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Title: Lamotrigine blocks apoptosis induced by repeated administration of high-dose methamphetamine in the medial prefrontal cortex of rats

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## **Abstract**

Lamotrigine (LTG) is sometimes co-administered with antipsychotic drugs for the treatment of schizophrenia. Nevertheless, the pharmacological basis of LTG use for schizophrenia has not been reported. Our group recently proposed a new psychostimulant animal model that might reflect the progressive pathophysiology of schizophrenia. Results obtained using that model show that LTG blocks the initiation and expression of repeated high-dosage methamphetamine-induced prepulse inhibition deficit in rats (Nakato et al., 2010, *Neurosci. Lett.*). Using the model, the effect of LTG (30 mg/kg) on methamphetamine (METH, 2.5 mg/kg)-induced increases in extracellular glutamate levels in the medial prefrontal cortex (mPFC) was examined in this study. Then the effect of repeated co-administration of LTG (30 mg/kg) on repeated METH (2.5 mg/kg)-induced apoptosis in this region of rats was investigated. Results show that LTG (30 mg/kg) blocked the METH (2.5 mg/kg)-induced glutamate increase in the mPFC. Repeated co-administration of LTG (30 mg/kg) blocked the development of apoptosis induced by repeated administration of METH (2.5 mg/kg) in the mPFC. The LTG blocks histological abnormalities induced by repeated administration of METH, which suggests a mechanism of LTG that protects against progressive pathophysiology in schizophrenia.

## Introduction

Many schizophrenic patients show progressive cortical atrophy in some brain regions [19, 31], but the precise mechanism of these progressive changes remains unknown. Postmortem reports have described no gliosis, but a high Bax / Bcl-2 ratio with a normal caspase 3 level in chronic schizophrenia, implying that an apoptotic process in the chronic patients plays an important role in progressive cortical atrophy from prodrome to several years after the first psychotic episode in schizophrenia [10, 15]. Smaller somal volume and decreased spine density, dendritic length, and terminals than in normal controls, which are seen in schizophrenia [10], are similarly induced in high-dose amphetamine-induced sensitized animals [29]. A toxic dosage regimen of METH induces apoptosis in cortical neurons in the medial prefrontal cortex (mPFC) [17].

Chronic abuse of METH, which increases extracellular dopamine (DA) levels by releasing cytoplasmic DA [7, 28], engenders positive schizophrenia-like symptoms such as hallucinations and delusions, but it rarely induces so-called negative symptoms such as flattened affect and social withdrawal. The METH-induced psychosis can therefore be used as a model for treatment-responsive schizophrenia [16] because positive symptoms are known to respond well to typical antipsychotics [30], which mainly antagonize DA D<sub>2</sub> receptors. However, a psychostimulant can induce psychotic symptoms, which are resistant to DA D<sub>2</sub> receptor antagonists [5].

Our group has demonstrated that a single administration of METH, at 2.5 mg/kg, increases the extracellular glutamate levels in the mPFC [2] and the nucleus accumbens (NAC) [13, 14]. Furthermore, repeated administration of METH (2.5 mg/kg) induces apoptosis in the mPFC. Atypical antipsychotics such as olanzapine and risperidone, which block METH (2.5 mg/kg)-induced extracellular glutamate increases in the mPFC, prevent apoptosis in this region [2], suggesting that the METH (2.5 mg/kg)-induced glutamate increase in the mPFC indicates apoptosis induced by repeated administration of METH (2.5 mg/kg) in this region.

Clinically, even if many schizophrenic patients respond well to DA D<sub>2</sub> receptor antagonist during their first psychotic episode, some later become resistant to

DA D<sub>2</sub> receptor antagonists [27]. The search for a treatment to prevent pathophysiological development in schizophrenia is important. We speculate that drugs such as those described above (olanzapine and risperidone) block METH-induced behavioral and histological changes in rats that are commonly observed in schizophrenia and thereby have effects that prevent the progressive pathophysiology associated with schizophrenia.

Lamotrigine (LTG) is known as an anticonvulsant [18] and mood stabilizer [12]. In addition, a clinical trial demonstrated that administration of LTG along with stable clozapine exhibited a beneficial effect on the psychopathological symptoms of clozapine-resistant schizophrenic patients [32]. Our recent report described that prepulse inhibition deficit induced by repeated METH is prevented by repeated LTG co-administration and that it is recovered by acute LTG administration [24]. However, the effects of LTG on METH-induced glutamate release and apoptosis remain unknown.

In this study, the effects of LTG (30 mg/kg) on METH (2.5 mg/kg)-induced increase in extracellular glutamate levels in the mPFC were first examined. Then, the effect of repeated co-administration of LTG (30 mg/kg) on apoptosis induced by repeated administration of METH (2.5 mg/kg) was examined. If LTG prevents METH-induced changes, then it might be a promising treatment strategy to prevent longitudinal pathophysiological progression of schizophrenia.

## **Materials and Methods**

Experiment 1 involved eight-week-old male Sprague–Dawley rats (SLC, Inc., Japan) weighing 230–250 g at the time of the experiment. Each group comprised 4–8 animals (24 animals total). Experiment 2 involved six-week-old male Sprague–Dawley rats (SLC, Inc., Japan) weighing 160–180 g at the start of the experiment. Each group comprised 6 animals (24 animals total). The animals were housed individually in plastic cages (30 × 25 × 18 cm) with a wire mesh top and sawdust bedding. The animal house was maintained under controlled conditions of light (6:30–18:30), temperature (24°C), and humidity (50%). The animals were provided a standard laboratory diet and tap water and handled daily for at least 3 days before the start of the experiment. This study was conducted in accordance with the guidelines for the care and use of laboratory

animals of the Hokkaido University Graduate School of Medicine and the National Institutes of Health guidelines on animal care.

For this study, METH (Dainippon Sumitomo Pharma Co. Ltd., Japan) was dissolved in sterile physiological saline and injected subcutaneously at a volume of 1 ml/kg; LTG (a gift from GlaxoSmithKline, U.K.) was dissolved in 10 ml of distilled water with 10 drops of 0.1 N HCl. It was then injected intraperitoneally at a volume of 4 ml/kg. Vehicles for METH and LTG were saline (1 ml/kg) and approximately 0.002 N HCl in distilled water (4 ml/kg), respectively. This study used dosages of METH (2.5 mg/kg) and LTG (30 mg/kg) that were chosen after considering the results obtained in previous studies [2, 24].

In experiment 1, we examined the effects of LTG (30 mg/kg) on METH (2.5 mg/kg)-induced delayed increases in glutamate levels in the mPFC. Rats were implanted stereotaxically under pentobarbital anesthesia (30 mg/kg, i.p.) with a G-4 guide cannula (Eicom Corp., Kyoto, Japan) leading to the surface of mPFC (A: +3.0 mm, L: 0.8 mm, DV: -1.8 mm) [26]. A dialysis probe (BDP-IV-03; Eicom Corp., Kyoto, Japan) was inserted into the guide cannula so that 3.0 mm of the probe was exposed to the tissue of the mPFC. On the next day, in freely moving rats, following initial perfusion for 1.5 h, samples were obtained every 20 min at the flow rate of 2  $\mu$ l/min. Then LTG was administered i.p. 120 min after METH s.c. At the end of the microdialysis study, rat brains were removed. Probe placement was verified microscopically; the success rate in this study was higher than 95%. Glutamate levels were measured and analyzed as described in previous reports [1, 3].

In experiment 2, we examined the effects of repeated administration of METH (2.5 mg/kg) on the expression of TUNEL-positive cells in the mPFC, 7 days after the last injection of METH, and examined the effects of post-treatment with LTG (30 mg/kg) after each METH injection on the expression of TUNEL-positive cells. LTG was administered i.p. 2 hr after METH s.c. After 21 administrations of METH and co-administrations of LTG and METH, animals were killed with a pentobarbital overdose (0.5–0.8 ml) before transcardial perfusion with phosphate buffer saline (PBS, 0.1 M phosphate containing 0.9% sodium chloride; pH 7.4) followed by 4% paraformaldehyde (in 0.1 M PBS).

Brains were removed, post-fixed overnight in the same fixative at 4°C, and stored in 30% sucrose solution at 4°C. The serial coronal sections of the brains were cut (30 µm sections) through the mPFC on a freezing microtome. Apoptotic changes were assessed using TUNEL method (In Situ Cell Death Detection Kit, POD; Roche Diagnostics Corp., Germany) according to the manufacturer's manual. Onto each section, 50 µl of Converter-POD was added, followed by incubation for 30 min at 37°C. Subsequently, 50 µl of the DAB substrate was added to each section for 10 min at room temperature. Slides from animals of different groups were randomized and coded such that all subsequent procedures were conducted blindly. The TUNEL-positive cell counts in the mPFC (Bregma +2.9–3.2 mm according to the atlas of Paxinos and Watson (1997)) were made bilaterally in six fields per coverslip using a grid in the microscope eyepiece [26]. The intensely labeled cells were counted within a 500 × 500 µm<sup>2</sup> grid area. Data were expressed as the number of intensely labeled cells per square millimeter.

Data from extracellular concentrations of glutamate were analyzed using repeated-measures three-way analysis of variance (ANOVA) (METH × LTG × time). Differences in absolute values measured at each time point of collection among the four groups were analyzed using Bonferroni's post-hoc test. Counts of TUNEL-positive cells were analyzed using a two-way ANOVA (METH × LTG) followed by Bonferroni's post-hoc test to determine significant differences between the different groups. Differences were considered significant at  $p < 0.05$ .

## Results

Results show that METH (2.5 mg/kg), but not saline, induced delayed increases in extracellular glutamate levels in the mPFC. Post-treatment with LTG (30 mg/kg) inhibited the METH (2.5 mg/kg)-induced delayed increases in glutamate levels, but had no effect on extracellular glutamate levels after saline injection. A repeated measures three-way ANOVA (METH × LTG × time) showed significant effects of METH [ $F(1, 23) = 6.91, p < 0.05$ ], LTG [ $F(1, 23) = 4.82, p < 0.05$ ], METH × LTG interaction [ $F(1, 23) = 17.24, p < 0.01$ ], METH × time interaction [ $F(16, 368) = 2.66, p < 0.05$ ], and METH × LTG × time

interaction [ $F(16, 368) = 3.17, p < 0.05$ ]. However, no significant effect of time [ $F(16, 368) = 1.18, p = 0.33$ ] or LTG  $\times$  time interaction [ $F(16, 368) = 2.10, p = 0.08$ ] was found. Bonferroni's post-hoc test revealed that LTG inhibited the METH-induced delayed increases in glutamate levels at 220–260 min, 300 min and 320 min (METH+Saline vs. METH+LTG,  $p < 0.05$ ).

Repeated administration of METH (2.5 mg/kg), but not saline, induced expression of TUNEL-positive cells in the mPFC after 7 days of withdrawal from the treatment. Treatment with LTG (30 mg/kg) after each METH injection completely inhibited the repeated administered METH-induced expression of TUNEL-positive cells in the mPFC. A two-way ANOVA (METH  $\times$  LTG) revealed significant effects of METH [ $F(1, 20) = 9.23, p < 0.01$ ] and METH  $\times$  LTG (30 mg/kg) interaction [ $F(1, 20) = 29.24, p < 0.01$ ], but no significant effect of LTG [ $F(1, 20) = 2.35, p = 0.14$ ] was found. Bonferroni's post-hoc test revealed that post-treatment with LTG completely inhibited the METH-induced expression of TUNEL-positive cells in the mPFC (METH+Saline vs. METH+LTG,  $p < 0.01$ ).

## Discussion

In the mPFC, METH (2.5 mg/kg) increased extracellular glutamate levels, which is consistent with the results of our previous studies that demonstrated METH-induced delayed increases in extracellular glutamate levels in the mPFC [2] and NAC [13, 14], which might play an important role in the development of repeated METH-induced PPI disruption and behavioral cross sensitization to an NMDA receptor antagonist, MK-801. In this study, LTG (30 mg/kg) administered 120 min after METH (2.5 mg/kg) injection blocked the METH-induced glutamate increase. Similarly, a report of a previous study described that that LTG reduced veratridine-evoked release of glutamate in the hippocampus [4]. Although the mechanism of this blockade by LTG remains unclear, LTG might reduce glutamate release by exerting an inhibitory effect on sodium channels [22, 33].

METH can cause cell death not only via apoptotic, but also via necrotic mechanisms [11]. In this study, repeated administration of METH induced apoptosis in the mPFC, consistent with previous studies [2, 9, 17]. The mechanism of METH-induced apoptosis is not well known; this apoptosis might result from many factors such as oxidants, reactive oxygen, and NO

radicals induced by glutamate and excess DA released by METH [2, 8]. As we reported previously [2], acute injection of 2.5 mg/kg, but not of 1 mg/kg, METH increased extracellular glutamate concentrations in the mPFC. In parallel with this, repeated METH-induced apoptosis in the mPFC was shown at a dose of 2.5 mg/kg, but not at 1 mg/kg [2]. Furthermore, this study showed that LTG prevented acute METH (2.5 mg/kg)-induced increases in extracellular glutamate concentrations in the mPFC and repeated METH-induced apoptosis in the mPFC. Although the effect of repeated METH on extracellular glutamate concentrations has not been examined, these results strongly suggest that METH-induced increases in extracellular glutamate concentrations induce apoptosis in the mPFC [2, 11]. Increased glutamate via  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors is suggested to be related to neurotoxicity and apoptosis induced by METH [11, 25]. In future studies, we will examine the effect of an NMDA receptor antagonist and an AMPA receptor antagonist on METH-induced apoptosis. Furthermore, behavioral changes, e.g. delayed response task, that reflect cell death in the medial prefrontal cortex should be examined.

Repeated co-administration of LTG (30 mg/kg) with METH (2.5 mg/kg) prevented METH-induced expression of TUNEL-positive cells, i.e. apoptotic cells, in the mPFC. Reportedly, LTG attenuates rotenone-induced or MPP+-induced mitochondrial damage leading to caspase-3 activation, increased oxidative stress and cell death [21]. It also prevents enhancement of NO generation and release [6, 23]. Therefore, not only the effect of LTG to block the METH-induced delayed increases in extracellular glutamate levels in the mPFC, but also other above-described effects might produce the blockade of apoptosis development in this area.

Associated with the overt onset of schizophrenia, progressive pathophysiology such as brain atrophy is strikingly advanced [20, 31]. However, the precise mechanism of these progressive changes remains unknown. These changes might be a peculiarity that is common to progressive pathophysiology in schizophrenia and METH-induced behavioral and histological changes, suggesting a possible mechanism for the use of LTG to protect against

progressive pathophysiology in schizophrenia. We speculate that LTG and other drugs, which can block the METH-induced behavioral and histological progressive abnormalities in rats, might prevent progressive pathophysiology in schizophrenia.

This is the first report describing that LTG (30 mg/kg) blocked the acutely injected METH (2.5 mg/kg)-induced increase in extracellular glutamate levels and the sub-chronically injected METH-induced apoptosis in the mPFC. These results suggest that LTG prevents longitudinal pathophysiological development.

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### **Author disclosure information**

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## Figure legends

Figure 1. Effects of LTG (30 mg/kg) on the METH (2.5 mg/kg)-induced delayed increases of glutamate in the mPFC. #,  $p < 0.01$ , METH + Vehicle vs. Saline + Vehicle. Each value represents the mean $\pm$ SEM. METH (2.5 mg/kg) and saline were each injected at 60 min. LTG (30 mg/kg) and vehicle were each injected at 180 min.

Figure 2. Effects of LTG (30 mg/kg) on the expression of TUNEL-positive cells in the mPFC induced by repeated administration of METH (2.5 mg/kg). #,  $p < 0.01$ , METH + Vehicle vs. Saline + Vehicle; a,  $p < 0.01$ , METH + Vehicle vs. METH + LTG.



