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Title	Biological basis of the anxiolytic-like effect of mirtazapine in the rat conditioned fear stress model
Author(s)	安, 燕
Description	配架番号：2131
Degree Grantor	北海道大学
Degree Name	博士(医学)
Dissertation Number	甲第11649号
Issue Date	2015-03-25
DOI	https://doi.org/10.14943/doctoral.k11649
Doc URL	https://hdl.handle.net/2115/58780
Type	doctoral thesis
File Information	An_Yan.pdf

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学位論文

Biological basis of the anxiolytic-like effect of mirtazapine in the rat conditioned fear stress model

（ラット恐怖条件付けモデルにおける mirtazapine の抗不安効果の生物学的基盤に関する研究）

2015 年 3 月

北海道大学

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1. **An, Y.,** Inoue, T., Kitaichi, Y., Izumi, T., Nakagawa, S., Song, N., Chen, C., Li, X., Koyama, T., Kusumi, I. Anxiolytic-like effect of mirtazapine mediates its effect in the median raphe nucleus. European journal of pharmacology 720:192-197, 2013.

2. **An, Y.,** Inoue, T., Kitaichi, Y., Izumi, T., Nakagawa, S., Song, N., Chen, C., Li, X., Kusumi, I. Anxiolytic-like effects of mirtazapine microinjection into the median raphe nucleus are enhanced by subchronic lithium carbonate.日本神経精神薬理学雑誌 34:57-58, 2014

3. **An, Y.,** Inoue, T., Kitaichi, Y., Nakagawa, S., Wang, C.,Chen, C.,Song, N., Kusumi, I. Subchronic lithium treatment increases the anxiolytic-like effect of mirtazapine on the expression of contextual conditioned fear. European journal of pharmacology 747:13-17, 2015.

4. Masuda, T., Inoue, T., **An, Y.,** Takamura, N., Nakagawa, S., Kitaichi, Y., Koyama, T., Kusumi, I. Effect of the coadministration of citalopram with mirtazapine or atipamezole on rat contextual conditioned fear. Neuropsychiatric disease and treatment 10: 289-295, 2014.

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1. **An, Y.,** Inoue, T., Kitaichi, Y.,Nakagawa, S.,Izumi, T., Song, N., Koyama, T.

Subchronic lithium carbonate potentiates the effect of mirtazapine treatment on the conditioned fear. 34th Japanese Society of Biological Psychiatry, September 28-30, 2012, Kobe, Japan.

2. **An, Y.,** Inoue, T., Kitaichi, Y.,Nakagawa, S.,Izumi, T., Song, N., Koyama, T., Kusumi, I. Anxiolytic effect of mirtazapine and its lithium augmentation on conditioned fear mediates its effect in the median rephe nucleus. Neuro 2013, June 20-23, 2013, Kyoto, Japan.

3. **An, Y.,** Inoue, T., Kitaichi, Y.,Izumi, T., Nakagawa, S.,Song, N., Chen, C., Kusumi, I. Anxiolytic effect of mirtazapine microinjection in the median raphe nucleus and subchronic lithium carbonate enhances this effect. The 3rd Congress of Asian College of Neuropsychopharmacology, September 11-14, 2013, Beijing, China.

Introduction

Mirtazapine is an antidepressant with a unique mechanism of action and has been categorized as a Noradrenergic and Specific Serotonergic Antidepressant (NaSSA). It preferentially blocks the noradrenergic α_2 auto- and hetero-receptors responsible for controlling noradrenaline and serotonin (5-hydroxytryptamine; 5-HT) release [1]. Recent clinical evidence has shown that mirtazapine is effective in the treatment of anxiety disorders in addition to depressive disorders[2-4]. Although its beneficial effects in relieving anxiety symptoms and curing anxiety disorders have been reported consistently, the mechanism how mirtazapine exerts its anxiolytic effect has not been fully clarified [2-4]. To further explore the anxiolytic mechanism of mirtazapine, in the first chapter, I investigated the brain area(s) in which mirtazapine exerts its anxiolytic-like effect.

Clinically, many augmentation strategies have been developed to increase the effectiveness of antidepressant drugs. One such approach used in psychiatric disorders is addition of lithium to antidepressant drug [5,6]. Previous studies have reported that both lithium and mirtazapine influence serotonergic systems, and have shown that the enhancement of serotonergic neurotransmission causes anxiolytic-like effect in the contextual fear conditioning model, a useful animal model of anxiety [7-11]. These suggest the possibility that the combination of lithium and mirtazapine may have better efficacies for anxiety disorders. Therefore, in the second chapter, the study was designed to assess the effectiveness of the combination of the subchronic lithium treatment and mirtazapine on the expression of conditioned freezing behavior.

Contextual fear conditioning is a form of Pavlovian conditioning, in which an environment previously paired with an aversive unconditional stimulus (US, usually a foot shock) elicits freezing behavior (immobility except for breathing). A number of studies have demonstrated the reliability of the fear conditioning test as a behavioral paradigm to clarify the mechanisms involved in fear and anxiety. Previous studies have shown that the fear and anxiety response of rats in the fear conditioning test is attenuated by standard anxiolytic drugs such as benzodiazepines as well as SSRIs [11]. Thus, the conditioned fear stress model is thought suitable to evaluate the efficacy of therapeutic agents of anxiety disorders.

List of Abbreviations

MAOIs	monoamine oxidase inhibitors
MRN	median raphe nucleus
MTZ	mirtazapine
NaSSA	Noradrenergic and Specific Serotonergic Antidepressant
SSRIs	selective serotonin reuptake inhibitors
5-HT	serotonin (5-hydroxytryptamine)

Chapter 1: Target brain sites of the anxiolytic-like effect of mirtazapine

Introduction

As mentioned above, mirtazapine, which blocks the noradrenergic α_2 auto- and hetero-receptors[1], is effective in the treatment of various anxiety disorders in addition to depressive disorders[2,3], but the mechanism how mirtazapine exerts its anxiolytic effect has not been fully clarified. Previous studies have found that systemic administration of mirtazapine increases the extracellular serotonin levels in the brain and this might be related to its anxiolytic-like effect in the contextual fear conditioning model [10,12], because a number of studies have demonstrated that the facilitation of 5-HT neurotransmission decreases the expression of contextual conditioned freezing [11]. To further explore the anxiolytic mechanism of mirtazapine, in the present study, I investigated the brain area(s) in which mirtazapine exerts its anxiolytic-like effect.

The amygdala and hippocampus are two main brain structures implicated in the acquisition, expression and retrieval of fear conditioning [13,14]. In addition, the median raphe nucleus (MRN) also has an important role in the acquisition and expression of conditioned fear [15-18]. The MRN receives noradrenergic afferents from the locus coeruleus and sends serotonergic efferents predominantly to the medial septum and hippocampus and less extensively to the amygdala [19]. Recent evidence indicated that serotonergic mechanisms of the MRN-dorsal hippocampus circuit might play a major role in the expression of contextual fear conditioning[15], and that the serotonergic pathway which goes from the MRN to the hippocampus might be a critical component of Gray's 'behavioral inhibition system'[20]. The MRN includes a great density of α_1-adrenoceptors and α_2-adrenoceptors besides the high density of 5-HT receptors [21-23]. The α_2-adrenergic agonist clonidine injected into the MRN decreases the level of 5-HT in this nucleus, while an α_2-adrenergic antagonist injected into the MRN enhances 5-HT release [24]. Although the role of α_2-adrenoceptors in mediating 5-HT release in the MRN has been elucidated, little is known about the influence of α_2-adrenoceptors in the MRN on anxiety-like behaviors.

Methods

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.

Drugs

Mirtazapine (obtained from Merck & Co. Inc., Whitehouse Station, NJ, U.S.A.) was suspended in 0.15% tartaric acid. The vehicle alone was administered as a control.Mirtazapine was injected intraperitoneally (i.p.) in a volume of 2 ml/kg.

In the microinjection study, 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 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.

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.

Fear conditioning and behavioral measures

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, the rats received a systemic or local infusion of mirtazapine. After the treatment, 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 [25]. During the observation period, the duration of freezing behavior was recorded using a modified time-sampling procedure as previously described[26]. 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.

Experimental design

Effect of acute systemic mirtazapine treatment on the expression of contextual conditioned freezing: a dose-response study and a study with different intervals between fear conditioning and exposure to conditioned fear

In the dose-response study, twenty-four hours after the footshock, the rats received a single intraperitoneal injection of mirtazapine at doses of 0, 1, 3 and 10 mg/kg 30 min before testing. In the study with different intervals, one and seven days after the footshock, the rats received a single intraperitoneal injection of mirtazapine (10 mg/kg) 30 min before testing.

Effect of local treatment of mirtazapine on the expression of contextual conditioned freezing

Twenty-four hours after footshock, mirtazapine (3 μg/site) was directly injected into three brain structures, the MRN, hippocampus or amygdala at 10 min before testing.

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 rats were housed individually for three days before testing. During the testing, rats were individually placed in a testing cage (38 × 33 × 17 cm), and motor activity was automatically recorded for 5 min as described previously [27] using infrared sensors between 08:00 and 13:00. Systemic or local mirtazapine administration was given 30 or 10 min before testing, respectively. 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.

Histology

At the end of the microinjection experiments, rat 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.

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, one- and two-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons as a post hoc test when the interaction was significant. The significance level was chosen at 0.05.

Results

Effect of acute systemic mirtazapine treatment on the expression of contextual conditioned freezing: a dose-response study

Acute systemic mirtazapine caused a dose-dependent reduction in freezing (Fig. 1). The higher doses of mirtazapine, 3 mg/kg ($P < 0.05$) and 10 mg/kg ($P < 0.05$), reduced the expression of contextual conditioned freezing significantly, while a lower dose, 1 mg/kg, showed no significant effect compared with the vehicle ($P = 0.27$).

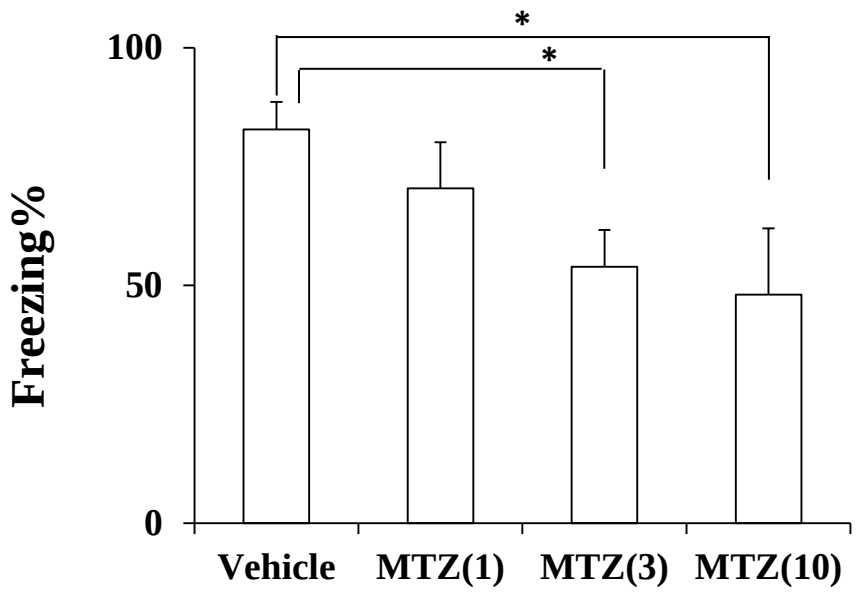


Fig.1 Effect of acute mirtazapine treatment on the expression of conditioned freezing. Mirtazapine was administered intraperitoneally 1 day after footshock and 30 min before conditioned fear stress. Data are represented as the mean ± S.E.M of freezing scored for a 5-min observation period. Behavior was sampled at 10-s intervals. * $P<0.05$. N=8-12 per group.

Effect of different intervals between fear conditioning and exposure to contextual conditioned fear on acute inhibition of conditioned freezing by mirtazapine

Two-way ANOVA revealed significant main effects of the interval and acute challenge of mirtazapine and a significant interaction [Effect of interval: $F(1,28)=4.62, P<0.05$; Effect of acute challenge of mirtazapine: $F(1,28)=8.39, P<0.05$; Effect of interaction: $F(1, 28)=6.81, P<0.05$] (Fig. 2). One day after the footshock, an acute challenge of mirtazapine (10 mg/kg) significantly reduced the expression of contextual conditioned freezing compared with the respective vehicle group ($P<0.01$). However, mirtazapine (10 mg/kg) did not affect contextual conditioned freezing compared with the respective vehicle group when the rats were exposed to conditioned fear seven days after footshock.

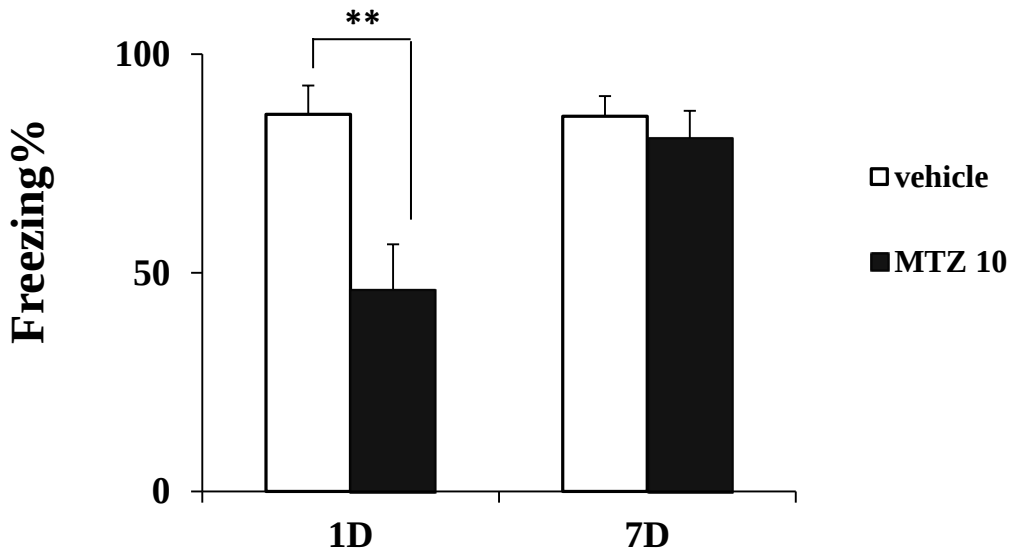


Fig. 2 Effect of acute mirtazapine treatment on the expression of conditioned freezing with various intervals (1 and 7 days) between fear conditioning (footshock) and testing. Mirtazapine (MTZ, 10 mg/kg) or the vehicle was administered intraperitoneally at 30 min before testing. Data are represented as the mean ± S.E.M of freezing scored for a 5-min observation period. Behavior was sampled at 10-s intervals.** $p<0.01$. N=8 per group. 1D, 1days; 7D, 7days.

Effect of mirtazapine microinjection into the MRN on the expression of contextual conditioned freezing (Fig. 3)

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].

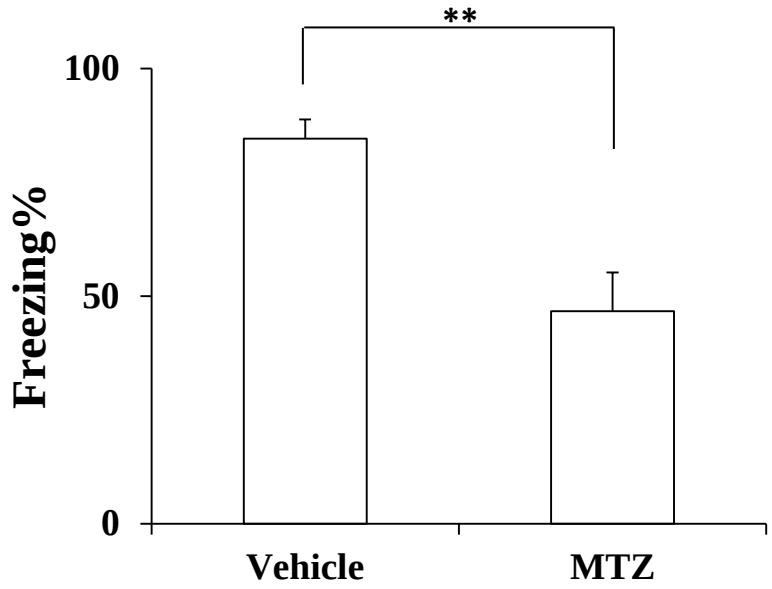


Fig. 3 The effect of mirtazapine (MTZ) microinjections (3 μg/site) into the MRN on freezing induced by conditioned fear. Mirtazapine was administered 24 h after footshock and 10 min before conditioned fear stress (testing). Freezing scored over a 5-min observation period is represented as the mean percentage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

Effect of mirtazapine microinjection into the dorsal hippocampus on the expression of contextual conditioned freezing (Fig. 4)

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.

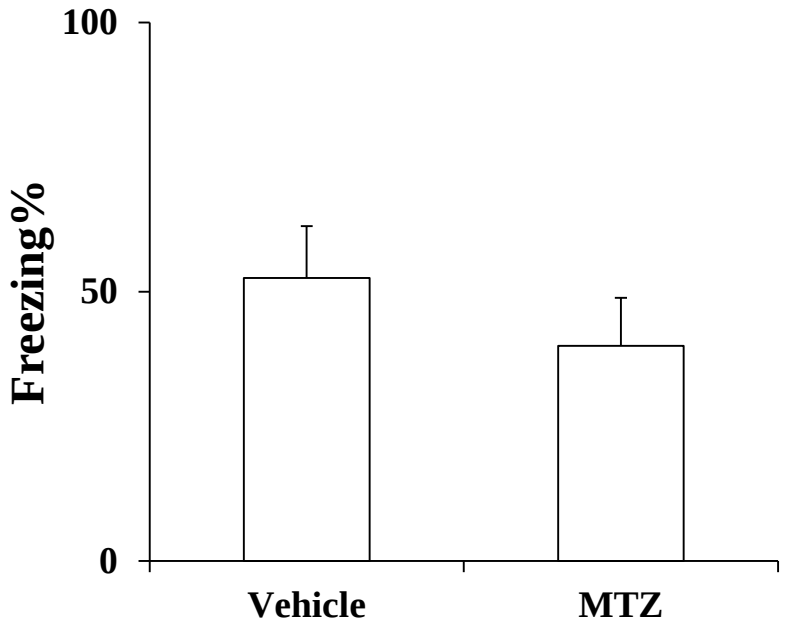


Fig.4 The effect of bilateral mirtazapine (MTZ) microinjections (3 μg/site) into the dorsal hippocampus on freezing induced by conditioned fear. Mirtazapine was administered 24 h after footshock and 10 min before conditioned fear stress (testing). Freezing scored over a 5-min observation period is represented as the mean percentage ± SEM. Behavior was sampled at 10-s intervals. N=9-10 rats.

Effect of mirtazapine microinjection into the amygdala on the expression of contextual conditioned freezing (Fig. 5)

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.

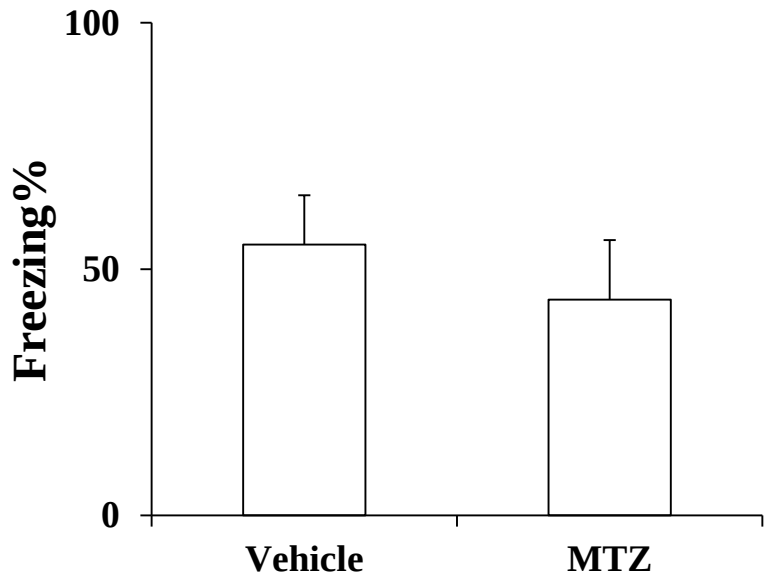


Fig.5 The effect of bilateral mirtazapine (MTZ) microinjections (3 μg/site) into the amygdala on freezing induced by conditioned fear. Mirtazapine was administered 24 h after footshock and 10 min before conditioned fear stress (testing). Freezing scored over a 5-min observation period is represented as the mean percentage ± SEM. Behavior was sampled at 10-s intervals. N=6-7 rats.

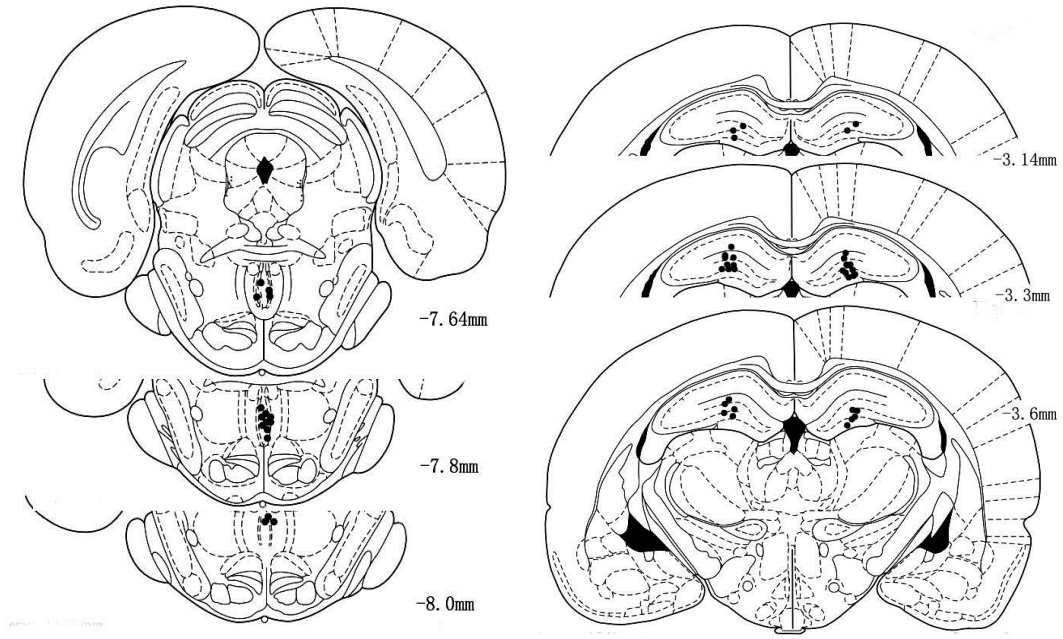
Effect of mirtazapine on locomotor activity

Acute systemic mirtazapine (10 mg/kg) treatment did not affect motor activity of unshocked rats during the 5-min testing in the home cages [vehicle 83.4±43.3 counts, mirtazapine 139.1±22.2 counts; t (14) =1.146, P=0.27, t-test]. Mirtazapine microinjection into the MRN did not change the motor activity of unshocked rats during the 5-min testing 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].

Histology

Tissue damage was not apparent in either the drug group or the vehicle group. Fig. 6 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).

(A) (B)



(C)

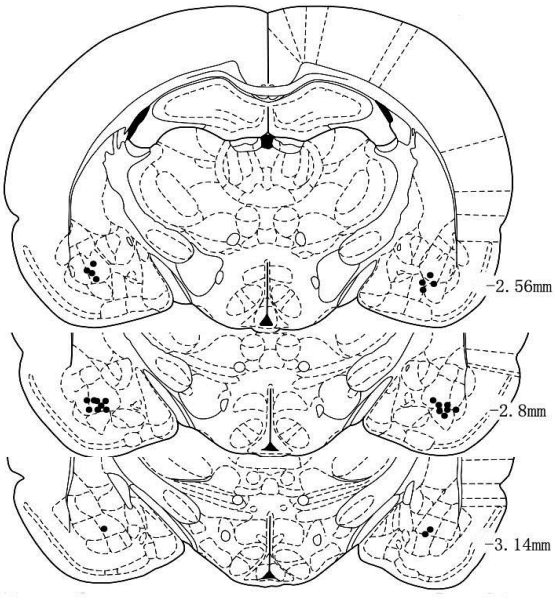


Fig.6 Diagrammatic representation of coronal sections through the rat brain showing the location of injection sites (solid circles) of the MRN (A), dorsal hippocampus (B) and amygdala (C). Figures represent coordinates from the Paxinos and Watson (1997) brain atlas, with respect to the bregma. The number of points in the figures is fewer than the total number

of rats used because of several overlaps.

Discussion

Acute systemic administration of mirtazapine dose-dependently reduced freezing one day after fear conditioning, whereas the anxiolytic-like effect of mirtazapine (10 mg/kg) was diminished when the interval between fear conditioning and testing was prolonged for seven days. The latter effect is consistent with our previous reports showing that the inhibitory effect of an acute challenge of SSRIs on conditioned freezing diminished by prolonging the interval between conditioning and testing [28-30]. Although the reason for the diminishment of the anxiolytic-like effect of SSRIs and mirtazapine by prolonging the intervals after fear conditioning remains unclear, memory consolidation processes after the fear acquisition may be involved in this effect [29,31].

In the present study, I 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. The inhibitory effect of intra-MRN mirtazapine treatment on freezing behavior indicates its anxiolytic-like effect.

The MRN is a main source of serotonergic innervation to the forebrain structures involved in anxiety regulation [19,32]. 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 [15,33,34]. The great density of α_1- and α_2-adrenoceptorsin the MRN shown by anatomical studies [21,23,35] 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 [24]. 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 [24]. Hence, intra-MRN infusion of mirtazapine, which is an α_2-adrenergic antagonist and has very weak α_1-adrenergic antagonistic action [1], is supposed to increase extracellular 5-HT levels in the nerve terminal areas such as the hippocampus. 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) [36]. Their results support my 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 serotonergic neurons.

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 [37]. 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 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.

Chapter 2: Augmentation strategies for the anxiolytic-like effect of mirtazapine

Introduction

Results in chapter 1 have shown that the anxiolytic-like effect of mirtazapine diminished 7 days after fear conditioning. This model with a long interval is consistent with clinical findings that antidepressants exert an anxiolytic effect only after chronic treatment [38,39]. The long interval between conditioning by footshock and exposure to conditioned fear stress reinforces fear memory and therefore may improve predictive and face validities of contextual conditioned freezing as an animal model of anxiety disorders [28,31]. Using this model, the effect of acute treatment with mirtazapine after subchronic lithium was investigated in this study. Clinical studies suggest that although most patients have a favorable response to antidepressant drugs for anxiety and mood disorders, approximately 30% to 40% would not respond to adequate first-line antidepressant medication [40,41]. Many augmentation strategies have been developed to increase the effectiveness of antidepressant drugs. One such approach used in psychiatric disorders is addition of lithium to antidepressant drugs [5,6].

Preclinical and clinical studies have shown that lithium modifies the serotonergic neurotransmission and lithium increases extracellular 5-HT levels through several mechanisms in the brain such as increased 5-HT synthesis, increased 5-HT turnover, and increased 5-HT release from nerve endings [42,43]. In vivo microdialysis studies have shown that lithium increases extracellular 5-HT concentrations in the medial prefrontal cortex and hippocampus [7-9,44]. Furthermore, clinical studies showed that lithium has been involved in the appearance of the 5-HT syndrome [45-47]. Therefore, it is suggested that the lithium augmentation of antidepressant effect may occur via a direct and/or indirect mechanism on 5-HT. Although a few clinical studies have shown that the combination of lithium and mirtazapine is safe and well-tolerated and may be more efficacious [48,49], to the best of my knowledge, the behavioral effects of adding lithium to mirtazapine have not yet been examined. Based on the mechanism of anxiolytic action of lithium and mirtazapine described above, the present study assessed the anxiolytic-like effect of the combination of the subchronic lithium treatment and acute systemic or local administration of mirtazapine in rats

using the contextual fear conditioning test as an animal model of fear and anxiety.

Methods

Animals

Male Sprague–Dawley rats weighing 260-320 g at the beginning of the experiments were used. 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 two-week period of acclimatization. The animals were maintained on a diet of standard laboratory rat chow, or rat chow containing 0.05% or 0.2% of Li_2CO_3 for 7 days. In the lithium experiments, the lithium-treated rats and the control rats were given 10 mM NaCl instead of tap water to prevent lithium-induced hyponatremia [50]. The rest of the time, all animals had free access to food and water. All experiments were performed between 08:00 and 13:00.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.

Drugs

Fear conditioning and behavioral measures

Stereotaxic surgery

Motor activity

Histology

The detailed methods of drugs, fear conditioning and behavioral measures, stereotaxic surgery, motor activity and histology are described in the Methods section of Chapter 1.

Experimental design

Effect of subchronic lithium with acute systemic mirtazapine treatment on the expression of contextual conditioned freezing

Immediately after the footshock, the rats received standard laboratory rat chow (0% Li_2CO_3) or rat chow containing 0.05% or 0.2% of Li_2CO_3 for 7 days. On the eighth day, the rats received a single intraperitoneal injection of mirtazapine at 10 mg/kg 30 min before testing.

Effect of subchronic lithium with local mirtazapine treatment on the expression of contextual conditioned freezing

Immediately after the footshock, the rats received standard laboratory rat chow (0% Li_2CO_3) or rat chow containing 0.05% or 0.2% of Li_2CO_3 for 7 days. On the eighth day, the rats were injected into hippocampus, amygdala and MRN with mirtazapine (3μg/site) at 10 mins before testing.

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, two-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons as a post hoc test when the interaction was significant. The significance level was chosen at 0.05.

Results

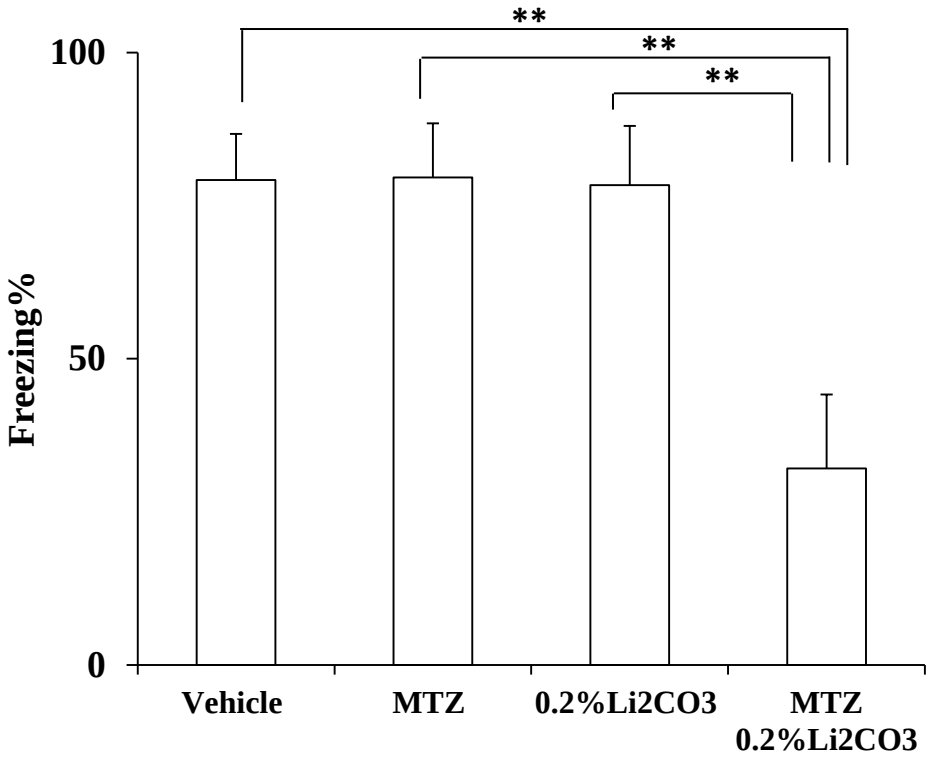
Effect of subchronic lithium with acute mirtazapine (10 mg/kg) treatment on the expression of contextual conditioned freezing

Subchronic treatment with 0.2% Li_2CO_3 significantly enhanced the effect of mirtazapine (10 mg/kg) on the expression of contextual conditioned freezing (Fig.7A). Two-way ANOVA revealed significant main effects of mirtazapine and 0.2% Li_2CO_3 on freezing behavior. Additionally, a significant interaction between mirtazapine and 0.2% Li_2CO_3 was identified [Effect of mirtazapine: $F(1,28)=5.62$, $P<0.05$; Effect of 0.2% Li_2CO_3: $F(1,28)=6.24$, $P<0.05$; Effect of interaction: $F(1,28)=5.82$, $P<0.05$]. *Post hoc* analysis showed that the mirtazapine-0% Li_2CO_3 or vehicle-0.2% Li_2CO_3treatments had no significant effect on freezing behavior compared with the untreated group, while the mirtazapine-0.2% Li_2CO_3

treatment significantly reduced freezing compared with the vehicle-0% Li_2CO_3 ($P<0.01$), mirtazapine-0% Li_2CO_3 ($P<0.01$) and vehicle-0.2% Li_2CO_3 ($P<0.01$) groups.

In contrast to the effect of subchronic treatment with 0.2% Li_2CO_3, subchronic treatment with 0.05% Li_2CO_3 did not change the inhibitory effect of mirtazapine on the expression of contextual conditioned freezing (Fig.7B).Two-way ANOVA revealed no significant effects of mirtazapine or 0.05% Li_2CO_3 and no significant interaction [Effect of mirtazapine: $F(1, 28)= 0.002$, $P=0.97$; Effect of 0.05% Li_2CO_3: $F(1, 28)= 0.15$, $P=0.71$; Effect of interaction: $F(1, 28)=3.11$, $P=0.09$].

(A)



(B)

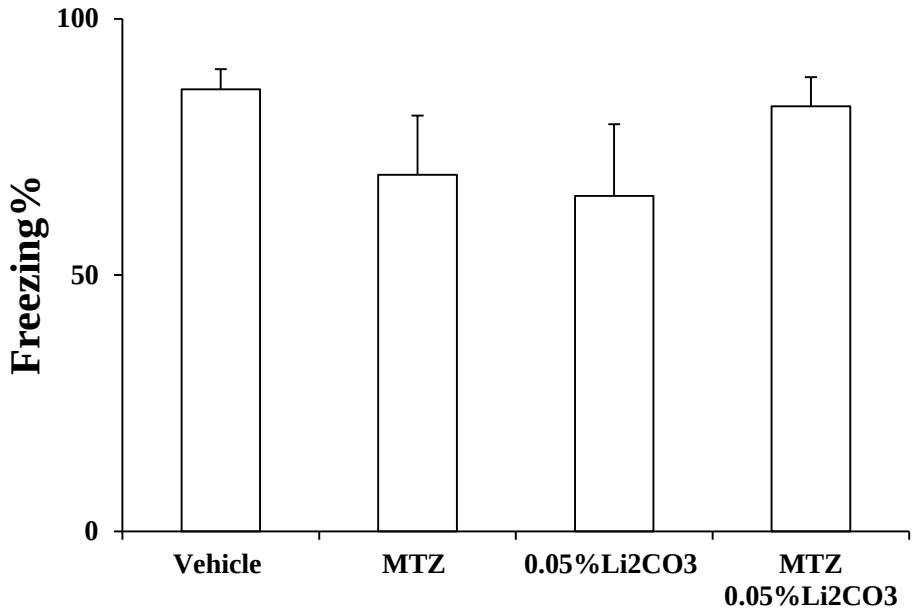


Fig.7. Effect of subchronic lithium treatment (A 0.2% Li_2CO_3 in diet; B 0.05% Li_2CO_3 in diet) on mirtazapine (MTZ, 10 mg/kg) - induced inhibition of the expression of conditioned freezing. $Li_2CO_3$was administered p.o. for 7 days after footshock. Mirtazapine 10 mg/kg was administered intraperitoneally 30 min before conditioned fear stress. Data are represented as the mean ± S.E.M of freezing scored for a 5-min observation period. Behavior was sampled at 10 s intervals. ** $p<0.01$. N=8.

Effect of subchronic lithium with acute mirtazapine microinjection on the expression of contextual conditioned freezing

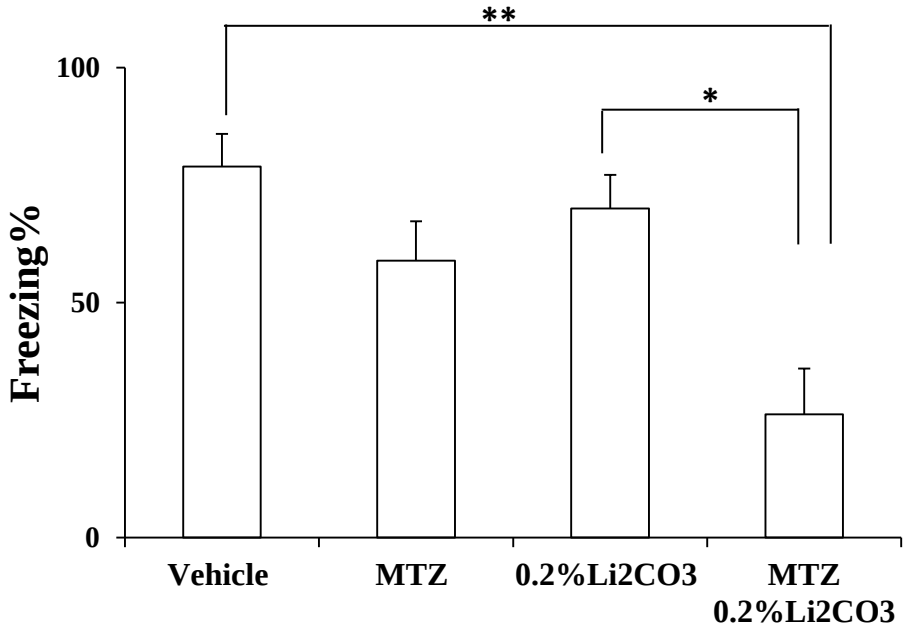
Fig.8A shows subchronic 0.2% Li_2CO_3 treatment significantly enhanced the effect of mirtazapine microinjection into the MRN on freezing behavior. Two-way ANOVA revealed significant main effects of mirtazapine and 0.2% Li_2CO_3 on freezing behavior. Additionally, a significant interaction between mirtazapine and 0.2% Li_2CO_3 was identified [Effect of mirtazapine: $F(1,22)=14.91$, $P<0.01$; Effect of 0.2% Li_2CO_3: $F(1,22)=6.33$, $P<0.05$; Effect of interaction: $F(1,22)=2.08$, $P=0.16$]. *Post hoc* analysis showed that the mirtazapine-0% Li_2CO_3 or vehicle-0.2% Li_2CO_3treatments had no significant effect on freezing behavior

compared with the untreated group, while the mirtazapine-0.2% Li_2CO_3 treatment significantly reduced freezing compared with the vehicle-0%Li_2CO_3 ($P < 0.01$), and vehicle-0.2%Li_2CO_3 ($P < 0.05$)groups.

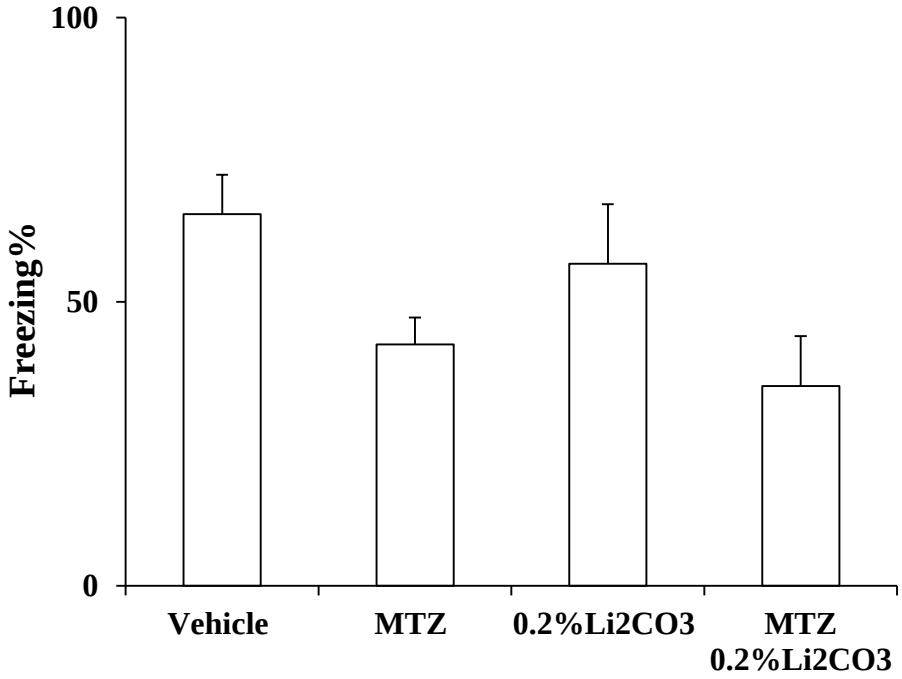
Fig.8B shows that subchronic 0.2% Li_2CO_3 treatment did not affect the effect of mirtazapine microinjection into the hippocampus on freezing behavior. Two-way ANOVA revealed significant main effects of mirtazapine on freezing behavior, but revealed no significant effects of 0.2% Li_2CO_3 and no significant interaction [Effect of mirtazapine: $F(1,29)= 7.5$, $P < 0.05$; Effect of 0.2% Li_2CO_3: $F(1, 29)= 0.98$, $P=0.33$; Effect of interaction: $F(1, 28)=0.01$, $P=0.93$].

As shown in Fig. 8C, subchronic 0.2% Li_2CO_3 treatment did not affect the effect of mirtazapine microinjection into the amygdala on freezing behavior. Two-way ANOVA revealed no significant effects of mirtazapine or 0.2% Li_2CO_3 and no significant interaction. [Effect of mirtazapine: $F(1,18)= 0.09$, $P=0.77$; Effect of 0.2% Li_2CO_3: $F(1, 18)= 1.59$, $P=0.22$; Effect of interaction: $F(1, 18)=1.0$, $P=0.33$].

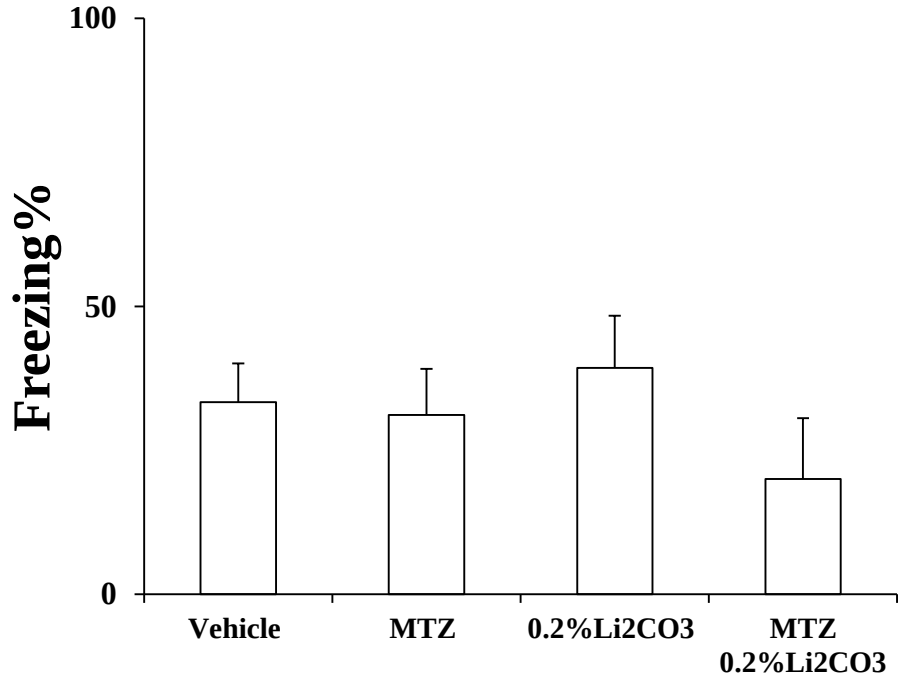
(A)



(B)


100
50
0
Freezing%
Vehicle
MTZ
0.2%Li2CO3
MTZ
0.2%Li2CO3

(C)


100
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Freezing%
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0.2%Li2CO3
MTZ
0.2%Li2CO3

Fig.8. Effect of subchronic lithium treatment 0.2% Li_2CO_3 with the acute mirtazapine (MTZ) microinjections (3 μg/site) into the MRN (A), hippocampus (B) and amygdala (C) on the expression of conditioned freezing. 0.2% $Li_2CO_3$was administered p.o. for 7 days after footshock. Mirtazapine (3 μg/site) was administered 10 min before conditioned fear stress. Data are represented as the mean ± S.E.M of freezing scored for a 5-min observation period. Behavior was sampled at 10 s intervals. ** $p<0.01$. N=7-9.

Motor activity

Acute mirtazapine (10 mg/kg) treatment and subchronic 0.2% Li_2CO_3 treatment alone did not affect motor activity in home cages. Furthermore, co-treatment with mirtazapine (10 mg/kg) and 0.2% Li_2CO_3 also did not affect motor activity in home cages compared with the control (Table 1). Two-way ANOVA revealed no significant effect of mirtazapine, 0.2% Li_2CO_3, or interaction. [Effect of mirtazapine, $F(1, 28)= 2.43$, $P=0.13$; Effect of 0.2% Li_2CO_3, $F(1, 28)= 2.90$, $P= 0.10$; Effect of interaction, $F(1, 28)=0.03$, $P=0.85$].

Table 1. The effect of the combination of mirtazapine (MTZ; 10mg/kg) and 0.2% Li_2CO_3 on spontaneous motor activity

Drug treatment	Motor activity (counts/5 min)
Vehicle- Vehicle	83.4±43.3
MTZ(10)- Vehicle	139.1±22.2
Vehicle-0.2% Li_2CO_3	34.9±22.7
MTZ (10)-0.2% Li_2CO_3	78.7±34.7

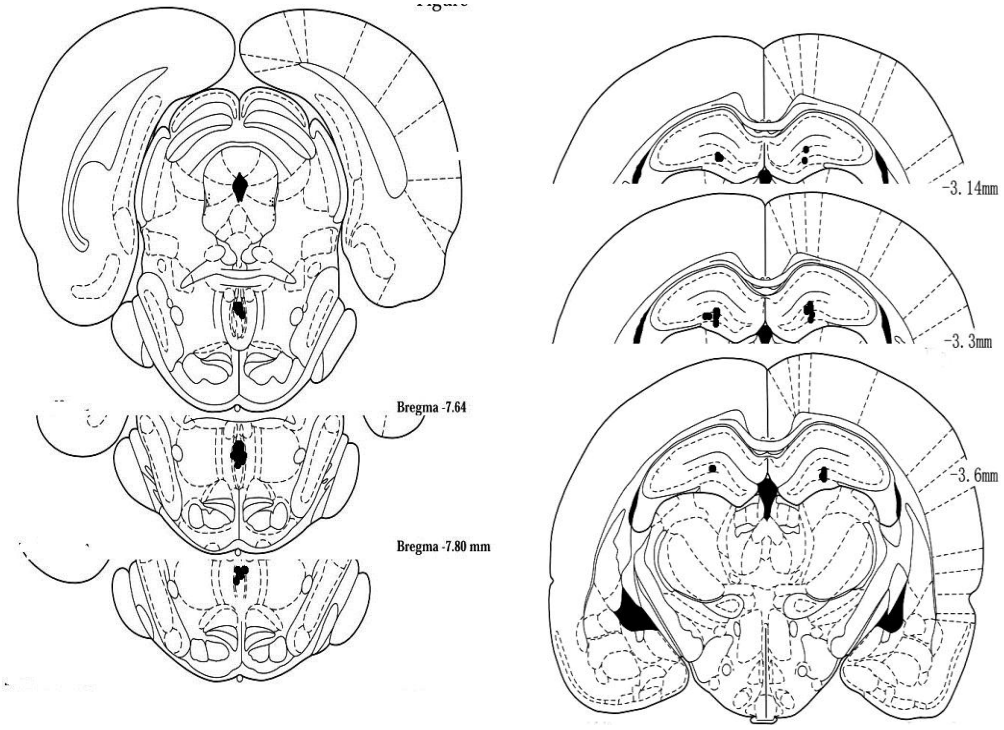
Two-way ANOVA showed no significant effect of mirtazapine, 0.2% Li_2CO_3 or interaction. All the data are represented as the means ± S.E.M. N=8 per group.

Histology

Tissue damage was not apparent in either the drug group or the vehicle group. Fig. 9 depicts the sites of drug injection into the MRN (A), hippocampus (B) and amygdala (C). The histological results were plotted on representative sections taken from the rat brain atlas of

Paxinos and Watson (1997).

(A) (B)



(C)

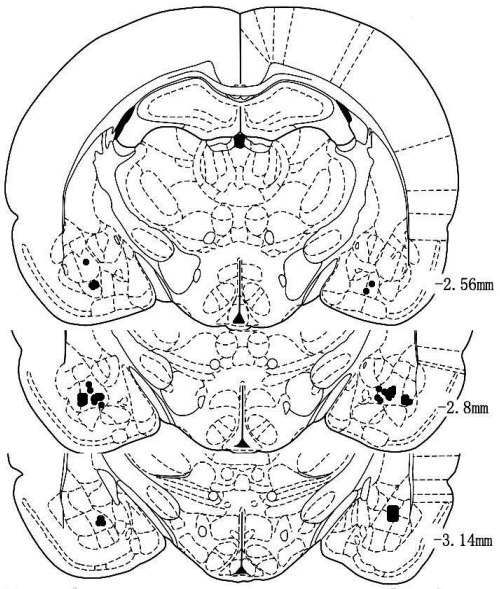


Fig.9 Diagrammatic representation of coronal sections through the rat brain showing the location of injection sites (solid circles) of the MRN (A), dorsal hippocampus (B) and amygdala (C). Figures represent coordinates from the Paxinos and Watson (1997) brain atlas,

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.

Discussion

In this study, subchronic 0.2% Li_2CO_3 treatment in the diet for one week significantly enhanced the inhibitory effect of mirtazapine on contextual conditioned freezing. Moreover, subchronic 0.2% Li_2CO_3 treatment with mirtazapine did not affect motor activity compared with the vehicle controls, thereby excluding the possibility of nonspecific motor interference as the main factor accounting for its effect in the conditioned fear test. These results indicate that subchronic 0.2% Li_2CO_3 treatment enhanced the anxiolytic-like effect of acute mirtazapine. *In vivo* microdialysis studies reported that the systemic administration of mirtazapine increased extracellular 5-HT concentrations in the hippocampus of rats [10]. In addition, subchronic lithium treatment increased extracellular 5-HT levels in the medial prefrontal cortex and hippocampus [7-9,44] and additively increased the elevating effect of citalopram and monoamine oxidase inhibitors (MAOIs) on extracellular 5-HT concentrations[8,9]. Furthermore, subchronic lithium increased the anxiolytic-like effect of citalopram and MAOIs in contextual conditioned fear [8,51]. Taken together, these data suggest that the augmenting effect of lithium on the anxiolytic-like effect of acute mirtazapine may also be associated with the enhancement of 5-HT neurotransmission. Future studies will be needed to confirm whether lithium enhances 5-HT neurotransmission increased by acute mirtazapine.

Subchronic treatment with 0.2% Li_2CO_3 significantly enhanced the effect of acute mirtazapine microinjection into the MRN, but not hippocampus or amygdala, on freezing. This result is in accordance with the result of Chapter 1, which demonstrated that the MRN plays an important role in the anxiolytic-like effect of mirtazapine in contextual conditioned fear. The anxiolytic-like effect of mirtazapine is suggested to be mediated by its action in the median raphe nucleus in the following hypothesis: mirtazapine blocks α_2-adrenoceptors in the median raphe nucleus, increases extracellular noradrenaline and stimulates the median raphe nucleus via α_1-adrenoceptors, and may increase 5-HT neurotransmission in the hippocampus,

the major projection area of the median raphe nucleus, leading to a reduction in anxiety [10,52]. Although no electrophysiological or *in vivo* microdialysis study has examined the effect of lithium on the neuronal activity of the median raphe nucleus, short-term (3 days), but not long-term (21 days), lithium administration facilitates the effect of electrical stimulation of the dorsal raphe nucleus on extracellular 5-HT levels in the hippocampus [53].Accordingly, it is hypothesized that subchronic lithium treatment stimulates the effect of mirtazapine on the median raphe5-HT neurons and enhances the anxiolytic-like effect of mirtazapine. Further studies are needed to determine whether lithium with mirtazapine stimulates the median raphe 5-HT neurons more.

Taken together, in the present study, I investigated the combined effect of subchronic lithium treatment and acute systemic or local administration of mirtazapine in the rat contextual conditioned fear stress model. Subchronic 0.2% Li_2CO_3 treatment significantly enhanced the anxiolytic-like effect of systemic and intra-MRN mirtazapine treatment on contextual conditioned fear. The results of this study provide further evidence for the lithium augmentation of mirtazapine, and suggests the hypothesis that the anxiolytic-like effect of mirtazapine and lithium augmentation of it is mediated by their action on the MRN.

Conclusion

In this study, I examined the anxiolytic-like effect of mirtazapine, an antagonist of central α_2-adrenergic autoreceptors and heteroreceptors, in the rat model of conditioned fear stress.

When the interval between conditioning and testing was 1 day, acute mirtazapine showed a dose-dependent reduction in freezing time, suggesting an anxiolytic-like effect. Intra-MRN injection of mirtazapine reduced freezing behavior significantly, while mirtazapine injections into the hippocampus or amygdala did not. These results demonstrated that the anxiolytic-like effect of mirtazapine was mediated by its action in the MRN. In the future, the effect of a selective α_2-adrenergic antagonist microinjection into the MRN on anxiety-like behaviors will be examined.

When the interval was 7 days, acute systemic mirtazapine 10 mg/kg treatment did not reduce the expression of conditioned freezing significantly, which suggests that the anxiolytic-like effect of mirtazapine was diminished. Therefore, I examined the augmentation strategies for the anxiolytic-like effect of mirtazapine. The combination of subchronic 0.2% Li_2CO_3 but not 0.05% Li_2CO_3 with acute mirtazapine reduced freezing significantly. Moreover, subchronic treatment with 0.2% Li_2CO_3 enhanced the effect of mirtazapine (3 μg/site) on freezing behavior significantly when mirtazapine was infused into the MRN but not hippocampus or amygdala. These results indicated that subchronic 0.2% Li_2CO_3 treatment enhanced the anxiolytic-like effect of mirtazapine. This augmentation therapy might provide a useful reference for the treatment of anxiety disorders. In addition, as the serotonergic pathway which goes from the MRN to the hippocampus plays a major role in the expression of contextual fear conditioning [15], the result of lithium-induced enhancement of the anxiolytic-like effect of the intra-MRN mirtazapine administration supports the hypothesis that this enhancement is mediated by the effect on serotonin. Further investigations are needed to clarify the mechanisms at the molecular level involved in the effect of lithium augmentation.

Acknowledgments

First of all, I would like to thank Professor Ichiro Kusumi for his kind mentorship and thoughtful comments on this thesis. Furthermore, I would like to thank for his understanding and supporting to my temporal absence due to the pregnancy and childcare. Moreover, I would like to thank Associate Professor Takeshi Inoue. He has given me thoughtful advices and numerous supports as my mentor. I also would like to thank Professor Emeritus Tsukasa Koyama for his support in my life and studies.

I would like to thank Dr. Yuji Kitaichi for his kind and appropriate advices on my studies. I would like to thank Dr. Shin Nakagawa, Dr. Takeshi Izumi, Professor Xiaobai Li, Dr. Minori Koga, Dr. Chong Chen and Dr. Ning Song for their kind supports and useful suggestions.
Finally, I would like to thank my parents and my husband Dr. Ce Wang for their kind and patient supports in childcare and housekeeping.

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