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Subchronic lithium treatment increases the anxiolytic-like effect of mirtazapine on the expression of contextual conditioned fear

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Abstract

Lithium not only has a mood-stabilizing effect but also the augmentation effect of an antidepressant, the mechanism of which remains unclear. Although lithium may augment the effect of mirtazapine, this augmentation has not been confirmed. Using a contextual fear conditioning test in rats, an animal model of anxiety or fear, we examined the effect of subchronic lithium carbonate (in diet) in combination with systemic mirtazapine on the expression of contextual conditioned fear. Mirtazapine (10 mg/kg) reduced freezing one day after fear conditioning dose-dependently, whereas the anxiolytic-like effect of mirtazapine (10 mg/kg) diminished seven days after fear conditioning. When the interval between fear conditioning and testing was seven days, only the combination of subchronic 0.2% Li_2CO_3 but not 0.05% Li_2CO_3 with acute mirtazapine (10 mg/kg) reduced freezing significantly. These results indicate that subchronic 0.2% Li_2CO_3 treatment enhanced the anxiolytic-like effect of systemic mirtazapine. This augmentation therapy might be useful for the treatment of anxiety disorders.

Keywords: mirtazapine; lithium; anxiety; freezing; contextual fear conditioning

1. Introduction

Most patients have a favorable response to antidepressant drugs for anxiety and depressive disorders, but approximately 30% to 40% do not respond adequately to first-line antidepressant medication (Kornstein and Schneider, 2001; Zamorski and Albucher, 2002). Recently, many augmentation strategies have been developed to increase the effectiveness of antidepressant drugs. One such approach used in the treatment of psychiatric disorders is the addition of lithium to antidepressant drugs (Bauer et al., 2010; Chenu and Bourin, 2006).

Preclinical and clinical studies have demonstrated that lithium modifies serotonergic neurotransmission and increases extracellular serotonin (5-hydroxytryptamine; 5-HT) levels in the brain through several mechanisms, such as increased 5-HT synthesis, increased 5-HT turnover, and increased 5-HT release from nerve endings (Eroglu and Hizal, 1987; Kitaichi et al., 2004, 2006; Muraki et al., 2001; Price et al., 1990; Wegener et al., 2003). Preclinically, the combinations of lithium with selective serotonin reuptake inhibitors (SSRIs) or monoamine oxidase inhibitors have been reported to increase the effects of these antidepressants on extracellular 5-HT concentrations and on anxiety-like behaviors in the contextual fear conditioning test (Kitaichi et al., 2006; Muraki et al., 1999; Muraki et al., 2001). Therefore, these findings indicate that lithium augmentation of the antidepressant effect may occur via a direct and/or indirect effect on 5-HT.

Recent clinical evidence has shown that mirtazapine is effective in the

treatment of anxiety disorders as well as depressive disorders (Davidson et al., 2003; Gambi et al., 2005). In addition, animal behavioral studies have shown that systemic administration and local administration to the median raphe nucleus of mirtazapine decreased contextual conditioned freezing behavior, an index of fear or anxiety (An et al., 2013; Kakui et al., 2009). An *in vivo* microdialysis study reported that mirtazapine increased extracellular 5-HT concentrations in the hippocampus of rats (Yamauchi et al., 2012). These results suggest that the anxiolytic and antidepressant effect of mirtazapine might be mediated by the facilitation of central 5-HT neurotransmission.

Although a few clinical studies have shown that the combination of mirtazapine and lithium is safe and well-tolerated and might be more efficacious (Bruijn et al., 1998; Sitsen et al., 2000), to the best of our knowledge, the behavioral effects of adding lithium to mirtazapine have not yet been examined. Based on the mechanism of the anxiolytic actions of lithium and mirtazapine described above, we hypothesized that the combination of lithium and mirtazapine would have a superior effect on anxiety-like behavior as measured in the contextual fear conditioning test. As described above, a number of studies have demonstrated the reliability of the use of the contextual fear conditioning test as a behavioral paradigm to show the anxiolytic-like effect of mirtazapine, other serotonergic anxiolytics/antidepressants and lithium augmentation and to clarify the mechanisms of drug interactions that are mediated by serotonin (Inoue et al., 2011). To verify the above hypothesis, the present study assessed the anxiolytic-like effect of the combination of the subchronic lithium and systemic mirtazapine treatment in rats using the contextual fear conditioning test

as an animal model of fear and anxiety.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 260–320 g at the beginning of the experiments were used. The rats were housed in polypropylene cages (four animals per cage) with wood shavings on the floor. The room temperature was kept at $22\pm 2^\circ\text{C}$. The subjects were maintained on a 12-h light/dark cycle (light phase: 06:30–18:30). The 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 seven 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 (Thomsen and Olesen, 1974). 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.

2.2. Drug

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.

The doses of mirtazapine used in this study were based on previous reports (Kakui et al., 2009; the company's drug information). The dose of mirtazapine (10 mg/kg) used in this study produces plasma mirtazapine concentrations that are comparable to or greater than the clinically therapeutic plasma concentrations observed after administration of the clinically maximal dose (45 mg/day) of mirtazapine (company's drug information). Accordingly, 10 mg/kg of mirtazapine in rats approximately corresponds to a clinically maximal dose.

The concentrations of lithium carbonate in the rat chow were chosen based on our previous study in which we found that plasma lithium levels are 0.71 ± 0.05 mEq/l after 1 week of 0.2% Li_2CO_3 treatment and 0.26 ± 0.01 mEq/l after 1 week of 0.05% Li_2CO_3 treatment (Muraki et al., 1999). The plasma levels of lithium after 1 week of 0.2% Li_2CO_3 treatments are within the recommended therapeutic range (0.5-1.2 mEq/l) (Suppes et al., 2008; Bauer et al., 2010).

2.3. Contextual conditioned fear stress model

In this study, 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. One or seven days after the electric footshock, the rats were again placed in the shock chamber and observed for 5 min, but no current was applied to the floor of the chamber. The behavior was videotaped and scored later by human observation. During the observation period, the duration of the freezing behavior was recorded using a modified time-sampling procedure as previously described (An et al., 2013). 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 with the exceptions of 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 the rats for successive 10-s periods over 5 min (i.e., 30 successive sampling periods). If a rat exhibited any activity during the 10-s sampling period, we considered that period as active. The percentage freezing score [freezing (%)] was computed as the proportion of the 10-s periods during which the animal remained frozen for the entire period.

2.4. Experimental design

2.4.1. 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

The rats were subjected to inescapable electric footshocks in a chamber with a grid floor. 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 at 10 mg/kg 30 min before testing.

2.4.2. 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.

2.5. Motor activity

Motor activity was measured for mirtazapine (10 mg/kg) with or without subchronic 0.2% Li₂CO₃ treatment in unshocked rats. 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 as described previously (Ohmori et al., 1994) using infrared sensors between 08:00 and 13:00.

Mirtazapine was administered i.p. at 30 min before testing for 5 min. 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. Data analysis

All the data are presented as the means ± S.E.M. of the individual values of the rats from each group. The statistical analyses of the data were performed using 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. Statistical significance was set at $P < 0.05$.

3. Results

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

Acute 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 ($P = 0.27$).

3.2. 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.

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

Subchronic treatment with 0.2% Li₂CO₃ significantly enhanced the effect of mirtazapine (10 mg/kg) on the expression of contextual conditioned freezing (Fig.3A). Two-way ANOVA revealed significant main effects of mirtazapine and 0.2% Li₂CO₃ on freezing behavior. In addition, a significant interaction between mirtazapine and 0.2% Li₂CO₃ was identified [Effect of mirtazapine: $F(1,28)=5.62, P<0.05$; Effect of 0.2% Li₂CO₃: $F(1,28)=6.24, P<0.05$; Effect of interaction: $F(1,28)=5.82, P<0.05$]. *Post hoc* analysis showed that the mirtazapine-0% Li₂CO₃ or vehicle-0.2% Li₂CO₃ treatments had no significant effect on freezing behavior compared with the untreated group, while the mirtazapine-0.2% Li₂CO₃ treatment significantly reduced freezing compared with the vehicle-0% Li₂CO₃ ($P<0.01$), mirtazapine-0% Li₂CO₃ ($P<0.01$) and vehicle-0.2% Li₂CO₃ ($P<0.01$) groups.

In contrast to the effect of subchronic treatment with 0.2% Li₂CO₃, subchronic treatment with 0.05% Li₂CO₃ did not change the inhibitory effect of mirtazapine on the expression of contextual conditioned freezing (Fig.3B). Two-way ANOVA revealed no significant effects of mirtazapine or 0.05% Li₂CO₃ and no significant interaction [Effect of mirtazapine: $F(1,28)=0.002, P=0.97$; Effect of 0.05% Li₂CO₃: $F(1,28)=0.15, P=0.71$; Effect of interaction: $F(1,28)=3.11, P=0.09$].

3.4. Motor activity

Both acute systemic mirtazapine (10 mg/kg) treatment alone and subchronic 0.2% Li₂CO₃ treatment alone failed to affect motor activity in the home cages. Furthermore, the combination of acute systemic mirtazapine (10 mg/kg) and subchronic 0.2% Li₂CO₃ also failed to affect motor activity in the home cages compared with the vehicle-0% Li₂CO₃ group (Table 1). Two-way ANOVA revealed no significant effect of mirtazapine or 0.2% Li₂CO₃, and there was no significant interaction [Effect of mirtazapine: $F(1, 28) = 2.43, P = 0.13$; Effect of 0.2% Li₂CO₃: $F(1, 28) = 2.90, P = 0.10$; Effect of interaction: $F(1, 28) = 0.03, P = 0.85$].

4. Discussion

In this study, subchronic 0.2% Li₂CO₃ treatment in the diet for one week significantly enhanced the inhibitory effect of mirtazapine on contextual conditioned freezing. Moreover, subchronic 0.2% Li₂CO₃ 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. Because freezing behavior induced by contextual conditioned fear has been used as an animal model of anxiety or fear (Inoue et al., 2011), these results indicate that subchronic 0.2% Li₂CO₃ treatment enhanced the anxiolytic-like effect of acute mirtazapine.

Generally, it is believed that the serotonergic system is involved in the pathophysiology and treatment of anxiety disorders (Graeff et al., 1996). Moreover, increased 5-HT neurotransmission decreases contextual conditioned fear in animal experiments (Inoue et al., 2011). *In vivo* microdialysis studies reported that the systemic administration of mirtazapine increased extracellular 5-HT concentrations in the hippocampus of rats (Yamauchi et al., 2012). In addition, subchronic lithium treatment increased extracellular 5-HT levels in the medial prefrontal cortex and hippocampus (Kitaichi et al., 2004, 2006; Muraki et al., 2001; Wegener et al., 2003) and additively increased the elevating effect of SSRI and MAOI on extracellular 5-HT concentrations (Kitaichi et al., 2006; Muraki et al., 2001). Furthermore, subchronic lithium increased the anxiolytic-like effect of SSRI and MAOI in contextual conditioned fear (Kitaichi et al., 2006; Muraki et al., 1999). Taken together, these data suggest that the increasing effect of lithium on the anxiolytic-like effect of acute mirtazapine may also be associated with the enhancement of 5-HT neurotransmission.

Much evidence indicates that 5-HT_{1A} receptors play a key role in the serotonergic mechanism associated with the etiology of stress-related disorders (Popova and Naumenko, 2013). The effect of mirtazapine on the serotonergic system is reportedly mainly dependent on 5-HT_{1A} receptor function (Rauggi et al., 2005) because mirtazapine blocks 5-HT_{2A}, 5-HT_{2C} and 5-HT₃ receptors (de Boer, 1996). Furthermore, our earlier study showed that the anxiolytic-like effect of the systemic administration of mirtazapine on contextual conditioned fear was inhibited by the co-administration of a selective 5-HT_{1A} receptor antagonist (Kakui et al., 2009),

which demonstrated the significant involvement of postsynaptic 5-HT_{1A} receptors in the anxiolytic-like effect of mirtazapine. Blier et al. (1987) has reported that lithium treatment enhanced the sensitivity of postsynaptic 5-HT_{1A} receptors. Haddjeri et al. (2000) demonstrated that the addition of lithium to antidepressant drugs induced a greater enhancement of the tonic activation of postsynaptic 5-HT_{1A} receptors in the rat dorsal hippocampus than any drug given alone. Our previous behavioral study also showed that the inhibitory effect of MKC-242, a selective 5-HT_{1A} receptor agonist, on contextual conditioned freezing was enhanced by subchronic 0.2% Li₂CO₃ treatment (Muraki et al., 1999). Subchronic lithium-induced enhancement of the anxiolytic-like effect of mirtazapine may also be mediated by the enhancement of postsynaptic 5-HT_{1A} receptor function.

Mirtazapine is extensively metabolized principally in the liver by the cytochrome P450 (CYP) isoenzymes CYP3A4, CYP2D6 and CYP1A2 (Holm and Markham, 1999). Lithium is purely renally excreted with no hepatic component and lacks any inhibitory or inductive capabilities (Sandson et al., 2005). Hence, based on the individual pharmacokinetic characteristics of each drug, drug interaction due to pharmacokinetic interactions between lithium and mirtazapine is unlikely to occur. Indeed, a clinical study reported that there is no pharmacokinetic interaction between lithium and mirtazapine (Sitsen et al., 2000). Therefore, a pharmacodynamic mechanism is likely involved in the increase of the effects of mirtazapine by the addition of lithium.

In conclusion, in the present study, we investigated the combined effect of subchronic lithium treatment and acute mirtazapine treatment in the rat contextual conditioned fear stress model. Subchronic 0.2% Li₂CO₃ treatment significantly enhanced the anxiolytic-like effect of mirtazapine on contextual conditioned fear, similar to how subchronic lithium enhanced the anxiolytic-like effect of the SSRI citalopram (Muraki et al., 1999). Our results provide further evidence for the lithium augmentation of mirtazapine, and this augmentation therapy may be demonstrated to be useful in the treatment of anxiety disorders.

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Figure legends

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

Fig. 2. Effect of acute mirtazapine treatment on the expression of contextual 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 30 min before testing. Data are represented as the mean \pm S.E.M of freezing scored for a 5-min observation period. Behavior was sampled in 10-s intervals. ** $P < 0.01$. N=8 per group. 1D, 1 day; 7D, 7 days.

Fig. 3. 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 contextual conditioned freezing. Li_2CO_3 was administered p.o. in the diet for 7 days after the footshock. Mirtazapine (10 mg/kg) was administered intraperitoneally 30 min before testing. Data are represented as the mean \pm S.E.M of freezing scored for a 5-min observation period. Behavior was sampled in 10-s intervals. ** $P < 0.01$. N=8 per group.

Figure 1

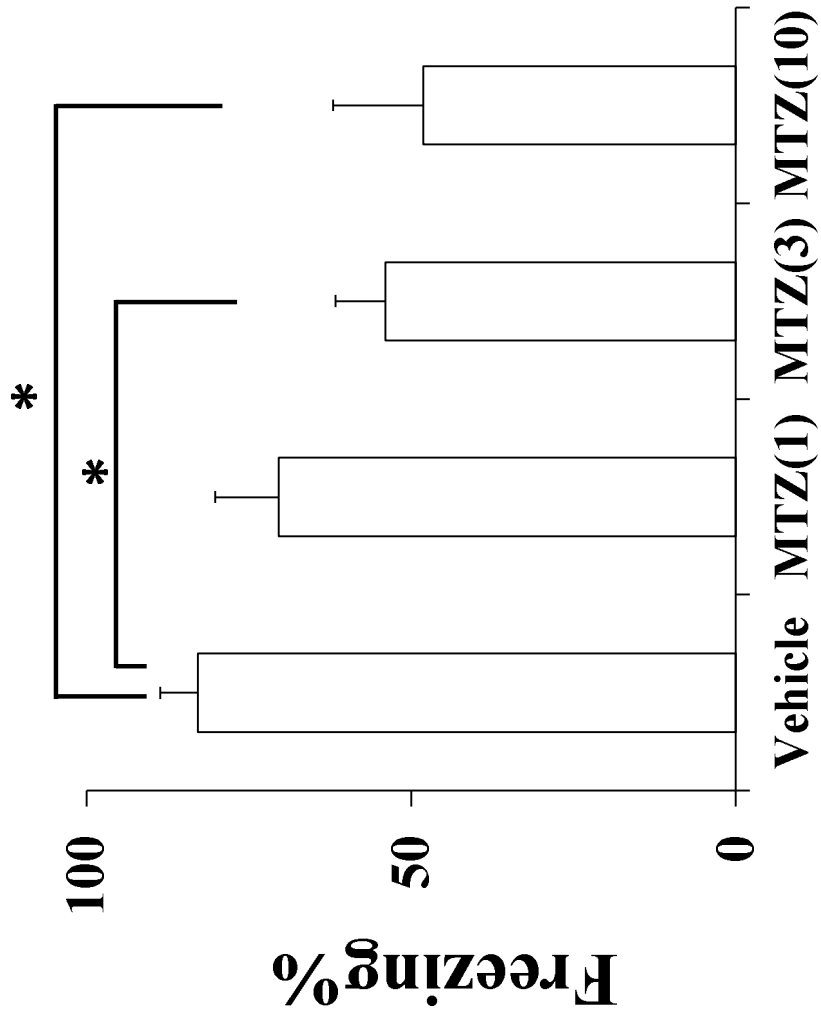


Figure 2

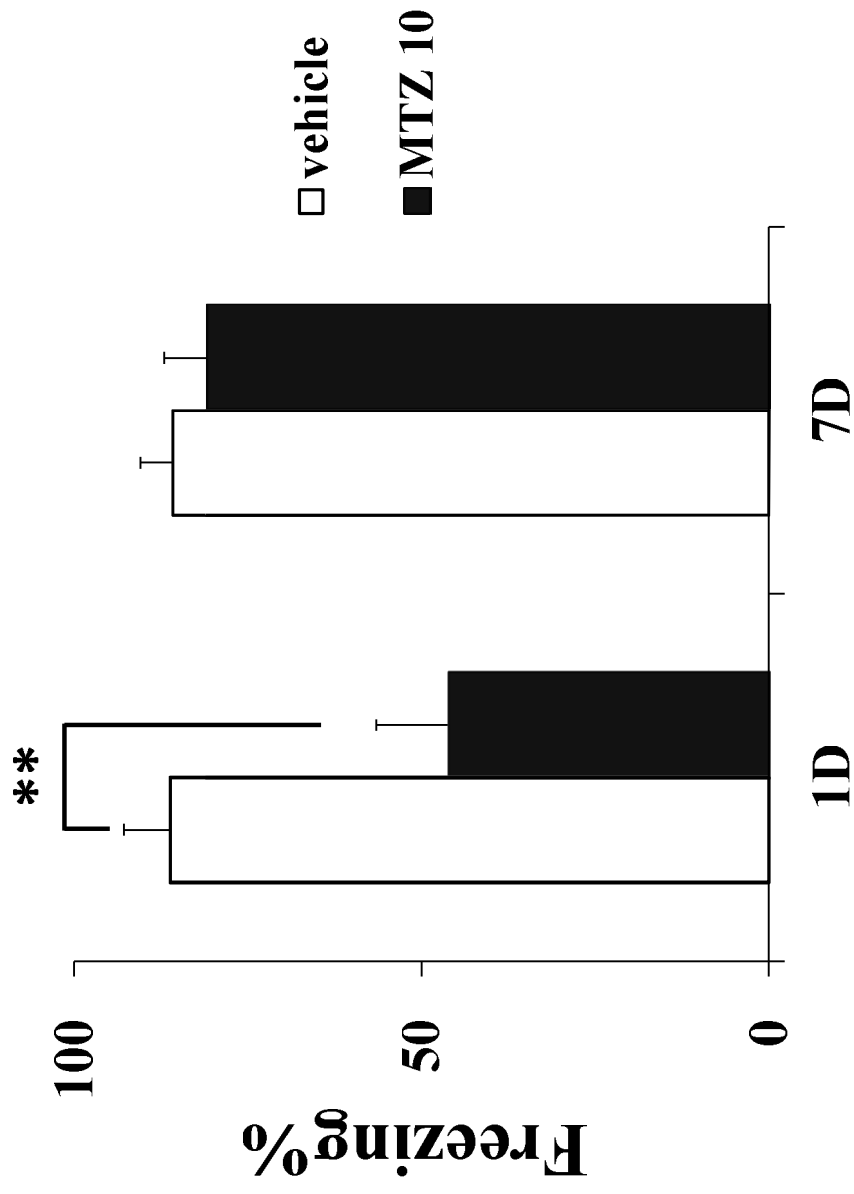


Figure 3

A)

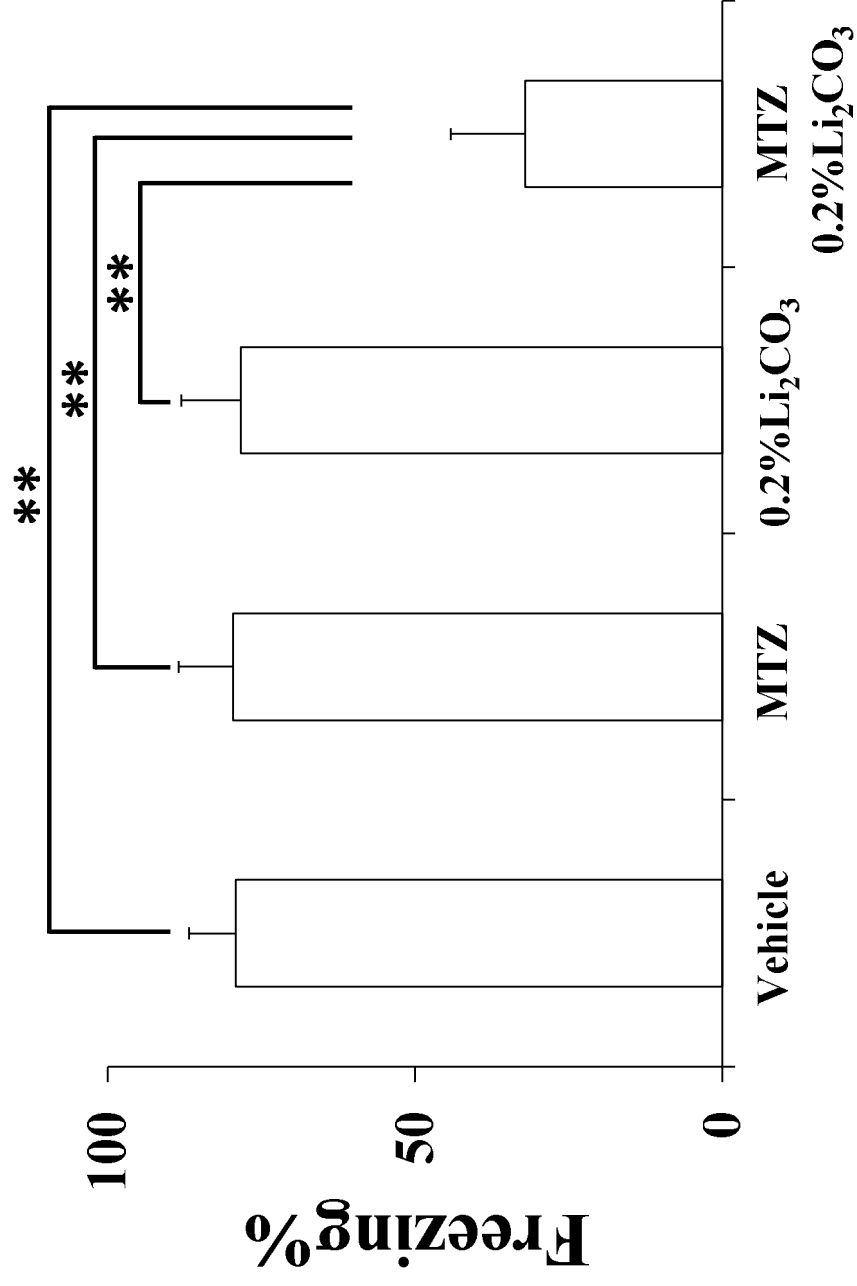


Figure 3

B)

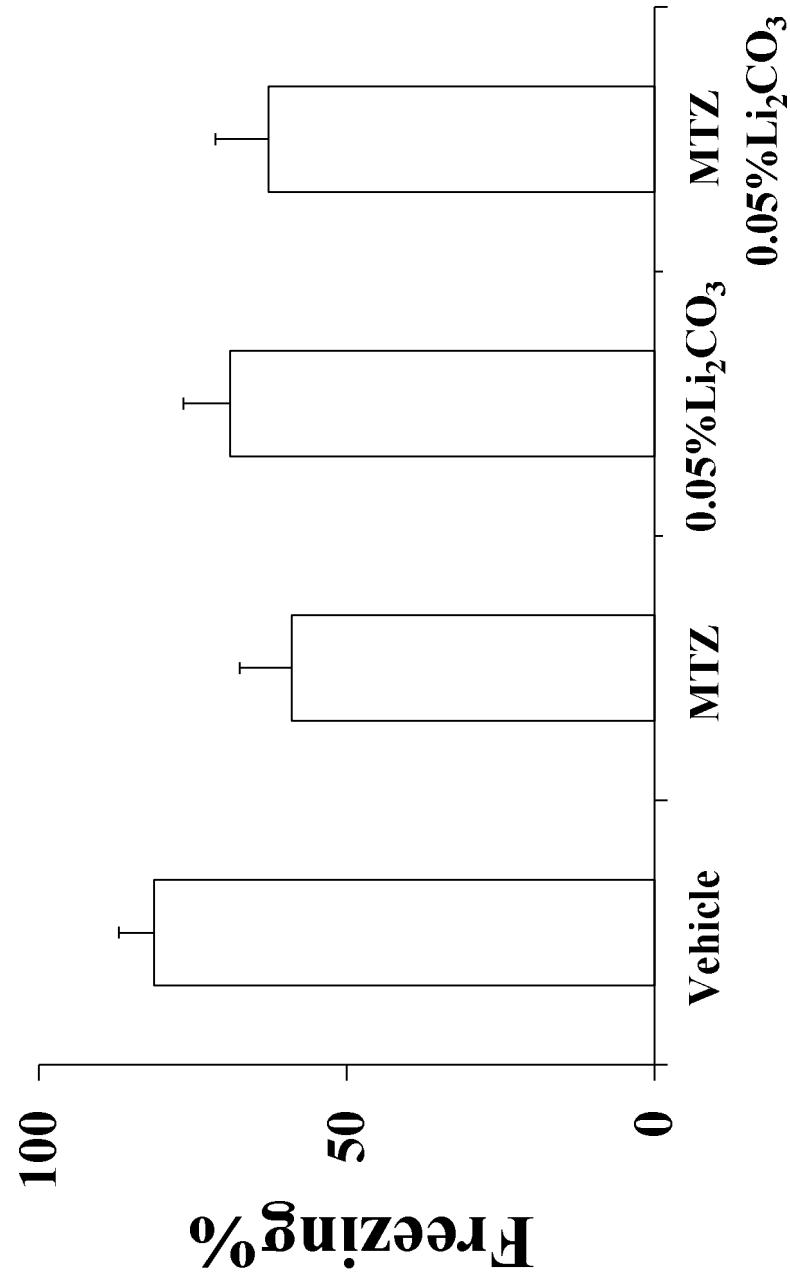


Table 1. The effect of the combination of mirtazapine (MTZ; 10 mg/kg) and 0.2% Li₂CO₃ on spontaneous motor activity.

Drug Treatment	Motor Activity (counts/5 min)
Vehicle-0% Li ₂ CO ₃	83.4 ± 43.3
MTZ (10)-0% Li ₂ CO ₃	139.1 ± 22.2
Vehicle-0.2% Li ₂ CO ₃	34.9 ± 22.7
MTZ (10)-0.2% Li ₂ CO ₃	78.7 ± 34.7

Two-way ANOVA showed no significant effect of mirtazapine, 0.2% Li₂CO₃ or interaction. All the data are represented as the mean ± S.E.M. N=8 per group.