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**5-HT<sub>1A</sub> receptor agonist affects fear conditioning through stimulations of the postsynaptic 5-HT<sub>1A</sub> receptors in the hippocampus and amygdala.**

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## **Abstract**

Evidence from preclinical and clinical studies has shown that 5-HT<sub>1A</sub> receptor agonists have anxiolytic actions. The anxiolytic actions of 5-HT<sub>1A</sub> receptor agonists have been tested by our previous studies using fear conditioning. However, little is known about the brain regions of anxiolytic actions of 5-HT<sub>1A</sub> receptor agonists in this paradigm. In the present study, we investigated the effects of bilateral microinjections of flesinoxan, a selective 5-HT<sub>1A</sub> receptor agonist, into the hippocampus, amygdala and medial prefrontal cortex on the expression of contextual conditioned freezing and the defecation induced by conditioned fear stress in rats. These results reveal that both intrahippocampal and intraamygdala injections of flesinoxan decreased the expression of conditioned freezing, while injections into the medial prefrontal cortex did not. In addition, intraamygdala injection of flesinoxan attenuated the increased defecation induced by conditioned fear, but injections into the hippocampus and medial prefrontal cortex failed. These results suggest that flesinoxan exerts its anxiolytic actions in the fear conditioning through stimulations of the postsynaptic 5-HT<sub>1A</sub> receptors in the hippocampus and amygdala.

**Key words:** Flesinoxan, 5-HT<sub>1A</sub> receptor agonist, fear conditioning, the hippocampus, the amygdala, the medial prefrontal cortex

## 1. Introduction

Serotonergic neurotransmission has an important role in the etiology, pathophysiology and treatment of anxiety disorders. Serotonin (5-HT) transporter is the primary target of the clinically successful class of drugs known as selective serotonin reuptake inhibitors, which have been demonstrated to be effective in the treatment of various anxiety disorders, in addition to depressive disorders (Eriksson and Humble, 1990; Lane et al., 1995). Apart from 5-HT transporter, 5-HT<sub>1A</sub> receptor is another potential target in the treatment of anxiety disorders (De Vry, 1995). 5-HT<sub>1A</sub> receptors are located presynaptically as somatodendritic receptors on the raphe nucleus and postsynaptically in particular limbic and cortical regions (Hoyer et al., 1986; Pazos et al., 1987; Hamon et al., 1990). Clinically, 5-HT<sub>1A</sub> receptor agonists, such as buspirone, have anxiolytic action (Traber and Glaser, 1987; Hindmarch et al., 1992).

Fear conditioning is one of the most extensively studied and reliable behavioral paradigms to clarify the mechanisms involved in fear and anxiety. Much evidence has shown that certain areas of the brain, predominantly the amygdala, hippocampus, and prefrontal cortex, consist of neural circuits responsible for the learning and memory of fear conditioning (Fendt and Fanselow, 1999; Fanselow, 2000). Anatomical and microdialysis studies showed that the hippocampus receives innervation from the median raphe nucleus (McQuade and Sharp, 1997), and 5-HT<sub>1A</sub> receptors have high densities in the hippocampus (Pazos and Palacios, 1985; Verge et al., 1985; Weissmann-Nanopoulos et al., 1985). Moreover, the amygdala, including the basolateral amygdala, also receives dense serotonergic innervations from the dorsal raphe nucleus (Fallon and Ciofi, 1992) and has several subtypes of serotonin receptors, such as 5-HT<sub>1A</sub> receptor (Radja et al., 1991; Wright et al., 1995). In addition, the

medial prefrontal cortex has dense reciprocal connection with the amygdala (McDonald et al., 1996) and lesions of the medial prefrontal cortex enhanced fear conditioning (Morgan and LeDoux, 1995). Studies using this paradigm have shown that 5-HT<sub>1A</sub> receptor agonists, ipsapirone and flesinoxan, reduced the conditioned fear (Rittenhouse et al., 1992; Inoue et al., 1996; Li et al., 2001). Although 5-HT<sub>1A</sub> receptor agonists have an evident role in decreasing anxiety, the synaptic sites of anxiolytic actions (i.e. pre- versus postsynaptic receptors) of 5-HT<sub>1A</sub> receptor agonists remain controversy (Kent et al., 2002) and, especially, little is known about the brain regions of anxiolytic action of 5-HT<sub>1A</sub> receptor agonists in fear conditioning.

The present study was undertaken to elucidate the brain regions where 5-HT<sub>1A</sub> receptor agonists act as anxiolytic in fear conditioning. In the present study, we investigated the effects of bilateral microinjection of flesinoxan, a selective 5-HT<sub>1A</sub> receptor agonist (Bosker et al., 1996), into the hippocampus, amygdala and medial prefrontal cortex on the expression of contextual fear conditioning in rats. To our knowledge, this is the first study to examine whether the anxiolytic effects of 5-HT<sub>1A</sub> receptor agonists are mediated by the postsynaptic receptor in the fear conditioning.

## **2. Method and materials**

### *2.1. Animals*

Male Sprague–Dawley rats obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), weighing 290–320 g at the time of conditioned fear test, were housed in groups of four per cage (38×33×17 cm), and maintained in a 12-h light–dark cycle (light phase: 0630–1830 h), temperature-controlled environment (22 ± 1 °C) with free

access to food and water. Experiments began after a one-week period of acclimatization. All experiments were performed between 0800 and 1300 h, except the surgery. In sum, 85 rats were used in this study, of which 16, 24 and 16 rats were used for the hippocampus, amygdala and medial prefrontal cortex administration, respectively, 14 and 15 rats for the measurement of locomotor activity of the hippocampus and amygdala administration, respectively.

All procedures were approved by the Hokkaido University School of Medicine Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine.

## 2.2. *Surgery*

Surgeries were performed under sodium pentobarbital (40 mg/kg, intraperitoneally) anaesthesia using aseptic conditions. The head position was adjusted to place the bregma and lambda in the same horizontal plane in a stereotaxic frame. Rats were stereotaxically implanted with bilateral 26-gauge stainless steel guide cannulae directed toward the amygdala (the basolateral nucleus of the amygdala), dorsal hippocampus or medial prefrontal cortex [coordinates relative to bregma: AP -2.8 mm, ML  $\pm$ 5.0 mm, V 7.6 mm for the amygdala; AP -2.8 mm, ML  $\pm$ 2.0 mm, V 2.6 mm for the dorsal hippocampus; AP +3.2 mm, ML  $\pm$ 0.5 mm, V 3.0 mm for the medial prefrontal cortex taken from the stereotaxic atlas of Paxinos and Watson (1997)]. When not used for injection, the guide cannulae were occluded with obturators made of 33-gauge stainless steel wire.

### 2.3. Drug

Bilateral infusions were made with 33-gauge injector cannulae connected by polyethylene tubing to motor-driven microsyringes. The microinjector delivered the injection solution 1 mm below the tip of the guide cannula. Flesinoxan (3 µg) (*R(+)-N*-[2[4-(2,3-dihydro-2-2-hydroxymethyl-1,4-benzodioxin-5-yl)-1-piperazinyl]ethyl]-4-fluorobenzoamide, Solvay-Seiyaku, Japan) was dissolved in 0.5 µl saline, and was bilaterally infused through each injector at a rate of 0.5 µl/min for 1 min (3 µg /side). The injectors were left in place for 30 sec after the infusion. Saline alone was administered as a control.

### 2.4. Fear conditioning and behavioral observations

As described previously (Inoue et al., 2004), for fear conditioning, the rats were individually subjected to inescapable electric footshocks for a total of 2.5 min [five footshocks (2.5-mA scrambled shock, 30-s duration) that were delivered at intershock intervals of 35–85 s (mean 60 s)] in a shock chamber with a grid floor (19×22×20 cm, Medical Agent, Japan). Electric shocks were administered with a Model SGS-02D Shock Generator (Medical Agent). This provides a circuit with resistance controlled by dial settings calibrated by the manufacturer in a short circuit current. At the setting of 2.5-mA, this generator gave 0.2-mA shock intensity to the rats (Inoue et al., 2004).

After a recovery period of at least 10 days from the surgery, the rats were contextually conditioned to a shock chamber. Twenty-four hours after fear conditioning, bilateral infusions were done simultaneously by using 33-gauge injector cannulae projecting 1.0 mm beyond the tips of guide cannulae. Ten min after the

infusion, the rats were again placed in the shock chamber and were observed for 5 min without shocks. With these procedures, conditioned fear, as measured by freezing, develops from the contextual stimuli of the conditioned chamber (Fanselow, 1980). During the observation period, freezing behavior was recorded using a time-sampling procedure (Fanselow, 1980) modified as previously described by Inoue et al. (2004). Freezing was defined as the absence of any observable movement of the skeleton and the vibrissae, except those related to respiration. All other behavior was scored as activity. The animal was classified as showing either freezing or active behavior according to its behavior throughout the entire 10-s period. The percentage score (% freezing) represented the number of 10-s periods during which the animal froze for the entire 10 s. During the testing sessions, the number of feces (bolus counts for 5 min) produced by each rat in each chamber was counted.

### *2.5 Motor activity*

To exclude the possibility that flesinoxan administration reduced freezing nonspecifically by increasing spontaneous activity, motor activity was measured in separate experiments. The rats were implanted with bilateral cannulae aimed at the amygdala and hippocampus, respectively. After a recovery period of 10 days, the rats were infused with 3  $\mu$ g flesinoxan or saline 10 min before testing. Their motor activity in Plexiglas boxes (38  $\times$  33  $\times$  17 cm) was recorded as described by Ohmori et al. (1994) automatically for 5 min by electronic digital counters with infrared cell sensors between 0800 and 1300 h. Horizontal movement was digitized and fed into a computer. Locomotion contributed predominantly to the count, but other body movements also contributed to the count when these movements contained substantial horizontal

components.

## *2.6. Data analysis*

All the data are the means  $\pm$  S.E.M of the individual values of the rats from each group. Statistical analysis of the data was conducted by using an unpaired t-test. The statistical significance was set at  $P < 0.05$ .

## *2.7. Histological verification*

Histological verification of injection sites was performed after behavioral testing. The injection sites were confirmed in each rat that received bilateral intrahippocampal or intraamygdala injections of cresyl violet solution. Then, rats were perfused across the heart with 0.1 M Phosphate-buffered saline (PBS) and then, followed by a 4% paraformaldehyde in 0.1 M PBS at pH 7.4. After extraction from the skull, brains were post-fixed in 4% paraformaldehyde in PBS solution for 24 h, at which time the solution was replaced with a 30% sucrose solution until sectioning. The brains of rats then were sectioned at 30  $\mu$ m thickness in a cryostat at  $-10$  °C, wet-mounted on microscope slides. Coronal slices of each rat brain were stained by cresyl violet. The location of injection cannulae placement was determined under a light microscope histologically. Only the data obtained from rats with verified cannulae tip locations were used.

## **3. Results**

### *3.1. Histology*

The photomicrographs in Figure 1 illustrate representative injection sites in the

dorsal hippocampus (A) and the amygdala (B). Figure 2 represents the injection sites for all rats included in the analysis in the dorsal hippocampus (A) and the amygdala (B). Two rats in the intrahippocampal injection and 1 rat in the intraamygdala injection was eliminated before statistical analysis because the cannula placement was misplaced.

### *3.2. Effect of flesinoxan injection into the dorsal hippocampus on the expression of conditioned freezing*

Bilateral flesinoxan injections into the dorsal hippocampus given 10 min before testing significantly reduced conditioned freezing compared with the saline-injection group [ $t(12) = 2.25, P < 0.05$ ] (Fig. 3A). Bilateral flesinoxan injections into the dorsal hippocampus did not significantly affect defecation (Fig. 3C) when compared with the saline group [ $t(12) = 0.45, P = 0.6600$ ].

### *3.3. Effect of flesinoxan injection into the amygdala on the expression of conditioned freezing*

Bilateral flesinoxan injections into the amygdala given 10 min before testing significantly reduced conditioned freezing compared with the saline-injection group [ $t(21) = 2.28, P < 0.05$ ] (Fig. 3B). Bilateral flesinoxan injections into the amygdala also significantly decreased defecation (Fig. 3D) when compared with the saline group [ $t(21) = 2.12, P < 0.05$ ].

### *3.4. Effect of flesinoxan injection into the medial prefrontal cortex on the expression of conditioned freezing*

Bilateral injection of flesinoxan into the medial prefrontal cortex given 10 min

before testing did not change conditioned freezing [ $t(14) = 0.72, P=0.4865$ ] or the defecation [ $t(14) = 0.30, P=0.7703$ ].

### 3.5. Motor activity

Local applications of flesinoxan into the hippocampus and amygdala given 10 min before testing failed to affect motor activity during the 5-min testing period compared with saline controls [Saline  $791.7 \pm 102.7$  counts, flexinoxan  $717.6 \pm 71.4$  counts in hippocampus administration,  $t(12) = 0.59, P=0.5650$ ; Saline  $806.1 \pm 91.6$  counts, flesinoxan  $779.0 \pm 97.6$  counts in amygdala administration,  $t(13) = 0.20, P=0.8425$ ].

## 4. Discussion

In the present study, we investigated the effects of direct injections of flesinoxan, a selective 5-HT<sub>1A</sub> receptor agonist, into the amygdala, hippocampus and medial prefrontal cortex on the expression of contextual fear conditioning. These results revealed that both intrahippocampal and intraamygdala injection of flesinoxan decreased the expression of conditioned fear, but did not affect locomotion which is consistent with our previous study (Li et al., 2001), indicating that the deficits in the expression of conditioned fear by flesinoxan are caused by its anxiolytic action, but are not due to increased spontaneous motor activity induced by flesinoxan, which may affect freezing nonspecifically.

5-HT<sub>1A</sub> receptors show high densities in the hippocampus including CA1, CA3 and dentate gyrus (Khawaja 1995; Raurich et al., 1999). Much evidence indicates that

5-HT<sub>1A</sub> receptors in the dorsal hippocampus contribute to the anxiolytic actions in several animal models of anxiety, such as the Vogel- and Geller-conflict tests (Kataoka et al., 1991; Schreiber et al., 1993; Przegalinski et al., 1994), light/dark exploratory model (Lopez-Rubalcava and Saldivar, 1992), the open-field and elevated plus-maze tests (Kostowski et al., 1989; Menard and Treit 1998). In addition, the hippocampus has also been suggested to be an important site in neural circuits in relation to Pavlovian conditioning (Fanselow, 2000). Lesions of the hippocampus abolished the expression of contextual fear conditioning significantly (Kim and Fanselow, 1992). Until now, there are few studies to investigate the role of 5-HT<sub>1A</sub> receptors of the hippocampus in fear conditioning. Stiedl and colleagues (2000) have microinjected a 5-HT<sub>1A</sub> receptor agonist, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), into the bilateral hippocampus in the conditioned fear in mice, showing that 8-OH-DPAT reduced the acquisition of fear conditioning. However, in their study, posttraining subcutaneous 8-OH-DPAT did not affect the expression of fear conditioning, which seems to be inconsistent with our results. One possible reason for the difference may be that administrations of 8-OH-DPAT in their study were performed immediately after training and 1h or 24 h before testing, so the time course of 8-OH-DPAT administration may reflect its effect on the consolidation of fear-related memory, rather than on the expression as measured in our study. Thus, it is concluded that 5-HT<sub>1A</sub> receptor agonists may affect both the acquisition and expression of fear conditioning via stimulations of the postsynaptic receptors in the hippocampus.

The amygdala has an essential role in the development and expression of conditioned fear (LeDoux, 2000), and its damage produces deficits in fear conditioning in rodents (LeDoux, 2000) and humans (Bechara et al., 1995; LeDoux, 2000). As with

the hippocampus, the amygdala also receives dense serotonergic innervations from the raphe nucleus (Fallon and Ciofi, 1992). However, results obtained from studies of animal models of anxiety about the role of 5-HT<sub>1A</sub> receptors in the amygdala are inconsistent, or opposite. 5-HT<sub>1A</sub> receptor agonists infused directly into the basolateral/lateral amygdala showed anxiogenic-like effects in the Geller-type conflict test (Hodges et al., 1987) and the social interaction test (Gonzalez et al., 1996), but were anxiolytic in shock-induced ultrasonic vocalization (Schreiber and De Vry, 1993). On the other hand, intra-basolateral amygdala injection of 8-OH-DPAT did not produce any significant behavioral changes in the elevated plus maze (Gonzalez et al., 1996). The different responses to 5-HT<sub>1A</sub> receptor agonists in various animal models of anxiety may indicate that these paradigms reflect different states of anxiety (Borsini et al., 2002). In the present study, flesinoxan reduced the expression of conditioned fear through stimulations of the postsynaptic 5-HT<sub>1A</sub> receptors in the amygdala. These results are consistent with the previous studies, which showed that systemic administration of 5-HT<sub>1A</sub> receptor agonists have anxiolytic actions in the conditioned fear (Rittenhouse et al., 1992; Inoue et al., 1996; Li et al., 2001). Moreover, the present results are partly supported by our recent findings showing that intraamygdala injections of citalopram, a selective serotonin reuptake inhibitor, impaired the expression of fear conditioning, because the anxiolytic actions of citalopram are considered to be due to an increase in extracellular concentrations of 5-HT through blockage of the 5-HT transporter, which consequently strengthened stimulations of the postsynaptic receptors, including 5-HT<sub>1A</sub> receptors (Inoue et al., 2004).

In this study, intraamygdala injections of flesinoxan significantly attenuated the increased defecations, as another index of fear, induced by conditioned fear. This

result is supported by the findings which showed that ipsapirone and buspirone, 5-HT<sub>1A</sub> receptor agonists, decrease the defecations induced by conditioned fear (Rittenhouse et al., 1992; Krysiak et al., 2000). In addition, lesions of the amygdala also diminish the defecations elicited by conditioned fear (Li et al., 2004). The intrahippocampus injections of flesinoxan, however, failed to affect the defecation. Taken together, it seems to suggest that 5-HT<sub>1A</sub> receptors in the amygdala participate in autonomic neural responses, whereas 5-HT<sub>1A</sub> receptors in the hippocampus do not participate, at least, in defecation.

Until now, the anxiolytic actions of 5-HT<sub>1A</sub> receptor agonists have been primarily ascribed to the activation to the presynaptic autoreceptors in the raphe nucleus (De Vry, 1995). For example, electrolytic and neurotoxic lesions of the median raphe nucleus disrupt the acquisition of freezing response in contextual conditioning fear (Avanzi et al., 1998; Avanzi and Branda 2001). The acute stimulations of the presynaptic receptors by 5-HT<sub>1A</sub> receptor agonists reduce the neuronal firing of serotonergic neurons (Blier and de Montigny, 1987; Sprouse and Aghajanian, 1987), which leads to the view that decreased serotonin neurotransmission is anxiolytic (De Vry, 1995). However, our previous study showed that the anxiolytic actions of 5-HT<sub>1A</sub> receptor agonists were mediated by stimulations of postsynaptic serotonin receptors (especially 5-HT<sub>1A</sub> receptors) in the nerve terminal areas but not in the raphe nucleus (Inoue et al., 1996), suggesting the postsynaptic 5-HT<sub>1A</sub> receptors are also involved in the anxiolytic action. This view is supported by the finding in various animal model of anxiety mentioned above and the results in this study, suggesting that the anxiolytic effects of 5-HT<sub>1A</sub> receptor agonists are mediated by both pre- and postsynaptic 5-HT<sub>1A</sub> receptors (De Vry et al., 1992).

In conclusion, the present study showed that flesinoxan exerts its anxiolytic actions in the fear conditioning through stimulations of the postsynaptic 5-HT<sub>1A</sub> receptors in the hippocampus and amygdala. These results extend our understanding of the mechanisms of action of 5-HT<sub>1A</sub> receptor agonists in the treatment of anxiety disorders.

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## Figure Legends

Fig.1. Injection sites in the dorsal hippocampus and amygdala. Photomicrograph showing cresyl violet-stained coronal sections from the brain of a rat with representative injection sites in the dorsal hippocampus (A) and amygdala (B), respectively.

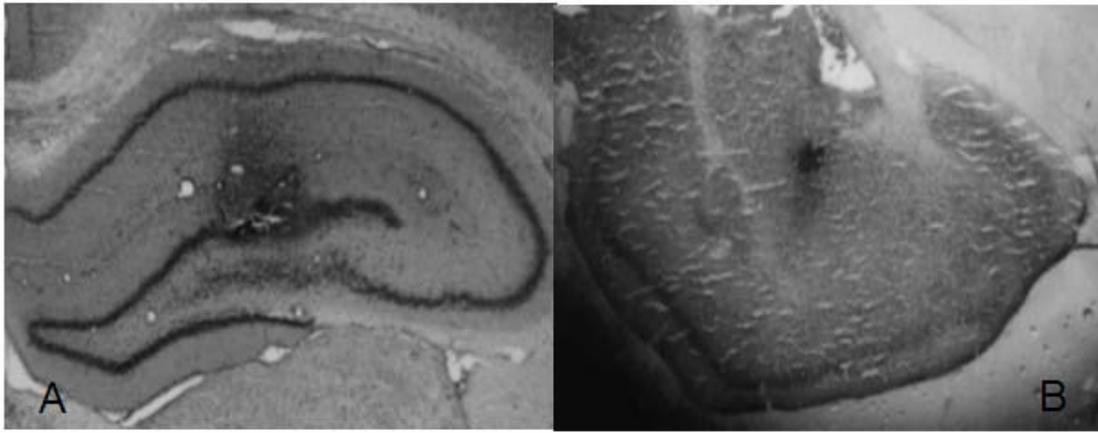


Fig.1 Li

Fig. 2. Coronal sections through the dorsal hippocampus (A) and amygdala (B) from the atlas of Paxinos and Watson (1997; 2.56–2.8 mm posterior to bregma). Solid circles represent location of bilateral injection cannula tips, which are from all rats included in the final analysis.

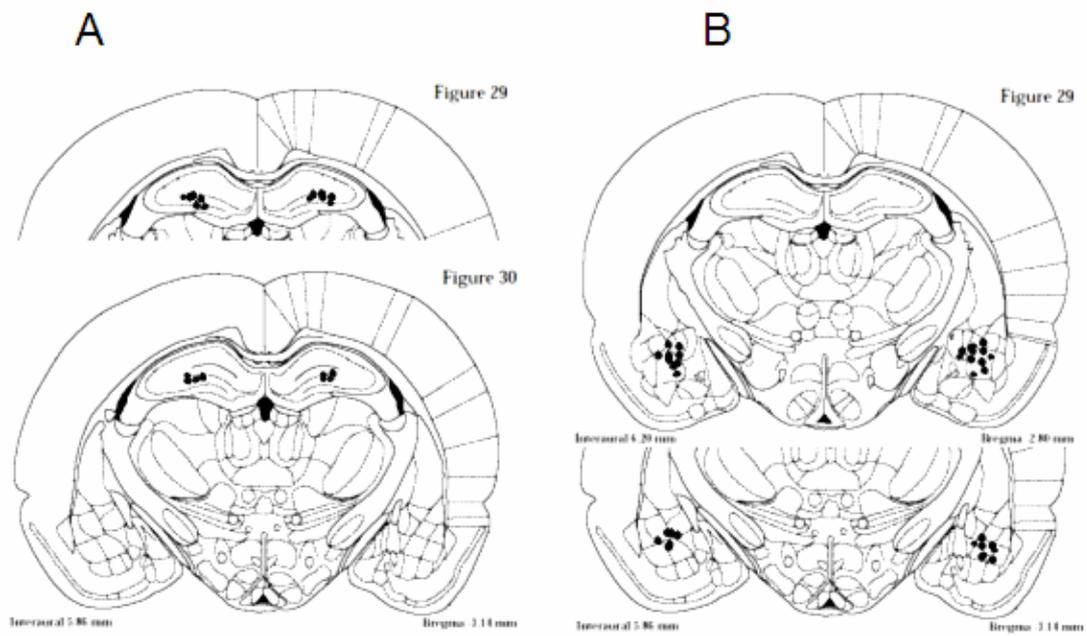
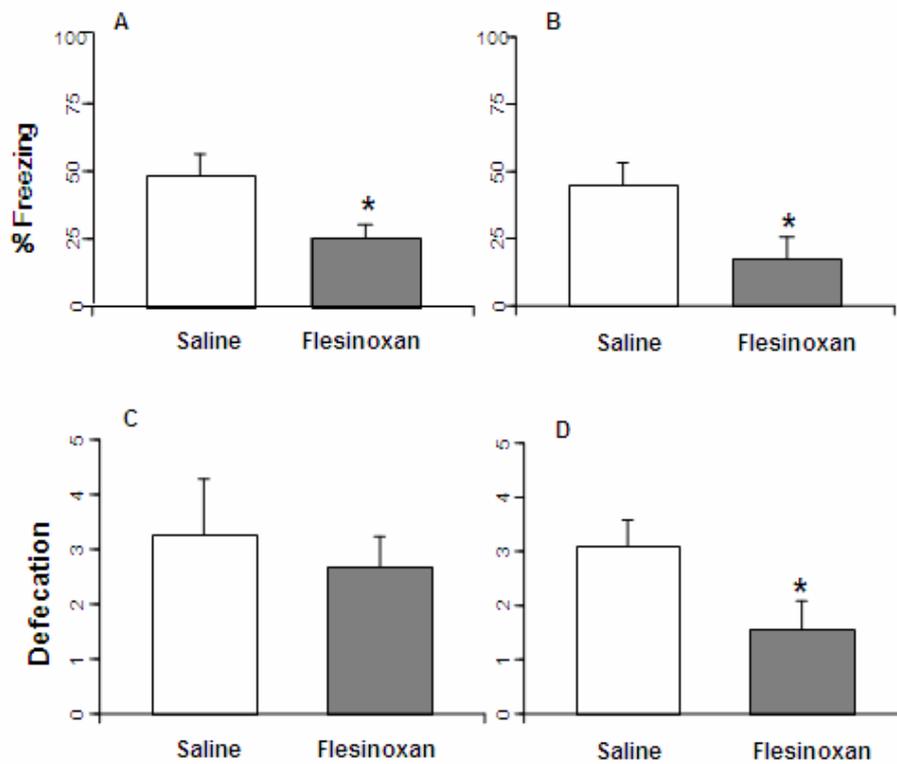


Fig.2 Li

Fig. 3. Effect of bilateral flesinoxan microinjections (3  $\mu\text{g}/\text{site}$ ) into the hippocampus (A, C) and amygdala (B, D) on freezing and defecation induced by conditioned fear. Flesinoxan was administered 24 h after footshock and 10 min before conditioned fear stress. Freezing scored over a 5-min observation period is represented as mean percentage  $\pm$  S.E.M. (A and B), while defecation is expressed as mean bolus counts  $\pm$  S.E.M. (C and D). Behavior was sampled at 10-s intervals. (A) \* $P < 0.05$  vs. saline controls. In (A) and (C),  $N=6-8$  rats; in (B) and (D),  $N=11-12$  rats.



**Fig. 3 Li**