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Title page

***In Vivo* Pharmacological Characterization of TAK-063, a Potent and
Selective Phosphodiesterase 10A Inhibitor with Antipsychotic-Like
Activity in Rodents**

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Running Title:

Pharmacological Property of a Novel PDE10A Inhibitor TAK-063

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AMPA, (\pm)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNS, central nervous system; CREB, cAMP response element-binding protein; EPS, extrapyramidal symptoms; GABA, γ -aminobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluR1, glutamate receptor subunit 1; MED, minimum effective dose; MSN, medium spiny neuron; pAMSF, p-amidinomethanesulfonyl fluoride; PCR, polymerase chain reaction; PDE, phosphodiesterase; PET, positron emission tomography; PKA, protein kinase A; PKG, protein kinase G; TAK-063, [1-[2-fluoro-4-(1H-pyrazol-1-yl)phenyl]-5-methoxy-3-(1-phenyl-1H-pyrazol-5-yl)-pyridazin-4(1H)-one].

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ABSTRACT

Phosphodiesterase 10A (PDE10A) is a cAMP/cGMP phosphodiesterase highly expressed in medium spiny neurons (MSNs) in the striatum. We evaluated the *in vivo* pharmacological profile of a potent and selective PDE10A inhibitor TAK-063 [1-[2-fluoro-4-(1*H*-pyrazol-1-yl)phenyl]-5-methoxy-3-(1-phenyl-1*H*-pyrazol-5-yl)-pyridazin-4(1*H*)-one]. TAK-063 at 0.3 and 1 mg/kg, p.o., increased cAMP and cGMP levels in the rodent striatum and upregulated phosphorylation levels of key substrates of cAMP-dependent and cGMP-dependent protein kinases. TAK-063 at 0.3 and 1 mg/kg, p.o., strongly suppressed MK-801-induced hyperlocomotion that is often used as a predictive model for antipsychotic-like activity in rodents. Upregulation of striatal cAMP/cGMP levels and the antipsychotic-like effect of TAK-063 were not attenuated after 15 days of pretreatment with TAK-063 in mice. The potential side effect profile of TAK-063 was assessed in rats using the clinical antipsychotics haloperidol, olanzapine, and aripiprazole as controls. TAK-063 did not affect plasma prolactin or glucose levels at doses up to 3 mg/kg, p.o. TAK-063 at 3 mg/kg, p.o., elicited a weak cataleptic response compared with haloperidol and olanzapine. Evaluation of pathway-specific markers (substance P mRNA for the direct pathway and enkephalin mRNA for the indirect pathway) revealed that TAK-063 activated both the direct and indirect

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pathways of MSNs. These findings suggest that TAK-063 represents a promising drug for the treatment of schizophrenia with the potential for a superior safety and tolerability profiles.

Introduction

The basal ganglia are a series of interconnected subcortical nuclei that integrate widespread cortical inputs with dopaminergic signaling to plan and execute relevant motor and cognitive patterns while suppressing unwanted or irrelevant patterns (Albin et al., 1989; Graybiel, 2000; McHaffie et al., 2005). Dysfunction in this circuit has been implicated in various central nervous system (CNS) disorders and diseases, including schizophrenia, Parkinson's disease, Huntington's disease, and addiction (Albin et al., 1989; Chesselet and Delfs, 1996; Hyman et al., 2006; Perez-Costas et al., 2010). Schizophrenia is a devastating neuropsychiatric syndrome that typically strikes in late adolescence or early adulthood (van Os and Kapur, 2009). Positive symptoms, including delusions and hallucinations, are the most apparent manifestation of the disorder. These emerge episodically and usually trigger the first hospitalization in early adulthood. Chronic aspects of the disorder include negative symptoms, such as social withdrawal, flattened affect, and anhedonia as well as pervasive cognitive deficits. The latter have been closely linked to a poor functional outcome and long-term prognosis (Green et al., 2004; Harvey et al., 2004). Current antipsychotics have been known to have clinical significant effects on positive symptoms that are mediated by dopamine D₂ receptor antagonism, but their efficacy is considerably limited for other aspects of the

disease, such as cognitive and negative symptoms. Typical antipsychotics are known to cause extrapyramidal symptoms (EPS) by excessive dopamine D₂ receptor antagonism in the striatum, and hyperprolactinemia by dopamine D₂ receptor antagonism in the pituitary gland (Krebs et al., 2006). Although atypical antipsychotics, such as olanzapine and risperidone, produce a lower incidence of EPS than typical antipsychotics, these drugs are still associated with hyperprolactinemia and serious metabolic side effects, including hyperglycemia, weight gain, diabetes, and an abnormal lipid profile (Krebs et al., 2006). Thus, novel drugs with potent efficacy against more symptoms of schizophrenia and a better safety profile would be of considerable therapeutic value.

Inhibition of cyclic nucleotide phosphodiesterase (PDEs) may provide a new therapeutic approach for the treatment of CNS disorders (Menniti et al., 2006; Menniti et al., 2007; Reneerkens et al., 2009). The PDE superfamily of enzymes is encoded by 21 genes and subdivided into 11 distinct families according to their structural and functional properties (Bender and Beavo, 2006). Identification of the entire PDE gene family and a greater understanding of the complex expression patterns of the different PDEs have renewed interest in the therapeutic potential of this important class of enzymes. Among PDE families, PDE10A is emerging as a promising target for CNS

disorders such as schizophrenia and Huntington's disease (Giampà et al., 2009; Giampà et al., 2010; Kehler and Nielsen, 2011). PDE10A mRNA expression was found to be particularly high in certain brain regions and testis in mice, rat, and humans (Fujishige et al., 1999; Soderling et al., 1999; Seeger et al., 2003; Lakics et al., 2010). Subsequent studies in multiple mammalian species indicated that PDE10A protein is highly expressed in the γ -aminobutyric acid (GABA)-containing medium spiny neurons (MSNs) in the mammalian striatum and substantia nigra, with restricted distribution in the periphery (Seeger et al., 2003; Coskran et al., 2006). MSNs are the principal input site for information integration in the basal ganglia of the mammalian brain, and they give rise principally to two pathways: a direct (striatonigral) pathway, which expresses dopamine D₁ receptors, and an indirect (striatopallidal) pathway, which expresses dopamine D₂ receptors (Graybiel, 1990; Graybiel, 2000). These pathways have competing effects on the striatal output. As PDE10A is expressed in both pathways, PDE10A inhibition and resulting elevation of striatal cyclic nucleotide levels would potentially have the effects of D₂ receptor antagonism, the standard treatment for psychosis, along with D₁ receptor agonism, which may minimize EPS liabilities. The results of preclinical studies of several PDE10A inhibitors suggest that PDE10A inhibition may be a novel therapeutic approach for the treatment of schizophrenia

(Schmidt et al., 2008; Grauer et al., 2009; Smith et al., 2013).

We recently discovered the novel PDE10A inhibitor TAK-063 [1-[2-fluoro-4-(1*H*-pyrazol-1-yl)phenyl]-5-methoxy-3-(1-phenyl-1*H*-pyrazol-5-yl)-pyridazin-4(1*H*)-one] and confirmed that TAK-063 can specifically bind to PDE10A under physiological conditions (Kunitomo et al., 2014; Harada et al., manuscript in preparation). In this study, we characterized the *in vivo* efficacy of TAK-063 as a novel treatment for schizophrenia. TAK-063 activates striatal signaling pathways, has antipsychotic-like activity in rodent models, and has the potential to demonstrate a superior side effect profile in the clinic. These findings suggest that TAK-063 is a promising drug for the treatment of schizophrenia.

Materials and Methods

Animals. Male ICR mice and Sprague–Dawley (SD) rats were supplied by CLEA Japan Inc. (Tokyo, Japan), and Charles River Laboratories Japan Inc. (Yokohama, Japan), respectively. These animals were housed in a light-controlled room (12-h light/dark cycle with lights on from 7:00 AM). Animals used in the experiments were 6–8 weeks old and had completed an acclimation period of at least 1 week. The Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited approved the care and use of animals and the experimental protocols used in this research.

Drug Administration. TAK-063 was synthesized by Takeda Pharmaceutical Company Limited (Fujisawa, Japan) (Kunitomo et al., 2014). TAK-063 and haloperidol (Sigma-Aldrich, St. Louis, MO) were suspended in 0.5% (w/v) methylcellulose in distilled water. The doses of TAK-063 were calculated as the free base. Olanzapine was extracted from Zyprexa[®] (Eli Lilly and Company, Indianapolis, IN) at KNC Laboratories Co. Ltd. (Kobe, Japan), and then dissolved in 1.5% (v/v) lactic acid. The pH of this solution was then adjusted to neutral using 1 M NaOH. Aripiprazole was

purchased from AK Scientific Inc. (Union City, CA), and suspended in a 1% (v/v) solution of Tween80 in distilled water. These compounds were administered orally (p.o.). (+)-MK-801 hydrogen maleate (MK-801, Sigma-Aldrich) was dissolved in saline and administered subcutaneously (s.c.). Rolipram (Sigma-Aldrich) was suspended in a 0.5% (w/v) solution of methylcellulose in saline and administered intraperitoneally (i.p.). To evaluate the activation of the direct and indirect pathways in MSNs, haloperidol was suspended in a 0.5% (w/v) solution of methylcellulose in saline. SKF82958 (Sigma-Aldrich) was dissolved in two drops of Tween80 and then diluted in saline. These compounds were administered i.p. All compounds were dosed at 20 mL/kg body weight in mice and 2 mL/kg body weight in rats.

Measurement of Cyclic Nucleotides and Phosphorylated Protein Levels in Various Brain Regions. Male ICR mice and SD rats were sacrificed using an MMW-05 focused microwave irradiation system (Muromachi Kikai Co. Ltd., Tokyo, Japan) 60 min after oral administration of TAK-063. Brain tissues were isolated, sonicated in 0.5 N HCl, and clarified by centrifugation. Cyclic nucleotide concentrations in the supernatants were measured using enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI). To assess the phosphorylation of cAMP

response element-binding protein (pCREB) and of AMPA receptor subunit GluR1 at Ser845 (pGluR1), microwaved brain tissues were sonicated in extraction buffer (Invitrogen, Carlsbad, CA) containing the protease inhibitor cocktail (Sigma-Aldrich) and 0.5 mM pAMSF (Sigma-Aldrich). After centrifugation, protein concentrations in the supernatant were determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Each sample (5 μ g protein) was subjected to electrophoresis through polyacrylamide gradient gels, followed by western blotting to nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, CA). The blots were incubated with antibodies to pCREB (Cell Signaling Technology Inc., Danvers, MA), total CREB (tCREB; Cell Signaling Technology), pGluR1 (PhosphoSolutions®, Aurora, CO), total GluR1 (Millipore, Temecula, CA), or β -actin (Sigma-Aldrich). Individual protein bands were visualized with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) followed by treatment with ECL western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK) and exposure to Hyperfilm ECL (GE Healthcare). Protein levels were calculated from densitometric analysis using a Bio-Rad GS-800 calibrated scanning densitometer. Densities of phosphorylated protein bands for each sample were normalized to the density of the corresponding total protein bands. These ratios are expressed as the

relative change divided by the average of the vehicle-treated samples. Amounts of pCREB and tCREB in the various brain regions were measured using the CREB [pS133] human ELISA kit and the CREB [total] human ELISA kit (Life Technologies, Carlsbad, CA), respectively.

MK-801-Induced Hyperlocomotion in Rodents. Locomotion was measured using a SUPERMEX spontaneous motor analyzer (Muromachi Kikai) for rats and an MDC system (BrainScienceIdea Co. Ltd., Osaka, Japan) for mice. Animals (male ICR mice or SD rats) were placed in locomotor chambers (length \times width \times height: 24 \times 37 \times 30 cm for rats and 36 \times 22 \times 13.5 cm for mice) for more than 60 min for habituation. Thereafter, animals were removed from each chamber and treated with either vehicle or test compounds and then quickly returned to the chamber. After an appropriate pretreatment time, animals were again removed from the chambers and treated with either vehicle (saline) or MK-801 (0.3 mg/kg as a salt, s.c.) and then quickly transferred to the test chamber. Activity counts were recorded in successive 1-min bins and then cumulative counts during 120 min after psychostimulant administration were calculated. A 60-min pretreatment time was used in the mouse study. Pretreatment times in rats varied according the pharmacokinetic profile (i.e., T_{max}) of each agent, resulting in a

90-min pretreatment time for TAK-063, a 30-min pretreatment time for olanzapine, and a 60-min pretreatment time for haloperidol and aripiprazole.

Measurement of Plasma Prolactin Levels. Male SD rats were orally administered either vehicle or test compounds after a habituation period of >30 min. Ninety minutes after administration, blood was collected from tail vein into a 1.5-mL Eppendorf tube containing 25 μ L of EDTA. Blood was immediately mixed with EDTA, placed on ice, and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were collected in another tube as plasma, and were stored in a deep-freezer until use. The prolactin concentrations in these plasma samples were measured using an ELISA kit (Bertin Pharma, Montigny le Bretonneux, France).

Measurement of Plasma Glucose Levels. Male SD rats were fasted overnight and were decapitated 150, 60, 60, and 90 min after administration of TAK-063, haloperidol, olanzapine, and aripiprazole, respectively. Trunk blood was collected into 50-mL centrifuge tubes. Plasma glucose levels were measured using a model 7180 Clinical Analyzer (Hitachi High-Technologies Inc., Tokyo, Japan).

Bar Test. The degree of cataleptic response in rats by antipsychotics such as haloperidol and ziprasidone was reported to increase as a function of both dose and time (Schmidt et al., 2008). The catalepsy-like behavior of male SD rats was measured 2 and 4 h after administration of test compounds in a blind manner, except where noted. Forelimbs were placed on a horizontal metal bar at 13-cm height and the length of time during which both forelimbs remained on the bar was determined with a stopwatch (Seiko Holdings Corporation, Tokyo, Japan). Animals not responding within 90 s were removed from the apparatus and assigned a latency of 90 s (cut-off value). The average of three trials was recorded as the duration of the cataleptic response.

Real-Time Quantitative PCR Expression Analysis. Total RNA from different brain regions was extracted using Isogen (Nippon Gene Co. Ltd., Toyama, Japan) and the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Real-time quantitative PCR was performed using an ABI PRISM 7900HT sequence detection system (Life Technologies) and TaqMan reagents (Eurogentec, Seraing, Belgium). RNA quantities were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan probes according to the manufacture's instruction. The following primers were used for mouse PDE10A analysis: forward primer,

5'-AAGAGACAGCAATGTGGATTTCAG-3'; reverse primer,
5'-TCTTCAACCTTCACGTTTCAGCTT-3'; TaqMan probe (MGB probe),
5'-CCCGGCGCCTAGCAAGAGCAC-3'. The following primers were used for mouse
PDE4D analysis: forward primer, 5'-GACGTGGCCTATCACAACAACA-3'; reverse
primer, 5'-GGTGTAGAGAGCAGCACGTGAGT-3'; TaqMan probe (MGB probe),
5'-CCATGCTGCAGACGTCGTCCAGTC-3'. The following primers were used for
enkephalin analysis: forward primer, 5'-GGACTGCGCTAAATGCAGCTA-3';
reverse primer, 5'-GTGTGCATGCCAGGAAGTTG-3'; TaqMan probe (MGB probe),
5'-CGCCTGGTACGTCCCGGCG-3'. The following primers were used for substance P
analysis: forward primer, 5'-CGCAAAATCCAACATGAAAATC-3'; reverse primer,
5'-GCAAACAGTTGAGTGGAACGA-3'; TaqMan probe (MGB probe),
5'-CGTGGCGGTGGCGGTCTTTTT-3'. The following primers were used for rat
GAPDH analysis: forward primer, 5'-TGCCAAGTATGATGACATCAAGAAG-3';
reverse primer, 5'-AGCCCAGGATGCCCTTTAGT-3'; TaqMan probe (MGB probe),
5'-TGGTGAAGCAGGCGGCCGAG-3'. The primers used for mouse GAPDH analysis
were from ABI (TaqMan Rodent GAPDH Control Reagents, VIC probe).

Statistics. The statistical significance of differences between two groups was assessed by the Student's *t*-test (for homogenous data) or the Aspin-Welch test (for non-homogenous data). Differences yielding *P* values ≤ 0.05 were considered significant. In the experiments that examined the effects of multiple doses of test compounds, statistical significance was determined using a one-tailed Williams' test (for homogenous data) or a one-tailed Shirley-Williams' test (for non-homogenous data). One-tailed differences yielding *P* values ≤ 0.025 were considered significant.

Results

Effect of TAK-063 on Striatal Cyclic Nucleotide Levels and Their Downstream

Signaling. PDE10A inhibition and the resulting elevation of cAMP and cGMP levels are known to activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) (Kehler and Nielsen, 2011). Activated PKA and PKG increase the phosphorylation of key substrates, such as CREB and AMPA-receptor GluR1 subunit (Lonze and Ginty, 2002; Wang et al., 2005; Serulle et al., 2007). To evaluate *in vivo* PDE10A inhibition by TAK-063, the effects of TAK-063 on cAMP and cGMP levels and phosphorylation levels of CREB and GluR1 were assessed. Oral administration of TAK-063 increased cAMP and cGMP levels in the mouse and rat striatum in a dose-dependent manner (Fig. 1A and B). The minimum effective dose (MED) of TAK-063 for the upregulation of cAMP and cGMP in the mouse striatum was 0.3 mg/kg, p.o. ($P \leq 0.025$). The phosphorylation of CREB and GluR1 in striatal extracts was evaluated by western blotting. TAK-063 increased phosphorylation levels of CREB and GluR1 in the mouse striatum in a dose-dependent manner, in the same dose range of TAK-063 that upregulated striatal cAMP and cGMP levels ($P \leq 0.025$, Fig. 1B, C, and D). These results suggest that TAK-063 suppressed PDE10A activity and activated downstream cAMP and cGMP signals in the striatum.

Striatal-Selective Upregulation of cAMP and CREB Phosphorylation by TAK-063. We investigated the upregulation of cAMP and pCREB by TAK-063 in various brain regions, such as the frontal cortex, striatum, thalamus, brainstem, hippocampus, and cerebellum, in mice. A quantitative PCR expression analysis revealed that PDE10A mRNA was expressed mainly in the striatal complex within these brain regions (Fig. 2A). TAK-063 at 0.3 mg/kg, p.o., selectively increased cAMP ($P \leq 0.01$, Fig. 2B) and pCREB levels ($P = 0.12$, Fig. 2C) in the striatal complex, where the PDE10A mRNA expression level was high. Rolipram (10 mg/kg, i.p.), a selective PDE4 inhibitor, increased cAMP levels in all brain regions tested, probably due to the broader expression pattern of PDE4 (Supplemental Fig. 1; Lakics et al., 2010; Johansson et al., 2012). These results indicate that TAK-063 selectively inhibits PDE10A *in vivo* under these conditions.

Effects of TAK-063 on MK-801-Induced Hyperactivity in Rodents. Hyperlocomotion induced by MK-801, an NMDA receptor antagonist, is commonly used as a model for acute psychosis based on the NMDA hypofunction hypothesis of schizophrenia (O'Neill and Shaw, 1999). TAK-063 produced dose-dependent

suppression of MK-801-induced hyperlocomotion in mice with an MED of 0.3 mg/kg, p.o. ($P \leq 0.025$, Fig. 3A). In rats, TAK-063 produced dose-dependent suppression of MK-801-induced hyperlocomotion with an MED of 0.1 mg/kg, p.o. ($P \leq 0.025$, Fig. 3B). Current clinically-used antipsychotics such as haloperidol, olanzapine, and aripiprazole also showed dose-dependent suppression of MK-801-induced hyperlocomotion. Their MEDs were 3, 30, and 100 mg/kg, respectively ($P \leq 0.025$, Fig. 3B). These data suggest that TAK-063 has a potent antipsychotic-like effect in rodents.

Effects of TAK-063 on Striatal Cyclic Nucleotide Levels and MK-801-Induced Hyperactivity After Repeated Administration. To assess whether the pharmacological effects of TAK-063 are altered after chronic treatment, we examined striatal molecular marker responses and antipsychotic-like effects of TAK-063 after repeated administration in mice. After 15 days of pretreatment with TAK-063 (0.5 mg/kg/day), TAK-063 at 0.5 mg/kg, p.o. significantly upregulated striatal cAMP and cGMP levels ($P \leq 0.05$ for cAMP, $P \leq 0.01$ for cGMP, Fig. 4A) and produced a suppression of MK-801-induced hyperlocomotion in mice ($P \leq 0.025$, Fig. 4B). These effects did not differ from those seen after administration of a single dose (Fig. 4A and B). These results suggest that the antipsychotic-like effects of TAK-063 are maintained

after repeated administration.

Evaluation of Potential Clinical Side Effects. The potential of TAK-063 to produce well-known side effects of current clinical antipsychotics, such as hyperprolactinemia, hyperglycemia and EPS, was evaluated in rats. TAK-063 did not affect plasma prolactin levels in male rats at doses up to 3 mg/kg, p.o., while current antipsychotics such as haloperidol, olanzapine, and aripiprazole significantly increased plasma prolactin levels. The MEDs of haloperidol, olanzapine, and aripiprazole for upregulation of plasma prolactin levels were 0.3, 3, and 30 mg/kg, p.o., respectively ($P \leq 0.025$, Fig. 5). It has been reported that the acute effects of antipsychotics on rodent plasma glucose levels are well-correlated with clinically observed effects (Assié et al., 2008). Oral administration of olanzapine significantly increased rat plasma glucose levels in a dose-dependent manner, with an MED of 10 mg/kg, p.o. ($P \leq 0.025$, Fig. 6). In contrast, TAK-063, haloperidol, and aripiprazole did not affect plasma glucose levels (Fig.6).

Next, the potential of these compounds to produce EPS was assessed by evaluating their cataleptogenic activities 2 and 4 h after administration in rats. All compounds tested produced a cataleptic response in time dependent manner; the MEDs for TAK-063, haloperidol, olanzapine, and aripiprazole were 3, 0.3, 10, 30 mg/kg, p.o.,

respectively (4 h after administration, $P \leq 0.025$, Fig. 7). Suppression of MK-801-induced hyperlocomotion and the potential to produce side effects in rats are summarized in Table 1. These results suggest that TAK-063 administration carries lower risk of the side effects assessed when compared with administration of current antipsychotics.

Activation of the Direct and Indirect Pathways by PDE10A Inhibition. It has been reported that PDE10A is expressed both in the direct (dopamine D₁ receptor-dependent, facilitate activity) and in the indirect (dopamine D₂ receptor-dependent, tonically inhibitory) striatal pathways of the striatum complex (Sano et al., 2008). To evaluate activation of the direct and the indirect pathways by a D₂ antagonist (haloperidol), a D₁ agonist (SKF82958), and a PDE10A inhibitor (TAK-063), we measured the expression of mRNA encoding substance P and enkephalin in the rat striatum. Substance P and enkephalin are markers of the direct and indirect pathways, respectively. RNAs encoding these neuropeptides are known to be upregulated via CREB response elements in the promoter regions of the respective genes (Gerfen et al., 1990; Simpson and McGinty, 1995). SKF82958 (2 mg/kg, i.p.) selectively increased the substance P mRNA level ($P \leq 0.05$, Fig. 8A), and haloperidol

(1 mg/kg, i.p.) selectively increased the enkephalin mRNA level in the rat striatum ($P \leq 0.01$, Fig. 8A). TAK-063 (1 mg/kg, p.o.) increased both enkephalin and substance P mRNA levels in the rat striatum ($P \leq 0.01$ for substance P and $P \leq 0.05$ for enkephalin). The indirect and direct pathways are thought to have competing effects on striatal output (Graybiel AM, 1990, 2000). The effect of D₁-direct pathway activation on the D₂-indirect pathway-mediated cataleptic response was evaluated using SKF82958 and haloperidol. Pretreatment with SKF82958 (2 mg/kg, i.p.) significantly decreased the magnitude of the haloperidol (1 mg/kg, i.p.)-induced cataleptic response in rats ($P \leq 0.05$, Fig. 8B). These results suggest that the reduced cataleptic response seen in rats after TAK-063 is due to modulation of both the direct and indirect pathways by TAK-063.

Discussion

We previously reported the discovery of a novel PDE10A inhibitor, TAK-063 (Kunitomo et al., 2014) that displays potent PDE10A inhibitory activity (hPDE10A IC_{50} = 0.30 nM) and high selectivity (>15000-fold) over other PDE families (PDE1–PDE11) in *in vitro* assays using recombinant human enzymes. TAK-063 also selectively binds to native PDE10A in the rodent brain (Harada et al., manuscript in preparation). In the present study, we described data validating the functional PDE10A selectivity of TAK-063. TAK-063 selectively (i.e., only in the striatum) upregulated cAMP level and phosphorylation levels of their downstream signaling molecules CREB and GluR1. This striatal-specific signal activation is consistent with the striatal-specific expression of PDE10A and its inhibition by TAK-063 in the mouse brain.

The antipsychotic-like effects of TAK-063 were evaluated through inhibition of MK-801-induced hyperlocomotion in rodents that is often used as a predictive assay for antipsychotic-like activity. In this model, pharmacological effects were observed at 0.3 mg/kg, p.o. This dose is consistent with doses shown to activate striatal signaling pathways in rodents. Furthermore, a dose of 0.3 mg/kg, p.o., of TAK-063 has been estimated to produce a striatal PDE10A occupancy level of 26% in rats (Harada et al., manuscript in preparation). Positron emission tomography (PET) occupancy studies

could be useful as a translational approach between preclinical and clinical studies of TAK-063. We developed a clinical PET radioligand targeting PDE10A, [^{11}C]T-773, and successfully estimated striatal PDE10A occupancy by TAK-063 using non-radiolabeled T-773 in rats (Harada et al., 2014; Harada et al., manuscript in preparation). Combined with the results of this study, investigation of the dose-occupancy relationship in humans will provide further characterization of the preclinical and clinical aspects of the pharmacological profile of TAK-063.

Activation of the indirect striatal pathway by blockade of the dopamine D₂ receptor is the hypothesized mechanism underlying the effect of current antipsychotics. The antipsychotic effects similar to those of current antipsychotics could be expected to be produced by TAK-063 through the upregulation of cAMP and concomitant activation of the indirect pathway. This hypothesis can be explored in future clinical studies.

Plasma glucose and prolactin levels and catalepsy were evaluated in rats dosed with TAK-063 as measures of potential adverse effects in the clinic. TAK-063, haloperidol, and aripiprazole did not affect plasma glucose levels, even at doses higher than those that produce antipsychotic-like effects, whereas olanzapine significantly increased plasma glucose levels in rats. These effects of current antipsychotics correlate well with clinical observations (Krebs et al., 2006). In addition, a dose of 3 mg/kg of TAK-063, at

which the striatal PDE10A occupancy level was 77% as determined by nonlinear regression analysis (Harada et al., manuscript in preparation), did not increase prolactin release in rats. TAK-063 showed a lower potential for producing a cataleptic response than haloperidol, olanzapine, and aripiprazole.

Given the unique pharmacological profile of TAK-063 relative to clinically-used antipsychotics and the high levels of PDE10A in MSNs, we examined the striatal pathways modulated by TAK-063. We confirmed that TAK-063 induces the expression of substance P (a marker of the direct striatal pathway) and enkephalin (a marker of the indirect striatal pathway) in rats. We also confirmed that activation of the direct pathway by SKF82958 (D₁ agonist) inhibited the indirect pathway-mediated cataleptic response to haloperidol (D₂ antagonist) in rats. The modulation of both the direct (D₁-dependent, facilitate activity) and indirect (D₂-dependent, tonically inhibitory) striatal pathways by PDE10A inhibitors (Siuciak et al., 2006) probably relates to their reduced cataleptic versus antipsychotic profile in animal models. Altogether these results suggest that the striatum-selective expression of PDE10A and the activation of both striatal pathways by PDE10A inhibition contribute to a superior side effect profile of TAK-063. Clinical studies, however, are critical to validate these predictions based on preclinical findings.

In the present study, we described results that demonstrated that TAK-063 is a potent and selective inhibitor of PDE10A *in vivo*. Additionally, the compound showed potent activity in rodent models of schizophrenic-like symptoms, but was less potent than clinically-used antipsychotics in their metabolic and cataleptic effects. PDE10A inhibitors have been reported to show efficacy in preclinical assays that test cognitive performance and possible models of negative symptoms in rodents (Grauer et al., 2009; Smith et al., 2013). Because cognitive and negative symptoms are not adequately treated by current antipsychotics, it is worthwhile to see the potential of TAK-063 in these domains.

In summary, these results suggest that TAK-063 could be a promising drug for the treatment of schizophrenia. This compound is currently in clinical development for the treatment of schizophrenia (ClinicalTrials.gov Identifiers: NCT01879722 and NCT01892189).

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Authorship Contributions

K.S, A.H., E.S., and H.K. are employees of Takeda Pharmaceutical Company Limited.

Participated in research design: Suzuki, Kimura.

Conducted experiments: Suzuki, Harada, Shiraishi.

Performed data analysis: Suzuki, Harada, Shiraishi, Kimura

Wrote or contributed to the writing of the manuscript: Suzuki, Kimura.

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Footnotes

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Legends and Figures

Fig. 1. Dose-dependent effects of TAK-063 on cyclic nucleotide levels and the phosphorylation of CREB and GluR1 in the striatum. A and B, Elevation of cAMP and cGMP levels in the mouse (A) and rat (B) striatum 60 min after administration of TAK-063. Data are presented as the mean + S.E.M. (n = 6–8). C, Dose-dependent increase of mouse striatal pCREB detected by western blotting. Representative western blot images are shown. Densities of pCREB bands for each sample were normalized to the corresponding tCREB band density. Data are presented as the mean + S.E.M. (n = 3). D, Dose-dependent effects of TAK-063 on the phosphorylation of mouse striatal GluR1 at Ser845. The phosphorylation level of GluR1 at Ser845 (pGluR1) was detected using western blotting. Representative western blot images are shown. The density of the pGluR1 band for each sample was normalized to the corresponding GluR1 band density. Data are presented as the mean + S.E.M. (n = 4). # $P \leq 0.025$ versus vehicle by one-tailed Williams' test. § $P \leq 0.025$ versus vehicle by one-tailed Shirley-Williams' test.

Fig. 2. PDE10A mRNA expression levels and the responses of molecular markers in the

various brain regions in mice. A, PDE10A mRNA expression levels in mouse brain regions were measured using real-time quantitative PCR analysis. B and C, Increases in cAMP from basal levels (vehicle-treated) and pCREB levels were measured in each brain region by ELISA 60 min after administration of vehicle or TAK-063 (0.3 mg/kg, p.o.). Data are presented as the mean \pm S.E.M. ($n = 3-8$). $**P \leq 0.01$ (vs vehicle, Aspin-Welch test). Fcx, frontal cortex; Str, striatum; Thal, thalamus; Bs, brainstem (pons and medulla); Hipp, hippocampus; Cb, cerebellum.

Fig. 3. Dose-dependent suppression of MK-801-induced hyperlocomotion in rodents. A, MK-801 (0.3 mg/kg, s.c.) was administered subcutaneously 60 min after oral administration of TAK-063 to mice. Accumulated activity counts during the 120 min following MK-801 treatment were calculated and are indicated as the mean + S.E.M. ($n = 8$). B, MK-801 was administered subcutaneously 90, 60, 30 or 60 min after oral administration of TAK-063, haloperidol, olanzapine, and aripiprazole, respectively, to rats. Accumulated activity counts during the 120 min following MK-801 treatment were calculated and are indicated as the mean + S.E.M. ($n = 6$). $**P \leq 0.01$ versus control by the Aspin-Welch test. $\#P \leq 0.025$ versus vehicle + MK-801 group by one-tailed Williams' test. $\$P \leq 0.025$ versus vehicle + MK-801 group by one-tailed

Shirley–Williams’ test.

Fig. 4. Effects on striatal cyclic nucleotide levels and antipsychotic-like effects after repeated administration of TAK-063 in mice. A, Vehicle or TAK-063 was administered once daily for 15 days (0.5 mg/kg/day, p.o.). On the next day, upregulation of cAMP and cGMP in the striatum were measured 60 min after administration of TAK-063 (0.5 mg/kg, p.o.). Data are presented as the mean + S.E.M. ($n = 7-9$). $*P \leq 0.05$ and $##P \leq 0.01$ versus vehicle by the Student’s *t*-test and the Aspin–Welch test, respectively. B, Vehicle or TAK-063 was administered to mice once daily for 15 days (0.5 mg/kg/day, p.o.). On the next day, the antipsychotic-like effect (suppression of MK-801-induced hyperlocomotion) was evaluated. MK-801 was administered subcutaneously 60 min after oral administration of TAK-063 (0.5 and 1.5 mg/kg, p.o.). Accumulated activity counts during 120 min following MK-801 treatment were calculated and indicated as the mean + S.E.M. ($n = 3-6$). $##P \leq 0.01$ versus control by the Aspin–Welch test. $\S P \leq 0.025$ versus vehicle + MK-801 group by one-tailed Williams’ test.

Fig. 5. Effects of TAK-063, haloperidol, olanzapine, and aripiprazole on plasma prolactin levels in rats. TAK-063, haloperidol, olanzapine, or aripiprazole was

administered orally to male SD rats, and blood was collected from the tail vein 90 min after compound administration. The plasma prolactin concentration was determined by ELISA. Data are presented as the mean + S.E.M. ($n = 6$). $\#P \leq 0.025$ versus vehicle by one-tailed Williams' test.

Fig. 6. Effects of TAK-063, haloperidol, olanzapine, and aripiprazole on plasma glucose levels in rats. TAK-063, haloperidol, olanzapine, or aripiprazole was administered orally to male SD rats, and then blood was collected by decapitation 150, 60, 60, or 90 min after administration of TAK-063, haloperidol, olanzapine, and aripiprazole, respectively. Plasma glucose concentrations were determined by colorimetric detection using a chemical analyzer. Data are presented as the mean + S.E.M. ($n = 5$). $\$P \leq 0.025$ versus vehicle by one-tailed Shirley-Williams' test.

Fig. 7. TAK-063 showed low cataleptic activity compared with clinically-used antipsychotics in rats. Duration of cataleptic response was measured using the bar test 2 and 4 h after administration. Data are presented as the mean + S.E.M. ($n = 6$). $\$P \leq 0.025$ versus vehicle by one-tailed Shirley-Williams' test. The dagger mark represents the occurrence of animals in a cataleptic position for more than 90 s (cut-off value).

Fig. 8. TAK-063 activated the D₁-direct and D₂-indirect pathways. A, Three hours after administration of SKF82958 (2 mg/kg, i.p.), haloperidol (1 mg/kg, i.p.), and TAK-063 (1 mg/kg, p.o.), mRNA expression levels of substance P (SP, a marker of D₁-direct pathway) and enkephalin (Enk, a marker of D₂-indirect pathway) in the rat striatum were measured by quantitative PCR analysis. The values in the graph represent expression levels relative to that of the vehicle-treated group. Data are presented as the mean + S.E.M. (n = 4–8). * $P \leq 0.05$, ** $P \leq 0.01$ versus vehicle by the Aspin–Welch test or the Student’s *t*-test. B, SKF82958 inhibition of haloperidol-induced cataleptic response in rats. Vehicle or SKF82958 (2 mg/kg, i.p.) was administered 5 min before administration of haloperidol (1 mg/kg, i.p.). Forty-five min after administration of haloperidol, the duration of cataleptic response was measured using the bar test. Data are presented as the mean + S.E.M. (n = 4–7). * $P \leq 0.05$, ** $P \leq 0.01$ versus vehicle by the Aspin–Welch test.

TABLE 1.

Minimum effective doses (mg/kg, p.o.) of TAK-063 and current antipsychotics haloperidol, olanzapine, and aripiprazole in rats.

	MK-801	Plasma	Plasma	Cataleptic
	hyperlocomotion	prolactin	glucose	response
TAK-063	0.1	No effect (up to 3)	No effect (up to 3)	3
Haloperidol	3	0.3	No effect (up to 3)	1
Olanzapine	30	3	10	10
Aripiprazole	100	30	No effect (up to 100)	30

Data are expressed as mg/kg, p.o. MK-801 hyperlocomotion; doses required to significantly suppress MK-801-induced hyperlocomotion (versus vehicle). Plasma prolactin; doses required to significantly upregulate plasma prolactin levels (versus vehicle). Plasma glucose; doses required to significantly upregulate plasma glucose levels (versus vehicle). Cataleptic response; doses required to significantly induce a cataleptic response 4 h after administration (versus vehicle).

Fig. 1

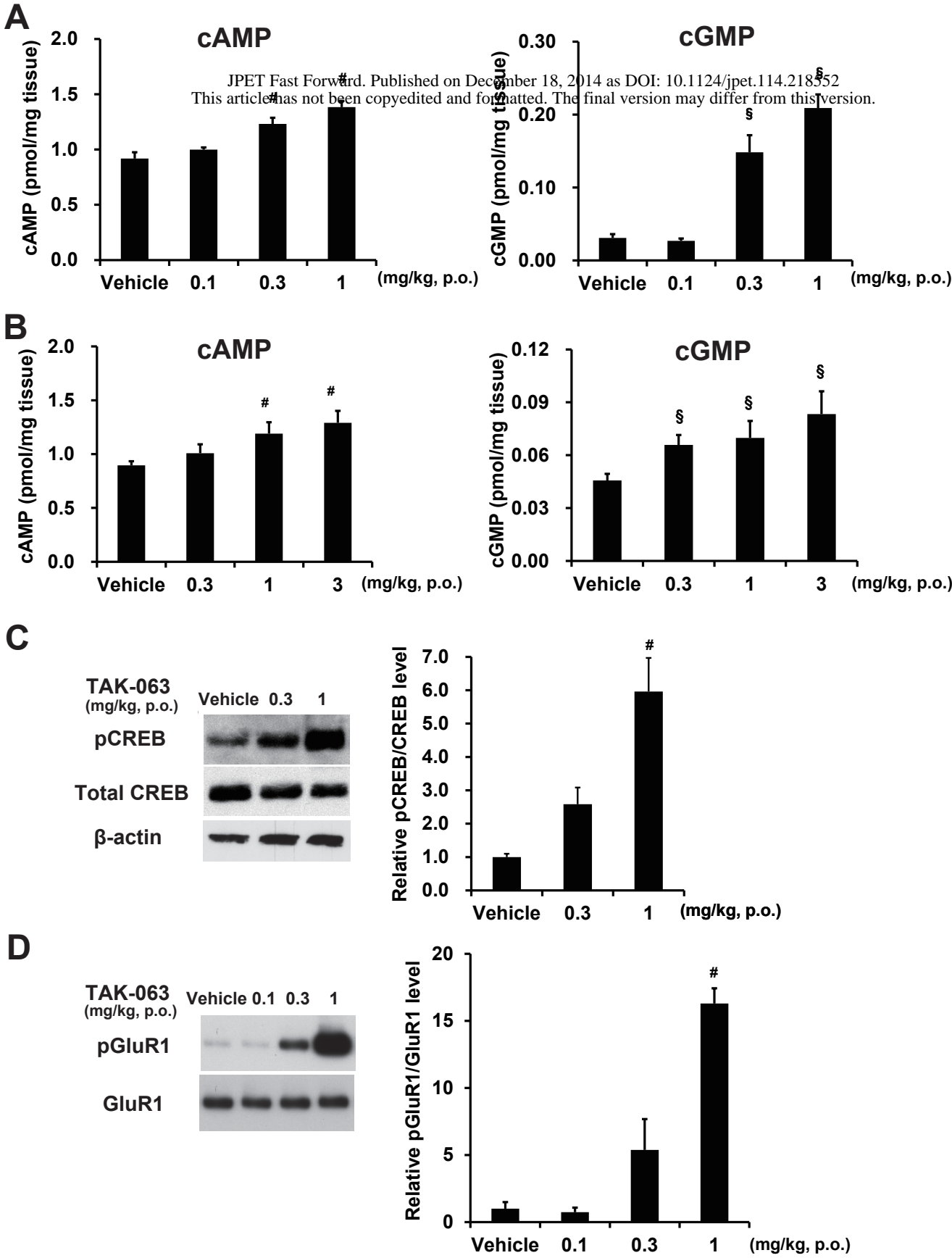


Fig. 2

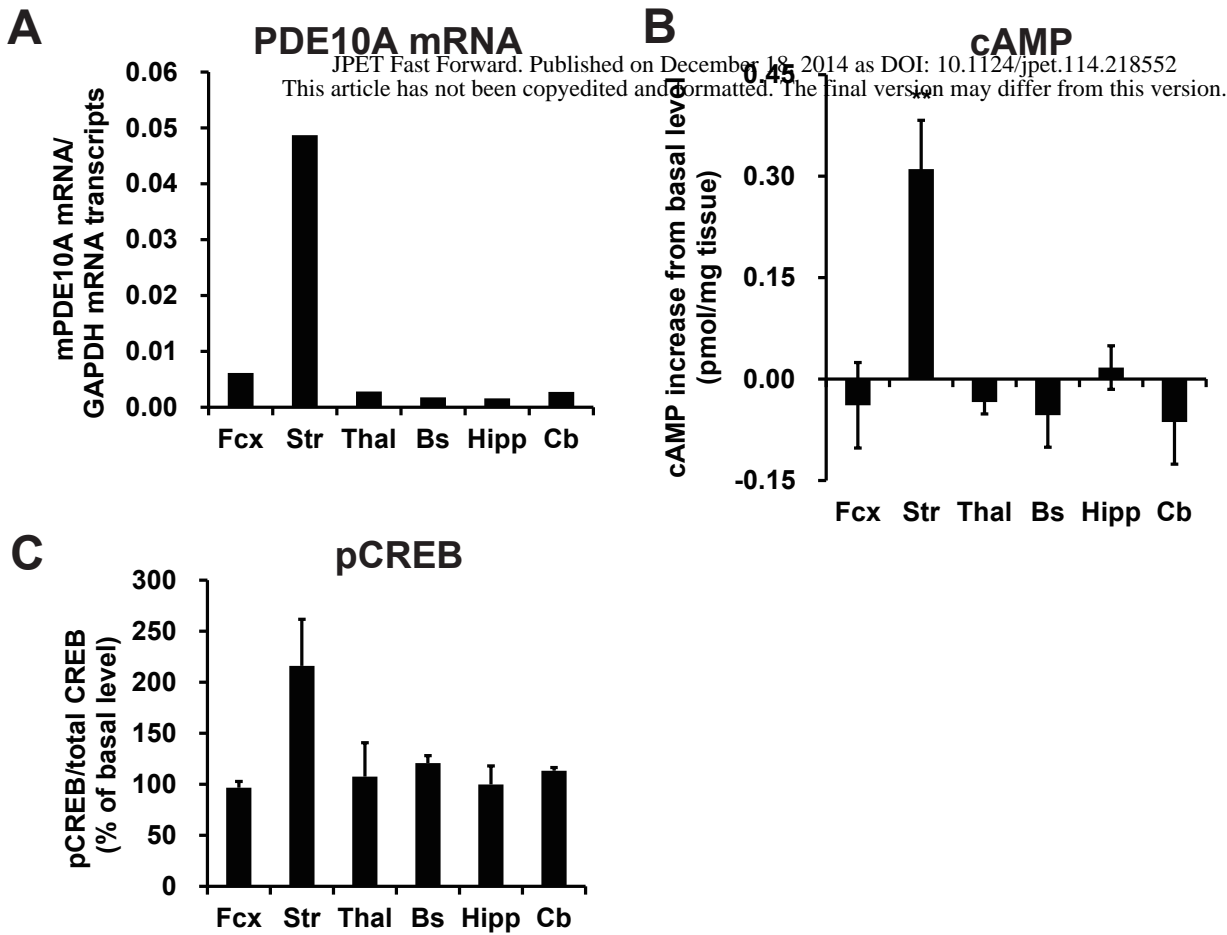
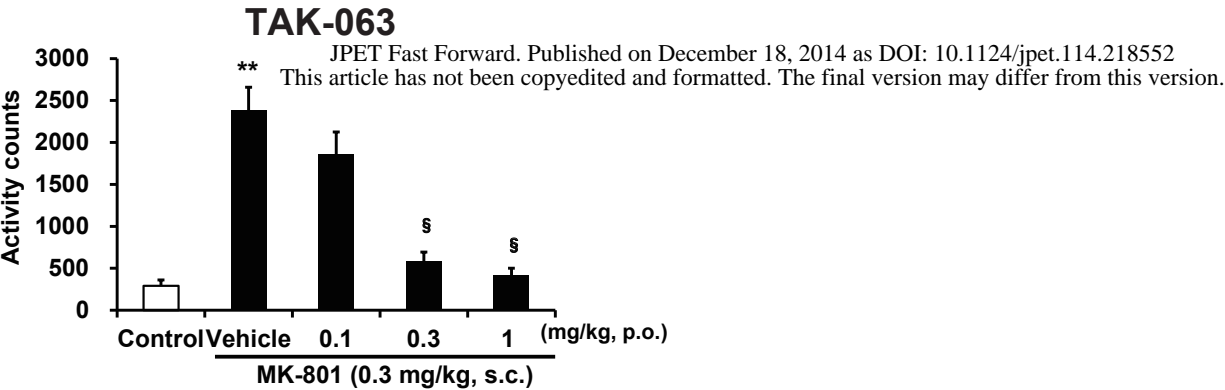


Fig. 3

A Mouse



B Rat

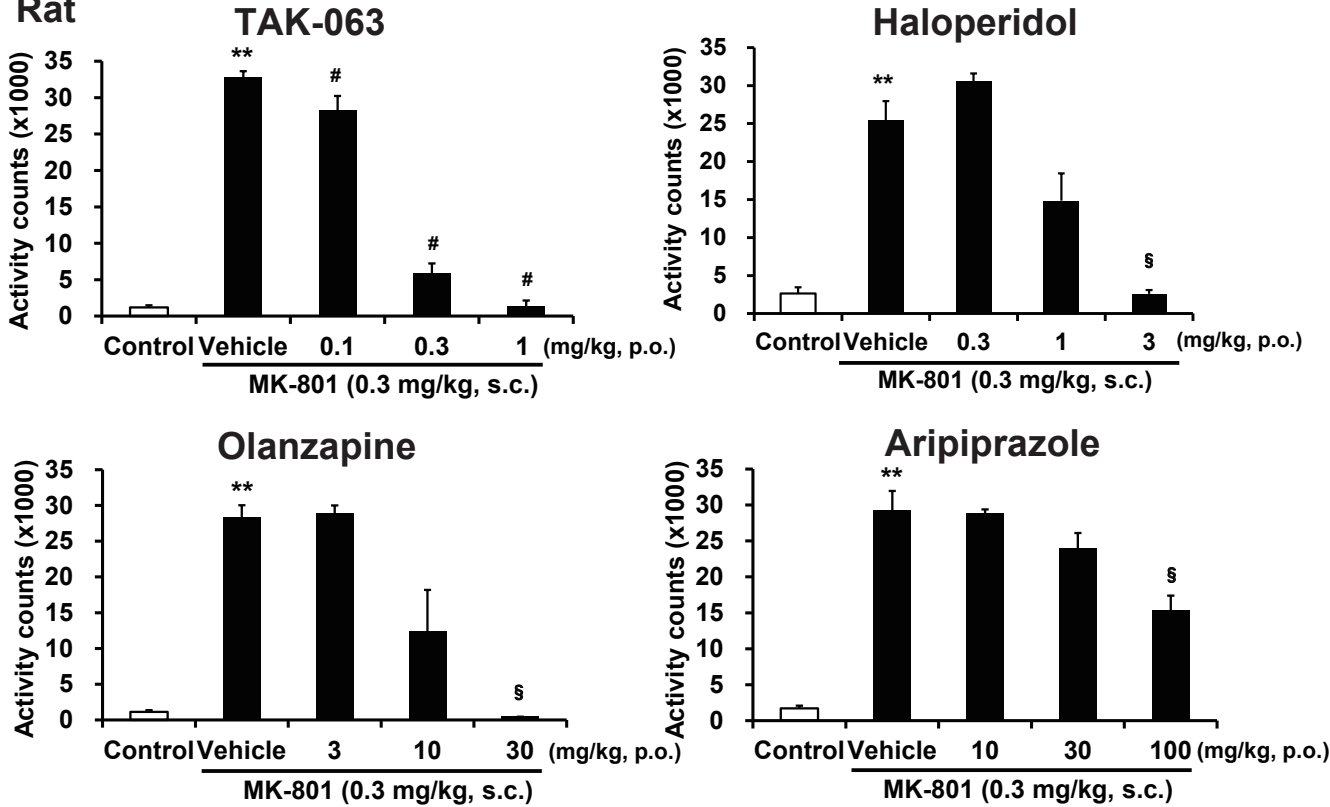
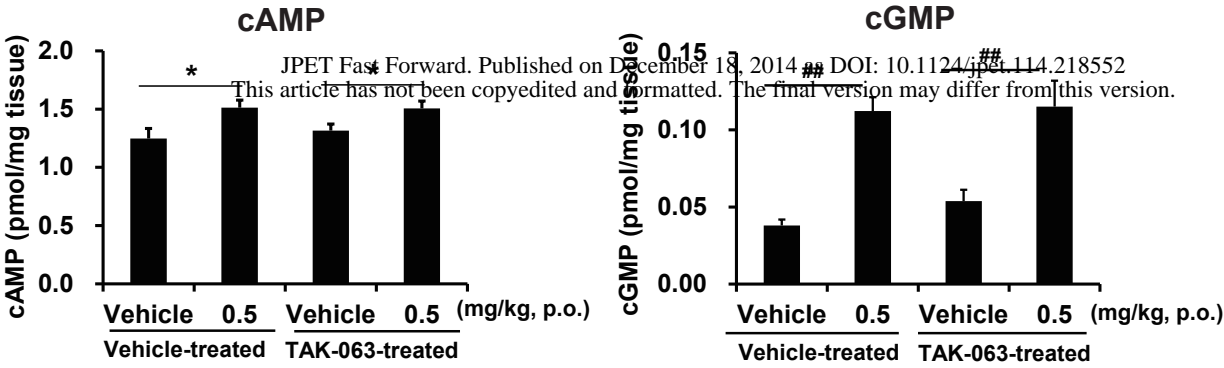


Fig. 4

A



B

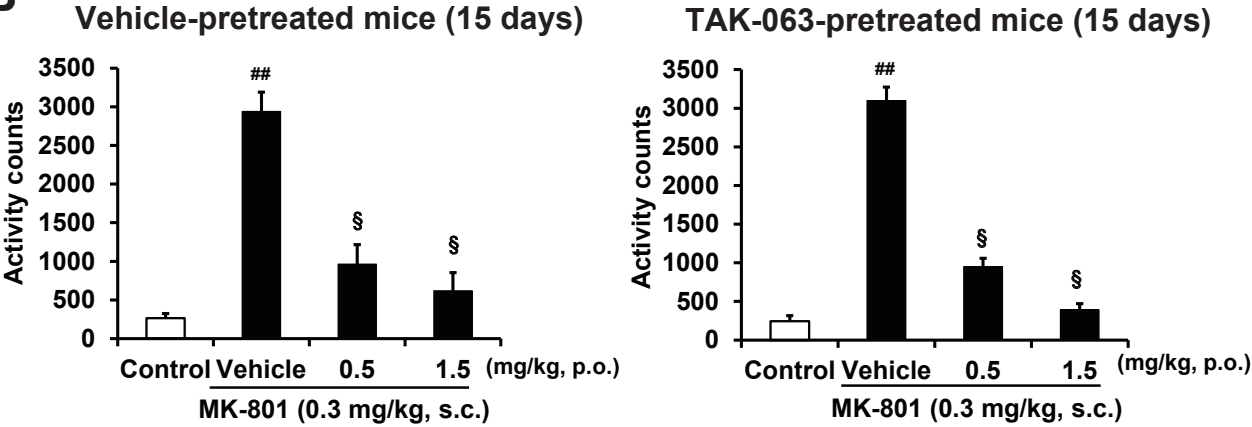


Fig. 5

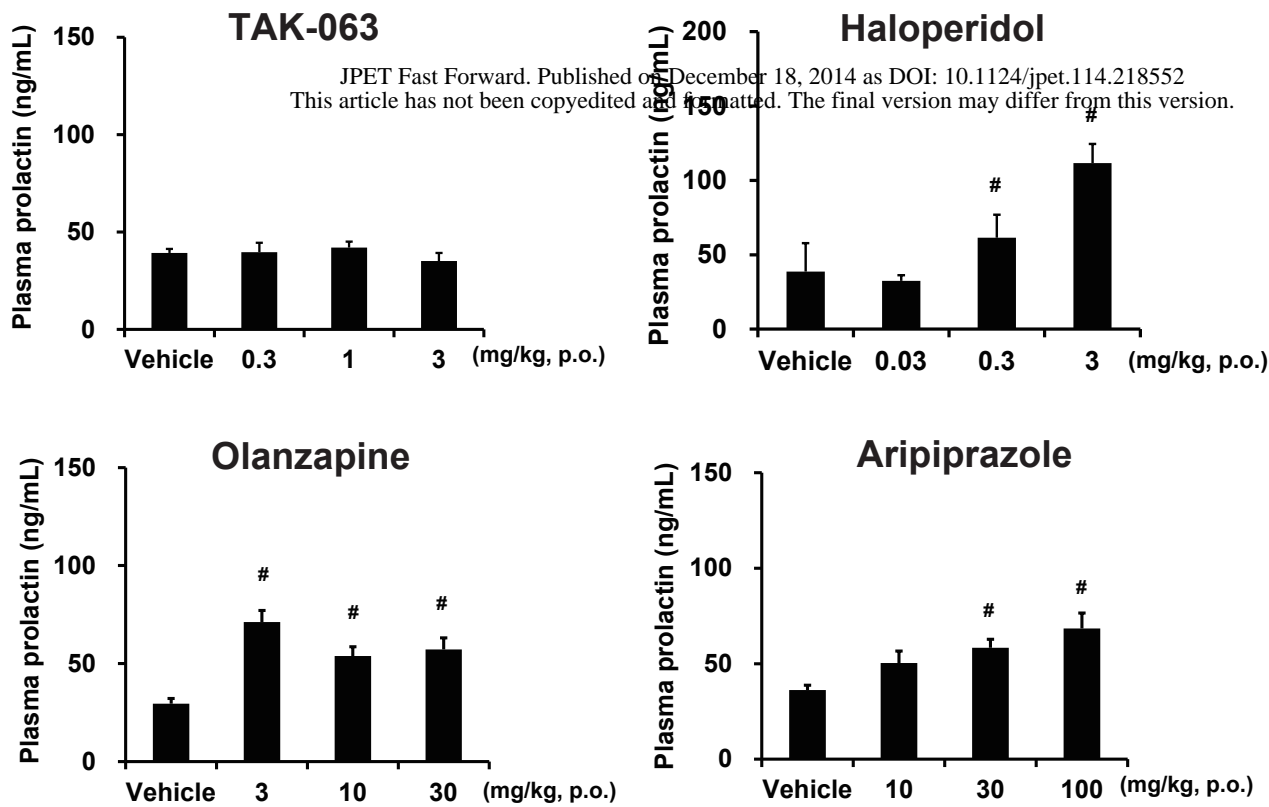
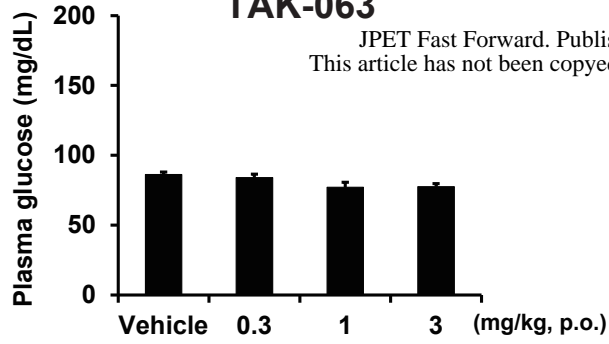


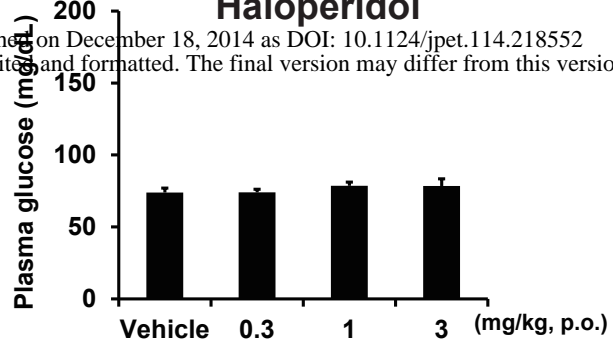
Fig. 6

TAK-063

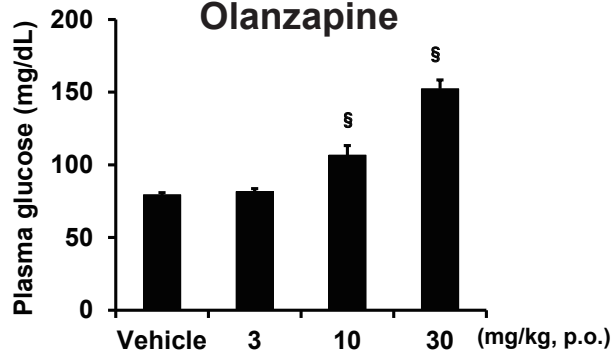
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Haloperidol



Olanzapine



Aripiprazole

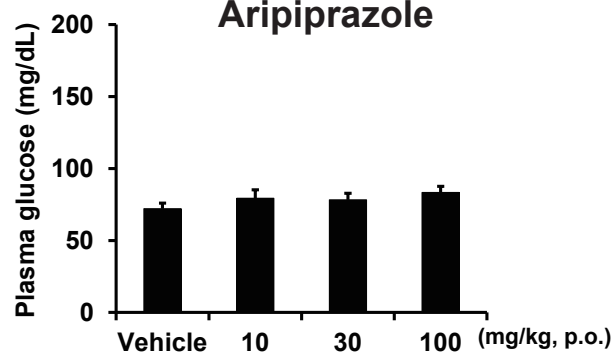


Fig. 7

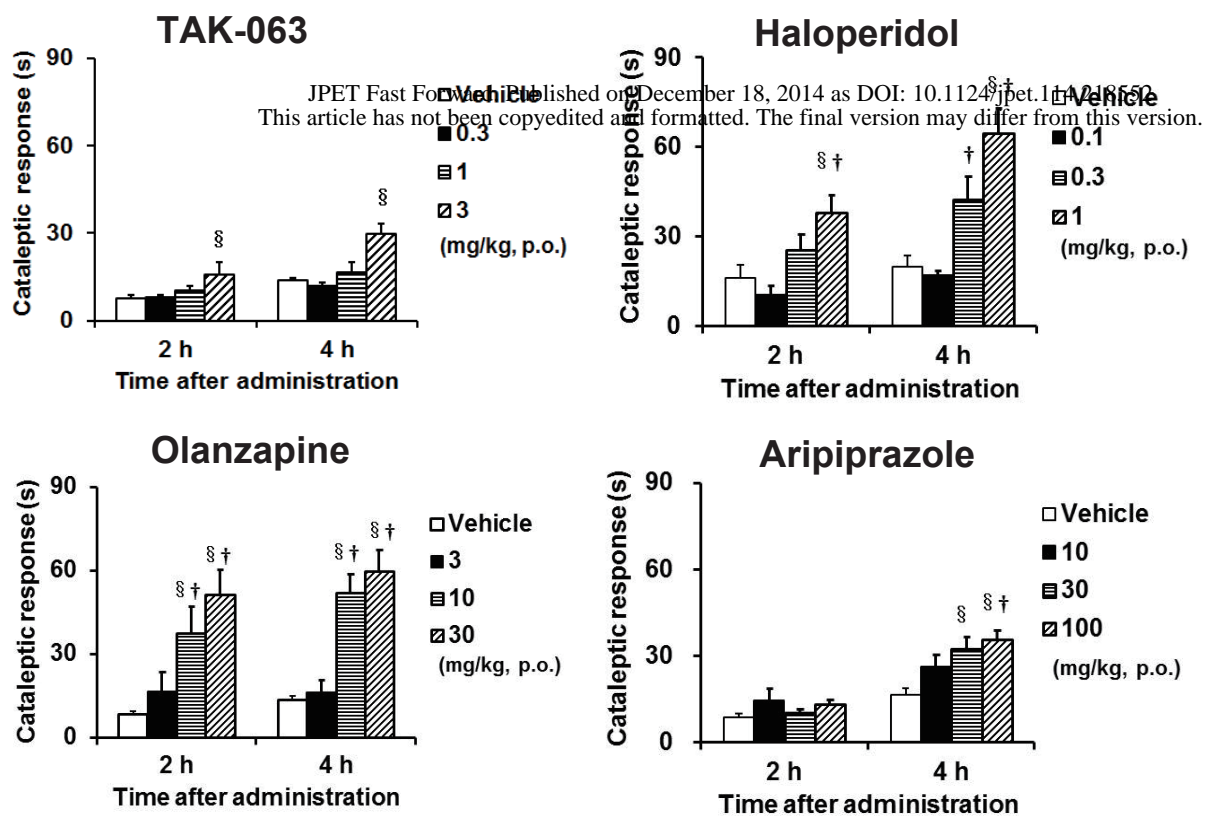
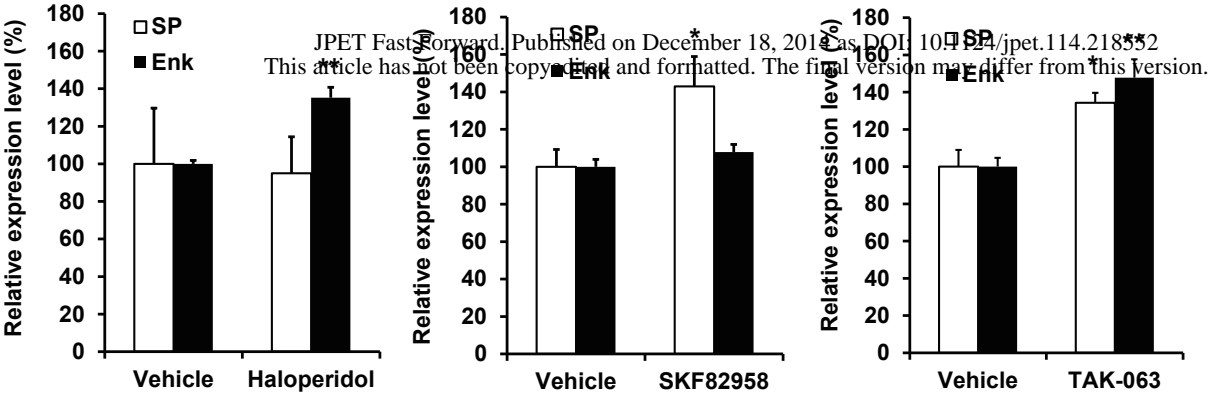


Fig. 8

A



B

