Behavioural Brain Research 385 (2020) 112556

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Prefrontal cortex is associated with the rapid onset of parental behavior in inexperienced adult mice (C57BL/6)

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ARTICLE INFO

Keywords:
c-Fos
Infanticidal
MPOA
Parental
Prelimbic cortex

ABSTRACT

There is significant variability in the immediate behavioral response displayed by inexperienced adult mice when exposed to pups for the first time. The aim of this study was to determine which brain regions were engaged (higher c-Fos-immunoreactivity, c-Fos-ir) when virgin females, that were exposed to pups for 15 or 60 min, displayed full parental behavior (FPB), partial parental behavior (PPB), or non-parental behavior (NPB), or virgin males displayed PPB or infanticidal behavior (IB). The number of c-Fos-ir neurons in the prelimbic cortex (PL) was higher in parental females than in the NPB group (after a 15-min exposure), and the group not exposed to pups (NE). C-Fos expression in the nucleus accumbens (NA) was increased in most groups of females exposed to pups compared to NE. Higher c-Fos-ir was also found in the shell subregion of the NA in infanticidal males, compared to males NE. The cortical (CoA) and medial (MA) amygdala also showed higher c-Fos-ir in parental females compared to NE animals. However, PPB and IB male groups also exhibited higher c-Fos-ir in the CoA and MA compared to the NE group. The expression of c-Fos in the different subregions of medial preoptic area and the ventromedial nucleus of the hypothalamus was not specifically associated with either parental or infanticidal behavior. No brain activation in males was specifically associated with infanticidal behavior. Our results suggest that 15 min of exposure to pups is enough to detect brain regions associated with parental behavior (PL) or pups processing (NA, MA, CoA) in mice. The PL might participate in the immediate onset of parental behavior in virgin females, coordinating and planning its rapid execution.

1. Introduction

Parental behavior in rodents involves several behavioral components such as pup retrieval, crouching postures, nest building and licking/grooming [38,51,52], which contribute to the survival of the offspring [41,45,46]. Although mothers are commonly responsible for taking care of the young, both males and females express parental behavior in different social contexts and hormonal/physiological states. In some species, pup-naive virgin females and males can display parental behavior in non-reproductive contexts [39,51,52]. The induction of parental behavior by continuous exposure to pups is commonly called *pup-induced* parental behavior or *sensitization* [40,52]. However, there are inter and intraspecific differences in the latency to display parental behavior among the different rodent species [1,19,33,47,52]. For example, while most CB57BL/6 female mice can display parental behavior almost immediately or sensitize rapidly after 30 min to 3 h of exposure to pups, males from most mouse strains are generally infanticidal or non-parental (ignore or neglect the pups) [1,16,17,25,31,38,43].

Thus, the immediate behavioral response toward pups is extremely variable in mice, and can be either completely parental (all behavioral components of parental behavior are displayed), partially parental (only some of the behavioral components of parental behavior are displayed), non-parental (only one or none of the behavioral components of parental behavior are displayed) or infanticidal (animals attack or hurt pups). Therefore, mice provide a good opportunity to discriminate brain regions that might be specifically engaged in parental or infanticidal behavior from those engaged in just the processing of pup-related stimuli and other social or non-social stimuli. Previous studies [7,58] investigated the expression of c-Fos only in female mice that showed full parental behavior and after 30 min of exposure to pups. Some of these studies also did not find behavioral variability in the immediate behavioral response to pups [7], something that differed from other studies, including ours [1,25,33]. Besides, all those previous studies [7,24,57–59] compared c-Fos expression between groups of parental/infanticidal mice and groups of mice not exposed to pups, or in different reproductive contexts. A control group of non-parental virgin mice exposed to pups was never

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https://doi.org/10.1016/j.bbr.2020.112556
Received 15 November 2019; Received in revised form 10 February 2020; Accepted 11 February 2020
Available online 19 February 2020
0166-4328/

used. Therefore, the expression of c-Fos found in those studies might not be specifically associated with parental or infanticidal behavior, but just with the exposure to pups or the presence of a different physiological state. Investigating the specificity of previous findings is one of the goals of the current study.

Mice also offer an opportunity to explore rapid neural changes that occur when animals are responding differently toward the same stimulus. The process of rapid sensitization gives us also the opportunity to compare brain regions engaged in the initial stages of the interaction with pups (first 15 min) from those engaged after longer interaction with them (60 min or several days). The immediate early gene *c-fos* is rapidly and transiently expressed in neurons in response to a variety of extracellular stimuli. Transcriptional activation of the gene occurs within minutes of stimulation, with levels of the nuclear protein c-Fos increasing 30–40 min later [4,9,20,35,36,54]. However, the temporal changes and maintenance of c-Fos expression depend on the areas of the brain studied [11,36,42,60]. For example, c-Fos mRNA expression was maximal in the dentate gyrus, cortical regions, accumbens, amygdala, septum, and lateral habenula before or at 60 min of an acute stress [9], morphine and convulsant administration [36,60]. Besides, other authors, that used similar time of sacrifice and procedures to us, found increased c-Fos in lateral habenula and raphe, but not in the medial preoptic nucleus, at 70 min after the beginning of the exposure to pups [11]. The expression of the c-Fos mRNA decline after that to low or basal levels at 1.5 or 2 h post stimulus [9]. The protein c-Fos was also observed to be maximal around 1 h in several brain regions [10,11]. Thus, the time of sacrifice and perfusion after stimulus presentation can differently affect the observed c-Fos expression in the different brain regions [11,60]. In the current study we decided to sacrifice animals after 1 h of the presentation of the stimuli (pups, object, or pups inside object) to detect brain regions rapidly, and perhaps transiently, engaged in the onset of parental behavior. Besides, we investigated for the first time if 15 min of exposure to pups was enough to increase c-Fos-ir neurons in the brain.

We analyzed several brain regions that included the prelimbic (PL) and infralimbic (IL) cortex, the medial preoptic area (MPOA), and the nucleus accumbens (NA). The PL and IL cortex might be critical for the recognition of stimulus, attentional selection, decision making, behavioral flexibility and planning of the immediate parental or infanticidal response [2,49,50]. The MPOA is critical for the expression of parental behavior in parturient females as well in sensitized females and males of several species [7,10,23,24,26,27,42,58,59]. However, the MPOA has several subareas and some of them have been associated also with infanticidal behavior or the inhibition of parental behavior [24,53]. Moreover, the NA has been involved in the processing of information related with the offspring and the facilitation of parental behavior [8,18,32,22,28,29,41,44,47,48]. However, this area has also been classically associated with the processing of novelty and the mediation of motivation to approach or avoid appetitive and aversive stimuli [5,34]. In the current study we investigated which brain regions expressed c-Fos associated with the rapid decision to take care of (parental behavior) or attack (infanticidal behavior) the pups. We expected that non-parental, parental and infanticidal animals would show different pattern of expression of c-Fos in these brain regions and those differences would be evident rapidly.

We found that 15 min of exposure to pups was enough to detect brain regions associated with parental behavior or pup processing. Besides, the PL was the only brain region specifically associated with parental behavior suggesting it plays a role in the immediate onset of parental behavior, perhaps coordinating and planning its rapid execution. Other areas of the brain, commonly associated with the stimulation (MPOA, NA), or inhibition of parental behavior (i.e. MA, CoA, and ventromedial nucleus of the hypothalamus, VMH) [6,14,37,57] were not specifically associated with parental or infanticidal behavior.

2. Experimental Procedures

2.1. Subjects

We used C57BL/6 mice originally obtained from Jackson Laboratory and inbred at the animal facility of the Facultad de Medicina (UdelaR, Montevideo, Uruguay). All animals were weaned at age 20–21 days. Subjects were housed and maintained in same-sex groups of 6–7 individuals per cage until adulthood. Cages were 45 cm x 25 cm x 15 cm, with transparent Plexiglas walls and wood shaving as bedding. Animals were kept under a 12:12 h light-dark cycle (light on from 6:00 am), at 22 ˚C, with ad libitum access to food (PMI nutrition international LabDiet®, Shoreview, Minnesota, USA) and water. Cages were regularly changed once a week. All procedures carried out in this study were approved by the local committee of ethics in animal research (CHEA, N˚ 070153-000979, May 2015) and followed the recommendations of the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health and the “Guidelines for Ethical Conduct in the Care and Use of Animals” [3].

2.2. Experimental design

To determine the brain areas engaged (express increased c-Fos) when female and male mice are exposed to pups for the first time and display parental, non-parental or infanticidal behavior, the following groups of pup and sexually naive adults (60–100 days of age) were used.

1) Females exposed to pups (n = 44): females exposed to two newborns for the first time.
2) Males exposed to pups (n = 26): males exposed to two newborns for the first time.
3) Males exposed to wire-mesh balls (n = 12): wire-mesh tea balls were used as novelty control in males, and to protect pups from injury in the 60-min infanticidal group (two new newborns pups were placed inside it after the first attack).
4) Females (n = 8) and males (n = 7) without exposure: females or males exposed to neither pups nor a novel object.

Different times of exposure to pups or novel object were used (see Fig. 1).

A.) Exposure to 15 min: pups (or the wire-mesh ball) were removed from the cage after 15 min and animals left without any disturbance until completing 60 min from the beginning of the test.
B.) Exposure to 60 min: pups (or the wire-mesh ball with or without pups) remained 60 min in the cage and were then removed.

2.3. Behavioral screening in a 15-min observation period

In all cases, the behavior displayed by the subjects was recorded during a observation period of 15 min (see Fig. 1). On the day of the test, animals were individually housed in a Plexiglas testing cage (27 cm x 21 cm x 14 cm, 370 cm^2 of area) and habituated for 60 min. The test consisted in placing two newborn pups (1–3 days of age), and nest material scattered in the side opposite to where the subject was located before opening the cage. During the 15-min test we recorded latency to approach to the pups, duration and frequency of the main components of parental behavior including licking/grooming, nest building, crouching postures, frequency of retrieval or pup transport, and other behaviors such as sniffing, immobility, and time away (> 10 cm) from the pups. The test was immediately interrupted when subjects attacked and/or hurt (accidentally or not) the pups. In that case, pups were immediately sacrificed.

Animals were categorized as showing full parental behavior (FPB) if they displayed all components of parental behavior: pups retrieval (at

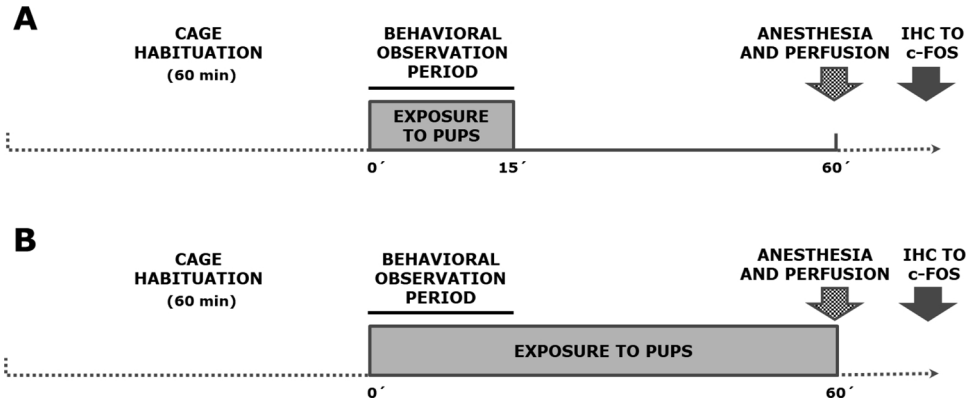

Fig. 1. Schematic representation of experimental design.

least one pup), licking (≥ 60 s), crouching over at least one pup (≥30 s), and nest building around or above the pups (≥ 60 s); partial parental behavior (PPB) if they showed 2 or 3 of the behavioral components of parental behavior; non-parental behavior (NPB) if they showed only one or none of the main components of parental behavior; and infanticidal behavior (IB) if they attacked the pups. The behavioral tests were carried out during the light phase. In previous studies, we showed that there was no difference in the behavior of parental and infanticidal animals when tested during the light or dark phase of the cycle [1,43]. All behavioral observations were recorded using the free software StopWatch http://www.cbn-atl.org/research/stopwatch.shtml.

2.4. Experimental groups

According to the different behavioral responses (FPB, PPB, NPB, IB) and duration of the exposure to pups (15- or 60-min), we created seven groups of females (Experiment I) and 7 groups of males (Experiment II).

A) Experiment I: Females exposed to pups during 15 or 60 min which had displayed FPB (15 min, n = 7; 60 min, n = 8); PPB (15 min, n = 7; 60 min, n = 8), or NPB (15 min, n = 8; 60 min, n = 6) during the behavioral screening test. We also used a group of naïve females that were not exposed to pups (non-exposure, NE, n = 8) as basal control. Animals in the NE group were placed in the same testing cages, habituated one hour as in the other groups, and left one additional hour there until sacrifice.

B) Experiment II: Males exposed to pups during 15 or 60 min which had displayed PPB (15 min, n = 6; 60 min, n = 5) or IB (15 min, n = 7; 60 min, n = 8) during the behavioral screening test. In the infanticidal group, males were exposed to pups until the first attack was registered (usually in few minutes). Immediately after the attack, the test was stopped and the pups removed (15-min Test). To complete the group of infanticidal animals exposed to pups for 60 min, the attacked pups were replaced by new pups, but this time they were placed inside a wire-mesh ball and put it back into the cage. Finally, we used males exposed to a wire-mesh ball (4.5 cm diameter) as novel object control (OE group; 15 min, n = 6; 60 min, n = 6), and males that were neither exposed to pups nor novel object (non-exposure, NE group, n = 6) as basal control. We did not find FPB animals, and the number of non-parental males (n = 3) was too small to make a group.

2.5. Immunohistochemistry (IHC)

Once the animals completed two hours in the cage, (one hour of habituation and one hour with or without the pups or the object) all subjects were deeply anesthetized (100 mg/Kg of ketamine with 14 mg/Kg of xylazine, i.p.) and perfused transcardially with 50 mL of cold phosphate-buffered saline (PBS), pH 7.4, 25 mM, followed by 100 mL of cold 4% paraformaldehyde in PBS 25 mM (see Fig. 1). Brains were removed from the skull, stored in fresh 4% paraformaldehyde for 24 h at 4 °C, and then transferred to a 15 % sucrose-PBS solution for 24–48 hr until sectioned. Three sets of serial coronal sections (30 μm) of the whole brain were cryo-sectioned and stored at −20 °C in an ethylene-glycol based cryoprotectant solution.

The IHC procedure started removing the sections from the freezer and leaving them floating in PBS at room temperature for 1 h. The free-floating sections were washed with five changes of PBS, bleached with 2% H_2O_2 with 10 % methanol in PBS for 15 min, rinsed with PBS, and placed in 0.3 % Triton X100-PBS and 4% normal goat (Vector laboratories, Burlingame, CA) for 1 h. Sections were then incubated in the primary antibodies against c-Fos (sc-52, a rabbit polyclonal antibody, Santa Cruz Biotechnology, CA) diluted (1:2000) in a 1% solution of normal goat serum in PBS at 4° for 40 − 45 hrs. Then six changes of PBS were made, and the sections were incubated in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, CA) diluted 1:200 in 1% normal goat serum-PBS for 1 h. Rinses with PBS were performed and the tissue placed for 1 h in avidin-biotin complex (ABC) reagent from an ABC Elite Kit (Vector Laboratories, CA). Then the tissue was rinsed in PBS and 100 mM sodium acetate buffer. The visualization reaction used a solution of nickel sulfate, diaminobenzidine (DAB), and hydrogen peroxide in sodium acetate buffer. After 2.5 min, the reaction was stopped by rinsing the sections in acetate buffer followed by rinses in 25 mM PBS. Sections were mounted on chrome alum-coated slides, air dried, dehydrated in graded alcohols, and coverslipped. Sections including the VMH were counterstained with neutral red to identify their different subdivisions. In order to avoid the variability among IHC assays, different behavioral groups were processed simultaneously on the same assay.

2.6. Microscopic analysis

The number of immunoreactive (ir) neurons were examined in all serial coronal sections (Fig. 2) of Prelimbic cortex (PL), Infralimbic cortex (IL), Nucleus Accumbens (NA) core and shell, medial amygdala (MA), cortical amygdala (CoA), subregions of the ventromedial nucleus of the hypothalamus (VMH) and medial preotic area (MPOA). The MPOA was divided in seven 0.04-mm^2 regions, two in the dorsal region (MPOAd, medial and lateral), two in the medial preoptic nucleus (MPN, upper and lower), two in lateral region (MPOAl, upper and lower), and one in the ventrolateral MPOA (MPOAvl). Brain areas were identified according to the atlas of the mouse brain of Franklin and Paxinos [15].

The microscopic analysis for c-Fos-ir neurons was performed by applying standard methods previously published [44]. Preparations were examined under a Nikon Optiphot microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon DS-2 M digital camera (Nikon Instruments Inc., Melville, NY, USA). The c-Fos-positive neurons were recognized by their nuclear dark purple stain. They were counted unilaterally within .04-mm^2 samples in 2 − 6 sections of each brain region of interest and averaged. For the VMH a smaller sample (.02-

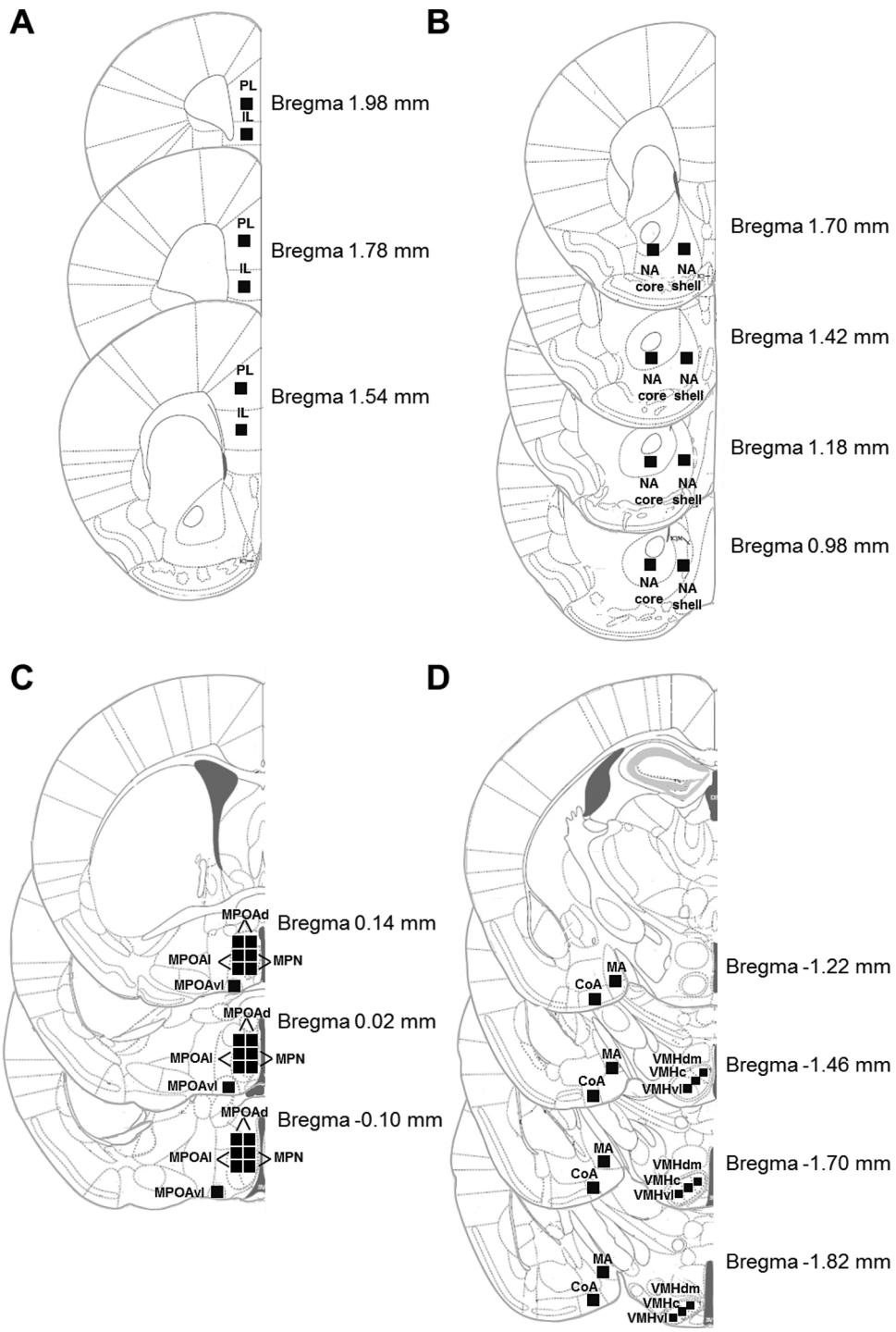

Fig. 2. Representative diagram of mice coronal sections showing the location of the samples at several level of the Prelimbic (PL) and Infralimbic (IL) cortex (A); Nucleus accumbens (NA) shell and core (B); different regions of medial preoptic area (MPOA), dorsal MPOA (MPOAd), medial preoptic nucleus (MPN) lateral MPOA (MPOAl) and ventrolateral MPOA (MPOAvl) (C); cortical amygdala (CoA), medial amygdala (MA) and ventromedial nucleus of the hypothalamus (VMH, D). The black box represents the sample of .04 mm^2 to PL, IL, NA, MPOA, MA, CA, or .02 mm^2 to VMH. Reprinted from: The mouse brain, 3th ed., K. Franklin and G. Paxinos, Copyright 2008.

mm^2) was taken from each subregion (dorsomedial: VMHdm, central: VMHc, and ventrolateral: VMHvl). The number of c-Fos-ir neurons were automatically counted using an image analysis system NIH (National Institute of Health, USA) ImageJ 1.52a software and confirmed visually.

2.7. Statistical analysis

Data of the number of c-Fos-ir neurons in each brain area were analyzed using the statistical package IBM SPSS statistics 22. All experimental data was tested for normality (Kolmorgorov-Smirnov test) and homogeneity of variance (Bartlett test). One-way analyses of variance (ANOVA; groups as factors) was performed, followed by Fisher's post hoc test for all group comparisons (2-tailed). The level of statistical significance was $p < .05$. For Bonferroni corrections, $p < .01$ is also shown.

3. Results

EXPERIMENT I: *Patterns of c-Fos immunoreactivity during immediate parental or non-parental behavioral responses in virgin female mice exposed to pups for the first time*

3.1. Groups of animals exposed to pups for 15 min

By definition, all subjects in the FPB ($n = 7$) group licked, retrieved the pups, adopted crouching posture, and built a nest. Those

percentages were 100 %, 0%, 75 %, and 87.5 % in the PPB (n = 7) group for each behavioral variable respectively, while in the NPB (n = 8) group those percentages were 14 %, 0%, 0%, and 14 % respectively.

There was a statistically significant difference in the number of c-Fos-ir neurons in the prelimbic cortex (PL, F = 7.8, df = 3, p < .001), while in the infralimbic cortex the difference did not reach significance level (IL, F = 2.6, df = 3, p = .07). Both PPB and FPB groups showed higher level of c-Fos expression in the PL (p < .01, p < .01 vs. NE n = 8, p < .04, p < .04 vs. NPB, Figure 3A and 4). Moreover, when FPB and PPB were pooled in a parental group, there was a significant difference in both brain regions (PL, F = 11.2, df = 2, p < .01; IL, F = 4.0, df = 2, p < .05). C-Fos-ir neurons in the PL (32.3 ± 1.3) and IL (26.6 ± 2.1) in the parental behavior (PB) group was higher than in the NE group (PL 20.6 ± 2.9; IL 18.5 ± 2.6, p < .01). PB group was also significantly different to NPB in the PL (24.0 ± 2.0, p < .01); but did not reach significant difference in the IL (22.3 ± 1.7, p = .1).

The number of c-Fos-ir neurons in the NA core (F = 3.6, df = 3, p < .03) and shell (F = 3.9, df = 3, p < .02) was statistically different among the groups. Most female groups exposed to pups for the first time (NPB, PPB, and FPB groups) showed higher number of c-Fos-ir neurons in both the NA core and NA shell compared with NE (Table 1).

Statistically significant differences among the groups were also observed in the pattern of c-Fos expression in the MA (F = 4.1, df = 3, p < .02) and CoA (F = 5.8, df = 3, p < .01). Females that displayed PPB showed significantly greater number of c-Fos-ir neurons in the MA (p < .01 vs. NE, Figure 3A and 5) and CoA (p < .001 vs. NE, p < .01 vs. NPB, p < .01 vs. FPB, Figure 3A and 5). No other difference was found among the groups Fig. 5.

The comparison of the four experimental groups by one-way analysis of variance did not show any statistically significant differences in the number of c-Fos-ir neurons in the MPOAd (medial; lateral, Table 1), MPN (upper; lower Table 1), MPOAl (upper; lower, Table 1) and MPOAvl (Table 1). There was also no difference among the groups in the total number of c-Fos-ir neurons in the MPOA (all subregions included).

No significant differences were found among the groups in the three subregions of the VMH (Table 1). No additional information was obtained from pooling PPB and FPB in the NA, MPOA, VMH, and MA.

3.2. Groups of animals exposed to pups for 60 min

By definition, all subjects in the FPB (n = 8) group licked, retrieved the pups, adopted crouching posture, and built a nest. In the PPB (n = 7) group those percentages were 100 %, 0%, 100 %, and 62.5 % for each behavioral variable respectively, while in the NPB (n = 6) group those percentages were 16 %, 0%, 0%, and 33.3 % respectively. One female attacked the pups and was discarded from the study. At the end of the 60 min all NPB females were found away from the pups, while FPB females were in the nest crouching over the pups. None of the PPB females retrieved the pups or built a nest, but 42 % were found crouching over at least one pup.

The number of c-Fos-ir neuron was significantly different in the PL (F = 3.3, df = 3, p < .04, Figs. 3B and 4) and IL (F = 4.0, df = 3, p < .02, Figs. 3B and 4). An increase in the number of c-Fos-ir neurons was found in animals that displayed FPB (p < .02) and NPB (p < .01) compared with animals NE (n = 8). Besides, when FPB and PPB were pooled in a parental group, there was a significant difference in both brain regions (PL F = 4.6, df = 2, p < .02; IL F = 5.2, df = 2, p < .01). C-Fos-ir neurons in the PL (31.1 ± 2.7) and IL (28.6 ± 2.7) was higher in the PB group than in the NE group (PL 20.6 ± 2.9, p < .02; IL 18.5 ± 2.7, p < .04). NPB group was also significantly different in the PL (PL 35.9 ± 4.3, p < .01) and IL (36.6 ± 5.9, p < .01) compared to NE.

Significant differences were found among the groups in the number of c-Fos-ir neurons in the NA core (F = 4.0, df = 3, p < .02) and shell (F = 4.7, df = 3, p < .01). Most of the groups of females exposed to pups showed higher number of c-Fos-ir neurons in the NA core and NA shell compared with NE group (Table 2).

C-Fos expression in the MA (F = 4.9, df = 3, p < .01) but not in CoA (F = 2.2, df = 3, p = .1) showed significant differences (Fig. 3B and Fig. 5). A significant increase in the number of c-Fos-ir neurons was found in females that displayed FPB (p < .01 vs. NE, Figure 3B and 5) and PPB (p < .01 vs. NE, Figure 3B and 5) in the MA. No other difference was found among the groups.

One-way ANOVA did not reveal any significant difference in the number of c-Fos-ir neurons in MPOAd (medial; lateral, Table 2), MPN (upper; lower, Table 2), MPOAl (upper; lower, Table 2) and MPOAvl (Table 2) among the groups. There was also no difference in the total number of c-Fos-ir neurons in the MPOA (all subregions included).

ANOVA analysis of the number of c-Fos-ir neurons in the VMH (Table 2) did not reveal significant differences among the groups. However, when PPB and FPB were pooled, ANOVA analysis was significant (p < .05) for VMHc and VMHvl. C-Fos in VMHc and VMHvl was higher in the PB group compared to NE (< .01 and < .02 respectively), but not when compared to NPB (p = .06 and p = .09 respectively). No additional information was obtained from pooling PPB and FPB in the NA, MPOA, MA, CoA or VMHdm.

EXPERIMENT II: *Patterns of c-Fos immunoreactivity during immediate parental or infanticidal behavioral responses in virgin male mice exposed to pups for the first time*

Table 1
Number of c-Fos immunoreactive neurons in virgin adult females exposed to pups for the first time for 15 min.

Brain area	NE n = 8	NPB n = 8	PPB n = 7	FPB n = 7	F (3,26)	P
NA core	14.8 ± 1.5	22.1 ± 2.2 *	23.1 ± 3.3 **	24.5 ± 1.8 **	3,6	< .03
NA shell	18.7 ± 1.8	24.4 ± 2.1 *	25.8 ± 2.3 **	27.6 ± 1.4 **	3,9	< .02
VMHdm	15.2 ± 1.3	14.3 ± 2.2	15.8 ± 2.9	11.6 ± 1.4	0,8	ns
VMHc	14.8 ± 1.3	15.7 ± 2.0	20.5 ± 3.1	14.7 ± 2.2	1,4	ns
VMHvl	13.7 ± 1.6	13.1 ± 1.5	18.1 ± 2.4	14.6 ± 2.4	1,1	ns
MPOAd medial	23.8 ± 3.7	26.2 ± 2.7	36.9 ± 5.0	27.6 ± 3.6	2,2	ns
MPOAd lateral	21.8 ± 3.9	20.4 ± 1.4	31.5 ± 6.1	22.8 ± 2.7	1,7	ns
MPN upper	27.9 ± 5.5	25.6 ± 1.6	36.9 ± 5.8	28.6 ± 3.7	1,2	ns
MPN lower	25.0 ± 5.7	25.9 ± 2.4	35.8 ± 7.5	28.9 ± 4.0	0,9	ns
MPOA upper	18.7 ± 3.9	19.6 ± 1.2	29.6 ± 4.5	24.7 ± 2.6	2,4	ns
MPOA lower	19.1 ± 3.9	21.1 ± 1.5	30.4 ± 4.7	25.0 ± 2.9	2,1	ns
MPOAvl	27.5 ± 2.0	31.8 ± 3.0	32.5 ± 4.8	29.9 ± 2.9	0,4	ns

NE (non-exposure), NPB (non-parental behavior), PPB (partial parental behavior), FPB (full parental behavior), NA (Nucleus accumbens), VMH (ventromedial nucleus of the hypothalamus), VMHdm (dorsomedial subdivision of the VMH), VMHc (central subdivision of VMH), VMHvl (ventrolateral subdivision of VMH), MPOA (medial preotic area), MPOAd (dorsal region of MPOA), MPN (medial preoptic nucleus), MPOAvl (ventrolateral region of MPOA), ns (nonsignificant). Data are expressed as mean ± SEM of number of c-Fos-immunoreactive neurons in 0.04 mm^2 (NA core, NA shell and subregions of MPOA) or 0.02 mm^2 (subregions of VMH) samples. F and p values for ANOVA are shown in the last two columns. *p < .03, **p < .01 vs. NE group, Fisher's post hoc test.

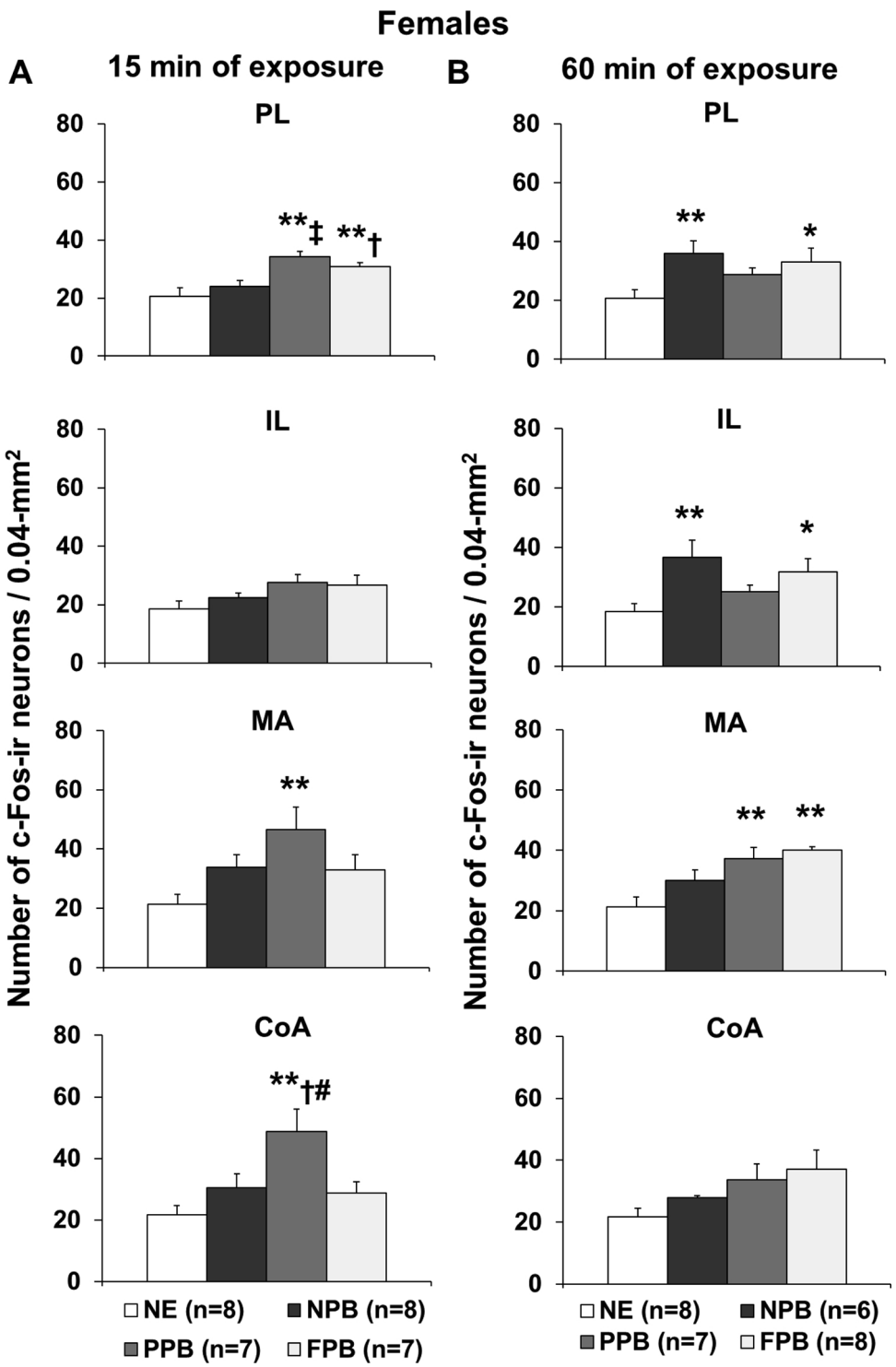

Fig. 3. Number of c-Fos immunoreactive neurons (Mean ± SEM) in the prelimbic (PL) and infralimbic (IL) cortex, medial amygdala (MA) and cortical amygdala (CoA) of virgin adult female mice exposed to pups for 15 (A) or 60 min (B). NE (non-exposure), NPB (non-parental behavior), PPB (partial parental behavior), FPB (full parental behavior). Statistically significant differences are indicated with *p < .05 and **p < .01 vs. NE, †p < .05 and ‡p < .01 vs. NPB, and #p < .01 vs. FPB, Fisher's post hoc test.

3.3. Groups of animals exposed to pups for 15 min

By definition, all males included in the IB (n = 7) group attacked the pups with a latency of 98.3 ± 1.6 s without showing any previous parental response, while in the PPB (n = 6) group, 100 % of males licked the pups, 0% retrieved the pups, 83.3 % crouched over the pups, and 16.6 % built a nest during the first 15-min test.

ANOVA analysis revealed no differences among the groups in the PL (Fig. 6A) and IL (Fig. 6A), in the VMHdm (Table 3), VMHc (Table 3), and VMHvl (Table 3), NA core (Table 3) and shell (Table 3); MPOAd (medial; lateral, Table 3), MPN (upper; lower, Table 3), MPOAl (upper; lower, Table 3), and MPOAvl (Table 3).

A significant difference was observed in the number of c-Fos-ir neurons in the CoA (F = 3.1, df = 3, p < .05), but no in the MA (F =

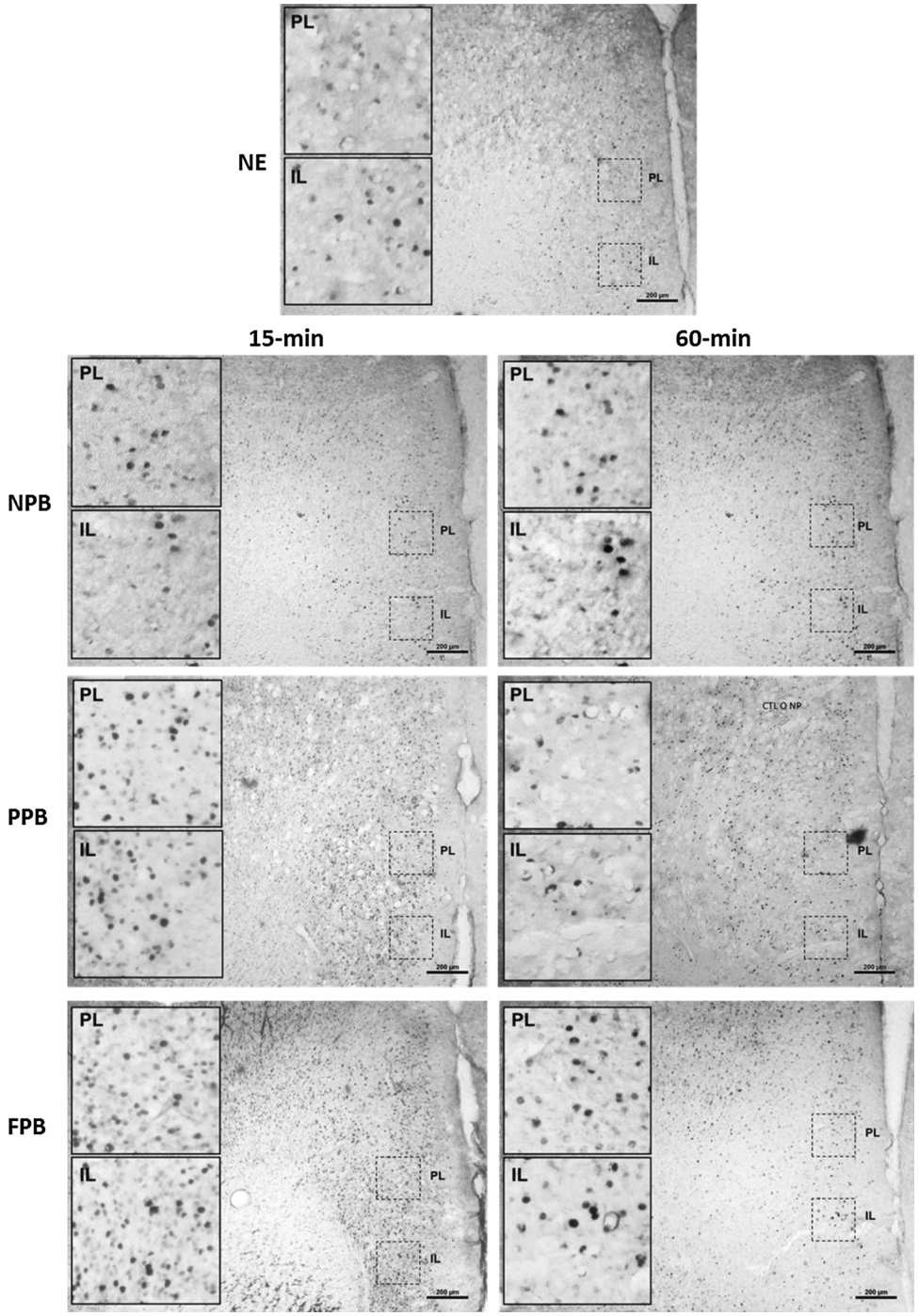

Fig. 4. Representative photomicrographs of c-Fos immunoreactive neurons in the prelimbic (PL) and infralimbic cortex (IL) in adult female mice exposed to pups during 15 (Left) or 60 min (Right). NE (non-exposure), NPB (non-parental behavior), PPB (partial parental behavior), and FPB (full parental behavior). Scale bar is 200 microns.

2.8, df = 3, p = .06). Males that displayed PPB, showed significantly greater number of c-Fos-ir neurons in the CoA (p < .03 vs. NE n = 7, p < .03 vs. OE n = 6, Fig. 6A).

3.4. Groups of animals exposed to pups for 60 min

By definition, all males included in the IB (n = 8) group attacked the pups with a latency of 120.9 ± 2.0 s without showing any previous parental response, while in the PPB (n = 5) group, 100 % of males licked the pups, 0% retrieved the pups, 80 % crouched over the pups, and 20 % built a nest during the first 15-min test. At the end of the 60-min test, 40 % of PPB males were found crouching over at least one pup, and one male transported a pup. In the IB group, 75 % of the males were found biting the wire-mesh ball.

There were no significant differences among the groups in the PL (Fig. 6B) and IL (Fig. 6B).

There was a significant difference in the number of c-Fos-ir neurons in the NA shell (F = 3.4, df = 3, p < .04), but not in the core (F = 1.2, df = 3, p = .32) among the groups. Males that displayed IB and were exposed to pups inside the wire-mesh ball, showed higher level of c-Fos in NA shell compared with NE (n = 7, p < .03, Table 4) and OE (n = 6, p < 0.2, Table 4) groups.

Table 2
Number of c-Fos immunoreactive neurons in virgin adult females exposed to pups for the first time for 60 min.

Brain area	NE n = 8	NPB n = 6	PPB n = 7	FPB n = 8	F (3,26)	P
NA core	14.8 ± 1.5	25.1 ± 5.2	25.9 ± 3.8 *	31.5 ± 3.9 **	4,0	< .02
NA shell	18.7 ± 1.8	28.7 ± 2.6 *	28.2 ± 3.4 *	32.6 ± 3.2 **	4,7	< .01
VMHdm	15.2 ± 1.3	20.3 ± 1.7	21.1 ± 3.5	25.5 ± 3.6	2,3	ns
VMHc	14.8 ± 1.3	20.2 ± 2.6	21.7 ± 2.4	22.0 ± 2.4	2,4	ns
VMHvl	13.7 ± 1.7	20.4 ± 2.3	21.7 ± 3.0	19.8 ± 2.2	2,4	ns
MPOAd medial	23.8 ± 3.7	31.5 ± 3.7	26.6 ± 2.7	33.9 ± 2.2	2,3	ns
MPOAd lateral	21.8 ± 3.9	29.4 ± 2.8	22.6 ± 1.9	31.6 ± 3.3	2,4	ns
MPN upper	27.9 ± 5.5	30.9 ± 5.4	26.6 ± 2.6	30.9 ± 2.7	0,2	ns
MPN lower	25.0 ± 5.7	20.6 ± 5.7	31.2 ± 4.0	34.0 ± 4.2	1,4	ns
MPOA upper	18.7 ± 3.9	28.2 ± 4.6	22.1 ± 1.4	27.0 ± 3.3	1,6	ns
MPOA lower	19.1 ± 3.8	24.5 ± 3.3	23.4 ± 3.9	28.4 ± 2.2	1,4	ns
MPOAvl	27.5 ± 2.1	38.4 ± 5.3	42.4 ± 7.4	48.3 ± 7.0	2,4	ns

NE (non-exposure), NPB (non-parental behavior), PPB (partial parental behavior), FPB (full parental behavior), NA (Nucleus accumbens), VMH (ventromedial nucleus of the hypothalamus), VMHdm (dorsomedial subdivision of the VMH), VMHc (central subdivision of VMH), VMHvl (ventrolateral subdivision of VMH), MPOA (medial preotic area), MPOAd (dorsal region of MPOA), MPN (medial preoptic nucleus), MPOAvl (ventrolateral region of MPOA), ns (nonsignificant). Data are expressed as mean ± SEM of number of c-Fos-immunoreactive neurons in 0.04 mm^2 (NA core, NA shell and subregions of MPOA) or 0.02 mm^2 (subregions of VMH) samples. F and p values for ANOVA are shown in the last two columns. *p < .04, **p < .01 vs. NE group, Fisher's post hoc test.

The expression of c-Fos in the amygdala (MA F = 3.7, df = 3, p < .03; CoA F = 8.0, df = 3, p < .01) showed significant differences (Fig. 6B) among the groups. Males that displayed PPB showed significantly greater number of c-Fos-ir neurons in the MA (p < .01 vs. NE, Fig. 6B) and CoA (p < .03 vs. NE, p < .03 vs. OE, Fig. 6B). Similarly, males that displayed infanticidal behavior, and were exposed to pups inside the wire-mesh ball, revealed significantly higher levels of c-Fos-ir neurons in the MA (p < .03 vs. NE, Fig. 6B) and CoA (p < .01 vs. NE, p < .01 vs. OE, Fig. 6B).

ANOVA did not reveal any significant difference in the number of c-Fos-ir neurons in the in the VMHdm (Table 4), VMHc (Table 4), VMHvl (Table 4), MPOAd (medial; lateral, Table 4), MPN (upper; lower, Table 4), MPOAl (upper; lower, Table 4) and MPOAvl (Table 4) among the groups.

4. Discussion

In the present study, we investigated which brain regions engaged (increased c-Fos immunoreativity) in adult inexperienced females and males exposed to pups for the first time (15 or 60 min) when displaying different behavioral responses. Our main contributions consist in showing that a) parental behavior in females was specifically associated with c-Fos expression in the PL during the first 15 min of exposure; b) NA activated non-specifically in response to the presence of the pups; c) MA and CoA activation in females and males that displayed partial parental behavior (PPB) after 15 min of exposure to pups, but also in infanticidal (IB) males (60 min of exposure), suggested that these subregions participated in the transition from non-parental to parental and/or infanticidal behavioral responses; d) higher c-Fos-ir in some of the subregions of the VMH in parental animals compared to NE, but not to the NPB group, revealed also a non-specific activation of these subregions or a different temporal pattern of activation in this area; e) the time of perfusion after pup exposure (1 h) was not sufficient to detect engagement of the MPOA in any behavior; e) there was no activation of c-Fos specifically associated with infanticidal behavior in any of the areas investigated. In the next paragraph, we will discuss our findings for each specific brain region in the context of previous work in mice and other species.

The role of the prefrontal cortex in maternal behavior has gained importance in recent years [13,45,46,49]. The prefrontal cortex is part of the limbic system and has been associated with the processing of emotions, learning, memory and the participation in the temporal organization of the components of parental behavior [13,45,55]. The classic work of Slotnick and Nigrosh [55] showed that electrolytic lesions of the prefrontal cortex in the mouse, including cingulate cortex, but also PL and IL, led to deficit in parental behavior, during the postpartum period. Moreover, the establishment of preference for offspring associated environments has been associated with higher levels of c-Fos expression in the prefrontal cortex [49]. Besides, inactivation of this area would affect organizational aspects of parental behavior during the postpartum period in the rat [49]. We found that activation of PL was associated with parental animals during the first 15 min of interaction with pups, whereas at 60 min, non-parental animals also showed some increase in the expression of c-Fos both in the PL and IL. Because female mice are induced to display parental behavior very quickly, one possibility is that non-parental animals at 60 min were starting to develop neural changes in the PL or IL cortex to become parental later. Therefore, PL and IL might regulate and/or coordinate temporally the different behavioral components of parental behavior and participate in the decision making or motivational processes that result in the execution of parental behavior [49,55]. Theses cortical subregions could be key to determine the immediate behavioral response to pups chosen by female mice. Although, c-Fos expression in the PL/IL cortical regions did not reach ANOVA significant difference in males, the PPB also showed higher c-Fos than control during the 15 min exposure. Perhaps if the PPB group were larger, the analysis would have passed the statistical requirement of ANOVA. Previous studies have found that temporal expression of c-Fos in the cortex (faster and shorter) is different from the expression in other areas of the brain [9,36,60]. This is an aspect that need to be considered when analyzing current results.

On the other hand, the NA showed consistent non-specific activation in females exposed to pups, suggesting a neural response associated with the processing of pups related information, regardless of the behavior displayed by the animal. Previous studies in rats have also associated activation of the NA with the novelty of the pups [32,44]. Infanticidal males also showed higher c-Fos in the shell subregion of the NA compared to both control groups (NE and OE). However, after 15 min of exposure to pups, c-Fos expression in the NA appeared to be lower than in females. Perhaps, different subpopulation of neurons could have been engaged in the female and male NA in response to pups. Different population of neurons in the shell has been previously associated with the processing of both rewarding and aversive responses [5]. Therefore, the NA might be involved in the processing of the incentive salience of pup-related stimulus that could result either in parental or infanticidal behavior.

The MA, CoA, and VMH are areas associated with the inhibition of parental behavior in rats. These areas are expected to activate during an aversive or neophobic response to pups. Lesions of the amygdala and the VMH facilitated the induction of parental behavior in rats

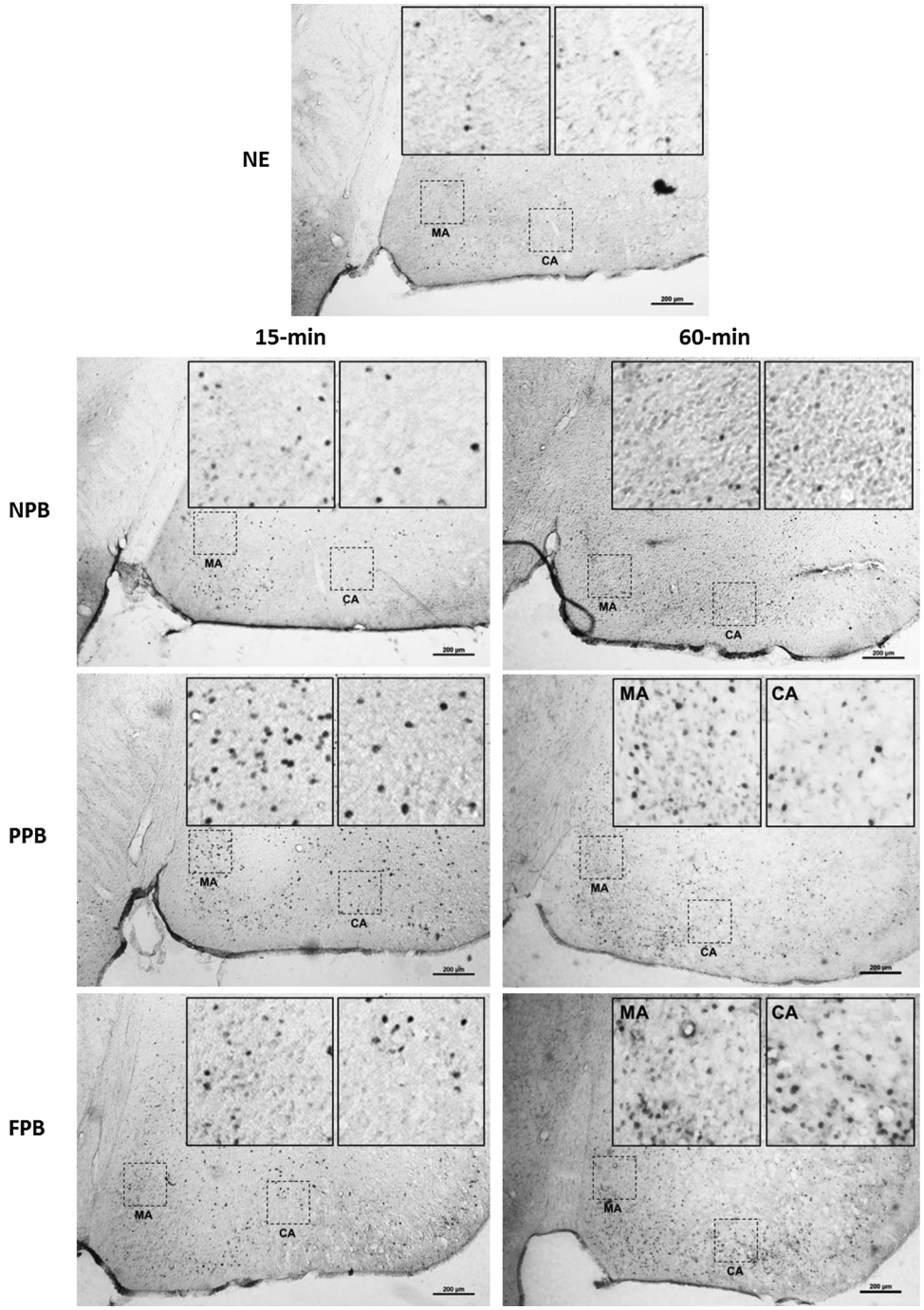

Fig. 5. Representative photomicrographs of c-Fos immunoreactive neurons in the medial (MA) and cortical amygdala (CoA) in virgin female mice exposed to pups during 15 or 60 min. NE (non-exposure), NPB (non-parental behavior), PPB (partial parental behavior), and FPB (full parental behavior). Scale bar is 200 microns.

[6,14,37]. However, the presence of offspring also induced higher c-Fos expression in the CoA/MA of parental female mice and prairie voles [7,23]. Besides, in contrast to rats, lesions of the MA and CoA in prairie voles disrupted parental behavior [23]. In our study, we found an increase in c-Fos expression in the MA and CoA in female and male animals that were partially parental during the first 15 min. However, both maternal females and infanticidal males showed increased c-Fos in these regions of the amygdala after 60 min of exposure. These findings partially agree with previous studies in rats in which non-parental and parental animals showed higher c-Fos in the MA and CoA [42,53]. However, rats and mice differ in how they respond and process pups initially. In contrast to rats, that show a typical approach/withdrawal behavioral response, mice explore pups immediately and intensively, independent of the final behavioral output. Our findings suggest that in the laboratory mice, MA and CoA might be critical for the normal induction of parental or infanticidal behavior, likely processing critical olfactory information that will trigger one or other behavioral response. However, the activation observed in PPB animals might also represent a conflict or transition period between two potential behavioral responses.

The MPOA is known to be critical for the induction and maintenance of parental behavior in mammals [41,45]. For example, parental behavior is associated with the expression of c-Fos and fosB in the MPOA in lactating rats, prairie voles, laboratory and California mice [7,10,23,24,26,27,42,58]. Sensitized rats also show higher c-Fos expression in MPOA [42,56]. A higher c-Fos expression in the dorsal

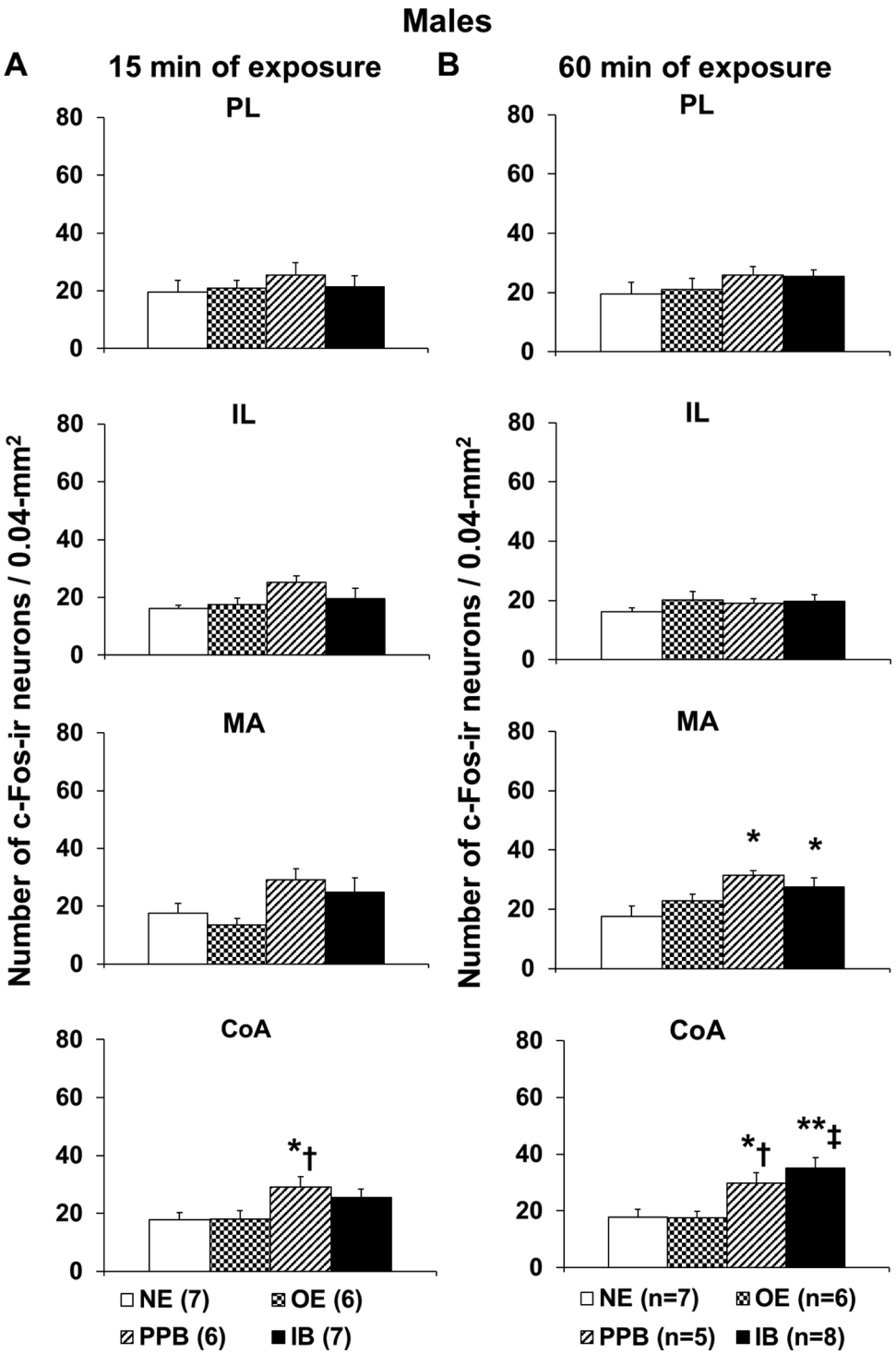

Fig. 6. Number of c-Fos immunoreactive neurons (Mean ± SEM) in the prelimbic (PL) and infralimbic (IL) cortex, medial amygdala (MA) and cortical amygdala (CoA) of virgin adult male mice exposed to pups by 15 (A) or 60 min (B). NE (non-exposure), OE (object exposure), PPB (partial parental behavior), IB (infanticidal behavior). Statistically significant differences are indicated with *p < .05 and **p < .01 vs. NE, †p < .05 and ‡p < .01 vs. OE, Fisher's post hoc test.

region of the MPOA has been previously associated with parental behavior in pregnant female rats [53] and virgin male mice [24]. Lesions in this brain region also induced transient attacks to pups in maternal virgin female mice [58]. In the present work, we did not find an association between this area of the brain and parental behavior. Tsuneoka et al. [58,59] showed that the central region of the MPOA, similar to the subregion we called MPOAl, was critical to display parental behavior in virgins, mother and father mice. Lesions in that brain subregion disrupted parental behavior in inexperienced, lactating female and father male mice [58,59], but also in postpartum mothers and fathers of California mice [26,27].

We must note that most previous works in mice did not use, as controls, animals that were exposed to pups but did not display parental behavior. Therefore, it is possible that previous findings were non-specific, something we revealed in the current study adding more groups. Besides, most previous studies sacrificed animals two hours after the exposure to pups, something that might be required to reach detectable levels in the MPOA. The MPOA has a different temporal

Table 3
Number of c-Fos immunoreactive neurons in virgin adult males exposed to pups for the first time for 15 min.

Brain area	NE n = 7	OE n = 6	PPB n = 6	IB n = 7	F (3,22)	P
NA core	12.8 ± 1.9	13.7 ± 1.6	22.0 ± 3.6	17.5 ± 4.1	1,7	ns
NA shell	17.6 ± 1.7	13.7 ± 2.6	23.3 ± 2.5	20.6 ± 4.7	1,5	ns
VMHdm	15.6 ± 2.2	16.5 ± 1.9	14.5 ± 2.0	15.8 ± 1.8	0,1	ns
VMHc	18.4 ± 2.3	17.1 ± 1.4	14.6 ± 2.2	17.0 ± 2.0	0,5	ns
VMHvl	15.7 ± 2.4	18.8 ± 1.2	14.2 ± 0.8	15.9 ± 1.6	1,2	ns
MPOAd medial	26.5 ± 2.1	31.7 ± 3.1	26.8 ± 3.6	36.9 ± 5.5	1,6	ns
MPOAd lateral	24.2 ± 2.0	23.9 ± 2.5	25.3 ± 4.3	25.1 ± 4.0	0,0	ns
MPN upper	26.9 ± 2.4	26.9 ± 3.6	28.4 ± 3.4	31.0 ± 5.6	0,2	ns
MPN lower	29.1 ± 3.5	27.5 ± 2.9	29.9 ± 4.1	29.5 ± 5.2	0,1	ns
MPOA upper	24.9 ± 2.6	23.2 ± 4.8	27.1 ± 5.0	21.6 ± 5.1	0,2	ns
MPOA lower	22.7 ± 3.4	23.0 ± 5.1	28.0 ± 4.3	22.5 ± 5.3	0,3	ns
MPOAvl	26.5 ± 3.3	38.5 ± 6.0	33.0 ± 5.9	39.5 ± 4.9	1,5	ns

NE (non-exposure), OE (object exposure), PPB (partial parental behavior), IB (infanticidal behavior), NA (Nucleus accumbens), VMH (ventromedial nucleus of the hypothalamus), VMHdm (dorsomedial subdivision of the VMH), VMHc (central subdivision of VMH), VMHvl (ventrolateral subdivision of VMH), MPOA (medial preotic area), MPOAd (dorsal region of MPOA), MPN (medial preoptic nucleus), MPOAvl (ventrolateral region of MPOA), ns (nonsignificant). Data are expressed as mean ± SEM of number of c-Fos-immunoreactive neurons in 0.04 mm^2 (NA core, NA shell and subregions of MPOA) or 0.02 mm^2 (subregions of VMH) samples. F and p values for ANOVA are shown in the last two columns.

pattern of expression than the cortical areas [56], and the fast killing and perfusion of the animals in the current experiment (1 h) may not be sufficient to detect differences in this brain region, commonly observed after 2 h of the exposure to pups. Thus, the lack of c-Fos activation in the MPOA does not exclude the participation of this brain region in the onset of parental behavior. We think that virgin mice rely mainly in cortical regions for the selection of the immediate behavioral response and then that information is transferred to the MPOA without a rapid (1 h) or significative activation of c-Fos expression in this region.

We must also note that juvenile rats, that also show rapid induction of parental behavior, do not express c-Fos in the MPOA [21,44]. However, other rodent species that shows rapid onset of parental behavior shows, in some cases, c-Fos in the MPOA but under different experimental conditions. Kirkpatrick et al. [23] found activation in the MPOA, but after 3 h of exposure to pups. These authors also sacrificed the animals 3 h after the stimulus was first presented. In accordance with our results, De Jong et al. [9,11] found that new fathers but not virgin or paired California mouse males showed activation of the medial preoptic nucleus when exposed to pups inside a wire mesh ball compared to an empty ball. Similar to our study, De Jong et al. studies [9,11] used a period of 1 h and 10 min from the time of exposure to pups to the time of sacrifice.

Previous studies also found greater c-Fos activation in the central subdivision of the VMH in non-parental rats compared to maternal or not exposed virgin females [53]. Greater c-Fos activation in the ventrolateral subdivision of the VMH was also found in non-parental [44], or in both parental and non-parental rats [53], depending of the study, compared to not exposed virgin females. Those results suggested a non-specific activation of the VMH to pup exposure, but also a possible inhibitory role of some of these subregions in parental behavior [44,53]. In addition, greater c-Fos-ir in the VMHvl was found in infanticidal male mice compared to sexually naïve males, or fathers not exposed to pups [57] supporting an inhibitory role of this subregion in parental behavior. In addition, other authors reported the stimulation of the ventrolateral subdivision of the VMH in mice promoted aggression towards males, females or even objects [12,30]. However, we did not find significant differences in the number of c-Fos-ir neurons in any of the subregions of VMH when the four groups were compared. When PPB and FPB were pooled, the analysis of the 60 min exposure revealed a significant increase in c-Fos-ir in the VMHc, and the VMHvl in parental animals that differed only from the NE group. That finding does not support a role for VMHc or the VMHvl in the inhibition of parental behavior in mice, but just a non-specific activation in response to pups, as also found in the VMHvl in a previous study in rats [53]. It is also possible that the VMH, as the MPOA, has a different temporal pattern of expression of c-Fos, and different activation could be found if animals were sacrificed after two hours of testing.

The lack of appropriate control groups in previous studies in mice, the different experimental designs, and the significant behavioral differences that exists in rats, mice and other rodent species make it

Table 4
Number of c-Fos immunoreactive neurons in virgin adult males exposed to pups for the first time for 60 min.

Brain area	NE n = 7	OE n = 6		PPB n = 5	IB n = 8	F (3,22)	P
NA core	12.8 ± 1.9	14.7 ± 1.6	17.1 ± 2.7		17.4 ± 1.7	1,2	ns
NA shell	17.6 ± 1.7	16.9 ± 1.1	23.9 ± 3.0		24.6 ± 2.5 *#	3,4	< .04
VMHdm	15.6 ± 2.2	24.0 ± 2.0	19.3 ± 3.5		21.6 ± 2.0	2,3	ns
VMHc	18.4 ± 2.3	24.1 ± 1.6	22.3 ± 4.4		24.0 ± 1.6	1,3	ns
VMHvl	15.7 ± 2.4	22.8 ± 1.6	21.6 ± 4.2		21.7 ± 2.4	1,9	ns
MPOAd medial	26.5 ± 2.2	23.6 ± 2.3	30.5 ± 4.6		25.3 ± 2.5	0,9	ns
MPOAd lateral	24.2 ± 2.0	22.9 ± 1.3	29.1 ± 2.8		24.3 ± 1.9	1,5	ns
MPN upper	26.9 ± 2.4	22.4 ± 2.4	27.4 ± 4.2		26.2 ± 1.2	0,8	ns
MPN lower	29.1 ± 3.5	20.0 ± 1.4	29.1 ± 5.7		24.8 ± 2.0	1,7	ns
MPOA upper	24.9 ± 2.6	21.5 ± 2.4	27.9 ± 5.4		21.3 ± 0.9	1,2	ns
MPOA lower	22.7 ± 3.4	19.9 ± 1.9	25.0 ± 3.0		23.7 ± 1.6	0,6	ns
MPOAvl	26.5 ± 3.3	28.7 ± 2.9	36.2 ± 2.9		35.7 ± 1.7	2,5	ns

NE (non-exposure), OE (object exposure), PPB (partial parental behavior), IB (infanticidal behavior), NA (Nucleus accumbens), VMH (ventromedial nucleus of the hypothalamus), VMHdm (dorsomedial subdivision of the VMH), VMHc (central subdivision of VMH), VMHvl (ventrolateral subdivision of VMH), MPOA (medial preotic area), MPOAd (dorsal region of MPOA), MPN (medial preoptic nucleus), MPOAvl (ventrolateral region of MPOA), ns (nonsignificant). Data are expressed as mean ± SEM of number of c-Fos-immunoreactive neurons in 0.04 mm^2 (NA core, NA shell and subregions of MPOA) or 0.02 mm^2 (subregions of VMH) samples. F and p values for ANOVA are shown in the last two columns. *p < .03 vs. NE group, #p < 0.02 vs. OE group, Fisher's post hoc test.

necessary to be cautious when translating results from one specie to another. In the current study we show for the first time that the prelimbic cortex specifically participates in the immediate onset of parental behavior in female mice. We also show for the first time that 15 min of exposure to pups is sufficient to detect associations between c-Fos and behavioral responses to pups. We also highlight the importance of using appropriate control groups to reveal the participation of the brain in the processing of pups, and the expression of parental or infanticidal behavior. Finally, analysis of the temporal engagement of the different brain regions can also reveal new and novel insight about adaptive neural changes that occur and prepare an animal to take rapid behavioral decisions. The different time of sacrifice might differently detect the engagement of brain regions implicated in the onset of parental behavior. If the animals were killed after two hours of testing, perhaps the PL/IL engagement could not be detected due to the rapid pattern of increase and decrease of the expression of the c-Fos protein in those cortical subregions [9]. Although optogenetic approaches are now helping to reveal the neural basis of behavior in mice, classic experimental approaches need also to be considered in order to understand the particularity of mice immediate behavioral responses to pups.

Acknowledments

The present study was supported by two research grants to D.E.O. and M.A. by the Comisión Sectorial de Investigación Científica (CSIC), UdelaR. We thank the staff of the Animal Facility of the Facultad de Medicina for providing care to our animals and the appropriate conditions to carry out these experiments.

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