dopamine levels in the medial prefrontal cortex, but the reason remains unclear. Possibly, only a few dopamine transporters were in the medial prefrontal cortex compared with those in the striatum (Sesack et al., 1998). Additionally, it is noteworthy that noradrenaline transporters have a large part in the reuptake inhibition of extracellular dopamine in the medial prefrontal cortex (Carboni et al., 1990). In comparison with sertraline, the inhibitory potency (Ki) of dopamine reuptake of paroxetine is very weak; fluvoxamine has only a negligible effect on that (260 nM, 1700 nM, and 42 000 nM, respectively) (Goodnick and Goldstein, 1998). A very high dosage of paroxetine might increase extracellular dopamine levels. However, we used the dosages of SSRIs corresponding to those used for the treatment of depression in humans based on *in vivo* or *ex vivo* inhibitory potencies for serotonin transporters. Results of this study suggest that clinical dosages of paroxetine and fluvoxamine are unlikely to increase extracellular dopamine levels in the brain.

All SSRIs (sertraline, paroxetine, and fluvoxamine) significantly increased extracellular noradrenaline levels in the nucleus accumbens. Paroxetine has a moderate affinity for noradrenaline transporters (Sánchez and Hyttel, 1999; Owens et al., 2001) and has the ability of noradrenaline reuptake inhibition (Goodnick and Goldstein, 1998). This noradrenaline reuptake inhibition can account for noradrenaline increases in the nucleus accumbens induced by paroxetine. As described above, sertraline inhibits dopamine reuptake from dopamine transporters (Goodnick and Goldstein, 1998). Some reports in the literature describe that reverse-dialysis of GBR 12935, a potent and selective dopamine reuptake inhibitor, into the ventral tegmental area, and nucleus accumbens increased extracellular noradrenaline levels in these areas (Chen and Reith, 1994; Li et al., 1996). Especially, extracellular noradrenaline levels in the nucleus accumbens were increased by low concentrations of GBR 12935 (Li et al., 1996), which suggests that dopamine reuptake inhibition results in increased levels of extracellular noradrenaline. Sertraline is likely to increase extracellular noradrenaline levels partly through its dopamine reuptake inhibition. Fluvoxamine administration only transiently increased extracellular noradrenaline levels in the nucleus accumbens. Fluvoxamine has very low affinities for both noradrenaline transporters and dopamine transporters. The mechanism by which noradrenaline is increased by fluvoxamine in the nucleus accumbens remains unclear; further studies might be necessary to elucidate it. On the other hand, no SSRI altered extracellular noradrenaline levels in the medial prefrontal cortex or striatum. Few studies have examined the effects of SSRIs on extracellular noradrenaline levels in the nucleus accumbens. However, administration of fluoxetine (10 mg/kg, subcutaneous injection) and citalopram (5 mg/ kg i.p.) had no effect on them (Li et al., 2002; Weikop et al., 2007). Their results are not consistent with ours. The reason remains uncertain except for the type of the SSRI.

The increases of extracellular serotonin levels in the nucleus accumbens and striatum after administrations of each SSRI continued until the measurement ended (6 hr after SSRI administration), although those in the medial prefrontal cortex continued only for a short time compared with those of other brain regions. Recent reports show that each SSRI has a sufficient inhibitory effect on serotonin reuptake until 6 h after administration (Koe et al., 1983; data on file, GlaxoSmithKline; Geldof et al., 2008). Therefore, we can explain the period of extracellular serotonin increases in the nucleus accumbens and striatum based on the above *in vivo* data, but the effects of SSRIs in the medial prefrontal cortex are shorter than periods predicted from *in vivo* data. Serotonin 1A stimulation in the medial prefrontal cortex exerts a distal feedback control of serotonergic activity, although such an effect in the nucleus accumbens and striatum is weak or nonexistent. Some reports have described that systemic or local administration of a serotonin 1A agonist decreased extracellular serotonin levels significantly in the medial prefrontal cortex (Celada et al., 2001; Casanovas et al., 1999). On the other hand, systemic administration of a serotonin 1A agonist had no effect on extracellular serotonin levels in the nucleus accumbens (Müller et al., 2003); local administration of it into the striatum had only an equivocal effect on extracellular serotonin levels (Casanovas et al., 1999). The reason why the increases of extracellular serotonin levels after SSRI administration disappeared in a short time might be that the inhibitory mechanism acting through the serotonin 1A receptor stimulation works more strongly in the medial prefrontal cortex than in other brain regions.

The biological basis of depression is hypothesized as a lack of neurotransmitters such as serotonin and noradrenaline. However, recent evidence suggests that dopamine might be related also to the pathogenesis and treatment of depression (Kapur and Mann, 1992; Papakostas, 2006). Previously, we reported that the addition of the dopamine agonists—bromocriptine and pergolide—in conjunction with antidepressants increased the antidepressant effect on treatment-resistant depression (Inoue et al., 1996; Izumi et al., 2000) and pointed out the possibility that the dopamine system is related to depression. Additionally, we reported the effect of co-administration of a serotonin–noradrenaline reuptake inhibitor and a dopamine agonist on extracellular monoamine levels in the medial prefrontal cortex (Kitaichi et al., 2008). Clinically, a double-blind study showed that pramipexole, a dopamine receptor agonist, has a significant antidepressant effect compared with placebo and that this effect was equal to that of fluoxetine (Corrigan et al., 2000). In a naturalistic study of treatment-resistant depression, pramipexole also exhibited an antidepressant effect (Lattanzi et al., 2002). Recently, the relation between dopamine and depression has increasingly attracted attention. In this study, sertraline increased extracellular dopamine levels in the nucleus accumbens and striatum. Cipriani et al. (2009) reported, based on their meta-analysis, that the antidepressant effect of sertraline is superior to those of other SSRIs. It is a unique property of sertraline to increase extracellular dopamine levels in the nucleus accumbens and striatum at clinical dosages; this property might be a reason that the antidepressant effect of sertraline is superior to that of other SSRIs. As described in the Introduction, some papers showed the possibility that dopaminergic neurons in the nucleus accumbens are related to depression and anhedonia (Nestler and Carlezon, 2006; Gorwood, 2008). The increases of extracellular dopamine levels by sertraline might affect the mesolimbic dopamine reward circuit so that sertraline might cause a unique antidepressant effect. Even for SSRIs classified into the same class, the pharmacological differences among SSRIs must be understood for their clinical application.

We measured extracellular monoamine levels in this study. However, Narita et al. (1996) reported that fluvoxamine has a higher affinity than other SSRIs for sigma 1 receptor. Sigma 1 receptor has a neuromodulatory role and is related to anxiety and depression (Bermack and Debonnel, 2005; Cobos et al., 2008). Consequently, fluvoxamine might produce an antidepressant effect through the effect on sigma 1 receptor, except for serotonin reuptake inhibition. Nevertheless, we did not examine this mechanism in our study. On the other hand, paroxetine inhibited noradrenaline reuptake in humans when a high dosage of paroxetine was administered (Gilmor et al., 2002). In addition, David et al. (2003) reported that single administration with a high dosage of paroxetine increased extracellular noradrenaline levels in the frontal cortex of mice using *in vivo* microdialysis. In the present study, paroxetine administration did not influence extracellular noradrenaline levels in the medial prefrontal cortex and striatum. Accordingly, a higher dosage of paroxetine might have induced significant increases in extracellular noradrenaline levels, although we selected the dose of paroxetine based on a comparison of clinical and animal studies.

In summary, we measured extracellular serotonin, dopamine, and noradrenaline levels after respective administrations of three SSRIs, sertraline, fluvoxamine, and paroxetine. Only sertraline at clinical dosages increased extracellular dopamine levels in the nucleus

accumbens and striatum. This effect on dopamine is a unique property of sertraline among SSRIs.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research No. 19591333 (T. Inoue) and No. 17790800 (Y. Kitaichi) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Schizophrenia Research 125 (2011) 77–87

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres

Effects of aripiprazole and haloperidol on progression to schizophrenia-like behavioural abnormalities and apoptosis in rodents

Tomohiro Abekawa [a,b,*], Koki Ito [b,1], Shin Nakagawa [b,1], Yasuya Nakato [b,1], Tsukasa Koyama [b,1]

[a] Kotoku-kai Aiko Hospital, Department of Psychiatry, Matsue 2-7-1, 243-0005, Kanagawa, Japan
[b] Hokkaido University, Graduate School of Medicine, Department of Psychiatry, Kita 15, Nishi 7, 060-8638, Sapporo, Japan

ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form 25 July 2010
Accepted 9 August 2010
Available online 15 September 2010

Keywords:
Aripiprazole
Haloperidol
Apoptosis
PPI
Methamphetamine
Schizophrenia

ABSTRACT

Aripiprazole (APZ) is considered a first-line medication for treating first and multiple episodes of schizophrenia, but its effect on preventing the progressive pathophysiology of schizophrenia remains unclear. This study examined the hypothesis that APZ blocks enhanced glutamate release in the medial prefrontal cortex (mPFC) during psychotic episodes of schizophrenia, thereby preventing progression of the pathophysiology. We examined effects of APZ on methamphetamine (METH)-induced increases in glutamate levels in the mPFC, and on repeatedly administered METH-induced progression to schizophrenia-like behavioural abnormalities involving cross-sensitization to the N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, deficit of prepulse inhibition (PPI), and expression of TUNEL-positive cells. Additionally, we compared the preventive effects of APZ to those of a conventional antipsychotic: haloperidol (HPD). Results show that APZ (1.0 and 3.0 mg/kg) and HPD (0.1 mg/kg) each blocked METH (2.5 mg/kg)-induced increases in glutamate levels in the mPFC. Furthermore, APZ (3.0 mg/kg) and HPD (0.1 mg/kg), when co-administered repeatedly with METH, each prevented progression to schizophrenia-like behavioural and neuropathological abnormalities. Repeated co-administration of APZ (3.0 mg/kg) with saline did not induce apoptosis, although HPD (0.1 mg/kg) with saline did induce apoptosis. These results indicate that APZ and HPD prevented progressive pathophysiology, which is related to increased glutamate levels, and indicate that repeated administration of HPD, but not APZ, induced apoptosis under conditions without increased glutamate levels. These findings suggest the importance of using APZ and HPD in the appropriate stages of the glutamate-related pathophysiology of schizophrenia.

1. Introduction

Our group (Abekawa et al., 2008) has developed a comprehensive animal model for the progressive pathophysiology of schizophrenia to clarify its mechanisms of progressive pathophysiology and to produce a strategy for preventing the progression. In the model, repeated administration of methamphetamine (METH) at a dosage of 2.5 mg/kg is used, at which dosage this psychostimulant can increase both extracellular glutamate and dopamine levels in the mPFC. It induces schizophrenia-related behavioural and histological abnormalities including cross-sensitization to the NMDA receptor antagonist, MK-801, a neuroplastic PPI deficit at the baseline state without challenge injection, and an apoptotic reaction in the mPFC. In contrast, at 1.0 mg/kg, METH merely increases the dopamine level. It does not induce behavioural or histological abnormalities. Some NMDA receptor antagonists such as MK-801 and phencyclidine (PCP) induce hyperlocomotion and the PPI deficit. These behavioural changes do not respond well to a dopamine D_2 receptor blocker, HPD, but they do respond to

* Corresponding author. Kotoku-kai Aiko Hospital, Department of Psychiatry, Matsue 2-7-1, 243-0005, Kanagawa, Japan. Tel.: +81 11 716 1161x5973; fax: +81 11 706 5081.
E-mail address: spg35k69@opal.ocn.ne.jp (T. Abekawa).
[1] Tel.: +81 11 716 1161x5973; fax: +81 11 706 5081.

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doi:10.1016/j.schres.2010.08.011

clozapine (Abekawa et al., 2003; Bakshi et al., 1994; Gleason and Shannon, 1997). In our preliminary study, this repeatedly administered METH-induced PPI deficit did not respond well to haloperidol, but did respond well to clozapine (data not shown). Taken together with these findings, we can speculate that this METH-induced development of cross-sensitization to MK-801 and the PPI deficit at the baseline state might reflect dopamine D_2 receptor antagonist-resistant pathophysiology. Our previous study (Abekawa et al., 2008) using a comprehensive animal model showed that olanzapine and risperidone, at dosages that completely blocked and markedly attenuated METH-induced glutamate increases, respectively inhibited the induction of cross-sensitization to MK-801, the neuroplastic PPI deficit, and the TUNEL-positive cells in the mPFC. Considering those findings, we formed the following hypothesis: A degree of increase in the dopamine release in the mPFC (putatively a pathophysiological change that occurs during psychotic episodes of schizophrenia) that is sufficiently high that it increases glutamate levels, if it changed dopamine-glutamate levels, would induce progression of the pathophysiology to cause resistance to dopamine D_2 receptor antagonists, neurocognitive deficits, and neuroanatomical volume reduction. Furthermore, we hypothesized that olanzapine and risperidone block the increases in glutamate levels, thereby preventing the progression of the pathophysiology.

Developed by Otsuka Pharmaceuticals (Lawler et al., 1999), APZ is regarded as a first-line atypical antipsychotic used for the treatment of first and multiple episodes of schizophrenia to improve positive- and/or negative-symptoms (Kane et al., 2003). Olanzapine, risperidone, and clozapine, which reportedly have neuroprotective effects in vitro (Bai et al., 2004; Ukai et al., 2004), can block progressive volume reduction in whole-brain grey matter (Chakos et al., 2005; Lieberman et al., 2005; Van Haren et al., 2007), although HPD, which reportedly induces apoptosis in vitro (Ukai et al., 2004), worsens progressive cortical atrophy (Lieberman et al., 2005). Although preclinical studies showed that APZ had neuroprotective effects on oxidative stress (Martins et al., 2008) and kainic acid-induced excitotoxicity (Cosi et al., 2005), no report describes neuroprotective effects against the progressive cortical atrophy of schizophrenic subjects or progressive pathophysiology in animal models of schizophrenia.

Considering that APZ inhibits terminal depolarization-induced increases in glutamate levels in the prefrontal cortex (Yang and Wang, 2008), it was hypothesized for the present study that APZ, by blocking increases in glutamate release during severe psychotic episodes, would prevent progression to resistance to dopamine D_2 receptor blockers, neurocognitive deficits, and cortical atrophy. To test this hypothesis, using our comprehensive animal model for the progressive pathophysiology of schizophrenia, we examined whether sufficient doses of APZ would bock METH (2.5 mg/kg)-induced increases in glutamate levels in the mPFC, and prevent repeatedly administered METH-induced induction of cross-sensitization to MK-801, neuroplastic PPI disruption at the baseline state, and TUNEL-positive cells in the mPFC. Furthermore, this study examined differences in the neuroprotective effects between APZ and HPD with respect to the progressive changes. Considering that HPD particularly worsens the progressive cortical atrophy of schizophrenic brains (Lieberman et al., 2005), and considering that HPD itself induces apoptosis in vitro (Ukai et al., 2004), the present study examined the effects of HPD on the METH-induced apoptosis and assessed whether repeated administration of HPD itself would induce apoptosis in vivo.

2. Experimental/materials and methods

2.1. Animals

Male Sprague–Dawley rats (SLC Inc., Japan) weighing 150–180 g at the start of the experiment were housed individually. The animal house was under controlled conditions of light (6:30 a.m.–6:30 p.m.), temperature (24 °C), and humidity (50%). This study was conducted in accordance with the Hokkaido University School of Medicine's guide for the care and use of laboratory animals and NIH guidelines on animal care.

2.2. Drugs and administration schedules

For use in this study, METH (Dainippon Sumitomo Pharma Co. Ltd., Japan) (2.5 mg/kg) was dissolved in saline. Dosages of METH are reported as hydrochloride salt form; METH was injected subcutaneously. The APZ (gift from Otsuka Pharmaceutical Co. Ltd., Japan) or HPD (gift from Dainippon Sumitomo Pharma Co. Ltd., Japan) was dissolved in 0.15% tartaric acid. This experiment used 3.0 and 1.0 mg/kg of APZ, and 0.1 and 0.01 mg/kg of HPD considering the results of earlier related studies (Abekawa et al., 2003; Li et al., 2004). Each dose of APZ or HPD was injected intraperitoneally. The APZ and each of the other drugs were administered, respectively, at respective volumes of 2.0 mL/kg and 1.0 mL/kg.

2.3. Experimental protocol

The ideal protocol for producing a comprehensive model of progressive pathophysiology is to use the same cohort for measuring all three phenotypes including the induction of cross-sensitization to locomotion-inducing effect of MK-801, PPI deficit at baseline state, and TUNEL-positive cells. However, this study used different cohorts in each of experiments 2 and 3 because the challenge injection of MK-801 can affect PPI and the expression of TUNEL-positive cells, each of which was evaluated at a baseline state without challenge injection.

2.4. Experiment 1 (microdialysis study)

Experiment 1 examined effects of APZ or HPD on METH (2.5 mg/kg)-induced increases in glutamate levels and extracellular glutamate levels in the mPFC emerging after saline injection.

2.5. Experiment 2 (behavioural cross-sensitization to MK-801)

Experiment 2 re-confirmed that repeated administration with METH (2.5 mg/kg, once a day, for 21 injections) develops behavioural cross-sensitization to locomotion-inducing effect of MK-801 (0.2 mg/kg), examined whether pretreatment with vehicle, APZ (3.0 mg/kg) or HPD (0.1 mg/kg) before

saline or METH (2.5 mg/kg) injection would block the development of this cross-sensitization to MK-801.

2.6. Experiment 3 (PPI study and TUNEL staining)

Experiment 3A examined the effects of repeated administration of METH (2.5 mg/kg, once a day, for 21 injections) on the development of PPI disruption after seven days of withdrawal from the METH treatment, and examined the effects of pretreatment with vehicle, APZ (3.0 mg/kg), or HPD (0.1 mg/kg) before saline or METH (2.5 mg/kg) injection on the development of repeated administered METH-induced PPI disruption.

Two days after finishing the PPI study, Experiment 3B examined the effects of repeated administration of METH (2.5 mg/kg, once a day, for 21 injections) on the expression of TUNEL-positive cells in the mPFC, and examined the effects of pretreatment with vehicle, APZ (3.0 mg/kg), or HPD (0.1 mg/kg) before saline or METH (2.5 mg/kg) injection on the expression of TUNEL-positive cells.

2.7. Experimental procedures

2.7.1. Microdialysis and biochemical measurement

Rats were implanted stereotaxically under pentobarbital anaesthesia (30 mg/kg, i.p.) with a G-4 guide cannula (Eicom Corp., Kyoto, Japan) leading to the surface of the mPFC (A, + 2.9–3.1 mm; L, 0.8 mm; DV, −1.8 mm in accordance with the atlas of Paxinos and Watson (1997)). 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. The next day, in freely moving rats, following the initial perfusion for 1.5 h, samples were obtained every 20 min at a flow rate of 2 μL/min. Brains were removed from the rats at the end of the microdialysis study. Probe placement was verified microscopically; a success rate of more than 95% found for this study. Glutamate and dopamine levels were measured and analyzed using a previously published method (Abekawa et al., 2006, 2003).

2.7.2. PPI

The startle response was assessed using SR-LAB systems (San Diego Instruments Inc.; San Diego, CA), as described by Sills (1999). Rats were placed in the startle apparatus, which had a 65 dB white noise in the background. Each rat was given 10 min to become accustomed to the equipment. After this period, each animal was presented with a series of five startle-pulse-alone trials to control for habituation of startle response. This series of stimuli was followed by 45 randomized trials that consisted of no pulse (0 dB), a startle pulse (120 dB, 40 ms), or three prepulse intensities (68 dB, 71 dB, and 77 dB, 20 ms) presented alone or 100 ms before a startle pulse. The times between trials were 10–20 s. Startle responses were measured every 1 ms for a 100 ms period that began from the onset of the startle stimulus. The percent PPI was calculated using the following formula: $PPI = 100 - ([P+S]/S) \times 100$ where "P + S" denotes the mean response amplitude for the prepulse-startle pulse trials and "S" signifies the mean response amplitude for the startle pulse only trials.

2.7.3. TUNEL-immunohistochemical staining

After 21 administrations of METH and co-administrations of APZ or HPD and METH, animals were killed with a pentobarbital overdose (0.5–0.8 mL) and subsequent transcardial perfusion with phosphate buffer saline (PBS, 0.1 M phosphate containing 0.9% sodium chloride; pH 7.4) and then 4% paraformaldehyde (in 0.1 M PBS). Brains were removed, post-fixed overnight in the same fixative at 4 °C, and stored in a 30% sucrose solution at 4 °C. Serial coronal sections of the brains were cut (30 μm sections) through the mPFC using a freezing microtome.

Apoptotic changes were assessed using the TUNEL method (In Situ Cell Death Detection Kit, POD; Roche Diagnostics Corp., Germany) according to the manufacturer's manual. After addition of 50 μL of Converter-POD, each section was incubated 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 to ensure that all subsequent procedures were conducted blindly. The manual counting of TUNEL-positive cells in the mPFC (Bregma + 2.9–3.2 mm according to the atlas of Paxinos and Watson (1997)) was performed by two blind raters bilaterally in a total of six fields per cover slip for each animal with the aid of a grid ($500 \times 500\ \mu m^2$ unit area) located in the microscope eyepiece. The data were expressed as the number of intensely labelled cells per square millimetre.

2.7.4. Locomotor activity

Measurement of locomotor activity began after a two-hour habituation period, using an apparatus with an infrared sensor that detects thermal radiation from animals (Supermex: Muromachi Kikai Co. Ltd., Tokyo, Japan). Horizontal movements of rat were digitized and fed into a computer every 10 min. Locomotion predominantly contributed to the count, but repetitive rearing and other nonspecific body movements might have contributed to the count when these movements had substantial horizontal components.

2.8. Statistical analyses

Data from extracellular concentrations of glutamate, locomotor activity were analyzed using a repeated two-way ANOVA using the treatment group as the between-subject factor and time as the repeated measure variable (defined as $p<0.05$). A post-hoc Duncan multiple range test was then used to determine which group significantly differed from the others ($p<0.05$). Data from PPI, the startle amplitude, and counts of TUNEL-positive cells were analyzed using one-way ANOVA followed by post-hoc Duncan multiple range testing to determine which groups differed significantly from the others ($p<0.05$).

3. Results

At both dosages of 3.0 mg/kg and 1.0 mg/kg, APZ blocked METH-induced increases in extracellular glutamate levels in the mPFC (Fig. 1A), and showed no effects on glutamate levels after saline injection (Fig. 1B). Results also show that HPD, at only 0.1 mg/kg but not at 0.01 mg/kg, blocked the METH-induced increases in glutamate levels (Fig. 1C), but it had no effect on glutamate levels after saline injection (Fig. 1D).

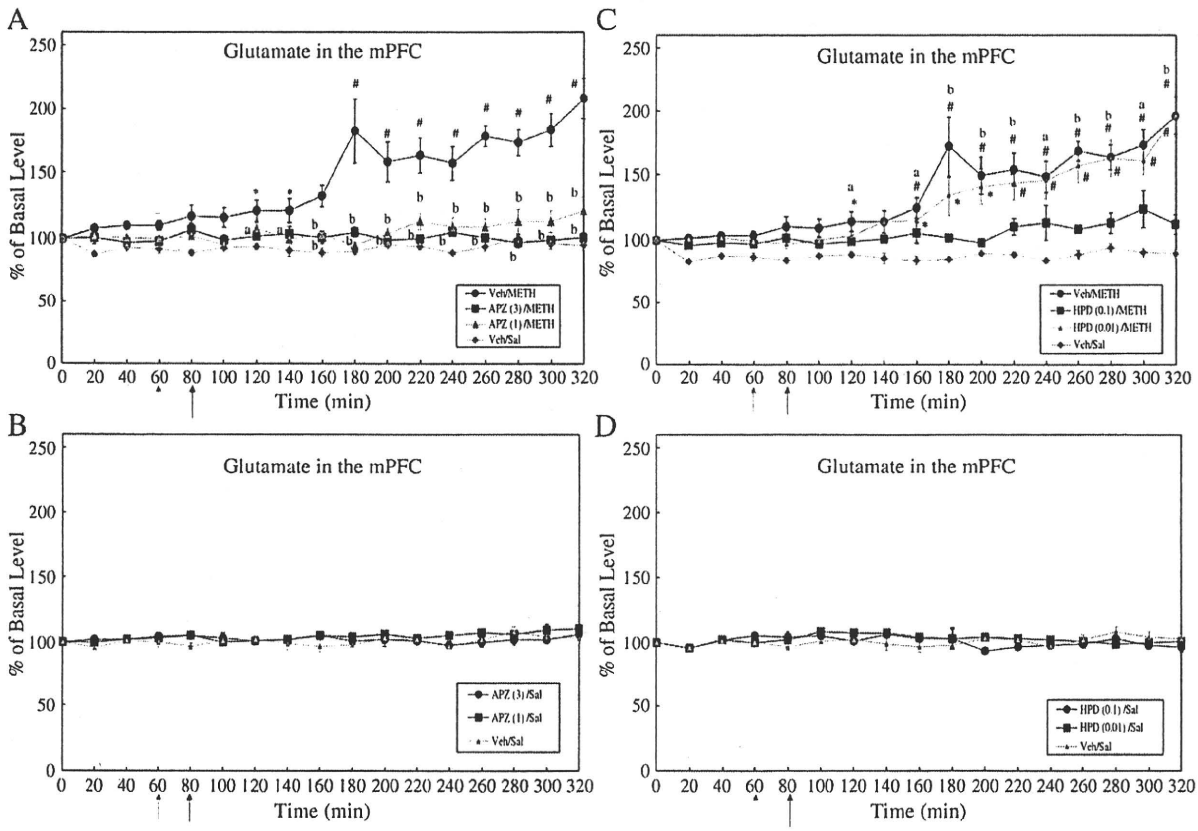

Fig. 1. A) Effects of APZ on METH-induced increases in glutamate levels in the mPFC. *: $p<0.05$, Veh/METH vs. Veh/Sal; #: $p<0.01$, Veh/METH vs. Veh/Sal; a: $p<0.05$, APZ (3)/METH vs. Veh/METH; b: $p<0.01$, APZ(3)/METH vs. Veh/METH, APZ(1)/METH vs. Veh/METH. A repeated two-way ANOVA revealed a significant effect for the treatment group vs. the time interaction analysis [$F(45, 420) = 7.35$, $p<0.01$], and for both the effect of group treatment [$F(3, 28) = 22.99$, $p<0.01$], and for the effect of time [$F(15, 420) = 12.35$, $p<0.01$]. The basal levels of glutamate (pmol/μL) in the Veh/METH group ($n=8$), APZ(3)/METH group ($n=8$), APZ(1)/METH group ($n=8$), and Veh/Sal group ($n=8$) were, respectively, 0.46 ± 0.06, 0.38 ± 0.05, 0.49 ± 0.07, and 0.50 ± 0.05. Each value represents the mean ± SEM. B) Effects of APZ on extracellular glutamate levels in the mPFC emerging after saline injection. A repeated two-way ANOVA revealed a significant effect for the treatment group vs. the time interaction analysis [$F(21, 315) = 0.70$, $p = 0.88$], and for both the effect of treatment group [$F(2, 21) = 0.52$, $p = 0.60$], and for the effect of time [$F(15, 315) = 1.37$, $p = 0.16$]. The basal levels of glutamate (pmol/μL) in the APZ(3)/Sal group ($n=8$), APZ(1)/Sal group ($n=8$), and Veh/Sal group ($n=8$) were, respectively, 0.33 ± 0.04, 0.50 ± 0.07, and 0.50 ± 0.05. Each value represents the mean ± SEM. C) Effects of HPD on METH-induced increases in glutamate levels in the mPFC. *: $p<0.05$, Veh/METH vs. Veh/Sal, HPD(0.01)/METH vs. Veh/Sal; #: $p<0.01$, Veh/METH vs. Veh/Sal, HPD(0.01)/METH vs. Veh/Sal; a: $p<0.05$, HPD(0.1)/METH vs. Veh/METH; b: $p<0.01$, HPD (0.1)/METH vs. Veh/METH. A repeated two-way ANOVA revealed a significant effect for the treatment group vs. the time interaction analysis [$F(27, 405) = 2.95$, $p<0.01$], and for both the effect of treatment group [$F(3, 27) = 15.50$, $p<0.01$], and for the effect of time [$F(15, 405) = 12.23$, $p<0.01$]. The basal levels of glutamate (pmol/μL) in the Veh/METH group (n = 8), HPD(0.1)/METH group ($n=7$), HPD(0.01)/METH group ($n=8$), and Veh/Sal group ($n=7$) were, respectively, 0.46 ± 0.06, 0.43 ± 0.02, 0.32 ± 0.09, and 0.50 ± 0.05. Each value represents the mean ± SEM. D) Effects of HPD on extracellular glutamate levels in the mPFC emerging after saline injection. A repeated two-way ANOVA revealed a significant effect for the treatment group vs. the time interaction analysis [$F(30, 270) = 1.39$, $p = 0.10$], and for both the effect of group [$F(2, 18) = 1.35$, $p = 0.28$], and for the effect of time [$F(15, 270) = 0.77$, $p = 0.71$]. The basal levels of glutamate (pmol/μL) in the HPD(0.1)/Sal group ($n=7$), HPD (0.01)/Sal group ($n=7$), and Veh/Sal group ($n=8$) were, respectively, 0.38 ± 0.05, 0.56 ± 0.01, and 0.50 ± 0.05. Each value represents the mean ± SEM.

Moreover, APZ (3.0 mg/kg) (Fig. 2A) or HPD (0.1 mg/kg) (Fig. 2B), co-administered repeatedly with METH, blocked progression to an enhanced response to locomotion-inducing effect of MK-801, as measured seven days after the treatment. Pretreatment with APZ (3.0 mg/kg) and HPD (0.1 mg/kg) before each METH injection prevented progression to PPI deficit at 68 dB, 71 dB, 77 dB (Fig. 3A), and at 68 dB (Fig. 3C), respectively, as measured seven days after the treatment with no challenge injection. Although repeated co-administration of vehicle and METH developed enhanced the startle amplitude around 68 dB and 71 dB of prepulse intensities, pretreatment with APZ (3.0 mg/kg) (Fig. 3B) or HPD (0.1 mg/kg) (Fig. 3D) before each METH injection showed no significantly greater enhancement of the startle amplitude than the Vehicle/ Saline control group did.

Marked expression of TUNEL-positive cells was detected in the slice of the positive control (Fig. 4B) but not in that the negative control (Fig. 4A). Repeated co-administration of vehicle with METH (Figs. 5B, 6) showed much more progression to marked expression of TUNEL-positive cells in the mPFC than the Vehicle/ Saline control group did (Figs. 5A, 6). Repeated co-administration of APZ (3.0 mg/kg) with METH (Figs. 5C, 6) or HPD (0.1 mg/kg) with METH (Figs. 5D, 6) showed remarkably more attenuated progression to expression of TUNEL-positive cells in the mPFC than the Vehicle/METH group showed (Figs. 5B, 6). Although repeated co-administration of APZ (3.0 mg/kg) with saline did not induce expression of TUNEL-positive cells (Figs. 5E, 6), HPD (0.1 mg/kg). Saline group induced marked expression of TUNEL-positive cells (Figs. 5F, 6).

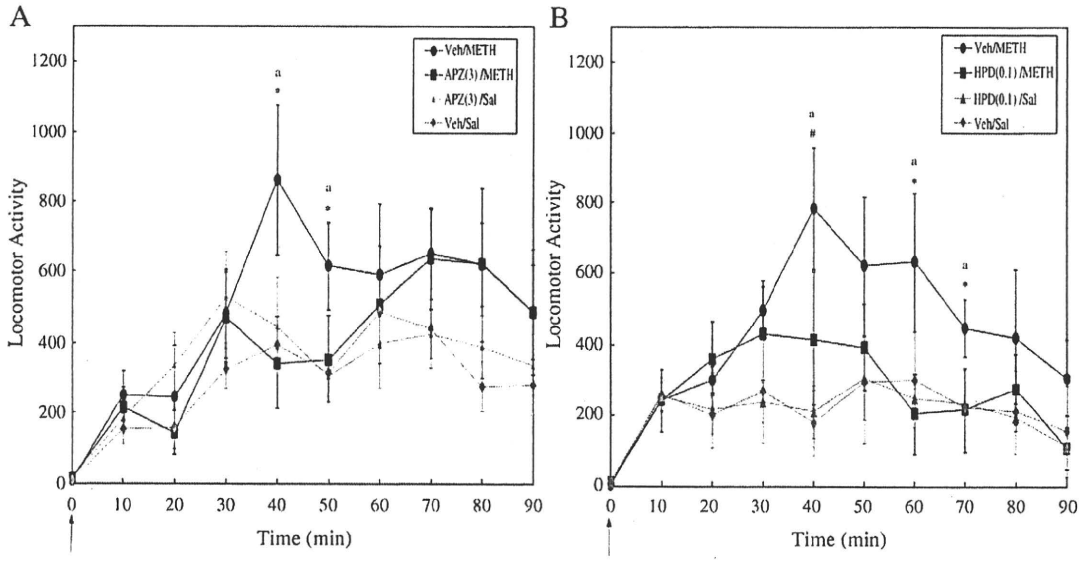

Fig. 2. A) Effect of a challenge injection of MK-801 (0.2 mg/kg) on locomotor activity after seven days of withdrawal from repeated co-administration of APZ (3 mg/kg) or saline with METH or saline. *: $p<0.05$, Veh/METH vs. Veh/Sal: a: $p<0.05$, APZ(3)/METH vs. Veh/METH. A repeated two-way ANOVA revealed a significant effect for the treatment group vs. the time interaction analysis [$F(27, 252)=3.24$, $p<0.05$], for both the effect of treatment group [$F(3, 28)=3.27$, $p<0.05$], and for the effect of time [$F(9, 252)=14.33$, $p<0.01$]. Animal quantities of each group are as follows: Veh/Sal ($n=8$), Veh/METH ($n=8$), APZ(3)/METH group ($n=8$), APZ(3)/Veh group ($n=8$). B) Effect of a challenge injection of MK-801(0.2 mg/kg) on locomotor activity after seven days of withdrawal from repeated co-administration of HPD(0.1 mg/kg) or saline with METH or saline. *: $p<0.05$, Veh/METH vs. Veh/Sal: a: $p<0.05$, HPD(0.1)/METH vs. Veh/METH. Repeated two-way ANOVA revealed a significant effect for the treatment group vs. the time interaction analysis [$F(27, 216)=3.09$, $p<0.05$], and for both the effect of treatment group [$F(3, 24)=3.03$, $p<0.05$], and for the effect of time [$F(9, 216)=6.81$, $p<0.01$]. Animal quantities of each group are as follows: Veh/Sal ($n=7$); Veh/METH($n=7$); HPD(0.1)/METH ($n=7$); HPD(0.1)/Sal ($n=7$).

4. Discussion

Co-administration of METH with APZ or HPD at doses that block the METH-induced increases in glutamate levels in the mPFC inhibited progression to cross-sensitization to MK-801, the neuroplastic PPI deficit at the baseline state, and the induction of TUNEL-positive cells in the mPFC.

4.1. Effects of APZ or HPD on METH-induced increases in glutamate levels

Results show that APZ at both 3.0 mg/kg and 1.0 mg/kg, and HPD at 0.1 mg/kg but not 0.01 mg/kg inhibited METH (2.5 mg/kg)-induced increases in glutamate levels in the mPFC. Yang and Wang (2008) showed that APZ blocks nerve terminal excitation-mediated glutamate release in the prefrontal cortex. They reported the possibility that this antipsychotic is neuroprotective. Taken together with these findings, we can postulate that APZ, in our comprehensive animal model for the progressive pathophysiology of schizophrenia (Abekawa et al., 2008), inhibits the induction of the METH-induced behavioural and histopathologic abnormalities. In the present study, because we specifically examined the difference between APZ and HPD in terms of neuroprotection against the progressive pathophysiology, the precise pharmacological mechanisms of these antipsychotics were not examined in light of profiles for modulating the function of receptors of several types (Lawler et al., 1999). The dopamine D_2 receptor antagonist HPD and the 5-HT_{1A} receptor antagonist WAY100635 cancelled the inhibiting effect of APZ on the terminal excitation-mediated glutamate release (Yang and Wang, 2008), suggesting that the effect of APZ is mediated by activation of dopamine D_2 and/or 5-HT_{1A} receptors. The same group (Yang and Wang, 2005) showed that HPD similarly inhibited the terminal excitation-mediated glutamate release. Therefore, APZ might inhibit glutamate release in the PFC, by partial agonism and/or complete blockade of dopamine D_2 receptors.

4.2. Effects of APZ or HPD on repeatedly administered METH-induced schizophrenia-like behavioural abnormalities

Each of APZ and HPD, at dosages that blocked the METH-induced increases in glutamate levels, inhibited the development not only of cross-sensitization to the locomotion-inducing effect of MK-801, but also of the neuroplastic PPI disruption at the baseline state without a challenge injection. In our preliminary experiment, this repeatedly administered METH-induced PPI deficit did not respond well to D_2 receptor antagonist, HPD, but it did respond to clozapine. Furthermore, NMDA receptor antagonists such as phencyclidine (PCP) and MK-801 induced hyperlocomotion and the PPI deficit. These behavioural abnormalities do not respond well to HPD, but they do respond to some atypical antipsychotics such as clozapine, olanzapine, and quetiapine (Abekawa et al., 2003; Gleason and Shannon, 1997; Bakshi et al., 1994; Swerdlow et al., 1996), which can stimulate NMDA receptor-mediated glutamatergic neurotransmission (Abekawa et al., 2006; Arvanov et al., 1997; Ninan et al., 2003). Therefore, hyperlocomotion and PPI disruption attributable to robust hypofunction of NMDA receptor-mediated glutamatergic neurotransmission induced by an acute single injection of NMDA receptor antagonist suggest a biological basis for the pathophysiology and

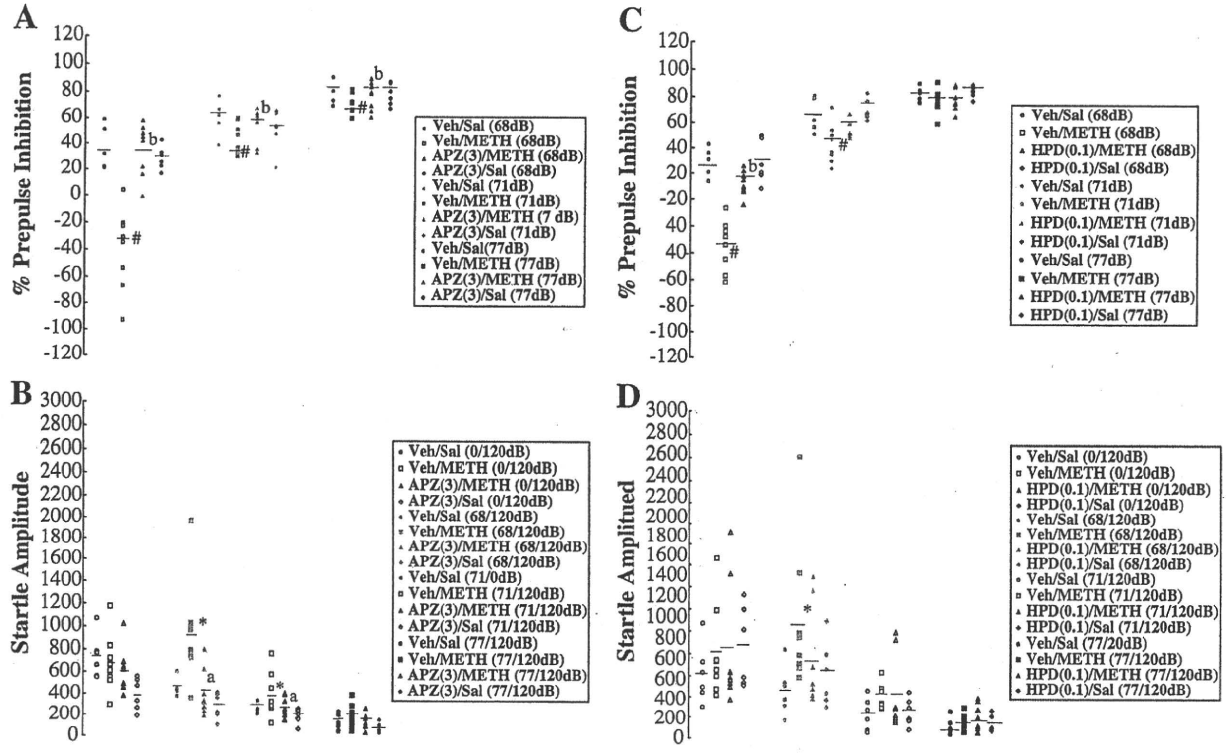

Fig. 3. A, B) Effects of co-administration of APZ (3.0 mg/kg) with METH on PPI and startle amplitude measured at baseline state with no challenge injection. *: $p<0.05$, Veh/METH (68/120 dB) vs. Veh/Sa (68/120 dB), Veh/METH (71/20 dB) vs. Veh/Sal (71/120 dB)l; #:$p<0.01$, Veh/METH (68 dB) vs. Veh/Sal (68 dB), Veh/METH (71 dB) vs. Veh/Sal (71 dB), Veh/METH(77 dB) vs. Veh/Sal (77 dB); a: $p<0.05$, APZ(3)/METH (68/120 dB) vs. Veh/METH (68/120 dB), APZ(3)/METH (71/120 dB) vs. Veh/METH (71/120 dB); b: $p<0.01$, APZ(3)/METH (68 dB) vs. Veh/METH (68 dB), APZ(3)/METH (71 dB) vs. Veh/METH (71 dB), APZ(3)/METH (77 dB) vs. Veh/METH (77 dB). Horizontal bars represent the mean. Animal quantities of each group are as follows. Veh/Sal ($n=6$), Veh/METH ($n=8$), APZ (3)/METH ($n=8$), APZ(3)/Sal ($n=6$). C, D) Effects of co-administration of HPD (0.1 mg/kg)) with METH on PPI and startle amplitude measured at baseline state with no challenge injection. *: $p<0.05$, Veh/METH (68/120 dB) vs. Veh/Sal (68/120 dB); #:$p<0.01$, Veh/METH (68 dB) vs. Veh/Sal (68 dB), Veh/METH (71 dB) vs. Veh/Sal (71 dB); b: $p<0.01$, HPD(0.1)/METH (68 dB) vs. Veh/METH (68 dB). Horizontal bars represent the mean. Animal quantities of each group are as follows. Veh/Sal ($n=6$), Veh/METH ($n=8$), HPD(0.1)/METH ($n=8$), HPD(0.1)/Sal ($n=6$).

neurocognitive deficit, which are resistant to dopamine D_2 receptor blockers and to neurocognitive deficits. However, in our study, cross-sensitization to MK-801 was evaluated as enhanced hyperlocomotion induced by a challenge injection of MK-801. In addition, the neuroplastic PPI deficit was detected with no challenge injection: the enhanced hyperlocomotion and the PPI deficit were evaluated at seven days' withdrawal from repeated METH administration.

Repeated administration of high doses of METH/amphetamine produces long-lasting decreases in immunoreactivity

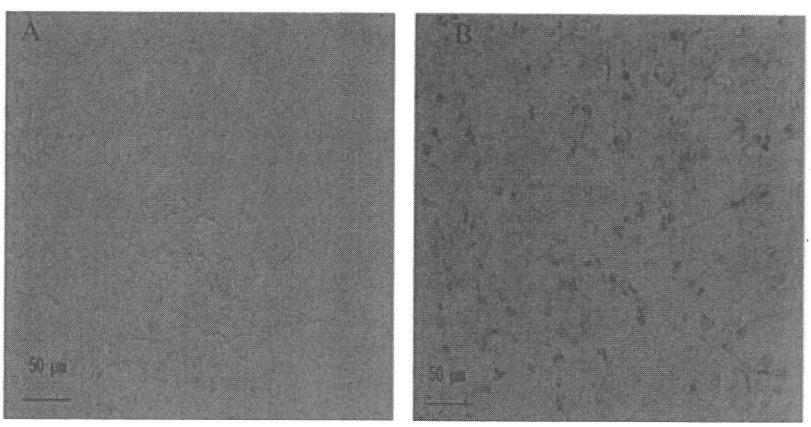

Fig. 4. Representative photographs of negative control (A) and positive control (B) of TUNEL-staining cells in the mPFC.

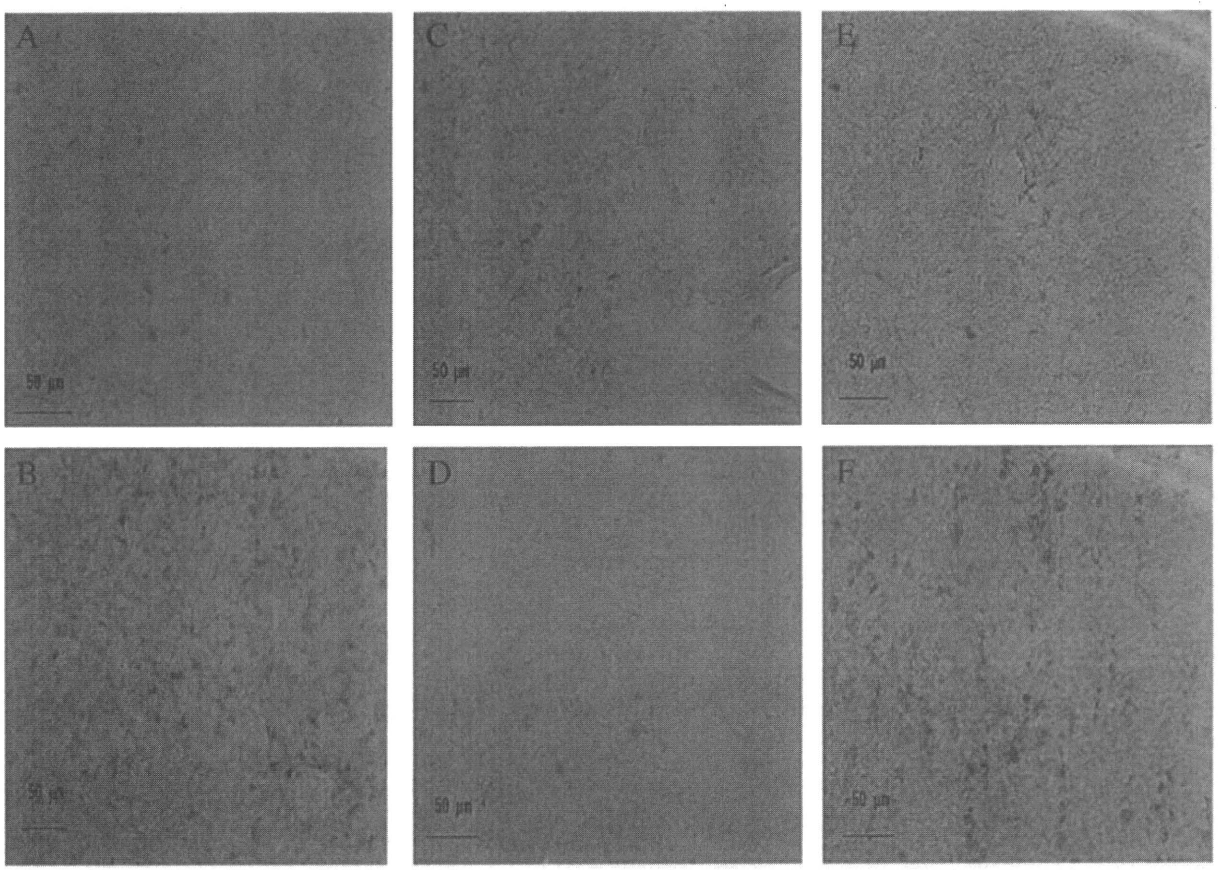

Fig. 5. Representative photographs of the expression of TUNEL-positive cells in the mPFC of Veh/Sal (A), Veh/METH (B), APZ(3)/METH (C), HPD(0.1)/METH (D), APZ(3)/Sal (E), and HPD/Sal (F).

of NMDA NR1 and NR2B subunits in the striatum (Yamamoto et al., 1999) and in mRNA levels of NMDA NR1 subunit in the PFC and the nucleus accumbens (Lu et al., 1999). The NMDA receptor antagonist (PCP)-induced hyperlocomotion is abolished by an ibotenic acid-induced lesion of the bilateral PFC (Jentsch et al., 1998). Bilateral lesions of the mPFC abolish NMDA receptor antagonist (MK-801)-induced PPI disruption but not a D_2 receptor agonist (apomorphine)-induced PPI

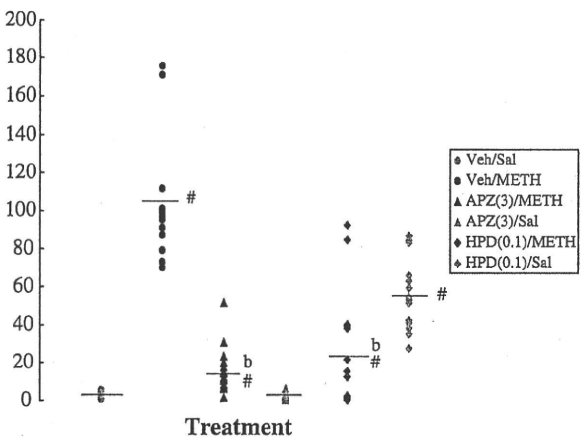

Fig. 6. Effects of co-administration of APZ (3.0 mg/kg) or HPD (0.1 mg/kg) with METH on the expression of TUNEL-positive cells in the mPFC, a brain region captured from brains of animals killed nine days after the repeated treatment. #: $p<0.01$, Veh/METH vs. Veh/Sal, APZ(3)/METH vs. Veh/Sal, HPD(0.1)/METH vs. Veh/Sal, HPD(0.1)/Sal vs. Veh/Sal; b: $p<0.01$, APZ(3)/METH vs. Veh/METH, HPD(0.1)/METH vs. Veh/METH. Each mark represents the average of counts for unilateral (left or right side) six unit areas (6×1 mm^2) per animal. A set of two marks (left and right sides) per animal is shown. Horizontal bars show the mean. Animal quantities of each group are as follows: Veh/Sal ($n=6$), Veh/METH ($n=7$), APZ(3)/METH ($n=8$), APZ(3)/Sal ($n=6$), HPD(0.1)/METH ($n=7$), HPD(0.1)/Sal ($n=8$).

deficit (Schwabe and Koch, 2004). Microinjection of MK-801 into the mPFC tends to disrupt PPI (Bakshi and Geyer, 1998). Considering these findings collectively, we speculate that, in the present study, repeated METH-induced increases in glutamate levels in the mPFC induce hypofunction of NMDA receptor-mediated neurotransmission and thereby induce the enhanced response to the locomotion-inducing effect of MK-801 and PPI disruption at the baseline state in this study.

In this study, APZ (3.0 mg/kg) and HPD (0.1 mg/kg) blocked the METH-induced increase in glutamate levels, engendering inhibition of the development of the neuroplastic behavioural changes induced by repeated METH administration. Similarly, in our previous study (Abekawa et al., 2008), olanzapine (1.0 mg/kg) and risperidone (0.1 mg/kg), which inhibited the METH-induced increases in glutamate levels, prevented induction of these behavioural abnormalities.

4.3. Effects of APZ or HPD on induction of TUNEL-positive cells in the mPFC

Results show that APZ (3.0 mg/kg) and HPD (0.1 mg/kg) markedly attenuated but did not completely prevent the induction of TUNEL-positive cells in the mPFC induced by repeated METH administration. As our previous study (Abekawa et al., 2008) showed, repeated administration of METH, but only at a dose where it was able to increase glutamate levels in the mPFC, induced expression of TUNEL-positive cells, which is a sensitive marker of apoptosis induction (Gavrieli et al., 1992). In addition, each of olanzapine and risperidone, when administered at a dosage that blocked increases in glutamate levels, prevented the expression of TUNEL-positive cells. Therefore, these findings suggest that repeated increases in glutamate levels are a trigger that induces apoptosis (Glantz et al., 2006), and that treatment with antipsychotics that can block METH-induced increases in glutamate levels might be useful for preventing the progressive neuropathological abnormalities. Although olanzapine (1.0 mg/kg) and risperidone (0.1 mg/kg) completely prevented the METH-induced expression of TUNEL-positive cells in the mPFC in our previous study (Abekawa et al., 2008), in the present study, APZ (3.0 mg/kg) and HPD (0.1 mg/kg) markedly attenuated, but did not completely block, the METH-induced histological changes. These findings suggest that partial agonism and/or complete blockade of dopamine D_2 receptors contributes to the neuroprotective effects. The differences in the types of antipsychotics and the timing of injections between the present study and our previous study might explain the discrepancy in the degree of the neuroprotective effects of antipsychotics. Although some difference exists between the METH-induced apoptosis and kainic acid-induced excitotoxicity, APZ reportedly prevents kainic acid-excitotoxicity via the stimulation of 5-HT_{1A} receptors (Cosi et al., 2005). Differently from that report, the present study limits clarification of the contribution of 5-HT_{1A} receptor partial agonism and /or 5-HT_{2A} receptor antagonism to the neuroprotection of APZ.

The other important finding of this study is that, compared to the vehicle/saline group, although the APZ/Saline group did not induce TUNEL-positive cells, the HPD/saline group did induce this apoptotic reaction. Because administration of saline did not increase glutamate levels in the mPFC, the finding described earlier suggests that, although HPD prevents the induction of apoptosis under the condition of METH-induced increases in glutamate levels, it does induce apoptosis with no increases in glutamate levels. Of the drugs examined by our group (olanzapine, risperidone, APZ, and HPD), only HPD-induced apoptosis when co-administered repeatedly with saline. Although we noted that an increase in glutamate levels is one of the triggers for apoptosis induction, mechanisms other than glutamatergic changes might contribute to these histological abnormalities because HPD itself did not increase glutamate levels. Activated AKT (protein kinase B) phosphorylates serine 9 on glycogen synthase kinase 3β (GSK-3β, engendering neuroprotection against apoptosis (Chen and Russo-Neustadt, 2005). Although APZ prevents induction of apoptosis by inhibiting GSK-3β activity followed by activation of Akt, HPD induces apoptosis by activating GSK-3β (Park et al., 2009). This HPD-induced change in Akt/GSK 3 signalling might contribute to the induction of apoptosis when HPD is co-administered repeatedly with saline.

4.4. Clinical significance

In schizophrenic subjects, the amphetamine-induced decrease in [^{123}I] IBZM ((−)-N-[[(2S)-1-ethyl-2-pyrrolidinyl] methyl]-2-hydroxy-3-iodo-6-methoxybenzamide) binding potential in the striatum--which results from increased dopamine release in this region in response to an amphetamine challenge--is significantly greater than in healthy controls (Laruelle et al., 1996). This dysregulation is observed in patients experiencing an onset and an episode of illness exacerbation, but it is not observed during remission (Laruelle et al., 1999). Although data obtained from schizophrenic subjects remain limited, AMPH challenge seems to increase dopamine release in several cortical regions of healthy control subjects (Narendran et al., 2009). Our previous study (Abekawa et al., 2008) showed that, although both 2.5 mg/kg and 1.0 mg/kg of METH significantly increased dopamine levels in the rat mPFC, with larger increases in dopamine levels with 2.5 mg/kg than with 1.0 mg/kg, as compared to saline challenge injection, only 2.5 mg/kg of METH enhanced glutamate release in this region. Taken together, if the degree of dopamine release in the frontal cortex at the onset or during a psychotic episode is severe, this intense dopaminergic hyperactivity might induce an increase in glutamate levels in this region.

In schizophrenia, repeated psychotic episodes reportedly induce resistance to dopamine D_2 receptor antagonists (Lieberman, 1999a,b; Sheitman and Lieberman, 1998), progressive cognitive deficits (Pukrop et al., 2006), and cortical atrophy (Kasai et al., 2003; Lieberman et al., 2005; Van Haren et al., 2007). Therefore, early intervention with antipsychotics that have neuroprotective effects might protect schizophrenic brains from this progression of pathophysiology. This neuroprotective pharmacological therapy should therefore be continued with good adherence.

A review of a workshop held after an annual American College of Neuropsychopharmacology (ACNP) meeting described that progressive brain-change associated schizophrenia is a real phenomenon, and that it occurs early in the illness (Borgwardt et al., 2009; DeLisi, 2008). Kasai et al. (2003)

reported a progressive decrease of left temporal gyrus grey matter volume within around 1.5 years in patients with first-episode schizophrenia. Whitford et al. (2006) showed progressive grey matter atrophy in frontal, parietal, and temporal cortices over the first 2–3 years of illness in first-episode schizophrenia. Progressive brain atrophy not only in the early stage but also during the prodromal phase to the full-blown stage of schizophrenia (Pantelis et al., 2003), and the possibility of underlying apoptosis (Glantz et al., 2006; Jarskog et al., 2005) and programmed cell death (Yuan and Yankner, 2000) have been reported. Jarskog et al. (2004) showed a higher Bax/Bcl 2 ratio with a normal caspase-3 level in chronic schizophrenia, suggesting vulnerability to apoptosis without activated apoptosis in this illness stage. Increased DNA fragmentation is not detected in post-mortem brains of patients with chronic schizophrenia (Benes et al., 2003; Buttner et al., 2007), suggesting that cortical apoptosis is not increased in chronic schizophrenia. However, these findings do not rule out the possibility that DNA breaks were increased during the progression from prodrome to the full-blown stage and at an earlier stage of illness because apoptotic cells are generally cleared rapidly and do not leave a residue (Hetts, 1998) and because TUNEL-positive cells might be difficult to detect in conditions where cell death occurs infrequently (Glantz et al., 2006; Jarskog et al., 2005). Smaller somal volume and decreased spine density, dendritic length, and terminals compared to normal controls, which are exhibited in schizophrenia (Glantz et al., 2006), are similarly induced in animals that are sensitized with a high dose of amphetamine (Selemon et al., 2006). Furthermore, a toxic dosage regimen of METH induces apoptosis in cortical neurons in the mPFC (Kadota and Kadota, 2004). These findings suggest that an apoptotic process plays a critical role in the progressive cortical atrophy. Furthermore, the METH-induced expression of TUNEL-positive cells in the rat mPFC, which is a sensitive marker of apoptosis induction (Gavrieli et al., 1992), might reflect the basis for progressive cortical volume loss because a gliotic reaction has not been detected in post-mortem schizophrenic brain studies.

Olanzapine can block progressive volume reduction in whole-brain grey matter (Lieberman et al., 2005; Van Haren et al., 2007), whereas HPD worsens progressive cortical atrophy in first-episode psychosis (Lieberman et al., 2005). Similarly, in the early schizophrenic group, atypical antipsychotics (olanzapine, clozapine, and risperidone) rather than haloperidol were associated with larger hippocampal volume Chakos et al., 2005). In schizophrenia subjects, the progression of left frontal density loss appears to be related to an increased number of psychotic episodes, with atypical antipsychotic medication (clozapine and olanzapine) attenuating these changes (Van Haren et al., 2007). These findings suggest that atypical antipsychotics attenuate the progressive volume reduction of the brain.

To date, the biological mechanisms for prevention of these progressive pathological abnormalities remain unclear. In the present study, APZ and HPD each blocked METH-induced increases in glutamate levels in the mPFC, leading to inhibition of repeated METH administration-induced behavioural abnormalities and apoptosis, as did olanzapine or risperidone in a previous study (Abekawa et al., 2008). Based on these findings, we might explain that at least the four typical and atypical antipsychotics described previously inhibit severe psychotic-episode-related glutamate increases in the brain to block the apoptosis-mediated progressive brain atrophy. Furthermore, attention should be devoted to the effects of HPD, which, under the condition of METH-induced glutamate increases, blocked this increase to prevent repeatedly administered METH-induced progression of pathophysiology. Repeated co-administration of HPD with saline, without increases in glutamate levels in the mPFC, induced apoptosis in this brain region, but not cross-sensitization to MK-801 or a neuroplastic PPI deficit. These findings suggest that HPD blocks glutamate release in the mPFC at the onset or during psychotic episodes of schizophrenia, leading to inhibition of ongoing progression of pathophysiology. They suggest that once the increased glutamate release has disappeared, repeated HPD administration might induce apoptosis in this region.

In summary, each of APZ (3.0 and 1.0 mg/kg) and HPD (0.1 mg/kg) blocked METH (2.5 mg/kg)-induced increases in glutamate levels in the mPFC. Furthermore, APZ (3.0 mg/kg) or HPD (0.1 mg/kg), when repeatedly co-administered with METH, prevented the progression to cross-sensitization to the locomotion-inducing effect of MK-801, the PPI deficit at the baseline state, and apoptosis in this brain region. Repeated co-administration of APZ (3.0 mg/kg) and saline did not induce apoptosis, but repeated co-administration of HPD (0.1 mg/kg) and saline did induce apoptosis. These results suggest that both APZ and HPD prevented progressive schizophrenia-like behavioural and pathological abnormalities, which were induced by repeated increases in glutamate levels, and indicate that repeated administration of HPD but not APZ induced apoptosis under conditions without increased glutamate levels. These findings suggest the importance of the use of APZ and HPD in the appropriate stages of the glutamate-related pathophysiology of schizophrenia. These findings might reflect the biological basis for the neuroprotective effects of APZ or HPD. They suggest the importance of continuing pharmacotherapy with APZ, but not HPD, after acute psychotic symptoms in the progressive stage of schizophrenia resolve. Continued administration of HPD during the remission or residual stage must be evaluated carefully.

Role for funding source

This study was supported in part by a Grant-in-Aid from the Mitsubishi Pharma Research Foundation. The Mitsubishi Pharma Research Foundation had no further role in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit this paper for publication.

Contributors

Tomohiro Abekawa and Tsukasa Koyama designed the study. Tomohiro Abekawa wrote the protocol. Tomohiro Abekawa, Yasuya Nakato, and Koki Ito managed the literature search and analyses. Tomohiro Abekawa and Koki Ito conducted experiments and statistical analysis. Tomohiro Abekawa wrote the first draft of the manuscript. All authors contributed to and have approved final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

Acknowledgements

The authors thank Akiko Kato and Aya Kakuta for their technical assistance.

Aripiprazole was a gift from Otsuka Pharmaceuticals. Haloperidol and methamphetamine were gifts from Dainippon Sumitomo Pharma Co. Ltd., Japan.

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Progress in Neuro-Psychopharmacology & Biological Psychiatry 35 (2011) 111–117

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Effects of mood stabilizers on adult dentate gyrus-derived neural precursor cells

Shuken Boku [a,*], Shin Nakagawa [a], Takahiro Masuda [a,b], Hiroyuki Nishikawa [b], Akiko Kato [a], Hiroyuki Toda [a], Ning Song [a,c], Yuji Kitaichi [d], Takeshi Inoue [a], Tsukasa Koyama [a]

[a] Department of Psychiatry, Hokkaido University Graduate School of Medicine, Sapporo, Japan
[b] Pharmaceutical Laboratories, Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan
[c] Department of Psychiatry, The First Hospital of China Medical University, Shengyang, China
[d] Department of Molecular Stressphysiology, Max Planck Institute of Psychiatry, Munich, Germany

ARTICLE INFO

Article history:
Received 14 July 2010
Received in revised form 25 September 2010
Accepted 26 September 2010
Available online 1 October 2010

Keywords:
Apoptosis
Dexamethasone
Differentiation
Hippocampus
Mood disorder
Neurogenesis
Proliferation
Retinoic acid
Staurosporine

ABSTRACT

Neurogenesis in the adult dentate gyrus (DG) is considered to be partly involved in the action of mood stabilizers. However, it remains unclear how mood stabilizers affect neural precursor cells in adult DG. We have established a culture system of adult rat DG-derived neural precursor cells (ADP) and have shown that lithium, a mood stabilizer, and dexamethasone, an agonist of glucocorticoid receptor, reciprocally regulate ADP proliferation. Neurogenesis constitutes not only proliferation of neural precursor cells but also apoptosis and differentiation. To develop further understanding of mood stabilizer effects on neural precursor cells in adult DG, we investigated and compared the effects of four common mood stabilizers—lithium, valproate, carbamazepine, and lamotrigine—on ADP proliferation, apoptosis, and differentiation. ADP proliferation, decreased by dexamethasone, was examined using Alamar Blue assay. Using TUNEL assay, ADP apoptosis induced by staurosporine was examined. The differentiated ADP induced by retinoic acid was characterized by immunostaining with anti-GFAP or anti-Tuj1 antibody. Lithium and valproate, but not carbamazepine and lamotrigine, recovered ADP proliferation decreased by dexamethasone. All four mood stabilizers decreased ADP apoptosis. Retinoic acid differentiated ADP into both neurons and astrocytes. Lithium and carbamazepine increased the ratio of neurons and decreased that of astrocytes. However, valproate and lamotrigine increased the ratio of astrocytes and decreased that of neurons. Therefore, these four stabilizers exhibited both common and differential effects on ADP proliferation, apoptosis, and differentiation.

1. Introduction

Drugs of a group including lithium (Li), valproate (VPA), carbamazepine (CBZ), and lamotrigine (LTG), known as mood stabilizers, are commonly used to treat bipolar disorder (Goodwin, 2003). Although the biochemical effects of mood stabilizers have been investigated extensively (Schloesser et al., 2007; Schloesser et al., 2008), the essence of their mood-stabilizing effects remains unclear.

Recently, neurogenesis has been confirmed to occur in the adult hippocampus (Kempermann, 2006; Gage et al., 2008). Stem cells (Type-1 cells) are located in the subgranular zone between the granular cell layer and hilus in the dentate gyrus (DG), and differentiate to amplify progenitor cells (Type-2a and Type-2b cells). Thereafter, numerous newborn cells die, presumably by apoptosis (Kempermann et al., 2003). New surviving neurons mature morphologically and electrophysiologically. They ultimately integrate into active neural circuits. Although the function of these newborn cells remains unclear, they are reportedly involved in the therapeutic action of antidepressants (Santarelli et al., 2003; Surget et al., 2008; David et al., 2009). Moreover, recent reports have described that Li and VPA affect neurogenesis through increasing cell proliferation and/or promotion of neuronal differentiation of neural precursor cells (Chen et al., 2000; Son et al., 2003; Hao et al., 2004; Hsieh et al., 2004; Kim et al., 2004b; Laeng et al., 2004; Wexler et al., 2008) and that Li blocks the effects of stress on depression-like behaviors through increasing hippocampal neurogenesis in adult rodent models (Silva et al., 2008). Results of these studies suggest that adult hippocampal neurogenesis plays an important role in the therapeutic action of mood stabilizers as well.

We have already established the culture system of adult DG-derived neural precursor cell (ADP), which approximately corresponds to Type-2a amplifying progenitor cells (Boku et al., 2009). Li reported a lack of an effect on ADP proliferation but recovered ADP proliferation decreased by dexamethasone (DEX), a specific agonist of glucocorticoid receptor (Boku et al., 2009). To expand knowledge about the effect of mood stabilizers on adult neurogenesis, we

Abbreviations: Li, lithium; VPA, valproate; CBZ, carbamazepine; LTG, lamotrigine; DG, dentate gyrus; ADP, adult rat DG-derived neural precursor cell; DEX, dexamethasone; STS, staurosporine; RA, retinoic acid; PHT, phenytoin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4′-6-diamino-2-phenylindole.

* Corresponding author. North 15, West 7, Kita-Ku, Sapporo, 060-8638, Japan. Tel.: +81 11 716 5160; fax: +81 11 706 5081.
E-mail address: shuboku@med.hokudai.ac.jp (S. Boku).

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doi:10.1016/j.pnpbp.2010.09.019

examined and compared the effects of four mood stabilizers—Li, VPA, CBZ, and LTG—on ADP proliferation, apoptosis and differentiation (cell-fate determination). Although these four stabilizers had varied effects on ADP proliferation and differentiation, all commonly decreased ADP apoptosis.

2. Materials and methods

2.1. Drugs

Dexamethasone (DEX) was purchased from Sigma Chemical Co. (St. Louis, MO). Retinoic acid (RA) was purchased from Invitrogen Corp. (Carlsbad, CA). Staurosporine (STS) was kindly donated by Asahi-Kasei Medical Co. Ltd. (Shizuoka, Japan). Lithium chloride (Li) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium valproate (VPA) was kindly donated by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). Carbamazepine (CBZ) was kindly donated by Nihon Ciba-Geigy K.K. (Tokyo, Japan). Lamotrigine was kindly donated by Glaxo SmithKline plc. (London, UK). Phenytoin (PHT) was kindly donated by Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan).

2.2. Isolation and culture of ADP

ADP was isolated from the dentate gyrus of adult male Sprague–Dawley rats (8 weeks old), as described in a previous report (Boku et al., 2009). The ADPs were maintained with Neurobasal (Invitrogen Corp., Carlsbad, CA)/B27 supplement minus vitamin A (Invitrogen Corp.)/1 mM L-glutamine (Invitrogen Corp.)/20 ng/ml bFGF (Invitrogen Corp.) (proliferation medium) at 37 °C on laminin (Invitrogen Corp.)-ornithine (Sigma)-coated dishes and fed with new medium every 2 or 3 days by replacing 50% of the medium. When cell confluency reached 80–90%, cells were passaged by trypsinization, and the cell density for plating was approximately 1×10^4 cells/cm^2.

2.3. Cell counting

Alamar Blue assay is a rapid and simple non-radioactive assay used to estimate the number of living cells (Ahmed et al., 1994). Alamar Blue dye is a fluorogenic redox indicator and is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent; fluorescence in Alamar Blue assay reflects the number of cells. Although BrdU-based assays are often used for cell counting, BrdU-positiveness reflects the duplication of DNA, but not the number of cells. Additionally, we confirmed that fluorescence in Alamar Blue assay is proportional to the simply counted number of ADPs (data not shown). Therefore, we used Alamar Blue assay to estimate the effects of drugs on the number of cells. First, 1×10^4 cells/well were put in laminin–ornithine coated 96-well plates in 100 μl/well of proliferation medium. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, 10 μl/well of Alamar Blue solution (Invitrogen Corp.) was added to medium, and cells were incubated at 37 °C for 3 h. Subsequently, 50 μl of medium was dispensed into plates and the fluorescence of samples were measured and calculated as described in the manufacturer's manual. Statistical analysis was performed using one-way ANOVA and Dunnet's post hoc test. Significance was inferred for $p<0.05$. Data are expressed as means ± SEM.

2.4. TUNEL assay

First, 2×10^4 cells/well were put in laminin–ornithine coated 8-chamber slides (Lab-Tek II; Nalge Nunc International, Naperville, IL) with proliferation medium. After overnight incubation, cells were treated in proliferation medium with STS, PHT, Li, VPA, CBZ, and/or LTG. After 2 days, cells were fixed in 4% paraformaldehyde for 15 min. Permeabilization was performed with PBS containing 0.2% Triton X-100 for 30 min. Subsequently, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with a DeadEnd Fluorometric TUNEL System (Promega Corp., Madison, WI), as described in the manufacturer's manual. Fluorescent signals were detected using a fluorescence microscope system (IX-71; Olympus Corp.). The quantities of both TUNEL and 4′·6-diamino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA) signals were counted in four randomly selected fields/well. Then the ratio of TUNEL signals/DAPI signals was calculated. Statistical analysis was performed using Student's *t*-test. Significance was defined as $p<0.05$. Data are expressed as means ± SEM.

2.5. Immunocytochemistry

First, 2×10^4 cells/well were put in laminin–ornithine coated Lab-TekII eight-chamber slides with proliferation medium without bFGF (differentiation medium). After overnight incubation, cells were treated in differentiation medium with 1 μM RA/0.5% fetal bovine serum (Invitrogen) and Li, VPA, CBZ, or LTG. After 7 days, cells were fixed 4% paraformaldehyde for 15 min. Permeabilization was performed with PBS containing 0.2% Triton X-100 for 30 min. Subsequently, samples were blocked in PBS containing 3% goat serum for 20 min at room temperature (RT), incubated in PBS containing 3% goat serum containing primary antibodies at 4 °C overnight, and incubated in PBS containing secondary antibodies for 1 h at RT. Samples were coverslipped with Vectashield containing DAPI. Fluorescent signals were detected using the IX-71 fluorescent microscope system described above. Primary antibodies were used at the following concentrations: mouse anti-nestin (1:2000; BD Biosciences, Franklin Lakes, NJ), rabbit anti-glial fibrillary acidic protein (GFAP) (1:2000; Dako, Glostrup, Denmark) and mouse anti-Tuj1 (1:5000; Covance Inc., Princeton, NJ). Secondary antibodies were used at the following concentrations: FITC-conjugated goat anti-mouse IgG antibody (1:100; Jackson Immuno Research Laboratories, Inc., West Grove, PA) and Cy3-conjugated goat anti-rabbit IgG antibody (1:100; Jackson Immuno Research Laboratories, Inc.). The quantities of signals of each marker gene and DAPI were counted in four randomly selected fields/well. Then the ratio of each marker gene-derived signals/DAPI signals was calculated. Statistical analysis was performed using Student's *t*-test. Significance was defined as $p<0.05$. Data are expressed as means ± SEM.

3. Results

3.1. Effects of mood stabilizers on ADP proliferation

Adult neurogenesis in DG is decreased in rodent models for stress-related disorders (Malberg and Duman, 2003; Jayatissa et al., 2006; Silva et al., 2008). Although it remains unclear how adult neurogenesis in DG is decreased in these models, reports of some studies have suggested that glucocorticoids are involved in them (Cameron and McKay, 1999; Kim et al., 2004a). We have already shown that Li has no effect on ADP proliferation but recovers ADP proliferation decreased by dexamethasone (DEX), a specific agonist of glucocorticoid receptor (Boku et al., 2009). Following our previous study, we used Alamar Blue assay to examine the effects of Li, VPA, CBZ and LTG on ADP proliferation in the absence or presence of 5 μM DEX. Results showed that ADP proliferation decreased significantly with 5 μM DEX. Furthermore, 0.3–3 mM Li and 30–1000 μM VPA showed no effect on ADP proliferation in the absence of DEX but 1–3 mM Li and 100–300 μM VPA recovered ADP proliferation decreased by 5 μM DEX in a dose-dependent manner (Fig. 1A and B). However, 3–30 μM CBZ and 30–300 nM LTG had no effect on ADP proliferation, either in the absence or presence of 5 μM DEX (Fig. 1C and D). The ADP

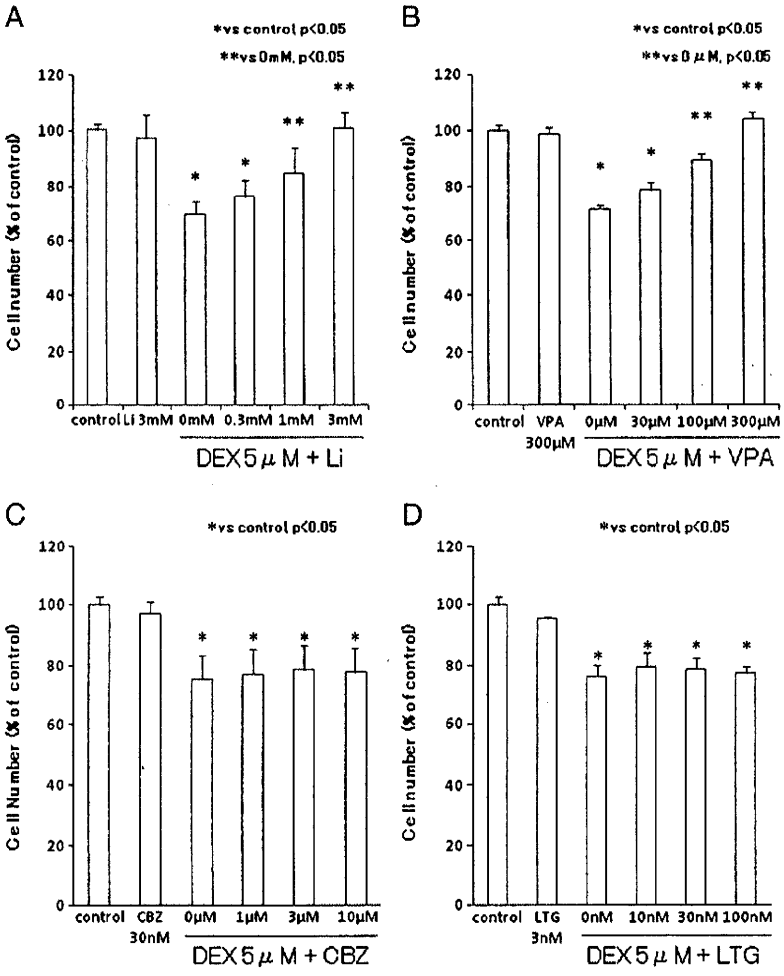

Fig. 1. Effects of mood stabilizers on ADP proliferation Li (A) and VPA (B) recovered ADP proliferation decreased by DEX, but not CBZ (C) and LTG (D). Alamar Blue assay was performed 3 days after drug treatments for four independent cultures. Data are shown as means ± SEM. *$p<0.05$, compared with control; **$p<0.05$, compared with 0 mM (A: Li), 0 μM (B: VPA).

proliferation was decreased remarkably more than 30 μM CBZ and 300 nM LTG (data not shown).

3.2. Effects of mood stabilizers on ADP apoptosis

Staurosporine (STS) is a common inhibitor of Protein Kinase C that is often used to induce apoptosis on culture cells (Sanchez et al., 1992). Apoptosis has two pathways: the internal pathway via mitochondria and external pathways via death receptors (Adams, 2003); STS is well known as an inducer of internal pathway (Ferrari et al., 1998). All four of these stabilizers increase the expression of Bcl-2, a key regulator of the internal pathway (Chen et al., 1999b; Chang et al., 2008). In addition, the internal pathway, but not the external pathway, is involved in the apoptosis of neural progenitor cells (Ekdahl et al., 2003; Ceccatelli et al., 2004). Our preliminary data showed that Tumor Necrosis Factor-α, a ligand of death receptors and an inducer of external pathway, did not induce apoptosis on ADP (data not shown). Therefore, we specifically examined the internal pathway in the present study. First, the effect of STS on ADP apoptosis was examined using TUNEL assay at 0, 100, 300 nM, and 1 μM. Only a few TUNEL signals were found at 0 and 100 nM STS. Most of the cells were removed from the bottom of the eight-well chamber at 1 μM STS (data not shown). However, around 70% of cells were TUNEL-positive with 300 nM STS (Fig. 2). Therefore, we investigated the effects of Li, VPA, CBZ, and LTG on ADP apoptosis induced by 300 nM STS. Next, the effects of Li (1, 3 and 10 mM), VPA (100, 300 μM and 1 mM), CBZ (1, 3, 10 and 30 μM), and LTG (10, 30, 100 and 300 nM) on ADP apoptosis induced by 300 nM STS were examined using TUNEL assay. All of 1 mM Li, 1 mM VPA, 3 μM CBZ, and 30 nM LTG decreased the ratio of TUNEL-positive cells to around 30% (Fig. 2A–D). Nevertheless, none of these four mood stabilizers had any effect on the ratio of TUNEL-positive cells or peeled off many cells from the bottom of the eight-well chamber at other doses (data not shown). We also examined the effect of 5 μM phenytoin (PHT), an antiepileptic drug that is not used as a mood stabilizer, on ADP apoptosis induced by 300 nM STS. It is noteworthy that 5 μM PHT had no effect on the ratio of TUNEL-positive ADPs (Fig. 2E).

3.3. Effects of mood stabilizers on ADP differentiation

Retinoic acid (RA) is widely used as a potent inducer of neural differentiation by multipotent cells of various types, such as neural stem cells, embryonal carcinoma cells, and embryonic stem cells *in vitro* (Takahashi et al., 1999; Soprano et al., 2007). Recent findings have shown that endogenous RA is involved in neural differentiation in adult hippocampus *in vivo* (Jacobs et al., 2006; McCaffery et al., 2006). We have already shown that RA induces ADP to both neuron and astrocyte (Boku et al., 2009). Moreover, ADP did not differentiate at all by only the depletion of bFGF, and all of Li, VPA, CBZ, and LTG had no effect on ADP differentiation without RA (data not shown).

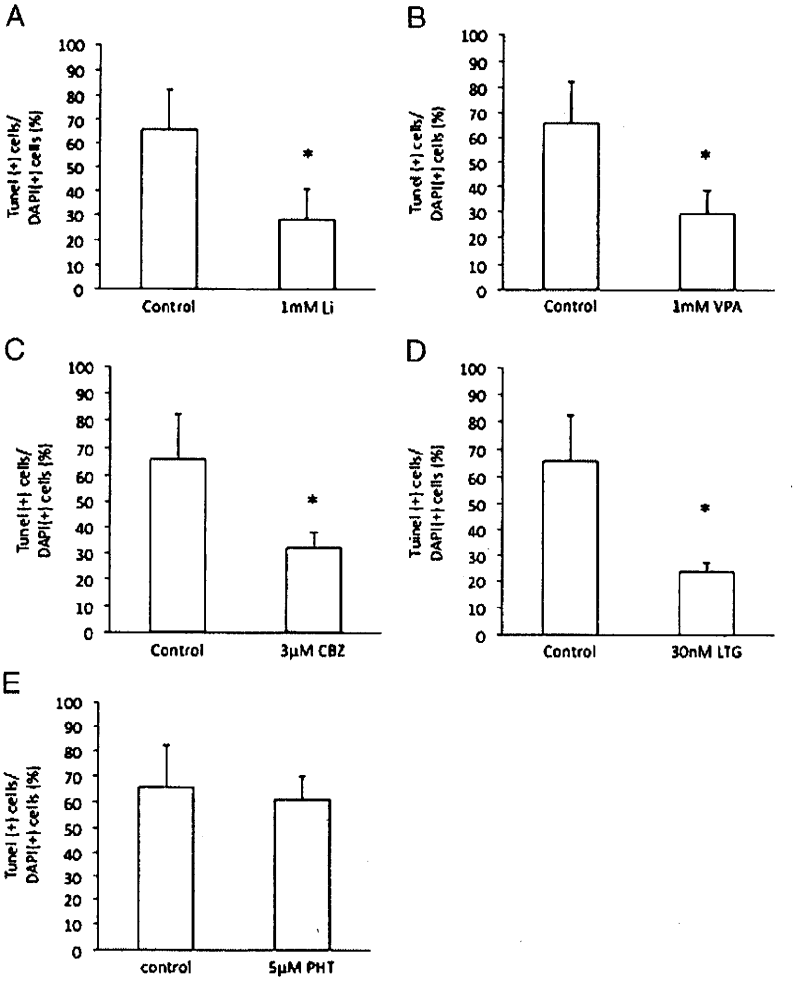

Fig. 2. Effects of mood stabilizers on ADP apoptosis. All four mood stabilizers decreased ADP apoptosis induced by staurosporine (STS) (A–D). Phenytoin (PHT), an anticonvulsant drug, did not affect staurosporine-induced apoptosis (E). TUNEL assay was performed 2 days after drug treatments for four independent cultures. The percentage of TUNEL (+)/DAPI (+) ADPs with STS is expressed as control. Data are shown as means ± SEM. $^*p<0.05$, compared with control.

Therefore, we examined the effects of Li (1, 3 and 10 mM), VPA (100, 300 μM and 1 mM), CBZ (1, 3, 10 and 30 μM), and LTG (10, 30, 100 and 300 nM) on ADP differentiation induced by RA. Results show that 1 μM RA differentiated around 50% of ADP into a neuron-like cell (Tuj1-positive cell) and around 12% of ADP into an astrocyte-like cell, which is GFAP-positive and which has larger size and a more spread shaped than that of ADP (Fig. 3A–C). Both 1 mM Li and 3 μM CBZ increased the ratio of neuron-like cells to around 70% and decreased the ratio of into astrocyte-like cells to around 5% (Fig. 3B and C). Both 1 mM VPA and 30 nM LTG decreased the ratio of neuron-like cells to around 10% and increased the ratio of astrocyte-like cell to around 25% (Fig. 3B and C). None of Li, VPA, CBZ, or LTG had any effect on the number of ADP in these doses (data not shown). None of these four mood stabilizers showed any effect on the ratio of Tuj1 or GFAP-positive cells or peeled off many cells from the bottom of the eight-well chamber at other doses (data not shown).

4. Discussion

Results show that four commonly used mood stabilizers—Li, VPA, CBZ, and LTG—have varying effects on ADPs (Table 1). Only four comparative studies have examined cells of other types, such as tumor-derived cell lines or primary neuron cultures. In addition, each study specifically addressed only a single phenomenon: neuronal differentiation or apoptosis (Li et al., 2002; Mai et al., 2002; Williams et al., 2002; Daniel et al., 2005). Therefore, the present report describes is the first comparative study of the effects of four mood stabilizers in adult DG-derived neural precursor cells on three phenomena which constitute neurogenesis: proliferation, apoptosis and differentiation.

Our present results differ from past studies in many points. In the case of proliferation, some reports have described that Li and VPA increased the proliferation of neural precursor cells (Kim et al., 2004b; Laeng et al., 2004; Wexler et al., 2008). In the case of differentiation, some reports have described that Li and VPA, but not CBZ, promote the differentiation of neural precursor cells into neurons (Hao et al., 2004; Hsieh et al., 2004; Kim et al., 2004b; Laeng et al., 2004). In these studies, neural precursor cells are derived from entire adult hippocampi, partly including the subventricular zone, and embryos. In contrast, our ADP is derived from dissected DG from adult hippocampi. Moreover, the proliferation potency of neural stem cells decreases according to age (Molofsky et al., 2006). Although neural stem cells in the early developmental stage tend to be differentiated into neurons by LIF, those in the late developmental stage tend to be differentiated into astrocytes (Takizawa et al., 2001). Moreover, in contrast to ADP-derived dissected DG, adult

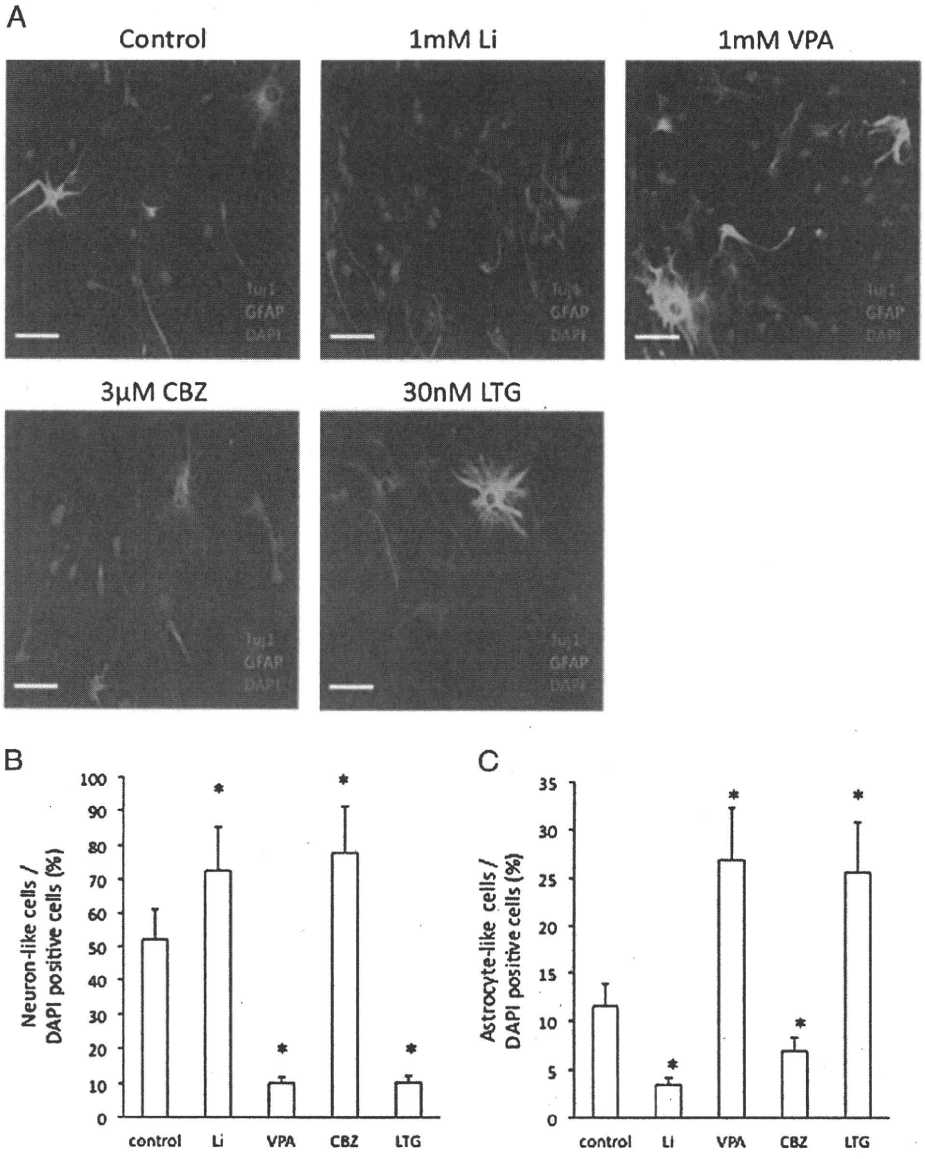

Fig. 3. Effects of mood stabilizers on ADP differentiation Li and CBZ increased the ratio of neuron-like cell induced by retinoic acid (RA). However, VPA and LTG decreased it and increased the ratio of astrocyte-like cell. Immunocytochemistry was performed 7 days after drug treatment for four independent cultures. A: Scale bar = 120 μm. B, C: Concentrations of mood stabilizers are as follows: Li, 1 mM; VPA, 1 mM; CBZ, 3 μM; LTG, 30 nM. Data are shown as means ± SEM. *$p<0.05$, compared with control.

hippocampal neural progenitors in past studies are derived from entire adult hippocampi. The culture condition of ADP also differs from that of adult hippocampal progenitors. Therefore, the reactivity to drugs of ADP might differ from those of embryonic neural stem cells and adult hippocampal neural progenitors. To confirm that point, some comparison is needed of the effects of mood stabilizers on proliferation, apoptosis and differentiation between ADP and neural precursor cells derived from other sources.

That GSK-3β and β-catenin/TCF pathways regulate cell proliferation is well known (Takahashi-Yanaga and Sasaguri, 2009). Regarding the effect of Li on ADP proliferation decreased by DEX, we have already shown part of its mechanism: DEX decreases ADP proliferation through activation of GSK-3β and following inhibition of β-catenin/TCF pathway; and Li reverses the inhibitory effect of DEX on ADP proliferation through inhibiting activated GSK-3β and following activation of β-catenin/TCF pathway (Boku et al., 2009). In the present

Table 1
The summary of the effects of mood stabilizers on ADPs.

	Lithium	Valproate	Carbamazepine	Lamotrigine
DEX-decreased proliferation	Recover	Recover	No effect	No effect
STS-induced apoptosis	Recover	Recover	Recover	Recover
RA-induced differentiation	Neuron↑ astrocyte↓	Neuron↓ astocyte↑	Neuron↑ astrocyte↓	Neuron↓ astrocyte↑

study, we showed that VPA recovered ADP proliferation decreased by DEX as in the case of Li. We also showed that the recovery effect of VPA on ADP proliferation decreased by DEX is reversed by quercetin, an inhibitor of β-catenin/TCF pathway, as in the case of Li (our unpublished data). In addition, some reports have described that VPA promotes β-catenin/TCF pathway through inhibition of GSK-3β (Chen et al., 1999a; Kim et al., 2005). These findings suggest that VPA also regulates ADP proliferation through GSK-3β and the β-catenin/TCF pathway. However, the other reports have described that the activity of GSK-3β is not inhibited by VPA (Williams et al., 2002; Kozlovsky et al., 2003; Ryves et al., 2005). Additionally, it has been shown that VPA actions are partly mediated by histone deacetylase (HDAC) inhibition (Phiel et al., 2001). Further investigation is necessary to elucidate how VPA recovers ADP proliferation decreased by DEX.

Our results of the effects of mood stabilizers on ADP differentiation induced by RA have shown that Li and CBZ increase neuronal differentiation and decrease astroglial differentiation and that VPA and LTG are vice versa. The opposite effects of Li/CBZ and VPA/LTG on ADP differentiation are so interesting that the investigation of the mechanism underlying these opposing effects might be expected to engender new aspects of the action mechanism of mood stabilizers. RA and brain-derived neurotrophic factor (BDNF) synergistically promote neuronal differentiation of neural precursor cells (Takahashi et al., 1999). In addition, RA and leukemia inhibitory factor (LIF) synergistically promotes astroglial differentiation of neural precursor cells (Asano et al., 2009). Therefore, Li/CBZ might affect BDNF pathway and VPA/LTG might affect the LIF pathway. This speculation is apparently interesting but has some problems. For example, the pathways cannot be merely separated from each other because some reports have described the existence of cross talk between BDNF and LIF pathways (Rajan et al., 1998; Lund et al., 2008; Yasuda et al., 2009). Although it has been shown that Li induces BDNF expression (Fukumoto et al., 2001), the effect of CBZ on BDNF pathway and the effects of VPA and LTG on LIF pathway remain poorly understood. However, our speculation might be worth further consideration. To confirm results of the effects of mood stabilizers on ADP differentiation induced by RA and to validate our speculation of the mechanism underlying them, further investigation using other physiological inducers of neural and glial differentiation, such as BDNF and LIF, is needed.

Although the effects of mood stabilizers on ADP proliferation decreased by DEX and ADP differentiation induced by RA differ among them, all of these four mood stabilizers decreased ADP apoptosis induced by STS (Table 1). In addition, phenytoin, an anticonvulsant but not mood stabilizer, had no effect on it. Therefore, their anti-apoptotic effects on neural precursor cell might be involved in a part of common mood-stabilizing effects. Results of some studies have suggested that internal pathway-related factors (e.g., GSK-3β, Bcl-2 and HSP70) might be candidates of the common anti-apoptotic effects of mood stabilizers (Chen et al., 1999b; Li et al., 2002; Jope and Bijur, 2002; Ren et al., 2003; Pan et al., 2005). Furthermore, Williams et al. showed that prolyl oligopeptidase is a candidate factor of the common effects of mood stabilizers on the collapse of sensory neuron growth cones (Williams et al., 2002); prolyl oligopeptidase is reportedly involved in apoptosis (Odaka et al., 2002). Therefore, we are going to investigate the mood-stabilizing effects of these molecules in ADP, which might engender elucidating the molecular mechanism of mood-stabilizing effects. However, staurosporine is not a physiological inducer of apoptosis. Therefore, further investigation using more physiological inducers of apoptosis, such as mitogen or insulin withdrawal and glucose deprivation, is necessary to confirm our results.

The differentiation stages of proliferative neural precursor cells have been proposed for the adult rodent hippocampus *in vivo* (Kempermann, 2006; Gage et al., 2008): the first stage (Type-1 cell), the second stage (Type-2a cell), and the third stage (Type-2b cell). Type-1 cell corresponds to stem cells. Type-2a cell and Type-2b cell correspond to amplifying progenitor cells. In these developmental stages, our ADP might correspond to Type-2a cell (Boku et al., 2009). Fluoxetine, an antidepressant, increases Type-2a-like cells in adult DG (Encinas et al., 2006); electroconvulsive seizure mainly increases Type-1-like cells in adult DG (Segi-Nishida et al., 2008). Our present study has elucidated the effects of mood stabilizers on Type-2a cell, but not on Type-1 cells and Type-2b cells. Therefore, it might be important to investigate the effects of specific drugs on precursor cells of other types.

Finally, results show that the four common mood stabilizers—Li, VPA, CBZ, and LTG—exert various effects on the actions of ADP, Type-2a like neural precursor cell in adult DG. However, *in vivo* functional significance of these results and mechanisms underlying them remain unclear. To investigate them might be beneficial to further the understanding of action mechanisms of mood stabilizers and the pathophysiology of mood disorders, which might engender the development of new therapeutic targets of mood disorder.

Acknowledgments

This work was supported in part by a Grant-in-aid No. 18591269 for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by a Grant from Mitsubishi Pharma Research Foundation, Japan. We thank Kihara M for expert technical assistance and Suzuki K, Tanaka T, and Masui T for their helpful critique.

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