higher magnification of labeled neurons in the MeA of a male hamster exposed to female odors. The black arrow is pointing to a Fos+ cell, the grey arrow is pointing to a CB+/Fos+ cell and the white arrow is pointing to a CB+ Cell.

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