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Social contact elicits immediate-early gene expression in dopaminergic cells of the male prairie vole extended olfactory amygdala

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Abstract

Male prairie voles (*Microtus ochrogaster*) are a valuable model in which to study the neurobiology of sociality because, unlike most mammals, they pair bond after mating and display paternal behaviors. Research on the regulation of these social behaviors has highlighted dopamine (DA) neurotransmission in both pair bonding and parenting. We recently described large numbers of dopaminergic cells in the male prairie vole principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd), but such cells were very few in number or absent in the non-monogamous species we examined, including meadow voles. This suggests that DA cells in these sites may be important for sociosexual behaviors in male prairie voles. To gain some insight into the function of these DAergic neurons in male prairie voles, we examined expression of the immediate-early genes (IEGs) Fos and Egr-1 in TH-immunoreactive (TH-ir) cells of the pBST and MeApd after males interacted or not with one of several social stimuli. We found that IEGs were constitutively expressed in some TH-ir neurons under any social condition, and that IEG expression in these cells decreased after a 3.5-hr social isolation. Thirty-min mating bouts (but not 6- or 24-hr bouts) that included ejaculation elicited greater IEG expression in TH-ir cells than did non-ejaculatory mating, interactions with a familiar female sibling, or interactions with pups. Furthermore, Fos expression in TH-ir cells was positively correlated with the display of copulatory, but not parental, behaviors. These effects of mating were not found in other DA-rich sites of the forebrain (including the anteroventral periventricular preoptic area, periventricular anterior hypothalamus, zona incerta, and arcuate nucleus). Thus, activity in DAergic cells of the male prairie vole pBST and MeApd is influenced by their social environment, and may be particularly involved in mating and its consequences, including pair bonding.

Keywords

dopamine; immediate-early gene; monogamy; olfaction; paternal behavior; sexual behavior

Prairie voles (*Microtus ochrogaster*) are an invaluable rodent model to study the neurobiology of sociality because they form life-long pair bonds after mating and show biparental behavior after the birth of pups (Carter et al., 1995). Release of numerous neurochemicals, including dopamine (DA), is required for the display of these social behaviors (Young et al., 2008). D1 receptor antagonism prevents the formation of partner preferences after mating in both sexes of prairie voles, while D2 stimulation induces partner preference formation in the absence of mating (Aragona et al., 2003, Wang et al., 1999). DA receptor antagonism also impairs parental responding in both sexes of prairie voles, with the mixed D1/D2 receptor antagonist haloperidol reducing voles' motivation to make contact with pups and lick them (Lonstein, 2002). The

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nucleus accumbens (NAcc) is one site where dopaminergic (DAergic) activity is critical for pair bond formation in prairie voles (Aragona et al., 2003, Gingrich et al., 2000), and this is probably also true for their biparental behaviors (see Numan and Stolzenberg, 2009).

The ventral tegmental area (VTA) is likely a major source of DA to the NAcc and other forebrain sites necessary for social behaviors in prairie voles (Curtis and Wang, 2005), but the involvement of other regions that may provide DAergic signaling necessary for sociality have not been investigated. We recently described novel populations of neurons expressing high levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, in the prairie vole principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) (Northcutt et al., 2007). These cells do not contain dopamine beta-hydroxylase and, thus, are likely DAergic (Northcutt et al., 2007). Few or no TH-immunoreactive (TH-ir) cells were found in these sites in three non-monogamous species that we examined, including the closely related meadow vole, suggesting that these cells may be involved in how DA influences social behaviors relatively unique to prairie voles. We also found that male prairie voles have 3-5-fold more TH-ir cells in these sites than do female prairie voles, indicating particular importance of these cells for social behaviors in males.

The pBST and MeApd have dense connections with the main and accessory olfactory systems, as well as with each other (Aldheid et al., 1995, Coolen and Wood, 1998, Davis et al., 1978, Scalia and Winans, 1975), and process and transmit olfactory information essential for conspecific identification and appropriate social responding in laboratory rats, hamsters, and mice (Newman, 1999). The same is true for male prairie voles, as indicated by the increased expression of the immediate-early gene (IEG) Fos in both the pBST and MeApd after male prairie voles cohabitate with a female, mate with her, or interact with pups (Cushing et al., 2003, Kirkpatrick et al., 1994b, Lim and Young, 2004). Furthermore, lesions of the entire MeA decrease males' paternal behaviors (Kirkpatrick et al., 1994a). How the DAergic cells of the pBST and MeApd are specifically involved in these effects is unknown.

To begin investigating potential roles for the DAergic cells of the male prairie vole pBST and MeApd, we examined Fos expression in TH-ir neurons in these brain sites after males interacted for 30 min with one of several social stimuli, including a sexually receptive female prairie vole or conspecific pups. We also examined Fos in other forebrain DAergic cell groups, to determine the selectivity of Fos expression in TH-ir cells in the pBST and MeApd after sociosexual interactions. Because we found that many, but not most, TH-ir cells in these two sites contained Fos immunoreactivity after social interactions, we carried out a subsequent experiment to determine if even more of these TH-ir cells would contain another IEG, Egr-1, after particular sociosexual interactions. Egr-1 was chosen because it is expressed in many brain sites after the display of sexual or maternal behaviors, including in cells that do not express Fos (Numan et al., 1998, Polston and Erskine, 1995, Wersinger and Baum, 1996).

Because the formation of partner preferences in male prairie voles requires rather long sexual interactions with a female (up to 24 h, Insel et al., 1995, Winslow et al., 1993), and surprisingly prolonged Fos expression can be seen after acute or chronic social or other stimulation (Bullitt et al., 1992, Matsuda et al., 1996, Quattrochi et al., 2005, Ricci et al., 2007, Stack and Numan, 2000, Wrynn et al., 2000, Xu et al., 2006), we also examined Fos expression in TH-ir cells of the pBST and MeApd after males cohabitated with a sexually receptive female for 6 or 24 hr.

Experimental Procedures

Subjects

Subjects were adult prairie voles (*Microtus ochrogaster*) that were born and raised in our colony at Michigan State University. The colony originated with voles originally captured in

Urbana, Illinois in 1994, and since maintained in laboratory environments and interbred with voles from the colonies of Drs. C. Sue Carter, Zuoxin Wang, Geert De Vries, and Betty MacGuire. This stock was brought to Michigan State University in 2002. Animals were housed in clear plastic cages (48×28×16 cm), containing wood chips, wood shavings, and a layer of hay. Food and water were provided *ad libitum*; food consisted of a mixture of cracked corn, whole oats, sunflower seeds, and rabbit chow (Teklad rodent diet #2031, Harlan, Madison, WI) mixed in a 1:1:2:2 ratio. The colony room was maintained with an ambient temperature of approximately 21°C and a 14:10 light:dark cycle. Litters were weaned from breeding pairs at 20 days of age and housed with their siblings in mixed-sex groups consisting of the entire litter until adulthood (60-120 days old) when they were used in one of the experiments. While exposure to male siblings affects sensitivity to estradiol in female prairie voles (Cushing and Carter, 1999), the effects of the postweaning social environment on males' reproductive potential are less pronounced (Mateo et al., 1994). In fact, we were able to retrospectively determine the sex ratio for 75% of our subjects' natal litters, and found that neither the total number of siblings nor the litter sex ratio were significantly correlated with any sociosexual behavior or the IEG expression found in any site examined in the present experiments (data not shown). The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23), as well as the Institutional Animal Care and Use Committee at Michigan State University.

Experiment 1a: Effects of 30-min social interactions on Fos expression

Sexually naïve male prairie voles were placed individually into a clean cage (48×28×16 cm) with bedding, food, and water, and transported to a nearby room illuminated with dim ambient light (20 lux) for behavioral testing. After a three-hour habituation to the testing cage and room, each subject was presented with a social stimulus (or nothing) for 30 min. After 30 min, the stimulus animal was removed and the male subject remained in the cage and testing room for one hour before being sacrificed. An additional control group was not placed in a novel cage or brought to the testing room, but instead sacrificed directly after removal from their home cage in the colony room. The social stimuli presented were:

Groups 1 and 2: Sexually receptive female—Males ($n = 40$) were allowed to interact with an adult female that was ovariectomized, allowed to recover for at least 2 weeks, and injected subcutaneously with estradiol benzoate (10 µg in 50 µl sesame oil; Sigma, St. Louis, MO) 48 and 24 h before testing to induce sexual receptivity (Carter et al., 1987a, Northcutt and Lonstein, 2008). Stimulus females were tested for receptivity with a sexually experienced male from our colony immediately before testing, and only females displaying lordosis when mounted were used. Because Fos expression in the pBST and MeApd of male rats is greatly increased after ejaculation, but this increase does not necessarily occur after mating bouts that do not include ejaculation (Coolen et al., 1997, Coolen et al., 1998, Veening and Coolen, 1998), male prairie voles interacting with receptive females were screened until two groups were filled: males that mated to ejaculation within the 30-min test (EJAC; $n = 8$) and males that did not ejaculate (NON EJAC; $n = 8$). Our previous work demonstrated that up to one-third of the sexually inexperienced males in our colony ejaculate during a 30-min sexual behavior test (Northcutt and Lonstein, 2008). After eight NON EJAC males were obtained, the subsequent males that were screened but did not ejaculate within 30 min were unnecessary, so were not sacrificed or included in the experiment.

Group 3: Pups—To compare the effects of mating versus exposure to other unfamiliar social stimuli on Fos expression, a group of parentally naïve virgin males was allowed to interact with two 2-3 day old conspecific pups (PUPS; $n = 9$). Pups were taken from breeding pairs in our colony just prior to testing, and were wiped clean with distilled water before being placed

in the testing cage with the subject for 30 min. One subject attacked the pups, so testing was terminated and this male was not included in the study (final $n = 8$).

Group 4: Familiar female sibling—To control for the effects of general social interaction on Fos expression, a fourth group of males interacted for 30 min with a familiar female sibling that he had been housed with until three hours before testing (SIBLING; $n = 9$). Female prairie voles are induced ovulators and do not come into behavioral estrus unless exposed to an unfamiliar male (Carter et al., 1980, Carter et al., 1987b). Incest is avoided in this species because females do not sniff the anogenital region of their familiar male siblings (Carter et al., 1980), which prevents estrus induction, so expectedly none of these males attempted to mate with his sibling.

Group 5: Alone control—To determine the effects of handling and habituation to a clean cage and the testing room, these males were removed from the colony but remained alone in the testing cage and room for 30 min (ALONE; $n = 8$). The cage lid was removed and a hand placed into the cage after the 3 h habituation, and again 30 min later, to mimic inserting and removing a social stimulus.

Group 6: Colony control—To determine baseline Fos expression in socially housed males, these subjects were removed from their home cages in the colony room, immediately overdosed with anaesthetic, and perfused (COLONY; $n = 8$).

All behavioral interactions for the socially stimulated groups were recorded using a Panasonic low-light-sensitive camera connected to a Panasonic VCR (Panasonic, Secaucus, NJ). Sexual behaviors, paternal behaviors, social contact, and non-social behaviors (including feeding, drinking, and exploring) were scored similarly to what our lab has previously described (Lonstein, 2002, Northcutt and Lonstein, 2008).

One hour after the stimulus was removed, or 4.5 hr after ALONE males were placed in the novel cage, subjects were overdosed with an anesthetic cocktail containing ketamine (62.5 mg/kg; Butler, Dublin, OH), xylazine (7.5 mg/kg; Butler), and acepromazine (0.8 mg/kg; Butler). They were perfused transcardially with 100 ml of 0.9% saline and 100 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer. After perfusion, brains were postfixed overnight in 4% paraformaldehyde, and then cryoprotected in a 20% sucrose solution for at least 2 days. Brains were sectioned into 35- μ m coronal sections on a freezing microtome and stored in cryoprotectant at -20°C until further processing. One series containing every other section throughout each brain was processed immunocytochemically for both tyrosine hydroxylase (TH) and Fos.

Fos and TH dual immunocytochemistry—Sections were rinsed three times in Tris-buffered saline (TBS; pH 7.6) between each step. First, sections were incubated in 0.1% sodium borohydride for 15 min, and then incubated in 1% hydrogen peroxide and 0.3% Triton X-100 in TBS for 10 min. Sections were blocked in 20% normal goat serum (NGS) and 0.3% Triton X-100 in TBS for 30 min before being placed in rabbit anti-Fos polyclonal primary antiserum (1:10,000; cat. #sc52; Santa Cruz Biotechnology, Santa Cruz, CA) in 2% NGS and 0.3% Triton X-100 in TBS at 4°C for 48 hr. Next, sections were incubated in a biotinylated goat anti-rabbit secondary antiserum (1:500; Vector Laboratories, Burlingame, CA) in 2% NGS and 0.3% Triton X-100 in TBS for 1 hr, followed by a 1-hr incubation in avidin-biotin complex (Vectastain Elite, Vector Laboratories). Fos was visualized by incubating sections in 3-3'-diaminobenzidine (Sigma) in TBS, which produced a brown nuclear reaction product. Brains were then blocked again in 20% NGS for 30 min, and were incubated in a mouse anti-TH monoclonal primary antiserum (1:2,000; cat. #MAB318; Chemicon, Temecula, CA) in 2% NGS and 0.3% Triton X-100 in TBS at room temperature overnight for ~ 18 hr. Sections were

then incubated in goat anti-mouse secondary antiserum (1:500; Vector Laboratories) in 2% NGS and 0.3% Triton X-100 in TBS for 1 h, followed by a 1-hr incubation in avidin-biotin complex. TH was visualized with Vector SG (Vector Laboratories) diluted in TBS, which resulted in a blue cytoplasmic reaction product. Cross-reactivity was not expected, given that the primary antisera were obtained from different species, and visual inspection of the tissue revealed that numerous single-labeled cells of both types were clearly visible in many brain areas. We also performed immunocytochemical controls that included omitting one or both of the primary and secondary antisera, which eliminated the corresponding specific labeling. After processing, brain sections were mounted onto slides, dehydrated, and coverslipped. Two immunocytochemical runs were performed, with a similar number of subjects from each group represented in each run.

Analysis of Fos and TH-immunoreactive cells—Slides were randomized and coded for analysis. Sections were analyzed by a single observer blind to subject condition (KVN). The number of Fos-immunoreactive (Fos-ir), TH-ir, and dual-labeled cells were counted in each site examined (see below). All cells expressing TH were counted at 200× magnification using a Nikon E400 light microscope with the aid of a reticle in one ocular lens. A cell was counted if it contained any blue cytoplasmic labeling. Fos-expressing cells were also examined at 200× magnification, and included in the analysis if they contained brown nuclear labeling. To determine the number of dual-labeled cells, TH-ir cells were re-examined at 400× magnification and were considered to also contain Fos immunoreactivity if they clearly contained a brown nuclear label surrounded by a blue cytoplasmic label.

Cells were examined bilaterally from four consecutive sections containing the principal nucleus of the bed nucleus of the stria terminalis (pBST; roughly corresponding to Swanson's plates 21-22; Swanson, 1998) and from seven sections containing the posterodorsal medial amygdala (MeApd; Swanson's atlas plates 28-30). To determine if the effect of social stimulation on IEG expression within TH-ir cells of the pBST and MeApd was unique, we also examined numerous other forebrain regions where TH-ir cells are found in prairie voles and other rodents (Bjorklund and Dunnett, 2007, Lansing and Lonstein, 2006, Smeets and Gonzalez, 2000). This involved bilaterally examining two sections containing the anteroventral periventricular nucleus of the preoptic area (AVPV) and ventrolateral region of the anteroventral preoptic area (AVP) (Swanson's atlas plate 19), periventricular anterior hypothalamus (PVA; atlas plate 24), zona incerta (ZI; atlas plate 28), and arcuate nucleus of the hypothalamus (ARH; atlas plate 30). For all regions, cells were counted from the entire area containing TH-ir cells, with the size of the area examined remaining constant across all subjects.

Statistical Analysis—Behavioral differences between EJAC and NON EJAC males were compared with unpaired *t*-tests. The duration of time that socially stimulated groups spent with their stimulus was compared with a one-way ANOVA, followed by Fisher's LSD post-hoc tests to compare differences between individual groups.

To analyze TH and IEG expression in each brain site, we first determined whether the data were normally distributed using the Shapiro-Wilk test. The TH immunoreactivity data from every site were normally distributed, so one-way ANOVAs were used to compare groups in the number of TH-ir cells. The IEG measures were not normally distributed, so data were subjected to a square-root transformation, and normality assessed again. If the transformation resulted in a normal distribution, one-way ANOVAs were used to analyze the transformed data. This was the case for Fos immunoreactivity in the AVP, ZI, and ARH, the number of cells containing both TH immunoreactivity and Fos immunoreactivity in the pBST, MeApd, AVP, PVA, and ZI, and the percentage of TH-ir cells containing Fos immunoreactivity in the pBST, MeApd, AVP, PVA, and ZI. If these omnibus ANOVAs were significant, Fisher's LSD post-hoc tests were then used to compare differences between individual groups. If square-root

transformation did not result in a normal distribution, Kruskal-Wallis tests were used to compare treatment groups, followed by Mann-Whitney tests to determine which individual groups differed from each another. This was the case for Fos immunoreactivity in the BST, MeA, AVPV, and PVa, the number of dual-labeled cells in the AVPV and ARH, and the percentage of TH-ir cells expressing Fos in the AVPV and ARH. Pearson's r was used to correlate the percentage of TH-ir cells expressing Fos in the pBST and MeApd with the latencies, frequencies, and durations of sexual behaviors displayed by EJAC and NON EJAC males, paternal behaviors in PUPS males, and total duration of all social behaviors in males tested with a stimulus animal. Based on the previous studies (Coolen et al., 1997, Coolen et al., 1998, Veening and Coolen, 1998), we expected EJAC males to have greater IEG expression than NON EJAC males, so one-tailed post-hoc tests were used to compare IEG expression between these two particular groups (significance indicated by $p \leq 0.10$). In all other cases, statistical significance was indicated by $p \leq 0.05$. Tissue from the AVPV and AVP of one NON EJAC male was damaged and could not be analyzed, resulting in a sample size of seven subjects for this group for these sites.

Experiment Ib: Effects of 30-min social interactions on Egr-1 expression in TH-ir cells

Egr-1 and TH immunocytochemistry and analysis—Because we found that mating to ejaculation was the most effective stimulus for eliciting Fos in many, but still not most, TH-ir cells in the pBST and MeApd (see Results), we determined if mating-induced modulation of even more TH-ir cells could be reflected by expression of another IEG, Egr-1. As noted above, Egr-1 is expressed in many brain sites after the display of social behaviors (Numan et al., 1998, Polston and Erskine, 1995, Wersinger and Baum, 1996), including in cells that do not express Fos. To do this, we examined the alternate series of brain sections for ALONE, SIBLING, NON EJAC, and EJAC males from Experiment Ia. Egr-1 and TH immunocytochemistry was performed identically to that described above for Fos and TH, except that the Fos primary antiserum was replaced with a rabbit polyclonal primary antiserum against Egr-1 (1:1,000; cat.#sc189; Santa Cruz Biotechnology). Immunocytochemical controls that omitted the primary or secondary antisera abolished specific labeling. The number of cells containing TH immunoreactivity, the number containing Egr-1 immunoreactivity, and the number containing both TH immunoreactivity and Egr-1 immunoreactivity were counted bilaterally from the two sections through the pBST and three sections through the MeApd that contained the most TH-ir cells in these sites.

Statistical Analysis—Statistical analyses of the number of TH-ir cells, the number of Egr-1-ir cells, and the percentage of TH-ir cells also expressing Egr-1 were performed as described for Experiment Ia. The number of TH-ir cells among groups was normally distributed, so one-way ANOVAs were used to analyze these data. The number of Egr-1-ir cells was normally distributed after square-root transformation of the data, so one-way ANOVAs were performed on these transformations. All other data sets were not normally distributed even after square-root transformation, so Kruskal-Wallis analyses were used to compare groups. Some tissue processed for TH and Egr-1 was damaged during immunocytochemical processing, resulting in final sample sizes for the pBST of ALONE and SIBLING $n = 8$, NON EJAC $n = 6$, EJAC $n = 5$ and for the MeApd of ALONE $n = 8$, SIBLING $n = 6$, NON EJAC $n = 5$, and EJAC $n = 6$.

Experiment II: Effects of 6- or 24-h social interactions on Fos expression

Prolonged bouts of mating up to 24 hr in duration are typically necessary to induce partner preferences in male prairie voles (Insel et al., 1995, Winslow et al., 1993). To determine if prolonged sexual interactions could induce persistently high levels of Fos expression in TH-ir cells of the pBST and MeApd that might reflect establishment of a pair bond, sexually naïve male prairie voles were exposed to sexually receptive females or a control stimulus for 6 or 24

h. Males were placed in a novel cage and moved to a testing room as described in Experiment Ia. After a 3-h habituation, one group of males interacted with a sexually receptive female (MATED; 6 h: $n = 12$, 24 h: $n = 9$). Receptivity was induced and verified before testing as described in Experiment Ia. Another group of males interacted with a familiar female sibling (SIBLING; 6 h: $n = 8$; 24 h: $n = 7$). Behavioral interactions were recorded at 5:1 time-lapse using a Panasonic video recorder, and were viewed to ensure that males mated with sexually receptive females within 2 h after the beginning of testing, but did not mate with their siblings. It was observed that five males in the MATED groups (four 6-h males, and one 24-h male) failed to mate in the first 2 h, and were excluded from the study, but the remaining males began mounting the stimulus female within 40 min of the beginning of the test (final $n = 8$ for both time points). None of the SIBLING males attempted to mate with their sisters. A third group of males remained alone in the observation cage (ALONE; 6 h: $n = 7$, 24 h: $n = 8$), but had the cage lid removed and a hand placed inside the cage after habituation to imitate inserting a stimulus animal. In the 6-h groups, six hours after the stimulus was introduced to the cage, it was removed (or a hand was placed into the cage for the ALONE group) and the male remained alone in the cage for one hour before perfusion. In the 24-h groups, the male subject was perfused 24 h after the stimulus was placed in the cage. Tissue collection, processing, and analysis, as well as statistical analyses, were identical to that described in Experiment Ia. TH immunoreactivity data was normally distributed, so one-way ANOVAs were used to analyze these data. All other data were not normally distributed, even after square-root transformation, so Kruskal-Wallis tests were used instead.

Results

Experiment Ia: Fos and TH expression after 30-min social interactions

Behavior—All males in the NON EJAC and EJAC groups mounted the stimulus female with a latency and frequency that did not significantly differ between the two groups (latency to mount: NON EJAC = 346 ± 78 s, EJAC = 182 ± 35 s, $t(14) = -1.93$, $p = 0.075$; frequency of mounts: NON EJAC = 19 ± 6 , EJAC = 34 ± 10 , $t(14) = 1.36$, $p = 0.194$). However, EJAC males displayed more thrusting bouts than did NON EJAC males (110 ± 19 vs. 30 ± 13 , $t(14) = 3.50$, $p = 0.004$). Males in the EJAC group ejaculated a mean of 1.9 ± 0.3 times, with the first ejaculation occurring 389 ± 182 s after the first mount and 383 ± 182 s after the first thrust.

All males in the PUPS group licked and hovered over pups. Males spent 777 ± 72 s licking pups, and began licking 64 ± 27 s after the beginning of testing. They spent 1061 ± 156 s huddled over pups, with an average latency of 99 ± 20 s after they began licking. Males retrieved pups an average of 2 ± 1 times, but 37% of males did not retrieve at all, which was not surprising (see Lonstein and De Vries, 1999).

The duration of time that males spent in physical contact with their stimulus (including the durations of sniffing, grooming, and huddling) significantly differed among groups ($F(3,29) = 4.47$, $p = 0.011$), and was greatest in the PUPS group (1207 ± 133 s). This was significantly more time spent with the stimulus than the duration of time that SIBLING and EJAC males spent in physical contact with their stimulus females (SIBLING: 682 ± 137 s, EJAC: 725 ± 53 s), but it did not differ from NON EJAC males (951 ± 103 s). SIBLING, NON EJAC, and EJAC males spent a similar amount of time in contact with their respective stimuli.

TH Expression—No significant differences were found between groups in the number of TH-immunoreactive (TH-ir) cells in the pBST, MeApd, AVPV, AVP, PVa, or ZI. Unexpectedly, EJAC and SIBLING males had significantly more TH-ir cells in the ARH (by approximately 30%) than the number of TH-ir cells found in the ARH of ALONE and NON EJAC males (Tables 1 and 2).

Fos expression—In the pBST and MeApd, as well as the AVPV and PVa, the number of Fos-ir cells significantly differed among groups of males (Figure 1 and Table 2; pBST - $H(5) = 39.96, p < 0.001$; MeApd - $H(5) = 38.46, p < 0.001$; AVPV - $H(5) = 35.72, p < 0.001$; PVa - $H(5) = 30.84, p < 0.001$). In the pBST, MeApd, and AVPV, EJAC males had 15-18 times more Fos-ir cells than ALONE, SIBLING, and COLONY groups, while NON EJAC and PUPS males had approximately eight times more Fos-ir cells than these three control groups. In the PVa, EJAC and PUPS males had approximately 5 times more Fos-ir cells, and NON EJAC males had approximately 4 times more Fos-ir cells, than that found in the three control groups.

For the other forebrain sites examined, males exposed to any novel social stimulus (EJAC, NON EJAC, and PUPS groups) generally had more Fos-ir cells compared to SIBLING, ALONE, and COLONY control males, with the pattern of group differences in Fos expression different in each brain site. Notably, the pattern of Fos expression seen in the pBST, MeApd, AVPV (*i.e.*, EJAC males having more Fos-ir cells than all other groups) was not found in any of the other brain sites we examined (see Table 2).

Fos expression within TH-immunoreactive cells—The percentage of TH-ir cells in the pBST and MeApd that also expressed Fos significantly differed among groups (Figures 2 and 3; pBST - $F(5, 43) = 5.44, p = 0.001$; MeApd - $F(5, 43) = 7.29, p < 0.001$). In both sites, EJAC males had a greater percentage of TH-ir cells expressing Fos (~6% of all TH-ir cells) than did ALONE, PUPS, and NON EJAC males. This pattern was unique, because EJAC males did not have a greater percentage of TH-ir cells expressing Fos than NON EJAC males in any of the other forebrain regions analyzed, including the AVPV and PVa (see Table 2). In addition, the percentage of TH-ir cells in the pBST and MeApd also expressing Fos was higher in all groups exposed to an adult social stimulus (COLONY, SIBLING, NON EJAC, and EJAC males) when compared to ALONE males.

The number of dual-labeled cells in the pBST and MeApd (Table 1) showed a very similar pattern as the percentage of TH-ir cells expressing Fos, with EJAC males having significantly more dual-labeled cells than all other groups in the pBST and more than the COLONY, ALONE, PUPS, and NON EJAC males in the MeApd.

Correlations between the percentage of TH-ir cells expressing Fos and behavior

—The percentage of TH-ir cells expressing Fos in the pBST and MeApd was significantly correlated with the expression of numerous sexual behaviors (see Figure 4 and Table 3). In the pBST, the percentage of TH-ir cells expressing Fos was positively correlated with the number of mounts, number of thrusting bouts, number of ejaculations, duration of time spent mounting, and total time engaged in all sexual behaviors. In the MeApd, the percentage of TH-ir cells expressing Fos was positively correlated with the number of thrusting bouts, duration of time spent mounting, duration of time spent thrusting, and total amount of time engaged in sexual behaviors. The percentage of TH-ir cells in the pBST that expressed Fos was also positively correlated with the latency to lick pups in PUPS males, but there were no significant correlations between the percentage of TH-ir cells expressing Fos in the MeApd and any paternal behaviors.

Experiment Ib: Egr-1 expression after 30-min social interactions

TH expression—The number of TH-ir cells in the pBST or MeApd did not differ among groups (pBST - $F(3, 23) = 0.119, p = 0.948$; MeApd - $F(3, 21) = 0.915, p = 0.451$) (Table 4).

Egr-1 expression—All groups of males had more Egr-1-expressing cells in the pBST and MeApd than the number of Fos-expressing cells they had in Experiment Ia. In both sites, Egr-1 expression differed among groups (Figure 5; pBST - $F(3, 23) = 53.52, p < 0.001$; MeApd - F

(3, 21) = 26.11, $p < 0.001$), with all groups being significantly different from one another. SIBLING males had approximately 1.5 times the number of Egr-1-ir cells than did ALONE males, and NON EJAC males had almost twice the number found in ALONE males. EJAC males had significantly more Egr-1 expressing cells than all other groups, including 1.5 – 2 times more than that seen in NON EJAC males.

Egr-1 expression within TH-immunoreactive cells—The percentage of TH-ir cells in the pBST and MeApd that also contained Egr-1 immunoreactivity significantly differed between groups (Figure 6; pBST - $H(3) = 9.58$, $p = 0.023$; MeApd - $H(3) = 18.96$, $p < 0.001$). In these regions, EJAC males had 9 and 23 times the percentage of TH-ir cells expressing Egr-1 than did ALONE males and twice that of NON EJAC males. Notably, the total percentage of TH-ir cells in these sites that also contained Egr-1 was higher than the percentage that contained Fos in Experiment 1a, but it was still quite moderate (up to ~10-15% of all TH-ir cells). The raw number of dual-labeled cells also differed among groups in both sites (Table 4), with a generally similar pattern as the percentage of TH-ir cells that were dual-labeled.

Correlations between the percentage of TH-ir cells expressing Egr-1 and behavior—The percentage of TH-ir cells in the pBST that also contained Egr-1 immunoreactivity was not significantly correlated with any measure of males' sexual or other social behaviors. In the MeApd, the percentage of TH-ir cells expressing Egr-1 was positively correlated with the number of ejaculations ($r(9) = 0.853$, $p < 0.001$) and negatively correlated with the latency to ejaculate after the first thrust ($r(4) = -0.814$, $p = 0.049$).

Experiment II: Fos expression after 6- or 24-h social interactions

TH expression—The number of TH-ir cells in the pBST and MeApd did not significantly differ among groups at either the 6- or 24-hr time point (Table 5).

Fos expression—After 6-h interactions, the number of Fos-ir cells in the pBST and MeApd significantly differed among groups (Figure 7; pBST - $H(2) = 15.05$, $p = 0.001$; MeApd - $H(2) = 14.71$, $p = 0.001$), with MATED males having five-fold more Fos-ir cells in both brain sites than did ALONE and SIBLING males.

Fos expression in the pBST and MeApd also differed among groups after 24-h interactions (Figure 7; pBST - $H(2) = 15.85$, $p < 0.001$; MeApd - $H(2) = 16.73$, $p < 0.001$). In both brain sites, MATED males had twice the number of Fos-ir cells than did SIBLING males, and SIBLING males had 2-3 times as many Fos-expressing cells as did ALONE males.

Fos expression within TH-ir cells—The percentage of TH-ir cells in the pBST and MeApd that also contained Fos immunoreactivity significantly differed among groups after 6-h interactions (Figure 8; pBST - $H(2) = 12.77$, $p = 0.002$; MeApd - $H(2) = 10.35$, $p = 0.006$), with MATED males having a significantly greater percentage than that found in ALONE males, although this percentage was very low (~2% of all TH-ir cells).

A similar pattern of Fos expression within TH-ir cells was seen after 24 h interactions with the stimuli. The percentage of TH-ir cells expressing Fos differed among all groups in the pBST and MeApd (Figure 8; pBST - $H(2) = 6.40$, $p = 0.041$; MeApd - $H(2) = 7.17$, $p = 0.028$), but was very low (less than 2% in all groups).

Discussion

IEG expression after sociosexual interactions

The pBST and MeApd are necessary for sexual and parental behaviors in many rodents (reviewed in Newman, 1999, Numan and Insel, 2003). Consistent with this, and similar to other studies of male prairie voles (Cushing et al., 2003, Kirkpatrick et al., 1994b, Lim and Young, 2004), we found increased IEG expression in these brain sites after male prairie voles interacted with either a sexually receptive female or pups. Our data are the first evidence in prairie voles, though, that mating to ejaculation induces even greater Fos and Egr-1 expression in these sites than does mating bouts that do not result in ejaculation. Similar results have been found in male rats, with Fos expression higher after ejaculation compared to that observed after only intromissions, even when the amount of mating stimulation required for ejaculation is drastically reduced (Baum and Everitt, 1992, Coolen et al., 1997, Veening and Coolen, 1998). This suggests that some cells in the pBST and MeApd may express IEGs only after males ejaculate and possibly reach sexual satiety.

The effects of social interactions on Fos expression in the two rostral periventricular regions we examined (AVPV and PVa) were very similar to what was found in the pBST and MeApd. This is similar to findings that Fos expression increases in the AVPV of other rodents after mating, and particularly after ejaculation (Simmons and Yahr, 2002). Because the AVPV receives projections from cells in the MeApd that also express Fos after ejaculation (Simmons and Yahr, 2002), the AVPV and possibly the PVa may work with other sites to regulate physiological or behavioral events occurring after ejaculation, such as hormone secretion or the sexual refractory period. Of the forebrain sites we examined, only in the pBST, MeApd, AVPV, and PVa did mating to ejaculation increase Fos expression above and beyond the effects of mating without ejaculation, so these sites may be part of a neural network regulating ejaculation-related events in male prairie voles, as is true in other rodents. Because mating greatly facilitates partner preference formation in male prairie voles (Insel et al., 1995, Winslow et al., 1993), these four neural sites may be particularly influential in the formation of social bonds with mates.

IEG expression within TH-expressing cells after sociosexual interactions

The behavioral or physiological functions of the hundreds of DAergic cells in the male prairie vole pBST and MeApd are unknown. Because cells containing immunocytochemically detectable levels of TH are few or non-existent in the pBST and MeApd of the non-monogamous species we examined (including meadow voles), we hypothesized that these cells in prairie voles contribute to their characteristic social behaviors (Northcutt et al., 2007). Our data support this hypothesis, although they further suggest that these cells may have a somewhat non-specific role in their sociality. Interaction with any social partner maintained at least baseline levels of Fos or Egr-1 within the TH-ir cells of the pBST and MeApd. In fact, social isolation (ALONE group) caused a significant decrease in IEG expression within TH-ir cells of these sites compared to that found in COLONY and SIBLING controls. These results highlight the importance of including a group of completely unmanipulated COLONY controls in studies such as these, because this group allowed us to discover the constitutive Fos expression in TH-ir cells of the pBST and MeApd of socially-housed prairie voles. Unexpectedly high constitutive expression of IEGs within TH-ir cells has also been found in the male zebra finch brain, although in zebra finches the number of dual-labeled cells decreases after social interactions rather than being maintained or increasing (Bharati and Goodson, 2006). A similar example of high constitutive expression of IEGs is provided by the visual cortex, where expression of these transcription factors decreases after adaptation to the dark (Worley et al., 1991). The pBST and MeApd were the only brain sites we examined where both COLONY and SIBLING males differed from ALONE males in Fos expression within

TH-ir cells, which highlights these regions as possibly unique among brain sites containing DAergic cells. These cells may be part of a neural network monitoring the ongoing status of a vole's social environment; their decreased activity resulting from even a few hours of social isolation may increase the motivation to seek out a social partner, or if unsuccessful, contribute to the stressful effects of social isolation in this highly gregarious species (Grippio et al., 2007; Kim and Kirkpatrick, 1996; Klein et al., 1997).

While any social contact maintained at least baseline levels of IEG expression within TH-ir cells of the pBST and MeApd, mating to ejaculation was particularly effective in inducing further IEG expression in these cells. The pBST and MeApd were the only forebrain sites we examined where EJAC males had more TH-ir cells expressing Fos than NON EJAC males. As noted above, EJAC males had more Fos-ir cells in the AVPV and PVa than did NON EJAC males, but not specifically within TH-ir cells. Thus, of the forebrain DA-rich sites we examined, IEG expression preceding or resulting from ejaculation was specific to the TH-ir cells of the pBST and MeApd. Furthermore, the percentage of TH-ir cells expressing Fos in both regions was positively correlated with males' sexual behaviors, but not males' paternal behaviors or total duration of social contact. In other rodents, the pBST and MeApd receive olfactory information from both the main and accessory olfactory systems, and process this information in a manner that promotes the appropriate expression of sociosexual behaviors (*e.g.*, Newman, 1999, Numan and Insel, 2003). TH-ir cells in these sites might help males establish olfactory-based memory of mates, which is necessary for the formation and maintenance of partner preferences (Curtis et al., 2001, Kirkpatrick et al., 1994c).

Mating bouts extending up to 24 h are typically necessary for establishing social bonds in prairie voles (Insel et al., 1995, Winslow et al., 1993). We hypothesized that prolonged sexual interactions may be even more effective than 30-min mating bouts in eliciting Fos expression in TH-ir cells of the pBST and MeApd. This was not the case, as males mating for 6 or 24 h had even less Fos expression in TH-ir cells than did males sacrificed after a 30-min bout (although MATED males did have a greater percentage of TH-ir cells expressing Fos than did ALONE males at both time points). Thus, mating elicits Fos expression in these TH-ir cells soon after initial mating and ejaculation, but does not appear to produce chronic or repeated waves of Fos expression, although it remains possible this might occur at times points we did not investigate. TH-ir cells in the pBST and MeApd may be involved in males' display of copulatory behaviors and initiating the neural cascades that lead to the development of social bonds after males ejaculate. Additional mating interactions over the following 24 h may instead more greatly affect downstream targets in this pathway, such as the nucleus accumbens and ventral pallidum, which may be involved in the solidification, rewarding qualities, and later expression of the social bond (Lim and Young, 2004).

Ejaculating males had the greatest percentage of TH-ir cells in the pBST and MeApd expressing IEGs, but it is clear that only a relatively small percentage of all TH-ir cells in either site was dual-labeled. This is completely consistent with studies in birds and rats investigating neural Fos expression after mating, which report that fewer than 5-10% of TH-ir cells in any site also express Fos (Balfour et al., 2004, Bharati and Goodson, 2006, Charlier et al., 2005). It is similarly clear that the far majority of IEG-expressing cells in the pBST and MeApd of ejaculating males do not synthesize TH and are not DAergic. Therefore, many neurons in the pBST and MeApd of yet unknown phenotype are also modulated in response to sociosexual interactions in male prairie voles. If the prairie voles are similar to gerbils, these other cells are probably glutamatergic or GABAergic (Simmons and Yahr, 2003).

DA and prairie vole sociosexual behaviors

Our data demonstrate that mating to ejaculation is more potent than some other social stimuli in eliciting IEG expression in TH-ir cells of the prairie vole olfactory extended amygdala. In

addition to being convenient markers for cellular modulation including depolarization (Hoffman and Lyo, 2002, Morgan and Curran, 1991), both Fos and Egr-1 can induce TH transcription, which may restore DA stores after cells have been active (Ghee et al., 1998, Guo et al., 1998, Nakashima et al., 2003, Stefano et al., 2006). It is possible these cells contribute to DAergic regulation of pair bonding, as D2 receptor activation is necessary for inducing partner preferences (Aragona et al., 2006). In addition, these cells may help upregulate D1 receptors in the NAcc and elsewhere after pair bond formation to prevent the formation of extra-pair bonds (Aragona et al., 2006). DAergic neurons in the ventral tegmental area (VTA) may be the predominant source of DA release in the NAcc during mating (Curtis and Wang, 2005), but we found very little Fos expression in either TH-ir or non-TH-ir cells of the VTA (or the substantia nigra; data not reported) in any group of males. This is similar to what is found in the VTA after lactating female rats display maternal behaviors (Lonstein and Stern, 1997), and indicates that not all DAergic cells involved in behavioral displays show increased IEG expression. Ascending projections from the VTA to the NAcc are surely involved in DAergic control of social behaviors in prairie voles, but it is intriguing to consider that that DA cells in the pBST and MeApd also project to the NAcc and other sites (*e.g.*, lateral septum, ventral pallidum, mPOA, each other) to modulate sociosexual behaviors in prairie voles.

These studies provide initial insight into how DAergic cells of the male prairie vole pBST and MeApd are influenced by, and might influence, social interactions. DA affects olfactory processing and increases olfactory discrimination in rats, mice, and primates (Miwa et al., 2004, Pavlis et al., 2006, Tillerson et al., 2006, Yue et al., 2004). Furthermore, DA is involved in learning about olfactory stimuli (Kruzich and Grandy, 2004, Rosenkranz and Grace, 2002, Weldon et al., 1982), including learning about socially-relevant odors (Cornwell-Jones and Bollers, 1983). Thus, a system of DAergic cells existing within the male prairie vole pBST and MeApd may be a unique neural mechanism through which these animals process socially relevant olfactory information, and then transmit this information to DA-sensitive areas of the brain necessary for social bonding. These could include sites essential for memory, reward, and emotional regulation. Current studies in our lab are examining the anatomical projections of TH-ir cells of the male prairie vole pBST and MeApd, which will determine if there is an anatomical basis to support this hypothesis.

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List of Abbreviations

ARH	arcuate nucleus of the hypothalamus
AVP	ventrolateral region of the anteroventral preoptic area
AVPV	anteroventral periventricular nucleus of the hypothalamus
DA	dopamine
DAergic	dopaminergic
Egr-1-ir	Egr-1-immunoreactive
Fos-ir	Fos-immunoreactive
IEG	immediate-early gene
MeApd	posterodorsal medial amygdale
NAcc	nucleus accumbens
NGS	normal goat serum
pBST	principal nucleus of the bed nucleus of the stria terminalis
PVa	periventricular anterior hypothalamus
SN	substantia nigra
TBS	Tris-buffered saline
TH	tyrosine hydroxylase
VTA	ventral tegmental area
ZI	zona incerta

Figure 1.

Number of Fos-ir cells (Mean ± SEM) in four sections through the pBST and seven sections through the MeApd after male prairie voles interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Figure 2.

Percentage of TH-ir cells expressing Fos (Mean $\pm$ SEM) in the pBST and MeApd after male prairie voles interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Figure 3.

Photomicrographs of the pBST (top row) and MeApd (bottom row) from representative males in the ALONE, NON EJAC, and EJAC groups in Experiment Ia. Small arrows in top left panel indicate cells that are single-labeled with TH immunoreactivity (blue cytoplasmic label). Large arrows in top middle panel indicate cells that are single-labeled with Fos immunoreactivity (brown nuclear label). Dual-labeled cells are highlighted at higher magnification in the two panels showing EJAC males. Scale bar in bottom left panel = 50 μ m. Scale bar in bottom right panel inset = 10 μ m.

Figure 4.

Correlations between the percentage of TH-ir cells in the pBST (left) and MeApd (right) that also contained Fos immunoreactivity and the duration of time spent mounting and thrusting by EJAC males (squares) and NON EJAC males (circles) during a 30-min test with a sexually receptive female. Time engaged in male sexual behavior (bottom row) is the summed duration of mounting, thrusting, and ejaculating. See Table 3 for correlations between other behaviors and the percentage of TH-ir cells that also contained Fos immunoreactivity.

Figure 5. Number of Egr-1-ir cells (Mean $\pm$ SEM) in two sections of the pBST and three sections through the MeApd after male prairie voles interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Figure 6.

Percentage of TH-ir cells (Mean $\pm$ SEM) in the pBST and MeApd that also contained Egr-1 immunoreactivity after male prairie voles interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Figure 7.

Number of Fos-ir cells (Mean $\pm$ SEM) in four sections through the pBST and seven sections through the MeApd after male prairie voles mated, interacted with a familiar female sibling, or remained alone for 6 or 24 h. Note that the Y-axis scales for both sites are the same as that in Figure 1. Bars with any of the same letters above them do not significantly differ from each other.

Figure 8.

Percentage of TH-ir cells also containing Fos immunoreactivity (Mean $\pm$ SEM) in the pBST and MeApd after male prairie voles mated, interacted with a familiar female sibling, or remained alone for 6 or 24 h. Note that the Y-axis scales for both sites are the same as that in Figure 2. Bars with any of the same letters above them do not significantly differ from each other.

Table 1

The number of cells containing TH immunoreactivity and the number of dual-labeled cells (Mean $\pm$ SEM) in the pBST and MeApd after males interacted with one of several social stimuli for 30 min. Groups with any of the same superscript letters do not significantly differ from each other.

		# of TH-ir cells	# of Dual-labeled cells
pBST	COLONY	154 $\pm$ 20	4 $\pm$ 1 ^b
	ALONE	184 $\pm$ 31	1 $\pm$ 0 ^a
	SIBLING	175 $\pm$ 21	4 $\pm$ 1 ^b
	PUPS	143 $\pm$ 26	2 $\pm$ 1 ^b
	NON EJAC	167 $\pm$ 22	5 $\pm$ 1 ^b
	EJAC	169 $\pm$ 12	9 $\pm$ 2 ^c
	<i>F</i> (5, 43)	0.425	5.72
	<i>p</i>	0.829	<0.001
MeApd	COLONY	336 $\pm$ 33	9 $\pm$ 3 ^{bc}
	ALONE	330 $\pm$ 33	1 $\pm$ 1 ^a
	SIBLING	334 $\pm$ 27	13 $\pm$ 3 ^{cd}
	PUPS	343 $\pm$ 52	4 $\pm$ 1 ^{ab}
	NON EJAC	289 $\pm$ 34	6 $\pm$ 2 ^{bc}
	EJAC	333 $\pm$ 42	23 $\pm$ 6 ^d
	<i>F</i> (5, 43)	0.278	7.26
	<i>p</i>	0.926	<0.001

Table 2

The number of cells containing TH immunoreactivity, Fos immunoreactivity, both Fos immunoreactivity and TH immunoreactivity, and the percentage of TH-ir cells also containing Fos immunoreactivity (Mean $\pm$ SEM) after subjects interacted with one of several social stimuli for 30 min. Groups with any of the same superscript letters do not significantly differ from each other.

		# of TH-ir cells	# of Fos-ir cells	# of Dual-labeled cells	% of TH-ir cells containing Fos immunoreactivity
AVP	COLONY	342 $\pm$ 15	25 $\pm$ 9 ^a	3 $\pm$ 1 ^b	1 $\pm$ 0 ^b
	ALONE	276 $\pm$ 18	18 $\pm$ 6 ^a	1 $\pm$ 0 ^a	0 $\pm$ 0 ^a
	SIBLING	293 $\pm$ 29	19 $\pm$ 2 ^a	1 $\pm$ 0 ^{ab}	0 $\pm$ 0 ^{ab}
	PUPS	297 $\pm$ 35	125 $\pm$ 23 ^b	3 $\pm$ 1 ^b	1 $\pm$ 0 ^{bc}
	NON EJAC	293 $\pm$ 27	181 $\pm$ 40 ^b	7 $\pm$ 3 ^b	2 $\pm$ 1 ^{bc}
	EJAC	277 $\pm$ 19	373 $\pm$ 57 ^c	7 $\pm$ 2 ^b	3 $\pm$ 1 ^c
		$F(5, 42) = 0.916$	$H(5) = 35.72$	$H(5) = 12.33$	$H(5) = 13.40$
<i>p</i>		0.480	<0.001	0.031	0.020
AVP	COLONY	185 $\pm$ 15	95 $\pm$ 22 ^a	6 $\pm$ 3	3 $\pm$ 1
	ALONE	166 $\pm$ 18	114 $\pm$ 16 ^a	2 $\pm$ 1	1 $\pm$ 0
	SIBLING	176 $\pm$ 22	114 $\pm$ 15 ^a	3 $\pm$ 1	2 $\pm$ 1
	PUPS	188 $\pm$ 15	384 $\pm$ 45 ^c	7 $\pm$ 1	4 $\pm$ 1
	NON EJAC	163 $\pm$ 25	242 $\pm$ 46 ^b	3 $\pm$ 1	2 $\pm$ 1
	EJAC	202 $\pm$ 18	314 $\pm$ 48 ^{bc}	6 $\pm$ 2	2 $\pm$ 1
		$F(5, 42) = 0.551$	11.85	1.54	1.58
<i>p</i>		0.736	<0.001	0.199	0.186
PV _a	COLONY	508 $\pm$ 19	117 $\pm$ 49 ^{ab}	5 $\pm$ 2 ^a	1 $\pm$ 0 ^a
	ALONE	449 $\pm$ 30	92 $\pm$ 18 ^a	5 $\pm$ 1 ^{ab}	1 $\pm$ 0 ^a
	SIBLING	498 $\pm$ 20	162 $\pm$ 26 ^b	8 $\pm$ 1 ^{bcd}	2 $\pm$ 0 ^{abc}
	PUPS	477 $\pm$ 37	472 $\pm$ 58 ^{cd}	7 $\pm$ 1 ^{abc}	1 $\pm$ 0 ^{ab}
	NON EJAC	430 $\pm$ 31	410 $\pm$ 44 ^c	10 $\pm$ 2 ^{cd}	2 $\pm$ 0 ^{bc}
	EJAC	480 $\pm$ 27	576 $\pm$ 91 ^d	14 $\pm$ 3 ^d	3 $\pm$ 1 ^c
		$F(5, 43) = 1.138$	$H(5) = 30.84$	$F(5, 43) = 4.03$	$F(5, 43) = 3.95$
<i>p</i>		0.355	<0.001	0.004	0.005
ZI	COLONY	732 $\pm$ 53	172 $\pm$ 38 ^a	20 $\pm$ 5 ^a	3 $\pm$ 1 ^a

	# of TH-ir cells	# of Fos-ir cells	# of Dual-labeled cells	% of TH-ir cells containing Fos immunoreactivity
ALONE	586 ± 58	208 ± 27 ^a	21 ± 4 ^a	4 ± 1 ^a
SIBLING	585 ± 46	242 ± 17 ^{ab}	24 ± 6 ^a	4 ± 1 ^a
PUPS	647 ± 29	347 ± 41 ^b	44 ± 3 ^b	7 ± 1 ^b
NON EJAC	575 ± 41	362 ± 62 ^b	37 ± 4 ^b	7 ± 1 ^b
EJAC	610 ± 33	266 ± 44 ^{ab}	24 ± 5 ^a	4 ± 1 ^a
<i>F</i> (5, 43)	1.800	3.08	4.50	5.60
<i>p</i>	0.133	0.004	0.002	0.001
ARH				
COLONY	194 ± 19 ^{abc}	31 ± 7 ^a	1 ± 1	1 ± 0
ALONE	152 ± 15 ^a	66 ± 12 ^{ab}	3 ± 1	2 ± 1
SIBLING	230 ± 12 ^c	62 ± 13 ^{ab}	2 ± 1	1 ± 1
PUPS	208 ± 12 ^{bc}	76 ± 10 ^b	3 ± 1	1 ± 0
NON EJAC	177 ± 25 ^{ab}	105 ± 25 ^b	6 ± 3	3 ± 2
EJAC	240 ± 20 ^c	103 ± 26 ^b	7 ± 3	3 ± 1
	<i>F</i> (5, 43) = 3.51	<i>F</i> (5, 43) = 2.83	<i>H</i> (5) = 3.98	<i>H</i> (5) = 3.94
<i>p</i>	0.010	0.027	0.552	0.558

Table 3

Correlations between the percentage of TH-ir cells containing Fos immunoreactivity and the expression of sexual behavior, paternal behavior, and all social behaviors during 30-min interactions with a sexually receptive female, pups, or a familiar female sibling. See Figure 4 for additional correlations associated with the duration of sexual behaviors.

	% TH-ir cells in pBST containing Fos immunoreactivity	% TH-ir cells in MeApd containing Fos immunoreactivity
Sexual behaviors		
<i>Latency (s)</i>		
Mount	-.481	-.184
Thrust from first mount	-.063	.112
Ejaculation from 1 st thrust	.469	.434
<i>Frequency</i>		
Mounts	.515*	.473
Thrusting bouts	.517*	.646**
Ejaculations	.516*	.410
Paternal behaviors		
<i>Latency (s)</i>		
Lick pups	.753*	-.328
Huddle from first lick	-.112	.390
<i>Frequency</i>		
Retrieve pups	-.160	.210
<i>Duration (s)</i>		
Lick pups	-.390	.537
Huddle over pups	-.309	.016
Time in contact with stimulus (s)	.141	.116

*
 $p \leq 0.05$,

**
 $p \leq 0.01$

Table 4

The number of cells containing TH immunoreactivity and the number of cells immunoreactive for both TH and Egr-1 (Mean $\pm$ SEM) in two sections through the pBST and three sections through the MeApd after males interacted with one of several stimuli for 30 min. Groups with any of the same superscript letters do not significantly differ from each other.

		# of TH-ir cells	# of Dual-labeled cells
pBST	ALONE	88 $\pm$ 6	1 $\pm$ 0 ^a
	SIBLING	82 $\pm$ 10	4 $\pm$ 1 ^b
	NON EJAC	92 $\pm$ 18	3 $\pm$ 1 ^b
	EJAC	87 $\pm$ 16	9 $\pm$ 4 ^b
		$F(3, 23) = 0.119$	$H(3) = 10.31$
	<i>p</i>	0.948	0.016
MeApd	ALONE	113 $\pm$ 12	1 $\pm$ 0 ^a
	SIBLING	80 $\pm$ 12	8 $\pm$ 3 ^b
	NON EJAC	112 $\pm$ 19	7 $\pm$ 1 ^b
	EJAC	97 $\pm$ 22	13 $\pm$ 4 ^b
		$F(3, 21) = 0.915$	$H(3) = 15.04$
	<i>p</i>	0.451	0.002

Table 5

The number of cells containing TH immunoreactivity and the number of cells immunoreactive for both TH and Fos (Mean $\pm$ SEM) in four sections through the pBST and seven sections through the MeApd after males interacted with one of several stimuli for 6 or 24 h.

		# of TH-ir cells	# of Dual-labeled cells
pBST	ALONE	236 $\pm$ 29	0 $\pm$ 0 ^a
6 h	SIBLING	226 $\pm$ 22	1 $\pm$ 0 ^a
	MATED	266 $\pm$ 18	6 $\pm$ 3 ^b
		$F(2, 20) = 0.851$	$H(2) = 13.27$
	<i>p</i>	0.442	0.001
pBST	ALONE	221 $\pm$ 19	0 $\pm$ 0 ^a
24 h	SIBLING	221 $\pm$ 17	4 $\pm$ 2 ^b
	MATED	285 $\pm$ 24	3 $\pm$ 1 ^b
		$F(2, 20) = 3.23$	$H(2) = 6.21$
	<i>p</i>	0.567	0.045
MeApd	ALONE	426 $\pm$ 12	0 $\pm$ 0 ^a
6 h	SIBLING	378 $\pm$ 25	6 $\pm$ 2 ^b
	MATED	363 $\pm$ 50	4 $\pm$ 2 ^b
		$F(2, 20) = 0.888$	$H(2) = 9.94$
	<i>p</i>	0.427	0.007
MeApd	ALONE	387 $\pm$ 18	0 $\pm$ 0 ^a
24 h	SIBLING	351 $\pm$ 32	5 $\pm$ 3 ^b
	MATED	413 $\pm$ 32	2 $\pm$ 1 ^b
		$F(2, 20) = 1.31$	$H(2) = 6.98$
	<i>p</i>	0.292	0.031