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Social novelty increases tyrosine hydroxylase immunoreactivity in the extended olfactory amygdala of female prairie voles

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Abstract

The monogamous social behaviors of prairie voles (*Microtus ochrogaster*) require olfactory inputs, which are processed by the posterodorsal medial amygdala (MeApd) and principal bed nucleus of the stria terminalis (pBST). The male prairie vole MeApd and pBST contain hundreds of cells densely immunoreactive for tyrosine hydroxylase (TH-ir). Female prairie voles have relatively few of these cells, but we previously found that the number of these TH-ir cells is greatly increased in females by exogenous estradiol. We here hypothesized that the number of TH-ir cells in the MeApd and pBST would also increase during the natural hormone surges associated with females' induced estrus. We found that the number of TH-ir cells in both sites did significantly increase after females cohabitated for two days with an unfamiliar male. However, this increase did not require the presence of ovaries and even tended to occur in the pBST of females cohabitating for two days with unfamiliar females. We then determined if the greater number of TH-ir cells after heterosexual pairing was transient by examining two groups of long-term pairbonded females (primiparous and multiparous), and found these females also had significantly more TH-ir cells in the pBST and/or MeApd compared to unmated controls. Thus, social novelty arising from cohabitation with unfamiliar conspecifics produces a reoccurring increase in the number of TH-ir cells in the female prairie vole extended olfactory amygdala. Ovarian hormones are not necessarily required. This increase in catecholaminergic cells may facilitate acquisition and retention of olfactory memories necessary for social recognition in this species.

Keywords

dopamine; maternal behavior; monogamy; olfaction; pair bonding; voles

The social organization of prairie voles (*Microtus ochrogaster*) is unusual compared to most other rodents because prairie voles are highly gregarious, live in extended family groups, display biparental care of offspring, and form post-copulatory pairbonds that can last a lifetime [1,2]. These social behaviors are regulated by olfactory inputs, as evidenced by the detrimental effects of olfactory bulbectomy or removal of the vomeronasal organ on mating, pairbonding, parenting, and other social interactions in prairie voles [3–7]. Downstream targets of main olfactory and vomeronasal inputs in rodents include the densely interconnected posterodorsal medial amygdala (MeApd) and principal bed nucleus of the stria terminalis (pBST) [8–12]. Both sites are rich with gonadal steroid hormone receptors [13–18], through which they integrate olfactory and hormonal information required for sociosexual behaviors [19,20]. Indeed, lesions of the MeApd or pBST disrupt odor-dependent social behaviors in laboratory rodents, including copulation [21–26], parenting [27,28] and aggression [29,30]. In male

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prairie voles, large lesions of the amygdala encompassing the MeApd decrease affiliative behaviors towards a mate or pups [31], while mating or parenting elicit immediate-early gene expression in the MeApd and pBST of both sexes [32–36].

Our laboratory recently reported a species difference in the expression of tyrosine hydroxylase (TH) in the prairie vole MeApd and pBST [37]. Male prairie voles have hundreds of TH-immunoreactive (TH-ir) cells in each site, while the polygamous species we examined (meadow voles, laboratory rats, and Syrian hamsters) have few or no TH-immunoreactive cells in these regions [37]. Because these cells do not express dopamine- β -hydroxylase [37], they are more likely dopaminergic rather than noradrenergic. We also found a sex difference in these TH-ir cell populations, with male prairie voles having 3–5-fold more TH-ir cells in each site than do females [37]. Gonadal hormones circulating during adulthood are mostly responsible for this sex difference, because castrating male prairie voles reduces the number of TH-ir cells in their MeApd and pBST almost to the levels found in females, while treating adult females with testosterone or estradiol benzoate increases their number of TH-ir cells almost to that of gonadally intact males [18,37]. Many TH-containing cells in the MeApd and pBST of both sexes of prairie vole are immunoreactive for AR and ER α [18], but the number of TH-ir cells in either sex is not affected by DHT [18], so ER activity may be particularly important for maintaining high TH content in these cells.

Our previous work investigating how gonadal hormones influence TH immunoreactivity in the MeApd and pBST involved experimental manipulations including gonadectomy and administration of supraphysiological doses of gonadal steroids [18,37]. It is unknown if similar effects occur under natural conditions. Because circulating levels of testosterone in males may [38,39] or may not [40,41] be significantly affected by reproductive state, and that the number of TH-ir cells in the male MeApd and pBST appears to be at a ceiling that cannot be increased even by supraphysiological levels of hormones [18,37], one might expect more reliable changes in the number of TH-ir cells in these sites to occur in females. This is because female prairie voles are induced ovulators [42] that have very low levels of ovarian hormones until being exposed to the urinary cues of an unfamiliar male [1]. A tremendous surge in ovarian estradiol occurs within one hour after exposure and levels remain elevated for at least 72 hr [43–46]. If these endogenous hormonal fluctuations also affect the number of TH-ir cells in females' MeApd and pBST, similar to what is observed when females are treated with exogenous gonadal hormones [18,37], there may be functional implications for the olfactory processing associated with pairbonding. To evaluate the likelihood of this, Experiment 1a examined the number of TH-ir cells in the MeApd and pBST of female prairie voles after they cohabitated for 48 hr with an unfamiliar male that can induce estrus, or with a familiar brother that cannot. To determine if surges in ovarian hormones and mating were necessary for any observed changes in the number of TH-ir cells, half of these females were ovariectomized before cohabitation. In Experiment 1b, we further investigated any capacity for non-sexual cohabitation with an unfamiliar vole to affect the number of TH-ir cells in the MeApd and pBST by studying groups of females that cohabitated for two days with an unfamiliar female or a familiar sister. Because a greater number of TH-ir cells in the extended olfactory amygdala of females cohabitating with males may be associated with the maintenance, not just the establishment, of new olfactory memories Experiment 2 examined TH-ir cells in the extended olfactory amygdala of females in established pairbonds that were sacrificed during two different endocrine states - primiparous (lactating and pregnant) and recently multiparous (lactating, recently parturient, and in a postpartum estrus).

General Methods

Subjects

Subjects were adult male and female prairie voles (*Microtus ochrogaster*) born and raised in our colony, from breeding stock originating from offspring of voles captured in 1994 in Urbana, Illinois. These voles were outbred in 2000 at the University of Massachusetts with voles of Illinois origin and were brought to Michigan State University in 2002. Animals were maintained on a 14:10-hour light: dark cycle with an ambient temperature maintained at 21° C. At all ages, animals were housed in clear plastic cages (48 × 28 × 16 cm) containing wood chips, wood shavings, and a substantial covering of hay. Voles were provided water and a food mixture containing cracked corn, whole oats, sunflower seeds, and rabbit chow (Tekland rodent diet No. 2031) in a ratio of 1:1:2:2. Subjects were weaned from their parents at 20 days of age and housed in mixed-sex sibling groups, where they remained until being used in an experiment when approximately 70–95 days old. Unlike laboratory rats, prairie voles are induced ovulators and subject to incest avoidance that prevents mating with siblings [1]. Mixed-sex housing after weaning was also advantageous in that it permitted us to use familiar brothers as the male stimulus for some of the control groups in the experiments below. All procedures were in accordance with the Institutional Animal Care and Use Committees at Michigan State University.

Perfusion, tissue collection, and immunocytochemistry

At the appropriate time points (detailed in each experiment below), subjects were overdosed with an anesthetic containing ketamine (62.5 mg/kg), xylazine (7.5 mg/kg), and acepromazine (0.8 mg/kg) and perfused through the heart with 100 mL 0.9% saline followed by 100 mL 4% paraformaldehyde in sodium phosphate buffer (NaPB; pH = 7.6). Brains were removed, post-fixed overnight in 4% paraformaldehyde in NaPB, and then submerged in a 20% sucrose/NaPB solution for at least three days before sectioning into 40-μm sections with a freezing microtome. Immunocytochemistry for TH was performed on every other section through the brain using methods previously described [18,37]. Briefly, sections were incubated in 0.1% sodium borohydride for 15 min, then 1% hydrogen peroxide in 0.3% Triton X-100 for 10 min, 20% normal goat serum in 0.3% Triton X-100 for 60 min, and then a rabbit anti-tyrosine hydroxylase polyclonal primary antiserum (AB152; 1:2,000; Chemicon, Temecula, CA) in 0.3% Triton × and 2% NGS overnight at room temperature. Sections were then incubated in a biotinylated goat anti-rabbit secondary antiserum (1:500; Vector Laboratories, Burlingame, CA) and then avidin-biotin complex (Vectastain Elite, Vector Laboratories). Vector SG was used as the chromagen (Vector Laboratories), which provided a blue cytoplasmic label. Sections were mounted on microscope slides, dehydrated, and coverslipped. Immunocytochemical control procedures included omission of the primary or secondary antisera, which abolished specific labeling.

Tissue Analyses

Slides were coded and randomized for analysis, which was performed by one person (BLC) using a Nikon E400 microscope at 200× magnification with the aid of a reticle placed in one of the ocular lenses. Three sections in the series through the MeApd and two sections through the pBST were analyzed bilaterally by eye and their totals used for statistical analysis. In Experiment 1a, the number of TH-ir cells in the zona incerta was also examined bilaterally from three consecutive sections in the series to determine if any cohabitation-induced changes in the number of TH-ir cells extended to all forebrain dopamine cell groups. All TH-ir somata, regardless of the intensity of immunoreactive labeling, were included in the quantification.

Experiment 1a - Effects of cohabitation with familiar or unfamiliar males, and ovariectomy before pairing, on TH-immunoreactive cells in the female prairie vole MeApd and pBST

Procedure: Adult female prairie voles were taken from their home cage, weighed, and anesthetized with an IP injection of the anesthetic mixture described above. Females were ovariectomized through a single midline ventral incision ($n = 32$), or received a sham surgery during which the ovaries were visualized but not removed ($n = 32$), and then returned to their home cage. Two weeks after surgery, female subjects and males from our colony were socially isolated for four days, which in the females promotes estrus induction when they are then housed with unfamiliar males; this includes a surge in estradiol, increased uterine weight, and lordosis [44,46]. Approximately half of the females from each surgery group (16 Sham, 15 OVX) were placed in a soiled cage containing an unfamiliar male. The other females (16 Sham, 17 OVX) were placed in the cage of a familiar male sibling with whom they had been housed until four days prior. Animals remained housed together until females were sacrificed 48 hr after pairing, which is more than enough contact time to induce estrus in female prairie voles [44]. The uterine horns of sham-operated females were removed, stripped of fat, and weighed just before perfusion as an indirect indicator of estrus [44,46].

The MeApd of a few females was lost or damaged during immunocytochemical processing and could not be analyzed, resulting in slightly reduced sample sizes. 2×2 ANOVAs using gonadal state and conspecific familiarity as factors was used to analyze data from each brain site. Pearson's r was used to correlate the number of TH-ir cells in the MeApd and pBST with females' uterine weights. Statistical significance was indicated by $p < 0.05$.

Results: In the MeApd, there was a significant main effect of reproductive status on the number of TH-ir cells ($F(1,54) = 9.75, p = 0.003$; Fig. 1), with females paired with unfamiliar males having more TH-ir cells than the number found in females paired with their brothers. Unexpectedly, there was no main effect of gonadal state ($F(1,54) = 0.16, p = 0.89$) and no significant interaction between mating status and gonadal state ($F(1,54) = 1.08, p = 0.30$) on the number of TH-ir cells in the MeApd. Sham-operated females paired with unfamiliar males had significantly heavier uterine horns compared to sham-operated females paired with their brothers ($t_{31} = 3.87, p = 0.0005$), indicative of higher circulating ovarian hormones in the former group. There was no significant correlation between the number of TH-ir cells in the MeApd and the uterine weights of the sham-operated females ($r = 0.29, p = 0.18$).

In the pBST, there was a significant main effect of reproductive status on the number of TH-ir cells ($F(1,60) = 8.38, p = 0.005$; Fig. 1), with females paired with unfamiliar males having significantly more TH-ir cells than did females paired with a familiar male sibling. Similar to the MeApd, there was no significant main effect of gonadal state ($F(1,60) = 0.16, p = 0.69$) nor a significant interaction between reproductive status and gonadal state ($F(1,60) = 0.48, p = 0.49$) on the number of TH-ir cells in the pBST. The number of TH-ir cells in the pBST tended to be positively correlated with the uterine weights of the sham-operated females ($r = 0.35, p = 0.08$).

In the zona incerta, there were no significant main effects of reproductive status ($F(1,60) = 1.44, p = 0.23$) or gonadal state ($F(1,60) = 0.02, p = 0.88$) on the number of TH-ir cells (data not shown; groups ranged from 859 ± 21 to 897 ± 21 TH-ir cells). There was also no significant interaction between these factors ($F(1,60) = 0.01, p = 0.95$).

Experiment 1b – Effects of intra-sexual pairing on TH-immunoreactive cells in the female prairie vole MeApd and pBST

Procedure: Gonadally intact adult females were taken from their home cage and socially isolated for four days. Half of the females were placed in a soiled cage containing an unfamiliar, similarly isolated adult female. The other half of the subjects was placed in the cage containing a familiar sister that was isolated in that cage for the four days before pairing. Animals remained housed together until sacrifice 48 hr later. Female pairs repeatedly observed to fight were separated and removed from the study. Unpaired *t*-tests were used to compare the number of TH-ir cells between the two groups for each brain site. Statistical significance was indicated by $p < 0.05$.

Results: In the MeApd, there was no significant difference between subjects housed with an unfamiliar or familiar female in the number of TH-ir cells ($t_{21} = 0.36$, $p = 0.73$), but there was a trend for females housed with unfamiliar females to have more TH-ir cells in the pBST compared with those housed with their sister ($t_{20} = 1.78$, $p = 0.089$; Figure 2).

Experiment 2 - TH-immunoreactive cells in the MeApd and pBST of long-term pairbonded female prairie voles

Procedure: Female prairie voles were taken from their home cage and socially isolated for four days. They were then placed in a soiled cage containing a similarly separated unfamiliar male or were placed in a soiled cage containing a similarly separated familiar brother. Starting 2–3 days before the expected day of parturition, cages were inspected throughout the day for the presence of pups. Females were sacrificed 12 days after giving birth to their first litter (so were lactating and pregnant; $n = 8$) or one day after giving birth to their second litter (so were lactating, recently parturient, in a postpartum estrus; $n = 11$). The previous litter of the females in the latter group remained in the home cage until dams were sacrificed (*i.e.*, they were not removed before the dam gave birth again). The control females paired with a brother were sacrificed contemporaneous with experimental females, corresponding to how long the experimental females were housed with their mate, which came to be between 35–40 days for the lactating and pregnant group (controls, $n = 8$), and 42–50 days for the lactating and recently parturient group (controls, $n = 10$). At the time of perfusion, it was confirmed that the uterine horns of the pregnant females contained fetuses, and that the uterine horns of both groups of brother-paired females contained no fetuses and no pups were living in the home cage. Because separate immunocytochemical runs were used for the two time points examined, the number of TH-ir cells in each site for each time point were analyzed with separate unpaired *t*-tests. Statistical significance was indicated by $p < 0.05$.

Results: Pairbonded females that were lactating and pregnant had a similar number of TH-ir cells in their MeApd compared to the number found in control females paired with their brothers ($t_{13} = 0.74$, $p = 0.48$). However, there was a significant difference between groups in the pBST ($t_{14} = 2.52$, $p = 0.02$; Fig. 3) with pairbonded females having more TH-ir cells than the unmated controls. Pairbonded females that were both lactating and recently gave birth to their second litter had 4–5-fold more TH-ir cells in the MeApd ($t_{12} = 2.28$, $p = 0.04$; Fig. 3) and pBST ($t_{19} = 2.59$, $p = 0.02$; Fig. 3) compared to unmated controls housed with a brother.

Discussion

Previous work from our laboratory demonstrated that gonadal hormones circulating during adulthood greatly affect the number of TH-ir cells in the MeApd and pBST of male and female prairie voles [18,37]. Because female prairie voles experience tremendous changes in ovarian hormone release during estrus induction [43–45], we hypothesized that these endogenous hormonal changes would reproduce the effects of exogenous hormones and upregulate the

number of TH-ir cells in the MeApd and pBST. We tested this in Experiment 1a by creating a social situation during which ovarian hormone surges and mating invariably occur in female prairie voles. We found that a two-day cohabitation with an unfamiliar male increased the number of TH-ir cells in the MeApd and pBST (but not in the zona incerta, a non-olfactory comparison site), but this was true whether or not females were gonadally intact. Furthermore, in Experiment 1b we even found a trend toward more TH-ir cells in the pBST when females were paired with unfamiliar females, a social environment that obviously does not induce estrus and mating. Thus, although ovarian hormones have the potential to naturally contribute to elevated TH immunoreactivity in these brain sites when female prairie voles cohabit with an unfamiliar male, hormones are clearly not required.

We have hypothesized that a greater number of TH-ir cells in the MeApd and pBST facilitates females' pairbonding with males, possibly related to establishing olfactory memories of conspecifics [18,37]. If so, it may not be completely surprising that cohabitation with an unfamiliar male increased the number of TH-ir cells in ovariectomized females. In the absence of estrus and mating, female prairie voles still display preferences for previously unfamiliar males, but forming these preferences takes longer than if mating occurs [47]. Similarly, ovarian hormones and mating are unnecessary for females' post-cohabitation increase in aggression toward unfamiliar voles [48]. Female prairie voles also form social bonds with unfamiliar females [49], which must be necessary when unrelated females live together in natural settings [50,51], and could be associated with our finding of a trend toward more TH-ir cells in the pBST after female-female cohabitation. These findings support previous work suggesting that social and sexual behaviors can be independently regulated in prairie voles [52] and lead to the conclusion that a greater number of TH-ir cells in the MeApd and pBST is more generally associated with social novelty, to possibly promote females' recognition of newly familiar voles of both sexes. If involved in establishing or maintaining social memories, the fact that male prairie voles have hundreds of TH-ir cells in these sites could mean that males are more prepared than females to form social bonds or discriminate among already familiar conspecifics. Alternatively, perhaps a large number of TH-ir cells in males renders them less likely to form social bonds, and that upregulation of TH-ir cells in paired females is part of the mechanism preventing females from forming social bonds with voles other than their primary mate [53].

Our studies exclude the possibility that ovarian hormones are responsible for the observed changes in the number of TH-ir cells in recently paired females. Hormones from other sources, however, need to be examined. For example, adrenal glucocorticoids might be released when females are socially isolated for four days and then paired/mated with a male. This could indicate that stress from these social manipulations is a relevant factor. However, basal glucocorticoid levels are very high in female prairie voles, and neither brief (1 hr) nor chronic (2–4 weeks) social isolation affect circulating corticosterone [41,54–57; but see 58]. Furthermore, cohabitation with an unfamiliar male decreases glucocorticoid levels in previously isolated female prairie voles, not increases them [54]. Thus, stress as indicated by increased glucocorticoid levels could not be responsible for the greater number of TH-ir cells found in heterosexually paired female prairie voles.

Similar to the increased number of TH-ir cells in the MeA and BST of paired female prairie voles, heterosexual cohabitation and exposure to male sensory cues also rapidly increase Fos expression in these sites [32,33,59,60]. These could be the same cells that eventually express TH two days later, particularly because Fos binds to the AP-1 site on the promoter region of the TH gene [61–63] to increase TH synthesis and possibly the number of immunocytochemically detectable cells. On the other hand, intrasexual cohabitation elicits Fos in females' MeA, but not BST [33], while we found upregulated TH-ir cells in the pBST, but not MeApd. Therefore, if early Fos is associated with later TH expression in cohabitating

female prairie voles, the relationship between the two neural events depends on the sex of the partner.

Experiment 2 revealed that an elevated number of TH-ir cells in the extended olfactory amygdala is not restricted to just the few days after females cohabitate with a novel conspecific, but also can be found in long-term pairbonded females. The endocrine profile of simultaneously pregnant and lactating prairie voles is unknown, but probably involves low circulating estradiol [64]. On the other hand, the females killed while lactating and recently parturient would have been in a postpartum estrus characterized by high circulating estradiol [45,65]. Higher numbers of TH-ir cells were found in the pBST of pairbonded females killed at both time points compared to their controls, further indicating that an elevated number of TH-ir cells (at least in the pBST) exists in paired female prairie voles even when circulating estradiol levels are low, consistent with the results of Experiment 1 revealing no effect of ovariectomy. Because the MeApd did not differ between mid-pregnant females and their controls during the first time point examined in Experiment 2, the number of TH-ir cells in this site may not be static but rises and falls across different reproductive states. A particularly notable increase in the number of TH-ir cells was found in the MeApd and pBST of the recently multiparous females. This might strengthen olfactory memories to bolster the established pairbond with their mate, but might also contribute to dams' recognition of their own offspring. This is particularly relevant for multiparous females because they live in complex social environments involving the 70–75% of their juvenile offspring that do not disperse from the natal nest [2,50,66]. Indeed, discriminating between their own offspring of reproductive age and unfamiliar young adults [67,68] is necessary to avoid incest, as well as to maintain selective aggression toward unfamiliar voles intruding upon the nest.

The TH-ir cells in the prairie vole MeApd and pBST are probably dopaminergic [37], which is consistent with a potential role in olfactory memory, as dopamine modulates olfactory detection, discrimination, and learning in other mammals [69–73]. The MeApd and pBST project densely to each other [8–10,12], and may also be a species-specific dopaminergic connection between olfactory sites and components of the mesolimbocortical system, which is essential for the formation and maintenance of pairbonds [53] and likely parenting [see 74] in prairie voles. Ongoing research in our lab is exploring the anatomical projections of the TH-ir cells in the prairie vole pBST and MeApd, which will help clarify how changes in the number of TH-ir cells in these sites in response to social stimuli could play a role in the olfactory regulation of social behaviors in this species.

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Figure 1.

Number (Mean $\pm$ SEM) of TH-ir cells in the MeApd and pBST of groups of sham-operated and ovariectomized female prairie voles who cohabitated for 48 hr with a familiar brother or an unfamiliar male. Data shown are collapsed across gonadal state, which had no significant effect. Sample sizes indicated at bottom of bars. * = Significant effect of male familiarity, $p < 0.05$.

Figure 2.

Number (Mean $\pm$ SEM) of TH-ir cells in the MeApd and pBST of female prairie voles after a 48-hr cohabitation with a familiar sister or an unfamiliar female. Sample sizes indicated at bottom of bars. + = Trend toward difference between groups within that site, $p < 0.09$.

Figure 3.

Number (Mean $\pm$ SEM) of TH-ir cells in the MeApd and pBST of female prairie voles cohabitating with a familiar brother or an unfamiliar male and sacrificed when the mated females were primiparous (simultaneously lactating and mid-pregnant; top panel) or recently multiparous (simultaneously lactating, recently parturient, and in postpartum estrus; bottom panel). Sample sizes indicated at bottom of bars. * = Significant difference between groups within that site, $p < 0.05$.