

JPET #245506

Title page

**The phosphodiesterase 2A inhibitor TAK-915 ameliorates
cognitive impairments and social withdrawal in
N-methyl-D-aspartate receptor antagonist-induced rat models
of schizophrenia**

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JPET #245506

Running title page

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In vivo profile of a novel PDE2A inhibitor, TAK-915

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Abbreviations:

AMPA, (\pm)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CSF,

cerebrospinal fluid; Enk, enkephalin; GAPDH, glyceraldehydes-3-phosphate

JPET #245506

dehydrogenase; METH, methamphetamine hydrochloride; MK-801, (+)-MK-801
hydrogen maleate; MP-10,
2-[4-(1-methyl-4-pyridin-4-yl-1*H*-pyrazol-3-yl)-phenoxy-methyl]-quinoline succinate;
MSNs, medium spiny neurons; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; PCP,
phencyclidine; PDE, phosphodiesterase; PDE2A, phosphodiesterase 2A; pGluR1,
phosphorylation of AMPA receptor subunit at serine 845; PKA, protein kinase A; PKG,
protein kinase G; SD, Sprague-Dawley; SNP, sodium nitroprusside; SP, substance P;
TAK-915,
N-{(1*S*)-1-[3-fluoro-4-(trifluoromethoxy)phenyl]-2-methoxyethyl-7-methoxy-2-oxo-2,3
-dihydropyrido[2,3-*b*]pyrazine-4(1*H*)-carboxamide.

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JPET #245506

Abstract

The pathophysiology of schizophrenia has been associated with glutamatergic dysfunction. Modulation of the glutamatergic signaling pathway, including *N*-methyl-D-aspartate (NMDA) receptors, can provide a new therapeutic target for schizophrenia. Phosphodiesterase 2A (PDE2A) is highly expressed in the forebrain, and is a dual substrate enzyme that hydrolyzes both cAMP and cGMP, which play pivotal roles as intracellular second messengers downstream of NMDA receptors. Here we characterize the *in vivo* pharmacological profile of a selective and brain penetrant PDE2A inhibitor, (*N*-{(1*S*)-1-[3-fluoro-4-(trifluoromethoxy)phenyl]-2-methoxyethyl}-7-methoxy-2-oxo-2,3-dihydropyrido[2,3-*b*]pyrazine-4(*1H*)-carboxamide) (TAK-915) as a novel treatment for schizophrenia. Oral administration of TAK-915 at 3 and 10 mg/kg significantly increased cGMP levels in the frontal cortex, hippocampus, and striatum of rats. TAK-915 at 10 mg/kg significantly upregulated the phosphorylation of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor subunit GluR1 in the rat hippocampus. TAK-915 at 3 and 10 mg/kg significantly attenuated episodic memory deficits induced by the NMDA receptor antagonist MK-801 in the rat passive avoidance test. TAK-915 at 10 mg/kg significantly attenuated working memory deficits induced by MK-801 in the rat radial arm maze test. Additionally, TAK-915 at 10 mg/kg prevented subchronic phencyclidine-induced social withdrawal in social interaction in rats. In contrast, TAK-915

JPET #245506

did not produce antipsychotic-like activity; TAK-915 had little effect on MK-801- or methamphetamine-induced hyperlocomotion in rats. These results suggest that TAK-915 has a potential to ameliorate cognitive impairments and social withdrawal in schizophrenia.

JPET #245506

Introduction

Schizophrenia is a severe psychiatric disorder characterized by three domains: positive symptoms, negative symptoms, and cognitive impairments. Current antipsychotic medications are effective in managing positive symptoms, but are limited in their ability to alleviate cognitive impairments and negative symptoms. Cognitive impairments are present at the onset of illness and persistent through the course of the disease, and appear to be strong predictors of functional outcome (Shamsi et al., 2011; Torgalsboen et al., 2015). Negative symptoms range from diminished expression such as blunted affect and poverty of speech, to motivational deficits characterized by avolition, anhedonia, and social withdrawal (Messinger et al., 2011; Foussias et al., 2014). Taken together, cognitive impairments and negative symptoms lead to a reduced quality of life and increased functional disability. Therefore, there remains an unmet clinical need and new therapeutic drugs are required for patients with cognitive impairments and negative symptoms in schizophrenia.

The pathophysiology of schizophrenia has been associated with dysfunction of glutamatergic neurotransmission (Goff and Coyle, 2001; Lin et al., 2012). Postmortem brain studies have shown changes in pre- and postsynaptic markers for glutamatergic neurons in patients with schizophrenia (Meador-Woodruff and Healy, 2000). Inhibition of glutamatergic transmission by *N*-methyl-D-aspartate (NMDA) receptor antagonists such as ketamine and phencyclidine

JPET #245506

(PCP) causes schizophrenia-like symptoms in humans (Javitt and Zukin, 1991; Olney and Farber, 1995; Coyle, 1996). In line with these clinical observations, reduction of nitric oxide (NO) and cGMP levels acting downstream of NMDA receptor has been observed in schizophrenia (Lee and Kim, 2008; Nakano et al., 2010). Interestingly, administration of the NO donor sodium nitroprusside (SNP) has shown a rapid improvement of multiple symptoms in schizophrenia (Hallak et al., 2013). These findings suggest that the modulation of glutamatergic transmission, which includes NMDA receptor/NO/cGMP pathway, can provide a new therapy for schizophrenia.

Phosphodiesterase 2A (PDE2A) is a dual substrate enzyme that hydrolyzes both cAMP and cGMP. PDE2A is abundant in the brain relative to peripheral tissues, and highly expressed in the forebrain, including the frontal cortex, hippocampus, and striatum, which are relevant to cognition (Stephenson et al., 2009; Stephenson et al., 2012). PDE2A is localized in axons and nerve terminals of principal neurons, suggesting that PDE2A plays an important role in the modulation of cyclic nucleotide-mediated signal transduction, synaptic neurotransmission, and plasticity in the forebrain. Thus, the role of PDE2A on cognition under physiological and pathological conditions has been investigated. Some PDE2A inhibitors, such as BAY 60-7550, have enhanced recognition memory in the novel object recognition task in rodents (Boess et al., 2004; Rutten et al., 2007; Reneerkens et al., 2013; Bollen et al., 2014; Redrobe et al.,

JPET #245506

2014; Bollen et al., 2015; Lueptow et al., 2016). However, these findings have not fully shown the relationship between brain PDE2A inhibition and pro-cognitive activities in rodents. Most of the studies using PDE2A inhibitors lack adequate evidence in terms of pharmacokinetics and pharmacodynamics. For example, there are no reports that investigate brain cyclic nucleotide levels after BAY-60-7550 administration. This compound does not show sufficient brain penetrant properties in rodents after oral administration (Reneerkens et al., 2013). Therefore, a PDE2A inhibitor with better pharmacokinetic profiles would be required to elucidate the role of PDE2A inhibition in the brain and clarify the relationship between brain cyclic nucleotide levels and behavioral outcomes.

We recently discovered a potent and selective PDE2A inhibitor TAK-915 (Mikami et al., 2017). TAK-915 inhibited human PDE2A3 enzyme activities with a 50% inhibitory concentration of 0.61 nmol/L, which exhibited more than 4100-fold selectivity against other phosphodiesterase (PDE) family members. In *in vitro* autoradiography studies in rodents, [³H]TAK-915 was accumulated in the frontal cortex, hippocampus, and striatum in rodents (Ito et al., manuscript in preparation), where PDE2A expression levels are high (Stephenson et al., 2009; Stephenson et al., 2012). This selective accumulation of [³H]TAK-915 was not observed in brain slices from *Pde2a* conditional knockout mice (Ito et al., manuscript in preparation). These findings indicate that TAK-915 selectively binds to a native PDE2A

JPET #245506

under physiological conditions.

In this report, we show *in vivo* pharmacological profile of a selective and brain penetrant PDE2A inhibitor TAK-915, as a potential treatment for schizophrenia. Firstly, to examine whether TAK-915 acts as a PDE2A inhibitor *in vivo*, we measured cyclic nucleotide contents and their downstream signaling in the brain. Secondly, we characterized the pro-cognitive properties of TAK-915 in NMDA receptor antagonist-induced deficit models. Thirdly, to investigate the potential of TAK-915 on social withdrawal, we evaluated social interaction in a subchronic PCP model. Finally, we performed psychostimulant-induced hyperlocomotion tests to predict the effect of TAK-915 on antipsychotic-like activity.

Materials and Methods

Animals. The care and use of the animals and the experimental procedures performed at Takeda Pharmaceutical Company Limited (Fujisawa, Japan) were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited. Experiments performed at Biotrial (Rennes, France) were approved by the Biotrial Ethical Committee. Specific details of strain and species are given within each section. The animals

JPET #245506

were housed in groups of 2-4 per cage under a 12-h light-dark cycle (lights on at 7:00 AM) with *ad libitum* food and water. After at least a 1-week habituation period, the animals were used for the experiment.

Drugs.

TAK-915

(*N*-{(1*S*)-1-[3-fluoro-4-(trifluoromethoxy)phenyl]-2-methoxyethyl}-7-methoxy-2-oxo-2,3-dihydropyrido[2,3-*b*]pyrazine-4(1*H*)-carboxamide) and MP-10 succinate (MP-10, 2-[4-(1-methyl-4-pyridin-4-yl)-1*H*-pyrazol-3-yl]-phenoxy-methyl-quinoline succinate) were synthesized at Takeda Pharmaceutical Company Limited (Grauer et al., 2009; Verhoest et al., 2009; Mikami et al., 2017). TAK-915 and MP-10 were suspended in 0.5% (w/v) methylcellulose in distilled water and administered orally (p.o.). Methamphetamine hydrochloride (METH, Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) and (+)-MK-801 hydrogen maleate (MK-801, Sigma Aldrich, Inc., St. Louis, MO) were dissolved in saline, and were administered subcutaneously (s.c.). Phencyclidine hydrochloride (PCP, Sigma Chemical Co., Saint Quentin Fallavier, France) was dissolved in saline, and was administered intraperitoneally (i.p.). The dosages of compounds were expressed as salt forms. The volume of administration was 2 mL/kg for p.o. and s.c., 5 mL/kg for i.p.

Pharmacokinetics Study. Eight-week-old male Long-Evans rats (Japan SLC Inc.,

JPET #245506

Hamamatsu, Japan) and male Sprague-Dawley (SD) rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used for sample collection. Blood and brain tissues were collected at 0.5, 1, or 2 h after administration of TAK-915. The plasma or brain homogenate samples were deproteinized with acetonitrile containing an internal standard and then centrifuged. The supernatant was diluted with solvents consisting of 10 mM ammonium acetate-acetonitrile-formic acid and centrifuged again. An LC-MS/MS system (API5000 or QTRAP5500, AB Sciex, Foster City, CA) was used to measure TAK-915 concentrations in the supernatant.

***In Vivo* Measurement of Cyclic Nucleotide Contents.** This assay was performed as previously described (Suzuki et al., 2015; Suzuki et al., 2016) with some modifications. Nine-week-old male Long-Evans rats were euthanized using a microwave irradiation system MMW-05 (Muromachi Kikai Co. Ltd., Tokyo, Japan) 2 h following oral administration of vehicle or TAK-915 (1, 3, or 10 mg/kg). Brain tissues were sampled, immediately frozen on dry ice, and stored at -80°C until use. To measure cyclic nucleotide contents, microwaved brain tissues were isolated and then homogenized in 0.5 N HCl followed by centrifugation. Cyclic nucleotide concentrations in supernatant were measured using enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI) in accordance with the manufacturer's protocol. Values were expressed as pmol per mg tissue weight.

JPET #245506

In Vivo Measurement of Protein Phosphorylation. Seven-week-old male Long-Evans rats were used for collecting the brain tissues. The hippocampus was immediately sampled 2 h after oral administration of vehicle or TAK-915 (1, 3, or 10 mg/kg) and put into 1.5-mL tubes. Samples were rapidly frozen in liquid nitrogen, and stored at -80°C until use. Whole hippocampus tissues were homogenized in ice-cold cell extraction buffer (Invitrogen, Carlsbad, CA) with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). After clarification by centrifugation, the supernatant fraction was collected and boiled in sample buffer solution with 3-mercapto-1,2-propanediol (Wako, Osaka, Japan). The protein content (0.5 µg) of each sample was loaded onto 7.5 to 15% SDS-PAGE gels (DRC, Tokyo, Japan). After electrophoresis, the proteins were transferred to PVDF membranes. Total protein levels and phosphorylation of (\pm)- α -amino-3-hydroxy-5-methylisoxasole-4-propionic acid (AMPA) receptor subunit at serine 845 (pGluR1) proteins were probed by immunoblotting with anti-total glutamate receptor subunit 1 (GluR1) antibody (diluted 1:5000, Millipore, Temecula, CA) and anti-pGluR1 (diluted 1:2000, Phosphosolutions, Aurora, CO), and were visualized with horseradish peroxidase-conjugated second antibodies (diluted 1:5000, GE Healthcare UK Ltd., Buckinghamshire, UK) followed by ECL prime western blotting detection reagents (GE Healthcare UK Ltd.). The membranes were scanned on a lumino-image analyzer,

JPET #245506

ImageQuant LAS4000 (Fujifilm, Tokyo, Japan). The amounts of total and pGluR1 proteins were quantified by measuring the density of blots using ImageQuant TL software (Fujifilm, Tokyo, Japan). The band densities of pGluR1 were normalized by those of total GluR1.

Step-Through Passive Avoidance Task. This task was conducted using 7-8-week-old male Long-Evans rats as previously described (Mikami et al., 2017). The task was carried out in an apparatus consisting of an illuminated ("light") compartment ($25 \times 10 \times 25$ cm) connected to a non-illuminated ("dark") compartment ($30 \times 30 \times 30$ cm) by a guillotine door (8×8 cm) (Brain Science idea, Osaka, Japan). In a habituation trial, each animal was gently placed in the light compartment. After 30 s, the guillotine door was opened and the rat was allowed to enter the dark compartment. Once the animal entered the dark compartment with all four paws, the door was closed. The animal was allowed to remain in the dark compartment for 30s before being taken to its home cage. An acquisition trial was conducted 4-6 h after the habituation trial. The rat was put into the light compartment, and the guillotine door was opened. Once the rat crossed into the dark compartment with all four paws, the door was closed and an electric shock (0.5 mA, 3 s) was delivered from the grid floor. After 30 s, the rat was removed from the dark compartment, and then returned to its home cage. One day after the acquisition trial, a retention test was performed to evaluate memory. After each rat was again put into the light compartment for 30 s, the door was opened. The retention test

JPET #245506

was terminated when the rat entered the dark compartment or remained in the light compartment for 300 s. During the retention trials, no electric shock was delivered from the grid floor. The time between the door being opened and the rat entering the dark compartment was defined as latency time. The maximum latency time for which the rat did not enter the dark compartment was 300 s. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) was administered 2 h prior, and saline or MK-801 (0.1 mg/kg, s.c.) was administered 30 min prior to the acquisition trial.

Radial Arm Maze Task. This task was assessed using 9-week-old male Long-Evans rats as previously described (Shiraishi et al., 2015; Nishiyama et al., 2017). An 8-arm radial maze with arms (50 x 10 x 40 cm) was mounted on platform, which was elevated 50 cm above the floor. Animals were fasted for 1 day before habituation of the maze. During the experimental period after the first day of habituation, animals were food-restricted to 85-90 % of free-feeding body weight for training. Reinforcement consisted of 3 food pellets (Dustless Precision Pellets, 45 mg, Bioserv Inc., Frenchtown, NJ), which were placed in a food cup at the end of each arm. Habituation: On the first day, three rats were placed in the maze and allowed to freely explore and retrieve the food pellets, which were placed near the entrance and at the mid-point of each arm for 8 min. On the second day, a single rat was allowed to explore and retrieve the pellets, which were then placed at the mid-point and in the food cup

JPET #245506

at the end of each arm for 5 min. Training: From the third day, reinforcement was placed in the food cup at the end of each arm. Each rat was allowed to explore until 5 min had elapsed, or the rat completed one entry in each arm. Entry into an arm previously chosen, and failure to get the pellets were counted as errors. Rats were trained until they achieved a criterion of ≤ 2 errors for 2 consecutive days. Vehicle or TAK-915 (1 or 10 mg/kg, p.o.) was administered 2 h, and saline or MK-801 (0.08 mg/kg, s.c.) was administered 30 min prior to the test.

Social Interaction Test. This experiment was carried out at Biotrial (Rennes, France) using male Long-Evans rats (JANVIER, Saint Berthevin, France) as previously described (Cayre et al., 2016). The experimental arena was a square wooden box (90 × 90 × 40 cm) painted dark blue, with black painted squares (15 × 15 cm). The arena was cleaned using water between each trial to avoid odor trails left by rats. The arena was placed in a dark room illuminated only by halogen lamps oriented towards the ceiling, which provided uniform dim light in the box. The day before the test, rats were placed in the box and allowed to habituate for 10 min. On experimental day, treated animals were placed with an unfamiliar animal in the experimental arena for a 10-min experimental session to allow them to interact freely. The experimenter then scored the time spent in social interaction for the treated rat. For each treated-rat, the total amount of time spent in active social behavior was recorded during the 10-min session. Active social behaviors were defined as sniffing, grooming, kicking,

JPET #245506

following, mounting, jumping on, boxing, wrestling, and crawling. The experimenter scoring the behavior was not aware of the animal treatment. Saline or PCP (5 mg/kg, i.p.) was administered twice daily (morning and afternoon) from day 1 to day 7. During the wash-out period from day 8 to day 15, animals were housed in their home cages without any treatment. On day 15, habituation was conducted. On day 16, vehicle or TAK-915 (3 or 10 mg/kg, p.o.) was administered 2 h before testing.

Hyperlocomotion Test. This test was assessed using 8-week-old male SD rats as previously described (Suzuki et al., 2015; Suzuki et al., 2016). A SUPERMEX spontaneous motor analyzer (Muromachi Kikai Co., Ltd., Tokyo, Japan) was used to measure locomotion. Rats were placed in locomotor chambers (24 × 37 × 30 cm) for more than 60 min for habituation. Thereafter, rats were injected with either vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) and then quickly returned to the chamber. After 2 h, rats were again taken out of the chambers and injected with either saline, MK-801 (0.3 mg/kg, s.c.), or METH (0.5 mg/kg, s.c.) and then quickly returned to the chamber. Activity counts were recorded every 1 min during 2 h after administration of psychostimulant.

Gene Expression Assay. This assay was conducted as previously described (Suzuki et al., 2015; Suzuki et al., 2016). Seven-week-old male SD rats were euthanized 3 h after oral

JPET #245506

treatment of vehicle, TAK-915 (10 or 100 mg/kg), or MP-10 (30 mg/kg). Striatum was collected and frozen on dry ice immediately and then stored -80°C until use. Total RNA from the striatum was extracted using Isogen (Nippon Gene Co., Ltd., Toyama, Japan) and an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was reverse-transcribed to cDNA using High-capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time quantitative polymerase chain reaction expression analysis was conducted using TaqMan reagents (Eurogentec, Seraing, Belgium) and ABI PRISM 7900HT sequence detection system (Life Technologies). In accordance with the manufacturer's instruction, quantities of RNA were normalized using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) TaqMan probes. The rat enkephalin (Enk) analysis was conducted using the following primers: forward primer, 5' -GGACTGCGCTAAATGCAGCTA-3' ; reverse primer, 5' -GTGTGCATGCCAGGAAGTTG-3' ; TaqMan probe (MGB probe), 5' -CGCCTGGTACGTCCCGGCG-3' . The rat substance P (SP) was conducted using the following primers: forward primer, 5' -CGCAAAATCCAACATGAAAATC-3' ; reverse primer, 5' -GCAAACAGTTGAGTGGAAACGA-3' ; TaqMan probe (MGB probe), 5' -CGTGGCGGTGGCGGTCTTTT-3' . The rat GAPDH analysis was conducted using the following primers: forward primer, 5' -TGCCAAGTATGATGACATCAAGAAG-3' ; reverse primer, 5' -AGCCCAGGATGCCCTTTAGT-3' ; TaqMan probe (MGB probe), 5'

JPET #245506

-TGGTGAAGCAGGCGGCCGAG-3' .

Statistical Analysis. The Aspin-Welch test (for nonhomogeneous data) or Student's *t*-test (for homogeneous data) was used for pairwise group comparison. In dose-response experiments, homogeneity of variances was using Bartlett's test, and then two-tailed Williams' test (for parametric data) or two-tailed Shirley-Williams test (for non-parametric data) was conducted. In the step-through passive avoidance test, two-tailed Wilcoxon's test was conducted. Value of $P \leq 0.05$ was considered significant.

Results

Effects of TAK-915 on cAMP and cGMP Levels in the Frontal Cortex, Hippocampus, and Striatum in Rats.

Two hours after oral administration of TAK-915, the concentration of TAK-915 in the brain of Long-Evans rats was 0.030 ± 0.021 $\mu\text{g/g}$ at 1 mg/kg, 0.160 ± 0.016 $\mu\text{g/g}$ at 3 mg/kg, and 0.331 ± 0.095 $\mu\text{g/g}$ at 10 mg/kg (Table 1). The brain concentration of TAK-915 dose-dependently increased, and the exposure was sustained for at least up to 2 h after dosing in rats. To assess the effect of PDE2A inhibition by TAK-915 in the brain, we measured

JPET #245506

cyclic nucleotide contents in the frontal cortex, hippocampus, and striatum in rats at 2 h after oral administration. Oral administration of TAK-915 dose-dependently increased cGMP levels in these brain regions. Significant increase in cGMP levels was observed at 10 mg/kg of TAK-915 in the frontal cortex (0.063 ± 0.004 pmol/mg, $P \leq 0.01$; Fig. 1A), at 3 and 10 mg/kg in the hippocampus (0.037 ± 0.002 , $P \leq 0.05$, at 3 mg/kg; 0.042 ± 0.002 pmol/mg, $P \leq 0.01$, at 10 mg/kg; Fig. 1B), and at 10 mg/kg in the striatum (0.047 ± 0.003 pmol/mg, $P \leq 0.01$; Fig. 1C). On the other hand, TAK-915 did not affect cAMP levels in the rat brain even at 10 mg/kg, p.o. In addition, TAK-915 at 30 mg/kg increased cGMP levels not only in the brain parenchyma, but also in the cerebrospinal fluid (CSF), and the time-dependent changes of cGMP levels in brain and CSF were well accorded with the changes of pharmacokinetics of TAK-915 (Supplemental Fig. 1).

Effects of TAK-915 on pGluR1 Levels in the Rat Hippocampus.

An increase in intracellular cyclic nucleotide levels induces the activation of protein kinase A (PKA) and protein kinase G (PKG) and results in upregulation of pGluR1 level (Wang et al., 2005; Serulle et al., 2007; Serulle et al., 2008). To assess the effect of TAK-915 on the downstream pathway of cyclic nucleotide signaling, we investigated the phosphorylation levels of GluR1 in the hippocampus following oral administration of TAK-915 at 1, 3, and 10 mg/kg. As shown in Figure 2, TAK-915 dose-dependently increased pGluR1 in the rat

JPET #245506

hippocampus, in the same dose range of TAK-915 that increased hippocampal cGMP levels; the relative phosphorylation levels at 1, 3, and 10 mg/kg of TAK-915 were $96 \pm 14\%$, $130 \pm 17\%$, and $148 \pm 10\%$, respectively. Significant increase in the pGluR1 levels was observed at 10 mg/kg, p.o. ($P \leq 0.05$; Fig. 2B).

Effects of TAK-915 on MK-801-Induced Episodic Memory Deficits in the Step-Through Passive Avoidance Task in Rats.

Among several cognitive domains, patients with schizophrenia have shown larger impairments in episodic memory (Schaefer et al., 2013). The NMDA receptor antagonist MK-801 has produced schizophrenia-like symptoms including episodic memory deficits in rodents (Neill et al., 2010; van der Staay et al., 2011). To assess the effects of TAK-915 on the episodic memory deficits induced by the NMDA receptor antagonist, we performed the passive avoidance task in MK-801-treated rats. As shown in Figure 3, subcutaneous treatment with MK-801 at 0.1 mg/kg significantly decreased the avoidance time in retention test compared with saline ($P \leq 0.01$). TAK-915 at 3 and 10 mg/kg significantly attenuated the MK-801-induced deficits in the avoidance time ($P \leq 0.01$ at 3 mg/kg; Fig. 3B, $P \leq 0.05$ at 10 mg/kg; Fig. 3C).

Effects of TAK-915 on MK-801-Induced Spatial Working Memory Deficits in Radial

JPET #245506

Arm Maze Task in Rats.

To evaluate the effect of TAK-915 on spatial working memory deficits, which are observed in patients with schizophrenia (Piskulic et al., 2007), we performed the radial arm maze task in rats with an MK-801-induced deficit. In the control group, all pellets in the 8 arms were effectively consumed within 2 errors (Fig. 4). Treatment of MK-801 at 0.08 mg/kg, s.c. significantly increased the number of errors (5.6 ± 0.8 , $P \leq 0.01$; Fig. 4). Pretreatment with TAK-915 at 10 mg/kg, p.o. significantly reduced the number of errors induced by MK-801 (3.4 ± 0.5 , $P \leq 0.05$; Fig. 4).

Effects of TAK-915 on Subchronic Phencyclidine-Induced Social Withdrawal in the Social Interaction Test in Rats.

Rodents treated subchronically with PCP have been used for investigating social withdrawal, a key sub-domain of negative symptoms (Wilson and Koenig, 2014). To assess the potential of TAK-915 for the treatment of negative symptoms, we evaluated the effect of TAK-915 on subchronic PCP-induced social withdrawal by using social interaction test in rats. Time spent in social interactions was significantly decreased in subchronic PCP-treated rats (30 ± 10 s) compared to control group rats (83 ± 10 s) ($P \leq 0.01$, Fig. 5). TAK-915 (3 or 10 mg/kg, p.o.) dose-dependently attenuated subchronic PCP-induced deficits in social interaction test (49 ± 6 s at 3 mg/kg, 72 ± 9 s at 10 mg/kg; Fig. 5). Significant effect in this test was observed at 10

JPET #245506

mg/kg, p.o. ($P \leq 0.05$; Fig. 5).

Effects of TAK-915 on MK-801 or METH-Induced Hyperlocomotion, and on Activation of Direct and Indirect Pathway Medium Spiny Neurons in Rats.

MK-801- or METH-induced hyperlocomotion test has been commonly used as an animal model for positive symptoms in schizophrenia (Andine et al., 1999; Jones et al., 2011). Current antipsychotics such as aripiprazole, olanzapine, and haloperidol, have attenuated psychostimulant-induced hyperlocomotion (Suzuki et al., 2015). As shown in Figure 6A, TAK-915 did not affect the MK-801-induced hyperlocomotion in rats even at 10 mg/kg. Likewise, TAK-915 did not suppress the METH-induced hyperlocomotion in rats (Fig. 6B). Activation of the indirect pathway medium spiny neurons (MSNs) by the blockade of dopamine D₂ receptors is thought to be a common mechanism of action of current antipsychotics (Kapur and Mamo, 2003; Agid et al., 2008; Kehler and Nielsen, 2011; Suzuki et al., 2015). To evaluate the effect of TAK-915 on the activation of direct and indirect pathway MSNs, we measured striatal mRNA expression levels of SP (a direct pathway marker) and Enk (an indirect pathway marker). MP-10, developed by Pfizer Inc., has been reported to be a potent and selective PDE10A inhibitor (Grauer et al., 2009; Verhoest et al., 2009). As a positive control, we used MP-10 which has been reported to activate both direct and indirect pathway MSNs (Suzuki et al., 2016). As previously reported, MP-10 at 30 mg/kg

JPET #245506

significantly increased the expression of both SP and Enk mRNA ($P \leq 0.01$ for SP and $P \leq 0.01$ for Enk; Fig. 6C). In contrast, TAK-915 did not affect the expression of either SP or Enk mRNA even at 100 mg/kg (Fig. 6C). Additionally, we examined whether TAK-915 causes side effects of antipsychotic medication such as hyperprolactinemia, hyperglycemia, or cataleptic response. TAK-915 did not increase prolactin (Supplemental Fig. 2A) or glucose levels (Supplemental Fig. 2B) in the rat plasma even at 100 mg/kg. Cataleptic response was assessed by the bar test at 2 h following administration of TAK-915. TAK-915 at 10 and 100 mg/kg did not significantly increase cataleptic response in this test (Supplemental Fig. 3).

Discussion

In the present study, we showed that TAK-915 works as a brain penetrant PDE2A inhibitor *in vivo*. Significant increases in cGMP levels in hippocampus were observed at 3 mg/kg and 10 mg/kg of TAK-915 (Fig. 1B). The PDE2A occupancy levels of TAK-915 in the hippocampus at 3 mg/kg and 10 mg/kg were 46.6% and 63.0%, respectively (Ito et al., manuscript in preparation). These results suggest that more than ~45% occupancy of PDE2A by TAK-915 would be sufficient to produce a significant increase in cGMP levels in the hippocampus. In contrast, TAK-915 did not affect cAMP levels in the brain under our experimental conditions (Fig. 1). There is a possibility that we could not detect a significant effect on cAMP levels by

JPET #245506

TAK-915 because of the high baseline for cAMP contents in brain tissues (Fig. 1). However, these observations are in accordance with previous reports that PDE2A inhibition mainly influences cGMP levels rather than cAMP levels (Suvarna and O'Donnell, 2002; Boess et al., 2004). Additionally, TAK-915 dose-dependently increased cGMP levels in rat primary neurons whereas a significant change of cAMP levels was not observed (Supplemental Fig. 4). These findings suggest that PDE2A plays an important role in degrading cGMP levels in the brain although PDE2A can hydrolyze both cAMP and cGMP. Other PDE family members with higher affinities for cAMP such as PDE4 and PDE10 might be responsible for degrading cAMP in the brain.

Cyclic AMP and cGMP are differentially involved in distinct phases of memory processing such as acquisition, and consolidation (Bernabeu et al., 1996; Rutten et al., 2007; Bollen et al., 2014; Akkerman et al., 2016; Lueptow et al., 2016). Cyclic GMP-PKG signaling mediates acquisition and early consolidation, whereas cAMP-PKA signaling mediates acquisition and late consolidation. In the passive avoidance task, oral administration of TAK-915 at 2 h prior to acquisition trial attenuated MK-801-induced episodic memory deficits (Fig. 3). As TAK-915 increased cGMP levels in the rat brain at 2 h after administration (Fig. 1), the enhancement of memory acquisition mediated by cGMP could contribute to the improvement of episodic memory observed in retention trial. Additionally, it has been reported that

JPET #245506

cGMP-PKG signaling mediates early consolidation, and late consolidation requires cAMP-PKA signaling (Bollen et al., 2014). Taking into account the PK profile of TAK-915, it is possible that TAK-915 affects not only early consolidation mediated by cGMP-PKG signaling, but also late consolidation mediated by cAMP-PKA signaling. Further experiments would be required to investigate the effects of TAK-915 on cyclic nucleotides during behavior tasks and to clarify the temporal contribution of cyclic nucleotides on memory formation.

Cyclic nucleotides play important roles in regulating various signal cascades including the NMDA receptor pathway, which are involved in synaptic plasticity such as long-term potentiation (Kleppisch and Feil, 2009). The hippocampal synaptic plasticity is known to be a key element of the neurobiological bases of cognitive function (Akhondzadeh, 1999). Elevated cAMP and/or cGMP lead to activation of several sequential cascades which phosphorylate target proteins (Lucas et al., 2000; Esteban et al., 2003; Kleppisch and Feil, 2009). Phosphorylation of GluR1 at serine 845 has been linked to AMPA receptor trafficking to the plasma membrane, which is thought to influence synaptic plasticity and cognition (Derkach et al., 2007; Serulle et al., 2007; Shepherd and Huganir, 2007; Citri and Malenka, 2008). Synaptic GluR1 delivery in the hippocampus has been reported to be required for the hippocampus-dependent learning in the passive avoidance task (Mitsushima et al., 2011). We

JPET #245506

investigated the effect of TAK-915 in the hippocampal pGluR1 levels which are associated with activation of downstream pathway of cyclic nucleotide signaling. TAK-915 at 10 mg/kg significantly increased hippocampal pGluR1 in rats (Fig. 2), indicating that TAK-915 activates the downstream pathway of cyclic nucleotide signaling in the hippocampus. The increased levels of pGluR1 by TAK-915 could enhance cognitive function via modulation of synaptic plasticity.

Among several cognitive domains that are commonly disrupted in schizophrenia, deficits in episodic memory have shown some of the largest effect size (Schaefer et al., 2013). Deficits in spatial working memory also have been consistently reported in schizophrenia patients (Piskulic et al., 2007) and emphasized as one of the key impairments in schizophrenia by the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative (Marder and Fenton, 2004). To explore the potential of TAK-915 on episodic memory and spatial working memory, we investigated the effects of TAK-915 on the performance of the passive avoidance task and radial arm maze task in MK-801-treated rats. In both tasks, TAK-915 attenuated memory deficits induced by MK-801 (Fig. 3 and 4). Based on *in vitro* autoradiography studies with rat brain slices, [³H]TAK-915 accumulated to high levels in CA3 mossy fibers and subiculum, a structure located between the hippocampus proper and entorhinal cortex (Ito et al., manuscript in preparation). These brain regions play

JPET #245506

pivotal roles in spatial and episodic information processing (O'Mara et al., 2009; Cerasti and Treves, 2010). In addition, TAK-915 significantly induced c-Fos protein expression, a marker of neuronal activity, in the subiculum and entorhinal cortex (Ito et al., manuscript in preparation). These results suggest that the pro-cognitive activities of TAK-915 in the passive avoidance task and radial arm maze task might be associated with neuronal activation in these brain regions.

Social withdrawal is one of the key components of negative symptoms in schizophrenia that generally persists through the course of the illness and contributes to poor psychosocial functioning (Pogue-Geile and Harrow, 1985; Morrison and Bellack, 1987; Puig et al., 2008). To investigate the potential for the efficacy of TAK-915 in social withdrawal, we used a subchronic PCP model. PCP is known to produce schizophrenia-like symptoms in humans (Allen and Young, 1978; Morris et al., 2005) and social interaction deficits produced by subchronic treatment with PCP in rodents resemble the negative symptoms, particularly social withdrawal (Jenkins et al., 2008; Neill et al., 2014). As shown in Figure 5, subchronic PCP treatment (5 mg/kg, i.p., twice daily for 7 days) to rats significantly reduced the time spent in social interaction followed by at least 8-day washout, indicating that this treatment may cause abnormalities in neural system and/or structures associated with social behaviors. TAK-915 at 10 mg/kg significantly attenuated this reduction of time spent in social

JPET #245506

interaction (Fig. 5). These results suggest that TAK-915 prevents social withdrawal in the subchronic PCP model relevant to schizophrenia.

Current clinically used antipsychotic drugs with dopamine D₂ blockade such as haloperidol lead to an activation of the indirect pathway in the striatal MSNs, which is thought to be the mechanism of their antipsychotic effects (Kapur and Mamo, 2003; Agid et al., 2008; Kehler and Nielsen, 2011; Suzuki et al., 2015). TAK-915 did not affect MK-801- or METH-induced hyperlocomotion, even at doses that produced a significant increase in cGMP levels in the striatum (Fig. 6A and 6B), and did not activate either direct or indirect pathway MSNs in the striatum (Fig. 6C). Our findings coincide with the observation that a PDE2A inhibitor, Lu AF64280, did not produce antipsychotic-like effects in PCP-induced hyperlocomotion in mice, or in the conditioned avoidance response in rats (Redrobe et al., 2014). In contrast to PDE2A inhibitors, a PDE10A inhibitor, MP-10, which showed antipsychotic-like effects in rodents (Grauer et al., 2009), activated both direct and indirect pathway MSNs in the striatum (Fig. 6C). Behavioral outcomes of PDE2A inhibition are different from those of PDE10A inhibition although PDE2A as well as PDE10A is highly expressed in striatal MSNs, and its inhibition increases cGMP levels in the striatum. As the cyclic nucleotides and their appropriate PDEs are confined to distinct cellular compartments (Francis et al., 2011), the specific PDEs may be regulating the distinct pools of cyclic nucleotides and different roles in

JPET #245506

the striatal MSNs.

Although the mechanism by which TAK-915 attenuates cognitive impairments and social withdrawal induced by NMDA receptor antagonists is still unclear, our findings suggest that TAK-915 provides a strategy for ameliorating these behavioral deficits through upregulation of cyclic nucleotides, mainly cGMP. Previous studies suggest that PDE2A inhibition can enhance the NMDA receptor/NO/cGMP pathway (Suvarna and O'Donnell, 2002; Boess et al., 2004). TAK-915 attenuated cognitive deficits and social withdrawal in rats at similar dosage that upregulated cGMP and pGluR1 levels in the rat brain. In line with this, Lu AF64280 also increased cGMP levels in the hippocampus, and attenuated cognitive deficits in animal models of schizophrenia (Redrobe et al., 2014). These findings support the potential of PDE2A inhibitors to impact cognitive function and social behavior through modulation of the NMDA receptor/NO/cGMP pathway in the forebrain. A single infusion of SNP which augments the levels of NO, a key molecule downstream of the NMDA receptor, significantly improved multiple symptoms of schizophrenia, with effects that lasted for up to 4 weeks (Hallak et al., 2013). These findings support the hypothesis that the modulation of the cGMP signaling pathway by TAK-915 may provide beneficial clinical effects in schizophrenia.

In conclusion, TAK-915 ameliorates cognitive impairments and social withdrawal induced by

JPET #245506

NMDA receptor antagonists in rodents. This selective PDE2A inhibitor has therapeutic potential for cognitive impairments and negative symptoms in schizophrenia.

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JPET #245506

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JPET #245506

Footnotes

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JPET #245506

Figure Legends

Figure 1. Effects of TAK-915 on cAMP and cGMP levels in the frontal cortex, hippocampus and striatum in rats. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) was administered 2 h before sampling. Cyclic nucleotide contents in the frontal cortex (A), hippocampus (B), and striatum (C) were measured using enzyme immunoassay kits. Data are expressed as mean + S.E.M., $n = 9$ per each group. $^*P \leq 0.05$, $^{**}P \leq 0.01$ (versus vehicle by two-tailed Williams' test), $^{\dagger\dagger}P \leq 0.01$ (versus vehicle by two-tailed Shirley-Williams' test).

Figure 2. Effects of TAK-915 on pGluR1 levels in the rat hippocampus. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) was administered 2 h before collecting the rat hippocampus. (A) Representative blots were probed with primary antibodies for pGluR1, GAPDH, and total GluR1. (B) The intensity of the pGluR1 band for each sample was normalized to the corresponding GluR1 band density. Data are expressed as mean + S.E.M., $n = 6$ per each group. $^*P \leq 0.05$ (versus vehicle by two-tailed Williams' test).

Figure 3. Effects of TAK-915 on MK-801-induced episodic memory deficits in the step-through passive avoidance task in rats. Vehicle or TAK-915 (A: 1 mg/kg, p.o., B: 3 mg/kg, p.o., C: 10 mg/kg, p.o.) was administered 2 h prior, and saline or MK-801 (0.1 mg/kg, s.c.) was administered 30 min prior to the acquisition trial. The latency to dark compartment

JPET #245506

was measured until the rat entered the dark compartment with all four paws or remained in the illuminated compartment for 300 s. One day after acquisition trial, retention trial was conducted. Data are expressed as mean + S.E.M., $n = 19$ for control group in the experiment of 3 mg/kg, $n = 20$ for other groups. $^{**}P \leq 0.01$ (versus control by two-tailed Wilcoxon's test), $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, (versus vehicle-MK-801 by two-tailed Wilcoxon's test).

Figure 4. Effects of TAK-915 on MK-801-induced working memory deficits in the radial arm maze task in rats. Vehicle or TAK-915 (1 or 10 mg/kg, p.o.) was administered 2 h prior to testing and saline or MK-801 (0.08 mg/kg, s.c.) was administered 0.5 h prior to testing. The numbers of errors are expressed as mean + S.E.M., $n = 6$ for control group, $n = 17$ for other groups. $^{**}P \leq 0.01$ (versus control by Aspin-Welch test). $^{\dagger}P \leq 0.05$ (versus vehicle-MK-801 by two-tailed Williams' test).

Figure 5. Effects of TAK-915 on subchronic phencyclidine-induced social withdrawal in the social interaction test in rats. Saline or phencyclidine (PCP, 5 mg/kg, i.p.) was administered twice daily (b.i.d.) from day 1 to day 7. After an 8-day wash-out period, vehicle or TAK-915 (3 or 10 mg/kg, p.o.) was administered 2 h before testing. Time spent in active non-aggressive social behavior during the 10-min session of the test was recorded. Data are expressed as means + S.E.M., $n = 12$ per each group. $^{**}P \leq 0.01$ (versus control group by

JPET #245506

Student's *t*-test). [†] $P \leq 0.05$ (versus vehicle-PCP by two-tailed Williams' test).

Figure 6. Effects of TAK-915 on MK-801- or METH-induced hyperlocomotion, and on activation of direct and indirect pathway medium spiny neurons in rats. (A and B) Effects of TAK-915 on MK-801 (A) or methamphetamine (METH) (B) induced hyperlocomotion in rats. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) was administered 2 h before the administration of MK-801 (0.3 mg/kg, s.c.) or METH (0.5 mg/kg, s.c.). Activity counts during the 2 h following the administration of MK-801 or METH were calculated. Data are expressed as mean + S.E.M., *n* = 3 for control, *n* = 6 for 1 mg/kg, *n* = 7 for other groups. ^{**} $P \leq 0.01$ (versus control by Student's *t*-test). (C) Vehicle, TAK-915 (10 or 100 mg/kg, p.o.), or MP-10 (30 mg/kg, p.o.) was administered 3 h prior before sampling. The rat striatal mRNA expression levels of substance P (SP, a direct pathway marker) and enkephalin (Enk, an indirect pathway marker) were analyzed using gene expression assays. Data are expressed as mean + S.E.M., *n* = 4 for MP-10 treated group, *n* = 6 for other groups, ^{**} $P \leq 0.01$ (versus vehicle by Student's *t*-test).

JPET #245506

Table 1. Plasma and brain concentrations of TAK-915 in rats 0.5, 1 and 2 h following the administration of TAK-915 (1, 3, or 10 mg/kg, p.o.). Data are expressed as mean \pm S.D., n = 3 each group.

Rat Strain	Dose (mg/kg, p.o.)	Time (h)	Plasma concentration ($\mu\text{g/mL}$)	Brain concentration ($\mu\text{g/g}$)
Long-Evans	1	0.5	0.033 \pm 0.002	0.028 \pm 0.003
		1	0.038 \pm 0.025	0.037 \pm 0.019
		2	0.023 \pm 0.013	0.030 \pm 0.021
	3	0.5	0.065 \pm 0.022	0.063 \pm 0.017
		1	0.098 \pm 0.053	0.114 \pm 0.055
		2	0.121 \pm 0.013	0.160 \pm 0.016
	10	0.5	0.227 \pm 0.067	0.192 \pm 0.042
		1	0.282 \pm 0.152	0.267 \pm 0.120
		2	0.326 \pm 0.093	0.331 \pm 0.095
Sprague-Dawley	10	0.5	0.119 \pm 0.040	0.090 \pm 0.051
		1	0.141 \pm 0.025	0.134 \pm 0.038
		2	0.272 \pm 0.099	0.280 \pm 0.114

JPET #245506

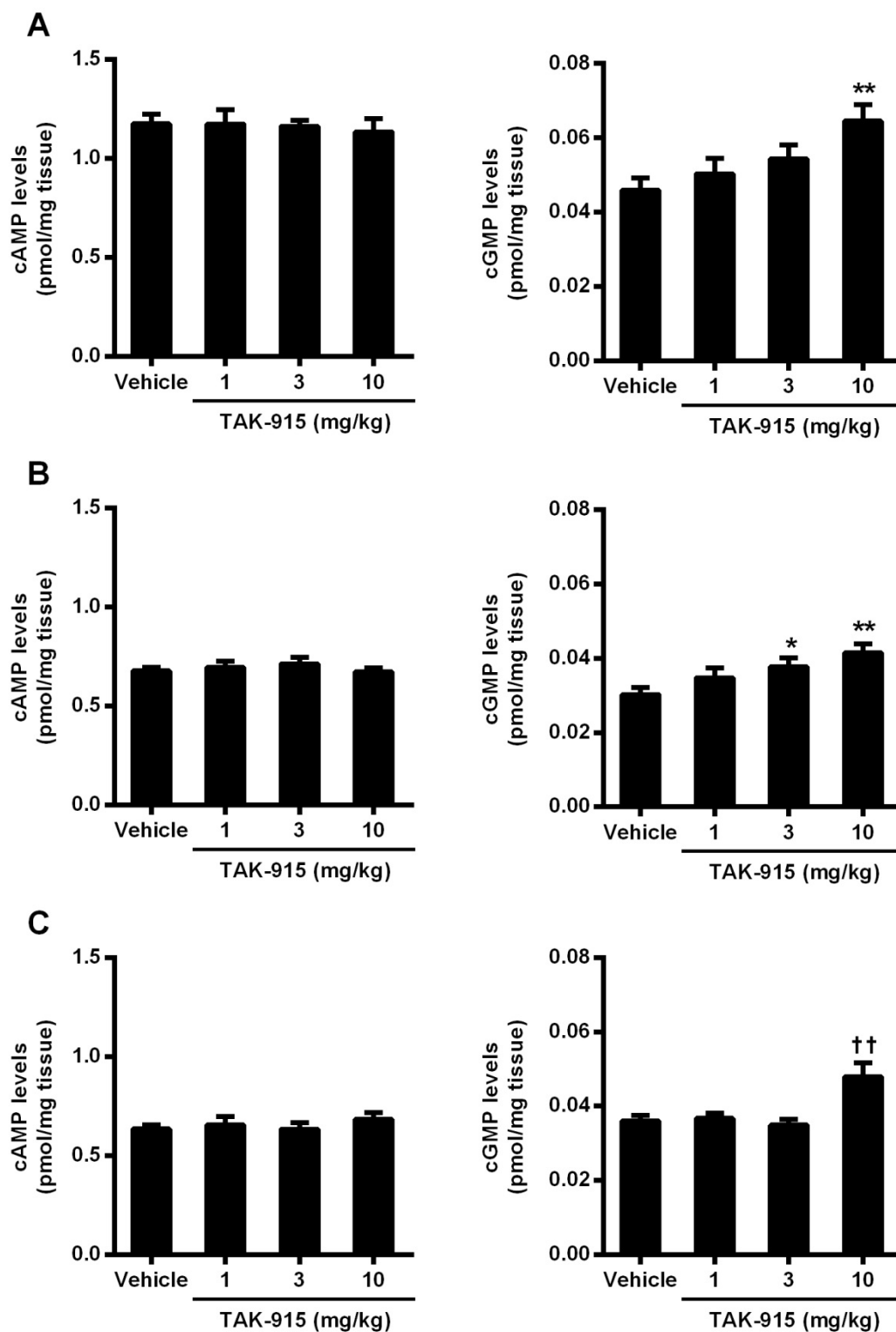


Figure 1

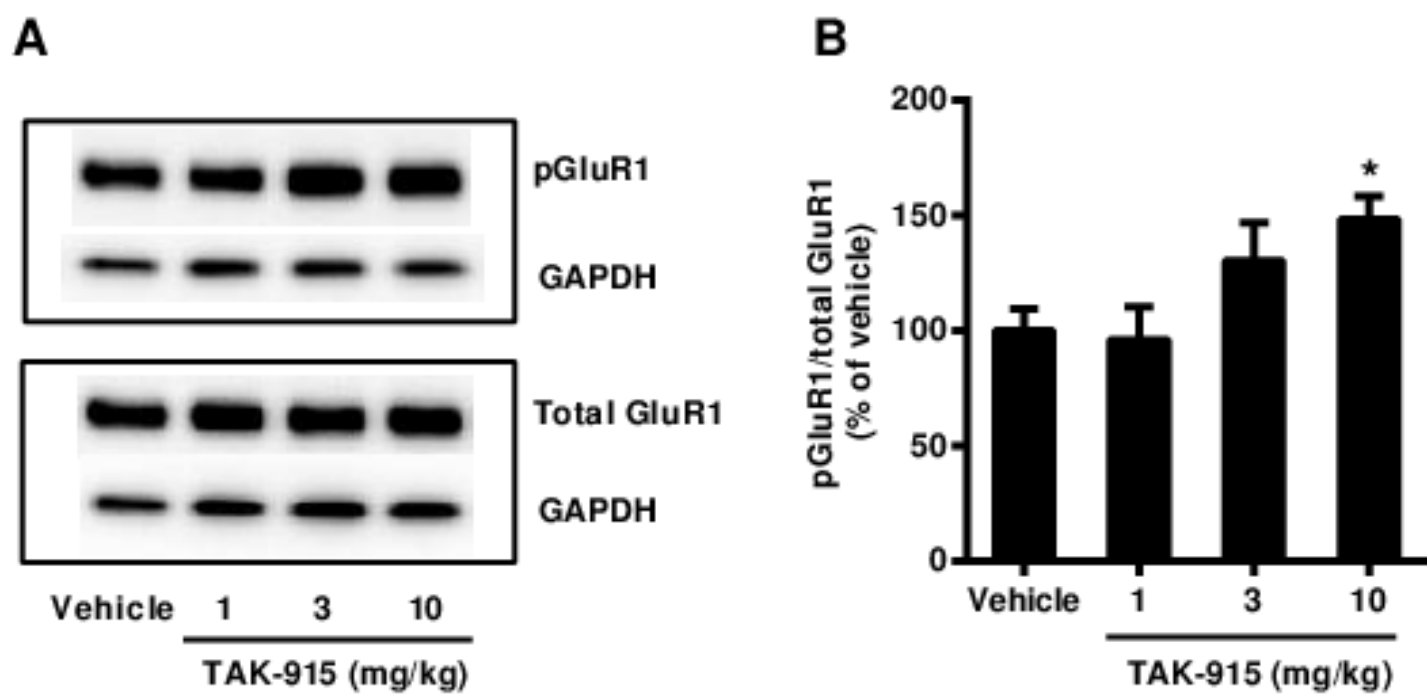


Figure 2

JPET #245506

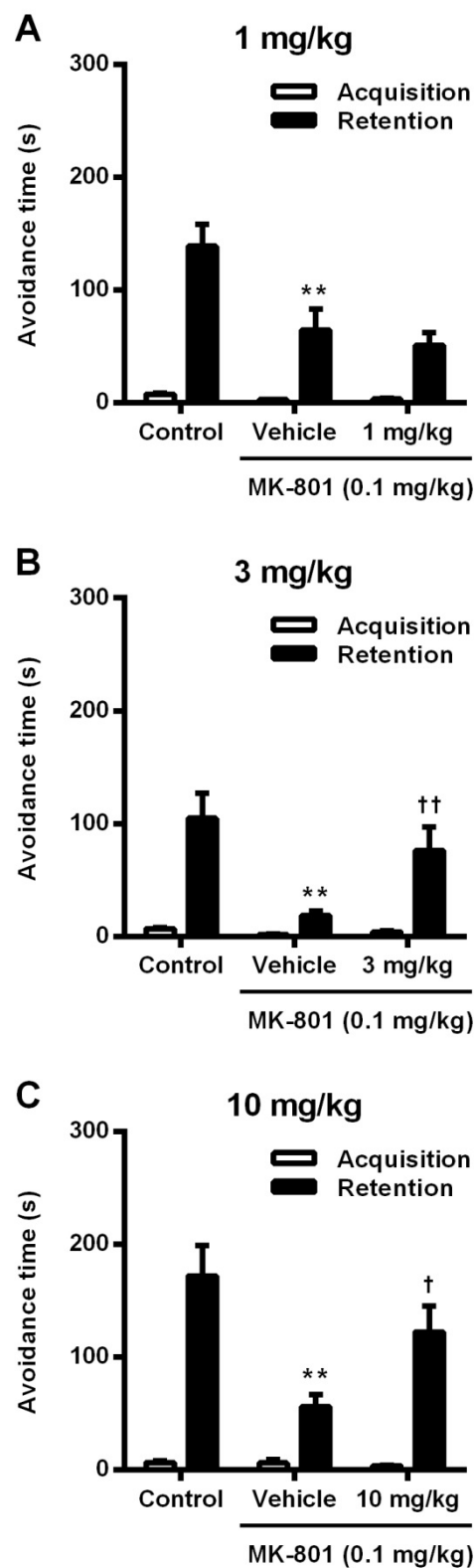


Figure 3

JPET #245506

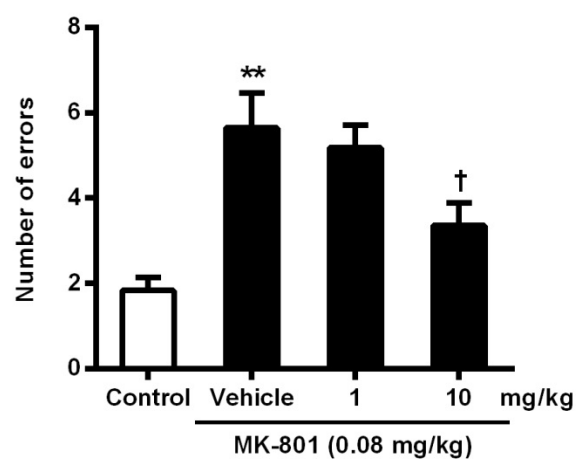


Figure 4

JPET #245506

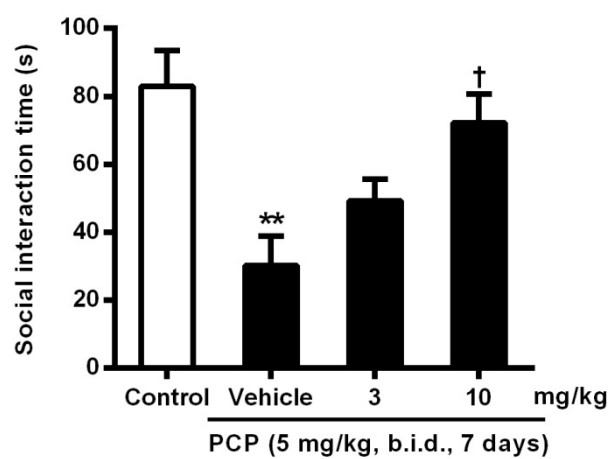


Figure 5

JPET #245506

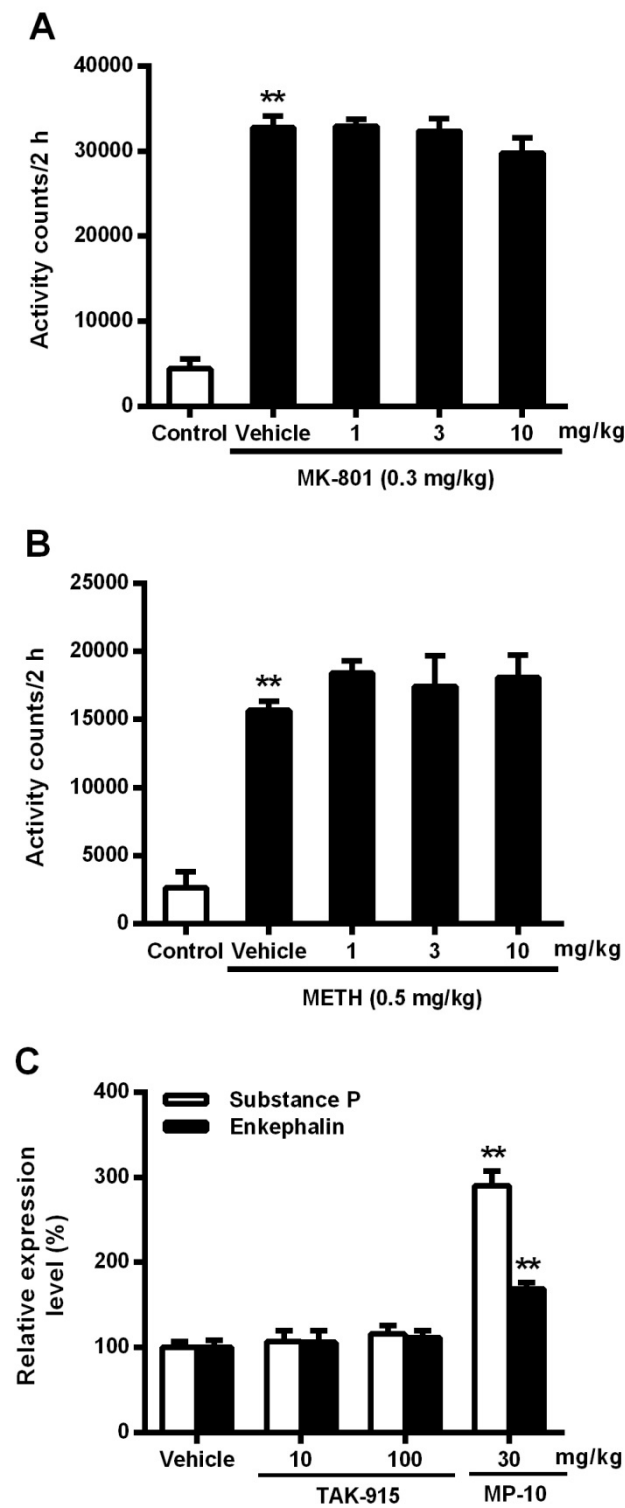


Figure 6