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Combined treatment with subchronic lithium and acute intracerebral mirtazapine microinjection into the median raphe nucleus exerted an anxiolytic-like effect synergistically

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Abstract

Although preclinical and clinical studies have established the efficacy of lithium augmentation of antidepressant drugs, the mechanism of action of lithium augmentation is not fully understood. Our previous study reported that subchronic lithium treatment enhanced the anxiolytic-like effect of systemic mirtazapine. In the present study, we examined the effect of subchronic lithium in combination with acute local intracerebral injection of mirtazapine on fear-related behaviors in a contextual fear conditioning test in rats to clarify the target brain region of lithium augmentation of mirtazapine. After conditioning by footshock, diet (food pellets) containing Li_2CO_3 at a concentration of 0.2% was administered for 7 days. Ten min before testing and 7 days after conditioning, mirtazapine (3 μg/site) in a volume of 0.5 μl was acutely injected into the median raphe nucleus (MRN), hippocampus or amygdala. The combination of subchronic lithium and acute mirtazapine microinjection into the MRN but not the hippocampus or the amygdala reduced fear expression synergistically. These results suggest that intra-MRN mirtazapine treatment with subchronic lithium exerts the anxiolytic-like effect through the facilitation of the MRN-5HT pathway.

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

1. Introduction

Enhancement of serotonergic neurotransmission has been regarded as the primary pharmacological target for the treatment of major psychiatric disorders, particularly those that involve depression and/or anxiety (Graeff et al., 1996). The median raphe nucleus (MRN), similar to the dorsal raphe nucleus, is known to be a major source of serotonin (5-hydroxytryptamine; 5-HT) innervation that sends serotonergic efferents predominantly to the hippocampus and, less extensively, to the amygdala (Vertes et al., 1999; Vertes and Linley, 2007), regions that have been implicated in anxiety and fear (Inoue et al., 2011). Moreover, the serotonergic pathway that goes from the MRN has been proposed to play an important role in contextual conditioned fear (i.e., re-exposure to an environment paired previously with inescapable electric footshock), an animal model of anxiety (Andrade et al., 2013).

Lithium, a mood stabilizer, has been reported to increase the effectiveness of antidepressant drugs clinically, but the mechanism of action of lithium augmentation needs to be further elucidated (Bauer et al., 2014). Animal studies have shown that subchronic lithium increased the anxiolytic-like effect of various serotonergic antidepressants in contextual conditioned fear (An et al., 2015; Kitaichi et al., 2006; Muraki et al., 1999). Further, subchronic lithium treatment not only increases extracellular 5-HT concentrations at baseline in various brain regions but also increases the elevating effect of various antidepressants on extracellular 5-HT concentrations additively, suggesting that the augmentation effect of lithium is mediated by the facilitation of 5-HT neurotransmission (Kitaichi et al., 2004, 2006; Muraki et al., 2001; Wegener et al., 2003). Because a recent study reported that lithium moderates the manic-like behavioral alterations induced by an electrolytic lesion of the MRN in mice (Pezzato et al., 2015), the MRN is a candidate region for lithium augmentation. However, no study to date has investigated the relationship between lithium and the behavioral effect of antidepressants injected into the MRN in the contextual fear conditioning.

Recently, we reported that the intra-MRN injection of mirtazapine, which increases extracellular

5-HT levels in the hippocampus innervated by the MRN (Yamauchi et al., 2012), reduced fear-related behavior, freezing, in contextual fear conditioning (An et al., 2013) and that subchronic lithium enhanced the anxiolytic-like effect of systemic mirtazapine without affecting general motor activity (An et al., 2015). Accordingly, considering reported evidence on the role of the MRN-5HT pathway and the mechanism of action of lithium on the serotonergic systems, we hypothesized that the behavioral effect induced by the stimulation of serotonergic neurons in the MRN can be increased by subchronic lithium treatment in an animal model of anxiety. Therefore, the study was designed to assess the effectiveness of subchronic lithium with acute local mirtazapine treatment in the MRN, hippocampus and amygdala in rats using the contextual fear conditioning test as an animal model of anxiety. Mirtazapine, which is an α_2-adrenergic antagonist, is considered to stimulate serotonergic neuronal firing through the stimulation of noradrenergic neurons in the MRN and by increasing the extracellular 5-HT levels in the forebrain (An et al., 2013; Kakui et al., 2009; Millan, 2006; Yamauchi et al., 2012).

2. Methods

2.1. Animals

A total of 93 male Sprague-Dawley rats (260-320 g) from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) were used. Animals were kept under controlled light (light phase: 06:30-18:30) and temperature (22±2°C) conditions. Experiments began after a two-week acclimatization period. The animals were maintained on a diet of standard laboratory rat chow or rat chow containing 0.2% of Li_2CO_3 for 7 days. In the lithium experiments, the lithium-treated rats and the control rats had free access to food and 10 mM NaCl instead of tap water to prevent lithium-induced hyponatremia. 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.

2.2. Drug administration

Bilateral or unilateral infusions of mirtazapine were given with a 33-gauge injector cannula connected by polyethylene tubing to motor-driven microsyringes. 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 vehicle alone was administered as a control.

The doses of mirtazapine were determined based on our previous experiments in which 3 μg of mirtazapine into the MRN showed the anxiolytic-like effect (An et al., 2013). The concentrations of lithium carbonate in the rat chow were chosen based on our previous studies, in which we observed that plasma lithium levels were 0.71±0.05 mEq/l after 7 days of 0.2% Li2CO3 treatment in diet (Muraki et al., 1999). This plasma lithium level is within the clinical therapeutic range (0.5–1.2 mEq/l) (Suppes et al., 2008; Bauer et al., 2010).

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 a unilateral or bilateral 26-gauge stainless steel guide cannula 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.

2.4. Fear conditioning and behavioral measures

To compare our results with the previous findings (An et al., 2015; Kitaichi et al., 2006), for fear conditioning, the rats were individually subjected to a total of 2.5 min of inescapable electric footshocks (five, 2.5 mA scrambled footshocks, pulse wave, 30 s duration) using a Model SGS-02D Shock Generator (Medical Agent, Kyoto, Japan) in a shock chamber with a grid floor (19 × 22 × 20 cm, Medical Agent). The shock 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 0.2-mA shock intensity to the rats. Seven days after surgery, rats were submitted to footshocks (fear conditioning). Immediately after the footshock, the rats received standard laboratory rat chow (0% Li_2CO_3) or rat chow containing 0.2% of Li_2CO_3 for 7 days until the beginning of the freezing behavior test session.

Seven days after the footshock test, 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 using a 33-gauge injector cannula projecting 1.0 mm beyond the tips of the guide cannula. The solution (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 to allow diffusion and avoid reflux. Ten min later, the rats were again placed in the shock chamber and were observed for 5 min without shocks. During the 5-min observation period, freezing behavior was recorded using a time-sampling procedure, in which the animal behavior was classified as either freezing or as activity at 10-s intervals as previously described (Inoue et al., 2004). Freezing was defined as the complete absence of movement, except movement related to respiration. All other behaviors were scored as activity. The percentage freezing score (freezing (%)) was computed as the proportion of 10-s periods during which the animal remained frozen the entire time.

The exact placement of the injector cannula tips was verified by the injection of Fast Green (2%; 0.5 μl) at the end of the experiments.

2.5. Data analysis

All data are presented as the mean ± S.E.M. of the individual values of the rats from each group. Statistical analysis of the data was conducted using one-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons as a post hoc test. The significance level was set at 0.05.

3. Results

The injection sites were verified to be localized inside the MRN, hippocampus and amygdala, as illustrated in Fig. 1. In 12 rats, the position of the cannula was located outside of the target area and these animals were excluded from statistical analysis.

Li_2CO_3 (0.2% in the diet) ingestion for 7 days significantly reduced freezing behavior in combination with acute mirtazapine microinjection into the MRN. One-way ANOVA revealed a significant difference among the vehicle, lithium alone, mirtazapine alone and lithium plus mirtazapine treatment groups ($F(3, 22) = 8.093$, $P < 0.001$). Post hoc analyses showed that the mirtazapine alone or 0.2% Li_2CO_3 alone had no significant effect on freezing behavior compared with the vehicle group, while the mirtazapine plus 0.2% Li_2CO_3 treatment significantly reduced freezing compared with the vehicle ($P < 0.01$) and 0.2% Li_2CO_3 alone ($P < 0.01$) groups (Fig. 2A).

Subchronic 0.2% Li_2CO_3 treatment with or without acute mirtazapine microinjection into the hippocampus revealed a nearly significant effect ($F(3,29) = 1.698$, $P = 0.053$), but a post-hoc analysis failed to detect any significant between-group effect (Fig. 2B).

Subchronic 0.2% Li_2CO_3 treatment did not affect freezing behavior with or without acute mirtazapine microinjection when mirtazapine was injected into the amygdala ($F(3, 18) = 0.823$, $P = 0.498$) (Fig. 2C).

4. Discussion

Using the contextual fear conditioning model, we have demonstrated that subchronic 0.2% Li_2CO_3 treatment, which alone had no effect, significantly reduced freezing behavior when administered in combination with acute mirtazapine microinjection into the MRN. This effect was not observed when mirtazapine was injected into the hippocampus or the amygdala, although the combination of subchronic 0.2% Li_2CO_3 treatment and mirtazapine microinjection into the hippocampus showed a nearly significant effect (P=0.053). It should be noted that this significant effect was observed when a seven-day interval between conditioning and testing was implemented, in which acute mirtazapine alone did not significantly reduce freezing (An et al., 2015). Because freezing behavior induced by contextual conditioned fear is used as an animal model of anxiety (Inoue et al., 2011), these results indicate that the combination of subchronic lithium and acute intra-MRN mirtazapine reduced fear expression synergistically.

The results of this study are consistent with the findings of a previous study that we conducted, which showed that the MRN is the target brain region for the anxiolytic-like effect of mirtazapine in contextual conditioned fear (An et al., 2013). Behavioral animal studies have confirmed that systemic administration of mirtazapine also exerts an anxiolytic-like effect in rats (An et al., 2015; Kakui et al., 2009), which represents supporting clinical evidence of the efficacy of mirtazapine treatment for anxiety disorders (Bandelow et al., 2008). In addition, Yamauchi et al. (2012) found that systemic administration of mirtazapine increases extracellular 5-HT levels in the dorsal hippocampus but not in the prefrontal cortex. Because the dorsal hippocampus receives projections primarily from the MRN (Vertes et al., 1999), these results support the hypothesis that the serotonergic pathway from the MRN underlies the anxiolytic-like effect of mirtazapine that stimulates the MRN and results in the elevated 5-HT levels in the hippocampus, which lead to the anxiolytic-like effect (Li et al., 2006).

However, in the present study, intra-MRN injection of mirtazapine did not significantly reduce contextual conditioned freezing, which is not consistent with our previous finding (An et al., 2013). One possible reason for this inconsistency is the difference in the intervals between conditioning by footshock and exposure to contextual conditioned fear in these studies. In the present study, because the interval was prolonged to seven days for lithium treatment, the long interval might reinforce fear memory and reduce the effect of mirtazapine, which is consistent with our previous results of acute mirtazapine with subchronic lithium treatment (An et al., 2015). A similar phenomenon was also observed in the effect of a selective serotonin reuptake inhibitor in studies of contextual fear conditioning (Hashimoto et al., 2009). Prolonging the interval between conditioning and testing up to 7-14 days diminishes acute effects of selective serotonin reuptake inhibitors and leads to the need for chronic treatment for anxiolytic-like effects (Inoue et al., 2011). Clinically, selective serotonin reuptake inhibitors and mirtazapine exert anxiolytic effects only after chronic treatment (Bandelow et al., 2008). Therefore, the lack of efficacy of acute mirtazapine on freezing suggests improved predictive and face validities of contextual fear conditioning as an animal model of anxiety disorders (Inoue et al., 2011).

Subchronic lithium with acute intra-MRN mirtazapine treatment exerted the inhibitory effect on freezing behavior synergistically. Although our study did not examine the pharmacological mechanism of action of lithium that may be responsible for its synergistic effects, previous studies have reported a variety of actions of lithium on 5-HT systems. For example, there is evidence that chronic lithium treatment enhances the release of 5-HT and the sensitivity of postsynaptic 5-HT_{1A} receptors in the hippocampus, a main projection region from the MRN (Treiser et al., 1981; Wegener et al., 2003). Based on this 5-HT hypothesis of lithium augmentation, we have shown in a reproducible fashion that subchronic lithium additively increased the anxiolytic-like effects of systemic serotonergic antidepressant drugs and increased extracellular 5-HT levels induced by such drugs in the prefrontal cortex, although we did not examine the effect in the hippocampus (Muraki et al., 1999, 2001; Kitaichi et al., 2006). Moreover, although no study has examined the effect of lithium on the neuronal activity

of the MRN, lithium administration for 3 days facilitates the effect of electrical stimulation of the dorsal raphe nucleus on extracellular 5-HT levels in the hippocampus (Sharp et al., 1991). Additionally, a recent report demonstrated that short-term (8 h) and long-term (14 days) treatment with lithium increases depolarization-induced 5-HT release in primary cultures of rat raphe neurons (Scheuch et al., 2010). Together with the effects of mirtazapine and lithium on the MRN serotonergic pathway and contextual fear conditioning, lithium may synergistically exert the anxiolytic-like effect with the intra-MRN mirtazapine administration by affecting the MRN serotonergic pathway.

In contrast to the results of the present study, a recent study reported that chronic lithium treatment had no effect on anxiolytic-like effects induced by the MRN lesion in the elevated plus maze and light/dark box tests (Pezzato et al., 2015). Variation in the dose and treatment duration of lithium, lesions or drug treatment to the MRN and the animal models used in these studies might account for the differences observed between the studies (Borsini et al., 2002; O'Donnell and Gould, 2007). Additional studies are needed to examine the anxiolytic-like effects of mirtazapine in different animal models of anxiety and the corresponding molecular mechanism of action of lithium.

It should to be noted that the freezing scores of control groups were not homogeneous in different experiments of this study. The difference in surgical locations and procedures (e.g., bilateral or unilateral infusion) may have contributed to this heterogeneity. Although groups for each experiment were treated and tested together, and the comparison in each experiment for each brain region was reliable, the possibility that low freezing scores of controls in the amygdala experiment masked a possible effect induced by the combined treatment cannot be obviated completely, which is a limitation of this study. In addition, the present study did not measure general motor activity that may affect conditioned freezing, which may be a limitation of this study. However, our recent study reported that subchronic lithium treatment enhanced the anxiolytic-like effect of acute systemic mirtazapine without affecting motor activity (An et al., 2015). Moreover, no significant change in motor activity was observed after the intra-MRN treatment with mirtazapine (An et al., 2013). Therefore, it seems unlikely

that the effect of subchronic lithium and intra-MRN injection mirtazapine on conditioned freezing is due to non-specific motor effects.

In conclusion, to the best of our knowledge, this study is the first to use a contextual fear conditioning model to demonstrate that acute intra-MRN mirtazapine treatment with subchronic lithium exerted an anxiolytic-like effect synergistically. Our findings, together with those of previous studies, indicate that the MRN-5HT pathway may be associated, at least in part, with the lithium augmentation of the anxiolytic-like effect of mirtazapine. Further studies are needed to clarify the specific mechanisms involved.

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Figures

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 with respect to the bregma from the brain atlas by Paxinos and Watson (1997). The number of points in the figures is fewer than the total number of rats used because of several overlaps.

Fig. 2. Effect of subchronic lithium treatment (0.2% Li_2CO_3) with acute mirtazapine (MTZ) microinjection (3 μg/site) into the MRN (A; n=6-7 per group), hippocampus (B; n=8-9 per group) and amygdala (C; n=5-6 per group) on the expression of conditioned freezing. Li_2CO_3 was perorally administered at a concentration of 0.2% in the diet for 7 days after conditioning by footshock. Mirtazapine (3 μg/site) was administered 10 min before conditioned fear testing. Data are presented as the mean ± S.E.M. of freezing scores recorded during a 5-min observation period. Behavior was sampled at 10-s intervals. ** $P < 0.01$ vs. vehicle group; $^{\#\#}$ $P < 0.01$ vs. 0.2% Li_2CO_3 alone group.

Fig 1

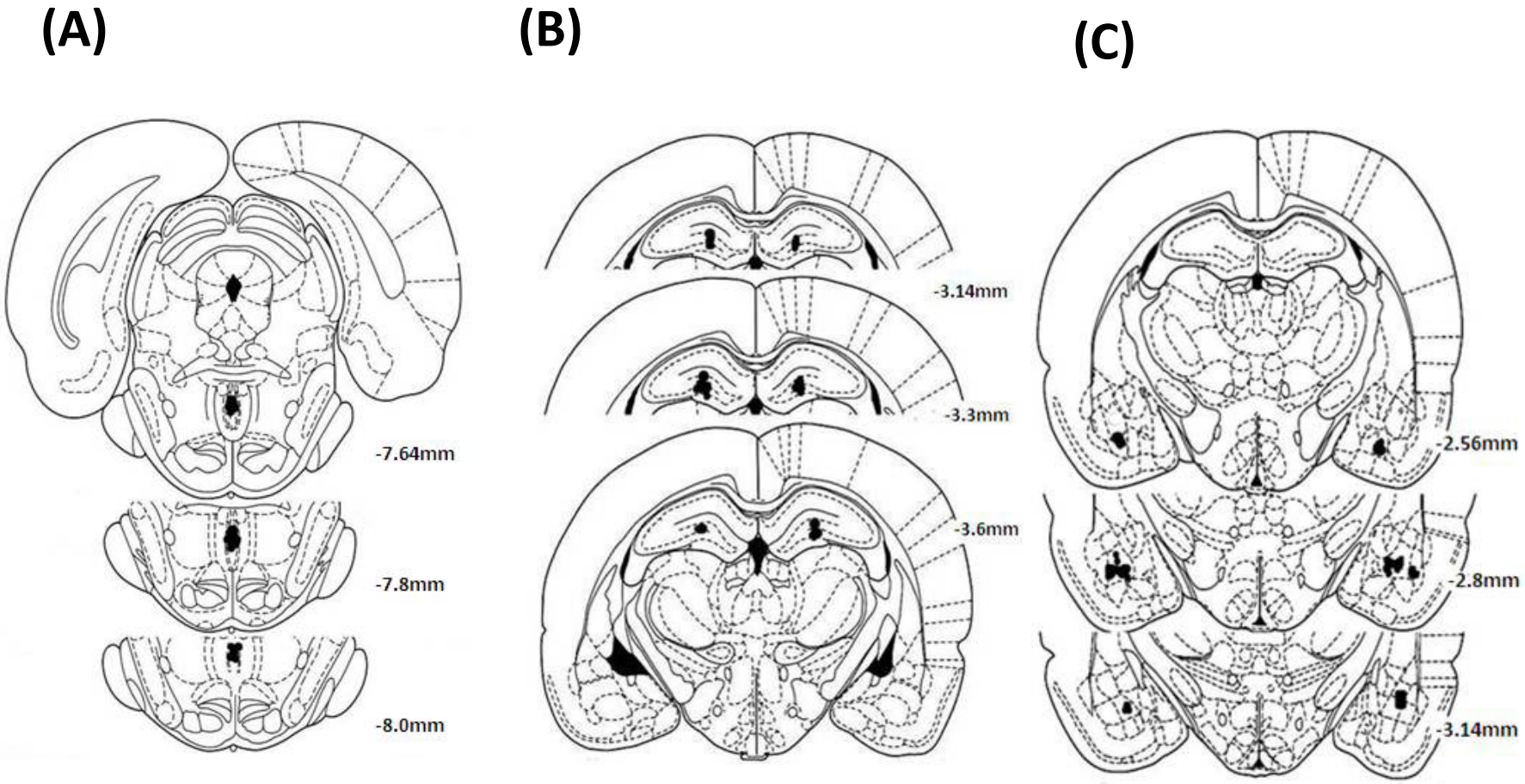


Fig 2 A

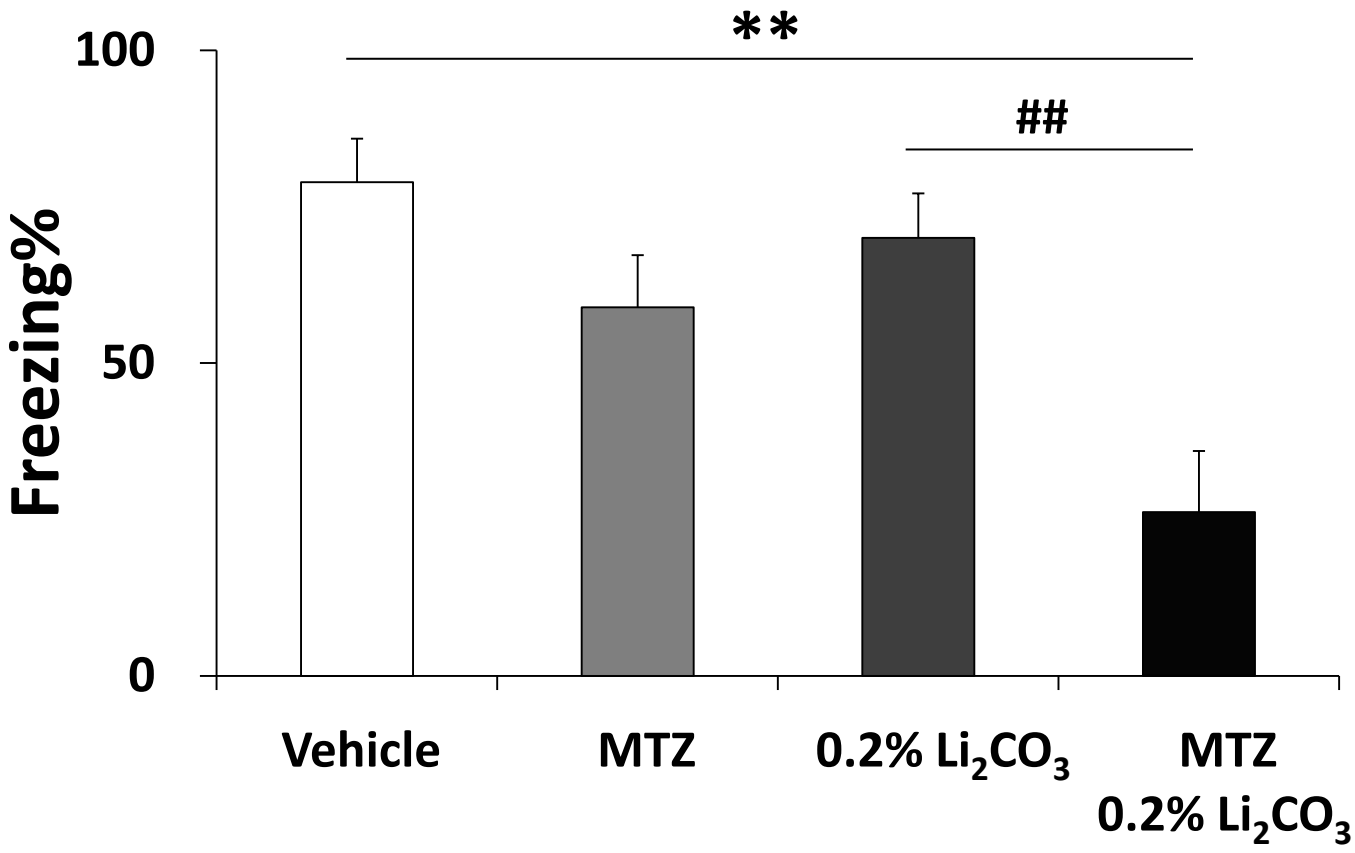


Fig 2 B

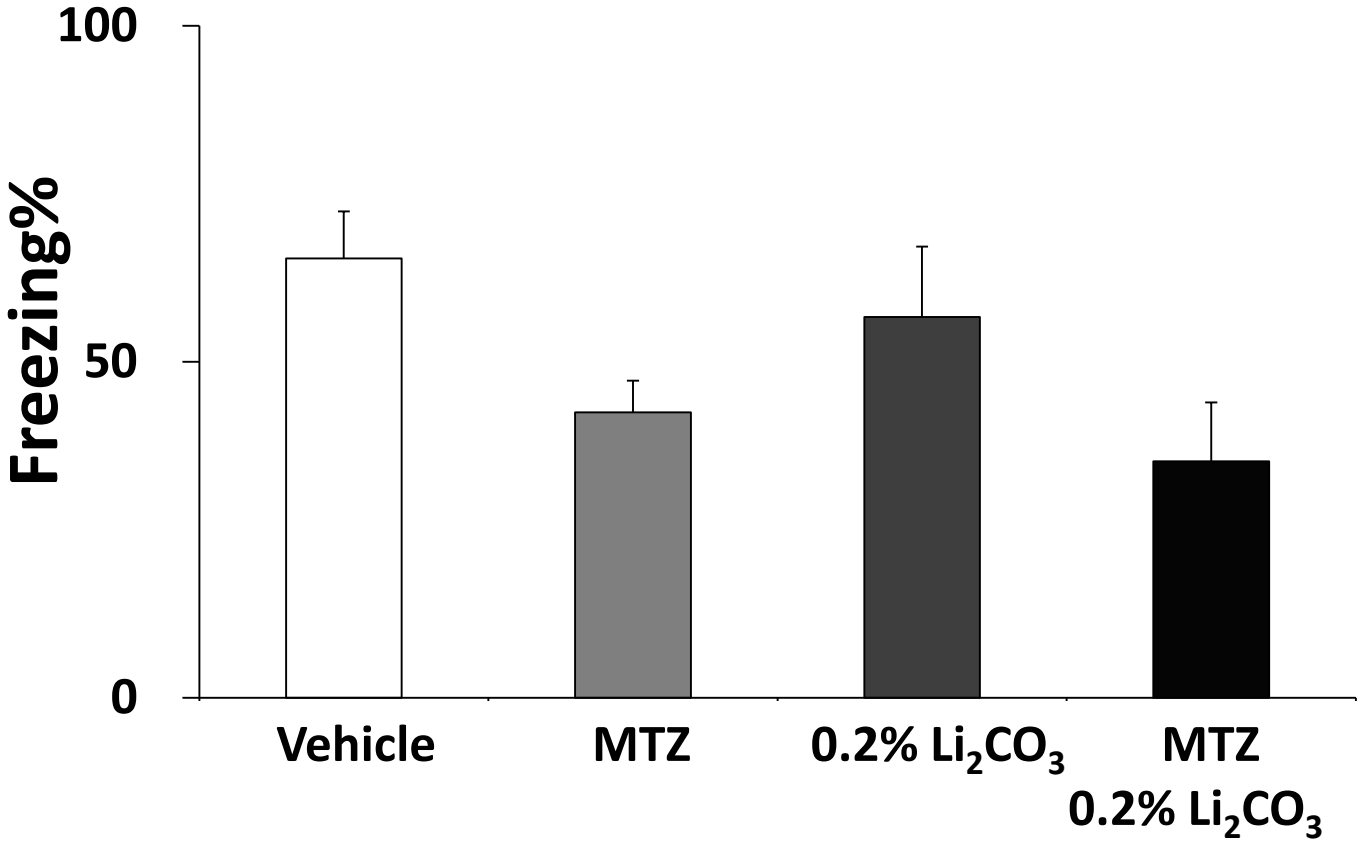


Fig 2 C

