Anxiolytic-like effect of mirtazapine mediates its effect in the median raphe nucleus

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Abstract

Mirtazapine, a noradrenergic and specific serotonergic antidepressant (NaSSA), blocks the α_2-adrenergic autoreceptors and heteroreceptors, which are responsible for controlling noradrenaline and 5-hydroxytryptamine (5-HT) release. Though preclinical and clinical studies have shown that mirtazapine exerts an anxiolytic action, its precise brain target sites remain unclear. In the present study, we investigated the brain area(s) in which mirtazapine exerts its anxiolytic-like effects on the expression of contextual conditioned freezing in rats. Mirtazapine (3 μg/site) was directly injected into three brain structures, the median raphe nucleus (MRN), hippocampus and amygdala. Freezing behavior tests were carried out 10 min after injections. Our results showed that the intra-MRN injection of mirtazapine reduced freezing significantly, whereas injections into the hippocampus or the amygdala did not. In addition, the intra-MRN injection of mirtazapine did not affect locomotor activity. These results suggest that the anxiolytic-like effect of mirtazapine might be mediated by its action on the MRN.

Keywords: mirtazapine; median raphe nucleus; hippocampus; amygdala; contextual fear conditioning

1. Introduction

Mirtazapine, an antagonist of central α_2-adrenergic autoreceptors and heteroreceptors, and postsynaptic 5-hydroxytryptamine$_2$ (5-HT$_2$) and 5-HT$_3$ receptors, achieves its unique therapeutic effect by enhancing noradrenaline and serotonin release (Millan, 2006; Yamauchi et al., 2012). Several clinical trials demonstrated the effectiveness of mirtazapine in the treatment of not only depression but also anxiety disorders, including post-traumatic stress disorder (Davidson et al., 2003), generalized anxiety disorder (Gambi et al., 2005), panic disorder (Ribeiro et al., 2001) and social anxiety disorder (Van Veen et al., 2002). Although its beneficial effects in relieving anxiety symptoms and curing anxiety disorders have been reported consistently, the mechanism by which mirtazapine exerts its anxiolytic effect has not been fully clarified.

Evidence from animal experiments also supports the anxiolytic-like effect of mirtazapine. The systemic administration of mirtazapine decreases conditioned freezing behavior in the contextual fear conditioning model (Kakui et al., 2009). A number of studies have demonstrated the reliability of the fear conditioning test as a behavioral paradigm with which to clarify the mechanisms involved in fear and anxiety and to evaluate the efficacy of therapeutic agents such as SSRIs and benzodiazepines in the treatment of anxiety disorders (Inoue et al., 2011). Studies using an in vivo microdialysis technique have found that the acute systemic administration of mirtazapine increases extracellular serotonin concentrations in the

hippocampus but not the prefrontal cortex of rats (Yamauchi et al., 2012), suggesting that the anxiolytic effect of mirtazapine might be mediated by the facilitation of central 5-HT neurotransmission in the hippocampus.

In contextual fear conditioning, the amygdala and hippocampus are two main brain structures implicated in the acquisition, expression and retrieval of fear conditioning (LeDoux, 2000; Maren, 2008). In addition, the median raphe nucleus (MRN) also has an important role in the acquisition and expression of conditioned fear (Almada et al., 2009; Avanzi et al., 2003; Borelli et al., 2005). Lesions or inactivation of the MRN were shown to reduce the expression of contextual fear conditioning (Borelli et al., 2005; Silva et al., 2004). The MRN includes a great density of α_1-adrenoceptors and α_2-adrenoceptors in addition to the high density of 5-HT receptors (Day et al., 1997; Kia et al., 1996; Rosin et al., 1993; Talley et al., 1996). Administration of the α_2-adrenergic agonist, clonidine, into the MRN decreases the level of 5-HT in this nucleus, whereas the administration of an α_2-adrenergic antagonist into the MRN enhances 5-HT release (Adell and Artigas, 1999). These results suggest that α_1- and α_2-adrenoceptors in the MRN may affect contextual conditioned fear by increasing 5-HT release. However, little is known about the sites of anxiolytic action of mirtazapine in the brain.

The aim of this study is to clarify the brain regions where mirtazapine acts as an anxiolytic in contextual conditioned fear. In this study, we examined the effects of intracranial injections of mirtazapine administered directly into the amygdala, hippocampus or MRN on the expression of contextual conditioned fear by using

freezing as an index of fear.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (260-320 g) were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). The rats were housed in polypropylene cages with wood shavings on the floor, four animals per cage, with free access to food and water. The room temperature was kept at 22±2°C. The subjects were maintained on a 12-h light/dark cycle (light phase: 06:30-18:30). Experiments began after a one-week period of acclimatization. All experiments were performed between 08:00 and 13:00, except for the surgery. All experiments 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.

2.2. Drug

Bilateral or unilateral infusions of mirtazapine were given with 33-gauge injector cannulae connected by polyethylene tubing to motor-driven microsyringes. The exact placement of the injector cannula tips was verified at the end of the experiments by standard histological methods. Mirtazapine (obtained from Merck & Co. Inc., Whitehouse Station, NJ, U.S.A.) was dissolved at a concentration of 6 μg/μl in 0.15% tartaric acid, and 0.5 μl was infused through each injector at a rate of 0.5 μl/min. The

injectors were left in place for 60 s after the infusion. The vehicle alone was administered as a control. In previous studies, the same systemic doses (10 mg/kg) of mirtazapine and citalopram reduced the expression of contextual conditioned freezing and increased the extracellular 5-HT concentrations in the brain (Hashimoto et al., 1999; Inoue et al., 2011; Kakui et al., 2009; Yamauchi et al., 2012). Because 3 μg/site of citalopram injected into the amygdala decreased the expression of contextual conditioned freezing (Inoue et al., 2004), the same intracranial dose (3 μg/site) was estimated to be effective and was chosen for mirtazapine on the contextual conditioned freezing.

2.3. Stereotaxic surgery

Surgeries were performed under sodium pentobarbital (40 mg/kg, intraperitoneally) anesthesia 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 unilateral or bilateral 26-gauge stainless steel guide cannulae directed toward the MRN (unilateral), amygdala (bilateral, the basal nucleus of the amygdala) or dorsal hippocampus (bilateral) [coordinates of injection sites relative to bregma: AP −7.8 mm, ML +0 mm, V 8.6 mm for the MRN; AP−2.8 mm, ML ±5.0 mm, V 8.4 mm for the amygdala; AP−3.3 mm, ML ±1.9 mm, V 2.9 mm for the dorsal hippocampus; taken from the stereotaxic atlas of Paxinos and Watson (1997)]. The guide cannulae for the MRN were unilaterally inserted at a lateral angle of 20° to avoid the sagittal sinus and cerebral aqueductal obstruction. After the surgery,

rats were housed individually. When not used for injection, the guide cannulae were occluded with obturators made of 33-gauge stainless steel wire. After 7 days, the animals were submitted to behavioral sessions.

2.4. Fear conditioning and behavioral measures

The animals received contextual fear conditioning (training sessions) 7 days after the stereotaxic surgery. As described previously (Inoue et al., 2004), for fear conditioning, the rats were individually subjected to a total of 2.5 min of inescapable electric footshocks [five footshocks (2.5 mA scrambled footshocks, pulse wave, 30 s duration) that were delivered at intershock intervals of 35 to 85 s (mean 60 s)] in a shock chamber with a grid floor (19 × 22 × 20 cm, Medical Agent, Kyoto, Japan). Electric shocks were produced by a Model SGS-02D Shock Generator (Medical Agent). This generator 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 delivered a 0.2-mA shock intensity to the rats. Twenty-four hours after training, bilateral or unilateral infusions of mirtazapine (3 μg/site, 6 μg/μl) or vehicle (0.5 μl of 0.15% tartaric acid) were performed for 1 min simultaneously using 33-gauge injector cannulae projecting 1.0 mm beyond the tips of the guide cannulae. Ten minutes after the infusion, the rats were again placed in the shock chamber and were observed for 5 min without shocks. The behavior was videotaped and scored later by human observation. With these procedures, conditioned fear, as measured by freezing, develops from the contextual stimuli of the conditioned chamber (Fanselow, 1980).

During the observation period, the duration of freezing behavior was recorded using a modified time-sampling procedure as previously described (Inoue et al., 2004). Every 10 s, the behavior in which the animal was currently engaged was classified as either "freezing" or "activity". Freezing was defined as the absence of any observable movement of the skeleton and the vibrissae, except those related to respiration. All other behaviors were scored as activity. The animal was classified as either freezing or active according to its behavior throughout the entire 10-s period. We observed rats for successive 10-s periods during 5 min (i.e., 30 successive sampling periods). If a rat showed any activity during the 10-s sampling period (including freezing for up to 9 s), we considered this period as active. The percentage freezing score [freezing (%)] was computed as the proportion of 10-s periods during which the animal remained frozen all of the time.

Several previous studies used a 24 h interval between footshock and testing to assess the effects of several drugs on contextual conditioned freezing (Inoue et al., 2011). The anxiolytic-like effect of systemic mirtazapine in contextual conditioned fear was reported using a 24 h interval (Kakui et al., 2009). To compare our results with the previous findings, a 24 h interval between footshock and testing was chosen.

2.5. Motor activity

To exclude the possibility that mirtazapine administration reduced freezing nonspecifically by increasing spontaneous activity, the motor activity of unshocked rats was measured in separate experiments. The motor activity of unshocked rats, not

shocked rats, was measured as examined in the previous microinjection study for contextual conditioned fear experiments (Inoue et al., 2004) because long-term reductions induced by a single inescapable shock session in rat motor activity are reversed by anxiolytic drugs (Van Dijken et al., 1992a, 1992b). Therefore, the effect of mirtazapine on motor activity in the shocked rats may be confounded by the therapeutic effect of the drug on stress influence and cannot be regarded as a pure motor effect.

The rats were implanted with unilateral cannulae aimed at the MRN. After a recovery period of 7 days, the rats were infused with 3 μg mirtazapine or vehicle 10 min before testing. During the testing, rats were individually placed in a testing cage (38 × 33 × 17 cm), and motor activity was automatically recorded as described by Ohmori et al. (1994) using infrared sensors between 08:00 and 13:00. Horizontal movement was digitized and uploaded to a computer. Locomotion was responsible for most of the count, though other body movements also contributed when they included a substantial horizontal component.

2.6. Histology

At the end of the experiments, each animal was deeply anesthetized with intraperitoneal sodium pentobarbital. Fast Green (2%; 0.5 μl) was then injected through the guide cannula. The brain was removed, and the dye spot was localized from 40-μm serial coronal sections stained with cresyl violet. The injection sites were histologically determined under a light microscope, with reference to the diagrams

from the stereotaxic atlas of Paxinos and Watson (1997). A total of 79 rats were used in the experiment, and 15 rats that received injections outside the aimed area were excluded from analysis.

2.7. Data analysis

All data are presented as the mean ± SEM of the individual values of the rats from each group. Statistical analysis of the data was conducted using an unpaired t-test. The significance level was chosen at 0.05.

3. Results

3.1. Histology

Tissue damage was not apparent in either the drug group or the vehicle group. Fig. 1 depicts the sites of drug injection into the MRN (A), dorsal hippocampus (B) and amygdala (C). The histological results were plotted on representative sections taken from the rat brain atlas of Paxinos and Watson (1997).

3.2. Effect of mirtazapine microinjection into the MRN on the expression of contextual conditioned freezing (Fig. 2A)

A unilateral mirtazapine microinjection into the MRN given 10 min before testing significantly reduced conditioned freezing compared with the vehicle group [$t(15)=3.996$, $P<0.01$].

3.3. Effect of mirtazapine microinjection into the dorsal hippocampus on the expression of contextual conditioned freezing (Fig. 2B)

Bilateral mirtazapine microinjections into the dorsal hippocampus given 10 min before testing did not affect conditioned freezing compared with the vehicle group [t (17)=1.26, P=0.22], indicating that mirtazapine did not affect anxiety-like behavior.

3.4. Effect of mirtazapine microinjection into the amygdala on the expression of contextual conditioned freezing (Fig. 2C)

Bilateral mirtazapine microinjections into the amygdala given 10 min before testing did not affect conditioned freezing [t (11) =0.698, P=0.5], indicating that mirtazapine did not affect anxiety-like behavior.

3.5. Effect of mirtazapine microinjection into the MRN on locomotor activity

Mirtazapine microinjection into the MRN did not change the motor activity of unshocked rats during the 5-min testing period compared with the vehicle group [vehicle 673.0 ± 226.3 counts, mirtazapine 735.6 ± 177.2 counts; t (13) =0.221, P=0.83, t-test].

4. Discussion

In the present study, we investigated the target brain sites where mirtazapine, a NaSSA, exerts its anxiolytic-like effect in the contextual fear conditioning test. The

local microinjection of mirtazapine (3 μg/site) into the MRN, but not into the hippocampus or amygdala, reduced the expression of contextual conditioned freezing significantly. Moreover, intra-MRN treatment with mirtazapine did not change motor activity compared with the vehicle controls, thereby excluding non-specific motor interference as the main factor accounting for its effect during conditioned freezing. Because freezing behavior induced by conditioned fear is used as an index of fear and anxiety and is decreased by several anxiolytic drugs, such as benzodiazepines, 5-HT_{1A} agonists and SSRIs (Inoue et al., 2011), the inhibitory effect of intra-MRN mirtazapine treatment on freezing behavior indicates its anxiolytic-like effect. The present study is in accordance with our previous studies, which demonstrated that the systemic administration of mirtazapine exerts an anxiolytic-like effect in the rat contextual fear conditioning test (Kakui et al., 2009).

The MRN, like the dorsal raphe nucleus, is a main source of serotonergic innervation to the forebrain structures involved in anxiety regulation (Vertes, 1991; Vertes et al., 1999). Recent evidence suggests that 5-HT neurons within the MRN play an important role in the regulation of anxiety-related behaviors in several animal models such as the social interaction test, elevated plus-maze test, light-dark transition test, elevated T-maze test and the conditioned fear test (Almada et al., 2009; File et al., 1996; Vicente et al., 2008). The great density of α_1- and α_2-adrenoceptors in the MRN shown by anatomical studies (Day et al., 1997; Rosin et al., 1993; Talley et al., 1996) reportedly modulates the firing of MRN serotonergic neurons: α_1-adrenoceptors directly stimulate serotonergic neuron firing and α_2-adrenoceptors indirectly inhibit it

through the inhibition of noradrenergic neurons (Adell and Artigas, 1999). An earlier study using in vivo microdialysis showed that the local perfusion of an α_1-adrenergic antagonist decreased 5-HT levels in the MRN, whereas that of an α_2-adrenergic antagonist increased them (Adell and Artigas, 1999). Hence, intra-MRN infusion of mirtazapine, which is an α_2-adrenergic antagonist and has very weak α_1-adrenergic antagonistic action (Millan, 2006), is supposed to increase extracellular 5-HT levels in the nerve terminal areas such as the hippocampus through activation of the MRN by α_2-adrenoceptor antagonism in the noradrenergic nerve terminals and subsequent α_1-adrenoceptor stimulation. Another hypothesis that α_2-adrenoceptor antagonism in the serotonergic nerve terminals in the MRN increases extracellular 5-HT and inhibits MRN activity should also be considered. However, a recent in vivo microdialysis study by Fukuyama et al. (2013) revealed that the local administration of mirtazapine or the α_2-antatgonist idazoxan into the MRN increased extracellular 5-HT levels in both the MRN and the entorhinal cortex (the projection area of the MRN). Their results support our hypothesis that local mirtazapine stimulates MRN activity by blocking α_2-adrenoceptors in the nerve terminals of noradrenergic neurons and increases the extracellular 5-HT levels in the nerve terminal areas of the MRN. Because increased 5-HT neurotransmission decreases contextual conditioned freezing (Inoue et al., 2011; Nishikawa et al., 2007), the above pharmacological profiles of mirtazapine may account for the mode of anxiolytic action of intra-MRN mirtazapine microinjection during the conditioned fear response. However, a reduction of 5-HT neurotransmission in the MRN as a result of local infusions of a 5-HT_{1A} agonist

significantly decreased the expression of contextual fear conditioning, although local infusions of a 5-HT_{1A} agonist into the dorsal hippocampus also decreased fear conditioning in a previous study (Almada et al., 2009). This result seems to be partly inconsistent with our results in this study. A possible explanation for this discrepancy is that both increased and abolished 5-HT neurotransmission might actually decrease the expression of contextual conditioned fear. Further studies are necessary for the elucidation of this bidirectional effect of the MRN.

The idea that the anxiolytic-like effect is induced by blocking the α_2-adrenergic receptors in the MRN is consistent with previous data. Mansur et al. (2010) reported that the microinjection of the α_2-adrenergic agonist, clonidine, into the MRN increased the total risk assessment frequency, an ethological parameter of the anxiogenic effect in the elevated plus-maze test. However, no study has reported the effect of α_2-adrenergic antagonist microinjection into the MRN on anxiety-like behaviors. In the future, the effect of a selective α_2-adrenergic antagonist microinjection into the MRN on anxiety-like behaviors should be examined.

In an earlier study, infusions of the α_1-adrenergic agonist, phenylephrine, into the MRN decreased risk assessment behavior in the elevated plus-maze, suggesting an anxiolytic profile (Mansur et al., 2011). Furthermore, blocking the α_1-adrenoceptors reduced the increased extracellular 5-HT levels induced by SSRIs (Rea et al., 2010) and the anxiolytic-like effect of the SSRIs (Takamura et al., 2012). More importantly, Kakui et al. (2009) showed that the anxiolytic-like action of mirtazapine was significantly reversed by the α_1-adrenergic antagonist prazosin, highlighting the

importance of α_1-adrenoceptor activation in the anxiolytic-like effect of mirtazapine in contextual conditioned freezing. Thus, the weak α_1-adrenoceptor antagonistic action of mirtazapine (Millan, 2006) may be essential for the exertion of an anxiolytic effect. In support of this idea, mianserin, which is a structural analogue of mirtazapine but inhibits both α_1 and α_2 receptors, was ineffective in decreasing contextual conditioned freezing (Kakui et al., 2009).

The hippocampus has an essential role in the development and expression of conditioned fear (LeDoux, 2000), and α_2-adrenoceptors are localized in several brain structures, including the hippocampus (Talley et al., 1996). An in vivo electrophysiological study suggests that systemic mirtazapine administration enhances 5-HT neurotransmission via the blockade of the presynaptic release-modulating α_2-heteroreceptor on 5-HT terminals in the dorsal hippocampus (Haddjeri et al., 1996). Systemic mirtazapine administration increases extracellular 5-HT release in the hippocampus (Yamauchi et al., 2012). These results of increased 5-HT neurotransmission in the hippocampus with systemic mirtazapine administration suggest that the anxiolytic-like effect of systemic mirtazapine on contextual conditioned fear is mediated by its effect on hippocampal 5-HT (Inoue et al., 2011; Kakui et al., 2009). The present study showed that mirtazapine microinjections into the hippocampus had no effect on the expression of contextual conditioned freezing. Using in vivo microdialysis, Bengtsson et al. (2000) reported that basal or clonidine-induced 5-HT release was not affected by the local perfusion of mirtazapine or an α_2-adrenergic antagonist in the hippocampus or frontal cortex. As cited

previously, the local administration of mirtazapine or the α_2-antatgonist idazoxan into the MRN reportedly increases the extracellular 5-HT levels in both the MRN and nerve terminal area of the MRN (the entorhinal cortex) (Fukuyama et al., 2013). The results of previous studies and our study suggest that the stimulating effect of systemic mirtazapine on extracellular 5-HT release in the hippocampus (Yamauchi et al., 2012) may be mediated by the blockade of α_2-adrenoceptors in the MRN and generates the anxiolytic-like effect of systemic mirtazapine in contextual conditioned fear.

The lack of significant decreases in freezing in subjects that received the intra-hippocampal or intra-amygdala injections of mirtazapine prior to testing should be interpreted with some caution because the percentage freezing scores of the control groups were different between the experiments (intra-MRN 84.6%±4.2, intra-hippocampal 52.6%±7.2 and intra-amygdala 55%±10.3). The differences in the damage by the guide cannulae to each region (location and bilateral or unilateral) may affect the %freezing of the control group. The control scores in conditioned freezing experiments were variable between experiments in previous studies (Inoue et al., 2011). Because the experiments for each region were performed simultaneously, it is unlikely that the control levels affect the significant effect of mirtazapine.

In conclusion, this study shows that the anxiolytic-like effect of mirtazapine in contextual conditioned fear is mediated by its action in the MRN, but not amygdala or hippocampus. The mechanism of the anxiolytic-like effect of mirtazapine is suggested to occur via the blockade of α_2-adrenoceptors in the MRN, which may

increase extracellular noradrenaline and stimulate the MRN via α_1-adrenoceptors.

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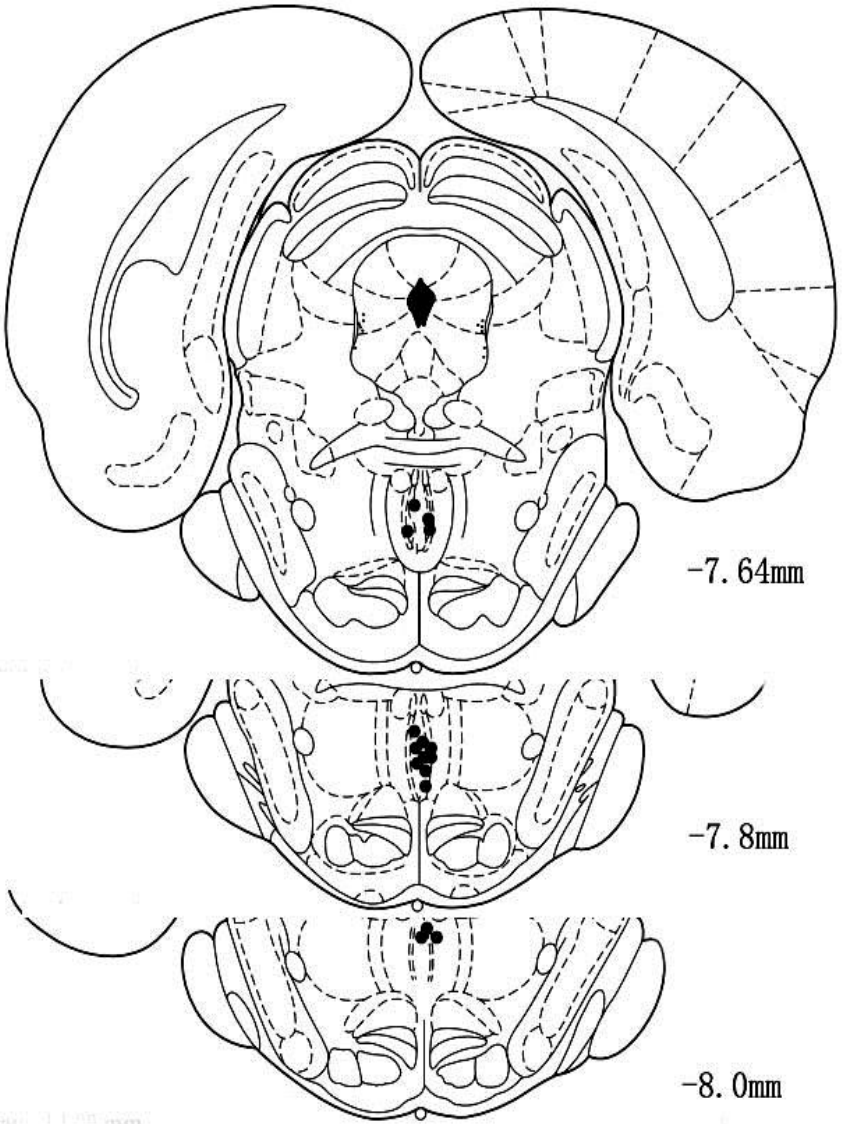
Figure legends

Fig. 1. Diagrammatic representation of coronal sections through the rat brain showing the location of injection sites (solid circles) of the MRN (A), dorsal hippocampus (B) and amygdala (C). Figures represent coordinates from the Paxinos and Watson (1997) brain atlas, with respect to the bregma. The number of points in the figures is fewer than the total number of rats used because of several overlaps.

Fig. 2. The effect of mirtazapine (MTZ) microinjections (3 μg/site) into the MRN (A), dorsal hippocampus (B) and amygdala (C) on freezing induced by conditioned fear. Mirtazapine was administered 24 h after footshock and 10 min before conditioned fear stress (testing). Freezing scored over a 5-min observation period is represented as the mean percentage ± SEM. Behavior was sampled at 10-s intervals. (**$P < 0.01$ when compared to the vehicle-treated rats). In (A), N=8-9 rats; in (B), N=9-10 rats; in (C), N=6-7 rats.

Figure 1

A)



B)

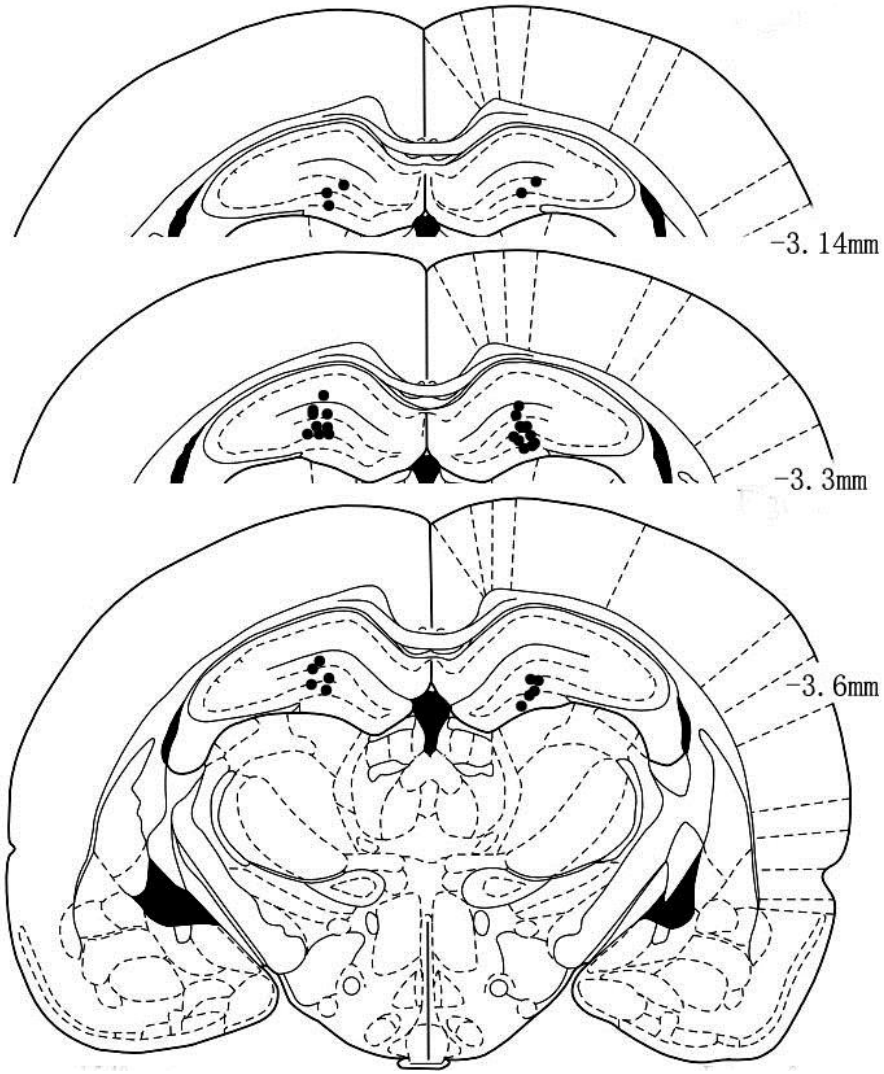


Figure 1

C)

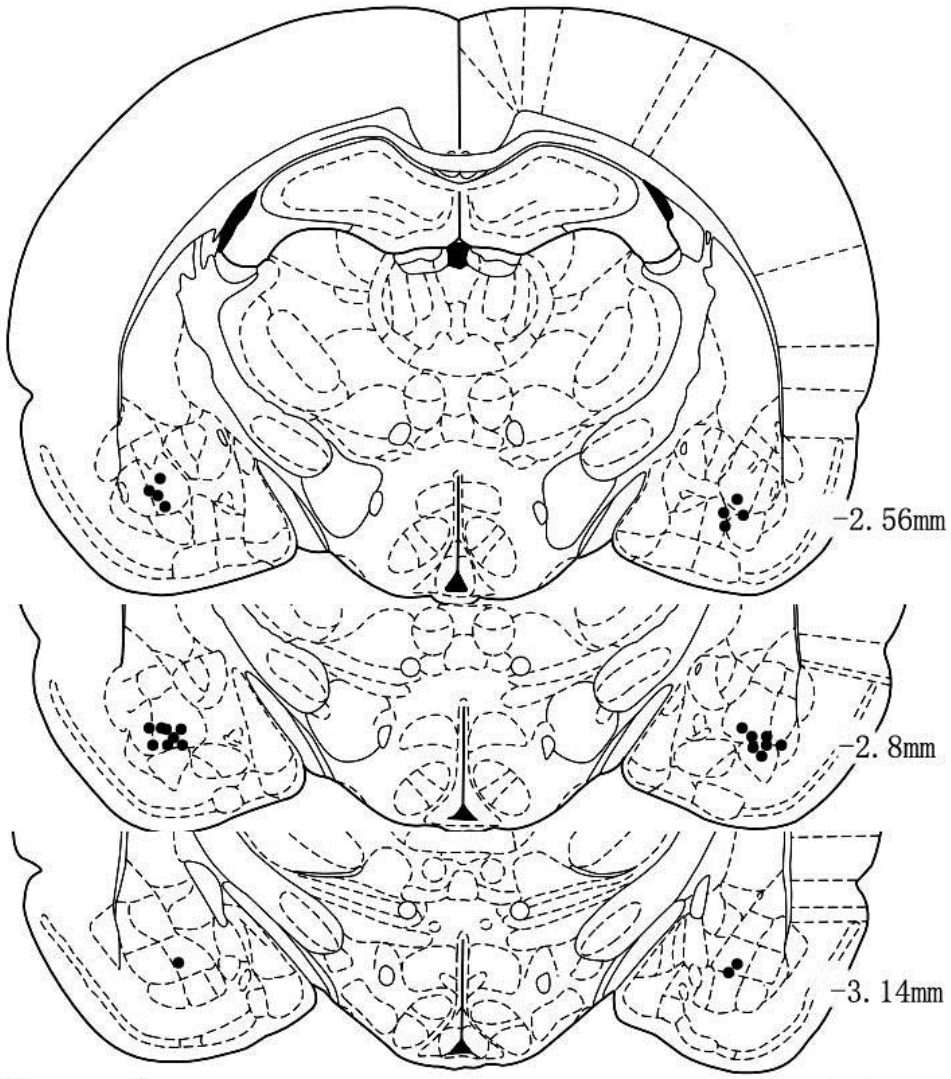


Figure 2

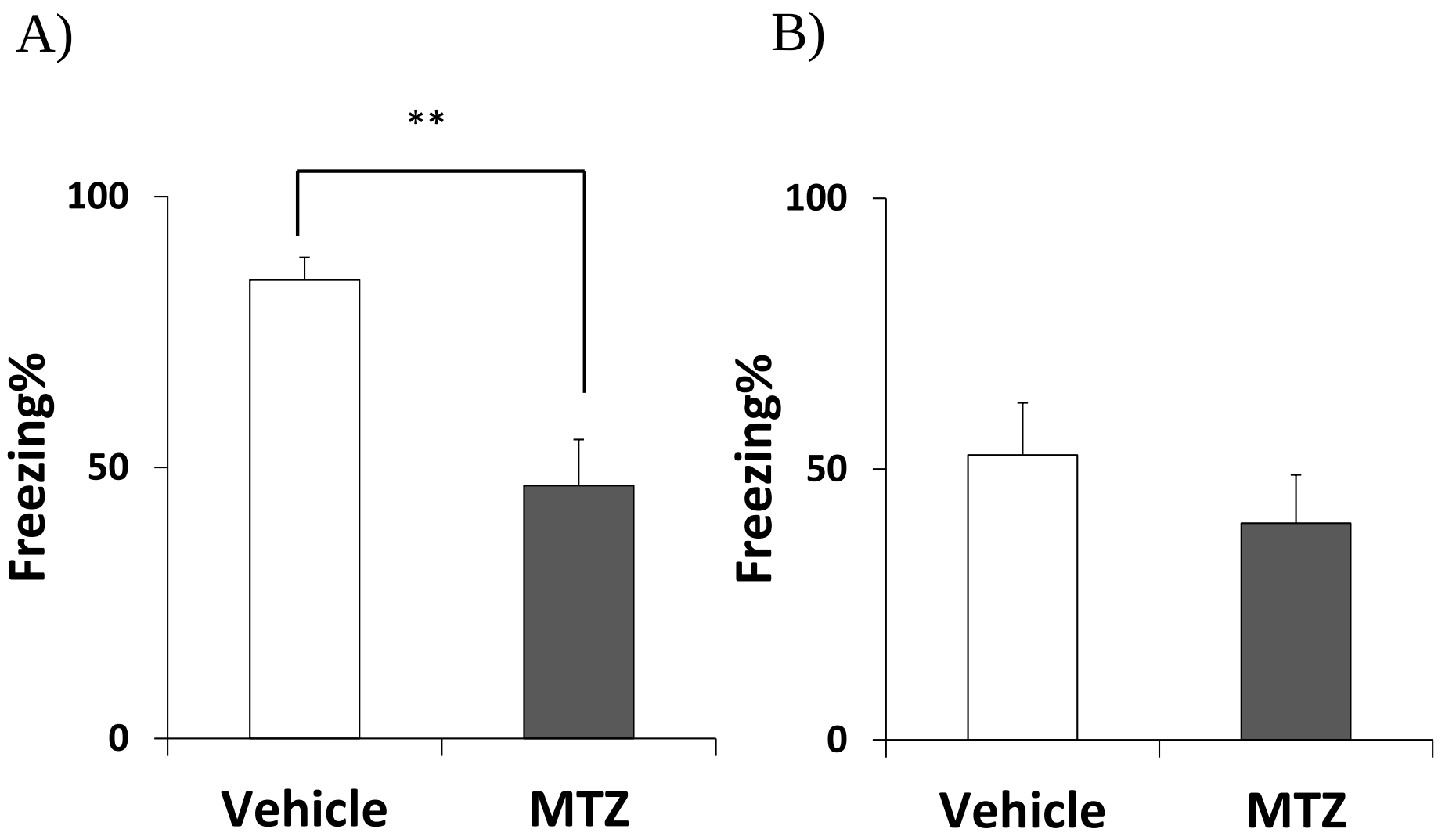

A)
**
100
50
0
Freezing%
Vehicle
MTZ
B)
100
50
0
Freezing%
Vehicle
MTZ

Figure 2

C)