Title: The Serotonergic Projection from the Median Raphe Nucleus to the Ventral Hippocampus is Involved in the Retrieval of Fear Memory via the Corticotropin-Releasing Factor Type 2 Receptor

Abbreviated title: Median Raphe-Hippocampus and Fear Memory

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Abstract

Several different studies have separately established that serotonin, corticotropin-releasing factor (CRF) receptors, and the hippocampus are involved in fear memory retrieval. The main aim of the present study is to connect these separate studies. To assess the levels of anxiety/fear, we employed the contextual fear conditioning test and the elevated plus maze test as memory-dependent and -independent tasks, respectively. We injected CRF receptor antagonists or vehicle into the median raphe nucleus (MRN) 10 minutes before behavioral tests. As a result, 1000 ng of astressin 2B (CRF_2 receptor antagonist), but not 250 ng of antalarmin (CRF_1 receptor antagonist), significantly suppressed the expression rate of freezing behavior in the contextual fear conditioning test. However, in the elevated plus maze test, there was no difference between astressin 2B-injected rats and saline-injected rats in time spent in the open arms. Neither the amount of exploratory behavior nor the moving distance in the EPM of astressin 2B-injected rats differed from that of vehicle-injected rats. Moreover, when we assessed the extracellular serotonin release in the ventral hippocampus in freely moving rats through *in vivo* microdialysis, it was shown that the blockade of the CRF_2 receptor in the MRN suppressed serotonin release in the ventral hippocampus during fear memory retrieval. These results indicated that endogenous CRF and/or related ligands released in the MRN could activate the CRF_2 receptor and stimulate serotonin release in the ventral hippocampus, thereby inducing fear memory retrieval.

Keywords: corticotropin releasing hormone; hippocampus; fear; memory; 5-HT; median raphe nucleus

INTRODUCTION

The retrieval of contextual fear memory is important in avoiding a previously encountered threat to life. However, patients with mental disorders, such as posttraumatic stress disorder and panic disorder, are often troubled by inappropriate retrieval of fear memory. Thus, the neural mechanism underlying fear memory retrieval should be elucidated to explore more efficient clinical treatments of these mental disorders.

Given that selective serotonin reuptake inhibitors are effective for treatment of posttraumatic stress disorder (Zohar and Westenberg, 2000; Irons, 2005; Robert *et al.*, 2006), serotonin is a strong candidate for involvement in the control of fear memory retrieval. In addition, it is well known that the hippocampus is a major brain region regulating contextual fear memory (Holt and Maren, 1999; Trivedi and Coover, 2004). However, to date there is only one study demonstrating the relationship between serotonin release in the ventral hippocampus and fear memory retrieval (Wilkinson *et al.*, 1996). Moreover, it remains unknown which brain regions and neurotransmitters modulate serotonin release in the hippocampus during the retrieval of fear memory.

In the present study, we focused on the median raphe nucleus (MRN), one of the origins of serotonergic projections to the forebrain. Avanzi *et al.* (1998) showed that the electrolytic lesion of the MRN remarkably reduced freezing behavior, which is a measure of fear memory retrieval. Moreover, the serotonergic neurons in the MRN project heavily to the hippocampus (Azmitia and Segal, 1978). Nevertheless, over the last 10 years, the involvement of the MRN in serotonin release in the hippocampus during the retrieval of fear memory and what modulates the activity of the MRN have remained to be elucidated, probably because of the difficulty of microinjection into a deeply located and small nucleus such as the MRN. This does not mean, however, that the MRN has a negligible role in fear memory retrieval.

One of the candidates for endogenous modulator of MRN activity is the

corticotropin-releasing factor (CRF), which is a 41-amino acid neuropeptide (Vale *et al.*, 1981). CRF_1 and CRF_2 receptors have thus far been identified in mammals (Hauger *et al.*, 2003), and it has been shown that both CRF_1 and CRF_2 receptor mRNAs are expressed in the MRN (Bittencourt and Sawchenko, 2000). Moreover, several psychiatric disorders, such as major depression (Nemeroff *et al.*, 1984) and posttraumatic stress disorder (Baker *et al.*, 1999), have been associated with increased concentrations of CRF in the cerebrospinal fluid.

Therefore, we examined the relationship between CRF receptors in the MRN and retrieval of fear memory by using CRF receptor antagonists. To assess the levels of anxiety/fear, we employed the contextual fear conditioning test and the elevated plus maze test as memory-dependent and -independent tasks, respectively. To discriminate the effects of CRF antagonists on the MRN from the effects of those on the dorsal raphe nucleus (DRN), which is another origin of serotonergic projections to the forebrain, we injected an effective antagonist into the DRN. Moreover, we examined the effects of an effective antagonist on extracellular serotonin release in the ventral hippocampus during fear memory retrieval by using *in vivo* microdialysis.

MATERIALS AND METHODS

Animals

The subjects were male adult Wistar rats (10-13 weeks old) supplied by Nippon SLC Co., Ltd. (Hamamatsu, Japan). They were housed in groups of two or three rats under an alternating light-dark cycle (light from 7 p.m. to 7 a.m.) at approximately 21 °C. All testing was performed in the dark period. The treatment of animals complied with the guidelines for the care and use of laboratory animals of the Animal Research Committee of the Hokkaido University Graduate School of Medicine.

Drugs

Antalarmin, a CRF_1 receptor antagonist, was dissolved in a solution of 5% camphor, 5% ethanol, and 90% saline. Astressin (nonselective CRF receptor antagonist) and astressin 2B (CRF_2 receptor antagonist) were dissolved in saline containing 0.1% bovine serum albumin (BSA). All drugs were purchased from Sigma (St. Louis, MO, USA). The doses of each antagonist were as follows: antalarmin, 250 ng; astressin, 250 ng; and astressin 2B, 1000 ng in 0.5 μl vehicle. These doses were determined based on previous studies (Sajdyk and Gehlert, 2000; Henry *et al.*, 2006; Lukkes *et al.*, 2008).

Surgical Procedure

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). Stainless-steel guide cannula (24 gauge, 13.5 mm long) was implanted 2 mm above the target sites at a 22° angle. The stereotaxic coordinates for the DRN and MRN were as follows: 7.8 mm posterior to the bregma (both sites), 2.6 and 2.9 mm lateral to the midline, 5.9 and 8.4 mm ventral to the dura, respectively (Paxinos and Watson, 2004). For microdialysis experiments (Experiment 4), a guide cannula (AG-8, Eicom Co. Ltd., Japan) was implanted into the ventral hippocampus: 5.3 mm posterior to the bregma, 5.0 mm lateral to the midline, and 4.2 mm ventral to the dura, in addition to the guide cannula for the intra-MRN injection. After surgery, rats were housed individually and allowed a 1-week recovery period prior to testing.

Microinjection Procedure

Ten minutes before the start of behavioral tests, the CRF antagonist or vehicle was injected into the MRN or DRN with a Hamilton microsyringe using a 30-gauge stainless-steel injector (15.5 mm long) attached to a polyethylene tube. The solution (0.5 μl) was infused over a period of 1

min at constant flow by a microinjection pump (CMA100, Carnegie Medicine, Sweden), and the injector was left in place for 1 min after injection to allow diffusion.

In Vivo Microdialysis

A dialysis probe (3mm long and 0.22 mm in outer diameter; A-I-8-03, Eicom Co. Ltd., Japan) was inserted through the guide cannula. The probe was perfused with artificial cerebrospinal fluid (aCSF) (2.7 mM KCl, 140 mM NaCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.3 mM NaH_2PO_4, and 1.7 mM Na_2HPO_4, pH 7.2) at a flow rate of 2 μl/min. Rats were placed in plastic observational cages (30×30×35 cm) and samples were collected every 10 min. After the serotonin levels were stabilized, three baseline samples were collected. It took at least 3 hours until serotonin levels were stabilized. The average of these samples was used as baseline. Intra-MRN drug injections were then conducted.

Serotonin and Dopamine Analysis

Serotonin and dopamine concentrations were measured in dialysates by using HPLC (Eicompak PP-ODS® 4.6 mm i.d. × 30 mm: Eicom) with electrochemical detection (ECD-300®, Eicom), as described previously (Yoshioka *et al.*, 1995; Matsumoto *et al.*, 2005, 2008). The mobile phase, which consisted of 2.1 mM sodium 1-decansulfonate, 0.1 mM EDTA-2Na/0.1 M phosphate buffer (pH 6.0), and 1% (v/v) methanol, was pumped at a rate of 1 ml/min. Data are expressed as the percentage of baseline, which was calculated as the average of three consecutive dialysates before drug injections. Areas under the curve (AUC) values for serotonin levels during the 20–40 min period were calculated.

Contextual Fear Conditioning Test

Each rat was acclimated in a footshock box (30.5×24.1×21.0 cm, Med Associates Inc.) for 5

min. This was followed by ten 2 s footshocks (shock intensity, 0.5 mA) administered at 30 s intervals, except for the no-footshock controls, which did not receive footshocks. After the last footshock, rats were returned to their home cage. Twenty-four hours later, drug injections were conducted. Ten minutes after the drug injections, the each rat was returned to the footshock box without being shocked. Freezing behavior was defined as a lack of movement except for respiration, accompanied by an arched back and retraction of the ears (Fanselow, 1980), and used as a measure of fear memory retrieval. In the 15-min testing period (Experiment 1, 2) or 30-min testing period (Experiment 4), the presence or absence of freezing was estimated by an automatic system (FreezeFrame, Actimetrics, USA) using a pixel difference method. The concordance between this automatic system and trained human observers is greater than 90%. (Actimetrics, USA). In Experiment 4, after the testing period, rats were returned to observational cages to continue microdialysis.

Elevated Plus Maze Test

The apparatus was made of wood and consisted of two open arms (50 x 10 cm) and two closed arms (50 x 10 cm) that extended from the central platform (10 x 10 cm). Closed arms were surrounded by 40 cm-high side walls. The maze was elevated 50 cm above the floor, and the illumination of the room was set to 200 lux. Rats were placed on the central platform facing an open arm. The behavior of each rat was monitored by a CCD camera over a 5-min testing period; and the number of entries for each arm, the distance moved in the maze, and the time spent in each arm were recorded and automatically analyzed by a software package (LimeLight, Actimetrics, USA). The total number of entries into the four arms and the distance moved in the maze were used as measures of locomotor activity. The time spent in and number of entries into the open arms were used as measures of memory-independent fear because rats innately avoid open spaces (Treit *et al.*, 1993). In this case, it is a little difficult to regard these parameters as an

index of anxiety because rats clearly avoid a specific situation. Although it may be a controversial issue, we regarded these parameters as measures of memory-independent fear in the present study. The time spent in the open arms was quantified as a percentage of the total time spent in the four arms. The number of entries into the open arms was quantified as a percentage of the total number of entries into the four arms.

Verification of Cannula and Dialysis Probe Placements

After the completion of experiments, rats were sacrificed under deep anesthesia (Urethane, 2 g/kg, i.p.). The brain was rapidly removed and frozen in liquid nitrogen. Coronal sections (50 μm thick) were cut on a cryostat and thaw-mounted onto slides. After drying, the sections were stained with toluidine blue, and cannula placements were verified under a microscope according to the atlas (Paxinos and Watson, 2004). Only data from rats with correct injection needle and probe placements were included in the final analysis (Figure 1).

Experiment 1: Effects of the Injection of CRF Receptor Antagonists into the MRN on Memory-Dependent Fear in the Contextual Fear Conditioning Test

To examine whether endogenous CRF in the MRN is involved in the retrieval of fear memory and to determine the subtype of CRF receptors in the MRN responsible for the effect of CRF on the retrieval of fear memory, we injected CRF receptor antagonists (antalarmin, 250 ng; astressin, 250 ng; and astressin 2B, 1000 ng) or vehicle (0.5 μl) into the MRN 10 minutes before the 15-min contextual fear conditioning test.

Experiment 2: Effects of the Injection of CRF_2 Receptor Antagonist into the DRN on Memory-Dependent Fear in the Contextual Fear Conditioning Test

To discriminate the effects of CRF antagonists on the MRN from the effects of those on the

DRN, we injected an effective antagonist (astressin 2B, 1000 ng) or vehicle (0.5 μl) into the DRN 10 minutes before the 15-min contextual fear conditioning test.

Experiment 3: Effects of the Injection of CRF_2 Receptor Antagonist into the MRN on Memory-Independent Fear in the Elevated Plus Maze Test

To discriminate fear memory retrieval (freezing behavior) from the increase in locomotor activity and memory-independent fear responses, we further employed the elevated plus maze test. We injected an effective antagonist (astressin 2B, 1000 ng) or vehicle (0.5 μl) into the MRN 10 minutes before the 5-min elevated plus maze test.

Experiment 4: Effects of Intra-MRN Injection of CRF_2 Receptor Antagonist on Extracellular Serotonin Release in the Ventral Hippocampus during Fear Memory Retrieval

To examine whether the CRF_2 receptor modulates extracellular serotonin release in the hippocampus during the retrieval of fear memory, we used *in vivo* microdialysis in freely moving rats. We injected the CRF_2 receptor antagonist (astressin 2B, 1000 ng) or vehicle (0.5 μl) into the MRN 10 minutes before the 30-min contextual fear conditioning test. To discriminate the effects of fear memory retrieval on serotonin release from the effects of unconditioned stimuli, such as handling and exposure to the footshock box, on serotonin release, we further employed no-footshock controls.

Data Analysis

In Experiment 1-3, comparisons between groups were performed by one-way analysis of variance (ANOVA). In Experiment 4, two-way repeated ANOVA was also conducted to examine the time effects and the treatment effects on freezing behavior. For microdialysis data

in Experiment 4, one-way ANOVA was employed. Multiple comparisons with Bonferroni's correction were also conducted following each ANOVA. The alpha level was set at 0.05 for all comparisons. All statistical procedures were conducted using SPSS (version 15.0 J).

RESULTS

Experiment 1: Effects of the Injection of CRF Receptor Antagonists into the MRN on Memory-Dependent Fear in the Contextual Fear Conditioning Test

The effect of antalarmin on freezing behavior was not significant (Figure 2A, see also Supplementary Figure S2), indicating that the CRF_1 receptor in the MRN is not involved in the retrieval of fear memory. However, one-way ANOVA indicated a significant main effect of intra-MRN injections of CRF antagonists (astressin and astressin 2B) on freezing behavior ($F(2, 24)=14.81$, $P<0.01$, see Figure 2C). Moreover, post hoc comparisons showed that astressin and astressin 2B significantly suppressed freezing behavior ($P<0.05$, see Figure 2C), indicating that the CRF_2 receptor in the MRN is involved in the retrieval of fear memory. The locations of the cannula placements within the MRN are shown in Figure 1A. The typical injection site is shown in Supplementary Figure S1.

Experiment 2: Effects of the Injection of CRF_2 Receptor Antagonist into the DRN on Memory-Dependent Fear in the Contextual Fear Conditioning Test

The effect of intra-DRN injection of astressin 2B (1000 ng) on freezing behavior was not significant (Figure 2B), indicating that the effects of intra-MRN injection of astressin 2B described above were not due to the leakage of astressin 2B to the DRN. The locations of the cannula placements within the DRN are shown in Figure 1A.

Experiment 3: Effects of the Injection of CRF_2 Receptor Antagonist into the MRN on

Memory-Independent Fear in the Elevated Plus Maze Test

In the present study, none of the parameters in the elevated plus maze test (time spent in the open arms, number of entries into the open arms, total number of entries into the four arms, and distance moved in the maze) were significantly affected by intra-MRN injection of astressin 2B (Table 1). The dose of astressin 2B (1000 ng) that had affected freezing behavior did not alter memory-independent fear expression or locomotor activity.

Experiment 4: Effects of Intra-MRN Injection of CRF_2 Receptor Antagonist on Extracellular Serotonin Release in the Ventral Hippocampus During Fear Memory Retrieval

One-way ANOVA indicated a significant main effect of treatment conditions on extracellular serotonin release in the ventral hippocampus ($F(2, 15)=22.52$, $P<0.001$, see Figure 3). Post hoc comparisons showed that the area under the curves (AUC) during re-exposure to the footshock box in the saline-treated footshock group was significantly higher than that of the saline-treated no-footshock controls ($P<0.05$, see Figure 3), consistent with a previous study (Wilkinson *et al.*, 1996). Moreover, the increase in serotonin release was significantly attenuated by the intra-MRN injection of astressin 2B ($P<0.05$, see Figure 3). The locations of the probe placements within the ventral hippocampus are shown in Figure 1B.

Experiment 4: Effects of the Injection of CRF_2 Receptor Antagonist into the MRN on Memory-Dependent Fear in the Contextual Fear Conditioning Test with Microdialysis

Two-way ANOVA revealed a significant time effect ($F_{2, 20} = 16.59$, $p < 0.001$, see Figure 4) and a significant time × conditions interaction ($F_{2, 20} = 6.44$, $p < 0.01$, see Figure 4) on freezing behavior, while a significant main effect of conditions was not observed. Post hoc comparisons showed that intra-MRN injections of astressin 2B suppressed freezing behavior only in the 0-10

min phase (P<0.05, see Figure 4).

DISCUSSION

In the present study, intra-MRN injections of astressin 2B (CRF_2 receptor antagonist) as well as astressin (nonselective CRF receptor antagonist) significantly suppressed memory-dependent fear expression in the contextual fear conditioning test, while neither intra-DRN injection of astressin 2B nor intra-MRN injection of antalarmin (CRF_1 receptor antagonist) had any effect. Although it seems that intra-DRN injection itself affected freezing behavior (Figure 2), it was likely due to damaging the ventral periaqueductal gray (De Oca *et al.*, 1998; Vianna *et al.*, 2001). Further, intra-MRN injection of astressin 2B did not affect memory-independent fear expression or locomotor activity in the elevated plus maze test. Ohmura *et al.* (2008) showed that intra-MRN injection of CRF did not affect memory-independent fear expression or locomotor activity in the elevated plus maze test. Andrade and Graeff (2001) demonstrated that serotonergic lesion of the MRN did not affect memory-independent fear expression in the elevated plus maze test, and Andrade *et al.* (2004) showed that serotonergic lesion of the MRN affected conditioned fear, but not unconditioned fear, in the T-maze test. The present findings are consistent with these previous studies. The results indicate that endogenous CRF could facilitate the retrieval of fear memory via the activation of the CRF_2 receptor. This is the first study to indicate the involvement of endogenous CRF within the MRN in fear memory retrieval and to identify the subtype of CRF receptors in the MRN responsible for fear memory retrieval.

We also demonstrated that serotonin release in the ventral hippocampus during the retrieval of fear memory increased, consistent with one previous study (Wilkinson *et al.*, 1996). Moreover, we found that the increase in serotonin release in the ventral hippocampus was significantly attenuated by intra-MRN injection of astressin 2B. Given that several previous studies have shown that the ventral hippocampus is involved in fear memory retrieval (Trivedi

and Coover, 2004; Hobin *et al.*, 2006; Burman *et al.*, 2006), these results indicate that endogenous CRF release could activate the CRF_2 receptor in the MRN and stimulate serotonin release in the ventral hippocampus, and thereby induce fear memory retrieval. This is the first study to indicate that the serotonergic projection from the MRN to the ventral hippocampus is involved in the retrieval of fear memory via the CRF_2 receptor in the MRN.

Supplemental data also support our findings. A higher dose of antalarmin did not show clear effects on fear memory retrieval (Figure S2). When we analyzed the data from animals that had cannula placements outside of the MRN, there were not clear effects of astressin 2B on freezing behavior (Figure S3). Dopamine release in the ventral hippocampus was not altered by exposure to the footshock box or intra-MRN injection of astressin 2B (Figure S4). Although dopamine receptors in the ventral hippocampus are involved in working memory and complex learning (Wilkerson and Levin, 1999; Umegaki *et al.*, 2001), they may not be involved in fear memory retrieval. Moreover, preliminary data showed that the serotonin release in the dorsal hippocampus was not altered by exposure to footshock box (Figure S5), though serotonin release in the dorsal hippocampus would be involved in stress responses other than fear memory retrieval (Matsuo *et al.*, 1996; Muchimapura *et al.*, 2002). These data also support our conclusion that the serotonergic projection from the MRN to the ventral hippocampus is selectively involved in the retrieval of fear memory via the CRF_2 receptor in the MRN.

Although promising, there are at least six unresolved issues in the present study. First, it is possible that endogenous CRF-related peptides such as urocortin, but not CRF itself, in the MRN (Bittencourt *et al.*, 1999) may induce fear memory retrieval via the activation of the CRF_2 receptor. It is necessary to assess the extracellular levels of CRF and/or urocortin in the MRN during fear memory retrieval to decipher this interesting question in future studies. Little is known about the role of urocortin in fear memory at this time (Pan and Kastin, 2008).

Second, we cannot completely exclude the possibility that CRF and/or related peptides in the

MRN affect memory-independent fear expression because the controllability of stress differs between the elevated plus maze test and the contextual fear conditioning test. The effect of CRF and/or related peptides in the MRN should be examined in different types of tests in future studies. However, it should be noted that Andrade *et al.* (2004) showed that serotonergic lesion of the MRN affected conditioned fear, but not unconditioned fear, in another type of test.

Third, it should also be noted that some studies have raised a question about the role of the MRN in fear memory retrieval because the microinjection of a serotonergic 5-HT_{1A} agonist into the MRN did not affect contextual fear-potentiated startle while it decreased freezing in the contextual fear conditioning test (Borelli *et al.*, 2005; Almada *et al.*, 2009). Several other types of tests should be conducted in future studies to settle this issue.

Fourth, we cannot completely deny the possibility that the CRF_2 receptor in the DRN as well as in the MRN is also involved in fear memory retrieval because previous studies have demonstrated that the CRF_2 receptor in the DRN is involved in fear conditioning. These studies, however, focused on conditioning and not memory retrieval (drug injections were conducted before fear conditioning; Hammack *et al.*, 2002, 2003). Although our present results showed that intra-DRN injection of astressin 2B had no effects on fear memory retrieval (Figure 2B), they do not completely rule out the possibility that the CRF_2 receptor in the DRN is involved in fear memory retrieval because it was shown that the effects of intra-DRN injection of CRF are subregion-dependent (Hammack *et al.*, 2002), and we did not intend to examine subregion-specific effects. We employed a larger volume (0.5 μl) for drug injections while Hammack *et al.* (2002) used a smaller volume (0.25 μl) to elucidate the site specificity. However, it is reasonable because our aim was to examine whether the effects of intra-MRN injection of astressin 2B in the present study were due to the leakage of astressin 2B to the DRN or not. It is also possible that the MRN is involved in the retrieval of fear memory, while the DRN is involved in the acquisition of fear memory.

Fifth, our present results did not directly prove the causal relationship between serotonin release in the ventral hippocampus and fear memory retrieval, although we demonstrated the association between them. However, the significant increase in serotonin release started soon after re-exposure to the footshock box (Figure 3A). Given that the concentration of serotonin assessed by microdialysis and HPLC reflects the serotonin release of several minutes before, it is likely that the increase in serotonin release preceded the retrieval of fear memory. Moreover, serotonin levels in the ventral hippocampus were still elevated in rats that had received footshock 24 hours before re-exposure and received an injection of saline on the testing day (FS-Saline group) even after animals started to stop freezing (Figure 3, 4). We speculate that rats still retrieved fear memory during 10-30 min phase. The reasons are twofold. First, the concentration of extracellular serotonin was decreased immediately after removing rats from the footshock box, which is a contextual stimulus (Figure 3). Second, rats in FS-Saline group alternated between freezing and exploratory behavior. Exploratory behavior during 10-30 min phase is probably coping behavior to the dangerous situation because no-footshock controls were in a resting posture and did not move during 10-30 min phase (data not shown). Further studies using serotonin receptor antagonists will be required to determine the causal relationship.

Finally, it should also be noted that intra-MRN injection of astressin 2B did not completely suppress freezing behavior (Figure 2, 4), while it almost completely suppressed serotonin release in the ventral hippocampus (Figure 3). This indicates that there are also other systems regulating the retrieval of fear memory. For example, many previous studies suggest that the amygdala is also involved in fear memory retrieval (for a review, LeDoux, 2000).

In conclusion, the present study suggests that the release of endogenous CRF and/or related ligands could activate the CRF_2 receptor in the MRN and stimulate serotonin release in the ventral hippocampus, thereby inducing fear memory retrieval. Although several different

studies have separately established that serotonin, CRF receptors, and the hippocampus are involved in fear memory retrieval, these distinct lines of study have not been linked to each other. We found that the MRN could be the key connecting these separate studies. The CRF_2 receptor in the MRN could play a pivotal role in fear memory retrieval and could be a target of drug development for the treatment of mental disorders involving fear memory, such as posttraumatic stress disorder.

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DISCLOSURE/CONFLICT OF INTEREST

The authors have no conflict of interest.

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Table 1. Memory-independent fear expression and locomotor activity in the elevated plus maze test.

	Treatment	
	Saline (n=7)	Astressin 2B (n=7)
Time spent in the open arms (%)	35.66 ± 9.04	21.42 ± 6.20
Entries into the open arms (%)	44.24 ± 7.89	38.98 ± 7.45
Total number of entries into the four arms	14.43 ± 2.82	17.29 ± 1.67
Moving distance on the maze (cm)	1107.63 ± 107.56	1056.57 ± 130.58

The mean ± S.E.M for each parameter is given. The dose of astressin 2B is 1000 ng.

Titles and legends to figures.

Figure 1 Cannula and probe placements. **(A)** The placements of the tip of the injectors in the DRN and MRN. **(B)** The placements of the microdialysis probe in the ventral hippocampus.

Figure 2 Effect of intra-MRN or DRN injection of CRF antagonists on freezing behavior in contextual fear conditioning test. Ten minutes before the start of behavioral tests, CRF antagonists or vehicles were injected. Bars represent mean freezing rate and lines represent S.E.M. **(A)** Effect of intra-MRN injection of antalarmin on freezing behavior **(B)** Effect of intra-DRN injection of astressin 2B on freezing behavior **(C)** Effects of intra-MRN injection of astressin and astressin 2B on freezing behavior

Figure 3 Effects of intra-MRN injection of astressin 2B on extracellular serotonin release in the ventral hippocampus. **(A)** The time course of extracellular serotonin level changes. Filled circles (n=6) represent rats that had received footshock 24 hours before re-exposure and received an injection of saline on the testing day (FS-saline). Open circles (n=6) represent rats that had received footshock and received an injection of astressin 2B (FS-Ast2B). Filled triangles (n=6) represent rats that had been placed in the footshock box without footschok and received an injection of saline (noFS-saline). Slightly high serotonin levels and extremely large S.E.M. in noFS-saline group during 70 min period are due to only one rat that showed extremely high serotonin level in the 70 min period. We did not exclude this rat from the data because this rat did not show any abnormal behavior and the serotonin levels in this rat returned to normal levels only 20 min after this time period (we continued to collect samples only in this rat). **(B)** The area under the curve (AUC) of extracellular serotonin levels during re-exposure to

footshock box. Data are given as mean AUC ± S.E.M. *P < .05.

Figure 4 Effects of intra-MRN injection of astressin 2B on freezing behavior in contextual fear conditioning test with microdialysis. Filled circles (n=6) represent rats that had received footshock 24 hours before re-exposure and received an injection of saline on the testing day. Open circles (n=6) represent rats that had received footshock and received an injection of astressin 2B. Filled triangles (n=6) represent rats that had been placed in the footshock box without footschok and received an injection of saline. We excluded the data on no-footshock controls in the statistical analysis because no-footshock controls rarely moved in the 10-30 min phase, and the automatic system could not discriminate between freezing and unmoving. Data are given as mean ± S.E.M. *P < .05.

Supplemental Figure 1. Photomicrographs showing coronal section of typical site of an (A, B) injection cannula and (C) microdialysis probe.

Supplemental Figure 2. Preliminary results of the effects of intra-MRN injection of a higher dose of antalarmin (1000 ng) on freezing behavior in contextual fear conditioning test. Ten minutes before the start of behavioral tests, CRF antagonist or vehicle was injected into the MRN. Bars represent mean freezing rate and lines represent S.E.M.

Supplemental Figure 3. Preliminary data from animals that had cannula placements outside of the MRN. Ten minutes before the start of behavioral tests, 1000 ng of astressin 2B was injected into outside of the MRN. Bars represent mean freezing rate

and lines represent S.E.M.

Supplemental Figure 4. The time course of extracellular dopamine levels changes in the ventral hippocampus. Filled circles represent rats that had received footshock 24 hours before re-exposure and received an injection of saline on the testing day (n=6). Open circles represent rats that had received footshock and an injection of astressin 2B (n=5). Filled triangles represent rats that had been placed in the footshock box without footshock and received an injection of saline (n=5). Some subjects were omitted because unknown peaks sometimes overlapped with the peak indicating dopamine. Data are given as mean ± S.E.M.

Supplemental Figure 5. Preliminary results (n=3) of the time course of extracellular serotonin levels changes in the dorsal hippocampus. For this experiment, a guide cannula (AG-4, Eicom Co. Ltd., Japan) was implanted into the dorsal hippocampus: 3.3 mm posterior to bregma, 2.2 mm lateral to the midline, 1.6 mm ventral to the dura. In the testing day, a dialysis probe (2 mm long and 0.22 mm in outer diameter; A-I-4-02, Eicom Co. Ltd., Japan) was inserted through the guide cannula. Rats had received footshocks 24 hours before re-exposure and received an injection of saline on the testing day. Other procedures were the same as the experiments of ventral hippocampus.

Figure 1

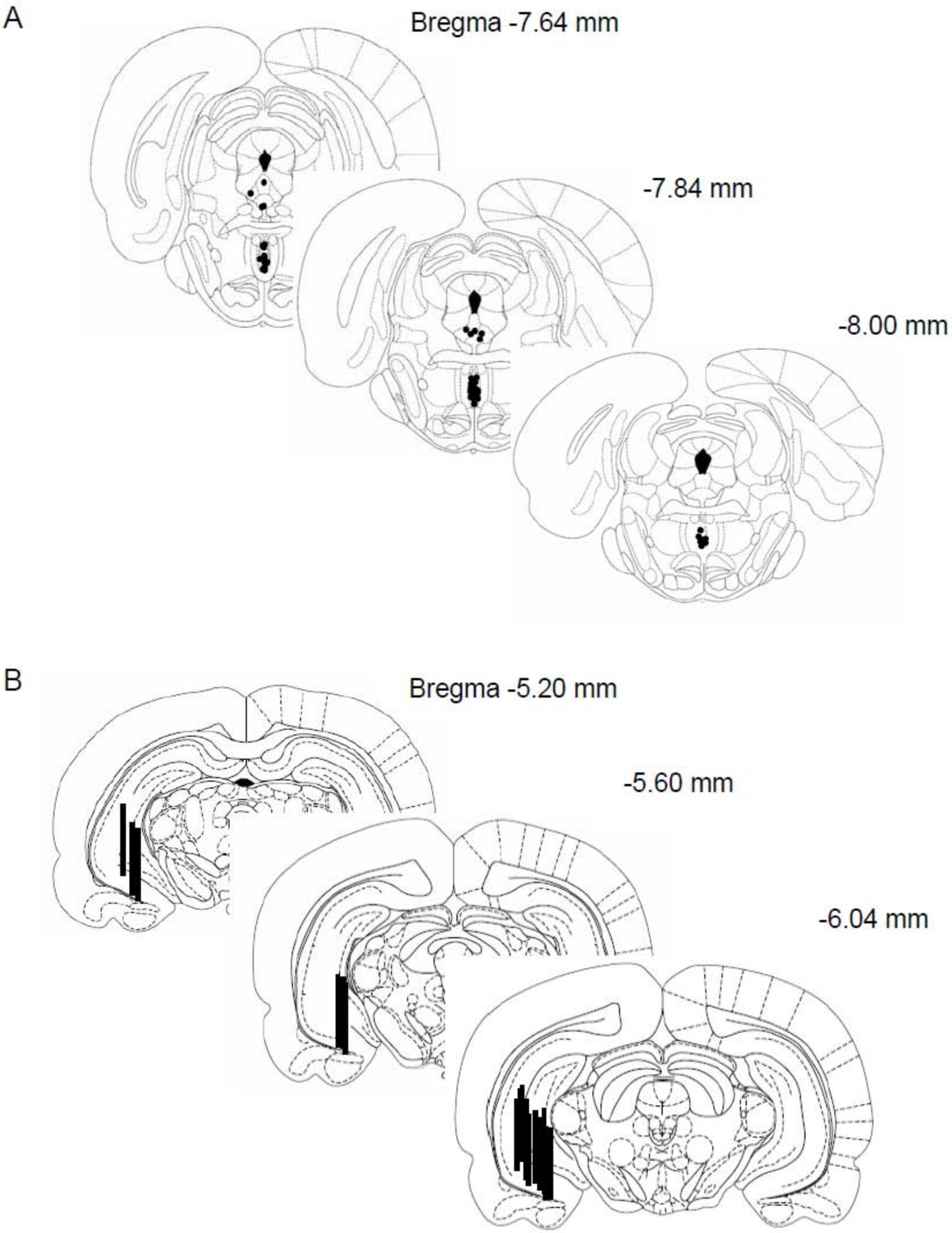
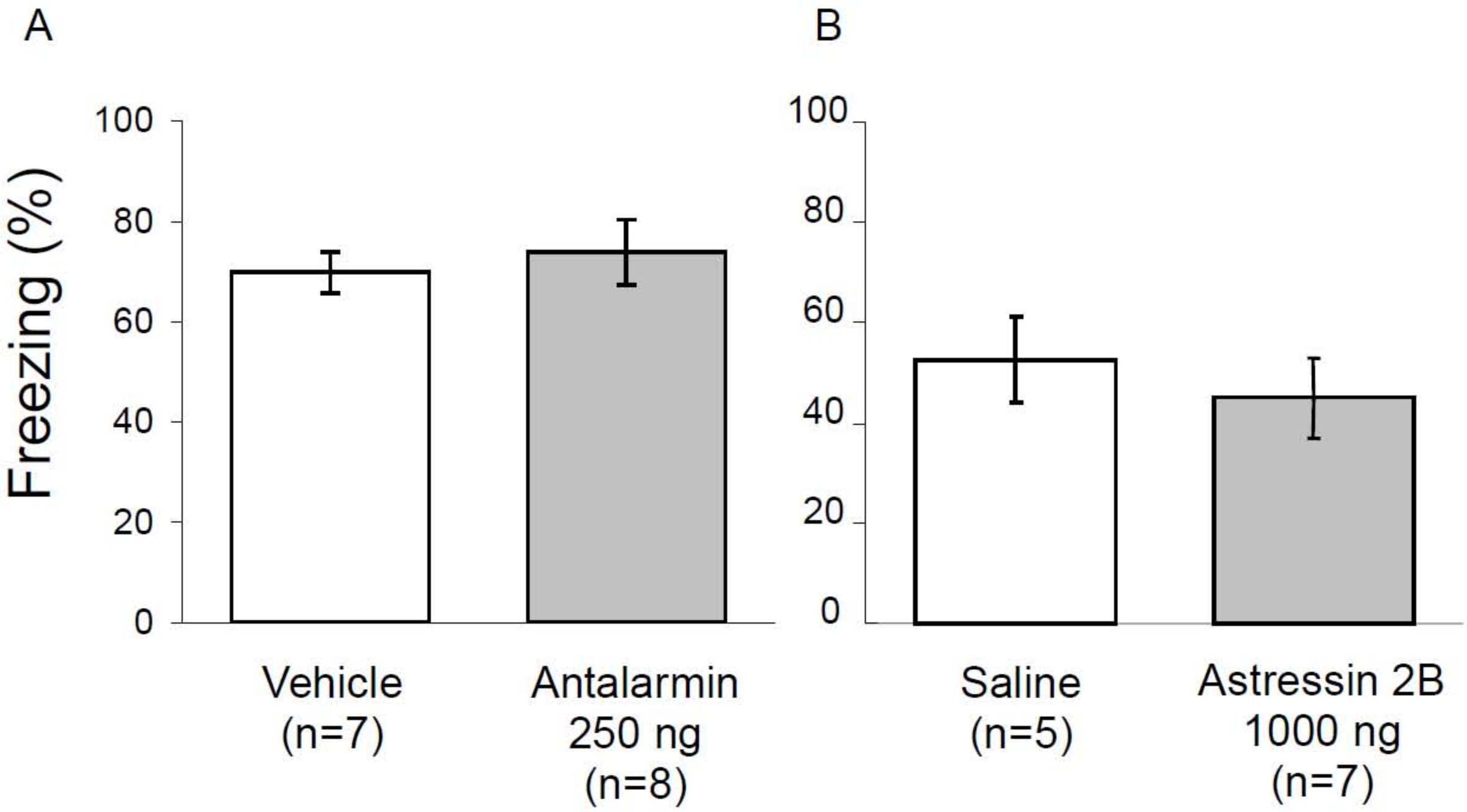


Figure 2


A
B
Freezing (%)
100
80
60
40
20
0
Vehicle (n=7)
Antalarmin 250 ng (n=8)
Saline (n=5)
Astressin 2B 1000 ng (n=7)

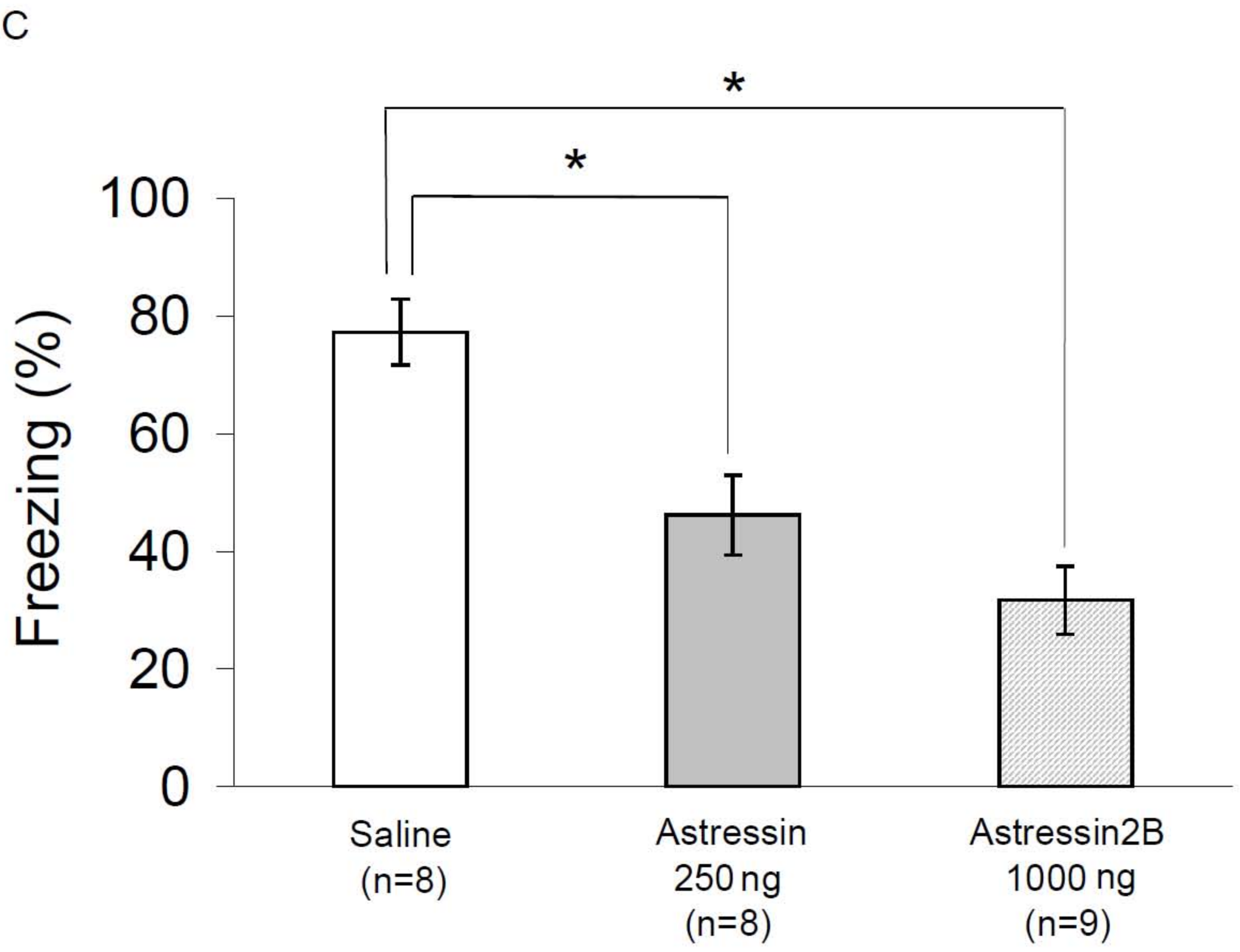
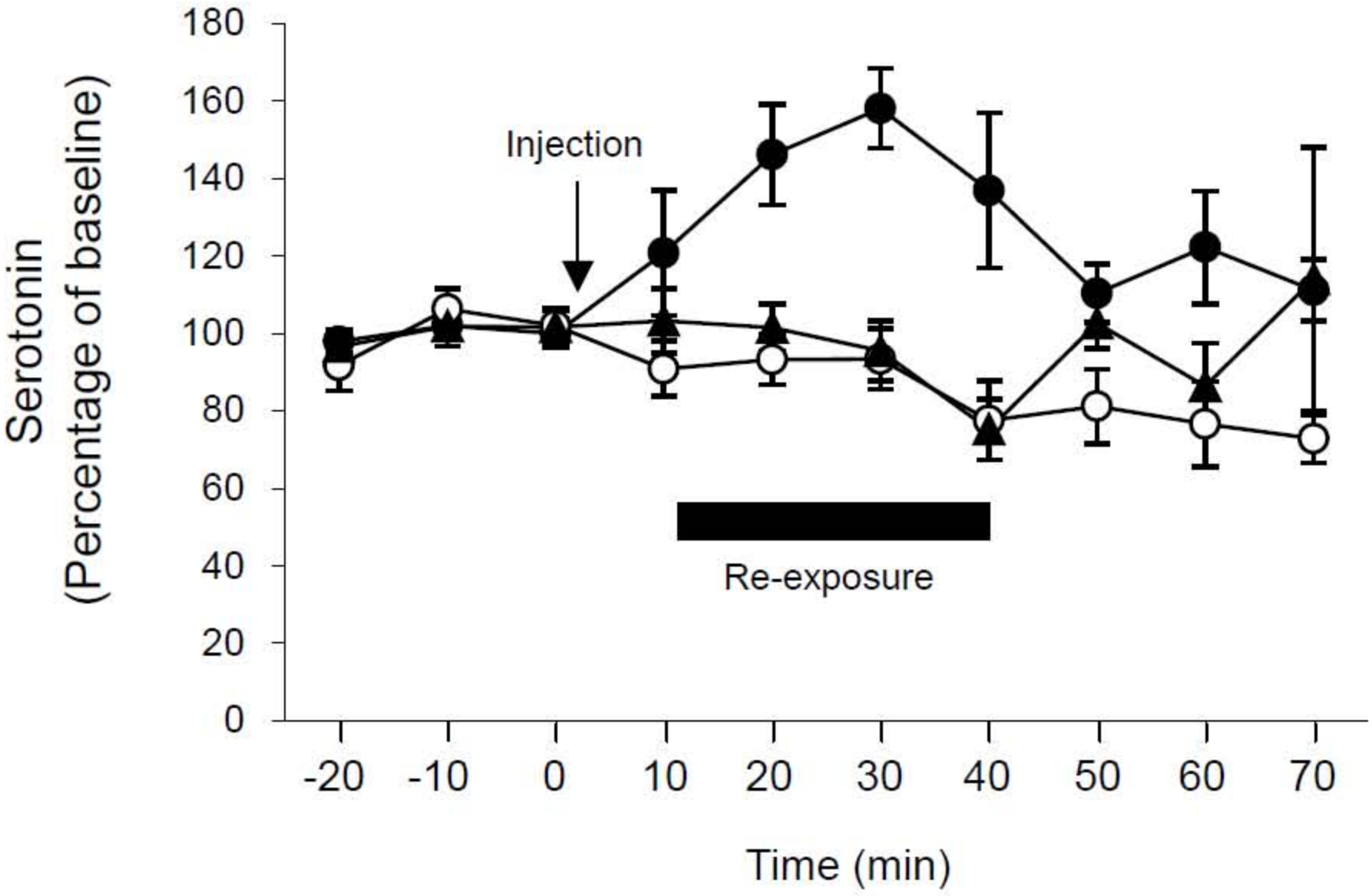

C
*
*
Freezing (%)
100
80
60
40
20
0
Saline (n=8)
Astressin 250 ng (n=8)
Astressin2B 1000 ng (n=9)

Figure 3

A


Serotonin
(Percentage of baseline)
180
160
140
120
100
80
60
40
20
0
Injection
Re-exposure
-20
-10
0
10
20
30
40
50
60
70
Time (min)

B

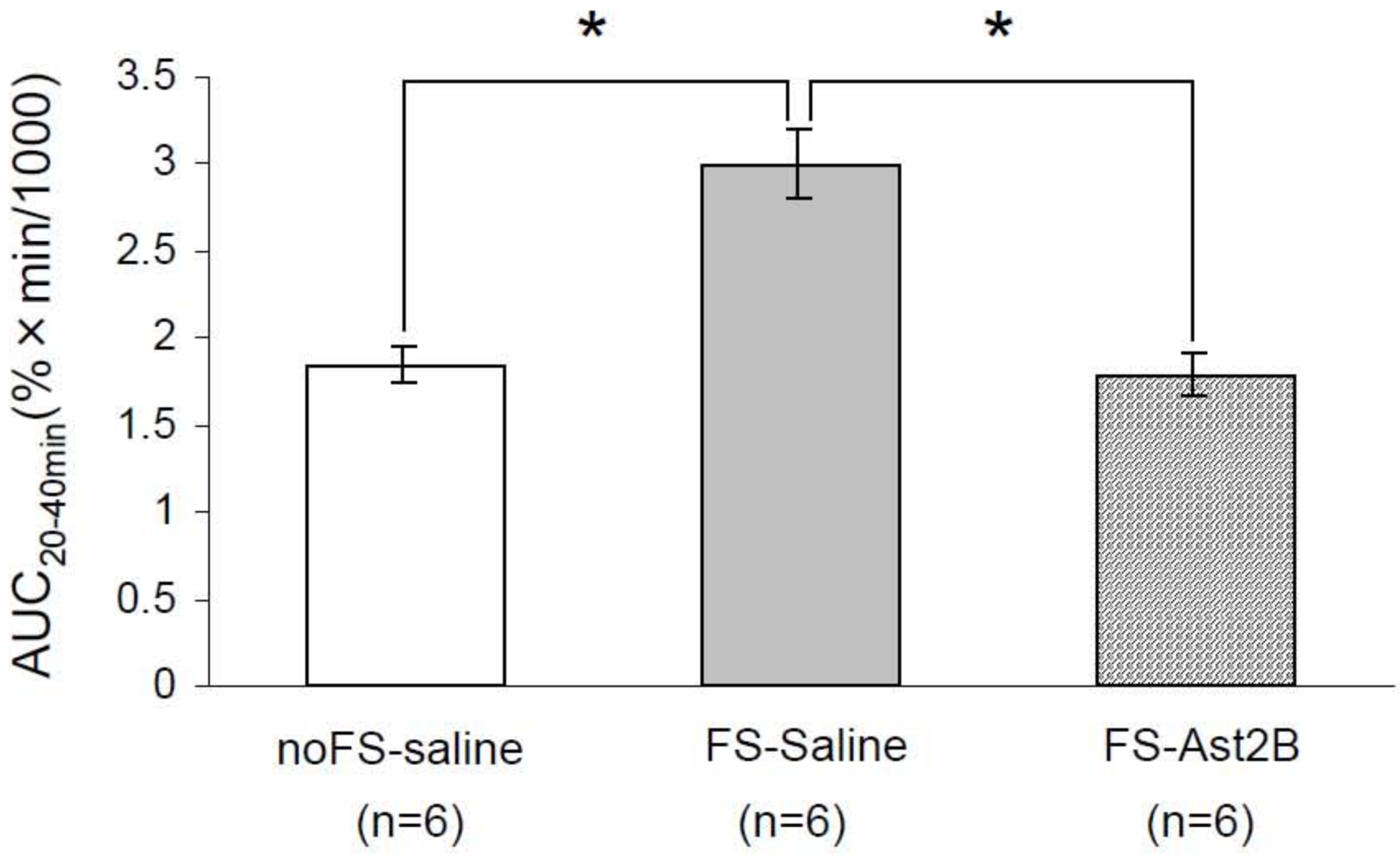

*
*
AUC20-40min(% x min/1000)
3.5
3
2.5
2
1.5
1
0.5
0
noFS-saline
(n=6)
FS-Saline
(n=6)
FS-Ast2B
(n=6)

Figure 4