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Effects of Formalin Pain on Hippocampal c-*Fos* Expression in Male and Female Rats

ILARIA CECCARELLI, ANDREA SCARAMUZZINO AND ANNA MARIA ALOISI

Institute of Human Physiology, University of Siena, Via Aldo Moro, I-53100 Siena, Italy

CECCARELLI, I., A. SCARAMUZZINO AND A. M. ALOISI. *Effects of formalin pain on hippocampal c*-Fos *expression in male and female rats.* PHARMACOL BIOCHEM BEHAV **64**(4) 797–802, 1999.—Immediate early genes are crucial intermediates in a cascade linking membrane stimulation to long-term alterations of neuronal activity. In the present experiment, we performed immunohistochemistry for c-*Fos* to determine the effects of persistent pain on cells of the hippocampus of male and female rats. Animals were subcutaneously injected with formalin (50 μl, 10%) and perfused: 2 h later, time 2; 24 h later, time 24; 24 h later after 20 min of the open-field test, time 24/OF. Controls were left undisturbed. In control, c-*Fos* was higher in females than in males in all hippocampal fields. In males at time 2, formalin increased c-*Fos* in the dentate gyrus (DG) and CA3 fields; at time 24, c-*Fos* returned to the control level; at time 24/OF, c-*Fos* was higher than in control in the DG, but not in the other fields. In the formalin-treated females at time 2 and at time 24, c-*Fos* levels were lower, or tended to be lower, than in control in all hippocampal fields; at time 24/OF, c-*Fos* levels in the DG were higher than in control and in males. In conclusion, persistent pain had different effects on c-*Fos* in the hippocampal subfields, depending on the time after treatment and the sex of the subject. © 1999 Elsevier Science Inc.

Sex differences Hippocampus c-*fos* Formalin test

AVERSIVE stimuli are known to leave long-lasting traces in the CNS that represent the basis of learning, a process essential for survival. However, there is growing evidence of the involvement of the same neuronal circuits mediating these functions—in particular, the limbic system—in the persistence of stress/pain effects, leading to the development of chronic disease (10). Thus, research on the molecular events evoked by aversive stimuli, and on their ability to persist in these brain structures, is one of the most powerful tools in the study of the development of these pathologies. Immediate early genes (IEG) have been shown to be rapidly induced following aversive stimuli in many CNS structures; it has been proposed that IEG proteins are crucial intermediates in a cascade-linking membrane stimulation to long-term alterations of neuronal activity (22,30). Fos is the most extensively studied IEG; its nuclear expression in response to stressful, as well as painful, stimuli is usually transient, with levels returning to baseline within a few hours (15). On the other hand, c-*Fos* has been reported to be involved in learning and memory (18), while persistent upregulation of c-*fos* mRNA and proteins, such as that induced by repeated stressful stimuli, has been associated with structural modifications in the brain (24,25).

The hippocampus, part of the limbic system, is affected by both phasic and longer lasting painful stimuli (1,19,26). We have found that subcutaneous injection of a dilute solution of formalin, able to induce an acute/persistent nociceptive input, produces changes in hippocampal cholineacetyltransferase (ChAT) activity and an increase in hippocampal c-*Fos* expression (3,4). Interestingly, in agreement with several anatomical and functional studies (1,9,12,17,20), we found sex differences in all these responses. Indeed, while in males formalin pain depressed ChAT activity, in females this enzyme remained at higher levels than in controls (3). In addition, the formalin-induced c-*Fos* increase, determined 90 min after treatment, was higher in females than males in all hippocampal subfields (4). However, these data refer only to males, and/or the determinations were performed within a few hours after the treatment. Therefore, the aim of the present experiments was to evaluate the longer lasting effects of the formalin-induced pain on the hippocampal neuronal activity of male and female rats. The formalin test was carried out and then immunohistochemistry for the protein product of the immediate early gene c-*fos* was performed in the dorsal hippocampus, 2 or 24 h later. A separate group of animals was subjected to a short (20-min) exposure to an open-field device 24 h after treatment, with the aim of evaluating the animal's status by the observation of spontaneous behavior.

Requests for reprints should be addressed to Dr. Anna Maria Aloisi, Istituto di Fisiologia Umana, Università degli Studi di Siena, Via Aldo Moro, 53100 Siena, Italy.

METHODS

Animals

Forty-eight experimentally naive male and female (of mixed estrous phases) Wistar rats (Harlan-Nossan, Milan, Italy) were used. They were all adult age-matched animals, the males weighing 240–260 g, and the females 200–220 g. After their arrival, they were housed in groups of four per cage, separated according to sex. To avoid social interference, the animals were isolated from 4 days before the beginning of the experiment until its conclusion. Room temperature was maintained at 23 ± 1°C, and the 12-h light/dark cycle was inverted (lights on at 1900 h). Food and water were freely available. In all experiments, attention was paid to the Ethical Guidelines for the investigation of experimental pain in conscious animals issued by the ad hoc Committee of the International Association for the Study of Pain (29). In particular, efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental Procedure

Experiments were carried out during the dark phase, between 1000 and 1600 h. On the day of testing, two animals at a time were transported to the experimental room and were assigned to the control or formalin groups. Controls (six males and six females) were not subjected to any treatment; they were only transported to the experimental room and deeply anesthetized. Animals belonging to formalin groups received a subcutaneous injection of dilute formalin (50 μl, 10%) in the dorsal and hind paw, and were immediately returned to their home cage. Formalin-treated animals were left in the experimental room for 2 h, and they were then divided into three groups (six animals each): 1) time 2: animals killed 2 h after treatment, and then immediately anesthetized (pentobarbital, 50 mg/kg, IP); 2) time 24: animals killed 24 h after treatment; 3) time 24/OF: animals killed 24 h after treatment and after 20 min of exposure to an open-field device. Two hours later, the time 2 group was anesthetized, while the other groups (time 24 and time 24/OF) were carried back to the animal house. Twenty-four hours later, the time 24 groups were transported to the experimental room and anesthetized, while the time 24/OF animals were introduced to an open-field apparatus and left there for 20 min. At the end of the open-field test they were anesthetized. For females, once anesthetized, their estrous cycle stage was determined by vaginal smears. In a room different from the experimental one, animals were perfused intracardially with 200 ml of phosphate-buffered saline, followed by 400 ml of 4% paraformaldehyde in phosphate buffer.

Open-Field Test (20 min)

The open field device consists of a transparent square cage (50 × 50 × 30 cm) with the floor divided into 25 equal squares for the assessment of locomotion (the 16 squares close to the walls = external crossing; the 9 squares in the middle = internal crossing). The following behaviors were recorded (in 5-min periods) during the open-field test: rearing frequency (raising the forepaw from the floor); internal, and external crossing frequency (forequarters entering a square of the floor); self-grooming duration (licking, washing, or scratching itself). One-way analysis of variance (ANOVA) was used to assess the group effects for each behavior.

Immunohistochemistry

After perfusion, the brains were removed, postfixed overnight, and cryoprotected in 30% sucrose. The immunohistochemical procedures followed those of Herdegen et al. (14). Briefly, the brains were cut on a freezing microtome (50 μm thick) and processed as free-floating sections. Sections were immunostained for Fos protein using a primary antibody and the avidin–biotin complex (ABC, Vector Laboratories, Burlingame, CA) method. The primary antibody was a rabbit polyclonal directed against residues 4–17 of human Fos (used at 1:10000). The dorsal hippocampal subfields (bregma −3.30/−4.00) were analyzed, reference being made to the rat brain atlas of Paxinos and Watson (21). Slices were coded so that the investigator had no knowledge of the experimental condition of any subject. Two-three slices per animal were counted. Fos-positive neurones appeared darkly stained against a background of lightly stained cells and fibers. Two-way ANOVA was used to assess the group effect with the factors Sex (two levels: male, female) and group (four levels: control, time 2, time 24, and time 24/OF) on counts of Fos-positive neurons within each hippocampal region [dentate gyrus (DG), CA1, and CA3]. Significant group effects ($p < 0.05$) were followed by appropriate post hoc comparisons for the assessment of group differences.

RESULTS

Control rats spent most of the time moving around the cage or lying on the bottom of the cage for the rest of the time, with short episodes of grooming. All formalin-treated animals showed the characteristic formalin-induced responses (licking, flexing, and jerking of the injected paw) repeatedly described in our laboratory (2). Other spontaneous behaviors were also present, but were continuously interrupted by pain-evoked responses. Both 2 and 24 h after treatment, the paw appeared to be swollen in all animals, but without any signs of ulceration.

c-Fos *Expression*

Two-way ANOVA applied to the number of cells counted in the three hippocampal subfields showed significant effects of the factors sex [dentate gyrus: $F(1, 58) = 5.84$, $p < 0.01$; CA1: $F(1, 57) = 4.33$, $p < 0.04$; CA3: $F(1, 57) = 22.4$, $p < 0.001$] and group [dentate gyrus: $F(3, 58) = 18.5$, $p < 0.001$; CA1: $F(3, 57) = 3.4$, $p < 0.02$; CA3: $F(3, 57) = 6.27$, $p < 0.001$] and the significant interaction sex × group [dentate gyrus: $F(3, 58) = 6.1$, $p < 0.001$; CA1: $F(3, 57) = 4.79$, $p < 0.004$; CA3: $F(3, 57) = 12.6$, $p < 0.001$]. Post hoc results are reported in Fig. 2.

Control Group

As shown in Figs. 1 and 2, the number of c-*Fos* labeled cells was higher in females than in males in all hippocampal regions.

Formalin Groups

Dentate gyrus (DG). In males, formalin treatment increased c-*Fos* expression at time 2. The c-*Fos* concentration returned to the control level at time 24, whereas exposure to the open field (time 24/OF) produced a new increase. In the formalin-treated females, at time 2 there was no significant difference in c-*Fos* concentration with respect to control, while at time 24 the c-*Fos* level was lower than control. At time 24/OF, the c-*Fos* level was higher than control and higher than the levels found at time 2 and time 24. Moreover, c-*Fos* at time 24/OF was higher in females than in males.

CA1. In males, there were no significant differences among the groups (Fig. 2). In females, c-*Fos* levels at time 2 were lower than control, and likewise at time 24 very few cells could be counted. At time 24/OF, c-*Fos* levels were still significantly lower than control, although they were significantly higher than the levels at time 2 and time 24.

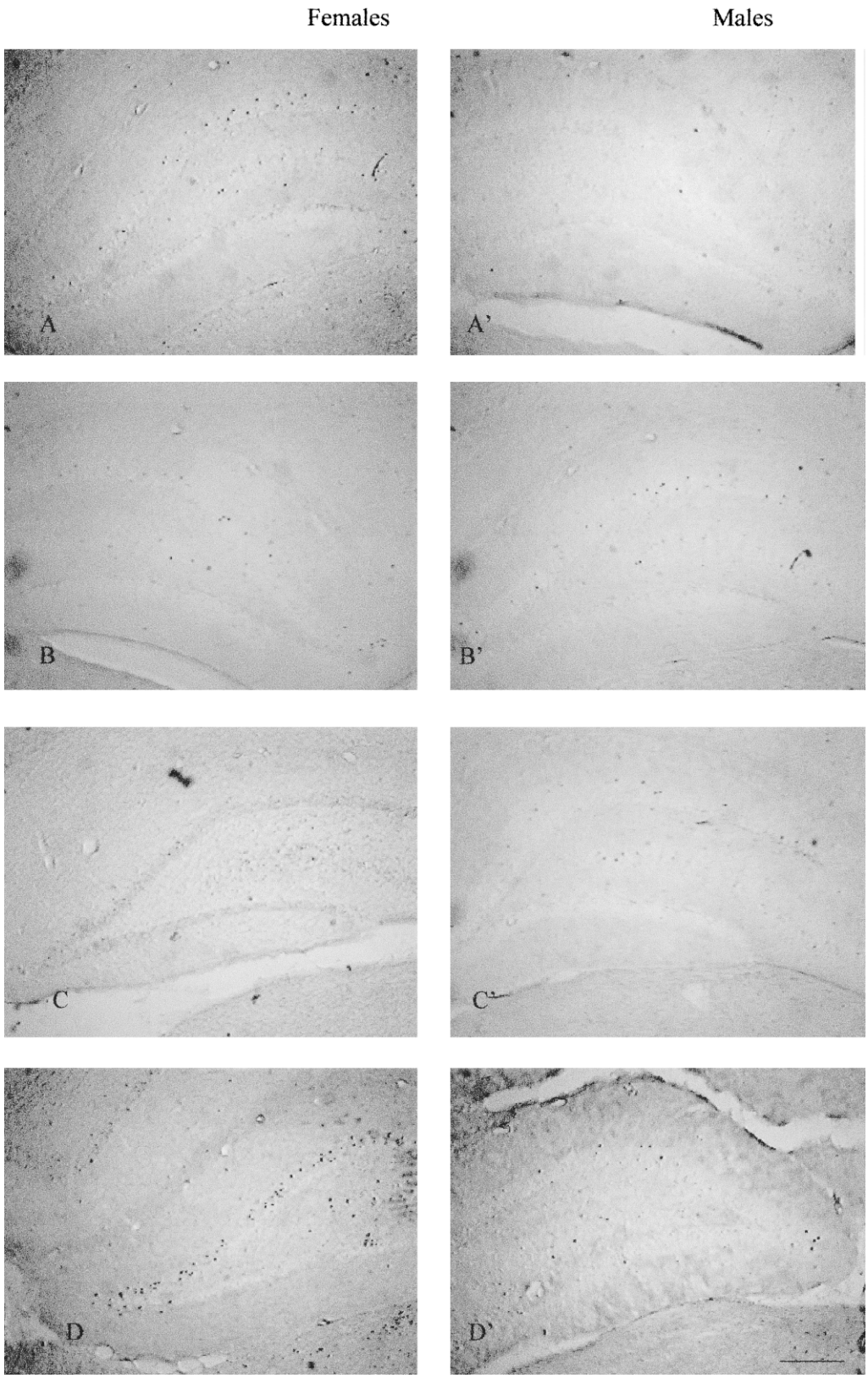

FIG. 1. Photomicrographs showing the pattern of noxious stimulus-evoked c-*Fos* expression in neurones from sections of the dentate gyrus of the dorsal hippocampus of female and male rats. Groups: control (A–A′); formalin-treated females and males killed at time 2 (B–B′), at time 24 (C–C′), or at time 24/OF (D–D′), immediately after 20 min of the open-field test. The scale bar represents 100 μm.

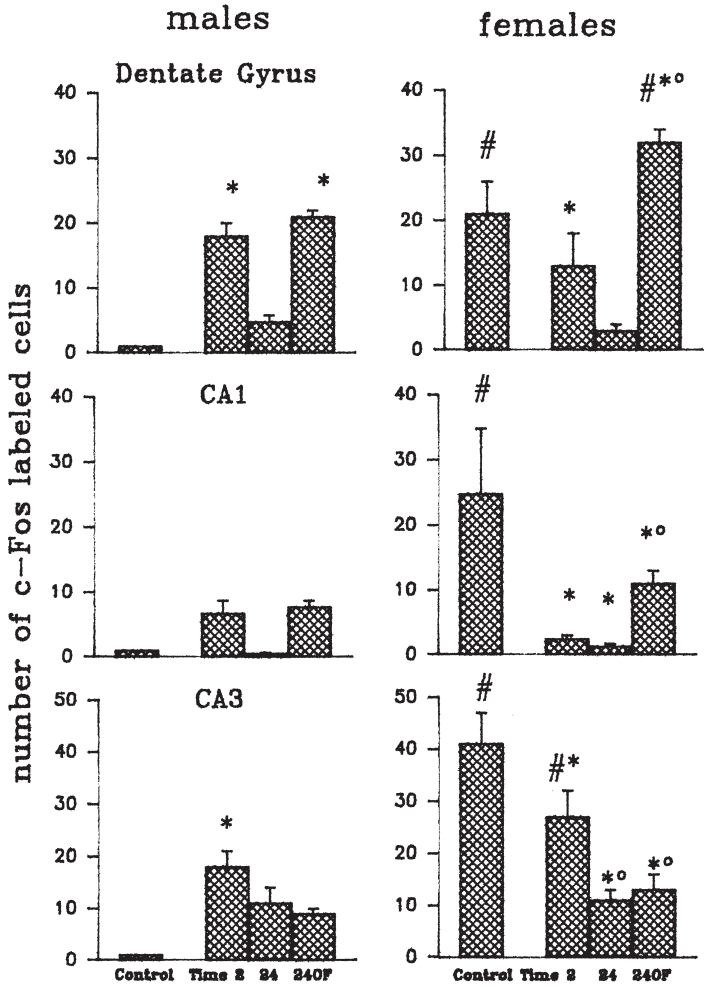

FIG. 2. Histogram representing the number of c-*Fos*-labeled neurons in the three hippocampal regions [dentate gyrus (DG), CA1, and CA3) in control and in formalin groups: time 2, time 24, and time 24/OF. Results are expressed as the mean (±SEM) number of c-*Fos* neurons per slice, per group. Significance is expressed as $p < 0.05$: * vs. control same sex; # vs. other sex-same treatment; ° vs. time 2 and time 24 groups same sex.

CA3. In males, the c-*Fos* levels at time 2 were higher than control. In females, the c-*Fos* levels at time 2 were lower than control, but were still higher than those in the males. The levels at time 24 and time 24/OF were lower than control and time 2.

Open-Field Test

As shown in Fig. 3, there were few differences between the two formalin-treated groups. The frequency of external crossing was significantly higher ($p < 0.03$) in females than males, while the frequencies of internal crossing and rearing did not differ between the sexes. Self-grooming tended to be higher in females; short licking episodes were recorded in all subjects.

Estrous Cycle Phases

Estrous phases determined at the end of the experiment in all females showed the following results: control: three in proestrus and three in diestrus; time 2: two in proestrus, one in diestrus, three in estrus; time 24: three in proestrus, three in diestrus; time 24/OF; two in proestrus, one in diestrus, two in estrus.

DISCUSSION

The immunohistochemical analysis demonstrated that all experimental phases (the preexperimental procedure, the formalin test, the time lag between treatment and killing, the open-field test) induced different c-*Fos* levels in males and females in most of the hippocampal fields.

The first thing to be emphasized is that there were already higher c-*Fos* levels in females than males in the control group. This is not the first time that we have found more c-*Fos*–labeled cells in the hippocampus of control females than in control males (4). However, in that case the difference was not significant, and the labeled cells in females remained at quite low levels (5–10 per slice, per field), in agreement with previous findings of very low levels of Fos and FosB proteins in the rat hippocampus under basal conditions (14). Isolation is a mild form of stress that can induce several central modifications (7,11). In the present experiment, we decided to isolate the animals to avoid the important influences of social interaction (23) on the coping strategies and thus on the hormonal, behavioral, and neuronal responses of each animal to the

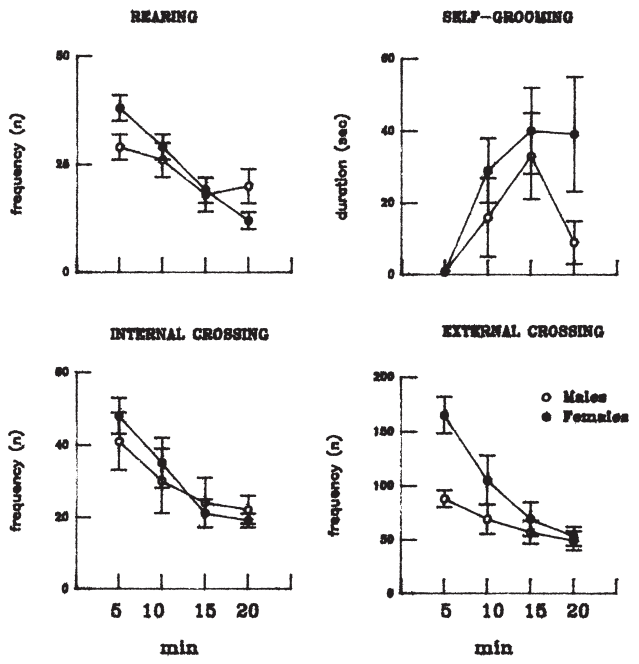

FIG. 3. Histogram representing the time course of the spontaneous behavior recorded in male and female rats during the open-field test (20 min). Data are expressed as mean (±SEM) of the frequency/duration of the behaviors during the four 5-min periods of the open-field test.

painful stimulation. The animals were allowed 4 days to become habituated to the isolation. However, while no sign of activation was found in the hippocampal c-*Fos* expression of males, the levels found in females appear to be too high to be considered basal. We have no data to explain this sex difference, although the result is in line with the finding of sexual dimorphism in the long-term potentiation effects induced in juvenile rats by neonatal isolation (8). On the other hand, the sex difference observed in control animals can be attributed to the acute reaction to the manipulation carried out before perfusion. Indeed, although control animals were not submitted to any specific treatment, they were transported to the experimental room, remained there for 2 h and were then anesthetized. These procedures could have been perceived as stressful by females, leading to the greater c-*Fos* expression in the hippocampus, in agreement with studies carried out with other methodological approaches, i.e., 2-deoxyglucose (9).

Two hours after the formalin treatment there were different changes in the c-*Fos* levels in the two sexes. In males, the levels increased in the DG and CA3, in agreement with previous results (4), while the increase in the CA1 did not reach significance. In females, the number of c-*Fos* labeled neurons at time 2 was also similar to the levels previously found (4), but lower than those found in the controls. This difference was present in all hippocampal fields. It appears that in females the isolation stress has a greater ability to activate the hippocampus than formalin pain. The analysis of c-*Fos* in the paraventricular nucleus of the thalamus (data not shown), in which the activity is more related to the neuronal input, showed higher formalin-induced c-*Fos* levels than control in both sexes.

In agreement with other studies (15), very few c-*Fos*–labeled nuclei were found in both sexes in all hippocampal fields 24 h after treatment. This could be explained by the disappearance of the acute effects related to the beginning of the nociceptive input. However, it should be emphasized that the short exposure to the open-field device induced higher levels of c-*Fos* in the DG in both sexes. Our explanation of these modifications is that the c-*Fos* depression present at 24 h masks a state of strong sensitization of the hippocampal neurons; thus, the simple exposure to a mild stress, for example, the novelty of the open-field, is able to induce in females an increase of c-*Fos* greater than that obtained with formalin pain. In this regard, further research should be conducted to study the kinds of new products obtained by the c-*Fos* binding with c-*Jun*. Indeed, members of the Jun family of IEGs can form dimers with c-*Fos* when binding with the AP-1 site. Because c-*Jun* is present in the hippocampus, a widespread induction of c-*Fos* would result in the ubiquitous formation of active c-*Fos*/c-*Jun* dimers.

Although the open-field-induced increase in c-*Fos* was present in both sexes, females showed the highest levels. Interestingly, the higher levels of c-*Fos* in females than in males were accompanied by greater locomotor activity (external crossing) in agreement with studies showing that the behavioral deficit commonly present after exposure to an uncontrollable aversive stimulus is greater in males than in females (2,6,13,28).

Finally, it must be noted that the three subregions of the hippocampal formation can be activated differently by formalin pain: DG and CA1 showed greater activation at time 24/OF, whereas CA3 showed the greater increase at time 2. These different changes could be explained by the different connections of one region or the other with other brain areas outside the hippocampus (5). In agreement with this finding, it was recently reported that there is a different c-*Fos* expression in the three hippocampal regions in response to novel or familiar items (27). Moreover, it has been reported that there is a different level of c-*fos* mRNA in the CA1 hippocampal subfield with respect to CA3 and the DG during exploration and odour discrimination learning (16).

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