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Research report

A natural scavenger of peroxynitrites, rosmarinic acid, protects against impairment of memory induced by $A\beta_{25-35}$

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Abstract

Peroxynitrite ($ONOO^-$)-mediated damage is regarded to be responsible for the cognitive dysfunction induced by amyloid beta protein (Aβ) in Alzheimer's disease (AD). In the present study, we examined the protective effects of rosmarinic acid (RA), a natural scavenger of $ONOO^-$, on the memory impairment in a mouse model induced by acute i.c.v. injection of $A\beta_{25-35}$. Mice daily received i.p. several doses of RA after the injection of $A\beta_{25-35}$. RA prevented the memory impairments induced by $A\beta_{25-35}$ in the Y maze test and novel object recognition task. RA, at the effective lowest dose (0.25 mg/kg), prevented $A\beta_{25-35}$-induced nitration of proteins, an indirect indicator of $ONOO^-$ damage, in the hippocampus. At this dose, RA also prevented nitration of proteins and impairment of recognition memory induced by $ONOO^-$-i.c.v.-injection. Co-injection of the non-memory-impairing dose of $ONOO^-$ with $A\beta_{25-35}$ blocked the protective effects of RA (0.25 mg/kg). These results demonstrated that the memory protective effects of RA in the neurotoxicity of $A\beta_{25-35}$ is due to its scavenging of $ONOO^-$, and that daily consumption of RA may protect against memory impairments observed in AD.
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Keywords: Rosmarinic acid; Amyloid beta (25–35); Peroxynitrite; Protein nitration; Memory impairment

1. Introduction

Complementary and alternative medicines, with the superiority as natural substances, have increasingly been used for health care and treatment for some chronic diseases. A number of herbs, including *Rosmarinus officinalis* L., *Borago officinalis*, *Melissa officinalis*, and *Salvia officinalis*, are used as an effective treatment against dementia in Traditional Uighur Medicine. Among these herbs, ethanol extracts of *M. officinalis* and *S. officinalis* have been reported to be effective in the management of mild to moderate AD in randomized double-blind clinical studies [1,2]. The principal ethanol-soluble constituent, with the therapeutic effects, of these herbs has been found to be rosmarinic acid (RA) [3,16,18] (**Fig. 1**). RA is regarded as a daily-consumed safe ingredient, due to its extensive use in food industry for flavouring.

The medicinal value of RA has also been well recognized, especially in regard to its antioxidant and anti-inflammatory activities [21,33,37]. RA displays a strong scavenger activity for $ONOO^-$ and other free radicals [8,21,32]. $ONOO^-$ is responsible for a wide spread biological damage in the brains of AD [6,35]. Aβ, the principal component of the senile plaques, is the main cause of increased $ONOO^-$ in the brain of AD [5,41]. In cell culture studies, RA protects against the reactive oxygen species induced by Aβ [12]. Further, RA not only inhibits the formation of beta-amyloid fibrils (fAβ), but also destabilizes preformed fAβ in vitro [27]. These reports suggest that RA have a beneficial role to reduce the neurotoxicity of Aβ in AD. However, no in vivo investigation on the effects of RA on the cognitive impairment in the neurotoxicity of Aβ has been reported. It is necessary to investigate the effects of RA and

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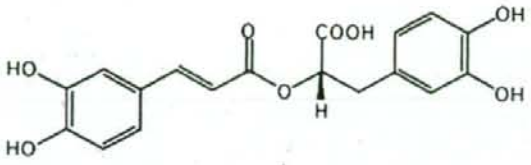

Fig. 1. Chemical structure of rosmarinic acid.

provide experimental basis of its use for Aβ neurotoxicity of AD.

The major component of senile plaques in the brain of AD is $A\beta_{1-40}$ and $A\beta_{1-42}$. The core toxic fragment of full-length $A\beta_{1-40}$ is $A\beta_{25-35}$. $A\beta_{25-35}$ shows faster and stronger, in the similitude with $A\beta_{1-40}$, neurotoxic effects such as oxidative damage, inflammatory responses and memory impairment [7,17,25,30,40,44,47]. In this study, we have therefore evaluated and characterized the antioxidant effects of RA in a mouse model of impaired memory established by the i.c.v.-injection of $A\beta_{25-35}$ [17].

2. Methods

2.1. Animals

Male ICR mice (Nihon SLC Co., Shizuoka, Japan) were used. The animals were housed in a controlled environment (23 ± 1 °C, 50 ± 5% humidity) and allowed food and water ad lib. The room lights were kept on between 8:00 a.m. and 8:00 p.m. All experiments were performed in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

2.2. Intracerebroventricular injection

$A\beta_{25-35}$ (Bachem, Bubendorf, Switzerland) was dissolved in sterile double-distilled water in a concentration of 1 mg/ml and stored at −20 °C before use. The dissolved $A\beta_{25-35}$ was incubated for aggregation at 37 °C for 4 days. The distilled water was incubated at the same condition as control. Aggregated $A\beta_{25-35}$ (3 μg/3 μl) or incubated distilled water (3 μl) was i.c.v. injected as described previously [17]. Briefly, a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long was used for all experiments. Mice were anesthetized lightly with ether, and the needle inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. A single-shot of the same volume (3 μl) of peptide or vehicle was delivered gradually within 3 s. Mice exhibited normal behavior within 1 min after injection. The administration site was confirmed in preliminary experiments. Neither insertion of the needle nor the volume of injection had a significant influence on survival, and behavioral responses or cognitive functions. $ONOO^-$ (Upstate, Lake placid, NY) was i.c.v. injected at a volume of 1 μl in the same way described above.

2.3. Experimental design

RA (Sigma, USA) was dissolved in physiological saline. Uric acid (UA) (Wako Chemicals, Osaka, Japan) was prepared as suspension in saline. Immediately after the injection of $A\beta_{25-35}$, mice were administrated with RA (0.05, 0.25, 1, 2, and 4 mg/kg day, i.p.) or UA (100 mg/kg, i.p.) daily for 9 consecutive days. Biochemical and behavioral investigations were performed at the indicated time points (see Fig. 2).

2.4. Y-maze test

The Y-maze task was carried out on Day 6 after the injection of $A\beta_{25-35}$, as described previously [45]. Briefly, the apparatus consisted of black-painted plywood. Each arm of the Y-maze was 50 cm long, 12 cm high and 4 cm wide and positioned at an equal angle. Each mouse was placed at the cross-points of arms and allowed to move freely through the maze for an 8-min session. The sequence of arm entries was recorded manually. Spontaneous alternation behavior was defined as the consecutive entry of a mouse into all three different arms (i.e., arm A, arm B, and arm C) to form a triplet of non-repeated components. The percent spontaneous alternation behavior was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries − 2) × 100.

2.5. Novel object recognition task (NORT)

This task, based on the spontaneous tendency of rodents to explore a novel object more often than a familiar one [10], was performed with a slight modification as described previously [9]. A plastic chamber (35 cm × 35 cm × 35 cm) was used in low light condition during the light phase of the light/dark cycle. The general procedure consisted of three different phases: a habituation phase, an acquisition phase, and a retention phase. On the 1st day (habituation phase), mice were individually subjected to a single familiarization session of 10 min, during which they were introduced in the empty arena, in order to become familiar with the apparatus. On the 2nd day (acquisition phase) animals were subjected to a single 10-min session, during which floor-fixed two objects (A and B) were placed in a symmetric position from the centre of the arena, 15 cm from each and 8 cm from the nearest wall. The two objects, made of the same wooden material with the similar color and smell, were different in shape but identical in size. Mice were allowed to explore the objects in the open field. A preference index for each mouse was expressed as a ratio of the amount of time spent exploring object A $(T_A \times 100)/(T_A + T_B)$, where T_A and T_B are the time spent on exploring

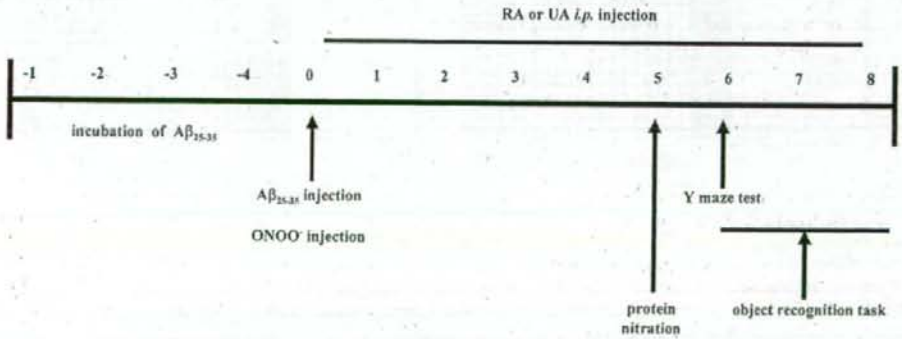

Fig. 2. Experimental schedule.

object A and object B, respectively. On the 3rd day (retention phase), mice were allowed to explore the open field in the presence of two objects: the familiar object A and a novel object C in different shape but in similar color and size (A and C). A recognition index, calculated for each mouse, was expressed as the ratio $(T_C \times 100)/(T_A + T_C)$, where T_A and T_C are the time spent during retention phase on object A and object C, respectively. The time spent exploring the object (nose pointing toward the object at a distance ≤1 cm) was recorded by hand.

2.6. Western blotting

Animals were decapitated on Day 5 after the injection of either $A\beta_{25-35}$ or peroxynitrite. The hippocampi were removed on ice-cold glass plate and stored at −80 °C. The hippocampal tissues were homogenized in ice cold extraction buffer (20 mM Tris–HCl buffer (pH 7.6) containing 150 mM NaCl, 2 mM EDTA·2Na, 50 mM sodium fluoride, 1 mM sodium vanadate, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mg/ml pepstatin, 1 mg/ml aprotinin, and 1 mg/ml leupeptin). Twenty microgram of equal amounts protein was resolved by a 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). Membranes were incubated in 3% skim milk in phosphate-buffered saline containing 0.05% (v/v) Tween-20 for 2 h at room temperature. Mouse anti-nitrotyrosine antibody, clone 1A6 (Upstate cell signaling, Lake Placid), and goat anti-actin antibodies (Santa Cruz Biotechnology Inc., Santa Crutz, CA) were used to detect nitrated protein and β-actin, respectively. The intensity of each protein band on the film, was analyzed with the Atto Densitograph 4.1 system (Atto, Tokyo, Japan), and was corrected with the corresponding β-actin level. The results were expressed as the percentage of that of the control.

2.7. Statistical analyses

The results are expressed as the mean ± S.E. Statistical significance was determined with one-way ANOVA followed by the Bonferroni multiple comparisons test. $p < 0.05$ was taken as a significant level of difference.

3. Results

3.1. Effects of RA on $A\beta_{25-35}$-induced memory impairment

Based on the reports of pharmacological studies of RA on central nervous system in mice [38,39] and the clinically applying doses of the herbs contained RA, we determined the dose range as 0.05–4 mg/kg day for the selection of optimum dose by behavioral investigation. In the Y-maze test, spontaneous alternation behaviors in $A\beta_{25-35}$ group were significantly less than that in vehicle group while the number of arm entry in each group was the same (**Fig. 3**A and B). Daily treatment with RA (0.25, 1, 2, and 4 mg/kg) after the injection of $A\beta_{25-35}$ increased the alternation behavior compared to $A\beta_{25-35}$ group (**Fig. 3**B). In the NORT, all groups explored the two different objects for a similar amount of time during acquisition phase (**Fig. 3**C). No differences among groups were observed with regards to overall object exploration when different doses of RA were administered (**Fig. 3**C). During retention phase, the group of

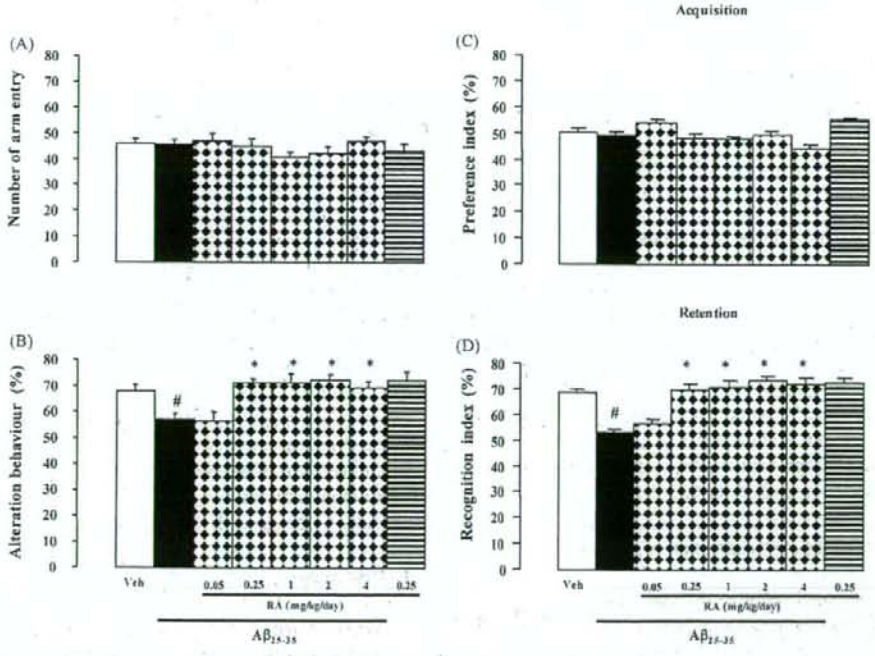

Fig. 3. Effects of rosmarinic acid on the impairment of memory induced by $A\beta_{25-35}$. Various doses of RA were administrated i.p. after i.c.v. injection of $A\beta_{25-35}$ (Day 0) for 9 consecutive days. Mice were subjected to the Y-maze test (A and B) on Day 6 and the NORT (C and D) on Days 6–8. The immediate working memory in the Y-maze test and the long-term memory in the NORT of $A\beta_{25-35}$-injected mice are significantly impaired than those in vehicle i.c.v.-injected mice. Treatment with RA at the doses of 0.25, 1, 2, and 4 mg/kg day significantly prevented the impairment of memory in $A\beta_{25-35}$-injected mice. RA treatment alone did not boost the memory in naive mice. Data were presented as the mean ± S.E. ($n = 10$). $^*p < 0.05$ vs. veh. $^\#p < 0.05$ vs. $A\beta_{25-35}$. Veh: vehicle, RA: rosmarinic acid, $A\beta_{25-35}$: amyloid beta (25–35).

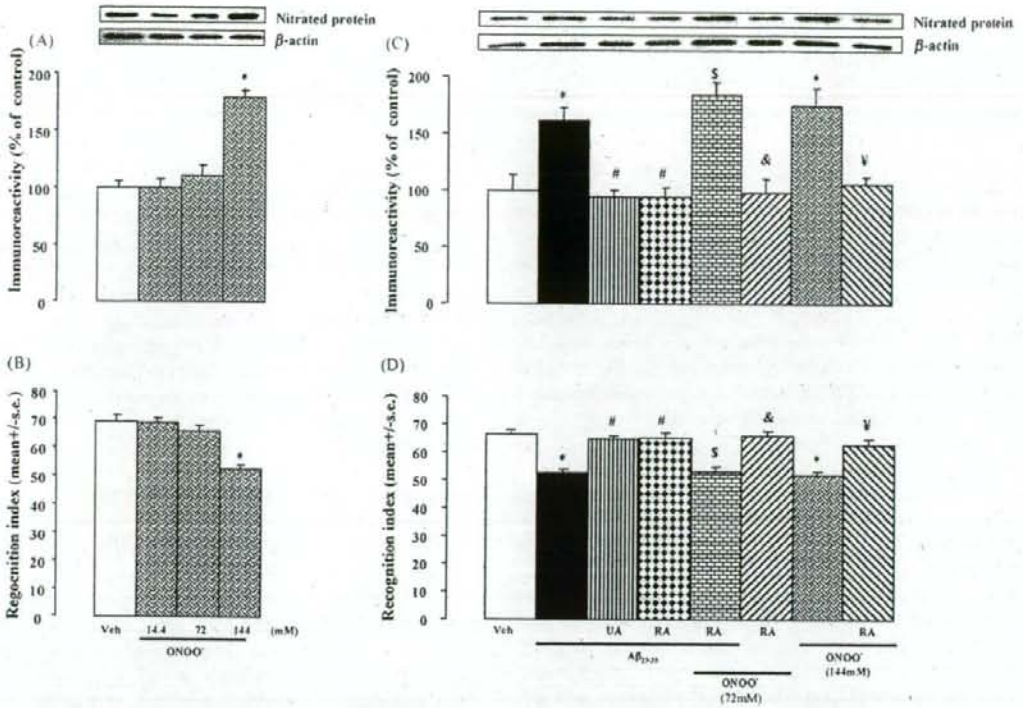

Fig. 4. The effects of rosmarinic acid on the nitration of proteins and the impairment of memory induced by $A\beta_{25-35}$ and $ONOO^-$. (A and B) i.c.v.-Injection of $ONOO^-$ nitrated hippocampal proteins and induced impairment of memory. (C and D) i.c.v.-Injection of $A\beta_{25-35}$ significantly increased the nitration of hippocampal proteins. RA (0.25 mg/kg day) manifested prevention of $A\beta_{25-35}$-induced protein nitration is not different from that of UA (100 mg/kg day), a scavenger of $ONOO^-$. Co-injection of a safe dose (72 mM) of $ONOO^-$ with $A\beta_{25-35}$ blocked the protective effects of RA. RA (0.25 mg/kg day) prevented the protein nitration and memory impairment induced by $ONOO^-$ (144 mM). Data were presented as the mean ± S.E. ($n = 10$). $^*p < 0.05$ vs. veh; $^\#p < 0.05$ vs. $A\beta_{25-35}$; $^\$p < 0.05$ vs. $A\beta_{25-35}$ + RA; $^\&p < 0.05$ vs. $A\beta_{25-35}$ + RA + $ONOO^-$; $^¥p < 0.05$ vs. $ONOO^-$. Veh: vehicle, RA: rosmarinic acid, $A\beta_{25-35}$: amyloid beta (25–35), UA: uric acid, $ONOO^-$: peroxynitrite.

$A\beta_{25-35}$-injected mice did not discriminate the novel and familiar objects by showing a significantly decreased exploration to the new object in a comparison with vehicle group. All doses (except 0.05 mg/kg) of RA significantly enhanced the new object discrimination ability of $A\beta_{25-35}$-injected mice (**Fig. 3**D). However, no dose dependent response was observed. The effective lowest dose (0.25 mg/kg) of RA did not boost memory function in normal mice (**Fig. 3**B and C), restricting its memory protecting effects to its anti-oxidant property. Therefore, the optimum dose of the memory protective effects of RA for the rest of the study was selected to be 0.25 mg/kg.

3.2. The effect of RA on the nitration of proteins

$ONOO^-$-mediated nitrative damage, which could be indirectly indicated by the nitration of proteins, seems to contribute much of the neurotoxicity of Aβ [4–6,34,35]. In vitro studies show that RA is a robust scavenger of $ONOO^-$ and other free radicals [8,32]. Thus, the protective effects of RA in our model seem to be involved in the scavenging of $ONOO^-$ induced by $A\beta_{25-35}$. To examine the direct involvement of $ONOO^-$ in the impairment of memory in our model, we tried the i.c.v.-injection of $ONOO^-$ itself. $ONOO^-$, at the concentration of 144 mM, but not at 72 mM, nitrated hippocampal proteins and induced impairment of memory (**Fig. 4**A and B). The i.c.v.-injection of $A\beta_{25-35}$ significantly increased the nitration of hippocampal proteins. Daily treatment with RA and uric acid (UA), a scavenger of peroxynitrite, prevented $A\beta_{25-35}$-induced protein nitration and memory impairment (**Fig. 4**C and D). However, co-injection of a non-memory-impairing dose (72 mM) of $ONOO^-$ with $A\beta_{25-35}$ blocked the preventive effects of RA in terms of protein nitration and impairment of memory. The protein nitration and memory impairment induced by $ONOO^-$ (144 mM) was prevented by the daily treatment with RA (**Fig. 4**C and D).

4. Discussion

AD is an age-related neurodegenerative disorder with progressive cognitive dysfunction and characterized by presence of senile plaques in the brain. Aβ is the major component of senile plaques and is considered to have a causal role in the development and progress of AD. *Intracerebroventricular* administration of $A\beta_{1-40}$ in rats causes memory deficits [20,24,25]. Etiological studies have indicated that oxidative

stress, an early event in the pathology of AD [26], is responsible for the onset of the cognitive dysfunction as well as the progression of the disease [5,14,34]. Elevated levels of Aβ, a natural antioxidant in physiological concentrations [15], induce oxidative stress which could mediate the damage seen in AD [4]. High levels of Aβ is responsible for the increased appearance of reactive oxygen species such as superoxide (O_2^-) and NO in AD [4,19,48]. The rapid interaction between O_2^- and NO produces $ONOO^-$, a general source of oxidative damage induced by Aβ in the brain of patients with AD [4,31]. $ONOO^-$ exhibits strong rapid damage including peroxidation of lipids and nitration of tyrosine residues of proteins, and thus affects cell function in many different ways. The $ONOO^-$-mediated damage, a wide spread phenomenon in AD brain, leads the onset of the disease [6,14,35]. Although the half-life of NO is extremely short, $ONOO^-$ is formed at a rate more than three times faster than the scavenging of superoxide by superoxide dismutase, suggesting criticality of the over-produced NO. Aβ induces the overproduction of NO via iNOS, and inhibition or ablation of the latter prevents the impairment of memory induced by $A\beta_{1-40}$ [23,41,42]. In the brains of both sporadic and familial AD patients, the number of inducible nitric oxide synthase (iNOS) positive neurons is conspicuously higher than in that of control patients [43]. It is obvious that the inhibition of iNOS to reduce $ONOO^-$ could be an ideal alternative to prevent the neurotoxicity of Aβ in AD. However, the involvement of iNOS in other pathological and physiological aspects of life makes the idea less feasible [11,13]. Therefore, the scavenging of peroxynitrites directly by using natural safe ingredients from the medicinal herbs could be a rational alternative of preventive and therapeutic interventions in the neurotoxicity of Aβ.

In this study, we have therefore investigated the effect of RA, a scavenger of $ONOO^-$, on the memory impairment induced by $A\beta_{25-35}$. We also examined the possible mechanism to explain the therapeutic property of RA through its $ONOO^-$-scavenging effects to prevent the nitration of proteins induced by $A\beta_{25-35}$. $A\beta_{25-35}$, at the dose of 3 μg/3 μl, did not produce the impairment of memory (in NORT) until Day 5 after its injection. The impairment of memory induced by $A\beta_{25-35}$ showed a gradual aggravation till Day 12 when evaluated in cue and contextual fear-conditioning test (unpublished data). The nitration of proteins was found relatively stronger (might be due to the rate of gradual formation and time-dependent clearance) on Day 5 than that on other time points and positively associated with the level of impairment of memory. When the nitration was prevented till Day 5, no impairment of memory was observed in behavioral tests on later time points (unpublished observation). Therefore, we selected the indicated time courses in this study. To determine the dose of RA which is sufficient to prevent the memory impairment induced by $A\beta_{25-35}$ in mice, several doses of RA were administered i.p. At the doses of 0.25, 1, 2, and 4 mg/kg, RA significantly prevented the $A\beta_{25-35}$-induced impairment of the alteration behavior as an immediate working memory in the Y maze test and the impairment of the ability of novel object discrimination as a long-term memory in the NORT. However, no dose-dependent response was observed. The lack of a dose-dependent effect of RA could be explained as follow: first, there is the ceiling effect of RA in the present behavioral tasks. Because it is hard to get 100% of alteration behavior and recognition index, since the chance level is 50% in both behavioral tasks. Second, RA is rapidly decreased in the blood circulation after intravenous administration ($t_{1/2} = 9$ min) [28]. A previous pharmacokinetic study has defined a number of metabolites of RA such as caffeic acid, ferulic acid and hydroxyphenylpropionic acid [22]. Among these, caffeic acid has been reported to inhibit the production and the release of NO in activated astrocytes and macrophages in culture studies [36,49]; ferulic acid, a further metabolite of caffeic acid, has been reported to protect against oxidative stress and memory impairment induced by Aβ [29,46]. These reports about the metabolism and metabolites of RA may explain the lack of dose response as well as the efficacy of RA at a very low dose of 0.25 mg/kg. Thus, the optimum dose of the protective effects of RA was selected to be 0.25 mg/kg for the rest of the study. The memory impairments seen in AD are contributed by Aβ-driven $ONOO^-$-mediated damages [34,35]. The memory protective effect of RA could be due to the capturing of the extremely noxious $ONOO^-$ induced of $A\beta_{25-35}$. To examine this, the involvement of $ONOO^-$ in the impairment of memory was confirmed by i.c.v.-injection of itself or daily treatment with UA, a scavenger of $ONOO^-$, after the injection of $A\beta_{25-35}$. $ONOO^-$ induced nitration of proteins and impaired the memory. UA prevented $A\beta_{25-35}$-induced nitration of proteins in the hippocampus and impairment of memory, indicating that the proxynitrites are the major contributors to the toxicity of $A\beta_{25-35}$. UA treatment in vehicle-treated group did not affect the memory (data not shown). RA prevented the $ONOO^-$-induced nitration of proteins and impairment of memory. Daily treatment with RA significantly prevented the nitration of proteins as well as memory impairment induced by $A\beta_{25-35}$. However, RA protection of $A\beta_{25-35}$ toxicity was abolished after the co-injection of non-memory impairing dose of proxynitrites to increase the proxynitrite burden. It was implied that the memory protecting effects of RA are due to its activity of scavenging proxynitrites. Indeed, proxynitrites-induced nitration of proteins and impairment of memory were prevented by daily treatment with RA. As for the increase of nitration of proteins by the co-injection, it might be that the pro-oxidative property of $A\beta_{25-35}$ may increase in the presence of proxynitrites, or the co-injected proxynitrites make the cells more vulnerable to the toxicity of $A\beta_{25-35}$. The results together indicated that the RA protects memory through the scavenging of $ONOO^-$ which induced by $A\beta_{25-35}$.

Although investigations about the activity of RA on central nervous system (CNS) are few, two lines of studies have shown the antidepressive-like activity of RA in mice exposed to conditioned fear stress and the forced swimming test [38,39]. Other unknown properties of RA and its metabolites in CNS may also contribute to its protective effects on the memory impairment induced by Aβ. Nevertheless, our study provided clear evidence that RA protects against the impairment of memory in the neurotoxicity of Aβ by preventing $ONOO^-$-mediated damage.

In summary, the results of this study and all other reported properties of RA strongly suggest its usefulness in the treatment of memory impairment observed in AD.

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Research report

Hypofunctional glutamatergic neurotransmission in the prefrontal cortex is involved in the emotional deficit induced by repeated treatment with phencyclidine in mice: Implications for abnormalities of glutamate release and NMDA–CaMKII signaling

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Abstract

In the present study, we investigated the involvement of prefrontal glutamatergic neurotransmission in the enhancement of immobility (emotional deficit) in a forced swimming test in mice treated with phencyclidine (PCP: 10 mg/kg/day for 14 days) repeatedly, which is regarded as an animal model for negative symptoms. A decrease in spontaneous extracellular glutamate release and increase in levels of the glutamate transporter GLAST, were observed in the prefrontal cortex (PFC) of PCP-treated mice, compared to saline-treated mice. NMDA receptor subunit 1 (NR1) and Ca^{2+}/calmoduline kinase II (CaMKII) were markedly activated in the PFC of saline-treated mice, but not PCP-treated mice, immediately after the forced swimming test. The facilitation of the function of NMDA receptors by D-cycloserine (30 mg/kg i.p.), an NMDA receptor glycine-site partial agonist, reversed the enhancement of immobility in the forced swimming test and impairment of CaMKII activation in the PCP-treated mice. Microinjection of DL-threo-β-benzyloxyaspartate (10 nmol/site/bilaterally), a potent blocker of glutamate transporters, into the PFC of PCP-treated mice also had an attenuating effect. In addition, activation of glial cells and a decrease of neuronal cell size were observed in the PFC of PCP-treated mice. These results suggest that repeated PCP treatment disrupts pre- and post-synaptic glutamatergic neurotransmission and induces morphological changes in the PFC and that such changes cause the emotional deficits exhibited in PCP-treated mice.
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Keywords: Phencyclidine; Emotional deficit; *N*-Methyl-D-aspartate; Prefrontal cortex; Glutamate; CaMKII

1. Introduction

Alternations of glutamatergic systems involving NMDA receptors are relevant to the pathophysiology of schizophrenia as demonstrated by pharmacological evidence that NMDA receptor antagonists reproduce some symptoms of the disease in normal individuals: phencyclidine (PCP), a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, has been shown to induce schizophrenia-like psychosis representing positive symptoms, negative symptoms, and cognitive deficits in humans [17], which persist several weeks after withdrawal from chronic PCP use [1,25,42]. D-Cycloserine, a partial NMDA receptor glycine-site agonist, provides a modest improvement of even the cognitive deficits and negative symptoms in schizophrenia [8,17]. Chronic PCP psychosis might be more consistent with schizophrenia than acute PCP psychosis [17,21].

We have previously reported that chronic treatment with PCP (10 mg/kg/day s.c. for 14 days) induces several schizophrenia-

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like behavioral abnormalities such as an enhancement of immobility in a forced swimming test [36–38], social deficits [41] in a social interaction test, associative learning impairment in cued and contextual fear conditioning [11] in mice. Therefore, PCP-treated mice might be a useful model of schizophrenia [11,19,22,28,36–38,41].

It is hypothesized that insufficient glutamate neurotransmission in the PFC is associated with the pathology of schizophrenia [3,4,13]. Abnormal glutamatergic systems in the PFC have been found in PCP-treated mice, i.e. altered expression of the NMDA receptor subunit or an associated protein and decreased metabolic activity [7]. NMDA receptors are composed of at least one NR1 subunit and one or more modulatory subunits and regulate various forms of neuronal plasticity. NR1 knockdown and NR2A knockout mice have been reported to show schizophrenia-like behavioral abnormalities such as hyperactivity and social withdrawal [15,31,32]. However, little attempt has been made to investigate the involvement of prefrontal glutamatergic neurotransmission via NMDA receptors in negative symptom-like emotional deficits in PCP-treated mice.

In this article, we describe the results of experiments designed to test the hypothesis that the negative symptom-like emotional deficit in a forced swimming test is mediated via impaired pre- and post-synaptic glutamatergic neurotransmission in the PFC of mice treated with PCP repeatedly. We attempted to investigate the changes in (1) NMDA-Ca^{2+}/calmodulin-dependent protein (CaMKII) signaling after the forced swimming test, since the NMDA receptor and CaMKII form a tight complex at synapses which may facilitate the activation of second messenger pathways [10,26], (2) extracellular glutamate levels, (3) the expression levels of glutamate transporters, and (4) morphology of neurons and glia in the PFC. We also examined the effect of (5) facilitation of the function of NMDA receptors by D-cycloserine, an NMDA receptor glycine-site agonist, and DL-threo-β-benzyloxyaspartate (DL-TBOA), a non-substrate glutamate transport inhibitor, on behavioral and biochemical abnormalities in PCP-treated mice.

2. Materials and methods

2.1. Animals

Male mice of the ddY strain (Japan SLC Inc., Shizuoka, Japan), weighing 30–33 g at the beginning of the experiments, were used. The animals were housed in plastic cages and kept in a regulated environment (23 ± 1 °C, 50 ± 5% humidity), with a 12/12 h light–dark cycle (lights on at 8:00 a.m.). Food (CE2; Clea Japan Inc., Japan) and tap water were available ad libitum. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine.

2.2. Drugs

Phencyclidine hydrochloride (PCP) was synthesized by the authors according to the method of Maddox et al. [27] and was checked for purity. D-Cycloserine (Sigma, MO) and PCP were dissolved in a 0.9% NaCl solution. DL-TBOA (Tocris, MO) was prepared as a stock solution of 100 mM in 50% dimethyl sulfoxide (DMSO) and 100 mM NaOH, and dissolved in phosphate-buffered saline (PBS) before the experiments. KN93 and KN92 were purchased from Sigma–Aldrich and dissolved in a 0.01% DMSO (dimethyl sulfoxide)-containing saline solution.

2.3. Drug treatment

The mice were administered saline or PCP (10 mg/kg/day s.c.) once a day for 14 consecutive days. They were injected with D-cycloserine (s.c.) or DL-TBOA 30 min and KN93 or KN92 10 min before the measurement of immobility in the forced swimming test. DL-TBOA (1 or 10 nmol/site/bilaterally), KN93 (10 nmol/site/bilaterally), and KN92 (10 nmol/site/bilaterally) were microinjected into the PFC [anteroposterior (AP): 1.7, mediolateral (ML): ±0.5 from bregma, dorsoventral (DV): 2 mm from the skull] according to the mouse brain atlas of Franklin and Paxinos [14]. Dual 27-gauge microinjectors attached to tubing were inserted through the guides, from which they protruded 2.5 mm. The other end of the tubing was connected to a 25 μl Hamilton syringe. The volumes were injected over a period of about 30 s, and the injector was left in place for 1 min to allow diffusion. All compounds except for DL-TBOA, KN93 and KN92 were administered in a volume of 0.1 ml/10 g body weight.

2.4. Forced swimming test

The forced swimming test was done according to previous reports [36–38] with a minor modification. The test was performed the day after the final treatment with PCP. Namely, each mouse was placed in a transparent glass cylinder (20 cm high, 15 cm in diameter), which contained water at 22–23 °C to a depth of 15 cm, and was forced to swim for 180 s. The duration of swimming was measured, by using a SCANET MV-10 AQ apparatus (Melquest Co. Ltd., Japan). The immobility time was calculated as follows: 180 (s) − swimming time (s) = immobility time (s).

2.5. Microdialysis analysis

On day 14 after the start of PCP treatment, the mice were anesthetized with pentobarbital Na (50 mg/kg i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, CA). A guide cannula (AG-6 EICOM, Japan) was implanted into the PFC [15° angle away from AP: +1.7, ML: −1.0 from bregma, DV: −1.5 mm from the skull] according to the atlas. On day 15 (24 h after the implantation of the guide cannula: 1 day after PCP withdrawal), a dialysis probe (A-I-6-01; membrane length 1 mm, EICOM, Japan) was implanted into the PFC and Ringer solution (147 mM, NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) was perfused at a flow rate of 1.0 μl/min. The dialysate was colleted every 10 min and the amount of glutamate in the dialysate was determined using an HPLC system (HTEC-500, EICOM, Japan) with electrochemical detection (ECD). Three samples were taken to establish baseline levels of extracellular glutamate. For depolarization stimulation, 100 mM KCl-containing Ringer solution was delivered through the dialysis probe for 30 min in order to induce the K^+-evoked release of glutamate. Then dialysate was collected for 70 min with Ringer solution. For the rescue study with DL-TBOA, a dialysis probe equipped with a microinjection tube (MIA-6-1; 1 mm membrane length, EICOM) was used. After the collection of baseline fractions, 10 nmol of DL-TBOA dissolved in 1 μl of PBS was injected bilaterally during a 10-min period through the microinjection tube into the PFC.

2.6. Western blotting

Western blotting was performed as described [11] with a minor modification. Immediately after the forced swimming test, the mice were sacrificed by decapitation. The PFC containing cingulate and prelimbic area (Bregma +2.96 to Bregma +1.34) defined as in Franklin and Paxinos was rapidly dissected out, frozen, and stored at −80 °C until used. The brain samples were homogenized in ice-cold buffer [50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 10 mM NaF; 10 mM EDTA, 1% NP-40; 1 mM sodium orthovanadate; 10 mM sodium pyrophosphate; 0.5 mM DTT; 0.2 mM PMSF; 4 μg/ml pepstatin, 4 μg/ml aprotinin, and 4 μg/ml leupeptin for measuring phospho-NR1, NR1, phospho-Ca^{2+}/calmoduline kinase II (CaMK II), CaMKII, GLT-1, GLAST and actin]. The lysate was centrifuged at 8000 × *g* for 10 min at 4 °C. The protein concentration of the supernatant was determined by a Bradford assay (Bio-rad, CA). Sample buffer [0.25% bromophenol blue/0.25% xylene cyanol 30% glycerol/20% 2× Tris–borate EDTA

(90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.4)] was added to the supernatant and the mixture was boiled at 95 °C for 5 min. Equivalent amounts of protein (20 μg) were electrophorated on SDS-polyacrylamide gels, transferred to PVDF membranes (Millipore, MA), and blocked with a Detector Block Kit (KPL, MD). The membranes were incubated with a rabbit anti-phospho-NR1 (S897), a rabbit anti-phospho CaMKII (1:1000; Upstate Biotechnology), a guinea-pig anti-GLAST (1:1000; Chemicon, CA), a guinea-pig anti-GLT-1 (1:1000; Oncogene, MA) or an anti-actin (C-11) (1:1000; Santa Cruz Biotechnology, CA) antibody at 4 °C overnight. For the analysis of phospho-CaMKII, CaMKII, GLAST and GLT-1 levels, each protein was boiled in a sample buffer and a 10% polyacrylamide gel and subsequently transferred to a polyvinylidine difluoride membrane (Millipore) and blocked with a Detector Block kit (KPL). The membranes were then washed with washing buffer (50 mM Tris–HCl, pH 7.4, and 150 mM NaCl and 0.05% Tween 20). After incubation with a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated IgG for 2 h, the membranes were washed with washing buffer. The immune complex was detected using an enhanced chemiluminscence (ECL) kit (Amersham Pharmacia Biotech Inc., NJ) and exposed to X-ray film. Images on the film were captured using a CCD camera (ATTO, Japan). The intensity of each band was analyzed quantitatively using the ATTO Densitograph Software Library Lane Analyzer (ATTO). To measure total (phospho- and non-phospho-) NR1 or CaMKII, membranes were stripped with stripping buffer (100 mM 2-melcaptoethanol; 2% SDS; and 62.5 mM Tris–HCl, pH 6.7) at 50 °C for 30 min, and incubated with a mouse anti-NR1 CT (1:1000; Upstate Biotechnology, NY) or a rabbit anti-CaMKII (1:1000; Sigma) antibody at 4 °C overnight. To evaluate NR-1 or CaMKII activation, phospho-NR-1 or CaMKII levels were normalized to total NR-1 or CaMKII levels in the same membranes, and then each phospho-/total-level was normalized to the basal phospho-/total-level in saline-treated mice. To evaluate the expression of GLAST and GLT-1, the GLAST and GLT-1 levels were normalized to the actin levels in the same membranes, and then GLAST/actin and GLT-1/actin levels were normalized to the GLAST/actin and GLT-1/actin levels in saline-treated mice.

2.7. Preparation of brain slice and staining

Histological procedures were performed as previously described with a minor modification [11,34]. Before and immediately after the forced swimming test, mice were anesthetized with chloral hydrate (200 mg/kg i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 2 h, and then soaked in 20% (w/v) sucrose in PBS. Coronal sections 20 μm thick were cut with a cryostat (CM 1850; Leica, Germany). For immunohistochemistry, the primary antibodies that were applied in the brain slices included anti-GLAST (1:300), a guinea-pig anti-GLT-1 (1:300; Chemicon), a rabbit anti-s100 (1:300; Dako, CA) and mouse anti-GFAP (1:300; Chemicon) antibody. Fluorescently conjugated secondary antibodies (Alexa 488, 546; Molecular Probes, OR) were used for detecting chromagen. Twenty sections at 80 μm intervals from Bregma +2.96 to Bregma +1.34 were used from each brain, and prelimbic area was defined as in Franklin and Paxinos. For counting the number and calculate the size of neuron, Cresyl Violet staining was performed and only neurons with a visible nucleus and in which the entire outline of the cell was apparent were counted in standardized area in the prelimbic area using computer-based image analysis system (Image J, Bethesda, MD). Using these measurements, the surface area (μm²) of s100-stained process and size of neuron, was calculated in standardized area in the layer II/III of the prelimbic area. Images were acquired with a confocal microscope (μ Radiance, Bio-Rad) provided by Division for Medical Research Engineering of Nagoya University or a light microscope (Axioskop2 plus; Carl Zeiss, Germany).

2.8. Statistical analysis

Statistical analysis was performed with a one-way analysis of variance (ANOVA) using Bonferroni's test. The unpaired *t*-test (Student's *t*-test) was used to compare two sets of data. A value of $p < 0.05$ was considered statistically significant. Data were expressed as the mean ± S.E.M.

3. Results

3.1. Ability to release glutamate in the prefrontal cortex

We examined the extracellular glutamate levels in the PFC of the PCP-treated mice by using an *in vivo* microdialysis technique. After the basal extracellular levels of glutamate reached a steady state, basal release of glutamate was monitored during 30 min of dialysis. PCP-treated mice showed a dramatically decreased basal level of glutamate in the PFC [PCP-treated mice: 0.905 ± 0.158 pmol/sample ($n = 6$), saline-treated mice: 4.26 ± 54.9 pmol/sample ($n = 5$)] (Fig. 1). We investigated the ability to release glutamate of high potassium (high K^+) (100 mM) in the PFC of the PCP-treated mice. The amount of glutamate released in the PFC of the PCP-treated mice was significantly less than that released the saline-treated mice ($p < 0.01$) [PCP-treated mice: 3.02 ± 0.28 pmol/sample ($n = 6$), saline-treated mice: 8.42 ± 0.90 pmol/sample ($n = 5$)] (Fig. 1), though the high K^+-induced extracellular glutamate release (% of baseline) in PCP-treated mice was about three-fold higher than that in saline-treated mice.

3.2. The changes of glutamate transporters, glial cells and neuronal morphology in the prefrontal cortex

Glial glutamate transporters, i.e. GLAST and GLT-1, play an important role in regulating glutamate transmission by rapidly

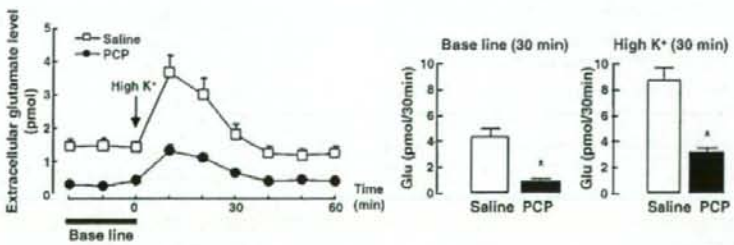

Fig. 1. Basal extracellular levels of glutamate and K^+-evoked glutamate release in the prefrontal cortex of mice treated with phencyclidine repeatedly. The mice were administered saline or phencyclidine (PCP: 10 mg/kg/day s.c.) for 14 days ($n = 5$ for saline-treated mice, $n = 6$ for PCP-treated mice). Basal extracellular levels of glutamate and K^+-evoked (100 mM) glutamate release in the prefrontal cortex (PFC) of saline- or PCP-treated mice were determined by a microdialysis method. Fractions were collected for 30 min. Basal extracellular glutamate levels were: PCP-treated mice, 0.91 ± 0.16 pmol/sample ($n = 6$); saline-treated mice, 4.26 ± 54.9 pmol/sample ($n = 5$). Values correspond to the mean ± S.E.M. $^*p < 0.05$ vs. saline-treated mice (Student's *t*-test). Glu: glutamate.

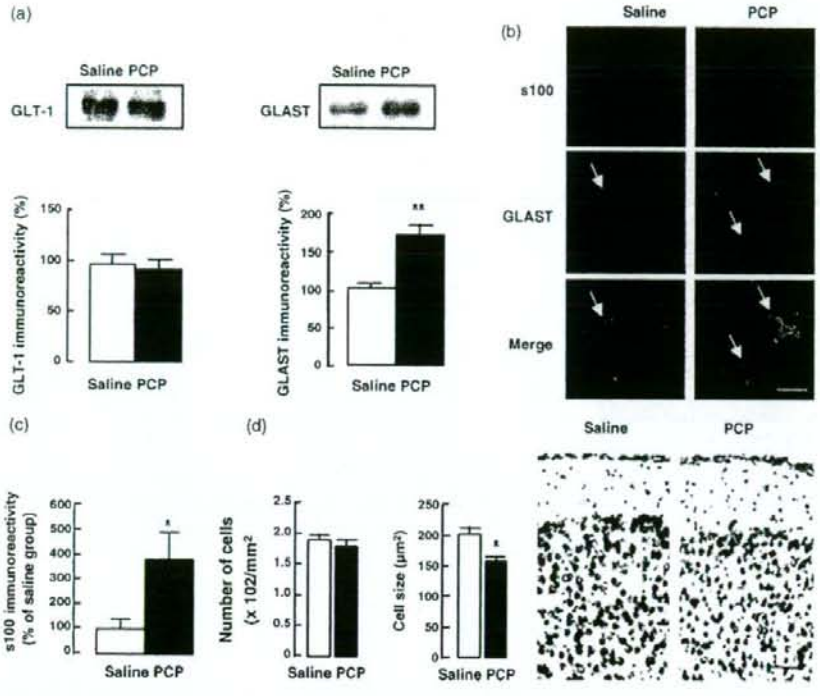

Fig. 2. The changes in expression of glutamate transporters and gross morphology in the prefrontal cortex of mice treated with phencyclidine repeatedly. The mice were administered saline or phencyclidine (PCP: 10 mg/kg/day s.c.) for 14 days. (a) Representative western blots and immunoreactivity to GLAST and GLT-1 in the prefrontal cortex (PFC) (GLAST: $n = 8$ in each group, GLT-1; $n = 6$ in each group). (b) Representative image of s100 (green) and GLAST (red) immunostained sections from the PFC of saline- and PCP-treated mice. Arrows indicates GLAST fluorescent puncta on the process of glia. Scale bar: 50 μm. (c) Stereological analysis of s100 immunoreactivity in the PFC of saline- and PCP-treated mice ($n = 4$ in each group). (d) Cresyl violet-stained coronal sections of the PFC and stereological analysis of cell number and cell size in the PFC of saline- and PCP-treated mice ($n = 5$ in each group). Scale bar: 200 μm. Values correspond to the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline-treated mice (Student's t-test). Glu: glutamate.

clearing glutamate from extracellular fluid [9]. In order to examine whether the decrease in the extracellular concentration of glutamate is due to changes of glutamate transporters, we investigated the protein levels of glial glutamate transporters in the PFC of PCP-treated mice by immunoblotting. Although there was no difference in the GLT-1 protein level between PCP-treated mice and saline-treated mice, the expression level of GLAST protein was higher in the PCP-treated mice ($p < 0.01$; **Fig. 2**a).

Since immunoblot analysis showed an increase of GLAST levels, the localization of GLAST in the PFC of PCP-treated mice was analyzed by immunohistochemical methods. GLAST was localized to the processes of cells positive for s100, a glial marker, and merged with s100, both in saline-treated and in PCP-treated mice (**Fig. 2**b). The morphology of s100-positive cells was altered to the activated form with thick processes in the PCP-treated mice (**Fig. 2**b and c).

Glia is activated in response to several pathological conditions, such as physical or biochemical damage to the nervous tissue. To investigate whether repeated PCP treatment induces neuronal damage in the PFC, we examined morphological changes in neurons by Cresyl Violet staining. No difference in the number of neurons in the layer II/III of the PFC was observed regardless of PCP treatment (**Fig. 2**d). However, a decrease in neuronal cell size was revealed in the layer II/III of the PFC of PCP-treated mice compared to saline-treated mice (**Fig. 2**d).

3.3. Intracellular signaling via NMDA receptors in the prefrontal cortex

We investigated the change in the activation of CaMKII signaling via NMDA receptors in the PFC after the forced swimming test. The phosphorylation levels of NR1, one of the subunit of NMDA receptors, and of CaMKII, in the PFC of the saline-treated mice were significantly increased immediately after the test, compared to those in the non-tested, saline-treated mice ($p < 0.01$; **Fig. 3**a and b). However, the phosphorylation levels of NR1 (p-NR1) and CaMKII (p-CaMKII) did not increase after the test in the PCP-treated mice (**Fig. 3**a and b). There was no significant difference in p-NR1 and p-CaMKII between non-tested, saline- and PCP-groups. No difference in total NR1 and CaMKII expression levels was observed between the saline- and PCP-treated mice [NR1: $F(3, 15) = 1.54$, $p = 0.26$; CaMKII: $F(3, 31) = 0.68$, $p = 0.56$] (data not shown).

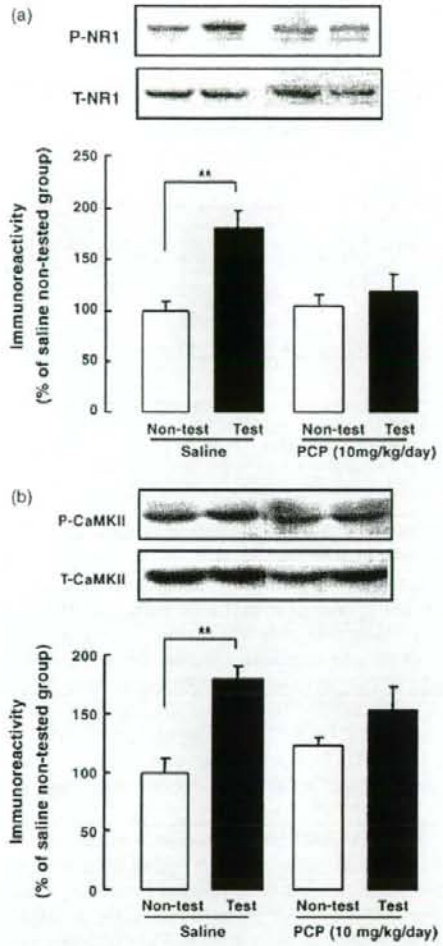

Fig. 3. NR1 and CaMKII activation in the prefrontal cortex after the forced swimming test. Immediately after the forced swimming test, western blotting was performed in the prefrontal cortex (PFC) of saline- or phencyclidine (PCP)-treated mice (10 mg/kg/day s.c. for 14 days). Representative western blots and phospho-NR1/total-NR1 ($n = 4$ in each non-tested or tested group) (a) or phospho-CaMKII/total-CaMKII (b) ($n = 8$ in each non-tested or tested group) immunoactivity in the PFC. Values correspond to the mean ± S.E.M. Results with the one-way ANOVA were: (a) $F(3, 15) = 7.12$ ($p < 0.01$) and (b) $F(3, 31) = 2.95$ ($p < 0.01$). $^{**}p < 0.01$ vs. the saline-treated mice. (Bonferroni's test (a), (b)) Glu: glutamate. Test: forced swimming test group. Non-test: without forced swimming test group. Phosho-: P-, total-: T-.

3.4. Relationship between enhancement of immobility and CaMKII activation

To examine the relationship between the activation of CaMKII and enhancement of immobility in the forced swimming test, we evaluated the effect of a CaMKII inhibitor, KN93, on the immobility induced by forced swimming in mice. The mice microinjected with KN93 (10 nmol/site/bilaterally) into the PFC before the test showed a significantly prolonged immobility time, compared with the mice treated with vehicle or KN92, an inactive inhibitor (**Fig. 4**a). KN93, but not KN92, treatment significantly decreased the level of p-CaMKII expression after exposure to swim stress (**Fig. 4**b) without affecting locomotor activities. A significant inverse relationship between immobility time and phosphorylation of CaMKII in the PFC was observed in the forced swimming test (data not shown).

3.5. Effects of D-cycloserine, a partial agonist of the glycine recognition site at NMDA receptors, and DL-TBOA, a potent glutamate transporter inhibitor, on enhancement of immobility and impairment of CaMKII activation induced by PCP

When PCP-treated mice were injected with D-cycloserine (30 mg/kg s.c.) or DL-TBOA (10 nmol/site/bilaterally) 30 min before the test, the effects of PCP on immobility were significantly attenuated compared to those in the vehicle-treated group ($p < 0.05$; **Fig. 5**a and c). Both saline- and PCP-treated mice showed an increase in the amount of glutamate released in the PFC after DL-TBOA treatment (**Fig. 5**e). However, D-cycloserine and DL-TBOA did not affect the mobility in either saline- or PCP-treated mice. Further, D-cycloserine (30 mg/kg, s.c.) and DL-TBOA (10 nmol/site/bilaterally) did not affect the immobility of saline-treated mice [D-cycloserine: $F(2, 35) = 0.79$, $p = 0.23$; DL-TBOA: $F(2, 31) = 0.55$, $p = 0.58$] (**Fig. 5**a and c).

To confirm whether impairment of CaMKII activation is facilitated after D-cycloserine or DL-TBOA injection, we measured the p-CaMKII levels of the PFC in D-cycloserine- or DL-TBOA-injected, PCP-treated mice. The impairment of CaMKII activation in the PFC of PCP-treated mice was improved by D-cycloserine and DL-TBOA ($p < 0.05$, **Fig. 5**b and d). These results suggested that the impairment of NMDA–CaMKII signaling in the PFC, at least in part, is involved in the emotional deficits in PCP-treated mice. A significant inverse relationship between immobility time and the phosphorylation of CaMKII in the PFC was observed in the forced swimming test on both D-cycloserine and DL-TBOA treatment (data not shown).

4. Discussion

The repeated administration of NMDA receptor antagonists induces negative symptom-like emotional deficits in healthy volunteers or schizophrenia **[17,33]**. As previously reported, repeated, but not single, treatment with PCP elicits some negative symptom-like behavioral deficits (emotional deficits) such as impairments of social behavior in the social interaction test and an enhancement of immobility in the forced swimming test in mice **[36–38,41]**. The effect of PCP on the immobility appears to be sensitive to atypical antipsychotic (clozapine and resperidone) treatment, but not to haloperidol and tricyclic antidepressant treatments **[36,37]**, which were consistent with the clinical findings that risperidone and clozapine, but not haloperidol and tricyclic antidepressants, improve negative symptoms in schizophrenia. Thus we have demonstrated that mice treated with PCP repeatedly provide a good animal model for emotional deficits relevant to negative symptoms of

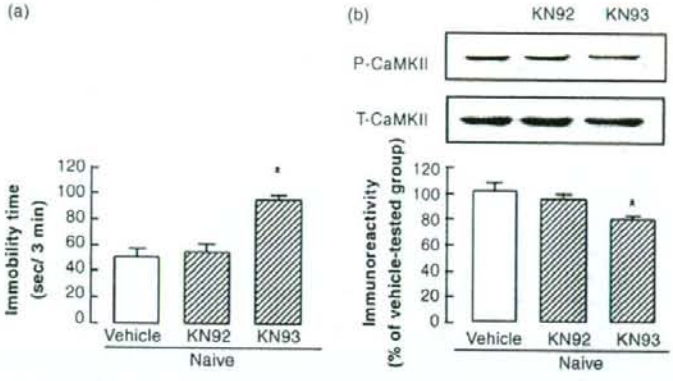

Fig. 4. The effect of CaMKII inhibitors on immobility in the forced swimming test in naïve mice. The naïve mice were microinjected with vehicle, KN92, or KN93 (10 nmol/site/bilaterally) into the prefrontal cortex (PFC). (a) The immobility time was measured for 3 min, 30 min after the microinjection ($n = 11$ in each group non-swimming or swimming). (b) Representative western blots and phospho-CaMKII/total-CaMKII ($n = 5$ in each group) in the PFC of naïve mice. Values correspond to the mean ± S.E.M. Results with the one-way ANOVA were: (a) $F(2, 33) = 16.67$ ($p < 0.01$) and (b) $F(2, 15) = 4.85$ ($p < 0.05$). $^{*}p < 0.05$ vs. the vehicle-treated mice. (Bonferroni's test (a), (b)) Glu; glutamate, P/T-: phospho/total-.

schizophrenia. Abnormal prefrontal glutamatergic neurotransmission has also been described in PCP-treated mice, i.e. altered expression of the NMDA receptor subunit or an associated protein and decreased metabolic activity [7]. Our previous study has shown an imbalance of prefrontal serotonergic and dopaminergic functions in mice treated with PCP repeatedly after forced swimming, which may disrupt the regulation of glutamatergic transmission [38]. In the present study, we found a decreased level of extracellular glutamate and high K^+-induced glutamate release in the PFC of PCP-treated mice. These findings suggest that the abnormal glutamatergic transmission in the PFC induces the emotional deficits in the forced swimming test in PCP-treated mice.

One explanation for the decrease in extracellular glutamate release in the mice treated repeatedly with PCP is the enhanced function of glutamate transporters. There are reports suggesting the involvement of malfunctioning glutamate transporters in the etiology of schizophrenia [9,24,35]. Further, it has been indicated that there is an increase in the number of binding sites and protein levels of glutamate transporters in the PFC of postmortum tissue of schizophrenics [44]. In addition, some antipsychotics inhibit or down-regulate the function of glutamate transporters [29,45]. Our results showed an increased level of GLAST in the PFC of PCP-treated mice. Furthermore, the increase of GLAST expression is detected in the PFC but not in the nucleus accumbens (NAc) (data not shown). These results suggest that the regulation of GLAST expression is restricted in some brain regions. Taken together, it is suggested that the increase in GLAST expression is somewhat related to the decrease in extracellular glutamate release in the PFC. Although we failed to detect any change in GLT-1 expression in neither the PFC nor the NAc, it is possible that the function of GLT-1 is activated without a change in expression. A potent glutamate transporter blocker, DL-TBOA, attenuated the enhancement of immobility and increased the extracellular glutamate level in the PFC of PCP-treated mice. Therefore, these results suggest that, at least in part, the changes in levels of glutamate transporter in the PFC are related to the induction of emotional deficits in mice treated with PCP repeatedly. However, the precise mechanism is still unknown and further investigation is needed.

Low basal levels of extracellular glutamate and the impairment of glutamate release in the PFC may affect the post-synaptic glutamatergic signaling. We found that the mice treated with PCP repeatedly showed the decreased phosphorylation of NR1 and CaMKII after forced swimming, indicating an impairment of NMDA–CaMKII signaling. The NMDA receptor and CaMKII form a tight complex at the synapse, which may facilitate the activation of second messenger pathways [10,12,26]. CaMKII has been demonstrated to be required for regulating emotion and learning and memory in rodents [10,30]. Since CaMKII phosphorylation is caused not only via NMDA receptor but also other receptors, the relation of other receptors cannot be excluded. We have previously reported that the activation of extracellular signaling-regulated kinase (ERK) is not induced by exogenous NMDA, glycine, or spermidine in slices of the hippocampus and amygdala prepared from PCP-treated mice, suggesting abnormal ERK signaling via NMDA receptors in these mice [11]. Further, a blockade of CaMKII signaling caused by the bilateral microinjection of KN93, a CaMKII inhibitor, into the PFC of saline-treated mice induced an enhancement of immobility in the forced swimming test without affecting motor function. It is suggested that the low basal level of extracellular glutamate in the PFC is related to the impairment of post-synaptic signaling via NMDA receptors, which is supported by experiments with DL-TBOA and D-cycloserine. Namely, the elevation of the extracellular glutamate level by DL-TBOA and activation of NMDA receptors by D-cycloserine attenuated the enhancement of immobility and impairment of NR1–CaMKII signaling in mice treated with PCP repeatedly. Furthermore, we confirmed the inverse relationship between CaMKII phosphorylation in the PFC and immobility time in this experiment (data not shown). D-Cycloserine has been reported to be a candidate for a drug to treat schizophrenia [8,18]. As far as we know, this is the first report that abnormalities of pre-synaptic

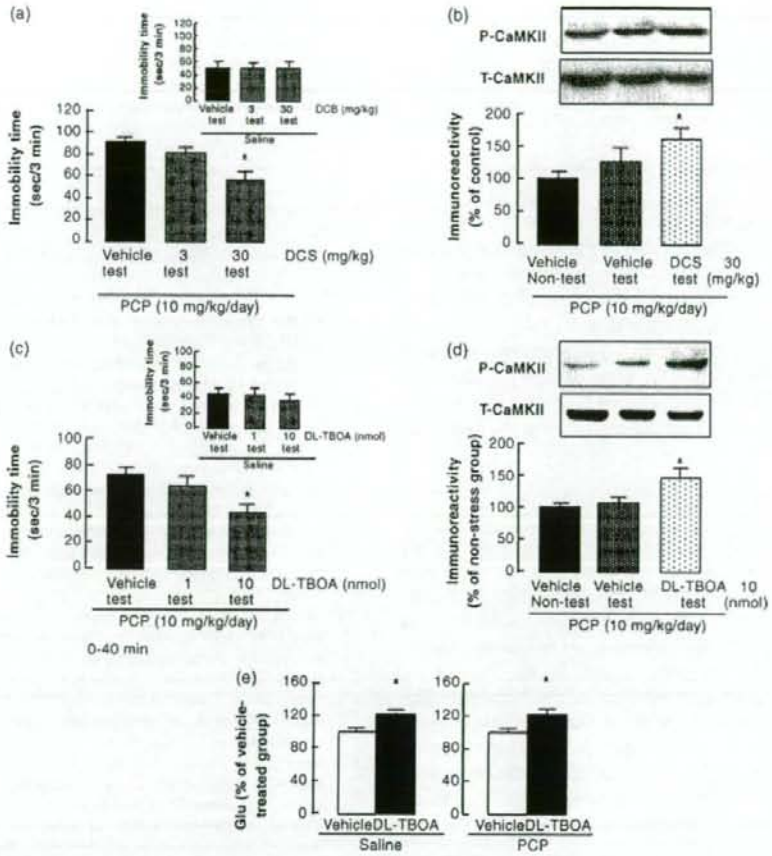

Fig. 5. Effects of D-cycloserine and DL-TBOA on enhancement of immobility and impairment of CaMKII activation induced by phencyclidine. (a) The saline- or phencyclidine (PCP)-treated mice were administered D-cycloserine (3 and 30 mg/kg s.c.) 30 min before the measurement of immobility ($n = 12$–15). (c) The saline- or PCP-treated mice were microinjected with DL-TBOA (1 and 10 nmol/bilaterally) into the PFC 10 min before the measurement of immobility ($n = 14$–20). Representative western blots and phospho-CaMKII/total-CaMKII immunoreactivity after D-cycloserine (b) and DL-TBOA (d) treatment in the PFC of PCP-treated mice ($n = 5$–7). (e) DL-TBOA-induced glutamate release in the PFC of saline- and PCP-treated mice. Fractions were collected for 40 min. Basal extracellular glutamate levels were: vehicle-injected saline-treated mice, 4.68 ± 2.80 pmol ($n = 4$); vehicle-injected PCP-treated mice, 0.83 ± 0.51 pmol ($n = 3$). Values correspond to the mean ± S.E.M. Results with the one-way ANOVA were: (a) $F(2, 41) = 7.92$ ($p < 0.01$); (b) $F(2, 15) = 3.998$ ($p < 0.05$); (c) $F(2, 53) = 4.98$ ($p < 0.05$); (d) $F(2, 19) = 5.515$ ($p < 0.05$). $^{*}p < 0.05$ vs. the vehicle-treated mice (Bonferroni's test (a)–(d) or Student's t-test (e)). DCS: D-cycloserine and Glu: glutamate. Test: forced swimming test group. Non-test: without forced swimming test group. P/T-: phospho/total-.

glutamatergic transmission and post-synaptic NMDA–CaMKII signaling are induced by repeated PCP treatment and are involved in the enhancement of immobility (emotional deficit) in the PCP-treated mice.

We found cell shrinkage and glial activation without cell death and activated s100-positive cells in the PFC after repeated PCP treatment. It has been reported that acute injection of PCP causes the increased release of glutamate in the PFC [2]. Increases in the extracellular synaptic glutamate concentration to excitotoxic levels lead to neuronal damage [5,6]. However, we could not detect apoptosis or a decrease in cell number, although the PCP-treated mice showed a decrease in cell size in the PFC as previously reported: NMDA antagonists induce not cell death but vacuolation if the severe NMDA receptor hypofunction is relatively brief [39,40]. Usually, reactive gliosis occurs after injury to the CNS [43]. In the present study, glial activation was observed with a decrease of neuronal cell size in the PFC of PCP-treated mice. Taken together with the previous reports that subchronic PCP treatment decreases the number of dendritic spine synapses in the rat PFC with astrogliosis [16], one explanation for the glial activation is that neuronal damage was occurred after repeated PCP treatment. S100, a calcium-binding protein, is expressed in astrocytes at high levels in brain lesions and used as a marker for identifying astrocytes especially in the damaged CNS [43]. Other reports have also indicated that chronic treatment with PCP activates astrocytes and damages neurons [20,23,46]. Although we did not examine the relation between glial activation and GLAST's up-regulation, glial function might

have changed during the PCP treatment. Further investigation is needed to elucidate the mechanism of activation of glia with the up-regulation of GLAST expression. Our results clearly suggest that repeated PCP treatment disrupts the pre-synaptic glutamatergic transmission and activation of CaMKII mediated via NMDA receptors.

In conclusion, our results suggest that activation of glia and up-regulation of glial transporters occurred following the neuronal damage caused by treatment with PCP for 14 days. We emphasize that this leads to altered glutamatergic functions via NMDA receptors involving pre-synaptic dysfunction in the PFC and induces emotional deficits in PCP-treated mice. Thus, PCP-treated mice are considered to be a good model with which to evaluate negative symptom-like emotional deficits and these results may support the hypothesis of glutamatergic hypofunction in schizophrenia. The study of PCP-treated mice should help us to develop medications for negative symptoms of schizophrenia.

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An Inducer for Glial Cell Line-Derived Neurotrophic Factor and Tumor Necrosis Factor–α Protects Against Methamphetamine-Induced Rewarding Effects and Sensitization

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Background: There are few efficacious medications for drug dependence. We investigated the potential of Leu-Ile, which induces the expression of glial cell line-derived neurotrophic factor (GDNF) and tumor necrosis factor-α (TNF-α), as a novel therapeutic agent for methamphetamine (METH)-induced dependence.

Methods: The levels of GDNF and TNF-α messenger RNA (mRNA) were determined by real-time reverse transcription polymerase chain reaction. Enzyme immunoassays and immunohistochemistry were employed to determine levels of these proteins. Effects of Leu-Ile on METH-induced rewarding effects and sensitization were investigated with conditioned place preference and locomotor activity tests. Extracellular dopamine (DA) levels and DA uptake into synaptosomes were examined with an in vivo microdialysis and trititated thymidine ([^{3}H]) DA uptake assay.

Results: Leu-Ile induced the expression of not only GDNF but also TNF-α. Pretreatment with Leu-Ile blocked the acquisition of METH-induced place preference and sensitization. Interestingly, post-treatment with Leu-Ile attenuated them even after their development. An inhibitory effect of Leu-Ile on METH-induced place preference was observed in neither GDNF heterozygous nor TNF-α knockout mice. Leu-Ile inhibited DA release in the nucleus accumbens and the decrease in synaptosomal DA uptake in the midbrain induced by repeated METH treatment.

Conclusions: These results suggest that Leu-Ile inhibits METH-induced rewarding effects and sensitization by regulating extracellular DA levels via the induction of GDNF and TNF-α expression.

Key Words: Dopamine (DA), glial cell line-derived neurotrophic factor (GDNF), methamphetamine (METH), rewarding effects, sensitization, tumor necrosis factor-α (TNF-α)

The abuse of substances such as psychostimulants, opiates, nicotine, and alcohol has become a significant social and public health concern worldwide. Activation of the mesocorticolimbic dopamine (DA) system has been implicated in the positive reinforcing (rewarding) effects of drugs of abuse (Robbins and Everitt 1999; Yamada and Nabeshima 2004). The psychostimulant effects of methamphetamine (METH), a typical drug of abuse, are associated with an increase in extracellular DA levels in the brain, by facilitating the release of DA from presynaptic nerve terminals and inhibiting reuptake (Giros *et al.* 1996; Heikkila *et al.* 1975; Seiden *et al.* 1993).

Neurotropic factors and cytokines, which are known to influence synaptic transmission and neuronal morphology (Boulanger and Poo 1999; Connor and Dragunow 1998; Neumann *et al.* 2002), might be involved in alterations of the morphology of dendrites and dendritic spines in the nucleus accumbens (NAc) and prefrontal cortex after repeated injection of psychostimulants (Robinson and Kolb 1997, 1999; Yamada *et al.* 2000). Glial cell line-derived neurotrophic factor (GDNF) inhibits the cocaine-induced upregulation of tyrosine hydroxylase activity in the ventral tegmental area (VTA) and blocks behavioral responses to cocaine (Messer *et al.* 2000). Furthermore, we have previously demonstrated that tumor necrosis factor-α (TNF-α) inhibits METH-induced dependence (Nakajima *et al.* 2004; Yamada and Nabeshima 2004). Taken together, GDNF and TNF-α would be candidates for therapeutic agents against drug dependence. However, there are serious obstacles to their therapeutic application: it is difficult to deliver GDNF from the periphery to the brain, because it is a macromolecule that cannot penetrate the blood-brain barrier (BBB) (Lin *et al.* 1993) and is easily broken down by proteases in the blood stream. In addition, TNF-α, an inflammatory cytokine, damages the peripheral tissues, because it triggers the expression of other cytokines (Bluthe *et al.* 1994). Therefore, GDNF and TNF-α cannot be used directly as therapeutic tools for drug dependence. We hypothesized that a low-molecular-weight compound that induces production of GDNF and TNF-α in the brain could be a novel therapeutic agent for drug dependence. Previous study has demonstrated that inflammatory stimuli such as TNF-α and lipopolysaccharide induces the synthesis of GDNF in cultured astrocytes from mouse brain (Appel *et al.* 1997). Furthermore, Leu-Ile, a hydrophobic dipeptide, induces GDNF synthesis both in vivo and in vitro (Nitta *et al.* 2004). Taken together, Leu-Ile is expected to induce the production of not only GDNF but also TNF-α and to inhibit drug dependence.

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In the present study, we examined: 1) whether Leu-Ile induces production of TNF-α, and 2) the effects of Leu-Ile on the rewarding actions and the sensitization to the locomotor-stimulating effects of METH and on the increase in extracellular DA levels and the decrease in DA uptake induced by METH.

Methods and Materials

Reagents

Glial cell line-derived neurotrophic factor and TNF-α were donated by Amgen (Thousand Oaks, California) and Dainippon Pharmaceutical (Osaka, Japan), respectively. Leu-Ile was purchased from Kokusan Chemical (Tokyo, Japan). All other materials used were of reagent grade.

Animals

Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23° ± 1°C; 50% ± 5% humidity; 12-hour light/dark cycle starting at 8:00 AM) and had ad libitum access to food and water, except during behavioral experiments. Animal care and use was in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-123, 1983) and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan. The behavioral experiment's schedule is shown in Figure 1.

The wild-type C57BL/6 mice were obtained from Slc Japan (Hamamatsu, Japan).

Male C57BL/6-GDNF heterozygous [GDNF-(±)] mice and C57BL/6-TNF-α knockout (TNF-α-[−/−]) mice, 8–12 weeks of age, were used in the experiments. The GDNF-(±) and TNF-α-(−/−) mice were generated as described previously (Nakajima *et al.* 2004; Pichel *et al.* 1996; Taniguchi *et al.* 1997); GDNF (−/−) homozygous knockout mice die shortly after birth (postnatal 7 days), but GDNF (±) mice are viable. Glial cell line-derived neurotrophic factor levels in the frontal cortex (Fc), NAc, striatum, and hippocampus (Hip) of GDNF-(±) mice are 54.8%, 65.4%, 59.0%, and 66.8 %, respectively, of those in littermate GDNF-(+/+) mice (Table 1). Littermate GDNF-(+/+) mice were used as control subjects in the behavioral experiments.

Drug Treatment

Mice were administered Leu-Ile (1.5 and 15 μmol/kg, IP) once/day 1 hour before METH (1 mg/kg, SC) treatment for 9 days. In the withdrawal experiment, mice were administered Leu-Ile or vehicle for 5 days after the withdrawal from METH after 9 successive days of METH administration. To determine trititated thymidine ([^{3}H]) DA uptake, messenger RNA (mRNA) expression, and protein levels, mice were decapitated 1, 2, and 24 hours after the last METH injection, respectively.

Enzyme Immunoassay of GDNF

Glial cell line-derived neurotrophic factor levels were measured with an enzyme immunoassay (EIA) with a minor modification (Nitta *et al.* 1999a, 1999b, 2004). Homogenate buffer (.1 mol/L Tris-HCl [pH 7.4] containing 1 mol/L sodium chloride (NaCl), 2% bovine serum albumin, 2 mmol/L ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), and .2% sodium nitride [Na3N]) was added to brain tissue at a ratio of 1 g wet weight / 19 mL of buffer, pulse-sonicated for 100 sec, and centrifuged at 100,000 *g* for 30 min. The supernatant was collected and used for the EIA.

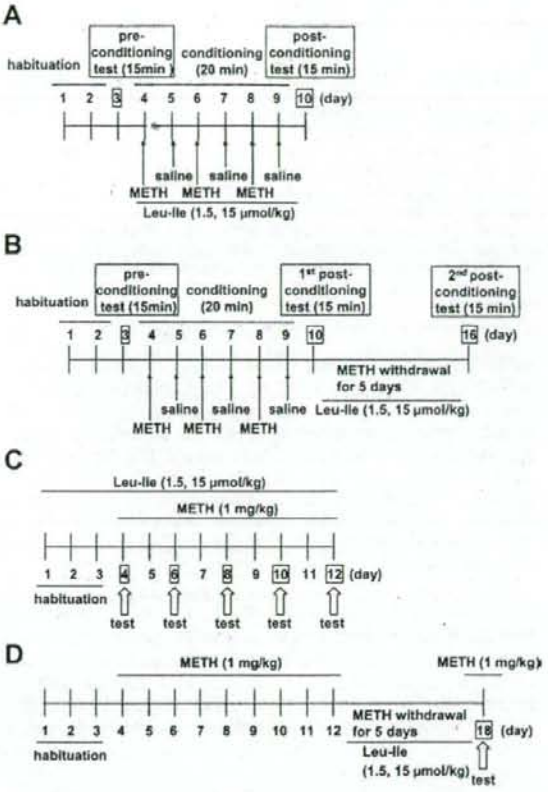

Figure 1. Experimental schedules. **(A)** Experimental schedule for the conditioned place preference task. Mice were co-treated with Leu-Ile and methamphetamine (METH) in the conditioning period. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before receiving METH (1 mg/kg, SC) or saline. Closed arrows indicate the days of METH or saline injection. **(B)** Experimental schedule for the conditioned place preference task to investigate the effects of Leu-Ile after the withdrawal from METH. Mice were not treated with Leu-Ile in the conditioning period. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days after withdrawal from METH. **(C)** Experimental schedule for measurement of locomotor activity. Mice were treated with Leu-Ile during the habituation period for 3 days and then co-treated with Leu-Ile and METH for 9 days. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, IP) 1 hour before the METH (1 mg/kg, SC) injection. Locomotor activity was measured for 2 hours after the METH treatment. Open arrows indicate the days when locomotor activity was measured. **(D)** Experimental schedule for measurement of locomotor activity to investigate the effects of Leu-Ile after the withdrawal from METH. Mice were treated with Leu-Ile after the establishment of METH (1 mg/kg, SC)-induced sensitization: mice were treated with METH for 9 days and then with Leu-Ile (1.5 and 15 μmol/kg, IP) for 5 days without METH. On day 18, mice were administered only METH (1 mg/kg, SC), and locomotor activity was measured for 2 hours after the METH treatment.

Semi-Quantative mRNA Analysis by Real-Time Reverse Transcription Polymerase Chain Reaction

Corti-hippocampal neurons from 18-day-old rat embryos were cultured as previously described (Nitta *et al.* 1999a, 1999b). More than 95% of the cells were positive for microtuble-associated protein-2 (MAP2) immunoreactivity. Corti-hippocampal neurons of 18-day-old rat embryos were cultured in serum-free

Table 1. The Difference of GDNF Levels in the Brain (Fc, NAc, Str, Hip) Between GDNF-(+/+) and GDNF-(+/−) Mice

Brain Region	GDNF Levels (pg/g Wet Tissue)		
	GDNF-(+/+) Mice	GDNF-(+/−) Mice	% of GDNF-(+/+) Mice
Fc	1285.0 ± 254.3	704.0 ± 26.4*	54.8
NAc	1253.4 ± 58.5	820.2 ± 58.4*	65.4
Str	2083.1 ± 231.0	1229.4 ± 178.2*	59.0
Hip	782.2 ± 99.9	522.5 ± 20.9*	66.8

Mice were decapitated without any treatment, and the brains were quickly removed. Glial cell line-derived neurotrophic factor (GDNF) levels were measured using an enzyme immunoassay. Values are means ± SE ($n = 10$).

Fc, frontal cortex; Hip, hippocampus; NAc, nucleus accumbens; Str, striatum.

*$p < .05$ versus GDNF-(+/+) mice.

defined medium containing Leu-Ile (.037, .37, 3.7, and 37 μg/mL) or TNF-α (2, 20, 100, and 200 ng/mL). Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted into complementary DNA with a SuperScriptTM First-Strand System for RT-PCR Kit (Invitrogen Life Technologies, Carlsbad, California). The rat GDNF primers used were as follows: 5'-AGCTGCCAGCCCAGAGAATT-3' (forward) and 5'-GCACCCCCGATTTTTGC-3' (reverse), and TaqMan probe: 5'-CAGAGGGAAAGGTCGCAGAGGCC-3'. The rat TNF-α primers used were as follows: 5'-ATTTGGCCCCATCCTTCC-3' (forward) and 5'-GCCTCCATGGCAGAGCC-3' (reverse), and TaqMan probe: 5'-TCCCAGGACATCAGGACTCTGTTCCC-3'. The 18S ribosomal RNA Kit was used as the internal control (PE Applied Biosystems, Foster, California). The amplification consisted of an initial step (50°C for 2 min and 95°C for 2 min) and then 40 cycles of denaturation for 15 sec at 95°C and annealing for 1 min at 60°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Tokyo, Japan). The expression levels were calculated as described previously (Wada *et al.* 2000).

Immunohistochemical Analysis

Polyclonal rabbit anti-GDNF (1:50; sc-328; Santa Cruz Biotechnology, Santa Cruz, California), polyclonal goat anti-TNF-α (1:100; R&D Systems, Minneapolis, Minnesota), monoclonal mouse anti-MAP2 (1:200; Sigma-Aldrich, Saint Louis, Missouri), and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:200, Chemicon International, Temecula, California) served as primary antibodies. Goat anti-rabbit Alexa Fluor 546 (1:1000, Molecular Probes, Eugene, Oregon) and goat anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes) served as secondary antibodies for GDNF immunostaining. Donkey anti-goat Alexa Fluor 546 (1:1000, Molecular Probes) and rabbit anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes) served as secondary antibodies for TNF-α immunostaining. Each stained tissue was observed under a fluorescence microscope (Axioskoop 2 plus: Carl Zeiss, Jena, Germany) and analyzed with Axiovision 3.0 systems (Carl Zeiss). The area with TNF-α-positive cells in the defined NAc region of mice was determined with the software WinROOF (Mitani, Fukui, Japan) (Kuwahara *et al.* 1999; Tsuji *et al.* 1999). We employed an immunostaining method with which one can analyze the distribution and levels of TNF-α protein in the present investigation, because it is too difficult to use Western blotting or an enzyme immunoassay to quantify the amount of TNF-α protein in brain tissue.

Measurement of the TNF-α Concentration in Peripheral Blood

Blood was collected into tubes containing 5% EDTA 1, 2, and 4 hours after the injection of TNF-α (4 μg/body, IP). Mice were treated with Leu-Ile (1.5 μmol/kg, IP), and their blood was collected into tubes containing 5% EDTA 0, 1, 2, 4, and 8 hours after the injection. The blood samples were centrifuged at 2000 × *g* for 20 min at 4°C. The supernatants were taken as the samples. The TNF-α concentration was assessed by using a specific human (QuantiGlo QTA00, R&D Systems) or mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Quantikine MTA00, R&D Systems), according to the instructions provided.

Behavioral Tests

Conditioned Place Preference. The place conditioning paradigm was performed by using previously established procedure with a minor modification (Nagai *et al.* 2004; Nakajima *et al.* 2004; Noda *et al.* 1998). The experimental schedule for the conditioned place preference (CPP) task is shown in Figures 1A and 1B. The mouse was allowed to move freely between transparent and black boxes for 15 min once/day for 3 days (days 1–3) in the preconditioning. On day 3, the time the mouse spent in each box was measured. On days 4, 6, and 8, the mouse was treated with METH and confined in either the transparent or black box for 20 min. On days 5, 7, and 9, the mouse was given saline and placed opposite the METH-conditioning box for 20 min. On day 10, the postconditioning test was performed without drug treatment, and the time the mouse spent in each box was measured for 15 min.

Locomotor Activity. Locomotor activity was measured with an infrared detector (Neuroscience, Tokyo, Japan) in a plastic box (32 × 22 × 15 cm high). Mice were administered METH (1 mg/kg, SC) or saline 1 hour after the Leu-Ile treatment, and the locomotor activity was measured for 2 hours immediately after the METH or saline administration (Figure 1C). In the withdrawal experiment, mice were administered Leu-Ile or vehicle for 5 days after the withdrawal from METH (days 13–17) after 9 successive days of METH administration. On day 18, the mice were administered only METH (1 mg/kg, SC), and locomotor activity was measured for 2 hours immediately after the administration (Figure 1D).

In Vivo Microdialysis

Mice were anesthetized with sodium pentobarbital, and a guide cannula (AG-8, EICOM, Kyoto, Japan) was implanted into the NAc (+1.1 mm anteroposterior, +1.0 mm mediolateral from bregma, and −4.0 mm dorsoventral to dura) according to the atlas of Franklin and Paxinos (1997) and secured to the skull with stainless steel screws and dental acrylic cement. One day after the operation, a dialysis probe (AI-8-1; 1-mm membrane length, EICOM) was inserted through the guide cannula and perfused continuously with artificial cerebrospinal fluid (aCSF; 147 mmol/L NaCl, 4 mmol/L potassium chloride, and 2.3 mmol/L calcium dichloride) at a rate of 1.0 μL/min. Dialysate was collected in 20-min fractions and injected into the HPLC system (EICOM) for the measurement of DA levels. Three samples were used to establish baseline levels of extracellular DA before the administration of Leu-Ile and METH.

Synaptosomal [^{3}H] DA Uptake

Midbrain synaptosomal [^{3}H] DA uptake was determined as previously described (Fleckenstein *et al.* 1997; Nakajima *et al.* 2004). Samples were incubated at 37°C for 4 min, and then ice-cold Krebs-Ringer's solution containing 10 μmol/L GBR12909, a specific DA uptake inhibitor, was added. Nonspecific values were determined in the presence of 100 μmol/L GBR12909. The radioactivity trapped on Whatman GF/B filters (Brandel, Gaith-