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Juvenile Rats Show Reduced c-*fos* Activity in Neural Sites Associated With Aversion to Pups and Inhibition of Maternal Behavior

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Abstract

Juvenile rats (18–23 days old) interact avidly with pups as novel stimuli and show maternal behavior after only 1–3 days of pup exposure; adults initially avoid pups and require 3–9 days of pup exposure. Upon exposure to pups as novel stimuli, adults had more c-Fos-immunoreactive neurons in the hypothalamus and amygdala—regions associated with aversion to pups—than adults exposed to familiar pup stimuli (maternal) or not exposed to pups ($p < .05$). In juvenile rats exposed to pups as novel stimuli, only the medial amygdala had a small significant increase of c-Fos neurons. In juveniles, this blunted engagement of c-Fos neurons may reflect the diminished activation of inhibitory neurons, facilitating the interaction of juveniles with pups as novel stimuli and onset of maternal behavior.

Keywords

accumbens; amygdala; hypothalamus; MPOA; development

Maternal behavior in the rat consists of an orderly sequence of behaviors, including nest building, retrieving pups to the nest, and then nursing them; anogenital cleaning; and defending the pups from intruders (Numan, 1994; Rosenblatt & Lehrman, 1963). Although parturient females immediately express maternal behavior after giving birth, rats that are not parturient can be induced to perform most aspects of maternal behavior by continuous exposure to foster pups. Pup-induced maternal behavior in virgin females, males, and juvenile rats includes an orderly sequence of robust maternal nest building, pup retrieval, and cleaning and adoption of nursing postures (Brunelli & Hofer, 1990; Kalinichev, Rosenblatt, & Morrell, 2000; Olazábal, Kalinichev, Morrell, & Rosenblatt, 2002; Rosenblatt, 1967; Stern, 1987). It is interesting that the onset of maternal behavior after pup exposure is substantially more rapid in juveniles than in adults. Preadolescent juvenile rats (18–24 days of age; also referred to as *late infant* stage) show maternal behavior immediately or after a brief exposure to pups (1–3 days), whereas adult rats (> 60 days of age) without benefit of parturitional hormones require 3–9 days of pup exposure to be induced into maternal behavior (Bridges, Zarrow, Goldman, & Denenberg, 1974; Brunelli, Shindledecker, & Hofer, 1985; Fleming & Luebke, 1981; Kalinichev, Rosenblatt, & Morrell, 2000; Mayer & Rosenblatt, 1979b; Olazábal et al., 2002; Stern, 1987).

The odor of pups and other pup-related stimulus properties initially induce an aversive or neophobic response in adult rats that is overcome only after several days of exposure to pups (Fleming & Luebke, 1981; Mayer & Rosenblatt, 1975). In contrast to adults, juveniles do not

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avoid pups; instead, they approach pups immediately, remain in contact with them, and subsequently start to perform maternal behavior much sooner (Brunelli et al., 1985; Mayer & Rosenblatt, 1979b; Reiss, Smith, & Morrell, 2003; Smith & Morrell, 2003; Stern, 1987). This avidity for pup stimuli may be linked to the rapid onset of maternal behavior in preadolescent juveniles and suggests they are less neophobic to novel stimuli.

We are exploring systematically the hypothesis that preadolescent juveniles are less neophobic to any novel stimulus. In other experiments (Reiss et al., 2003; Smith, Gonzalez, & Morrell, 2003), we found that preadolescent juveniles (18–21 days old) were statistically significantly more likely to approach any novel stimulus across such diverse categories as social, object, or food stimuli, and they were more likely to engage longer with novelty than adults. From among all the novel stimuli we offered them, the pup as a novel stimulus elicited the longest initial engagement from both adults and juveniles; this was a statistically significantly greater interaction with live pups (adults, 16% of test time) that was much more continuous than with warm dead pups or warm pup-sized objects (adults, 3% of test time), age peers, or objects with a variety of sizes or interactive capacities. However adults and juveniles differed markedly on the initial phases of interaction with live pups. Although both adults and juveniles rapidly investigated live pups, the adults initially engaged with the pups for very limited time (16% of test period) and thereafter avoided pups for days. Meanwhile juveniles had a prolonged initial interaction (66% of test time) that continued unabated. The initial preference for pups became a marked and adult-like initial aversion to pups as preadolescent juveniles matured into the earliest pubertal phase of adolescence (24–26 days of age). At this age, the length of pup exposure required to overcome aversion to pups as novel stimuli and induce maternal behavior started to increase toward adult-like exposure times because of unknown developmental changes (Brunelli et al., 1985; Mayer, Freeman, & Rosenblatt, 1979; Mayer & Rosenblatt, 1979a; Zaias, Okimoto, Trivedi, Mann, & Bridges, 1996).

The components of the neural circuits that mediate response to novel stimuli, including the neophobic responses of the adult, are not known. For an initial analysis of some of the structures that might mediate novelty or neophobic responses to pups, we considered brain regions known to mediate the inhibitory and, for comparison, the facilitatory aspects of maternal behavior. The medial amygdala (MA) and cortical amygdala (CA), the dorsal and anterior hypothalamic nuclei (DH/AH), and the ventromedial nuclei of the hypothalamus (VMH) have been associated with the inhibition of maternal behavior and, thus, hypothetically with the initial aversive response to pups in adult rats (Bridges, Mann, & Coppeta, 1999; Fleming, Vaccarino, & Luebke, 1980; Morgan, Watchus, & Fleming, 1997; Numan, Numan, & English, 1993). When lesions were made in these brain regions, maternal behavior was induced more rapidly; that is, fewer days of pup exposure were required for the full expression of maternal behavior (Bridges et al., 1999; Fleming et al., 1980; Numan et al., 1993). The MA and AH/VMH are thought to function in series in a circuit that underlies inhibition of maternal behavior or aversive– defensive responses to pups (Sheehan, Paul, Amaral, Numan, & Numan, 2001). Recently, Numan and colleagues (Sheehan, Cirrito, Numan, & Numan, 2000) found that these and other regions show an increased number of neurons that express c-Fos immunoreactivity (c-Fos-ir) when nonmaternal females are exposed to pups for the first time. A reasonable interpretation of these data is that the initial c-Fos response in these structures occurs in neurons that are part of the proposed inhibitory circuit. There are two corollary hypotheses: first, that in inhibitory areas, there would be less c-Fos responsiveness in the maternal state after presentation of pups and, second, after pups become familiar stimuli as evidenced by maternal responsiveness, different brain regions might well be more responsive. These are likely to be brain regions already understood to mediate maternal responsiveness, for example the nucleus accumbens (NAC) or the medial preoptic area (MPOA).

The oncogene c-*fos* is member of the *fos* immediate early gene (IEG) family, which is activated in response to a variety of stimuli and conditions (Ebert, Gernert, Loscher, & Richter, 1996; Fleming & Walsh, 1994; Gréco, Edwards, Michael, & Clancy, 1998; Harris, 1998; Luckman, 1995; McCarthy, Besmer, Jacobs, Keidan, & Gibbs, 1997; Numan & Numan, 1994; Park & Carr, 1998; Wang, Guldenaar, & McCabe, 1997; Wirtshafter, Stratford, & Shim, 1998). This particular IEG has been used extensively to study the neural circuit that mediates the active expression of maternal behavior in postpartum females or adult females that have been induced to display maternal behavior by exposure to pups (Brown, Ye, Bronson, Dikkes, & Greenberg, 1996; Fleming & Walsh, 1994; Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Lonstein, Simmons, Swann, & Stern, 1998; Numan & Numan, 1994; Sheehan et al., 2000). We and others have found that juveniles engaged in maternal behavior show a different pattern of neurons expressing c-*fos* than do adults engaged in maternal behavior (Gonzalez & Fleming, 2002; Kalinichev et al., 2002b). These studies and others that are based on lesions suggest that in juveniles, the components of the neural circuit that mediate maternal behavior do not have the same level of function as in the fully matured adult (Gonzalez & Fleming, 2002; Kalinichev, Rosenblatt, & Morrell, 2000; Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Olazábal et al., 2002; Oxley & Fleming, 2000).

In this study, we continued to use c-Fos-ir as a marker for neurons engaged in behavioral processes, and we compared the pattern and number of neurons engaged during the response of preadolescent juveniles and adults to pups as novel stimulus versus pups as familiar stimuli. We hypothesized that the differential behavioral response of the juveniles and adults will result in a differential response of neurons engaged in this behavioral response. We examined both juveniles and adults that had been (a) exposed to pups for the first time, (b) were induced into maternal state by continuous pup exposure, or (c) were not exposed to pups (controls). We chose pups as novel stimuli on the basis of data from our experiments on novelty (Smith et al., 2003; Smith & Morrell, 2003) for several reasons. First, pups are the novel stimulus that most engages both adults and juveniles compared with a considerable repertoire of other novel stimuli, making it likely to be a sufficient stimulus to evoke a differential c-Fos response. Second, although the pup stimulus is novel, it results in a very different response from juveniles (rapid initial investigation, then continued engagement) versus adults (rapid initial investigation, then avoidance) that, as the stimulus becomes familiar, results in a similar final response pattern: maternal behavior with significant interaction with the stimulus. Thus, both the response to pups as novel and pups as familiar are likely to evoke a response measurable by c-Fos.

We hypothesized that in juveniles, brain areas that mediate inhibition of maternal behavior (e.g., MA, CA, AH, VMH) will be differentially engaged compared with adults when they are exposed to pups for the first time, similar to the findings of Sheehan et al. (2000). Unique to this study, we compared juveniles and adults. There may be either more engagement of neurons that inhibit interaction with pups in the adult or more engagement of neurons that disinhibit interaction with the pup in the juvenile. For additional comparisons, we analyzed c-Fos expression in two brain regions, the MPOA and the NAC, that have been proven to have significant engagement during the expression of maternal behavior (Fleming, Suh, Korsmit, & Rusak, 1994; Lee, Clancy, & Fleming, 1999; Numan, Rosenblatt, & Komisaruk, 1977). The MPOA has also been shown to respond in a manner that suggests facilitatory and inhibitory responses exist side by side (Sheehan et al., 2000), suggesting that a higher order of integrative complexity exists in this region.

Method

Subjects

The rats used in these experiments were obtained from our colony maintained in the Rutgers University Laboratory Animal Facility (Newark, NJ), which is accredited by the Association for the Accreditation and Assessment of Laboratory Animal Care. This colony consists of offspring of rats originally purchased from Charles River Laboratories (Wilmington, MA); the colony is maintained as genetically consistent with their Sprague-Dawley CD strain by regular and frequent purchase of males and females to serve as breeders.

Female rats were bred with males recently purchased from the same source. Adult rats were housed in groups of 2 adults until the experiment, when they were individually housed in cages measuring 53 × 36 × 25 cm, with transparent Plexiglas walls. Juveniles were weaned at the age of 19–21 days and individually housed until the next day when the experiment started. All females were kept on a 12-hr light– dark cycle and in a stable environmental temperature of 22 °C with ad-lib access to food and water. Wood shavings (BETA CHIP and NEPCO, North Eastern Products Corp., Warrensburg, NY) were used to cover the floor of the cages. All procedures used in this study followed the standards approved by the National Institutes of Health (1986)*Guide for the Care and Use of Laboratory Animals* and the Rutgers University Animal Care and Facilities Committee.

Fifteen virgin adult females (60–100 days of age) and 13 juvenile females (19–21 days of age) were submitted to one of the following behavioral treatments.

No pup exposure—These females were housed in isolation with no exposure to pups for ~5 days (6 adults and 5 juveniles).

One exposure to pups (i.e., pups as novel stimuli)—These females were exposed to six pups for the first time in their home cage for 2 hr. After 2 hr of pup exposure, the rats were killed. None of these females showed any of the components of maternal behavior (4 adults and 4 juveniles).

Continuous exposure to pups until maternal, and pup reexposure (i.e., pups as familiar stimuli)—These females were induced into maternal behavior by continuous exposure to pups in their home cages. After they showed complete maternal behavior for 4 consecutive days, pups were removed and the subjects isolated overnight. The next morning, six pups were returned to their home cage, allowing the females to engage in maternal behavior for 2 hr, and after that they were killed (5 adults and 4 juveniles). This sequence was applied to the maternal animals because the separation of pups from maternal animals and their return for 2 hr before killing have been successfully used to demonstrate the brain regions that are engaged by maternal behavior; continuous exposure to pups in maternal animals blunts the c-Fos response (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Stack & Numan, 2000).

The average number of days of pup exposure required to induce maternal behavior was 4.8 ± 1.1 days (*Mdn* = 3 days) for adults and 1.7 ± 0.5 days (*Mdn* = 1.5 days) for juveniles; the more rapid onset of maternal behavior in the juveniles was expected and agreed with our previous findings (Olazábal, Abercrombie, Rosenblatt, & Morrell, 2004) for this age group. Thus, when they were killed, maternal adults had been exposed to pups on average for 8.8 days (*Mdn* = 7 days) and were maternal for 4 of those days, with the last day of maternal behavior being a 2-hr exposure followed by killing. When they were killed, the maternal juveniles had been exposed to pups on average for 5.7 days (*Mdn* = 5.5 days), with the last day of maternal behavior being a 2-hr exposure to pups followed by killing, at Days 25–27. It is important to point out that by the time juveniles that were initially exposed to pups as preadolescents entered a period

of development (24–28 days) in which aversion to pups on the first exposure to pups occurs, all the juveniles were already maternal. Maternal rats do not show any aversive or inhibitory response to pups, as shown in previous studies (Mayer & Rosenblatt, 1979b).

Maternal Behavior Testing

This procedure has been described in detail in our previous studies (Kalinichev, Rosenblatt, & Morrell, 2000; Olazábal et al., 2002) and consists of continuous exposure of females to pups in their home cages. Females were supplied daily with newborn pups (2–7 days old) that remained in the cages until the next morning. Pups were exchanged daily with freshly fed pups provided by donor mothers, not included in these experimental groups. These donor mothers nursed the pups and kept them healthy and warm. Shredded paper towels were provided as nesting material. Maternal behavior was assessed by a daily maternal care test consisting of an analysis of qualitatively and quantitatively scored components of maternal behavior according to our previously published protocol (Kalinichev, Rosenblatt, & Morrell, 2000; Olazábal et al., 2002). Briefly, pups that had spent the night with the experimental animals were removed before the test, and the overnight state of the nest was recorded. Immediately thereafter, freshly cared for foster pups were placed in the cage in a corner diagonally opposite the original nest site. The following behavioral responses were recorded: retrieving, crouching, and pup licking. Retrieving required that females pick pups up in their mouth and carry them back to the nest where they licked them anogenitally and on the body. Crouching was assessed during a 20-min observation period and was measured as the time spent hovering over at least two pups, usually in the maternal nest. Nests were destroyed, nest material was scattered in all quadrants of the cage, and nest building was evaluated again the next day. Quality of nest was rated as 0 (*there was no nest*), 1 (*the nest was poor and not all the nest material was used*), 2 (*the nest was flat but all the paper was used*), 3 (*the nest had in addition low or medium walls*), or 4 (*the nest had high walls*). A nest score of 2 was accepted as a maternal nest. The first of 2 consecutive days in which the animals exhibited maternal behavior was considered the first day of maternal behavior and was used in our calculation of how many days of pup exposure were required to induce maternal behavior.

Immunocytochemistry

To identify the number of cells that express c-Fos-ir, the following immunoreactive procedures, previously published by our laboratory (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000), were used. At the appropriate point of the behavioral procedures, all animals were deeply anesthetized with sodium pentobarbital, 0.2 mL heparin (1,000 units/mL) was injected in the left ventricle of the heart, followed by systemic perfusion with 100 mL phosphate-buffered saline (PBS), pH 7.4, followed by 400 mL of 4% paraformaldehyde in the same PBS. In this and all additional procedures, the PBS was 25 mM. Brains were removed from the skulls and stored in the same fixative medium at 4 °C for 24 hr. After that, brains were placed in 15% sucrose–PBS solution until sectioning, within 48 hr of killing. A sliding microtome with a freezing platform was used to cut 40-μm-thick frozen sections of the brains. Five sets of serial sections of the brain regions of interest were collected in an ethylene-glycol based cryoprotectant solution (Watson, Wiegand, Clough, & Hoffman, 1986) and stored at −20 °C.

On the first day of the immunocytochemical procedure, the sections were removed from the freezer, allowed to equilibrate to room temperature for 1 hr, rinsed with five changes of PBS, and then placed in 4% normal goat serum (Vector Laboratories, Burlingame, CA) diluted in 0.3% Triton X-100/PBS (pH 7.2–7.4) for 1 hr to block nonspecific binding of the antibody. Sections were then incubated in the primary antibody anti-c-*Fos* (Ab-5, a rabbit polyclonal antibody; Oncogene Research Products, San Diego, CA), diluted (1:2,000) in a 1% solution of normal goat serum in PBS at 4 °C for 48 hr, and then rinsed in PBS and incubated for 1 hr in biotinylated goat anti-rabbit secondary antibody (diluted 1:400 in 1% normal goat serum/

PBS). Then sections were rinsed with PBS and placed for 1 hr in avidin–biotin complex (ABC) reagent from an ABC Elite Kit (Vector Laboratories). The tissue was rinsed in PBS and 100 mM sodium acetate buffer. The final reaction used a solution of nickel sulfate, diamino benzidine, and hydrogen peroxide in aqueous sodium acetate. After 2 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, and coverslipped. To control for and assess the variability among different assays of the immunocytochemical runs, the same number of animals from each behavioral group was processed simultaneously in each run. Pilot immunostaining assays were performed to assess the best dilutions and optimal reaction conditions. Additional details are described elsewhere (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000).

Microscopic Analysis

The analysis of the immunoreactive neurons was performed by applying our standard previously published methods (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000). Brain sections were examined microscopically for c-Fos-ir neurons recognized by their nuclear black or dark purple stain. The number of neurons that expressed c-Fos within 0.142-mm^2 samples that were taken from approximately four sections of each brain region of interest was counted and averaged. To count cells in the different subregions of the VMH (central, dorsomedial, and ventrolateral), 0.055-mm^2 samples were used instead. Brain sections from individual rats from different experimental groups were processed simultaneously so that the impact of technical variability from batch-to-batch differences in immunocytochemistry was limited. For each brain, a complete series of sections through the areas of interest was always processed as a unit of tissue; in addition, for many brains, multiple series of sections were processed to allow appropriate comparisons across experimental groups within a given immunocytochemistry (ICC) batch. This ICC processing strategy allowed us to prove empirically that the number of c-Fos-ir positive neurons found in any given area for a particular experimental condition was stable in series of sections processed in different ICC batches. This tissue-processing strategy also resulted, however, in some differences in the total number of sections analyzed across subjects; the number of sections analyzed for each subject in a particular brain region thus varied from a minimum of two and up to six sections. The labeled cells were counted only on one side of the section.

Our criterion for a labeled neuron was one in which the immunoreactive c-Fos staining was considerably darker than background staining as judged by eye using a Zeiss (Thornwood, NY) microscope. Then, we used densitometric methods to measure precisely the density of the immunoreactive neurons compared with the density of the surrounding background in the region of the immunoreactive neurons by using our automated computer-based image analysis system, the Microcomputer Imaging Device (MCID M4; Imaging Research, Inc., Catharines, Ontario, Canada). Those neurons identified as immunoreactive by eye had c-Fos-ir in their nuclei that was on average at least two to three times the level of immunoreactivity found in the surrounding background neuropil or other nonimmunoreactive neurons; this criterion made the inclusion of false-positive neurons extremely unlikely. All other details of our quantitative methods and production of the photomicrographs were the same as previously specified (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000).

Statistical Analysis

Data on the number of immunoreactive neurons in each brain region were tested for the requirements of normality and homogeneity of variance, and then by two-way analysis of variance (ANOVA; age and group as factors) followed by Fisher's protected least significant difference post hoc test. A *t* test was used for limited specific comparisons when a significant

difference was found for group or age. The level of statistical significance was set at .05. Data were analyzed using the StatView statistical package (SAS Institute, Inc., Cary, NC).

Results

Patterns of c-Fos Immunoreactive Neurons in the Amygdala and Hypothalamus

Adult behavioral group comparisons—The MA, CA, AH as well as the ventrolateral and dorsomedial subdivisions of the VMH all responded similarly in that first exposure to pups increased the number of c-Fos-ir neurons, whereas exposure to pups, once the adults were maternal, did not. Adult rats that were exposed to pups for the first time (not maternal) had significantly more neurons that expressed c-Fos in the MA, ($F = 14.0$, $df = 2$, $p < .0001$; CA, $F = 3.6$, $df = 2$, $p < .05$); and the ventrolateral subregion of the VMH (VL), ($F = 7.9$, $df = 2$, $p < .005$) compared with adults with no exposure to pups or maternal adult females exposed to pups ($p < .05$; Figures 1, 2, 3, and 4). These rats also had more neurons that expressed c-Fos-ir in the AH ($F = 6.5$, $df = 2$, $p < .01$) and the dorsomedial subregion of the VMH ($F = 4.3$, $df = 2$, $p < .05$), but they were significantly different only respect to the rats that had no exposure to pups ($p < .05$; Figures 1, 2, 3, 4, and 5). This increase in the number of c-Fos-ir neurons ranged from 50% greater in the VL, to 95% greater in the CA, to a maximal increase in the MA of 125% over adults with no exposure to pups or maternal adults exposed to pups. Adults with no exposure to pups and maternal adults exposed to pups did not differ in the number of c-Fos-ir neurons in the MA, CA, VMH, or AH. There were no statistically significant differences among any of the adult behavioral groups in the number of c-Fos–labeled cells in the central subregion of the VMH (Figures 1 and 4).

Juvenile behavioral group comparisons—Only the MA of juvenile rats that were exposed to pups for the first time (not maternal) had significantly more neurons expressing c-Fos-ir compared with the other two juvenile groups ($p < .05$; Figure 2). This c-Fos expression was 57% greater than that of juveniles with no exposure to pups or maternal juveniles exposed to pups. There were no statistically significant differences among number of c-Fos-ir neurons in the AH, CA, or VMH across the three juvenile groups (Figures 3, 4, and 5). In the VL subregion of the VMH, note that juveniles that were exposed to pups for the first time had more c-Fos–labeled cells than the no-pup-exposure group but this increase did not reach statistical significant ($p = .06$).

Adults compared with juveniles—The MA, CA, dorsomedial subdivision of the VMH, and AH showed the same trend: The adults had more c-Fos-ir neurons after first exposure to pups than did the juveniles, and maternal groups exposed to pups and those without pup exposure were not different from each other in adults or juveniles. Adults exposed to pups for the first time had more c-Fos-ir neurons in the MA, CA, and the dorsomedial subdivision of the VMH than did juveniles, but these differences were not statistically significant. There was an age effect for the AH ($F = 6.5$, $df = 2$, $p < .01$). The AH of all adult groups had significantly more c-Fos-ir neurons than did the comparable juvenile groups ($p < .05$; Figure 3). There were no adult versus juvenile differences in the central or VL subregions of the VMH.

Patterns of c-Fos Immunoreactive Neurons in the Accumbens

Adult behavioral group comparisons—Exposure to pups for the first time resulted in a statistically significantly greater number of c-Fos-ir neurons in the NAC shell ($F = 8.4$, $df = 2$, $p < .05$) and core ($F = 4.4$, $df = 2$, $p < .05$). In the shell, the number of c-Fos-ir neurons was 137% higher in adults exposed to pups for the first time compared with isolates or maternal adults ($p < .005$; Figure 6). The NAC core of adults exposed to pups for the first time also showed more neurons that expressed c-Fos-ir compared with adults with no exposure to pups or with maternal adults exposed to pups; only the comparison with maternal adults was

statistically significant ($p < .05$). As in other regions, no difference was found in either the shell or core between adults with no pup exposure and maternal groups.

Juvenile behavioral group comparisons—There were no differences in the number of c-Fos-ir neurons in the NAC shell across all three juvenile groups. In the NAC core, juveniles exposed to pups for the first time showed slightly more neurons that expressed c-Fos-ir compared with juveniles with no pup exposure or maternal juveniles, but this difference was not statistically significant.

Adults compared with juveniles—The number of c-Fos-ir neurons in NAC shell was statistically different across the two age groups ($F = 6.2$, $df = 2$, $p < .05$). Again, in contrast to juveniles, which showed no change, in the adults the first exposure to pups resulted in substantially more c-Fos-ir neurons (130%; $p < .05$) in the shell. This trend continued in the core, where there were more reactive neurons in the adults first exposed to pups; however, this was not statistically different from the juveniles. The number of c-Fos-ir neurons in the shell and core was the same in the juvenile and adult groups that were not exposed to pups or were maternal.

Patterns of c-Fos Immunoreactive Neurons in the MPOA

Adult behavioral group comparisons—Comparison of the three experimental groups demonstrated a statistically different number of c-Fos-ir neurons in the MPOA ($F = 3.5$, $df = 2$, $p < .05$). Maternal adults had statistically significantly more neurons expressing c-Fos-ir than did adults with no pup exposure ($p < .05$; Figure 7). Adults exposed to pups for the first time also showed similar higher numbers of neurons expressing c-Fos-ir compared with isolates, but this did not reach statistical significance ($p = .056$). Maternal adults and those exposed to pups for the first time were not different from each other in the number of neurons expressing c-Fos-ir in the MPOA.

Juvenile behavioral group comparisons—Juveniles exposed to pups for the first time showed higher numbers of neurons that express c-Fos-ir in the MPOA compared with juveniles with no exposure to pups or maternal juveniles, but this difference did not reach statistical significance ($p = .07$; see Figure 7). Juveniles with no pup exposure and the maternal group had the same number of c-Fos-ir neurons.

Adults compared with juveniles—Both juveniles and adults showed similar higher numbers of c-Fos-ir neurons after first exposure to pups compared with animals not exposed to pups. Maternal adults tended to have higher number of c-Fos-ir neurons than did maternal juveniles, which were not different from juveniles with no pup exposure. Neither difference was statistically significant.

Discussion

Given that juvenile rats are avidly interested in pups as novel stimuli and that adult rats, after brief investigation, actively avoid them, we hypothesized that particular brain areas would be differentially engaged when juveniles and adults were exposed to pups as novel stimuli. We found this differential responsiveness with first exposure to the pup stimulus in the accumbens, amygdala, VMH, and AH. Adults had more c-Fos-ir neurons and were more inhibited in their initial interaction with pups. In general, juveniles had a blunted response of c-Fos-ir neurons in these regions, even though the MA in particular did have increased c-Fos-ir neurons with first pup exposure. Taken together, the data suggest that the c-Fos–responsive neurons are behaviorally inhibitory and that the behavioral differences between adult and juvenile rats are explained by increased inhibition in the adult, not disinhibition in the juvenile. Because the

juveniles were not inhibited in their interactions with pups as novel stimuli, even with c-Fos response in some of these regions, this component of the data suggests that a concerted inhibitory response from all of these regions is needed for adult-like behavioral inhibition to emerge.

After the pup stimulus became familiar and the rats were in a maternal state, these regions no longer responded. The response of the MPOA was unique in two dimensions. First, this was the only brain region that we examined that had more c-Fos–positive neurons in response to pups as familiar stimuli, and this was the case only in the adults. The fact that the adult MPOA responded to the pup both as a novel and as a familiar stimulus suggests important dual functions in this region, which has been established as critical for the integration of responses leading to maternal behavior (Numan & Insel, 2003). Second, the MPOA of both the juvenile and adult rat responded to the initial presentation of pups as novel stimuli to an equal extent. Consistent with our interpretation of the responses in the regions such as AH and VMH, as discussed above, we hypothesize that these are inhibitory responses in the MPOA. Sheehan et al. (2000) also found patterns that suggested that facilitatory and inhibitory responses exist within the MPOA. Lonstein and DeVries (2000) found GABA-ergic, c-Fos–responsive neurons in the MPOA of lactating females, which suggests that this type of neuron could participate in these inhibitory or disinhibitory functions within the MPOA. Thus, our data and those of Sheehan et al. (2000) suggest that within the adult MPOA, both inhibitory and facilitatory functions may exist side by side, within different subsets of neurons.

Most of the brain regions had similar levels of basal responsiveness of c-Fos-ir neurons in animals that had no pup exposure, a finding that agrees with our prior findings in juveniles compared with adults (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000). The exception to this was the AH, where all juvenile groups had fewer c-Fos-ir neurons than all the adult groups. The uniformity of this difference suggests that in the AH, more general developmental changes have yet to occur.

Our data support the general view that the AH, dorsomedial VMH, MA, and CA amygdaloid nuclei participate in either the inhibition of maternal behavior or the aversive– defense responses on first exposure to pups but do not facilitate the expression of maternal behavior in the adult. Our data agree well with that of others who have demonstrated that nonmaternal females had more neurons expressing c-Fos in the MA, VMH (both dorsomedial and ventrolateral subdivisions), and AH than did maternal females (Sheehan et al., 2000), and electrical stimulation of the MA reduced maternal responsiveness (Morgan et al., 1997; Morgan, Watchus, Milgram, & Fleming, 1999). Our interpretation also agrees with the hypothesis of Sheehan et al. (2001), who proposed a neural circuit involving MA and AH–VMH for the inhibition of maternal behavior. The CA has received less attention but several studies show activation of c-Fos in this brain region by pup stimulation or the expression of maternal behavior (Fleming et al., 1994; Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Lonstein et al., 1998; Numan & Insel, 2003; Walsh, Fleming, Lee, & Magnusson, 1996). This brain region may be critical for the processing of olfactory information that can be threatening or aversive for the adult.

In accordance with the roles of the MA and CA in the processing of sensory information relevant for maternal behavior (Fleming & Walsh, 1994; Walsh et al., 1996) and in the inhibition of maternal behavior (Fleming et al., 1980; Numan et al., 1993), we expected and found that as the rats adapted to pup stimuli as they become maternal, the inhibition of maternal behavior and the expression of c-Fos in these inhibitory regions would decrease. Indeed, c-Fos expression in the MA, CA, and NAC has been shown to depend on sensory stimuli (Fleming & Walsh, 1994; Walsh et al., 1996), and the number of neurons that express c-Fos in the MA declines to control values after 12 hr of continuous pup exposure (Stack & Numan, 2000).

Similarly, others (Nakahara et al., 1999; Rosen, Chuang, & Iadarola, 1994) have reported desensitization of c-Fos expression in several brain regions, including the NAC and amygdala, after repeated self-stimulation or chronic treatment with the same stimuli.

Similar to the response pattern in the amygdala, VMH, and AH, the NAC, particularly the shell, was very responsive to first exposure to pups in adult rats. A role for the nucleus accumbens has been proposed in the mediation of neophobic and anxiety responses: It acts as an interface between emotional stimuli and behavioral responses (Barrot et al., 2002; Burns, Annett, Kelley, Eberitt, & Robbins, 1996; Roozendaal & Cools, 1994). Although there are no data from others that are specific to the initial response of the nucleus accumbens to the first contact with a pup stimulus, in the maternal adult, pup exposure increases the number of c-Fos-ir neurons in the shell (Lonstein et al., 1998; Stack, Balakrishman, Numan, & Numan, 2002). We did not find this increase with pups as familiar stimuli; in our study, the shell was responsive only to pups as novel stimuli. Our data agree with that of Stack et al. (2002) that there is no c-Fos responsiveness in the core of the accumbens in the maternal adult.

Most of our findings in the response of adults in the maternal state to pups as familiar stimuli confirm our previous work and that of others, suggesting that we document a fundamental and stable neural basis for maternal behavior. We are, however, not surprised that some differences emerge across different laboratories and experiments. For example Numan and Numan (1994), Fleming et al. (1994), and Kalinichev, Rosenblatt, Nakabeppu, and Morrell (2000) found a greater number of neurons expressing c-Fos in the MA, CA, and accumbens (as discussed above) in maternal adult females. Although we do not know which of the experimental variables is key to generating the differences across the studies, several variables that could contribute are the period of separation from pups before testing maternal behavior (24 hr, 48 hr, or 76 hr), the time the subjects performed maternal behavior before being killed (2 hr, 2 days, or 4 days), the duration of the interaction between the subjects and the pups on the day they were killed (1 hr vs. 2 hr), the different hormonal status of the subjects (lactating, hormonally stimulated, intact, or ovariectomized females), the latencies to become maternal, and the number of days that nonmaternal versus maternal animals were exposed to pups (Fleming et al., 1994; Gonzalez & Fleming, 2002; Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Numan & Numan, 1994; Sheehan et al., 2000; Stack & Numan, 2000).

In the present study, we chose to use animals that performed maternal behavior for 4 days prior to final test so that maternal animals would be completely adjusted to the pups. We considered it possible that adults in which the maternal state induced by pup exposure was emerging might not yet be at a stable maternal state if they were maternal for fewer days. In these animals, the overnight pup deprivation and then reexposure called for in our paradigm may still induce some negative reactivity to pups. This reactivity may occur only during the first few days of pup-induced maternal behavior but not if the deprivation and next-day reexposure is performed after many days of maternal experience. Because our goal was to compare the central nervous system response with a completely novel or a completely familiar pup stimulus, we used only adults that had been fully maternal for 4 days. Maternal subjects were then deprived of pups overnight prior to the final pup exposure of 2 hr, a sequence of experimental manipulations that has previously been shown to produce optimal c-*fos* expression (Gonzalez & Fleming, 2002; Kalinichev, Rosenblatt, & Morrell, 2000; Stack & Numan, 2000).

In this study, we focused on comparing the response of juveniles and adults with live pups as novel versus familiar stimuli, and we will in future studies examine in more detail the question of the interaction with nonsocial stimuli that are either novel or familiar. Others have compared the c-Fos response of virgin adults to pups as novel stimuli with their response to objects (marbles) as novel stimuli and have found a greater response to the pups as novel stimuli (Sheehan et al., 2000). This differential c-Fos response could be due to the fact that the virgins

interact quantitatively less (3% of test time in our data, as discussed in the introduction) with objects than they do with live pups (16% of the test time in our data) or because there are qualitative differences in the nature of the stimulus, temperature, and olfactory and somatosensory aspects that result in different interactions (Smith & Morrell, 2003). It is known that the intensity of the stimulus also affects c-Fos expression (Campeau & Watson, 1997; Kovács, 1998). Although it is possible that novel social stimuli such as live pups do engage more or different neurons than do novel objects, this distinction would minimally require analysis of neurons that were engaged after an equal duration of behavioral interaction, and with consideration of sensory quality of the object stimulus. One way to ensure a comparable interaction that would reveal the uniqueness of the pup versus the object interaction, and not simply a predictable difference in response that was due to a difference in interaction time, would be to use the novelty shuttle approach (Ambert & Struthers, 2003) in which different novel objects are provided so that the time spent interacting with various stimuli is of the same duration.

The c-Fos response of juveniles that were responding to pups as novel stimuli was generally very blunted compared with adult responses, with the exception of the response in the MA and MPOA. These two regions had increases in c-Fos-ir neurons after first pup exposure. We propose that the avid interaction (or reduced inhibitory response) with pups as novel stimuli in juveniles compared with adults may be mediated in part by these blunted responses in the inhibitory regions of the neural circuit for maternal behavior. Furthermore, we propose that the change from the juvenile level of interaction to the full behavioral inhibition of adults requires inhibitory responses in all components of the neural circuit. How these regions might participate in the generally less neophobic response of juveniles to other novel stimuli, which has been suggested by previous studies (Reiss et al., 2003; Smith & Morrell, 2003; Spear, 2000), remains to be determined.

Our data suggest that in juvenile rats, there is incomplete development of these brain regions in terms of their engagement in the inhibition of maternal behavior. This may be similar to stressor-induced Fos immunoreactivity in the CA and MA, which is also not completely developed in 28-day-old rats (Kellogg, Awatramani, & Piekut, 1998). Furthermore, in the juvenile, the partial responsiveness of the MA to first exposure to pups suggests that its inhibitory function is only partially developed, as proposed by Oxley and Fleming (2000).

The data from the MPOA in the juvenile rat showed the most complex picture overall, from the developmental perspective. First, our data agree with and extend to the juvenile rat the hypothesis of Sheehan et al. (2000) that perhaps this area has a role in the aversion or inhibitory response (in both adults and juveniles) when pups are presented as novel stimuli and, in contrast to most other areas we examined, that the inhibitory role of this region may be completely developed in juveniles.

In contrast, the response of the MPOA to pups as a familiar stimulus to juveniles in the maternal state did not include increased c-Fos-ir neurons, whereas in adults there was an increase. This difference in the maternal rats, according to their age, confirms our previous data and supports the hypothesis that the neural circuit that mediates the expression of maternal behavior or the sensory and physiologic mechanisms supporting the induction of maternal behavior are not completely mature in juvenile rats (Kalinichev, Rosenblatt, & Morrell, 2000; Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Olazábal et al., 2002). As discussed below, the MPOA may still be critical for maternal behavior, as suggested by the effect of very large lesions (Kalinichev, Rosenblatt, & Morrell, 2000), but the immature stage of development seems either to require a differential temporal activation of the c-*fos* gene or does not require a continuous expression of this gene after several days of maternal experience.

Our data on the MPOA and MA brain regions also confirm that the juvenile rat brain can respond with an increase in c-Fos-ir neurons, as seen in the case of the juvenile response to pups as novel stimuli, but that there are other development differences, which we believe are related to the neurotransmitter systems, as discussed below. The finding of Gonzalez and Fleming (2002) in regards to the number of c-Fos-ir neurons of the juvenile rat's MPOA also showed an increased number of c-Fos-ir neurons with pups as stimuli.

Our findings differ from Gonzalez and Fleming (2002) in that neither in our current or prior work (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000) did we find increases in the MPOA in maternal juvenile rats. Careful examination of the paradigms across the laboratories suggests a possible explanation for this. Gonzalez and Fleming demonstrated increased c-Fos-ir in juvenile MPOA that had 4 days of exposure to pups, but in contrast to our juveniles, most of their juveniles were not maternal after 4 days of pup exposure. Gonzalez and Fleming considered number of days of pup exposure rather than maternal or nonmaternal condition to group their animals. The finding by Gonzalez and Fleming that, after 4 days of pup exposure, most juveniles were not maternal and showed c-Fos expression in the MPOA may be similar to the trend in c-Fos found in females exposed to pups for the first time in our study; that is, perhaps this is a continuation of the response to pups as novel stimuli. Subsequently, after 8 days of pup exposure, Gonzalez and Fleming's rats were almost all maternal and c-Fos in the MPOA was higher. We did not examine animals after this lengthy exposure to pups.

This complex mosaic of developmental differences within the MPOA suggests that perhaps specific neuronal subsets within each region may underlie these different phases of the state-dependent behavioral responses to pup stimuli. Presumably different neurochemistry and interconnections within the MPOA develop in slightly different time frames, such that the adult state of facilitation emerges somewhat later postnatally than the adult state of inhibition, because of a thus far unknown process. The initial processing of pup sensory information by the MPOA on first pup exposure appears to be completely developed in juveniles. One possibility is that juveniles do not need continuous c-Fos expression to maintain responsiveness to pups. Other transcription factors or a different and immature neurochemical system may be able to maintain the reinforcement of pups without continuous c-Fos activation in the MPOA after repeated pup presentation.

In these experiments, we kept all of our pup stimulus exposure procedures exactly the same for juveniles and adults. Although it is theoretically possible that a different temporal course of pup exposure would induce c-Fos in juveniles, the fact remains that we have established a material difference in the response of juveniles compared with adults both to pups as novel and pups as familiar stimuli (when animals are maternal). These data show that juveniles have a differential response to the stimulus, and this suggests that they have an immature stage of neural development in their response to pups. What that difference is remains to be determined in future work.

We hypothesize that the key adult–juvenile differences lie in the developmental state of the afferent stimulation of c-Fos responsive neurons. These developmental differences may be very specific. We and others have documented differences in neurotransmitter components of both the serotonergic and dopaminergic system in juveniles of this age and adults. These differences are in the density of dopamine receptors and dopamine content (Olazábal et al., 2004; Tarazi & Baldessarini, 2000; Tarazi, Tomasini, & Baldessarini, 1998) in the NAC. We have also found more serotonin in the MPOA of the juvenile compared with the adult rat (Olazábal et al., 2004). Dopamine participates in the mediation of the novelty response in the NAC (Bardo, Donohew, & Harrington, 1996; Burns et al., 1996), whereas the role of serotonin in the MPOA on pup stimuli processing remains to be investigated.

Consider the response of the MPOA, which in the juvenile has similar adult c-Fos responsiveness when presented with pups as a novel stimulus but has virtually no response when the pup has become a familiar stimulus and the juvenile is maternal. Certainly the MPOA neurons have the molecular capacity for Fos responsiveness, but it is possible that certain neurotransmitter-specific afferents are not yet developmentally complete. The *N*-methyl-$_{D}$-aspartate (NMDA)–dopamine interaction has been shown to be critical for c-Fos expression in some of the brain regions studied here (Hussain, Flumerfelt, & Rajakumar, 2001) and is proposed to be critical in the synaptic changes involved in reinforcement and associative learning. It is possible that there is a different NMDA–dopamine interaction in juveniles compared with adults explaining the differential Fos response specific to a very particular behavioral response.

c-Fos immunoreactivity in neurons is generally accepted as a marker of neuronal engagement during a variety of processes, including complex behavioral processes, sensory and motor processes, and the effects of drugs on the central nervous system. The specific molecular mechanism of the action of the c-Fos protein in the context of the response to novelty or maternal behavior is not known, as is the case for most stimulus–response processes that include a c-Fos response. Better understanding of these phenomena and the neurochemistry associated with maternal behavior may reveal the role of c-Fos expression in the different regions of the neural circuit that supports or inhibits maternal behavior.

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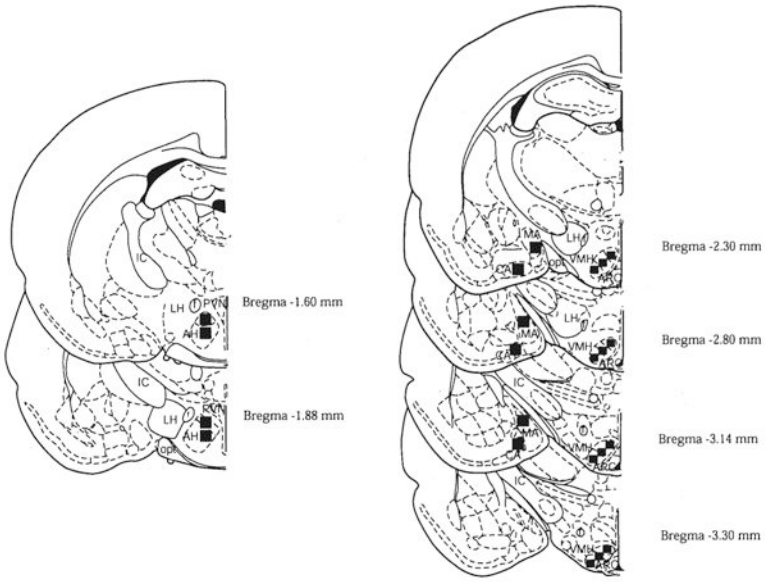

Figure 1.

Diagrams of the rat brain showing the location of the samples at several levels of the anterior hypothalamus (AH), medial amygdala (MA), cortical amygdala (CA), and the ventromedial nucleus of the hypothalamus (VMH) where number of neurons were counted. The black box represents the sample. IC = internal capsule; f = fornix; PVN = paraventricular nucleus of the hypothalamus; opt = optic chiasm; LH = lateral hypothalamus; ARC = arcuate nucleus. Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Figure 11, Copyright 1998, with permission from Elsevier.

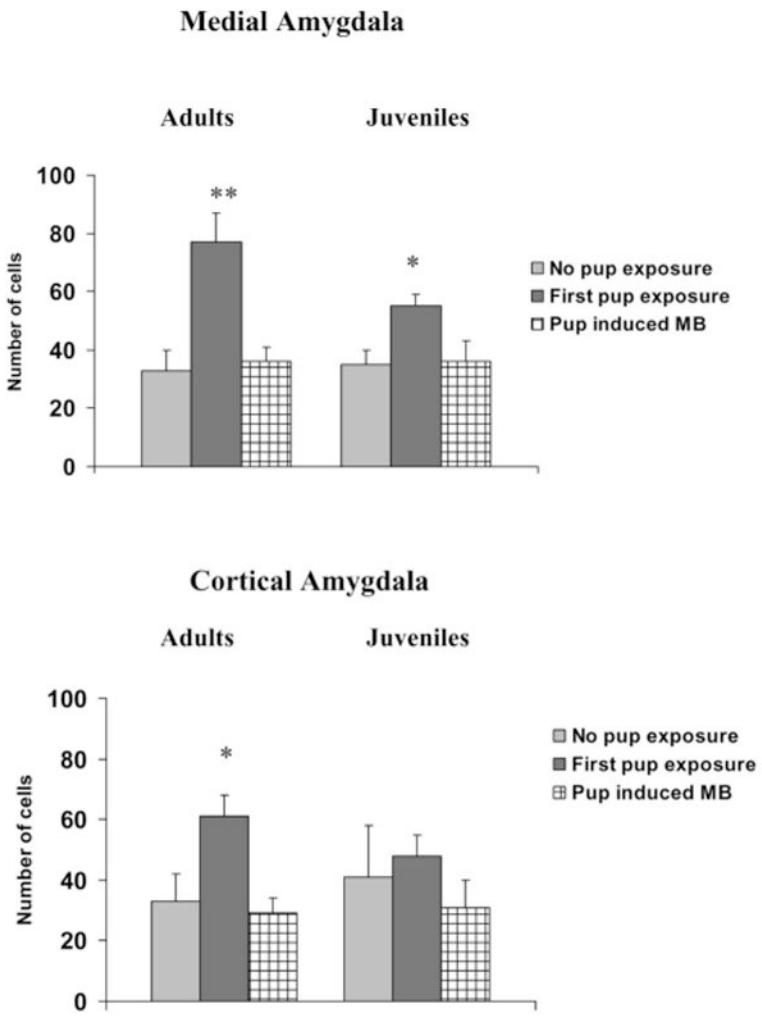

Figure 2.

The graphs indicate the number of c-Fos–immunoreactive (ir) neurons in the medial amygdala (top) and cortical amygdala (bottom) in adult and juvenile rats with no pup exposure, after first pup exposure, or after induction of maternal behavior by long-term pup exposure. Statistically significant differences with respect to the other adult or juvenile groups are indicated by * ($p < .05$) and ** ($p < .01$). Bars in the histograms show the average number of Fos-ir neurons (± *SEM*) in a 0.142-mm^2 sample.

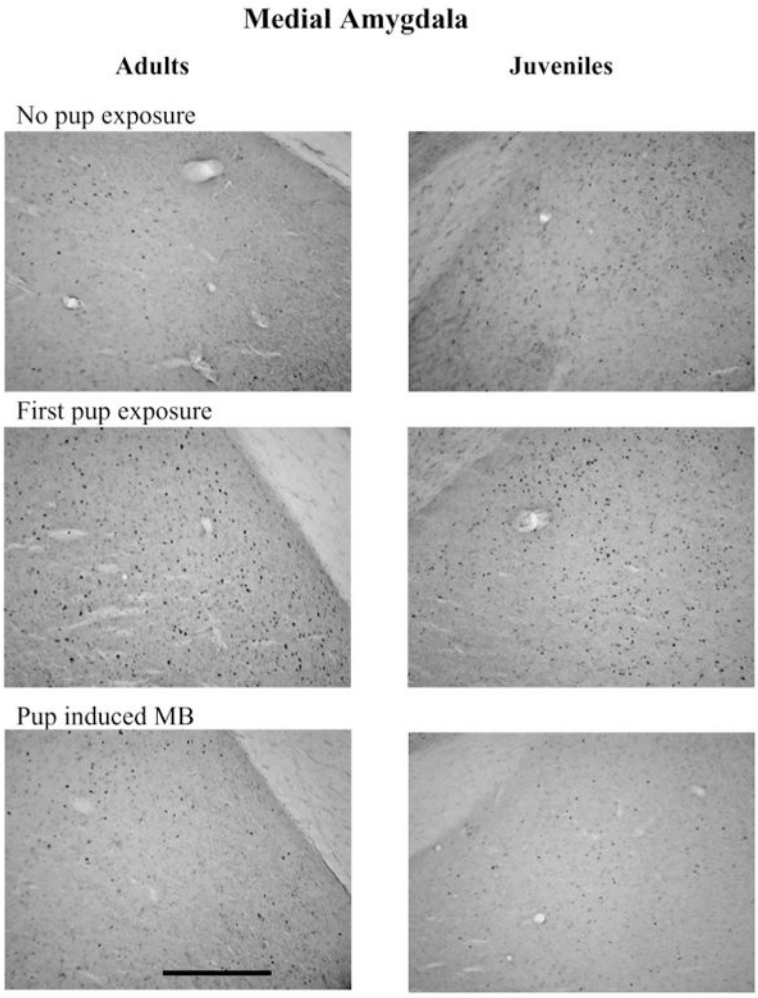

Figure 3.
Representative photomicrographs of neuronal nuclei that are positive for c-Fos-immunoreactivity in the medial amygdala in adult and juvenile rats with no pup exposure, first pup exposure, or after pup-induced maternal behavior (MB). Scale bar is 300 microns.

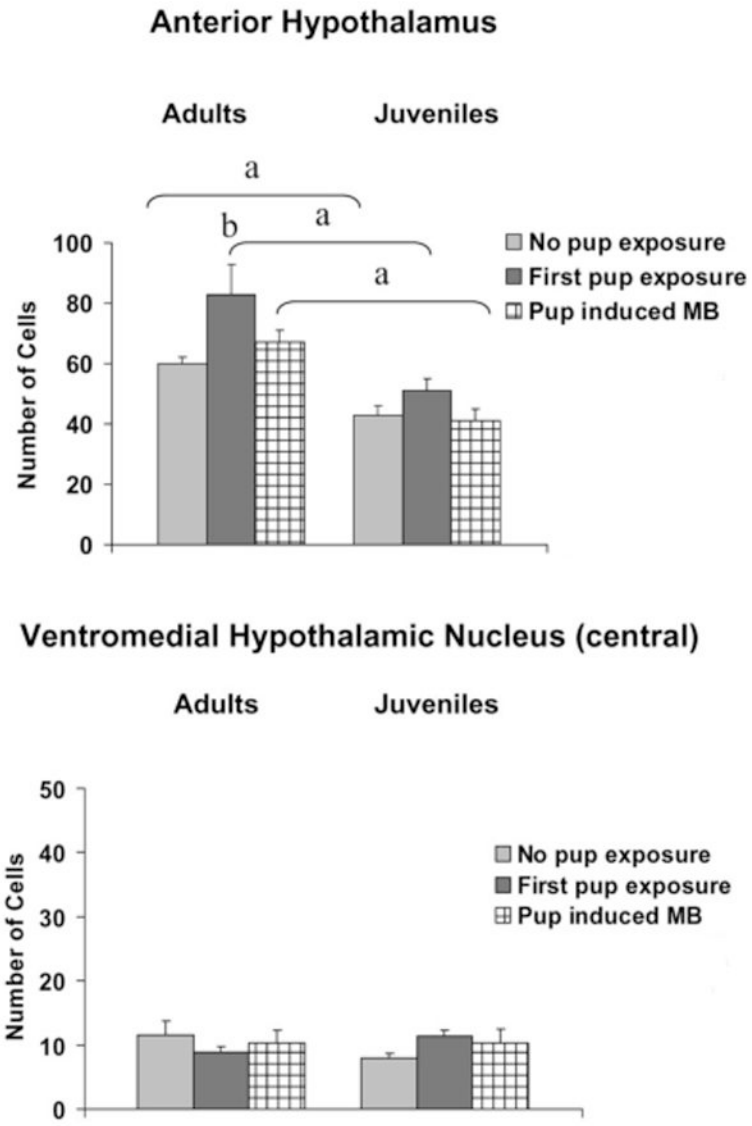

Figure 4.

The graphs indicate the number of c-Fos–immunoreactive (ir) neurons in the anterior hypothalamus (AH; top) and central subregion of the ventromedial hypothalamic nucleus (VMH; bottom) in adult and juvenile rats with no pup exposure, first pup exposure, or pup-induced maternal behavior (MB). A statistically significant difference with respect to the isolated group with no exposure to pups is indicated with *b* ($p < .05$). A statistically significant difference between adults and juveniles of comparable behavioral groups is indicated with *a* ($p < .05$). Bars in the histograms show the average number of c-Fos-ir neurons (± *SEM*) in 0.142-mm^2 (AH) or 0.055-mm^2 (central VMH) samples.

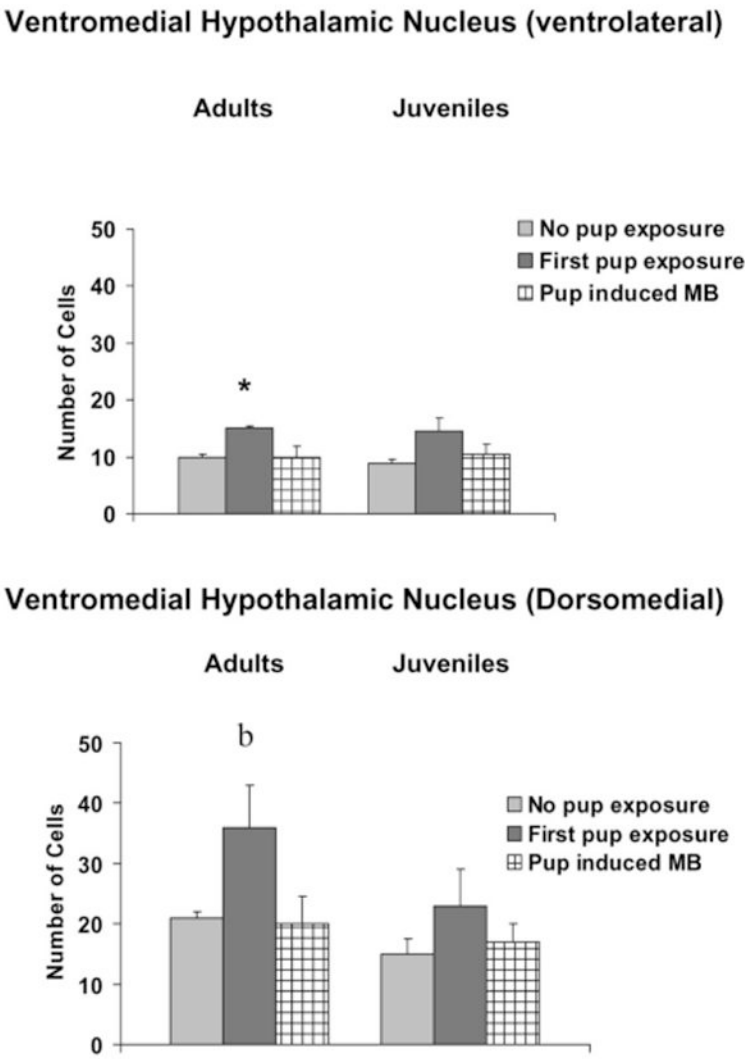

Figure 5.

The histograms indicate the number of c-Fos–immunoreactive (ir) neurons in the ventrolateral (top) and dorsomedial (bottom) subregions of the ventromedial hypothalamic nucleus of the hypothalamus in adult and juvenile rats with no pup exposure, after first pup exposure, or pup-induced maternal behavior (MB). A statistically significant difference within a subregion across the behavioral groups is indicated with * ($p < .05$). A statistically significant difference between pup-exposed and isolated groups (no pup exposure) is indicated with *b* ($p < .05$). Histogram bars show the average number of c-Fos-ir neurons (± *SEM*) in a 0.055-mm^2 sample.

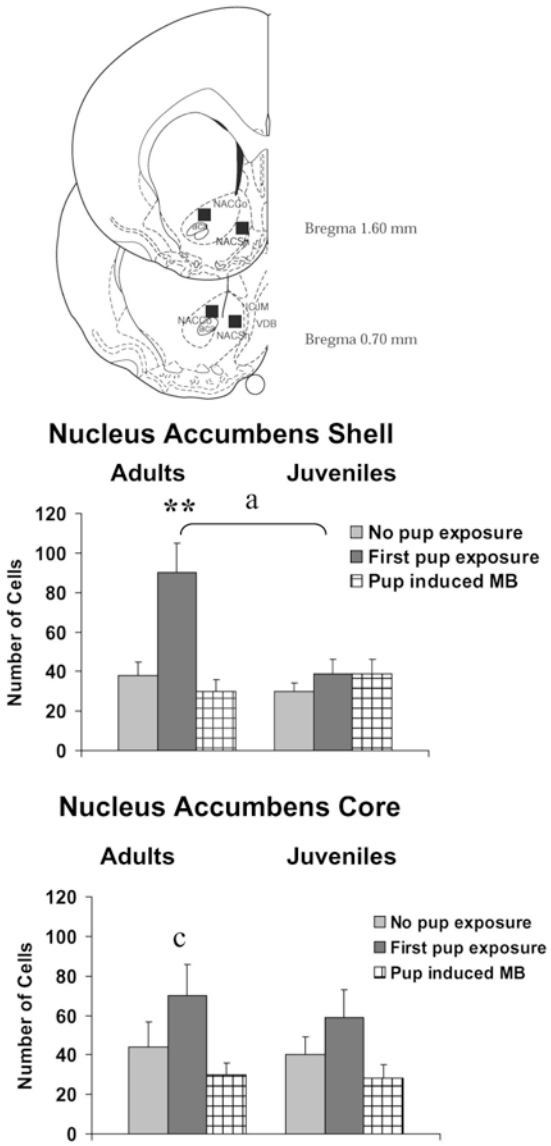

Figure 6.

Top: Diagram of the rat brain showing the location of the samples at several levels of the nucleus accumbens (NAC) where the number of c-Fos–immunoreactive (ir) neurons was counted. The black box represents the sample size. Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Figure 11, Copyright 1998, with permission from Elsevier. Histograms at the bottom represent the number of neurons (± *SEM*) that expresses c-Fos-ir in the NAC shell and core. A statistically significant difference between adult groups is indicated by ** ($p < .01$). A statistically significant difference between nonmaternal and maternal groups is indicated with *c* ($p < .05$). A statistically significant difference between adults and juveniles across comparable behavioral groups is indicated with *a* ($p < .05$). Histogram bars show the average number of c-Fos-ir neurons (± *SEM*) in a 0.142-mm^2 sample.

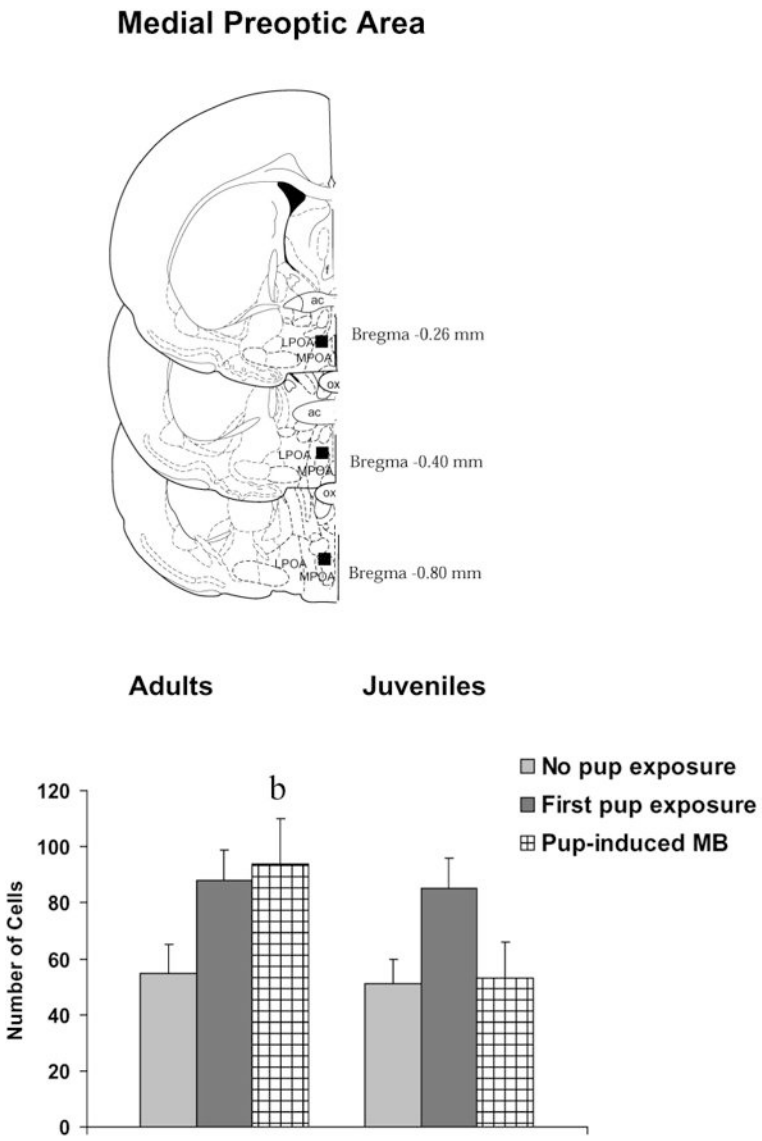

Figure 7.

Top: Diagrams of the rat brain showing the location of the samples at several levels of the medial preoptic area (MPOA) where the number of neurons was counted. The black box represents the sample size. Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Figure 11, Copyright 1998, with permission from Elsevier. Histograms at the bottom indicate the number of c-Fos–immunoreactive (ir) neurons in the MPOA in adult and juvenile groups with no pup exposure, first pup exposure, or pup-induced maternal behavior (MB). A statistically significant difference with respect to the isolated, adult group never exposed to pups is indicated by *b* ($p < .05$). Histogram bars show the average number of c-Fos-ir neurons (± *SEM*) in a 0.142-mm^2 sample.