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Exposure to female pheromones stimulates a specific type of neuronal population in the male but not female magnocellular division of the medial preoptic nucleus (MPN mag) of the Syrian hamster☆

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ABSTRACT

The magnocellular division of the medial preoptic area (MPN mag) integrates pheromonal and hormonal signals to play a critical role in the expression of male typical sex behavior. The MPN mag contains two morphologically distinct neuronal populations; the percentage of each type within the nucleus is sex specific. Males have more neurons with a single nucleolus whereas females have more with multiple nucleoli. To determine which neuronal subtype mediates pheromonal induction of copulation, tissue from male and female hamsters exposed to female pheromones was immunolabeled for the immediate early protein (EGR-1). Subsequently the tissue was counterstained and the number of ERG-1 neurons with one or two nuclei was determined. The results indicate that pheromones stimulate neurons with single nucleoli in males but fail to stimulate either neuronal subtype in females suggesting that synaptic input to the MPN mag is sexually differentiated.

Introduction

Most mammalian species display an array of sex specific behaviors that include aggression, parenting and mating. Indeed, mating is one of the most "sex typical" behaviors. The act of copulation is highly sex specific with males and females adopting strikingly different motor patterns. More importantly the stimuli that initiate copulation differ significantly between the sexes. In Syrian hamsters, copulation is readily induced in males following exposure to pheromones found in the vaginal secretions of their conspecifics (hamster vaginal secretions; HVS) (Murphy, 1973). Females detect the pheromones and utilize them to mark and identify territories (Petrulis and Johnston, 1997) but exposure to female pheromones does not trigger sex behavior in females suggesting that the neural pathway mediating pheromonal response is sex specific.

The neural pathway conveying pheromonal signals to central targets has been well documented in the hamster (Baum and Kelliher, 2009; Swann et al., 2003). Chemical signals from conspecifics are processed in the vomeronasal organ (VNO) and olfactory epithelium (OE) (Keller et al., 2009). Receptors from these areas project to the main and accessory olfactory bulbs, which relay signals to the medial amygdala (Me) and the bed nucleus of the stria terminalis (BST) (Lehman and Winans, 1982). Both the BST and Me send projections to the preoptic area (Maragos et al., 1989). Sex differences in size and neuronal number have been identified in several of these structures including in the olfactory bulbs (Miranda et al., 2000; Segovia et al., 2006), amygdala (Guillamon and Segovia, 1997) and preoptic area (Govek et al., 2003; Richendrfer and Swann, 2010). Interestingly, exposure to pheromones stimulates neurons in the olfactory bulbs, amygdala and BST of both sexes of the hamster (Fiber and Swann, 1996). But only the neurons in the preoptic area show sex specific stimulation as discussed below.

The preoptic area is a likely site for integration of sensory input and hormones (Swann et al., 2003). Results from our lab and others suggest that the magnocellular division of the medial preoptic area (MPN mag) plays a critical role in the expression of male sex behavior in the hamster. For example, lesions that include the MPN mag have

Abbreviations: IIIv, 3rd ventricle; BST, Bed nucleus of the stria terminalis; BSTpm, Bed nucleus of the stria terminalis posterior medial subdivision; BSTpi, Bed nucleus of the stria terminalis posterior intermediate subdivision; f, Fornix; HVS, Hamster vaginal secretions; MPN mag, Magnocellular division of the medial preoptic nucleus; MPOA, Medial preoptic area; Me, Medial nucleus of the amygdala; MeA, Medial nucleus of the amygdala anterior subdivision; MeP, Medial nucleus of the amygdala posterior subdivision; ot, Optic tract; sm, Stria medullaris.

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been shown to eliminate male sex behavior in naïve animals (Powers et al., 1987) (although more recent evidence suggests that other areas may be involved in more experienced males) (Been and Petrulis, 2010). Exposure to female pheromones stimulates neurons in the MPN mag but only in the presence of testosterone (Fiber and Swann, 1996). More intriguingly, pheromones fail to stimulate neurons in the MPN mag in females, including those treated with testosterone as adults. These results are consistent with the finding that males have more neurons in the MPN mag (Govek and Swann, 2007; Govek et al., 2003) but are confounded by a more recent study describing an additional, morphologically distinct, neuronal type (Richendrfer and Swann, 2010). The second type of cell, neurons that display multiple nucleoli, is more abundant in the female MPN mag. When this additional neuronal type is included in the analysis the sex difference in neuron number is eliminated. The role of these two populations in the regulation of sex behavior remains to be determined.

The goal of the present study is to reexamine sex differences in pheromonal stimulation of the MPN mag that would aid in the delineation of neuronal subtypes. The current investigation utilized the expression of immediate early gene protein, EGR-1, as a marker for neuronal stimulation to evaluate this hypothesis. Previous studies in our lab have examined pheromonal stimulation of the chemosensory pathway by immunolabeling for the immediate early protein from the c-fos gene (Fiber et al., 1993). The studies in this paper utilized an alternative immediate early gene product EGR-1. Like fos the protein is translated immediately after the gene is turned on through mechanical or chemical stimulation. Moreover, in our hands the antibodies to EGR-1 have proved more reliable than those commercially available for c-fos. The EGR-1 antibody has been successfully used to identify stimulated neurons in varying brain regions, including those within chemosensory pathways, of mice and rats (Dardou et al., 2010; Faria et al., 2008).

Experiment 1 Sex differences in pheromonal stimulation of ERG-1 in the chemosensory pathway

EGR-1, like c-fos, has been shown to respond to chemosensory stimulation. However the induction of c-fos and EGR-1 can differ significantly among the nuclei of the chemosensory pathways specifically in the BST, amygdala and MPOA (Yang et al., 2007; Salmaso et al.; Dardou et al., 2006). Therefore, sex differences in pheromone-induced EGR-1 expression in the extended amygdala and MPN mag were examined to ensure that the marker responded to pheromonal stimulation in the relevant areas of the pathway.

Materials and methods

Male and female Syrian hamsters were purchased from Harlan laboratories (Somerville, NJ, USA). Animals were group housed and maintained on a 14:10 h light/dark cycle, and given ad libitum access to food and water and allowed to habituate/entrain to the light/dark cycle for a several weeks before testing. All protocols for maintenance and sacrifice were approved by the IACUC board at Lehigh University.

Table 1
Statistical measures obtained in comparing the total area of the region examined in experiment 1.
Note: there were no significant differences in size among any of the areas.

Source	Sex			Treatment			Sex*Treatment		
Region	F	p	η_p^2	F	p	η_p^2	F	p	η_p^2
BSTpm	0.076	0.787	0.005	3.226	0.091	0.168	0.438	0.517	0.027
BSTpi	0.987	0.335	0.05	0.841	0.372	0.05	1.019	0.328	0.06
MeA	0.116	0.738	0.007	0.442	0.516	0.027	2	0.176	0.111
MePD	0.003	0.959	0	0.195	0.665	0.012	0.302	0.59	0.019
MPNmag	1.01	0.33	0.059	0.141	0.712	0.009	0.024	0.88	0.001

Tissue collection

After the animals had reached 60 days of age they were singly housed, at least twenty four hours before testing, in order to reduce extraneous EGR-1 expression created by interactions with cage mates. Within the first four hours of the dark cycle, each animal was given either a clean cotton swab or a swab containing vaginal secretions (HVS) from Syrian hamsters. (Secretions were collected without regard to the day of the cycle but most likely came from those that had ovulated as these secretions are copious.) Both males and females were observed to investigate, chew and often put the swab in a check pouch. One hour after presentation of the stimulus, each animal was sacrificed by an overdose of pentobarbital (19.5 mg/100 g body weight) and perfused intracardially with 0.1 M phosphate buffered saline (PBS; pH = 7.5) followed by 4% paraformaldehyde in 0.1 M PBS. Each brain was removed and postfixed in 4% paraformaldehyde for 48–72 h and then cryoprotected in 30% sucrose in PBS with .01% Thimerosal until sectioned at 40 μm on a freezing stage (Physitemp) attached to a sliding microtome (Leica SM 200R). Sections were stored at 20 °C until being processed for immunocytochemistry or mounted and stained with cresyl violet for anatomical reference.

Immunolabeling

Every third section of each brain was labeled for EGR-1 using free floating immunocytochemistry. Sections were washed in PBS 3 × 5 min and then incubated in rabbit EGR-1 antibody (Santa Cruz Biotechnology sc-189; 1:4500) for 48 h at 4 °C. Sections were then washed again in PBS for 3 × 5 min and incubated in biotin-SP-conjugated goat anti-rabbit (Jackson Immunoresearch, Westgrove, PA, USA; 1:600) for 1 h at room temperature. Sections were removed, washed for 3 × 5 min in PBS and incubated in avidin–biotin–HRP complex (Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 h at room temperature. Sections were rinsed for 3 × 5 min and incubated in .01% H_2O_2 for 10 min. The tissue was rinsed again and then incubated in Vector SG peroxidase for 7 min (Vector SG Substrate Kits sk-4700, Vector Laboratories, Burlingame, CA, USA). The sections were washed for 3 × 5 min and stored at 4 °C in PBS with .01% Thimerosal until mounted on superfrost plus slides (VWR West Chester, PA, USA). Sections were allowed to dry for at least 24 h in the oven at 37 °C. Slides were then dehydrated in an ethanol series and coverslipped with Eukitt. Every third section from the remaining tissue was mounted on slides and stained with cresyl violet in order to visualize conventional neural areas.

Analysis

Slides were coded so that the experimenter was blind to the sex and treatment of the animal. Cresyl-violet stained sections containing the posterior medial and posterior intermediate subdivisions of the BST (BSTpm, BSTpi respectively), the anterior and posterior subdivisions of the medial amygdala. (MeA, MeP respectively) and MPNmag were identified in tissue from each subject using the hamster brain atlas (Morin and Wood, 2001). One section per brain through each structure was chosen, and the area of each nucleus was outlined. The adjacent brain section, labeled for EGR-1, was found and both images were captured using the microscope imaging software, NIS-Elements, and saved as jpeg files. The number of immunolabeled neurons was determined for each brain area using the particle counting software in ImageJ. The area of the region of interest was also measured using this software and the numbers of cells per area were obtained for each brain.