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ORIGINAL ARTICLE

Phosphorylation of cAMP response element-binding protein in the extended amygdala of male rats is induced by novel environment and attenuated by estrous female-bedding

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Abstract

OBJECTIVE: We examined whether female pheromone, which would be contained in female-soiled bedding, affected the expression of phosphorylated cAMP response element-binding protein-like (pCREB) immunoreactive cells in the extended amygdala.
METHODS: Male rats were exposed to following conditions: maintained in their home cage (home cage group), or relocated to a cage containing clean bedding (clean-bedding exposed group), ovariectomized (OVX) rat-soiled bedding (OVX-bedding exposed group) or estrogen-treated OVX rat-soiled bedding (OVX+E_2-bedding exposed group). Rats were sacrificed 10–20 min after exposure and brain sections were subject to immunocytochemical processing.
RESULTS: In the medial subdivision of the bed nucleus of the stria terminalis (BST) and the central amygdala (CeA), the number of pCREB immunoreactive (pCREB-ir) cells in the clean-bedding exposed group was significantly larger than in the home cage group, while the number of pCREB-ir cells in the OVX+E_2-bedding exposed group did not differ from that in the home cage group. The bedding soiled by OVX rats was less effective. No significant difference in the number of pCREB-ir cells was detected in the other regions of the extended amygdala among all groups.
CONCLUSIONS: The present study suggests that the exposure of clean bedding to male rats induces the expression of pCREB-ir in the medial BST and the CeA; exposure to female pheromone attenuates this expression.

INTRODUCTION

Pheromones induce neuroendocrine responses and are important for social and sexual communication in many species, including rodents (Halpern 1987; Keverne 2004), likely via activation of specific brain regions. Studies using Fos immunoreactivity as a marker have demonstrated that male rats exposed to bedding soiled by estrous female rats exhibit a significantly increased number of Fos immunoreactive neurons in brain areas related to pheromone responses, including the medial amygdala, the bed nucleus of the stria terminalis (BST) and the preoptic area (Bressler & Baum 1996; Kippin *et al.* 2003; Nishitani *et al.* 2004). These results suggested that female pheromone, which would be present in the bedding, activated the neuronal system involved in pheromone response in the male rat brain.

One report, on the other hand, showed that brief preexposure of males to a female reduced all predator odor and novelty elicited anxiety, fear and stress responses (Kavaliers *et al.* 2001). This finding suggests that female pheromone not only stimulates but also attenuates brain response to certain stimuli. Furthermore, dog-appeasing pheromone has been suggested to be useful in reducing anxiety and fear in puppies (Sheppard & Mills 2003). Also in humans, it has been found that maternal odors could have a soothing effect on crying newborn infants (Sullivan & Toubas 1998; Nishitani *et al.* 2009). Additionally, among human adults, sex-steroid derived compound, which acts as a $GABA_A$ receptor modulator (Kaminski *et al.* 2006), has been shown to have parasympathetic effects in men (Bensafi *et al.* 2004). Whether female pheromone can attenuate the activations of the neuronal system involved in the certain responses is not yet known.

To address this question, we examined the phosphorylation of cAMP response element-binding protein (pCREB) instead of Fos. Because CREB activation/inactivation occurs via phosphorylation, the average time course for its activation/inactivation is much faster than that of c-Fos (Ji & Rupp 1997). In fact, fasting-induced pCREB expression decreases within 5 minutes (Mogi *et al.* 2005). Further, stimulation-induced Fos expression pattern is sometimes different from pCREB expression (Chen *et al.* 1999; Stanciu *et al.* 2001). We first identified the brain areas that were activated via pCREB phosphorylation in response to a simple stimulus and focused on the BST and the amygdala, so called the extended amygdala (Shammah-Lagnado *et al.* 2000), because those are related to both the pheromone responses and the emotion (Winans & Scalia 1970; Lehman *et al.* 1980; Walker *et al.* 1997).

MATERIAL AND METHODS

Animals

Intact male and female Wistar rats (Charles River, Yokohama, Japan) at 7–8 weeks of age were housed under controlled lighting conditions (light, 5:00–19:00) with food and water available ad libitum. Male rats were housed individually in plastic cages (25×50×15 cm) and handled 5 min daily for 1 week prior to the experiment. Female rats were ovariectomized (OVX) and two weeks later, a subset of the OVX rats were subcutaneously implanted with a silicon tube (15 mm length, i.d. 1.5 mm, o.d. 3.0 mm, Dow Corning, Midland, Mich., USA) containing 20% 17β-estradiol (Sigma Chemical Co., St. Louis, USA). This estrogen treatment induces the luteinizing hormone surge and sexual behavior including the lordosis. The remaining OVX rats were implanted with a silicon tube containing 100% cholesterol. The bedding soiled by female rats was stored for 1 week. Rats received implantation of a cannula for an anesthesia into the jugular vein under ether anesthesia the day prior to the experiment.

On the morning of the experiment, male rats were divided into 4 groups and treated as follows. The first group of male rats were kept in their home cage, and handled and sacrificed in their home cage (n=5, home cage group). The second group of male rats were removed from their home cages and placed in a new cage containing clean bedding (n=7, clean bedding-exposed group). The third group of male rats were removed from their home cages and placed in a cage in which an OVX rat had stayed for 1 week (n=6, OVX bedding-exposed group). The final group of male rats were removed from their home cages and placed in a cage in which an OVX rat exposed to 17β-estradiol ($OVX+E_2$) had stayed for 1 week (n=6, $OVX+E_2$ bedding-exposed group). For all groups exposed to new bedding (clean bedding, OVX bedding and $OVX+E_2$ bedding), male rats were sacrificed 10 to 20 min after exposure to the bedding. All rats were sacrificed by intravenous injection of an overdose of sodium pentobarbital (100 mg/rat) and subjected to immunocytochemical processing.

All animal housing and surgical procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Yokohama City University School of Medicine.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Kudo *et al.* 2004; Mogi *et al.* 2005). Briefly, rats were perfused immediately through the cardiac ventricle with 2% paraformaldehyde and 4% acrolein in phosphate buffer (pH 7.5) at approximately 4 °C. Thirty µm frozen coronal sections were cut with a Bright cryostat. The sections were incubated with 1% sodium borohydrite and H_2O_2 in 20% methanol, and then incubated overnight with rabbit polyclonal antibody to pCREB diluted 1:800 (Cell Signaling Technology, Inc, Beverly, USA). Sections were incubated with biotinylated anti-rabbit IgG diluted 1:200, followed by streptavidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vectastain Lab, Burlingame, USA). The sections were then incubated for 30 min in 0.05%

3,3'-diaminobenzidine (DAB) with H_2O_2. The control samples without primary antibodies resulted in absence of staining as expected. Caution was taken to treat all sections similarly, including exposure time to DAB, to ensure reliable results for the quantification of pCREB immunoreactivity.

Histological analysis

The number of cells positive for pCREB-immunoreactivity in brain regions (Paxinos & Watson 1986) (as shown in Figure 1) was counted by visual inspection performed by an investigator blinded to the experimental groups. The number of pCREB-positive cells in each 30 μm section, i.e., the sum of pCREB-positive cells in the right and left hemispheres of each section, was first determined for each area, and then the average number of cells expressing pCREB per area was calculated by dividing the sum of all pCREB-positive cells by the number of sections counted. Sections were carefully matched across all animals in all experimental groups.

The data were analyzed by one-way ANOVA followed by Fisher's protected LSD post-hoc test; differences were considered statistically significant at $p<0.05$.

RESULTS

We first assessed pCREB expression in brain sections of rats either maintained in their home cages or exposed to various kinds of beddings. Among all the groups, pCREB-positive cells (as indicated by blue-black nuclei) were clearly visible in the BST (Figure 2) and the amygdaloid nuclei (Figure 3). However, only a small number of cells expressing pCREB were found in the paraventricular nucleus, irrespective of groups (data not shown), suggesting that the experimental condition used in the present study was not a strong stress for male rats.

As shown in Figure 2, we detected some expression of pCREB in both the medial and lateral subdivision of the BST, and strong expression was detected in rats exposed to clean bedding. The number of pCREB-positive cells appeared to increase in the medial subdivison of the BST (BSTM) upon exposure to clean-bedding. In contrast, this increase in pCREB-positive cells in the BSTM was not evident in the OVX+E_2-bedding exposed group (Figure 2a). One-way ANOVA ($F_{3,20}$=4.41, $p<0.05$) showed that the number of pCREB-positive cells in the BSTM (Figure 2b) was significantly greater in the clean-bedding exposed group than in the home cage group (post-hoc, $p<0.05$), suggesting that the process of transferring male rats into a new cage with a clean bedding induced pCREB expression in the BSTM. There was no significant difference between the number of cells expressing pCREB in the OVX-bedding exposed and the clean-bedding exposed groups; the number of pCREB-positive cells in the OVX+E_2-bedding exposed group was, however, significantly smaller than that in the clean-bedding exposed and

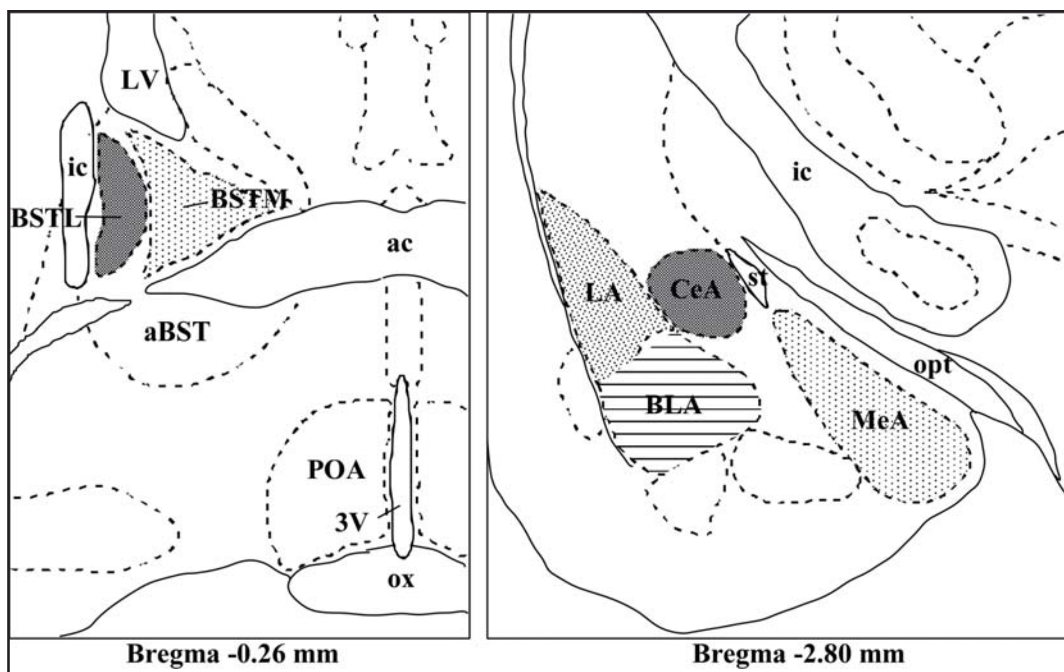

Fig. 1. Schematic illustration of a coronal section showing the brain area in which pCREB cells were quantified (modified from Paxinos and Watson, 1986). ac, anterior commissure; BLA, basolateral nucleus of the amygdala; BSTL, lateral subdivision of the anterior part of the bed nucleus of the stria terminalis; BSTM, medial subdivision of the anterior part of the bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; ic, internal capsule; LA, lateral nucleus of the amygdala; LV, lateral ventricle; MeA, medial nucleus of the amygdala; opt, optic tract; ox, optic chiasm; POA, preoptic area; st, stria terminalis; 3V, third ventricle.

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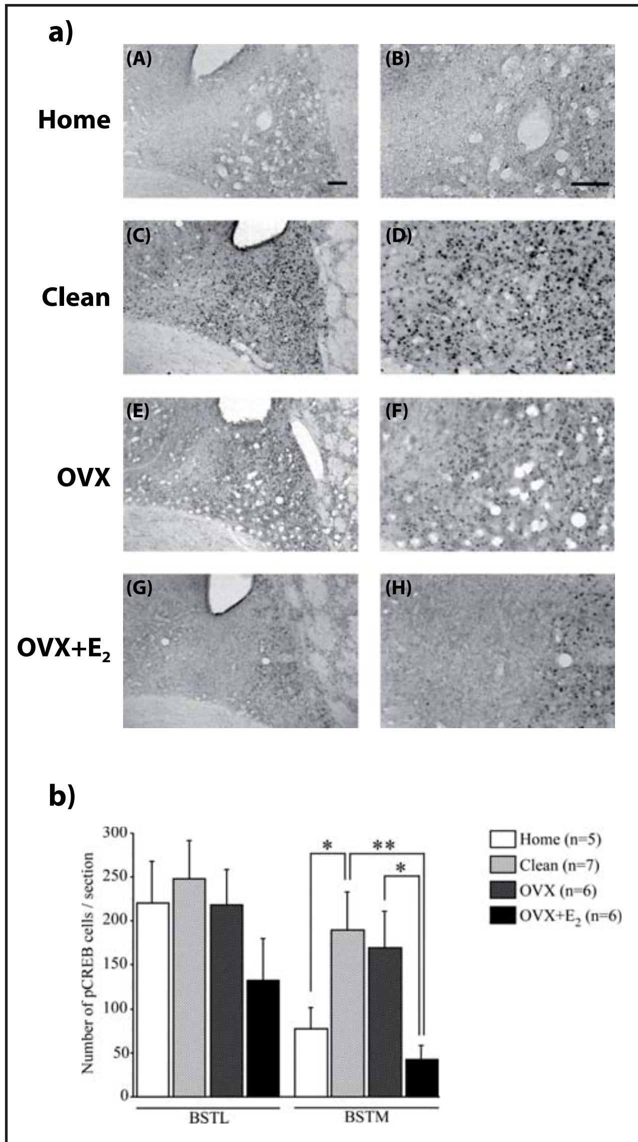

Fig. 2. a) Photographs showing examples of pCREB cells in the BST of rats kept in the home cage (A and B), or exposed to clean bedding (C and D), OVX rat-soiled bedding (E and F) or OVX+E_2 rat-soiled bedding (G and H). Scale bar=150 µm. b) The mean number of pCREB cells in the BSTL and BSTM sections of rats exposed to various beddings. * indicates $p<0.05$. ** indicates $p<0.01$.

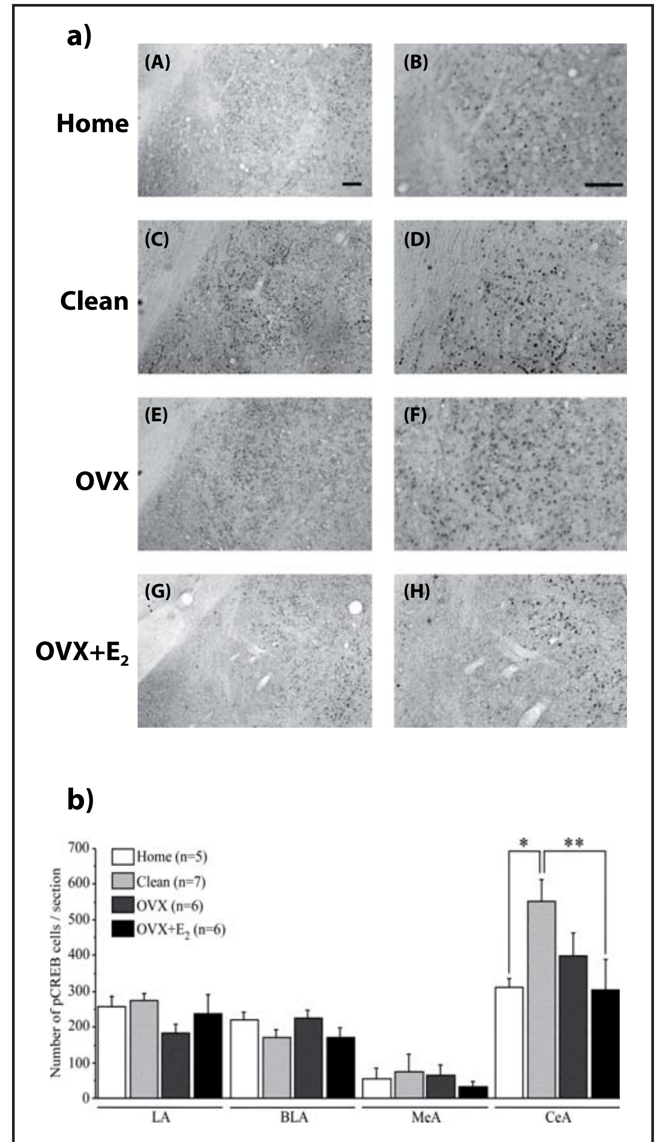

Fig. 3. a) Photographs showing examples of pCREB cells in the CeA of rats kept in the home cage (A and B), or exposed to clean bedding (C and D), OVX rat-soiled bedding (E and F) or OVX+E_2 rat-soiled bedding (G and H). Scale bar=150 µm. b) The mean number of pCREB cells in the LA, BLA, MeA and CeA sections of rats exposed to various beddings. * indicates $p<0.05$. ** indicates $p<0.01$.

the OVX-bedding exposed groups ($p<0.01$), but was not significantly different from the home cage group ($p>0.5$). In contrast, we did not see any statistically significant changes in the number of pCREB-positive cells in the lateral subdivision of the BST (BSTL) among the four groups ($F_{3,20}=1.365$, $p>0.2$).

The expression of pCREB in the central amygdala (CeA) of the clean-bedding exposed group was most evident within amygdaloid nuclei (Figure 3a). One-way ANOVA ($F_{3,20}=3.58$, $p<0.05$) followed by post-hoc comparison showed that the number of pCREB-positive cells in the clean-bedding exposed group was significantly greater than in the home cage group (Figure 3b, $p<0.05$). There was no significant difference between the number of pCREB-positive cells in the OVX-bedding exposed and the clean-bedding exposed groups. In contrast, significantly less pCREB-positive cells were detected in the OVX+E_2-bedding exposed group compared to the clean-bedding exposed group ($p<0.01$); furthermore, no significant difference was detected in comparison with the home cage group. There was no significant difference in the number of pCREB-positive cells in the lateral amygdala (LA) (($F_{3,20}=1.507$, $p>0.2$) or the basolateral amygdala (BLA) ($F_{3,20}=1.982$, $p>0.1$) or the medial amygdala (MeA) ($F_{3,20}=0.305$, $p>0.8$) among the four groups.

DISCUSSION

The present study showed that the process of removing male rats from their home cage and relocating to another cage containing clean bedding induced an increase of pCREB expression in the BSTM and the CeA. Notably, this increase in pCREB expression was not observed if the cage contained bedding from OVX rats treated with estrogen. These results suggest that female pheromone, which would be present in the bedding from OVX rats treated with estrogen, interfered with brain response to the procedure used in this study.

The stimulus and mechanism by which the procedure induced pCREB expression in the extended amygdala is not known in the present study. However, several factors may be involved, such as the new cage alone or in combination with clean bedding may act as a novel environment. A previous report showed that new environmental factors can induce the expression of c-fos mRNA in the extended amygdala (Day *et al.* 2001), which is also known to induce Fos in response to stimuli such as a predatory odor (Dielenberg *et al.* 2001). Thus, it is conceivable that a novel environment may induce the activation of a variety of brain areas including the BST and the amygdala. A fear conditioning study showed that mice exposed to the context only, that might act as a novel environment, exhibited an increase in the expression of pCREB and Fos in several regions including the amygdala (Stanciu *et al.* 2001), though these changes were statistically not significant. Furthermore, CREB-deficient mice exhibited altered behavior in novel environments, suggesting that CREB is involved in the response to novel environments (Hebda-Bauer *et al.* 2007). It has been suggested that detection of novelty is associated with an increase in phosphorylated CREB protein levels in the hippocampus (Winograd & Viola 2004). Together, these results led us to propose that brain response to a novel environment could be demonstrated by expression of pCREB. We thus speculate that the procedure used in the present study (placing male rats into a cage containing clean bedding) acts as a novel environmental stimulus to result in an increase in pCREB expression in the BSTM and CeA.

If this is the case, the absence of pCREB expression in rats exposed to OVX+E_2-soiled bedding suggests that female pheromone, which would be present in the bedding soiled by estrogen-treated female rats, exposure in male rats attenuates brain response by a novel environment. Both the BST (Gray *et al.* 1993; Walker *et al.* 1997; Onaka & Yagi 1998) and the amygdaloid nuclei (Buijs & Van Eden 2000; Stanciu *et al.* 2001) are well known to be involved in many different types of stress responses in the brain. Recently, it has been suggested that the extended amygdala is involved in the brain response to fear and anxiety, respectively (Walker *et al.* 1997). In addition, cat odor-induced anxiety has been shown to increase Fos expression in the BSTM (McGregor *et al.* 2004). Our results, therefore, are consistent with the results from these studies. In support of the present study, exposure of male mice to female urine has been shown to reduce anxiety (Aikey *et al.* 2002).

The bioactive substances present in female-soiled bedding that affect the expression of pCREB have yet to be identified. The identity of the mammalian pheromones is not well understood, but production of pheromones is often regulated by gonadal steroid hormones (Dulac & Torello 2003). Indeed, in male rats, the attractiveness of female rat odor varies with the estrous cycle, and odors from female rats at proestrus are the most attractive to male rats (Lucas *et al.* 1982). In addition, sexually active male rats preferred OVX rats with estrogen replacement than those without estrogen replacement (Xiao *et al.* 2004). These reports suggested that the production of pheromone by female rats depended on the presence of estrogen. Our findings demonstrating that OVX+E_2-soiled bedding was more effective than OVX-soiled bedding is in good accord with these previous reports, and further suggests the presence of female pheromone in the bedding.

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REFERENCES

1 Aikey JL, Nyby JG, Anmuth DM, James PJ (2002). Testosterone rapidly reduces anxiety in male house mice (Mus musculus). Horm Behav **42**: 448–60.
2 Bensafi M, Tsutsui T, Khan R, Levenson RW, Sobel N (2004). Sniffing a human sex-steroid derived compound affects mood and autonomic arousal in a dose-dependent manner. Psychoneuroendocrinology **29**: 1290–9.
3 Bressler SC, Baum MJ (1996). Sex comparison of neuronal Fos immunoreactivity in the rat vomeronasal projection circuit after chemosensory stimulation. Neuroscience **71**: 1063–72.
4 Buijs RM, Van Eden CG (2000). The integration of stress by the hypothalamus, amygdala and prefrontal cortex: balance between the autonomic nervous system and the neuroendocrine system. Prog Brain Res **126**: 117–32.
5 Chen B, Wang JF, Hill BC, Young LT (1999). Lithium and valproate differentially regulate brain regional expression of phosphorylated CREB and c-Fos. Brain Res Mol Brain Res **70**: 45–53.
6 Day HE, Badiani A, Uslaner JM, Oates MM, Vittoz NM, Robinson TE, *et al.* (2001). Environmental novelty differentially affects c-fos mRNA expression induced by amphetamine or cocaine in subregions of the bed nucleus of the stria terminalis and amygdala. J Neurosci **21**: 732–40.
7 Dielenberg RA, Hunt GE, McGregor IS (2001). "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. Neuroscience **104**: 1085–97.
8 Dulac C, Torello AT (2003). Molecular detection of pheromone signals in mammals: from genes to behaviour. Nat Rev Neurosci **4**: 551–62.

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9 Gray TS, Piechowski RA, Yracheta JM, Rittenhouse PA, Bethea CL, Van de Kar LD (1993). Ibotenic acid lesions in the bed nucleus of the stria terminalis attenuate conditioned stress-induced increases in prolactin, ACTH and corticosterone. Neuroendocrinology **57**: 517–24.
10 Halpern M (1987). The organization and function of the vomeronasal system. Annu Rev Neurosci **10**: 325–62.
11 Hebda-Bauer EK, Luo J, Watson SJ, Akil H (2007). Female CREBalphadelta- deficient mice show earlier age-related cognitive deficits than males. Neuroscience **150**: 260–72.
12 Ji RR, Rupp F (1997). Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. J Neurosci **17**: 1776–85.
13 Kaminski RM, Marini H, Ortinski PI, Vicini S, Rogawski MA (2006). The pheromone androstenol (5 alpha-androst-16-en-3 alpha-ol) is a neurosteroid positive modulator of GABAA receptors. J Pharmacol Exp Ther **317**: 694–703.
14 Kavaliers M, Choleris E, Colwell DD (2001). Brief exposure to female odors "emboldens" male mice by reducing predator-induced behavioral and hormonal responses. Horm Behav **40**: 497–509.
15 Keverne EB (2004). Importance of olfactory and vomeronasal systems for male sexual function. Physiol Behav **83**: 177–87.
16 Kippin TE, Cain SW, Pfaus JG (2003). Estrous odors and sexually conditioned neutral odors activate separate neural pathways in the male rat. Neuroscience **117**: 971–9.
17 Kudo K, Qiao CX, Kanba S, Arita J (2004). A selective increase in phosphorylation of cyclic AMP response element-binding protein in hippocampal CA1 region of male, but not female, rats following contextual fear and passive avoidance conditioning. Brain Res **1024**: 233–43.
18 Lehman MN, Winans SS, Powers JB (1980). Medial nucleus of the amygdala mediates chemosensory control of male hamster sexual behavior. Science **210**: 557–60.
19 Lucas PD, Donohoe SM, Thody AJ (1982). The role of estrogen and progesterone in the control of preputial gland sex attractant odors in the female rat. Physiol Behav **28**: 601–7.
20 McGregor IS, Hargreaves GA, Apfelbach R, Hunt GE (2004). Neural correlates of cat odor-induced anxiety in rats: region-specific effects of the benzodiazepine midazolam. J Neurosci **24**: 4134–44.
21 Mogi K, Funabashi T, Mitsushima D, Hagiwara H, Kimura F (2005). Sex difference in the response of melanin-concentrating hormone neurons in the lateral hypothalamic area to glucose, as revealed by the expression of phosphorylated cyclic adenosine 3',5'-monophosphate response element-binding protein. Endocrinology **146**: 3325–33.
22 Nishitani S, Miyamura T, Tagawa M, Sumi M, Takase R, Doi H, *et al.* (2009). The calming effect of a maternal breast milk odor on the human newborn infant. Neurosci Res **63**: 66–71.
23 Nishitani S, Moriya T, Kondo Y, Sakuma Y, Shinohara K (2004). Induction of Fos immunoreactivity in oxytocin neurons in the paraventricular nucleus after female odor exposure in male rats: effects of sexual experience. Cell Mol Neurobiol **24**: 283–91.
24 Onaka T, Yagi K (1998). Role of noradrenergic projections to the bed nucleus of the stria terminalis in neuroendocrine and behavioral responses to fear-related stimuli in rats. Brain Res **788**: 287–93.
25 Paxinos G, Watson C (1986). The rat brain in sterotaxic coordinates. Australia: Academic Press.
26 Shammah-Lagnado SJ, Beltramino CA, McDonald AJ, Miselis RR, Yang M, de Olmos J, *et al.* (2000). Supracapsular bed nucleus of the stria terminalis contains central and medial extended amygdala elements: evidence from anterograde and retrograde tracing experiments in the rat. J Comp Neurol **422**: 533–55.
27 Sheppard G, Mills DS (2003). Evaluation of dog-appeasing pheromone as a potential treatment for dogs fearful of fireworks. Vet Rec **152**: 432–6.
28 Stanciu M, Radulovic J, Spiess J (2001). Phosphorylated cAMP response element binding protein in the mouse brain after fear conditioning: relationship to Fos production. Brain Res Mol Brain Res **94**: 15–24.
29 Sullivan RM, Toubas P (1998). Clinical usefulness of maternal odor in newborns: soothing and feeding preparatory responses. Biol Neonate **74**: 402–8.
30 Walker DL, Cassella JV, Lee Y, De Lima TC, Davis M (1997). Opposing roles of the amygdala and dorsolateral periaqueductal gray in fear-potentiated startle. Neurosci Biobehav Rev **21**: 743–53.
31 Winans SS, Scalia F (1970). Amygdaloid nucleus: new afferent input from the vomeronasal organ. Science **170**: 330–2.
32 Winograd M, Viola H (2004). Detection of novelty, but not memory of spatial habituation, is associated with an increase in phosphorylated cAMP response element-binding protein levels in the hippocampus. Hippocampus **14**: 117–23.
33 Xiao K, Kondo Y, Sakuma Y (2004). Sex-specific effects of gonadal steroids on conspecific odor preference in the rat. Horm Behav **46**: 356–61.