Effects of Administering a Nitric Oxide Synthase Inhibitor into the Medial Preoptic Area on Retrieval Behavior and Maternal Aggression in Postpartum Rats

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ABSTRACT

Effects of Administering a Nitric Oxide Synthase Inhibitor into the Medial Preoptic Area on Retrieval Behavior and Maternal Aggression in Postpartum Rats

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Previous research has shown that suppressing the production of nitric oxide (NO) by administering an inhibitor of nitric oxide synthase (NOS), L-NAME, into the third ventricle disrupts pup retrieval and maternal aggression in postpartum rats. The current experiments were carried out to investigate the sites of action of NO in the control of maternal behavior. We first identified a dose of L-NAME that was ineffective when administered into the third ventricle. Results obtained from this experiment (Experiment 1) indicated that L-NAME at the two highest doses (250 μg and 200 μg) but not at lower doses (0, 20 μg, 80 μg, and 160 μg) increased the latency to retrieve the first pup as well as reduced the number of pups retrieved by day 4 postpartum rats. These two high doses of L-NAME were also effective in reducing the percentage of females aggressing against a male intruder. In experiment 2, doses of L-NAME that were shown to be ineffective when injected into the 3rd ventricle in Experiment 1 were administered bilaterally into the MPOA of rats on Day 4 postpartum. These rats were then tested for pup-retrieval and maternal aggression. Results from this study demonstrated that L-NAME at doses of 20 μg and 40μg/side significantly increased the latency to retrieve the first pup and reduced the total number of pups retrieved. Interestingly, aggression towards a male intruder in the home cage was much less affected by these treatments. All rats in both experiments showed normal maternal behavior 24h after drug administration. These data suggest that NO acts within the MPOA to play a critical role in the facilitation of retrieval behavior but not maternal aggression in lactating rats.

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General Introduction

In many species, parental care is essential for the survival of the young. Paternal care is common in birds and fish, and has been reported in some mammalian species. In most mammals, however, it is typically females that provide most of the care and protection for their young as well as milk for their nourishment. The critical role that maternal behavior plays in the development of the young has provided the impetus for many studies on its neurobiological substrate. Much of this work has been carried out using animal models, such as sheep, and rodents. A great deal of information has accumulated on the role that gonadal and peptide hormones play in the onset and maintenance of maternal behavior as well as in the neural circuits that control it (See Numan and Insel for review, 2003). The neurochemistry of these circuits, however, is less well understood and the experiments described in this thesis were carried out to investigate the role of one neurotransmitter, nitric oxide (NO), in the control of maternal behavior in the rat.

In the rat, maternal behavior includes building a nest for the litter, picking up and returning pups to the nest site (retrieval), nursing, as well as licking and grooming the pups, and hovering over them to keep them warm (Numan, 1988). Maternal females also protect their pups and will attack an intruder to the nest site (Albert, Jonik &Walsh, 1992). Maternal behavior in the rat is particularly impressive because it appears fully formed with the birth of the first pup even though prior to pregnancy, female rats do not show any of these behaviors. In fact, virgin rats ignore pups and move as far away from them as possible (Fleming and Rosenblatt, 1974b).

Interestingly, repeated exposure of rat pups to virgin female rats over several days, a procedure called sensitization (Rosenblatt, 1967), has been shown to induce all the characteristics of maternal behavior (except milk delivery) that are seen in postpartum females. It has been suggested that during the sensitization period virgin rats develop tolerance to the pups through habituation to pup-odor, which inhibits maternal behavior in non-sensitized virgins (Rosenblatt, 1967).

Brain areas implicated in the control of maternal behavior in lactating rats include the olfactory bulb (OB) (Flemings and Rosenblatt, 1974b), the medial amygdala (MeA) (Fleming et al, 1983; Numan, Numan and English, 1993), the ventral region of bed nucleus of the stria terminalis (v/BNST) and the medial preoptic area (MPOA) (Numan, 1974; Stack et al, 2002). Collectively, these areas are sometimes referred to as "the mammalian maternal circuit" (Numan and Sheehan, 1997).

Afferent connections between neurons of the OB and the MeA play an important role in facilitating mother-young recognition in sheep (Gonzalez-Mariscal and Poindron, 2002) and the development of tolerance for pups in rats (Flemings, Vaccarino, Tambosso and Chee, 1979; Numan and Insel, 2003). Damage to this pathway immediately after parturition, for example, has been shown to cause ewes to abandon their lambs (Levy, Keller and POindron, 2004). Conversely, olfactory-bulbectomy promotes maternal behavior in virgin rats (Fleming and Rosenblatt, 1974a). The MeA projects to the MPOA via the vBNST and has an inhibitory role in the onset of maternal behavior (Sheehan et al, 2001). Excitotoxic lesions of neurons of the MeA that project to the MPOA dramatically shorten the period of sensitization in virgin rats confirming the notion of an inhibitory effect of the MeA on the MPOA (Numan, Numan and English, 1993).

Conversely, lesions of vBNST/MPOA prevent the onset of maternal behavior in pregnant females and sensitization in virgin rats (Numan, Rosenblatt and Komisaruk, 1977).

Recent evidence has indicated that the maternal circuit interacts with neural areas typically associated with reward to facilitate maternal responsiveness in postpartum rats. Neurotoxic lesions of the NA disrupt retrieval behavior but, interestingly, do not affect quiescent nursing behavior in postpartum rats (Hansen et al, 1991b) suggesting that the NA influences some, but not all, components of maternal behavior. Numan has argued that output from the MPOA either directly or through the VTA acts to inhibit NA output resulting in the disinhibition of the ventral pallidum (VP), which allows active maternal behavior to occur (Numan et al, 2005). Evidence in partial support of this notion comes from a study by Stack et al. (2002), who used a combined lesion and c-fos immunocytochemistry (ICC) approach to examined activation of areas that received inputs to the MPOA when rats were engaged in maternal behavior. Unilateral lesion of the MPOA, which does not interfere with maternal behavior (Numan, 1974) resulted in a reduction of c-Fos in the shell of the NA and lateral septum (LS) on the side ipsilateral to the lesion (Stack et al, 2002). There was no effect of the lesion on Fos immunoreactivity in the core of the NA or in the VTA.

In support of the idea that the MPOA-VP interaction is critical for maternal behavior, Numan et al (2005) have shown recently that bilateral administration of a gamma aminobutyric acid (GABA) agonist, muscimol, into the VP disrupts maternal behavior in postpartum rats. A similar effect was seen when unilateral muscimol administration into the VP was paired with contralateral NMDA lesions to the MPOA. Neither unilateral lesion to the MPOA nor unilateral administration of muscimol into the

VP alone interfered with maternal behavior indicating that the MPOA interact with the VP to regulate maternal behavior in postpartum rats.

The rapid onset of maternal behavior seen in primiparous rats at parturition has been shown to result from the action of steroid and peptide hormones within the maternal circuit. Researchers have simulated the changes in ovarian hormonal profile that occur over the course of pregnancy and were able to show that increased circulating estrogen (E) with a background of falling progesterone (P) is sufficient to stimulate the rapid onset of maternal behavior in virgin rats (Bridges and Russell, 1981). This steroid treatment also induces an increase in prolactin (PRL) (Bridges and Ronsheim, 1990) and oxytocin (OT) levels (Popeski, Amir, Diorio and Woodside, 2003) that are also seen in late pregnant rats suggesting that both peptide and gonadal hormones may interact within specific neural areas to stimulate maternal behavior.

Direct injections of either PRL (Bridges et al, 1990), or OT (Pedersen, Ascher and Monroe, 1982) into the MPOA stimulates the onset of maternal behavior in virgin rats, whereas, suppression of endogenous PRL with bromocriptine blocks the rapid onset of maternal behavior in postpartum rats (Bridges et al, 1985). Similarly, infusion of an OT antagonist in the VTA and the MPOA during parturition disrupts the immediate onset of maternal behavior in lactating female rats (Pedersen et al, 1994). Estrogen implants into the MPOA also facilitate the onset of maternal behavior in both pregnant female and ovariectomized virgin rats (Numan, Rosenblatt, and Komisaruk, 1977). Because E levels are relatively high during pregnancy compared to the postpartum period in the rat, it has been suggested that the inhibitory effect of the MeA on maternal behavior is lifted in the presence of high levels of E.

Interestingly, the maintenance of maternal behavior is largely independent of circulating hormones, since postpartum rats in which maternal behavior has already been established still show all aspects of maternal behavior when the sources of both E and P have been removed (Rosenblatt, 1967; Bridges, Feder and Rosenblatt, 1977). Removal of the pituitary hormones and hence PRL, by hypophysectomy, is also without effect on maternal behavior in postpartum rats (Bridges et al, 1985). Moreover, central administration of an OT receptor antagonist had no effect on ongoing maternal behavior in postpartum rats (Lipschitz, Crowley, and Bealer, 2003) supporting data from other studies on the role of hormones in the maintenance of maternal behavior.

Although hormones are not necessary for ongoing maternal behavior, there is convincing evidence that it does depend on the integrity of the maternal circuit. Lesions of the MPOA/vBNST disrupt established maternal behavior in postpartum rats (Numan, 1974), as do lateral knife cuts which interrupt the projections from the MPOA to the VTA (Numan an Smith, 1984). In addition, lesions of the NA produce deficits in retrieval behavior (Lee et al, 1999). Other studies have shown that it is the shell of the NA which plays a role in the normal expression of pup-retrieval in postpartum rats (Li and Fleming, 2003).

Thus far, most studies examining the neurochemistry of the maternal circuit have focused on the role of the opioid and dopaminergic systems. Endogenous opioid tone varies across pregnancy and lactation. During pregnancy, opioid levels as well as μ opioid receptors in the MPOA/vBNST are elevated but during lactation, there is a dramatic reduction in both the receptor and its ligand (Bridges and Ronsheim, 1987; Hammer and Bridges, 1987). Microinjections of morphine, an opiate agonist, into the

MPOA (Rubin and Bridges, 1984) disrupt established maternal behavior in postpartum rats. Rubin et al (1984) also showed that both the effects of morphine on maternal behavior and on maternal aggression could be reversed by naloxone administration. One way in which opiates might modulate maternal behavior is by changing responses to olfactory inputs (Kinsley et al, 1994).

In support of such a role Kinsley and Bridges (1990) showed that vehicle-treated rats tested on Day 5 postpartum preferred an area associated with pup odor to one associated with male odor or to a control area. This preference was reversed in the morphine-treated group. Interestingly, morphine treatment induced a preference for pup-related odors in ovarectomized virgin rats. Other studies investigating the role of opioids on maternal behavior have shown that inhibition of opiate system resulted in a deficit in maternal aggression in postpartum rats (Kinsley and Bridges, 1986).

Manipulating the dopaminergic system within the maternal circuit also affects maternal behavior. Infusions of *cis*-flupenthixol, a mixed D1/D2 dopamine (DA) antagonist, into the NA inhibits nest building, pup licking and pup-retrieval in postpartum rats (Keer and Stern, 1999) but does not affect nursing. The MPOA expresses D1 and D2 receptor subtypes (Bakowska and Morrell, 1995) and the level of intracellular DA in the MPOA fluctuates during pregnancy and lactation, with the highest levels in lactation (Lonstein et al, 2003). Miller and Lonstein (2005) have shown that activation of the D1 but not the D2 receptor subtype in the MPOA mediates the normal expression of pup-retrieval, consistent with the notion that DA activities in the maternal circuit play a role in mediating some components of maternal behavior.

Recently, data from this laboratory has suggested a role for NO in the control of maternal behavior. Nitric oxide is a gaseous transmitter molecule synthesized in both peripheral and neural tissues from the conversion of the amino acid L-arginine to L-citrulline. Nitric Oxide acts both as an intra- and inter- cellular messenger (Murad, 1994) and the production of NO from its precursor requires the enzyme nitric oxide synthase (NOS) and, in neurons and epithelial cells, the presence of calcium. The synthesis of NO can be suppressed dose-dependently by a NOS inhibitor, L-nitro argenine methyl ether (L-NAME) (Bhat, Mahesh, Aguan, & Brann, 1996).

Nitric oxide synthase is widely distributed within the central nervous system and has being implicated in a number of different functions. Interestingly, neuronal NOS (nNOS) is observed in many brain areas involved in the regulation of parturition, lactation and maternal behavior including the magnoocellular and parvocellular regions of the paraventricular nucleus (PVN), the supra optic nucleus (SON), (Popeski, Amir, and Woodside, 1999) and the MPOA (Okamura et al, 1994).

In the PVN, nNOS is colocalized with OT, Corticotropin releasing hormone (CRH) and arginine-vassopressin (AVP) (Yamada, Emson and Hokfelt, 1996). Stimulating the OT system either by water deprivation (Summy-Long et al, 1993), osmotic stimulation (Neumann et al, 1995), late pregnancy or lactation (Popeski , Amir and Woodside, 1999) increases NOS and hence the capacity for NO production within both the PVN and SON. Ovariectomized virgin rats given estrogen and progesterone to mimick the hormonal profile of pregnancy also show an increase in NOS within the PVN and SON (Popeski et al, 1999). Further, either suppression of PRL with bromocryptine or adminstration of an oxytocin receptor antagonist reduced the number of NOS positive

cells in the PVN (magnocellular subdivision) and the SON (Popeski, Amir, Diorio, and Woodside, 2003). Interestingly, exogenous OT administration was able to restore NOS in rats treated with either an OT receptor blocker or bromocryptine. These data confirmed the positive relationship between OT and NOS and also lend additional support to the hypothesis that PRL interacts with OT to contribute to the increased in NOS observed in the PVN and the SON of late pregnant rats (Popeski et al, 2003).

Studies from our laboratory have shown that suppression of NO production interferes with some components of maternal behavior in postpartum rats. Specifically, inhibition of NO production by administration of L-NAME into the third ventricle eliminates pup retrieval in females treated on Day 4 postpartum (pp) and reduces maternal aggression (Popeski and Woodside, 2004). Interestingly, maternal aggression was also disrupted in females treated on Day 10 pp without affecting their willingness to interact with pups that approach them. Apparently, it is the characteristics of the pups rather than the dams that produced this change in response to NO inhibition. Mothers tested on Day 10 pp. also failed to retrieve Day 4 pups but again interacted with 10 day old pups that were capable of initiating maternal contact. We have also demonstrated that ICV treatment with L-NAME increase the latency to approach pups and decrease the time females spent investigating their pups but does not affect the amount of time spent investigating another appetitive stimulus, fruit loops (Woodside, Service and Popeski, *neuroscience abstract 2002)*. These behavioral deficits were associated with a significant reduction in c-fos immunoreactivity in both the MeA and the MPOA but not in other areas of the maternal circuit.

An important question arising from these studies is the identity of the brain where NO acts to produce such profound changes in maternal behavior in postpartum rats. Because lesion and pharmacological studies have shown that retrieval behavior is one component of maternal behavior that is consistently affected by manipulations of the MPOA in postpartum rats this was the site we investigated. Therefore, the experiments described in this thesis examined the role of NO in the MPOA on pup-retrieval and maternal aggression. First, however, it was necessary to identify a dose of the NOS inhibitor, L-NAME, that did not affect either pup retrieval or maternal aggression when injected into the third ventricle. Thus in Experiment 1 the dose response relationship between administration of L-NAME into the third ventricle and disruption of retrieval behavior and maternal aggression was investigated. In Experiment 2 a dose response curve was obtained for the effect of bilateral administration of L-NAME into the MPOA on maternal behavior.

Experiment 1

Methods

Subjects

Forty-five virgin Wistar rats, weighing from 220- 240g, when obtained from Charles Breeding Farms, (St. Constant, Quebec) were subjects in this experiment. On arrival in the laboratory, rats were housed in groups of five in stainless steel cages (50 cm x 20 cm x 15 cm). Two days later, a stud male was placed into each group-cage. Approximately eighteen days later, females showing signs of pregnancy were housed individually in polypropylene cages (37 cm x 33 cm x 17 cm) with beta chip bedding. All rats were maintained on a 12/12 light cycle with lights on at 8: 00 am, and at a room temperature of 21 $^{\pm}2^{\circ}$C. Rat-chow and water were available *ad libitum* throughout the experiment. All procedures were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Apparatus and Materials

Testing Apparatus: Testing was carried out in the females' home cage. For the test, the top of the home cage was removed and was replaced by a rectangular frame structure (L: 19 " x W: 13.5 " x H: 11.5 ") with each side constructed with wire mesh. This removable top of the apparatus was constructed with 1" cubic wooden strip framed around the wire mesh so that any activity in the home cage was visible. A groove was made in the wood around the base of the apparatus such that it attached securely to the top of the rat's home cage. Female-pup interaction was recorded with a Digital HD Panasonic video camera connected to a television (Toshiba) and a VHS DA SEARS Electronics VCR

Drugs: Nitro^{w}-$_{L}$-Argenine Methyl Ether (L-NAME) purchased from Sigma Chemical Co. St. Louis, MO was prepared at concentrations of 0, 5 mg, 10 mg, 20 mg, 40 mg, 80 mg, 100 mg and 125 mg / ml of 0.9% saline solution. One of these concentrations was administered unilaterally into the 3rd ventricle (ICV) at a volume of 2 µl to give doses of 0, 10µg, 20 µg, 80 µg, 160 µg, 200 µg or 250 µg of L-NAME.

Procedure

Stereotaxic surgery: On the day after birth, Day 1 postpartum (pp), all litters were culled to 8 pups and each female was weighed, and injected with a mixture of ketamine /xylazine (5.7 mg of ketamine and 0.86 mg xylazine/ 100g of body weight). Once the animal was completely anaesthetized, the fur on the surface of the skull was shaved, cleaned with 70% ethanol and then the rat was placed in a stereotaxic apparatus in a flat skull position. A 26 gauge stainless steel cannula (Plastic One) was implanted in the third ventricle of the brain using coordinates (AP: -0.09 mm from bregma, DV: -6.4 mm) based on the Paxinos and Watson atlas (1986). The cannula was held in place by a head cap made of dental acrylic that was anchored to the skull by four stainless steel screws. When the cannula was securely fixed in place, a blocker was place within the guide to prevent any particles from getting into the brain. At the end of each surgery, a topical antibiotic (Cicatrin ^{tm}) was applied to the incision. The rat was removed from the stereotaxic apparatus, wrapped up in paper towel in order to be kept warm and then was returned to its home cage to recover from surgery.

Habituation: On Days 2 and 3 pp, a candy (Tootsie roll ^{tm}) was placed into the home cage of each female rat, and was removed immediately after the rat picked it up with their mouth and transported it to a different location.

On the same days (Day 2 and 3 pp.), litters were removed from the home cage and separated from dams for approximately 20 minutes. During this period, females were habituated to the injection procedure by removing the blocker from the cannula and inserting the injector for 2 minutes. The injector through which the drug and vehicle were administered extended 1mm below the tip of the cannula. Immediately following habituation to the injection procedure, the testing procedure was simulated by fitting the apparatus around the female's home cage for fifteen minutes. This process was performed in order to reduce any potential stress to the animal that can be provoked by the injection procedure or exposure to novelty. After exposure to the testing apparatus, mother and pups were reunited by placing the pups in the nest site of the home cage.

Behavioral testing: On Day 4 pp (Test 1), pups were removed from the home cage and were placed in a small plastic container with soft paper towel in order to keep them warm. The female was immediately transported in the home cage to the testing room, the top of the home cage was removed and the apparatus was attached to it. At this point the female was not able to hear, see, smell or interact with the pups. Ninety minutes after separation, each female received one dose of L-NAME depending on group assignment. After the injection was completed, the injector was left in place for 2 minutes to ensure that the drug was completely deposited into the target site. Thirty minutes later, pups were scattered individually around the home cage and mother-pup interaction was videotaped for thirty minutes. Immediately following the pup-retrieval test, a candy was placed in the home cage and taping continued for a maximum of 10 minutes.

Once the retrieval tests were completed, an adult male was introduced into the home cage with the female and pups for a maximum time of fifteen minutes. The male

was removed from the female's home cage either as soon as the male was attacked by the female or after 15 minutes. Following completion of all testing, the apparatus was removed, the cage lid was replaced and dams and litters were returned to the colony.

The day after testing (Test 2), females were separated from their litters for 30 minutes. Pups were then scattered in the home cage and this mother-pup interaction was recorded for 10 minutes.

Measures (*Test 1*): Videotapes were observed, and the latency to approach the first pup, latency to retrieve the first pup as well as the total number of pup retrieved within 30 minute was recorded for each female. Whether or not females retrieved the candy and attacked the male intruder as well as the latency to aggress against the male was also recorded.

Test 2.

The latency to retrieve the first pup as well as the total number of pups retrieved within the 15 minute test was recorded for each female.

Histology: Each rat was injected intraperitoneally with an overdose of sodium pentobarbital (Somnotol 30 mg/ 100 g body weight) and was perfused transcardially with approximately 150 ml of 0.9% saline followed by 150 -200 ml of 10% formaldehyde. Each brain was carefully removed from the rat's skull, was place in a vial with 15 ml of 30% sucrose/ 10% formaldehyde solution and was kept in the refrigerator at -4°C until the brain sank. Brains were transferred to the -80° C freezer until they were processed for cannula placement.

To check the accuracy of cannula placements for each brain, 40 μm sections were obtained on a cryostat. Sections were obtained from where the cannula tract first

appeared until it completely disappeared. Each set of sections was placed free-floating in Trizma Buffered Saline (TBS; Ph 7.2) solution and subsequently was individually mounted on gelatin-coated slides. The slides were left to dry for a minimum of three days before they were stained with cresyl violet (using a standard protocol), cover slipped with permount and were left to dry for at least 48 hours. Sections were visualized using the microscope. Only data from animals identified as having cannula placements directly into the third ventricle were included in the statistical analyses.

Statistical analyses: A one-way between groups analysis of variance (ANOVA) was employed in order to compare groups on latency and frequency measures. When appropriate, post hoc analyses were carried out using the Tukey least significant difference test (LSD).

In addition, Chi square analyses were performed to compare the number of females in each treatment condition that retrieved at least one pup, the number of females in each group that aggressed against an adult male intruder and the number of females that retrieved candies

Results

Test 1.

Latency to approach first pup

There were no significant group differences in the latency to approach first pup (see Figure 1) in the home cage.

Latency to retrieve first pup

The mean latency to retrieve the first pup for all 7 groups can be seen in Figure 2. Data analysis revealed significant differences across treatment conditions ($F(6, 38) = 9.408$, $p < 0.05$). Post hoc tests indicated that ICV infusion of L-NAME at doses of 200 μg (n = 6) and 250 μg (n = 7), but not at 0 (n = 6), 20 μg (n = 6), 80 μg (n = 6) or 160 μg (n = 6), increased the latency to retrieve the first pup.

Total number of pups retrieved

As can be seen in Figure 3, the average number of pups retrieved varied across treatment conditions ($F(6, 38) = 6.460$, $p < 0.05$). Results obtained from post hoc analysis indicated that L-NAME treatment at doses of 200 μg and 250 μg reduced the number of pups that females retrieved compared to all other groups

Percentage of female retrieving at least one pup

The percentage of females across treatment conditions that retrieved at least one of eight pups can be seen in figure 4. Chi square analysis revealed a significant difference among groups ($\chi^2(6, 45) = 24.698$, $p < 0.05$). Females treated with L-NAME at 200 μg and 250 μg retrieved significantly fewer pups than females in all other groups.

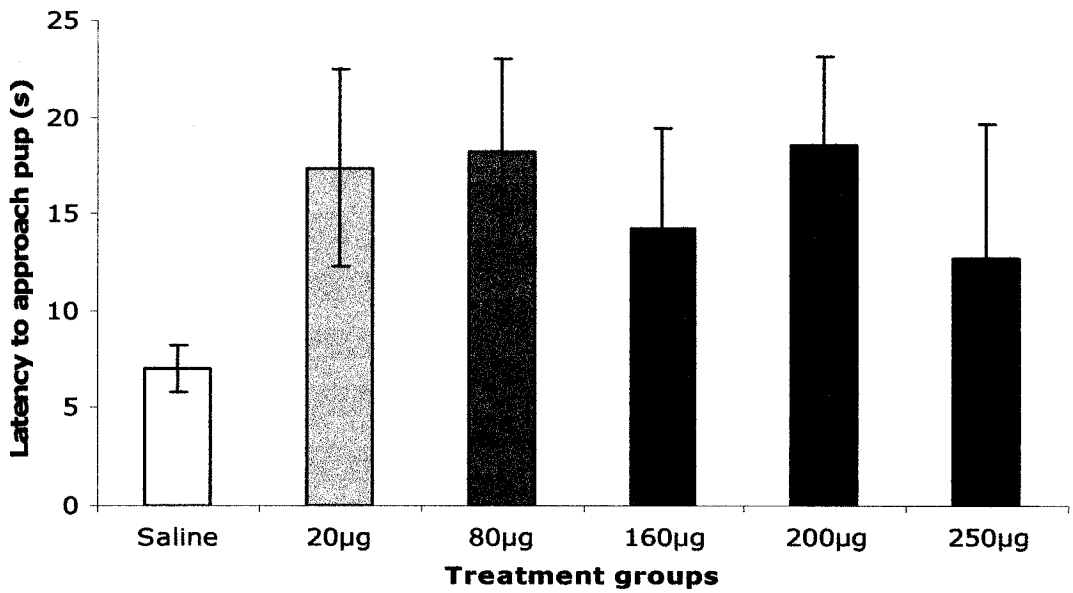

Figure 1. Latency to approach the first pup for all groups (mean ± standard error) during Test 1.

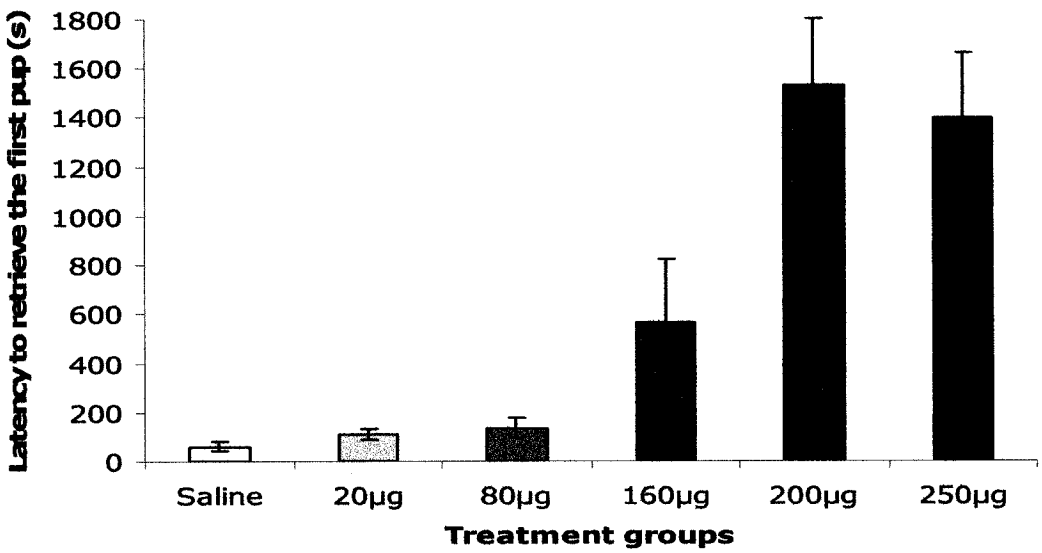

Figure 2. Latency to retrieve the first pup for all groups (mean ± standard error) during Test 1.

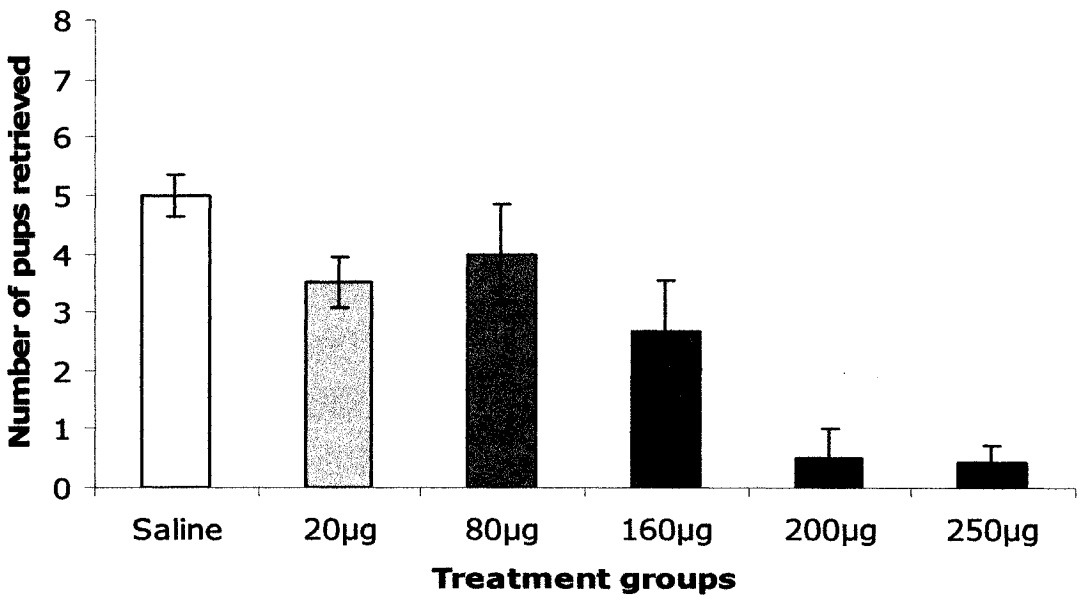

Figure 3. Average number of pups retrieved for all groups (mean ± standard error) during Test 1.

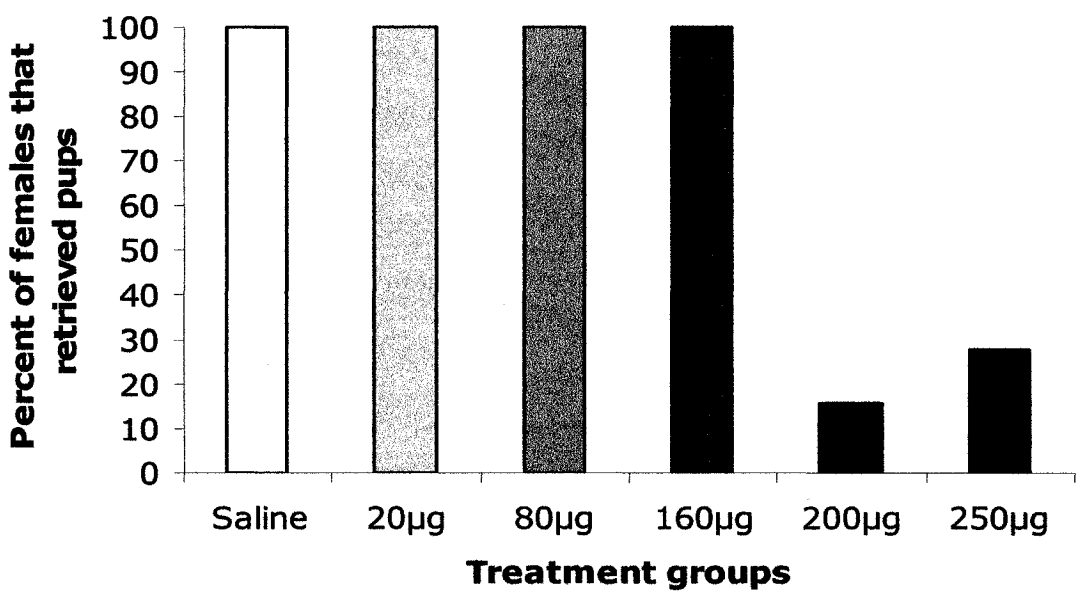

Figure 4. Percentage of females for all groups that retrieved at least one pup during Test 1.

Candy retrieval

There were no differences across groups in the percentage of rats that retrieved candy (see Figure 5) in the home cage.

Latency to contact a male intruder

There were no differences across groups in the latency to contact a male intruder into the home cage (see Figure 6).

Latency to aggress a male intruder

The mean latency to aggress against a male intruder for all 7 groups can be seen in Figure 7. Analysis revealed significant differences across groups ($F(6, 38) = p < 0.05$). Post hoc tests indicated that ICV infusion of L-NAME at a dose of 250 μg (n = 7) and but not at 0 (n = 3), 20 μg (n = 6), 80 μg (n = 6), 160 μg (n = 6), or 200μg (n = 6) increased the latency to aggress a male intruder.

Percentage of female that aggressed towards a male intruder

Figure 8 shows the percentage of females in each group that attacked a male intruder in the home cage. Chi square analysis revealed a significant difference between groups ($\chi^2(6, 45) = 13.437, p < 0.05$). Infusion of L-NAME at a dose of 250 μg resulted in a reduction of the proportion of females aggressing against a male intruder compared to all other groups.

Test 2.

There were no group differences in the latency to retrieve the first pup or the total number of pups retrieved when females were tested on the drug-free day (see figure 9 and 10)

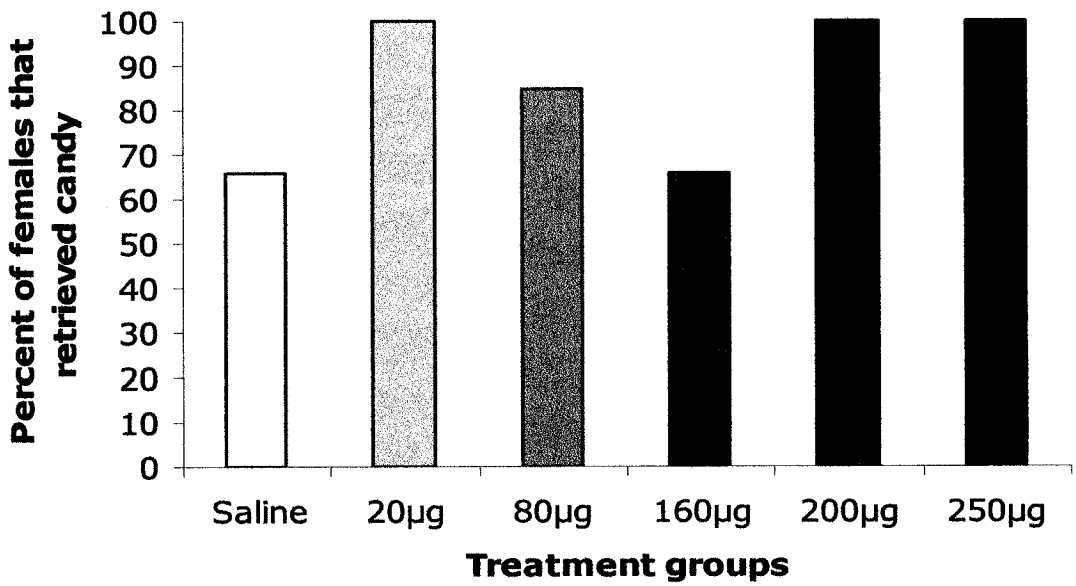

Figure 5. Percentage of females in all groups that retrieved candy during Test 1.

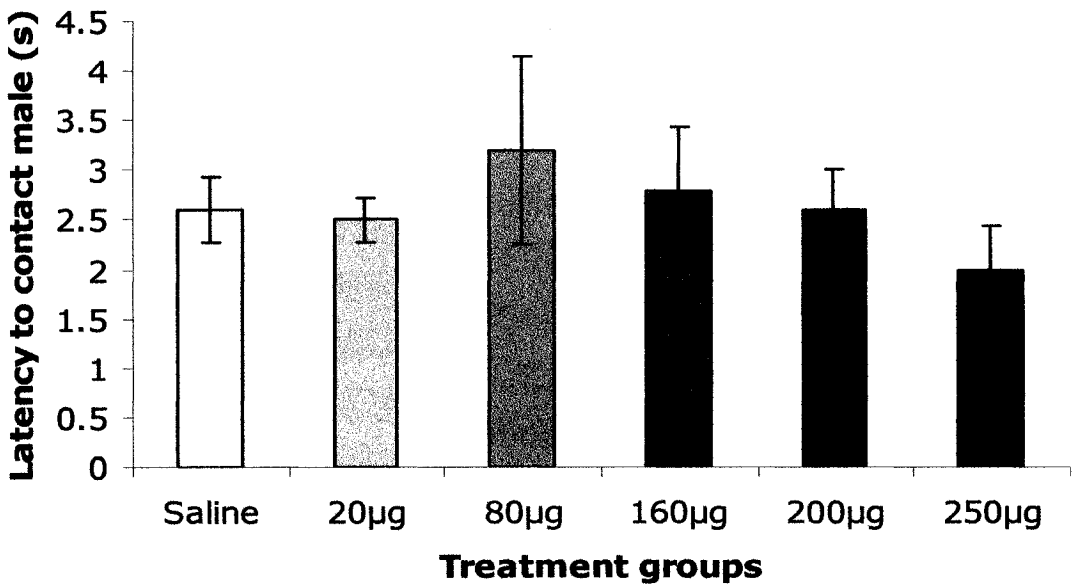

Figure 6. Latency to first contact a male intruder for all groups (mean ± standard error) during Test 1.

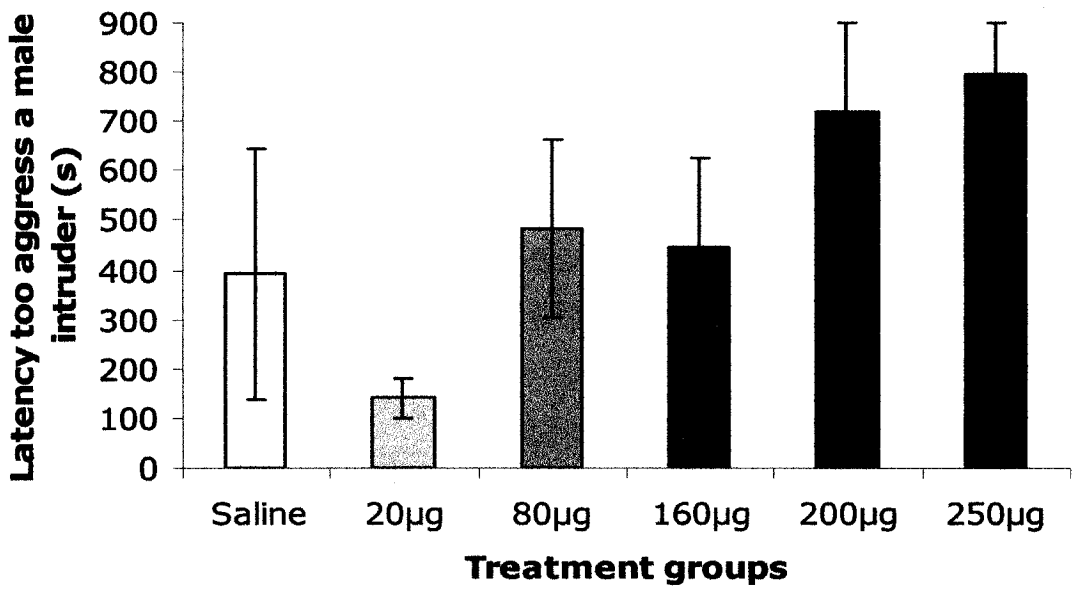

Figure 7. Latency to aggress a male intruder for all groups (mean ± standard error) during Test 1.

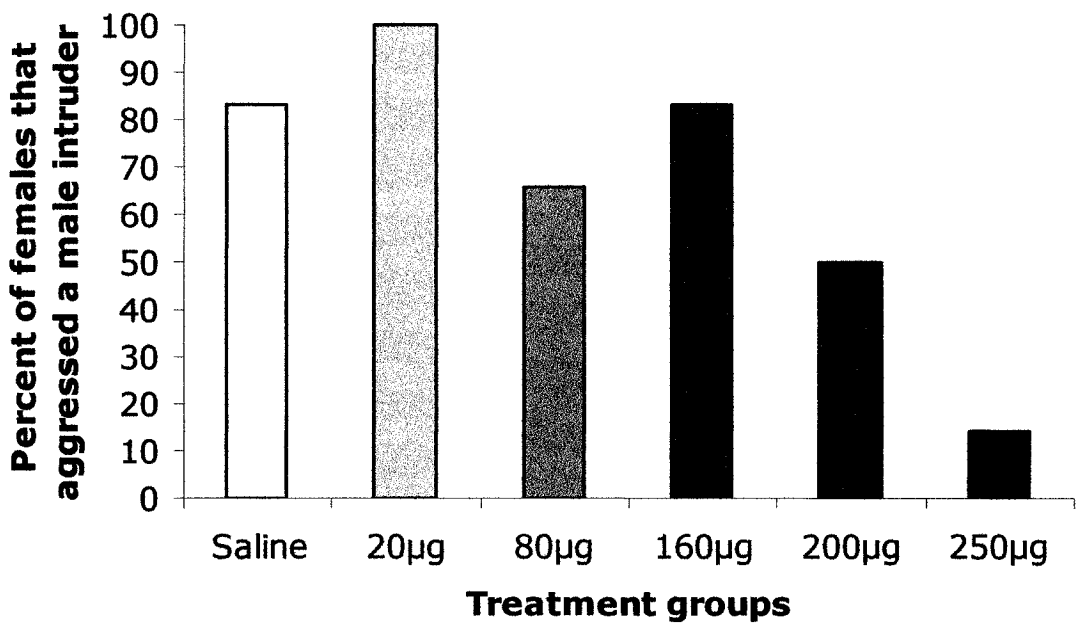

Figure 8. Percentage of females in all groups that show aggression toward a male intruder during Test 1.

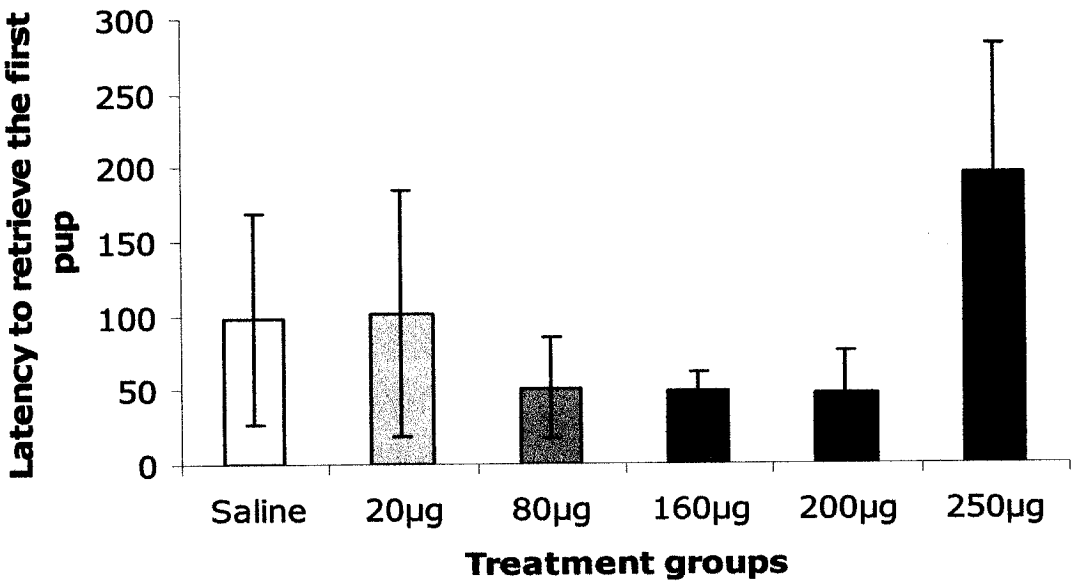

Figure 9. Latency to retrieve the first pup for all groups (mean ± standard error) during Test 2.

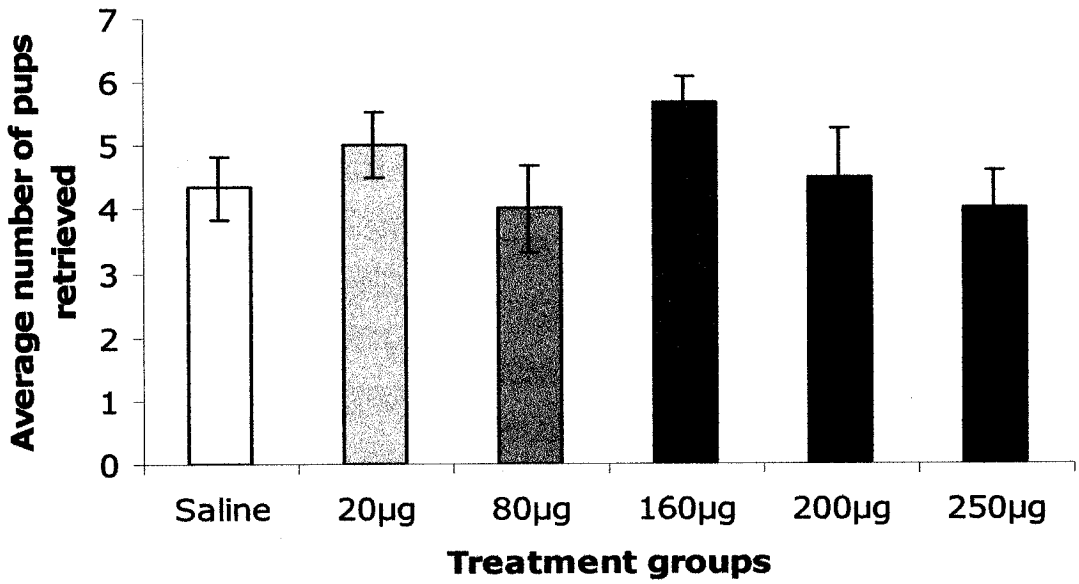

Figure 10. Average number of pups retrieved for all groups (mean ± standard error) during Test 2.

Discussion

The results of Experiment 1 demonstrate that administration of L-NAME at doses of 200 μg and 250μg into the third ventricle disrupts some aspects of maternal behavior. These results are similar to previous findings by Popeski and Woodside (2004) showing that suppression of NO production with L-NAME at a dose of 250μg administered ICV disrupted both pup-retrieval and maternal aggression in lactating rats. A key difference between the Popeski et al (2004) study and the present experiment, however, is that a variety of doses of L-NAME were used in the current study.

Administration of L-NAME at 200 μg and 250 μg into the third ventricle increased the latency for female rats to retrieve the first pup, but did not affect their latency to approach them when compared to all other groups. Furthermore, females treated with the two highest doses of L-NAME also retrieved fewer pups than females in all other conditions. In fact, only one female from the 200 μg group (n = 6) and two females from the 250μg group (n = 7) retrieved any pups at all.

Females in all groups retrieved the candy to a different location in the home cage suggesting that their ability to retrieve was not impaired; rather this impairment in retrieval was specific to pups. Moreover, as shown in figure 9 and 10, females across all groups both approached and retrieved their pups the day after L-NAME treatment suggesting that it had no permanent effect on maternal behavior.

Similar to results obtained by Popeski et al, (2004) findings of Experiment 1 showed that ICV treatment of L-NAME at the two highest doses also reduced maternal aggression in day 4 postpartum rats. Although females in these two groups did not differ from any other groups in their latency to first contact the male, they had an increased

latency to aggress against him. Moreover, fewer females in the 250 μg group directed any aggression to the male and there was a similar trend (P = 0.07) for rats in the 200 μg group.

Consistent with report from previous research (Popeski et al, 2004), the present experiment show that NO activity during lactation within the rat brain is critical in mediating these pup retrieval and maternal aggression. The two lowest doses used in this experiment did not produce any behavioral effects and in Experiment 2, we investigated the effects of infusing these two ineffective doses of L-NAME into both hemisphere of the MPOA on both pup-retrieval and maternal aggression.

Experiment 2

Methods

The general methods, surgical procedures, testing, histological procedures, measures and statistical procedures were the same as described earlier for experiment 1. In this experiment however, a 26 gauge stainless steel bilateral cannula (width: 1.5 mm; Plastic One) was implanted aimed at the MPOA using coordinates (AP: -0.01 mm from bregma; ML: -0.03 mm; DV: -0.70 mm from top of skull) from the Paxinos and Watson (1986). In an additional group of females (dorsal control, DC), similar bilateral cannulae were implanted in an area above the MPOA using the same anterior/posterior (AP) and medial/lateral (ML) coordinates

Rats were assigned to one of five groups. Rats in the first four groups received bilateral injections of 0, 10 μg, 20 μg, or 40 μg of L-NAME per side into the MPOA in a volume of 0.5 μl. The dorsal control group (DC) received bilateral injections of 20 μg/side of L-NAME. Only data from animals identified as having cannula placements directly into the MPOA or a site dorsal to the MPOA were included in the statistical analyses (see Figure 11 and 12 for injection sites).

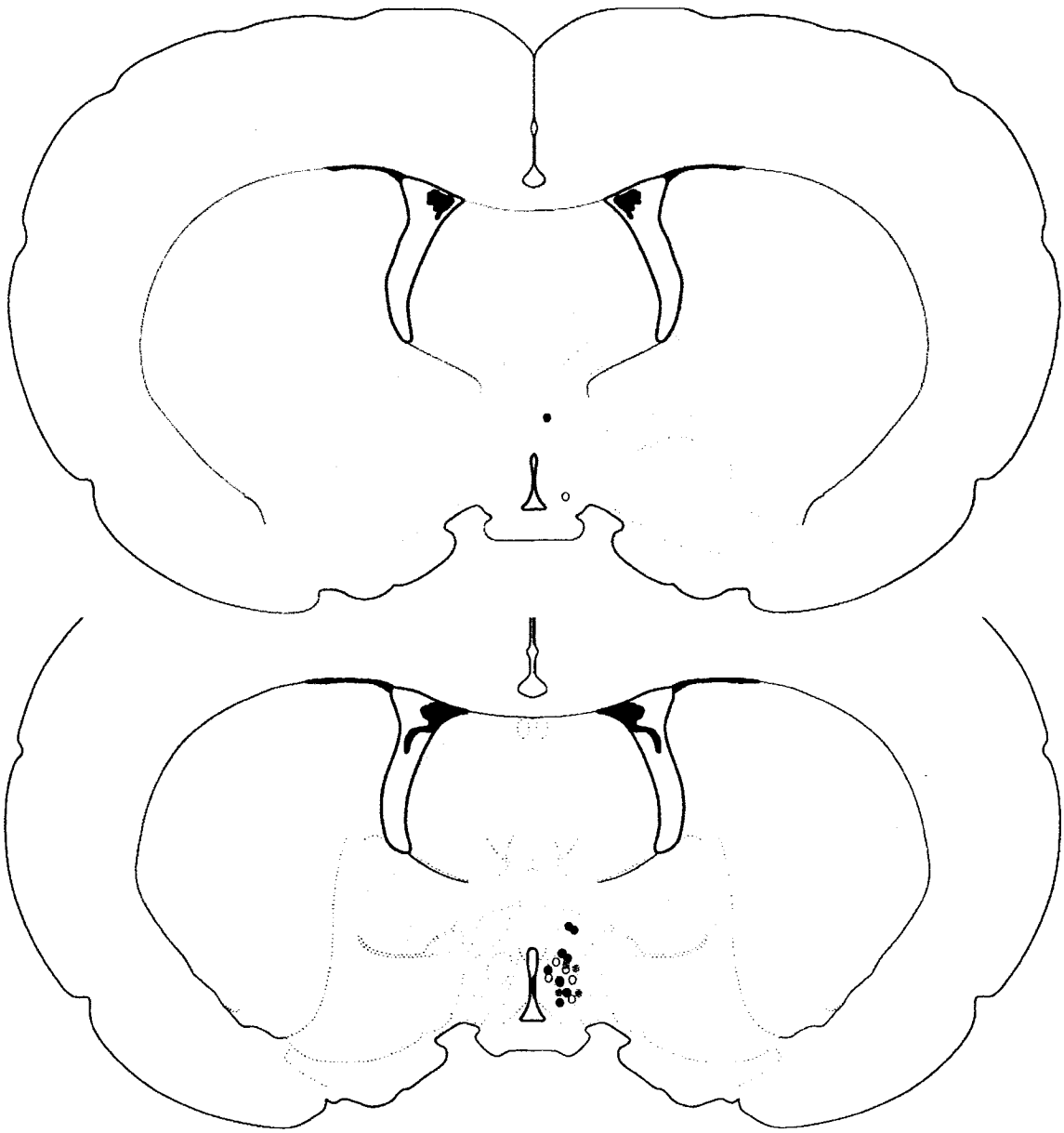

Figure 11. Coronal sections taken from the Swanson atlas depicting the injection sites for all groups (corresponding to the different colors).

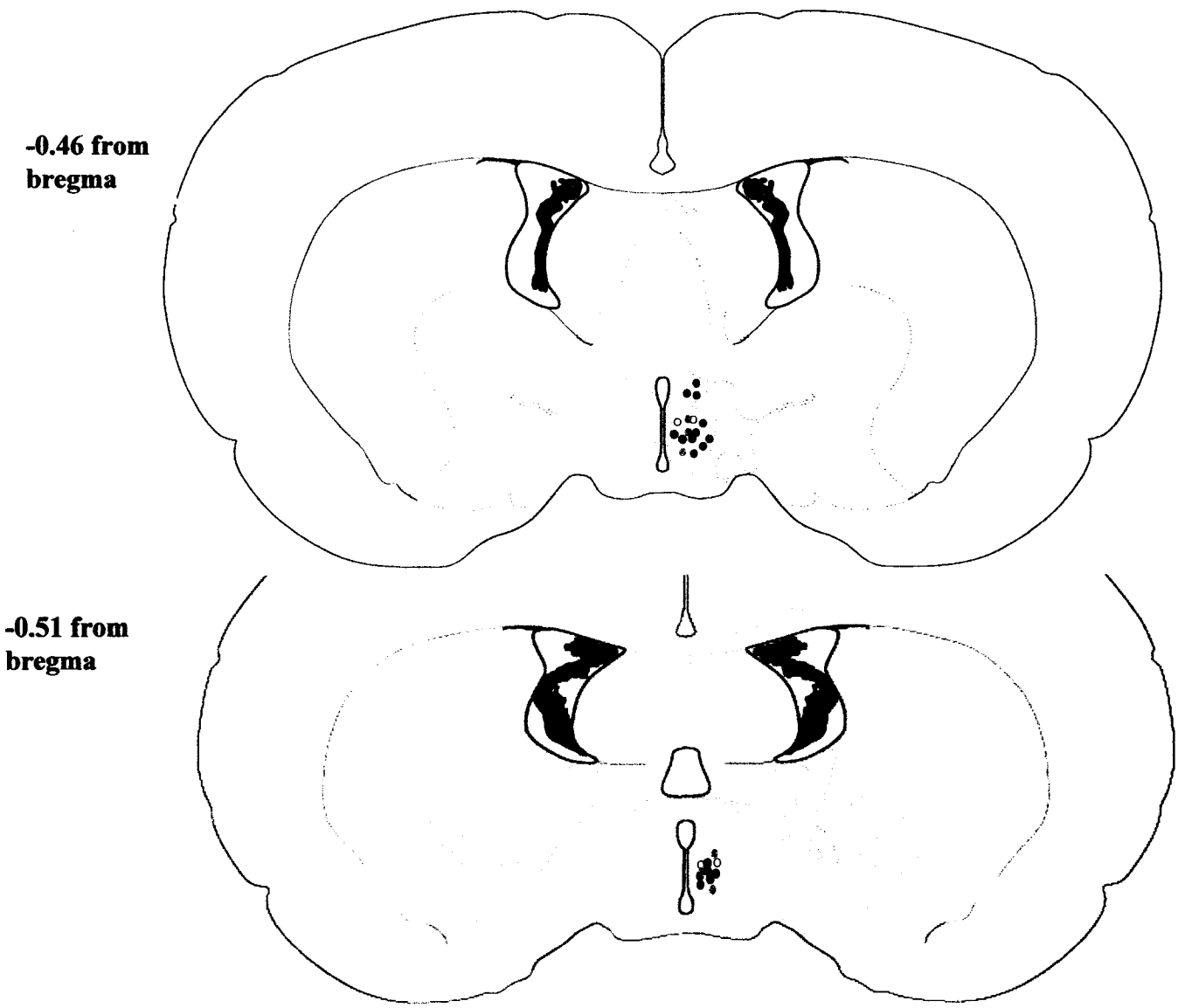

Figure 12. Coronal sections taken from the Swanson atlas depicting the injection sites for all groups (corresponding to the different colors).

Results

Test 1.

Latency to contact first pup

There were no significant differences across groups in the latency to approach the first pup (see Figure 13).

Latency to retrieve first pup

As can be seen in Figure 14, the mean latency to retrieve the first pup differed across groups (F (4, 39) = 6.867, $p < 0.05$). Post-hoc analysis indicated that females in the L-NAME group treated with doses of 20 μg (n = 10) or 40 μg (n = 10) took significantly longer to retrieve the first pup than females in the other groups saline (n =10), 10μg (n = 10), and DC at 20 μg (n = 6).

Number of pups retrieved

Figure 15 shows the average number of pups retrieved for each treatment group. Data analysis revealed a significant difference among treatment groups (F (4, 39) = 5.884, $p < 0.05$). Post-hoc analysis indicated that L-NAME treatment in the MPOA at 20 μg and 40 μg significantly reduced the number of pup retrieved compared to all other groups.

Percentage of female retrieving at least one pup

The percentage of female retrieving at least one pup varied across groups (χ^2 (4, 46) = 19.550, $p < 0.0006$) (see Figure 16). Infusions of L-NAME at 20 μg and 40 μg administered into the MPOA caused a reduction in the proportion of females that retrieve at least one pup compared to all other conditions.

Candy retrieval

As can be seen in Figure 17, the percentage of rats that retrieved candy did

not differ across groups.

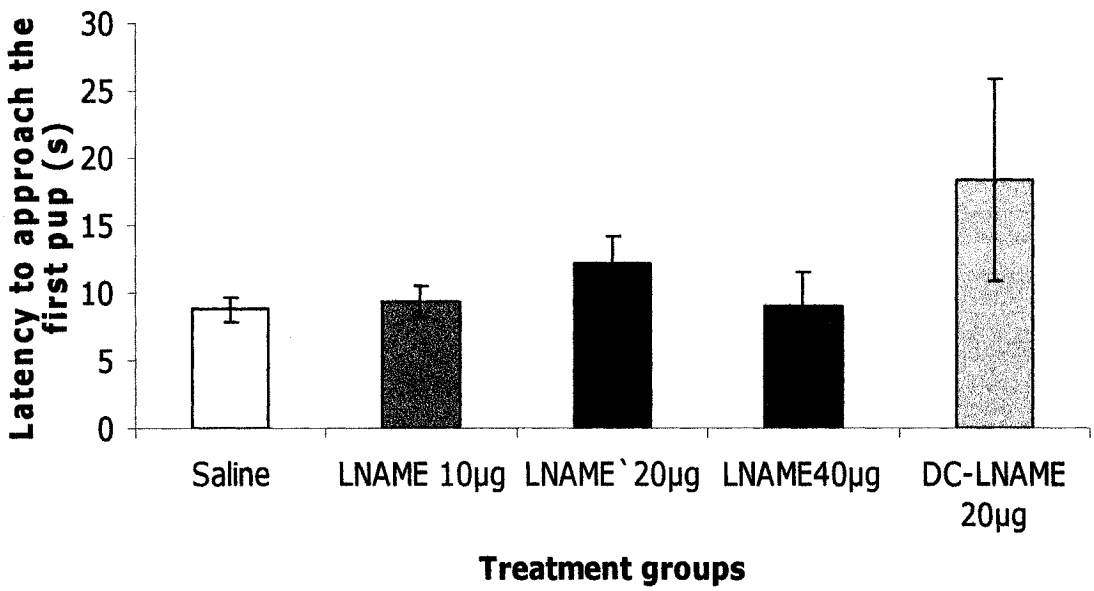

Figure13. Latency to approach the first pup for all groups (mean ± standard error) during Test 1.

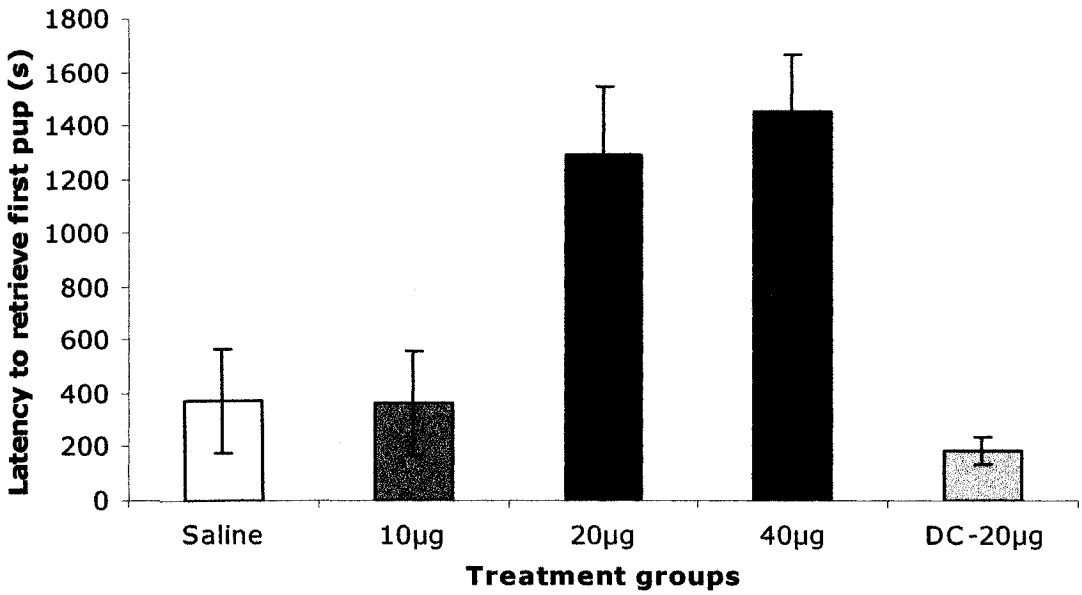

Figure 14. Latency to retrieve the first pup for all groups (mean ± standard error) during Test 1.

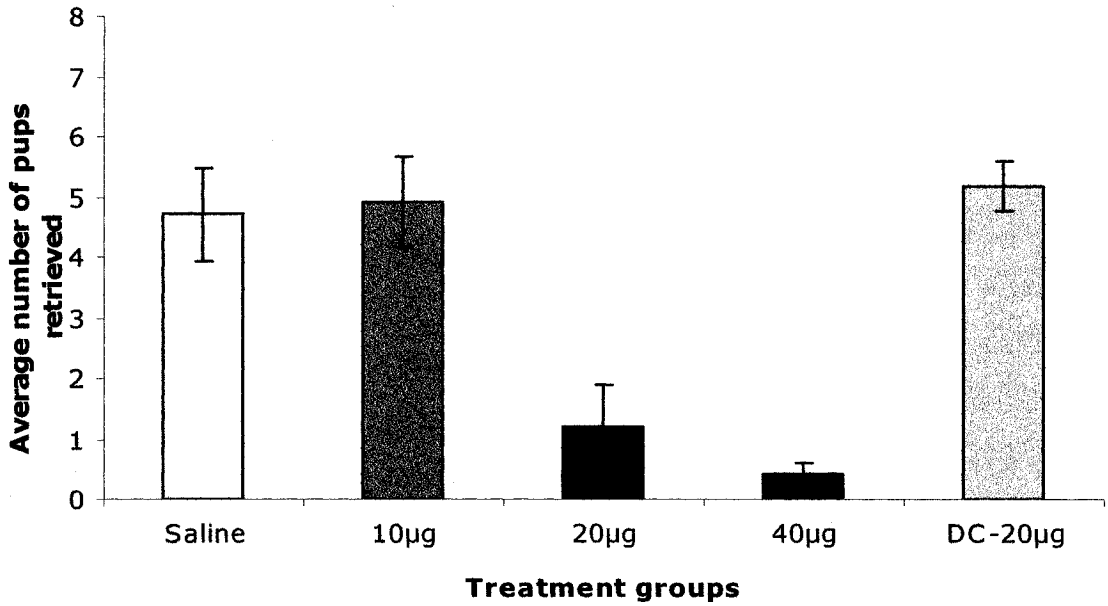

Figure 15. Average number of pups retrieved for all groups (mean ± standard error) during Test 1.

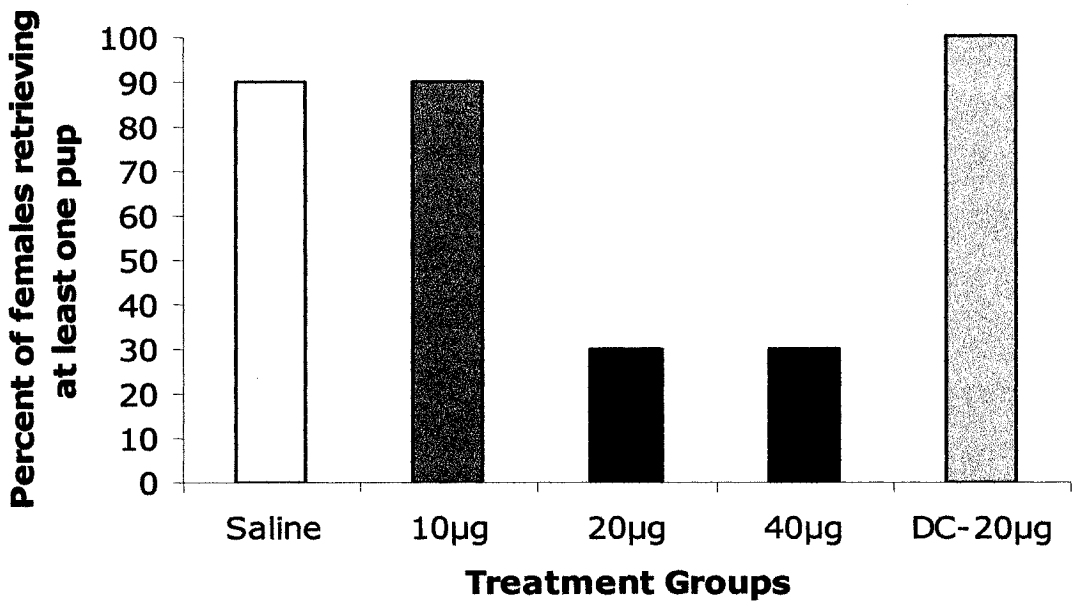

Figure 16. Percentage of females for all groups that retrieved at least one pup during Test 1.

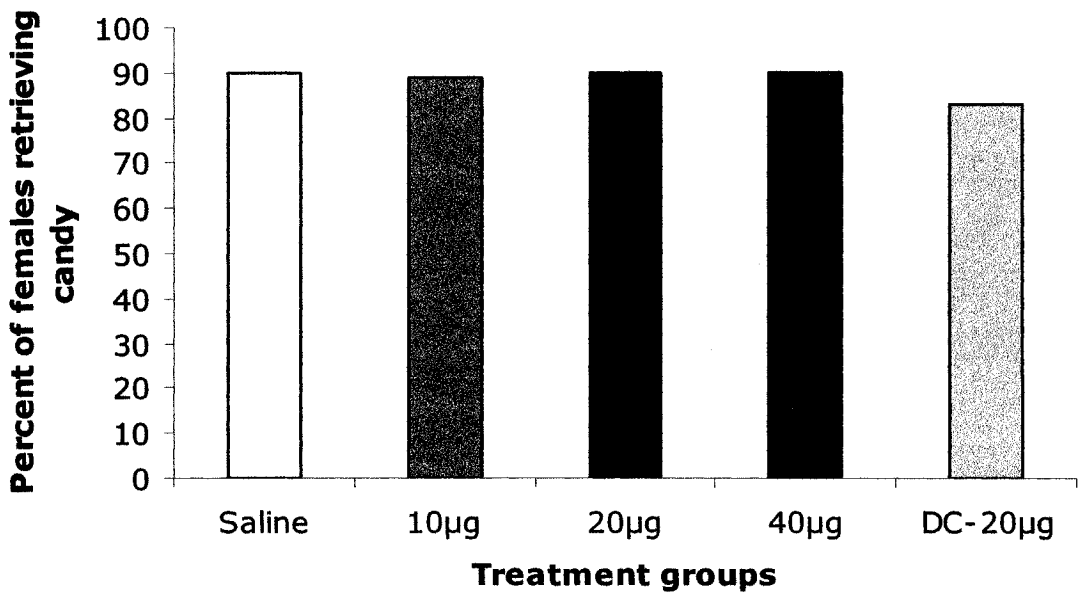

Figure 17. Percentage of females across all conditions that retrieved candy during Test 1.

Latency to contact a male intruder

There were no significant differences across groups in the latency to contact a male intruder in the home cage (see Figure 18).

Latency to aggress a male intruder

As can be seen in Figure 19, the latency to aggress against a male intruder differed among groups (F (4, 40) = 4.454, $p < 0.05$). Post hoc analysis indicated that females treated with 40 μg took significantly longer than females treated with saline to show aggression toward the male intruder. There were no significant differences among groups that receive L-NAME treatment.

Percentage of female that aggressed towards a male intruder

Figure 20 shows the percentage of female who directed aggression toward a male to the nest site. Chi square analysis revealed no significant differences across groups although there was a trend (P =.076) for fewer animals in the 40 μg and the DC to show a reduction in maternal aggression compared to all other groups.

Test 2.

There were no differences across groups in either the latency to retrieve the first pup or the total number of pups retrieved when females were tested the day after the drug treatment (see figure 21 and 22).

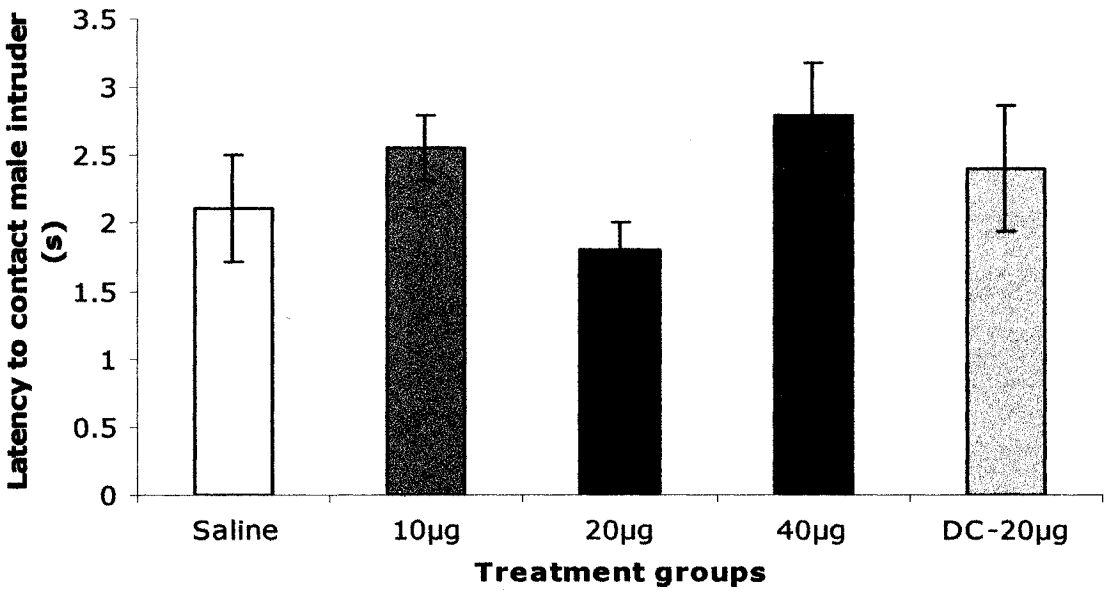

Figure 18. Latency to contact a male intruder for all groups (mean ± standard error) during Test 1.

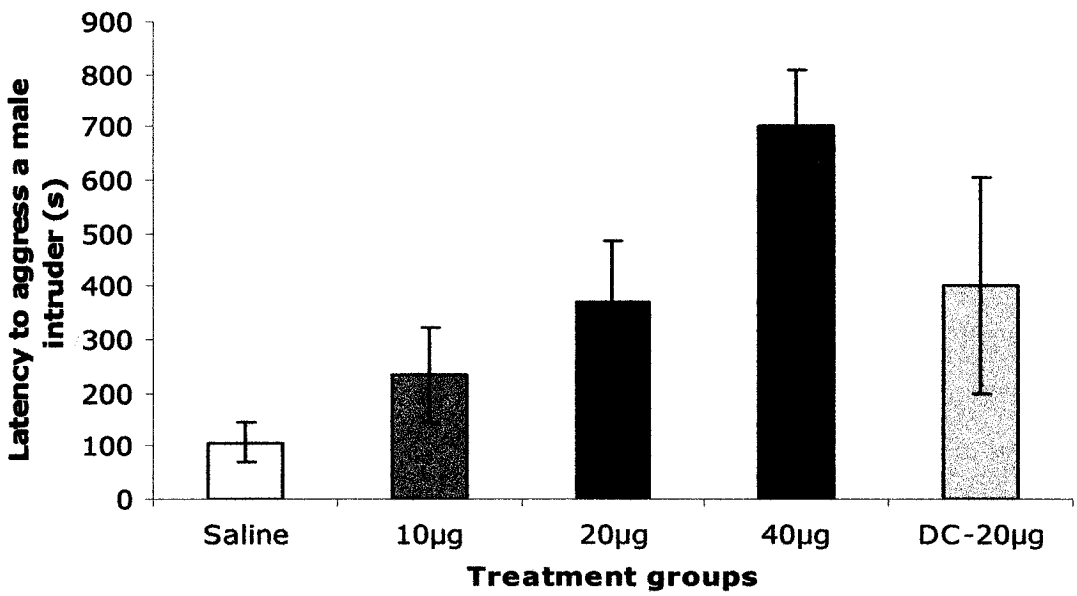

Figure 19. Latency to show aggression to a male intruder (mean ± standard error) during Test 1.

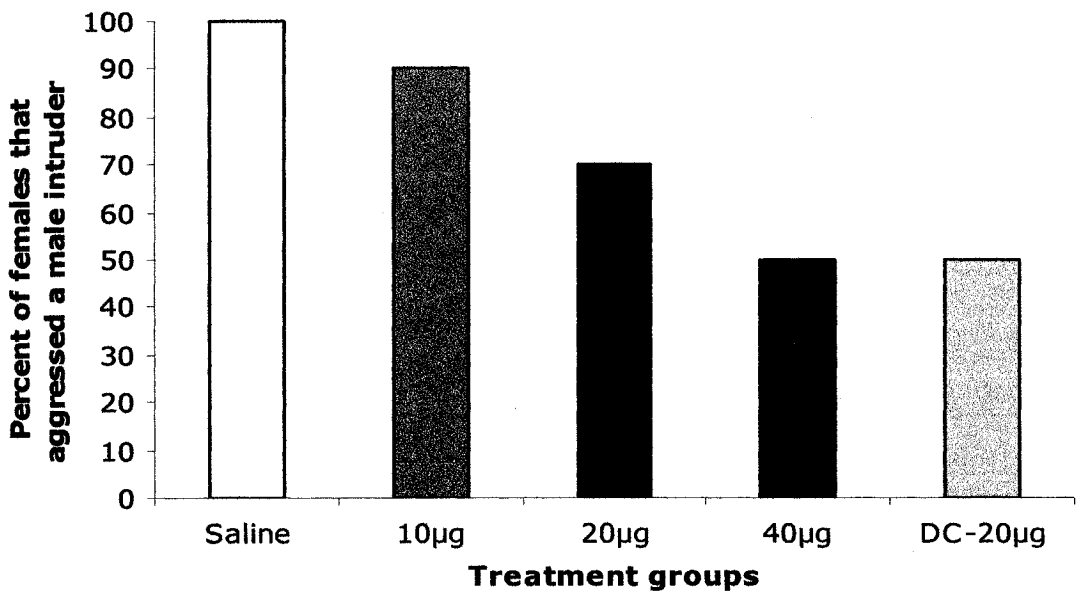

Figure 20. Percentage of females for all groups that showed aggression to a male intruder during Test 1.

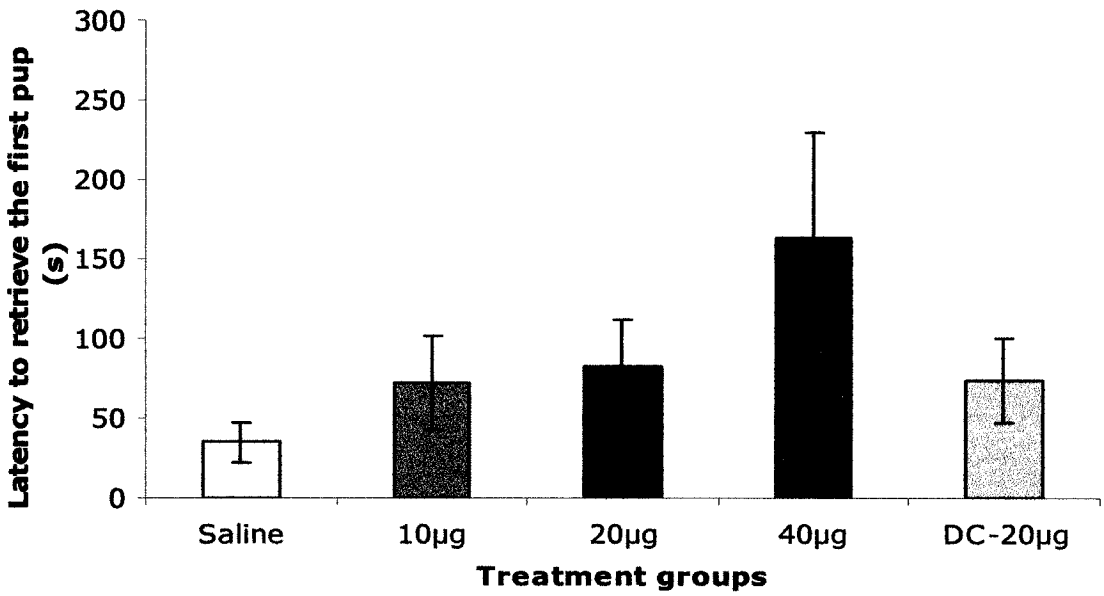

Figure 21. Latency to retrieve the first pup for all groups (mean ± standard error) during Test 2.

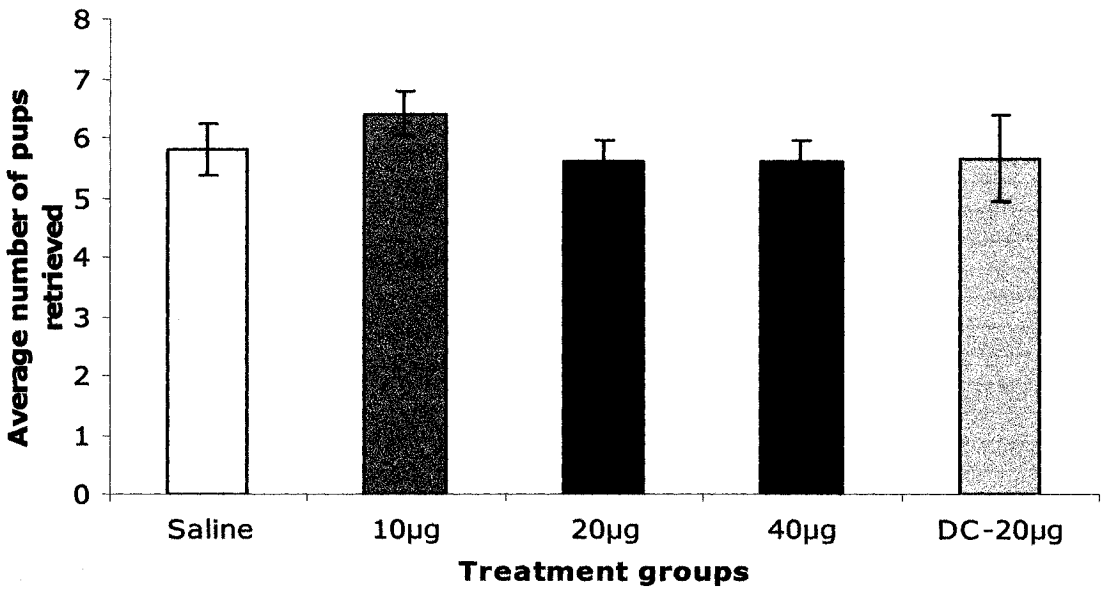

Figure 22. Total number of pup retrieved for all groups (mean ± standard error) during Test 2.

General Discussion

Results obtained from the experiments described in this thesis demonstrate a role for NO acting within the MPOA to facilitate aspects of ongoing maternal behavior. Data obtained from Experiment 1 are consistent with previous findings showing that suppression of NO production with L-NAME administered into the third ventricle disrupts both pup-retrieval and maternal aggression in lactating rats (Popeski and Woodside, 2004). These findings were elaborated upon in experiment 2 where it was shown that NOS activity within the MPOA mediates pup-retrieval but interestingly had little effect on maternal aggression in lactating rats on day 4 postpartum.

As in the Popeski et al study (2004) the effect of L-NAME administration on pup retrieval was quite specific. All females across different groups retrieved candy to the nest site suggesting that the ability to retrieve was not impaired. Similarly, although L-NAME at doses of 200 μg and 250μg increased the latency for female rats to retrieve the first pup, it did not affect their latency to approach their young suggesting that investigatory behavior was similar across all the groups. Interestingly, there were no differences across groups in pup retrieval on the day after treatment suggesting only a transient effect of L-NAME on retrieval behavior.

There was a marked similarity between the effects on pup retrieval observed following 3rd ventricle L-NAME treatment in experiment 1 and those obtained with L-NAME infusions into the MPOA in Experiment 2. Again there were no group differences in either the latency to approach the pups or in the ability to retrieve candy but as is shown in figures 12 and 13, respectively, suppression of NO synthesis with L-NAME at doses of 20μg/side and 40μg/side into the MPOA increased the latency to retrieve the

first pup and reduced the amount of pup retrieved compare to all other groups. Importantly similar to results of experiment 1, there were no groups differences in either latency to retrieve the first pup or number of pups retrieved on the day after treatment (see figure 19 and 20). Because bilateral lesions to the MPOA impair ongoing maternal behavior (Numan, 1974), the fact that all females in this experiment retrieved their pups to the nest site in Test 2 suggests that only limited damage to this area was caused by bilateral cannula implantation.

One possible mediator of NO's effects in the MPOA is dopamine. In fact, administering the NO precursor, L-arginine, into the MPOA has been shown to increase extracellular DA in male rats (Lorrain and Hull, 1993). In addition, Miller and Lonstein (2005) have recently reported that infusion of a D1 receptor antagonist into the MPOA impaired retrieval in postpartum rats without interfering with approach behavior or nursing. Moreover, NO has been shown to increase DA receptor activation by blocking DA transporter (Pogun et al, 1994). The specific impairment in pup-retrieval observed in Experiment 2 may very well be the result of a disruption in the NO-DAergic system in the MPOA. In fact, preliminary results obtained from a study currently being conducted in our laboratory indicate that the effects of bilateral L-NAME administration into the MPOA can be reversed with co-infusion of a D1 agonist, SKF3893.

In the context of their work on male sexual behavior, Elaine Hull and her colleagues have provided interesting evidence on another way in which NO might influence dopaminergic activity within the MPOA. They showed that reverse dialysis of the neurotransmitter glutamate into the MPOA increased extracellular DA levels (Dominguez et al, 2004) and that this effect was reversed with simultaneous infusion of

L-NAME but not its inactive isomer D-NAME. Although these effects were observed in males, similar neurochemical mechanisms may very well facilitate aspects of maternal behavior such as the impairment on pup-retrieval observed in Experiment 2.

Whether L-NAME injections into the MPOA modulate retrieval behavior through DA receptor activity or some other pathway how such changes within the MPOA modulate other brain areas to produce these behavioral effects remains to be determined. Lesions of the NA shell as well as cutting the lateral pathways from the MPOA to the VTA also disrupt retrieval behavior suggesting that projections from the MPOA to these areas may be critical for maternal behavior.

In postpartum rats, both the MPOA/vBNST and the NA show an increase in c-fos activation in response both to the simple presence of pups (Lonstein et al, 1998) and as a result of mother litter interaction (Fleming et al, 1994; Stack and Numan, 2000). Further, unilateral lesion of the MPOA was reported to result in a reduction in FOS activation on the ipsilateral side of the shell of NA during mother-pup interaction (Stack et al, 2002). Considered together, this evidence indicates a functional relationship between the MPOA and the NA and it is possible that NO activity modulates the output from the MPOA to the NA to facilitate aspects of maternal behavior including pup-retrieval. According to Numan et al (2005) the influence of the MPOA on the NA ultimately modulates its output to the VP, which is responsible for controlling the motor output that leads to retrieval behavior.

There is a considerable amount of evidence, which suggests that pups and pup-odor are rewarding to maternal rats. One paradigm that is commonly used to examine reinforcement and reward properties of stimuli is the place preference paradigm. Using

this paradigm, Mattson et al (2001) showed that during the early postpartum period (day 8 pp) rats show a preference for a chamber associated with pup-cues over a chamber associated with cocaine. This preference was reversed in the late postpartum period (day 16 pp and on day 10 pp there was equipreference for either the pup-cue or the cocaine cued chamber. These data suggest that there is a shift in the reward properties associated with pups across the postpartum period. Other studies have shown that lactating rats press a bar to gain access to pups and that this behavior is increased after as little as one hour of pup deprivation (Lee, Clancy and Fleming, 2000). Bar-pressing for pups, like other a maternal behavior is significantly reduced in postpartum rats with MPOA lesions. Whether manipulating NO production within the MPOA would also change the mother's willingness to bar press for pups or her preference for a chamber associated with pup-related cues remains to be determined.

Although there were marked similarities in the effects of administration of L-NAME into the 3rd ventricle and the MPOA on retrieval behavior, the effects of these two manipulations on maternal aggression differed somewhat. Bilateral suppression of NOS into the MPOA had little effect on both the latency to contact or to show aggression toward the male intruder. However, as can be seen in figure 20, the percentage of rats that actually show aggression to the male intruder decreased as the dose increased, although this effect did not reach statistical significance. It is possible that a higher a dosage of L-NAME bilaterally infused into the MPOA would be sufficient to disrupt maternal aggression. However, as Figure 20 shows there is a remarkable similarity in aggressive behavior between the DC group and rats treated with the highest dose infused into the MPOA although there were marked differences in retrieval behavior between these two

groups. These data raise the possibility that sites dorsal to the MPOA are critical for maternal aggression. One area of interest that lies within the same rostral-caudal plane as the MPOA and is located dorsolateral to the MPOA and ventral to the anterior commissure is the vBNST. Interestingly, it has been shown that microinjections of OT into the vBNST disrupt maternal aggression in postpartum rats. Whether the OT system within the vBNST is modulated by NO and how that might affect maternal aggression is yet to be determined.

Another site that has been implicated in the control of maternal aggression is the PVN. There is an elevation in both OT and NOS in the PVN during late pregnancy as well as during lactation (Popeski et al, 1999). Recently, Bosch et al (2005) showed that OT is released in the PVN during maternal aggression and individual differences in OT in this region correlate with levels of aggression. Furthermore, injections of OT into the PVN increase maternal aggression suggesting that OT activity in this site plays an important role to maternal aggression. Release of OT from the magnocellular compartment of the PVN has been shown to be modulated by NO. Whether NO modulates the OT circuits that contribute to maternal aggression as it does OT release in response to osmotic stimulation is as yet unknown.

The interpretations of the results described in this thesis rest on the assumption that the effects of L-NAME administration were limited to the MPOA. The fact that we observed no effect of L-NAME on retrieval in the dorsal control group suggests that dorsal sites are unlikely to be involved in the effects of L-NAME that we observed in this behavior. However, lateral knife cuts that sever the connections between the MPOA and the LH have been shown to disrupt maternal behavior and it may be that lateral spread of

L-NAME is important for the effects on retrieval behavior that we observe. Future studies investigating the effects of L-NAME administration at more lateral sites would test this possibility.

L-NAME itself suppresses both neuronal and endothelial NOS. therefore, the administration of L-NAME results both in changes to neural chemistry and the state of constriction of the microvasculature. It is conceivable, therefore, that the changes that we have attributed to neuronal function are mediated by a more general effect on blood flow to these brain areas. The site specificity of our effects together with the fact that studies using the specific inhibitor of nNOS, 7 nitroindazole, have similar effects on maternal aggression to L-NAME make this possibility unlikely.

The studies described in this thesis generate many other questions and hypotheses. One of these is whether NO interacts with the dopamine system in the MPOA to modulate maternal behavior as it does to modulate male sexual behavior. As mentioned above, in an on-going experiment, we are investigating the potential interaction of NO and dopamine in the MPOA on maternal behavior. Preliminary results indicate that the effects of L-NAME administration into the MPOA can be reversed with a D1 agonist suggesting that NO-DA interactions are indeed important for retrieval behavior.

Other studies are needed to better characterize the precise deficit(s) in maternal behavior produced by L-NAME administration into the MPOA. The data obtained by Popeski and Woodside (2004), as well as other preliminary data obtained after MPOA administration of L-NAME suggest that these treatments do not change nursing behavior. It may be that inhibiting NO production prevents the appropriate response of the female

to distal cues from pups and that the more reflexive response to proximal stimuli is not affected by these manipulations.

In conclusion, bilateral administration of a nitric oxide inhibitor, L-NAME, impaired retrieval behavior in day 4 postpartum rats. This effect was transient as all rats show normal retrieval behavior the day after the treatment was administered. Interestingly, L-NAME administration into the MPOA affected on maternal aggression only at the highest dose and then to only at a similar extent to that produced at a lower dose in the dorsal controls. These data suggest that NO activity within the MPOA mediates some components of maternal behavior and suggest that separate neural circuitry plays the critical role in maternal aggression.

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