Role of tyrosine kinase-dependent phosphorylation of NR2B subunit-containing NMDA receptor in morphine reward

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Hideaki Kato

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This dissertation is dedicated to my parents, my sister, my wife and my son.

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Abbreviations

Chemical substances and Drugs

AMPA: α-Amino-3-hydroxy-5-methylisoxazole propionic acid

DMSO: Dimethyl sulfoxide

DNQX: 6,7-Dinitroquinoxaline-2,3-dione

EDTA: Ethylendiaminetetraacetic acid

EGTA: Ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid

Ifenprodil tartrate: (1*RS*,2*SR*)-4-[2-(4-Benzylpiperidin-1-yl)-1-hydroxypropyl]phenol hemi-(2*R*,3*R*)-tartrate

Ketamine: (*R*,*S*)-2-(2-Chlorophenyl)-2-methylaminocyclohexanone monohydrochloride

MK-801 (Dizocilpine maleate): (5*R*,10*S*)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[*a*,*d*] cyclohepten-5,10-imine hydrogen maleate

MPEP: 2-Methyl-6-(phenylethynyl)pyridine hydrochloride

Morphine hydrochloride: (5*R*,6*S*)-7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol monohydrochloride

NMDA: *N*-Methyl-D-aspartate

PP2: 4-Amino-5-(4-chlorophenyl)-(*t*-butyl)pyrazolo[3,4-D]pyrimidine

Ro25-6981: (*R*,*S*)-Alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperidinepropanol hydrochloride

SDS: Sodium dodecyl sulfate

Brain regions

N.Acc.: Nucleus accumbens

VTA: Ventral tegmental area

Receptors and transporters

DAT: Dopamine transporter

mGluR: Metabotropic glutamate receptor

Buffers and mediums

DMEM: Dulbecco's modified Eagle's medium

PBS: Phosphate-buffered saline

TBS: Tris-buffered saline

TTBS: TBS containing 0.1% Tween 20

Endogeneous substances

GFAP: Glial fibrillary acidic protein

IgG: Immunoglobulin G

Enzymes and intracellular messengers

PKC: Protein kinase C

Serum

BSA: Bovine serum albumin

NHS: Normal horse serum

Injection routes

i.c.v.: Intracerebroventricular

i.p.: Intraperitoneal

s.c.: Subcutaneous

Others

ANOVA: Analysis of variance

$[Ca^{2+}]i$: Intracellular calcium concentration

CNS: Central nerveous system

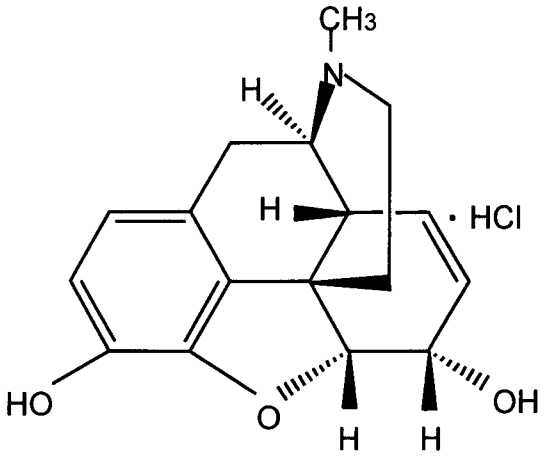
CPP: Conditioned place preference

LTP: Long-term potentiation

PCP: Phencyclidine

Structures of drugs used in the present study

Morphine hydrochloride:



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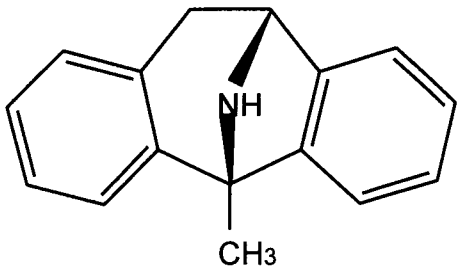

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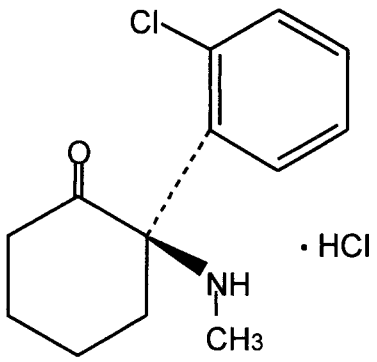

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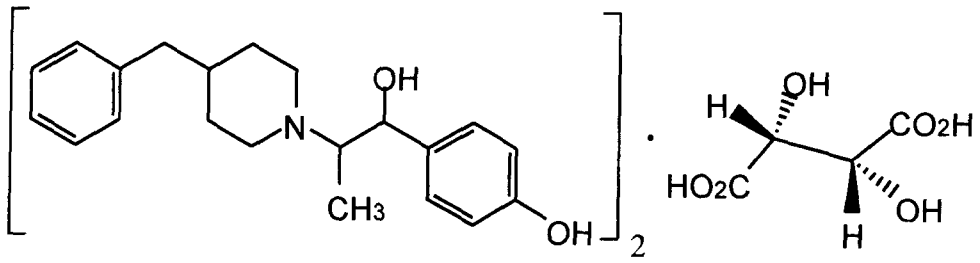

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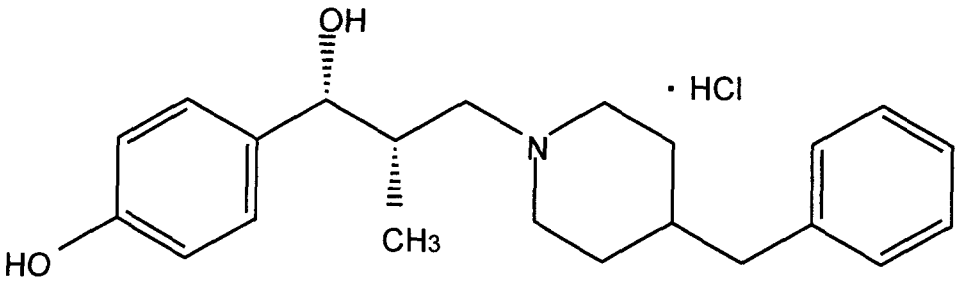
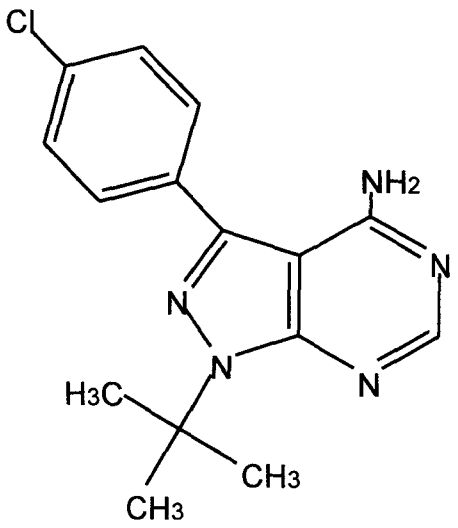


PP2:



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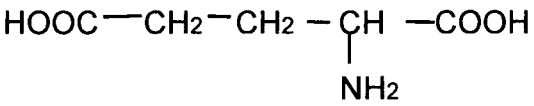

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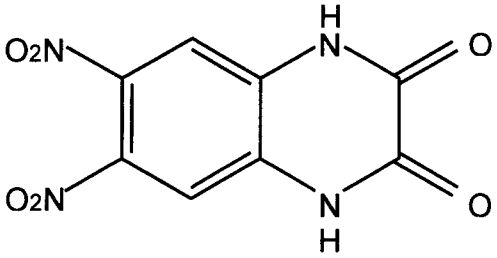

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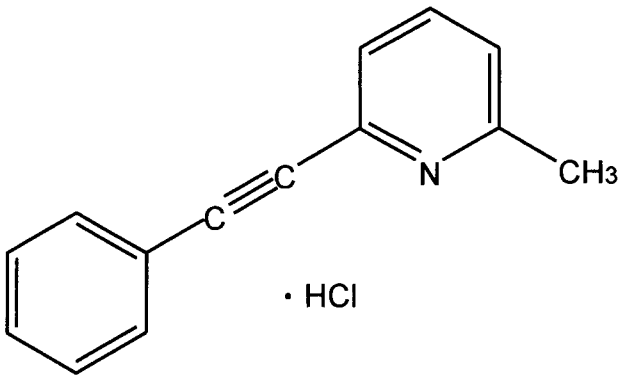

General Introduction

Rewarding property of usefulness of "conditioned place preference" paradigm

Morphine is the prototypical μ-opioid receptor agonist that serves as the standard analgesic for the severe pain. However, repeated morphine administration produces undesirable effects such as psychological dependence. Three main types of procedures have been used to assess the rewarding properties of morphine in animals: drug self-administration, intracranial electrical self-stimulation and conditioned place preference (CPP). The CPP procedure is used to evaluate motivational properties, such as rewarding or aversive properties of drugs. Several investigators have demonstrated that the rewarding effects of addictive drugs, such as morphine, methamphetamine and cocaine, can be evaluated by the CPP paradigm [1-3]. The CPP paradigm has become the most frequently used method to assess drug-induced motivational effects. In this procedure, the association that develops between the presentation of a drug and a previously conditioned stimulus (e.g., differently colored compartments of a shuttle-box) is evaluated. Several of the advantages of the CPP paradigm have been discussed by Carr et al. [4]. First, the rewarding effects of drugs are measured in animals that drug-free. Thus, any changes in unconditioned motor activity do not directly influence the development variable. Second, in general, the rewarding effects of drugs can be assessed more quickly than with other operant paradigms such as self-administration. The CPP paradigm is therefore now more frequently used than the self-administration paradigm. In addition to providing information on the rewarding properties of drugs, the

CPP paradigm also enables detection of aversive properties of drugs. Several investigators have demonstrated that the reinforcing effects of a typical μ-opioid receptor agonist morphine can be evaluated by the CPP paradigm [1-3].

Major pathway of morphine reward

Brain dopamine systems have been the focus of histochemical, biochemical, and pharmacological research into psychological dependence on opioids, such as morphine. A growing body of evidence indicates that the mesolimbic dopaminergic pathway projecting from the ventral tegmental area (VTA) to the nucleus accumbens (N.Acc.) plays a critical role in the initiation of psychological dependence on morphine. As well as dopaminergic system, the involvement of non-dopaminergic neurotransmitter and neuromodulator systems in rewarding effect induced by morphine has been recently documented. Of these systems, a role for glutamatergic system, especially *N*-methyl-D-aspartate (NMDA) receptors, in opioid rewarding effects can be proposed [3].

Implication of glutamate receptors in the development of morphine reward

Glutamate receptors have been divided into NMDA and non-NMDA (α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) and kainate) receptors based on their pharmacological properties. The molecular cloning of NMDA receptor has identified a variety of NMDA receptor subunits, common NR1 subunit and four distinct NR2 (NR2A, NR2B, NR2C and NR2D) subunits. The NMDA receptor is a heteromeric ligand-gated ion channel that interacts with multiple intracellular proteins. The

obligatory NR1 subunits form ion channels and multiple NR2 subunits regulate the properties of the channel [5]. Interestingly, recent behavioral studies have revealed that NMDA receptors are involved in the development of rewarding effect caused by chronic administration of morphine.

Regulation of NMDA receptors by tyrosine kinases

It is well documented that tyrosine kinases increase the NMDA receptor phosphorylation and potentiate NMDA receptor function [6]. The function of these receptors is regulated by a series of phosphorylation and dephosphorylation processes mediated by protein kinases and protein phosphatases, respectively [6,7]. Numerous studies have demonstrated the important role of protein tyrosine kinases, such as tyrosine kinases of Src family, in the regulation of NMDA receptors [8]. Several protein tyrosine kinases have been shown to phosphorylate NR2 subunits at their tyrosine residues [9,10] and may potentiate NMDA receptor function both by modulation of the channel gating and by an increase in the number of NMDA receptors on the neural cell surface [11,12].

Increased Ca^{2+} influx through NMDA receptors

The NMDA receptor is a subclass of ionotropic glutamate receptors in the mammalian brain. The NMDA receptors exhibit several channel properties distinct from other non-NMDA glutamate receptors, including a high Ca^{2+}/Na^{+} permeability ratio, a voltage-dependent Mg^{2+} block, and a requirement for glycine as co-agonist [13-16]. Ca^{2+}

influx through NMDA receptors plays a key role in synaptic transmission, neuronal development, and plasticity. In addition, a recent new insight into the physiology of neuron/glia has emerged, leading to a different view of its role in the central nervous system (CNS), i.e., "active regulation of neuronal function". The glutamate-evoked increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in cultured neuron/glial cocultures and purified astrocytes can be evaluated using a Ca^{2+} imaging technique.

Recent approach for drug addiction: possible role of astrocytes

Glial cells have important physiological properties as they relate to CNS homeostasis [17]. Astrocytes are a subpopulation of glial cells in the CNS. They have traditionally been considered to be structural elements within the CNS with the main function of maintaining nerve tissue. Astrocytes have a large variety of receptors for neurotransmitters and hormones, including glutamate receptors and dopamine receptors, which are coupled to various intracellular signaling cascades [18-20]. They undergo a process of proliferation, morphological changes, and enhancement of glial fibrillary acidic protein (GFAP) expression, which has been referred to as the activation of astrocytes or astrogliosis [21-23]. Glutamate is major fast excitatory neurotransmitter in the CNS, and glutamate receptors have been shown to play important role in synaptic plasticity such as long-term potentiation (LTP), which is thought to underlie physiopathological phenomena, neural development, synaptic plasticity and synaptogenesis. Therefore, it is likely that the long-lasting synaptic adaptations in glutamatergic neurotransmission provide a neural framework for altered behavioral

processing underlying the development of psychological dependence on drugs of abuse.

Aim and Scope

The main aim of the present study was to investigate the change in tyrosine phosphorylation of NR2B subunit-containing NMDA receptor on the morphine-induced rewarding effect. In my experimental approach, behavioral, neurochemical and biochemical analysis were employed.

The specific aims of the proposed research are:

In Chapter 1:

The present study was undertaken to clarify the role of NMDA receptor including NR2B subunit-containing NMDA receptor in the morphine-induced rewarding effect using the conditioned place preference paradigm. Additionally the availability of ifenprodil as adjuvant analgesic of morphine was examined. The present study focused on the effect of NR2B subunit-containing NMDA receptor antagonist ifenprodil on morphine-induced antinociception.

In Chapter 2:

The present study was aimed to clarify the role of tyrosine kinases of Src family, which regulate the NR2B subunit-containing NMDA receptors, in the rewarding effect induced by morphine. I also investigated the changes in immunoreactivities to phospho-

Tyr-416 Src family kinases, which are known to active form of Src family kinases, in the N.Acc. of mice shown the morphine-induced rewarding effect.

In Chapter 3:

The final chapter was designed to determine the role of NMDA receptors in glutamate-evoked Ca^{2+} influx into neurons and astrocytes. Furthermore, I investigated whether NR2B subunit-containing NMDA receptor antagonists could suppress the astrocytic activation, as detected by GFAP (as a specific marker of astrocyte)-like immunoreactivities in the mouse cortical astrocyte.

Ethics

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study.

Chapter 1

Effects of NMDA receptor antagonists on the morphine-induced rewarding effect and antinociception in mice

Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Glutamate receptors have been divided into *N*-methyl-D-aspartate (NMDA), non-NMDA (α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) and kainite and G-protein-coupled metabotoropic glutamate receptors (mGluRs) based on their pharmacological and physiological properties [14,15,24,25]. It has been reported that ketamine, an antagonist of non-competitive NMDA receptor, which is one of the principal excitatory amino acid receptors in the CNS [26], is useful as an adjunctive medication for cancer pain, especially neuropathic pain for which morphine is ineffective. Ketamine is an intravenous or intramuscular anesthetic that produces profound analgesia in human and animals [27-30], and has been used clinically for analgesia and anesthesia. However, there are some problems associated with its use, since ketamine has some side effects. The chronic use of ketamine has been somewhat limited by its psychotomimetic action and psychological dependence at moderate and high doses [31-34]. Moreover, various side effects, including perceptual alterations, vivid dreams, and delirium, are associated with waking from ketamine anesthesia [34-36]. Ketamine has been shown to inhibit NMDA receptors in a non-competitive manner [37,38]. Some actions of ketamine are related to the blockade of NMDA receptors. NMDA receptor antagonists such as phencyclidine (PCP) and MK-801 (dizocilpine maleate) have been shown to produce a significant place preference using the conditioned place preference (CPP) paradigm in animals [39]. The CPP paradigm is now commonly used to

study the rewarding properties of various drugs. However, there were no reports on the ketamine-induced rewarding effect using the CPP paradigm. In the present study, therefore, I investigated the rewarding effects of non-competitive NMDA receptor antagonists, such as ketamine and MK-801.

Although morphine is a potent analgesic that is widely used for the relief of cancer pain, it produces psychological dependence. It has been reported that the NMDA receptor is involved in the development of antinociceptive tolerance and physical dependence induced by chronic administration of morphine [40]. A variety of NMDA receptor subunits have been revealed by molecular cloning studies. There are two families of NMDA receptor subunits, NR1 and NR2 (A, B, C and D), and the expression of NR1 along with different NR2 subunits yields receptors with distinct pharmacological characteristics. Non-competitive NMDA receptor antagonists, such as ketamine, MK-801 and memantine, show high affinity for recombinant heteromeric NR1/NR2A and NR1/NR2B subunits-containing NMDA receptors [41]. Several lines of evidence have suggested that ifenprodil, which is clinical used, selectively inhibits NR2B subunit-containing NMDA receptors [41,42]. Thus, ifenprodil may be a useful tool for elucidating the role of NR2B subunit-containing NMDA receptors. In the present study, I examined the effects of NMDA receptor antagonists, especially a selective NR2B subunit-containing NMDA receptor antagonist ifenproodil, on the morphine-induced rewarding effect in mice. Furthermore, I investigated the effect of a selective NR2B subunit-containing NMDA receptor antagonist ifenproodil on the morphine-induced antinociception in mice.

Experiment 1-1

Effects of NMDA receptor antagonists, such as ketamine, MK-801 and ifenprodil on the morphine-induced rewarding effect in mice

Materials and Methods

Animals

Male ddY mice (Tokyo Experimental Animals Co. Ltd., Tokyo, Japan), weighting 20-30 g, were housed in a temperature-controlled room (23±1°C). The animals were maintained on a 12-hr light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum during the experimental period.

Place conditioning

Place conditioning studies were conducted using the apparatus consisted of a shuttle box (15 x 30 x 15 cm, w x l x h), which was made of an acrylic resin board and divided into two equal-sized compartments. One compartment was white with a texture floor, and the other was black with a smooth floor to create equally inviting compartments. Only animals that did not exhibit a significant preference for either the white or black compartment were used and divided randomly into each separate group of 8-16 mice. For conditioning, mice were confined to one compartment after drug injection and to the other compartment after saline injection. Conditioning sessions (3

days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after subcutaneous (s.c.) injection of morphine (1-5 mg/kg), animals were placed in the other compartment for 1 hr. On alternate days, animals receiving with saline were placed in the other compartment for 1 hr. On day 7, tests of conditioning were performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900 sec session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusho Co., Tokyo, Japan). All sessions were conducted under conditions of dim illumination (28 lux lamp) and white noise. Morphine (1-5 mg/kg, s.c.) and NMDA receptor antagonists (ketamine; 1-10 mg/kg, MK-801; 0.2 mg/kg, ifenprodil; 5-20 mg/kg, i.p.) were injected on alternate days. In the combination study, mice were pretreated with NMDA receptor antagonists (ketamine; 1-10 mg/kg, MK-801; 0.1 and 0.2 mg/kg, ifenprodil; 5-20 mg/kg, i.p.) 30 min before each morphine (5 mg/kg, s.c.) injection.

Drugs

The drugs used in the present study were morphine hydrochloride (Sankyo Co. Ltd., Tokyo, Japan), ketamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), (5*R*,10*S*)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) (dizocilpine maleate; Merck/Banyu, Tokyo, Japan) and

ifenprodil tartrate (Grelan Pharmaceutical Co. Ltd., Tokyo, Japan). Ifenprodil tartrate was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Co. Ltd., Tokyo, Japan) and diluted in 5% DMSO with 9% Tween 80/saline before use. Other drugs were dissolved in saline. All drugs were then injected in a volume of 10 mL/kg.

Data analysis

All data are expressed as mean ± S.E.M. The statistical significance of differences between groups was assessed with an analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test or Student's *t*-test.

Results

Morphine-induced place preference

The place preference induced by morphine is shown in Fig. 1. The saline-conditioning control mice exhibited no preference for either compartment of the box. Morphine (1-5 mg/kg, s.c.) caused a dose-related place preference ($F_{3,59} = 4.13$, $p<0.01$), and a significant place preference was observed at all doses (1, 3 and 5 mg/kg; $p<0.01$).

Ketamine- or MK-801-induced place preference

As shown in Fig. 2A and B, the saline-conditioning control mice exhibited no preference for either compartment of the box. MK-801 (0.2 mg/kg, i.p.) caused a significant place preference ($p<0.05$). Ketamine (3 and 10 mg/kg, i.p.) also caused a significant place preference ($p<0.05$).

Effects of ketamine and MK-801 on the morphine-induced place preference

Morphine (5 mg/kg, s.c.)-induced place preference was suppressed by pretreatment with MK-801 (0.1 and 0.2 mg/kg, i.p.; $p<0.01$) (Fig. 3A). Furthermore, morphine-induced place preference was also suppressed by pretreatment with ketamine (10 mg/kg, i.p.; $p<0.01$) (Fig. 3B).

Effect of ifenprodil on the morphine-induced place preference

As shown in Fig. 4A, vehicle exhibited no preference for either compartment of the

box. Ifenprodil (5-20 mg/kg, i.p.) alone produce a neither preference nor aversion for the ifenprodil-associated place. The morphine (5 mg/kg, s.c.)-induced place preference was suppressed by pretreatment with ifenprodil in a dose-dependent manner ($F_{3,44}$ = 4.26 $p<0.01$, 5 mg/kg; $p<0.05$, 10 and 20 mg/kg; $p<0.01$) (Fig. 4B).

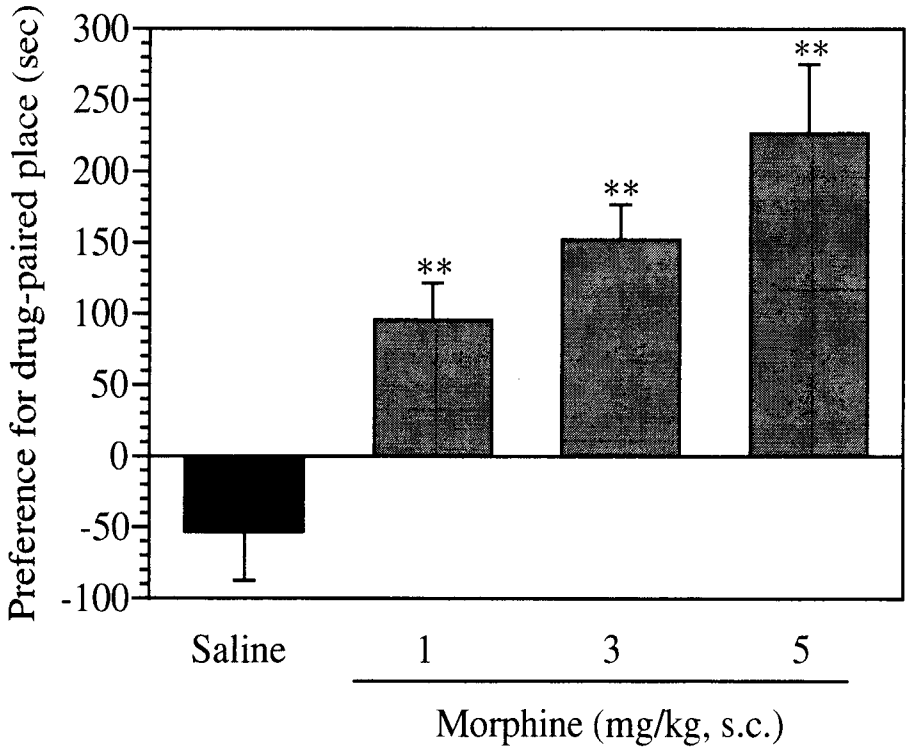

Fig. 1 Place preference induced by morphine (1, 3 and 5 mg/kg, s.c.) in mice. Ordinate: mean difference(s) between times spent on drug- and saline-paired sides of the test box. Each column represents the mean with S.E.M. of 15-16 mice.
**$p<0.01$ vs. saline-conditioning control group.

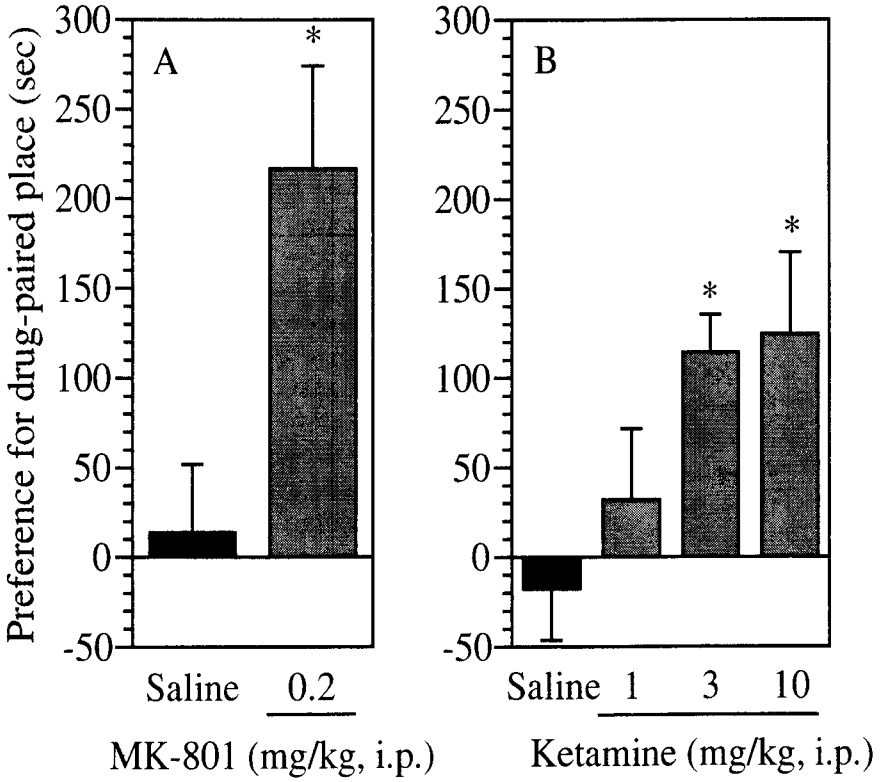

Fig. 2 Place preference induced by (A) MK-801 (0.2 mg/kg, i.p.) and (B) ketamine (1, 3 and 10 mg/kg) in mice. Ordinate: mean difference(s) between times spent on drug- or saline-paired sides of the test box. Each column represents the mean with S.E.M. of 8 mice. *$p<0.05$ vs. saline-conditioning control group.

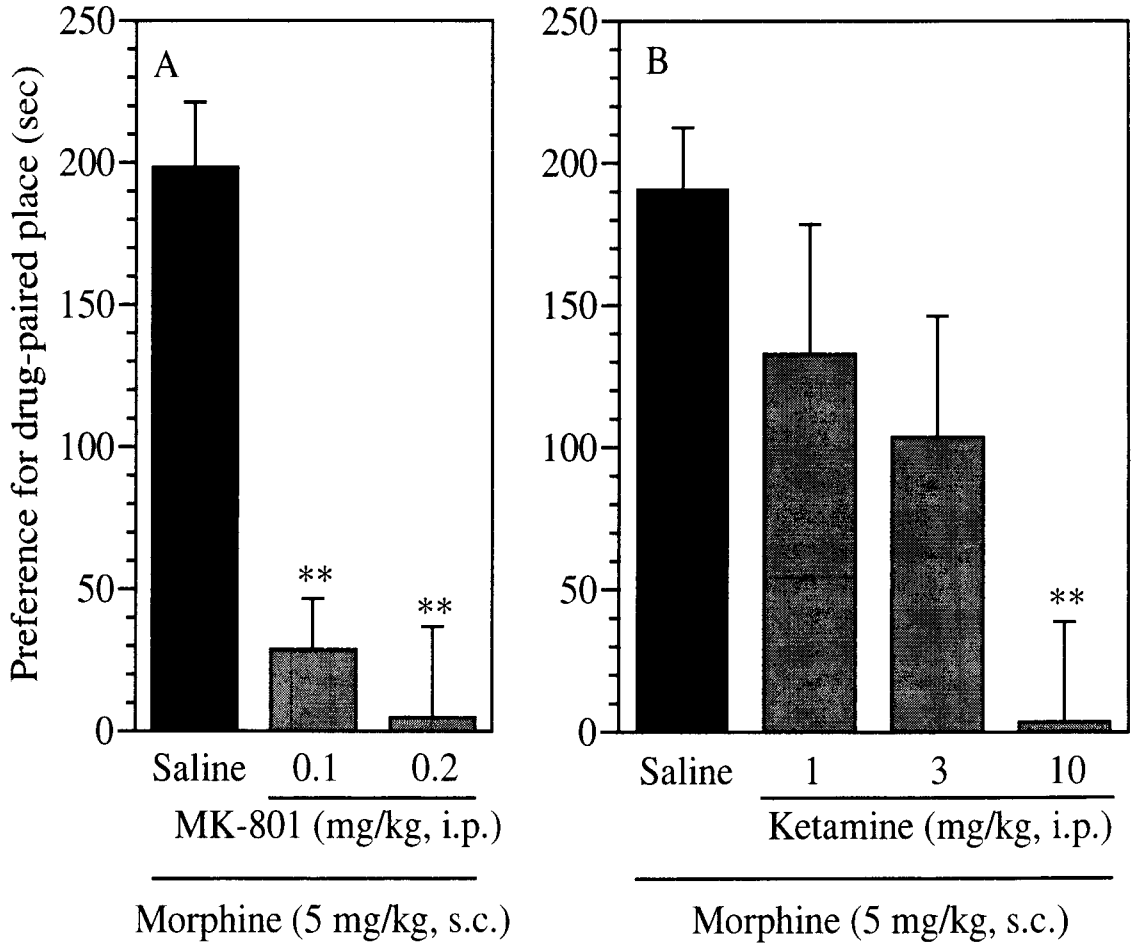

Fig. 3 Effect of pretreatment with (A) MK-801 (0.1 and 0.2 mg/kg, i.p.) or (B) ketamine (1, 3 and 10 mg/kg, i.p) on the place preference induced by morphine (5 mg/kg, s.c.) in mice. Mice were pretreated with ketamine or MK-801 30 min prior to each morphine injection. Each column represents the mean with S.E.M. of 8 mice.
**$p<0.01$ vs. morphine alone-conditioning group.

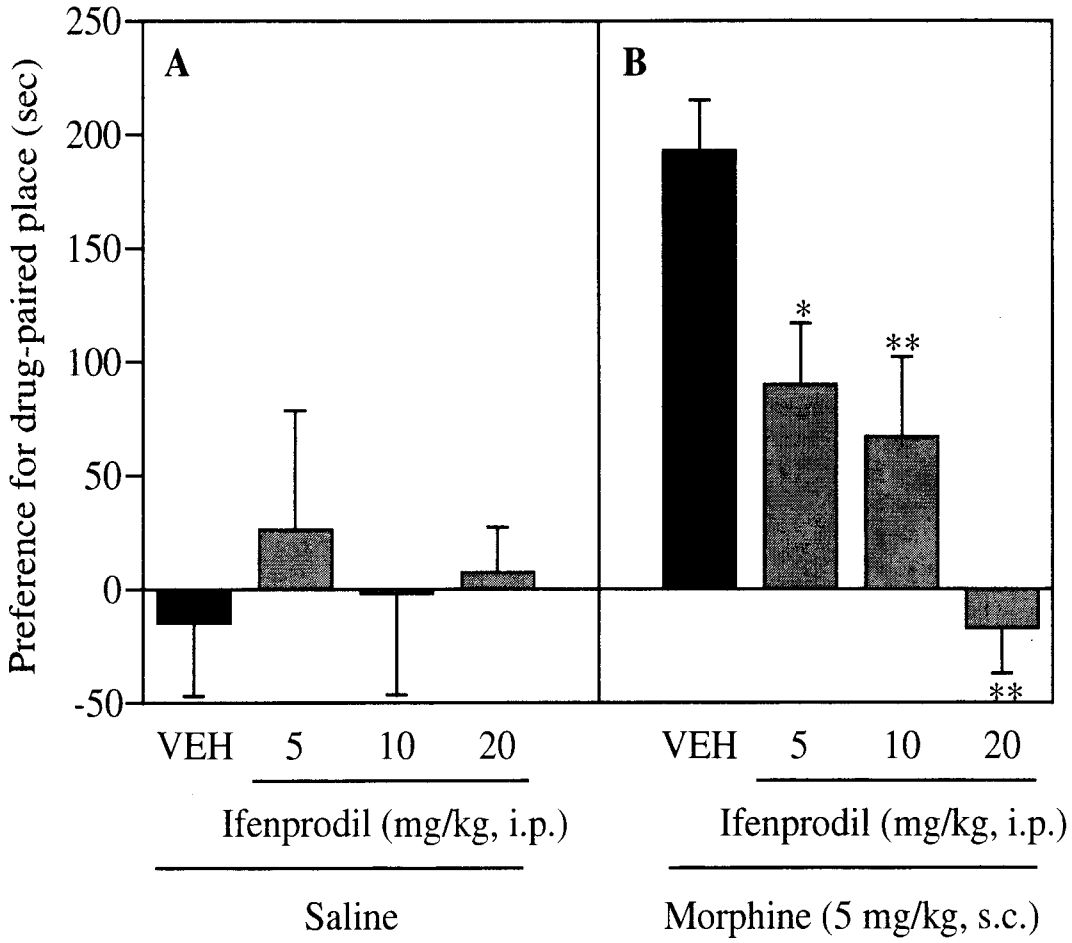

Fig. 4 Effect of pretreatment with ifenprodil (5, 10 and 20 mg/kg, i.p.) on the place preference induced by morphine (5 mg/kg, s.c.) in mice. Mice were pretreated with vehicle (VEH) or ifenprodil 30 min prior to saline (A) or morphine (B) injection. Each column represents the mean with S.E.M. of 8 (A) and 16 (B) mice.

* $p<0.05$, ** $p<0.01$ vs. morphine alone-conditioning control group.

Experiment 1-2

Effect of NMDA receptor antagonist ifenprodil on the morphine-induced antinociception in mice

Materials and Methods

Animals

Male ddY mice (Tokyo Experimental Animals Co. Ltd., Tokyo, Japan), weighting 20-30 g, were housed in a temperature-controlled room (23±1°C). The animals were maintained on a 12-hr light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum during the experimental period.

Assessment of antinociception

Formalin test

Formalin (2%, 20 μL) was injected into the plantar surface of the right mouse hindpaw. The mice were observed for 30 min and the time that the mice spent licking the injected right hindpaw was recorded. The nociceptive scores normally peaked 0 to 10 min after formalin injection (early phase) and 10 to 30 min (late phase) after the injection, representing the neurogenic and inflammatory pain response, respectively. The time spent licking the injected paw was recorded and data were expressed as total licking time in the early phase and late phase. Mice were treated with MK-801 (0.025

and 0.05 mg/kg, i.p.) or ifenprodil (10 and 20 mg/kg, i.p.) 30 min before formalin injection.

Tail-flick test

Morphine-induced antinociceptive response was determined with tail-flick test [43]. For measurement of the latency of the tail-flick response, mice were gently held with one hand with the tail positioned in the apparatus (model MK330B; Muromachi kikai Co., Tokyo, Japan) for radiant heat stimulation. The tail-flick response was elicited by applying radiant heat to the dorsal surface of the tail. The intensity of the heat stimulus in the tail-flick test was adjusted that the animal flicked its tail within 3 to 5 sec. The latency of the tail-flick responses was measured before (T_0) and at various times after (T_1) s.c. injection of morphine. The inhibition of the tail-flick responses to morphine was expressed as a percentage of the maximum possible effect (% antinociception), which was calculated as $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where the cut-off time, T_2, was set at 15 sec for the tail-flick response. Morphine was administered immediately after vehicle or ifenprodil injection. The antinociceptive effect of morphine (3 mg/kg, s.c.) was measured for 120 min after drug injection.

Drugs

The drugs used in the present study were morphine hydrochloride (Sankyo Co. Ltd., Tokyo, Japan), MK-801 (dizocilpine maleate; Merck/Banyu, Tokyo, Japan), formalin (Wako Pure Chemical Co. Ltd., Tokyo, Japan) and ifenprodil tartrate (Grelan

Pharmaceutical Co. Ltd., Tokyo, Japan). Ifenprodil tartrate was dissolved in DMSO (Wako Pure Chemical Co. Ltd., Tokyo, Japan) and diluted in 5% DMSO with 9% Tween 80/saline before use. Formalin was diluted in saline. Other drugs were dissolved in saline. Formalin was injected in a volume of 20 μL. Other drugs were then injected in a volume of 10 mL/kg.

Data analysis

All data are expressed as mean ± S.E.M or mean alone. The statistical significance of differences between groups was assessed with an ANOVA followed by the Dunnett's multiple comparison test or Student's *t*-test.

Results

Effects of MK-801 and ifenprodil on the formalin-induced nociceptive response in mice

The nociceptive response induced by formalin is shown in Fig. 5A and B. Formalin-induced nociceptive responses were significantly suppressed by pretreatment with MK-801 and ifenprodil at the early and late phases.

Effect of ifenprodil on the morphine-induced antinociception in mice

The antinociception of morphine is shown in Fig 6. The morphine (3 mg/kg, s.c.)-induced antinociception was significantly increased by co-treatment ifenprodil (10, 20 and 30 mg/kg, i.p.) with morphine.

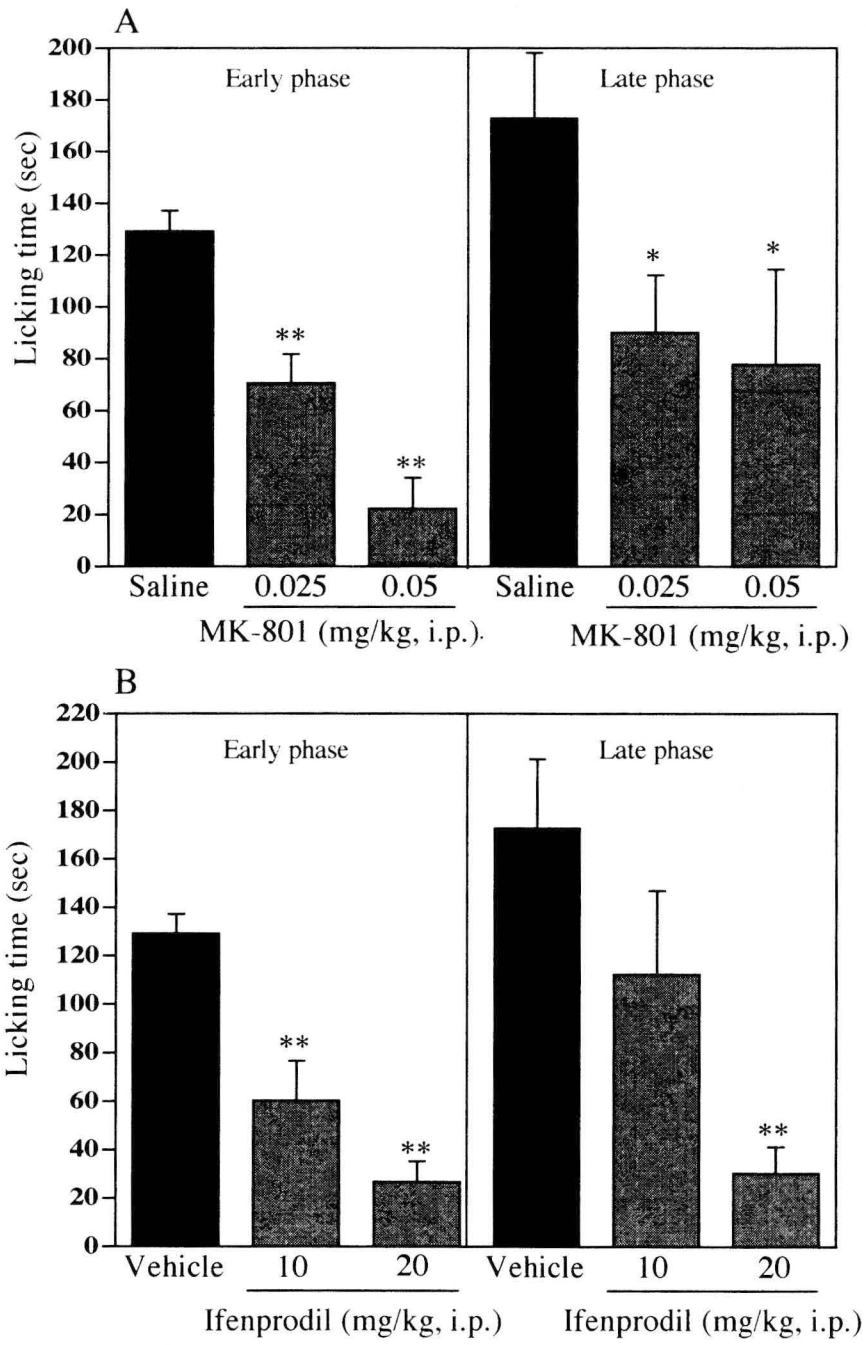

Fig. 5 Effects of MK-801 (A) and ifenprodil (B) on formalin-induced nociceptive response in mice. Mice were treated with MK-801 (0.025 and 0.05 mg/kg, i.p.) or ifenprodil (10 and 20 mg/kg, i.p.) 30 min before formalin injection. The time spent licking the injected paw was recorded and data were expressed as total licking time in the early phase (0 to 10 min) and late phase (10 to 30 min). Each column represents the mean with S.E.M. of 8-18 mice.
*p<0.05, **p<0.01 vs. vehicle or saline-treated control group.

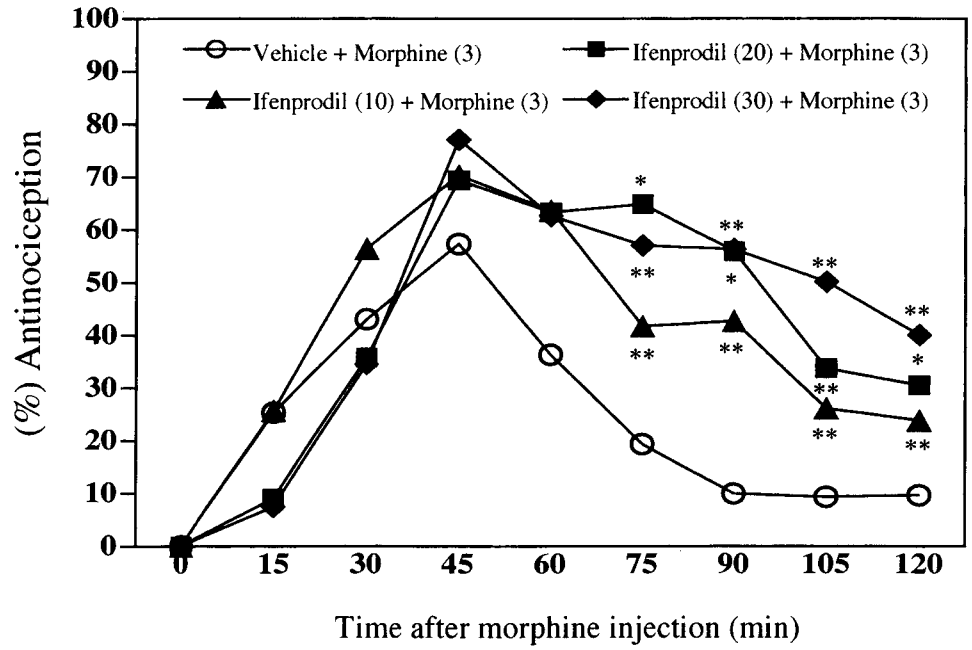

Fig. 6 Effect of ifenprodil on morphine-induced antinociception with time course in mice. Mice were treated with morphine (3 mg/kg, s.c.). Ifenprodil (10, 20 and 30 mg/kg, i.p.) or vehicle was co-administered with morphine. Each point represents the mean of 8-10 mice.
*p<0.05, **p<0.01 vs. vehicle + morphine treated group

Discussion

A variety of NMDA receptor subunits have been revealed by molecular cloning studies. There are two families of NMDA receptor subunits, NR1 and NR2 (A, B, C and D), and the expression of NR1 along with different NR2 subunits yields NMDA receptors with distinct pharmacological characteristics. Many non-competitive NMDA receptor antagonists, such as MK-801, PCP and memantine, show a high affinity for recombinant heteromeric NR1/NR2A and NR1/NR2B subunits-containing NMDA receptors [41,42]. On the other hand, several lines of evidence have suggested that ifenprodil, which is clinically used in USA as a neuroprotective agent for head ischemia, Parkinson's disease and stroke, inhibits the NR2B-subunit containing NMDA receptors [41,42].

In the present study, I demonstrated that the non-selective NMDA receptor antagonists ketamine and MK-801, which prefer both NR2A subunit- and NR2B subunit-containing NMDA receptors, induced a rewarding effect. On the other hand, I found that the selective NR2B subunit-containing NMDA receptor antagonist ifenprodil produces neither rewarding nor aversive effects under these conditions. The morphine-induced rewarding effect was markedly suppressed by pretreatment with non-selective NMDA receptor antagonists and a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil. These findings indicate that the inhibition of NR2A subunit-containing NMDA receptors may be critical for the NMDA receptor antagonist-induced rewarding effect. In contrast, the inhibition of NR2B-subunit containing NMDA

receptor may possess no psychomimetic effect. This fact provides evidence that the stimulation of NR2B subunit-containing NMDA receptor could positively regulate the morphine-induced rewarding effect.

Based on behavioral, neurochemical and electrophysiological studies, it is well-known that activation of the mesolimbic dopamine system may be responsible for the morphine-induced place preference [44]. However, it is unclear at present whether ifenprodil shows a suppressive effect on the morphine-induced activation of dopamine transmission. Many investigators have indicated that mesolimbic dopamine neurons may be controlled through an NMDA receptor in the ventral tegmental area (VTA) and the nucleus accumbens (N.Acc.) [45,46]. Interestingly, the density of NR2B subunit mRNA in the N.Acc. is higher than that of NR2A subunit mRNA [47]. Therefore, these findings suggest the possibility that NMDA receptor antagonists, such as ketamine, MK-801 and ifenprodil, may attenuate the activation of the mesolimbic dopamine system by morphine through the blockade of NR2B subunit-containing NMDA receptors in the N.Acc. On the other hand, the present data support the idea that the NMDA receptor antagonist-induced rewarding effect mostly results from the activation of the mesolimbic dopamine system through the inhibition of NR2A subunit-containing NMDA receptors. It has been also reported that during recovery from ketamine anesthesia, a high dose of ketamine increases dopamine turnover in several brain regions [48]. Moreover, a low subanesthetic dose of ketamine in the early state increases dopamine metabolism in the N.Acc. [48]. MK-801 has been shown to increase dopamine release in the N.Acc. [49]. NMDA receptor antagonists, which have different affinity for

recombinant heteromeric NR1/NR2A and NR1/NR2B subunits-containing NMDA receptors, may modulate the mesolimbic dopamine system via different mechanism. Considering their distinct distribution, it is likely that different regional distribution of NMDA receptor subunits in the CNS contributes to the different modulation of the mesolimbic dopamine system.

Furthermore, the present study focused on the effect of NR2B-containing NMDA receptor antagonist ifenprodil on the morphine-induced antinociception, and the availability of ifenprodil as adjuvant analgesic of morphine was examined. The formalin-induced licking response has been used as a model for evaluating new analgesics. The duration of the nociceptive response induced by formalin can be divided into two phases. The two phases have obvious differential properties. The nociceptive response in the early phase is evoked by the direct stimulation of the nerve fibers, while that in the late phase is due to the inflammatory response. Here I found that ifenprodil inhibited the nociceptive response induced by formalin at both the early and late phases. In addition, the morphine-induced antinociception was potentiated by co-treatment with ifenprodil, as detected by the tail-flick assay. The restricted localization of the NR2B subunit-containing NMDA receptor in the dorsal horn of the spinal cord [50] can explain the possibility that NR2B subunits contribute to nociceptive processing via glutamate sensory transmission. Furthermore, it has been reported that mice overexpressing NR2B subunits in the forebrain exhibit an enhancement of nociceptive behaviors [51], suggesting that drugs targeting NR2B subunit-containing NMDA receptors in pain-relevant structures, such as the dorsal horn of the spinal cord or forebrain, could serve as a new

class of medicine for managing pain in humans. Furthermore, these findings suggest that the blockade of NR2B subunit-dependent signaling in the dorsal horn of the spinal cord and forebrain, which may be associated with the dramatic inhibition of glutamate-induced nociceptive transmission, could be a critical mechanism that ifenprodil can potentiate the morphine-induced antinociception.

In conclusion, I demonstrated that the non-selective NMDA receptor antagonists ketamine and MK-801, which have similar affinity for NR2A and NR2B subunit-containing NMDA receptors, induce the rewarding effect. In contrast, it is worth noting that the selective NR2B subunit-containing NMDA receptor antagonist ifenprodil, which alone does not produce rewarding effect, dramatically suppresses the morphine-induced rewarding effect. These findings suggest that NR2B subunit-containing NMDA receptor may be involved in morphine-induced rewarding effect. Furthermore, I found that ifenprodil potentiated the morphine-induced antinociception. Considering its availability and advantages, ifenprodil may be useful as an adjuvant analgesic.

Chapter 2

Implication of tyrosine kinase-dependent phosphorylation of NR2B subunit-containing NMDA receptor in the rewarding effect of morphine in mice

Introduction

Various studies provide arguments to support substantial roles for the mesolimbic dopaminergic transmission, which originates from the VTA projecting mainly to the limbic forebrain including the N.Acc., in rewarding effect induced by opioids [52-54]. In addition, the N.Acc. is terminal and intermedia area for glutamatergic neurons associated with drug addiction [55]. I previously demonstrated that the NMDA receptor antagonist, especially a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil, suppressed the morphine-induced rewarding effect [56,57].

NMDA receptors are heteromeric assemblies composed of multiple subunits; these include NR1 subunits and a family of four distinct NR2 subunits (A, B, C and D) [58-60]. Several lines of evidence have suggested that the C-terminal region of the NR2B subunit is directly phosphorylated by protein kinase C (PKC) and protein tyrosine kinases [9,10,61]. It is well documented that tyrosine kinases increase the NMDA receptor phosphorylation and potentiate NMDA receptor function [6]. The function of these receptors is regulated by a series of phosphorylation and dephosphorylation processes mediated by protein kinases and protein phosphatases, respectively [6,7].

Numerous studies have demonstrated that protein tyrosine kinases, such as Src family kinase, play an important role in the regulation of NMDA receptors [8]. Several protein tyrosine kinases have been shown to phosphorylate NR2 subunits at their tyrosine residues [9,10] and may potentiate NMDA receptor function both by modulation of the channel gating and by an increase in the number of NMDA receptors at the neural

cell surface [11,12]. Several of the intracellular C-terminal tyrosine residues on the NR2B subunit, including residue 1472, are phosphorylated by Src family kinase [62]. Non-receptor type tyrosine kinases of the Src family have a critical role in a fundamental process for regulating the ion channels, neurotransmitter receptors, and signaling molecules in the synaptic junction [63]. Members of Src family kinase, namely Src, Fyn, Yes, Lyn and Lck, are known to be highly expressed in the CNS, and have been suggested to have an important function in many regions [63]. Src family members respond to a number of receptor-mediated signals, both by changing in kinase activity and by changes in intracellular localization [63].

In the present study, I therefore investigated the change in levels of phospho-Tyr-1472 NR2B subunit-containing NMDA receptor, phospho-Ser-1303 NR2B subunit-containing NMDA receptor and phospho-Tyr-416 Src family kinase in the mouse limbic forebrain containing the N.Acc. region of mice that had shown the rewarding effect induced by morphine. I also investigated the effect of pretreatment with a selective Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-(*t*-butyl)pyrazolo[3,4-D]pyrimidine (PP2) [64], on rewarding effect and dopamine-related behavior, hyperlocomotion, induced by morphine in mice. Furthermore, I examined the changes in phosphorylated-Src family kinase (phospho-Tyr-416), which is an active form of Src family kinase, in the N.Acc. of mice shown the morphine-induced rewarding effect.

Experiment 2-1

Change in Src family kinase-dependent phosphorylation of NR2B subunit-containing NMDA receptor in the rewarding effect of morphine

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan), weighting 20-30 g, were housed in a temperature-controlled room (23±1°C). The animals were maintained on a 12-hr light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum during the experimental period.

Place conditioning

The procedure for conditioned place preference (CPP) was performed following the method described in Chapter 1. Morphine (5 mg/kg) and saline (10 mL/kg) were injected s.c. on alternate days.

Western blotting

For membrane preparation, the limbic forebrain (containing the N.Acc.) of morphine- or saline-conditioned mice was rapidly removed following the test session. In addition, the limbic forebrain (containing the N.Acc.) of acute morphine- or saline-

treated mice was removed 30 min after morphine or saline injection. Each sample was homogenized in ice-cold buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM ethylendiaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin and 0.1 mg/mL aprotinin. Protein concentration in the samples was assayed by the method of Bradford (1976) [65]. An aliquot of tissue sample was diluted with an equal volume of 2 x electrophoresis sample buffer (Protein Gel Loading Dye-2 x; Amresco, Solon, OH, USA) containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol with 0.2 M dithiothreitol. Proteins (10-20 μg/lane) were separated by size on 4-20% SDS-polyacrylamide gradient gel using the buffer system, and then transferred to nitrocellulose membranes in Tris-glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection, membranes were blocked in Tris-buffered saline (TBS) containing 0.3% non-fat dried milk with 0.1% Tween 20 (Bio-Rad Laboratories, Hercules, CA, USA) for 1 hr at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS containing 0.1% Tween 20 (TTBS) [1:1000 or 1:1500 rabbit anti-phospho-Tyr-1472 NR2B subunit antibody (Chemicon international, Inc., Temecula, CA, USA), 1:1000 rabbit anti-phospho-Ser-1303 NR2B subunit antibody (Upstate, Lake Placid, NY, USA), 1:1000 rabbit anti-phospho-Tyr-416 Src family kinase antibody (Cell Signaling Technology Inc., MA, USA), 1:1000 goat anti-phospho-Thr-12 Fyn antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)], containing 0.3% non-fat dried milk, overnight, at 4°C. The membrane was washed in TTBS, followed by 2 hr incubation at room temperature with

horseradish peroxidase-conjugated anti-rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) or horseradish peroxidase-conjugated anti-goat IgG (Zymed Laboratories, South San Francisco, CA, USA) diluted 1:10000 in TTBS containing 0.3% non-fat dried milk. To control for validation in loading, the expression of housekeeping gene GAPDH was also assayed by Western blot analysis. After incubation with primary and secondary antibodies, the membrane was then re-probed with mouse anti-GAPDH polyclonal antibody (1:400000; Chemicon international, Inc., Temecula, CA, USA, in TTBS containing 0.3% non-fat milk) for 1 hr, followed by incubation with anti-mouse secondary antibody conjugated with horseradish peroxidase (1:10000) for 2 hr at room temperature. The antigen-antibody peroxidase complex was finally detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacture's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL, USA).

Immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion fixed with 4% paraformaldehyde, pH 7.4. The brains were quickly removed after perfusion, and the brain coronal sections including N.Acc. were postfixed in 4% paraformaldehyde for 2 hr. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored -30°C until use. Frozen 8-μm-thick coronal sections were cut with a cryostat (CM1510; Leica,

Heidelberg, Germany) and thaw mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 10% normal horse serum (NHS) in 0.01 M phosphate-buffered saline (PBS) for 1 hr at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% NHS [1:200 goat anti-NR2B subunit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:3000 rat anti-dopamine transporter (DAT) antibody (Chemicon international, Inc., Temecula, CA, USA)] and incubated for 2 day at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibodies conjugated with Alexa^{TM} fluo 546 (1:800; for NR2B subunit) and Alexa^{TM} fluo 488 (1:1500; for DAT) for 2 hr at room temperature. The slides were then coverslipped with PermaFlluor Aqueous mounting medium (Immunon, Pittsburgh, PA, USA). All sections were observed with a light microscope (Olympus BX-80) and photographed with a digital camera (CoolSNAP HQ; Olympus).

Drugs

The drug used in the present study was morphine hydrochloride (Sankyo Co. Ltd., Tokyo, Japan). Morphine hydrochloride was dissolved in physiological saline.

Statistical analysis

All data are expressed as mean ± S.E.M. The statistical significance of differences between groups was assessed with an ANOVA followed by the Dunnett's multiple comparison test or Student's *t*-test.

Results

Immunohistochemical approach for the determination of NR2B subunit in the N.Acc.

Immunoreactivities for NR2B subunit and DAT were observed in the N.Acc. Double-labeling experiments showed that NR2B subunit-immunoreactivity was not colocalized with the selective presynaptic dopamine neuron marker DAT in the N.Acc. (Fig. 7).

Protein levels of phospho-Tyr-1472 and phospho-Ser1303 NR2B subunits in the mouse limbic forebrain after the morphine conditioning

A significant increase in levels of phospho-Tyr-1472, but not phospho-Ser1303, NR2B subunit-like immunoreactivities in membranes of the limbic forebrain (including N.Acc.) of mice showing morphine (5 mg/kg, s.c.)-induced rewarding effect was noted as compared with that of saline-conditioned mice ($p<0.01$; Fig. 8).

No change in phospho-Tyr-1472 NR2B subunit after acute morphine treatment

There was no significant difference between saline- and morphine (5 mg/kg, s.c.)-treated groups in levels of phospho-Tyr-1472 NR2B subunit-like immunoreactivities in membranes of the limbic forebrain (Fig. 9).

Change in phospho-Tyr-416 Src family kinase and phospho-Thr-12 Fyn after the morphine conditioning

A significant increase in levels of phospho-Tyr-416 Src family kinase-, but not phospho-Thr-12 Fyn, like immunoreactivities in membranes of the limbic forebrain of mice showing morphine (5 mg/kg, s.c.)-induced rewarding effect was noted as compared with that of saline-conditioned mice ($p<0.05$; Fig. 10).

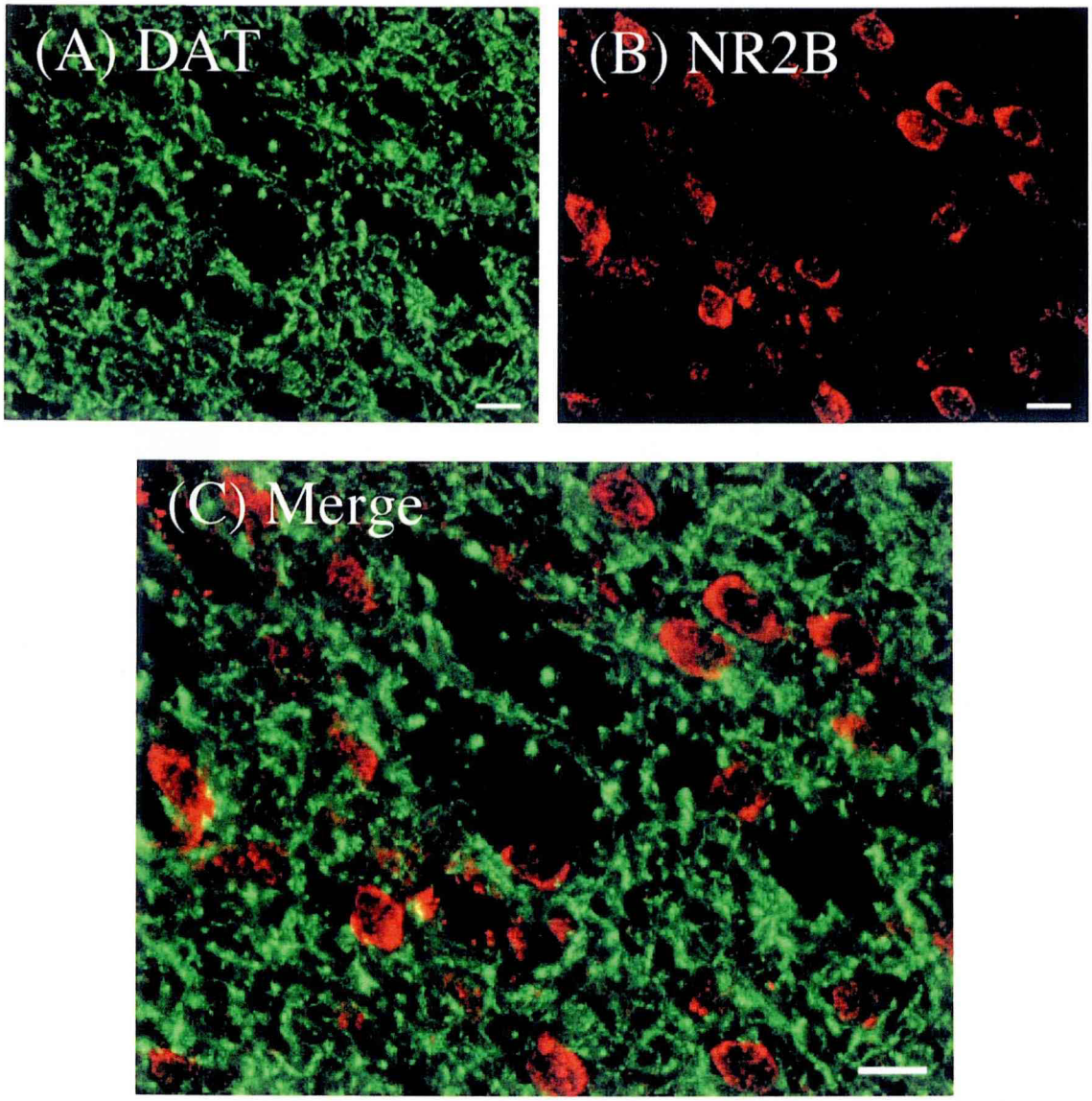

Fig. 7 Immunostaining for dopamine transporter (DAT) (A) is visible in many N.Acc. neurons that show no immunoreactivity for NR2B subunit (B). (C) No expression of NR2B subunit in dopamine neurons of the N.Acc. Scale bars=10 μm.

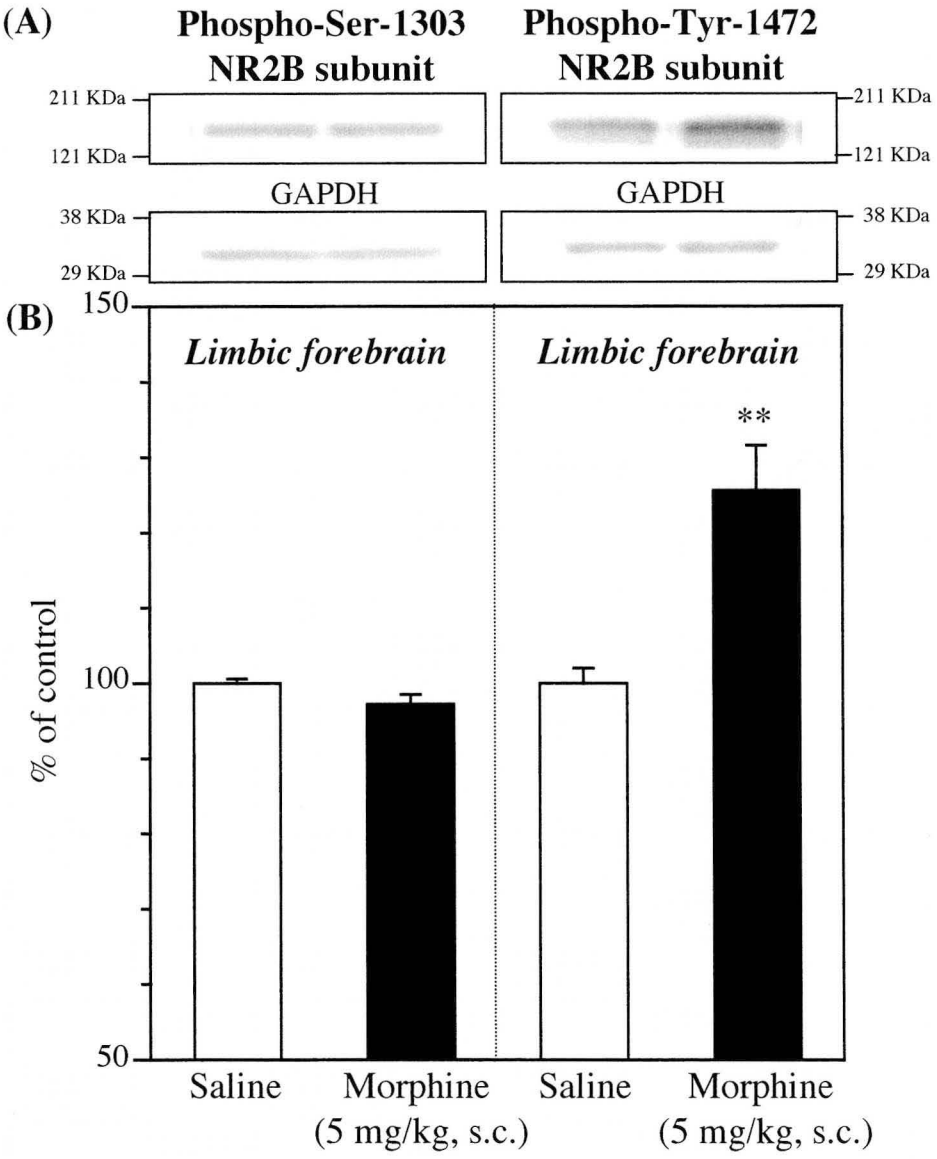

Fig. 8 (A) Representative immunoblot analysis of membranous phospho-Tyr-1472 or phospho-Ser-1303 NR2B subunit. (B) Changes in phospho-Tyr-1472 and phospho-Ser-1303 NR2B subunits immunoreactivities in membranous fractions of the limbic forebrain obtained from saline- or morphine (5 mg/kg, s.c.)-conditioned mice. The sample was prepared 24 hr after the conditioning. Each column represents the mean with S.E.M. of at least three independent experiments.

** $p<0.01$ vs. saline-conditioning group.

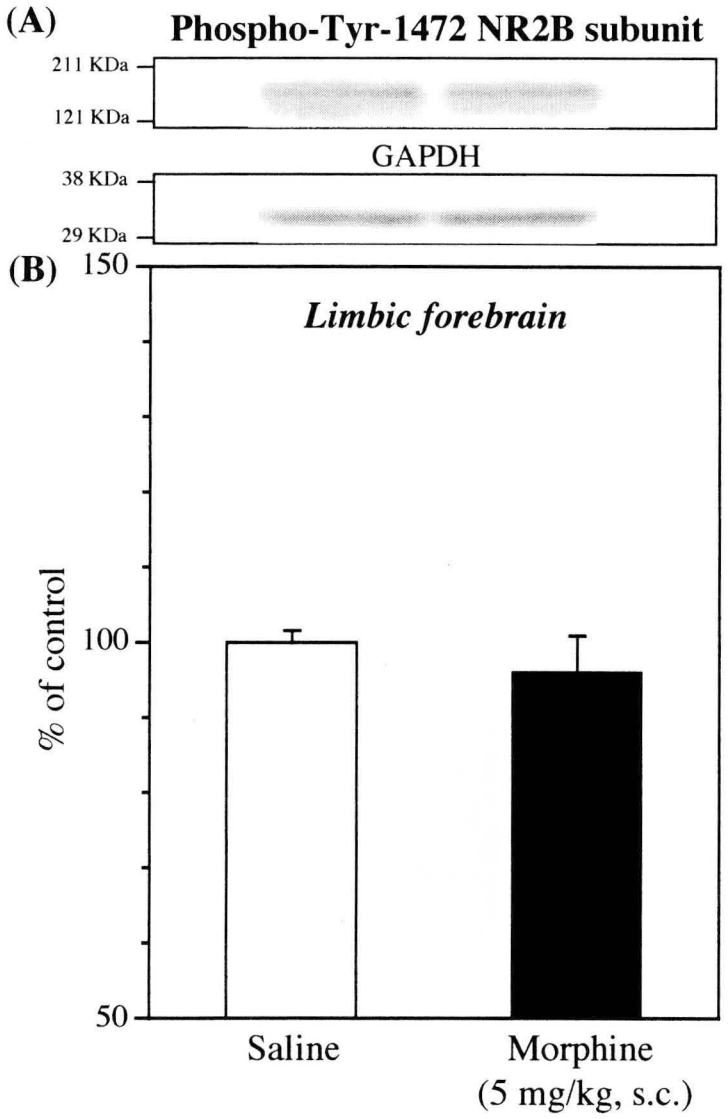

Fig. 9 (A) Representative immunoblot analysis of membranous phospho-Tyr-1472 NR2B subunit. (B) Change in phospho-Tyr-1472 NR2B subunit immunoreactivities in membranous fractions of the limbic forebrain obtained from acute saline- or morphine (5 mg/kg, s.c.)-treated mice. The sample was prepared 30 min after the treatment. Each column represents the mean with S.E.M. of at least three independent experiments.

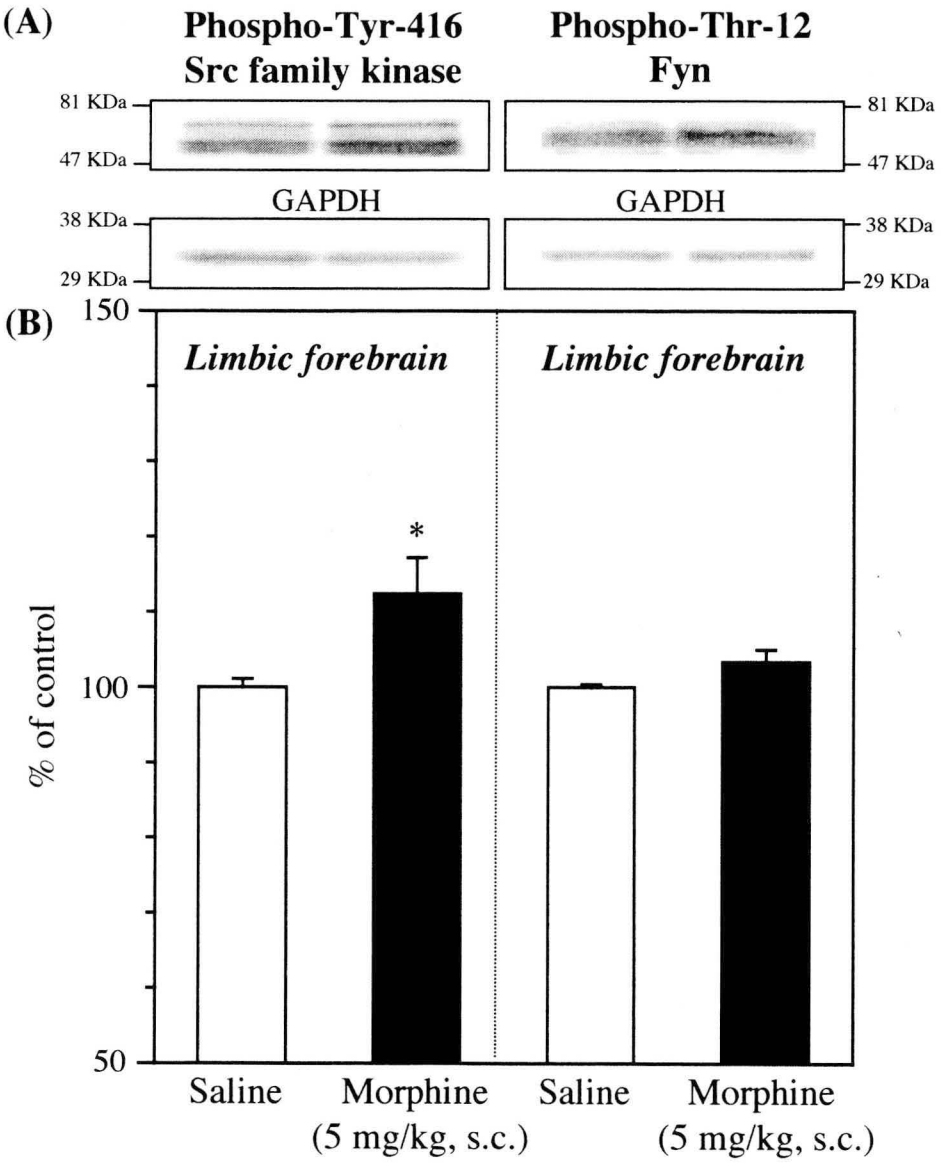

Fig. 10 (A) Representative immunoblot analysis of membranous phospho-Tyr-416 Src family kinase or phospho-Thr-12 Fyn. (B) Changes in phospho-Tyr-416 Src family kinase and phospho-Thr-12 Fyn immunoreactivities in membranous fractions of the limbic forebrain obtained from saline- or morphine (5 mg/kg, s.c.)-conditioned mice. The sample was prepared 24 hr after the conditioning. Each column represents the mean with S.E.M. of at least three independent experiments.
* $p<0.05$ vs. saline-conditioning group.

Experiment 2-2

Role of Src family kinase in the morphine-induced rewarding effect and hyperlocomotion

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan), weighting 20-30 g, were housed in a temperature-controlled room (23±1°C). The animals were maintained on a 12-hr light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum during the experimental period.

Place conditioning

The procedure for conditioned place preference (CPP) was performed following the method described in Chapter 1. Morphine (1, 3 and 5 mg/kg) and saline (10 mL/kg) were injected s.c. on alternate days. In the combination study, mice were pretreated with vehicle (4 μL/mouse, i.c.v.) or 4-amino-5-(4-chlorophenyl)-(*t*-butyl)pyrazolo[3,4-D]pyrimidine (PP2; 0.1, 1 and 10 nmol/mouse, i.c.v.) 30 min before each morphine (5 mg/kg, s.c.) injection.

Immunohistochemistry

For the immunohistochemical assay, the place conditioning sessions were first conducted for a 60 min period once daily for six days. Mice were injected s.c. with morphine (5 mg/kg) and saline (10 mL/kg) on alternate days. On day 7, conditioning tests were performed. Immediately after the test, the saline- and morphine-conditioning mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion-fixed with 4% paraformaldehyde (pH 7.4). The frozen 10 μm thick coronal sections including the N.Acc. region cut with a cryostat (Leica CM1510, Leica Microsystems, Heidelberg, Germany) and thaw-mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 10% normal horse serum (NHS) in 0.01 M phosphate-buffered saline (PBS) for 1 hr at room temperature (about 23°C). Sections were incubated with a primary antibody to phospho-Tyr-416 Src family kinase (1:50, ALEXIS Biochemicals, CA, USA) two overnights at 4°C. For labeling, Alexa™ fluo 488-conjugated goat anti-rabbit IgG for phospho-Tyr-416 Src family kinase was diluted 1:400 in PBS containing 10% NHS. Fluorescence immunolabeling was detected using a U-MNIBA filter cube (Olympus, Tokyo, Japan) for Alexa™ fluo 488.

Digitized images of N.Acc. sections were captured at a resolution of 658 x 517 pixels with camera. The density of phospho-Tyr-416 Src family kinase labeling was measured with a computer-assisted imaging analysis system (NIH Image). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and non-immunoreactive material as white pixels. A standardized rectangle was

positioned over the N.Acc. of saline-treated mice. The area and density of pixels within the threshold value representing immunoreactivity were calculated and the integrated density was the product of the area and density. The same box was then 'dragged' to the corresponding position on the N.Acc. of morphine-treated mice and the integrated density of pixels within the same threshold was again calculated.

Locomotor assay

The locomotor activity of mice was measured by an ambulometer (ANB-M20, O'hara Co. Ltd., Tokyo, Japan) as described previously [66]. Briefly, a mouse was placed in a tilting cage of 20 cm in diameter and 19 cm in height. Any slight tilt of the activity cage caused by horizontal movement of the animal was detected by microswiches. Total activity counts in each 10 min segment were automatically recorded for 30 min prior to the injections and for 180 min following injection of saline (10 mL/kg, s.c.) or morphine (10 mg/kg, s.c.). Mice were pretreated with vehicle (4 μL/mouse, i.c.v.) or PP2 (0.1, 1 and 10 nmol/mouse, i.c.v.) 30 min before morphine injection.

Intracerebroventricular (i.c.v.) injection

The i.c.v. administration was performed following the method described previously [67]. In order to make a hole in the skull for the injection, 2-3 days before each experiment, mice were briefly anesthetized with ether, and a 2-mm double-needle (Natsume Seisakusho Co. Ltd., Tokyo, Japan) attached to a 25-μL Hamilton microsyringe was inserted into the unilateral injection site using V-shaped holder to

hold the head of the mouse. The injection volume was 4 μL for each mouse.

Drugs

The drugs used in the present study were morphine hydrochloride (Sankyo Co. Ltd., Tokyo, Japan) and a selective Src family kinase inhibitor PP2 (Calbiochem, San Diego, CA). Morphine hydrochloride was dissolved in physiological saline. PP2 was dissolved in 100% DMSO and added to physiological saline immediately before use (50% DMSO in saline).

Statistical analysis

All data are expressed as mean ± S.E.M. The statistical significance of differences between groups was assessed with an ANOVA followed by the Dunnett's multiple comparison test or Student's *t*-test.

Results

Effect of a Src family kinase inhibitor PP2 on the morphine-induced rewarding effect in mice

The place preference induced by morphine is shown in Fig. 11A. The saline-conditioning control mice exhibited no preference for either compartment of the box. Morphine (1, 3 and 5 mg/kg, s.c.) caused a dose-related place preference, and a significant place preference was observed at doses of 3 and 5 mg/kg ($p<0.01$). The effect of PP2 on the morphine-induced place preference is shown in Fig. 11B. A selective Src family kinase inhibitor PP2 (10 nmol/mouse, i.c.v.) induced neither place preference nor place aversion. On the other hand, i.c.v. pretreatment with PP2 (1 and 10 nmol/mouse) significantly suppressed the morphine (5 mg/kg, s.c.)-induced place preference (1 nmol/mouse; $p<0.05$, 10 nmol/mouse; $p<0.01$).

Effect of PP2 on the morphine-induced hyperlocomotion in mice

The effect of PP2 on the morphine-induced increase in locomotor activity is shown in Fig. 12A and B. Morphine (10 mg/kg, s.c.) produced a significant increase in locomotor activity as compared to that of vehicle + saline-treated control group ($p<0.01$; Fig. 12B). PP2 (10 nmol/mouse, i.c.v.) had no effect on the locomotion as compared to that of vehicle + saline-treated control group. In combination study, PP2 (10 nmol/mouse, i.c.v.) significantly suppressed the increase in locomotor activity produced by morphine ($p<0.05$; Fig. 12B).

Increase in the level of Src family kinase-like immunoreactivity in the mouse N.Acc. under the morphine reward

As shown in Fig. 13A, B, C and D, the phospho-Tyr-416 Src family kinase-like immunoreactivity was seen in the mouse N.Acc. of both saline (Fig. 13A and C) and morphine (5 mg/kg, s.c.; Fig. 13B and D)-conditioned mice. The phospho-Tyr-416 Src family kinase-like immunoreactivity in the N.Acc. of morphine-dependent mice was markedly increased as compared to that of saline-conditioned mice ($p<0.01$; Fig. 13D).

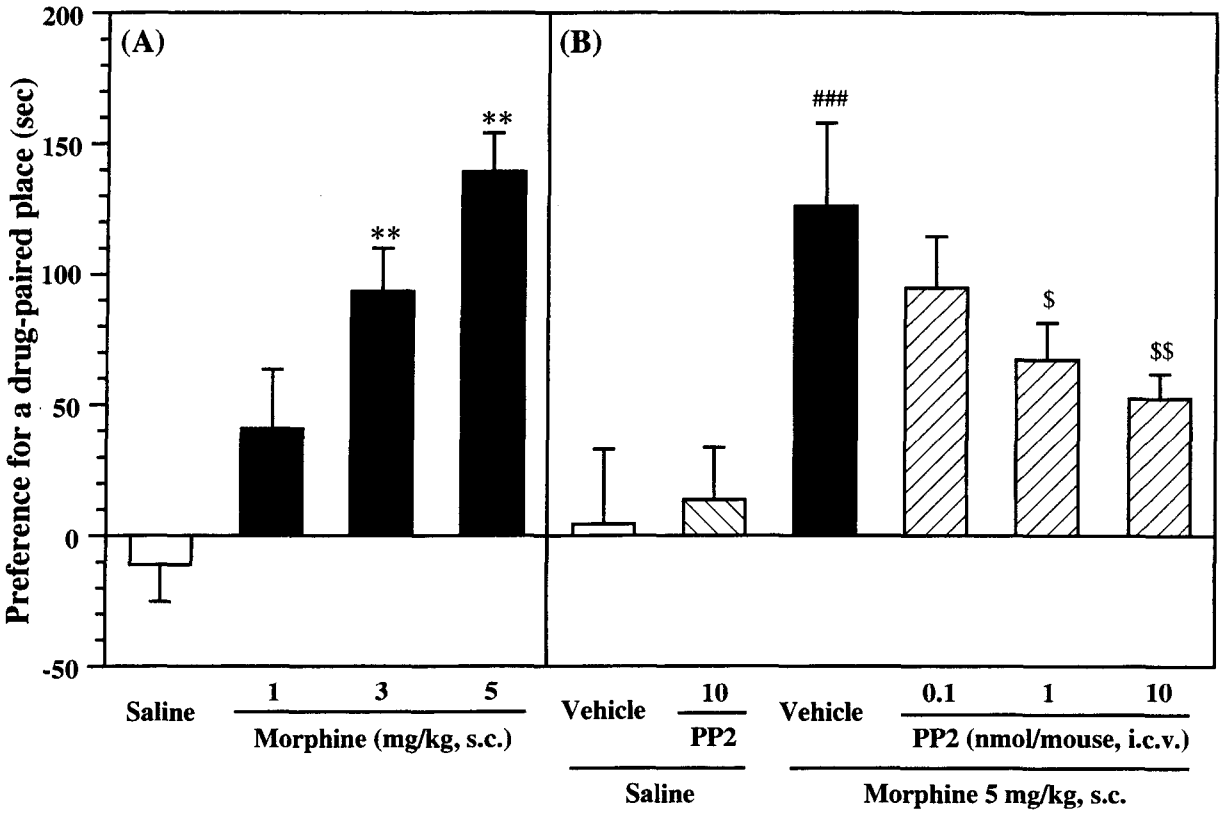

Fig. 11 (A) Place preference induced by morphine (1-5 mg/kg) in mice. (B) Effect of a selective Src family kinase inhibitor PP2 on morphine (5 mg/kg, s.c.)-induced place preference in mice. Groups of mice were pretreated with vehicle or PP2 (0.1-10 nmol/mouse, i.c.v.) 30 min before morphine injection. Ordinate: mean difference(s) between the times spent on the drug- and saline-associated sides of the test box. Each column represents the mean with S.E.M. of 7-8 mice.

** $p<0.01$ vs. saline-conditioning control group. ### $p<0.001$ vs. vehicle + saline-conditioning group. $ $p<0.05$, $$ $p<0.01$ vs. vehicle + morphine-conditioning group.

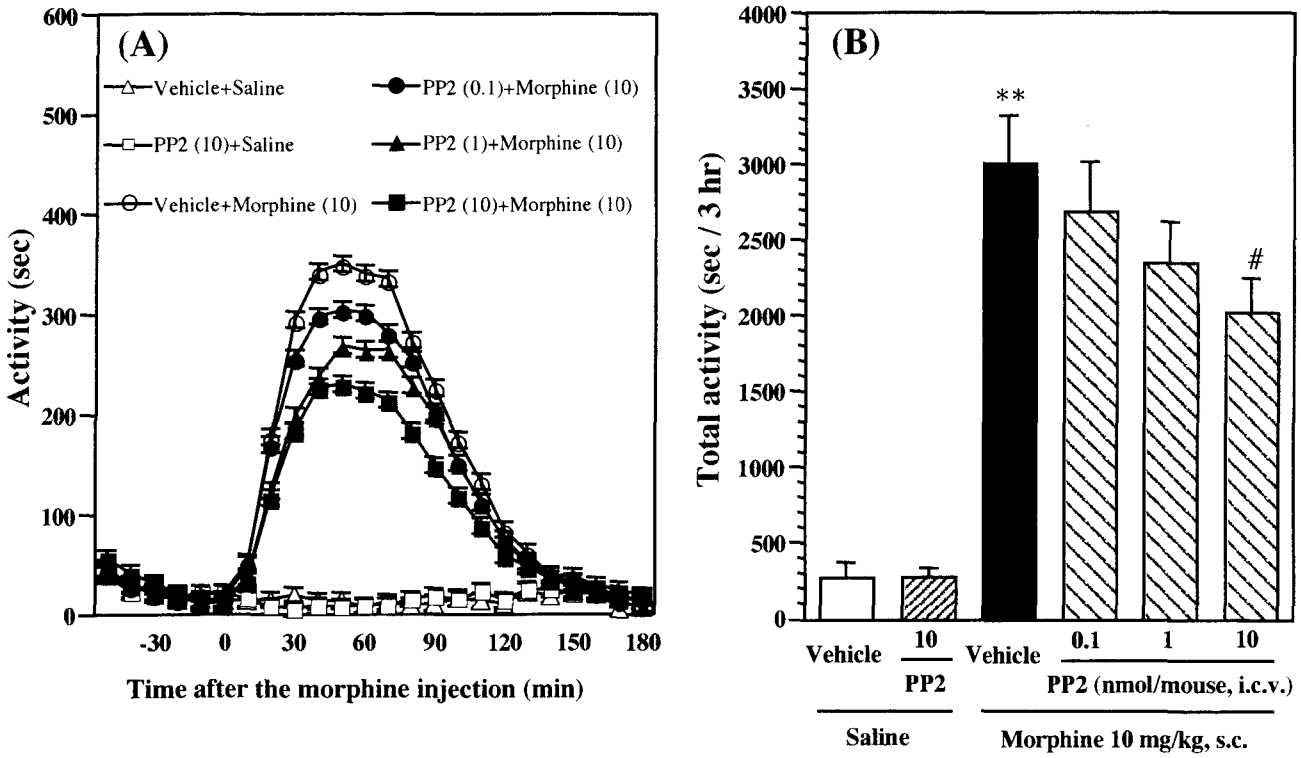

Fig. 12 Effect of a selective Src family kinase inhibitor PP2 on the time-course changes (A) or total activity (B) of morphine (10 mg/kg, s.c.)-induced hyperlocomotion. (A) Each point represents the mean activity counts for 10 min with S.E.M. of 8-20 mice. (B) Each column represents the total activity counts for 3 hr with S.E.M. of 8-20 mice. Groups of mice were pretreated with vehicle or PP2 (0.1-10 nmol/mouse, i.c.v.) 30 min before morphine injection.
** $p<0.01$ vs. vehicle + saline-treated control group. # $p<0.05$ vs. vehicle + morphine-treated group.

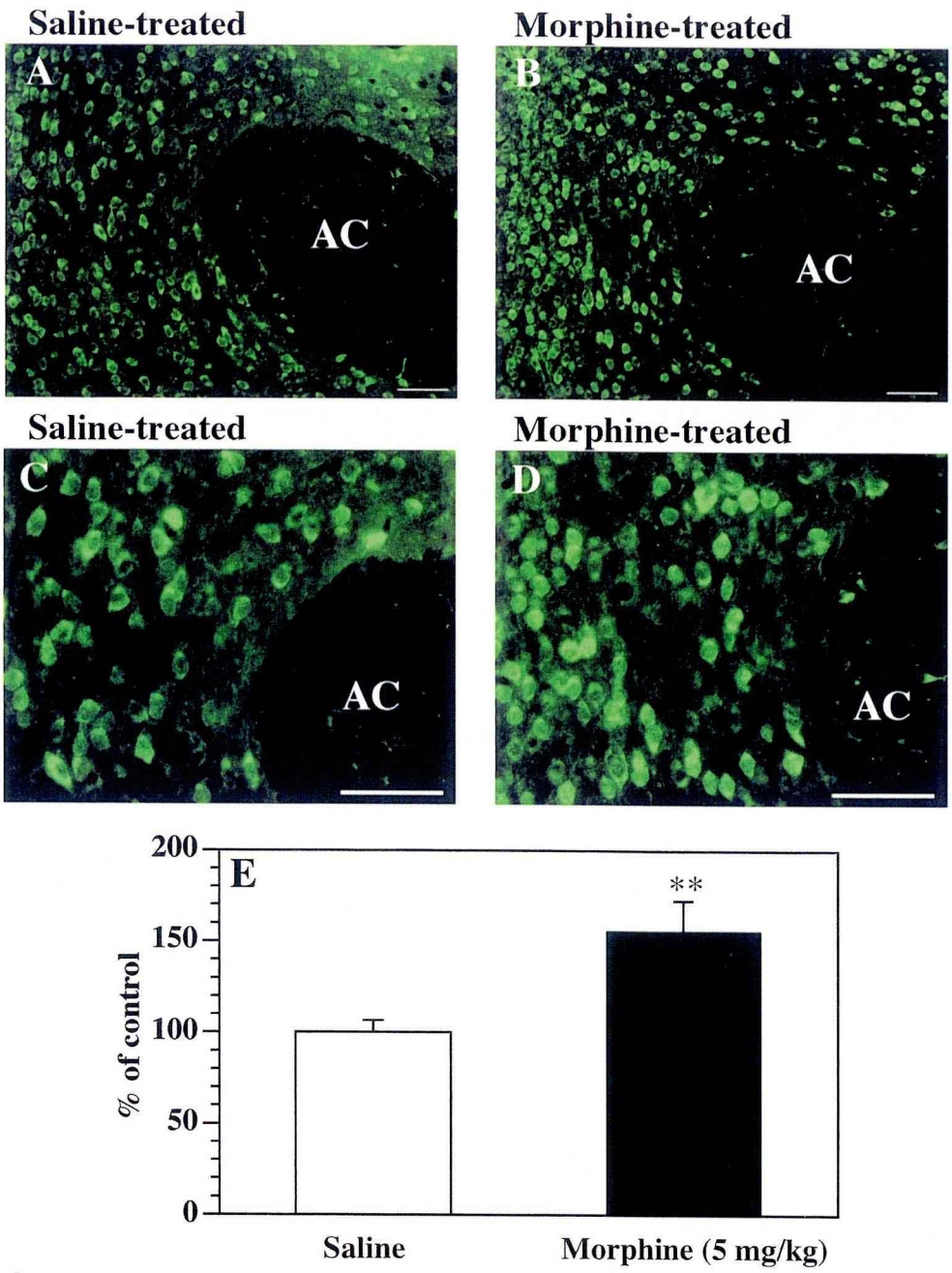

Fig. 13 Immunofluorescence photomicrographs of the mouse coronal sections stained with anti-phospho-Tyr-416 Src family kinase antibody in saline- and morphine-conditioning mice. The brain slices were prepared immediately after the test session on day 7 of in (A), (C) saline- or (B), (D) morphine (5 mg/kg)-conditioning mice. AC; anterior commissure. Scale bars=50 μm. (E) Subquantification of change in phosphorylated-Src family kinase-immunoreactivity in the N.Acc. of morphine (5 mg/kg, s.c.)-dependent mice.
** $p<0.01$ vs. saline-conditioning mice.

Discussion

It has been widely accepted that tyrosine phosphorylation of the NMDA receptor produces potentiation of its receptor function [6]. There are many tyrosine residues, including residue Tyr-1472, on the NR2B subunit that could be subjected to phosphorylation site of Src family kinase [62,68,69]. It is also well-known that C-terminal region of the NR2 subunit possesses PKC-dependent phosphorylation sites including Ser-1303 [70,71]. The PKCs are a family of phospholipids-dependent serine/threonine protein kinases that are highly concentrated in the brain. In the present study, I found that the level of phospho-Tyr-1472, but not phospho-Ser-1303, NR2B subunit, was increased in the limbic forebrain containing the N.Acc. of mice showing the morphine-induced rewarding effect. On the other hand, acute morphine treatment failed to affect the phosphorylation of Tyr-1472 on NR2B subunit protein. I also demonstrated that morphine-induced rewarding effect was suppressed by a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil. In addition, most of NR2B subunit immunoreactivities in the N.Acc. showed hardly any co-localization with the presynaptic dopamine neuron maker DAT immunoreactivities. These findings suggest that phospho-Tyr-1472 NR2B subunit-containing NMDA receptor at the postsynaptic site in the N.Acc. may play an important role in the rewarding effect of morphine.

Tyrosine phosphorylation of NR2B subunit-containing NMDA receptor is regulated by tyrosine kinases of Src family [62]. Various studies have provided the argument to support that Src family kinases are essential components intermediated in

many cellular pathways in the synaptic junction. The Src family kinases are a subset of enzymes known as the non-receptor type protein tyrosine kinase, which transfer phosphate groups onto the tyrosine residue in proteins. Many protein tyrosine kinases span the membrane encapsulating a cell, and are activated only by binding of one specific extracellular molecule. In addition, Src family kinases are considered to be associated with the cell membrane but reside totally inside the cell, and are activated in response to a variety of extracellular stimuli. Activity of Src family kinases is regulated by tyrosine phosphorylation at two sites with opposing effects. The non-phosphorylated form of Src family kinase is inactive [72]. The measure autophosphorylation site, Tyr-416, lies in the activation segment, a flexible portion of the catalytic domain near the active site, and leads to enhance catalytic activity of Src family kinase [72]. In contrast, another phosphorylation site of Tyr-527, which was identified in the C-terminal tail, has been known as an inhibitory tyrosine phosphorylation site [73-75]. In order to investigate whether Src family kinase could be activated by morphine, I used an antibody to phospho-Tyr-416 Src family kinase, which is known to active form of Src family. As a result, phospho-Tyr-416 Src family kinase was significantly increased in the limbic forebrain of mice showing the morphine-induced rewarding effect. In contrast, Fyn, which is one of Src family kinases, was not affected in the limbic forebrain of mice showing the morphine-induced rewarding effect. In the present study, I also found that a selective Src family kinase inhibitor PP2 administered with morphine significantly suppressed the morphine-induced rewarding effect and hyperlocomotion. I demonstrated here for the first time that repeated treatment with morphine following a

conditioning leads to a dramatical increase in phospho-Tyr-416 Src family kinase-like immunoreactivity in the N.Acc. region as compared to those in saline-conditioning mice. These findings suggest that Src family kinase-, excluding Fyn, dependent phosphorylation of NR2B subunit-containing NMDA receptor in the N.Acc. may be involved in the rewarding effect of morphine.

In summary, I report here that Tyr-1472 phosphorylation of NR2B subunit-containing NMDA receptor via activation of Src family kinase in the limbic forebrain including the N.Acc. may be involved in the morphine-induced rewarding effect. These findings provide novel evidence for the critical role of Src family kinases in the regulation of morphine dependence.

Chapter 3

Role of neuronal NR2B subunit-containing NMDA receptor-mediated Ca^{2+} influx and astrocytic activation in cultured mouse cortical neurons and astrocytes

Introduction

Changes in the intracellular calcium concentration ($[Ca^{2+}]i$) mediate a variety of biological responses in both excitable and nonexcitable cells. In the CNS, the mechanism of calcium signaling has been investigated extensively in neurons [76], whereas less attention has been granted to other CNS cells such as glial cells [77]. Astrocytes are the predominant glial cell type in the CNS and are intimately associated with neurons. A growing body of evidence suggests that astrocytes also respond to a variety of extracellular stimuli, such as glutamate, with increases in intracellular calcium concentration [78].

Glutamate, the major excitatory transmitter in the mammalian CNS, activates three classes of ionotropic receptors: AMPA, kainate, and NMDA receptors and mGluR that can be separated on biophysiological and pharmacological grounds [79]. The mGluRs are heterotrimeric G-protein-coupled receptors and are believed to modulate transmitter release [80], calcium oscillations [81], and some use-dependent changes in synaptic currents [82,83]. AMPA and kainate receptors are functional at negative membrane potentials and mediate synaptic transmission at resting membrane potentials. Overactivation of NMDA receptors plays a critical role in animal models of ischemic brain damage, and several different types of NMDA receptor antagonists have attracted interest in recent years as neuroprotective compounds [84-88]. Ifenprodil was the first neuroprotective compound found to be selective for NMDA receptors containing NR2B subunits [89-91]. Accumulating evidence suggests that ifenprodil is the effective neuroprotective agent *in*

vitro and *in vivo* but is devoid of many of the side effects that have limited the therapeutic usefulness of other types of NMDA receptor antagonists [92,93]. In chapter 1, I demonstrated that *in vivo* ifenprodil treatment dramatically blocked the development of rewarding effect of morphine [56].

A major step in understanding the mechanism underlying the excitation of neurons and astrocytes regulated by glutamate through NMDA receptors in several brain functions has been achieved by demonstrating that Ca^{2+} signaling, morphological changes in astrocytes and apoptotic neuronal cell death. In this chapter, I investigated whether NR2B subunit-containing NMDA receptor could participate in Ca^{2+} influx in mouse cortical neurons and astrocytes. I also examined the role of NR2B subunit containing NMDA receptor in the glutamate-induced astrocytic activation in neuron/glia cocultures and purified astrocyte.

Materials and Methods

Tissue Processing

Cortical neuron/glia cocultures were grown as follows; cerebral cortices were obtained from newborn ICR mice (Tokyo Laboratory Animals Science, Tokyo, Japan), minced, and treated with papain (9 U/mL, Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate (Sigma-Aldrich, St. Louis, MO, USA), 0.5% glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02% bovine serum albumin (BSA; Wako Pure Chemicals, Osaka, Japan). After enzyme treatment at 37°C for 15 min, cells were seeded at a density of 2 x 10^6 cells/cm^2. The cells were maintained for 7 days in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carsbad, CA, USA) supplemented with 10% precolostrum newborn calf serum (Invitrogen, Carsbad, CA, USA), 10 U/ml penicillin and 10^{-5} g/mL streptomycin.

Purified cortical astrocytes were grown as follows; cerebral cortices were obtained from newborn ICR mice, minced, and treated with trypsin (0.025%, Invitrogen; Carsbad, CA, USA) dissolved in PBS containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% BSA. After enzyme treatment at 37°C for 15 min, cells were dispersed by gentle agitation through a pipette and plated on a flask. One week after seeding in DMEM supplemented with 5% precolostrum newborn calf serum, 5% heat-inactivated (56°C, 30 min) horse serum (Invitrogen, Carsbad, CA, USA), 10 U/mL penicillin and 10^{-5} g/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, the flask was shaken for 12 hr at 37°C to remove non-astrocytic cells. The cells were seeded

at a density of 1 x 10^5 cells/cm^2. The cells were maintained for 3 to 10 days in DMEM supplemented with 5% precolostrum newborn calf serum, 5% horse serum, 10 U/ml penicillin and 10^{-5} g/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Confocal Ca^{2+} imaging

Cells were loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo molecular Technologies, MD, USA) for 90 min at room temperature. After a further 20-30 min of de-esterification with the acetoxymethyl ester, the coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000; BioRad Richmond, CA, USA). Fluo-3 was excited with the 488 nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cell was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (ratio: $Rs = F/F_0$). MK-801, ifenprodil, (*R*,*S*)-alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperid inepropanol hydrochloride (Ro25-6981), 6,7-dinitroquinoxaline-2,3-dione (DNQX) or 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) was incubated for 5 min before glutamate (10 μM, 30 sec) treatment.

Immunohistochemistry

Mouse cortical neuron/glia cocultures or purified cortical astrocytes were treated with normal medium (control), glutamate (0.1 μM) or glutamate (0.1 μM) + MK-801

(0.1 μM) or glutamate (0.1 μM) + ifenprodil (10 μM) for 24 hr. The cells were then identified by immunofluorescence using mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:1000; Chemicon, International, CA, USA), followed by incubation with Alexa 488-conjugated goat anti-mouse IgG (1:4000) for mouse anti-GFAP antibody. Images were collected using a Radiance 2000 laser-scanning microscope (BioRad, Richmond, CA, USA).

The intensity of GFAP-like immunoreactivity was measured with a computer-assisted system (NIH image). The upper and lower threshold intensity ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and nonimmunoreactive material appearing in white pixels. The area and intensity of pixels within the threshold value representing immunoreactivity were calculated.

Evaluation of glutamate-induced apoptotic neuronal cell death

To evaluate the apoptotic neuronal cell death, cortical neuron/glia cocultures were treated with normal medium, glutamate (0.1 μM), or NMDA receptor antagonist (MK-801; 0.1 μM or ifenprodil; 10 μM) + glutamate (0.1 μM) for 24 hr. The cells were then identified by immunofluorescence, using rabbit-cleaved caspase-3 antibody (1:100, Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488-cojugated goat anti-rabbit antibody (1:10000).

Drugs

The drugs used in the present study were L-glutamate (Wako Pure Chemicals, Osaka, Japan), MK-801 (Sigma-Aldrich, St. Louis, MO, USA), Ro25-6981 (Sigma-Aldrich, St. Louis, MO, USA), DNQX (Acros Organics One Reagent Lane, Fairlawn, NJ, USA), MPEP (Sigma-Aldrich, St. Louis, MO, USA) and ifenprodil maleate (Grelan Pharmaceutical, Co., Ltd., Tokyo, Japan).

Statistical analysis

The data are presented as the mean ± S.E.M. The statistical significance of differences between groups was assessed by an ANOVA followed by Dunnett's multiple comparison test or Student's *t*-test.

Results

Glutamate-mediated Ca^{2+} influx in mouse cortical neurons and astrocytes

Glutamte (0.1-10 μM) produced the increase in levels of $[Ca^{2+}]i$ in a concentration-dependent manner in both neurons and astrocytes (data not shown). Glutamate (10 μM) produced a transient increase in the $[Ca^{2+}]i$ in cultured cortical neurons (Fig. 14A) and astrocytes (Fig. 15A). Either a non-selective NMDA receptor antagonist MK-801 (0.1-1 μM) or selective NR2B subunit-containing NMDA receptor antagonists ifenprodil (0.1-30 μM) and Ro25-6981 (0.1-1 μM) significantly inhibited the glutamate-evoked Ca^{2+} influx into neurons (Fig. 14A, B), but not into astrocytes (Fig. 15A, B). On the other hand, the glutamate-evoked increase in levels of $[Ca^{2+}]i$ was significantly inhibited by pretreatment with either an AMPA/kainate receptor antagonist DNQX (10 μM) or an mGluR5 antagonist MPEP (100 μM) in cultured neurons (Fig. 14A, B) and astrocytes (Fig. 15A, B).

Glutamate-induced astrocytic activation

Astrocytes are activated in response to brain tissue damages, which are characterized by stellate morphology and increased the immunoreactivity of GFAP. Thus, I investigated whether treatment with glutamate to mouse cortical neuron/glia cocultures and purified astrocytes could cause any morphological changes in the adjacent astrocytes. As shown in Fig. 16 and 17, treatment with glutamate (0.1 μM) for 24 hr caused a robust activation of astrocytes in mouse cortical neuron/glia cocultures

and purified astrocytes, as detected by a stellate morphology and an increase in the level of GFAP-like immunoreactivity compared to that in normal medium-treated cells. These increases in the level of GFAP-like immunoreactivities induced by glutamate were dramatically suppressed by co-treatment with a non-selective NMDA receptor antagonist MK-801 (0.1 μM) or a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil (10 μM) in cortical neuron/glia cocultures (Fig. 16A, B), but not in purified astrocytes (Fig. 17A, B).

Glutamate-induced apoptotic neuronal cell death

I also confirmed that treatment with glutamate in cortical neuron/glia cocultures caused the activation of cleaved caspase-3, which is a marker of apoptosis. As shown in Fig. 18, treatment with glutamate (0.1 μM) for 24 hr in cortical neuron/glia cocultures caused the robust activation of cleaved caspase-3. Apoptotic neuronal cell death induced by treatment with glutamate was clearly blocked by co-treatment with a non-selective NMDA receptor antagonist MK-801 (0.1 μM) and a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil (10 μM).

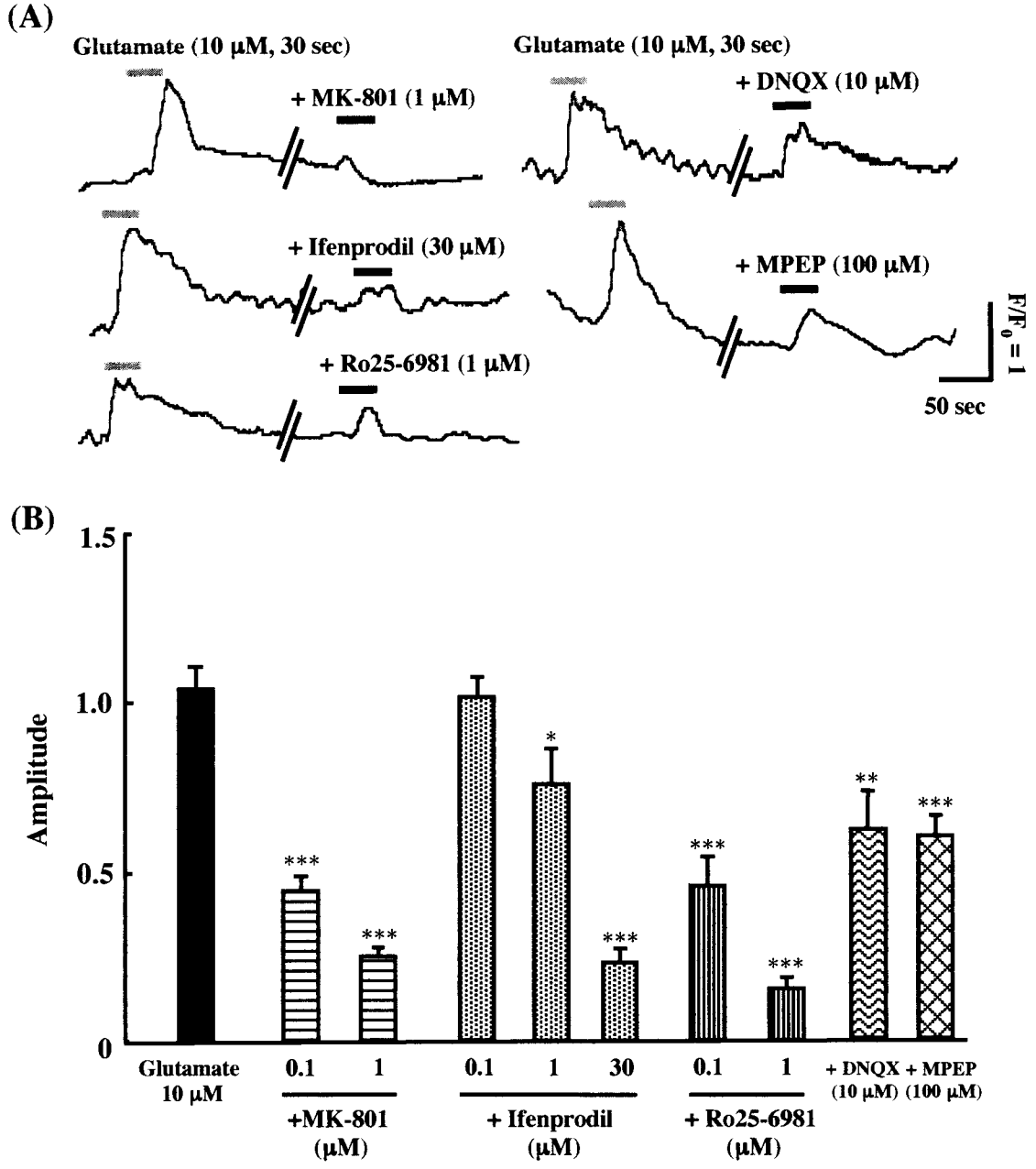

Fig. 14 The glutamate-mediated Ca^{2+} influx in mouse cortical neurons. Characterization of glutamate-evoked increase in intracellular Ca^{2+} concetration in mouse cortical neurons. (A) Typical traces show the glutamate-evoked increase in intracellular Ca^{2+} concetration, showing the effects of a non-selective NMDA receptor antagonist MK-801 (1 μM), selective NR2B subunit-containing NMDA receptor antagonists ifenprodil (30 μM) and Ro25-6981 (1 μM), an AMPA/kainate receptor antagonist DNQX (10 μM) and an mGluR5 antagonist MPEP (100 μM). Glutamate (10 μM) was applied to the neurons for 30 sec twice separated by 10 min. Cells were then pretreated with these antagonists for 5 min. (B) The glutamate-mediated Ca^{2+} influx in neurons are summarized. Data represent the mean ± S.E.M. of 36-186 cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. glutamate control.

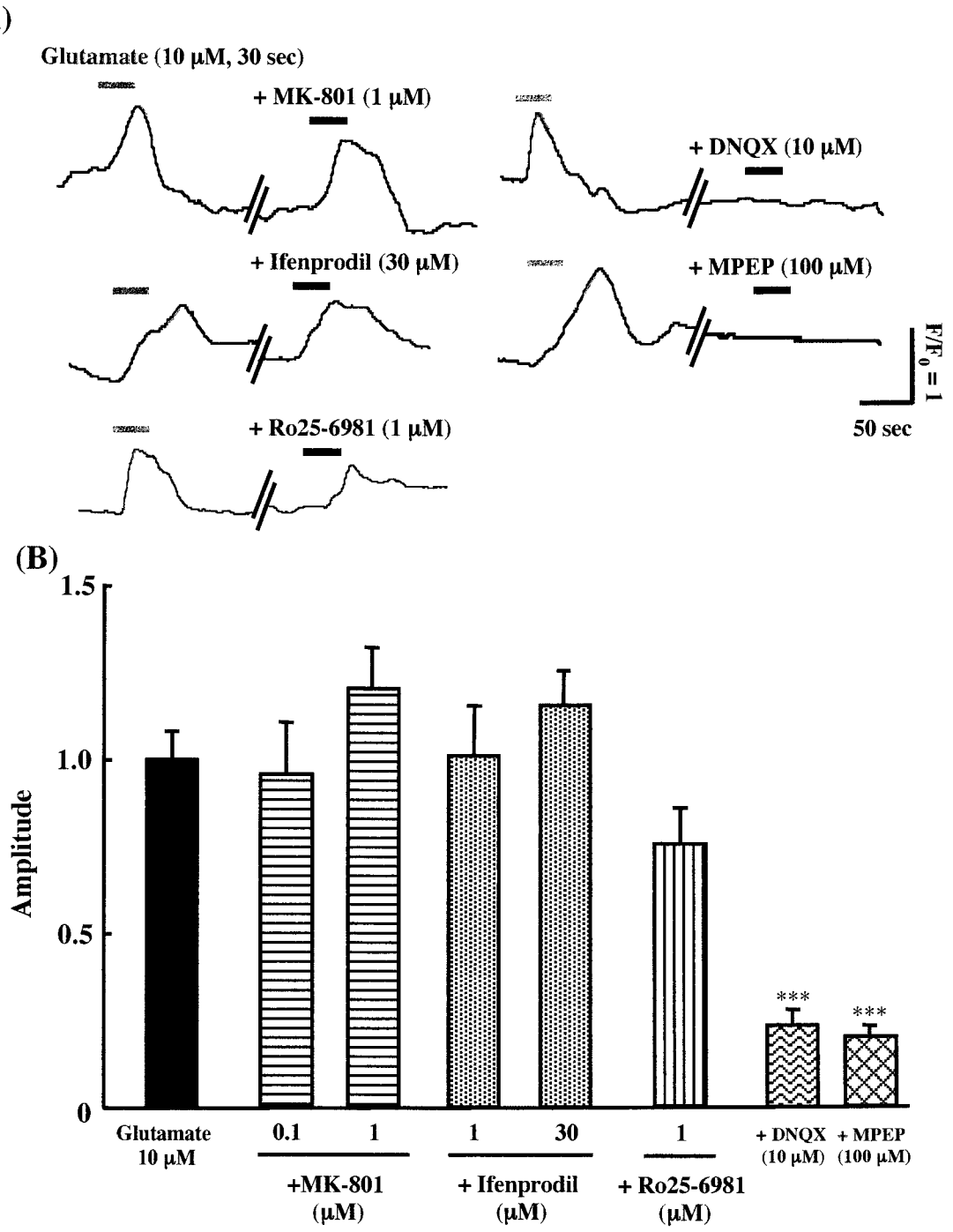

Fig. 15 The glutamate-mediated Ca^{2+} influx in mouse cortical astrocytes. Characterization of glutamate-evoked increase in intracellular Ca^{2+} concetration in mouse cortical astrocytes. (A) Typical traces show the glutamate-evoked increase in intracellular Ca^{2+} concetration, showing the effects of a non-selective NMDA receptor antagonist MK-801 (1 μM), selective NR2B subunit-containing NMDA receptor antagonists ifenprodil (30 μM) and Ro25-6981 (1 μM), an AMPA/kainate receptor antagonist DNQX (10 μM) and an mGluR5 antagonist MPEP (100 μM). Glutamate (10 μM) was applied to the astrocytes for 30 sec twice separated by 10 min. Cells were then pretreated with these antagonists for 5 min. (B) The Ca^{2+} responses to glutamate in neurons are summarized. Data represent the mean ± S.E.M. of 36-144 cells.
***: $p<0.001$ vs. glutamate control.

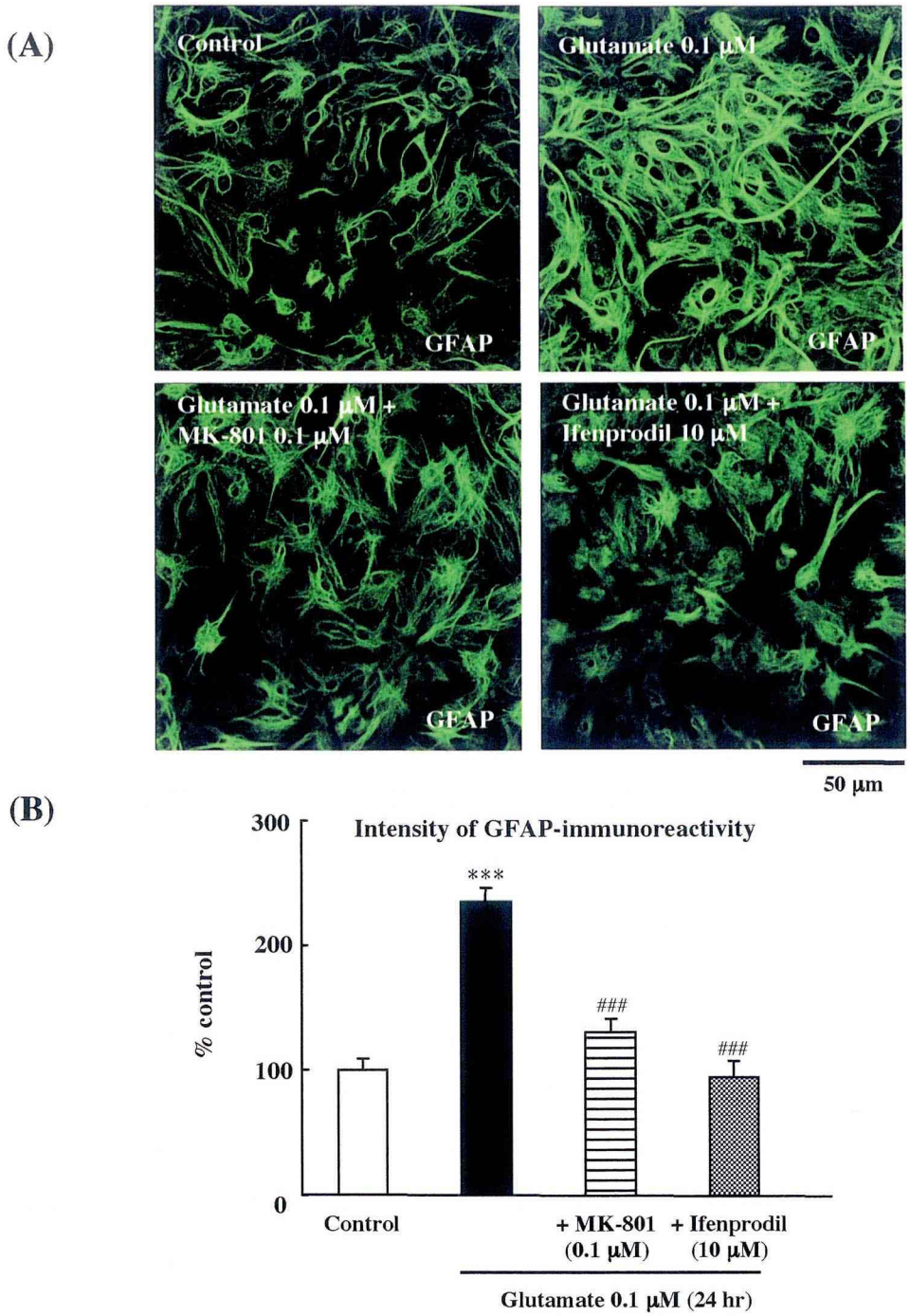

Fig. 16 Effects of NMDA receptor antagonists on glutamate-induced astrocytic activation under the statement of neuron/glia cocultures. Astorocytic activation induced by treatment with glutamate (0.1 μM) for 24 hr was blocked by co-treatment with a non-selective NMDA receptor antagonist MK-801 and a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil. (A) Cortical neuron/glia cocultures were treated with normal medium (control), glutamate (0.1 μM), MK-801 (0.1 μM) or ifenprodil (10 μM) + glutamate (0.1 μM). The cells were stained with an antibody to GFAP. (B) The density of GFAP-like immunoreactivity of each image was measured using NIH image. The levels of GFAP-like immunoreactivities on glutamate-treated cells are expressed as a percent increase (mean ± S.E.M.) with respect to that on control cells.

*** $p<0.001$ vs. control cells. ### $p<0.001$ vs. glutamate alone-treated cells.

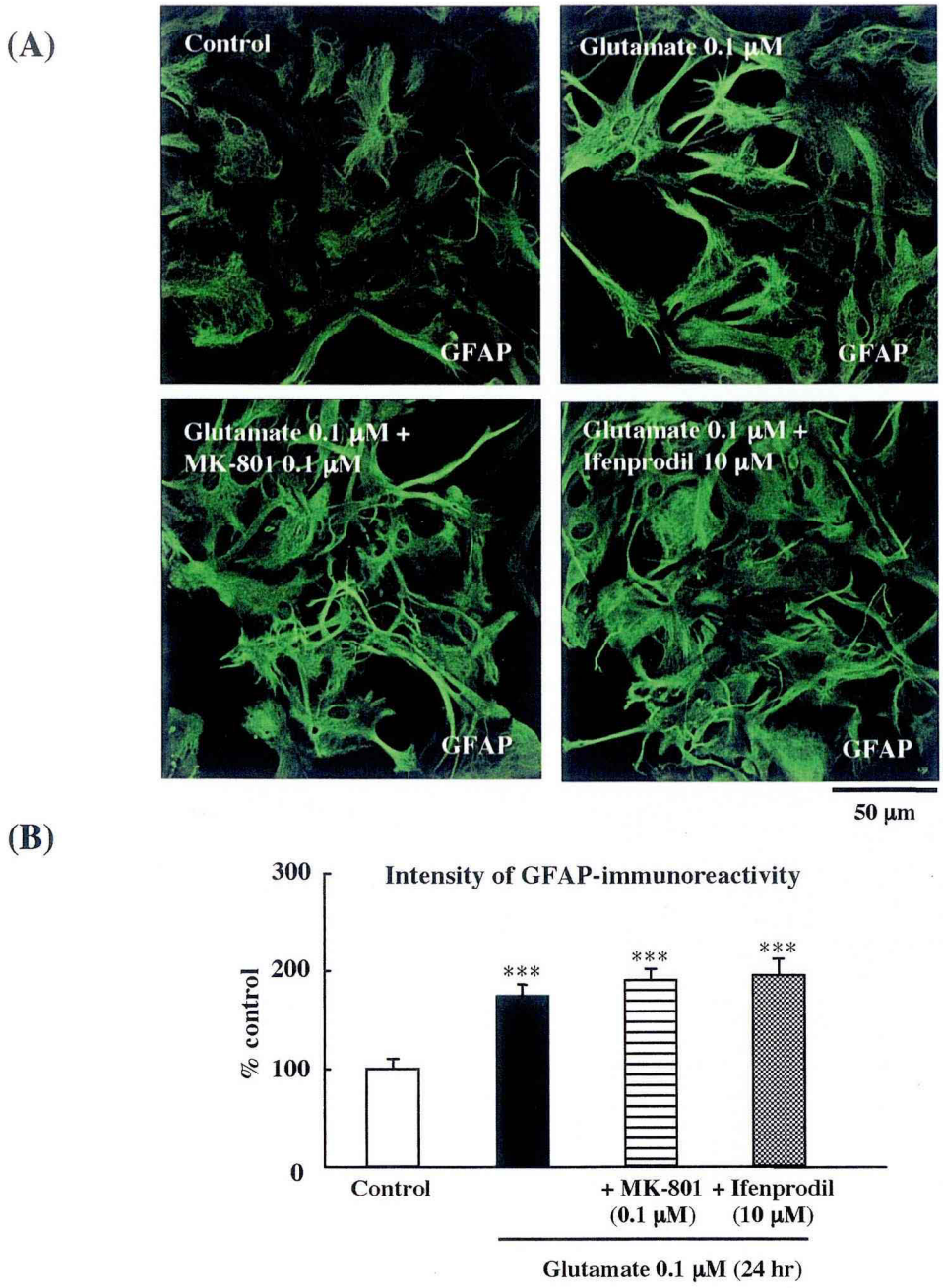

Fig. 17 Effects of NMDA receptor antagonists on glutamate-induced purified astrocytic activation. Purified astorocytic activation induced by treatment with glutamate (0.1 μM) for 24 hr was blocked by co-treatment with a non-selective NMDA receptor antagonist MK-801 and a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil. (A) Cortical purified astrocytes were treated with normal medium (control), glutamate (0.1 μM), MK-801 (0.1 μM) or ifenprodil (10 μM) + glutamate (0.1 μM). The cells were stained with an antibody to GFAP. (B) The density of GFAP-like immunoreactivity of each image was measured using NIH image. The levels of GFAP-like immunoreactivities on glutamate-treated cells are expressed as a percent increase (mean ± S.E.M.) with respect to that on control cells.

*** $p<0.001$ vs. control cells.

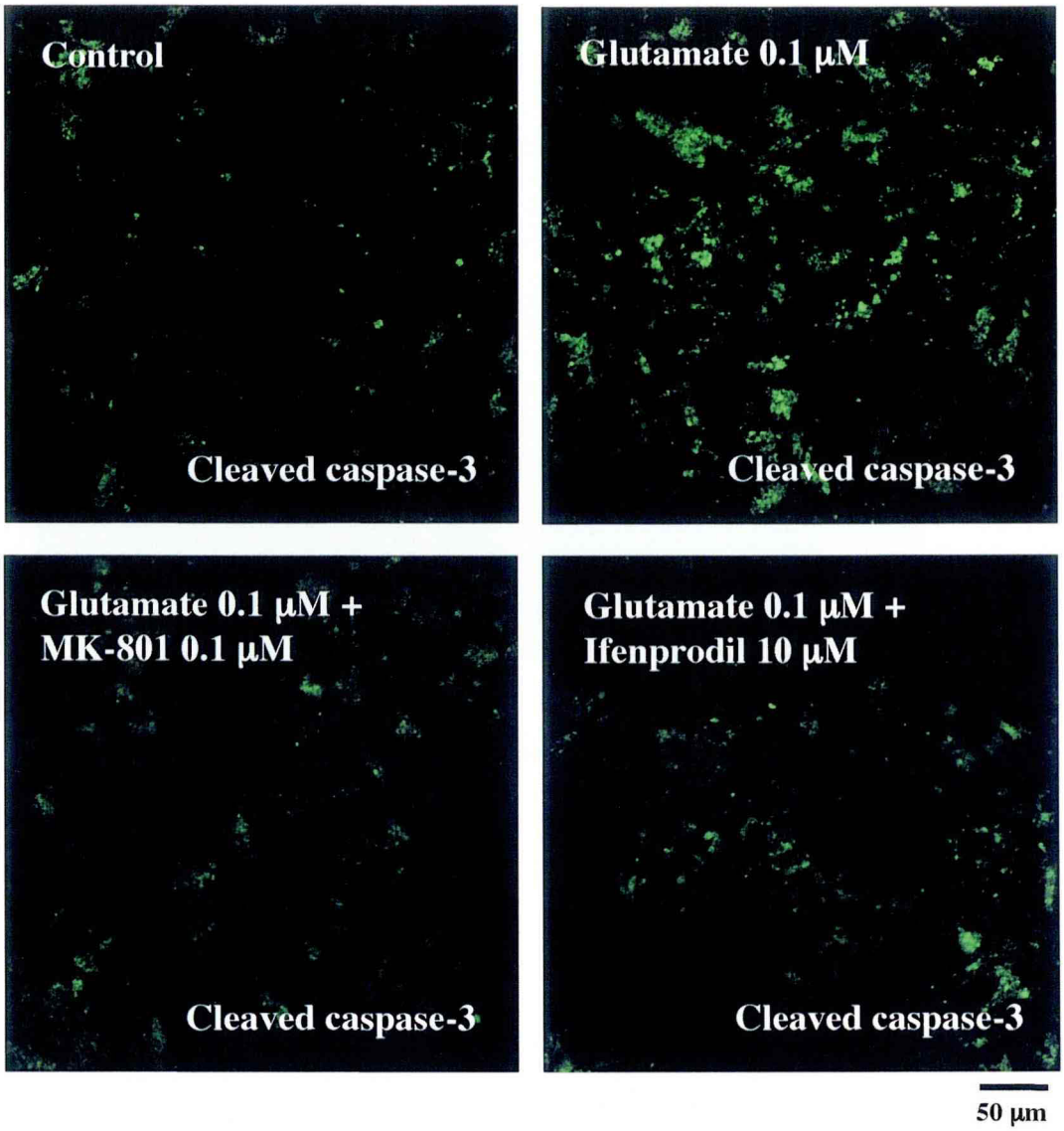

Fig. 18 Effects of NMDA receptor antagonists on glutamate-induced apoptotic neuronal cell death. Apoptotic neuronal cell death induced by treatment with glutamate (0.1 μM) for 24 hr was blocked by co-treatment with a non-selective NMDA receptor antagonist MK-801 and a selective NR2B subunit-containing NMDA receptor antagonist ifenprodil. Cortical neuron/glia cocultures were treated with normal medium (control), glutamate (0.1 μM), MK-801 (0.1 μM) or ifenprodil (10 μM) + glutamate (0.1 μM). The cells were stained with an antibody to cleaved caspase-3.

Discussion

The NMDA receptors exhibit several channel properties distinct from other non-NMDA receptors, including a high Ca^{2+}/Na^{+} permeability ratio, a voltage-dependent Mg^{2+} block, and a requirement for glycine as co-agonist [13-16]. The Ca^{2+} influx through NMDA receptors plays key roles synaptic transmission, neuronal development, and plasticity. A recent new insight into the physiology of neuron/glia has emerged, leading to a different view of its role in the CNS, i.e., "active regulation of neuronal function". Using a Ca^{2+} imaging technique, I investigated the glutamate-evoked increase in intracellular Ca^{2+} concentration in cultured mouse cortical neurons and astrocytes and clarified mechanisms underlying the $[Ca^{2+}]i$ elevation. Glutamate produced the increase in levels of $[Ca^{2+}]i$ in a concentration-dependent manner in both neurons and astrocytes. The glutamate-evoked $[Ca^{2+}]i$ elevation was inhibited by a non-selective NMDA receptor antagonist MK-801 and selective NR2B subunit-containing NMDA receptor antagonists ifenprodil and Ro25-6981 in neurons, but not in astrocytes. Ro25-6981 is more potent and selective blocker of NR2B subunit-containing NMDA receptor than ifenprodil [94]. These data suggest that Ca^{2+} influx into neurons evoked by glutamate is mainly regulated through the activation of NMDA receptors, including NR2B subunit-containing NMDA receptors. Glutamate receptors are the most ubiquitous receptor types expressed in neuronal synapses and astrocytes. The excitatory neurotransmitter glutamate released from activated neurons stimulates two types of glutamate receptors, ionotropic glutamate receptors, which are ligand-gated ion channels and modulate

synaptic response, and mGluRs [95]. Two members of the mGluR family, mGluR3 and mGluR5 subtypes, have been detected in glial cell by antibody staining and in situ hybridization [96,97]. It was demonstrated that the Ca^{2+} responses to glutamate were mediated by the mGluR5 subtype in astrocytes [98]. Therefore, I investigated whether other types of glutamate receptors, such as AMPA/kainate and mGluRs, could suppress the glutamate-evoked Ca^{2+} influx into neurons and astrocytes. In both neurons and astrocytes, glutamate-evoked Ca^{2+} influx into neurons and astrocytes was suppressed by either an AMPA/kainate receptor antagonist DNQX or an mGluR5 antagonist MPEP, suggesting that AMPA/kainate and mGluRs are also responsible for Ca^{2+} influx evoked by glutamate into neurons and into astrocytes. These inhibitory effects of the AMPA/kainate receptor antagonist and the mGluR5 antagonist were more potent in astrocytes than in neurons. These results suggest that AMPA/kainate receptors and mGluRs may be critical for glutamate-evoked Ca^{2+} influx into astrocytes.

Recently, astrocytes have been reported to induce and/or stabilize CNS synapses [99] and may be capable of integrating neuronal inputs and modulating synaptic activity. GFAP is an intermediate filament protein expressed predominantly in the astrocytes of the CNS. The morphological changes that occur in astrocytes produce what are collectively known as reactive astrocytes, which are characterized by specific changes such as the accumulation of intermediate-filament GFAP and hypertrophy [100-102]. Ifenprodil, which is clinically used in USA as a neuroprotective agent for head ischemia, Parkinson's disease and stroke, blocks NR2B subunit-containing NMDA receptor in a non-competitive, voltage-independent and activity-dependent manner. However, little is

known about the roles of NR2B subunit-containing NMDA receptor on glutamate-induced astrocytic activation. In the present study, treatment with glutamate in cortical neuron/glia cocultures and astrocytes with for 24 hr caused the astrocytic activation, as detected by a stellate morphology, an increase in the levels of GFAP-like immunoreactivities. These effects were reversed by either a non-selective NMDA receptor antagonist or selective NR2B subunit-containing NMDA receptor antagonists in neuron/glia cocultures. In purified astrocytes, glutamate-induced astrocytic activation was not suppressed by the co-treatment with NMDA receptor antagonists. It is, therefore, likely that NR2B subunit-containing NMDA receptor in neurons, but not in astrocytes contributed to the glutamate-induced astrocytic activation.

Furthermore, glutamate-mediated Ca^{2+} influx via NR2B subunit-containing NMDA receptor may, at least in part, contribute to neuron-to-astrocyte signaling. Additionally, treatment of cortical neuron/glia cocultures with glutamate caused the apoptotic neuronal cell death, as detected by an increase in the levels of cleaved caspase-3-like immunoreactivities. These effects were reversed by the treatment with selective NR2B subunit-containing NMDA receptor antagonist ifenprodil, suggesting that NR2B subunit-containing NMDA receptor may be involved in the glutamate-induced apoptotic neuronal cell death.

In conclusion, the present data indicate that NR2B subunit-containing NMDA receptor plays a critical role in glutamate-evoked Ca^{2+} influx into neurons, but not into astrocytes. Furthermore, neuronal NR2B subunit-containing NMDA receptor can modulate the glutamate-induced astrocytic activation and apoptotic neuronal cell death.

General Conclusion

The above findings lead to the following conclusions (Fig. A):

In Chapter 1:

I demonstrated that the non-selective NMDA receptor antagonists ketamine and MK-801, which have similar affinity for NR2A and NR2B subunit-containing NMDA receptors, induce the rewarding effect. In addition, it is worth noting that the selective NR2B subunit-containing NMDA receptor antagonist ifenprodil, which alone does not produce rewarding effect, dramatically suppresses the morphine-induced rewarding effect. These findings suggest that NR2B subunit-containing NMDA receptor may be, at least in part, responsible for the morphine-induced rewarding effect. Furthermore, I found that ifenprodil, which is clinical used, potentiates the morphine-induced antinociception. Considering its availability and advantages, ifenprodil may be useful as an adjuvant analgesic.

In Chapter 2:

The present study was undertaken to further clarify the role of tyrosine phosphorylation of NR2B subunit-containing NMDA receptor in the morphine-induced rewarding effect in mice. The level of phospho-Tyr-1472, but not phospho-Ser-1303, NR2B subunit was significantly increased in the mouse limbic forebrain containing the

N.Acc. of mice that had shown the morphine-induced rewarding effect. In addition, the level of phospho-Tyr-416 Src family kinase was also increased in the limbic forebrain of mice that had shown the morphine-induced rewarding effect. Furthermore, an i.c.v. pretreatment with a selective Src family kinase inhibitor PP2 significantly suppressed the morphine-induced rewarding effect. I also found a significant increase in the immunoreactivity for phospho-Tyr-416 Src family kinase in the N.Acc. of mice shown the morphine-induced rewarding effect. Furthermore, the increased immunoreactivity for phospho-Tyr-416 Src family kinase was clearly noted in the N.Acc. of mice shown the morphine-induced rewarding effect. These findings suggest that phospho-Tyr-1472 NR2B subunit-containing NMDA receptor associated with activation of Src family kinase in the limbic forebrain, especially N.Acc., may be critical for the expression of the morphine-induced rewarding effect.

In Chapter 3:

The excitatory neurotransmitter glutamate has been shown to mediate such bidirectional communication between neurons and astrocytes. In this chapter, I determined the role of NMDA receptors on glutamate-evoked Ca^{2+} influx into neurons and astrocytes. Either a non-selective NMDA receptor antagonist MK-801 or selective NR2B subunit-containing NMDA receptor antagonists ifenprodil and Ro25-6981 significantly inhibited the glutamate-evoked Ca^{2+} influx into neurons, but not into astrocytes. Furthermore, I investigated whether NR2B subunit-containing NMDA

receptor antagonists could suppress the astrocytic activation induced by glutamate, as detected by GFAP (as a specific marker of astrocyte)-like immunoreactivities in mouse cortical astrocytes. I demonstrated here that the increased level of GFAP-like immunoreactivity induced by glutamate was dramatically suppressed by co-treatment with ifenprodil in cortical neuron/glia cocultures, but not in purified astrocytes. These results suggest that NR2B subunit-containing NMDA receptor plays a critical role in glutamate-evoked Ca^{2+} influx into neurons, but also glutamate-induced astrocytic activation.

Based on the findings noted, I propose here that Tyr-1472 phosphorylation of NR2B subunit-containing NMDA receptor, which can regulate the glutamate-evoked Ca^{2+} influx into neurons, at the postsynaptic site in the N.Acc. may play an important role in the rewarding effect of morphine. This Tyr-1472 phosphorylation of NR2B subunit-containing NMDA receptor is associated with activation of Src family kinases.

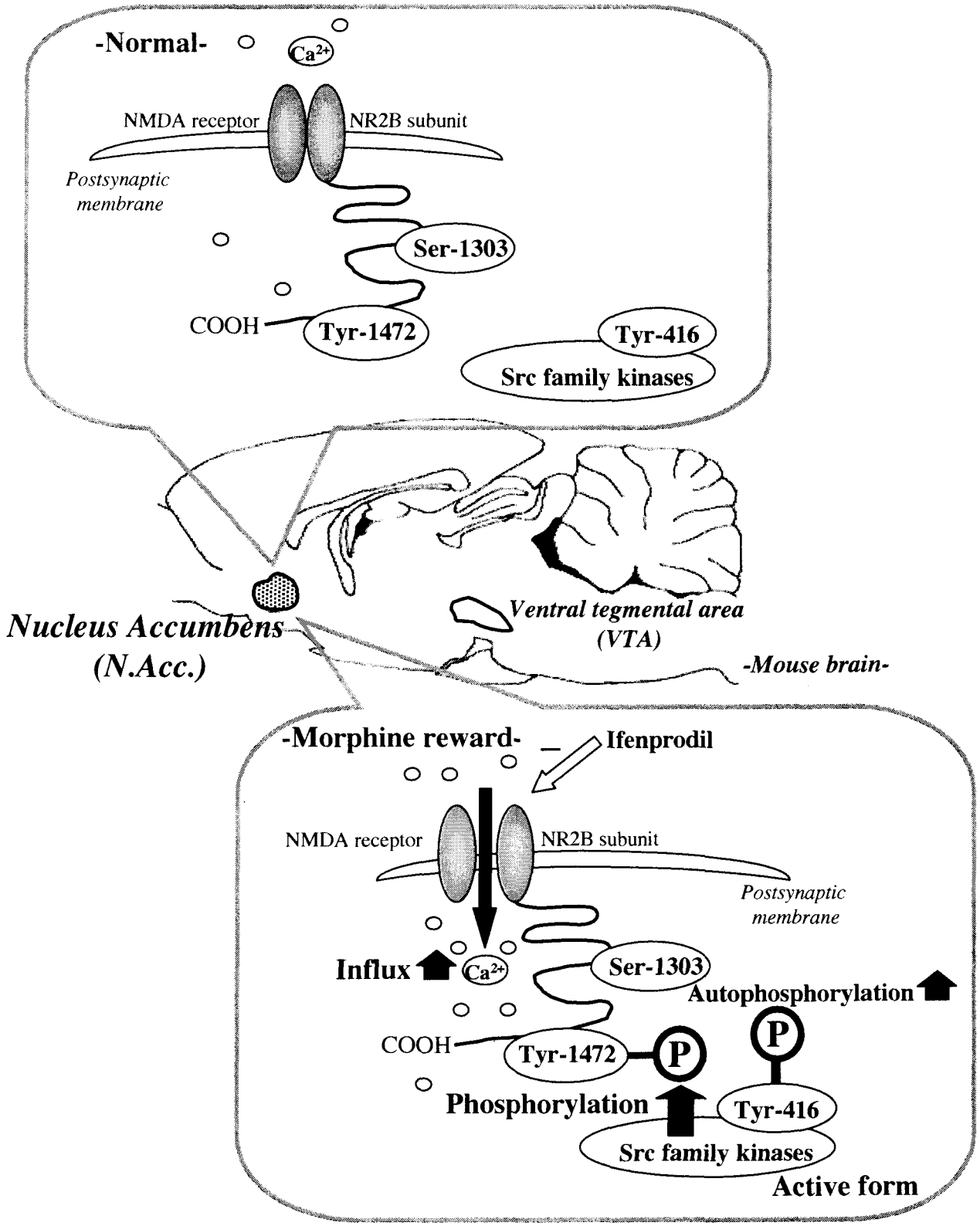

Fig. A Schematic model of morphine reward in the nucleus accumbens. The mesolimbic dopaminergic pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens (N.Acc.). Tyr-1472 phosphorylation of NR2B subunit-containing NMDA receptor at the postsynaptic site in the N.Acc. may play an important role in the rewarding effect of morphine. This Tyr-1472 phosphorylation of NR2B subunit-containing NMDA receptor is associated with activation of Src family kinases.

List of Publications

This dissertation is based on the following original publications:

1. Tsutomu Suzuki, Hideaki Kato, Makoto Tsuda, Hajime Suzuki and Miwa Misawa: Effects of the non-competitive NMDA receptor antagonist ifenprodil on the morphine-induced place preference in mice. *Life Sci* **64**, 151-156 (1999): **Chapter 1**

2. Tsutomu Suzuki, Hideaki Kato, Takeshi Aoki, Makoto Tsuda, Minoru Narita and Miwa Misawa: Effects of the non-competitive NMDA receptor antagonist ketamine on morphine-induced place preference in mice. *Life Sci* **67**, 383-389 (2000): **Chapter 1**

3. Hideaki Kato, Mihoko Metoki, Yoshinori Yajima, Minoru Narita and Tsutomu Suzuki: Usefulness of the NMDA receptor antagonist ifenprodil as adjuvant analgesic of morphine. *Kanwairyougaku* **2**, 85-95 (2000): **Chapter 1**

4. Hideaki Kato, Minoru Narita, Kan Miyoshi, Michiko Narita, Megumi Asato, Nana Hareyama, Hiroyuki Nozaki, Tomoe Takagi, Masami Suzuki and Tsutomu Suzuki: Implication of Src family kinase-dependent phosphorylation of NR2B subunit-containing NMDA receptor in the rewarding effect of morphine. *Jpn J Neuropsychopharmacol* **26**, 119-124 (2006): **Chapter 2**

5. Minoru Narita, Hideaki Kato, Akiko Kasukawa, Michiko Narita, Masami Suzuki, Tomoko Takeuchi and Tsutomu Suzuki: Role of Src family kinase in the rewarding effect and hyperlocomotion induced by morphine. *Neuroreport* **17**, 115-119 (2006): **Chapter 2**

6. Hideaki Kato, Minoru Narita, Mayumi Miyatake, Yoshinori Yajima and Tsutomu Suzuki: Role of neuronal NR2B subunit-containing NMDA receptor-mediated Ca^{2+} influx and astrocytic activation in cultured mouse cortical neurons and astrocytes. *Synapse* **59**, 10-17 (2006): **Chapter 3**

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