Research report

3-Bromo-7-nitroindazole, a neuronal nitric oxide synthase inhibitor, impairs maternal aggression and citrulline immunoreactivity in prairie voles

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Abstract

Lactating female rodents are aggressive against intruders when they are rearing and protecting pups. In prairie voles, Microtus ochrogaster, females exhibit a dramatic increase in citrulline immunoreactivity (citrulline-IR) in the paraventricular nucleus (PVN), but not in control regions of the brain, in association with maternal aggression. Citrulline is an indirect indicator of nitric oxide (NO) synthesis and it is possible that NO release in the PVN is an important element in the control of maternal aggression in prairie voles. In this study, we sought to examine the role of NO in maternal aggression by selectively inhibiting neuronal nitric oxide synthase (nNOS) in lactating prairie voles. Intraperitoneal injections of the nNOS inhibitor, 3-bromo-7-nitroindazole (3-Br-7NI) (20 mg/kg), three times per day over 4 days resulted in significant impairment of the expression of maternal aggression in terms of the average time in aggressive encounters, the average number of attacks, and the average latency to first attack. These behavioral deficiencies were observable beginning two days following the onset of drug treatment. The average time spent sniffing the intruder was indistinguishable between the 3-Br-7NI- and oil-treated females. In 3-Br-7NI-treated relative to oil-treated females, the number of citrulline-positive cells was reduced by 70% in the PVN and by 50% in the anterior amygdaloid area, a control region of the brain. Taken together, these results indicate that 3-Br-7NI effectively inhibits maternal aggression and NO production in prairie voles and suggest that the central release of NO may play an important role in the production of maternal aggression in prairie voles.

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1. Introduction

Female rodents express aggression towards intruders, termed maternal aggression, when they are rearing pups [22,25,31]. Maternal aggression is conserved among mammals and likely increases offspring survival and, hence, maternal fitness [31]. The hormonal and neural bases of maternal aggression are complex. In rats, steroid hormones released during pregnancy enable females to express maternal aggression [2,18,24] and sensory input from nursing pups facilitates the continued expression of maternal aggression during lactation [6,23]. The neuromodulators involved in maternal aggression include oxytocin [11] and serotonin [14]. The expression of maternal aggression is not impaired by the serotonin-specific reuptake inhibitor, fluoxetine, in prairie voles, suggesting that serotonin does not inhibit maternal aggression in this species as it does male aggression [27]. Contrary to the inhibitory role of the gas, nitric oxide (NO), in the control of male aggression in mice [3,19], indirect evidence that NO may play an excitatory role in the production of maternal aggression in mice [8] and in prairie voles [9] has been reported. Mice lacking the gene for neuronal nitric oxide synthase (nNOS−/−) exhibit dramatic deficiencies in the production of maternal aggression, although other features of maternal behavior are normal [8]. In mice and...
prairie voles, a dramatic increase in immunoreactivity for citrulline, an indirect indicator of NO synthesis, occurs in cells within the hypothalamus only in association with maternal aggression [8,9]. In lactating prairie voles, the increase in citrulline-positive cells during maternal aggression occurs in the paraventricular nucleus (PVN) [9], whereas in lactating mice the increase occurs in the medial preoptic area, the suprachiasmatic nucleus, and the subparaventricular zone [8].

In this study we sought to examine, whether, or how maternal aggression was affected in lactating prairie voles that were exposed to a specific nNOS inhibitor, 3-bromo-7-nitroindazole (3-Br-7-NI). If NO is released as an indirect by-product of maternal aggression, then an nNOS inhibitor should not affect maternal aggression. If, though, the release of NO somehow facilitates the expression of maternal aggression, then it would be expected that an nNOS inhibitor would impair maternal aggression. In this study we injected lactating prairie voles with either oil or the specific nNOS inhibitor, 3-Br-7-NI, to examine the effects of an nNOS inhibitor on maternal aggression and citrulline-immunoreactivity (citrulline-IR).

2. Materials and methods

2.1. Animals

Adult male and female prairie voles (Microtus ochrogaster) (>50 days) were used. Animals were housed in polycarbonate cages with ad libitum access to food and tap water throughout the study. All animals were born and maintained in long day lengths (with a 24-h 16:8 light/dark light schedule). All efforts were made to minimize animal pain and discomfort.

2.2. Maternal aggression behavioral testing and drug treatment

Female voles were paired with conspecific males. Following impregnation the females were housed individually. Females, between the fifth and eighth day postpartum were pre-tested for maternal aggression by exposing each female to a group housed, sexually naive intruder male vole for 10 min between 12:00 and 16:00 h. The pups were removed from the cage 3 min prior to the behavioral test and each test session with a male was recorded on videotape and subsequently analyzed off-line to quantify aggressive behaviors. In mice and hamsters, removal of the pups from a mother just before an aggressive test does not diminish the expression of maternal aggression [22,26]. Only females exhibiting ≥5 s of aggression were used in the study. Five females were assigned to each of the two groups, such that the pretest levels of aggression were approximately equivalent. Following the pre-test (day 1), females received intraperitoneal (i.p.) injections of either oil (0.1 ml) or 3-bromo-7-nitroindazole (3-Br-7NI) (Calbiochem, San Diego, CA) (20 mg/kg) dissolved in oil (0.1 ml) at 00:00, 08:00, and 16:00 h for four consecutive days. Each female was again tested for maternal aggression around 16:00 h on day 3 (following 2 days of injections) and on day 5 (following the final injection). On days 3 and 5 each animal was tested exactly 30 min following either the oil or 3-Br-7-NI injection. For comparisons of the behaviors of animals in the two groups, an unpaired Student’s t-test was used for each of the 3 days of behavioral testing. For analysis of latency to first attack, if an animal exhibited no aggression, then it was assigned a conservative latency time of 600 s, the maximum amount of time of the test.

2.3. Immunocytochemistry

Immediately following the final behavioral tests for each 3-Br-7NI-treated animal and for three of the five oil-treated animals, the tested voles were briefly anesthetized for 1 min with methoxyflurane vapor (Mallinckrodt Veterinary, Mundelein, IL) and further anesthetized with an overdose of sodium pentobarbital. Animals were perfused through the heart with an oxygenated Krebs–Heinzleit buffer (118 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose; pH 7.4) followed by a 5% glutaraldehyde/0.5% formaldehyde solution containing 0.2% Na₂S₂O₅ in 0.1 M PBS [5]. Following the perfusions, the brains were removed, post-fixed overnight at 4°C and placed in a 20% glycerol cryoprotectant for 2 days. The brains were frozen on dry-ice immediately before sectioning at 40 μm on a cryostat. The brain sections were collected in PBS and reduced for 45 min with 0.5% NaBH₄ and 0.2% Na₂S₂O₅ in 10 mM PBS with 0.19 mM NaCl (pH 7.4). Subsequently, the sections were washed in PBS in the presence of 0.2% Triton X-100 (PBS-X), blocked in 5% normal goat serum for 1 h, and incubated for 2 days at 4°C with either rabbit anti-citrulline antibodies (1:5000) that had been preabsorbed against arginine [5,20], or rabbit anti-nNOS (C-terminal) antibodies (1:15 000) (Diasorin, Stillwater, MN). After washes in PBS-X, the sections were incubated overnight at 4°C in biotinylated goat anti-rabbit secondary antibodies (1:1000), washed in PBS-X, exposed to an avidin–biotin complex (Vector Laboratories, Burlingame, CA) for 1 h, washed again in PBS-X, and visualized using diaminobenzidine as a chromagen. The sections were mounted and dehydrated before coverslips were applied.

2.4. Cell counting

From each animal, nine consecutive frontal brain sections taken at 80-μm intervals containing the PVN were identified and citrulline-positive cells within the PVN region for each section were counted. The control region for examining overall citrulline-IR was a square region...
with an area of 800 \( \mu \text{m}^2 \) placed at the most ventral region of the anterior amygdaloid area at the level of the brain equivalent to the first appearance of the optic chiasm. All cell counting was performed by eye at \( \times200 \) magnification under a microscope. Cell counting was performed by an individual uninformed about experimental treatments or expectations. For statistical analysis of the number of cells with citrulline-IR in the two groups, an unpaired Student’s \( t \)-test was employed. Because the distance between the representative sections was greater than 80 \( \mu \text{m} \), a size greater than the average size of cell bodies in these regions of the brain, it is not likely that any cells were counted twice. Images shown were scanned at a resolution of 720 dpi using a digital scanning camera (Leaf Systems, Southborough, MA).

3. Results

3.1. Effects of 3-Br-7-NI on maternal aggression in prairie voles

During the pre-test on day 1, lactating prairie voles were reliably aggressive towards intruder males and only three out of 13 females were excluded from the study based on a lack of maternal aggression on day 1. On three measures of aggression on day 1, the means between animals in the oil- and 3-Br-7NI-treated females were approximately equivalent (Fig. 1A–C). When tested for maternal aggression 2 days following the onset of drug treatment (day 3), though, animals injected with 3-Br-7NI exhibited significant inhibition of maternal aggression in terms of time in aggressive encounters, number of attacks, and time to first attack (Fig. 1A–C). These significant deficiencies in maternal aggression were also observed 4 days following drug treatment (day 5), in all categories except average number of attacks where the oil-treated animals exhibited a mean of 13.4±4.2 (±S.E.) attacks compared to the 3-Br-7NI-treated animals that exhibited a mean of 3.8±2.1 (±S.E.) attacks. These differences were not statistically significant according to our criteria (\( P=0.079 \)). On day 5 only three of five 3-Br-7NI-treated animals exhibited aggression compared to five of five oil-treated animals. One of the greatest differences between the two groups occurs in terms of time to first attack. Over the three test days the oil-treated females exhibited a decreased latency to first attack while the 3-Br-7NI animals exhibited an increased latency (Fig. 1C). Further, the latency to first attack is approximately 20-fold longer for 3-Br-7NI-treated animals. The decrease of maternal aggression was not due to a general decrease in mobility of the 3-Br-7NI-treated animals because no differences in movement or interaction of the mothers with the pups were observed either before or after each test. The levels of sniffing the intruder during each test were equivalent during each test day (Fig. 1D).

3.2. Pattern of citrulline-IR in the brain of oil- and 3-Br-7NI-treated lactating female voles following exposure to a male intruder

Citrulline-IR is specifically elevated in the PVN during maternal aggression in prairie voles, but does not change in the anterior amygdaloid area [9], suggesting that NO is released in the PVN during maternal aggression. In the present study, a specific inhibitor of nNOS, 3-Br-7NI, was injected i.p. to inhibit the production of NO by nNOS in lactating prairie voles. Following 4 days of 3-Br-7-NI injections (three injections per day), analysis of brains revealed a significant decrease, but not complete absence of citrulline-IR in drug-treated animals compared to oil injected controls following behavioral testing (Fig. 2). As indicated above, 3-Br-7NI-treated females also expressed
Fig. 2. Citrulline-IR is significantly altered in the PVN of lactating prairie voles treated with 3-Br-7NI relative to oil-treated animals following a 10-min behavioral test with an intruder male. Representative photomicrographs showing high-level citrulline-IR in the PVN of an oil-treated female (A) and a 3-Br-7NI-treated (B) female following the behavioral test. Oil-treated females exhibited high maternal aggression, while 3-Br-7NI-treated females exhibited low or no maternal aggression. The number of citrulline-positive cells is also higher in the anterior amygdaloid area in oil-treated (C) relative to 3-Br-7NI-treated (D) lactating females. The average number citrulline-positive cells in the PVN and anterior amygdaloid area for the two female groups are shown in (E). Bars represent means ± S.E. For the PVN, the total number of citrulline-positive cells was counted from nine sections per animal. For the anterior amygdaloid area cells was counted from one section. Statistically significant differences are shown with an asterisks. *P < 0.05; **P < 0.01, unpaired Student’s t-test.

impaired levels of maternal aggression. In this study, relative to oil injected controls, we observed a 50% decrease in the number of citrulline-positive neurons in the anterior amygdaloid area and a 70% decrease in the PVN in 3-Br-7-NI-treated animals (Fig. 2C–E). The decrease in the number of citrulline-positive cells in the PVN was reduced by drug treatment (Fig. 2E), suggesting that 3-Br-7NI treatment effectively impaired the rapid activation of NO synthesis in the PVN.

To determine whether the number of citrulline-positive cells in the PVN increased with levels of maternal aggression, we examined the correlation coefficient of time spent attacking the intruder and total number of citrulline-positive cells in the PVN. As seen in Fig. 3, using data from both oil- and 3-Br-7NI-treated animals, a significant positive correlation exists between these two variables with a correlation coefficient of 0.851 (P < 0.01; Pearson product moment correlation).

We confirmed that nNOS expression was not disrupted by 3-Br-7NI by examining nNOS levels in the PVN and anterior amygdaloid area of oil- and 3-Br-7NI-treated females. As seen in Fig. 4, no differences are observable in
between NO and maternal aggression has not been established. In this study, we pharmacologically inhibited NO synthesis in lactating prairie voles and found significant deficits in maternal aggression and citrulline-IR. The results of our study are consistent with the hypothesis that NO plays a role in the regulation of maternal aggression in prairie voles.

4.1. Specificity of the nNOS inhibitor, 3-Br-7NI

7-Nitroindazole (7-NI) is a potent inhibitor of the three different isoforms of the NOS enzyme [1]. An advantage of 3-Br-7NI, a derivative of 7-NI, is that it has greater inhibitory activity than 7-NI on nNOS and it is a more selective inhibitor of nNOS than the other two NOS isoforms [1]. In previous studies, acute i.p. injections of 7-NI result in a maximal inhibition of NOS in the brain between 15 and 30 min following injection, but this effect is transient [16] and full NOS activity is restored within 2 h of injection [15]. Chronic i.p. injections of 7-NI can result in approximately 50% [16] or complete inhibition of brain NOS activity [3]. 3-Br-7NI, the derivative of 7-NI that most specifically inhibits nNOS, was used in this study to limit the unnecessary and possibly confounding inhibition of either endothelial NOS or inducible NOS. Citrulline-IR was used to assay the effectiveness of 3-Br-7NI. Citrulline is the breakdown product when NO is enzymatically cleaved from arginine by NOS that remains in the cell and provides a reliable indirect measurement of NO production [5]. The approximately 50% decrease in the number of citrulline-positive cells in the anterior amygdaloid area (Fig. 2C–E) in 3-Br-7NI injected animals suggests that injection of the nNOS inhibitor was effective in reducing production of NO by nNOS within neurons. Previous work indicates that citrulline (and, indirectly,
NO) is actively synthesized during maternal aggression in prairie voles in the PVN [9]. An almost 70% decrease in the number of citrulline-positive cells in the PVN suggests that 3-Br-7NI was able to dampen this phasic increase in NO production in the PVN that accompanies maternal aggression. It is possible that the 3-Br-7NI treatment inhibited citrulline-IR by inhibiting expression of nNOS, but the absence of a difference in nNOS immunoreactivity between the drug and oil injected groups (Fig. 4) does not support this scenario.

4.2. Possible role of NO in maternal aggression in prairie voles

Female nNOS−/− mice show significant deficits in maternal aggression [8], yet male nNOS−/− mice are hyperaggressive [19], and it is possible that the role of NO is different in maternal and male aggression in mice. The finding that mice and prairie voles both exhibit increases in citrulline-IR in the hypothalamus during maternal aggression [8,9], is consistent with the hypothesis that NO has an excitatory role on maternal aggression in rodents. The results from this study indicate that both citrulline-IR and maternal aggression are impaired by an nNOS inhibitor and suggests an excitatory role for NO in maternal aggression. The significant positive correlation between the number of citrulline-positive cells in the PVN and levels of maternal aggression (Fig. 3) is also consistent with a role for NO in maternal aggression. Interestingly, the 3-Br-7NI injected animal that showed the least response to the drug in terms of inhibition of citrulline also exhibited the highest level of aggression among the drug-treated animals (see arrow, Fig. 3). A caveat of this study, though, is that because NO was inhibited in a number of brain regions, it is possible that the deficits in maternal aggression reflect a disabling of a brain function not specific to maternal aggression, but necessary to its proper expression. Thus, in future experiments it will be valuable to specifically target the site of NO inhibition (e.g., in the PVN) to determine how the reduction of NO in a specific site affects maternal aggression.

In contrast to other rodents, male prairie voles normally express low levels of aggression, but exhibit a dramatic increase in aggression towards intruders following mating, termed mating-induced aggression [13,29,30]. We previously found that mating-induced aggression in male prairie voles is also associated with significant increases in citrulline-IR in the PVN [9]. Thus, it is possible that maternal and mating-induced aggression in prairie voles share a common neural basis. Because i.p. injections of 7-NI result in decreased anxiety in male rats [4] and an increase in aggression in male mice [3], it would be interesting to determine how 3-Br-7NI influences mating-induced aggression in prairie voles. If NO has an excitatory role on mating-induced aggression, then it would be expected that this form of aggression would be inhibited by an nNOS inhibitor.

How NO might act in the PVN during maternal aggression remains undetermined. In the PVN nNOS is coexpressed in neurosecretory cells with a range of neuropeptides including oxytocin [32], vasopressin [28], and corticotropin-releasing factor (CRF) [12,21,32]. If NO released in the PVN facilitates maternal aggression, then NO may act by modifying the release or actions of these, or other, neuropeptides. In rats, oxytocin release in the PVN may inhibit maternal aggression [10]. A role for vasopressin in maternal aggression has not been established, but vasopressin has an excitatory role in male aggression [7]. In lactating rodents, a decrease in anxiety is associated with pregnancy and lactation [17], suggesting that decreased anxiety and inhibition of the CRF pathway may facilitate maternal aggression. Whether, or how, NO released during maternal aggression interacts with oxytocin, vasopressin, or CRF remains to be determined.

Maternal aggression in prairie voles is associated with a dramatic elevation of citrulline, an indirect marker for NO production in the PVN that accompanies maternal aggression [8,9], is consistent with the hypothesis that NO has an excitatory role on maternal aggression in rodents. The excitatory role in the production of maternal aggression. In this study we injected lactating prairie voles with a specific nNOS inhibitor and observed significant deficits in both maternal aggression and citrulline-IR. Thus, the results from our work suggest that NO plays an excitatory role in maternal aggression in prairie voles.

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