

Maternal and Mating-Induced Aggression Is Associated With Elevated Citrulline Immunoreactivity in the Paraventricular Nucleus in Prairie Voles

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ABSTRACT

Lactating female rodents are fiercely aggressive against intruders when they are rearing and protecting pups. In monogamous prairie voles, *Microtus ochrogaster*, males are parental and exhibit a dramatic increase in aggression, termed mating-induced aggression, in association with reproduction. In mice, the gas, nitric oxide (NO), inhibits male aggression, but may have an excitatory role in the production of maternal aggression. In this study, we combined aggressive behavioral testing of female and male prairie voles with immunohistochemistry for citrulline, a marker of NO synthesis, to examine NO synthesis indirectly during maternal and mating-induced aggression. A significant increase in the number of citrulline-positive cells was identified in the paraventricular nucleus (PVN) of the hypothalamus in aggressive lactating females compared with unstimulated lactating females. A significant increase in the number of citrulline-positive cells was also observed in the PVN of aggressive mated males compared with nonaggressive unmated males and unstimulated mated males. Both nonaggressive unmated males and unstimulated mated males show similar levels of citrulline immunoreactivity in the PVN. In other regions of the brain, no changes in the number of citrulline-positive cells were observed. These results suggest that NO is released specifically in the PVN during both maternal and mating-induced aggression in prairie voles. *J. Comp. Neurol.* 418:182–192, 2000. © 2000 Wiley-Liss, Inc.

Indexing terms: nitric oxide; hypothalamus; behavior

Female rodents express fierce aggression toward intruders when they are lactating and rearing pups (Siegel et al., 1983; Wolff, 1985; Svare, 1990). This temporal expression of aggression, termed maternal aggression, is conserved among mammals and likely increases the fitness of the offspring (Wolff, 1985). Prairie voles, *Microtus ochrogaster*, are unusual rodents because they are monogamous and both parents contribute to the rearing of pups (Carter and Getz, 1993; Carter et al., 1995). Compared with other male rodent species, male prairie voles are relatively non-aggressive, but they exhibit a dramatic increase in aggression toward intruders after mating, termed mating-induced aggression (Winslow et al., 1993; Insel et al., 1995; Wang et al., 1997), which likely helps to protect the pups. Aggression in most male rodents depends on testosterone, but recent work in prairie voles indicates that some forms of male aggression are independent of testosterone (Demas et al., 1999). Female prairie voles increase aggression after mating and display a strong maternal

aggression toward intruders (Getz et al., 1981; Villalba et al., 1997). In terms of their responsiveness to some pharmacological agents, maternal and mating-induced aggression can be dissociated because the serotonin-specific reuptake inhibitor, fluoxetine, impairs mating-induced aggression but has no effect on maternal aggression (Villalba et al., 1997). Nonetheless, because maternal and mating-induced aggression are both expressed in associa-

Grant sponsor: National Institutes of Health; Grant numbers: MH 57535 and MH 57760; Grant sponsor: National Institute of Mental Health National Research Service Award; Grant number: MH 12371-01.

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Received 12 August 1999; Revised 12 November 1999; Accepted 16 November 1999

tion with mating and reproduction, it is possible that they share some common neural elements.

The mechanisms controlling maternal aggression are complex and are best understood in rats. Steroid hormones, such as estradiol and progesterone, released during pregnancy, enable female rats to express maternal behaviors (Stern and McDonald, 1989; Bridges, 1996), including maternal aggression (Mann et al., 1984), and the sensory input produced by the suckling of pups contributes to the activation and maintenance of maternal aggression (Stern and Kolunie, 1993). This latter action may result from suckling-induced increases in central serotonin production and release (Kordon et al., 1973). Serotonin agonists, though, can have either excitatory or inhibitory actions on maternal aggression in rats, depending on the site of injection (De Almeida and Lucion, 1997). The neuropeptide, oxytocin, may act centrally to facilitate (Ferris et al., 1992) or inhibit (Giovenardi et al., 1998) maternal aggression in rats. Although the gas, nitric oxide (NO), which can act as a neuromodulator within the central nervous system (CNS) (Bredt and Snyder, 1992), appears to have an inhibitory role in the control of male aggression in mice (Nelson et al., 1995; Demas et al., 1997), we recently conducted work with transgenic mice that indicates that NO may play an excitatory role in the production of maternal aggression (Gammie and Nelson, 1999). As part of this research, we combined behavioral testing of wild-type mice with immunohistochemistry for citrulline, an indirect marker of NO synthesis, and found a dramatic increase in citrulline immunoreactivity (citrulline-IR) in cells in the hypothalamus only in association with maternal aggression (Gammie and Nelson, 1999). This result suggested that NO was produced in association with maternal aggression.

Whether or how NO plays a role in the control of maternal aggression of mice is currently being investigated, but in the present study we sought to examine, indirectly, the pattern of NO release in the prairie vole brain in association with both maternal and mating-induced aggression by combining behavioral testing with citrulline immunohistochemistry. A role for NO in any prairie vole behavior has not previously been examined. Because maternal aggression is highly conserved, we hypothesized that citrulline (and NO) might also be synthesized in prairie voles as in mice during the production of maternal aggression. We further hypothesized that if NO plays a similar role in maternal and mating-induced aggression in the prairie vole, then the site of increased synthesis of citrulline (and NO) during the production of both types of behavior would be similar.

MATERIALS AND METHODS

Animals

Adult male and female prairie voles (*Microtus ochrogaster*) (>50 days old) were used. All animals were sexually inexperienced and reared with littermates before matings. 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-hour light-dark schedule of 16:8). The Johns Hopkins animal care and use committee approved all experiments and all research conformed to National Institutes of Health guidelines.

Maternal aggression behavioral testing

Ten female voles were paired with males. After impregnation the females were housed individually. The date of birth was considered postpartum day 0. On day 8, five females were exposed to an intruder male for 10 minutes between 1200 and 1600 hours. The pups were removed from the cage 3 minutes before the behavioral test, and each test session with a male was recorded on videotape and subsequently analyzed off-line to quantify aggressive behaviors by the female. In mice and hamsters, removal of the pups from a mother just before an aggressive test does not diminish the expression of maternal aggression (Svare et al., 1981; Siegel et al., 1983). The intruder males were sexually naive, group-housed voles. For the control females, the pups were also removed, but no male intruder was introduced during the 10-minute period.

Mating-induced aggression behavioral testing

Five males were paired with females. After 2 weeks of cohabitation, the female was removed from the cage, and the mated male was exposed to an intruder male for 10 minutes between 1200 and 1600 hours. Each test session was recorded on videotape and subsequently analyzed off-line to quantify aggressive behaviors by the mated male toward the intruder by individuals who were uninformed about experimental treatments or conditions. Five age-matched unmated males were also exposed to an intruder male, and the aggressive behaviors toward the intruders were also analyzed.

Immunocytochemistry

Immediately after the behavioral tests described above, each test animal was briefly anesthetized for 1 minute with methoxyflurane vapor (Mallinckrodt Veterinary, Inc., 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 (pH, 7.4) (Eliasson et al., 1997). Five lactating females and six males that had been mated for 2 weeks were also perfused as described, but no behavioral test was conducted before fixation. All perfusions were performed between 1200 and 1600 hours. After the perfusions were performed, the brains were removed, postfixed 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 phosphate-buffered saline (PBS) and reduced for 30 minutes 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 hour, and incubated for 2 days at 4°C with either rabbit anti-citrulline antibodies (1:10,000) that had been preabsorbed against arginine (Pasqualotto et al., 1991; Eliasson et al., 1997) or rabbit anti-oxytocin antibodies (1:5,000; Diasorin, Stillwater, MN). After washes in PBS-X, the sections were incubated overnight at 4°C in biotinylated goat anti-rabbit secondary antibodies (1:1,000), washed in PBS-X, exposed to an avidin-biotin

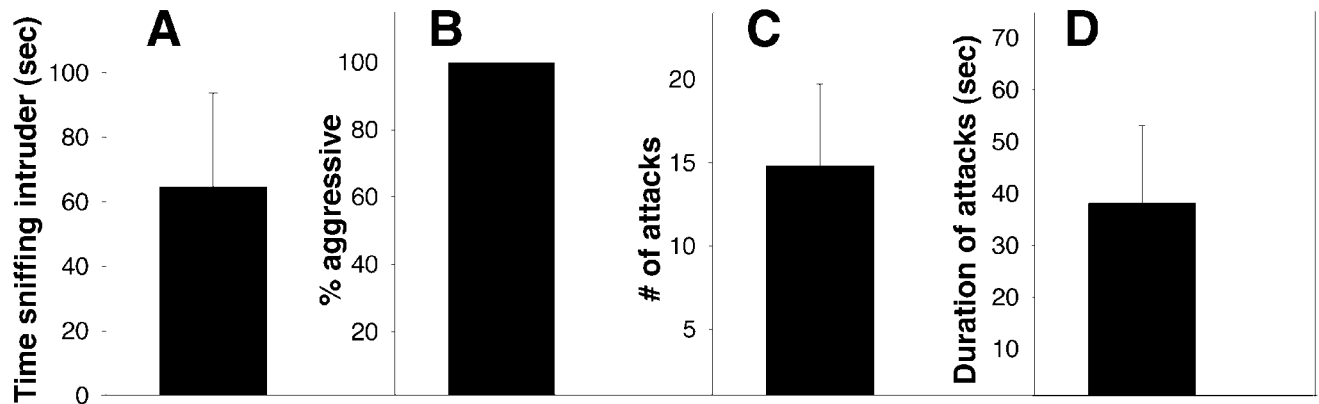


Fig. 1. Lactating female prairie voles exhibit maternal aggression toward intruder males. By using a resident-intruder test, a behavioral profile of lactating female actions toward an intruder is provided in (A) average amount of time sniffing the intruder, (B) percent of the

females that were aggressive, (C) the average number of attacks per 10-minute test period, and (D) the average amount of time spent engaged in an agonistic encounter. Bars represent means \pm SE.

complex (Vector Laboratories, Burlingame, CA) for 1 hour, washed again in PBS-X, and visualized by using diaminobenzidine as a chromagen. The sections were mounted and dehydrated before coverslips were applied.

Cell counting, statistical analysis, and preparation of photomicrographs

From each animal, nine consecutive frontal brain sections taken at 80- μ m intervals containing the paraventricular nucleus (PVN) were identified and 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 μ m² 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 \times 200 magnification under a microscope. The counting of cells with citrulline-IR was performed by two individuals blind to the experimental conditions. An average of these two counts was used for statistical analysis. For statistical analysis of the number of cells with citrulline-IR in aggressive and unstimulated females, an unpaired Student's *t*-test was used. For analysis of cell numbers in males, a one-way analysis of variance (ANOVA) was used. Because the distance between the representative sections was $>$ 80 μ 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 by using a digital scanning camera (Leaf Systems, Southborough, MA).

RESULTS

Maternal aggression of lactating prairie voles

We examined the levels of maternal aggression in lactating prairie voles. For the maternal aggression tests, lactating females were individually housed with their pups, and the pups were removed 3 minutes before the introduction of a sexually naive male intruder in the home cage. Each aggression test lasted 10 minutes. The female

voles were reliably aggressive toward the intruder and 5 of 5 lactating females attacked the intruder (Fig. 1). During the 10-minute test period, the average amount of time spent sniffing the intruder was 64.8 ± 28.7 seconds (\pm SE), the average number of attacks was 14.8 ± 4.9 (\pm SE), and the average duration of time spent attacking the intruder was 38.2 ± 15.0 seconds (\pm SE) (Fig. 1).

Pattern of citrulline-IR in the brain of aggressive and nonaggressive lactating voles

In these studies, we examined the levels of citrulline-IR (an indirect marker of NO release) in the brains of lactating female prairie voles immediately after the behavioral test for maternal aggression and compared those with levels in an unstimulated lactating female control. As seen in Figure 2, when a lactating female prairie vole was exposed to an active intruder male and produced aggression, a significant increase in the number of cells exhibiting citrulline-IR occurs within the PVN of the hypothalamus relative to the nonaggressive controls. In addition to the increase in the number of neurons exhibiting citrulline-IR, aggressive lactating females also exhibited an increase in the number of cells with detectable levels of citrulline-IR within their neuronal processes (Fig. 2D). The unstimulated lactating females exhibited almost no citrulline-IR in the PVN (Fig. 2A,C), whereas the aggressive females exhibited citrulline-positive cells throughout the PVN. Figure 3 shows a representative camera lucida drawing of the distribution of citrulline-positive cells throughout the length of the PVN. The outline of the PVN shown represents the boundary of oxytocin cells in the PVN that were observed in alternate sections of the same animal. As shown in Figure 3, the highest number of citrulline-positive cells occurs in the middle sections of the PVN, and fewer cells are observed in the more rostral and caudal sections. A summary of the distribution of citrulline-positive cells along the length of the PVN is also shown in Figure 7. The combined total number of citrulline-positive cells in the PVN was used for most analyses, and the means from the two female groups are shown in Figure 2G.

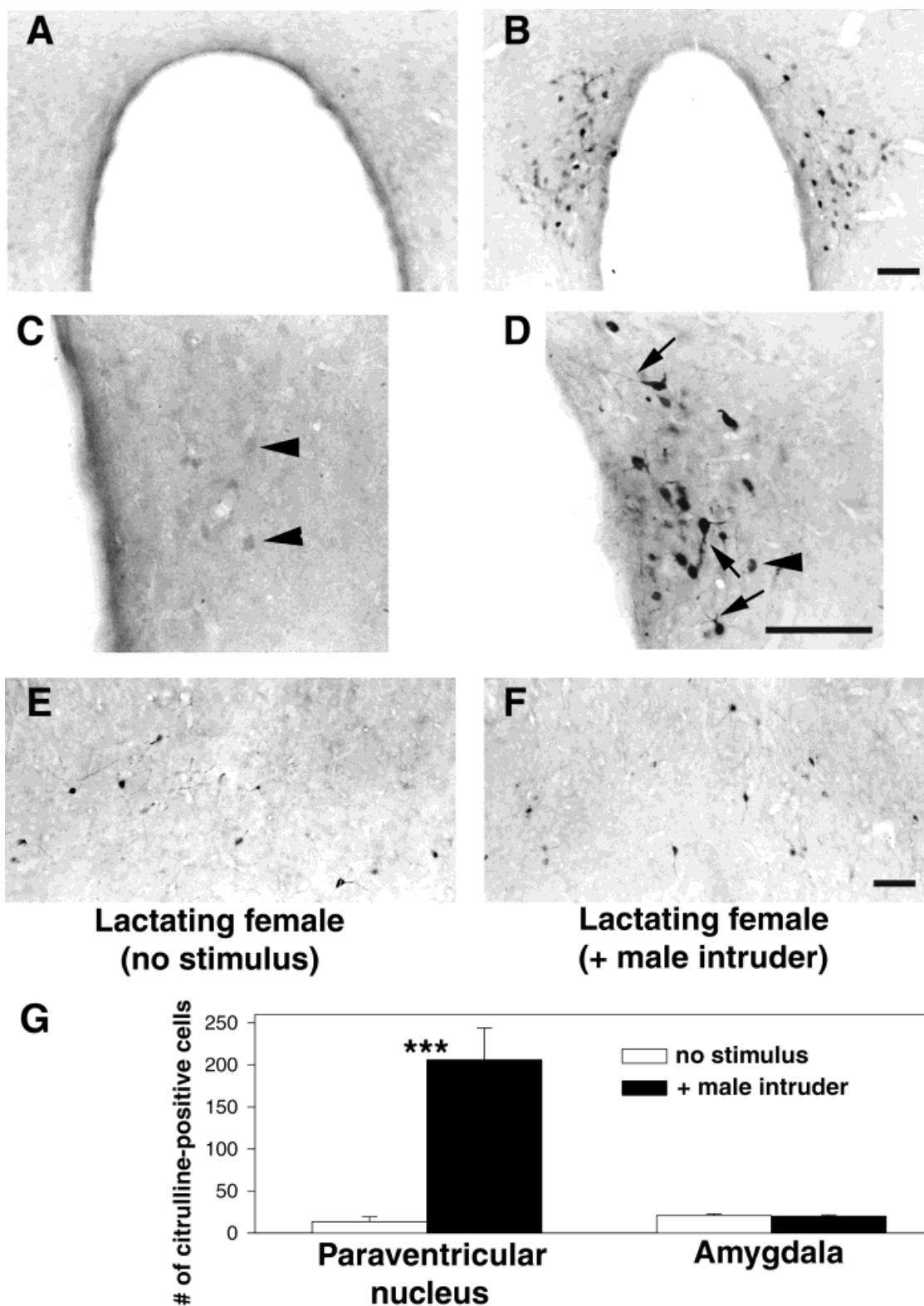


Fig. 2. Citrulline-IR is significantly altered in the PVN of aggressive lactating prairie voles relative to unstimulated lactating voles. Representative photomicrographs showing low-level citrulline-IR in the PVN of an unstimulated lactating female (A,C) and a lactating female exposed to an active male intruder that triggered an aggressive response (B,D). Higher power representative photomicrographs showing different levels of citrulline-IR in the neuronal processes in the PVN of (C) unstimulated lactating females and (D) aggressive lactating females. Arrowheads indicate cells with citrulline-IR in the cell bodies only, and arrows correspond to cells with citrulline-IR

within neuronal processes. The number of citrulline-positive cells is equivalent in the anterior amygdaloid area in the unstimulated (E) and aggressive (F) lactating females. The average number citrulline-positive cells in the PVN and anterior amygdaloid area for the two female groups are shown in (G). Bars represent means \pm SE. For the PVN, the total number of citrulline-positive cells were counted from nine sections per animal. For the anterior amygdaloid area, cells were counted from one section per animal. Statistically significant differences are shown with asterisks. *** = $P < 0.001$, unpaired Student's t-test. Scale bars = 100 μ m.

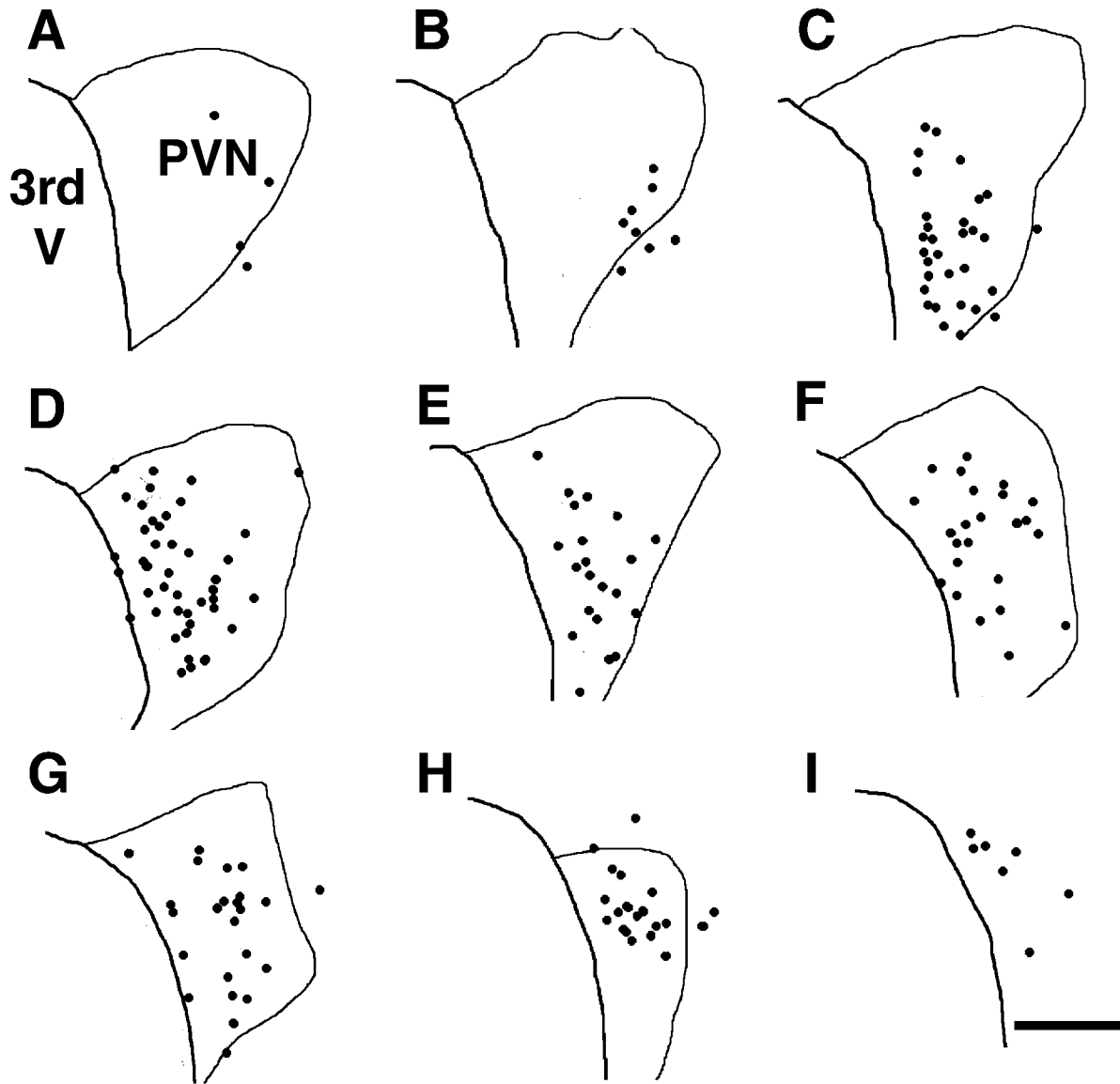


Fig. 3. **A-I:** Camera lucida drawing of the distribution of citrulline-positive cells along the length of the PVN of an aggressive lactating female. The boundary of the PVN was drawn from the boundary of oxytocin cells in the PVN taken from alternate sections of

the same animal. The circles represent citrulline-positive cells drawn from a 40- μ m-thick section of the PVN. Only every other 40- μ m-thick section is shown. The sections are ordered in sequence from rostral to caudal. Scale bar = 100 μ m.

In mice, the regions of the hypothalamus exhibiting the greatest increases in citrulline-IR in association with maternal aggression include the medial preoptic area (MPOA), the suprachiasmatic nucleus (SCN), and the subparaventricular zone (SPa) (Gammie and Nelson, 1999), but in prairie voles no changes in citrulline synthesis were seen in these regions (data not shown). In contrast to the changes in the PVN, the number of cells with citrulline-IR was unaltered in the anterior amygdaloid area. An example of citrulline-IR in the anterior amygdaloid area of the brain in an aggressive and control lactating female is shown in Figure 2E,F. The mean number of citrulline-positive cells in this region did not differ significantly (Fig. 2G).

Mating-induced aggression in male prairie voles

In this study, we tested males for aggression that were mated and had been cohabiting with their partner for 14 days by using the resident-intruder paradigm. We also tested males for aggression that were nonmated. Group-housed male prairie voles were used as intruders. As seen in Figure 4, mated males were reliably aggressive toward intruders and five of five males exhibited aggression. In contrast, the unmated males exhibited no aggression but showed approximately equal levels of sniffing the intruder male as the mated males.

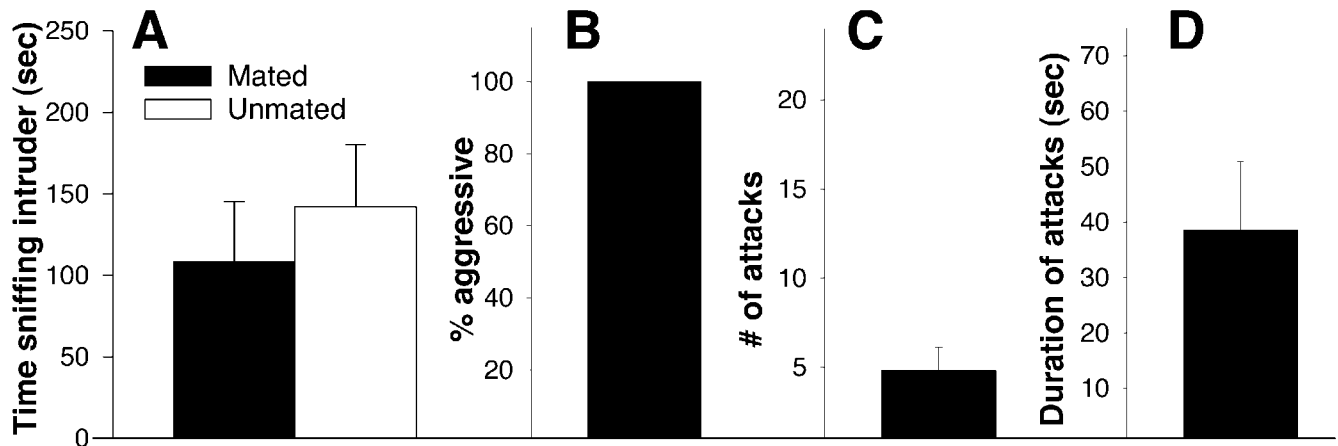


Fig. 4. Mated male prairie voles exhibit mating-induced aggression toward intruder males. By using a resident-intruder test, a behavioral profile of mated and unmated males toward an intruder was examined in (A) average amount of time sniffing the intruder, (B) percent of the males that were aggressive, (C) the average number of

attacks per 10-minute test period, and (D) the average amount of time spent engaged in an agonistic encounter. Because none of the unmated males used in this study were aggressive, no bars are shown in terms of aggressive behavior. Bars represent means \pm SE.

Pattern of citrulline-IR in the brain of aggressive and nonaggressive male prairie voles

To determine whether mated, aggressive males also increase citrulline-IR in the hypothalamus compared with nonaggressive, unmated males, we examined the brains of males for citrulline-IR after resident-intruder aggressive testing. Because NO release in the PVN of rats has been implicated in noncontact penile erections (Melis et al., 1998), we also examined citrulline-IR in mated male voles that were unstimulated. As seen in Figure 5, the mated, aggressive males exhibited a significantly higher number of citrulline-positive cells in the PVN than either of the two control groups. This increase in the mated, aggressive males was accompanied by an increase in the number of cells exhibiting citrulline-IR within their neuronal processes (Fig. 5F). The numbers of citrulline-positive cells in the PVN of the two male control groups are equivalent, are more than twofold fewer than aggressive, mated males, but are higher than the levels observed in the unstimulated lactating females (see Figs. 2A,C; 5A,B,D,E).

A representative camera lucida drawing of the distribution of citrulline-positive cells along the length of the PVN in unstimulated mated males and aggressive mated males is shown in Figure 6. As with the aggressive females, the highest number of citrulline-positive cells occurs in the middle sections of the PVN, and fewer cells are observed in the rostral and caudal sections. A summary of the number of citrulline-positive cells in the male and female groups is shown in Figure 7.

DISCUSSION

Maternal and mating-induced aggression in prairie voles

In this study, five of five lactating females exhibited maternal aggression, and five of five mated males exhibited mating-induced aggression (Figs. 1 and 4). Maternal aggression has been hypothesized to increase the likeli-

hood of survival of the pups (Wolff, 1985). Because prairie voles are a monogamous species and both males and females provide parental care, it is likely that mating-induced aggression also increases the fitness of the offspring. Because both forms of aggression are expressed temporally in association with reproduction and the rearing of pups, it is possible that they share a common neural basis. In nonmonogamous rodent species, different mechanisms seem to control male and maternal aggression. Male aggression is usually androgen dependent, and serotonin and NO have inhibitory actions (Nelson et al., 1995; Olivier et al., 1995; Demas et al., 1997; Kriegsfeld et al., 1997). Maternal aggression is androgen independent, and serotonin and NO may have excitatory actions (Kordon et al., 1973; Gammie and Nelson, 1999). In this study both maternal and mating-induced aggression in prairie voles are associated with increased synthesis of citrulline (and, indirectly, NO) in the PVN.

Citrulline-IR as an indirect indicator of NO activity

This is the second study to combine behavioral testing with citrulline-IR to provide an indirect snapshot of NO release during a behavior. This technique was previously used to examine NO production indirectly during maternal and male aggression in mice (Gammie and Nelson, 1999). Because citrulline is the breakdown product when NO is cleaved enzymatically from arginine by nitric oxide synthase (NOS), it can be analyzed chemically and immunohistochemically as an indirect measurement of NO production (Eliasson et al., 1997; Moroz et al., 1999). The use of citrulline-IR complements the use of NO-specific probes (e.g., (Luo et al., 1993; Clough et al., 1998; Loeb et al., 1998), because the specific cells producing NO can be identified (Gammie and Nelson, 1999). Three lines of evidence support citrulline-IR as a reliable, indirect indicator of NO release in the CNS: 1) citrulline-IR is only found in mice neurons that also contain nNOS (Eliasson et al., 1997); 2) pharmacological inhibitors of nNOS and the deletion of the nNOS gene eliminate citrulline-IR from the

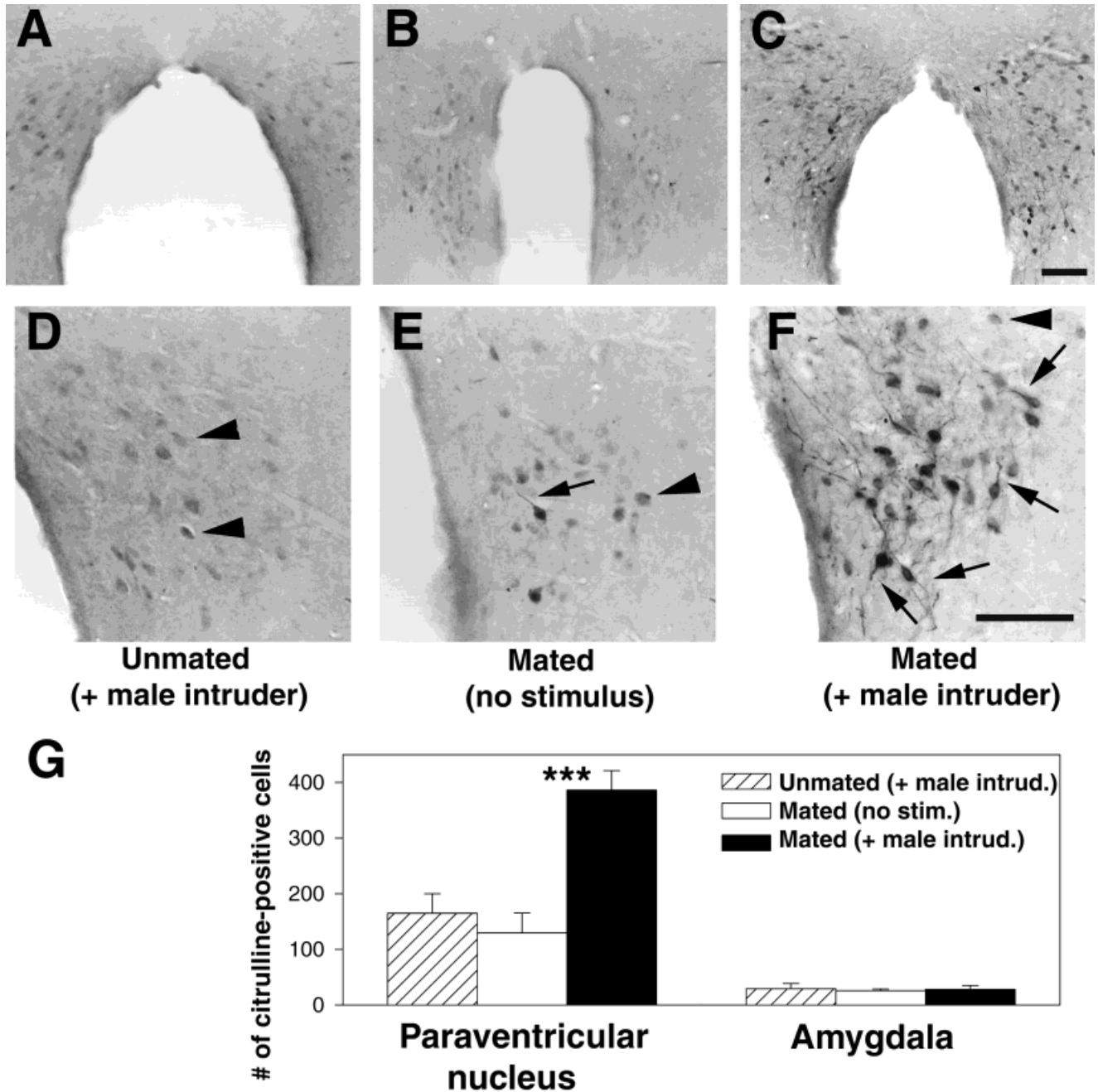


Fig. 5. Citrulline-IR is significantly altered in the PVN of aggressive mated male prairie voles compared with control males. Representative photomicrographs showing citrulline-IR in the PVN of an unmated nonaggressive male (A,D), a mated unstimulated male (B,E), and an aggressive mated male (C,F). The respective higher power representative photomicrographs showing different levels of citrulline-IR in the neuronal processes in the PVN are shown in D–F. Arrowheads indicate cells with citrulline-IR in the cell bodies only, and arrows correspond to cells with citrulline-IR within neuronal

processes. The average number citrulline-positive cells in the PVN and anterior amygdaloid area for the three male groups are shown in (G). Bars represent means \pm SE. For the PVN, the total number of citrulline-positive cells were counted from nine sections per animal. For the anterior amygdaloid area cells were counted from only one section per animal. The number of citrulline-positive cells in the aggressive mated males differs significantly from the other two male groups in the PVN, but not the anterior amygdaloid area. *** = $P < 0.001$, one-way ANOVA. Scale bars = 100 μ m.

mouse brain (Demas et al., 1997; Eliasson et al., 1997; Gammie and Nelson, 1999); and 3) no detectable levels of the messenger ribonucleic acid (mRNA) of an important urea cycle enzyme are found in the mouse brain (Eliasson

et al., 1997). Consequently, it is likely that the citrulline-IR observed in the prairie vole brain reflects NO synthesis. Because this is the first study to examine a possible role for NO in behaviors in prairie voles, in future

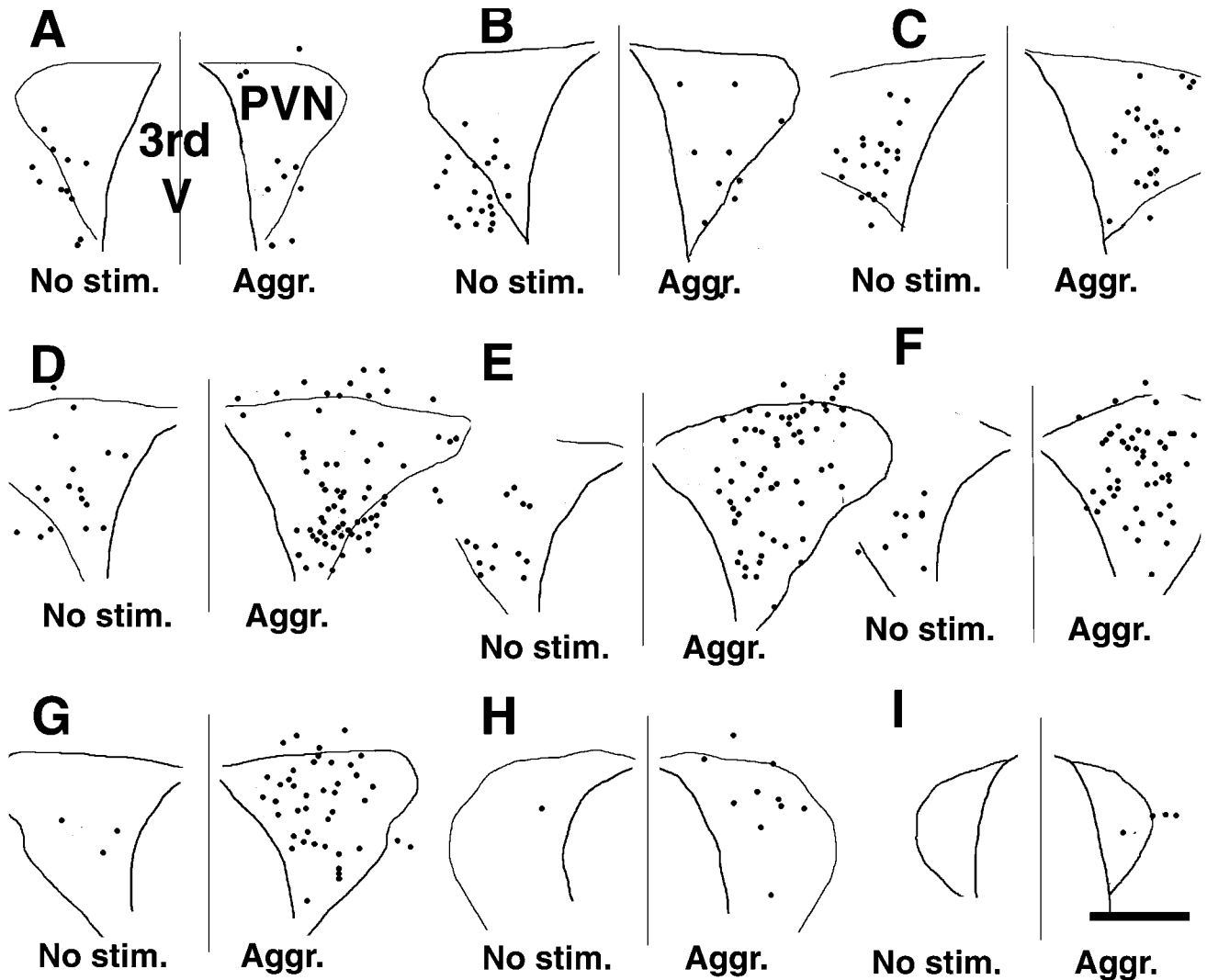


Fig. 6. Camera lucida drawing of the relative distribution of citrulline-positive cells along the length of the PVN of a mated unstimulated male (left sections) and an aggressive mated male (right sections). The boundary of the PVN was drawn from the boundary of oxytocin cells in the PVN taken from alternate sections of the aggres-

sive mated male. The circles represent citrulline-positive cells drawn from a 40- μ m-thick section of the PVN. Only every other 40- μ m-thick section is shown. The sections are ordered in sequence from rostral to caudal. Scale bar = 100 μ m.

work it will be useful to use NO-specific probes during the production of a behavior to quantify properly and confirm NO production.

Possible significance of increased numbers of citrulline-positive cells in the PVN during maternal and mating-induced aggression

This study indicates that an increase in citrulline-IR in the PVN, but not in other regions of the brain (Figs. 2 and 5), is associated with maternal aggression and mating-induced aggression and suggests that NO is released in the PVN during both of these types of aggression in prairie voles. Whether this increased synthesis of citrulline (and NO) is directly, indirectly, or not at all related to maternal aggression and mating-induced aggression remains unspecified. In mice, a significant elevation of the number of citrulline-positive cells occurs in the MPOA, SCN, and

SPa regions of the hypothalamus in association with maternal aggression (Gammie and Nelson, 1999). In cats, electrical stimulation of similar hypothalamic regions elicits defensive rage, which is thought to be equivalent to maternal aggression (Siegel et al., 1999). In the prairie voles, though, these hypothalamic areas do not exhibit citrulline-IR either during maternal or mating-induced aggression (data not shown). It is interesting that some of the cells that exhibit citrulline-IR in the mouse hypothalamus in association with maternal aggression are thought to contain arginine vasopressin (AVP) and to project to the PVN (Gammie and Nelson, 1999) where they could influence maternal aggression (Vrang et al., 1995).

Lesion studies in the rat implicate the PVN and the ventromedial hypothalamus as being involved in the control of maternal aggression (Hansen, 1989; Giovenardi et al., 1998). The PVN contains a large number of neurose-

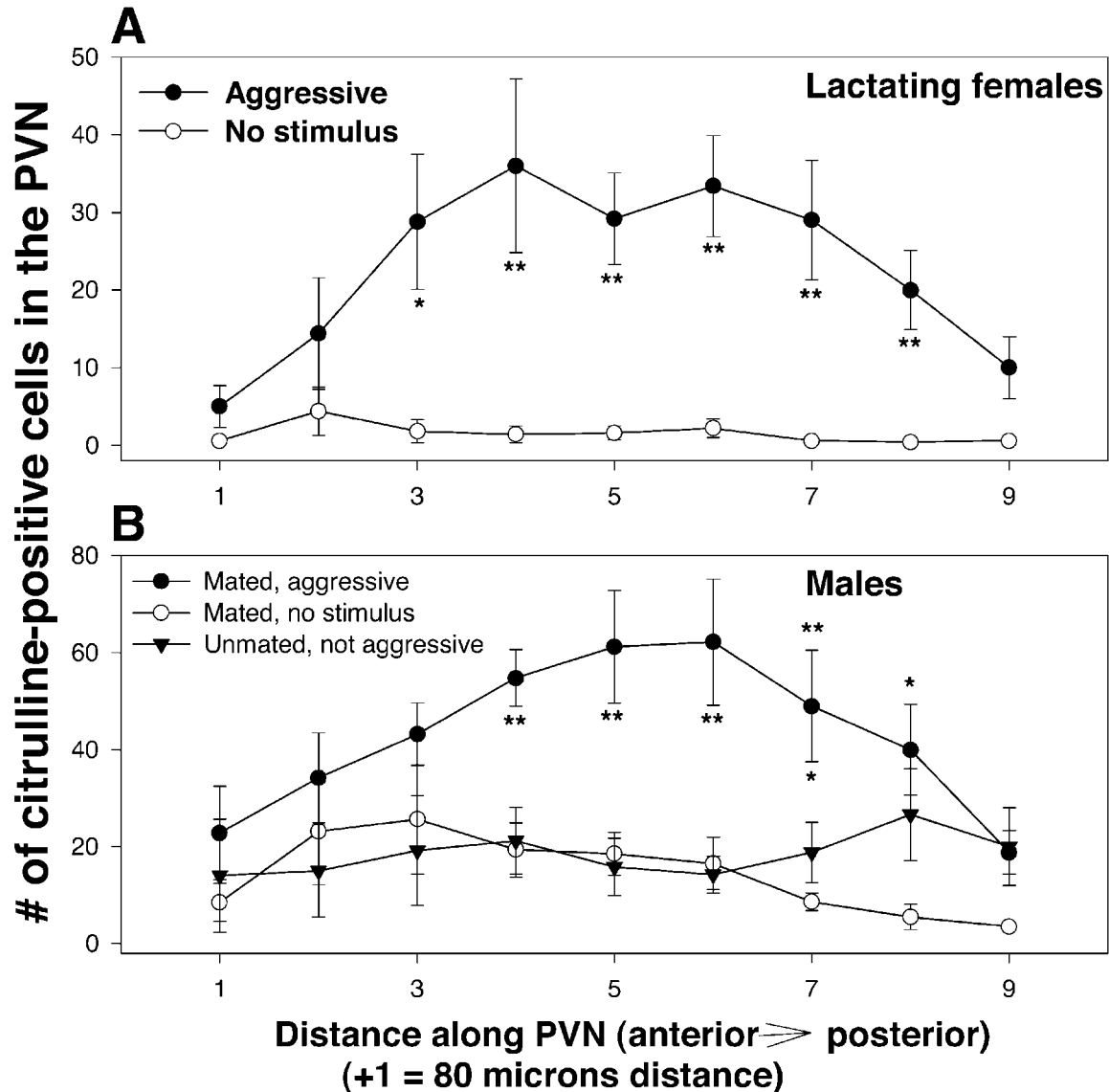


Fig. 7. Composite line graph showing the relative number of citrulline-positive cells along the length of the PVN in (A) females and (B) males. Statistically significant differences are shown. For the male groups in (B), when asterisks are above and below the line for mated, aggressive males, the asterisks above the line indicate significant difference from the mated, no stimulus males, and those below

the line indicate significant differences from the unmated, nonaggressive males. When the differences between the mated, aggressive males are equally significantly different from both control groups, then only one asterisk appears below the line for mated, aggressive males. * = $P < 0.05$; ** = $P < 0.01$, unpaired Student's *t*-test for (A) and one-way ANOVA for (B).

cretory cells that coexpress nNOS and a range of neuropeptides including oxytocin (Yamada et al., 1996), AVP (Villar et al., 1994; Hatakeyama et al., 1996), CRF (Siaud et al., 1994; Yamada et al., 1996; Harada et al., 1999), and enkephalin (Yamada et al., 1996). In rats, oxytocin may act centrally to facilitate (Ferris et al., 1992) or inhibit maternal aggression (Giovenardi et al., 1998). AVP has an excitatory role in male hamster aggression (Ferris et al., 1997), but a role for this neuropeptide in maternal aggression has not been established. In addition, AVP is critical during pair bonding and in facilitating the subsequent expression of mating-induced aggression in male prairie voles (Winslow et al., 1993), but it is not known whether

AVP plays an active role in the production of mating-induced aggression. Consequently, double-labeling experiments using citrulline and neuropeptides, or neurotransmitters, will be useful to identify specifically which cells are producing NO in the PVN in both males and females.

An intriguing possibility is that elevated citrulline expression colocalizes with CRF or that NO release in adjacent cells may play a role in inhibiting the central release or action of CRF during maternal and mating-induced aggression. The central release of CRF has been implicated in the defeat response of rats (Heinrichs et al., 1992), and inhibition of central CRF release by aggressive resident male rats may suppress anxiety and facilitate

aggression. Furthermore, in rodents, a decrease in anxiety is associated with pregnancy and lactation (Maestriperi and D'Amato, 1991), suggesting that decreased anxiety and inhibition of the CRF pathway may facilitate maternal aggression. The role of NO in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis has been studied, but the results are ambiguous because some reports find that NO can prevent increases in CRF release from hypothalamic explants (Costa et al., 1993), whereas others find that NO can trigger the *in vitro* release of CRF from hypothalamus (Raber et al., 1995). Future work will be needed to clarify the role for NO in the central release of CRF in the PVN.

It will be important to determine whether or how NO and neuropeptides interact in the PVN of prairie voles during maternal and mating-induced aggression. NO can act as either an intracellular, intercellular signal, or both (Holscher, 1997; Park et al., 1998), and NO could modulate the action of any neuropeptide that is coreleased or could inhibit or activate the release of neuropeptides from neighboring cells. The most common pathway for NO signaling is the activation of soluble guanylate cyclases and future studies using cyclic guanosine monophosphate (cGMP) immunohistochemical and double-labeling techniques will also be useful in identifying possible targets of NO in the PVN.

By using NO-specific probes, NO was recently shown to be released in the PVN during noncontact penile erections in rats (Melis et al., 1998), and this could account for the citrulline levels observed in the mated, unstimulated males. The elevated citrulline in the nonmated, nonaggressive males is equal to the mated, unstimulated males, however, and hence the citrulline levels in both may reflect a male-specific physiological function that is independent of aggression.

Female rats show a dramatic increase in nNOS synthesis in the PVN during pregnancy and lactation (Popeski et al., 1999), and this increase may also occur during pregnancy and lactation in prairie voles. If this is the case, then it is tempting to speculate that citrulline synthesis (and NO release) plays a role in maternal aggression because high levels of citrulline-IR were not observed in any of the control females. If NO release in the PVN played a role in milk release or some other maternally related behavior not including maternal aggression, then we would expect to see higher citrulline levels in some of the control females because in all cases, the experimental and control females were nursing and tending to their pups just before separation, testing (or lack of testing), and perfusion. It is also tempting to speculate that because the site of increased citrulline synthesis is in similar regions of the PVN in males and females, that maternal and mating-induced aggression may share a common neural basis that is linked to NO.

During maternal aggression in mice, a significant elevation of citrulline occurs in cells of the hypothalamus that appear to contain AVP and to send projections to the PVN (Gammie and Nelson, 1999). From an evolutionary perspective, if NO release is indeed involved in regulating maternal aggression, it would be interesting if during the divergence of mice and prairie voles that the site of control of aggression was shifted so that NO now regulates a more upstream control site in the mice and a more downstream control site in the prairie voles. Such lines of speculation,

though, will require that a link between NO and maternal aggression is established.

CONCLUSIONS

Work in mice suggests that NO inhibits male aggression (Nelson et al., 1995; Demas et al., 1997), but that female mice may use NO to activate maternal aggression (Gammie and Nelson, 1999). We find in this study that the synthesis of citrulline (and, indirectly, NO) increases dramatically within the PVN of prairie voles in association with both maternal and mating-induced aggression. Thus, the neural mechanisms underlying maternal and mating-induced aggression in this monogamous species may be similar. Whether or how the actions of these cells of the PVN contribute to maternal or mating-induced aggression is currently being determined. This work further indicates that the technique of combining behavioral testing and citrulline immunohistochemistry can be used for comparative studies between species and between sexes to gain, indirectly, a better understanding of where NO synthesis occurs during behaviors.

ACKNOWLEDGMENTS

We thank Drs. M.J.L. Eliasson and S.H. Snyder for use of the anti-citrulline antibodies. We also thank W. Grueber, L.J. Kriegsfeld, S. Mozzicato, U.B. Olaghere-da Silva, B.D. Spar, and C.Y. Wan for advice and technical assistance.

LITERATURE CITED

- Bredt DS, Snyder SH. 1992. Nitric oxide, a novel neuronal messenger. *Neuron* 8:3–11.
- Bridges RS. 1996. Biochemical basis of parental behavior in the rat. In: Rosenblatt JS, Snowden CT, editors. *Parental care: evolution, mechanisms, and adaptive significance*. San Diego: Academic Press. p 215–237.
- Carter CS, DeVries AC, Getz LL. 1995. Physiological substrates of mammalian monogamy: the prairie vole model. *Neurosci Biobehav Rev* 19:303–314.
- Carter CS, Getz LL. 1993. Monogamy and the prairie vole. *Sci Am* 268: 100–106.
- Clough GF, Bennett AR, Church MK. 1998. Measurement of nitric oxide concentration in human skin *in vivo* using dermal microdialysis. *Exp Physiol* 83:431–434.
- Costa A, Trainer P, Besser M, Grossman A. 1993. Nitric oxide modulates the release of corticotropin-releasing hormone from the rat hypothalamus *in vitro*. *Brain Res* 605:187–192.
- De Almeida RM, Lucion AB. 1997. 8-OH-DPAT in the median raphe, dorsal periaqueductal gray and corticomedial amygdala nucleus decreases, but in the medial septal area it can increase maternal aggressive behavior in rats. *Psychopharmacology (Berl)* 134:392–400.
- Demas GE, Eliasson MJ, Dawson TM, Dawson VL, Kriegsfeld LJ, Nelson RJ, Snyder SH. 1997. Inhibition of neuronal nitric oxide synthase increases aggressive behavior in mice. *Mol Med* 3:610–616.
- Demas GE, Moffatt CA, Drazen DL, Nelson RJ. 1999. Castration does not inhibit aggressive behavior in adult male prairie voles (*Microtus ochrogaster*). *Physiol Behav* 66:59–62.
- Eliasson MJ, Blackshaw S, Schell MJ, Snyder SH. 1997. Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc Natl Acad Sci USA* 94:3396–3401.
- Ferris CF, Foote KB, Meltser HM, Plenby MG, Smith KL, Insel TR. 1992. Oxytocin in the amygdala facilitates maternal aggression. *Ann NY Acad Sci* 652:456–457.
- Ferris CF, Melloni RH Jr, Koppel G, Perry KW, Fuller RW, Delville Y. 1997. Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. *J Neurosci* 17:4331–4340.

- Gammie SC, Nelson RJ. 1999. Maternal aggression is reduced in neuronal nitric oxide synthase-deficient mice. *J Neurosci* 19:8027–8035.
- Getz LL, Carter CS, Gavish L. 1981. The mating system of prairie voles, *Microtus ochrogaster*. Field and laboratory evidence for pair-bonding. *Behav Ecol Sociobiol* 8:189–194.
- Giovenardi M, Padoin MJ, Cadore LP, Lucion AB. 1998. Hypothalamic paraventricular nucleus modulates maternal aggression in rats: effects of ibotenic acid lesion and oxytocin antisense. *Physiol Behav* 63:351–359.
- Hansen S. 1989. Medial hypothalamic involvement in maternal aggression of rats. *Behav Neurosci* 103:1035–1046.
- Harada S, Imaki T, Chikada N, Naruse M, Demura H. 1999. Distinct distribution and time-course changes in neuronal nitric oxide synthase and inducible NOS in the paraventricular nucleus following lipopolysaccharide injection. *Brain Res* 821:322–332.
- Hatakeyama S, Kawai Y, Ueyama T, Senba E. 1996. Nitric oxide synthase-containing magnocellular neurons of the rat hypothalamus synthesize oxytocin and vasopressin and express Fos following stress stimuli. *J Chem Neuroanat* 11:243–256.
- Heinrichs SC, Pich EM, Miczek KA, Britton KT, Koob GF. 1992. Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. *Brain Res* 581:190–197.
- Holscher C. 1997. Nitric oxide, the enigmatic neuronal messenger: its role in synaptic plasticity. *Trends Neurosci* 20:298–303.
- Insel TR, Preston S, Winslow JT. 1995. Mating in the monogamous male: behavioral consequences. *Physiol Behav* 57:615–627.
- Kordon C, Blake CA, Terkel J, Sawyer CH. 1973. Participation of serotonin-containing neurons in the suckling-induced rise in plasma prolactin levels in lactating rats. *Neuroendocrinology* 13:213–223.
- Kriegsfeld LJ, Dawson TM, Dawson VL, Nelson RJ, Snyder SH. 1997. Aggressive behavior in male mice lacking the gene for neuronal nitric oxide synthase requires testosterone. *Brain Res* 769:66–70.
- Loeb AL, Raj NR, Longnecker DE. 1998. Cerebellar nitric oxide is increased during isoflurane anesthesia compared to halothane anesthesia: a microdialysis study in rats. *Anesthesiology* 89:723–730.
- Luo D, Knezevich S, Vincent SR. 1993. N-methyl-D-aspartate-induced nitric oxide release: an in vivo microdialysis study. *Neuroscience* 57:897–900.
- Maestripieri D, D'Amato FR. 1991. Anxiety and maternal aggression in house mice (*Mus musculus*): a look at interindividual variability. *J Comp Psychol* 105:295–301.
- Mann MA, Konen C, Svare B. 1984. The role of progesterone in pregnancy-induced aggression in mice. *Horm Behav* 18:140–160.
- Melis MR, Succu S, Mauri A, Argiolas A. 1998. Nitric oxide production is increased in the paraventricular nucleus of the hypothalamus of male rats during non-contact penile erections and copulation. *Eur J Neurosci* 10:1968–1974.
- Moroz LL, Gillette R, Sweedler JV. 1999. Single-cell analyses of nitric oxide neurons in simple nervous systems. *J Exp Biol* 202:333–341.
- Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson VL, Dawson TM, Snyder SH. 1995. Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature* 378:383–386.
- Olivier B, Mos J, van Oorschot R, Hen R. 1995. Serotonin receptors and animal models of aggressive behavior. *Pharmacopsychiatry* 28 (Suppl 2):80–90.
- Park JH, Straub VA, O'Shea M. 1998. Anterograde signaling by nitric oxide: characterization and in vitro reconstitution of an identified nitric oxide synapse. *J Neurosci* 18:5463–5476.
- Pasqualotto BA, Hope BT, Vincent SR. 1991. Citrulline in the rat brain: immunohistochemistry and coexistence with NADPH-diaphorase. *Neurosci Lett* 128:155–160.
- Popeski N, Amir S, Woodside B. 1999. Changes in NADPH-d staining in the paraventricular and supraoptic nuclei during pregnancy and lactation in rats: role of ovarian steroids and oxytocin. *J Neuroendocrinol* 11:53–61.
- Raber J, Koob GF, Bloom FE. 1995. Interleukin-2 (IL-2) induces corticotropin-releasing factor (CRF) release from the amygdala and involves a nitric oxide-mediated signaling; comparison with the hypothalamic response. *J Pharmacol Exp Ther* 272:815–824.
- Siaud P, Mekaouche M, Ixart G, Balmeffre M, Givalois L, Barbanel G, Assenmacher I. 1994. A subpopulation of corticotropin-releasing hormone neurosecretory cells in the paraventricular nucleus of the hypothalamus also contain NADPH-diaphorase. *Neurosci Lett* 170:51–54.
- Siegel A, Roeling TA, Gregg TR, Kruk MR. 1999. Neuropharmacology of brain-stimulation-evoked aggression. *Neurosci Biobehav Rev* 23:359–389.
- Siegel HI, Giordano AL, Mallafre CM, Rosenblatt JS. 1983. Maternal aggression in hamsters: effects of stage of lactation, presence of pups, and repeated testing. *Horm Behav* 17:86–93.
- Stern JM, Kolunje JM. 1993. Maternal aggression of rats is impaired by cutaneous anesthesia of the ventral trunk, but not by nipple removal. *Physiol Behav* 54:861–868.
- Stern JM, McDonald C. 1989. Ovarian hormone-induced short-latency maternal behavior in ovariectomized virgin Long-Evans rats. *Horm Behav* 23:157–172.
- Svare B. 1990. Maternal aggression: hormonal, genetic, and developmental determinants. In: Krasnegor NA, Bridges RS, editors. *Mammalian parenting: biochemical, neurobiological, and behavioral determinants*. New York: Oxford University Press. p 118–132.
- Svare B, Betteridge C, Katz D, Samuels O. 1981. Some situational and experiential determinants of maternal aggression in mice. *Physiol Behav* 26:253–258.
- Villalba C, Boyle PA, Caliguri EJ, De Vries GJ. 1997. Effects of the selective serotonin reuptake inhibitor fluoxetine on social behaviors in male and female prairie voles (*Microtus ochrogaster*). *Horm Behav* 32:184–191.
- Villar MJ, Ceccatelli S, Ronnqvist M, Hokfelt T. 1994. Nitric oxide synthase increases in hypothalamic magnocellular neurons after salt loading in the rat. An immunohistochemical and in situ hybridization study. *Brain Res* 644:273–281.
- Vrang N, Larsen PJ, Mikkelsen JD. 1995. Direct projection from the suprachiasmatic nucleus to hypophysiotropic corticotropin-releasing factor immunoreactive cells in the paraventricular nucleus of the hypothalamus demonstrated by means of Phaseolus vulgaris-leucoagglutinin tract tracing. *Brain Res* 684:61–69.
- Wang Z, Hulihan TJ, Insel TR. 1997. Sexual and social experience is associated with different patterns of behavior and neural activation in male prairie voles. *Brain Res* 767:321–332.
- Winslow JT, Hastings N, Carter CS, Harbaugh CR, Insel TR. 1993. A role for central vasopressin in pair bonding in monogamous prairie voles. *Nature* 365:545–548.
- Wolff JO. 1985. Maternal aggression as a deterrent to infanticide in *Peromyscus leucopus* and *P. maniculatus*. *Anim Behav* 33:117–123.
- Yamada K, Emson P, Hokfelt T. 1996. Immunohistochemical mapping of nitric oxide synthase in the rat hypothalamus and colocalization with neuropeptides. *J Chem Neuroanat* 10:295–316.