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MPOA Cytotoxic Lesions and Maternal Behavior in the Rat: Effects of Midpubertal Lesions on Maternal Behavior and the Role of Ovarian Hormones in Maturation of MPOA Control of Maternal Behavior

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Small neurotoxin lesions in the medial preoptic area (MPOA) block maternal behavior (MB) in adults but large lesions are required to produce the same effect in juvenile rats (23–27 days of age). To study the maturation of MPOA control of MB, in Experiment I, we compared the effects of small versus large neurotoxin MPOA lesions at midpuberty (38 days of age) on MB. Midpubertal females with large MPOA lesions showed severe impairment in MB affecting retrieving, crouching, and nest building, but 85% of females with small MPOA lesions exhibited all components of MB and performed like control females without MPOA lesions. To study the role of ovarian hormones during puberty on the maturation of MPOA mediation of MB (Experiment IIA), females were ovariectomized either before or after puberty and small MPOA cytotoxic lesions were made at 53 days of age. At 60 days of age both groups showed similar deficits in MB which indicated that the maturation of the MPOA mediation of MB is not dependent on pubertal ovarian hormones. In Experiment IIB, we administered estradiol benzoate (sc) and this overcame the deficit in MB after small MPOA lesions in females that had been deprived of estrogen for shorter periods (30 days) but had not been deprived for longer periods (60 days). In addition, ovary-intact females with circulating estrogen and small lesions in the MPOA at 53 days of age did not show deficits in MB.

Key Words: **estrogen; hormones; maternal behavior; MPOA; ovaries; puberty; rat.**

Several studies have shown that the medial preoptic area (MPOA) is necessary for the expression of all components of maternal behavior in adult (53 days old) female rats (Kalinichev, Rosenblatt, and Morrell, 2000a; Numan, 1974, 1994; Rosenblatt, Hazelwood, and Poole, 1996). Lesions in the MPOA disrupt both hormonally and pup-induced maternal behavior in females (Jacobson, Terkel, Gorski, and Sawyer, 1980; Numan, 1974, 1994; Numan, Rosenblatt, and Komisaruk, 1977).

Maternal behavior can be elicited in juvenile females (18 to 29 days of age) by presenting them with young pups, a procedure which is called sensitization (Bridges, Zarrow, and Denenberg, 1974; Brunelli, Shindledecker, and Hofer, 1985; Brunelli and Hofer, 1990; Cosnier and Couturier, 1966; Gray and Chesley, 1984; Mayer and Rosenblatt, 1979; Rosenblatt, 1967; Stern, 1987; Wiesner and Sheard, 1933). This procedure has enabled us to study the role of the MPOA in juvenile maternal behavior. After exposure to pups, prepubertal rats express the typical behavioral pattern shown by maternal adults (nestbuilding, retrieving, licking pups, and crouching postures). Initially, around day 18 postpartum, a few components of maternal behavior are exhibited and over the next 2 weeks the number increases until the behavior of juveniles more closely resembles the behavior exhibited by adults and is organized in adult-like sequences, with fewer intrusions of nonmaternal play behaviors (Brunelli and Hofer, 1990; Mayer, Freeman, and Rosenblatt, 1979). Early juvenile females and males

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(<24 days) show maternal behavior faster than adults do when exposed to pups (~2.0 vs ~4.0 days; Mayer *et al.*, 1979; Mayer and Rosenblatt, 1979; Stern, 1987). However, at the beginning of puberty (30 days of age) latencies to express maternal behavior increase as the juveniles begin to avoid the pups (Fleming and Luebke, 1981; Mayer and Rosenblatt, 1975; Mayer *et al.*, 1979; Stern, 1987).

Our previous work has shown that in 23-day-old females the MPOA appears to be necessary only for the expression of retrieval behavior and nest building, whereas in adult females the MPOA also controls crouching over pups and licking them, which are also components of maternal behavior. Specifically, we found that very large lesions of the MPOA were capable of blocking retrieval behavior and nest building in 23-day-old females but other components of maternal behavior (licking, crouching posture) were unaffected (Kalinichev *et al.*, 2000a). In sensitized adult females even very small lesions in the MPOA blocked the induction of all components of maternal behavior (Gray and Brooks, 1984; Jacobson *et al.*, 1980; Rosenblatt *et al.*, 1996) but these small lesions were totally ineffective in juveniles (Kalinichev *et al.*, 2000a). In the present series of experiments we utilized the differential response to small cytotoxic lesions of the MPOA in juveniles and adults to study the maturation of MPOA control of maternal behavior.

There is an association in adults between the performance of maternal behavior and the activation of the immediate early genes *c-fos* and *fosB* in the MPOA and other brain areas implicated in the circuitry mediating maternal behavior (i.e., ventral bed nucleus of the stria terminalis and the medial and cortical nuclei of the amygdala; Numan and Numan, 1994). Juvenile females, by contrast, exhibited no increase in these measures of activation in these brain regions; the only exception was in the lateral habenula, which exhibited increases in *c-fos* associated with the performance of maternal behavior (Kalinichev, Rosenblatt, Nakabeppu, and Morrell, 2000b). Together with the data based on the lesion approach, these results suggest that developmental changes during puberty contribute to the maturation of the neural circuitry underlying the performance of adult maternal behavior.

In Experiment I we extended our previous work investigating the maturation of the role of the MPOA in the regulation of pup-induced maternal behavior, i.e., sensitized maternal behavior. Our previous work demonstrated that in the adult the MPOA had to be virtually intact for normal maternal behavior to occur: in the adult even quite small lesions of the MPOA severely disrupt such maternal behavior. However, in the prepubertal female only very large lesions impair the behavior and it could be maintained even in the absence of much of the MPOA. In the present study we investigate specifically the developmental status of the MPOA at midpuberty by determining the effect of MPOA lesions on sensitized maternal behavior. We found that the behavior could not be sustained at any age with large lesions in the MPOA. However, because smaller lesions in midpubertal females did not impair maternal behavior we concluded that MPOA control of maternal behavior was not yet mature.

In Experiment IIA we hypothesized that the maturation of MPOA with respect to its specific role in the regulation of pup-induced maternal behavior is dependent upon ovarian hormones during puberty. Females were ovariectomized before or after puberty and subjected to small MPOA lesions postpubertally. We found that ovarian hormones did not mediate the development of these behavioral functions of the MPOA. That is, the adult role of the MPOA in mediating sensitized maternal behavior developed normally, regardless of the presence or the absence of ovarian hormones during puberty.

Our attention then shifted to the role of postpubertal estrogen on the behavioral regulation of the young adult MPOA. In Experiment IIB we hypothesized that estrogen would overcome the impairment of maternal behavior produced by small MPOA lesions in adulthood, since the behavior was restored by estrogen replacement to non-brain- lesioned ovariectomized females (Mayer and Rosenblatt, 1980). Adult females with small lesions were given acute estrogen replacement at varying times after ovariectomy. Estrogen restored short latency maternal behavior in females up to 1 month after ovariectomy; however, 2 months after ovariectomy acute estrogen replacement was not sufficient to restore the behavior.

MATERIALS AND METHODS

Animals and Care

The animals used in these experiments were from our colony of the Sprague–Dawley strain. The parents of these animals were originally purchased from Charles River Laboratories (Wilmington, DE) and were bred in the Rutgers University Laboratory Animal Facility in Newark, which is accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC). All females were main-

tained under a 12/12 h light/dark cycle and a stable, environmental temperature of 22°C, with *ad libitum* access to food and water. Wood shavings (BETA CHIP®, NEPCO, North Eastern Products Corp., NY) was used to cover the floor of the cages. All procedures used in this study followed the standards approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Newborn pups 2–7 days old were obtained daily from a pool of lactating donor females and were used to induce maternal behavior in the experimental subjects.

In Experiment I we used 32 females that were 38 days old at the start of the study, weighed between 120 and 170 g, and were weaned at 25–30 days of age. Juvenile females used in this experiment came from 10 different litters. Only 3 or 4 females per litter were used and no more than 2 subjects in an experimental group came from the same litter. Females were housed in groups of 6 until age 38 days and then individually housed in translucent Plexiglas cages measuring 25 × 45 × 20 cm.

The animals used in Experiments IIA and IIB were 48 females that were 23 days old at the start of the experiment. Juveniles were housed in groups of four until age 35 days, groups of two from 35 to 50, and then individually housed in cages as described above. Females used in these experiments came from 14 litters. Only 3 or 4 animals per litter were used and no more than 2 subjects in an experimental group came from the same litter.

Surgery and Groups

Experiment I: Midpuberty MPOA lesions and ovariectomy. At 38 days of age, females were anesthetized with ketamine (100 mg/kg of body weight) and Rompun (xylazine, 10 mg/kg of body weight) anesthesia for ovariectomy and placed in a Kopf stereotaxic apparatus for neurotoxin injection. The stereotaxic coordinates relative to bregma were determined using the rat brain atlas of Paxinos and Watson (1998). Animals received bilateral injections at coordinate sites anterior–posterior −0.4 mm, medial–lateral ±0.5 mm, and dorsal–ventral −7.4 mm using a horizontal skull.

Ovariectomies were performed at the same time as the MPOA lesions. Since at this time we did not know the effect of ovarian hormones during puberty on the MPOA, our aim was to maintain the developmental status of the females as of day 38, midpuberty, and to prevent the possible further maturation of the MPOA control of maternal behavior under the influence of ovarian hormones. In this way we could specify the hormonal role in the maturation of the MPOA up to day 38.

All females were ovariectomized and randomly assigned to one of four groups. One group ($n = 8$) was not submitted to any type of brain surgery to exclude any nonspecific effect on maternal behavior induced by the surgical procedure (needle placement, skull perforation). The other three groups received bilateral injections into the MPOA of one of the following three solutions: 0.6 μl of 25 mM phosphate-buffered saline solution ($n = 6$, PBS, pH = 7.4); 24 μg of *N*-methyl-D-aspartate ($n = 13$, NMDA, excitatory neurotoxin, Sigma Chemical Co., St. Louis, MO) dissolved in 0.6 μl of PBS or 24 μg of *N*-methyl-DL-aspartate ($n = 5$, NMDLA, racemic mixture of the dextro and levo forms of *N*-methyl-aspartate, Sigma) dissolved in 0.6 μl of PBS to more reliably produce small MPOA lesions (Watkins, 1962) and to enable us to compare our results with those of Kalinichev *et al.* (2000a) in adults. The solution was injected with a 1-μl Hamilton syringe at a rate of 0.1 μl/min. After injection, the needle remained in place for 5 min. NMDA was chosen as the neurotoxin, because previous studies had shown the effectiveness of this drug to induce lesions in the MPOA (Kalinichev *et al.*, 2000a). NMDLA has been reported to have less excitatory potency by Watkins (1962). After ovariectomy and neurosurgery were performed, the animals were returned to their home cages in the observation room and allowed to recover for 1 week. Females were tested for maternal behavior beginning at 45 days of age for 12 days.

Adult MPOA-lesioned females were not included in this experiment because animals were drawn from the same rat colony, the procedures followed for producing cytoxic lesions by injecting the animals with NMDA were the same as those used in our previous study (Kalinichev *et al.*, 2000a) by the same experimenters, and testing for maternal behavior and nonmaternal behaviors also employed the same methods.

Experiment II: Adult MPOA lesion and ovariectomy before or after puberty. In Experiment IIA 22 females were randomly assigned to one of two groups. Females in the group OV23 ($n = 11$) were ovariectomized when they were 23 days old to remove the source of ovarian hormones during the peripubertal period. Females in the group OV53 ($n = 11$) were ovariectomized at 53 days of age. When females of all groups were 53 days old they were anesthetized with ketamine and Rompun for placement in a Kopf stereotaxic apparatus for neurotoxin injection. The ste-

reotaxic coordinates relative to bregma were the same as those cited above. Cytotoxic lesions were made using the procedure described above for NMDA. Females were tested for maternal behavior beginning on day 60 for 12 days.

In Experiment IIB those animals that did not show maternal behavior after pup exposure (Experiment IIA) were subcutaneously injected with 100 μg/kg of estradiol benzoate (EB) at 85 days of age (OV23/EB85, n = 9/11; OV53/EB85, n = 8/11) and were reexposed to pups and retested beginning at day 86 of age. The pup exposure and observational period lasted from days 86 to 93 (8 days). The OV23/EB85 group was without the ovaries for 2 months (23–85) and the OV53/EB85 group for 1 month (53–85). A control group of females was ovariectomized on day 23 and was administered EB on day 59 to allow about 1 month from ovarian removal to EB administration (OV23/EB59, n = 10), and they were tested at 60 days of age, as in Experiment IIA. This group received MPOA lesions (the same procedure as that in Experiment IIA) on day 53. Females of this group (OV23/59) were compared with OV53/85 females. Any difference between these two groups would be due to the duration of the absence of the ovaries but not by the period during which the ovaries were absent (e.g., during puberty and postpuberally). An additional group of 16 females were sham ovariectomized (ovary-intact), MPOA lesioned at age 53 days, and tested at 60 days, as the previous group. This ovary-intact group was included to determine whether the estrogen that is present during proestrus of regular estrous cycles is effective in preventing the deficit induced by small neurotoxin lesions in the MPOA.

General Testing Procedure

In Experiment I behavioral testing started when the females were 45 days old (1 week after surgery) and lasted 12 days. In Experiment IIA, females were tested for 12 days starting at 60 days of age and in Experiment IIB they were tested at 86 days of age for 8 days.

Sensitization. This procedure involved the continuous exposure of females to pups in their home cages to induce maternal behavior (Rosenblatt, 1967). All females were supplied daily with three pups (2–7 days old) that remained in the cages until the next morning. The pups were exchanged daily with three freshly fed pups from donor mothers. The criterion for full maternal behavior was that females build a good nest, retrieve and lick the three pups, and adopt a crouching posture over them on 2 consecutive days. The first of the 2 days (including this day in the counting) was used to calculate the latencies in days to display full maternal behavior. The behavior patterns that were tested and recorded are described as follows:

Retrieving and licking. Retrieval tests lasting 10 min started each morning between 900 and 1000 h, shortly after freshly fed pups were placed in the cage, diagonally opposite the nest site. 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. The occurrence of anogenital and body licking of the pups was scored only when the subject was clearly observed to perform the behaviors. Additional observations separated by intervals of 1 to 2 h were also performed during the day to see if females had retrieved pups to the nest. However, retrieving latencies were recorded only for retrieving occurring during the 10-min tests.

Nest building. Each afternoon, between 1700 and 1800 h, the quality of the nest was scored. Nests were destroyed, and nest material was scattered in all cage quadrants immediately after the retrieval test (1000 h) so that nest building could be evaluated in the late afternoon. New nest material, two paper towels (26 $\times$ 33 cm) cut into lengthwise pieces, was provided every 3 days after the old nest material was removed from the cage.

Nests were rated as described by Numan and Callahan (1980): 0 = no nest, paper is scattered across the cage; 1 = poor nest, not all the nest material is used, and paper is gathered into a flat bed with no walls; 2 = fair nest, all paper is used to build a flat nest; 3 = good nest, all of the paper is used, and nests with low or medium walls are constructed; 4 = excellent nest, all of the nest material is used to build high walls. On the basis of our experience, those nests having scores of 2 or better were considered maternal nests.

Nursing posture. Each afternoon (1700–1800 h), the occurrence of crouching was assessed during a 20-min observation period. Each minute a female was observed for 5 s, and a score of 1 was allotted each time the female was observed to hover over or to lie adjacent to and in contact with at least one pup. We did not use the more refined categories of nursing posture (i.e., kyphosis) proposed by Stern and Johnson (1990) and more recently by Lonstein, Wagner, and De Vries (1999). Females could obtain a maximum score of 20 on each test day. In addition, each morning during the retrieval test, we recorded the occurrence of the above behaviors and if they occurred in or out of the nest.

General Measures of Health and Activity

The locomotor activity, motor performance, exploratory behavior, and physical condition of these animals were observed to identify any nonspecific behavioral effect induced by the treatment applied.

Locomotor activity and exploratory behavior. Every 4 days, the locomotor activity and exploratory behavior of the females were evaluated in computerized photocell activity boxes (Digiscan Animal Activity Monitor, Model RXYZCM, Omnitech Electronics, Columbus, OH) at 1300 h. After a short period (10 min) of adaptation to the chamber, activity was recorded for 10 min. This apparatus measures movement in the horizontal and vertical planes by the combination of inputs from photocell beams. Total distance traveled, horizontal and vertical activity, time spent in central and marginal regions, number of movements, movement time, and clockwise or counterclockwise revolutions were also recorded, among other activity parameters.

Motor performance. The motor performance of the females carrying candies (Tootsie Rolls) of similar size and weight as pups was also tested. Three candies were placed in the cage diagonally opposite the nest site and both the number of candies retrieved by the females during a 10-min test period and the latencies to retrieve each piece of candy were recorded.

Physical parameters. Female body weight was determined every 3 days from before surgery to the end of the experiment. Body temperature was recorded with the same frequency using a rectal thermometer. These measurements were recorded to assess the health of the animals and to exclude possible changes in thermal regulation induced by MPOA lesions. Previous studies have shown that lesions in the MPOA anterior hypothalamic region induce hypothermia and less effective thermoregulatory responses (Hammel, 1968). Overall condition and health were evaluated daily by visual examination, particularly after surgery. Animals that were sick or unhealthy were sacrificed with CO_2.

Histology, Lesion Analysis, and Lesion Reconstruction

All females were deeply anesthetized with pentobarbital (250 mg / kg of body weight), injected with 0.1 ml of heparin into the left ventricle of the heart, and perfused intracardially with 200 ml of PBS (25 mM, pH = 7.4) and 300 ml of 4% paraformaldehyde in PBS. Brains were removed from the skull, stored at 4°C in the fixative solution for 48 h, and then transferred to sucrose dissolved in PBS (15%) for 24 h. They were frozen with powdered dry ice and sections (30 μm) were cut with aid of a Bright cryostat. All sections around the areas of study (rostral–caudal extension from bregma 1 mm to −2.12 mm, Paxinos and Watson, 1998) were saved, mounted on slides, and stained with cresyl violet.

By means of a microprojector and a Zeiss microscope with a camera lucida attached, the rostral–caudal extent of each lesion and the track left by placement of the needle were drawn on photocopies of frontal plane diagrams taken from the atlas of Paxinos and Watson (1998). All sections were examined (without reference to behavioral scores) and the boundaries of the damaged area were outlined on each drawing.

A lesion was identified by the presence of gliosis and the absence of neurons with apparent rounded healthy shape. The area of the lesion and the percentage of the MPOA damaged were calculated using a Bioquant System endowed with a digitizing pad and morphometry program (R & M Biometrics, Inc., Nashville, TN). Area measurements and rostral-to-caudal sectioning records were used to determine the volume of the lesions, as previously described by Bloch and Gorski (1988), and used in our laboratory by Corodimas, Rosenblatt, Canfield, and Morrell (1993) and Kalinichev *et al.* (2000a).

In Experiment II, an additional procedure was used to determine the extent of the MPOA NMDA lesion. After all behavioral observations were done, females from all groups, all subjected to MPOA lesions, were randomly selected to be submitted to fluorogold injection unilaterally in the ventral tegmental area (the brain area that receives projections from the MPOA) to aid in the analysis of the damage induced by lesions in the MPOA. Nine females ovariectomized at 23 days and lesioned at 53 days were included as well as 8 females ovariectomized and lesioned at 53 days and 6 nonovariectomed females lesioned at 53 days. No unlesioned females were injected with fluorogold. The presence or the absence of neurons labeled with fluorogold at the MPOA was used to determine the area damaged (Corodimas, Rosenblatt, Matthews-Felton, and Morrell, 1995). These females were anesthetized again as described above and submitted to surgery. The coordinates used for the fluorogold injection were AP −5.7, ML −0.8, and DV −8 using a horizontal skull. Females were injected with 0.6 μl of a 4 % solution of fluorogold (Fluorochrome, Inc., Denver, CO) dissolved in distilled water. The solution was

injected with a 1-μl Hamilton syringe at a rate of 0.1 μl/min.

One week after the fluorogold injection, all rats were deeply anesthetized and perfused to fixate and remove the brain as described above. Brains were frozen with powdered dry ice and sections (30 μm) were cut with aid of a Bright cryostat. All sections around the areas of study (rostral–caudal extension from bregma 1 mm to −2.12 mm, Paxinos and Watson, 1998) were saved, mounted on slides, and stained with cresyl violet or analyzed using ultraviolet light (360 nm) (HBO 50-V mercury bulb source, Zeiss UV-G 365 excitation filter, epifluorescence system with Neofluar objectives). Sections around the area of fluorogold injection were also saved to determine the exact location of the injection.

A lesion was identified by the absence of neurons labeled with fluorogold or cresyl violet staining, the presence of gliosis, and the absence of neurons with a rounded healthy shape. Neurons retrogradely labeled with fluorogold were identified by the brilliant white fluorescence in their somas and proximal processes. Labeling of cells in sections of the injection site in the ventral tegmental area was found to be confined to that area alone. The area of the lesion and the percentage of the MPOA damaged were calculated using the Bioquant System endowed with a digitizing pad and morphometry program (R & M Biometrics, Inc.). Area measurements and rostral-to-caudal sectioning records were used to determine the volume of the lesions (Kalinichev *et al.*, 2000a).

Data Analysis

Groups for statistical analysis in Experiment I were constituted according to size of the MPOA lesions in individual females. In Experiements IIA and IIB all lesions were about 10% of the MPOA; therefore, data were analyzed according to preestablished groups. Group behavioral data were statistically evaluated by nonparametric Kruskal–Wallis analysis of variance and paired comparisons by the Mann–Whitney *U* test. Body weight and temperature passed the requirements for homogeneity of variance and were analyzed with parametric statistics (ANOVA repeated measures). All proportion data (percentage of animals retrieving, building nest, crouching, and carrying candies) were evaluated with the χ^2 test (Siegel and Castellan, 1988). *P* values ≤ 0.05 were accepted as significant.

TABLE 1
Volume of the Lesions and Percentage of MPOA Damaged

Group	Total brain volume lesioned (mm^3)	Volume of the MPOA lesioned (mm^3)	Percentage of MPOA lesioned
MPOA-Large ($n = 5$)	3.20 ± 0.49	0.88 ± 0.11	20 ± 2.6
MPOA-Small ($n = 13$)	0.68 ± 0.10	0.14 ± 0.03	3.3 ± 0.8
Control ($n = 14$)	0.40 ± 0.12	0.00	0.00

Note. Volumes are means ± SE.

RESULTS

Experiment I

MPOA Lesion Analysis

After histological analysis of the brain damage, rats were included in one of four groups. The criterion for postmortem categorization of group members was based on several factors. First the animals were separated into two groups, those with lesions and those without lesions. Second, the lesion size was evaluated and categorized by a histogram distribution analysis that showed basically a bimodal distribution of the lesions. Lesions that included more than 10% of the MPOA were in one mode, and these were categorized as large lesions. Lesions that were smaller than 10% but were also larger than the 0.5%, maximum lesion size induced by a needle tract alone, were categorized as small lesions. Eight females did not receive brain surgery (Intact), 6 females suffered only the damage produced by the needle (less than 0.5% of the MPOA) or had small lesions outside the MPOA (Sham), 13 had small lesions in the MPOA occupying more than 0.5% but in no case exceeding 10% of the MPOA (MPOA-Small), and 5 had lesions larger than 10% of the MPOA (MPOA-Large). All animals with larger lesions in the MPOA had been injected with NMDA. Of the 13 animals with smaller lesions in the MPOA, 7 were injected with NMDA, 4 with NMDLA and 2 with PBS. Of the 6 animals categorized as Sham, 1 was injected with NMDLA, 4 with PBS, and 1, which had a small lesion dorsal to the paraventricular nucleus of the hypothalamus, with NMDA.

The bilateral volume of the MPOA calculated in this study using intact control animals was 4.14 mm^3. Table 1 shows the percentage of the MPOA volume

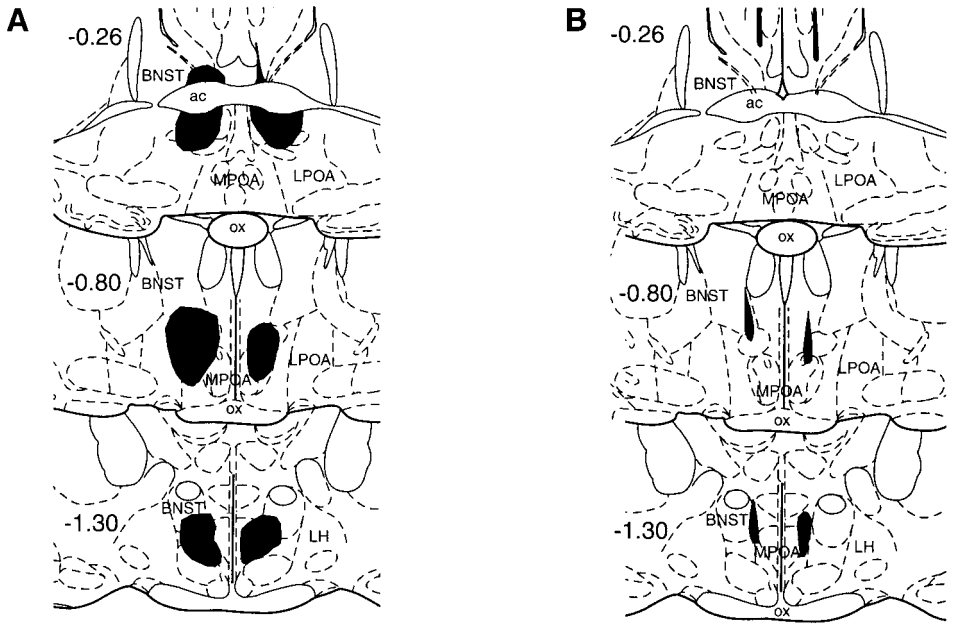

FIG. 1. Placement of the lesions represented at three stereotaxic levels of the MPOA. The dark areas represent the typical damage sustained by animals in the MPOA-Large (A) and the MPOA-Small (B) lesioned groups. ac, anterior commissure; BNST, bed nucleus of the stria terminalis; LH, lateral hypothalamic area; LPOA, lateral preoptic area; ox, optic chiasm.

affected by the lesion, the volume of the MPOA damaged, and the total volume of the lesion.

The area of the lesion was recognized through gliosis and the absence of healthy neurons. Lesions in other areas, such as the lateral preoptic area, the bed nucleus of the stria terminalis, the anterior hypothalamic area, and the paraventricular nucleus of the hypothalamus, were observed in some of the animals that were characterized as having larger or smaller lesions of the MPOA. Reconstructed diagrams of representative cresyl violet-stained sections of the damage sustained by the groups MPOA-Large and MPOA-Small are shown in Fig. 1.

The locations of the needle path and the lesions were similar in all groups, suggesting that the size, not the location, of the lesion in the MPOA was determinant in this case. Both the larger (20%, 0.88 mm^3) and the smaller (3.3%, 0.14 mm^3) lesions of the MPOA in this experiment were smaller than those produced in adult females (larger, 43.2%; smaller, 6.7%) by Kalinichev *et al.* (2000a). However, this difference in the size of the lesions cannot account for the lack of maternal impairment found after smaller lesions in midpubertal females. Previous studies (Numan *et al.*, 1977; Rosenblatt *et al.*, 1996) showed that just the placement of the needle in the MPOA induces deficits in maternal behavior in ovariectomized adult females; hence a lesion occupying 3.0% of the volume of the MPOA (larger than that induced by the needle itself, $<$0.5%) seems to be sufficient to induce such an effect in adults. Furthermore, a number of the small lesions in the Kalinichev *et al.* (2000a) experiment that did impair adult maternal behavior were within the range of small lesion sizes in this study.

Behavioral results. All the females with large lesions in the MPOA showed severe impairment of maternal behavior and did not display any of its components (retrieving, crouching, nestbuilding) for 2 consecutive days to meet the criterion for sensitization.

Sixty-one percent (8 of 13) of the females with small lesions in the MPOA exhibited full maternal behavior (displaying all components of maternal behavior for at least 2 consecutive days) by the eighth day of testing and were not different from the control groups. Eighty-five percent showed maternal behavior at the end of the testing period. Their median latency ($\pm$IQR) was 7.0 $\pm$ 2.6 days (excluding two animals that did not show maternal behavior during the testing period).

Unoperated females and those that had lesions smaller than 0.5% or no lesions did not differ from each other and were combined to form a single control group. Control animals exhibited full maternal behav-

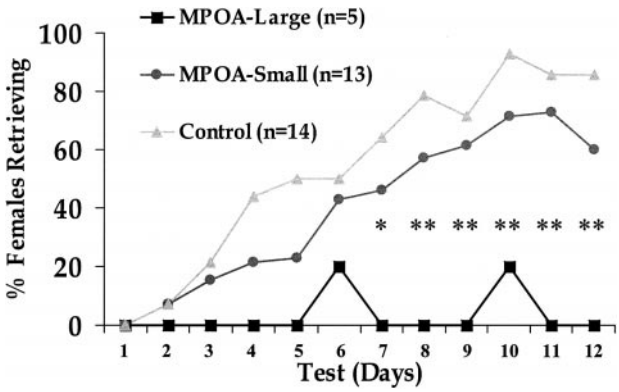

FIG. 2. Percentage of females retrieving three pups during the 10-min retrieval test. There was a significant difference in the percentage of females that retrieved pups across the groups when comparisons where made from the days 7 to 12 (χ^2; $^*P < 0.05$, $^{**}P < 0.01$, $df = 2$). The percentage of females that retrieved pups was significantly lower in the MPOA-Large lesion group in comparison with MPOA-Small lesion and control groups.

ior with a median latency of 5.0 ± 2.5 days. Eighty-six percent (12 of 14) of the animals of the control group had already exhibited full maternal behavior by the eighth day and 100% by the end of the testing period.

Retrieving. There was a significant difference across the groups in the percentages of females that retrieved the three pups from day 7 of testing to the end of the experiment ($P < 0.05$, $df = 2$). Post-hoc analyses showed that from days 7 to 12 of testing, fewer females with large lesions in the MPOA retrieved pups than either those with small lesions or the controls females ($P < 0.05$, $df = 1$) (see Fig. 2). In the group with larger lesions in the MPOA, only one animal retrieved the three pups on days 6 and 10 but did not retrieve on the other days. There was no significant difference in the percentages of females retrieving pups in the control and small MPOA lesion groups. The median latencies to retrieve the first pup on the first day of being maternal were 90 (±114) and 150 (±105) s among control females and those with small lesions.

Licking and cleaning. Licking was also significantly different across the groups ($P < 0.05$, $df = 2$; data not shown). Post-hoc analyses showed that the occurrence of licking and cleaning in the group with larger lesions was less when compared with those in the control group ($P < 0.05$, $df = 1$). The group with large lesions had a tendency to lick less in comparison with the group with small lesions ($P < 0.07$, $df = 1$). No difference was found between animals with small lesions and controls.

Nest building. There was a significant difference in scores of the quality of the nests built by the different groups over the 7- to 12-day period of testing (Fig. 3; H = 12.3, $P < 0.01$, $df = 2$). The group with large lesions in the MPOA built nests that were inferior to the maternal nests of either the MPOA-Small ($U = 0$, $P < 0.01$, $df = 1$) or the control ($U = 0$, $P < 0.01$, $df = 1$) groups during days 7–12 of testing, which did not differ from one another. During the previous 1–6 days the nest scores were not significantly different among the groups.

Females with large lesions, from day 7 onward, had significantly smaller percentages, with nest scores ≥2.0, than females with small lesions (days 7 and 9, $P < 0.05$, $df = 1$) and the control group on days 7, 9, and 11 ($P < 0.05$, $df = 1$).

Crouching posture. None of the five females with larger lesions adopted the crouching postures during the morning 10-min period of the retrieval test, whereas the percentage of females with smaller lesions and controls that exhibited crouching postures increased gradually throughout the 12 days of testing and reached 50% by day 12 ($P < 0.05$, $df = 1$). The proportions of females with smaller lesions and the control females adopting a crouching posture were not significantly different.

When crouching over pups was tested for a 20-min period in the afternoon, no significant differences

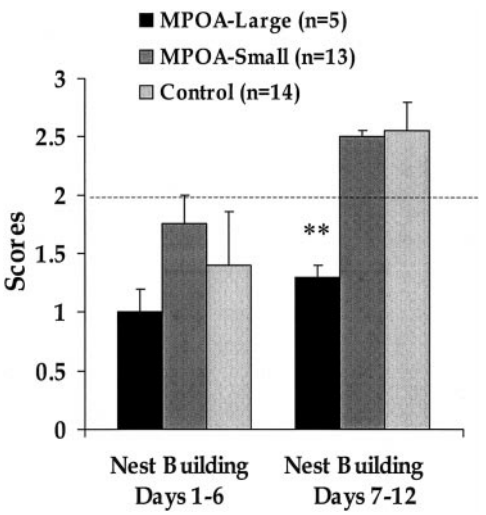

FIG. 3. Scores of nest building exhibited during the intervals 1–6 and 7–12 days of testing (median ± IRQ presented). A score of 2 or higher (above the dashed line) was considered as maternal nest. Females with larger lesions in the MPOA received lower scores than MPOA-Small lesion and control females during the interval 7–12 days ($^{**}P < 0.01$, $df = 2$). No differences among the groups were observed at the interval 1–6 days.

were found among the groups. All animals spent similar periods in the crouching posture. During days 1–6 crouching occurred for a median duration of 2.5 to 5.5 min and during days 7–12 for median durations of 5.5 to nearly 12 min; large variability in the time spent adopting the crouching posture was observed in all groups. The percentage of animals that spent half or more of the 20-min testing period adopting the crouching postures was also not different. For example, on day 7, 40% of the animals with larger lesions, 54% of those with smaller lesions, and 43% of the control animals spent 10 min or longer adopting a crouching position.

General Measures of Health and Activity

Females in all groups were in good health and showed no deficits in locomotor activity and exploratory behavior (i.e., total distance traveled and all other measures), no motor impairments, no loss of oromotor capability, no differences in body weight (except immediate ones following surgery), and no changes in body temperature.

Experiment II

MPOA Lesion Analysis

By design, all experimental animals included in this experiment had small lesions in the MPOA that affected about 10% of the volume of the MPOA. The size and location of the lesions were similar in all experimental groups.

The area of the lesion, as indicated above, was recognized through gliosis and the absence of healthy neurons and in this study, in contrast to Experiment I, also by the absence of fluorogold-labeled neurons. Lesions extended to other areas, such as the lateral preoptic area, the bed nucleus of the stria terminalis, the anterior hypothalamic area, and the paraventricular nucleus of the hypothalamus in some of the animals.

The results of retrograde fluorogold labeling and of the gliosis, which was confined almost entirely to the MPOA, indicated that the NMDA lesions were sufficiently localized to test the effect of the small MPOA lesions on maternal behavior in the experimental groups. We did not find fluorogold labeling in any areas that showed gliosis, but we found fluorogold labeling surrounding the gliosis site and in the anterior part of the MPOA that was not damaged. There were no differences among or within the groups in the absence of fluorogold labeling and the presence of gliosis in the MPOA and there was no evidence of damage to fibers of passage. Moreover, the site of the injections of fluorogold was confined to the ventral tegmental area. The brain damage induced by the injection of NMDA occupied about 10% of the MPOA. No differences in the size or location of lesion were found among the different groups. These lesions affected principally the medial and posterior regions of the MPOA, leaving the anterior portion intact. The small amount of damage of brain areas surrounding the MPOA was also similar in all groups.

Although, in the present study, several larger lesions in the MPOA also affected surrounding areas (for example, the medial region of the bed nucleus of the stria terminalis), implicated in maternal behavior (Numan and Numan, 1996), it is unlikely that the deficit in maternal behavior can be the consequence of this damage. Both small and large lesions affected different surrounding areas, whereas the deficit in maternal behavior was clearly correlated only with the size of damage in the MPOA. In addition, previous studies showed that lesions in most surrounding areas of the MPOA do not block maternal behavior (Gray and Brooks, 1984).

Behavioral Analysis

Experiment IIA. Lesions affecting about 10% of the MPOA induced severe impairment of maternal behavior in ovariectomized females (OV23 and OV53 groups). No differences were found between females OV at 23 ($n = 11$) or 53 ($n = 11$) days in the effectiveness of small neurotoxin lesions to block maternal behavior. Only 18 and 27% of the females ovariectomized at 23 or 53 days, respectively, showed maternal behavior (retrieving, crouching, nestbuilding) during the 12-day testing period. The remaining females in both groups OV23 and OV53 did not show maternal behavior. The two maternal females in the group OV23 showed a latency of 5 days, while the individual latencies of the three maternal females in the group OV53 were 3, 7, and 9 days.

Experiment IIB. The animals that were not maternal in the period of testing on day 60 in the groups ovariectomized at 23 ($n = 9/11$) and 53 ($n = 8/11$) days of age all received 100 μg/kg of EB (sc) at 85 days of age, 1 day before testing. Most of the MPOA-lesioned females that had been ovariectomized postpuberally on day 53 (OV53/EB85) showed all components of maternal behavior, with a median (and IQR)

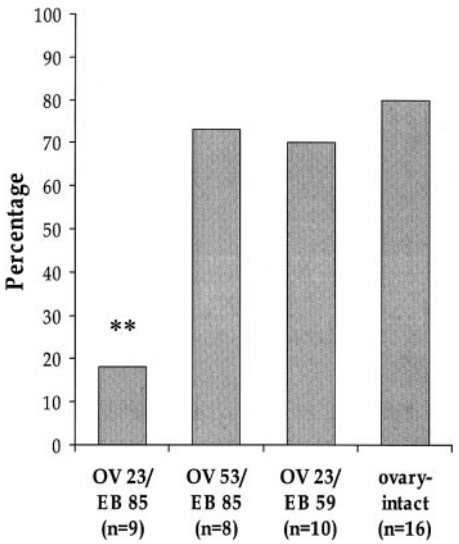

FIG. 4. Percentage of females with MPOA lesions that showed maternal behavior after pup exposure or hormonal treatment plus pup exposure. Note that maternal females from Experiment IIA were also included in this figure. These females were not injected with EB because they showed maternal behavior after MPOA lesion. The percentage of maternal females in the group OV23/EB85 (n = 11) was much lower and statistically significantly different (χ^2; $^{**}P < 0.01$, $df = 3$) compared with the other three groups (OV53/EB85, OV23/EB59, and ovary-intact). There were no differences among the groups OV53/EB85 (n = 11), OV23/EB59 (n = 10), and ovary-intact (n = 16).

latency of 3.0 ± 1.0 days (Fig. 4). However, in the group ovariectomized on day 23 and not given EB until day 85 (OV23/EB85), MPOA lesions were highly effective in blocking maternal behavior. None of the females exhibited maternal behavior ($P < 0.01$, df = 1). These females had been without estrogen for 62 days, twice as long as the day 53 group, before they were given a single injection of estrogen and started in the sensitization procedure.

In contrast, females ovariectomized prepubertally, MPOA lesioned on day 53 of age, and given estrogen before testing on day 60 (OV23/EB59) were highly responsive to pups (Fig. 4). These females were without EB for 36 days, similar to the OV53 group. All components of maternal behavior were exhibited by a majority of females, with a median latency of 2.0 ± 0.37 days. The proportion of females in this group that were maternal was significantly greater than that in the group of prepubertally ovariectomized females (OV23/EB85) of this study tested at day 86 of age ($P <$ 0.01, df = 1) but did not differ from the postpuberally ovariectomized females (OV53/EB85) tested at that age.

Similarly, most of the females sham-ovariectomized prepubertally (Fig. 4), subjected to MPOA lesions on day 53, and tested starting on day 60 exhibited all components of maternal behavior, with a median latency of 4.0 ± 1.25 days.

When we compare the percentages of animals showing maternal behavior after pup and hormonal stimulation in all groups, a significantly lower percentage of females showing maternal behavior can be seen only in the group OV23/EB85 with respect to the other three groups, OV23/59, OV23/85, and ovary-intact ($P < 0.01$, $df = 3$).

General Measures of Health and Activity

All groups showed good health and similar performance in all the activity patterns studied in this experiment. The locomotory and exploratory behavior, motor performance, and general physical condition were similar among all of the groups, as in the first study. No changes in body temperature were observed during the experiment.

DISCUSSION

Our first study led to the conclusion that there was no interference with the performance of sensitized maternal behavior in midpubertal females with small cytotoxic lesions of the MPOA and this was unlike their response to large MPOA lesions, which produced severe deficits in maternal behavior. During the 3 weeks after midpuberty the MPOA matures so that in 60-day-old females small lesions produce severe behavioral deficits. It is not known if this change occurs gradually or abruptly. In adult females with small MPOA lesions severe deficits in maternal behavior were not prevented by their being ovariectomized from prepuberty to adulthood. The deficits could be overcome, however, if MPOA-lesioned females were administered estrogen shortly before testing, but this was the case only if they had not been without estrogen for too long. The females that were without estrogen for 2 months did not overcome the effects of the small MPOA lesions and as a result they continued to exhibit severe deficits in sensitized maternal behavior.

MPOA Lesions and Maternal Behavior

In this experiment we produced small lesions so that the effects could be compared with the results found in previous studies in adult females (Kalinichev *et al.*, 2000a). All females injected with NMDLA and

61% of those injected with NMDA had small lesions. In this case, smaller neurotoxic lesions of the MPOA in midpubertal females did not affect the display of any of the components of maternal behavior at puberty. These results are similar to those found in juveniles (Kalinichev *et al.*, 2000a), but contrast with the severe deficit induced by small lesions in adult females. Several groups (Jacobson *et al.*, 1980; Numan *et al.*, 1977; Rosenblatt *et al.*, 1996; Kalinichev *et al.*, 2000a) found that very small lesions, or even the placement of the needle in the MPOA, induce severe deficits in maternal behavior in adult females. The results of Experiment I, therefore, indicate that the role played by the MPOA in the support of maternal behavior in adults is not completely developed even at midpuberty.

In midpubertal females with larger lesions crouching results differed in the morning and afternoon tests in that the females did not show crouching in the morning but in the afternoon test several of the them hovered over one or two pups that were scattered on the cage floor. Kalinichev *et al.* (2000a) found similar results in 27-day-old MPOA-lesioned females. Since the females did not retrieve the pups it is likely that their behavior toward the pups was not specifically maternal but more generally social. This has been reported of juvenile rats in other studies (Kalinichev *et al.*, 2000a, and Mayer and Rosenblatt, 1979). Nonmaternal juveniles and pubertals appear to spend more time in contact with the pups than adults, making the behavioral observation more complex. Perhaps the use of the different categories (hover over, kyphosis, prone, and hunched) described recently by Lonstein *et al.* (1999) would show more clearly whether complete disruption of crouching is present. We speculate that these categories might not all be present with the same frequency in ovariectomized pubertal animals as in adults.

Overall activity and exploratory levels were similar among the three groups, showing that no interference in the expression of maternal behavior due to changes in other behaviors or activity patterns was present. Females of the three groups displayed similar ability to carry candies, demonstrating that oral-motor deficit was not responsible of the poor retrieval scores exhibited by animals with larger lesions in the MPOA. Normal weight gain, temperature, and health condition were found in all groups, suggesting no sign of health problem or dysfunction in these females.

Considering what is known concerning the effective MPOA lesion size, or the damage to the MPOA, which is sufficient to induce a deficit in sensitized maternal behavior in rats 23, 38, 53, and 60 days old (data for 23- and 53-day old females are from Kalinichev *et al.*, 2000a; data for 60-day-old females are estimated from Numan *et al.*, 1977, and Rosenblatt *et al.*, 1996), we can conclude that large cytoxic or radiofrequency lesions in the MPOA are necessary to block maternal behavior in young animals. With increasing age, however, smaller lesions are equally effective in blocking maternal behavior.

The present and previous studies (Kalinichev *et al.*, 2000a; Numan, 1974; Rosenblatt *et al.*, 1996) suggest a differential role for the MPOA in the modulation of the different components of maternal behavior during the life of the rat. Retrieval behavior and nest building are blocked in juvenile, pubertal, and adult females after larger lesions (Kalinichev *et al.*, 2000a; Numan, 1974; Rosenblatt *et al.*, 1996). However, very small lesions block these behaviors only in adults (Kalinichev *et al.*, 2000a; Numan *et al.*, 1977; Rosenblatt *et al.*, 1996). Therefore, the MPOA appears to play an entirely necessary role in the expression of retrieval behavior and nest building during adulthood. The unique role played by the MPOA in the modulation of retrieval behavior, crouching, and nest building appears to be only partially established in juveniles and pubertal animals, and this role is further crystallized during the second half of the puberty.

In Experiment II small MPOA lesions produced severe deficits in maternal behavior at 60 days whether ovarian hormones were absent throughout puberty or not, suggesting that the maturation of the adult responses to small lesions is not dependent on these hormones. Females ovariectomized before puberty that received small lesions in the MPOA at 53 days of age were as deficient in their maternal behavior as females with the same lesions, but ovariectomized after pubertal development had been completed.

When estrogen was administered to females that failed to show maternal behavior, shortly before testing, it restored their responding despite the small MPOA lesions. This was effective only if females had not been without estrogen for too long a period. A single EB injection after estrogen deprivation for only 1 month enabled females to overcome the effects of the lesions and to respond maternally. The longer estrogen deprivation of 2 months (day 23 to 85) resulted in failure of estrogen to restore maternal behavior at 86 days in the females with lesions in the MPOA. Age at the time of estrogen administration was not a factor since females with lesions in the MPOA, ovariectomized at 53 days, and given estrogen at day 85 were able to respond maternally in 62% of cases. The 1-month period without ovarian hormones from day

23 to 59 with EB administered on day 59 and testing on day 60 also resulted in 70% females exhibiting maternal behavior. In addition, 81% of the ovary-intact females showed maternal behavior after small MPOA lesions, suggesting that circulating estrogen present during proestrus of the regular estrous cycle is sufficient to prevent the deficit on maternal behavior induced by small MPOA lesions.

The deficit in maternal behavior found after NMDA-induced lesions in the MPOA was not a consequence of changes in locomotor activity, motor performance, poor health, or differences in body temperature or the weight gain among the groups. The animals were able to retrieve candies of similar size and weight as pups. Although two groups were exposed to pups twice, the opposite response shown by them suggests that this was not an important factor.

The importance of ovarian hormones in the stimulation of maternal behavior in adults has been known for a long time (Fleming and Sarker, 1990; Moltz, Lubin, Leon, and Numan, 1970; Rosenblatt and Ceus, 1988; Rosenblatt, Mayer, and Giordano, 1988; Siegel and Rosenblatt, 1975; Zarrow, Gandelman, and Denenberg, 1971). It is also known that ovarian secretions (i.e., estrogen) also play a role during puberty and afterward in the maintenance of short-latency sensitized maternal behavior in females (Mayer and Rosenblatt, 1980). Stern (1987) had shown that ovarian hormones did not play a role in maternal behavioral changes leading up to puberty. According to our findings, the maturation in females of behavioral deficits that are produced by small MPOA lesions is not ovarian hormone dependent. This does not preclude the fact that a single injection of estrogen can overcome the effects of these lesions.

An alternative source of developmental influence on the MPOA may be the neurochemical changes that occur around puberty. One type of neurochemical change that is likely to be important is the influence of neurotransmitters in the MPOA. Currently we are measuring the content and metabolism of dopamine and serotonin comparing adult and juvenile rats that are performing maternal behavior or are not maternal. Both sensitized (i.e., nonhormonal) females and parturient females are being studied.

The reduction in responsiveness to EB stimulation in the group OV23/EB85 (i.e., ovariectomized on day 23 and given an injection sc of estradiol benzoate on day 85) with small lesions in the MPOA could be due to changes in second messengers or gene expression mediated directly or indirectly by estrogen receptor activation. The long absence of ligand acting on estrogen receptor can change the normal or basal concentration of those factors, which mediate the facilitatory role of EB on maternal behavior. A single systemic injection of EB would not be sufficient to recover the basal levels of these mediators of estrogen action.

An alternative explanation for finding a lack of effect of estrogen stimulation on maternal behavior could be unknown changes in the dynamics of the estrogen receptor after long absence of ligand. Previous studies found that MPOA estrogen receptor concentrations increase or stay unchanged a few days up to 4 weeks after ovariectomy (Morrell, Krieger, and Pfaff, 1986; Shughrue, Bushnell, and Dorsa, 1992; Yuri and Kawata, 1991). Measures of estrogen receptor protein and mRNA after gonadectomy also show a marked upregulation in the absence of circulating hormones (Morrell, Wagner, Malik, and Lisciotto, 1995).

In conclusion, ovarian hormones during puberty do not play a role in the maturation of the adult response to small cytotoxic lesions of the MPOA in the control of pup-stimulated maternal behavior. The effects of these small MPOA lesions can be overcome by estrogen administered in adulthood. Short-term estrogen replacement is effective in this respect only in females that have been deprived of ovarian hormones for about 1 month, including the pubertal period, or during the postpubertal period alone. It is not effective in females that are deprived of ovarian hormone for about 2 months that include the pubertal period.

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