Mapping the neural circuit activated by alarm pheromone perception by c-Fos immunohistochemistry

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Abstract

We previously reported that the alarm pheromones released from stressed male rats exaggerated both behavioral and autonomic (stress-induced hyperthermia) responses in recipient rats that were introduced into a novel environment. Subsequent experiments provided evidence that these alarm pheromones could be divided into two functionally different categories based on the site specificity and testosterone dependency of their production. However, the neural mechanisms underlying these behavioral and physiological responses remain unknown. In the present study, we examined Fos expression in 26 brain sites of the recipient rat 60 minutes after the exposure to the pheromone that aggravated stress-induced hyperthermia. The alarm pheromone-exposed rats showed a concurrent increase in Fos expression, in contrast to control odor-exposed rats in the anterior division lateral and medial group of the bed nucleus of the stria terminalis, paraventricular nucleus, dorsomedial hypothalamic nucleus, anterodorsal medial, lateral and basolateral amygdaloid nucleus, ventrolateral periaqueductal gray, laterodorsal tegmental nucleus, and locus coeruleus. These results provide information about the neural mechanisms in response to a non-sexual pheromone, i.e., an alarm

pheromone, and suggest that the perception of the alarm pheromone is related to

stress-responsive brains structures, including the hypothalamus and brainstem, as well

as to the amygdaloid nuclei.

Classification terms

Theme: Other systems of the CNS

Topic: Limbic system and hypothalamus

Key words: Alarm pheromone, Stress-induced hyperthermia, Brain mapping, Rat,

Medial amygdala, the bed nucleus of the stria terminalis

Introduction

Chemical communication plays important roles in various social interactions among mammals, including sexual [52], territorial [20], and maternal behaviors [33]. Alarm chemosignals, which alert animals to the proximity of conspecific individuals [1,4,53], are considered to be used widely in the animal kingdom. In rodent species, it was reported that rats could distinguish between the odors released from stressed and non-stressed conspecifics [51]. These odors were shown to change the behavior [1,34,35] and immune responses of the recipient [11].

We previously reported that the alarm pheromones released from male rats receiving foot shocks augmented both behavioral (increased sniffing, walking and rearing, and decreased resting behavior) and autonomic (stress-induced hyperthermia, SIH) responses in recipient male rats [26]. We then found that these alarm pheromones could be divided into two functionally different categories based on the androgen-dependent production and the area of the body surface from which they were released, namely, one category of alarm pheromone modifying recipient behavior is released from the whisker pad of the male in a testosterone-dependent manner, whereas

the other category of alarm pheromone influencing the autonomic response is released

from the perianal region in a testosterone-independent manner [27,28]. Considering that

the importance of propagating the notice of a dangerous situation to family or group

members is not limited to male and that the intensity of SIH reflects the animal's

anxiety status [32], it appears reasonable to postulate that the testosterone-independent

pheromone that aggravates SIH is biologically more important as compared with the

other one. We have therefore decided to focus more attention on this category of alarm

pheromone in our subsequent investigation including the present study.

Although behavioral and physiological responses to alarm pheromones have

been clearly demonstrated [27-29], the neural mechanism underlying these responses

remains largely unknown, with the exception of our preliminary finding that exposure to

alarm pheromones increased Fos protein expression in the mitral cell layer of the

accessory olfactory bulb [26]. Along with responses elicited due to intra-species

chemical communication, a fear or anxiety response has also been shown to be elicited

by inter-species chemical communication, e.g., by being exposed to predator odor. It is

well known that cat odor can innately elicit a fearful response and risk assessment

behaviors in rats [3,38]. C-Fos immunohistochemistry has been most widely used for functional anatomical mapping in laboratory animals, and previous reports have suggested that several brain regions play especially important roles in the stress response in reaction to fear and anxiety [2,31,47]. Recently, a systematical brain mapping study was conducted in rats that had been exposed to the cat odor using c-Fos immunohistochemical analysis [14,37]. It is therefore of interest to compare the neural circuit activated by intra-species chemical communication (e.g., via an alarm pheromone from conspecifics) with that activated by inter-species chemical communication (e.g., by predator odor).

In the present study, we examined Fos expression in 26 brain regions of subjects 60 minutes after exposure to an alarm pheromone that aggravated the autonomic response [26-28]. To the best of our knowledge, this is the first report to examine Fos expression in response to a non-sexual pheromone in rats. In comparison with the results obtained using control animals, we attempted to map the neural circuit activated by the perception of the alarm pheromone.

Experimental procedures

Animals

Experimentally naïve male Wistar rats were purchased from Clea Japan (Tokyo, Japan) and were used at the age of 10 weeks. Three animals per cage were housed under constant temperature (24±1°C) and humidity (45±5%) until they were implanted with a telemetry transmitter (see below). Food and water were available *ad libitum*, and the animals were maintained under a 12 h light/12 h dark cycle (lights on at 0800) throughout the experiment. The animals were cared for in accordance with "Policies Governing the Use of Live Vertebrate Animals", set by the University of Tokyo, and based on "The Public Health Service Policy on Humane Care and Use of Laboratory Animals" (revised in 1985) and "the National Institutes of Health's Guide for the Care and Use of Laboratory Animals". The subject animals were implanted with a telemetry transmitter (TA10TA-F40; Data Sciences International, St. Paul, MN) intraperitoneally under anesthesia with ether 10-11 days before the experiment. After surgery, the animals were housed individually on an antenna board (RLA1020 RPC-1; Data Sciences International) in a soundproof chamber (36 cm×54 cm×35 cm;

Muromachi Kikai, Tokyo, Japan) located in another room that was maintained at a

constant temperature (22±1°C) under a 12 h light/12 h dark cycle (lights on at 0800).

All animals were handled for 5 minutes each day beginning 6 days before the

experiment, and then each animal was exposed to the test box (see below) for 20

minutes each day beginning 4 or 5 days before the experiment in order to minimize the

effects of novelty stress.

Pheromone exposure

The general procedures were basically the same as those used in our previous

study [27]. Adult male Wistar rats were selected as the pheromone donors and were

anesthetized with sodium pentobarbital (50 mg/kg, i.p., Nembutal; Abbott Laboratories,

North Chicago, IL). Anesthetized donor rats bearing two intra-dermal needles (27G) for

electrical stimulation of the neck or perianal region were placed in the acrylic test box

(27.5 cm×20 cm×27 cm) for 15 minutes; during this period, the donor rats received 15

electrical stimulations (10V for 1 second) at 1 minutes intervals. The electrical

stimulation to the perianal region induced the alarm pheromone release that aggravated

the SIH response in the other rats [27]. A box in which the neck region of the donor had been electrically stimulated was used as a control box in an attempt to provide the subject with similar amount of the olfactory stimuli derived from the body surface following local stimulation. The neck region was chosen based on our previous finding that the presence of this odor in the test box modify neither the SIH nor behavioral response as compared with the one that were exposed to odors released from the donor receiving no stimulation [27].

After stimulation, the donor rat was removed and the empty box was brought into the room in which the subject animals were maintained after the surgery. Then, the test box was installed on an antenna board in the soundproof chamber and the body temperature (BT) and behavioral responses of each subject rat were recorded. To ensure stable baselines for BT, subject rats were placed in the test box only after showing a BT of less than 37.5°C for at least 5 minutes before the experiment in their homecage, and then they were held there for 60 minutes. The subject rats were randomly assigned to one of two treatments groups, i.e., exposed to either an alarm pheromone (n=7) or a control odor (n=8). After being placed in the test box, the BT and activity were

transmitted via the antenna board and the signals were recorded by a data acquisition

system (Dataquest® LabPRO 3.10; Data Sciences International). Activity was a

continuous measure of body movements, which was automatically extracted by the

software from changes in orientation of the radio-telemetric probe [10]. After the

experiment, the test box was washed in hot water with a cleanser to remove any odors

that might influence the next measurement. The pheromone donors were used 3-4 times

with at least a one-week interval between uses and the pheromone exposure trials were

conducted between 0900 and 1800.

Immunohistochemistry

After the 60 minutes exposure period, each subject rat was deeply

anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago,

IL) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in

0.1M phosphate buffer (PB). The brain was removed and immersed overnight in 4%

paraformaldehyde in 0.1M PB and then placed in 30% sucrose/PB for cryoprotection.

The olfactory bulb was cut into 30 μm thick sagittal sections and the rest of the brain

was cut into 30 μm thick coronal sections. Six successive sections were collected and

the second and fifth of these sections were stained with Cresyl Violet in order to

confirm the location of the nucleus. The remaining sections were floated in 0.05M

phosphate buffered saline with 0.3% Triton X-100 (PBST) and in 3% H_2O_2 in methanol

for 15 minutes in order to eliminate endogenous peroxidase activity. Thereafter, these

sections were incubated with blocking serum (Vectastain Elite ABC kit; Vector

Laboratories, Burlingame, CA) in PBST containing 10 % BlockAce (Dainippon

Pharmaceutical, Tokyo, Japan) for 30 minutes, and then, for 60 hours with the antibody

to Fos protein (Ab-5, Oncogene Research Products, Cambridge, MA; diluted 1:7500) in

PBST containing 10% BlockAce and at 4°C. After being washed in PBST, the sections

were incubated in biotinylated secondary antibody, anti-rabbit IgG (VECTASTAIN®

elite ABC kit) for 2 hours, followed by incubation in an avidin and biotinylated

horseradish peroxidase macromolecular complex (VECTASTAIN® elite ABC kit) for 2

hours. Thereafter, the sections were rinsed with PBST, washed twice with 175 mM

sodium acetate buffer, incubated with 0.004% 3,3' diaminobenzidine solution with

nickel intensification. The sections were then mounted on subbed slides, dehydrated in

ascending concentrations of ethanol, xylene cleared, and coverslipped.

Quantification

We analyzed Fos immunoreactive cells in 26 sites that had been selected generally based on a previous study [14]. The regions of interest were confirmed by subsequent sections stained with Cresyl Violet and evaluated according to a brain atlas [45]. Four sections at each region were captured using a microscope equipped with a digital camera (DP-12, Olympus, Tokyo, Japan). The numbers of Fos immunoreactive cells in a 0.5 mm square was counted unilaterally by an experimenter who was blind to the experimental groups using NIH image 1.63 software. The accessory olfactory bulb (AOB) was divided according to the mitral/tufted cell layer and the granule cell layer, and the center of each layer was analyzed. Because the boundary between the mitral cell layer and the granule cell layer was ambiguous in the main olfactory bulb, the immunoreactive cells were counted inclusively in its dorsal anterior region, as was also the case in our previous study [26]. Analyzed areas at other sites are shown in Figure 1. In some cases, the designated area containing cells to be counted was smaller than the

boundaries of a 0.5 mm square. In such cases, only the cells in the region of interest, but

not those in the extraneous areas, were counted.

Statistical analyses

Data analysis was performed with Stat View J 5.0 software (Abacus Concepts,

Berkeley, CA) and values of $p<0.05$ were considered to indicate statistical significance

for all tests. Body temperature was recorded continuously and the values were stored as

an average for a 5 seconds period in each minute. The individual baseline values were

defined as the averaged BT values recorded at the home cage during the 5 minutes

period just prior to the introduction to the test box. The SIH was expressed as the

change from the baseline for group comparison, and was analyzed by repeated two-way

analysis of variance (ANOVA). Activity was recorded continuously each minute and

was analyzed by repeated two-way ANOVA. For the immunohistochemical analysis, the

mean numbers of 4 sections in each region was used for the group comparison and the

results were analyzed by one-way ANOVA.

Results

The effects of the alarm pheromone on SIH and behavioral response

The rats exposed to the alarm pheromone showed a significantly aggravated SIH ($F(1,13)=16.7$, $p<0.01$), as compared to those rats exposed to the control odor (Fig. 2, top). The body temperature also significantly changed with time ($F(65, 845)=52.4$, $p<0.0001$) and there was a significant interaction between group and time ($F(65,845)=6.04$, $p<0.0001$). In contrast to the effects on SIH, the alarm pheromone did not exert any influence on the activity of the subjects. The activity of the subject animals changed significantly with time ($F(65,845)=9.58$, $p<0.001$), but there was no interaction between the group and time (Fig. 2, bottom).

The effects of the alarm pheromone on Fos expression

The mean numbers of Fos immunoreactive cells in 26 brain regions was summarized in Table 1. No significant between-group difference was observed in the olfactory bulb. In the forebrain and hypothalamus, on the other hand, the exposure to the alarm pheromone significantly increased Fos expression in the anterior division

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lateral group ($F_{(1,13)}=44.5$, $p<0.0001$) and medial group ($F_{(1,13)}=6.39$, $p<0.05$) of the

bed nucleus of the stria terminalis, the paraventricular nucleus ($F_{(1,13)}=23.2$, $p<0.001$),

and the dorsomedial hypothalamic nucleus ($F_{(1,13)}=17.0$, $p<0.01$), as compared to the

effects associated with the control odor only (Fig. 3). Significant differences between

the alarm pheromone and control odor groups were also found in the amygdaloid nuclei,

including the anterodorsal medial amygdaloid nucleus ($F_{(1,13)}=11.0$, $p<0.01$), lateral

amygdaloid nucleus ($F_{(1,13)}=14.7$, $p<0.01$), and the basolateral amygdaloid nucleus

($F_{(1,13)}=29.0$, $p<0.001$), as shown in Fig. 4. In the brainstem, the alarm pheromone

increased Fos expression in the ventrolateral periaqueductal gray ($F_{(1,13)}=29.6$,

$p<0.001$), laterodorsal tegmental nucleus ($F_{(1,13)}=40.7$, $p<0.0001$) and the locus

coeruleus ($F_{(1,13)}=17.0$, $p<0.01$)(Fig. 5). In other regions, no significant difference was

observed between the two groups, although Fos expression in the alarm pheromone

group tended to increase in five regions, namely, in the lateral hypothalamic area

($F_{(1,13)}=3.41$, $p=0.0876$), central amygdaloid nucleus ($F_{(1,13)}=3.71$, $p=0.0762$),

posteroventral medial amygdaloid nucleus ($F_{(1,13)}=3.54$, $p=0.0824$), dorsolateral

periaqueductal gray ($F_{(1,13)}=3.70$, $p=0.0768$) and the lateral parabrachial nucleus

$(F(1,13)=3.86, p=0.0723)$.

Discussion

In the present study, it was observed that the alarm pheromone released from the perianal region of anesthetized donor rats by local electrical stimulation aggravated the SIH but not the behavioral response of recipient animals. These results were consistent with our previous findings [27]. It therefore appears likely that the activation of neural circuit herein described can be ascribed to the effect of the alarm pheromone.

Fos expression in the olfactory bulb

It is well known in rodents that pheromonal signals are processed by the vomeronasal (or the accessory olfactory) system, which involves the vomeronasal organ and the AOB [25,40]. In the present study, we did not see any differences in Fos expression between the main and the accessory olfactory bulbs. These results were inconsistent with our previous finding that exposure to alarm pheromones resulted in an increase in Fos protein expression, specifically in the mitral/tufted cell layer of the AOB

[26]. This discrepancy may be due to experimental procedures employed in the two studies, e.g., differences in the timing of sample collection. In the present study, brain samples were collected 60 minutes after the start of pheromone exposure, and this time point was twice as long as that in our previous study [26]. If we observe at different time point, we may be able to detect the difference in Fos expression in the AOB, because Fos protein is known to be expressed its maximal level between 1 and 3 hours and then gradually disappears by 4-6 hours after stimulation [31]. Taken together, it seems likely that the time point of brain sampling may have disturbed the comparison of Fos expression between the groups in this study.

Fos expression in the forebrain and hypothalamus

Lesion and stimulation studies have shown that the bed nucleus of the stria terminalis (BNST) is involved in anxiety and stress responses [8,48,55,57]. The anterior BNST can be divided into subdivisions, including the anterior division medial group (BNSTam) and anterior division lateral group (BNSTal), and each subdivision appears to play different roles in the stress responses [19]. The BNSTal was densely connected

to the central and basolateral amygdaloid nucleus (BLA), whereas the BNSTam received light input from the medial amygdaloid nucleus (Me) [16-18]. Increased Fos expression in the BNSTam due to pheromone exposure suggests that the BNSTam receives input from the Me and alarm pheromone can elevate a recipient's general state of arousal [30]. In the present study, the effect of the alarm pheromone was more conspicuous at the BNSTal, from which the neural connections are known to project to the ventrolateral periaqueductal gray (VLPAG) and less heavily to the paraventricular nucleus (PVN) [16,18]. Fos expression was also enhanced in these areas, as described below, and it seems likely that the information communicated by alarm pheromone is transmitted from the amygdaloid nuclei to the deeper part of the diencephalons regulating the anxiety and stress responses via the BNST (especially the BNSTal).

The observed increased Fos expression in the dorsomedial hypothalamic nucleus (DMH) and the PVN due to exposure to the alarm pheromone was consistent with previous findings that the DMH provides a dense input to the PVN via CRFergic neurons [9]. In addition, it is also known that the DMH plays an important role in brown adipose tissue thermogenesis [41,56] and that the DMH itself mediates the stress

response as well as anxiety [15,23].

Increased Fos expression in these three regions was observed in response to a wide variety of stressors [5,6,36,42,44]. Taken together, the present results suggest that exposure to the alarm pheromone serves as a stressor.

Fos expression in the amygdaloid nucleus

The exposure to the alarm pheromone increased Fos expression in the anterodorsal medial amygdaloid nucleus (MeAD). These results also support the view that the alarm pheromone is transmitted via the vomeronasal pathway. Along with other subdivisions, the MeAD receives direct projections from the AOB [54] and plays an important role in the transmission of sexual pheromonal signals [43]. However, there remains a possibility that increased Fos expression is simply part of stress responses because the Me is functionally related to the activation of the hypothalamic-pituitary-adrenal axis [13].

Increased Fos expression was observed in the BLA and lateral amygdaloid nucleus (La) after exposure to the alarm pheromone. These regions play an important

role in the convergence of information regarding conditioned stimuli (CS) and aversive

unconditioned stimuli (US) [22]. Such results have indicated the possibility of an

involvement of learned associations in response to the alarm pheromone because

previous studies showed that odor could serve as CS in fear-conditioning experiments

[46], and that the BLA was shown to play an important role in this CS-US association

[12]. As regards Fos expression, an exposure to a reward-conditioned neutral odor

induced Fos expression in the BLA [50]. This possibility was also supported by a report

showing that repeated exposure was necessary for female mice to be attracted to sexual

pheromones, i.e., chemically naive females were not attracted to the volatile compound

of male sexual pheromones [39]. Further research is still needed to clarify whether

learning is involved in the response evoked by the alarm pheromone.

Fos expression in the brain stem

The periaqueductal gray (PAG) is known for its role in the integration of

defensive behavior [21]. Moreover, increased Fos expression was shown at the

ventrolateral region of the periaqueductal gray in response to CS associated to aversive

US [7]. In addition to its role in the fear response, this region is also postulated to modulate the emotional response to inescapable stressors [24]. The present results were found to be in agreement with such findings, because the alarm pheromone evoked anxiety, as assessed by SIH, under conditions in which the subject could not escape from the test box.

It is well known that the locus coeruleus (LC) provides the majority of noradrenergic neurons in the brain and is activated in response to a variety of acute stressful stimuli [49]. The stress-evoked activation of LC neurons was shown to mediate the activation of other stress-related brain regions [44]. The present results demonstrated that exposure to the alarm pheromone also increased Fos expression in the LC, as was the case with a variety of acute stressful stimuli [36,42,44].

Comparison to cat odor

The comparison of the present results with the Fos expression patterns induced by exposure to cat odor [14,37] provided us information regarding differences between the neural circuit related to inter- and intra-species communication of fear,

although the laterodorsal tegmental nucleus was investigated only in response to the alarm pheromone. The regions activated by both olfactory signals are thought to be related to anxiety and/or stress responses (e.g., the BNSTam, PVN, DMH, VLPAG, and LC). The observation that both the alarm pheromone and cat odor induced Fos expression in the Me was consistent with previous findings that Me plays important roles in the mediation of vomeronasal information as well as in stress responses [13,54]. The alarm pheromone induced Fos expression in the anterodorsal subdivision whereas the cat odor increased Fos expression in the posteroventral subdivision. It therefore appears likely that these subdivisions of the Me play different roles in mediating intra- and inter-specific olfactory stimuli.

The alarm pheromone induced Fos expression in the BNSTal, La and BLA, whereas cat odor induced Fos expression in the lateral septum, medial and lateral preoptic area, the ventromedial hypothalamic nucleus, and the dorsomedial and dorsolateral periaqueductal gray. These results suggest that intra- and inter-species communications of fear are probably transmitted in distinct neural circuits; for example, the amygdaloid nuclei may be more closely related to the transmission of alarm

pheromones, whereas the cat odor signal may be more related to hypothalamic regions.

Further research will still be necessary in order to answer this question, as well as to

reveal the meaning of these differences.

Implication of this study

Based on the present findings, we hypothesizes the neural circuit of the alarm

pheromone perception as follows. When the rat is placed in the test box containing

alarm pheromone, the pheromone information is perceived by the vomeronasal system

and transmitted through the AOB to the Me, and then to the BNST. At the same time,

the main olfactory system might also transmit some volatile information because even

an experimenter can discriminate the odor released from perianal region of anesthetized

donor [27]. The information perceived by the main olfactory system is then transmitted

through the main olfactory bulb to the La and BLA, and then to the BNST. At the BNST,

the information from the both systems is integrated, and then pheromone signal is

transmitted to several nuclei in the hypothalamus and the brain stem such as PVN,

DMH, PAG and LC that are involved in the regulation of anxiety as well as stress

responses.

Literature references

[1]   Abel, E.L. and Bilitzke, P.J., A possible alarm substance in the forced swimming

        test, *Physiol Behav*, 48 (1990) 233-9.

[2]   Bisler, S., Schleicher, A., Gass, P., Stehle, J.H., Zilles, K. and Staiger, J.F.,

        Expression of c-Fos, ICER, Krox-24 and JunB in the whisker-to-barrel pathway

        of rats: time course of induction upon whisker stimulation by tactile exploration

        of an enriched environment, *J Chem Neuroanat*, 23 (2002) 187-98.

[3]   Blanchard, R.J. and Blanchard, D.C., Antipredator defensive behaviors in a

        visible burrow system, *J Comp Psychol*, 103 (1989) 70-82.

[4]   Boissy, A., Terlouw, C. and Le Neindre, P., Presence of cues from stressed

        conspecifics increases reactivity to aversive events in cattle: evidence for the

        existence of alarm substances in urine, *Physiol Behav*, 63 (1998) 489-95.

[5]   Briski, K. and Gillen, E., Differential distribution of Fos expression within the

        male rat preoptic area and hypothalamus in response to physical vs.

        psychological stress, *Brain Res Bull*, 55 (2001) 401-8.

[6]   Carr, K.D., Park, T.H., Zhang, Y. and Stone, E.A., Neuroanatomical patterns of

        Fos-like immunoreactivity induced by naltrexone in food-restricted and ad

        libitum fed rats, *Brain Res*, 779 (1998) 26-32.

[7]   Carrive, P., Leung, P., Harris, J. and Paxinos, G., Conditioned fear to context is

        associated with increased Fos expression in the caudal ventrolateral region of the

        midbrain periaqueductal gray, *Neuroscience*, 78 (1997) 165-77.

[8]   Casada, J.H. and Dafny, N., Restraint and stimulation of bed nucleus of the stria

        terminalis produce similar stress-like behaviors, *Brain Res Bull*, 27 (1991)

        207-12.

[9]   Champagne, D., Beaulieu, J. and Drolet, G., CRFergic innervation of the

        paraventricular nucleus of the rat hypothalamus: a tract-tracing study, *J

        Neuroendocrinol*, 10 (1998) 119-31.

[10]   Clement, J.G., Mills, P. and Brockway, B., Use of telemetry to record body

        temperature and activity in mice, *J Pharmacol Methods*, 21 (1989) 129-40.

[11]   Cocke, R., Moynihan, J.A., Cohen, N., Grota, L.J. and Ader, R., Exposure to

conspecific alarm chemosignals alters immune responses in BALB/c mice,

Brain Behav Immun, 7 (1993) 36-46.

[12]   Cousens, G. and Otto, T., Both pre- and posttraining excitotoxic lesions of the

basolateral amygdala abolish the expression of olfactory and contextual fear

conditioning, *Behav Neurosci*, 112 (1998) 1092-103.

[13]   Dayas, C.V., Buller, K.M. and Day, T.A., Neuroendocrine responses to an

emotional stressor: evidence for involvement of the medial but not the central

amygdala, *Eur J Neurosci*, 11 (1999) 2312-22.

[14]   Dielenberg, R.A., Hunt, G.E. and McGregor, I.S., "When a rat smells a cat": the

distribution of Fos immunoreactivity in rat brain following exposure to a

predatory odor, *Neuroscience*, 104 (2001) 1085-97.

[15]   DiMicco, J.A., Stotz-Potter, E.H., Monroe, A.J. and Morin, S.M., Role of the

dorsomedial hypothalamus in the cardiovascular response to stress, *Clin Exp*

Pharmacol Physiol, 23 (1996) 171-6.

[16]   Dong, H., Petrovich, G.D. and Swanson, L.W., Organization of projections from

the juxtacapsular nucleus of the BST: a PHAL study in the rat, *Brain Res*, 859

(2000) 1-14.

[17]   Dong, H.W., Petrovich, G.D. and Swanson, L.W., Topography of projections

from amygdala to bed nuclei of the stria terminalis, *Brain Res Brain Res Rev*, 38

(2001) 192-246.

[18]   Dong, H.W., Petrovich, G.D., Watts, A.G. and Swanson, L.W., Basic

organization of projections from the oval and fusiform nuclei of the bed nuclei

of the stria terminalis in adult rat brain, *J Comp Neurol*, 436 (2001) 430-55.

[19]   Dunn, J.D., Plasma corticosterone responses to electrical stimulation of the bed

nucleus of the stria terminalis, *Brain Res*, 407 (1987) 327-31.

[20]   Eichmann, F. and Holst, D.V., Organization of territorial marking behavior by

testosterone during puberty in male tree shrews, *Physiol Behav*, 65 (1999)

785-91.

[21]   Farook, J.M., Wang, Q., Moochhala, S.M., Zhu, Z.Y., Lee, L. and Wong, P.T.,

Distinct regions of periaqueductal gray (PAG) are involved in freezing behavior

in hooded PVG rats on the cat-freezing test apparatus, *Neurosci Lett*, 354 (2004) 139-42.

[22]   Fendt, M. and Fanselow, M.S., The neuroanatomical and neurochemical basis of conditioned fear, *Neurosci Biobehav Rev*, 23 (1999) 743-60.

[23]   Inglefield, J.R., Schwarzkopf, S.B. and Kellogg, C.K., Alterations in behavioral responses to stressors following excitotoxin lesions of dorsomedial hypothalamic regions, *Brain Res*, 633 (1994) 151-61.

[24]   Keay, K.A. and Bandler, R., Parallel circuits mediating distinct emotional coping reactions to different types of stress, *Neurosci Biobehav Rev*, 25 (2001) 669-78.

[25]   Keverne, E.B., The vomeronasal organ, *Science*, 286 (1999) 716-20.

[26]   Kikusui, T., Takigami, S., Takeuchi, Y. and Mori, Y., Alarm pheromone enhances stress-induced hyperthermia in rats, *Physiol Behav*, 72 (2001) 45-50.

[27]   Kiyokawa, Y., Kikusui, T., Takeuchi, Y. and Mori, Y., Alarm pheromones with different functions are released from different regions of the body surface of male rats, *Chem Senses*, 29 (2004) 35-40.

[28]   Kiyokawa, Y., Kikusui, T., Takeuchi, Y. and Mori, Y., Modulatory role of

testosterone in alarm pheromone release by male rats, *Horm Behav*, 45 (2004)

122-7.

[29]   Kiyokawa, Y., Kikusui, T., Takeuchi, Y. and Mori, Y., Partner's stress status

influences social buffering effects in rats, *Behav Neurosci*, 118 (2004) 798-804.

[30]   Kollack-Walker, S. and Newman, S.W., Mating and agonistic behavior produce

different patterns of Fos immunolabeling in the male Syrian hamster brain,

Neuroscience, 66 (1995) 721-36.

[31]   Kovacs, K.J., c-Fos as a transcription factor: a stressful (re)view from a

functional map, *Neurochem Int*, 33 (1998) 287-97.

[32]   Lecci, A., Borsini, F., Volterra, G. and Meli, A., Pharmacological validation of a

novel animal model of anticipatory anxiety in mice, *Psychopharmacology (Berl)*,

101 (1990) 255-61.

[33]   Leon, M. and Moltz, H., Maternal pheromone: discrimination by pre-weanling

albino rats, *Physiol Behav*, 7 (1971) 265-7.

[34]   Mackay-Sim, A. and Laing, D.G., Discrimination of odors from stressed rats by

non-stressed rats, *Physiol Behav*, 24 (1980) 699-704.

[35]   Mackay-Sim, A. and Laing, D.G., The sources of odors from stressed rats,

Physiol Behav, 27 (1981) 511-3.

[36]   Martinez, M., Phillips, P.J. and Herbert, J., Adaptation in patterns of c-fos

expression in the brain associated with exposure to either single or repeated

social stress in male rats, *Eur J Neurosci*, 10 (1998) 20-33.

[37]   McGregor, I.S., Hargreaves, G.A., Apfelbach, R. and Hunt, G.E., Neural

correlates of cat odor-induced anxiety in rats: region-specific effects of the

benzodiazepine midazolam, *J Neurosci*, 24 (2004) 4134-44.

[38]   McGregor, I.S., Schrama, L., Ambermoon, P. and Dielenberg, R.A., Not all

'predator odours' are equal: cat odour but not 2,4,5 trimethylthiazoline (TMT;

fox odour) elicits specific defensive behaviours in rats, *Behav Brain Res*, 129

(2002) 1-16.

[39]   Moncho-Bogani, J., Lanuza, E., Hernandez, A., Novejarque, A. and

Martinez-Garcia, F., Attractive properties of sexual pheromones in mice: innate

or learned?, *Physiol Behav*, 77 (2002) 167-76.

[40]   Mori, K., Nagao, H. and Yoshihara, Y., The olfactory bulb: coding and

processing of odor molecule information, *Science*, 286 (1999) 711-5.

[41]   Morrison, S.F., Central pathways controlling brown adipose tissue

thermogenesis, *News Physiol Sci*, 19 (2004) 67-74.

[42]   Palmer, A.A. and Printz, M.P., Strain differences in Fos expression following

airpuff startle in Spontaneously Hypertensive and Wistar Kyoto rats,

Neuroscience, 89 (1999) 965-78.

[43]   Paredes, R.G., Lopez, M.E. and Baum, M.J., Testosterone augments neuronal

Fos responses to estrous odors throughout the vomeronasal projection pathway

of gonadectomized male and female rats, *Horm Behav*, 33 (1998) 48-57.

[44]   Passerin, A.M., Cano, G., Rabin, B.S., Delano, B.A., Napier, J.L. and Sved, A.F.,

Role of locus coeruleus in foot shock-evoked Fos expression in rat brain,

Neuroscience, 101 (2000) 1071-82.

[45]   Paxinos, G. and Watson, C., *The rat brain in stereotaxic coodinates*, 2nd edn.,

Academic Press, Inc., Tokyo, Japan, 1998.

[46]   Richardson, R., Vishney, A. and Lee, J., Conditioned odor potentiation of startle

in rats, *Behav Neurosci*, 113 (1999) 787-94.

[47]   Sagar, S.M., Sharp, F.R. and Curran, T., Expression of c-fos protein in brain:

metabolic mapping at the cellular level, *Science*, 240 (1988) 1328-31.

[48]   Schulz, D. and Canbeyli, R.S., Lesion of the bed nucleus of the stria terminalis

enhances learned despair, *Brain Res Bull*, 52 (2000) 83-7.

[49]   Singewald, N., Kaehler, S.T. and Philippu, A., Noradrenaline release in the locus

coeruleus of conscious rats is triggered by drugs, stress and blood pressure

changes, *Neuroreport*, 10 (1999) 1583-7.

[50]   Tronel, S. and Sara, S.J., Mapping of olfactory memory circuits: region-specific

c-fos activation after odor-reward associative learning or after its retrieval,

Learn Mem, 9 (2002) 105-11.

[51]   Valenta, J.G. and Rigby, M.K., Discrimination of the odor of stressed rats,

Science, 161 (1968) 599-601.

[52]   Vandenbergh, J.G., Acceleration and inhibition of puberty in female mice by

        pheromones, *J Reprod Fertil Suppl*, 19 (1973) 411-9.

[53]   Vieuille-Thomas, C. and Signoret, J.P., Pheromonal transmission of an aversive

        experience in domestic pig, *J Chem Ecol*, 18 (1992) 1551-1557.

[54]   von Campenhausen, H. and Mori, K., Convergence of segregated pheromonal

        pathways from the accessory olfactory bulb to the cortex in the mouse, *Eur J

        Neurosci*, 12 (2000) 33-46.

[55]   Walker, D.L., Toufexis, D.J. and Davis, M., Role of the bed nucleus of the stria

        terminalis versus the amygdala in fear, stress, and anxiety, *Eur J Pharmacol*, 463

        (2003) 199-216.

[56]   Zaretskaia, M.V., Zaretsky, D.V., Shekhar, A. and DiMicco, J.A., Chemical

        stimulation of the dorsomedial hypothalamus evokes non-shivering

        thermogenesis in anesthetized rats, *Brain Res*, 928 (2002) 113-25.

[57]   Zhu, W., Umegaki, H., Suzuki, Y., Miura, H. and Iguchi, A., Involvement of the

        bed nucleus of the stria terminalis in hippocampal cholinergic system-mediated

activation of the hypothalamo--pituitary--adrenocortical axis in rats, *Brain Res*,

916 (2001) 101-6.

Figure Legends

Fig. 1. Schematic diagrams showing the location of brain regions (open square containing numbers) in which Fos immunoreactive cells were mapped and counted. Adapted from [45]. For an explanation of the abbreviations, see the corresponding numbers in Table 1.

Fig. 2. Top: Time-dependent changes in the body temperature of recipient rats introduced into a test box containing either the odor released from the neck region (Control) or perianal region (Alarm pheromone) of an anesthetized donor by regional electrical stimulation (mean±S.E.M.). Bottom: Time-dependent changes in the activity of recipient rats introduced into a test box containing either the odor released from the neck region (Control) or the perianal region (Alarm pheromone) of an anesthetized donor by regional electrical stimulation (mean±S.E.M.). * P <0.05 with repeated two-way ANOVA.

Fig. 3. Photomicrographs showing Fos immunoreactive cells in the bed nucleus of the

stria terminalis (A,D), paraventricular nucleus (B,E), and dorsomedial hypothalamic nucleus (C,F) from male rats sacrificed 60 minutes after being exposed to either the control odor (A-C) or the alarm pheromone (D-E). Horizontal bar indicates 200 μm.
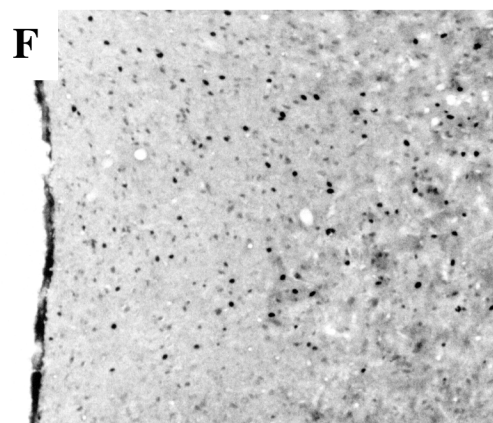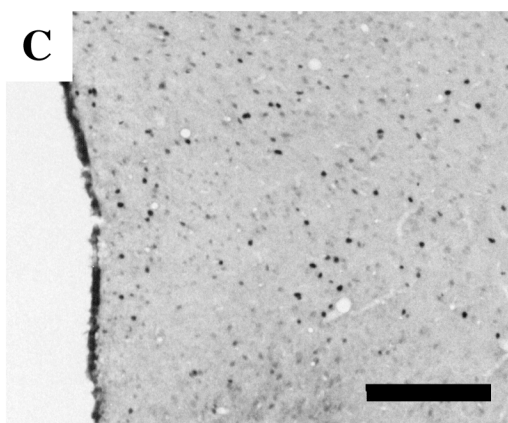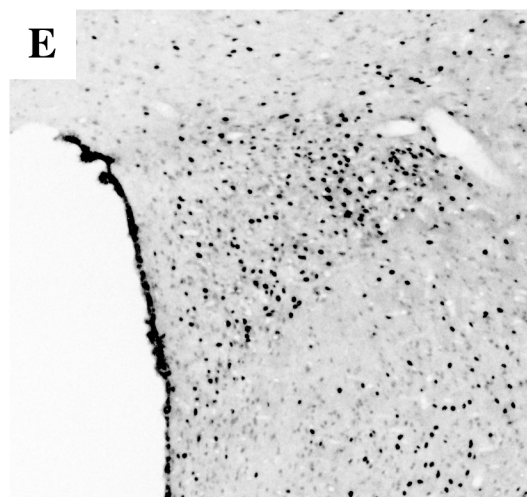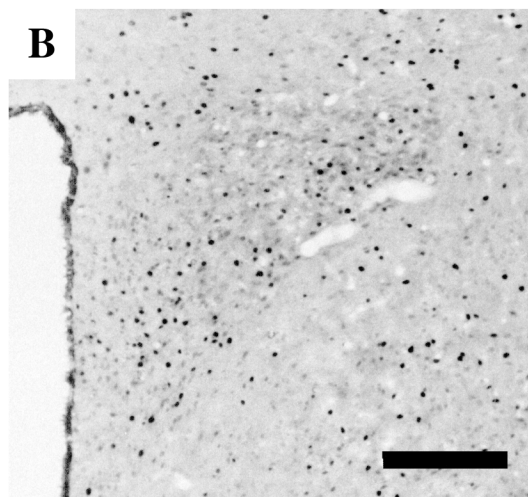
Fig. 4. Photomicrographs showing Fos immunoreactive cells in the anterodorsal medial amygdaloid nucleus (A,C) and lateral and basolateral amygdaloid nucleus (B,D) from male rats sacrificed 60 minutes after being exposed to either the control odor (A,B) or the alarm pheromone (C,D). Horizontal bar indicates 200 μm.
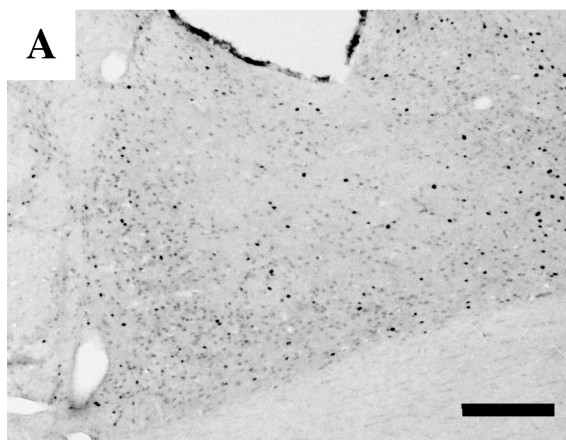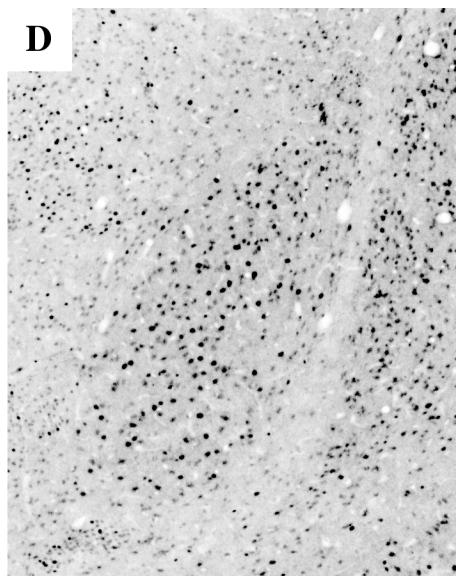
Fig. 5. Photomicrographs showing Fos immunoreactive cells in the ventrolateral periaqueductal gray (A,D), laterodorsal tegmental nucleus (B,E), and locus coeruleus (C,F) from male rats sacrificed 60 minutes after being exposed to either the control odor (A-C) or the alarm pheromone (D-F). Horizontal bar indicates 200 μm.
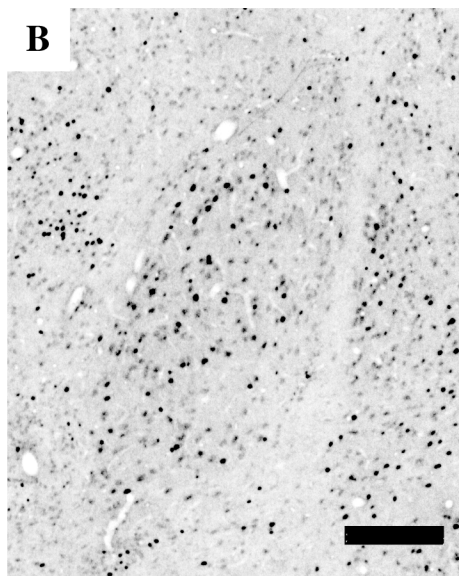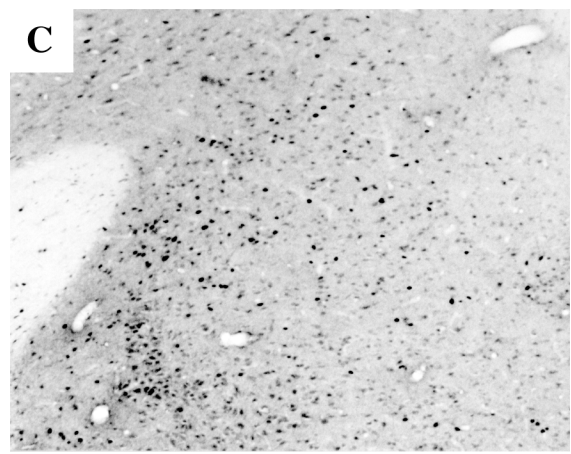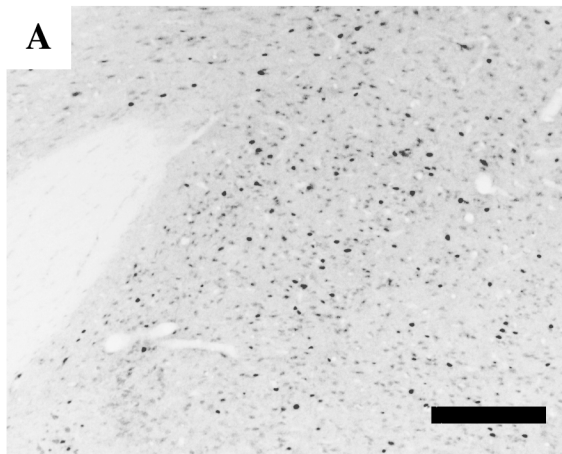
Bregma -0.26mm

LS
[6]
BNSTam   BNSTal
[4] [5]
PVN   LH
[9]   [10]
[7]   [8] LPO
MPA

Bregma -1.80mm

BAOT   ACo
[13] [14]

Bregma -2.30mm

La
[17]
Ce
[16]
MeAD [15]   [18]
BLA

Bregma -2.80mm

MePD
[19]
DMH
[11]
MePV
[12] VMH   [20]

DMPAG
[21] [22]
DLPAG

Bregma -6.30mm

Bregma -8.30mm

VLPAG
[23]

[25]   [24] LPB
LDTg

Bregma -9.30mm

[26] LC

Bregma -9.68mm

Control
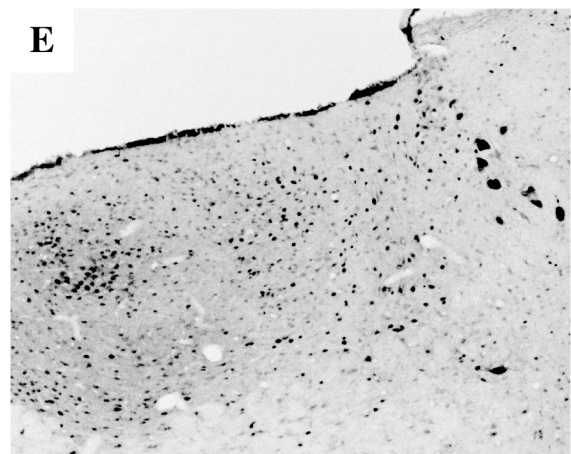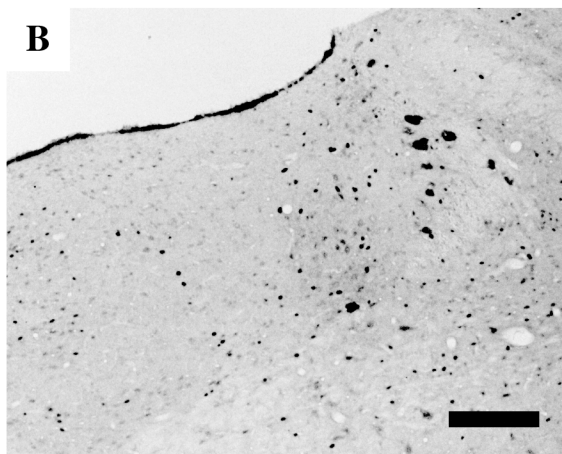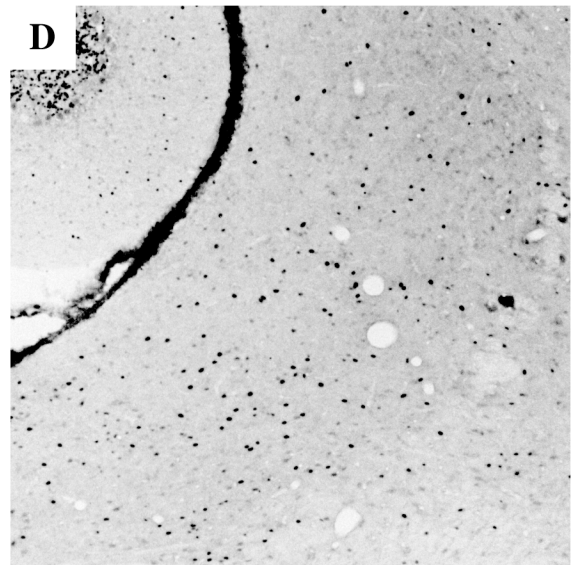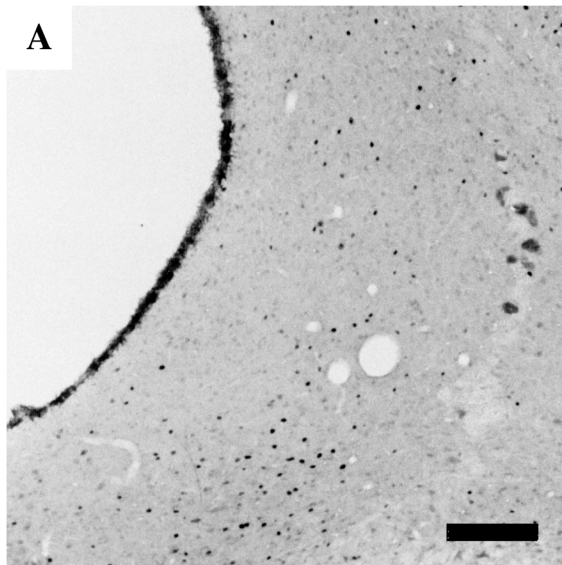Alarm pheromone

Temperature

Activity
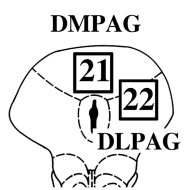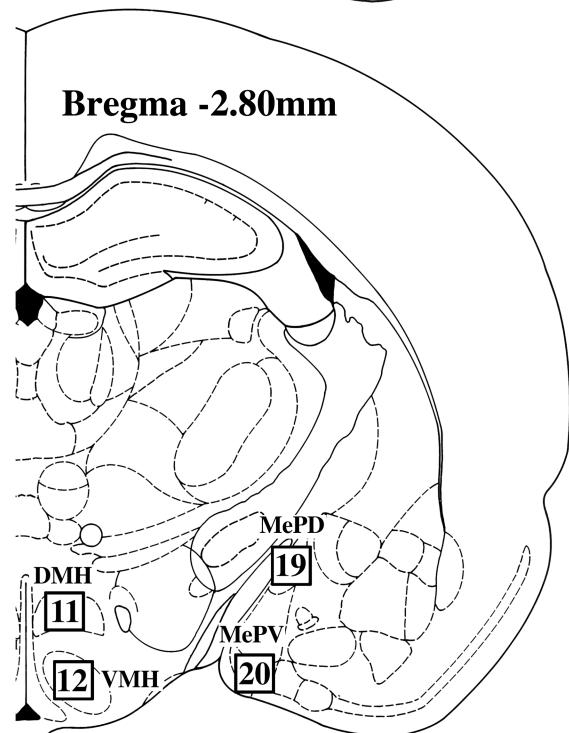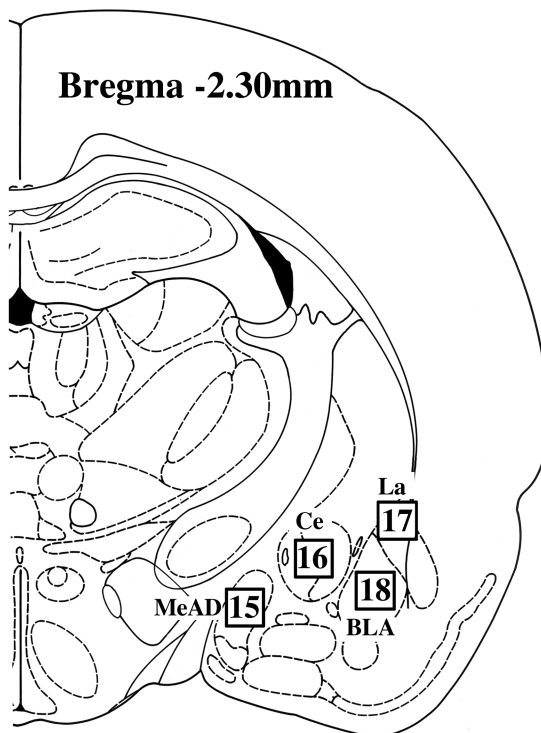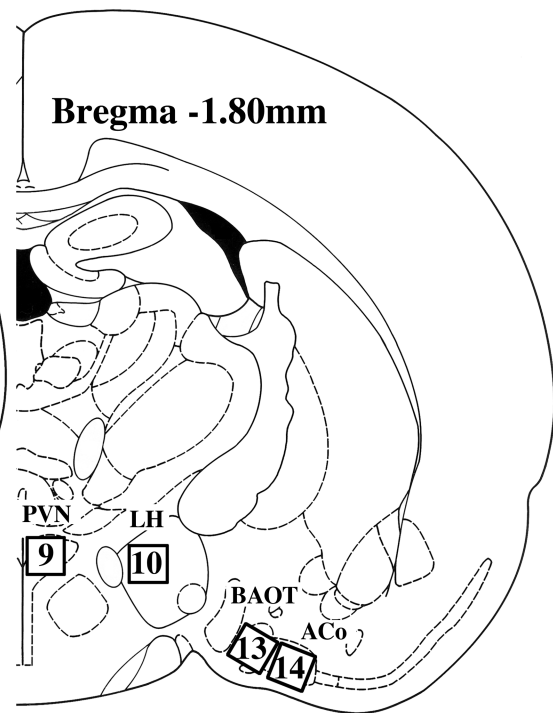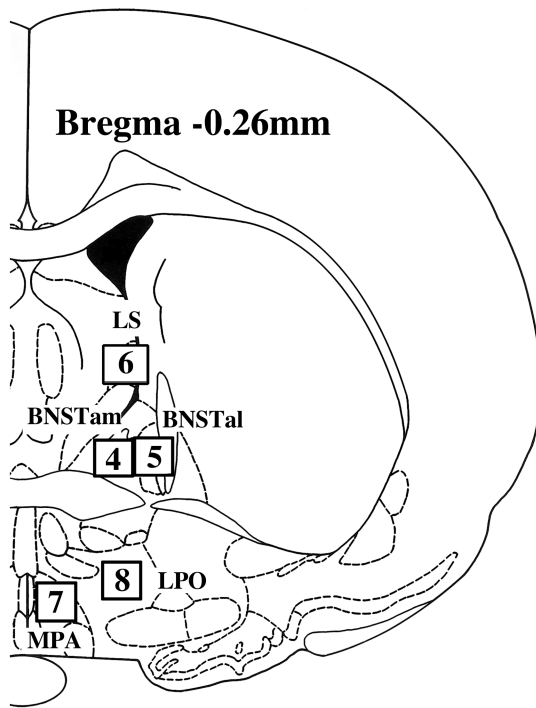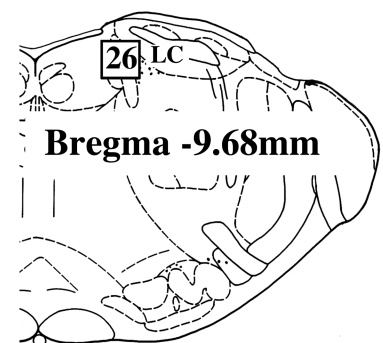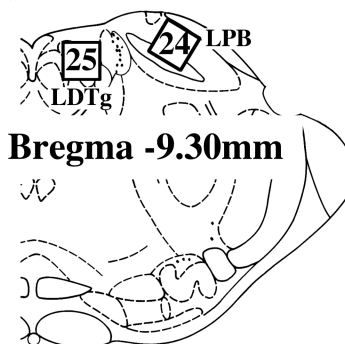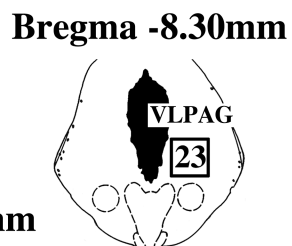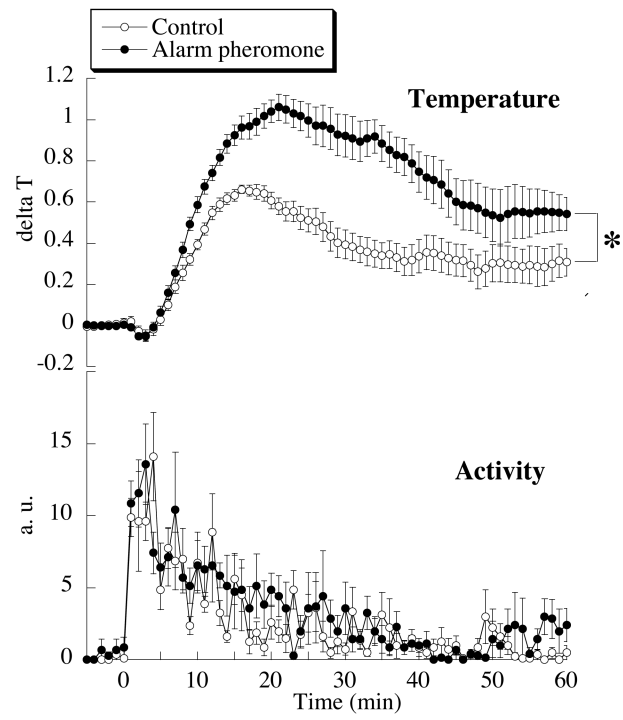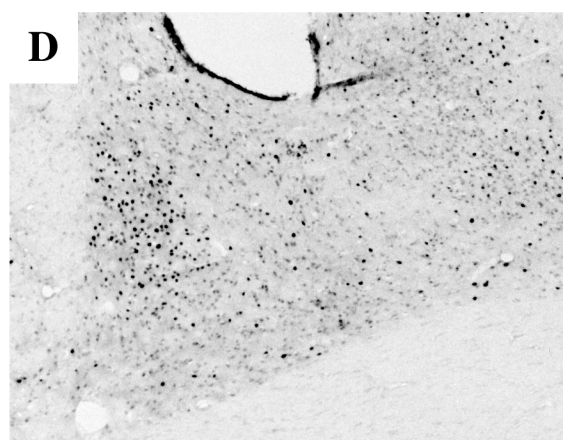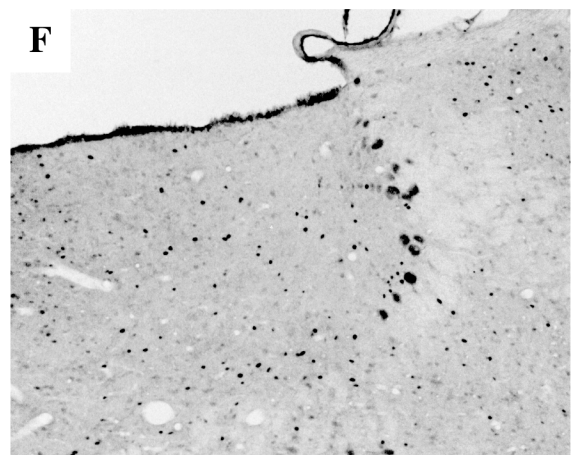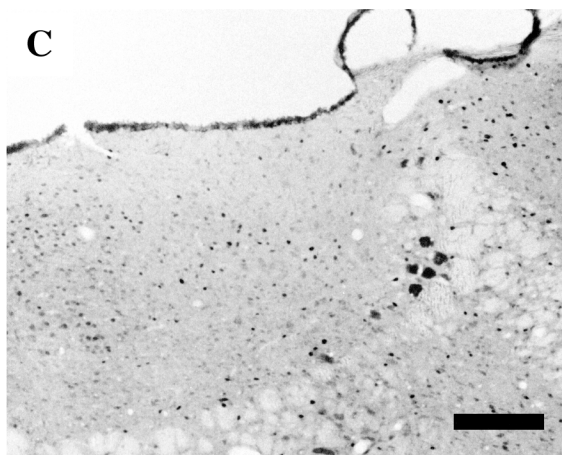
Table 1. Number of Fos immunoreactive cells/0.25 mm^2 in various brain regions of either exposed to control odor or alarm pheromone.

Region	Control (n=8)			Alarm pheromone (n=7)			
Olfactory bulb							
1. Accessory olfactory bulb (mitral/tuffted cell layer)	30.4	±	0.8	30.8	±	1.4	
2. Accessory olfactory bulb (granule layer)	57.4	±	4.0	69.4	±	6.0	
3. Main olfactory bulb	64.1	±	5.5	57.8	±	3.6	
Forebrain and Hypothalamus							
4. Bed nucleus of the stria terminalis, anterior division medial group	14.5	±	1.1	19.6	±	1.8	*
5. Bed nucleus of the stria terminalis, anterior division lateral group	26.4	±	2.3	52.1	±	3.2	*
6. Lateral septal nucleus (ventral)	15.9	±	1.6	20.2	±	2.0	
7. Medial preoptic area	11.3	±	0.9	12.6	±	1.0	
8. Lateral preoptic area	24.5	±	3.0	28.8	±	3.4	
9. Paraventricular nucleus	57.6	±	3.0	84.8	±	5.0	*
10. Lateral hypothalamic area	22.7	±	1.8	28.3	±	2.5	
11. Dorsomedial hypothalamic nucleus	34.0	±	2.6	46.3	±	1.1	*
12. Ventromedial hypothalamic nucleus	8.8	±	1.3	9.8	±	2.1	
Amygdaloid nuclei							
13. Bed nucleus of the accessory olfactory tract	29.8	±	3.5	39.0	±	4.2	
14. Cortical amygdaloid nucleus (anterior)	53.2	±	4.8	61.7	±	4.6	
15. Medial amygdaloid nucleus (anterodorsal)	25.0	±	1.9	33.3	±	1.5	*
16. Central amygdaloid nucleus	19.4	±	3.6	34.4	±	7.3	
17. Lateral amygdaloid nucleus	14.4	±	1.0	22.9	±	2.0	*
18. Basolateral amygdaloid nucleus	21.2	±	1.6	37.1	±	2.5	*
19. Medial amygdaloid nucleus (posterodorsal)	19.6	±	2.6	23.3	±	3.0	
20. Medial amygdaloid nucleus (posteroventral)	37.3	±	3.8	46.6	±	3.1	
Brainstem							
21. Dorsomedial periaqueductal gray	10.7	±	1.3	13.1	±	2.0	
22. Dorsolateral periaqueductal gray	8.5	±	1.6	13.1	±	1.8	
23. Ventrolateral periaqueductal gray	15.4	±	0.6	29.5	±	2.7	*
24. Lateral parabrachial nucleus	25.6	±	2.8	33.4	±	2.8	
25. Laterodorsal tegmental nucleus	12.5	±	0.9	22.4	±	1.3	*
26. Locus coeruleus	4.4	±	0.3	6.8	±	0.5	*

Data are expressed as means ± SEM. The number of subjects is given in parentheses.
*P<0.05 with ANOVA as compared to Control group.