Neurobiology of Learning and Memory 146 (2017) 31–36

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

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

Olfactory stimulation induces cerebellar vermis activation during sexual learning in male rats

Zuleyma Samaria Hernández-Briones[a], Paulina García-Bañuelos[b], María Elena Hernández[b], María-Leonor López[b], Armando Martínez Chacón[c], Porfirio Carrillo[c], Genaro Coria-Avila[b], Jorge Manzo[b], Luis Isauro García[b,*]

[a] Doctorado en Investigaciones Cerebrales, Universidad Veracruzana, Xalapa, Ver., Mexico
[b] Centro de Investigaciones Cerebrales, Universidad Veracruzana, Xalapa, Ver., Mexico
[c] Instituto de Neuroetología, Universidad Veracruzana, Xalapa, Ver., Mexico

ARTICLE INFO

Keywords:
Cerebellum
Fos immunoreactivity
Olfaction
Sex
Learning
Conditioning

ABSTRACT

The cerebellum is a complex structure mainly recognized for its participation in motor activity and balance, and less understood for its role in olfactory processing. Herein, we assessed Fos immunoreactivity (Fos-IR) in the cerebellar vermis following exposure to different odors during sexual training in male rats. Males were allowed to copulate for either one, three or five sessions. One day after the corresponding session they were exposed during 60 min to woodshaving that was either: clean (Control), sprayed with almond scent (Alm) or from cages of sexually receptive females (RF). The vermis of the cerebellum was removed, cut in sagittal sections and analyzed for Fos-IR to infer activation. Our results showed that the cerebellum responded with more Fos-IR in the Alm and RF groups as compared to Control. More copulatory sessions resulted in more odor-induced Fos-IR, especially in the RF group. Accordingly, we discuss possible mechanisms on how the cerebellum mediates processing of both unconditioned and conditioned odors, and how sexual experience accelerates such process.

1. Introduction

The cerebellum represents less than 10% of the total volume of the brain, but contains about 80–85% of the total number of neurons in the brain (Ghez & Thach, 2000; Herrup & Kuemerle, 1997). Its structure is complex, due to its laminar cytoarchitecture with uniform and highly specific microcircuits, which makes it an ideal organ for the study of neural complex systems (Middleton & Strick, 1998). The cerebellum participates in movement and posture (Andreasen et al., 1999) reflexes (Wiggs, Weisberg, & Martin, 1998) memory (Apps & Garwicz, 2005; Ito, 1989) emotions (Sacchetti, Scelfo, Tempia, & Strata, 2004), sexual responses (Holstege & Georgiadis, 2004; Manzo et al., 2008), language (Baillieux, De Smet, Paquier, De Deyn, & Mariën, 2008), planning (Bastian, 2006) and also integrates different types of sensory information (Sobel et al., 1998; Vaillancourt, Mayka, & Corcos, 2006; Zhu, Yung, Kwok-Chong Chow, Chan, & Wang, 2006). For instance, human studies using functional magnetic resonance imaging (fMRI) have reported that the cerebellum is activated during olfactory stimulation and that such responses are different during sniffing (mere motor response) than during smelling (the actual recognition of olfactory information) (Sobel et al., 1998). However, not much is known about how the cerebellum may process olfactory inputs. Likewise, a study using fMRI in monkeys showed increased activation of the cerebellum during olfactory stimulation with urine scent of periovulatory females (Ferris et al., 2004), and sexually-experienced male rats also respond with more Fos immunoreactivity (Fos-IR) in the cerebellar vermis following non-contact exposure to receptive females (Manzo et al., 2008). Interestingly, when sexually-naïve males are exposed to either food odors or scent from sexually receptive females they express similar Fos-IR in the vermis (García et al., 2015). Accordingly, it appears that regardless of their nature exposure to odors result in higher Fos-IR in the cerebellum only after a male has gained sexual experience (García et al., 2015). Thus, in the present study we assessed Fos-IR following exposure to unconditioned (female scent) or conditioned (almond scent) in male rats with different amount of sexual experience. We hypothesized that odor-induced Fos-IR would mainly depend on sexual experience, rather than the nature of the odor.

2. Material and methods

2.1. Subject

We used Wistar male rats (n = 54) and ovariectomized females

* Corresponding author at: Centro de Investigaciones Cerebrales, Universidad Veracruzana, Médicos y Odontólogos S/N, Unidad del Bosque, Xalapa, Ver. C.P. 91010, Mexico.
E-mail address: luisgarcia@uv.mx (L.I. García).

http://dx.doi.org/10.1016/j.nlm.2017.11.003
Received 7 June 2017; Received in revised form 27 September 2017; Accepted 1 November 2017

(n = 20) (adults between 200 and 250 g), raised and kept at the colony room of the Centro de Investigaciones Cerebrales at Universidad Veracruzana, Xalapa, Mexico. Animals were housed by sex in groups of five per cage (44 × 34 × 20 cm) containing woodshaving bedding (Ristmart, Mexico) and kept in a room with inversed light/dark cycle (12 × 12 h) at room temperature. Access to drinking water (Xallapan®) and rat feed (Purina rodent Chow®) was *ad libitum*. All surgical procedures and manipulations of rats were carried out under the Norma Oficial Mexicana (NOM-062-ZOO-1999) and the policies of the Society for Neuroscience on the use of experimental animals.

2.2. Training of sexual behavior

In order to reach sexual experience, males were allowed to copulate with ovariectomized, sexually-receptive females that received estradiol benzoate (10 μg) and progesterone (2 mg) 48 and 4 h respectively, before every copulatory session. Hormones were dissolved in sesame oil and injected subcutaneously in a volume of 0.1 ml per rat. During each session each male was allowed to interact to one ejaculation with one receptive female in a transparent circular acrylic chamber that contained woodshaving as bedding. Then, the female was gently removed from the cage, ending the copulatory session. Three groups were formed depending on the number of copulatory sessions: Group S1 (n = 18) received 1 session of sexual experience, Group S3 (n = 18) received three sessions, and Group S5 (n = 18) received five sessions. Each copulatory session occurred every 48 h. Then, 24 h after the last session all groups of males received olfactory stimulation. Each group (S1, S3 and S5) was divided into three subgroups according to the type stimulus they were exposed to: Control (clean woodshaving), Almond scent (Alm) and Sexually receptive female scent (RF).

2.3. Olfactory stimulation

Olfactory stimulation occurred after sexual experience and the protocol was carried out as described by García et al. (2015). The experimental subjects were placed in a transparent acrylic chamber (30 × 30 × 30 cm) with a double bottom, during 10 min for habituation. The floor of the cage had a central area with ten holes (5 mm θ) that allowed males to smell the olfactory stimuli located underneath in the lower compartment inside a small glass container. After habituation, they were exposed during 60 min to clean woodshaving for controls (Ctrl, n = 6), almond-sprayed bedding for one subgroup (Alm, n = 6), and woodshaving from cages of sexually receptive females for the last subgroup (RF, n = 6). In all cases sterilized woodshaving was used (Rismart México) as a vehicle (80 g for each sample). In the case of Alm and RF groups sterilized woodshaving was used, sprayed with almond odor (3 ml) or from the bedding of ovariectomized females (hormone-primed that remained 24 h after receptivity), respectively.

2.4. Fos immunohistochemistry: collection and processing of the tissue

After 60 min of olfactory stimulation, each male rat received an over dose of sodium pentobarbital (Sedalpharma, México 120 mg/kg, intraperitoneally) and then processed for transcardial perfusion with 400 ml of 0.9% saline, followed by the same volume of 4% paraformaldehyde in a phosphate buffer (pH 7.4) through a perfusion pump (Masterflex® L/S 77200-62) with flow parameters: 8 ml/min through a 16 gauge needle placed into the left heart ventricle. Once perfused, animals were decapitated, their cerebellum removed and immersed during 24 h in 4% paraformaldehyde (in order to strengthen fixation). The vermis was isolated and cryoprotected in successive solutions of 10%, 20% and 30% sucrose (PB 0.1 M, 10 mL, 24 h respectively) for three days. Sagittal slices of 40 μm from the vermis area were obtained using a cryostat (Leica CM1850) at a temperature of −24 °C. Immunoreactivity tests were carried out for Fos. Sections were co-incubated for 48 h at 4 °C with the first polyclonal Fos antibody (rabbit anti-Fos Santa Cruz Biotechnology SC-52G) diluted at 1:2000 in a 0.3% PBT solution (Triton X-100) and 0.3% normal goat serum (Vector Laboratories S-100). Subsequently, added the anti-rabbit secondary antibody (Vector Laboratories AI-100) in 0.3% PBT diluted at 1:250 and incubated the sections for 90 min in constant agitation at room temperature. The avidin-biotin complex (Vectastain Elite ABC-Peroxidase Kit Standard, Vector Laboratories PK-6100) was then applied in PB 0.1 M diluted at 1:200 for 90 min in constant agitation at room temperature. Immunoreactivity was revealed in 20 ml PB 0.1 M with 5 drops of diaminobenzadine (0.1 mg/100 ml DAB Substrate Kit, Vector Laboratories SK-4100) as chromogen, 4 drops of nickel (0.05 mg/5 m, Fisher Scientific), and 3 drops of 30% hydrogen peroxide for 6–7 min at which point sections showed a dark purple staining. Finally, sections were mounted on gelatin-coated slides and left to dry for 1 week, after which they were dehydrated with ascending alcohol concentrations (70, 90 100%), cleaned with xylene (Fisher Scientific) and finally coverslipped (Corning Inc.) and fixed permanently with Permount (Fisher Scientific). All cerebellar sections were processed under similar conditions to minimize any differences that possibly affected the immunoreaction.

2.5. Histological data

The slides were observed under a microscope (AX70 Olympus Optical Co., LTD, Japan) connected to a computer that received the scanned image, then viewed through the Image Pro Plus 6.7 (Media Cybernetics) software. All sections were observed using a 40x objective, defining an area of 20,000 μm^2 in the distal region of each lobe. The filter tool recorded the cells that expressed intense immunoreactivity in this area automatically. In order to standardize cell type by size the software was programmed to account exclusively those cells with a diameter ≤7 μm (Eccles, Ito, & Szentágothai, 1967). By doing this we maximized the possibility of accounting granule cells, and minimized the possibility of accounting other type of cells in the granule layer, such as Golgi (> 10 μm θ) Palay & Chan-Palay, 1974.

2.6. Statistical analysis

The number of Fos-IR cells was analyzed using a nested analysis of variance (ANOVA) following a generalized linear model (GLM). The following model represents the analyzed variables: y = Group + Sessions + $Animal_{[lobe]}$ + Lobe + G × S + G × L + S × L + G × S × L + Error, where y, is the variable response, and G, S and L are the Group, Sessions and Lobes, respectively. The pseudoreplication model (nesting factor within brackets) was part of the variation as suggested (García et al., 2015; Herrera-Meza, Manzo, Hernández, Miquel, & García, 2014; Lazic, 2010). Subsequently, a *post hoc* Tukey test was performed to determine lobes differences between groups over sessions with an alpha level of $p < .05$. All the analyses were performed in JMP (V 9.0.1 SAS Institute, Inc. Cary NC 1999-2010) and the assumptions of normality and homogeneity of variances of the response variables were verified.

3. Results

Fos-IR was induced by olfactory stimulation in the three groups (Ctrl, Alm, RF). Interestingly, males allowed to copulate more times (S5), as well as males exposed to receptive female scent (RF) expressed higher number of Fos-IR cells at the granule layer of the cerebellar vermis (Fig. 1). The overall analysis by session showed that the average of Fos-IR granule cells at the vermis was significantly different (Fig. 2, Table 1). Olfactory stimulation of male rats showed a significant increase in the number of Fos-IR cells after each consecutive session of sexual training (S1, S3, S5). However sexually-experienced males (S5) showed higher values as compared with less experienced males (S1 and S3). Thus, the overall analysis showed that the more sexual experience,