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Fos expression at the cerebellum following non-contact arousal and mating behavior in male rats

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Abstract

The cerebellum is considered a center underlying fine movements, cognition, memory and sexual responses. The latter feature led us to correlate sexual arousal and copulation in male rats with neural activity at the cerebellar cortex. Two behavioral paradigms were used in this investigation: the stimulation of males by distant receptive females (non-contact sexual stimulation), and the execution of up to three consecutive ejaculations. The vermis area of the cerebellum was removed following behavioral experiments, cut into sagittal sections, and analyzed with Fos immunohistochemistry to determine neuronal activation. At the mid-vermis region (sections from the midline to 0.1 mm laterally), non-contact stimulation significantly increased the activity of granule neurons. The number of activated cells increased in every lobule, but lobules 1 and 6 to 9 showed the greatest increment. In sexual behavior tests, males reaching one ejaculation had a high number of activated neurons similar to those counted after non-contact stimulation. However, two or three consecutive ejaculations showed a smaller number of Fos-ir cells. In contrast to the mid-vermis region, sections farthest from the midline (0.1 to 0.9 mm laterally) revealed that only lobule 7 expressed activated neurons. These data suggest that a well-delineated group of granule neurons have a sexual biphasic response at the cerebellar vermis, and that Fos in them is under an active degradation mechanism. Thus, they participate as a neural substrate for male rat sexual responses with an activation-deactivation process corresponding with the sensory stimulation and motor performance occurring during copulation.

Keywords

Granule cells; sexual behavior; sensory stimulation; cerebellar cortex; ejaculation

1. Introduction

The cerebellum, or little brain, is located posterior to the brain hemispheres covering the dorsal part of the brainstem. It is a symmetrical structure with a remarkable stereotyped neuronal

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organization in its cortex. The dorsal view shows that its geometry is a hemispherical ellipse with the major axis in a coronal position and the minor in a sagittal position, and allows the recognition of a central region known as the vermis, and two hemispheres at each side. The cerebellar surface appears undulated in the rostrocaudal direction, which results from the presence of transverse fissures. Some of the transverse fissures extend until the lateral border of the cerebellum. Additionally, some of the fissures are deeper than others. The arrangement of fissures led to the recognition of 10 different lobules of the cerebellar cortex [1,2], which are composed of three different layers organized in a characteristic manner: 1) the uppermost layer is called the Molecular Layer, 2) the middle layer is known as the Purkinje Perikarya Layer, and 3) the bottom layer is referred to as the Granule Cell Layer.

The neurons and axons in these layers of the cerebellar cortex are arranged in complex circuits that seem to have just one aim: the control of Purkinje neurons that constitute the only cortical output [3]. It has been suggested that this arrangement serves at least two purposes, being the control of fine movements and the regulation of time in the execution of movements [4,5]. In humans with cerebellar lesions, studies have shown the loss of accurate movements associated with the opening of fingers when releasing a ball during throwing tests [6] or the failure to adapt arm movements for the purpose of adjusting the hand when catching balls with different weights [7]. In animals, a timing function for the cerebellar cortex was hypothesized following the first histological drawings made by Santiago Ramon y Cajal [8], who described the now well-known distribution of granule and Purkinje somas and the parallel and climbing fiber inputs to Purkinje's dendritic tree. This anatomical arrangement led to various investigations of cerebellar function, such as the hypothesis of parallel fiber beam [9], the proposal of a radial organization of granule axons [10], the role of cannabinoids in regulating neurotransmitter release [11], and the long-term depression process for cognitive functions [12]. Although a number of these studies have analyzed cerebellar physiology using different techniques, correlations with natural behaviors were absent. Thus, it was our purpose to correlate sexual arousal and copulation of male rats with the neural activity of the cerebellar cortex.

The sexual behavior of male rats is highly stereotyped. It includes the three well-described patterns known as mount, intromission and ejaculation [13]. A male displays several mounts and intromissions until reaching ejaculation; after a refractory period the male can display another group of patterns and a second ejaculation, and so on until reaching a state of exhaustion after several consecutive ejaculations. Whether this behavior and the repetitive ejaculations are represented in the cerebellum is still unknown. However, some authors have presented data concerning the possible role of the cerebellum in sexual responses in humans and animals [14]. Thus, to investigate the role of the cerebellum during male rat sexual behavior, we correlated its activity in two behavioral contexts: non-contact stimulation, and up to three consecutive ejaculations [15,16].

2. Methods

2.1. Subjects and housing

Sexually experienced Wistar male rats and ovariectomized females were used (200–300 g/bw), living in the same room with an inverted light-dark cycle (12–12 hr, lights off at 0800 hrs). Males selected for the experiment had ejaculated in the last two of four copulatory tests (two tests per week) and exhibited ejaculation latencies of less than 10 min. Receptivity in females was induced with exogenous steroids diluted in sesame oil. Accordingly, subcutaneous injections of estradiol benzoate (10 µg) and then progesterone (2 mg) were administered 48 and 4 hrs before tests, respectively. Rats were housed in plastic cages (50 × 30 × 20 cm; Five per cage) containing wood chip bedding and rooms were under a controlled temperature (22 ± 2°C), with food (Harlan Mexico rodent chow) and water available *ad libitum*. Every surgical

intervention and manipulation of rats was guided by the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

2.2. Non-contact arousal tests

This set of experiments was designed to determine Fos expression at the cerebellar cortex of males after being stimulated by sexual cues from estrous females [17]. Sexual stimulation of males by nearby receptive females occurred in a glass chamber (60 × 30 × 50 cm) divided in half by a mesh screen (1.5 cm²; 2.5 cm wide) that prevented direct contact between rats on either side but permitted other modes of communication. Males (n=8) were placed alone for 5 min into one side of the testing chamber, after which a receptive female was introduced into the other side. Tests ended 30 min after the female was introduced. Any indication of non-contact penile erection, continuous nose poking through the grid, genital grooming or urine marking behavior was considered indicative of sexual arousal [18,19]. A second group of males (controls, n=6) was used with a non-receptive female introduced on the other side.

2.3 Sexual behavior tests

The experimental paradigm of these tests was used to determine the effect of consecutive ejaculations (up to 3) on the level of Fos expression at the cerebellar cortex of males. Experiments were carried out under red light between 12:00 and 16:00 hrs. Briefly, a male rat was taken from its cage and placed into a Plexiglas arena (60 cm diameter × 60 cm high), where a receptive female was introduced five minutes later. Parameters detailing the sexual behavior of the male were recorded immediately (not shown here, but similar to those we reported elsewhere [20]). The number of consecutive ejaculations marked the assigned group of the animal. Males from group E1 (n = 8) were allowed to ejaculate once, Group E2 (n = 8) was allowed to ejaculate twice, and group E3 (n = 8) was allowed three consecutive ejaculations before analysis of cerebellar Fos. A control group (n=6) involved a male being placed in the arena for five minutes without the introduction of a female.

2.4. Fos immunohistochemistry

An hour after ending behavioral tests, males were anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg). A transcardial perfusion was then performed with saline solution (0.9%; 200 ml), followed by 4% paraformaldehyde in a phosphate buffer (PB, pH 7.4; 400 ml). The skull was removed and the whole cerebellum obtained and post-fixed for 12 hrs in the paraformaldehyde. The vermis area was then isolated and cryoprotected successively in 10, 20 and 30% sucrose in PB 0.1M, and then sectioned sagittally at 50 µm in a Leica cryostat. The width of the vermis was about 2.5 mm and sagittal cuts were collected at known distances from the midline. Thus, sections around 0.1, 0.3, 0.5 and 0.9 mm from the midline were separated and processed immunohistochemically for Fos (see Fig. 6). Briefly, tissue was incubated in a polyclonal primary Fos antibody (rabbit anti-Fos Santa Cruz SC-52G) diluted 1:2500 in PBSGT (PBS, 1% goat serum and 0.3% Triton X-100) at 4°C for 72 hrs. The tissue was then incubated in biotinylated secondary antibody (goat anti-rabbit serum; Vector Laboratories) 1:250 in PBSGT for 2 hrs at room temperature, followed by incubation in avidin-biotin complex (0.9% avidin and 0.9% biotin solutions; Vector Laboratories) in PBSGT. Fos-antibody-peroxidase complex was stained with a solution of 0.05% diaminobenzidine (Polyscience) in the presence of nickel sulfate (10 mg/ml, Fisher Scientific), cobalt chloride (10 mg/ml, Fisher Scientific) and 0.01% hydrogen peroxide. Finally, the sections were mounted onto gelatin-subbed slides, dehydrated and cleared with xylene (Fisher Scientific), and coverslipped with Permount. Control sections were processed as above, but the primary antibody was omitted.

2.5. Photomicrography and Cell Counting

Digital images of sections were obtained using an Olympus Provis AX70 microscope, with a camera sending photomicrographs to a computer system containing Image-Pro Plus software (Media Cybernetics). A grid of 8 squares, $40 \times 40 \mu\text{m}^2$ each and totalling $12,800 \mu\text{m}^2$, was superimposed on the granule cell area of the micrographs, and Fos-ir cells located inside the grid were counted. Three sections of each point from the midline were counted per animal. The average of these sections was used as the animal value. Data were analyzed with a one-way ANOVA followed by a *post hoc* application of Dunnett's test when the F value showed significant differences at $p < 0.05$.

3. Results

3.1. Non-contact stimulation

The distant stimulation of males for 30 min significantly increased the activation of the granule cell layer, which is noticeable with photomicrograph comparisons (Fig. 1). No reactivity was reliably found in other layers. The counting of Fos-immunoreactive cells (Fos-ir) revealed a significant increase in the number of activated granule cells in every lobule ($F[1,9] = 3.16$, $p < 0.01$). However, the peak quantity depended on the lobule. Lobule 1 and lobules 6 to 9 had the greatest number of Fos-ir neurons when observed at the 0.1-mm cut (Fig. 2). Sagittal sections of the vermis at different distances from the midline (0.5 to 0.9 mm) showed that only lobule 7 remained highly activated, while the others steadied with a moderate level of activity (Fig. 3).

3.2. Sexual behavior

The sexual behavior of males also resulted in a significant increase in the number of Fos-ir neurons at the granule layer (Fig. 4). In males reaching one ejaculation, the profile of activated neurons resembled that obtained after non-contact stimulation ($F[1,9] = 2.79$, $p < 0.01$). However, two ($F[1,9] = 2.01$, $p < 0.05$) or three consecutive ejaculations resulted in a smaller increase in the number of Fos-ir cells that did not produce statistically significant differences after the third ejaculation ($F[1,9] = 0.64$, NS) when compared to controls (Fig. 5). As in non-contact males, the vermis showed distinct activity if analyzed in the midline or in more lateral regions. Lobule 7 expressed the greatest number of Fos-ir neurons when considering the whole width of the cerebellar vermis (Fig. 6).

4. Discussion

The cerebellum is activated during male rat sexual behavior. This investigation revealed a specific region at the mid vermis area accentuated in lobule 7 that responded significantly to both sensory stimuli and motor performance. Human neuroimaging studies already demonstrated increased activity in the vermis during ejaculation [14,21] and sexual arousal [22]. In these studies, cerebellar activity became associated with emotions and expectations, and was not related directly to motor performance. Indeed, cerebellar activation was also observed during heroin rush [23], monetary reward [24] and pleasant response to music [25]. The present pattern of cerebellar activity could be then connected with emotional and cognitive changes during sexual behavior in rats. Unfortunately, with the Fos immunohistochemistry it is not possible to separate the cerebellar activity related to the behavior from the cerebellar activity related to the emotional and cognitive aspects during ejaculation [26].

In the present study, sensory stimulation in non-contact tests produced activation of a high number of granule cells, indicating that cues from estrous females trigger the activity of those neurons. The activity could be evoked by visual and auditory stimulation or airborne scents, being the latter the one with more impact on the observed sex-related behavior after non-contact

sexual stimulation [17]. Although a neural pathway from the olfactory system to the cerebellum is unknown, it is well-known that non-contact stimulation activates a number of central structures that produce complex responses such as penile erection or micturition behavior [18,19]. Thus, this olfactory-spinal path could also activate the cerebellum in the rat. The existence of such a pathway in humans and other animal models is supported by studies that consistently found activation of the cerebellum by stimulation of the olfactory system [27, 28]. Here we showed that distant stimulation with sexual cues seems to produce a similar effect in male rats. However, it is important to notice that this activation could result not from the non-contact stimulation *per se*, but from the facial or pelvic movements resulted as consequence. The activation does not affect the whole of the vermis. Hence, in spite of the stereotyped histological organization of cerebellar cortex neurons, there is significant heterogeneity of functional activity among granule cells.

The execution of sexual behavior revealed that the number of activated granule cells after one ejaculation increased to a level equivalent to that produced after non-contact stimulation. This finding and the non-contact tests suggest that this activity could result from the sensory stimulation and that motor performance did not produce a further effect on the activation. Furthermore, the execution of the second and third consecutive ejaculations seemed to contribute to a reduction in the number of Fos-ir cells relative to the first ejaculation. Thus, the combined data from non-contact stimulation and consecutive ejaculations suggest the triggering of two different mechanisms modulating granule cell activity, namely, the first (sensory) to stimulate and the second (motor) to inhibit granule cells. The sensory pathway is activated by different cues from females [17], which in turn activates several projections down to the spinal cord and perhaps includes participation of the mossy fibre system supplying those areas with the granule cells. However, the reduction in Fos-ir neurons after two or three ejaculations indicates a more complex mechanism.

Previous reports observed an induction of Fos-ir after one, two, and up to seven ejaculations in the ventromedial hypothalamus, the medial nucleus of the amygdala, the dorsal part of the bed nucleus of the stria terminalis, the parvocellular part of the suprafascicular nucleus and the medial preoptic nucleus [29-32]. Our results showed, however, that Fos expression in the cerebellum during sexual behavior follows a different pattern. A central question here relies on the functional meaning of the reduction in the number of Fos-ir cells in the cerebellum. As suggested for the medial preoptic nucleus [30], Fos-ir in the cerebellum probably reflects a motivational state following the convergence of different sensory inputs that in turn activate a motor system related to sexual displays. Therefore, the reduction in Fos-ir we observed after repeated ejaculations could be due to changes in the motivational state of the subject, as approaching sexual satiety. Very recently, it was analyzed the expression of Fos-ir in several brain areas after four consecutive ejaculations and after male rats attained sexual satiety [32]. Surprisingly, areas mediating masculine sexual behaviour from copulating males expressed the same level of Fos-ir regardless of whether they do or do not reach sexual satiety. The bed nucleus of the stria terminalis, posterodorsal medial amygdala, medial preoptic area, nucleus accumbens, suprachiasmatic nucleus and supraoptic nucleus showed a clear increase in Fos-ir, which showed no correlation with the number of ejaculations. However, the pattern of Fos-ir in the piriform and entorhinal cortex was related to satiety due to its increase after four ejaculations and its decrease after satiety [32]. Hence, it seems that Fos expression after consecutive ejaculations follows a different pattern depending on the central structure. Here we showed that the cerebellar cortex has a biphasic, increase-decrease, response after consecutive ejaculations.

The passive degradation of Fos after being produced by a neuron requires a length of time that is longer than that required for three consecutive ejaculations, indicating that the smaller number of Fos-ir cells results from synaptic inhibitory interactions or an active-proteolytic

mechanism in the cell triggered by the sexual motor pattern [33]. A mechanism that could underlie the active degradation of Fos seems to involve a parallel fiber-Purkinje dendrite interaction (PF-PC), considering that the parallel fibers are axons from granule cells. It is widely known that this special synapse system plays a key role in the process of long-term depression and provides a foundation mechanism for learning and memory [34]. The process has been reported for PF-PC of the molecular layer at the cerebellar cortex [35]. Briefly, the retrograde inhibition of neurotransmitter release at the presynaptic parallel fiber occurs by messengers released by the postsynaptic Purkinje dendrite, with endocannabinoids acting as retrograde messengers [36]. Thus, our proposal is that the retrograde inhibition of neurotransmitter release could send this situation further backward, via some unknown mechanism, to the granule cell soma and activate the proteolytic degradation of Fos.

On the other hand, it is also known that Fos has an important function in activating gene expression, and its prolonged accumulation produces pathological modifications of the cell [33], suggesting that the active degradation of Fos after consecutive ejaculations could be a regulated mechanism for the control of the Fos-induced expression of those genes in normal neurons. Also, other complex mechanisms for the proteolysis of Fos are known. However, in this study we focus on that produced by steroid hormones. Some authors have reported the degradation of Fos triggered by certain hormones [37]. This is an important issue for our investigation because the execution of sexual behavior in rats produces a significant increase in the serum level of androgens [38], and the cerebellum is an important center for steroid action [39]. Thus, we hypothesize that the sexual stimulation of the male triggers the expression of Fos, and the execution of behavior produces the increase in androgen levels that activate the proteolytic process for Fos degradation. All these processes still deserve further research.

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Figure 1.

Cerebellar vermis and cells showing Fos-ir after non-contact sexual stimulation. Top Left: sagittal section of the mid-vermis showing numerical nomenclature of lobules; square in Lobule 1 indicates the amplification area for bottom row micrographs. Top Right: amplification of a lobule to identify the three layers of the cerebellar cortex. The molecular layer (ML), the Purkinje Perikarya layer (PL), and the granule cell layer (GL); inner region shows white matter (wm). This cortical organization is maintained in all lobules. Bottom row: micrographs showing an amplified cerebellar lobule of a non-contact male with a non-receptive female (Ctrl) and 30-min non-contact stimulated male with a receptive female (NCS). An increased number of Fos-ir neurons at the granule cell layer was observed in NCS subjects. Bar = 100 μ m.

Figure 2.

Number of Fos-ir cells after non-contact stimulation. The graph represents the number of Fos-ir cells in each lobule at 0.1-mm sections. Every lobule showed a significant increase in the number of active neurons after non-contact stimulation with a receptive female (NCS) when compared to its respective control value after non-contact with a non-receptive female (Ctrl). However, lobules 1 and 6 to 9 showed the greatest number of cells. Each point represents the mean $\pm$ SEM. * = $p < 0.05$, ** = $p < 0.01$.

Figure 3.

Number of Fos-ir cells after non-contact stimulation. 3-D graph showing the number of activated granule cells after non-contact stimulation. The graph shows the number of cells per lobule at different distances from the midline. Lobule 7 possessed the highest number of active cells for lateral areas of the vermis region.

Figure 4.

Fos-ir cells after sexual behavior. The sexual behavior of males increased the number of active neurons after one ejaculation (1E) relative to experienced males that remained in the arena alone (Ctrl). The second (2E) and third (3E) consecutive ejaculations also increased the number of activated cells, but to a lesser degree when compared to the increase after one ejaculation. Bar = 100 μ m.

Figure 5.

Number of Fos-ir cells after sexual behavior. The graph represents the number of Fos-ir cells in each lobule at 0.1-mm sections. Every lobule showed a significant increase in the number of active neurons after one ejaculation (E1) when compared to experienced males that remained in the arena alone (Ctrl). The second (2E) consecutive ejaculation produced a smaller significant increase in the number of active neurons, while the third (3E) consecutive ejaculation produced an increase that was not significantly different from Ctrl values. Each point represents the mean; SEM values were removed for clarity. * = $p < 0.05$, ** = $p < 0.01$.

Figure 6.

The sexual vermis. The drawing represents the dorsal view of the vermis area, showing the surface of the lobules that can be seen after removal of the skull, i.e., lobules 6 (L6) to 9 (L9). The topographic shadow represents areas that were activated during non-contact stimulation and sexual behavior. Bar below indicates the average number of neurons counted per region. Lobule 7 expressed the greatest number of Fos-ir neurons around midline and in the whole width of the cerebellar vermis.