

Species differences in anxiety-related responses in male prairie and meadow voles: The effects of social isolation

Jennifer R. Stowe^a, Yan Liu^a, J. Thomas Curtis^a, Marc E. Freeman^b, Zuoxin Wang^{a,*}

^a Department of Psychology and Program in Neuroscience, Florida State University, Tallahassee, FL 32306, USA

^b Department of Biological Sciences and Program in Neuroscience, Florida State University, Tallahassee, FL 32306, USA

Abstract

Prairie (*Microtus ochrogaster*) and meadow voles (*M. pennsylvanicus*) are closely related species that differ in life strategy and social behaviors, and thus provide an excellent comparative model for the study of neuronal and hormonal mechanisms underlying behavior. In the present study using the elevated plus maze (EPM) test, we found that male prairie voles entered the open arms of the EPM more and remained there longer, and showed a higher level of overall locomotor activity than did male meadow voles. In addition, two weeks of social isolation induced an increase in open arm entries in prairie, but not meadow, voles. Prairie voles also had a higher level of circulating corticosterone compared to meadow voles, and the EPM test increased circulating corticosterone in prairie voles. Finally, social isolation coupled with the EPM test influenced Fos-immunoreactive expression in several brain areas, including the medial preoptic area, ventromedial hypothalamus, amygdala, and prefrontal cortex differently between the two species. Together, these data indicate a neural circuit involved in mediating anxiety-associated behavior in voles, and that the functioning of this circuit is influenced by social environment differently between social and non-social species.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Elevated plus maze; Corticosterone; C-fos; Amygdala; VMH; MPOA

1. Introduction

Pathological anxiety is defined as fear without a relevant corresponding object or event. Animal models of anxiety are widely sought in an attempt to analyze pathological anxiety states based on the assumption that some anxiety mechanisms are essential for survival and are a feature of all mammals [1]. The elevated plus maze (EPM) is one of the most commonly used behavioral paradigms for testing animal anxiety; it relies on a rodent's relative aversion to venture onto the open arms of the maze versus the safer closed arms [2,3]. Animals that spend more time in the open arms are thought to be less anxious. It has been suggested that the EPM test is sensitive to alterations in anxiety produced by ecologically relevant stimuli [4].

Activation of the hypothalamic–pituitary–adrenal (HPA) axis is a physiological marker of stress and is typically measured by an increased level of plasma corticosterone (CORT) in rodents [5]. The EPM test is known to increase CORT levels in rats, particularly if the animals are confined to the open arms of the maze [3]. Fluctuations in CORT levels are also seen in response to changes in social interactions. Social isolation, for example, is considered stressful or anxiety producing and plays a role in influencing behavior on the EPM in a strain- and/or gender-specific manner [6,7]. Socially isolated rats show an increase in anxiety-like behavior on the EPM that is associated with increases in circulating CORT [8].

Microtine rodents have proven to be a useful model for studying the effects of social interactions on physiology and behavior. For example, prairie voles (*Microtus ochrogaster*) are highly social, demonstrating the characteristics of monogamy [9]. Prairie voles display high levels of social affiliation and mating induces pair bond

* Corresponding author. Department of Psychology, Florida State University, Tallahassee, FL 32306-1270. Tel.: +1 850 644 5057; fax: +1 850 644 7739.

E-mail address: zwang@psy.fsu.edu (Z. Wang).

formation between the mates [10]. Social isolation has been found to increase CORT levels in female prairie voles [11], and increased CORT seems to facilitate pair bonding in male, but not female, prairie voles [12,13]. In contrast, meadow voles (*Microtus pennsylvanicus*) are promiscuous, asocial, display low levels of affiliative behavior, and mating does not induce pair bond formation [14,15]. Together, these data suggest that social environment may differentially influence endocrine responses and behavior of closely related vole species with different life strategies. Therefore, comparing these species provides an excellent opportunity for the study of hormonal and neurochemical mechanisms underlying behavior [16,17].

As social environment has significant impacts on anxiety [6,7,11], we hypothesized that social isolation may affect anxiety-related behaviors and the underlying hormonal and neuronal mechanisms differently in monogamous and promiscuous vole species. To test this hypothesis, the present study was conducted 1) to compare anxiety behavior and associated circulating CORT and regional brain activation between male prairie and meadow voles, and 2) to examine the effects of social environment, in particular acute or chronic social isolation, on those measures. We used the EPM test to study anxiety behavior and radioimmunoassay (RIA) to measure plasma levels of CORT. Fos is a protein product of the immediate early gene *c-fos* that is rapidly induced following sensory stimulation and can be used as a marker of neuronal activation [18,19]. Therefore, we used Fos immunoreactivity (Fos-ir) to label brain areas activated following EPM testing.

2. Materials and methods

2.1. General methods

2.1.1. Animals

Subjects were sexually naive male prairie and meadow voles that were the offspring from a laboratory breeding colony. All voles were weaned at 21 days of age and placed in same sex sibling pairs in plastic cages (29 × 18 × 13 cm) that contained cedar chip bedding. Food and water were provided *ad libitum*. All cages were maintained in a 14L:10D photoperiod with lights on at 0700 h while temperature was maintained at about 21 °C. All subjects were 3–4 months of age at the beginning of experiments and each subject was randomly assigned to either treatment or control group. All groups were placed in the testing room 24 h or 2 weeks prior to the onset of behavioral testing to allow for habituation (see below).

2.1.2. Social isolation

In the acute social isolation situation, voles were isolated from their cage mates for a period of 24 h [11] whereas in the chronic social isolation situation, voles were housed individually for a period of 2 weeks prior to

behavioral testing. During these two periods of social isolation, animals were placed in the testing room, and an opaque divider was placed between cages to eliminate visual cues. All animals were maintained under the same food, photoperiod, and temperature conditions as in the colony room.

2.1.3. Elevated plus maze test

The elevated plus maze (EPM) has been validated in the study of both rats [3] and mice [2] and has been successfully employed in work with voles [20]. Briefly, the EPM (Columbus Instruments, Columbus, OH) is comprised of two open arms (35 cm (L) × 6.5 cm (W)) and two closed arms (35 cm (L) × 5 cm (W) × 15 cm (H)) that cross in the middle, and is elevated 45 cm off the ground. The EPM test was carried out between 0900 and 1100 h under standard lighting conditions.

The subjects were individually placed on the intersection of the EPM facing an open arm and then observed for 10 min. Number of entries into the open or closed arms, time spent in each arm or in the center, rears, and falls were recorded by an experimenter. A rear was defined as standing on the hind paws with the front 2 paws placed on the walls of the maze. An entry into an arm was counted only when all four paws of an animal crossed from the center panel onto the arm. After the behavioral test, each subject was returned to a clean cage for 2 h without any further disturbance. The maze was cleaned thoroughly with soapy water between animals.

2.1.4. CORT radioimmunoassay

Two hours following the EPM testing, subjects were anesthetized with sodium pentobarbital (1mg/10 g body weight) and decapitated. Pilot experiments were conducted to compare plasma samples obtained by rapid decapitation, sodium pentobarbital followed by decapitation, or sodium pentobarbital followed by heart puncture, and the data indicated no significant group differences in CORT levels. Therefore, sodium pentobarbital administration followed by decapitation was used in all experiments. Approximately 1.0 ml of trunk blood was collected in a tube containing 50 µl of EDTA to prevent clotting. Blood samples were centrifuged for 20 min at 4 °C at 3000 RPM, and plasma was extracted for use in the radioimmunoassay. Plasma samples were analyzed for CORT using a Coat-A-Count[®] assay kit (Diagnostic Products Corp., Los Angeles, CA).

Because prairie voles are known to have relatively high glucocorticoid levels, plasma was diluted 1:10 in assay buffer (0.1 M phosphate-buffered saline (PBS), pH 7.4) with 1% sodium azide) to insure that results would reliably fall within the standard curve fit by linear regression [38]. Meadow vole plasma was also diluted in the same fashion to obtain an accurate comparison. All plasma samples were run in duplicate. Interassay variance was less than 10%.

2.1.5. *c-fos* immunocytochemistry

Following anesthesia and blood extraction, brains were rapidly removed and placed into 4% paraformaldehyde in 0.1 M PBS) at 4 °C for fixation, with the solution changed at 6 h. After 24 h of fixation, brains were stored in 30% sucrose in PBS, and then sliced into 40 µm coronal sections using a microtome, and stored in 0.1 M PBS containing 1.0% sodium azide.

Floating brain sections at 120 µm intervals were processed for *c-fos* immunocytochemistry using established procedures [22,23]. Briefly, sections were rinsed in 0.05M Tris–NaCl buffer (pH 7.6); incubated in 3.0% methanol and 0.5% H₂O₂ in the same buffer for 30 min; and then incubated in 10% normal goat serum (NGS) in 0.05 M Tris–NaCl with 0.5% Triton X-100 (pH 7.6) for 1 h. Sections were then incubated in rabbit polyclonal IgG antibody for *c-fos* (1:30,000, Jackson Immuno) in 0.05 M Tris–NaCl with 0.5% Triton X-100 and 2.0% NGS (pH 7.6) for 72 h at 4 °C and then one hour at room temperature, followed by incubation with the biotinylated goat anti-rabbit secondary antibody (1:300, Jackson Immuno) for 2 h. Sections were incubated in Vector Elite ABC complex in the Tris–NaCl buffer for 90 min, stained with Nickel-DAB (Vector Laboratories, Inc. Burlingtone, CA), mounted on microscope slides, and then cover slipped. To control for variability all sections within each experiment were processed simultaneously.

2.1.6. Data quantification and analysis

For all animals, plasma samples and brain slides were coded to conceal group identity until all samples were analyzed. The *c-fos* labeled cells were examined in the medial (MeA), anterior cortical (ACo), and central (CeA) subnuclei of the amygdala (AMYG); bed nucleus of the stria terminalis (BST; including the anterior dorsal and anterior ventral parts); lateral septum (LS; intermediate); paraventricular nucleus (PVN); ventromedial hypothalamus (VMH); medial preoptic area (MPOA); anterior hypothalamus (aHYP); and prefrontal cortex (PFC). These areas were chosen because they have been implicated in anxiety and social behaviors [23–27], activated following an EPM test [26,28], and/or involved in regulation of the HPA axis activity in rodents [29].

Brain sections were matched between animals with 3–4 sections per brain area being examined. Fos-ir cells were visualized under 10× magnification using a Zeiss Axioskop II microscope, and the images were captured using a computerized image program (NIH Image 1.60). All Fos-ir cells in the sampling area were quantified and analyzed bilaterally. The average number of cells containing Fos-ir staining from both sides of each brain area was used to provide individual means for data analysis. All behavioral, radioimmunoassay, and *c-fos* immunostaining data were analyzed by either one-way or two-way analysis of variance (ANOVA), and significant effects were further evaluated using a Student Newman–Keul's (SNK) post-hoc test.

2.2. Experiment 1: does social isolation influence anxiety behavior on the EPM and subsequent CORT and neuronal activation differentially between male prairie and meadow voles?

Prairie and meadow voles show remarkable differences in life strategies and social behaviors [9,15,30]. As social environment has significant impacts on anxiety [6,7,11], we hypothesized that in response to social isolation these two species may differ in anxiety-related behaviors and the underlying hormonal and neuronal mechanisms. In the present experiment, we studied anxiety behaviors in the EPM and examined the subsequent circulating CORT and neuronal activation in male prairie and meadow voles that were either housed in pairs or socially isolated for 24 h or for 2 weeks.

2.2.1. Design

Male prairie and meadow voles were randomly assigned into one of 4 experimental groups. In the no social isolation group (no isolation; *n*=9 prairie, *n*=8 meadow), subjects were housed with a same-sex sibling and transferred to the testing room for 24 h prior to being tested. In the 24 h social isolation group (24 h; *n*=9 prairie, *n*=6 meadow), subjects were housed individually for 24 h in the testing room. In the 2 weeks social isolation group (2 wks; *n*=9 prairie, *n*=6 meadow), subjects were housed individually for a period of 2 weeks in the testing room. At the end of treatment, subjects from all above three groups were individually tested on the EPM for 10 min. A fourth group of animals served as controls for handling (Control; *n*=8 prairie, *n*=6 meadow). For this group, subjects were housed with a sibling and handled such that they were picked up from the home cage, placed alone into a clean cage and picked up again 10 min later although no EPM test was conducted. After the EPM test or being handled, subjects in all groups were placed individually into clean cages and left undisturbed for 2 h. The 2 h time period was chosen based on our pilot data showing clear Fos-ir induction in the prairie vole brains following social stimulation. Thereafter, subjects were anesthetized, trunk blood was collected for CORT radioimmunoassay, brains were harvested, placed into 4% paraformaldehyde for 24 h of fixation, and brain sections were processed for *c-fos* immunocytochemistry. Behavioral and CORT data were analyzed by a 2-way ANOVA followed by an SNK post-hoc test. Group differences in the number of Fos-ir cells in each species were examined by a one-way ANOVA followed by an SNK post-hoc test.

2.3. Experiment 2: does the EPM test elevate CORT time-dependently in male prairie voles?

Social isolation is anxiety producing for prairie voles as it elevates plasma CORT in both pups [31] and adult

females [11]. However, in our experiment socially isolated voles showed more entries on the open arms of the EPM, which may indicate less anxiety in lab rodents [2,3] but not in wild animals [32]. Unfortunately, our CORT data 2 h after the EPM test were not conclusive. In rats, the EPM test induces a significant increase in circulating CORT approximately 20 min post-test [33]. Therefore, it remained possible that the EPM test could also induce an increase in plasma CORT in male prairie voles, but the 2-h waiting period, which was needed for Fos-ir induction, may have been too long to detect such a change in plasma CORT. To test this hypothesis, a time course experiment was performed to examine CORT elevation following the EPM test.

2.3.1. Design

As above described, male prairie voles were tested on the EPM for 10 min, put in a clean cage, and then sacrificed at 10 ($n=8$), 20 ($n=8$), 30 ($n=7$), or 60 min ($n=8$) following the EPM test. All subjects were sacrificed between 1100 and 1300 h as in Experiment 1. Trunk blood was collected and processed for CORT measurement using RIA. Handled control animals, without the EPM test, were also sacrificed at different time points. As no differences were found in their CORT levels, they were pooled into one control group ($n=19$) for statistical analysis. CORT levels were analyzed using a one-way ANOVA followed by an SNK post-hoc test.

2.4. Experiment 3: does social isolation alone alter basal levels of neuronal activation in male prairie voles?

Data from Experiment 1 demonstrated that socially isolated prairie voles had significant increases in the number of Fos-ir cells in several brain regions following the EPM test, but this increased Fos-ir expression was not found in non-isolated animals. It was not clear, however, if increased Fos-ir staining was due to increased brain responsiveness to the EPM test or to an increased basal level of neuronal activation resulting from social isolation. Therefore, several additional groups of animals were processed in the present experiment to test the hypothesis that social isolation alone might elevate the basal level of neuronal activation in the brain of prairie voles.

2.4.1. Design

Male prairie voles were randomly assigned into one of 3 experimental groups. Two groups were established based on length of social isolation: socially isolated for 24 h ($n=8$) or for 2 weeks ($n=8$). Control animals were housed with a male cage mate ($n=8$). After each treatment, subjects were anesthetized and sacrificed between 1100 and 1300 h, and their brain sections were processed for Fos-ir staining as above described. Group differences in Fos-ir staining in selected brain areas were analyzed by a one-way ANOVA followed by an SNK post-hoc test.

3. Results

3.1. Elevated plus maze behavior

Species differences were found in behaviors on the EPM between male prairie and meadow voles (Table 1). Prairie voles entered the open arms more, and remained there longer, than did meadow voles. Prairie voles also entered the closed arms more and demonstrated greater overall activity as indicated by the total arm entries than did meadow voles. Meadow voles, on the other hand, spent more time in the closed arms and displayed rearing behavior more frequently than prairie voles.

Significant species by treatment effects were also found: generally prairie, but not meadow, voles' behaviors were affected by social isolation (Fig. 1). There was a significant species by treatment interaction ($F_{(2, 42)}=5.66, p<0.05$) in which prairie voles that were isolated for 2 weeks entered to the open arms of the EPM more frequently than prairie voles that were not isolated or were isolated for 24 h. Prairie voles that were isolated for 2 weeks also had a greater number of total arm entries on the EPM than those that were isolated for 24 h ($F_{(2, 42)}=3.23, p<0.05$). Social isolation experience did not alter male meadow vole behavior on the EPM.

3.2. Plasma CORT concentration

A significant species difference was found in circulating levels of plasma CORT: prairie voles had higher levels of CORT than did meadow voles ($F_{(1, 53)}=171.0, p<0.001$; Table 2). Further, prairie, but not meadow, voles showed approximately a 25% increase in the level of plasma CORT 2 h after the EPM test but this increase did not reach statistical significance. Acute or chronic social isolation experience did not alter plasma CORT levels in either species 2 h post EPM.

3.3. Fos-ir induction following the EPM test

When analyzed by a two-way ANOVA, prairie voles showed persistent higher levels of Fos-ir labeling than meadow voles in all brain areas measured. To focus on the effects of the EPM test and social isolation on neuronal activation, we then analyzed Fos-ir data using a

Table 1
Species differences in behavior on the elevated plus maze (mean±SEM)

Behavior on the EPM		Prairie vole	Meadow vole	<i>p</i>
Frequency	Open arms	7.9±1.0	1.4±0.5	0.001
	Closed arms	20.3±2.8	10.2±1.7	0.01
	Total entries	28.2±3.5	11.6±1.9	0.001
	Rears	14.9±1.3	23.5±2.9	0.01
	Falls	1.1±0.2	0.1±0.1	0.001
Duration (sec)	Open arms	136.9±16.7	29.4±13.7	0.001
	Closed arms	370.5±20.7	503.0±31.9	0.001
	Center	42.5±8.3	36.5±18.8	ns

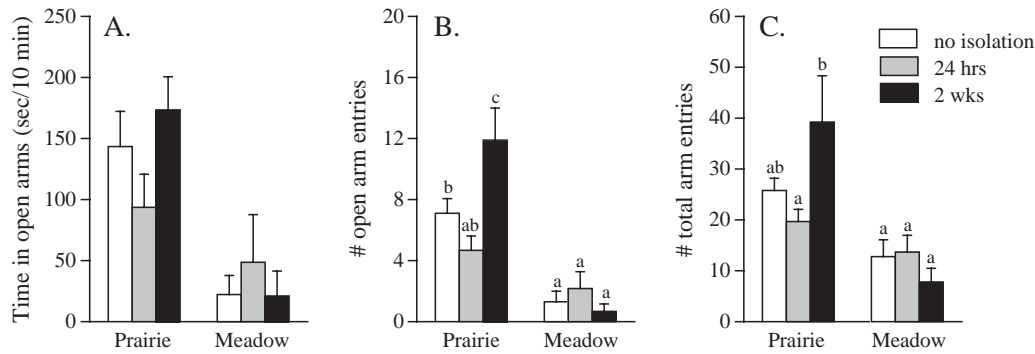


Fig. 1. Prairie voles spent more time on the open arms of the EPM (A), entered the open arms more frequently (B) and showed a higher level of overall locomotor activity indicated by the total arm entries (C) than did meadow voles. Further, prairie voles that were socially isolated for 2 weeks entered the open arms more than did the other two groups (B) and showed a higher level of locomotor activity compared to the prairie voles socially isolated for 24 h (C). Social isolation had no significant effects on any of the behaviors measured. Error bars indicate SEM.

one-way ANOVA within each species. Cells expressing Fos-ir were found in several regions of the hypothalamus, and social isolation influenced the number of Fos-ir cells following the EPM test in a region-specific manner. In the MPOA, the EPM test increased the number of Fos-ir cells in prairie voles that experienced social isolation ($F_{(3,29)}=7.23$, $p<0.001$), but this effect was not found in meadow voles (Figs. 2 and 3A). In VMH, the EPM test increased the number of Fos-ir cells in socially isolated prairie ($F_{(3,30)}=5.65$, $p<0.01$) and meadow voles ($F_{(3,24)}=6.95$, $p<0.01$; Fig. 3B). No treatment effects were found in the PVN (Fig. 3C), BST (Fig. 3D), or CeA (Fig. 3E) of either species. However, in the MeA (Fig. 4), prairie voles isolated for 2 weeks had more Fos-ir cells than the control group ($F_{(3,30)}=3.13$, $p<0.05$), whereas meadow voles isolated for either 24 h or 2 weeks had increased numbers of Fos-ir cells following the EPM test ($F_{(3,24)}=14.46$, $p<0.001$; Fig. 3F).

Treatment did not influence Fos-ir labeling in the ACo or aHYP of prairie voles. However, meadow voles that were socially isolated for 24 h showed increased numbers of Fos-ir cells following the EPM test in both areas (for ACo: $F_{(3,24)}=9.14$, $p<0.001$, Fig. 3G; For aHYP: $F_{(3,24)}=6.91$, $p<0.01$, Fig. 3H). In the lateral septum, meadow voles that were socially isolated for either 24 h or 2 weeks had more Fos-ir cells following the EPM test than did control animals ($F_{(3,24)}=5.09$, $p<0.01$; Fig. 4I). Finally, animals that experienced 24 h of social isolation showed an increase in the number of Fos-ir cells in the prefrontal cortex (Fig. 3J) in both prairie ($F_{(3,29)}=3.99$, $p<0.05$) and meadow voles ($F_{(3,24)}=6.15$, $p<0.01$).

Table 2
Plasma levels of corticosterone (ng/ml, mean \pm SEM) in prairie and meadow voles

	Prairie vole	Meadow vole
Control	1517.1 \pm 190.9	462.9 \pm 441.1
No isolation- EPM	2023.6 \pm 180.8	401.8 \pm 51.1
24-h isolation- EPM	2002.4 \pm 147.8	416.3 \pm 43.4
2-wk isolation- EPM	2000.5 \pm 221.0	418.6 \pm 38.0

3.4. Time dependent increase in plasma CORT following the EMP test

In Experiment 2, a significant group difference was found in the levels of plasma CORT in male prairie voles ($F_{(4,45)}=5.5$, $p<0.001$; Fig. 5). Twenty minutes after the EPM test, males had a level of plasma CORT significantly higher than that of control males. At 1 h post EPM test, plasma CORT had returned to the baseline level.

3.5. Basal level Fos-ir expression associated with social isolation

In Experiment 3, in comparison to control animals that were housed in pairs, increased Fos-ir staining was observed in several brain regions of male prairie voles following 24 h or 2 weeks of social isolation in the absence of the EPM test (Table 3). Animals that experienced 24 h of social isolation had higher basal levels of Fos-ir expression in the MPOA, VMH and aHYP relative to controls and to those animals isolated for 2 weeks. In addition, animals that experienced 2 weeks of social isolation had fewer Fos-ir cells in LS compared to the control animals and animals isolated for 24 h. No group differences were found in any other brain areas.

4. Discussion

Voles are a group of rodents that show remarkable diversity in social organizations and behaviors, and thus provide a useful comparative model for studying the neuronal and hormonal bases of behavior [15–17,34]. Here, we compared highly social, monogamous prairie voles with non-social, promiscuous meadow voles to examine anxiety-related behaviors, circulating CORT concentrations, and neuronal activation in the brain, and to study the effects of social isolation experience on these markers. Our data demonstrate that social isolation is more stressful for social prairie voles than for non-social meadow voles, and exerts stimulus- and species-specific effects on anxiety-related

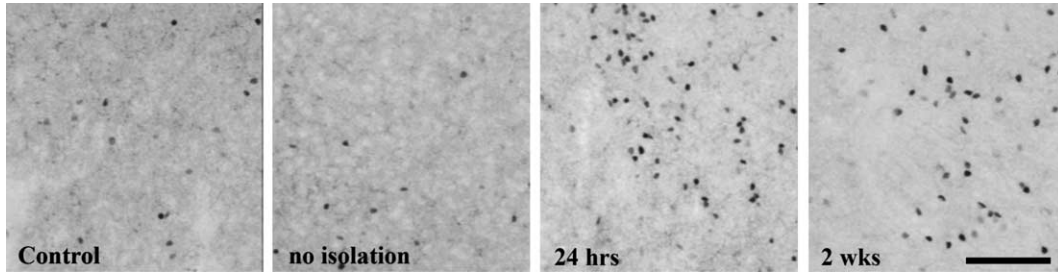


Fig. 2. Photomicrographs displaying Fos-ir stained cells in the medial preoptic area of male prairie voles that were housed with a same sex sibling without the EPM test (control) or with the EPM test (no iso.), or that were socially isolated for 24 h or for 2 wks. Bar=100 μ m.

behavior and associated neuronal activation. In addition, the EPM test elevates circulating CORT in prairie, but not meadow, voles independent of their social experience.

4.1. Anxiety-related behavior on EPM

Male prairie voles entered the open arms of the EPM more and remained there longer than did male meadow voles. Prairie voles also showed greater overall activity as demonstrated by the total number of arm entries on the EPM. In a pilot experiment, male prairie and meadow voles did not differ in locomotor activities during an open field test (Stowe JR and Wang ZX, unpublished data). Further, male voles performed similarly in the Morris water maze [35] and in a series of seven symmetrical mazes [36]. Together, these data suggest that greater activity in prairie voles, compared to meadow voles, in the present study were not due to general species differences in locomotor activities or spatial abilities. Rather they were specific to the EPM.

Apparently, prairie and meadow voles may have different coping strategies when placed on the EPM. Given that laboratory rodents are generally considered to be less anxious if they spend more time on the open arms of EPM [2,3], our data seem to indicate that prairie voles are less anxious than meadow voles. However, wild and laboratory animals may express different behavioral reactions under similar conditions [1], and they may act differently on the EPM test. Indeed, wild male mice tested on the EPM explored the open arms more than laboratory Swiss mice, demonstrating high reactivity and escape behavior rather than low levels of anxiety [32]. Like these wild mice, more time spent on the open arms by prairie voles may reflect their high reactivity associated with anxiety.

In previous studies in rats, chronic (4 weeks) social isolation was found to be anxiety producing; it increased the plasma CORT concentration and induced anxiety-like behaviors on EPM [8,37]. Social isolation is also known

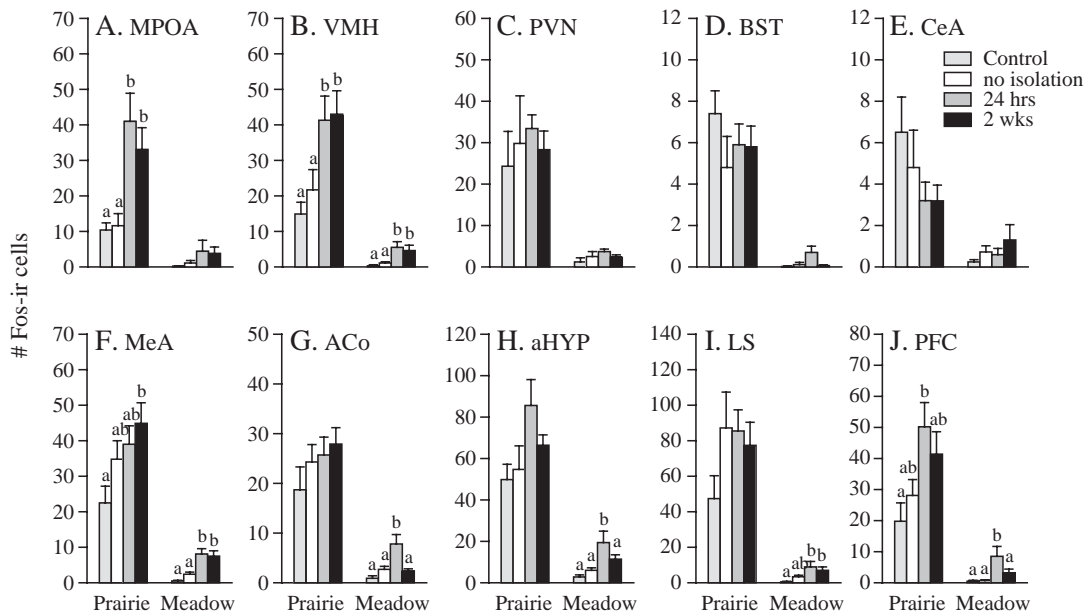


Fig. 3. Group differences in the number of Fos-ir stained cells in the medial preoptic area (A. MPOA), ventromedial hypothalamus (B. VMH), paraventricular nucleus (C. PVN), bed nucleus of the stria terminalis (D. BST), central (E. CeA), medial (F. MeA), and anterior cortical subnuclei (G. ACo) of the amygdala, anterior hypothalamus (H. aHYP), lateral septum (I. LS), and prefrontal cortex (J. PFC) of male prairie and meadow voles. The voles were housed with a same sex sibling without (Control) or with the EPM test (no iso.), or were housed socially isolated for 24 h or for 2 weeks. Data were analyzed by one-way ANOVA within each species. Dissimilar letters illustrate group differences based on the SNK post-hoc test. Error bars indicate SEM.

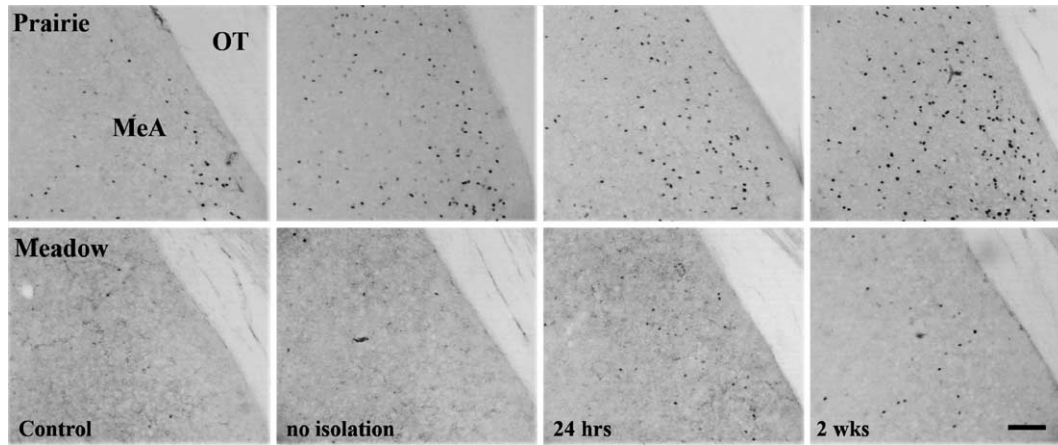


Fig. 4. Photomicrographs displaying Fos-ir stained cells in the amygdala of male prairie (top panels) and meadow voles (bottom panels) that were housed with a same sex sibling without (Control) or with the EPM test (no iso.) or were housed socially isolated for 24 h or for 2 wks. OT: optic tract, MeA: medial nucleus of the amygdala. Bar=100 μ m.

as a stressor for prairie voles as it increases circulating CORT and ultrasonic vocalization in pups [31] and elevates CORT in adult females [11]. Our data indicated that social isolation resulted in modest alterations on the prairie vole’s EPM performance; only voles that were socially isolated for 2 weeks had a significant increase in open arm entries. Nevertheless, these data provide evidence to support the notion that prairie vole’s performance on the open arms of the EPM reflects their high reactivity associated with anxiety. Social isolation may evoke less distress for non-social voles as it neither altered the meadow vole’s behaviors on the EPM, nor increased CORT and ultrasonic vocalization in montane vole (*M. montanus*) pups [31] — a species that shows similar life strategy and social behaviors as meadow voles [9,20]. These data suggest that social isolation exerted species-specific effects on anxiety-related behaviors on EPM in voles. Similar differences have also been found in different strains of laboratory rodents [6,7].

4.2. Plasma CORT levels following the EPM test

The low levels of plasma CORT found in meadow voles were similar to that reported for promiscuous montane voles [38]. Previous studies also suggested that prairie voles are glucocorticoid resistant: they have high levels of basal CORT titers and their adrenal axis is refractory to dexamethasone challenge [21,38]. Our finding that prairie voles had a very high level of plasma CORT provides further evidence supporting the notion that this species is glucocorticoid resistant. In our first experiment, 2 h after the EPM test, CORT levels did not change in meadow voles but increased by approximately 25% in prairie voles although this increase did not reach statistical significance. The subsequent time course experiment in prairie voles illustrated a 2-fold increase in CORT 20 min following the EPM test. This finding is in agreement with the finding in rats that showed a significant increase in plasma CORT 20–30 min following the EPM test [5,33]. In a previous study in male prairie voles, a peak of plasma CORT appeared 15–30 min following a swimming test, and then this CORT concentration gradually decreased and returned to the baseline level

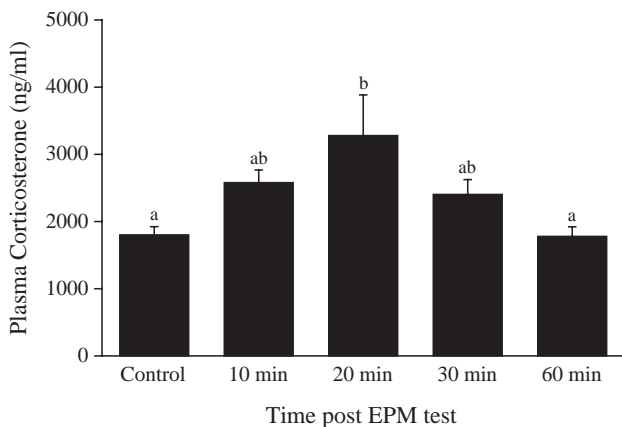


Fig. 5. Group differences in the plasma corticosterone concentrations between male prairie voles that did not experience the EPM test (Control), or experienced the EPM test and then sacrificed at 10, 20, 30, or 60 min post-test. Dissimilar letters illustrate group differences based on a SNK post-hoc test following ANOVA. Error bars indicate SEM.

Table 3
Basal levels of *c-fos* expression in male prairie voles following social isolation (mean \pm SEM)

Brain region	Control	24 h isolation	2 wk isolation	<i>p</i>
MPOA	18.6 \pm 3.1 ^{a*}	32.4 \pm 4.4 ^b	13.9 \pm 2.9 ^a	0.01
VMH	7.0 \pm 1.1 ^a	12.2 \pm 1.6 ^b	5.5 \pm 1.0 ^a	0.01
PVN	2.9 \pm 0.8	6.2 \pm 1.9	3.6 \pm 0.6	ns
BST	2.4 \pm 0.5	3.0 \pm 0.4	1.1 \pm 0.6	0.06
CeA	0.7 \pm 0.2	1.5 \pm 0.5	1.5 \pm 0.4	ns
MeA	7.4 \pm 0.6	7.0 \pm 1.1	6.0 \pm 0.8	ns
ACo	3.3 \pm 0.5	3.5 \pm 0.6	4.0 \pm 0.9	ns
aHYP	18.9 \pm 2.5 ^a	28.7 \pm 2.2 ^b	21.1 \pm 3.5 ^{a,b}	0.05
LS	8.9 \pm 1.8 ^b	11.4 \pm 3.2 ^b	2.4 \pm 0.6 ^a	0.05
PFC	2.1 \pm 0.5	5.4 \pm 2.2	2.2 \pm 1.1	ns

*Dissimilar letters illustrate group differences based on a SNK post hoc test following ANOVA.

[38]. Together, these data suggest that the EPM test likely induced a significant increase in circulating CORT in male prairie voles and this increased CORT had returned to the baseline levels 2 h later. It has been demonstrated that either confinement to or voluntarily increasing time on the open arms, but not the closed arms, of the EPM increases circulating CORT significantly in rats [3]. Thus, a greater amount of time spent on the open arms by prairie voles, compared to meadow voles, may account for the increased CORT found in the prairie voles. Alternatively, because prairie voles are CORT resistant [21,38], this increase in CORT may indicate an increase in arousal rather than stress levels. Interestingly, all three groups of prairie voles showed similar increases in circulating CORT levels following the EPM test, indicating that previous social experience did not influence CORT responses to the EPM test. It should also be noted that in the absence of time course measurements of CORT in meadow voles, we cannot exclude a possibility that the EPM test induced a transient increase in circulating CORT that returned to the baseline level 2 h post test in meadow voles as found in prairie voles.

4.3. Neuronal activation following the EPM test

A guiding assumption in our study was that the EPM test and/or manipulation of social environment produce changes in neuronal activity that can be detected by increased Fos-ir staining in an anatomically discrete circuit. Although some differences were found between the two species, our data in general illustrated a pattern of neuronal activation similar to that found in other species of rodents following the anxiety tests [26,28]. In the prairie voles, socially isolated males showed elevated Fos-ir staining in the MPOA and VMH following the EPM test, indicating an increased regional neuronal activation. Data from Experiment 3 further revealed stimulus-specific effects of social isolation on the basal levels of neuronal activation; increased Fos-ir staining in the MPOA and VMH was found following 24 h, but not 2 weeks, of social isolation in the absence of the EPM test. Lack of increased basal levels of Fos-ir staining for the 2-week group may reflect an adaptive process [39]. Importantly, these data suggest that in the MPOA and VMH, increased Fos-ir expression in the prairie voles socially isolated for 2 weeks was primarily due to responses to the EPM test. On the other hand, increased basal levels of neuronal activation in the voles socially isolated for 24 h contributed to their increased Fos-ir expression seen following the EPM test.

The notion that social isolation alters emotional brain responses in a stimulus-specific manner is further supported by our data showing that the EPM test increased Fos-ir staining in the PFC of the prairie voles that were socially isolated for 24 h and in the MeA of the voles that were socially isolated for 2 weeks. Therefore, acute and chronic social isolation exerted varying effects on different brain areas in mediating their responses to the subsequent EPM

test. Previous studies in other rodent species have demonstrated an involvement of these brain areas in the regulatory control of the HPA axis [40,41]. Our data implicate these brain areas as components of functional circuits mediating emotional responses in voles, as has been suggested in other rodents [26]. It is interesting to note that in most of these brain areas, neuronal activation is associated with receiving and processing socially relevant cues and in mediating social behaviors in prairie voles [22,23,42] as well as in other species of rodents [24,25,27,43]. Therefore, local neuron populations in these brain areas may comprise a common neural pathway that could be activated by a variety of social and non-social stimuli.

Social isolation coupled with the EPM test also increased Fos-ir expression in a region- and stimulus-specific manner in the meadow voles. Meadow voles that were socially isolated for 24 h or for 2 weeks showed an increase in Fos-ir expression in the VMH, MeA, and LS following the EPM test. In addition, 24 h of social isolation coupled with the EPM test increased Fos-ir staining in the PFC, ACo, and aHYP in the meadow vole brains. Although it is unknown whether this pattern of increased Fos-ir staining was due to increased basal levels of neuronal activation resulted from the social isolation experience, or enhanced brain responses to the EPM test, or both, these data in general support the notion that these brain areas are involved in mediating stress and emotional responses [26]. It is important to note that the prairie and meadow voles differ in life strategy and behavior and thus may respond to social environment differently [9,15]. Differences in the patterns and magnitudes of neuronal activation between the two species may reflect their differences in brain responses to anxiety-associated cues. Certainly, further studies are needed to address the functional significance of regional neuronal activation in species-specific behavioral and hormonal responses to stress.

Several aspects of the Fos-ir staining in the vole brains are worthy of further discussion. First, increased Fos-ir staining following the social isolation experience and EPM test was found in the MeA, but not CeA, in both species. Although most studies examining the role of amygdala in the regulation of endocrine stress responses have been focused on the CeA [29,44], it has been shown that *c-fos* expression elicited by emotional stressors is more consistent in the MeA than in other subnuclei of the amygdala, including CeA [43,45,46]. Our data are in agreement with these findings and provide further evidence to support the notion that the MeA is involved in HPA mediated stress responses [29,43].

Second, the PVN has also been implicated in stress responses as it contains neurons that secrete corticotrophin releasing factor and thus is crucial for central regulation of the HPA axis. Previous studies have shown that the PVN can be activated in an animal's response to stress [28,46]. In the present study, however, the social isolation experience and EPM test did not elevate Fos-ir expression in PVN in

either species. Therefore, the vole's PVN appears less sensitive to the induction of Fos-ir staining in response to behavioral tests of anxiety—a finding demonstrated in an early study in rats [26]. It is also possible that other immediate early genes are involved in the PVN responses to emotional stressors in voles.

Third, in our study (Experiment 1) only socially isolated voles, but not the controls housed with siblings, showed increased Fos-ir staining following the EPM test. Although these data support the notion that social isolation alters brain responses to anxiety-associated cues, lack of Fos-ir responses to the EPM test itself in intact voles is in direct contrast to what has been shown in rats [26,28]. The EPM test may not be as anxiety producing in intact voles as in typical laboratory rodents [2,3]. However, this is not supported by CORT data from our Experiment 2. Certainly, this issue needs to be addressed in further studies.

Finally, it should be noted that the results from Fos-ir staining are not conclusive. Data for the meadow voles were somewhat difficult because stained cell counts were so low in almost all brain areas examined. We found that male prairie voles had more Fos-ir cells in every brain area examined than did male meadow voles. Although these data may indicate species differences in brain responses to the EPM associated stimuli, we cannot exclude a possibility that there may be species differences in the binding affinity of the antibody. Further, although prairie and meadow voles are taxonomically close to each other, neurons involved in processing multi-modal stimuli may express different immediate early gene products that were not examined in the present study [18], and thus, Fos-ir staining may not provide an reliable insight into neural mechanisms underlying species differences in EPM induced anxiety. Finally, while increased Fos-ir staining may indicate regional neuronal activation, the physiological significance of Fos-ir induction must be determined through other techniques in further studies.

4.4. Conclusion

In conclusion, we have shown that prairie and meadow voles differ dramatically in behavioral profiles on the EPM, circulating CORT concentrations, and regional neuronal activation following the EPM test. Further, the influence of social isolation on anxiety-associated behaviors and neuronal activation markers is stimulus- and species-specific. Unlike studies in rats, one notable finding in the present study was a marked disassociation among neuronal, hormonal, and behavioral responses. In male prairie voles, neuronal and hormonal measures seemed to be more sensitive than the behavioral response to the EPM test, and social isolation experience affected neuronal, but not hormonal, response to the EPM test. Such disassociation was even more evident in male meadow voles that only showed some neuronal activation without any visible responses in behavior or plasma CORT. This disassociation

cannot easily be interpreted in terms of changes in emotional and stress responses. Nevertheless, our data support the notion that while the EPM test induces neuronal and endocrine changes, causation will need to be taken to study anxiety-related behavior on EPM in wild animals [32].

Acknowledgments

We thank Mike Smeltzer, Kyle Gobrogge, and Jacqui Detwiler for their critical reading of this manuscript. This research was supported by NIH grants MH58616 and MH66734 to ZW, HD 40722 to JTC, and DK43200 and HD38551 to MEF.

References

- [1] Hendrie CA, Weiss SM, Eilam D. Exploration and predation models of anxiety: evidence from laboratory and wild species. *Pharmacol Biochem Behav* 1996;54:13–20.
- [2] Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* 1987;92:180–5.
- [3] Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985;14:149–67.
- [4] Rodgers RJ, Cole JC. Anxiety enhancement in the murine elevated plus maze by immediate prior exposure to social stressors. *Physiol Behav* 1993;53:383–8.
- [5] File SE, Zangrossi H Jr, Sanders FL, Mabbutt PS. Raised corticosterone in the rat after exposure to the elevated plus-maze. *Psychopharmacology (Berl)* 1994;113:543–6.
- [6] Berton O, Ramos A, Chaouloff F, Mormde P. Behavioral reactivity to social and nonsocial stimulations: a multivariate analysis of six inbred rat strains. *Behav Genet* 1997;27:155–66.
- [7] Rodgers RJ, Cole JC. Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. *Physiol Behav* 1993;54:729–36.
- [8] Serra M, Pisu MG, Littera M, Papi G, Sanna E, Tuveri F, et al. Social isolation-induced decreases in both the abundance of neuroactive steroids and GABA(A) receptor function in rat brain. *J Neurochem* 2000;75:732–40.
- [9] Getz LL, Carter SC, Gavish L. The mating system of the prairie vole, *Microtus ochrogaster*: field and laboratory evidence for pair-bonding. *Behav Ecol Sociobiol* 1981;8:189–94.
- [10] Carter CS, Getz LL. Monogamy and the prairie vole. *Sci Am* 1993;268:100–6.
- [11] Kim JW, Kirkpatrick B. Social isolation in animal models of relevance to neuropsychiatric disorders. *Biol Psychiatry* 1996;40:918–22.
- [12] DeVries AC, DeVries MB, Taymans SE, Carter CS. The effects of stress on social preferences are sexually dimorphic in prairie voles. *Proc Natl Acad Sci U S A* 1996;93:11980–4.
- [13] De Vries AC, Gupta T, Gardillo S, Cho M, Carter CS. Corticotropin-releasing factor induces social preferences in male prairie voles. *Psychoneuroendocrinology* 2002;27:705–14.
- [14] Lim M, Wang Z, Olazaba D, Ren X, Terwilliger E, Young L. Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. *Nature* 2004;429:754–7.
- [15] Madison DM. An integrated view of the social biology of *Microtus pennsylvanicus*. *Biologist* 1980;62:20–33.
- [16] Aragona BJ, Wang ZX. The prairie vole: an animal model for behavioral neuroendocrine research on pair bonding. *Inst Lab Anim Res* 2004;45:35–45.

- [17] Young LJ, Wang ZX, Insel TR. Neuroendocrine bases of monogamy. *Trends Neurosci* 1997;21:71–5.
- [18] Hoffman GE, Smith MS, Verbalis JG. *c-fos* and related immediate early gene products as markers of activity in neuroendocrine systems. *Front Neuroendocrin* 1993;14:173–213.
- [19] Sheng M, Greenberg ME. The regulation and function of *c-fos* and other immediate early genes in the nervous system. *Neuron* 1990;4:477–85.
- [20] Insel TR, Preston S, Winslow JT. Mating in the monogamous male: behavioral consequences. *Physiol Behav* 1995;57:615–27.
- [21] Hastings NB, Orchinik M, Aubourg MV, McEwen BS. Pharmacological characterization of central and peripheral type I and type II adrenal steroid receptors in the prairie vole, a glucocorticoid-resistant rodent. *Endocrinology* 1999;140:4459–69.
- [22] Curtis JT, Wang ZX. Mating-induced *c-fos* expression in the forebrain of female prairie voles (*Microtus ochrogaster*): implication in pair bonding. *Physiol Behav* 2003;80:95–103.
- [23] Wang ZX, Hulihan TJ, Insel TR. Sexual and social experience is associated with different patterns of behavior and neural activation in male prairie voles. *Brain Res* 1997;767:321–32.
- [24] Baum MJ, Everitt BJ. Increased expression of *c-fos* in the medial preoptic area after mating in male rats: role of afferent inputs from the medial amygdala and midbrain central tegmental field. *Neurosci* 1992;50:627–46.
- [25] Kollack-Walker S, Newman SW. Mating and agonistic behavior produce different patterns of fos immunolabeling in the male syrian hamster brain. *Neurosci* 1995;66:721–36.
- [26] Duncan GE, Knapp DJ, Breese GR. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. *Brain Res* 1996;713:79–91.
- [27] Heeb MM, Yahr P. *C-fos* immunoreactivity in the sexually dimorphic area of the hypothalamus and related brain regions of male gerbils after exposure to sex-related stimuli or performance of specific sexual behaviors. *Neuroscience* 1996;72:1049–71.
- [28] Silveira MC, Sandner G, Graeff FG. Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze. *Behav Brain Res* 1993;56:115–8.
- [29] Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis. *Trends Neurosci* 1997;20:78–84.
- [30] Carter CS, Witt DM, Thompson G, Carlstead K. Effects of hormonal, sexual, and social history on mating and pair bonding in prairie voles. *Physiol Behav* 1987;44:691–7.
- [31] Shapiro LE, Insel TR. Infant's response to social separation reflects adult differences in affiliative behavior: a comparative developmental study in prairie and montane voles. *Dev Psychobiol* 1990;23:375–93.
- [32] Holmes A, Parmigiani S, Ferrari PF, Palanza P, Rodgers RJ. Behavioral profile of wild mice in the elevated plus-maze test for anxiety. *Physiol Behav* 2000;71:509–16.
- [33] Hennessy JW, Levine S. Stress, arousal and the pituitary-adrenal system: a psychoendocrine hypothesis. In: Sprague JM, Epstein AN, editors. *Progress Psychobiol Physiol Psychol*, vol. 8. New York: Academic Press; 1979.
- [34] Carter CS, Getz LL, Cohen-Parsons M. Relationships between social organization and behavioral endocrinology in a monogamous mammal. *Adv Study Behav* 1986;16:109–44.
- [35] Sawrey DK, Keith JR, Backes RC. Place learning by three vole species (*Microtus ochrogaster*, *M. montanus*, and *M. pennsylvanicus*) in the Morris swim task. *J Comp Psychol* 1994;108:179–88.
- [36] Gaulin SJ, FitzGerald RW, Wartell MS. Sex differences in spatial ability and activity in two vole species (*Microtus ochrogaster* and *M. pennsylvanicus*). *J Comp Psychol* 1990;104:88–93.
- [37] Parker V, Morinan A. The socially isolated rat as a model for anxiety. *Neuropharmacology* 1986;25:663–4.
- [38] Taymans SE, DeVries AC, DeVries MB, Nelson RJ, Friedman TC, Castro M, et al. The hypothalamic-pituitary-adrenal axis of prairie voles (*Microtus ochrogaster*): evidence for target tissue glucocorticoid resistance. *Gen Comp Endocrin* 1997;106:48–61.
- [39] Stamp J, Herbert J. Corticosterone modulates autonomic responses and adaptation of central immediate-early gene expression to repeated restraint stress. *Neuroscience* 2001;107:465–79.
- [40] Cullinan WE, Helmreich DL, Watson SJ. Fos expression in forebrain afferents to the hypothalamic paraventricular nucleus following swim stress. *J Comp Neurol* 1996;368:88–99.
- [41] Feldman S, Conforti N, Saphier D. The preoptic area and bed nucleus of the stria terminalis are involved in the effects of the amygdala on adrenocortical secretion. *Neuroscience* 1990;37:775–9.
- [42] Cushing BS, Mogeckwu N, Le WW, Hoffman GE, Carter CS. Cohabitation induced Fos immunoreactivity in the monogamous prairie vole. *Brain Res* 2003;965:203–11.
- [43] Dayas CV, Buller KM, Day TA. Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. *Eur J Neurosci* 1999;11:2312–22.
- [44] Feldman S, Conforti N, Weidenfeld J. Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli. *Neurosci Biobehav Rev* 1995;19:235–40.
- [45] Arnold FJ, De Lucas Bueno M, Shiers H, Hancock DC, Evan GI, Herbert J. Expression of *c-fos* in regions of the basal limbic forebrain following intracerebroventricular corticotropin-releasing factor in unstressed or stressed male rats. *Neuroscience* 1992;51:377–90.
- [46] Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 1995;64:477–505.