Forebrain c-*fos* expression under conditions conducive to pair bonding in female prairie voles (*Microtus ochrogaster*)

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Abstract

Repeated mating over a period of 6 h facilitates pair-bond formation in monogamous prairie voles. Using this paradigm, we examined fos expression in brain areas implicated in social behavior in voles. We hypothesized that the presence of the fos protein after a period of time sufficient for pair bonding to occur may indicate brain areas that are especially important in pair bond formation. We found elevated levels of fos immunoreactivity in the medial and cortical amygdala, medial preoptic area (MPOA), and bed nucleus of the stria terminalis (BNST) in females that mated several times over a 6-h period as compared to a variety of unmated controls. No treatment effects were found in the central amygdala, nucleus accumbens (NAcc), or lateral septum (LS). We suggest that areas that show evidence of fos expression after sufficient time for pair bonding to occur may be important in the formation of associations between the partner and mating stimuli.
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1. Introduction

Many species, including humans, display strong social attachments, with pair bonds between individuals being perhaps the most fundamental form of social attachment. Pair bonds typically are associated with reproduction and, in fact, reproductive success can be influenced by the stability of the pair bond [1]. Given the close association between reproduction and pair bonding, it is not surprising that sexual contact often is important in the formation and/or maintenance of social attachments. The monogamous prairie vole (*Microtus ochrogaster*) is an excellent model for examining the behavioral, neural, and physiological bases of social attachment. After mating, both sexes display pair bonds that extend beyond the breeding season, share a common nest, vigorously defend their mate from other conspecifics, and provide parental care [2,3], behaviors that are not seen prior to mating.

Such complex and long-lasting behavioral changes exhibited by prairie voles after mating suggest changes in central activity. Efforts have been made to examine the neural circuits underlying pair bonding. For example, lesion and pharmacological studies have indicated brain areas important for the neurochemical regulation of pair bonding [4–7]. However, such studies are of limited value in determining where pair bonding occurs because these methods are not capable of distinguishing between (1) areas that are important for pair bonding since they are parts of necessary pathways and (2) those where the actual associations that underlie pair bonding may occur.

It is likely that the formation of a pair bond involves learning and memory processes [6,8,9]. Although typically associated with acute neuronal activation, expression of the immediate early gene c-*fos* has been implicated in learning and memory. In birds, there is a positive correlation between fos expression and song learning [10] and, in rats, fos expression is enhanced during acquisition of a motor learning task [11] or an avoidance task [12]. Further, Cammarota et al. [13] showed that fos, but not other immediate early gene products such as Fra-1, Fra-2, or Jun, was elevated after avoidance learning. Finally, Watanabe et al. [14] concluded that expression of immediate early genes, particularly c-*fos*, was important in the mechanisms whereby short-term changes in neuronal activity result in long-term modification of neuronal structure and function.

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In the present study, we examined fos expression under conditions conducive to pair bond formation in female prairie voles. We hypothesized that continued expression of fos may be an indication of ongoing processes underlying the formation of associations between salient stimuli and the partner that may be critical for pair bond formation. We chose to examine fos expression rather than other fos-related antigens since fos expression typically is short-lived (c.f. Refs. [15,16]) but, in some situations, can be sustained for many hours [17,18]. Since the brain areas examined [amygdala (Amyg), lateral septum (LS), nucleus accumbens (NAcc), bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA)] are likely to serve multiple functions, it is unlikely that simple measures of acute activation will be sufficient to identify areas solely involved in pair bonding. As such, our goal was to identify in which of these areas fos expression continued for a sufficient period of time for pair bonding to occur.

2. General methods

2.1. Subjects

Subjects were the female offspring of the F3 generation of a laboratory colony of prairie voles (*Microtus ochrogaster*) originating from Illinois. After weaning at about 21 days of age, pups were kept in same-sex sibling pairs until used in experiments. All animals were housed (plastic shoebox style cages, $29 \times 19 \times 13$ cm) under a 14:10 light/dark cycle with ad libitum food and water. All subjects were about 70 days of age at the time of the experiments.

2.2. Estrogen treatment

Females were treated with estradiol benzoate (EB) for 3 days (0.1 μg/day in 100 μl sesame oil). This treatment induces sexual receptivity in a majority of female prairie voles [19,20]. Since an estrous cycle does not occur in this species [21], ovariectomy is not considered necessary to ensure consistent levels of estrogen between animals [22]. Further, unlike rats, voles do not require progesterone to induce sexual receptivity [23].

2.3. Experimental protocol

Williams et al. [24] showed that estrogen-treated female prairie voles formed pair bonds after as few as 6 h if mating occurred. Similarly treated females that did not mate failed to form pair bonds. We employed this paradigm to examine fos expression under conditions conducive to pair bonding in prairie voles. EB-treated sexually naive, adult females were randomly assigned to one of three groups: (1) remained with their cage-mates to serve as a control for handling ($n = 5$); (2) paired with an unfamiliar female to control for novelty ($n = 5$); or (3) paired with sexually experienced males and allowed the opportunity to mate ad libitum ($n = 14$). A fourth group of females ($n = 6$) was injected with sesame oil alone and remained with their cage-mate throughout to serve as a control for estrogen treatment.

Despite being exposed to a sexually receptive female, some male prairie voles will not mate. Time-lapse videotapes [Panasonic time-lapse video recorder (12:1 compression) and low-light camera] of the cohabitation period were analyzed for the presence and frequency of mating. A typical mating sequence involves 4–10 mounts with pelvic thrusting by the male over several minutes, followed by genital grooming by both sexes [25]. In preliminary studies, sperm were consistently present in vaginal smears after such a sequence. Females were assigned to an unmated subgroup if mating did not occur during the 6 h of exposure to a male ($n = 8$), while those that mated were assigned to a mated subgroup ($n = 6$). On the test day, all animals were transferred to clean cages and allowed to interact for 6 h. For females exposed to unfamiliar females or to males, the subject female was transferred to the clean cage first, followed after 5 min by the stranger female or the male. After 6 h, all females were perfused and their brains processed for fos immunocytochemistry.

2.4. Perfusion and fos immunocytochemical processing

Immediately after experimental manipulations, animals were overanesthetized with sodium pentobarbital (1 mg/10 g body weight) and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS; pH 7.6). Brains were postfixed for 2 h in the same fixative and then immersed in 30% sucrose in PBS. Brains were sectioned at 40 μm on a cryostat and processed immunocytochemically for visualization of c-fos protein following established procedures [26]. Briefly, floating sections were rinsed in 0.05 M Tris–NaCl (TBS, pH 7.6), incubated in 0.5% H_2O_2 for 30 min, rinsed in TBS, and then incubated in TBS with 0.5% Triton X-100 (TBS-t) containing 10% normal goat serum (NGS) for 1 h. Tissue was incubated in the primary antibody (rabbit anti-rat fos, diluted 1:30,000 in TBS-t; Santa Cruz, cat. #SC-52) overnight at 4 °C, followed by rinses in TBS-t containing 2% NGS. Tissue was placed in the secondary antibody (biotinylated goat anti-rabbit, diluted 1:300 in TBS-t containing 2% NGS) for 2 h followed by rinses in TBS and then in PBS. Sections were incubated in an avidin–biotin–peroxidase conjugate (Vector Laboratories) in PBS and then rinsed in PBS. Staining was visualized by reacting the tissue with 3,3′-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate followed by rinses in PBS. Sections were mounted on slides, allowed to dry, cover slipped using Permount, and examined microscopically.

2.5. Analysis of fos expression

We examined c-*fos* expression in forebrain areas previously implicated in social behavior in voles [4,5,22]: NAcc, MPOA, Amyg, BNST, and LS. Fos-positive cells were quantified using computerized software (NIH image). Particle density and maximum and minimum particle size were adjusted based on comparisons with visual counts. Briefly, fos-ir nuclei were counted by eye on random sections and the results compared with those from computer analysis. The computer counting parameters were then adjusted such that similar counts were produced. This process was repeated until the visual counts and computer generated counts were consistently similar. For each animal, representative sections from each area (three to four sections per area, matched between animals) were analyzed and the mean number of fos-positive cells for each area was calculated. Differences in the mean numbers of fos-positive nuclei in each area among treatment groups were analyzed using ANOVA with post hoc comparisons made using Student Newman Keul's (SNK) analysis.

3. Results

Among females that mated, the mean number of mating bouts was 6.1 ± 0.5. All mated during the first hour of cohabitation with the male (mean 2.5 ± 0.3 mating bouts during first hour). The remaining mating bouts were distributed across the remaining 5 h.

Several forebrain areas that have previously been implicated in pair bonding in prairie voles were examined. Significant treatment effects were found in the medial [$F(4,23) = 3.55$, $P < .05$], and cortical [$F(4,25) = 4.47$, $P < .01$] subnuclei of the amygdala, in the dorsal bed nucleus of the stria terminalis (dBNST, the portion dorsal to the anterior commissure), [$F(4,25) = 2.72$, $P = .05$] and in the MPOA [$F(4,27) = 5.80$, $P < .01$]. Mated females had significantly more fos-positive cells in the medial amygdala (Figs. 1A and 2) than did estrogen or oil-injected controls ($P < .05$). In this area, exposure to strangers of either sex tended to increase the number of activated cells, although the trend did not quite reach statistical significance. A similar pattern emerged for the cortical amygdala (Fig. 1B), with mated females having more fos-positive cells than did either control group ($P < .05$) and a tendency for exposure to a stranger to increase fos expression. In the BNST as a whole, there was a trend toward an increase [Fig. 1B; $F(4,25) = 2.58$, $P = .06$] in fos expression in females that had mated over that of both the EB- and oil-treated baseline control groups. When the dorsal portion of the nucleus was considered separately (Fig. 1D), there were no differences between the control groups and the stranger-exposed groups, while the mated females had significantly higher numbers of activated cells ($P < .05$). Mating significantly elevated fos expression in the MPOA ($P < .01$) over that

found in any of the other groups, which in turn did not differ from each other (Figs. 1E and 3).

No treatment effects were found in the central subnucleus of the amygdala [Fig. 1F; $F(4,21) = 0.94$, $P = .46$], LS [Fig. 1G; $F(4,25) = 0.56$, $P = .67$], or in NAcc [Fig. 1H; core, $F(4,25) = 1.73$, $P = .17$; shell, $F(4,25) = 0.96$, $P = .45$; total, $F(4,25) = 1.34$, $P = .28$]. There were no differences in the numbers of fos-positive cells between oil-treated and EB-treated controls in any of the areas examined.

4. Discussion

Prairie voles form long-term monogamous pair bonds under both natural and laboratory conditions and mating facilitates the formation of such bonds. Females form pair bonds after as few as 6 h if mating occurs, while 6 h of cohabitation without mating does not induce partner preferences [24]. Here we used immunocytochemistry to examine fos expression under circumstances that are conducive to pair bonding in voles. We hypothesized that brain areas that display fos expression after 6 h of mating may be important in pair bond formation. It is important to note that the use of an extended stimulus period likely resulted in differences in the exact timing of various interactions between animals and, as a result, increased variability may mask biologically important differences. Given this caveat, therefore, we are not advocating that areas activated by mating in other species but that show no differences in the present study play no role in mating per se in voles. Rather, we discuss our results in terms of their implications for pair bonding.

Brain areas that display fos expression after 6 h of mating may be important in pair bond formation. Of particular interest in this regard is the fact that fos levels were elevated in the amygdala even after 6 h. This area is capable of responding in a graded fashion to repeated sexual stimuli [27] and has been strongly implicated in pair bond formation in voles [4,26]. In addition to its role in mating, the amygdala is part of the accessory olfactory system and receives input from the vomeronasal organ [28]. Accessory olfactory inputs to the amygdala are found primarily within the medial and cortical subnuclei in female prairie voles, and exposure to urine from males has previously been shown to cause fos expression in these subnuclei [29]. Interestingly, these two subnuclei, but not the central subnucleus of the amygdala, showed significant increases in fos labeling in the present study. Oboh et al. [30] suggested that fos expression in the medial amygdala of females after mating arose from a combination of inputs from the olfactory and genitosensory systems. Thus, the amygdala may be important in integrating mating associated stimuli with other sensory inputs. In prairie voles, lesions of either the corticomedial amygdala [4] or the vomeronasal organ [25] disrupt pair bonding, the latter despite the fact that the animals mate normally if treated with estrogen to induce sexual receptivity. It also has been suggested that the corticomedial
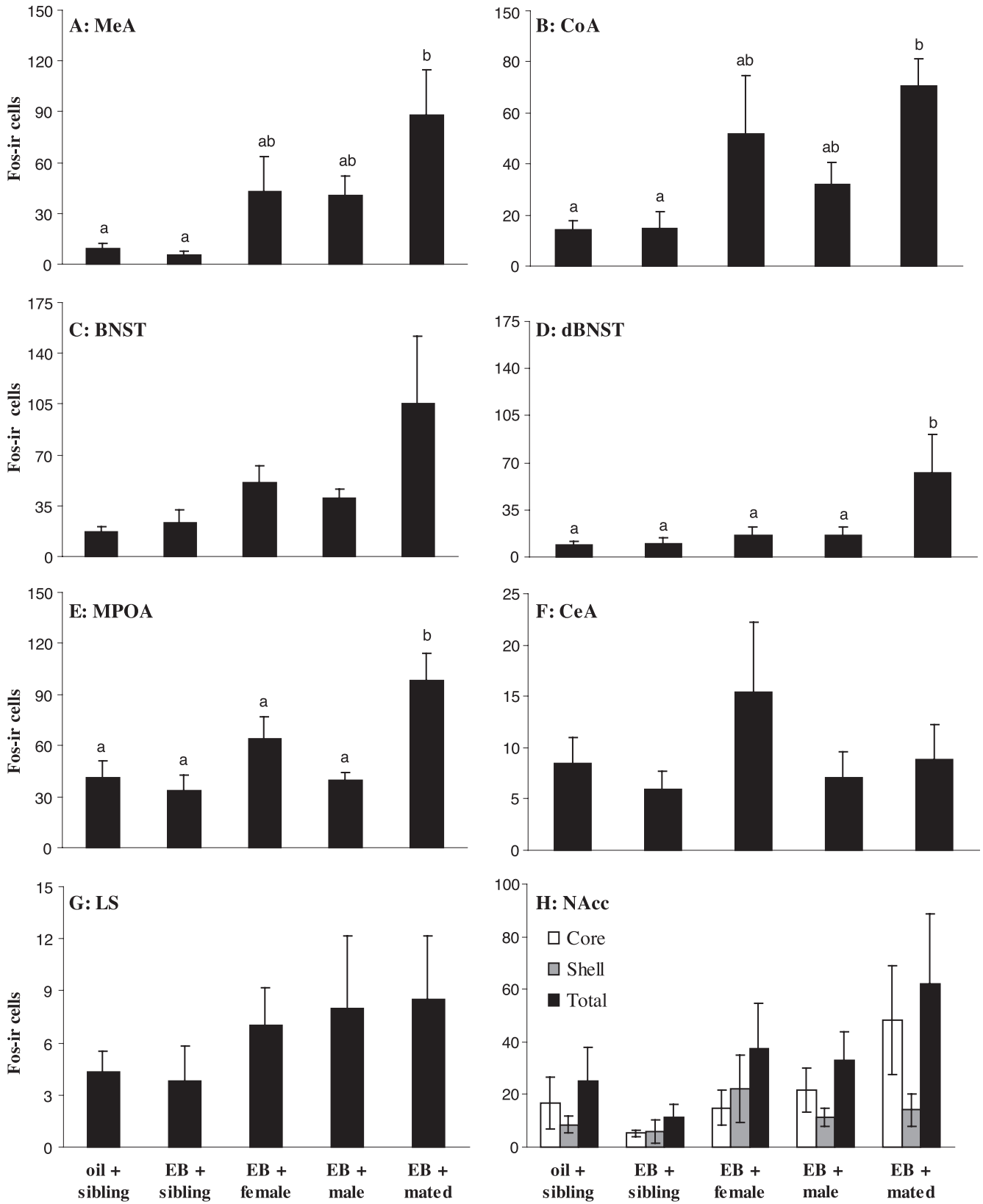
Fig. 1. Numbers of fos-immunoreactive cells in female prairie voles in selected brain areas. Females were treated with either oil or estrogen and paired for 6 h with either a same-sex sibling cage-mate, with an unfamiliar female, or with an unfamiliar male with or without mating. Areas included are medial amygdala (MeA), cortical amygdala (CoA), bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), central amygdala (CeA), lateral septum (LS), and nucleus accumbens (NAcc). Groups with shared letters do not differ from each other. Data are means ± S.E.
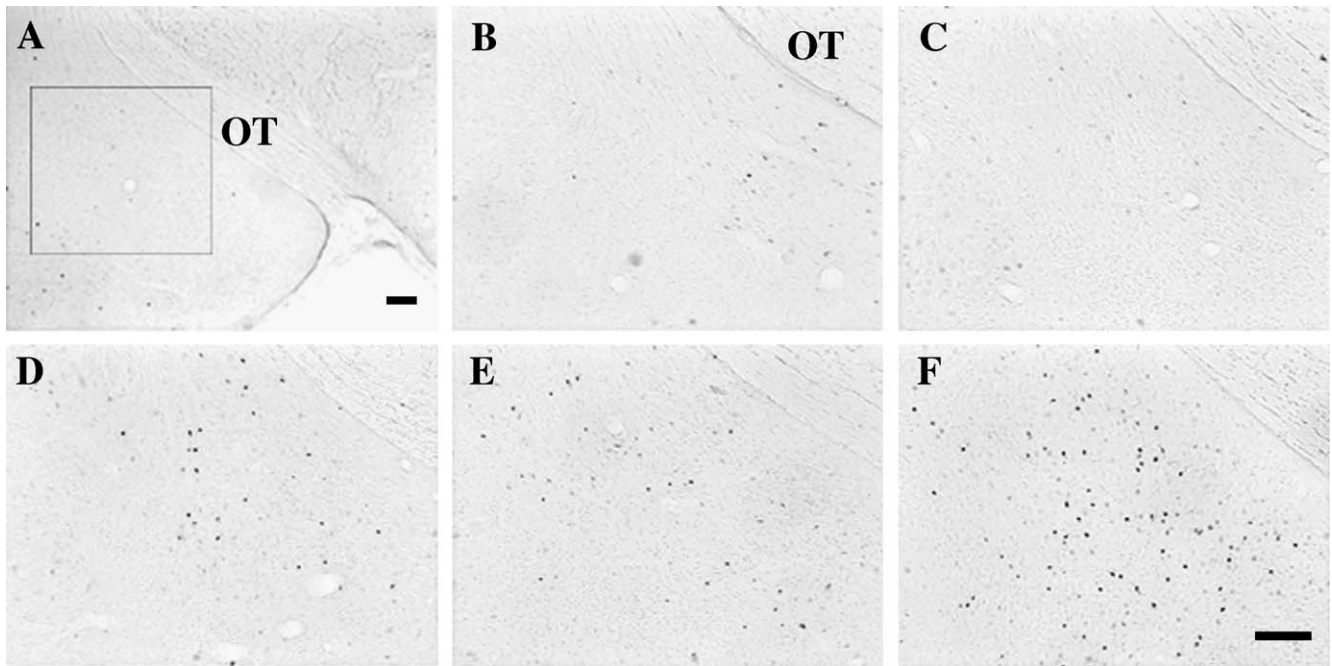
Fig. 2. Micrographs showing fos-immunoreactive cells in the medial amygdala (A) in female prairie voles after oil treatment and exposure to a same-sex sibling cage-mate (B); or after estrogen treatment and exposure to: a same-sex sibling cage-mate (C); an unfamiliar female (D); an unfamiliar male without mating (E); or an unfamiliar male with ad libitum mating (F). All exposures lasted for 6 h. B–F are higher-magnification micrographs of the area outlined in A. OT = optic tract; scale bar = 100 μm.

amygdala is important in social learning [31]. Prairie voles are known to exhibit the Bruce Effect [32], the termination of a pregnancy after exposure to a novel male. Demas et al. [33] showed that female prairie voles with amygdala lesions also displayed the Bruce Effect when reexposed to their mate after a period of separation. The fact that similar results were not found after hippocampal lesions suggests that the amygdala may be the brain region where mate recognition is mediated. Therefore, continued fos expression in the amygdala may indicate an important aspect of pair bonding, the formation of an association between a positive reinforcer (sexual contact Ref. [34]) and the partner.

As with the amygdala, the BNST is capable of responding to sexual stimuli in a graded fashion [27] and comprises part of the accessory olfactory system. Further, the BNST also has been strongly implicated in pair bonding via vasopressin (AVP) projections to the LS. In pair-bonded voles there appears to be no change in AVP receptor density in the LS (Wang, unpublished data) while the BNST projections display enhanced vasopressin release [44,45]. These data suggest that the critical factor that changes in this circuit is on the presynaptic side. The promoter region of the AVP gene contains an AP1 binding region [35] and thus AVP gene expression may be regulated, at least in part, by fos via its interaction with jun proteins in the form of the AP1 hetero-dimer. Kovacs et al. [36] showed that the timing of fos expression could alter the timing of AVP expression. Thus, the expression of fos in the BNST may be related to regional
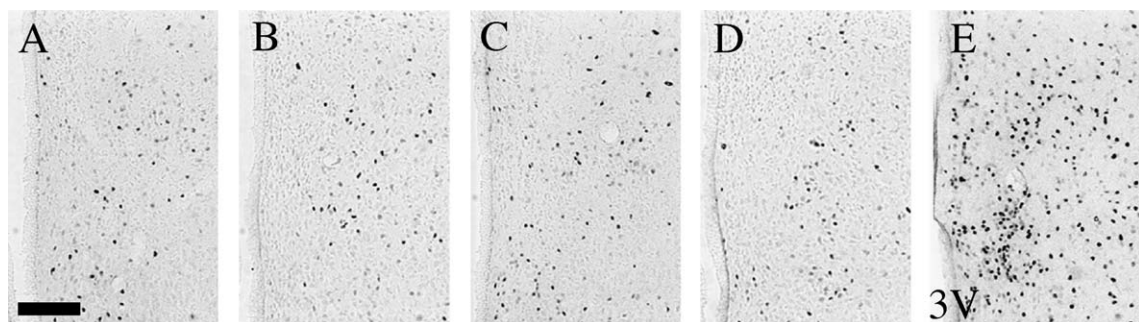

Fig. 3. Micrographs showing fos-immunoreactive cells in the medial preoptic area in female prairie voles after oil treatment and exposure to a same-sex sibling cage-mate (A), estrogen treatment and 6 h of exposure to a same-sex sibling cage-mate (B), estrogen treatment and exposure to an unfamiliar female (C), estrogen treatment exposure to an unfamiliar male without mating (D), estrogen treatment and exposure to an unfamiliar male with ad libitum mating (E). 3V = third ventricle; scale bar = 100 μm.

changes in AVP expression that are involved in pair bonding, a notion that needs to be tested in further experiments.

It must be noted that estrogen is implicated in the regulation of AVP expression in the BNST [37] and most animals in the present study were estrogen primed. How this may alter the responses relative to those found under natural circumstances is not known at this point. Nonetheless, it is important that several of the control groups also were exposed to estrogen, while only the mated group displayed an increase in fos expression in BNST, suggesting that the increased fos was not solely the result of estrogen treatment.

The MPOA has been consistently shown to be involved in mating, but unlike the amygdala and BNST, MPOA does not seem to be capable of a graded response to repeated mating stimuli [27]. The MPOA, however, receives projections from the medial amygdala and BNST [38]. As such, the long-term mating effect seen here in the MPOA may be secondary to that in the amygdala [39] and its continued fos expression may reflect a role in pair bonding. This area also has been implicated in the onset of hormonal changes associated with pseudopregnancy in female rats [40]. The fact that MPOA responds to repeated mating stimuli in female voles could also suggest a role in the induction of ovulation.

The forebrain areas we examined have previously been shown to be activated by mating in other species and/or implicated in pair bonding in voles. Why then did we fail to find fos expression in the NAcc and LS? Although species and sex differences may contribute to the discrepancies, one likely explanation involves differences in timing of mating stimuli between the present and previous studies. Sustained fos expression for many hours is possible in NAcc under certain conditions [17,18]. In other cases, however, it has been shown that NAcc may be activated only by the initial exposure to a stimulus but nonresponsive to subsequent exposures to the same stimulus [41]. This latter observation may explain the lack of elevated fos expression despite the fact that voles mated repeatedly in the present study.

Alternatively, the lack of effect in NAcc may reflect differences in mating patterns between voles and rats. Given the opportunity, female rats avoid the male between copulations (pacing) and NAcc has been implicated in pacing behavior [42]. Mating increases dopamine release in NAcc [43] and this increase is independent of changes in locomotor activity [43] but is associated with the timing of mating [44,45]. The mating behavior in prairie voles appears to differ from that of rats. Female voles appear to make no effort to avoid the male, and in contrast, a pair typically huddles quietly together between mating bouts. Further, the pace of mating is much slower in voles (30–40 min, Ref. [25]) than in rats (about 100 s, Ref. [44]). Finally, the lack of fos expression in NAcc may reflect a preponderance of inhibitory processes. Fos may not be expressed in response to inhibition and it is well established that NAcc role in pair bonding is via D2 dopamine receptors [6,7] which are usually associated with inhibition.

The LS has been implicated in pair bonding in male voles [5]. However, as has been reported for other rodents [46], fos expression in the LS was not significantly increased by any of the social interactions in the present study. The lack of fos expression suggests that the LS may be important for partner preference expression, but not formation, as LS plays an important role in social memory [47].

Our results complement those from a recent study [48] that examined fos expression in many of the same brain regions after 1 h of social interactions between sexually naïve voles. Although there are important methodological differences, in terms of pair bonding, comparing the results of the two studies is useful. Cushing et al. [48] found that exposure to unfamiliar animals tended to increase fos expression in the central nucleus of the amygdala while no such change was found after 6 h under any of the social conditions in the present study. This comparison shows that, in at least some brain areas, novelty-induced elevations in fos are not necessarily continued for several hours (although the anxiolytic effects of estrogen [49] may have influenced this result). In contrast, the elevations of fos expression seen by Cushing et al. [48] in medial amygdala and BNST appear to be sustained for several hours, further supporting their contention that these areas are particularly important in pair-bond formation in voles.

In summary, we have used immunocytochemical visualization of fos to identify vole brain areas that display evidence of fos expression for a period of time sufficient for pair bonding to occur. These results further strengthen arguments that the amygdala and BNST may be particularly important in pair-bond formation in addition to other functions. Since fos expression has been linked to learning and memory, areas such as these that display continued fos activation might be sites where critical associations between sensory stimuli and the partner are formed.

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