In Vivo Studies on the Regulation of Neuropsychiatric Behaviors via CRF and AMPA Receptor Signaling

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Abstract

In the central nervous system (CNS) of vertebrates, neurotransmitters such as glutamate, gamma aminobutyric acid, and monoamines, mediate signal transmission via synapses. In addition, various peptides also transmit cell signaling and are referred to as neuropeptides or hormones. Emotions and behaviors are regulated and diversified by the expression patterns or levels of those neurotransmitters and their receptors. The regulation of neurotransmission is critical to the maintenance of physical and mental health and its abnormality in the CNS is considered as one of the main causal factors of neurodegenerative and neuropsychiatric diseases. Therefore, investigation of the relationship between neural signals and behavioral outcomes is expected to improve the understanding of disease mechanisms. In this study, two kinds of neurotransmission were selected to investigate those physiological and functional roles in animal models: corticotropin releasing factor (CRF) and α−amino-3-hydroxy-5-methyl-4 -isoxazole-propionic acid (AMPA).

In the first chapter, two aspects of the function of CRF, which is known not only as a hormone but also as a neurotransmitter in the brain, were analyzed: 1) the hypothalamus-pituitary-adrenal (HPA) axis by measuring the adrenocorticotropin (ACTH) in plasma, and 2) locomotion and anxiety behaviors. In mammals, CRF plays a role as a neurotransmitter in the brain. Its signal is mediated by two kinds of receptors, CRF_1 and CRF_2. CRF_1 receptor expresses broadly and mainly in the cortico-limbic system in the brain whereas CRF_2 receptor expresses in a limited brain area, such as the lateral septum. CRF signaling also mediates signaling in the endocrine and gastrointestinal system in the peripheral nervous system (PNS). The major function of CRF is in the signal start of the HPA axis which is recognized as a key stress signal. CRF is released from the paraventricular nucleus of the hypothalamus (PVN) in the brain to the anterior pituitary expressing CRF_1 receptors. This triggers CRF signaling in the pituitary which stimulates the release of ACTH in circulation. ACTH reaches the adrenal cortex to stimulate the release of cortisol (in humans) or corticotropin (in rodents). The response of the HPA axis is affected by the feedforward and feedback effects of CRF and cortisol, respectively. The overactivation of the HPA axis and CRF signaling occurs in depression, anxiety disorder, and posttraumatic stress disorder. It remains to be clarified how CRF regulates the function of the HPA axis and behavioral outcomes in the CNS and PNS. To clarify the role of peripheral and central CRF signaling on the HPA axis and on behaviors such as locomotion and anxiety behaviors, CRF was challenged from the peripheral route (intravenously (i.v.)) or the central route (intracerebroventricularly (i.c.v.)) in rats, followed by experiments using two small molecules with different brain permeabilities to understand CRF_1 antagonism in the peripheral and CNS regions. Plasma ACTH concentration increased significantly in both administration routes of CRF but hyperlocomotion and anxiety behavior were induced only by the i.c.v. route. In the drug discovery of CRF_1 receptor antagonists, I identified two types of compounds, A and B, which antagonized peripheral CRF-induced HPA axis activation to the same extent but showed different effects on the central CRF signal. These compounds had similar *in vitro* binding

affinities to the CRF_1 receptor (15 and 10 nM) and functional activities in the reporter gene assay (15 and 9.5 nM). In the *ex vivo* binding assay using tissues of the rat pituitary, oral treatment with compound A and compound B at 10 mg/kg inhibited [^{125}I]-CRF binding, whereas in the assay using tissues of the rat frontal cortex, treatment of compound A but not compound B inhibited [^{125}I]-CRF binding, indicating that only compound A inhibited central [^{125}I]-CRF binding. In the peripheral CRF challenge via the i.v. route, an increase in plasma ACTH concentration was significantly suppressed by both compound A and compound B. In contrast, compound A inhibited the increase in locomotion induced by the central CRF challenge whereas compound B did not. Compound A also reduced central CRF challenge-induced anxiety behavior and c-fos immunoreactivity in the cortex and the hypothalamic paraventricular nucleus. These results indicate that the central CRF signal, rather than the peripheral CRF signal, would be related to anxiety and other behavioral changes, and CRF_1 receptor antagonism in the CNS may be critical for identifying drug candidates for anxiety and mood disorders.

In the second chapter, the role of AMPA receptor signaling was investigated to understand the relationship with the behavioral outcomes, especially those which are related to schizophrenia. Glutamate is the major neurotransmitter for excitatory neurotransmission in the brain and is related to neural plasticity, memory, and cognitive functions. Abnormal glutamate neurotransmission is related to neuropsychiatric diseases such as schizophrenia, depression, and autism. Especially in schizophrenia, the state of hypoglutamate is indicated to cause positive, negative, and cognitive symptoms, and the enhancement of glutamate signaling is expected to improve those symptoms; however, the direct activation of glutamate neurons through exogenously applied agents causes cellular toxicity or seizures via hyperexcitability, which makes it difficult to investigate the function of glutamate signals in disease models. The AMPA receptor is the main receptor to conduct fast excitatory neurotransmission and to induce the long-term potentiation of neurons, which is the key function of memory and cognition. The importance of the AMPA receptor has been reported in genetically modified animal models. Mice lacking the GluR1 subunit of the AMPA receptor exhibit hyperexcitability of striatal dopaminergic neurons and schizophrenia-like behaviors. In addition, a reduction of the expression level of AMPA receptors has been reported in the postmortem brain analysis of schizophrenic patients. Therefore, an investigation of the relationships between AMPA receptor signaling and behavioral outcomes would help to understand the signal mechanism in schizophrenia. In this chapter, a new AMPA receptor potentiator, TAK-137 (9-(4-phenoxyphenyl)-3,4-dihydropyrido[2,1-c][1,2,4]thiadiazine 2,2-dioxide), was used to investigate behavioral changes in animal models of schizophrenia for positive, negative, and cognitive symptoms. TAK-137 activates AMPA receptors only in the presence of glutamate and reduces the risk of seizure compared with other compounds reported so far. In this study, rodents and non-human primates were used to assess the efficacy of TAK-137 in the naïve state or in

drug-induced disease models with methamphetamine (METH) or N-methyl-D-aspartate (NMDA) antagonists, such as MK-801, ketamine, and phencyclidine (PCP). At 10 mg/kg per os (p.o.), TAK-137 partially inhibited METH-induced hyperlocomotion in rats, and at 3, 10, and 30 mg/kg p.o., TAK-137 partially inhibited MK-801-induced hyperlocomotion in mice, suggesting weak effects on the positive symptoms of schizophrenia. At 0.1 and 0.3 mg/kg p.o., TAK-137 significantly ameliorated MK-801-induced deficits in the social interaction of rats, demonstrating potential improvement of impaired social functioning, which is a negative symptom of schizophrenia. The effects of TAK-137 were evaluated on multiple cognitive domains—attention, working memory, and cognitive flexibility. TAK-137 enhanced attention in the five-choice serial reaction time task in rats at 0.2 mg/kg p.o., and improved working memory both in rats and monkeys: 0.2 and 0.6 mg/kg p.o. ameliorated MK-801-induced deficits in the radial arm maze test in rats, and 0.1 mg/kg p.o. improved the performance of ketamine-treated monkeys in the delayed matching-to-sample task. At 0.1 and 1 mg/kg p.o., TAK-137 improved the cognitive flexibility of subchronic PCP-treated rats in the reversal learning test. Since current medication with dopamine D2 receptor antagonist effect has limited efficacy on negative and cognitive symptoms, TAK-137-type AMPA receptor potentiators with low intrinsic activity may offer new therapies for schizophrenia.

In summary, this study clarified the role of CRF signaling via the CRF_1 receptor, especially in the CNS, and the role of AMPA receptor signaling in behavioral changes related to schizophrenia. Findings related to neurotransmission provide not only insight into the function of each type of signaling but also help to understand the mechanism underlying neuropsychiatric diseases, which are usually difficult to detect as morphological changes in the living state.

Abbreviations

ACTH, adrenocorticotropin;

AD, Alzheimer's disease;

ADHD, attention deficit hyperactivity disorder;

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

ANOVA, the analysis of variance;

BBB, blood-brain barrier;

BDNF, Brain-derived neurotrophic factor;

Bed nucleus of the stria terminalis (BNST)

BSA, bovine serum albumin;

CeA, central nucleus of the amygdala;

CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate;

CHO, Chinese hamster ovary

CNS, central nervous system;

CRF, corticotropin-releasing factor;

DMTS, delayed matching-to-sample;

D2R, dopamine D_2 receptor;

EDTA, ethylenediaminetetraacetic acid;

EEG, electroencephalogram;

EPM, elevated plus maze;

FBS, fetal bovine serum

FDG, fluoro-2-deoxy-D-glucose;

FR1, fixed ratio 1;

GABA, gamma aminobutyric acid;

GI, gastrointestinal;

GlyT1, glycine transporter type 1;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

HPA, hypothalamic-pituitary-adrenal;

HTS, high-throughput screening;

i.c.v., intracerebroventricularly;

i.m., intramuscularly;

i.p., intraperitoneally;

ITI, intertrial interval;

i.v., intravenously;

LSD, least significant difference;

MAM, methylazoxymethanol acetate;

METH, methamphetamine;

$MgCl_2$, magnesium chloride;

NDI, novelty discrimination index;

NMDA, *N*-methyl-D-aspartate;

NORT, Novel Object Recognition Test;

NSB, nonspecific binding;

PCP, phencyclidine;

PD, Parkinson's disease;

PET, positron emission tomography;

PMSF, phenylmethylsulfonyl fluoride;

PNS, peripheral nervous system;

p.o., per os;

Poly(I:C), polyriboinosinic-polyribocytidylic acid;

PTSD, posttraumatic stress disorder;

PV, parvalbumin;

PVN, paraventricular nucleus;

RAM, radial arm maze;

SB, specific binding;

SI, social interaction;

SSRI, selective serotonin reuptake inhibitors;

s.c., subcutaneously;

TAK-137, 9-(4-phenoxyphenyl)-3,4-dihydropyrido[2,1-c][1,2,4]thiadiazine 2,2-dioxide;

TB, total binding;

Tris-HCl, tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl);

5-CSRTT, five-choice serial reaction time task;

General Introduction

1. The functions of neuronal transmissions

One of the key mechanisms in the regulation of physiological and psychological functions is neuronal transmission. The nervous systems involved in neuronal transmission can be classified into two categories: peripheral and central nervous systems. The peripheral nervous system (PNS) includes the somatic nervous system to transmit motor perceptions and the autonomic nervous system to regulate the homeostasis of internal organs [1]. The central nervous system (CNS) consists of neural network systems in the brain to regulate cognition and emotion, and the sensory nerves which regulate the movement of the face, eyes, and tongue. The CNS also connects to the peripheral internal and sensory organs [2]. Those nervous systems form the network system for signal transmission and utilize neuronal transmitters as messengers to surrounding neurons via synapses. Neuronal cells regulate the expression and release of those neuronal transmitters based on external signals. Generally, in the CNS of vertebrates, various kinds of the neuronal transmitters are used, and the choice depends on whether the neuronal cell is excitatory or inhibitory, and/or on the brain region. The major neuronal transmitters have been categorized into acetylcholine, monoamines, amino acids, and peptides, and those are transmitted from neuron to neuron across synapses [3]. In addition, some neurotransmitters, especially neuropeptides and hormones, circulate in body fluids such as blood or cerebrospinal fluid to induce cellular and organic responses which are usually remote from the original cells [4]. The signaling of neurotransmitters results in the certain output in the living body, such as behavioral and emotional responses. The functions of neurotransmitters have been diversified by using several subtypes of receptors for each neurotransmitter, or by changing their expression patterns or levels according to the species, sexes, or development and aging throughout life. Therefore, investigation of the impact of specific neurotransmitters on biological and behavioral outputs is important to understand neural functions. Indeed, abnormal functions in neurotransmission are related to various CNS diseases, such as in neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), or psychiatric diseases, including schizophrenia, depression, anxiety, and attention-deficit hyperactivity disorder [5]. A common characteristic of these diseases is abnormality in some specific neurons. For example, in PD, there is a significant loss of dopaminergic neurons [6]. In contrast, overactivation of the striatal dopaminergic neurons, especially in the dopamine D2 receptor-expressing neurons, have been reported in schizophrenia [7]. The loss of cholinergic neurons is regarded as a cholinergic hypothesis in AD [8]. These characteristics of neuronal abnormalities result in specific symptoms, such as loss of motor functions or cognitive functions. Although some key features in neuronal functions in CNS diseases have been determined, detailed analyses of specific neurotransmission to investigate the scope of functional outcomes would deepen an understanding of neuronal function and disease biology and would extensively lead to the generation of medicines with new mechanisms of action. In the first chapter of this study, using rats, I investigated the impact of corticotropin-releasing factor

(CRF) on the response of the hypothalamus-pituitary-adrenal (HPA) axis as plasma adrenocorticotropic hormone (ACTH) increased and on behavioral outcomes as locomotor and anxiety behaviors to classify the key signals in PNS and CNS. In the second chapter, I focused on the relationship between glutamate neurons, especially α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor signals, and schizophrenia in behaviors related to positive, negative, and cognitive symptoms by using rodents and non-human primates.

2. CRF signaling

2.1. Stress response and regulation

A living body always receives external stimuli, and the physiological responses to those stimuli are referred to as stress responses [4]. The factors which induce physiological and psychological changes in the body are called stressors, including environmental or physical stressors such as the natural environment or infectious viruses, and psychological stressors such as family or working environments. Adequate responses in the body induce the transient activation of neuronal or immunological responses to cope with, or to resist, stress. Several signaling systems are involved in stress responses: rapid activation of the autonomic nervous system to enhance the release of noradrenaline and adrenaline for the activation of the sympathetic nerve system. Another system is the activation of the HPA axis [9]. This signal is activated by the increased release of CRF from the paraventricular nucleus of the hypothalamus (PVN). CRF binds to CRF receptors, especially to CRF_1 receptors located on the anterior pituitary where this triggers the release of ACTH. ACTH reaches the adrenal cortex in peripheral organs and cortisol or corticotropin is released in humans and rodents, respectively. Cortisol or corticotropin work as effectors of the stress response to modulate immunological responses. CRF stimulates not only the HPA axis but also the limbic brain system including amygdala, hippocampus, and cortical neurons where emotional and cognitive functions are highly regulated [10]. In addition, the stimulation of peripheral CRF signaling induces intestinal contraction and other abdominal neurons. The hyperactivation of CRF signaling is related to psychiatric diseases such as depression, anxiety, posttraumatic stress disorder (PTSD), and gastrointestinal (GI) diseases such as inflammatory bowel disease [11].

2.2. CRF peptide family and its receptors

CRF is a polypeptide consisting of 41 amino acid [12]. There are several other peptides which are categorized as being members of the CRF peptide family, such as urocortin 1, 2, and 3. The affinities of CRF peptide family members to CRF_1 and CRF_2 receptors, as well as their expression patterns, are diversified in the brain and throughout the entire body [13]. This fact indicates that the CRF peptide family involves diversified physiological roles especially in coordinating endocrine, immune,

autonomic, and behavioral responses to stress. The binding affinity (Ki) of CRF to CRF_1 receptor was found to be 3.3 nM, which is about 10-fold higher than that to the CRF_2 receptor [14]. CRF is the most well-studied peptide regarding its physiological function, and CRF signaling via the CRF_1 receptor is considered as a key function in the stress response. There are distinct but overlapping expression patterns in the brain between CRF_1 and CRF_2 receptors. The CRF_1 receptor is widely expressed in brain regions that are related to sensory information processing and motor control, such as cortex, hippocampus, bed nucleus of the stria terminalis (BNST), basal ganglia, and hypothalamic nuclei [4]. It also expresses outside the brain especially in the anterior and intermediate lobes of the pituitary, where the binding of CRF to the CRF_1 receptor triggers the secretion of ACTH to the circulatory system. Expression of the CRF_2 receptor is much more restricted to subcortical structures in the brain, especially in the lateral septal nucleus and in the skeletal muscle and heart. Coextension of the CRF_1 and CRF_2 receptors seems to be limited. There is a distinct expression pattern of the CRF_2 receptor among species [15], which indicates that the receptor and signal functions may have diversified during evolution.

2.3. Pharmacological and physiological studies on stress response by modulating CRF signaling

The accumulation of evidence of the function of CRF has indicated that the CRF plays a key role in stress responses. The initial experiment exogenously administered CRF [16]. Most of the reported studies applied an intracerebroventricular (i.c.v.) injection of CRF, resulting in the detection of the response of the HPA axis and changes to locomotion and anxiety behaviors. Recently, the application of genetic mouse models and viral applications to modulate the expression level of CRF peptide families or CRF receptors have also been reported [17]. These have also resulted in the functional classification of CRF peptide families and receptors [18]. Based on those findings, CRF signaling via the CRF_1 receptor seems to be the major route of neurotransmission in the CNS and the main trigger of the activation of the HPA axis at the pituitary.

3. Glutamate signaling

3.1. Glutamate neuron and its receptors

The glutamate neuron is the major excitatory neuron throughout the entire brain region [19]. The excitation of glutamate neurons is indispensable for brain functions especially in neurocognitive domains such as memory, learning, and cognition. Glutamate receptors are classified into two categories: ionotropic and metabotropic receptors. Ionotropic receptors including N-methyl-D-aspartate (NMDA), AMPA, and kainite receptors conduct fast excitatory signaling [20]. Metabotropic receptors, including mGluR1-8, are also involved with presynaptic and postsynaptic transmission to regulate learning and memory. Among glutamate receptors, the AMPA receptor plays a key role in learning and memory [21]. AMPA receptor subunit GluR1-knockout mice exhibited

cognitive impairment, hyperdopaminergia, and psychosis-like behaviors [22]. The AMPA receptor is involved in the regulation of NMDA receptor activation, which induces ion influx into cells (Na^+ and Ca^{2+}), triggering the release of channel-blocking magnesium ion from the NMDA receptor [20]. This results in the activation of NMDA receptor signaling through an increase in NMDA receptor-mediated calcium influx [23].

3.2. Glutamate neuron and diseases

Several CNS diseases are reportedly involved with abnormal glutamate signaling such as epilepsy, schizophrenia, depression, and autism. Especially in schizophrenia, antagonists of NMDA receptors such as ketamine and phencyclidine (PCP) were accidentally discovered to induce symptoms which mimic schizophrenia, such as psychosis, abnormal social interaction, or cognitive impairment in healthy people [24]. In addition, when people with schizophrenia were treated with those agents, their symptoms worsened [25-27]. Schizophrenia consists of a spectrum of symptoms: positive symptoms (hallucinations and delusions), negative symptoms (blunted affect and deficits in social functioning), and cognitive symptoms (deficits in attention, working memory, and cognitive flexibility) [2]. The current hyperdopamine hypothesis postulates that excessive activation of dopaminergic neurons in the subcortical regions of the brain is deeply involved in the pathophysiology of the positive symptoms of schizophrenia [7]; however, this hypothesis cannot fully elaborate the mechanism of negative and cognitive symptoms. On the other hand, based on clinical and preclinical findings, NMDA hypofunction supports key hypotheses for various symptoms of schizophrenia [28]. The functions of glutamate signaling have also been studied by the pharmacological application of agonists, antagonists or genetically-modified animal models [29]. Mice with a knocked-out NMDA receptor subunit NR1 showed schizophrenia-like behaviors, whereas the overexpression of NR1 enhanced cognitive performance [30]. The enhancement of NMDA receptor signaling was investigated as a strategy for novel treatment of schizophrenia by using small molecules [31-34]. To avoid excitotoxicity by agonists, the application of a co-agonist like D-serine or glycine, or the inhibitor of those degradation enzymes was studied in clinical studies which resulted in the improvement of negative symptoms in phase II studies. Therefore, approaches to enhance glutamate signaling should be studied further to understand the relationship between signaling enhancement and behavioral outcomes.

3.3. AMPA receptor positive allosteric modulator

In view of the function of the AMPA receptor to trigger AMPA and NMDA signal activation, potentiation of the AMPA receptor is expected to offer a new therapeutic strategy for schizophrenia by enhancing glutamate signaling. Enhancement of AMPA receptor signaling by small molecules, the AMPA receptor potentiators, has been studied for a long time. However, reported AMPA receptor

potentiators such as LY451646 ((R)-N-(2-(4-cyanobiphenyl-4-yl)propyl) propane-2-sulfonamide), LY451395, and S18986 demonstrated a bell-shaped response in their various pharmacological effects [35-37]. Recently, a novel AMPA receptor potentiator—TAK-137 (9-(4-phenoxyphenyl)-3,4-dihydropyrido[2,1-c][1,2,4]thiadiazine 2,2-dioxide) was discovered. TAK-137 presented lower risks of a bell-shaped dose response and seizure owing to its low agonistic activity [38, 39]. In the molecular characterization of TAK-137 and LY451646, it was found that LY451646 showed an agonist-like effect in the absence of glutamate in rat primary neurons, whereas TAK-137 did not. In addition, LY451646 induced seizures at a 3.1-fold higher concentration in the brain based on a calculation of the area under the curve (AUC_{brain}), whereas TAK-137 showed a safety margin from the induction of seizures with a 116-fold higher AUC_{brain}. Therefore, it is hypothesized that the agonistic effect by this compound is strongly related to the induction of seizures. An electrophysiological study by using whole-cell voltage-clamp recordings was performed on cultured neurons of the hippocampus [40]. The result also showed that LY451646 induced slowly, developing large inward currents in the absence of the AMPA-R agonist, whereas TAK-137 did not impact baseline holding currents, which supports the lower agonistic properties of TAK-137 than LY451646. Therefore, TAK-137 can be a highly differentiated AMPA receptor potentiator without inducing seizures; however, it has never been proven that AMPA receptor potentiators such as TAK-137 still exert efficacy on modulation of cognition and other behaviors related to schizophrenia. In this study, I used TAK-137 to evaluate the impact of AMPA receptor potentiation on the behaviors related to schizophrenia without agonist-like outputs such as seizure.

Chapter I

Characterization of CRF1 receptor antagonists with differential peripheral vs central actions in CRF challenge in rats

Abstract

The aim of this study was to investigate peripheral and central roles of corticotropin-releasing factor (CRF) in endocrinological and behavioral changes. Plasma adrenocorticotropin (ACTH) concentration was measured as an activity of hypothalamic-pituitary-adrenal (HPA) axis. As behavioral changes, locomotion and anxiety behavior were measured after CRF challenge intravenously (i.v.) for the peripheral administration or intracerebroventricularly (i.c.v.) for the central administration. Plasma ACTH concentration was significantly increased by both administration routes of CRF; however, hyperlocomotion and anxiety behavior were induced only by the i.c.v. administration. In the drug discovery of CRF_1 receptor antagonists, I identified two types of compounds, Compound A and Compound B, which antagonized peripheral CRF-induced HPA axis activation to the same extent but showed different effects on the central CRF signal. These had similar in vitro CRF_1 receptor binding affinities (15 and 10 nM) and functional activities in reporter gene assay (15 and 9.5 nM). In the ex vivo binding assays using tissues of the pituitary, oral treatment with Compound A and Compound B at 10 mg/kg inhibited [^{125}I]-CRF binding, whereas in the assay using tissues of the frontal cortex, treatment of Compound A but not Compound B inhibited [^{125}I]-CRF binding, indicating that only Compound A inhibited central [^{125}I]-CRF binding. In the peripheral CRF challenge, increase in plasma ACTH concentration was significantly suppressed by both Compound A and Compound B. In contrast, Compound A inhibited the increase in locomotion induced by the central CRF challenge while Compound B did not. Compound A also reduced central CRF challenge-induced anxiety behavior and c-fos immunoreactivity in the cortex and the hypothalamic paraventricular nucleus. These results indicate that the central CRF signal, rather than the peripheral CRF signal would be related to anxiety and other behavioral changes, and CRF_1 receptor antagonism in the central nervous system may be critical for identifying drug candidates for anxiety and mood disorders.

1. Introduction

Corticotropin-releasing factor (CRF) is a 41-amino acid polypeptide that plays a central role in coordinating the endocrine, immune, autonomic, and behavioral responses to stress [12, 41], especially in physiological regulation of the endocrine system related to the hypothalamic–pituitary–adrenal (HPA) axis. When physiological or psychological stress occurs, CRF is synthesized and secreted from the hypothalamic paraventricular nucleus (PVN) and binds to CRF receptors in the pituitary gland. This triggers secretion of adrenocorticotropin (ACTH) from the anterior lobe of the pituitary. ACTH stimulates glucocorticoid secretion from the adrenal glands into blood, and glucocorticoid exerts a negative feedback on the activity of HPA axis [12, 42, 43]. In the disease state, hyperactivation of HPA axis and hypersecretion of CRF have been reported to be related to the pathogenesis of anxiety and depression [44-46], and modulation of CRF signaling has been investigated as a new treatment strategy for those mood disorders. Two CRF receptors have been identified, named as CRF_1 and CRF_2. CRF_1 receptors are expressed in the mammalian GI tracts, anterior pituitary located outside of the blood-brain barrier (BBB), and the brain regions related to emotion and cognitive processes, such as amygdala, hippocampus, and cerebral cortex [47-49], whereas the distribution of CRF_2 receptors is more restricted [10, 15]. In addition, CRF_1 receptor knockout mice show less behaviors related to anxiety and depression compared with those in wild-type mice [50-52]. Thus, CRF_1 receptor antagonists have been recognized as promising candidates for new anxiolytics and antidepressants.

In the preclinical evaluation of CRF_1 receptor antagonists, effects were mainly focused on the regulation of hyperactivated HPA axis and the modulation of anxiety or depressive-like behaviors in CRF challenge [53-55] or stress models [54, 56, 57]. However, it remains to be concluded whether the antagonism of CRF_1 receptor in the peripheral, central, or both systems, is required to exert anxiolytic or antidepressive efficacy. Recently, multiple reports have indicated that central, especially limbic, CRF_1 receptors modulate anxiety-related behavior independent of endocrinological HPA regulation. Conditional knockout mice of limbic CRF_1 receptors reduced the anxiety behavior, but not the stress-induced increase of ACTH secretion [50]. Transient over-expression of CRF in the forebrain induced neuroendocrinological and behavioral changes [58]; however, the overexpression of CRF only in the pituitary did not induce anxiety behavior [59]. The CRF_1 receptor antagonist SSR125543 attenuated long-term cognitive deficit induced by acute inescapable stress independent of the regulation of the HPA axis [60].

In this study, to investigate the roles of peripheral or central CRF signaling in endocrinological and behavioral changes, I measured the activity of HPA axis as plasma ACTH concentration, locomotion, and anxiety behavior after peripheral or central CRF challenges. Moreover, two CRF_1 receptor antagonists, Compound A and Compound B, were identified from my drug discovery research. They were found to have different binding profiles for central CRF_1 receptors indicated in

the ex vivo binding assay with rat cortex homogenate and were used to study the in vivo effects of antagonism of peripheral and central CRF_1 receptors.

2. Materials and Methods

2.1 Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited. Animal care followed the Guide for Care and Use of Laboratory Animals. Male Wistar rats (CLEA Japan, Inc., Tokyo, Japan) aged 7–8 weeks weighing from 240 to 280 g at the beginning of the experiments were used. Animals were housed in groups of 4 or 5/cage and maintained under a 12:12 light:dark cycle (light on from 7:00 am to 7:00 pm), with food (CLEA Rodent Diet CE-2 purchased from CLEA Japan Inc., Tokyo, Japan) and water provided ad libitum and habituated for more than 7 days in the laboratory before experiments.

2.2 Materials

CRF_1 receptor antagonists Compound A [(N-(4-bromo-2-methoxy-6-methylphenyl)-4-chloro-1-methyl-7-(pentan-3-yl)-1H-benzimidazol-2-amine)] and Compound B [4-chloro-2-(2-chloro-6-methyl-4-(methylthio)phenoxy) -1-methyl-7-(pentan-3-yl)-1H –benzimidazole] were synthesized in the laboratories of Takeda Pharmaceutical Company Limited (Kanagawa, Japan). Compounds were suspended in water containing 0.5% methyl cellulose (MC, Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) and administered orally in a volume of 2 mL/kg. Other reagents were purchased from local vendors. Human/ rat (h/r) CRF or ovine CRF (Peptide Institute, Inc., Osaka, Japan) was used for CRF challenge tests. The h/r CRF was dissolved in phosphate buffered saline (PBS) (Sigma-Aldrich Japan, Tokyo, Japan) at 10 μg/μL and diluted with PBS to 0.2 μg/μL for i.c.v. injection or with saline to 10 μg/mL for i.v. injection. Further dilution for dose response studies of CRF was conducted using PBS for i.c.v. or saline for i.v. Diluted CRF was kept on ice just before injection. PBS for i.c.v. and saline for i.v. were administered as vehicle. Ovine CRF was dissolved and diluted with 0.05% bovine serum albumin (BSA) (Sigma-Aldrich Japan, Tokyo, Japan) in saline to 0.1 μg/mL. The injection volume was 5 μL/rat for i.c.v. and 1 mL/kg for i.v. administration.

2.3 Surgery

2.3.1. Anesthetics

The rats were anesthetized with intraperitoneally administered pentobarbital (50 mg/kg) (Sumitomo Dainippon Pharma, Osaka, Japan) during the operation.

2.3.2. Intracerebroventricular (i.c.v) cannula implantation and administration

Anesthetized rats were placed in a stereotaxic frame (David Kopf Instruments, California, U.S.A). A guide cannula (AG-4, Eicom, Kyoto, Japan) was implanted in the lateral ventricle and anchored to

the skull with 2 stainless steel screws and dental cement. Stereotaxic coordinates were tooth bar +3.3 mm above interaural zero, −0.8 mm posterior to bregma, +1.6 mm lateral, and −3.5 mm below the surface of the dura [61]. A dummy cannula was kept inserted into the guide cannula until vehicle or drug injection. Animals were undisturbed during a postsurgical recovery period of at least 7 days. At the time of injection, inserted dummy cannula was removed and clean microinjection cannula needles jointed with Teflon tube (0.1 mm ID, 50 cm) were inserted. CRF or vehicle was infused at a rate of 10 μL/min using auto-injector. After infusion, the microinjection needles were kept in place for an additional 1 min to allow for diffusion of the substances into the surrounding tissue, and then the injector was removed.

2.3.3. Intravenous (i.v.) cannulation and administration

The examination for i.v. injection of CRF was performed under the conscious state. Each rat was held in the apparatus (ICM Co., Ltd., Ibaraki, Japan) for 30 s to 1 min and vehicle or CRF was administered from the tail vein. The animals were immediately backed to the home cage after the injection. In the evaluation of efficacy of compounds on peripheral ovine CRF challenge, the experiment was performed as per a published method [62]. A heparinized i.v. cannula (TERUMO SURFLO I.V. CATHETER 22G, Terumo Corporation, Tokyo, Japan) was acutely inserted into one side of the femoral vein of rat under pentobarbital anesthesia which lasted until decapitation. The catheter was secured in place with a bonding agent and flushed with saline just after cannulation. Local anesthetic (Bupivacaine Hydrochloride Hydrate, AstraZeneca K.K, Osaka, Japan) was administered at the incision to reduce pain of noxious stimuli. Rats were placed onto a heat carpet to maintain normal body temperature until decapitation.

2.4 Measurement of plasma ACTH levels

To examine the dose response of CRF on plasma ACTH levels, h/r CRF was i.v. or i.c.v. administered to rats (n = 5–7) under the conscious state. Blood was collected 30 min after CRF administration. Plasma was separated from whole blood by centrifugation (3,000 rpm for 15 min) and stored in 1.5 mL Eppendorf tubes at −20ºC until the measurement of ACTH concentration. Compounds were orally administered, followed by i.p. delivery of pentobarbital 65 min later (n = 6–8). CRF was injected from the cannula 55 min after anesthesia and the cannula was flushed with saline. Blood was collected into EDTA tubes 5 min after the CRF injection (60 min after anesthesia) by decapitation. Plasma was separated from whole blood by centrifugation (15,000 rpm for 10 min) and stored in 1.5 mL Eppendorf tubes at −20ºC until the measurement of ACTH concentration. ACTH concentration was measured using an immunoradiometric assay kit (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) or EIA Kit EKE-001-21 (Phoenix Pharmaceuticals, Inc, US). The assay could detect 5-1000 pg/mL ACTH concentration. The intra-assay variation was less than

30%, and the inter-assay variation was less than 10%. To examine the effect of compounds without CRF on plasma ACTH levels compounds were administered orally 65 min before anesthesia (n = 8). 125 min after the CRF injection, plasma was separated from whole blood by centrifugation (15,000 rpm for 10 min) and stored in 1.5 mL Eppendorf tubes at -20ºC until measurement of ACTH concentration. ACTH concentration was measured using.

2.5 Behavioral assays

2.5.1. Measurement of locomotion

One day before the testing, rats were placed into a transparent Plexiglas®-covered cage (38 x 25 x 32 cm) at 5:00 pm, and the room was maintained under a 12:12 light:dark cycle (light on from 7:00 am to 7:00 pm) with food and water provided ad libitum. Locomotion was automatically counted using an infrared sensor system (SuperMex, Muromachi Kikai, Tokyo, Japan). The infrared sensor was positioned at a hole in the center of the top cover to detect locomotion. Food and water were available ad libitum. Locomotion was measured for 3 h after CRF or vehicle administration. Compounds or vehicle were orally administered 2 h before i.c.v. injection of CRF (1 μg) or vehicle. All data were analyzed and stored in a personal computer using analytical software (Comp ACT AMS, Muromachi Kikai, Tokyo, Japan). Results were presented as total locomotion for 3 h after administration of CRF.

2.5.2. Elevated plus maze (EPM) test

Anxiety behavior was evaluated by the EPM test. The EPM apparatus was made of black Plexiglas® and consisted of 4 arms (50 cm long, 10 cm width): 2 arms had 40-cm high black walls (closed arms) and other 2 arms had 1.5-cm high edges (open arms). The maze was elevated to a height of 40 cm. Rats were weighed and transferred to a darkened testing room (approximately 5–10 lux at the open arms of the maze). The animals were allowed to habituate for at least 1 hour before testing. Vehicle or a compound was orally administered 2 h before the administration of CRF. After vehicle or CRF injection, animals were returned to the home cages for 30 min (i.v. administration) or 1 hour (i.c.v. administration). At the beginning of the EPM test, rats were placed individually at the center of the maze, facing one of the closed arms. The time spent on open arms during a 5-minute testing session was counted manually by stopwatch, and number of arm entries was counted visually. The apparatus was wiped clean after the testing of each subject. The results were expressed as the mean time spent on open arms and the number of open, closed, and total arm entries.

2.6. In vitro binding assay

A receptor binding experiment was carried out using a human CRF receptor expressing Chinese hamster ovary (CHO) cellular membrane fraction and ovine CRF, [^{125}I]-Tyr^{0}([^{125}I]-CRF). Various

concentrations of a Compound A or Compound B were incubated with 1 μg of human CRF receptor expressing CHO cellular membrane fraction and 50 pM of [^{125}I]-CRF in a binding assay buffer, which included 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl), 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM magnesium chloride ($MgCl_2$), 0.05% 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), 0.1% BSA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 μg/ml pepstatin, and 20 μg/ml leupeptin, pH 7.5. In addition, for measuring nonspecific binding (NSB), 0.1 μM unlabeled human urocortin was incubated with 1 μg protein of human CRF receptor expressing CHO cellular membrane fraction and 50 pM of [^{125}I]-CRF in a binding assay buffer. Binding reaction was performed at room temperature for 90 min, and the membrane was then entrapped on a glass filter (UniFilter-96 GF/C, PerkinElmer, Inc., Massachusetts, U.S.A) by suction filtration using a cell harvester (PerkinElmer, Inc., Massachusetts, U.S.A), and washed with ice-cooled 50 mM Tris–HCl (pH 7.5). After drying the glass filter, a liquid scintillation cocktail (MicroScint™-O, PerkinElmer, Inc., Massachusetts, U.S.A) was added, and the radioactivity of [^{125}I]-CRF remaining on the glass filter was measured using TopCount NXT™ (PerkinElmer, Inc., Massachusetts, U.S.A). To obtain the binding inhibition rate under the presence of various concentrations of each test substance, (Total binding (TB) − -Specific binding (SB))/(TB-NSB) × 100 (SB: radioactivity when a compound is added, TB: maximum binding radioactivity, NSB: nonspecific binding radioactivity) was calculated. The IC_{50} values were calculated using GraphPad Prism software (GraphPad Software, California, U.S.A.).

2.7. In vitro CRF antagonistic activity

CRF antagonistic activity was obtained by measuring inhibition of adenylate cyclase by the use of a CRE-luciferase reporter gene assay. CHO cells with a CRE-luciferase gene expressing human CRF receptor were inoculated on a 96-well plate at 40,000 cells/well and allowed to grow for 24 h. After cultivation, the culture medium was removed and the cells were treated with various drug concentrations in 100 μL of assay buffer [20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), Ham F-12, 0.1% BSA, pH 7.2] containing 1 nM human CRF for 4 h. Following drug exposures, the cells were lysed, and luciferase activity was measured using a Steady-Glo® Luciferase Assay System (Promega Corporation, Wisconsin, U.S.A.) Light output was detected by ARVO-SX (Wallac, PerkinElmer, Inc., Massachusetts, U.S.A). The IC_{50} values were calculated using GraphPad Prism software.

2.8. Ex vivo binding assay

2.8.1. Preparation of brain membrane homogenates

Compound A, Compound B, or the corresponding vehicle was administered orally to rats at 10 mg/kg (n = 3). Rats were decapitated and organs (frontal cortex and pituitary) were removed 1 or 2 h

later. Brain sections were homogenized at 4°C using PHYSCOTRON (MICROTEC CO., LTD., Chiba, Japan) for 10 s in lysis buffer (50 mM Tris–HCl pH 7.0, 10 mM $MgCl_2$, 2 mM EDTA, and 100 KU/mL aprotinin). Frontal cortex was diluted with the lysis buffer to a final concentration of 5 mg wet tissue/mL. Pituitary was homogenized in 2.5 mL of the lysis buffer and diluted with the lysis buffer to a final concentration of 5 mg It tissue/mL.

2.8.2. Binding assay

[^{125}I]-CRF (ovine) binding was performed with rat membrane homogenates in the presence of 100 pM of [^{125}I]-CRF (ovine) in lysis buffer containing 0.1% BSA, 0.5% DMSO, and 0.05% CHAPS in a final volume of 200 mL. After incubation at room temperature for 2 h, the incubation mixture was filtered on Whatman® glass microfiber filters, Grade GF/C (Sigma-Aldrich Corporation, Missouri, U.S.A.) presoaked in 0.3% polyethyleneimine. The filters were washed 6 times with ice-cold wash buffer (PBS containing 0.05% CHAPS, 0.01% Triton X-100) and dried. The radioactivity was determined with a gamma scintillation counter. Results were expressed as percentage of [^{125}I]-CRF (ovine) binding with the count of vehicle-treated group defined as 100%, with in vitro determination of the nonspecific binding using 1 μM of the selective CRF_1 receptor antagonist R121919.

2.9. Immunohistochemistry

2.9.1. Perfusion and tissue processing

Compound A (10 mg/kg) was orally administered 2 h before i.c.v. administration of CRF (1 μg) or vehicle to rats (n = 3). The dose of CRF was selected based on my results of locomotion and anxiety behavior and published data [63]. The animals used for immunohistochemistry were prepared independently from those used in other experiments (as shown in Fig. 1-6 and 1-7). Rats were deeply anesthetized with ether and perfused with 0.1 M PBS followed by 4% formaldehyde prepared from 20% paraformaldehyde via an intracardiac cannula [64] 2 h after i.c.v. administration. The brain was immediately removed and kept in 4% formaldehyde in PBS for 16–18 h, followed by immersion into 20% sucrose in PBS at 4°C for 3 days. The brain was set to the stage of a cryostat (CM3050S, Leica Microsystems GmbH, Wetzlar, Germany) with Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan). A rat brain atlas was used for selecting coronal brain sections (20 μm thickness) containing the PVN (1.5– −2.1 mm posterior from the bregma) and the cortex and amygdala (1.70– −2.80 mm posterior from the bregma).

2.9.2. Immunohistochemistry of c-fos

Immunohistochemistry was performed with an avidin–biotin–peroxidase method using specific anti-c-Fos polyclonal antiserum (1:5,000, Ab-5, Oncogene, California, U.S.A or 9F6, #2250 Cell Signaling Technology, 1:1000 in blocking solution). Brain sections were washed in 0.1 M PBS and

placed into 3% fetal bovine serum (FBS) for 1 h. Sections were washed in PBS and then transferred into a vial containing anti-c-fos rabbit polyclonal antibody for 16–18 h at 4°C with shaking. After washing with PBS, brain sections were incubated in a solution of biotinylated anti-rabbit IgG of VECTASTAIN® (Vector Laboratories, California, U.S.A) (1:1000, in 0.3% Triton X in PBS) for 1 hour at room temperature, followed by rinsing in PBS. Then brain sections were incubated in an avidin–biotin complex solution (0.1% avidin, 0.1% biotinylated enzyme in 0.1 M PBS, VECTASTAIN® Elite ABC Kit of rabbit IgG) for 16–18 h (overnight) at 4ºC. Brain sections were rinsed with 0.05M Tris–HCl (pH 7.6) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and then transferred to a solution of 0.2 mg/mL diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris–HCl (pH 7.6). For reaction, 30% H_2O_2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used, and the reaction was stopped by transferring the section to 0.05 M Tris–HCl (pH 7.6). After washing the brain sections with 0.05 M Tris–HCl (pH 7.6), they were adhered to glass slides. Ethanol (70, 80, 90, 95, and 99.5%) and xylene (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used for dehydration before enclosure. C-fos-immunopositive cells were checked with microscope and counted by Image J (NIH). Bilateral brain areas of the PVN, cortex, and amygdala were counted from 4–7 (PVN) or 6–12 brain (cortex and amygdala) slices in each rat. The numbers of immunopositive cells were calculated as an average of 3 rats.

2.10. Statistical analysis

Data and statistical analysis were performed using Microsoft Excel, Preclinical C Package modified from SAS Ver. 8.2 (SAS Institute Japan Ltd., Tokyo, Japan), and EXSUS (CAC EXICARE Corporation, Tokyo, Japan) software. All results were presented as means ± standard error of the mean (S.E.M.). Statistical analyses of two-group comparisons of independent samples were performed using Student's *t*-test or Aspin‑Welch test and the statistical significance was accepted at $P \leq 0.05$. Statistical analyses of multiple comparisons of independent samples were performed using Dunnett's or Steel test, and statistical significance was accepted at $P \leq 0.05$. To examine the dose-responses of CRF or compounds, statistical analyses were performed using one-tailed Williams' test or Shirley–Williams' test, for which statistical significance was accepted at $P \leq 0.025$. Student's *t*-test or Aspin‑Welch test was conducted to compare two groups, vehicle (p.o.) – vehicle (i.v. or i.c.v.) and vehicle (p.o.) – CRF (i.v. or i.c.v.) treatment (in Fig. 6, 7, and 8). The compound-treated groups with CRF were compared with vehicle (p.o.) – CRF (i.v. or i.c.v.) - treated group by one-tailed Williams' test or Shirley–Williams' test for dose response analysis of one compound or by Dunnett's or Steel test for multiple comparison with Compound A and Compound B.

3. Results

3.1. The effects of CRF challenges

3.1.1. Plasma ACTH concentration

CRF was i.v. or i.c.v. injected as peripheral or central CRF challenge under the conscious state and blood was collected 30 min after CRF challenges. Plasma ACTH concentration was increased dose-dependently by both administration routes of CRF (Fig. 1-1A and 1-1B). In i.v. administration, plasma ACTH concentration was increased by CRF dose-dependently and was significant at 0.4 μg/kg (187.2 ± 13.0 pg/mL), 1 μg/kg (268.92 ± 21.83 pg/mL), 4 μg/kg (421.29 ± 41.97 pg/mL), and 8μg/kg (495.81 ± 53.93 pg/mL). The percentage of increase from vehicle-treated group was 254.36% ± 17.59%, 365.39% ± 29.66%, 572.42% ± 57.03%, and 673.67% ± 73.28% at 0.4, 1, 4, and 8 μg/kg, respectively (n = 7, $P \leq 0.025$, Shirley–Williams' test, Fig. 1-1A) compared with vehicle-treated group (73.6 ± 15.5 pg/mL). Dose-dependent increase in ACTH concentration was also observed in i.c.v. administration of CRF and was significant at 0.15 μg (164.5 ± 22.5 pg/mL), 0.3 μg (247 ± 43.13 pg/mL), 0.5 μg (284.24 ± 42.01 pg/mL), and 1 μg (621 ± 33.37 pg/mL) (n = 5−7, $P \leq 0.025$, Shirley–Williams' test, Fig. 1-1B) compared with vehicle-treated group (48.1 ± 5.3 pg/mL). The percentage of increase from vehicle-treated group was 342.18% ± 54.11%, 514.21% ± 103.58%, 591.15% ± 100.90%, and 1107.34% ± 193.65% at 0.15, 0.3, 0.5, and 1 μg, respectively.

3.1.2. Locomotion

Locomotion under habituated environment was measured, and total locomotion for 3 h after CRF challenge is presented in Fig. 1-2 (n = 6–8). The i.v. administration of CRF (5 μg/kg) did not show significant effect on locomotion (Fig. 1-2A and Table 1-2) throughout the measurement (Fig. 1-2C and 1-2D). The i.c.v. administration of CRF (0.03–10 μg) dose-dependently increased locomotion, and its effect reached significance at 0.1 μg compared with that of the vehicle-treated group ($P \leq 0.025$, one-tailed Williams' test, Fig. 1-2B and Table 1-3). The increase in locomotor lasted for more than 2 h (Fig. 1-2C and 1-2D).

3.1.3. Anxiety behavior

Anxiety behavior was evaluated by EPM test after peripheral or central CRF challenges (n = 8–14). Time spent on open arms (Fig. 1-3A and 1-3C) and numbers of arm entries (Fig. 1-3B and 1-3D) were measured during 5 min-trial for each rat. The shorter time spent on, or the less numbers of entries to open arms is regarded as high anxiety. In i.v. administration of CRF (4 and 12 μg/kg), time spent on open arms did not change compared with vehicle-treated group (59.9 ± 6.4 s, Fig. 1-3A). The time spent on open arms was 69.1 ± 7.1 and 61.6 ± 4.3 s in 4 and 12 μg/kg of CRF-treated group, respectively. The numbers of arm entries did not change between groups either (Fig. 1-3B). In contrast, i.c.v. administration of CRF (0.3, 0.5, and 1 μg) dose-dependently decreased time spent on

open arms and was significant at 0.5 and 1 μg compared with that in the vehicle-treated group. The time spent on open arms was 56.2 ± 7.3, 24.9 ± 4.9, and 21.0 ± 7.4 s in vehicle and 0.5 and 1 μg of CRF-treated group, respectively ($P \leq 0.025$, one-tailed Williams' test, Fig. 1-3C). The number of arm entries was also decreased by i.c.v. treatment of CRF and significant at 0.5 and 1 μg compared with vehicle-treated group (4.6 ± 0.6, 7.3 ± 0.3, and 11.9 ± 0.8 for open, closed, and total arm entries, respectively) (Fig. 1-3D). Number of open, closed, and total arm entries was 2.0 ± 0.4, 5.5 ± 0.6, and 7.5 ± 0.8 in 0.5 μg of CRF and 1.5 ± 0.4, 3.4 ± 0.5, and 4.9 ± 0.7 in 1.0 μg of CRF-treated group ($P \leq 0.025$, one-tailed Williams' test).

3.2. In vitro profiles of CRF_1 receptor antagonists

From my in vitro screening of CRF_1 receptor antagonist, I identified Compound A and Compound B (Fig. 1-4A and 1-4B), which had similar binding affinities with IC_{50} values of 15 and 10 nM, respectively (Table 1-1). There are no significant differences between the potency of Compound A and Compound B. Reporter gene assay with luciferase method using Cre-luc CRF_1 protein also showed similar inhibition activity, and IC_{50} values of Compound A and Compound B were 15 and 9.5 nM, respectively (Table 1-1).

3.3. Ex vivo binding assay of CRF_1 receptor antagonists

Ex vivo binding assay was conducted using brain tissues of the frontal cortex and the pituitary in rats after oral administration of 10 mg/kg of Compound A or Compound B (n = 3). Competition between [^{125}I]-CRF and compounds was measured in each brain tissue. Both Compound A and Compound B inhibited binding of [^{125}I]-CRF in pituitary to 16 ± 7% and 41 ± 6% at 1 hour and to 12 ± 3% and 22 ± 6% at 2 h after oral administration of compounds compared with [^{125}I]-CRF binding in the drug-free condition (expressed as time point zero), taken as 100% in calculation (Fig. 1-5A). On the other hand, binding of [^{125}I]-CRF in the frontal cortex was inhibited only by Compound A to 51 ± 13% at 1 hour and 42 ± 10% at 2 h, and not by Compound B (109 ± 9% at 1hour and 110 ±11% at 2 h, Fig. 1-5B).

3.4. Effects of CRF_1 receptor antagonists on endocrinological and behavioral changes by CRF challenge

3.4.1. Effects of compounds on peripheral and central CRF challenges

The effects of 10 mg/kg of Compound A or Compound B on the increase in plasma ACTH concentration induced by i.v. administration of CRF (0.1 μg/kg) were examined (n = 6–8). In the evaluation of compounds on i.v.-administrated CRF, i.v. injection was performed under anesthesia to eliminate physiological stress on HPA axis through the activation of CRF signal in CNS [19]. When compounds were administered 2 h before i.v. injection of CRF, both compounds significantly

suppressed increase in plasma ACTH concentration. The plasma ACTH concentration in the vehicle–vehicle- and vehicle-CRF-treated group was 59.4 ± 9.3 and 161.5 ± 24.5 pg/mL, respectively ($P \leq 0.05$, Aspin–Welch test, versus vehicle-vehicle-treated group). Those in Compound A and Compound B–CRF-treated groups were 79.1 ± 10.1 pg/mL ($P \leq 0.05$, Steel test) and 87.7 ± 7.7 pg/mL ($P \leq 0.01$, Steel test, Fig. 1-6A). The effects of two compounds on increase in locomotion by i.c.v. administration of CRF (1 μg) were studied (n = 6-12). Compound A (10 mg/kg) significantly suppressed the CRF-induced increase of locomotion (Fig. 1-6B) and the suppression was observed throughout the recording period (Fig. 1-6C). Total locomotion for 3 h after CRF administration was 18763.7 ± 1259.9 counts in vehicle–CRF-treated group ($P \leq 0.01$, Aspin–Welch test, versus vehicle–vehicle-treated group, Fig. 1-6B) and that in the Compound A–CRF-treated group was significantly reduced to 11185.0 ± 592.6 counts ($P \leq 0.01$, Dunnett' s test, Fig. 1-6B). The Compound B–CRF-treated group (17881.7 ± 1385.8 counts) did not show significant suppression of locomotion compared with vehicle–CRF-treated group (Fig. 1-6B and 1-6C). Compound A or Compound B did not show effect on the plasma ACTH concentration or locomotion in rats without CRF challenge (Fig. 1-7).

3.4.2. Effect of Compound A on anxiety behavior

The effect of Compound A on anxiety behavior induced by i.c.v. administration of CRF (1 μg) was studied using the EPM test (n = 17–20). Compound B did not show binding affinity to cortex and did not suppress locomotion (Fig. 1-6B). In addition, anxiety behavior was reported to have relationship with cortical CRF signaling [16]. Therefore, I did not examine Compound B because cortical suppression may be critical for the central-modulated changes by CRF [54]. Compound A at 5 and 10 mg/kg significantly increased time spent on open arms (51.0 ± 8.5 and 62.1 ± 13.3 s at 5 and 10 mg/kg, respectively) compared with that in vehicle–CRF-treated group (12.0 ± 3.0 s) ($P \leq 0.025$, one-tailed Williams' test, Fig. 1-8A). The numbers of arm entries were also significantly increased by Compound A treatment (Fig. 1-8B). The number of open arm entries in groups of vehicle and Compound A at 5 and 10 mg/kg- treatment with CRF was 1.1 ± 0.3, 3.5 ± 0.5, and 3.5 ± 0.5, respectively. The number of total arm entries of each group was 4.7 ± 0.8, 9.5 ± 0.6, and 8.2 ± 0.9, respectively. In contrast, compound A did not show the effect on the behavior in the EPM test in rats without CRF challenge (Fig. 1-8C and 1-8D).

3.5. Effect of Compound A on c-fos immunohistochemistry after central CRF challenge

For further analysis of effects of Compound A on central CRF signals in vivo, I measured the number of c-fos-immunopositive cells in the cortex, PVN, and amygdala after i.c.v. administration of CRF (1 μg, n = 3). CRF induced a significant increase in the number of c-fos-immunopositive cells in these brain areas compared with that in vehicle–vehicle-treated group (Fig. 1-9A-D). The numbers

of c-fos-immunopositive cells in vehicle–vehicle-treated group in the cortex, PVN, and amygdala were 736.6 ± 59.3, 182.0 ± 14.0, and 16.2 ± 0.5, respectively. Those in vehicle–CRF-treated group were 1775.9 ± 49.0 ($P \leq 0.01$, Student's *t*-test), 410.7 ± 30.9 ($P \leq 0.05$, Aspin–Welch test), and 94.3± 4.3 ($P \leq 0.01$, Aspin–Welch test), respectively. Compound A (10 mg/kg), administered orally 2 h before i.c.v. administration of CRF, significantly decreased the number of CRF-induced c-fos-immunopositive cells to 1046.8 ± 145.2 in the cortex ($P \leq 0.01$, Student's *t*-test, compared with vehicle–CRF-treated group, Fig. 1-9A and 1-9B) and to 236.8 ± 14.7 in the PVN ($P \leq 0.05$, Aspin–Welch test, versus vehicle–CRF-treated group, Fig. 1-9A and 1-9C); however, it was not decreased in the amygdala (66.4 ± 16.3, Fig. 9A and 9D). Compound A did not show the effect on the number of c-fos-immunopositive cells in rats without CRF challenge (Fig. 1-10).

2. Discussion

Mood disorders such as depression and anxiety are medicated by selective serotonin reuptake inhibitors (SSRI) and benzodiazepine anxiolytics in the current clinical situation [65]. SSRIs enhance monoamine neurotransmission by inhibiting its reuptake. Benzodiazepine works by activating gamma aminobutyric acid $(GABA)_A$ receptors. Various SSRIs and Benzodiazepines have been launched so far; however, there are still high unmet needs in the early onset of efficacy and safety. CRF_1 receptor antagonists have been expected to be one candidate of the new drug for mood disorders with new mechanism of action, however, clinical trials conducted so far have not succeeded in showing the expected efficacy [66]. Nevertheless, as stress is related to mood disorders [67], CRF_1 receptor antagonists may still have opportunities to provide efficacy on stress-related mental diseases, such as anxiety and PTSD.

This study confirmed that the central CRF challenge, but not peripheral challenge, induced behavioral changes in locomotion and anxious behavior, whereas endocrinological response in the HPA axis can be activated by both central and peripheral CRF challenges. These results are in agreement with the reported findings in rats [68] and indicate that the regulation of CRF signaling related to locomotion and anxiety in the CNS may be independent from HPA axis regulation. The other was that a CRF_1 receptor antagonist, Compound A, which inhibits binding of [^{125}I]-CRF in the frontal cortex and the pituitary, decreased behavioral changes induced by central CRF challenge, whereas Compound B, another CRF_1 receptor antagonist which inhibits binding of [^{125}I]-CRF only at the pituitary located outside the BBB, did not. These results indicate that the central CRF_1 receptor antagonism would be involved in anxiolytic and other behavioral effects.

Although the relationship between central and peripheral regulation of CRF signaling is yet to be elucidated, an accumulation of findings indicates independent regulation between central and peripheral CRF signaling. For example, i.c.v. administration of CRF to hypophysectomized rats induced locomotor activation [69] and anxiety behavior [17], but not HPA axis activation. Pryce et al. also compared central and peripheral responses of CRF in endocrinological and behavioral changes [70]. In this report, i.c.v. CRF induced the activation of HPA axis and locomotor inactivity in mice, and the hyper-HPA axis was blocked only by i.p.-injected astressin, and not by i.c.v.-injected astressin, although locomotion was blocked only by i.c.v.-injected astressin [70]. This indicates that the HPA axis may be regulated by CRF at the pituitary level. Results from conditional knockout mice of Crhr1 (CRF_1 receptor) only in the anterior forebrain using calcium/calmodulin-dependent kinase Iiα promoter suggest that the mice showed reduced anxiety with the normal system of HPA axis, although the feedback process of the HPA axis would be impaired by causing the absence of cortical CRF_1 receptor [50]. In this experiment, peripheral or central challenges of CRF were conducted to understand endocrinological and behavioral changes induced by CRF. Although HPA activation was induced by both peripheral and central CRF challenges (Fig. 1-1), increase in

locomotion (Fig. 1-2) and anxiety behavior (Fig. 1-3) were induced only by central CRF challenge, which are consistent with the earlier reported results. Preclinical effects of CRF_1 receptor antagonists have been reported in behavioral assays with exogenously administered CRF [54, 71]. Central challenges of CRF have been known to induce direct peripheral effects on stimulation of the pituitary corticotropic cell, located outside the BBB [72, 73]. Therefore, the central administration of CRF activates HPA axis via direct activation of CRF_1 receptors in the anterior pituitary, which is outside the BBB [74]. In contrast, most reported studies do not show evidence of direct central effects after peripheral CRF challenge [75]. A report by Kastin et al. regarding blood-to-brain entry of [^{125}I]-CRH indicates that the influx of CRF into the brain was low and there would be a saturable efflux system out of the brain [76], which indicates that the activation of HPA axis after i.v. administration of CRF can be induced only by CRF receptor activation in the anterior pituitary. In this aspect, evaluating CRF_1 receptor antagonists on behavioral or neurochemical changes by central CRF challenges may provide key information for identifying potential drug candidates as novel anxiolytics or antidepressants. Further research, especially regarding the regulation of CRF signaling under these physiological conditions, may be important because it is reported that permeability of the BBB may be changed by peripheral CRF activation under acute stress [77] or according to developmental stages [78].

Compound A and Compound B (Fig. 1-4) were found in the in vitro screening assays and have similar CRF_1 receptor in vitro profiles (Table 1-1). When binding was investigated using [^{125}I]-CRF in ex vivo assay, Compound A inhibited binding of [^{125}I]-CRF both in the frontal cortex and the pituitary; however, Compound B antagonized [^{125}I]-CRF only in the pituitary and not in the frontal cortex, maybe due to its low brain permeability (Fig. 1-5). The results of Compound A and Compound B on the activation of HPA axis by peripheral CRF challenge indicate that HPA axis regulation can be accomplished by antagonizing CRF_1 receptors in the pituitary (Fig. 1-6A). In the comparison of i.v. and i.c.v. injected CRF on HPA axis, CRF was injected under conscious state and it was the same condition for behavioral analysis. In the evaluation of compounds on HPA axis, i.v. administration of CRF was conducted under anesthesia [62] because the physiological stress, holding in the apparatus for i.v. injection under conscious state, may be involved in the activation of CRF signaling in the CNS [79] and, with this condition, the effect of compounds on the peripheral CRF signaling may not be evaluated.

From the results of behavioral analysis, antagonizing central CRF_1 receptors seems to be essential to suppress increase in locomotion and anxiety behaviors because Compound B did not reduce locomotion induced by central CRF challenges (Fig. 1-6B). Compound A decreased locomotion (Fig. 1-6B) and anxiety behavior (Fig. 1-8) induced by the central CRF challenge. The independent regulation of anxiolytic effect and HPA axis by a compound has been reported by Philbert et al. using CRF_1 antagonist SSR125543A, and it attenuated long-term cognitive deficit

induced by acute inescapable stress even under the condition in which HPA axis was blunted using dexamethasone [60].

In addition, Compound A inhibited c-fos expression in the cortex and the PVN induced by central CRF challenge (Fig. 1-8). Because c-fos plays an important role in signal transmission [80], it is used as a marker of neural activation [81]. Recently, Takahashi et al. reported that CRF_1 receptor antagonist antalarmin inhibited the c-fos expression induced by central challenge of CRF in the PVN, but not in the central nucleus of the amygdala (CeA) [82]. Consistent with their report, Compound A inhibited c-fos expression in the PVN but not in the amygdala, although we did not segment the amygdala. One cannot exclude the possibility of subtle change in the subregion of amygdala, or the possibility of the different time course of c-fos expression changes in the amygdala. In contrast, the regulation of c-fos expression may not be the same between the central CRF challenge and acute stress, because antalarmin reduced the number of c-fos-immunopositive cells in the CeA under acute stress [82]. Therefore, the evaluation of CRF_1 receptor antagonists on biochemical changes induced by stress is also important to enable an understanding of their roles under these physiological conditions.

Although there are many reports that suggest efficacy of CRF_1 receptor selective antagonists on HPA axis activity, anxiety, and other behaviors in preclinical studies [53, 83, 84], successful results have not been reported in the clinical studies from R121919, the first CRF_1 receptor selective antagonist that entered into a clinical study [85], to Pexacerfont [66]. Further investigation will be needed regarding the compensation system in CRF signaling, pathophysiology of anxiety disorders, and others, to understand the discrepancy between non-clinical and clinical results. One of the key experiments for this point may be translational research to ensure target engagement such as receptor occupancy in the brain. In view of the specific distribution patterns of CRF_1 receptors in the brain areas related to anxiety and stress, such as PVN, cortex, and limbic systems, it may be possible to analyze receptor occupancy by positron emission tomography (PET). There are a few reports about a radio-labeled ligand for CRF_1 receptor [86, 87]. In the PET studies in baboons, [^{11}C]SN003 penetrated the BBB; however, regional variation in total binding could not be observed due to the rapid metabolism or the small number of CRF_1 receptors [86]. Further efforts have been continuously made to identify reasonable PET ligands [88, 89] and target occupancy of CRF_1 receptor antagonists will enable us to predict active doses for clinical studies. So far, because ideal radiotracers for clinical use have not been obtained, effects of CRF_1 receptor antagonist R317573 on regional cerebral glucose metabolism were studied using [^{18}F]fluoro-2-deoxy-D-glucose (FDG) PET in healthy subjects [90]. The results showed dose-dependent increase in cerebral glucose metabolism by acute dosing in frontal cortical regions and decrease in the putamen and right amygdala, indicating the pharmacologically active doses in humans. Thus, preclinical studies for receptor occupancy or neural modulation in the brain by a

compound would provide key information for the selection of a clinical candidate in addition to efficacy on central CRF-induced and stress-induced biochemical and behavioral changes.

In conclusion, the current study showed that central CRF signals have crucial roles in behavioral changes, such as locomotor activity and anxiety behavior. In contrast, the hormonal regulation of CRF through HPA axis can be primarily modulated at the peripheral level. CRF_1 receptor antagonists that block CRF signaling in the CNS, like Compound A, are expected to show anxiolytic effects.

Tables & Figures

Table 1-1.

In vitro binding affinities to CRF_1 receptor and functional activity in reporter gene assay.

	CRF_1 Binding assay IC_{50} (nM)	Reporter gene assay IC_{50} (nM)
Compound A	15	15
Compound B	10	9.5

Table 1-2.

The counts of locomotion for 3 h after CRF i.c.v. administration represented in the Fig. 1-2A.

i.v. CRF (μg/kg)	Counts	% increase from Vehicle
Vehicle	6307.3 ± 339.0	100.0 ± 0.7
5	6491.6 ± 591.6	105.6 ± 1.1

Table 1-3.

The counts of locomotion for 3 h after CRF i.c.v. administration represented in the Fig. 1-2B.

i.c.v. CRF (μg)	Counts	% increase from Vehicle
Vehicle	4201.0 ± 489.5	100.0 ± 1.7
0.03	5289.9 ± 706.4	125.9 ± 2.4
0.1	10605.6 ± 1858.8	252.5 ± 6.3
0.3	10992.2 ± 755.4	224.3 ± 2.6
1	18014.0 ± 2487.4	428.8 ± 8.5
3	31422.3 ± 1825.0	748.0 ± 6.2
10	30179.3 ± 1304.9	615.8 ± 4.4

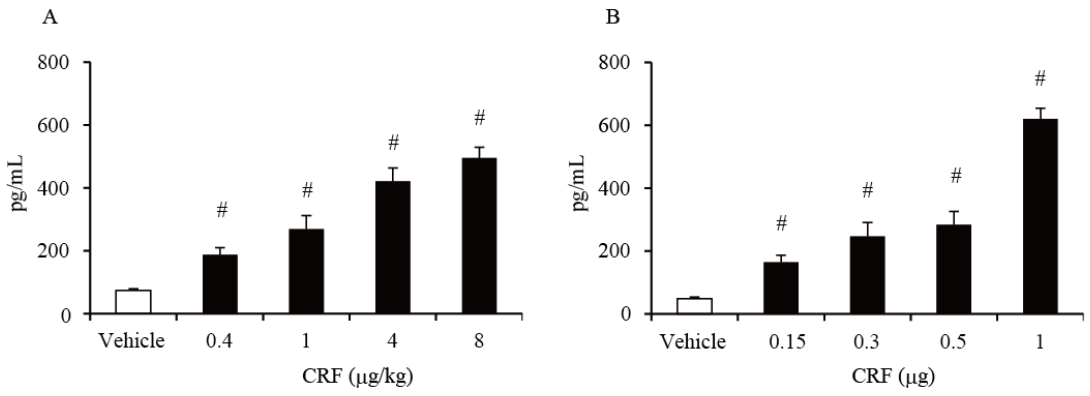


Fig. 1-1. Effects of peripheral and central CRF administration on plasma ACTH concentration in rats. CRF was administered (A) i.v. (μg/kg) and (B) i.c.v. (μg). Blood was collected 30 min after administration. Bars represent mean ± S.E.M. of plasma ACTH concentration (n = 5–7). #$P \leq 0.025$, Shirley–Williams' test.

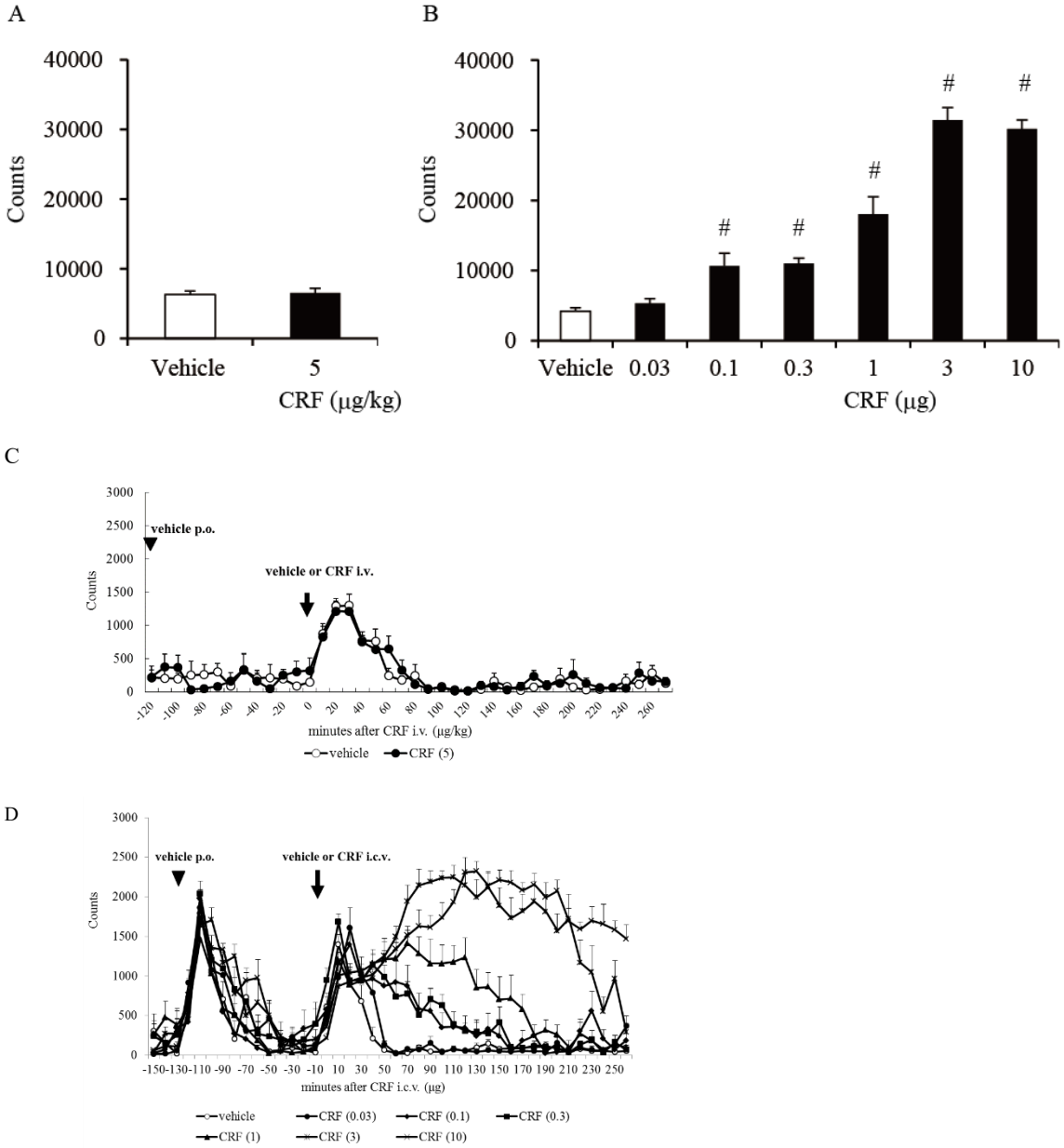


Fig. 1-2. Effects of peripheral and central CRF administration on locomotion under habituated environment in rats. Locomotor activity was counted for 3 h after (A) i.v. or (B) i.c.v. injection of CRF by infrared sensor. (C) Time-course changes of in locomotion in Fig. 1-2A. (D) Time-course changes of in locomotion in Fig. 1-2B. Rats were habituated from 1 day before the experiment in the experimental apparatus with food and water ad libitum. Vehicle was orally administrated 120 min before CRF administration. A plot and bars represent mean ± S.E.M. of counts of (A) and (B); total for 3 h or (C) and (D); 10-bin locomotion (n=6–8). #$P \leq 0.025$, one-tailed Williams' test.

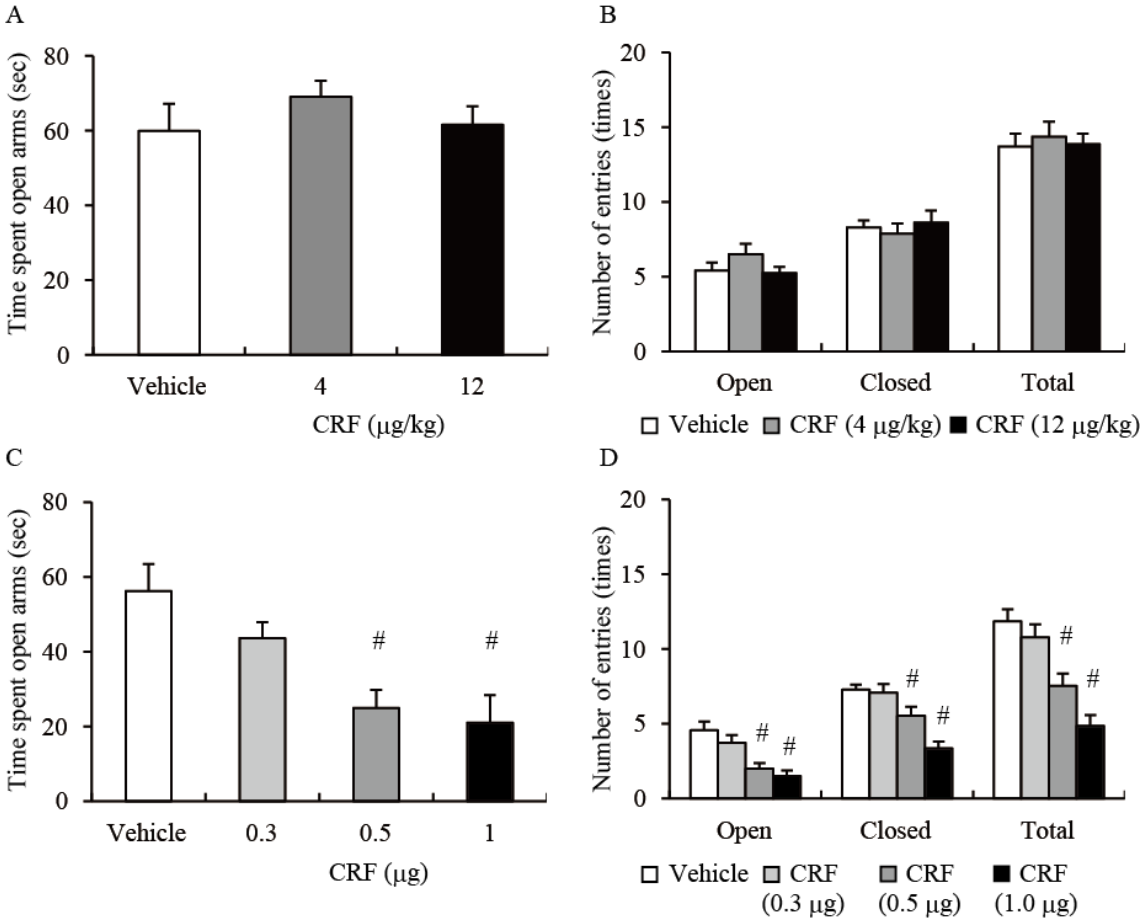


Fig. 1-3. Effects of peripheral and central CRF administration on anxiety behavior in rats. Anxiety behavior was evaluated by the elevated plus maze (EPM) test. (A, B); CRF (4 and 12 μg/kg) was i.v. administered 30 min before the EPM test. (C, D); CRF (0.3, 0.5, and 1 μg) was i.c.v. administered 1 hour before the EPM test. Bars represent mean ± S.E.M. of time spent on open arms (A and C) or mean ± S.E.M. of open, closed or total arm entries (B and D) (n = 8–14). #$P \leq 0.025$, one-tailed Williams' test.

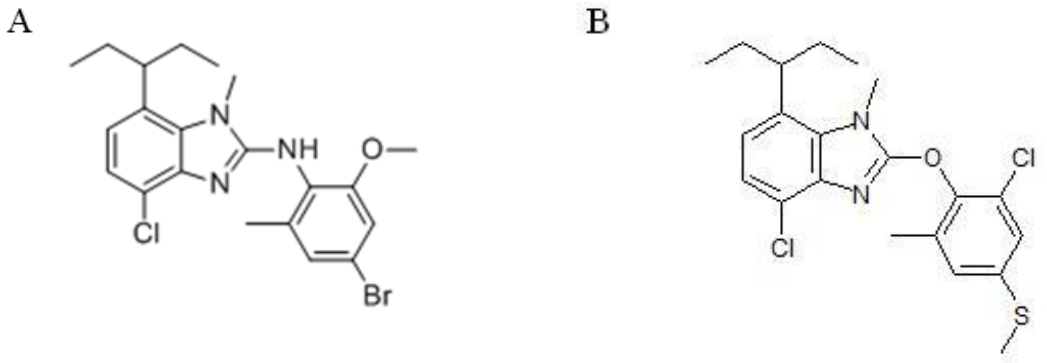


Fig. 1-4. Chemical structures of Compound A and Compound B. Compound A (A), [*N*-(4-bromo-2-methoxy-6-methylphenyl)-4-chloro-1-methyl-7-(pentan-3-yl)-1*H*-benzimidazol-2-amine] and Compound B (B), [4-chloro-2-(2-chloro-6-methyl-4-(methylthio)phenoxy) -1-methyl-7-(pentan-3-yl)-1*H* – benzimidazole], were synthesized in-house.

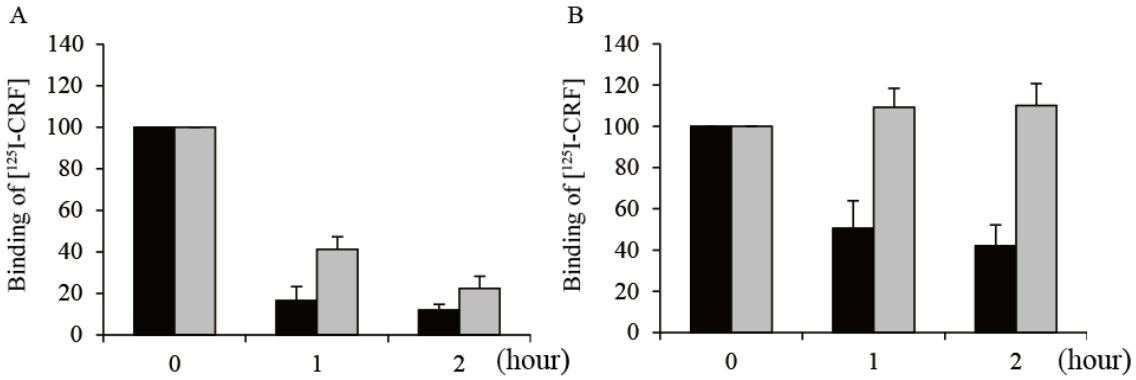


Fig. 1-5. [^{125}I]-CRF binding in membranes of (A) the pituitary and (B) the frontal cortex 1 and 2 h after oral administration of 10 mg/kg of Compound A (black column) or Compound B (gray column). Tissues were homogenized and membranes were prepared. Binding of [^{125}I]-CRF in the rat brain tissues under drug-free condition was defined as 100% binding. Data at 1 and 2 h were represented as mean ± S.E.M. of the percentage of [^{125}I]-CRF binding in the presence of compounds (n = 3).

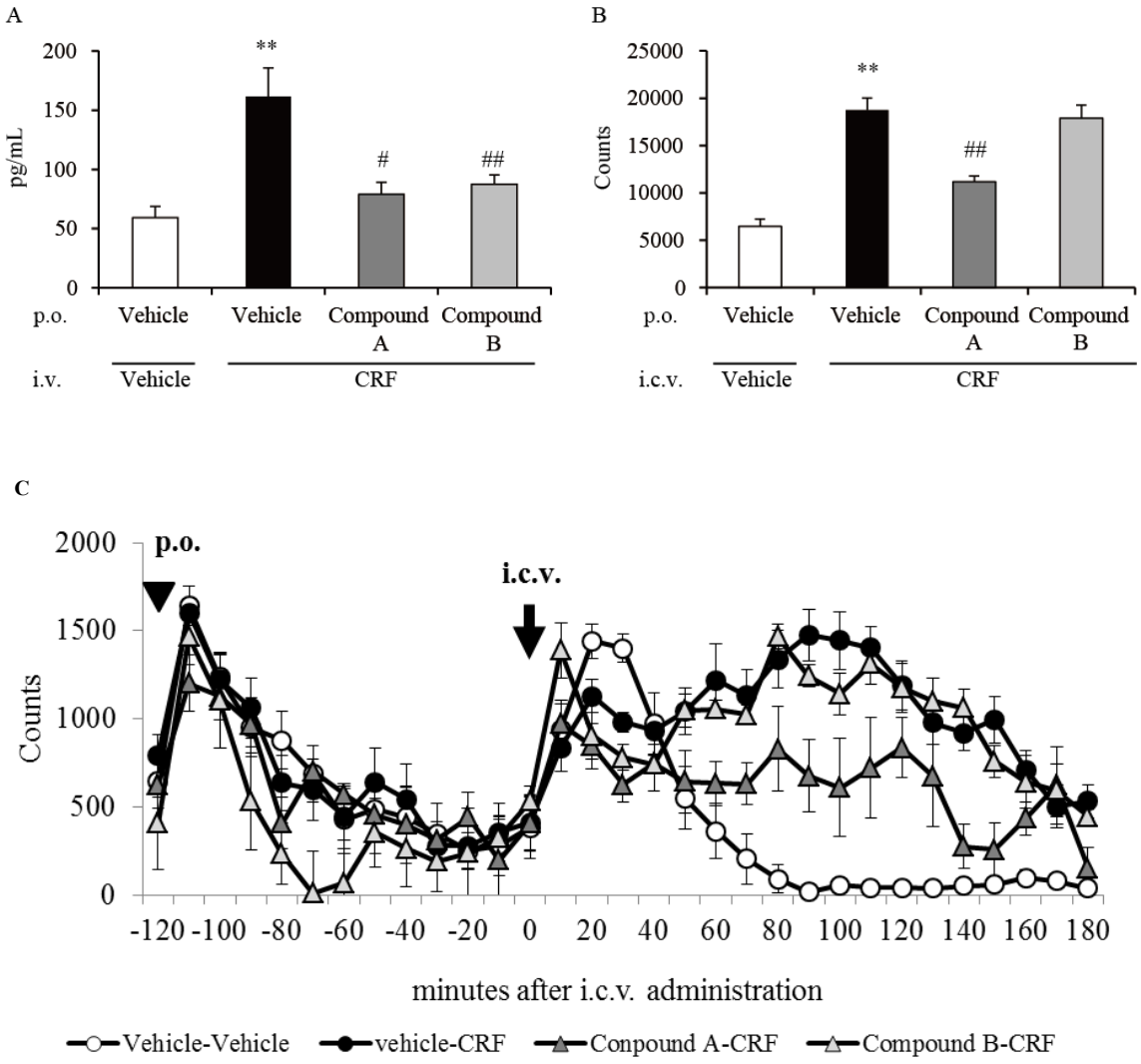


Fig. 1-6. Effects of compounds on HPA-axis activity and locomotion in rats. (A) Plasma ACTH concentration after oral administration of 10 mg/kg of compounds and i.v. administration of CRF (0.1 μg/kg). Compounds were administered 2 h before CRF injection (n = 6–8). Bars represent mean ± S.E.M. of plasma ACTH concentration. $^{**}P \leq 0.05$ (versus vehicle-treated group, Aspin–Welch test); # $P \leq 0.05$, ## $P \leq 0.01$ (versus vehicle-CRF treated group, Steel test). (B) Locomotor activity after oral administration of 10 mg/kg of compounds and i.c.v. administration of CRF (1 μg). Compounds were administered 2 h before CRF injection (n = 6–12). Locomotion was counted for 3 h after CRF injection. (C) Locomotion after oral administration of 10 mg/kg of compounds followed by i.c.v. administration of CRF (1 μg) was expressed as time course changes. A plot represents mean ± S.E.M. of counts of 10-bin locomotion. Bars represent mean ± S.E.M. of locomotion. $^{**}P \leq 0.05$ (versus vehicle–vehicle-treated group, Aspin–Welch test); ##$P \leq 0.01$ (versus vehicle–CRF-treated group, Dunnett' s test).

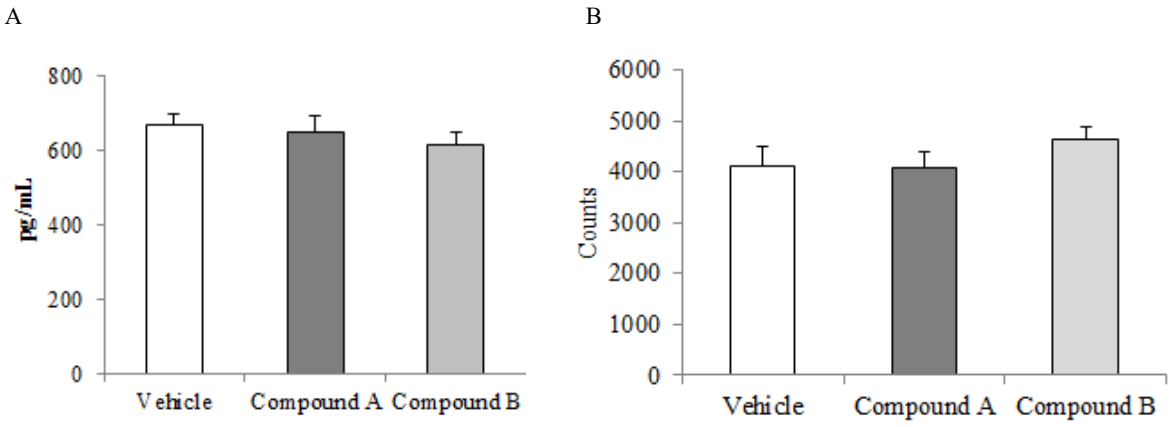


Fig. 1-7. Control experiments of Compound A or Compound B without CRF administration in plasma ACTH concentration and locomotion in rats. Bars represent mean ± S.E.M. of (A) plasma ACTH concentration (n=8) and (B) locomotion (n=10).

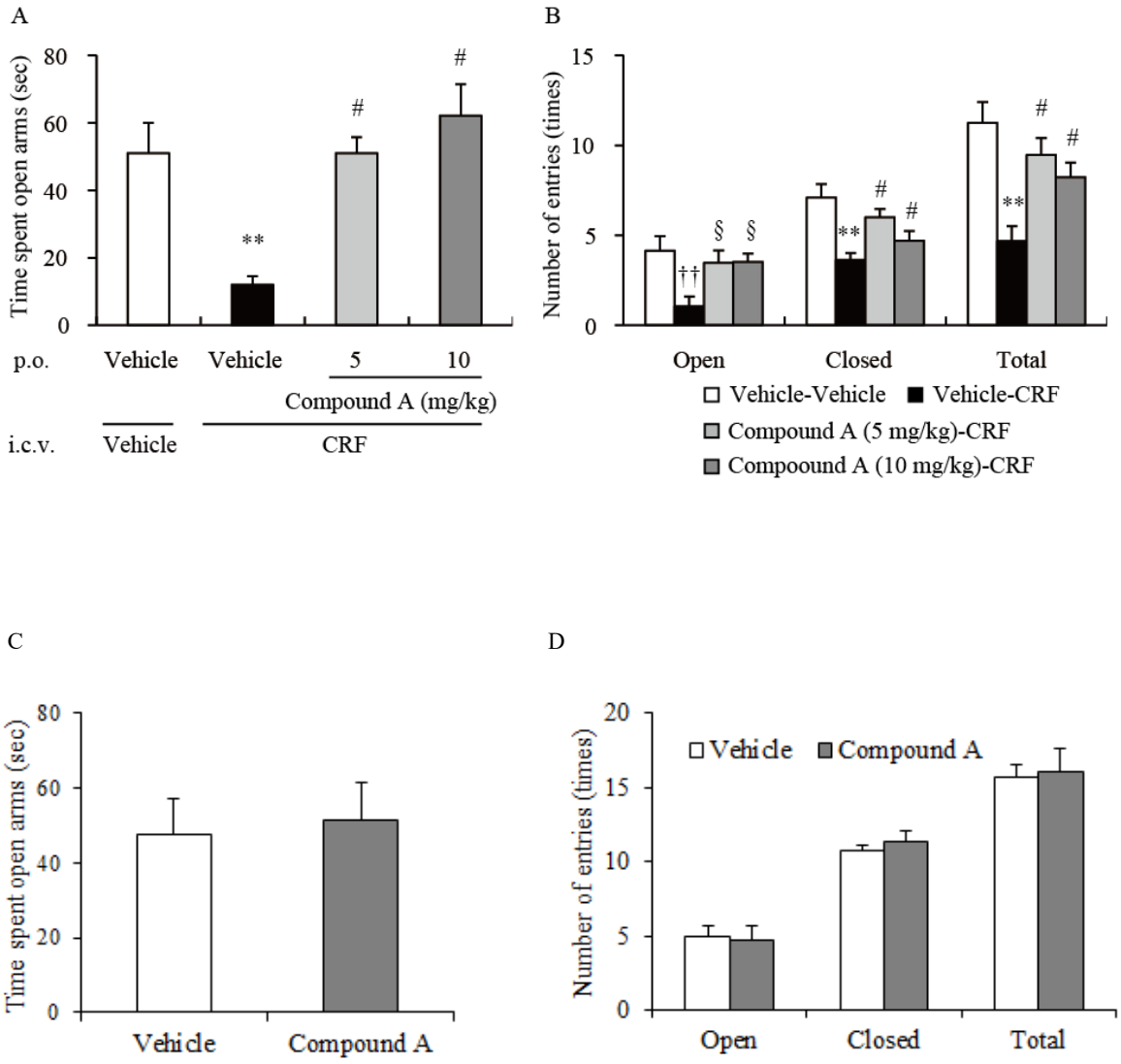


Fig. 1-8. Effect of Compound A on anxiety behavior induced by central CRF challenge. Anxiety behavior was evaluated by elevated plus maze test 1 hour after i.c.v. administration of CRF (1 μg). Compound A (5 and 10 mg/kg) was orally administered 2 h before CRF administration (n = 17–20). Bars represent mean ± S.E.M. of time spent on open arms (A). Number of arm entries (open, closed, and total arm entries) was expressed as mean ± S.E.M. in each treatment group (B). □, vehicle–vehicle; ■, vehicle–CRF; ■, Compound A (5 mg/kg) –CRF; and ■, Compound A (10 mg/kg) –CRF. (C) Time spent open arms and (D) number of arm entries by Compound A or B administration without CRF administration in elevated plus maze test in rats (n=10). Bars represent mean ± S.E.M.

**$P \leq 0.01$, Student's *t*-test (versus vehicle–vehicle-treated group). ††$P \leq 0.01$, Aspin–Welch test (versus vehicle–vehicle treated group). §$P \leq 0.025$, Shirley-Williams' test (versus vehicle–CRF-treated group). #$P \leq 0.025$, one-tailed Williams' test (versus vehicle–CRF-treated group).

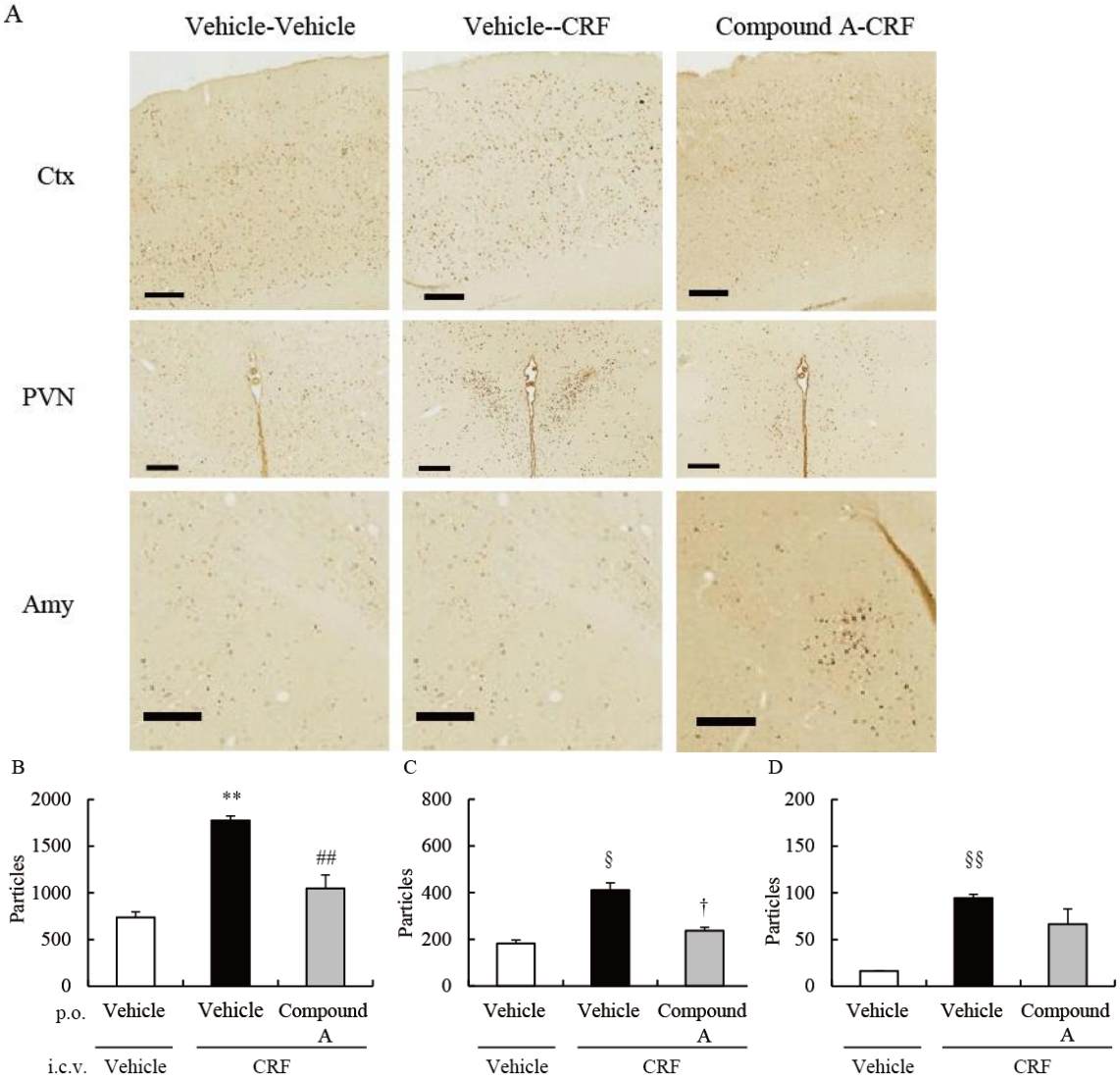


Fig. 1-9. Effects of Compound A on c-fos expression induced by central CRF challenge. (A) Representative slides of the rat cortex and PVN after oral administration of vehicle or Compound A (10 mg/kg) followed by i.c.v. administration of CRF (1 μg). Ctx: cortex, PVN: paraventricular nucleus of hypothalamus, Amy: amygdala. Compound A was administered 2 h before i.c.v. administration of CRF (n = 3). The scale bars represent 500 μm. Result of quantification of immunopositive cells in (B) cortex, (C) PVN, and (D) amygdala. C-fos-immunopositive cells were counted by software Image J (NIH). Each brain area was counted bilaterally in 4–7 (PVN) or 6–12 (cortex and amygdala) brain sections of each rat. The numbers of immunopositive cells were calculated as mean ± S. E. M. of those sections. $^{**}P \leq 0.01$, Student's *t*-test (versus vehicle–vehicle group). $^{\#\#}P \leq 0.01$, Student's *t*-test (versus vehicle–CRF-treated group). $^{\S}P \leq 0.05$, $^{\S\S}P \leq 0.01$, Aspin–Welch test (versus vehicle–vehicle-treated group). $^{\dagger}P \leq 0.05$, Aspin–Welch test (versus vehicle–CRF-treated group).

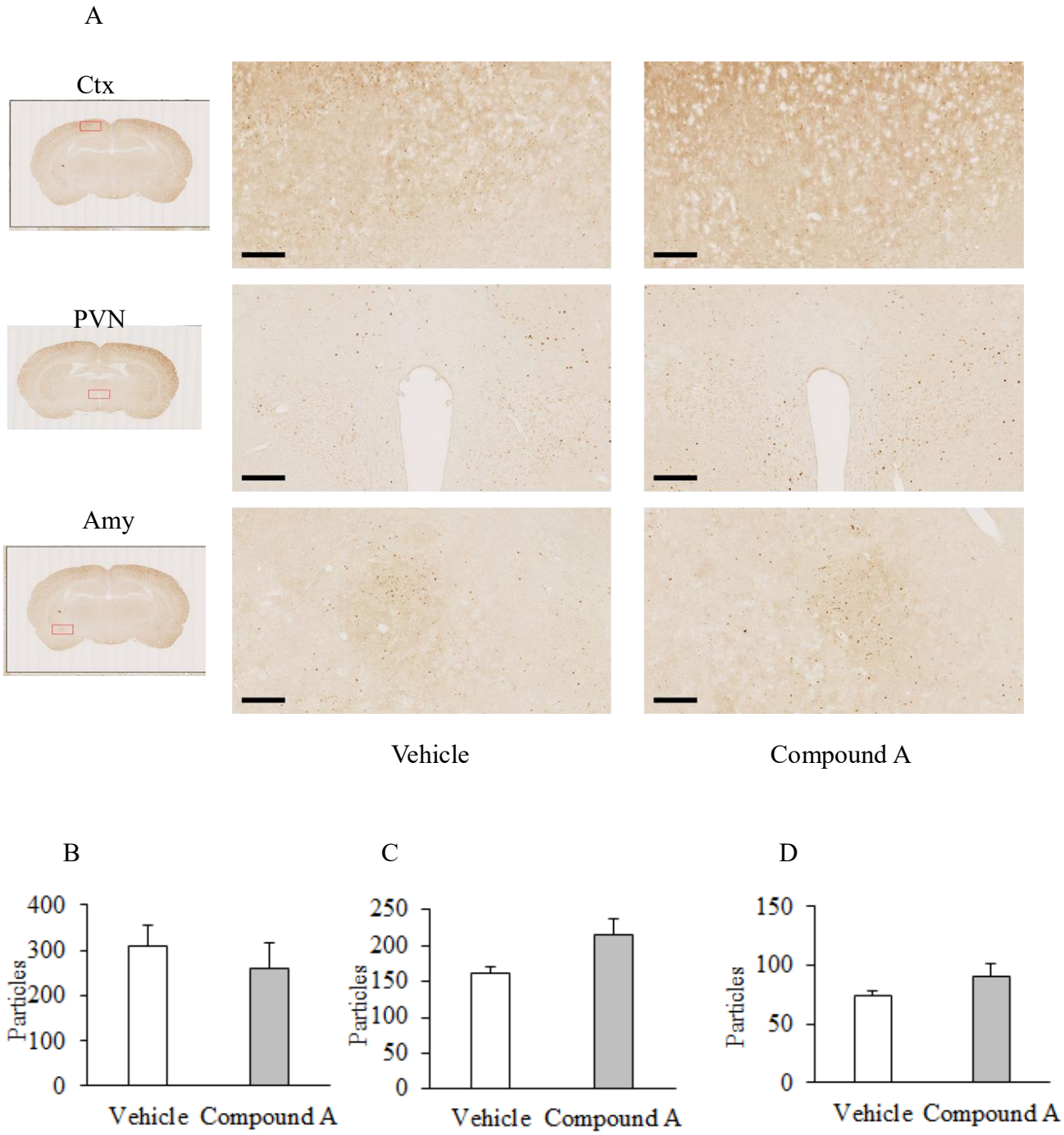


Fig. 1-10. Control experiment of Compound A without CRF in immunohistochemistry of c-fos in rats. The scale bars represent 200 µm. Result of quantification of immunopositive cells in cortex (B), PVN (C), and amygdala (D). C-fos-immunopositive cells were counted by software Image J (NIH). Each brain area was counted bilaterally in 4-7 (PVN) or 6-12 (cortex and amygdala) brain sections of each rat (n=3). The numbers of immunopositive cells were calculated as mean ± S.E.M. of those sections.

Chapter II

Preclinical Characterization of AMPA Receptor Potentiator TAK-137 as a Therapeutic Drug for Schizophrenia

Abstract

The downregulation of the glutamate system may be involved in positive, negative, and cognitive symptoms of schizophrenia. Through enhanced glutamate signaling, the activation of the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor, an ionotropic glutamate receptor, could be a new therapeutic strategy for schizophrenia. TAK-137 is a novel AMPA receptor potentiator with minimal agonistic activity; in this study, I used rodents and non-human primates to assess its potential as a drug for schizophrenia. At 10 mg/kg p.o., TAK-137 partially inhibited methamphetamine-induced hyperlocomotion in rats, and at 3, 10, and 30 mg/kg p.o., TAK-137 partially inhibited MK-801-induced hyperlocomotion in mice, suggesting weak effects on the positive symptoms of schizophrenia. At 0.1 and 0.3 mg/kg p.o., TAK-137 significantly ameliorated MK-801-induced deficits in the social interaction of rats, demonstrating potential improvement of impaired social functioning, which is a negative symptom of schizophrenia. The effects of TAK-137 were evaluated on multiple cognitive domains—attention, working memory, and cognitive flexibility. TAK-137 enhanced attention in the five-choice serial reaction time task in rats at 0.2 mg/kg p.o., and improved working memory both in rats and monkeys: 0.2 and 0.6 mg/kg p.o. ameliorated MK-801-induced deficits in the radial arm maze test in rats, and 0.1 mg/kg p.o. improved the performance of ketamine-treated monkeys in the delayed matching-to-sample task. At 0.1 and 1 mg/kg p.o., TAK-137 improved the cognitive flexibility of subchronic phencyclidine-treated rats in the reversal learning test. Thus, TAK-137-type AMPA receptor potentiators with low intrinsic activity may offer new therapies for schizophrenia.

1. Introduction

Schizophrenia consists of a spectrum of symptoms: positive symptoms (hallucinations and delusions), negative symptoms (blunted affect and deficits in social functioning), and cognitive symptoms (deficits in attention, working memory, and cognitive flexibility) [2, 91]. The hyperdopamine hypothesis postulates that excessive activation of dopaminergic neurons in the subcortical regions of the brain is deeply involved in the pathophysiology of the positive symptoms of schizophrenia [7]. Current antipsychotics, based on dopamine D2 receptor (D2R) antagonism, are effective against the positive symptoms of schizophrenia; however, the efficacy against the negative and cognitive symptoms is limited [7, 92] . In addition, their side effects such as extrapyramidal symptoms and metabolic changes, limit their clinical application [93]. Owing to the limitations of the efficacy and safety of the current medications, there is a large unmet need for novel therapeutic strategies for schizophrenia.

As postulated by the hypoglutamate hypothesis, the downregulation of glutamate signaling, especially the dysfunction of the *N*-methyl-D-aspartate (NMDA) receptor in the medial prefrontal cortex plays an important role in schizophrenia [30, 94, 95]. NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, induce not only psychotic symptoms, but also negative and cognitive symptoms in healthy subjects [24, 96, 97]. Thus, the activation of the NMDA receptor may offer a potential therapeutic approach against schizophrenia. Bitopertin (RG1678), a glycine transporter type 1 (GlyT1) inhibitor, which can activate the NMDA receptor by increasing the level of the NMDA receptor co-agonist (glycine) [33], improved the negative symptoms of schizophrenia [34]. Moreover, glycine or D-serine—agonists at the glycine site of the NMDA receptor, significantly improved the negative symptoms of schizophrenia [31, 32]. However, strategies that directly activate NMDA function have not been successful, possibly owing to the excitotoxic side effects [98, 99] or the desensitization of the NMDA receptors [100].

The α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor is an ionotropic glutamate receptor that plays a key role in learning and memory [21]. Glutamate receptor subunit 1-knockout mice exhibited cognitive impairment, hyperdopaminergia, and psychosis-like behaviors [22]. The AMPA receptor is involved in the regulation of NMDA receptor activation, which induces ion influx into cells, triggering the release of channel-blocking magnesium ion from the NMDA receptor. This results in the activation of NMDA receptor signaling through an increase in NMDA receptor-mediated calcium influx [23]. Therefore, the potentiation of the AMPA receptor is expected to offer a new therapeutic strategy for schizophrenia through the enhancement of glutamate signaling.

Recently a novel AMPA receptor potentiator—TAK-137 (9-(4-phenoxyphenyl)-3,4-dihydropyrido[2,1-c][1,2,4]thiadiazine 2,2-dioxide) has been discovered. TAK-137 presented lower risks of a bell-shaped dose response and seizure owing to its low agonistic

activity [38, 39]. TAK-137 enhanced cognitive function in I rats and non-human primates. In this study, I characterized TAK-137 as a therapeutic drug for schizophrenia by using behavioral test batteries for the positive, negative, and cognitive symptoms of schizophrenia in rodents and non-human primates.

Methamphetamine (METH) enhances dopamine release and activates dopaminergic neurons in the subcortical regions of the brain, which causes psychotic symptoms that resemble the positive symptoms of schizophrenia [101]. Based on hyperdopamine and hypoglutamate hypotheses for psychosis, METH and MK-801 (an NMDA receptor antagonist) were used, respectively. As for cognitive functions, I evaluated the effects of TAK-137 on multiple cognitive domains associated with schizophrenia, such as attention, working memory, and cognitive flexibility, using behavioral test batteries in rats and monkeys [2, 102]. The data suggest that TAK-137-type AMPA receptor potentiators with low intrinsic activity may be a promising treatment for the multiple symptoms of schizophrenia, especially for negative and cognitive symptoms.

2. Materials and Methods

2.1. Drug administration

TAK-137 was synthesized by Takeda Pharmaceutical Company Limited (Fujisawa, Japan) and suspended in 0.5 % (w/v) methylcellulose in distilled water; oral administration (p.o.) was conducted at a volume of 2 mg/kg in rats, 20 mg/kg in mice, and 5 mg/kg in monkeys. A solution of 0.5 % methylcellulose was administered as the vehicle control. Methamphetamine hydrochloride (METH, Sumitomo Dainippon Pharma, Osaka, Japan) at 0.5 mg/kg and (+)-MK-801 hydrogen maleate (MK-801, Sigma-Aldrich St Louis, MO) at 0.08 or 0.1 mg/kg were dissolved in 0.9 % saline and subcutaneously administered (s.c.) to rats at a volume of 2 mg/kg. Phencyclidine hydrochloride (PCP, Sigma-Aldrich, Poole, UK) (2 mg/kg) was dissolved in 0.9 % saline and administered intraperitoneally (i.p.) to rats at a volume of 1 mg/kg twice per day for 7 days. Ketamine hydrochloride (KETALAR®, Daiichi Sankyo Propharma Co. Ltd, Tokyo, Japan) was dissolved in 0.9 % saline and administered intramuscularly (i.m.) to monkeys at a volume of 5 mg/kg.

2.2 Animals

Animal species and strains were selected based on the previous publications for each experiment. Male Sprague-Dawley (SD) rats were purchased from Charles River Laboratories, Japan (Yokohama, Japan) for the measurements of locomotion (7-week-old rats) [103]. Male ICR mice and Wistar rats were purchased from CLEA Japan Inc. (Tokyo, Japan) for the measurement of locomotion (6-week-old mice) [103] and the social interaction (SI) test (6-week-old rats), respectively. Male Long-Evans rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) for the five-choice serial reaction time task (5-CSRTT) (6-week-old rats) and the radial arm maze (RAM) test (8-week-old rats) [104]. Female Lister Hooded rats (21 days postnatal) were supplied by Harlan Laboratories UK, Ltd. (Bicester, UK) for the reversal learning test [105]. Female rats were used for reversal learning because females are highly sensitive to PCP [106] and showed more robust performance in certain cognitive tasks compared with male rats [107, 108]. Rats and mice were housed in groups of 4 or 5 per cage in a light-controlled room (12 h light/dark cycle; lights on at 7:00 AM) with free access to food and water. The room temperature and humidity were 20–25 °C and 40–60 %, respectively, and the animals were given a minimum acclimation period of 1 week prior to the experiment. The animals were randomly assigned to the vehicle- or compound-treated groups.

The delayed matching-to-sample (DMTS) task was evaluated in 4–6-year-old male cynomolgus monkeys (*Macaca fascicularis*) weighing 4–6 kg (Keari Co. Ltd., Osaka, Japan). The monkeys were housed individually in cages stored at a room temperature of (24 ± 1) °C and a humidity of (55 ± 15) %, with a 12 h light/dark cycle (lights on at 7:00 AM). The monkeys were fed a complete, nutritionally balanced diet with fruit once daily (approximately 3:00–4:00 PM) and water was available *ad libitum*. All monkeys were housed and handled in strict accordance with good animal

practice under the supervision of veterinarians. They received environmental enrichment and were monitored for evidence of disease and changes in attitude, appetite, or behavior suggestive of illness. The care and use of the animals, and the experimental protocols used in this research except the reversal learning test, were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited. The reversal learning test was carried out at b-neuro™ (University of Manchester, UK) in accordance with the Animals Scientific Procedures Act, UK, and was approved by the Ethical Review Panel of the University of Bradford.

2.3 Behavioral assays

2.3.1. Locomotion measurement in rats

Locomotion was automatically evaluated using an infrared sensor system (SuperMex, Muromachi Kikai, Tokyo, Japan). The infrared sensor was placed on the center of the cover on the cage with a hole to detect locomotion. Each rat was placed in a Plexiglas covered cage (38 × 25 × 32 cm) for more than 2 h before the experiment was started to allow acclimation to the experimental environment. Food and water were available *ad libitum*. After acclimation, TAK-137 or the corresponding vehicle was administered p.o. 4 h prior to METH administration (0.5 mg/kg, s.c.). The numbers of animals used were as follows—vehicle/vehicle: 6, vehicle/METH: 12, TAK-137 (0.1 mg/kg)/METH: 11, TAK-137 (1 mg/kg)/METH: 13, and TAK-137 (10 mg/kg)/METH: 13. The data were collected as an accumulation of the counts of infrared sensors in each 3 min block after METH administration and are presented as the mean ± standard error of the mean (S.E.M.) of the changes over time and the accumulation of activity counts for 120 min after METH administration. All the data were stored in a personal computer and analyzed by appropriate software (Comp ACT AMS, Muromachi Kikai, Tokyo, Japan).

2.3.2 Locomotion measurement in mice

Locomotion was automatically counted with an infrared sensor automated activity monitoring system. Each of the 36 test cages (30 × 40 × 20 cm) was equipped with a pyroelectric infrared sensor. Each mouse was allowed to acclimate to the test cage for more than 2 h before the experiment was started. After acclimation, TAK-137 or the corresponding vehicle was administered p.o. 2 h before the s.c. administration of MK-801 (Sigma-Aldrich St Louis, MO) at 0.3 mg/kg in 0.9 % saline or the corresponding vehicle. The numbers of animals used were as follows—vehicle/vehicle: 6, vehicle/MK-801: 13, TAK-137 (1 mg/kg)/MK-801: 14, TAK-137 (3 mg/kg)/MK-801: 13, TAK-137 (10 mg/kg)/MK-801: 16, and TAK-137 (30 mg/kg)/MK-801: 15. The data were collected as accumulation of counts of infrared sensors in each 3 min block after MK-801 administration and presented as the mean ± S.E.M. of the changes over time and the accumulation of activity counts for 120 min after MK-801 administration.

2.3.3 SI test

The experiment was performed as described previously [109] with some modifications. One day before the experiment, the rats were placed in the experimental room for 2 h and orally administered distilled water for acclimation. On the day of the experiment, the rats were again placed in the experimental room for 2 h for acclimation. After acclimation, TAK-137 (0.1 or 0.3 mg/kg) or the corresponding vehicle was administered p.o., followed by s.c. injection of MK-801 (0.1 mg/kg) or the corresponding vehicle at 4 h before testing (n = 14 per group). Two rats in the same drug treatment group, from the different home cages but with a body weight difference of less than 15 g were placed diagonally opposite in a test box (60 × 60 × 60 cm, 35–40 lux). The SI between 2 rats was determined from the total time spent participating in social behaviors such as sniffing, genital investigation, chasing, and fighting. The interaction time within a 10 min test period was measured by a researcher through the monitoring of the CCD camera viewing the test box under blind condition. Between each session, the test box was cleaned with 10 % ethanol. In addition to the interaction time, the locomotor activity of each animal was measured by the tracking system in the same software (TopScan, CleverSys Inc., Reston, VA). The data are presented as the mean ± S.E.M. of the interaction time and distance moved during the test.

2.3.4 5-CSRTT

The experiment consisted of 2 sessions: training and testing. The training session started with 7-week-old male Long-Evans rats, and 13 rats were used for the test at 12-13 months of age. These rats were also used to assess the effects of drugs other than TAK-137 on 5-CSRTT performance between ages 6 and 12 months. They had a 2-week washout period prior to the start of the study with TAK-137. From the training session, food was restricted to 80–85 % of the animals' free-feeding body weight, throughout the experimental period. The training and testing were conducted by using 4 operant chambers enclosed in sound-attenuating boxes (Med Associates Inc., St Albans, VT). Each chamber contained a curved wall with 5 contiguous apertures. Food pellets were supplied automatically into a magazine located in the opposite wall of the 5 contiguous apertures in the chamber, and a photocell beam was used to detect head entries into the magazine. In the training session, a pellet was delivered into the magazine at the start of each session to initiate the first trial. After a 5 s intertrial interval (ITI), a light stimulus was presented in one of the 5 apertures, followed by a 5 s limited hold in the absence of light stimulus. The duration of the light stimuli was set to 5 s and gradually decreased during training to 2 s. The rats were required to nose-poke into the illuminated aperture. The correct responses (nose-poke responses in the illuminated aperture during a light stimulus and limited hold) resulted in the delivery of a food pellet into the magazine, with sound and light signals above the magazine occurring for 2 s. Rats with incorrect responses

(nose-poke responses in non-illuminated apertures), omissions (failure to respond during the limited hold), and premature responses (responses occurring prior to the presentation of the stimulus) were punished by a 5 s time-out period, with extinction of the house light and no delivery of food. Each session lasted for 35 min or until 100 trials were completed. From a group of 15 trained rats, 13 rats that achieved the performance criteria (> 75 % correct responses and < 20 omissions) over three consecutive days were used for the experiment. In the testing session, the duration of light stimuli on the aperture was set to 0.5 s. Vehicle or 0.2 mg/kg TAK-137 was orally administered to the rats 4 h prior to testing in a crossover fashion, with a washout period of 1 week. The data are indicated as the mean ± S.E.M. of the number of correct responses, omissions, and premature responses.

2.3.5 RAM test

The experiment was performed as previously described [110, 111] with a minor modification. The dimensions of each arm were 50 cm × 10 cm × 40 cm (length × width × height); the maze was elevated 50 cm above the floor. After a 24 h fast, the rats' food intake was restricted to 80–85 % of the free-feeding body weight on the first day of exposure to the maze and throughout the experimental period. The RAM test consists of 2 sessions: training and testing. In the training session, rats were acclimated to the maze and then trained. Reinforcement consisted of 3 food pellets (45 mg each) in a food cup. On the first day of acclimation to the maze, reinforcements were placed near the entrance and at the mid-point of each arm. Three rats were placed on the maze once and allowed to explore and consume the pellets for 8 min. On the second day of acclimation, each rat was placed on the maze and allowed 5 min to consume the pellets in the food cups placed at the mid-point and at the end of each arm. From the third day, reinforcements were placed in a food cup at the end of each arm. The rats were well-trained to collect the pellets placed on the edge of each arm. The learning criterion for the testing session was defined as 2 errors or fewer for 2 consecutive days. From a group of 50 rats which were trained for 15 days, 36 rats that achieved the performance criterion were used for the experiment. On the previous day of the testing session, the baseline level of performance of the rats was assessed to select the rats that would complete the collection of all the pellets placed in the 8 arms with 2 errors or fewer. In the testing session, each rat was placed on the maze facing away from the researcher and facing the fixed arm at the start of the trial. The entry of rats into each arm was recorded in sequence. The rats were allowed to explore until all the pellets in the 8 arms were consumed, or 5 min had elapsed; entry into an arm previously chosen was counted as an error. If an animal left some of the 8 arms unexplored during the 5 min session, the number of unexplored arms was also counted as an error. TAK-137 (0.2 and 0.6 mg/kg, p.o.) or corresponding vehicle was administered 4 h before the testing session. MK-801 (0.08 mg/kg, s.c., as a salt) or saline was administered 30 min before the testing session. The numbers of animals used were as follows— n = 7 in the vehicle/vehicle-treated group, n = 9 in the vehicle/MK-801-treated group, n =

11 in the TAK-137 (0.2 mg/kg)/MK-801-treated group, and n = 9 in the TAK-137 (0.6 mg/kg)/MK-801-treated group. The data are presented as the mean ± S.E.M. of errors in the testing session.

2.3.6 DMTS task

The experiment was performed on 4–6-year-old male cynomolgus monkeys. The monkeys were maintained on 80 % of their free-feeding body weight throughout the experiment. 4 monkeys were trained to perform the DMTS task by using the Cambridge Neuropsychological Test Automated Battery system (CeNes, Cambridge, UK) [112]. Briefly, a trial was initiated by the presentation of an image of a sample object on the screen. The monkey had to touch this object on the screen within 30 s. Subsequently, the sample object diminished from the screen, followed by a variable delay (0, 4, 8, or 16 s) before the re-appearance of the sample object together with 3 other objects. The monkey had to choose the sample object from the 4 objects and a correct choice was rewarded by food. The ITI was 5 s and one session consisted of 96 trials (24 trials with delays of 0, 4, 8, and 16 s). The variable delay durations were randomly presented within the 96 trials. The criterion for the experimental use of monkeys was 70 % or more of correct responses over the 96 trials. In the testing session, TAK-137 (0.1 mg/kg, p.o.) or corresponding vehicle were administered to monkeys 2 h before DMTS testing. Ketamine (1.0 mg/kg i.m., as a salt) or corresponding vehicle was administered 15 min before DMTS testing. The administration followed a crossover design (n = 4). The dose of ketamine and the number of animals were determined on the basis of previous reports [39, 113] and I confirmed that the ketamine-induced deficits in the DMTS task was detectable under the experimental condition of n = 4. The correct responses were recorded for all the trials during the test sessions. The data were subdivided by delay interval, which consisted of 24 trials in each session, and are presented as the mean ± S.E.M. of the percentage of correct trials out of the 96 trials per session.

2.3.7 Reversal learning test

The reversal learning test was conducted by b-neuro™ (University of Manchester, UK). The training and testing methods have been previously described [105]. All rats were tested in operant chambers with 2-lever Skinner boxes. The details of the apparatus were described in the previous report [114]. The boxes were controlled by Med-PC software (Version 2.0 for DOS or Med-PC for Windows, Med Associates, Inc. Lafayette, IN). Programs were written using Medstate notation. At 12 weeks post-weaning, the female Lister Hooded rats (n = 60) were initially trained to respond to food (45 mg Noyes pellets, PJ Noyes Co Inc., Sandown Chemical Ltd) on a fixed ratio 1 (FR1) schedule of reinforcement in standard 2-lever Skinner boxes for 30 min. In the FR1 schedule, one press of either lever resulted in the delivery of a food pellet. The rats were food-restricted to approximately 85 % of

free-feeding body weight, maintained throughout the training and testing by feeding them approximately 12 g of standard lab chow per rat per day. Following a stable level of response to the FR1 schedule, the rats were trained to respond to food in the presence or absence of a visual cue in the form of a light stimulus above the lever. At the start of each session, the house light was turned on; both levers were introduced into the chamber, and the activation of one lever resulted in the delivery of a food pellet. One half of the animals were trained to respond in the presence of visual cue and the other half in the absence of the cue. Following a response on one lever, the house light was turned off and both levers were retracted for a period of 3 s; subsequently, the cycle was repeated. The rats were tested until 128 responses were obtained, or until the experimental session was terminated 30 min after the initiation of the training. The active lever was changed from session to session according to a pseudorandom Gellerman schedule. The rats participated in approximately 10–15 sessions over 2–3 weeks of training on the initial reward contingency and I ensured that they met the criterion on this initial task; i.e., achieved at least 90 % of correct responses with each lever active in at least 2 sessions. In the next step, they were trained on the opposite reward contingency, the reversal task. Once the criterion (90 % of correct responses) was achieved on the reversal task, PCP (2 mg/kg, as a salt) or vehicle was administered (i.p.) at a volume of 1 mg/kg twice per day for 7 days [115]. During the PCP treatment period, the animals did not receive training to avoid association between drug treatment and cognitive performance. After 1-week drug-free period without training [116], the reversal testing was carried out on the animals. One day before the reversal testing, the rats were trained to respond to food using a randomly assigned contingency (i.e., in the presence or absence of a cue). The session was terminated after the consumption of 128 pellets. Both PCP- and vehicle-treated groups were required to achieve 90 % of correct responses. If the animals failed to reach this criterion, they were subjected to further training until they were able to sustain 90 % of the correct responses. The reversal testing consisted of 2 experimental phases: initial and reversal. In the initial phase, a consistent reward contingency like that of the previous training day was presented to the rats for 5 min or until the rats had earned 20 pellets. Following the initial phase, there was a time-out period of 2 min (the house light was turned off). The animal stayed in the Skinner box during this time-out period and the reversal phase was then initiated. In the reversal phase, the reward contingency was reversed so that the animals must respond in the opposite way from the initial test. The reversal test was also performed for 5 min. TAK-137 (0.01, 0.1, or 1 mg/kg, p.o.) or vehicle was administered 2 h before the initial phase of the reversal testing. The numbers of animals used were as follows: n = 10 in the vehicle/vehicle-treated group, n = 8 in the vehicle/PCP-treated group, and n = 8 in the TAK-137/PCP-treated groups. The data are presented as the mean ± S.E.M. of the percentage of correct responses in the initial and reversal phases of the reversal testing.

2.3.8 Novel Object Recognition Test (NORT)

On day 1, male Long-Evans rats at 6 weeks old (Japan SLC Inc., Hamamatsu, Japan) were allowed to habituate to the behavioral test room environment for over 1 h, and then they were allowed to habituate to the empty test box [a gray-colored polyvinyl chloride box (40 × 40 × 50 cm)] for 10 min individually. Testing was composed of 2 trials; the acquisition and the retention trials (3-min per trial). These trials were separated by a 48-h inter-trial interval (ITI). On day 2, during the acquisition trial, rats were allowed to explore 2 identical objects (A1 and A2) for 3 min. On day 4, in the retention trial, rats were allowed to explore a familiar object (A3) and a novel object (B) for 3 min. Exploration of an object was defined as licking or touching the object with forelimbs while sniffing. Leaning against the object to look upward, and standing, or sitting on the object were excluded. The exploration time for each object (A1, A2, A3, and B) in each trial was scored manually. A novelty discrimination index (NDI) was calculated as the novel object interaction time / total interaction time × 100 (%).

2.3.9 Measurement of cataleptic response

The experimental procedure was performed in accordance with the method reported by Hoffman and Donovan [1217]. On the day before the experiment, the rats were trained to grab a horizontal metal bar at a 13 cm height with their forelimbs for approximately 30 s. Catalepsy-like behavior was measured 4 h after the administration of vehicle or TAK-137 (0.1, 1, and 10 mg/kg, p.o.) in blinded condition (n = 12). A rat was placed in front of the metal bar, with its forelimbs placed on the bar, and the time until the rat removed both forelimbs from the bar was recorded. Animals with sustained grabbing posture for 90 s were removed from the apparatus and assigned a latency time of 90 s. The average of three consecutive trials was recorded to determine the duration of the cataleptic response. The data are presented as the mean ± S.E.M. of the average latency time before the removal of both forelimbs from the bar in three consecutive trials.

2.4 Measurement of plasma prolactin levels

TAK-137 (0.1, 1, and 10 mg/kg, p.o.) was administered 4 h before decapitation and whole blood samples were collected (n = 6). The collected samples were stored on ice in EDTA-containing tubes. Plasma was obtained by the centrifugation of whole blood (4 °C, 12000 rpm, and 15 min). The plasma supernatant was collected, transferred to another tube, and stored in a deep-freezer until use. The plasma concentrations of prolactin were measured by using an enzyme-linked immunoassay kit (Bertin Pharma, Montigny le Bretonneux, France).

2.5 Measurement of plasma or brain concentration of compounds

Male Sprague-Dawley rats (Charles River Laboratories Japan) were used in this experiment. The rats

were administered TAK-137 (0.1 mg/kg, p.o.), and were sacrificed by decapitation after 2 h for the collection of blood and brain tissues. The plasma was separated from the blood samples by centrifugation. The brain tissue was homogenized with saline. The concentrations of TAK-137 in the plasma and brain were determined by using liquid chromatography-tandem mass spectrometry.

2.6 Combination effects of olanzapine and TAK-137 in locomotion, NORT, cataleptic response, and plasma prolactin levels

Olanzapine was extracted from Zyprexa® (Eli Lilly and Company, Indianapolis, Indiana, US) at KNC Laboratories Co., Ltd. (Kobe, Japan) and dissolved in 1.5 % (v/v) lactic acid. The pH of the solution was adjusted to neutral using 1 M NaOH. Olanzapine was administered (p.o.) at a volume of 2 mg/kg in rats. After the habituation, TAK-137 (0.1, 1, and 10 mg/kg, p.o.), olanzapine, or corresponding vehicle was orally administered 4 h (TAK-137) or 1 h (olanzapine) before METH administration (0.5 mg/kg, s.c.). In NORT, TAK-137 (1 mg/kg) and olanzapine (3 mg/kg) were orally administered 2 h or 1 h prior to the acquisition and the retention trials, respectively. (n = 10). Catalepsy-like behavior was measured 4 h after the administration of vehicle, TAK-137 (0.1, 1, and 10 mg/kg, p.o.), or olanzapine (3 mg/kg, p.o.) in blinded condition (n =12). For the measurement of plasma prolactin levels, TAK-137 (0.1, 1, and 10 mg/kg, p.o.) was administered 4 h before, and olanzapine (3 mg/kg, p.o.) was administered 1.5 h before decapitation to collect whole blood (n = 6).

2.7 Statistical analysis

The data are presented as the mean ± S.E.M. Statistical analysis was performed by EXSUS (CAC Croit Corporation). The *F* test, followed by Student's *t*-test (for data with homoscedasticity with $P \geq 0.2$ by *F* test) or Aspin-Welch test (for data with heteroscedasticity with $P < 0.2$ by *F* test) with multiplicity adjustment by Bonferroni correction were used for comparisons between two groups: vehicle/vehicle and vehicle/METH (Fig. 2-1A and 2-1B), vehicle/MK-801, and vehicle/PCP treatments. The level of significance in each *t* test was designated by the values of $P < 0.05$. Bartlett's test was performed to test the equality of variances, followed by a one-tailed Williams' test (for parametric data, $P \geq 0.05$ by Bartlett's test) or one-tailed Shirley-Williams' test (for non-parametric data, $P < 0.05$ by Bartlett's test) to assess the dose-dependent effects of TAK-137 compared with the vehicle/METH- or vehicle/MK-801-treatment groups and differences were considered significant for *P* values of < 0.025. The analysis of variance (ANOVA) followed by post-hoc analysis of contrast test (5-CSRTT), Bonferroni/Dunnett multiple comparisons (DMTS task), or a least significant difference (LSD) test (reversal learning test), which was used for crossover or repeated design experiments. The effect of TAK-137 was compared with vehicle-, vehicle/ketamine-, or vehicle/PCP-treatment groups and the differences were considered significant for *P* values < 0.05. The effect of olanzapine (3 mg/kg) was also compared with

vehicle/METH-treated group by *F* test followed by Student's *t*-test (Fig. 2-7). In the experiments that examined the effects of multiple doses of test compounds, statistical significance was analyzed using Bartlett's test to test for the homogeneity of variances, followed by one-tailed Williams' test (for parametric data, $P > 0.05$ by Bartlett's test) or one-tailed Shirley-Williams' test (for non-parametric data, $P \leq 0.05$ by Bartlett's test) for the comparison of the dose-dependent effects of the multiple doses of the test compounds with the vehicle group. Differences at P values < 0.025 were considered statistically significant. In the NORT, the effect of combination of TAK-137 and olanzapine was assessed by using two-way ANOVA followed by Bonferroni/Dunnet multiple comparisons with significance set at $P < 0.05$.

3. Results

3.1. Effect of TAK-137 on the positive symptoms of schizophrenia

The total count of locomotion over 120 min after METH administration in the METH-treated group (14470 ± 1136 counts) was significantly higher than that in the vehicle-treated group (2154 ± 588.7 counts) (P = 0.0394, F test followed by P < 0.01, Aspin–Welch test; Fig. 2-1A). TAK-137 significantly decreased the METH-induced hyperlocomotion to 10328 ± 943.5 counts in the TAK-137 (10 mg/kg)/METH group (P = 0.9298, Bartlett's test followed by P < 0.025, one-tailed Williams' test, compared with the vehicle/METH-treated group). The efficacy of TAK-137 was most prominent between 90 and 120 min after METH administration. TAK-137 at 0.1, 1, and 10 mg/kg reduced the total counts of locomotion in the TAK-137/METH-treated group to 1062 ± 449.7, 797.0 ± 287.9, and 653.4 ± 254.8, respectively in comparison with that in the vehicle/METH-treated group (2328 ± 521.3 counts) (P = 0.0917 in Bartlett's test followed by P < 0.025, one-tailed Williams' test; Fig. 2-1B).

The effect of TAK-137 on hyperlocomotion induced by MK-801 (0.3 mg/kg as a salt, s.c.) was also investigated in mice (Fig. 2-1C). The total count of locomotion over 120 min after MK-801 administration in the MK-801-treated group (1910 ± 197.8 counts) was significantly higher than that in the vehicle-treated group (317.7 ± 63.67 counts) (P = 0.0035, F test followed by P < 0.01, Aspin–Welch test; Fig. 2-1C). TAK-137 (3, 10, and 30 mg/kg) significantly decreased MK-801-induced hyperlocomotion to 1290 ± 180.3, 1469 ± 119.0, and 1164 ± 128.8 counts respectively in the TAK-137/MK-801-treated group in comparison with that in the vehicle/MK-801-treated group (1910 ± 197.8 counts) (P = 0.2894 in Bartlett's test followed by P < 0.025, one-tailed Williams' test). In contrast to the result of METH-induced hyperlocomotion, TAK-137 did not induce prominent effects on locomotion between 90 and 120 min after MK-801 administration; a significant effect was observed only at 30 mg/kg (P = 0.0131 in Bartlett's test followed by P < 0.025, one-tailed Shirley-Williams' test; Fig. 2-1D).

Unlike reported antipsychotics with dopamine D2 antagonism, TAK-137 did not induce a significant cataleptic response and increase of prolactin and glucose levels in plasma (Fig. 2-6).

3.2. Effect of TAK-137 on the negative symptoms of schizophrenia

3.2.1. Social behavior

The interaction time in the vehicle/MK-801 (0.1 mg/kg, as a salt)-treated group (43.6 ± 4.33 s) was significantly lower than that in the vehicle/vehicle-treated group (90.33 ± 5.71 s, P = 0.3285, F test followed by P < 0.01, Student's t-test; Fig. 2-2A). The interaction time was significantly increased by the administration of 0.1 and 0.3 mg/kg TAK-137 to 65.5 ± 4.78 and 58.9 ± 4.30 s, respectively (P = 0.9146 in Bartlett's test followed by P < 0.025, one-tailed Williams' test; Fig. 2-2A). MK-801 administration did not affect the total distance moved. The total distances moved, in the presence and

absence of MK-801 were 5796 ± 149 cm and 6205 ± 155 cm, respectively. TAK-137 treatment did not significantly increase the distance moved in the presence of MK-801 (7048 ± 276 cm and 6171 ± 324 cm at 0.1 and 0.3 mg/kg with MK-801, respectively) (Fig. 2-2B).

3.3 Effects of TAK-137 on the cognitive symptoms of schizophrenia

The plasma and brain concentrations of TAK-137 under fasted conditions were 67 % and 71 % of those under the fed conditions (Table 2-1). Thus, 0.2 and/or 0.6 mg/kg of TAK-137 were used in the experiments requiring restricted food consumption.

3.3.1 Attention

The vehicle-treated group achieved 59.1 ± 3.63 correct responses in 100 trials; this was significantly increased by TAK-137 (0.2 mg/kg, p.o.) to 69.0 ± 2.61 responses in 100 trials ($F(1, 11) = 5.28$, $P < 0.05$, cross over ANOVA; Fig. 3A). There was no significant difference in the number of omissions and premature responses between the vehicle-treated group and the TAK-137-treated group. The number of omission responses recorded was 23.8 ± 3.73 and 15.5 ± 2.54 in the vehicle-treated group and the TAK-137-treated group, respectively ($F(1, 11) = 4.56$, cross over ANOVA, $P = 0.056$; Fig. 2-3B). The number of premature responses (a response prior to light stimulation) was 5.15 ± 1.57 and 6.92 ± 1.00 in the vehicle-treated group and the TAK-137-treated group, respectively (Fig. 2-3C).

3.3.2. Working memory

In the testing session of the RAM test, treatment with MK-801 (0.08 mg/kg, as a salt, s.c.) disrupted the performance of the well-trained rats in the collection of the pellets on the maze and increased the number of errors from 1.43 ± 0.81 to 10.33 ± 1.72 ($P = 0.0461$, F test followed by $P < 0.001$, Aspin–Welch test, compared with the vehicle-treated group; Fig. 2-4A). TAK-137 (0.2 and 0.6 mg/kg) significantly reduced the number of errors to 4.00 ± 0.85 and 3.44 ± 1.02, respectively ($P = 0.1488$ in Bartlett's test followed by $P < 0.025$, one-tailed Williams' test, compared with the vehicle/MK-801-treated group; Fig. 2-4A).

In the DMTS task, the monkeys were trained to correctly identify a sample object from 4 objects projected on the monitor after various delay intervals of 0 (no delay), 4, 8, and 16 s. Ketamine treatment significantly decreased the percentage of correct responses compared with vehicle treatment, with values of (96.88 ± 1.99) % and (82.63 ± 4.29) % recorded with 0 s interval, (89.58 ± 2.08) % and (76.99 ± 6.82) % recorded with 4 s interval, (87.50 ± 1.70) % and (64.18 ± 1.48) % recorded with 8 s interval, and (70.8 ± 9.77) % and (55.0 ± 3.00) % recorded with 16 s interval after the administration of vehicle and ketamine, respectively ($F(1, 24) = 18.39$, $P < 0.001$, two-way ANOVA; Fig. 2-4B). The plasma concentration of TAK-137 at 0.1 mg/kg in monkeys (40

ng /mL as C_{max}) was comparable to that at 0.1 mg/kg (25 ng/mL as C_{max})—the dose which showed pharmacological efficacies, in rats (Kunugi et al., 2019). At 0.1 mg/kg, TAK-137 significantly ameliorated ketamine-induced cognitive deficits, with percentage accuracy of (89.3 ± 6.51) %, (89.5 ± 6.54) %, (81.4 ± 6.97) %, and (71.5 ± 8.16) % at 0, 4, 8, and 16 s interval memory test, respectively ($F(1, 24) = 7.29$, $P < 0.05$, two-way ANOVA; Fig. 2-4B).

3.3.3. Cognitive flexibility

I evaluated the effects of TAK-137 on subchronic PCP-induced deficits in a reversal learning task. In the initial phase of the testing session, there were no significant differences in the percentage of correct responses in all the groups: (77.7 ± 4.30) % in the vehicle/vehicle-treated group, (80.0 ± 3.19) % in the vehicle/PCP-treated group ($P = 0.68$, Student's *t*-test, compared with the vehicle/vehicle-treated group), (77.3 ± 4.07) % in the TAK-137 (0.01 mg/kg)/PCP-treated group, (82.0 ± 2.73) % in the TAK-137 (0.1 mg/kg)/PCP-treated group, and (77.7 ± 3.60) % in the TAK-137 (1 mg/kg)/PCP-treated group ($F(3, 28) = 0.3992$, $P = 0.75$, one-way ANOVA; Fig. 2-5). In the reversal phase, the percentage of correct responses was significantly lower in the vehicle/PCP-treated group than in the vehicle-treated group ((44.9 ± 7.63) % vs. (73.1 ± 5.20) %) ($P = 0.4394$, *F* test followed by $P < 0.01$, Student's *t*-test; Fig. 2-5). TAK-137 significantly ameliorated the PCP-induced deficits and increased the percentage of correct responses to (68.5 ± 7.28) % at 0.1 mg/kg and (71.3 ± 7.34) % at 1 mg/kg ($F(3, 28) = 3.41$, $P < 0.05$ and $p < 0.01$, at 0.1 and 1 mg/kg, respectively, one-way ANOVA).

3.4. Effects of TAK-137 under the combination with olanzapine

I studied the effects of the combination of olanzapine (3 mg/kg), one of the antipsychotics, and TAK-137 (0.1, 1, and 10 mg/kg) on METH-induced hyperlocomotion, cataleptic response, and plasma prolactin level (Fig. 2-7). The results showed that the inhibitory effect of olanzapine on METH-induced hyperlocomotion was not affected by TAK-137 (Fig. 2-7A). TAK-137 did not exacerbate the cataleptic response and plasma prolactin level (Fig. 2-7B and 2-7C, respectively). In addition, co-treatment of TAK-137 (1 mg/kg, p.o.) and olanzapine (3 mg/kg, p.o.) did not affect the effect on cognitive improvement in novel object recognition test in rats (Fig. 2-7D).

4. Discussion

Schizophrenia is a chronic psychiatric disorder with a spectrum of symptoms: positive, negative, and cognitive symptoms. Several hypotheses for the pathophysiology of schizophrenia have been indicated based on clinical findings [3]. The hyperdopamine hypothesis is considered to indicate the main cause of positive symptoms, and the hypoglutamate hypothesis has been proposed not only for positive symptoms but also for negative and cognitive symptoms [118]. Recent genetic findings in genome-wide association studies revealed that genes related to the NMDA receptor signaling complex are associated with an increased risk of schizophrenia [119]. Moreover, NMDA receptor antagonists are reported to induce schizophrenia-like psychosis, social dysfunction, and cognitive impairments in healthy volunteers, and exacerbate symptoms in patients with schizophrenia [25-27]. Indeed, several small molecules that enhance NMDA signaling, such as D-serine and a GlyT1 inhibitor, have been reported to improve negative and cognitive function in clinical trials [31, 34]. Thus, modulation of NMDA function is likely a promising approach for the treatment of patients with schizophrenia.

In the hypoglutamate state, NMDA receptor signaling on the parvalbumin (PV)-positive GABA interneurons is decreased in the cortex and hippocampus [120]. PV-positive GABA interneurons play a key role in cognitive function through the production of neural oscillation, especially gamma oscillation [121]. Moreover, a significantly low level of gamma oscillation during cognitive tasks is reported in patients with schizophrenia [122]. Thus, the clinical characterization of gamma oscillation could be a promising biomarker.

Schizophrenia is recognized as a neurodevelopmental disease and altered fetal or neonatal environments, such as viral infection, are indicated to be risk factors [123]. Methylazoxymethanol acetate (MAM) exposure [124] and polyriboinosinic-polyribocytidylic acid (Poly(I:C)) injection [125] have been reported to mimic maternal mitotoxin or viral exposure during pregnancy in rodents and these manipulations result in the hypofunctioning of NMDA signaling in PV-positive GABA interneurons [126]. These preclinical results further support the association of the hypoglutamate state with the pathophysiology of schizophrenia. To evaluate the effects of TAK-137 on glutamate hypofunction, I used both acute and sub-chronic treatments with NMDA receptor antagonists. The acute treatment of MK-801 and ketamine can inhibit NMDA receptor signaling in PV-positive neurons, and the sub-chronic treatment of PCP is reported to reduce PV expression [96].

The activation of the AMPA receptor leads to increased NMDA receptor function; thus, AMPA receptor potentiators have potential as therapeutic drugs for schizophrenia. However, risks such as the bell-shaped response and the induction of seizures have been indicated in previously reported compounds. The in vivo studies of LY451646 and LY404187 revealed their steeply bell-shaped responses. For example, c-fos induction in rats was detected only at 0.5 mg/kg after 0.05, 0.5, and 5 mg/kg treatments [37]. Brain-derived neurotrophic factor (BDNF) mRNA in the rat hippocampus

was induced only at 0.5 mg/kg after 7-day dosing at 0.125, 0.25, 0.5, and 1 mg/kg [127]. Such a bell-shaped response makes it difficult to select the optimal dosage in clinical studies given the heterogeneity of drug metabolizing enzymes in humans. In addition, LY451646 showed narrow safety margins between exposure at the effective dose in rat NORT and at the maximum dose in the absence of seizures, i.e., 3.1- and 7.5-fold in the AUC_{brain} and brain C_{max}, respectively [39]. Thus, it can be presumed that the doses of AMPA receptor potentiators in previous clinical studies could not be increased to the exposure level required for efficacy owing to their narrow safety margin against seizure. Therefore, compounds with improved bell-shaped responses and reduced seizure risk should be generated.

It is indicated that the bell-shaped response is related to the agonistic property of compounds, which was detected only in primary cells and not in recombinant cells [38]. Thus, the agonistic property of each compound should be characterized using the respective physiological receptor, although such complex conditions may not be appropriate for a high-throughput screening (HTS) assay. Therefore, I established an original screening strategy, which included a unique binding assay for HTS, and identified TAK-137 [39]. In the DMTS test in naive monkeys, TAK-137 enhanced cognitive performance at 0.03, 0.1, and 1 mg/kg, whereas LY451656 enhanced performance only at 0.1 mg/kg when dosed at 0.03, 0.1, and 1 mg/kg. The safety margins between the exposure yielding cognitive enhancement in NORT and the maximum exposure in absence of seizure were 116- and 43.7-fold in the AUC_{brain} and brain C_{max}, respectively. Therefore, TAK-137 is superior to LY451646 in terms of the bell-shaped responses and the safety margin.

In this study, I investigated effects of TAK-137, a compound with low agonistic properties, on animal models of schizophrenia. At 10 mg/kg, TAK-137 significantly inhibited METH-induced hyperlocomotion in rats to 33.6 ± 7.66% of that in the control (Fig. 2-1A and 2-1B); however, the percentage of inhibition was lower than that of antipsychotics, which exhibited greater than 50% inhibition under the 60% occupancy of D2R [103, 128]. TAK-137 may therefore have limited efficacy against the positive symptoms of schizophrenia. Negative symptoms are rarely improved by current antipsychotics [129]. In this study, TAK-137 at 0.1 and 0.3 mg/kg significantly ameliorated MK-801-induced deficits in SI (Fig. 2-2A) without a significant increase in locomotion (Fig. 2-2B). This indicated that the effect was not a secondary effect of an increase in locomotion and that TAK-137 has the potential to improve the social behavior associated with NMDA receptor hypofunction. Further characterization should be considered using other in vivo tests, such as the sucrose preference test, as several neuronal networks may be related to the negative symptoms [2, 130, 131]. The effects of TAK-137 on cognitive symptoms, especially attention, working memory, and executive control, were investigated as these were recognized as the key cognitive domains impaired in schizophrenia by the Measurement and Treatment Research to Improve Cognition in Schizophrenia initiative [132, 133]. The 5-CSRTT contains aspects of the continuous performance

task in humans [134], the RAM test in rats can be adapted to mimic the N-back task in humans [135], and the monkey DMTS task can be translated to the human DMTS task [136]. A reversal learning task is regarded as the evaluation of the cognitive flexibility required for rule generation and selection [137, 138]. TAK-137 improved these cognitive dimensions in animal models with acute or sub-chronic NMDA receptor antagonists (Fig. 2-3, 2-4, and 2-5), indicating that it may be effective for the treatment of multiple cognitive domains in patients with schizophrenia. Recently, an AMPA receptor potentiator, PF-04958242, was reported to significantly reduce ketamine-induced impairment in immediate recall and working memory tasks in healthy human subjects at plasma concentrations similar to that in non-human primates [139]. The data further supported that AMPA receptor activation can counteract the hypoglutamate state induced by NMDA inhibitors in humans.
The positive symptoms of schizophrenia are well-controlled by current antipsychotics compared with other symptoms. Considering the reported findings and the results in this study, TAK-137 may be characterized by particular efficacy against the negative and cognitive symptoms. Therefore, I investigated the impact of the concomitant use of TAK-137 and an antipsychotic (Fig. 2-7). TAK-137 did not interfere with the effects of olanzapine on METH-induced hyperlocomotion and did not exacerbate the cataleptic response and the plasma prolactin level. In addition, co-treatment of olanzapine did not alter the effects of TAK-137 on cognition. Thus, the combination of TAK-137 with the currently available antipsychotics may be beneficial in treating multiple symptoms of schizophrenia. A synergistic enhancement of efficacies was not detected in this study, which may be a result of the different mechanisms of action of D2R antagonism by olanzapine and enhancement of the glutamate signal by TAK-137.

Another critical issue in the development of drugs for schizophrenia is the heterogeneity of the disease etiology and biology of schizophrenia. Indeed, the importance of patient segmentation by biophenotype has been suggested [140]. Patients in the hypoglutamate state may be determined, for example, by mismatch negativity or gamma oscillations [141, 142]. The proof of concept should be investigated in the clinical studies of patients selected by such biomarkers.

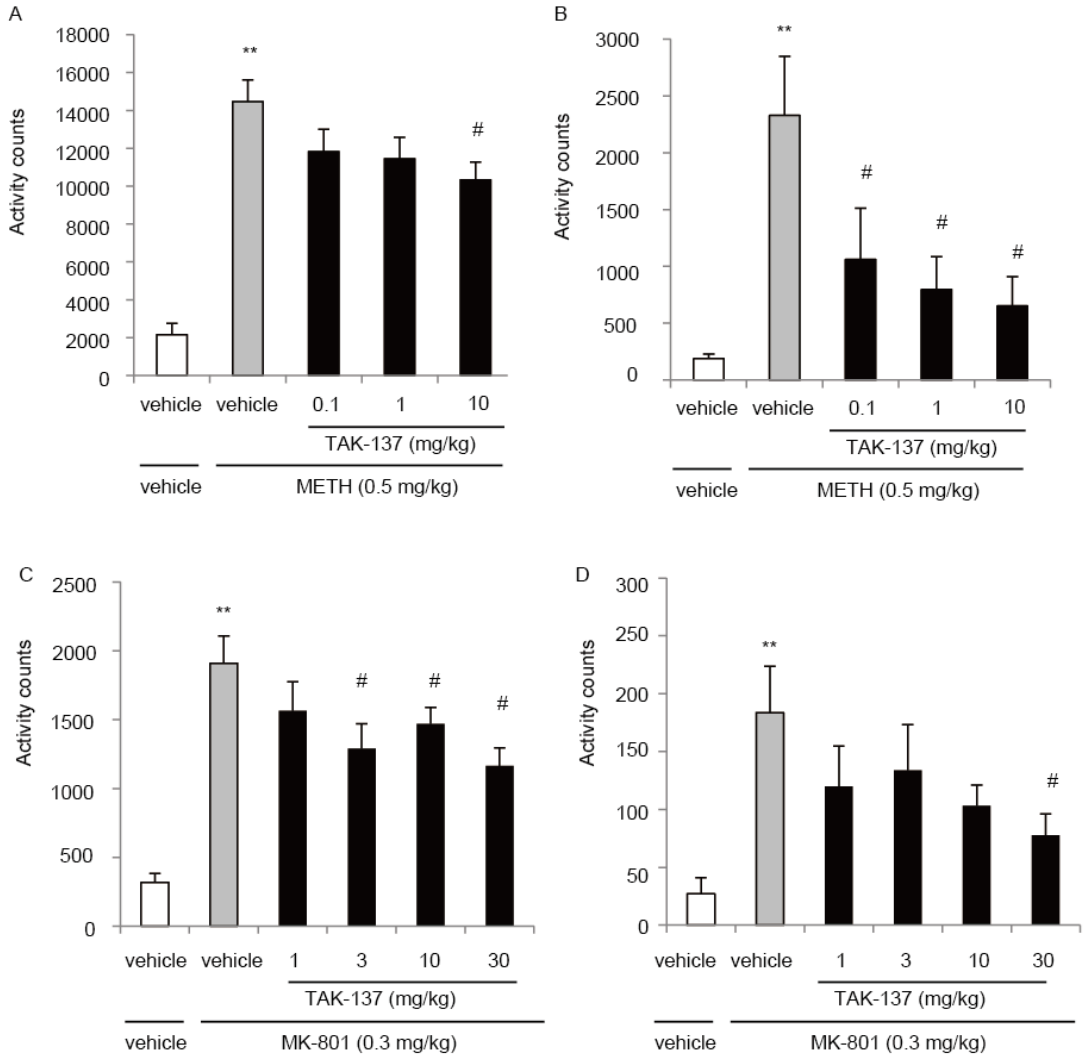
In conclusion, TAK-137, an AMPA receptor potentiator with a low agonistic activity, broader effective dose range, and greater safety margin against seizure, was shown to be efficacious in various animal models of schizophrenia. Thus, TAK-137-type AMPA receptor potentiators may be promising therapeutic options in neuropsychiatry and neurological diseases.

Tables & Figures

Table 2-1.

Pharmacokinetic profile of TAK-137 in rats under non-fasted and fasted condition. TAK-137 at 0.1 mg/kg was orally administered to rats (n = 3).

	Dose (mg kg^{-1}, p.o.)	Concentration (ng mL^{-1} or g^{-1})	Non-fasted	Fasted
TAK-137	0.1	Plasma	21	14
		Brain	7	5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Activity counts
18000
16000
14000
12000
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6000
4000
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vehicle
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0.1
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TAK-137 (mg/kg)
vehicle
METH (0.5 mg/kg)
B
Activity counts
3000
2500
2000
1500
1000
500
0
**
#
#
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vehicle
vehicle
0.1
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10
TAK-137 (mg/kg)
vehicle
METH (0.5 mg/kg)
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Activity counts
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vehicle
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3
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30
TAK-137 (mg/kg)
vehicle
MK-801 (0.3 mg/kg)
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Activity counts
300
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200
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100
50
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#
vehicle
vehicle
1
3
10
30
TAK-137 (mg/kg)
vehicle
MK-801 (0.3 mg/kg)

Fig. 2-1. (A) The effect of TAK-137 on METH-induced hyperlocomotion in rats. The rats were acclimated in Plexiglas-covered cages for at least 2 h before the start of the experiment. TAK-137 (0.1, 1, or 10 mg/kg, p.o.) was administered 4 h before METH administration (0.5 mg/kg, s.c.). The data are presented as the mean ± S.E.M. of cumulative locomotion for 0–120 min. The numbers of animals (n) = 6 in the vehicle/vehicle-treated group, n = 12 in the vehicle/METH-treated group, n = 11 in the TAK-137 (0.1 mg/kg)/METH-treated group, n = 13 in the TAK-137 (1 mg/kg)/METH-treated group, and n = 13 in the TAK-137 (10 mg/kg)/METH-treated group. $^{**}P < 0.01$, statistically significant compared with the vehicle/vehicle-treated group by Aspin–Welch test, $^{\#}P < 0.025$, statistically significant compared with the vehicle/METH-treated group by one-tailed Williams' test. (B) Data between 90 and 120 min after METH administration. $^{**}P < 0.01$, statistically significant compared with the vehicle/vehicle-treated group by Aspin–Welch test. $^{\#}P < 0.025$, statistically significant compared with the vehicle/METH-treated group by one-tailed Williams' test. (C) The effect of TAK-137 on MK-801-induced hyperlocomotion in mice. The mice were acclimated in cages for at least 2 h before the start of the experiments. TAK-137 (1, 3, 10, or 30 mg/kg, p.o.) was administered 2 h before MK-801 administration (0.3 mg/kg, s.c.). The data are presented as the mean ± S.E.M. of cumulative locomotion for 120 min after MK-801 administration, n = 6 in the vehicle/vehicle-treated group, n = 13 in the vehicle/MK-801-treated group, n = 14 in the TAK-137 (1 mg/kg)/MK-801-treated group, n = 13 in the TAK-137 (3 mg/kg)/MK-801-treated group, n = 16 in the TAK-137 (10 mg/kg)/MK-801-treated group, and n = 15 in the TAK-137 (30 mg/kg)/MK-801-treated group. $^{**}P < 0.01$, statistically significant compared with the vehicle/vehicle-treated group by Aspin–Welch test; $^{\#}P < 0.025$, statistically significant compared with the vehicle/MK-801-treated group by one-tailed Williams' test. (D) Data between 90 and 120 min after MK-801 administration. $^{**}P < 0.01$, statistically significant compared with the vehicle/vehicle-treated group by Aspin–Welch test; $^{\#}P < 0.025$, statistically significant compared with the vehicle/MK-801-treated group by one-tailed Shirley-Williams' test.

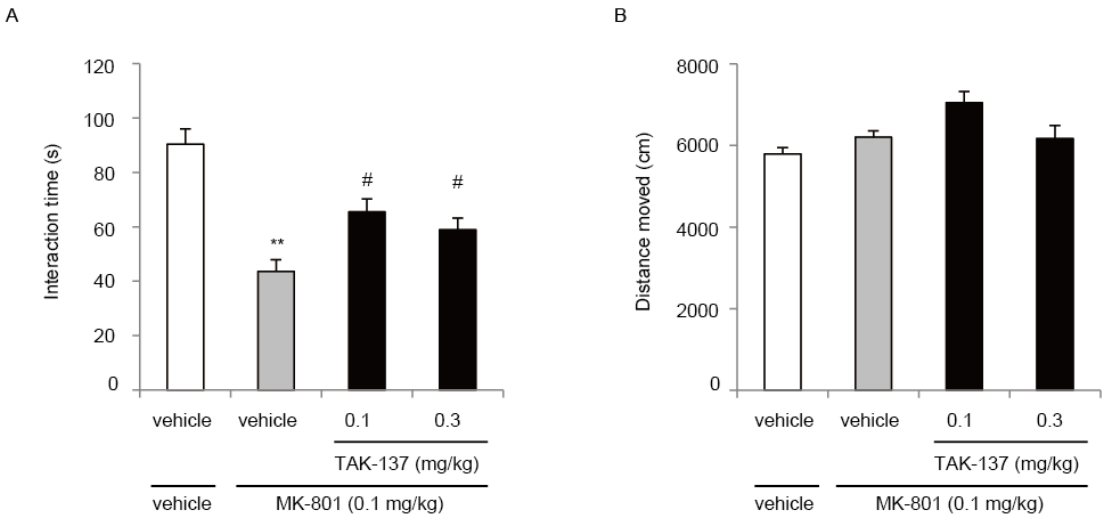

Fig. 2-2. Effect of TAK-137 on MK-801-induced deficits in the social behavior evaluated by the social interaction test in rats. (A) TAK-137 (0.1 or 0.3 mg/kg, p.o.) or corresponding vehicle, and MK-801 (0.1 mg/kg, s.c.) or corresponding vehicle were administered to rats 4 h before testing. The interaction time of each treatment group is presented as the mean ± S.E.M. (n = 14). $^{**}P < 0.01$, statistically significant compared with the vehicle/vehicle-treated group by Student's *t*-test. $^{\#}P < 0.025$, statistically significant compared with the vehicle/MK-801-treated group by one-tailed Williams' test. (B) The distance traveled during testing over 10 min was measured and is presented as the mean ± S.E.M. (n = 14).

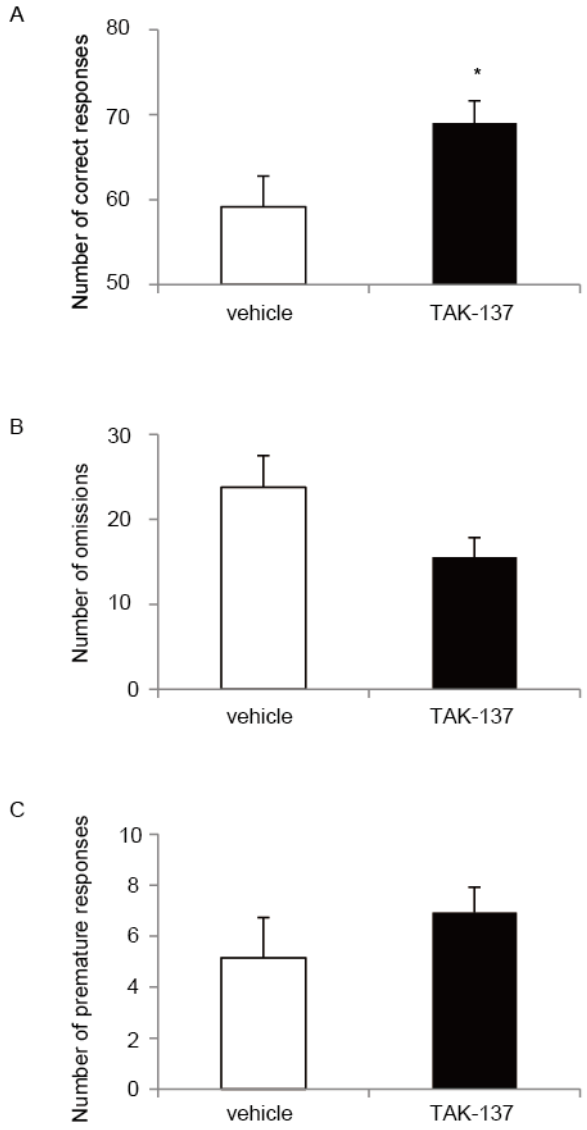

Fig. 2-3. The effect of TAK-137 on attention in the five-choice serial reaction time task in rats. TAK-137 (0.2 mg/kg, p.o.) was administered 4 h prior to the trial. (A) Correct responses are the total number of nose-pokes in an illuminated aperture within the limited hold. (B) Omission responses are the number of non-responses during the limited hold. (C) Premature responses represent the number of responses that occurred prior to stimulus presentation. The data are presented as the mean ± S.E.M. (n = 13). Significant differences from the vehicle-treated group are indicated by $*P < 0.05$ in the crossover ANOVA followed by a contrast test.

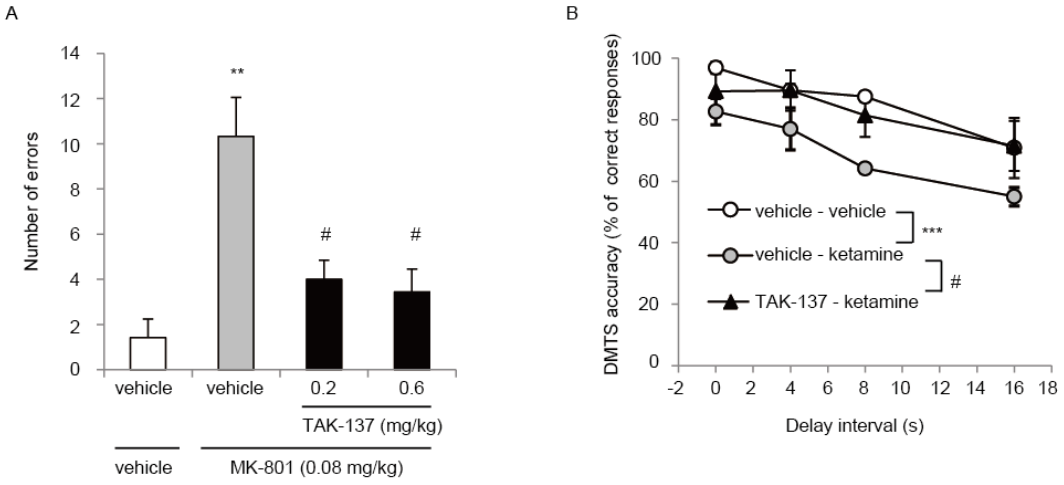

Fig. 2-4. The effects of TAK-137 on MK-801- or ketamine-induced deficits in working memory in the radial arm maze test in rats or delayed matching-to-sample task in monkeys. (A) TAK-137 (0.2 or 0.6 mg/kg, p.o.) or corresponding vehicle was administered to rats 2 h before MK-801 (0.08 mg/kg, s.c.) or corresponding vehicle administration. The rats were placed on the maze 30 min after MK-801 administration and entries into the arms were recorded. The data are presented as the mean ± S.E.M. of the number of errors. The numbers of animals (n) = 7 in the vehicle/vehicle-treated group, n = 9 in the vehicle/MK-801-treated group, n = 11 in the TAK-137 (0.2 mg/kg)/MK-801-treated group, and n = 9 in the TAK-137 (0.6 mg/kg)/MK-801-treated group. $^{**}P < 0.01$, statistically significant compared between the vehicle/vehicle-treated and the vehicle/MK-801-treated group by Aspin–Welch test. $^{\#}P < 0.025$, statistically significant compared with the vehicle/MK-801-treated group by one-tailed Williams' test.

(B) TAK-137 (0.1 mg/kg) or corresponding vehicle was orally administered 2 h prior to ketamine or corresponding vehicle administration (1.0 mg/kg, i.m.) in monkeys. The DMTS task was conducted 15 min after ketamine administration. Each plot represents the mean ± S.E.M. of the percentage of correct responses from 96 trials per session (n = 4). The statistical analysis was performed using two-way ANOVA followed by Bonferroni/Dunnet multiple comparisons, with significance set at $^{***}P < 0.001$ (the vehicle/vehicle-treated group versus the vehicle/ketamine-treated group) and at $^{\#}P < 0.05$ (the TAK-137/ketamine-treated group versus the vehicle/ketamine-treated group).

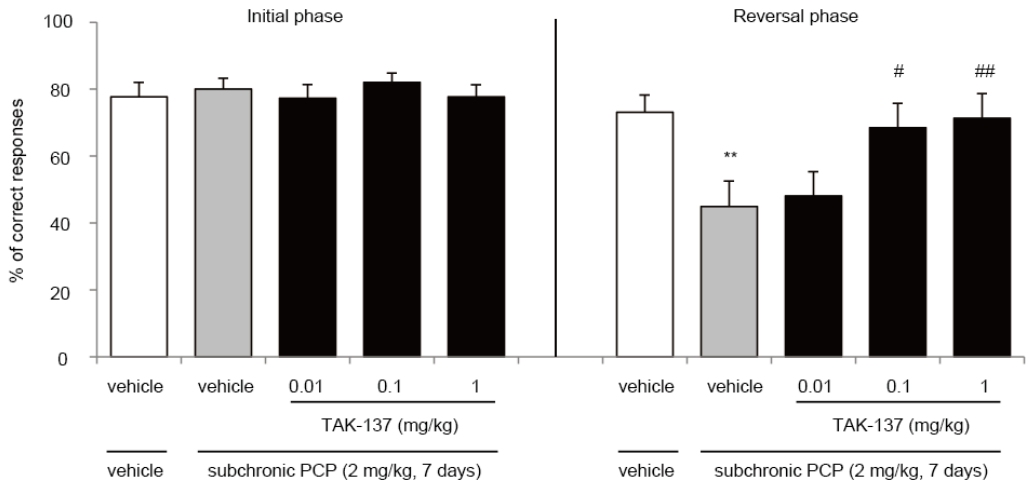

Fig. 2-5. The effect of TAK-137 on subchronic PCP-induced deficits in cognitive flexibility in a reversal learning test in rats. The rats were trained to achieve 90 % criterion of the correct response, followed by the administration of PCP (2 mg/kg, i.p.) twice per day for 7 days without training. In the reversal testing, TAK-137 (0.01, 0.1, or 1 mg/kg, p.o.) or vehicle was administered 2 h before the initial phase. The reversal phase was examined for 2 min immediately after the completion of the initial phase. The data are presented as the mean ± S.E.M. of the percentage of correct responses in the initial and reversal phases. The numbers of animals (n) = 10 in the vehicle/vehicle-treated group, n = 8 in the vehicle/PCP-treated group, and n = 8 in the TAK-137/PCP-treated groups. $^{**}P < 0.01$, statistically significant compared with the vehicle/PCP-treated group by Student's *t*-test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, statistically significant compared with the vehicle/PCP-treated group by one-way ANOVA followed by LSD test.

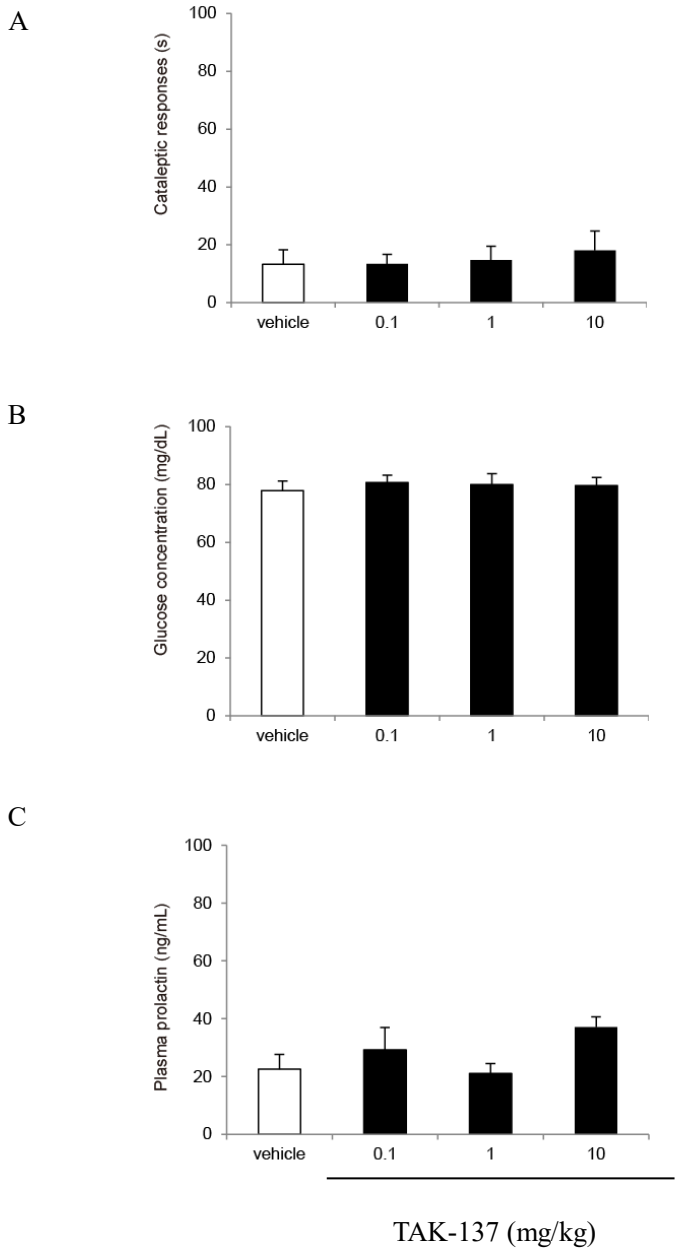

Fig. 2-6. The effects of TAK-137 on cataleptic response, and plasma glucose and prolactin level in rats. (A) TAK-137 (0.1, 1, or 10 mg/kg) was administered 4 h before test. The duration of grabbing the bar was measured to evaluate cataleptic response and is presented as mean ± S.E.M. (n = 6). (B) TAK-137 (0.1, 1, or 10 mg/kg) was administered 4 h before blood collection. Data are presented as mean ± S.E.M. of plasma prolactin levels (n = 5). (C) TAK-137 (0.1, 1, or 10 mg/kg) was administered 4 h before blood collection. Data are presented as mean ± S.E.M. of plasma prolactin levels (n = 6-7).

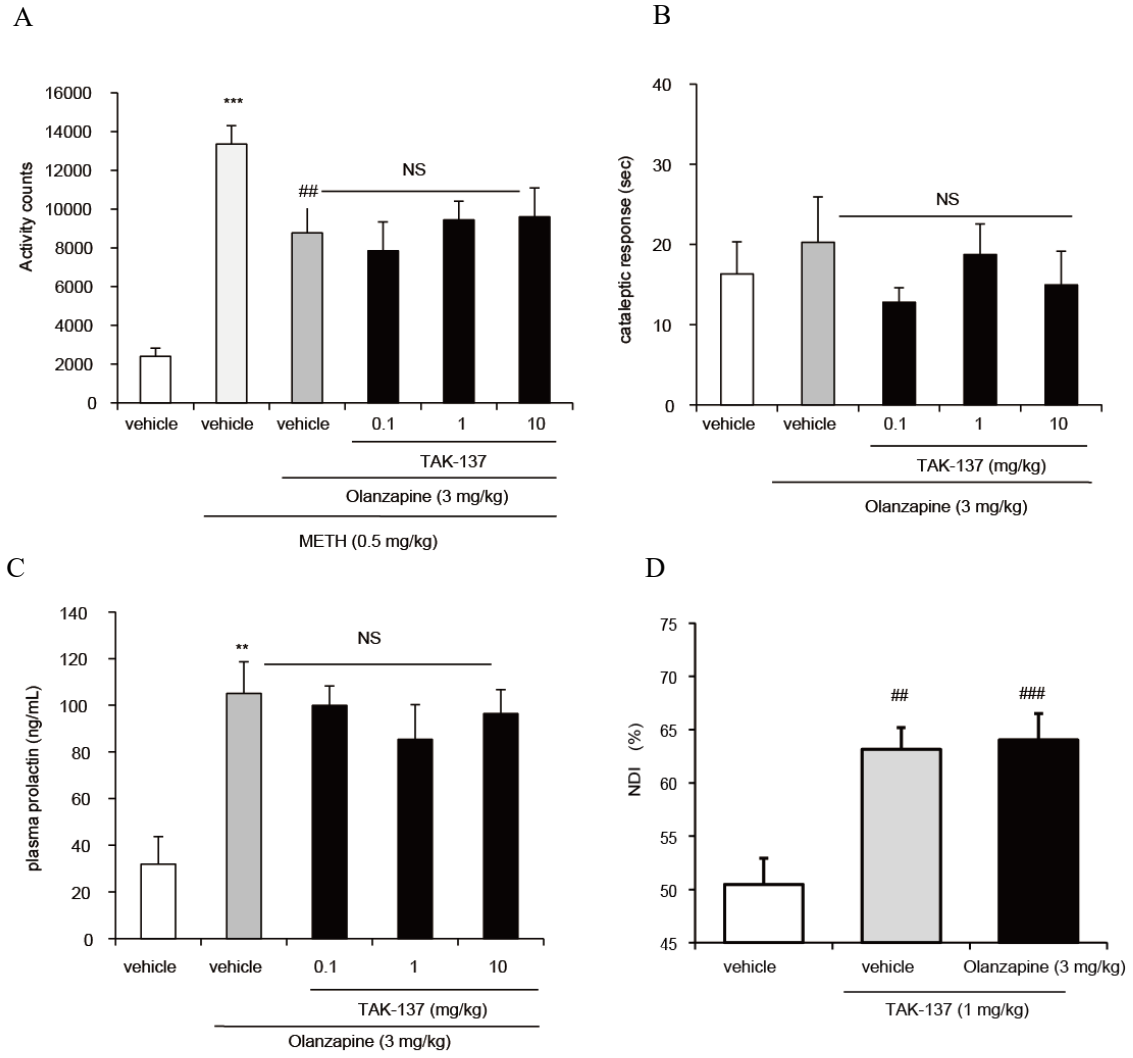

Fig. 2-7. Effects of TAK-137 on the pharmacological effects and side effects of olanzapine in METH-induced hyperlocomotion, NORT, cataleptic response, and plasma prolactin level in rats. (A) TAK-137 (0.1, 1, or 10 mg/kg, p.o.) and olanzapine (3 mg/kg, p.o.) was administered 4 h and 1 h respectively, before METH administration (0.5 mg/kg, s.c.). Total locomotor activities for 120 min after METH administration are presented as mean ± S.E.M. The numbers of animals used were 6, 12, 13, 8, and 9 in the vehicle-treated group, vehicle/METH-treated group, olanzapine/METH-treated group, olanzapine/TAK-137 (0.1 or 1 mg/kg)–METH-treated group, and olanzapine/TAK-137 (10 mg/kg)/METH-treated group respectively. ***P < 0.001, statistically significant compared with vehicle-treated group by Aspin–Welch test. ##P < 0.01, statistically significant compared with vehicle/METH-treated group by Aspin–Welch test. NS, not significant (versus olanzapine/METH-treated group; one-tailed Williams' test). (B) TAK-137 (0.1, 1, or 10 mg/kg) and olanzapine (3 mg/kg) were administered 4 h before testing. The duration of grabbing the bar was measured to evaluate cataleptic response and is presented as mean ± S.E.M. (n = 12). NS, not

significant (versus vehicle-olanzapine group; one-tailed Williams' test). (C) TAK-137 (0.1, 1, or 10 mg/kg) was administered 4 h, and olanzapine (3 mg/kg) was administered 1.5 h before blood collection. Data are presented as mean ± S.E.M. of plasma prolactin levels (n = 6). $^{**}P < 0.01$, statistically significant compared with vehicle-treated group by Aspin–Welch test. NS, not significant (versus vehicle-olanzapine- treated group; one-tailed Williams' test). (B) TAK-137 (1 mg/kg) and olanzapine (3 mg/kg) were orally administered 2 h or 1 h prior to the acquisition and the retention trials, respectively (n = 10). A novelty discrimination index (NDI) was calculated as the novel object interaction time / total interaction time × 100 (%) and are presented as mean ± S.E.M. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ statistically significant compared with vehicle-treated group by ANOVA followed by Bonferroni/Dunnet multiple comparisons.

General discussion

In the first chapter, this study clarified that CRF signaling in the CNS is the main route of locomotion and anxiety-like behavior. As compounds with the same main chemical structure were confirmed to be selective to the CRF_1 receptor compared with the CRF_2 receptor [143], the anxiogenic effect of CRF can be regarded as being blocked by CRF_1 receptor inhibition. In view of the receptor expression patterns and the effect of the CRF_1 receptor antagonist in rodents, this indicates that blocking of CRF signaling via the CRF_1 receptor may be a new treatment strategy for anxiety. CRF signaling in acute and chronic stress responses still needs further investigation. Stress loading would induce neuronal transmission much more broadly than exogenous CRF injection. In addition, the acute and chronic stress response would be different in terms of epigenetic changes of the synaptic function via changes in the expression of neurotransmitters or receptors.

In the second chapter, AMPA receptor signaling was indicated to be involved in the behavioral modulation of multiple symptoms of schizophrenia, especially in negative and cognitive symptoms. It was also indicated that the combination therapy with current medication mainly for positive symptoms would be beneficial since a combination with an AMPA receptor potentiator and olanzapine did not affect side effects. This study used NMDA receptor antagonist-induced behavioral deficits in animal models. Since schizophrenia is considered as a developmental disease, further studies can be conducted in other animal models such as Poly(I:C) or MAM models [124, 125].

The experiments in this study indicate two main points. The first is that it is important to investigate the function of neurotransmitters via specific receptors in disease models to clarify the scope of their efficacy. This will provide further applications to consider drug candidates. The current medication for schizophrenia, anxiety, and depression was accidentally found in clinical evidences. For example, antipsychotics are based on the dopamine D2 receptor antagonism, followed by additional affinities to other receptors such as serotonin and noradrenaline receptors to reduce side effects, especially of extrapyramidal symptoms [144]. The first generation of antidepressants is based on the selective serotonin reuptake inhibitor followed by serotonin noradrenaline reuptake dual inhibitor to reduce side effects or for additional benefits on pain. These past facts indicate that drug discovery targeting multiple molecules would be benefit for efficacy and safety. On the other hand, a multi-targeting approach should be based on a precise and deep understanding of each signal function to consider the key biological signals for each symptom in a disease [145]. Therefore, an investigation that focuses on one specific form of neuronal transmission would yield fundamental biological findings that would allow a comprehensive approach to be considered.

The second point is the importance of translational tools for three reasons. The first rationale is understanding the relationship between the extent of signal modulation and the efficacy of the outcome. Although this study indicated the key roles of CRF and AMPA receptor signaling on

anxiety and schizophrenia-like behavior, respectively, further translational studies would offer a better understanding of how much CRF_1 receptor should be antagonized or how much AMPA receptor should be activated for efficacy to be achieved. This can be analyzed by receptor occupancy of the ligand using PET or by assessing changes in electrophysiological signal with the electroencephalogram (EEG). There might be some limitations in the application of PET studies in terms of the difficulties in PET ligand generation and the level of expression and distribution of its target. Ionotropic receptors such as AMPA and NMDA receptors recycle quickly at the postsynaptic site and their expression levels change according to neuronal excitation, which makes it difficult to develop a clinically applicable PET ligand although several candidates have been reported in preclinical research [146]. The second rationale is based on the difficulty in visualizing the CNS diseases. Especially for neuropsychiatric diseases, significant morphological changes in neurons or synapses cannot be detected between the normal and diseased state. The imaging tools mentioned above might be one candidate to detect changes in the expression of a target or in ligand binding in a living body. There might also be some proteins in body fluids that are related to the function of specific neurotransmitters. The third reason is based on the heterogeneity of neuropsychiatric diseases in terms of their etiology, symptoms, and functional abnormality in multiple neurons, which makes it difficult to treat a disease and specific neuron signaling as a one-to-one response. If patients can be classified according to the most affected neurons or signals, this would enhance understanding of the relationship between key receptor signaling or pathways and clinical symptoms. In fact, the concept of a biophenotype, which classifies patients according to biomarkers, including biochemical, imaging, and electrophysiological features, has been developed. This concept is supported by such biomarker tools. In addition, such patient stratification would help clinical studies of specific drug targets. The CRF_1 receptor antagonist can be administered to patients who show overactivated CRF signaling. The AMPA receptor potentiator should be administered to people who show dysfunctional NMDA receptor signaling. Dysfunctional NMDA receptor signaling would be translationally evaluated as a reduction of gamma-oscillation by an EEG.

As a next step, a network analysis would be important to investigate the role of neuronal connectivity in functional outcomes, especially in cognitive functions, which are classified into many categories such as working memory, attention, and cognitive flexibility. Many types of neurons and cells are involved in a single cognitive domain. This would also be important to consider when assessing potential combination therapies to treat diversified neuronal dysfunctions in neuropsychiatric diseases based on an understanding of the functions of each neurotransmitter.

In summary, this study found that CRF_1 receptor signaling in the CNS plays a critical role in locomotion and anxiety behaviors, and that AMPA receptor activation improves behavioral deficits which are related to positive, negative, and cognitive symptoms in schizophrenia. This indicates that an investigation of specific neurotransmitter signaling is important to identify

functional roles. In addition, these results will add value when considering the treatment of neuropsychiatric diseases in terms of monotherapy and combination therapies with other drugs by modulating different neurotransmitters based on a scientific understanding of each signal function. In the future, further analysis of the neuronal network and the level of signal activation by imaging or electrophysiological analysis with translational tools such as PET ligand and EEG would be key to understand the role of neurotransmitters and their interaction with other neurons in the diseased state.

Acknowledgements

Chapter I.

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Chapter II.

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