Study on the Effect of Phosphodiesterase 2A Inhibition in Cognitive Impairment

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Abstract

Cognitive impairment in the elderly, and in psychiatric and neurodegenerative diseases is a serious health problem worldwide, resulting in reduced quality of life, and a social and economic burden on the patients families and caregivers. There is an unmet medical need for novel therapeutic drugs that can slow down or restore cognitive impairment. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) have been suggested to regulate synaptic neurotransmission and plasticity, which are associated with multiple cognitive functions. Phosphodiesterase 2A (PDE2A), highly expressed in the forebrain, is one of the key phosphodiesterase enzymes that hydrolyses both cAMP and cGMP. Therefore, PDE2A inhibition has the potential to ameliorate cognitive impairment in diseases by modulating the neural circuit via the up-regulation of cAMP and cGMP levels in the brain. In the first chapter, to probe the potential of PDE2A inhibition as a treatment for schizophrenia, I investigated the effect of a brain penetrant and selective PDE2A inhibitor, TAK-915, in rat models of schizophrenia based on *N*-methyl-$_{D}$-aspartate (NMDA) receptor hypofunction. Oral administration of TAK-915 ameliorated cognitive impairment and social withdrawal induced by NMDA receptor antagonists in rats. This selective PDE2A inhibitor has therapeutic potential in cognitive impairment and the negative symptoms in schizophrenia. In the second chapter, the effect of PDE2A inhibition on the cognitive functions associated with aging were evaluated. The selective PDE2A inhibitor TAK-915 ameliorated age-related cognitive deficits in rats. Based on these findings, PDE2A inhibition demonstrated a beneficial effect on multiple cognitive domains, such as spatial learning, episodic memory, and attention, and may provide a new therapeutic option in patients with cognitive impairment.

Abbreviations:

5-CSRTT, 5-choice serial reaction time task

AMPA, (±)-α-amino-3-hydroxy-5-methylisoxasole-4-proprionic acid

AD, Alzheimers disease

cAMP, cyclic adenosine monophosphate

cGMP, cyclic guanosine monophosphate

CSF, cerebrospinal fluid

Enk, enkephalin

GAPDH, glyceraldehydes-3-phosphate dehydrogenase

LTP, long-term potentiation

mHb, medial habenula

METH, methamphetamine hydrochloride

MK-801, (+)-MK-801 hydrogen maleate

MP-10, 2-[4-(1-methyl-4-pyridin-4-yl-1*H*-pyrazol-3-yl)-phenoxymethy]-quinoline succinate

MSNs, medium spiny neurons

MWMT, Morris water maze task

NDI, novelty discrimination index

NMDA, *N*-methyl-$_{D}$-aspartate

NO, nitric oxide

NORT, novel object recognition test

NREM, non-rapid eye movement

PCP, phencyclidine

PDE, phosphodiesterase

PDE2A, phosphodiesterase 2A

pGluR1, phosphorylation of AMPA receptor subunit at serine 845

PKA, protein kinase A

PKG, protein kinase G

REM, rapid eye movement

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*)-carboxamid

General Introduction

Cognitive impairment in the elderly, psychiatric and neurodegenerative diseases is a serious health problem worldwide, reducing the quality of life and creating a social and economic burden on patients families and caregivers. In schizophrenia, cognitive impairments are present at the onset of illness, persistent through the course of the disease, and have been associated with long-term functional disability (Shamsi et al., 2011; Torgalsboen et al., 2015). Current antipsychotic medications are effective in managing positive symptoms, but are limited in their ability to alleviate cognitive impairment and negative symptoms such as social withdrawal. Impairments in the cognitive domains are also observed in the elderly, patients with mild cognitive impairment, and Alzheimers disease (AD). Acetylcholinesterase inhibitors, such as donepezil, are commonly used as the standard of care for symptomatic AD. However, the efficacy of acetylcholinesterase inhibitors is limited in patients with mild to moderate AD (Thompson et al., 2004; Deardorff et al., 2015; Anand et al., 2017). Additionally, the long-term use of these medications is problematic owing to the adverse effects, including nausea, diarrhea, and insomnia (Raina et al., 2008; Tan et al., 2014). Therefore, there is still an unmet medical need for new therapeutic drugs that slow down or restore cognitive impairment in the elderly and in patients with psychiatric and neurodegenerative diseases.

Second messengers propagate and amplify signals at receptors on the cell surface, and are important components in signal transmission cascades. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are intracellular second messengers produced by adenylate cyclase or guanylate cyclase,

respectively. Adenylate cyclase is activated by receptors that are coupled to Gs proteins. The activity of guanylate cyclase is regulated by *N*-methyl-$_{D}$-aspartate receptor (NMDA) receptor, and nitric oxide (NO). Cyclic AMP and cGMP activate cAMP and cGMP-dependent protein kinases, thus playing critical roles in various cellular functions including synaptic neurotransmission, plasticity, and neuroprotection.

Phosphodiesterase (PDE) is an enzyme that hydrolyzes cyclic nucleotides. The PDEs are classified into 11 families from PDE1 to PDE11 on the basis of their tissue distributions, amino acid sequence homology, and regulatory properties (Beavo, 1995; Lugnier, 2006). Additionally, based on substrate specificity, the PDE family is mainly divided into three categories: the cAMP-specific PDEs (PDE4, PDE7 an PDE8); the cGMP specific PDEs (PDE5, PDE6 and PDE9), the dual substrate PDEs (PDE1, PDE2, PDE3, PDE10 and PDE11) degrading both cAMP and cGMP with different affinities. Clinically, several PDE inhibitors have been used for treatments of acute heart failure, chronic obstructive pulmonary disease, and pulmonary arterial hypertension (Maurice et al., 2014). Since some PDE families are selectively expressed in specific brain regions, selective PDE inhibitors are attracting attention as promising drug targets for brain disorders.

Phosphodiesterase 2A (PDE2A) is a dual-substrate enzyme that hydrolyzes both cAMP and cGMP. It is also termed as cGMP-stimulated cAMP PDE as the binding of cGMP to the GAF domain of PDE2A allosterically stimulates its cAMP hydrolyzing activity. This suggests that PDE2A plays a key role in regulating the cross-talk between cAMP and cGMP signaling. PDE2A is highly expressed in the brain regions such as frontal

cortex, hippocampus, striatum, and amygdala, which regulate memory and emotion, whereas its expression in peripheral tissues is relatively low (Stephenson et al., 2009; Stephenson et al., 2012). Therefore, PDE2A inhibition can be expected to exert brain-specific actions without side effects on the periphery. As cyclic nucleotides have been suggested to regulate synaptic neurotransmission and plasticity associated with cognitive functions, a selective PDE2A inhibitor may demonstrate the potential to ameliorate cognitive impairments associated with psychiatric and neurodegenerative diseases by modulating the neural circuit via up-regulation of cAMP and cGMP levels in the brain. Pharmaceutical companies have investigated PDE2A inhibitors for brain disorders, however their candidate agents displayed problems including the potency of PDE2A inhibitory activity, selectivity against other PDE families, and pharmacokinetics profiles including brain penetration. Although ND-7001, developed by Neruo3D, advanced to the phase I study, its clinical development has been discontinued. In fact, ND-7001 was found to have an insufficient profile for both PDE2A inhibitory activity and PDE selectivity. Similarly, PF-999, developed by Pfizer, was not successful, and the reasons for discontinuation and the pre-clinical profiles have not yet been disclosed (Gomez and Breitenbucher, 2013). In preclinical studies, BAY-60-7550 is often used as a commercially available PDE2A inhibitor. However, this compound does not demonstrate sufficient brain-penetrant properties in rodents after oral administration (Reneerkens et al., 2013). Additionally, PDE2A constitutive knockout mice are not available for behavioral assays to investigate the role of PDE2A in cognitive functions due to their embryonic lethality (Assenza et al., 2018). Therefore, a PDE2A inhibitor with better PDEs selectivity and pharmacokinetic profile is necessary to elucidate the precise role of PDE2A inhibition in the brain.

The aim of this research was to characterize the effects of PDE2A inhibition on cognitive impairment in preclinical models using a selective and brain penetrant PDE2A inhibitor. In the first chapter, the effect of PDE2A inhibition on cognitive impairment have been demonstrated in rat models of schizophrenia. The second chapter illustrates the effect of PDE2A inhibition on cognitive decline associated with aging in rodent models.

Chapter I: The Phosphodiesterase 2A Inhibitor TAK-915 Ameliorates Cognitive Impairments and Social Withdrawal in *N*-methyl-D-aspartate Receptor Antagonist-Induced Rat Models of Schizophrenia

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 I characterized 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(1*H*)-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 up-regulated the phosphorylation of a-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptor subunit GluR1 in the rat hippocampus. TAK-915 at 3 and 10 mg/kg significantly attenuated the 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 the subchronic phencyclidine-induced social withdrawal in rats. In contrast,

TAK-915 did not produce antipsychotic-like activity; TAK-915 had little effect on MK-801- or methamphetamine-induced hyperlocomotion in rats. These results indicate the potential of TAK-915 to ameliorate cognitive impairment and social withdrawal in schizophrenia.

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 impairment and negative symptoms. Cognitive impairment is present at the onset of illness, and persistent through the course of the disease, and appears to be strong predictor 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). Collectively, 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 agents are required for patients with cognitive impairment 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 NMDA receptor antagonists such as ketamine and phencyclidine (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 the 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 in 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 therapeutic strategy in schizophrenia.

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., 2014; Bollen et al., 2015; Lueptow et al., 2016). However, these findings have not fully demonstrated the relationship between brain PDE2A inhibition and pro-cognitive activities in rodents. Most studies using PDE2A inhibitors lack adequate evidence in terms of pharmacokinetics and pharmacodynamics. For example, there are no reports available investigating brain cyclic nucleotide levels after BAY-60-7550 administration. This compound does not show sufficient brain penetrating properties in rodents after oral administration (Reneerkens et al., 2013). Therefore, a PDE2A inhibitor with better

pharmacokinetic profiles is required to elucidate the role of PDE2A inhibition in the brain and to clarify the relationship between brain cyclic nucleotide levels and behavioral outcomes.

A potent and selective PDE2A inhibitor, TAK-915 was recently discovered (Mikami et al., 2017). TAK-915 inhibited human PDE2A enzyme activities with a 50% inhibitory concentration of 0.61 nmol/L, which exhibited more than a 4100-fold selectivity against other PDE family members. In rodents, *in vitro* autoradiography studies revealed that [^{3}H]TAK-915 accumulated in the frontal cortex, hippocampus, and striatum (Ito et al., manuscript in preparation), where PDE2A expression levels are high (Stephenson et al., 2009; Stephenson et al., 2012). This selective accumulation of [^{3}H]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 under physiological conditions.

In this thesis, the *in vivo* pharmacological profile of a selective and brain penetrant PDE2A inhibitor, TAK-915, has been demonstrated as a potential treatment for schizophrenia. Firstly, to examine whether TAK-915 acts as a PDE2A inhibitor *in vivo*, the cyclic nucleotide contents and their downstream signaling in the brain were evaluated. Secondly, the pro-cognitive properties of TAK-915 were characterized in the NMDA receptor antagonist-induced deficit models. Thirdly, to investigate the potential of TAK-915 in social withdrawal, social interaction was evaluated in a subchronic PCP model. Finally, psychostimulant-induced hyperlocomotion tests were performed 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 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)-phenoxymethy]-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.). Olanzapine was extracted from Zyprexa® (Eli Lilly and Company, Indianapolis, IN) at

KNC Laboratories Co. Ltd. (Kobe, Japan). Olanzapine was dissolved in 1.5% (v/v) lactic acid. The pH of this solution was then adjusted to neutral using 1 M NaOH and administered p.o. 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., 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.

Measurement of CSF. Six-week-old male Long-Evans rats (Japan SLC Inc., Hamamatsu, Japan) were used for determination of cGMP levels in the CSF and hippocampus. Under pentobarbital anesthesia, CSF was collected from the cisterna magna 2, 4, 8, 16 or 24 h after the administration of vehicle or TAK-915 (30 mg/kg, p.o.). CSF samples were snap-frozen and maintained at -80 °C until use. After collecting CSF samples, the animals were euthanized by a focused microwave irradiation system MMW-05 (Muromachi Kikai Co. Ltd., Tokyo, Japan). Brain tissues were sampled and immediately frozen on dry ice. Samples were 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. Concentrations of cyclic nucleotides in samples were measured using enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI) in accordance with the manufacturer's instructions. Values were expressed as pmol per μL for CSF or pmol per mg tissue weight for the brain tissue.

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 (±)-α-amino-3-hydroxy-5-methylisoxasole-4-proprionic 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, 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 × 10 × 25 cm) connected to a non-illuminated ("dark") compartment (30 × 30 × 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 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., 2016; Nishiyama et al., 2017). An 8-arm radial maze with arms (50 × 10 × 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 at the end of each arm for 5 min. 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, 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 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 manufacturers 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 manufacturers 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′-CGTGGCGGTGGCGGTCTTTTT-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′-TGGTGAAGCAGGCGGCCGAG-3′.

Measurement of Plasma Prolactin Levels. Eight-week-old male SD rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were administered either vehicle or

TAK-915 (10, 30, or 100 mg/kg, p.o.) after a habituation period of >30 min. Two hours after administration, blood was collected from tail vein into a 1.5-ml Eppendorf tube containing 25 μL 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 the plasma samples were measured using an ELISA kit (Bertin Pharma, Montigny le Bretonneux, France).

Measurement of Plasma Glucose Levels. Seven-week-old male SD rats were fasted overnight and were euthanized 2 h after the administration of vehicle or TAK-915 (10, 30, or 100 mg/kg, p.o.). 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 catalepsy-like behavior of 7-week-old male SD rats was measured 2 h after the administration of vehicle, olanzapine (10 mg/kg, p.o.), or TAK-915 (10 or 100 mg/kg, p.o.) in a blind manner. Forelimbs were placed on a horizontal metal bar at a 13-cm height and the length of time during which both forelimbs remained on the bar (cataleptic response) was measured with a maximum limit of 90 s. The procedure was repeated 3 times and the cataleptic response time was averaged for each rat.

Cell Culture. Primary cortical neurons were prepared from fetuses of rats, which were extracted from a mother animal at 17-18 days of gestation. Cells were isolated using nerve-cell dispersion solutions (Sumitomo Bakelite, Tokyo, Japan) containing papain,

following the manufacturer's instructions. Isolated primary cells were suspended in neurobasal medium (Life technologies, CA) with B-27 supplement, Penicillin-Streptomycin and L-Glutamine (Life technologies), and plated onto poly-L-lysine-coated 96-well culture plates (Sumitomo Bakelite) at a density of 5×10^4 cells/ 100 μL/well. The plates were incubated at 37 °C under 5% CO_2. Medium (100 μL) was added to each well on day in vitro (DIV) 3 or 4 and half of the medium was renewed on DIV 6. For expression analysis of PDE1C, 2A, 4B, 4D, 8B, and 10A, rat cortical primary neurons were isolated on DIV 11. Tissues were homogenized in QIAzol Lysis Reagent (QIAGEN, UK), followed by total RNA extraction using RNeasy 96 Kit (QIAGEN). Complementary DNA was synthesized from 1000 ng of the total RNA using High Capacity cDNA Reverse Transcription Kit (Life technologies). Quantitative real-time PCR was performed with an ABI PRISM 7900HT sequence detection system (Life technologies) and qPCR MasterMix Plus without UNG (Eurogentec, Belgium). Primers, probes and standards for PDE1C, 2A, 4B, 4D, 8B, and 10A were purchased from Sigma-Aldrich. All procedures were performed in accordance with the manufacturers instructions.

Measurement of Intracellular Cyclic Nucleotides in Primary Cortical Neurons. TAK-915 and 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO), and were then diluted in neurobasal medium. All the solutions of TAK-915 and its vehicle contained 0.1% DMSO. All the solutions and vehicle were dispensed in a polypropylene 96-well plate and incubated at 37 °C until just before use. For evaluating the effects of TAK-915, rat neurons on DIV 11 were rinsed with Hanks balanced salt solution (Life technologies), and were incubated with

TAK-915 for 30 min at 37 °C. To inhibit the activity of other endogenous PDEs, 10 μM of IBMX was also added with TAK-915. Finally, all the solutions were decanted, and the cells were dissolved in 200 μL/well of lysis buffer. After being shaken on a plate shaker for 30 min, the cell lysates were transferred to a new polypropylene plate. Intracellular cyclic nucleotide concentration in the lysates was measured using the cAMP/cGMP enzyme immunoassay system (GE healthcare, UK), in accordance with the manufacturer's instructions.

Statistical Analysis. The Aspin-Welch test (for nonhomogeneous data) or Students *t*-test (for homogeneous data) was used for pairwise group comparison. In dose-response experiments, homogeneity of variances was using Bartletts 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 the oral administration of TAK-915, the concentration of TAK-915 in the brain of Long-Evans rats was 0.030 ± 0.021 μg/g at 1 mg/kg, 0.160 ± 0.016 μg/g at 3 mg/kg, and 0.331 ± 0.095 μg/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, the cyclic nucleotide contents were measured in the frontal cortex, hippocampus, and striatum in rats 2 h after oral administration. Oral administration of TAK-915 dose-dependently increased cGMP levels in these brain regions. Significant increase in cGMP level 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). However, 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 in cGMP levels in brain and CSF were well correlated with the changes in pharmacokinetics of TAK-915 (Fig. 2)

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

An increase in the 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, the phosphorylation levels of GluR1 in the hippocampus were investigated following oral administration of TAK-915 at 1, 3, and 10 mg/kg. As shown in Figure 3, TAK-915 dose-dependently increased pGluR1 in the rat 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 ± 14%, 130 ± 17%, and 148 ± 10%, respectively. A significant increase in the pGluR1 level was observed at 10 mg/kg, p.o. ($P \leq 0.05$; Fig. 3B).

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, the passive avoidance task in MK-801-treated rats was performed. As shown in Figure 4, the subcutaneous treatment with MK-801 at 0.1 mg/kg significantly decreased the avoidance time in the 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. 4B, $P \leq 0.05$ at 10 mg/kg; Fig. 4C).

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

Radial 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), the radial arm maze task was performed 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. 5). Treatment with MK-801 at 0.08 mg/kg, s.c. significantly increased the number of errors (5.6 ± 0.8, $P \leq 0.01$; Fig. 5). 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. 5).

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). The potential of TAK-915 in the treatment of negative symptoms was evaluated on subchronic PCP-induced social withdrawal by using the social interaction test in rats. Time spent in social interactions was significantly decreased in subchronic PCP-treated rats (30 ± 10 s) compared to the control group rats (83 ± 10 s) ($P \leq 0.01$, Fig. 6). TAK-915 (3 or 10 mg/kg, p.o.) dose-dependently attenuated subchronic PCP-induced deficits in the social interaction test (49 ± 6 s at 3 mg/kg, 72 ± 9 s at 10 mg/kg; Fig. 6). A significant effect in this test was observed at 10 mg/kg, p.o. ($P \leq 0.05$; Fig. 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.

MK-801- or METH-induced hyperlocomotion test has commonly been 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 the psychostimulant-induced hyperlocomotion (Suzuki et al., 2015). As shown in Figure 7A, 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. 7B). Activation of the indirect pathway MSNs by the blockade of dopamine D_2 receptors is thought to be a common mechanism of action of the 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, the striatal mRNA expression levels of SP (a direct pathway marker) and Enk (an indirect pathway marker) were measured. MP-10, developed by Pfizer Inc., is a potent and selective PDE10A inhibitor (Grauer et al., 2009; Verhoest et al., 2009). MP-10 was used as a positive control, 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 significantly increased the expression of both SP and Enk mRNA ($P \leq 0.01$ for SP and $P \leq 0.01$ for Enk; Fig. 7C). In contrast, TAK-915 did not affect the expression of either SP or Enk mRNA even at 100 mg/kg (Fig. 7C). Additionally, TAK-915 was evaluated for any possible side effects of antipsychotic medications such as hyperprolactinemia, hyperglycemia, or cataleptic response. TAK-915 did not increase prolactin (Fig. 8A) or glucose levels (Fig. 8B) in the rat plasma even at 100 mg/kg. Cataleptic response was assessed by the bar test 2 h following the administration of TAK-915. TAK-915 at 10 and 100 mg/kg did not significantly increase the cataleptic response in this test (Fig. 9).

Discussion

In the present study, I demonstrated that TAK-915 works as a brain penetrant PDE2A inhibitor *in vivo*. As shown in Figure 1B, a significant increase in cGMP levels in the hippocampus were observed at 3 mg/kg and 10 mg/kg of TAK-915. 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 suggested 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 the experimental conditions (Fig. 1). There is a possibility that I failed to detect a significant effect of cAMP levels by TAK-915 owing to the high baseline for cAMP contents in brain tissues (Fig. 1). However, these observations are in accordance with previous reports that PDE2A inhibition in the brain mainly influences cGMP levels rather than cAMP levels (Suvarna and ODonnell, 2002; Boess et al., 2004). Additionally, TAK-915 dose-dependently increased cGMP levels in rat primary neurons whereas a significant change in cAMP level was not observed (Fig. 10). 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 primarily responsible for degrading cAMP in the brain, and play a greater role in regulating cAMP hydrolysis in the brain compared to PDE2A.

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 TAK-915 administered 2 h prior to the acquisition trial attenuated MK-801-induced episodic memory deficits (Fig. 4). As TAK-915 increased cGMP levels in the rat brain 2 h after administration (Fig. 1), the enhancement of memory acquisition mediated through cGMP could contribute to the improvement in episodic memory observed in the retention trial. Additionally, it has been reported that cGMP-PKG signaling mediates early consolidation, and late consolidation requires cAMP-PKA signaling (Bollen et al., 2014). Considering 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 are 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 is involved in synaptic plasticity such as long-term potentiation (LTP) (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 leads to an 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, 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 is reportedly required for hippocampus-dependent learning in the passive avoidance task (Mitsushima et al., 2011). The effect of TAK-915 on the hippocampal pGluR1 levels, associated with activation of downstream pathways of cyclic nucleotide signaling, was investigated. TAK-915 at 10 mg/kg significantly increased hippocampal pGluR1 in rats (Fig. 3), 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 the 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 have also been consistently reported in schizophrenia patients (Piskulic et al., 2007) and are 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, the effects of TAK-915 were assessed in 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. 4 and 5). Based on *in vitro* autoradiography studies using rat brain slices, [^{3}H]TAK-915 accumulated to high levels in the 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 pivotal roles in spatial

and episodic information processing (OMara 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 the negative symptoms in schizophrenia, generally persisting through the course of the illness and contributing to poor psychosocial functioning (Pogue-Geile and Harrow, 1985; Morrison and Bellack, 1987; Puig et al., 2008). To investigate the potential efficacy of TAK-915 in social withdrawal, the subchronic PCP model was used. 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 resembles the negative symptoms, particularly social withdrawal (Jenkins et al., 2008; Neill et al., 2014). As shown in Figure 6, subchronic PCP treatment (5 mg/kg, i.p., twice daily for 7 days) in rats significantly reduced the social interaction time followed by at least 8-day washout, indicating that this treatment may cause abnormalities in the neural system and/or structures associated with social behaviors. TAK-915 at 10 mg/kg significantly attenuated this reduction in time spent in social interaction (Fig. 6). These results suggest that TAK-915 prevents social withdrawal in the subchronic PCP model relevant to schizophrenia.

Currently, the antipsychotic drugs used in clinical practice demonstrate dopamine D_2

blockade, such as haloperidol, and lead to an activation of the indirect pathway in the striatal MSNs, which is thought to be the mechanism of the observed 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. 7A and 7B), and did not activate either direct or indirect pathway MSNs in the striatum (Fig. 7C). Similarly, 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. 7C). Behavioral outcomes of PDE2A inhibition are different from those of PDE10A inhibition, although PDE2A and PDE10A are both highly expressed in striatal MSNs, and their 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 the striatal MSNs.

Although the mechanism by which TAK-915 attenuates cognitive impairment and social withdrawal induced by NMDA receptor antagonists remains unclear, our findings suggest that TAK-915 provides a strategy for ameliorating these behavioral deficits through the up-regulation of cyclic nucleotides, mainly cGMP. Previous studies suggest that PDE2A inhibition can enhance the NMDA receptor/NO/cGMP pathway (Suvarna and ODonnell, 2002; Boess et al., 2004). TAK-915 attenuated cognitive

deficits and social withdrawal in rats at a similar dosage that up-regulated 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 impact of PDE2A inhibitors on 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, the effects of which 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.

Conclusion

TAK-915 ameliorates cognitive impairment and social withdrawal induced by NMDA receptor antagonists in rodents. This selective PDE2A inhibitor demonstrates therapeutic potential for cognitive impairment and the negative symptoms in schizophrenia.

Table and Figures

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 ± S.D., n = 3 each group.

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

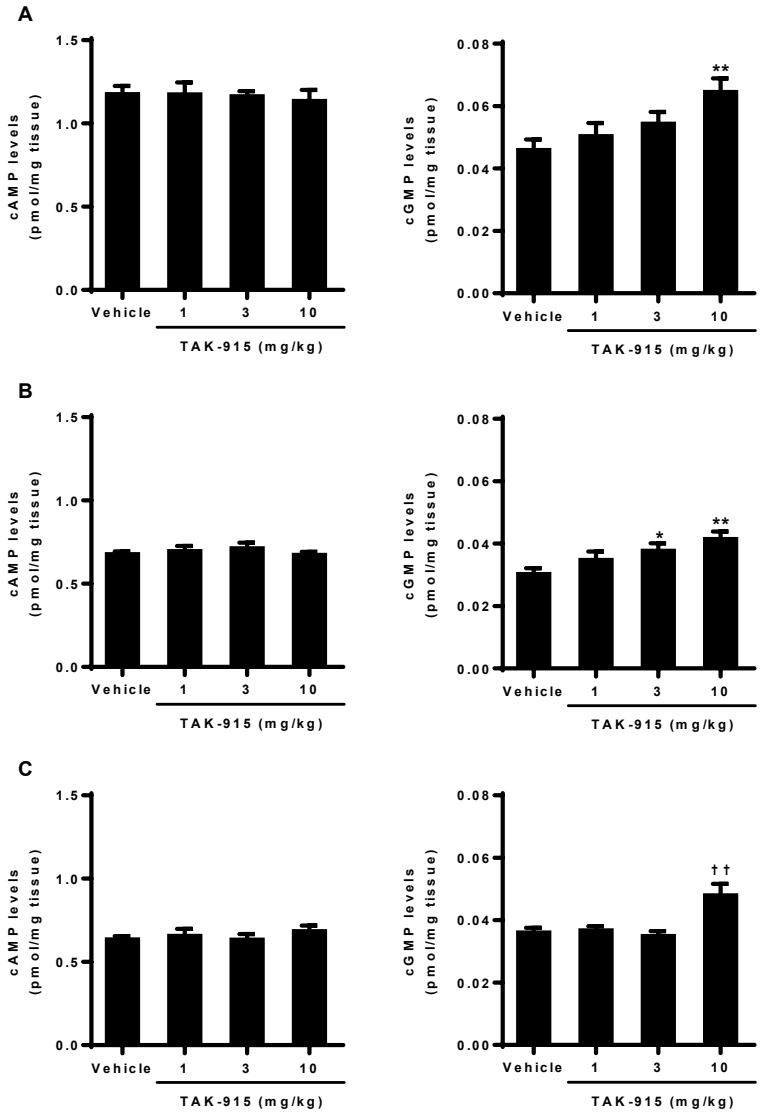

Figure 1. Effects of TAK-915 on cAMP and cGMP levels in the frontal cortex, hippocampus and striatum in rats. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) was administered 2 h before sampling. Cyclic nucleotide contents in the frontal cortex (A), hippocampus (B), and striatum (C) were measured using 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).

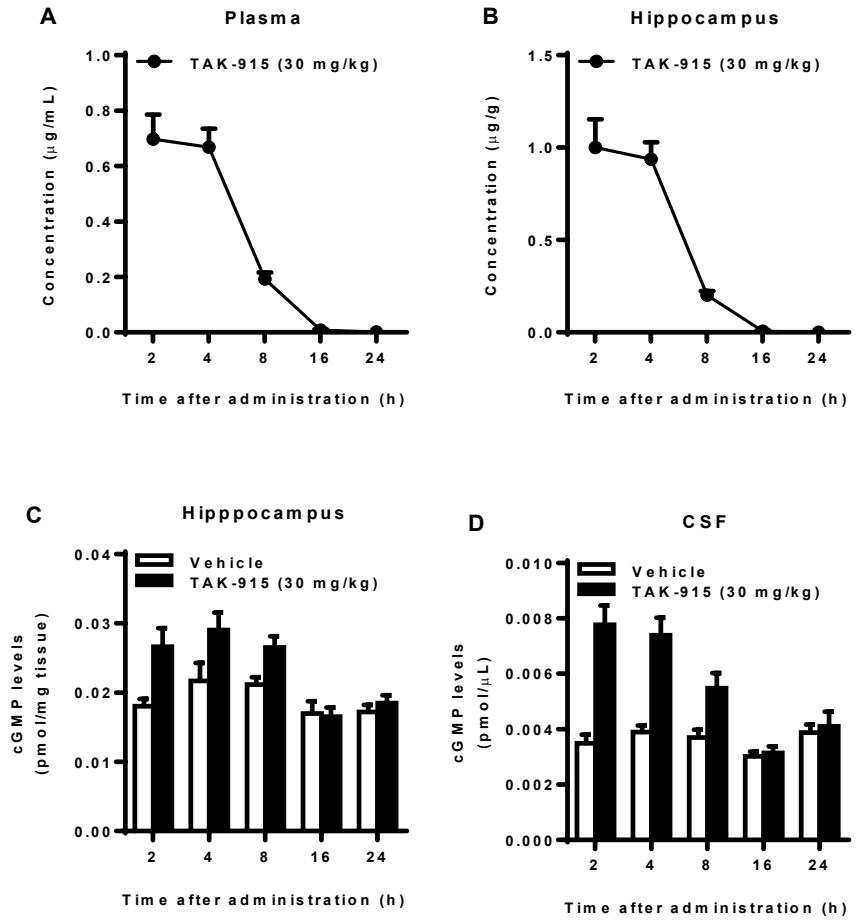

Figure 2. Time course of pharmacokinetics profiles and cGMP levels in brain following administration of TAK-915 in rats. The concentration of TAK-915 in plasma (A) and hippocampus (B), and cGMP levels in the hippocampus (C) and in the CSF (D) are shown. Samples were collected 2, 4, 8, 16, and 24 h following the administration of TAK-915 (30 mg/kg, p.o.). cGMP level was measured using enzyme immunoassay kits. Data are expressed as mean + S.E.M., n = 7 per each group.

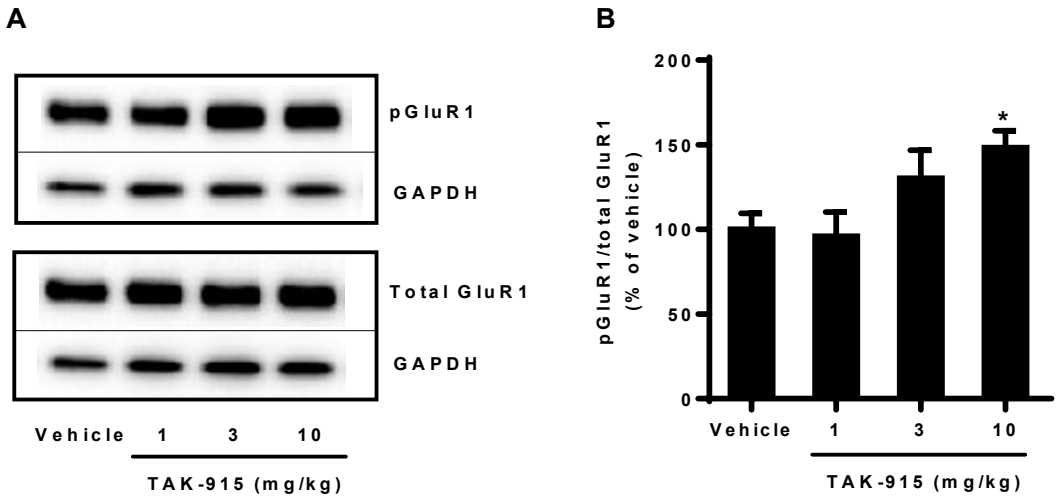

Figure 3. Effects of TAK-915 on pGluR1 levels in the rat hippocampus. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) was administered 2 h before collecting the rat hippocampus. (A) Representative blots were probed with primary antibodies for pGluR1, GAPDH, and total GluR1. (B) The intensity of the pGluR1 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).

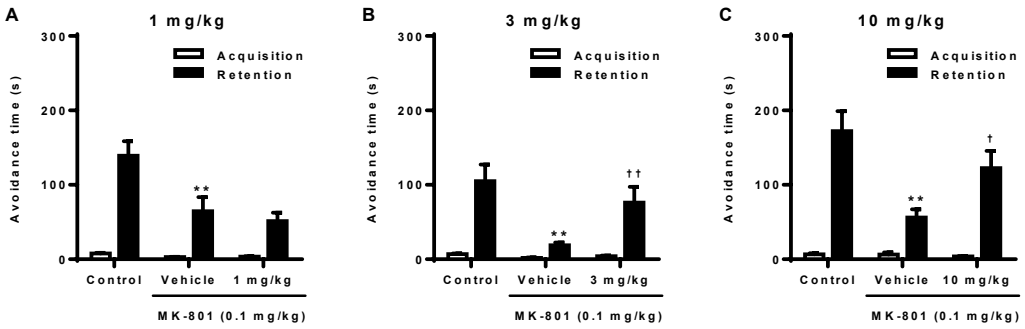

Figure 4. Effects of TAK-915 on MK-801-induced episodic memory deficits in the step-through passive avoidance task in rats. Vehicle or TAK-915 (A: 1 mg/kg, p.o., B: 3 mg/kg, p.o., C: 10 mg/kg, p.o.) was administered 2 h prior, and saline or MK-801 (0.1 mg/kg, s.c.) was administered 30 min prior to the acquisition trial. The latency to dark compartment 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 Wilcoxons test), $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, (versus vehicle-MK-801 by two-tailed Wilcoxon's test).

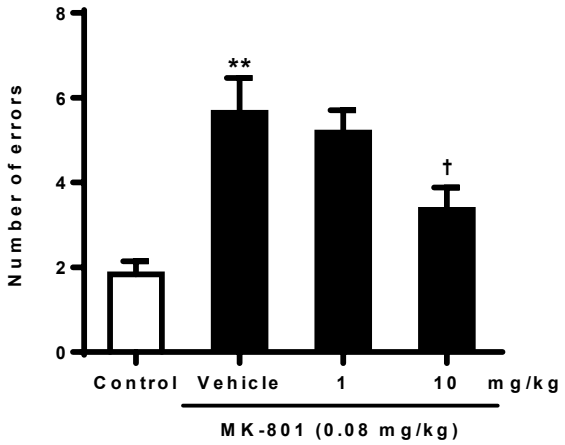

Figure 5. Effects of TAK-915 on MK-801-induced working memory deficits in the radial arm maze task in rats. Vehicle or TAK-915 (1 or 10 mg/kg, p.o.) was administered 2 h prior to testing and saline or MK-801 (0.08 mg/kg, s.c.) was administered 0.5 h prior to testing. The numbers of errors are expressed 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).

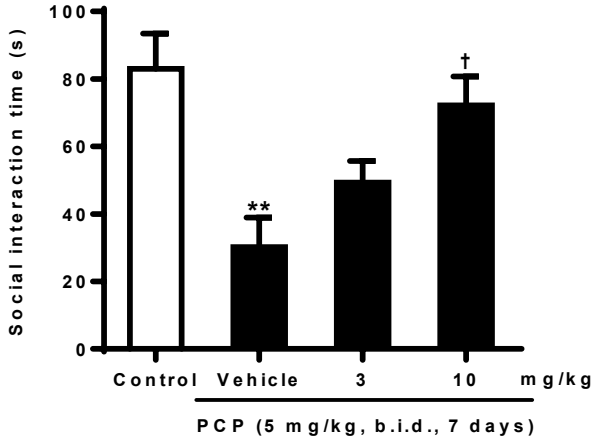

Figure 6. Effects of TAK-915 on subchronic phencyclidine-induced social withdrawal in the social interaction test in rats. Saline or phencyclidine (PCP, 5 mg/kg, i.p.) was administered twice daily (b.i.d.) from day 1 to day 7. After an 8-day wash-out period, vehicle or TAK-915 (3 or 10 mg/kg, p.o.) was 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 Student's *t*-test). $^{\dagger}P \leq 0.05$ (versus vehicle-PCP by two-tailed Williams' test).

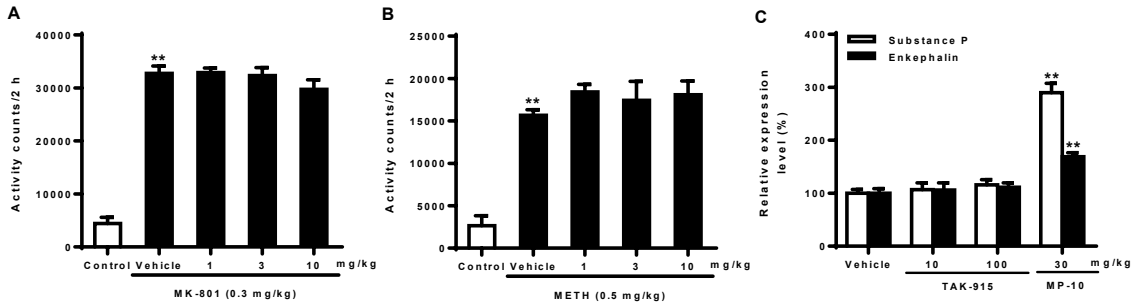

Figure 7. Effects of TAK-915 on MK-801- or METH-induced hyperlocomotion, and on activation of direct and indirect pathway medium spiny neurons in rats. (A and B) Effects of TAK-915 on MK-801 (A) or methamphetamine (METH) (B) induced hyperlocomotion in rats. Vehicle or TAK-915 (1, 3, or 10 mg/kg, p.o.) 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 SP (a direct pathway marker) and 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).

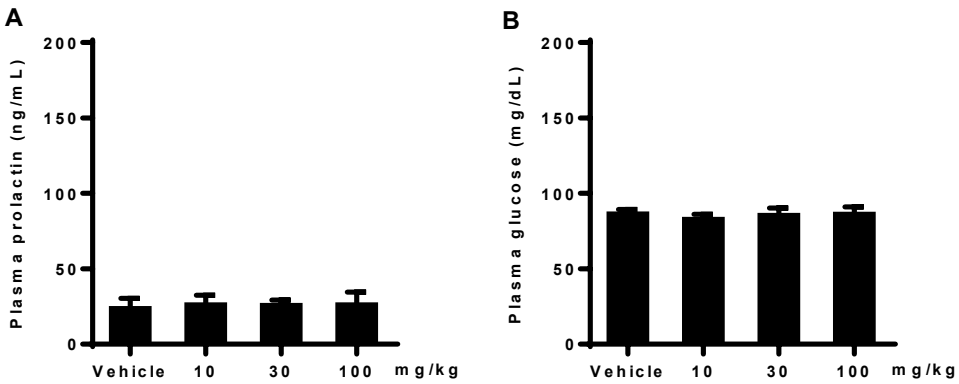

Figure 8. Effects of TAK-915 on plasma prolactin and glucose levels in rats. Blood samples were collected from the tail vein 2 h after the administration of TAK-915 (10, 30, or 100 mg/kg, p.o.). (A) The plasma prolactin concentration was determined by enzyme immunoassay kits. (B) The plasma glucose concentration was determined by colorimetric detection using a chemical analyzer. Data are expressed as mean + S.E.M., n = 5 per each group.

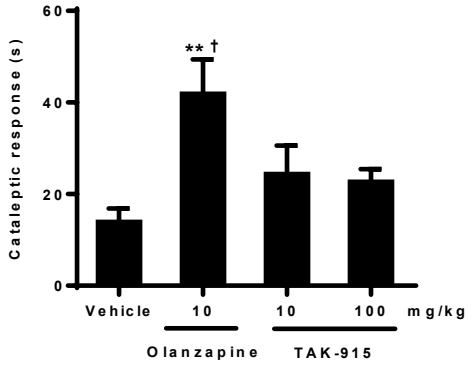

Figure 9. Effects of TAK-915 on cataleptic response in rats. Duration of cataleptic response was measured using the bar test 2 h after the administration of TAK-915 (10 or 100 mg/kg, p.o.) and olanzapine (10 mg/kg, p.o.). Data are expressed as mean + S.E.M., n = 8 for each group, $^{**}P \leq 0.01$ (versus vehicle by Student's *t*-test). †Occurrence of animals in a cataleptic position for more than 90 s.

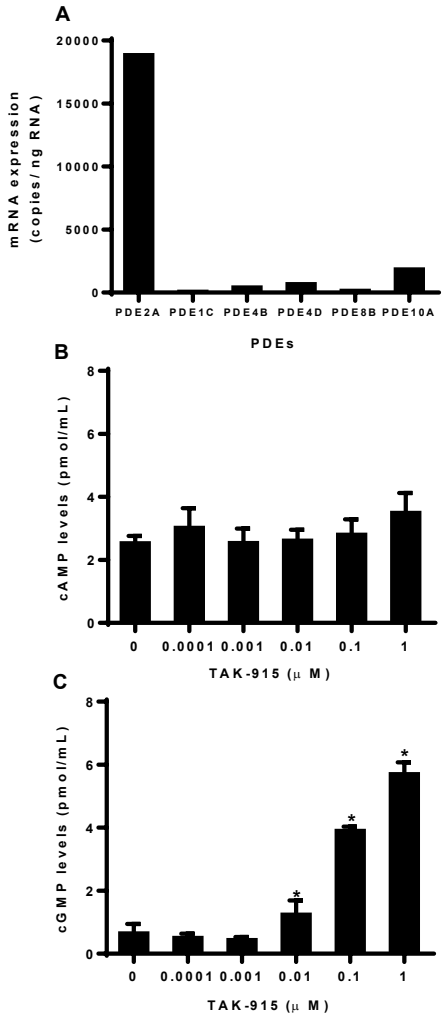

Figure 10. Effects of TAK-915 on cAMP and cGMP levels in the rat primary cortical neurons. (A) Expression of mRNA for PDE2A, PDE1C, PDE4B, PDE4D, PDE8B, and PDE10A in the rat cortical neurons on day in vitro (DIV) 11 were measured using quantitative real-time PCR. (B and C) cAMP (B) and cGMP (C) levels in TAK-915 (0.0001-1 μM)-treated neurons were measured using enzyme immunoassay kits. Data are expressed as mean + S.E.M., n = 3. $^{**}P \leq 0.01$ (versus vehicle by two-tailed Williams' test).

Chapter II: TAK-915, a Phosphodiesterase 2A Inhibitor, Ameliorates the Cognitive Impairment Associated with Aging in Rodent Models

Abstract

Changes in the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) signaling are implicated in older people with dementia. Drugs that modulate the cAMP/cGMP levels in the brain might therefore provide new therapeutic options for the treatment of cognitive impairment in aging and elderly with dementia. Phosphodiesterase 2A (PDE2A), which is highly expressed in the forebrain, is one of the key phosphodiesterase enzymes that hydrolyze cAMP and cGMP. In this study, the effects of PDE2A inhibition on the cognitive functions associated with aging, such as spatial learning, episodic memory, and attention, in rats with a selective, 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(1*H*)-carboxamide (TAK-915) were investigated. Repeated treatment with TAK-915 (3 mg/kg/day, p.o. for 4 days) significantly reduced the escape latency in aged rats in the Morris water maze task compared to the vehicle treatment. In the novel object recognition task, TAK-915 (1, 3, and 10 mg/kg, p.o.) dose-dependently attenuated the non-selective muscarinic antagonist scopolamine-induced memory deficits in rats. In addition, oral administration of TAK-915 at 10 mg/kg significantly improved the attention in middle-aged, poorly performing rats in the 5-choice serial reaction time task. These findings suggest that PDE2A inhibition in the brain has the potential to ameliorate the age-related cognitive decline.

Introduction

Aging is a major risk factor in dementia, often accompanied by a cognitive decline. Impairments in the cognitive domains, such as spatial learning, episodic memory, and attention, are observed in the elderly, patients with mild cognitive impairments, and the early stages of AD, although individual differences have been reported (Reid et al., 1996; Perry et al., 2000; Lithfous et al., 2013; Huntley et al., 2017; Mortamais et al., 2017). The cognitive decline in the elderly contributes to gradual loss of daily functioning and decrease in quality of life, resulting in considerable costs to society and family caregivers. Therefore, cognition-enhancing drugs are needed as an approach to prevent age-related cognitive impairments and reduce the incidence of dementia.

Changes in the cAMP and cGMP signaling in the brain are associated with aging and AD. This is supported by findings including, a reduced adenylyl cyclase activity in the brain of patients with AD (Cowburn et al., 1992), reduced NO-soluble guanylyl cyclase in the brains of older people aged 60-90 years old (Ibarra et al., 2001), as well as the correlation between changes in the cyclic nucleotide levels in the CSF and the cognitive performance in patients with AD (Ugarte et al., 2015). Multiple cognitive domains such as spatial learning, episodic memory, and attention are to be regulated by cAMP/cGMP and their downstream signaling cascades, which are mediated by PKG and PKA (Taylor et al., 1999; Paine et al., 2009; Bollen et al., 2014; Heckman et al., 2015). The increase in the cAMP/cGMP levels plays an important role in neurotransmitter release (Schoffelmeer et al., 1985; Arancio et al., 1995; Neitz et al., 2011) and in neural plasticity changes, such as enhancement of LTP (Kleppisch and Feil, 2009). Therefore, enhancing cAMP/cGMP signaling may be a promising strategy

to ameliorate impairments in multiple cognitive domains in age-related diseases.

PDEs are enzymes that degrade the intracellular cyclic nucleotides. They are currently classified into 11 types. PDE2A is a dual substrate enzyme that hydrolyzes both cAMP and cGMP. PDE2A is highly expressed in the forebrain, which is associated with cognitive function, whereas its expression in the peripheral tissues is relatively low (Stephenson et al., 2009; Stephenson et al., 2012). The cognition-enhancing effects of PDE2A inhibition, under physiological and pathological conditions, have previously been investigated. The PDE2A inhibitor BAY 60-7550 demonstrated enhanced cognitive effects in aged rats (Domek-Lopacinska and Strosznajder, 2008), in a APPsw/PS1dE9 mouse model of AD (Sierksma et al., 2013). These effects are also supported by the finding that PDE2A inhibition has been shown to modulate the short and/or long terms of synaptic plasticity in rodents, including LTP and paired-pulse facilitation, linked to memory formation (Boess et al., 2004; Fernandez-Fernandez et al., 2015). Taken together, PDE2A inhibition is expected to ameliorate the cognitive impairments associated with aging. Several PDE2A inhibitors, such as ND-7001 and PF-999, have been tested in clinical trials. These PDE2A inhibitors, however, were not successful and the reasons for their discontinuation have not yet been disclosed (Gomez and Breitenbucher, 2013). TAK-915 is a selective PDE2A inhibitor that exhibits more than 4100-fold higher selectivity for PDE2A than for other PDEs, has good brain penetration, and an attractive overall pharmacokinetic profiles (Mikami et al., 2017; Nakashima et al., 2018). In a previous study, I demonstrated that oral administration of TAK-915 increased the cGMP levels and up-regulated the phosphorylation of a-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA)

receptor subunit GluR1, which is one of the downstream targets of cyclic nucleotides in the rat hippocampus (Nakashima et al., 2018). TAK-915 ameliorated deficits in several cognitive domains, including episodic memory and working memory in rat models of schizophrenia.

In this study, I investigated the effect of PDE2A inhibition on the cognitive functions associated with aging using TAK-915. First, I assessed the effect of TAK-915 on spatial learning in aged rats using the Morris water maze task (MWMT). Second, the effect of TAK-915 on episodic memory deficits induced by scopolamine was evaluated using the novel object recognition test (NORT). Finally, I conducted the 5-choice serial reaction time task (5-CSRTT) to investigate the effect of TAK-915 on attention in middle-aged rats.

Materials and Methods

All studies were performed in accordance with the guidelines of the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited, Shonan Health Innovation Park, Fujisawa, Japan, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Animals. Specific details of the strain and species are given within each section. The animals 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. Animals were allowed a habituation period of one-week or greater before initiation of experiments.

Drugs. TAK-915 was synthesized at Takeda Pharmaceutical Company Limited, Fujisawa, Japan (Mikami et al., 2017). Donepezil hydrochloride (Megafine Pharma (P) Ltd., Mumbai, India) and scopolamine hydrobromide (Tocris Bioscience, Minneapolis, MN, USA) were used in this study. TAK-915 and donepezil were suspended in 0.5% (w/v) methylcellulose in distilled water and administered orally (p.o.). Scopolamine was dissolved in saline and administered subcutaneously (s.c.). The dosage of the compounds corresponds to molecular weight of salt. The volume of administration was 2 mL/kg body weight.

Morris Water Maze Task. This task was assessed in 10-week-old and 26-month-old male F344 rats (Charles River Laboratories Japan Inc., Tokyo, Japan) as previously described (Takahashi et al., 2010), with some modifications. In order to reduce the individual difference in the swimming abilities, the visible platform test was conducted before the hidden platform test. Each rat was trained in a circulator tank (120 cm in diameter and 45 cm in depth), which was then filled with water (24 ± 1 °C) to a depth of 35 cm. A platform (10 × 10 cm) covered with green-painted wire mesh was located in the center of the circulator tank, and its surface was exposed 2 cm above the water surface. Each trial was started when the rat was placed in the water facing the wall of the water tank. The sequence of starting points was selected randomly in one of four equally spaced start locations. In the visible platform test, each rat was given four trials in a day, and the latency which each rat required to escape onto the visible platform was recorded. Based on the averaged escape latency, the aged rats were allocated into two groups for the hidden platform test. In the hidden platform test, a transparent

platform (10 × 10 cm) was located in the middle of one quadrant, equidistant from the center and edge of the tank, 1 cm below the surface of the water. The tank was placed in an experimental room with many visible cues, which were all kept constant during the test period. Each rat underwent two trials daily with a 1-h inter-trial interval (ITI) for 4 consecutive days. After reaching the hidden platform, the rat was allowed to remain on it for about 10 s. Rats that failed to find the platform within 120 s were guided to it by hand. The escape latency and the swimming speed of the rats to find the hidden platform were automatically recorded by a computer analyzing system (CompACT VAS Ver.3.0, Muromachi Kikai Co., Ltd., Tokyo, Japan). The value of the data was the mean of two trials. During the hidden platform test (days 1- 4), a vehicle or TAK-915 (3 mg/kg) was administered p.o. 1 h before the testing. The probe test was conducted 24 h after the hidden platform test. During the probe test, the platform was not present, and each rat was allowed to swim for 60 s. The percent of time spent in the target quadrant was recorded and analyzed.

Novel Object Recognition Test. This test was assessed in 7-week-old male Long-Evans rats (CLEA Japan Inc., Tokyo, Japan) as described previously (Bevins and Besheer, 2006; Shiraishi et al., 2016), with minor modifications. On day 1, the rats were habituated to the experimental room for over 1 h, and then each animal was allowed to habituate to the empty test box (a gray-colored polyvinyl chloride box of 40 × 40 × 50 cm) for 10 min. This test consists of both acquisition and retention trials. These trials were separated by a 4-h ITI. In the acquisition trial on day 2, the rats were allowed to freely explore two identical objects (A1 and A2) for 3 min. In the retention trial, rats were again allowed to explore a familiar object (A3) and a novel object (B)

for 3 min. The object exploration was defined with the rats' licking, sniffing, or touching the object with the fore-limbs while sniffing. Leaning against the object to look upward and standing or sitting on the object was excluded. The exploration time was scored manually for each subject (A1, A2, A3, and B). The data of the rats with an object exploration totaling less than 10 s were excluded. Novelty discrimination index (NDI) was calculated as follows: novel object interaction time/total interaction time × 100 (%). Vehicle or TAK-915 (1, 3 or 10 mg/kg) was administered p.o. 2 h before the acquisition trial. Saline or scopolamine (0.1 mg/kg) was administered s.c. 30 min before the acquisition trial.

Measurements of Acetylcholine Release in the Hippocampus of Rats. The microdialysis studies were conducted using 7-week-old male SD rats (Charles River Laboratories Japan). The rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and fixed on a stereotaxic frame (Narishige, Tokyo, Japan). A dummy cannula was implanted through the hippocampus (coordinates: 3.8 mm posterior to the bregma, 1.8 mm lateral to the midline, and 2.2 mm ventral to the bregma). Brain dialysis was performed 48 h after the implantation of the dummy cannula in the animals following replacement of the cannula with a microdialysis probe (A-I-4-02, depth; 4.0 mm, membrane length; 2.0 mm, Eicom, Kyoto, Japan). Ringer solution (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) was pumped through the dialysis probe at a constant rate of 1 μL/min. The baseline for acetylcholine release (average of 3 consecutive 20-min samples immediately before treatment) was obtained. The dialysates were collected for 4 h following oral administration of a vehicle, TAK-915 (10 mg/kg), or donepezil (1 mg/kg). These samples (20 μL) collected every 20 min were subjected to

high-performance liquid chromatography using an electrochemical detector for determination of the acetylcholine levels. The column (Eicompak AC-GEL [2.0 × 150 mm], Eicom AC-ENZYMPAK [3.0 × 4 mm], Kyoto, Japan) was used with a mobile phase (50 mM $KHCO_3$, 300 mg/L sodium 1-decanesulfonate, and 50 mg/L EDTA-2Na). The flow rate of the mobile phase was set at 150 μL/min using a pump and Ringer solution was injected using a microsyringe pump (Eicom, Kyoto, Japan). The detector was set at +450 mV versus Ag/AgCl. After the measurements, the animals were sacrificed, and the brains were removed. Trypan blue stain solution (Wako, Tokyo, Japan) was injected from the end of the guide cannula, and the placement of the tip of the probe guide cannula was confirmed.

5-choice Serial Reaction Time Task. This task was assessed in 10-month-old Long-Evans rats (CLEA Japan Inc., Tokyo, Japan) as previously described (Mohler et al., 2010; Shiraishi et al., 2016), with some modifications. During the experimental period, animals were food-restricted to a limit of 85-90% of their free-feeding body weight. Training and testing were conducted using eight operant chambers in sound-attenuating boxes (Med Associates Inc., St Albans, VT). Each chamber had a concave curved wall with five adjacent apertures. The light above each aperture was used as the discriminative stimulus. Food pellets (Bioserve, Dustless precision 45-mg pellets) were delivered into a food magazine. Each session started with illumination of the house light and delivery of an initial food pellet into the food magazine. Each trial was started by a nose poke into the food magazine by the rat. After a 5-s ITI, a light stimulus was presented in one of the five apertures. Correct responses (responses in the illuminated aperture) resulted in the delivery of a food pellet into the food tray.

Incorrect responses (responses in the non-illuminated apertures), omissions (failure to respond during the limited hold), and premature responses (responses occurring before stimulus presentation) were punished by a 5-s timeout, turning off of the house light, and no delivery of pellets. Each session lasted 35 min or until 100 trials had been completed. The duration of the light stimulus was initially set at 30 s and gradually decreased throughout training to 2 s. The duration of the limited hold was set at 5 s during the experiments. The rats received one training session per day until they achieved criterion performance (>75% accuracy and <20 omissions) over 3 consecutive days. On the testing day, the stimulus duration was 0.5 s. On other days, the rats received a daily maintenance training with the standard version (stimulus duration, 2 s). Vehicle and TAK-915 (3 and 10 mg/kg) were administered p.o. 2 h before the testing in a crossover design with a week washout period. In order to investigate the sensitivity of the "poor" performing group to TAK-915, the rats were separated into "poor" (n = 8) and "good" (n = 8) performing groups of equal size, with a split at the median performance. Parameters of this task were as follows; accuracy (percentages of correct responses) [correct responses/ (correct + incorrect responses)]; omission errors (the number of omissions); premature responses (the number of nose pokes into any stimulus aperture during an ITI); and magazine latency (the mean time between a correct response and a nose poke into the food magazine).

Measurement of Sleep-Wake State. Eight-week-old male SD rats were used for the recording of electroencephalography (EEG) and electromyography (EMG). The animals were anesthetized using pentobarbital (50 mg/kg i.p.) and were fixed on a stereotaxic frame (Narishige, Tokyo, Japan). The transmitter (F40-EET, Data Sciences

International, St. Paul, MN, USA) was placed in the subcutaneous cavity along the dorsal flank, between the forelimb and hind limb, and the leads were routed along a head/neck incision. For the detection of EEG signals, one pair of leads (negative lead placed +2.2 mm anterior and -3 mm lateral to bregma, positive lead placed +1 mm anterior and -4 mm lateral to lambda) was fixed to the skull using a dental cement. Additionally, another set of pair of leads was sutured into the nuchal muscles to monitor the EMG activity. After the surgery, the rats were singly housed under a 12-h light-dark cycle (lights on at 7:00 AM) with food and water *ad libitum* and were allowed to recover from the implantation surgery for at least one week. Vehicle and TAK-915 (3 and 10 mg/kg) were administered p.o. at around 11:30 AM in a crossover design, with a two- or three-day washout period. The EEG/EMG signals were continuously recorded using the Dataquest ART system (Data Sciences International, St. Paul, MN, USA). The recorded data were automatically scored in 4 s epochs as wakefulness, non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep using a sleep scoring software (Sleepsign ver.3, Kissei Comtec, Nagano, Japan). Data were expressed as the time spent in each state during 3 h following the oral administration.

Measurement of Intracellular Cyclic Nucleotides in the Primary Cortical Neurons. Primary cortical neurons were prepared from fetuses of SD rats, which were extracted from a mother animal at 17-18 days of gestation. Cells were isolated using nerve-cell dispersion solutions (Sumitomo Bakelite, Tokyo, Japan) containing papain, following the manufacturer's instructions. The isolated primary cells were suspended in a neurobasal medium (Life technologies, CA) with a B-27 supplement,

Penicillin-Streptomycin and L-Glutamine (Life technologies), and were plated onto poly-L-lysine-coated 96-well culture plates (Sumitomo Bakelite) at a density of 5 × 104 cells/100 μL/well. The plates were incubated at 37 °C under 5% CO_2. Medium (100 μL) was added to each well on day in vitro (DIV) 3 or 4. TAK-915 and 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO), and were then diluted in a neurobasal medium. All the solutions of TAK-915 and its vehicle contained 0.1% DMSO. All the solutions and the vehicle were dispensed in a polypropylene 96-well plate and incubated at 37 °C until just before use. For evaluating the effects of TAK-915 in the presence of NMDA or SNP, on DIV 7, the cells were rinsed with a Hanks balanced salt solution and incubated with 1 μM of TTX for 50 min at 37 °C. After addition of 10 μM IBMX and TAK-915, cells were incubated for 10 min at 37 °C. Then, NMDA or SNP was added, and the cells were incubated for 15 min at 37 °C. Finally, all the solutions were decanted, and the cells were dissolved in 100 μl/well of lysis buffer. After shaken on a plate shaker for 30 min, the cell lysates were transferred to a new polypropylene plate. Intracellular cyclic nucleotide concentration in the lysates was measured using cAMP/cGMP EIA system (GE healthcare, UK), in accordance with the manufacturer's instructions.

Statistical Analysis. The Aspin-Welch test (for nonhomogeneous data) or Students *t*-test (for homogeneous data) was used for pairwise group comparison. In the dose-response experiments, homogeneity of the variances was evaluated using the Bartlett's test, followed by the two-tailed Williams' test (for parametric data) or two-tailed Shirley-Williams test (for non-parametric data). A value of $P \leq 0.05$ was considered significant. In the MWMT study, a pairwise group comparison was

performed using two-way analysis of variance (ANOVA) for repeated measures followed by Bonferronis t -test with significance set at $P \leq 0.0125$ or one-way ANOVA followed by Bonferronis t -test with significance set at $P \leq 0.05$. In the 5-CSRTT and sleep studies, the statistical differences between the vehicle-treated group and TAK-915-treated group were analyzed using a crossover analysis of variance followed by a contrast test with significant set at $P \leq 0.05$.

Results

In the previous study, an increase in the hippocampal cGMP levels was observed after the oral administration of 3 and 10 mg/kg of TAK-915 (Nakashima et al., 2018). Therefore, TAK-915 doses of 3 and 10 mg/kg were primarily used to induce PDE2A inhibition in the following behavioral tasks.

Effects of Repeated Administration of TAK-915 on the Performance of Aged Rats in the Morris Water Maze Task.

To investigate the effects of PDE2A inhibition on the spatial memory deficits associated with aging, I orally administered TAK-915 to aged rats during the learning session for 4 days and tested their performance to find the hidden platform in the MWMT. Initially, the visible platform test was conducted to investigate whether there were any differences in the swimming ability and motivation to escape from the water between the young and aged rats. The mean latencies to escape onto the visible platform in the young and aged groups were 13.2 ± 1.6 s (n =15) and 54.0 ± 5.5 s (n = 30), respectively. The latency of the aged rats to reach the visible platform in the water

tank was significantly longer than the latency in the young rats ($P \leq 0.01$). Based on the results of the visible test, the aged rats were randomly allocated into two groups before the treatments, a control group (54.1 ± 8.1 s, n = 15) and TAK-915-treated group (53.8 ± 7.6 s, n = 15). In the hidden platform test, significant differences were observed between the young and aged-control rats in the escape latency from day 2 to day 4 ($P \leq 0.0125$, Fig. 11A). The aged group receiving 3 mg/kg of TAK-915 showed a significantly reduced latency to find the platform compared to the vehicle-treated group on day 3 ($P \leq 0.0125$, Fig. 11A). There was no significant difference in the mean velocity of swimming during the MWMT between the vehicle-treated aged rats and TAK-915-treated aged rats on each day ($P > 0.0125$, Fig. 11B), suggesting that TAK-915 did not affect the motor functions. In the probe test, significant differences were observed between the young and aged-control rats in the percent of time spent in the target quadrant ($P \leq 0.05$, Fig. 12). The aged group receiving 3 mg/kg of TAK-915 did not show a significant effect on the percent of time spent in the target quadrant compared with the vehicle-treated aged group ($P > 0.05$, Fig. 12).

Effects of TAK-915 on the Scopolamine-Induced Deficit in the Novel Object Recognition Task in Rats.

To evaluate the effect of PDE2A inhibition on the cholinergic deficits, I used the NORT with a scopolamine-induced deficit. A one-way analysis of variance revealed that there were no differences among the groups in the total exploration time of the two identical objects in the acquisition trials ($P > 0.05$, Fig. 13A). After a 4-h ITI, the vehicle-pretreated rats spent more time exploring the novel object in the retention trial than the familiar object ($P \leq 0.01$, Fig. 13B). This effect was disrupted by the

administration of 0.1 mg/kg of scopolamine treatment. The scopolamine-treated groups orally administered TAK-915 (1, 3, and 10 mg/kg) explored the novel object significantly longer than groups treated with a vehicle ($P \leq 0.05$ for 1 mg/kg, $P \leq 0.01$ for 3 and 10 mg/kg, Fig. 13B). TAK-915 at a dose of 3 and 10 mg/kg significantly increased the NDI ($P \leq 0.05$, Fig. 13C). Based on these results, I investigated the possibility that TAK-915 enhanced the cognitive function by increasing the acetylcholine release in the hippocampus. As shown in Figure 14, the oral administration of donepezil at a dose of 1 mg/kg significantly increased the acetylcholine release in the hippocampus compared to that of a vehicle ($P \leq 0.05$, Fig. 14B). In contrast, TAK-915 at a dose of 10 mg/kg did not affect the acetylcholine release under these experimental conditions ($P > 0.05$, Fig. 14B).

Effects of TAK-915 on the Performance of the 5-choice Serial Reaction Time Task in Middle-aged, Poorly Performing Rats.

Attention deficits are observed in middle-aged rats, indicating that the attention deficit is an early marker of cognitive decline (Guidi et al., 2015). In this study using the 5-CSRTT, the effects of PDE2A inhibition on attention and impulsivity in middle-aged, poorly performing rats were assessed. TAK-915 at a dose of 10 mg/kg, p.o., significantly enhanced the performance accuracy in poorly performing subjects compared to the vehicle ($P \leq 0.05$, Fig. 15A). TAK-915 at a dose of 10 mg/kg showed a tendency to reduce the number of omissions ($P = 0.066$, Fig. 15B) and premature responses ($P = 0.059$, Fig. 15C). There were no significant differences among the three groups with respect to the latency to collect rewards from the food magazine following a correct response (magazine latency) ($P > 0.05$, Fig. 15D). These results suggest that

TAK-915 can improve attention and attenuate impulsivity without affecting the motor function in poorly performing rats. In good performers, TAK-915 did not show any significant effects on the accuracy, number of omissions, premature responding, and magazine latency (Fig. 16). To investigate whether this pro-cognitive effect by PDE2A inhibition is associated with sedation or arousal, the amount of sleep and wakefulness following the administration of TAK-915 in the rats was measured. Figure 17 shows the mean time spent in wakefulness, NREM sleep, and REM sleep during 3 h after treatment. TAK-915 at all tested doses did not affect the duration of wakefulness, NREM sleep, and REM sleep ($P > 0.05$, Fig. 17).

Discussion

Age-related cognitive decline influences the ability of daily functioning and increases the risk of developing dementia. PDE2A has been proposed as one of the attractive targets for cognitive enhancement in aging. In the present study, I demonstrated that PDE2A inhibition can ameliorate the age-related cognitive deficits in rats.

Deficits in spatial memory, which are associated with hippocampal function, have been observed in aging (Moffat, 2009; Lithfous et al., 2013). Similar to the clinical findings, aged rats show deficits in spatial memory that depend on the integrity of the hippocampus and related brain regions, such as the subiculum (Lindner, 1997; Leutgeb et al., 2005; Witter and Moser, 2006). Accordingly, PDE2A immunostaining in the hippocampus was especially strong in the CA3 mossy fibers and subiculum (Stephenson et al., 2012), and TAK-915 at 3 and 10 mg/kg, p.o., significantly increased

the cGMP levels in the hippocampus of rats (Nakashima et al., 2018). Cyclic GMP/PKG signaling has been reported to be required for the improvement of hippocampus-dependent spatial memory by PDE inhibitors (Hosseini-Sharifabad et al., 2012), and phosphorylation of GluR1 plays critical roles in synaptic plasticity, including LTP and spatial memory (Lee et al., 2003; Serulle et al., 2007). Interestingly, in addition to the cGMP levels, TAK-915 also up-regulated the pGluR1 levels in the rat hippocampus (Nakashima et al., 2018). Considering these findings, the increases in cGMP and pGluR1 levels in the hippocampus induced by PDE2A inhibition may result in the amelioration of cognitive deficits in aged rats through the modulation of synaptic plasticity. In aged rats, repeated administration of TAK-915 significantly reduced escape latency, measured as an animal's ability to learn the spatial location of a hidden platform across multiple sessions without intervening confounding factors such as differences in swim speed (Fig. 11). In the hidden platform test, rodents usually use a combination of hippocampus-dependent (spatial learning) and -independent systems although the mechanism of switching between the two systems and their relative contributions are not well understood (Moghaddam and Bures, 1996; McGauran et al., 2005). Based on these findings, I believe that TAK-915 has the potential to accelerate spatial learning in rodents. In the probe test, I could not detect any significant effect induced by TAK-915 (Fig. 12). To detect a significant effect by TAK-915 in the probe test, I may need to perform TAK-915 treatment immediately prior to the probe test since cAMP/cGMP levels play important roles in memory recall (Lueptow et al., 2016). In addition, spatial memory consolidation in aged rats may require a much higher increase in cyclic nucleotides by high dose treatment with TAK-915. To finely characterize the effect of PDE2A inhibition on spatial learning, further investigations

using different protocols in the MWMT, such as a cue-based platform test, will be required.

Cholinergic dysfunction in the forebrain and hippocampus is observed in older people with dementia (Mesulam, 2004). Blockade of the cholinergic transmission using scopolamine, across multiple species, has been shown to induce cognitive impairments similar to those seen in aging (Tariot et al., 1996; Buccafusco et al., 2008). The oral administration of TAK-915 dose-dependently ameliorated the acquisition memory deficits induced by scopolamine in rats in the NORT (Fig. 13). As PDE2A is highly expressed at the synapse terminal of neurons, it may be involved in the regulation of neurotransmitters, including acetylcholine (Stephenson et al., 2009; Hu et al., 2012; Stephenson et al., 2012). However, under the present experimental conditions, TAK-915 at 10 mg/kg did not induce any changes in the acetylcholine release in the hippocampus in contrast to the increased acetylcholine release induced by donepezil (Fig. 14). Thus, this dose-dependent amelioration in scopolamine-induced cognitive deficits could not be explained by an increase in acetylcholine release. However, further investigations to measure acetylcholine release under the scopolamine-treated condition or during the performance of NORT will be required. A possible explanation is that PDE2A inhibition may ameliorate scopolamine-induced acquisition memory deficits by affecting postsynaptic cholinergic function. Interestingly, the ability of cholinergic neurons to produce cGMP levels is decreased during aging (Domek-Lopacinska et al., 2005), implying that cholinergic dysregulation underlying age-related cognitive dysfunction is associated with cGMP levels. Cyclic AMP and cGMP are known to be differentially involved in the memory processes, including

acquisition, consolidation, and retention (Bollen et al., 2014; Akkerman et al., 2016; Lueptow et al., 2016), and both cAMP and cGMP mediate the acquisition processes (Akkerman et al., 2016). Pretreatment with TAK-915 may facilitate acquisition memory at the same doses that increase the cGMP levels in the hippocampus (Fig. 12C). In addition, cGMP signaling is also important for the regulation of synaptic plasticity (Kleppisch and Feil, 2009), as demonstrated by reports that PDE2A inhibition was able to enhance the induction of LTP in the rat hippocampus (Boess et al., 2004). These findings suggest that the increased cGMP levels mediated by PDE2A inhibition play a crucial role in ameliorating the acquisition memory deficits by cholinergic dysfunction.

Attention deficits, which occur in aging and early AD, can precede impairments in other cognitive domains and lead to difficulties in day-to-day living, including safe driving (Parasuraman and Nestor, 1991; Perry et al., 2000; Huntley et al., 2017). In the 5-CSRTT, TAK-915 improved the accuracy in middle-aged, poorly performing rats (Fig. 15A). In contrast, TAK-915 had no impact on the accuracy in good performers (Fig. 16A), which is likely due to ceiling effects on cognitive enhancement in these models. Similar ceiling effects are also observed in attentional improvement following nicotine administration in the 5-CSRTT, where the enhancement was detected in poor performers, but not in good performers (Mohler et al., 2010). Additionally, an infusion of cAMP analog or dopamine D1 receptor agonist into the medial prefrontal cortex, which increases the cAMP levels, improved the attention only in poor performers in the 5-CSRTT (Granon et al., 2000; Paine et al., 2009). These findings support the enhancing effects of PDE2A inhibition on attention in poor performers.

Agents that enhance the attentional functions, such as nicotine and amphetamine, possess the risk of increasing impulsivity and inducing psychotic symptoms. Interestingly, TAK-915 demonstrated the tendency to reduce premature responses in poorly performing rats ($P = 0.059$, Fig. 15C). This can be interpreted as a non-specific effect associated with sedation or decreased activity. However, this possibility could be ruled out as there were no differences in the magazine latencies (to collect food reward from the magazine following a correct response) between the vehicle-treated and TAK-915-treated groups. Additionally, TAK-915 did not affect the time spent in wakefulness, NREM sleep, and REM sleep at doses that showed the pro-cognitive activity in rats (Fig. 17). TAK-915 may therefore have a very low risk of inducing non-specific effects such as sedation and decreased activity. These results also suggest that the attentional enhancing activity of TAK-915 is different from that induced by wake-promoting drugs, such as amphetamines, which is linked to psychosis and hyperactivity. It has been reported that genetically modified mice with selective ablations in the medial habenula (mHb)-interpeduncular nucleus pathway, exhibited impulsive behaviors (Kobayashi et al., 2013). PDE2A is highly expressed in the mHb, which plays a pivotal role in regulating learning, memory, and attention (Lecourtier and Kelly, 2007; Stephenson et al., 2009; Stephenson et al., 2012). Although the role of PDE2A in the mHb is not well characterized, it is possible that this enzyme may regulate impulsive behaviors by modulating the mHb function.

The mechanism of PDE2A inhibition-induced amelioration of the age-related cognitive impairment is still unknown. One possible explanation could be the possible

modulation of the glutamatergic pathways in the brain. Changes in the glutamatergic signaling are seen in aging. For example, patients with dementia showed reduced glutamate levels in the CSF (Martinez et al., 1993) and in post-mortem brain (Lowe et al., 1990). Preclinical studies also suggest that NMDA receptor hypofunction is associated with cognitive impairment in aging. Aged rats showed a reduction in NR2B expression, which correlated with their cognitive performance in the MWMT (Clayton et al., 2002). Moreover, the increase in cGMP levels mediated by NMDA receptor activation was attenuated in the hippocampus of aged rats (Vallebuona and Raiteri, 1995; Chalimoniuk and Strosznajder, 1998), suggesting that the impaired downstream signaling of the NMDA receptor is associated with aging. In terms of the enhanced NMDA receptor signaling in age-related cognitive decline, D-cycloserine reportedly ameliorated social memory and spatial reversal learning in aged rats (Portero-Tresserra et al., 2018). The previous studies have demonstrated that TAK-915 can ameliorate the cognitive impairment and social withdrawal in NMDA receptor antagonist-induced rat models (Nakashima et al., 2018). Consistent with the reports that PDE2A inhibition can enhance the NMDA receptor/NO/cGMP pathway in the primary neuronal cultures (Suvarna and ODonnell, 2002; Boess et al. , 2004), TAK-915 also enhanced the cGMP production in the presence of NMDA or NO donor (Fig. 18). Collectively, these findings support the possibility that modulation of the NMDA receptor/NO/cGMP pathways by PDE2A inhibition could contribute to the amelioration of cognitive impairments associated with aging.

Conclusions

TAK-915, the selective PDE2A inhibitor, ameliorated age-related cognitive deficits in

rats. PDE2A inhibition showed a beneficial effect on multiple cognitive domains, such as spatial learning, episodic memory, and attention, and may provide a new therapeutic option in patients with cognitive impairments associated with aging.

Figures

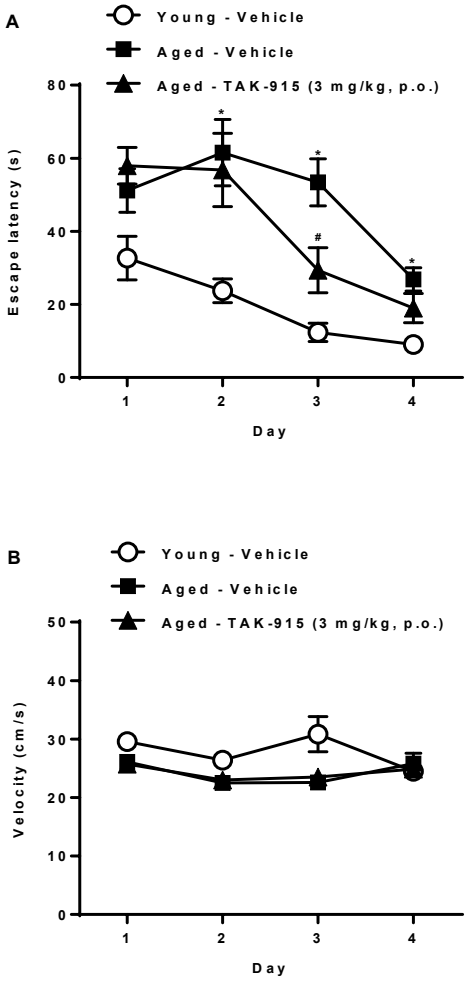

Figure 11. Effects of Repeated Administration of TAK-915 on the Performance of Aged Rats in the Morris Water Maze Task.

Vehicle or TAK-915 (3 mg/kg) was administered p.o. 2 h prior to the hidden platform test. The mean the escape latency to the hidden platform (A) and swimming velocity (B) on each day of testing is plotted as day 1 to day 4. Data are expressed as mean ± S.E.M., n = 15 per each group. $^{*}P \leq 0.0125$ (vs. vehicle-treated young group by Student's t-test after Bonferroni correction), $^{\#}P \leq 0.0125$ (vs. vehicle-treated aged group by Student's t-test after Bonferroni correction).

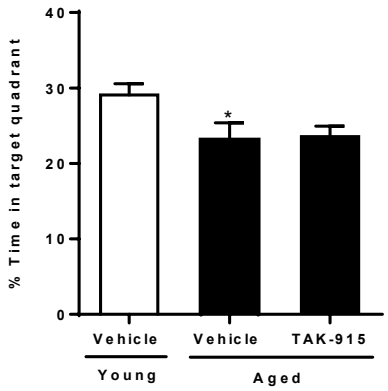

Figure 12. Effects of Repeated Administration of TAK-915 on the Performance of Aged Rats in the Probe Test in the Morris Water Maze Task.

Vehicle or TAK-915 (3 mg/kg) was administered p.o. 1 h prior to the hidden platform test for 4 days. The probe test was conducted 24 h after the hidden platform test. During the probe test, each rat was allowed to swim for 60 s. The percent of time spent in the target quadrant is expressed as mean + S.E.M.; n = 14-15 per each group. *$P \leq$ 0.05 (vs. vehicle-treated young group by Bonferronis t -test).

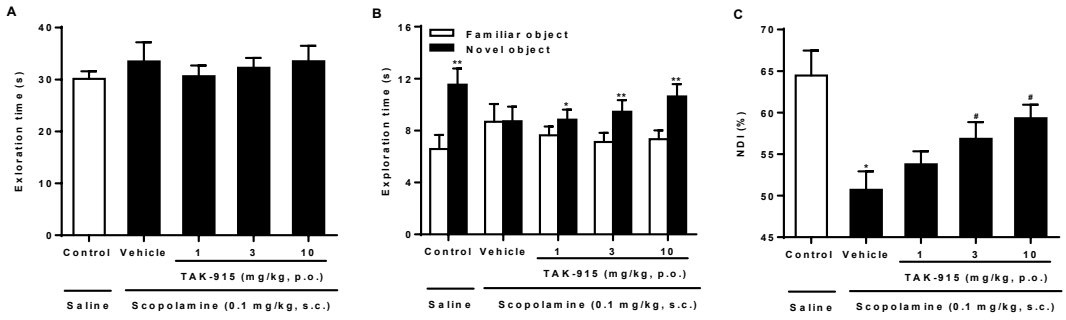

Figure 13. Effects of TAK-915 on the Scopolamine-Induced Deficit in the Novel Object Recognition Task in Rats.

Vehicle or TAK-915 (1, 3 or 10 mg/kg) was administered p.o. 2 h prior to the acquisition trials. Saline or scopolamine (0.1 mg/kg) was administered s.c. 0.5 h prior to the acquisition trials. The exploration time in the acquisition trial (A) and the retention trial (B) was scored manually. Novelty discrimination index (C) in the retention trial was calculated as follows: novel object interaction time/total interaction time × 100 (%). Data are expressed as mean + S.E.M., n = 11 for vehicle-treated group, n = 12 for other groups. $*P \leq 0.05$, $**P \leq 0.01$ (by paired t-test), $^{\#}P \leq 0.05$ (vs. vehicle-scopolamine by two-tailed Williams' test).

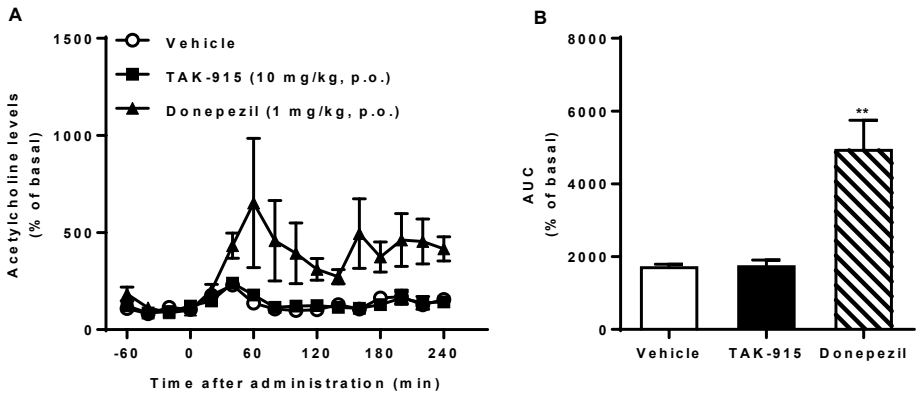

Figure 14. Effects of TAK-915 on the Hippocampal Acetylcholine Release in Rats.

Vehicle, TAK-915 (10 mg/kg), or donepezil (1 mg/kg) was administered p.o. after obtaining baseline acetylcholine. The time-course of the percentage of baseline acetylcholine levels (A), and the area under the curve for 4 h following treatments (B) are expressed as mean ±S.E.M., n = 11 for vehicle, n = 12 for TAK-915, n = 6 for donepezil. $^{**}P \leq 0.01$ (vs. vehicle by Aspin-Welch t-test).

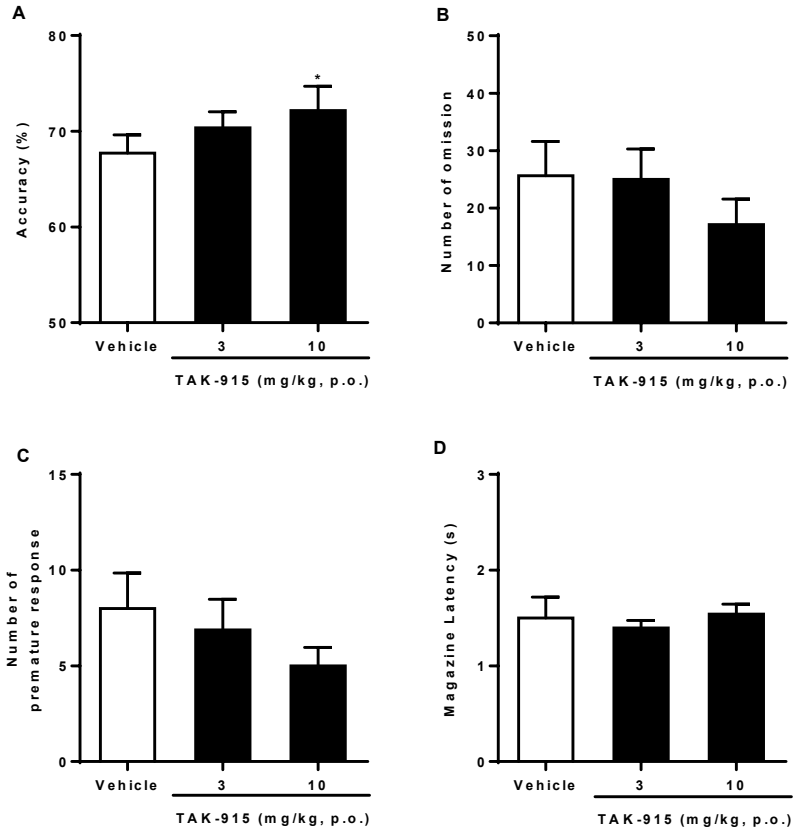

Figure 15. Effects of TAK-915 on the Performance of the 5-choice Serial Reaction Time Task in Middle-aged, Poorly Performing Rats.

Vehicle and TAK-915 (3 and 10 mg/kg) were administered p.o. 2 h prior to testing. The percent of accuracy (A), number of omissions (B), number of premature responses (C), and magazine latency (D) are expressed as mean + S.E.M., n = 8 per each group. *$P \leq$ 0.05 (vs. vehicle by crossover analysis of variance followed by a contrast test).

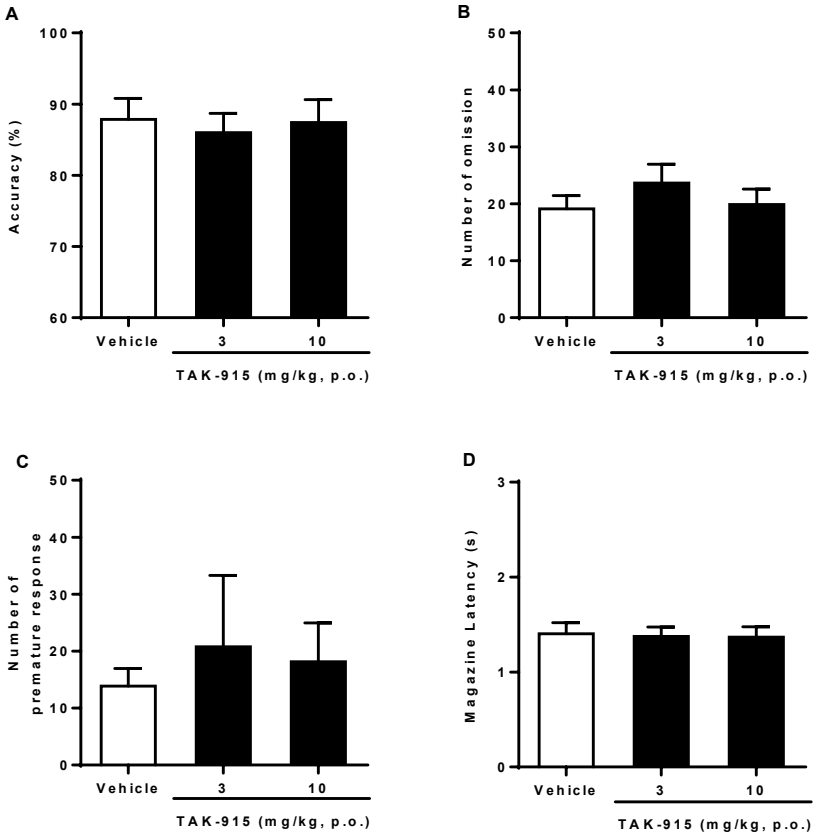

Figure 16. Effects of TAK-915 on the Performance in the 5-choice Serial Reaction Time Task in Good Performing Rats.

Vehicle and TAK-915 (3 and 10 mg/kg) were administered p.o. 2 h prior to the testing. The percent of accuracy (A), number of omissions (B), number of premature responses (C), and magazine latency (D) are expressed as mean + S.E.M.; n = 8 per each group.

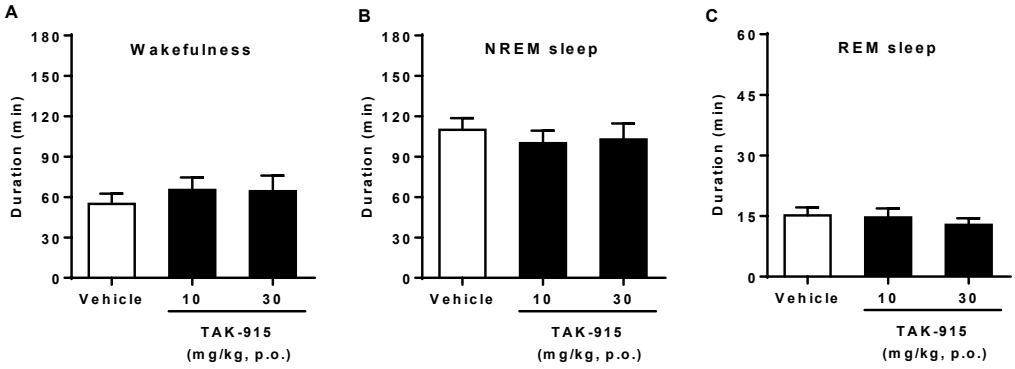

Figure 17. Effects of TAK-915 on Wakefulness, NREM sleep, and REM sleep During the Light Phase in Freely Moving Rats.

Vehicle and TAK-915 (3 and 10 mg/kg) were administered p.o. during the light phase. EEG data was assessed for 3 h following oral administration. Data show the average of time spent in each stage of wakefulness, NREM sleep, and REM sleep with S.E.M.; n = 8 per each group.

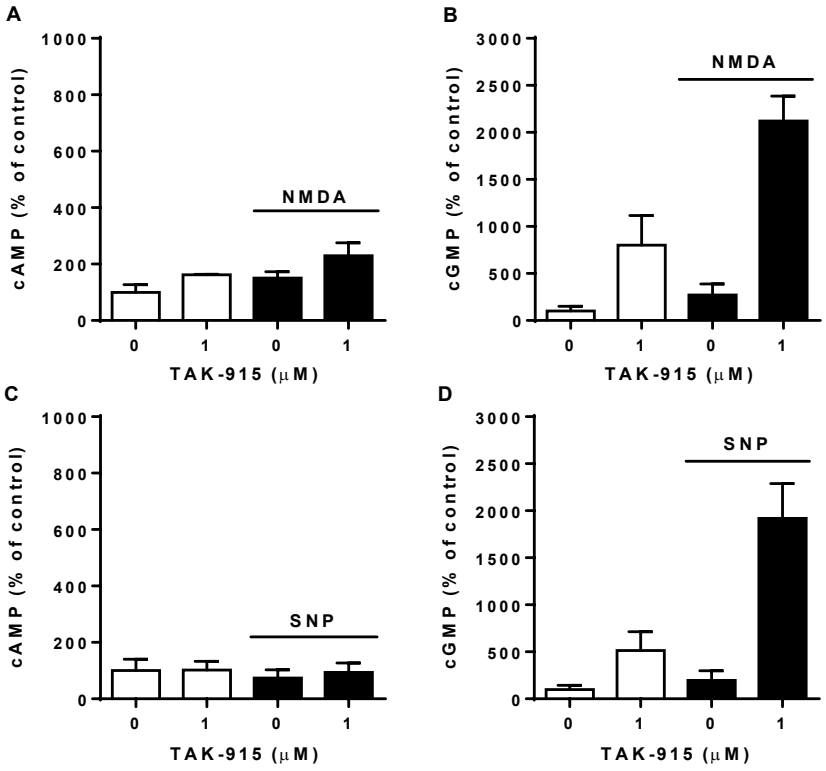

Figure 18. Effects of TAK-915 on the cAMP and cGMP Levels in the Rat Primary Cortical Neurons in the Presence of NMDA or NO Donor.

The rat primary cortical neuron on 7 days in vitro was treated with TAK-915 (1 μM) before NMDA (300 μM) or sodium nitroprusside (SNP) (3μM). Cyclic AMP (A and C) and cGMP (B and D) levels were measured using ELISA. Data are expressed as mean + S.E.M.; n = 3 per each group.

General Discussion

PDE inhibitors are currently considered attractive therapeutic targets for the treatment of cognitive dysfunctions in psychiatric and neurodegenerative diseases. In order to select suitable CNS drug targets among all PDE subfamilies, the impact of selective PDE2A inhibition in the brain, particularly, cognitive function must be considered. Initial studies have demonstrated that PDE2A is highly expressed in the forebrain regions such as the frontal cortex, hippocampus, and striatum (Stephenson et al., 2009; Stephenson et al., 2012). PDE2A is also reported to be localized in axons and nerve terminals of neurons (Stephenson et al., 2009; Stephenson et al., 2012), and is detected in membrane rafts and synaptosomal membranes (Russwurm et al., 2009). These findings suggest that PDE2A plays an important role in the modulation of cyclic nucleotides directly related to synaptic neurotransmission and plasticity. In fact, PDE2A inhibition has shown to facilitate the short and/or long term of synaptic plasticity such as paired-pulse facilitation and LTP (Boess et al., 2004; Fernandez-Fernandez et al., 2015). Immunoreactivity studies also revealed that a few brain regions such as mHb or raphe nuclei show somatic staining (Stephenson et al., 2009; Stephenson et al., 2012). The unique distribution pattern indicates the various roles of PDE2A in brain, which suggests that neuronal-circuit modulation by PDE2A inhibition might affect multiple cognitive domains.

In this study, I have demonstrated that PDE2A inhibition can ameliorate cognitive impairment in preclinical models. In the first chapter, I investigated the effects of selective PDE2A inhibition on the positive symptoms, negative symptoms, and cognitive impairment in a rat model of schizophrenia. The pathophysiology of

schizophrenia has been associated with the dysfunction of glutamatergic neurotransmission, suggesting that glutamatergic modulation including the NMDA receptor pathway can provide a new therapeutic treatment in schizophrenia. PDE2A inhibition has the potential to suppress the degradation of cAMP/cGMP, which plays an important role in the downstream signaling of NMDA receptors, and results in enhancement of NMDA receptor-mediated signaling. In order to probe the possibility of a PDE2A inhibitor as a therapeutic target in schizophrenia, the selective and brain penetrant PDE2A inhibitor TAK-915 was used to investigate the effect of PDE2A inhibition in rat models of schizophrenia associated with NMDA receptor hypofunction. Oral administration of TAK-915 attenuated episodic and working memory deficits induced by MK-801 (Fig. 4 and 5). TAK-915 prevented social withdrawal in the subchronic PCP models relevant to schizophrenia (Fig. 6). In contrast, TAK-915 did not produce an antipsychotic-like activity; TAK-915 had little effect on MK-801- or methamphetamine-induced hyperlocomotion in rats (Fig. 7). Side effects such as extrapyramidal symptoms, hyperprolactinemia, and impaired glucose tolerance observed with olanzapine were not detected (Fig 8 and 9). These findings suggest that TAK-915 has the potential to ameliorate cognitive impairments and social withdrawal in schizophrenia without producing side effects.

In the second chapter, I investigated the effects of PDE2A inhibitors in cognitive decline associated with aging. Aging is a major risk factor in dementia, often accompanied with cognitive decline. Impairment in the cognitive domains, such as spatial learning, episodic memory, and attention, are observed in the elderly, patients with mild cognitive impairments, and the early stages of AD (Reid et al., 1996; Perry et

al., 2000; Lithfous et al., 2013; Huntley et al., 2017; Mortamais et al., 2017). Changes in cAMP/cGMP signaling have been observed in the elderly and Alzheimers patients, implying that drugs that modulate cAMP and cGMP levels in the brain may present new therapies for aging associated cognitive impairment. In this study, I investigated the effects of TAK-915 on cognitive functions associated with aging, such as spatial learning, episodic memory, and attention, in rats. Repeated treatment with TAK-915 significantly reduced escape latency in aged rats in the MWMT compared to vehicle treatment (Fig. 11). In the NORT, TAK-915 dose-dependently attenuated scopolamine-induced memory deficits in rats (Fig. 13). In addition, the oral administration of TAK-915 significantly improved the attentional performance in middle-aged, poorly performing rats in the 5-CSRTT (Fig. 15). These findings suggest that PDE2A inhibition in the brain has the potential to ameliorate the age-related cognitive decline.

Although PDE2A inhibition clearly plays a critical role in cognitive impairment associated with diseases, the relationship between PDE2A and cognitive disorders has not been fully elucidated. As for the genetic links with human cognitive performance, several PDEs including PDE2A has implicated plausible links to cognitive function although further investigation of the genetic link to diseases is imperative (Gurney, 2019). Limited evidence is available regarding the change in PDEs expression levels related to pathological states. In aged rats, the PDE2A mRNA expression levels were not altered in the hippocampus and cortex (Kelly, 2018). In a study using human brains, the expression of PDE2A mRNA was not significantly different in AD patients compared with normal elderly subjects (Reyes-Irisarri et al., 2007). Moreover, no

changes in PDE2A mRNA expression were observed in the brain of patients with aging and/or AD. However, it may be difficult to simplify or predict the changes associated with diseases owing to the compensatory mechanisms by other PDEs, and/or the complexity of subtype and splice variants. As PDEs and their isoforms are localized in specific cellular compartments, they may regulate distinct pools of cAMP/cGMP and their signaling (Francis et al., 2011). Recent reports indicate that the subcellular compartmentalization of the PDE9 isoform changes across the lifespan in mice (Patel et al., 2018), although PDE9 mRNA expression in the total brain homogenates was not altered (Kelly, 2018; Patel et al., 2018). The changes in the PDE2A isoforms, in subcellular compartments, may play an important role in regulating the distinct pool of cyclic nucleotides in psychiatric and neurodegenerative diseases although further characterization will be needed.

The mechanism by which TAK-915 ameliorates cognitive impairment in schizophrenia and aging is still unknown. One possible explanation implicates the modulation of the glutamatergic pathways in the brain. Changes in glutamatergic signaling are seen in schizophrenia and aging. For example, the postmortem brain of patients with schizophrenia have shown changes in pre- and postsynaptic markers for glutamatergic neurons (Meador-Woodruff and Healy, 2000). In line with these clinical findings, NO and cGMP levels, acting downstream of the NMDA receptor, have been reduced in schizophrenia (Lee and Kim, 2008; Nakano et al., 2010). Interestingly, NO donors, which increase cGMP levels, rapidly improved multiple symptoms in schizophrenia (Hallak et al., 2013). Patients with dementia showed reduced glutamate levels in the CSF (Martinez et al., 1993) and in the post-mortem brain (Lowe et al., 1990).

Additionally, preclinical studies have indicated age-related cognitive impairments associated with NMDA hypofunction. Aged rats demonstrated a reduction in NR2B expression, which correlated with their cognitive performance in the MWMT (Clayton et al., 2002). Moreover, the increase in cGMP levels mediated by NMDA receptor activation was attenuated in the hippocampus of aged rats (Vallebuona and Raiteri, 1995; Chalimoniuk and Strosznajder, 1998), suggesting that the impaired downstream signaling of the NMDA receptor is associated with aging. As for the enhancement of NMDA receptor signaling in age-related cognitive decline, D-cycloserine reportedly ameliorated social memory and spatial reversal learning in aged rats (Portero-Tresserra et al., 2018). In the present study, TAK-915 ameliorated the cognitive impairment and social withdrawal in NMDA receptor antagonist-induced rat models (Fig. 4-6). Consistent with the reports that PDE2A inhibition can enhance the NMDA receptor/NO/cGMP pathway in the primary neuronal cultures (Suvarna and ODonnell, 2002; Boess et al., 2004), TAK-915 also enhanced the cGMP production in the presence of NMDA or NO donor (Fig. 18). Therefore, the evidence supports that the possibility that modulation of the NMDA receptor/NO/cGMP pathways by PDE2A inhibition may contribute to the amelioration of the cognitive impairment associated with schizophrenia and aging.

Translational research for the development of CNS drugs is critical to determine the appropriate dose in clinical studies. Positron emission tomography (PET) analysis can measure brain PDE2A enzyme occupancy levels of drugs, which allows us to confirm the relationship between plasma concentration and target engagement. In fact, a PDE2A PET ligand PF-05270430 has been characterized in clinical studies (Naganawa

et al., 2016). In preclinical studies using the same ligand, PDE2A occupancy of TAK-915 was demonstrated in a dose-dependent inhibition and saturated at doses higher than 10 mg/kg. In a phase I study, TAK-915 has been used to investigate the brain PDE2A enzyme occupancy levels as assessed by using the PDE2A ligand to determine the appropriate dosing schedule (ClinicalTrials.gov Identifiers: NCT02584569). The present studies will contribute to understanding the relationship between cognition-enhancing effects by PDE2A inhibition and PDE2A enzyme occupancy in future clinical trials.

In conclusion, PDE2A inhibition can ameliorate cognitive impairment associated with schizophrenia and aging. These findings contribute to the understating of the role of PDE2A in the brain under pathological conditions and provide the possibility of TAK-915 as new therapeutic option in cognitive disorders.

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Nakashima M, Imada H, Shiraishi E, Ito Y, Suzuki N, Miyamoto M, Taniguchi T and Iwashita H (2018) Phosphodiesterase 2A Inhibitor TAK-915 Ameliorates Cognitive Impairments and Social Withdrawal in *N*-Methyl-$_D$-Aspartate Receptor Antagonist-Induced Rat Models of Schizophrenia. *The Journal of pharmacology and experimental therapeutics* **365**:179-188.

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