Molecular mechanisms of the development of tolerance to morphine-induced antinociception following repeated treatment with morphine in the mouse spinal cord

2006

Masami Suzuki

A dissertation submitted in partial fulfillment of the requirements leading to the degree of Doctor (Pharmacy) presented to the Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, Tokyo, Japan

This dissertation is dedicated to my parents, my grandmother and my brothers.

Table of Contents

Abbreviations

Chemical substances and drugs

BCA: Bicinchoninate

DAMGO: [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin

DHPG: 3,5-Dihydroxyphenylglycine

EDTA: Ethylenediaminetetraacetic acid

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

PPF: Propentofylline

SDS: Sodium dodecyl sulfate

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

Buffers

PBS: Phosphate-buffered saline

TBS: Tris-buffered saline

TTBS: Tween 20-TBS

Endogenous substances

ATP: Adenosine 5'-triphosphate

BDNF: Brain-derived neurotrophic factor

DAG: Diacylglycerol

IL: Interleukin

IP_3: Inositol (1,4,5)-triphosphate

PG: Prostaglandin

TNF: Tumor necrosis factor

Enzymes and intracellular messengers

GRK: G-protein coupled receptor kinase

PKC: Protein kinase C

PLC: Phospholipase C

p-cPKC: Phosphorylated-conventional PKC

MAPK: Mitogen-activated protein kinase

Receptors

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate

GPCR: G-protein coupled receptor

iGluR: Ionotropic glutamate receptor

mGluR: Metabotropic glutamate receptor

NMDA: N-methyl-D-aspartate

Serum

NGS: Normal goat serum

NHS: Normal horse serum

Injection route

i.t.: Intrathecal

s.c.: Subcutaneous

Others

ANOVA: Analysis of variance

ACM: Astrocyte-conditioned medium

B_{max}: Binding maximum

DMEM: Dulbecco's modified Eagle's medium

EGFP: Enhanced green fluorescent protein

GFAP: Glial fibrillary acidic protein

IR: Immunoreactivity

K_d: Constant dissociation

LSN: Lateral spinal nucleus

LTD: Long term depression

LTP: Long term potentiation

MAP2a/b: Microtubule associated protein 2a/b

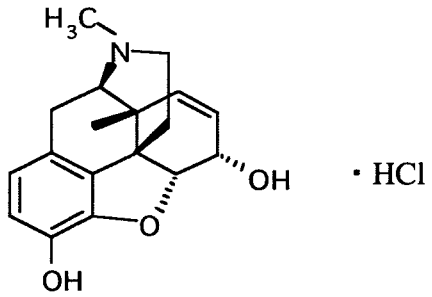
MPE: Maximum possible effect

NeuN: Neuronal nuclei

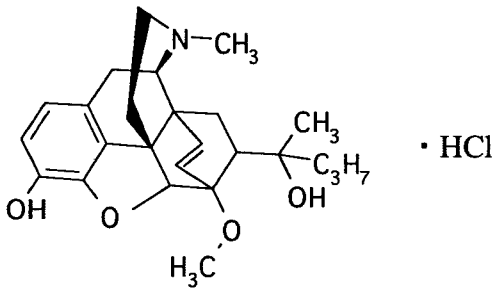
SEM: Standard error mean

Structures of drugs used in the present study

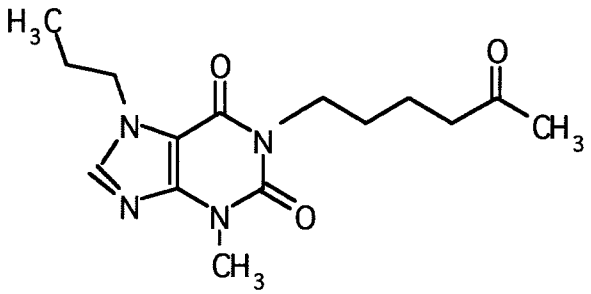
Morphine hydrochloride



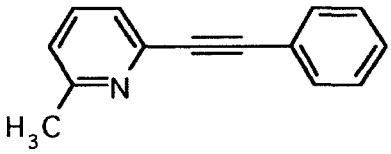
Etorphine hydrochloride



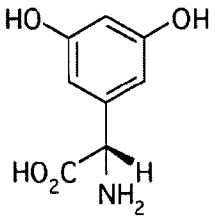
Propentofylline: 3-Methyl-1-(5-oxohexyl)-7-propylxanthine



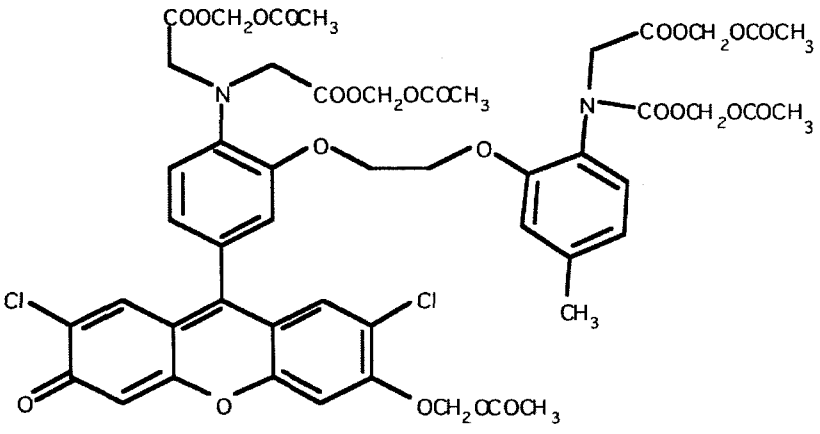
MPEP: 2-Methyl-6-(phenylethynyl)-pyridine hydrochloride



S-3,5-DHPG: (S)-3,5-Dihydroxyphenylglycine



Fluo-3 acetoxymethyl ester



General Introduction

Opioid receptors

The plant alkaloids derived from the opium poppy will be referred to as opiates (e.g., opium, heroin, morphine) and the broader class of related endogenous peptides and receptors as opioids. Current research has shown that the administration of opioids results in a variety of physiological processes such as analgesia, changes in mood, respiratory depression, decreased gastrointestinal motility, nausea, vomiting and alterations of neuroendocrine function and cardiovascular control. These diverse effects of the opioid system are mediated by the activation of specific membrane receptors in both neuronal and non-neuronal tissues.

Pharmacological studies using peptide and alkaloid opioid ligands have identified several classes of opioid receptors, including μ, δ and κ receptors [1-3]. In 1992, a δ-opioid receptor was first cloned by two independent groups, Evans *et al.* [4] and Kieffer *et al.* [5]. Following the cloning of the δ-opioid receptor, μ- and κ-opioid receptors have been cloned in the past several years [6-9]. Studies on cDNA clones encoding structurally related receptors with amino acid have suggested the similarity of these three receptor genes as high as 65 % [10,11]. The cloning of these opioid receptor types revealed that they are members of the G-protein-coupled receptor (GPCR) superfamily. Opioid receptors are coupled to pertussis toxin-sensitive Gi/o protein, which is linked functionally to the inhibition of adenylyl cyclase activity, the activation of receptor-operated K^+ currents, and the suppression of voltage-gated Ca^{2+} currents [12-14].

Recently, it has been demonstrated that opioid receptors may be coupled to other second-messenger systems, including the activation of mitogen-activated protein (MAP) kinases and the phospholipase C (PLC)/protein kinase C (PKC)-mediated cascade, through the βγ subunits of their G protein [15-17].

Opioid tolerance

Opioids have been recommended as the drug of choice for the management of patients with chronic cancer pain by Cancer Pain Relief Program of the World Health Organization Cancer Unit [18]. However, opioids also have undesirable effects, such as tolerance, physical and psychological dependence.

Tolerance can be described as an adaptation of a biological system to the continued or repeated effects of a drug and defined as a loss of potency of a drug after its repeated administration. In the field of opioids, the concept of tolerance is usually equated with the phenomenon termed "chronic tolerance", i.e., tolerance which (1) is induced by the repeated administration of opioid agonist and (2) refers to a state in which it is necessary to increase the dose to achieve the original effects.

The development of chronic tolerance is characteristic of all opioids with agonist activity, regardless of the type of receptor with which they interact. Tolerance to opioids first becomes evident as a shortening of the duration of drug action and a diminution of the peak effects. The rate at which tolerance develops depends on the pattern of use and the characteristics of the opioids used. Significant tolerance only develops when there is a continuous drug action.

μ-Opioid receptor desensitization

Desensitization is defined as the progressive loss of receptor function under continued exposure to an agonist. Receptor activation often leads to the removal of receptors from the cell surface by internalization, and less often, to recruitment of new receptors to the cell surface. Internalized receptors can be recycled to the cell surface (resensitization) for further duty or targeted for degradation in lysosomes (down-regulation). Prolonged stimulation generally leads to a profound receptor loss from the cell surface [19,20]. One major mechanism controlling GPCR responsiveness is the activation-dependent regulation of receptors, which is called homologous desensitization [21,22]. Other mechanisms also contribute to the intrinsic regulation of GPCR signaling. These include receptor activation-independent regulation of receptors, called heterologous desensitization, which results from regulating and altering the signaling efficiency of downstream effectors [19, 23].

Like other GPCRs, μ-opioid receptor can undergo rapid agonist-mediated internalization via a classic endocytic pathway. Opioid-receptor internalization is mediated, at least partially, by the actions of G-protein coupled receptor kinase (GRK). GRKs selectively phosphorylate agonist-bound receptors thereby promoting interactions with β-arrestin, which interfere with G protein coupling and promote receptor internalization [24-27]. Although a great deal has been learned about the mechanism mediating the initial endocytosis of certain GPCRs including μ-opioid

receptor from the plasma membrane, a little is known about mechanisms underlying the development of tolerance to morphine-induced antinociception.

Protein kinase C (PKC)

PKC, which is activated by 1,2-diacylglycerol in the presence of Ca^{2+} and phospholipids, acts as a key enzyme for signal transduction in various physiological processes [28-30]. The protein phosphorylation catalyzed by PKC may exert profound modulation of various processes, such as the release of neurotransmitters, cell proliferation and differentiation, potentiation and desensitization of several kinds of receptor systems [28-30]. Recent studies have revealed a family of closely related proteins subdivided on the basis of certain structural and biochemical similarities: Ca^{2+}-dependent or conventional isoforms (PKCα, βI, βII and γ; cPKCs), Ca^{2+}-independent or novel isoforms (PKCδ, ε, η and θ; nPKCs), and atypical isoforms (PKCλ and ζ; aPKCs) [31,32]. PKCα, βI, βII, γ, ε δ and ζ isoforms have been identified in the brain and spinal cord. The various isoforms appear to be differentially distributed in the mammalian central nervous system, with cell type and cellular component specificity [33,34].

Considerable evidence suggests that the activation of PKC in the spinal cord modulates the enhancement of neuronal excitability, which results in nociceptive pain perception [35-38]. It has been reported that activation of PKCγ isoform in the dorsal horn of the spinal cord is involved in the development of tolerance to morphine-induced antinociception and the enhancement of nociceptive responses [39-41]. These findings

strongly suggest that PKCγ isoform in the spinal cord may play an important role in modulating nociceptive transmission and neuronal plasticity.

Astrocytes

Cell interactions are obviously the basis of function of the nervous system as much as of the function of any other body system. It was thought for a long time that the relevant cell interactions for brain function were those of neurons with other neurons. Now it is more and more evident that taking into consideration the interactions of neurons with glial cells is essential to understand how the brain works. Neuron-glia cross talk appears to be fundamental for the most basic phenomenon in the nervous system, that is, the transfer of information by chemical synapses [42].

Astrocytes are the principal type of glial cell and are known to support the proliferation, survival and maturation of developing neurons. In the mature mammalian brain, astrocytes constitute nearly half of the total cells, providing structural, metabolic and trophic support for neurons [43]. There is a large body of evidence indicating that astrocytes possess a wide variety of voltage-gated ion channels and functional receptors for neurotransmitters [44-46]. Activation of astrocytes receptors induces the synthesis and release of substances capable of modulating the surrounding cells, including neurons [47,48]. This evidence suggests the idea that astrocytes are responsive to their environment and that they have the potential to modulate neuronal activity in response to neuronal and synaptic activities. Furthermore, considerable evidence suggests that the glial network may be quite important in maintaining the

basal level of excitability of neuronal circuit, shaping its properties in an integrated fashion. Perhaps the purpose of the glial network is to maintain the neuronal network in a functional state that is adjusted from the previous experience of the glial and neuronal networks. These findings suggest that two-way communication between neurons and astrocytes plays a substantial role in the regulation of neuronal activity and synaptic transmission.

Glutamate receptor

Glutamate is the major excitatory neurotransmitter of the central nervous system. The interaction of glutamate with its receptors is essential for the normal function of the central nervous system, including cognition, sensation and memory. Glutamate activates two major classes of receptors: ionotropic glutamate receptor (iGluR) and metabotropic glutamate receptor (mGluR). iGlu receptors are glutamate-gated ion channels that, when activated, increase cationic flux (mainly Na^+ and K^+ and to a lesser extent Ca^{2+}) across the neuronal membrane and thereby increase cellular excitability [49]. iGluRs consist of the following receptor subtypes: N-methyl-D-aspartate (NMDA) receptors containing NR1, NR2A-D and NR3A-B subunits; α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors containing GluR1-4 subunits; and kainate receptors containing GluR5-7, KA1 and KA2 subunits. Initially, it was believed that iGluRs were exclusively involved in gating glutamate-mediated transmission. However, in the mid-1980s glutamate was shown to activate intracellular signaling cascades in a GPCR-dependent manner [50], suggesting the

existence of "metabotropic" glutamate receptors. Subsequently, eight mammalian subtypes of mGluRs have been identified and classified into three groups (I, II and III) [51,52]. Group I mGluRs (mGluR1 and mGluR5) are predominately located in postsynaptic neurons where they couple to Gq proteins to activate phospholipase C (PLC). Group II mGluRs (mGluR2 and mGluR3) are found both pre- and postsynaptically and are coupled to Gi/o proteins to negatively regulate the activity of adenylyl cyclase [52]. Group III mGluRs (mGluR4, mGluR6, mGluR7 and mGluR8) are predominately located presynaptically where they act as autoreceptors, and are also coupled to Gi/o proteins to decrease adenylyl cyclase activity [52,53]. Recently, a growing body of evidence suggests that changes in glutamate transmission are associated with a number of central nervous system pathologic conditions, including chronic pain, opioid tolerance and dependence.

Aim and Scope

The aim of the present study was to investigate the molecular mechanism of the development of tolerance to morphine-induced antinociception in the mouse spinal cord. Behavioral, neurochemical and biochemical experiments were conducted.

The specific aims of the proposed research are:

In Chapter 1:

To clarify the distinct mechanisms of the effects of μ-opioid receptor agonists, etorphine and morphine, on the development of tolerance to antinociception, I investigated whether repeated *in vivo* administration of etorphine and morphine could recruit receptor trafficking proteins to the plasma membrane by translocation from the cytoplasm. Furthermore, I examined whether chronic *in vivo* or *in vitro* treatment with etorphine or morphine could affect spinal astrocytes, which could be related to the development of tolerance to etorphine- or morphine-induced antinociception.

In Chapter 2:

This study was to investigate whether repeated *in vivo* treatment with morphine could affect the immunoreactivity for PKCγ in the dorsal horn of the mouse spinal cord following repeated treatment with morphine. In addition, I examined the change in expression of glial fibrillary acidic protein (GFAP) following repeated *in vivo* treatment

with morphine using the transgenic mice with GFAP promoter-controlled enhanced green fluorescent protein (EGFP) expression. Furthermore, a direct approach with PKCγ knockout mice was used to investigate the influence of the PKCγ gene deletion in the astroglial response following repeated *in vivo* treatment with morphine in the dorsal horn of the mouse spinal cord.

In Chapter 3:

To clarify whether metabotropic glutamate receptor 5 (mGluR5) within the spinal cord could be involved in the development of tolerance to morphine-induced antinociception, the present study was undertaken to investigate the effect of selective mGluR5 antagonist on the development of morphine tolerance in mice. I also examined the changes in the number of and the immunoreactivity (IR) for mGluR5 in the spinal cord of morphine-tolerant mice. In addition, I investigated the effect of chronic treatment with morphine on the increase in the intracellular Ca^{2+} concentration induced by a selective group I mGluR agonist in primary spinal neurons.

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 the 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

μ-Opioid receptor internalization-dependent and -independent mechanisms of the development of tolerance to μ-opioid receptor agonists: Comparison between etorphine and morphine

Introduction

The administration of opioids produces a powerful antinociception/analgesia. It is well known that long-term exposure to opioids results in tolerance to opioids-induced antinociception, which is related to the receptor desensitization. Studies on β-adrenergic receptors [54,55], later extended to μ-opioid receptor, have shown that the process of receptor desensitization results from a series of events beginning with receptor phosphorylation, uncoupling with G-proteins and ending with receptor internalization [25-27]. Agonist-induced activation of GPCR specifically activates G protein-coupled receptor kinases (GRKs), probably through liberated G βγ subunits [25-27]. GRKs phosphorylate serine and threonine in the carboxy terminus of the μ-opioid receptor. This conformational change of the receptor increases its affinity for β-arrestin. The binding of β-arrestin prevents the receptor from interacting with G proteins, leading to desensitization. Clusters of receptor-β-arrestin complexes segregate on the cell surface and internalize by a clathrin- and dynamin-dependent process [56]. These reports suggest that receptor desensitization results from several events ending with receptor internalization. However, a number of observations have indicated that receptor desensitization can occur in the absence of internalization. In addition, opioid agonists exhibit remarkable differences in their ability to desensitize the μ-opioid receptor [57-60]. Recently, it has been reported that chronic treatment with etorphine produces μ-opioid receptor down-regulation associated with the increase in dynamin II protein in the spinal cord, whereas chronic treatment with morphine does not change μ-opioid receptor

density and trafficking proteins [61,62]. Considering these backgrounds, the detailed molecular events underlying this differential regulation of the μ-opioid receptor by distinct agonist remain unclear.

In the central nervous system, there are two categories of cells: neurons and glial cells including astrocyte, microglia and oligodendrocyte. Two-way communication between neurons and glial cells is considered to be essential for axonal conduction and synaptic transmission [63]. Expression of glial fibrillary acidic protein (GFAP), a cytoskeletal intermediate filament protein, is found almost exclusively in mature astrocytes, and its specificity and abundance has made it the most commonly used marker for astrocytes. While GFAP is known to be present in normal astrocytes, the activated forms of astrocytes called reactive astrocytes are well known by the high level of GFAP expression [64]. A growing body of evidence suggests that the activation of spinal cord astrocyte is sufficient to create exaggerated pain states and identifies a potential neuron-to-glia signal capable of driving pathological pain [65]. Recently, quantitative changes in level of GFAP can be observed in the spinal cord after chronic administration of morphine [66,67]. These findings suggest that astrocytes may play an active role in the development of tolerance to morphine-induced antinociception and the induction of neuronal plasticity.

In the present study, I investigated whether repeated *in vivo* administration of μ-opioid receptor agonists, etorphine and morphine, could recruit GRK 2, dynamin II, β-arrestin 2 and phosphorylated-conventional PKC (p-cPKC), an active form of cPKC, to the plasma membrane by translocation from cytoplasm. Furthermore, I also

investigated whether chronic *in vivo* and *in vitro* treatment with etorphine or morphine could affect the spinal astrocytes, which is related to the development of tolerance to etorphine- or morphine-induced antinociception.

Materials and Methods

Animals

Male ICR mice weighing about 25 g (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) at the beginning of the experiments were used in the present study. Animals were housed in a room maintained at 23 ± 1 °C with an alternating 12 hr light-dark cycle. Food and water were available *ad libitum* during the experimental period.

Drugs

The drugs used were morphine hydrochloride (Sankyo Co., Tokyo, Japan), a selective μ-opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO, Sigma Chemical Co., St. Louis, MO) and propentofylline (PPF, Sigma Chemical Co.). Etorphine hydrochloride was synthesized by Dr. T. Iwamura (Gifu Pharmaceutical University, Gifu, Japan). All drugs were dissolved in 0.9% sterile saline.

Intrathecal injection

Intrathecal (i.t.) injection was performed as described by Hylden and Wilcox [68] using a 25-μL Hamilton syringe with a 30 1/2-gauge needle. The needle was inserted into the intervertebral space between L5 and L6 of the spinal cord. A reflexive flick of the tail was considered to be a sign of the accuracy of each injection. The injection volume was 4 μL for i.t. injection.

Assessment of antinociception

To investigate the development of antinociceptive tolerance following repeated treatment with etorphine or morphine, mice were repeatedly s.c. injected with etorphine (10 μg/kg), morphine (10 mg/kg) or saline (10 mL/kg) once a day for 7 consecutive days. The etorphine- or morphine-induced antinociceptive response was evaluated by recording the latency to paw licking or tapping in the hot-plate test (55 ± 0.5 °C, Muromachi Kikai Co., Ltd., Tokyo, Japan). The hot-plate latencies were measured 15 min after saline or etorphine and 30 min after saline or morphine injection. To investigate the effect of the glial-modulating agent, propentofylline (PPF, 5.0 mg/kg, i.p.), on the development of tolerance to morphine- or etorphine-induced antinociception, groups of mice were treated with PPF or saline once 30 min before every morphine, etorphine or saline treatment. To examine the role of astrocyte-released soluble factors and astrocytes in the development of tolerance to morphine- or etorphine-induced antinociception, the cultured spinal astrocytes with astrocyte-conditioned medium (astrocytes/ACM) mixture was injected into the spinal cord. Twenty-four hr after a single i.t. injection of astrocytes/ACM mixture or Dulbecco's modified Eagle's medium (DMEM) treatment, mice were repeatedly injected with morphine or etorphine for 7 consecutive days.

To examine the functional change of μ-opioid receptor in the spinal cord following repeated treatment with etorphine or morphine, mice were repeatedly s.c. injected with etorphine (10 μg/kg), morphine (10 mg/kg) or saline (10 mL/kg) once a day for 7 consecutive days. Twenty-four hr after the last injection, mice were i.t. injected with a

selective μ-opioid receptor agonist DAMGO. To ascertain the spinal antinociception, DAMGO-induced antinociception was determined by the tail-flick test (Muromachi Kikai Co., LTD., Tokyo). The tail-flick latencies were measured 10 min after a single i.t. injection of DAMGO. For measurement of the latency of the tail-flick response, mice were held gently by hand with their tail positioned in an apparatus for radiant heat stimulation on the dorsal surface of the tail.

Antinociception was calculated as percentage of the maximum possible effect (% MPE) according to the following formula: % MPE = (test latency – pre-drug latency)/ (cut-off time – pre-drug latency) x 100. The cut-off time that was set at 30 sec for the hot-plate test or 10 sec for the tail-flick test to prevent tissue damage. Antinociceptive response is expressed as the mean % MPE with S.E.M. The ED_{50} value was calculated by GraphPad Prism Programs version 3.0 (GraphPad Software Inc., CA).

Western blotting

Mice were repeatedly injected with etorphine (10 μg/kg s.c.), morphine (10 mg/kg s.c.) or saline (10 mL/kg s.c.) once a day for 7 days. Twenty-four hr after the last injection, mice were sacrificed by decapitation. Their whole spinal cords were removed quickly and homogenized in 10 volumes of ice-cold buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*,-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL of leupeptin, 0.1 mg/mL of aprotinin and 0.32 M sucrose using a Potter-Elvehjem tissue grinder with a Teflon pestle. The

homogenate was centrifuged at 1,000 x g for 10 min at 4 °C and the supernatant was ultracentrifuged at 100,000 x g for 30 min at 4 °C. The pellets were then re-homogenized and re-centrifuged at 100,000 x g for 30 min at 4 °C. The resulting pellets were retained as membrane fractions for subsequent analysis.

An aliquot of tissue sample was diluted with an equal volume of electrophoresis sample buffer (Protein Gel Loading Dye-2x; 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 and transferred to a nitrocellulose membrane in a buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection of GRK 2, dynamin II, β-arrestin 2 and p-cPKC, membranes were blocked in Tris-buffered saline (TBS) containing 5 % nonfat dried milk (Nakarai Tesque, Inc., Kyoto, Japan) for 1 hr at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS containing 5 % nonfat dried milk [1:1000 GRK 2, dynamin II (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), 1:500 β-arrestin 2 (Santa Cruz Biotechnology, Inc.), and 1:1,000 p-cPKC (Cell Signaling Technology Inc., Beverly, MA, USA)] overnight at 4 °C. The membranes were washed in Tween 20-TBS (TTBS) and then incubated for 2 hr at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), which was diluted 1:10,000 in TBS containing 5 % nonfat dried milk. After this incubation, the membranes were washed in TTBS. The antigen-antibody-peroxidase complex was then finally detected by

enhanced chemiluminescence (Pierce, Rockford, IL, USA) and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL, USA). Film autoradiograms were analyzed and quantified by computer-assisted densitometry using NIH Image.

Immunohistochemistry using spinal cord-slice sections

Mice were repeatedly injected with etorphine, morphine and saline once a day for 7 consecutive days. Twenty-four hr after the last injection, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and intracardially perfusion-fixed with freshly prepared 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the lumbar spinal cord was quickly removed, post-fixed in 4 % paraformaldehyde for 2 hr, and permeated with 20 % sucrose in 0.1 M PBS for 1 day and 30 % sucrose in 0.1 M PBS for 2 days with agitation. The L5 lumbar spinal cord segments were then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at -30 °C until use. Frozen spinal cord segments were cut with a freezing cryostat (Leica CM 1510, Leica Microsystems AG, Wetzlar, Germany) at a thickness of 10 μm and thaw-mounted on poly-L-lysine-coated glass slides.

The spinal cord sections were blocked in 10 % normal goat serum (NGS) in 0.01 M PBS for 1 hr at room temperature. The primary antibody was diluted in 0.01 M PBS containing 10 % NGS [1:250 glial fibrillary acidic protein (GFAP, Chemicon International, Inc., Temecula, CA, USA)] and incubated for 48 hr at 4 °C. The

antibody was then rinsed and incubated with an appropriate secondary antibody-conjugated with Alexa 546 (Molecular Probes, Inc., Eugene, OR, USA) for 2 hr at room temperature. Since the staining intensity might vary between experiments, control sections were included in each run of staining.

The slides were then cover-slipped with PermaFluor Aqueous mounting medium (Immunon™; ThermoShandon, Pittsburgh, PA, USA). All sections were observed with a light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus). Digitized images of superficial laminae of the spinal dorsal horn sections were captured at a resolution of 1,316 x 1,035 pixels with camera.

Immunohistochemistry using spinal cord neuron/glia cocultures

Spinal cord neuron/glia cocultures were grown as follows; whole spinal cords were obtained from newborn ICR mice (Tokyo Laboratory Animals Science), minced, and treated with papain (9 units/mL, Worthington Biochemical, Lakewood, NJ) dissolved in PBS solution containing 0.02 % L-cysteine monohydrate, 0.5 % glucose and 0.02 % bovine serum albumin. 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 DMEM (Invitrogen, Carsbad, CA) supplemented with 10 % precolostrum newborn calf serum, 10 U/mL penicillin and 10 μg/mL streptomycin. In order to evaluate the astrocytic activation, the cells were treated with etorphine (10 μM), morphine (10 μM) or DMEM for 3 days. The cells were then identified by immunofluorescence using rabbit anti-GFAP (Chemicon International, Inc.) followed by incubation with Alexa 488-

conjugated goat anti-rabbit IgG. Stained cells were mounted on glass slides and viewed using a Radiance 2000 laser-scanning microscope (BioRad, Richmond, CA, USA). For each cover slip, four randomly chosen fields were measured (about 85 cells in each field), and the density of GFAP-IR was measured with a computer-assisted system (NIH Image). The upper and lower threshold density ranges were adjusted to encompass and match the IR to provide an image with immunoreactive material appearing in black pixels, and non-immunoreactive material as white pixels. The area and density of pixels within the threshold value representing immunoreactivity were calculated. Each experimental condition was repeated from three independent culture preparations. The percentage of GFAP-IR was expressed as mean ± S.E.M. The statistical analysis was performed using Student's *t*-test.

Preparation of astrocyte-conditioned medium (ACM) mixture

Purified spinal cord astrocytes were grown as follows; whole spinal cords obtained from newborn ICR mice were minced and treated with trypsin (0.025 %, Invitrogen, Carlsbad, CA) dissolved in PBS solution containing 0.02 % L-cysteine monohydrate, 0.5 % glucose and 0.02 % bovine serum albumin. 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, 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 7 days in DMEM, supplemented with 5 % precolostrum newborn calf serum, 5 % heat-inactivated (56 °C, 30 min) horse serum, 10 U/mL penicillin and

10 μg/mL streptomycin. For preparation for ACM, astrocytes were grown to confluence. Cells were washed once with DMEM and then covered with an equal volume of serum-free medium for 24 hr at 37 °C. The supernatant was collected 1 day after changing to the serum-free medium culture and centrifuged at 1,000 x g for 20 min. The mixture of the collected cells and final supernatant was used as astrocytes/ACM.

Statistical analysis

The behavioral data are presented as the mean ± S.E.M. The statistical significance of differences between groups was assessed with one-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn multiple comparison test. Differences in integrated immunoreactivity to GRK 2, dynamin II, β-arrestin 2 and p-cPKC in the spinal cord obtained from morphine-treated mice were expressed as a percent increase (mean ± S.E.M.) with respect to that of saline-treated mice. The statistical analysis was performed using Student's *t*-test.

Results

Development of tolerance to etorphine- or morphine-induced antinociception

The development of tolerance to etorphine- or morphine-induced antinociception was assessed using hot-plate test. Groups of mice were repeatedly injected s.c. with etorphine (10 μg/kg), morphine (10 mg/kg) or saline once a day for 7 consecutive days. The injection of etorphine or morphine produced about 70-90 % antinociceptive effect on the first day. However, this antinociception was significantly decreased during consecutive exposure to etorphine or morphine, indicating the development of tolerance to etorphine- or morphine-induced antinociception (Fig. 1A: $p<0.05$ and $p<0.001$ vs. the first day of etorphine group; Fig.1C: $p<0.01$ and $p<0.001$ vs. the first day of morphine group).

The functional change of μ-opioid receptor in the spinal cord following repeated treatment with etorphine or morphine was evaluated by tail-flick test. Groups of mice were repeatedly injected with saline, etorphine (10 μg/kg, s.c.) or morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. Twenty-four hr after the last repeated injection, groups of mice were challenged i.t. with a selective μ-opioid receptor agonist DAMGO (1.0-10 pmol/mouse). Repeated s.c. treatment with etorphine or morphine significantly attenuated the antinociceptive effects induced by DAMGO compared to that of saline-treated mice (Fig. 1B, D: $p<0.01$). The ED_{50} value (with 95 % confidence range) for DAMGO-induced antinociception in saline-treated mice was increased from 3.0 (2.0-4.0) to 7.2 (5.3-9.1) pmol/mouse by repeated treatment with

etorphine or 3.0 (2.0-4.0) to 6.2 (2.8-9.6) pmol/mouse by repeated treatment with morphine, respectively.

Change in protein levels of membrane-bound GRK 2, dynamin II, β-arrestin 2 and p-cPKC in the whole spinal cord by repeated treatment with etorphine or morphine

The change in protein levels of membrane-bound GRK 2, dynamin II, β-arrestin 2 and p-cPKC in the mouse spinal cord following repeated treatment with etorphine or morphine is shown in Fig. 2. Western blots showed that protein levels of membrane-bound GRK 2, dynamin II, β-arrestin 2 and p-cPKC in the spinal cord were significantly increased by repeated treatment with etorphine compared to those in the saline-treated mice (Fig. 2A: GRK 2; 178.9 ± 5.3 % of control, dynamin II; 121.3 ± 3.4 % of control, β-arrestin 2; 173.4 ± 9.2 % of control, and p-cPKC 161.2 ± 8.1 % of control, $p<0.001$ vs. saline-treated group). In contrast, protein levels of membrane-bound GRK 2, dynamin II, β-arrestin 2 and p-cPKC in the spinal cord were not altered by repeated treatment with morphine (Fig. 2B).

Change in GFAP immunoreactivity in the mouse spinal cord by repeated treatment with etorphine or morphine

Astrocytes in the dorsal horn of the spinal cord were stained with GFAP antibody. These astrocytes were sparsely distributed in saline-treated mice (Fig. 3A). Repeated treatment with morphine produced a robust increase in GFAP immunoreactivity

(GFAP-IR) in the L5 lumbar spinal dorsal horn of the mouse spinal cord (Fig. 3C). It was apparent that each individual astrocyte labeled by GFAP was hypertrophied with an enlarged cell body (Fig. 3C). In contrast, GFAP-IR was not changed by repeated treatment with etorphine (Fig. 3B) compared to that of saline-treated mice (Fig. 3A). Based on the data presented above, I next investigated the effect of etorphine or morphine on astrocytes using the spinal neuron/glia cocultures. As shown in Fig. 4, *in vitro* treatment with morphine (10 μM) for 3 days caused a robust astrocytic activation in spinal neuron/glia cocultures (Fig. 4C, D), as detected by a hypertrophy and an increase in the level of GFAP-IR compared to that in normal medium-treated cells (Fig. 4A). In contrast to morphine, treatment with etorphine (10 μM) for 3 days failed to activate GFAP-positive astrocytes in spinal neuron/glia cocultures (Fig. 4B, D).

Delay of the development of tolerance to morphine-induced antinociception by pretreatment with propentofylline

The effect of pretreatment with the glial-modulating agent propentofylline (PPF) on the development of tolerance to morphine- or etorphine-induced antinociception was assessed by the hot-plate test. At first, I confirmed whether pretreatment with PPF could affect acute morphine- or etorphine-induced antinociception. The dose-response curves for the antinociceptive effects of either morphine or etorphine were not affected by pretreatment with PPF (Fig. 5A, C). In saline-pretreated mice, either morphine- or etorphine-induced antinociception was significantly decreased during consecutive exposure to morphine or etorphine, respectively, indicating the

development of tolerance to morphine- or etorphine-induced antinociception (Fig. 5B: $p<0.01$ and $p<0.001$ vs. the first day of saline-pretreated morphine group, Fig. 5D: $p<0.01$ and $p<0.001$ vs. the first day of saline-pretreated etorphine group). The development of tolerance to morphine-induced antinociception, but not to etorphine-induced antinociception, was significantly delayed by pretreatment with PPF (Fig. 5B: $p<0.01$, $F_{1,29} = 10.6$, PPF-pretreated morphine group vs. saline-pretreated morphine group).

Acceleration of the development of tolerance to morphine-induced antinociception following a single i.t. injection of cultured spinal cord astrocytes with astrocyte-conditioned medium (ACM) mixture

The next study was undertaken to examine the role of astrocyte-released soluble factors and astrocytes in the development of tolerance to morphine- or etorphine-induced antinociception. The spinal astrocytes and astrocytes-conditioned medium (astrocytes/ACM) mixture, which were obtained from cultured astrocytes of the newborn mouse spinal cord, was injected into the spinal cord. Twenty-four hr after i.t. injection of astrocytes/ACM mixture or DMEM, mice were repeatedly injected with morphine or etorphine for 7 consecutive days. A single intrathecal injection of astrocytes/ACM mixture produced a significant decrease of antinociceptive effect on the third day, indicating the acceleration of the development of tolerance to morphine-induced antinociception (Fig. 6B: $p<0.05$, $F_{1,11} = 7.93$, astrocytes/ACM-treated morphine group vs. DMEM-treated morphine group). However, the development of

tolerance to etorphine was not affected by intrathecal injection of astrocytes/ACM mixture. Under the condition, an intrathecal injection of astrocytes/ACM mixture had no effects on the acute etorphine- or morphine-induced antinociception (Fig. 6A, C) and basal hot-plate latency after s.c. treatment with saline (Fig. 6B, D).

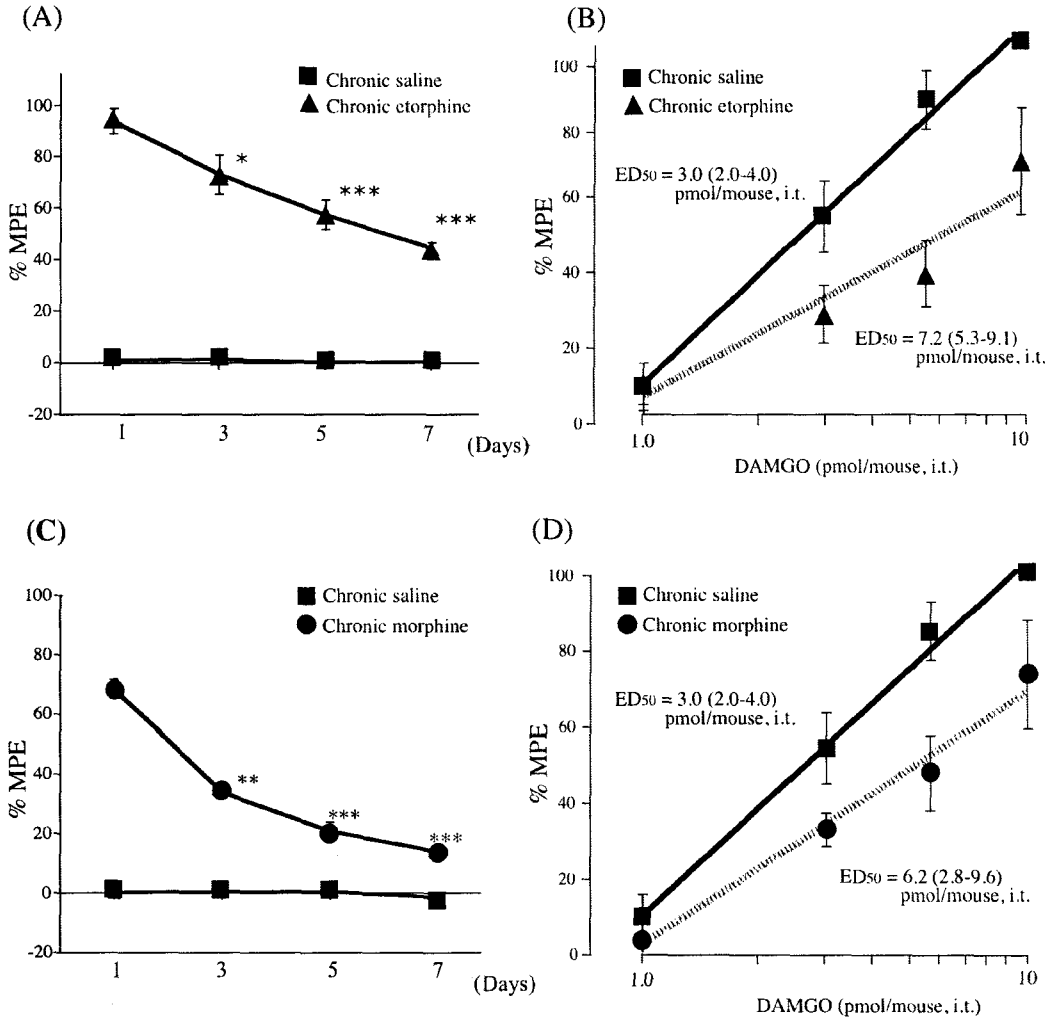


Fig. 1 Development of tolerance to etorphine- or morphine-induced antinociception assessed by hot-plate test (A, C). Groups of mice were injected with saline, etorphine (10 μg/kg, s.c.) or morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. The antinociceptive effect induced by etorphine or morphine on the first day significantly decreased during consecutive exposure to etorphine or morphine. (Fig. 1A: *p<0.05 and ***p<0.001 vs. the first day of etorphine group; Fig.1C: **p<0.01 and ***p<0.001 vs. the first day of morphine group). Effect of repeated administration of etorphine or morphine on spinal antinociception produced by i.t. injection of a selective μ-opioid receptor agonist DAMGO assessed by tail-flick test (B, D). Twenty-four hr after the last repeated injection of saline, etorphine or morphine, the mice were treated with DAMGO (1.0-10 pmol/mouse, i.t.). The dose-response curves for the antinociceptive effects produced by i.t. injection of DAMGO in mice treated repeatedly with saline (square, ED_{50}: 3.0 pmol/mouse, i.t.), etorphine (triangle, ED_{50}: 7.2 pmol/mouse, i.t.) and morphine (circle, ED_{50}: 6.2 pmol/mouse, i.t.) were shown. The data represent the mean with S.E.M. Each group used 6-12 mice.

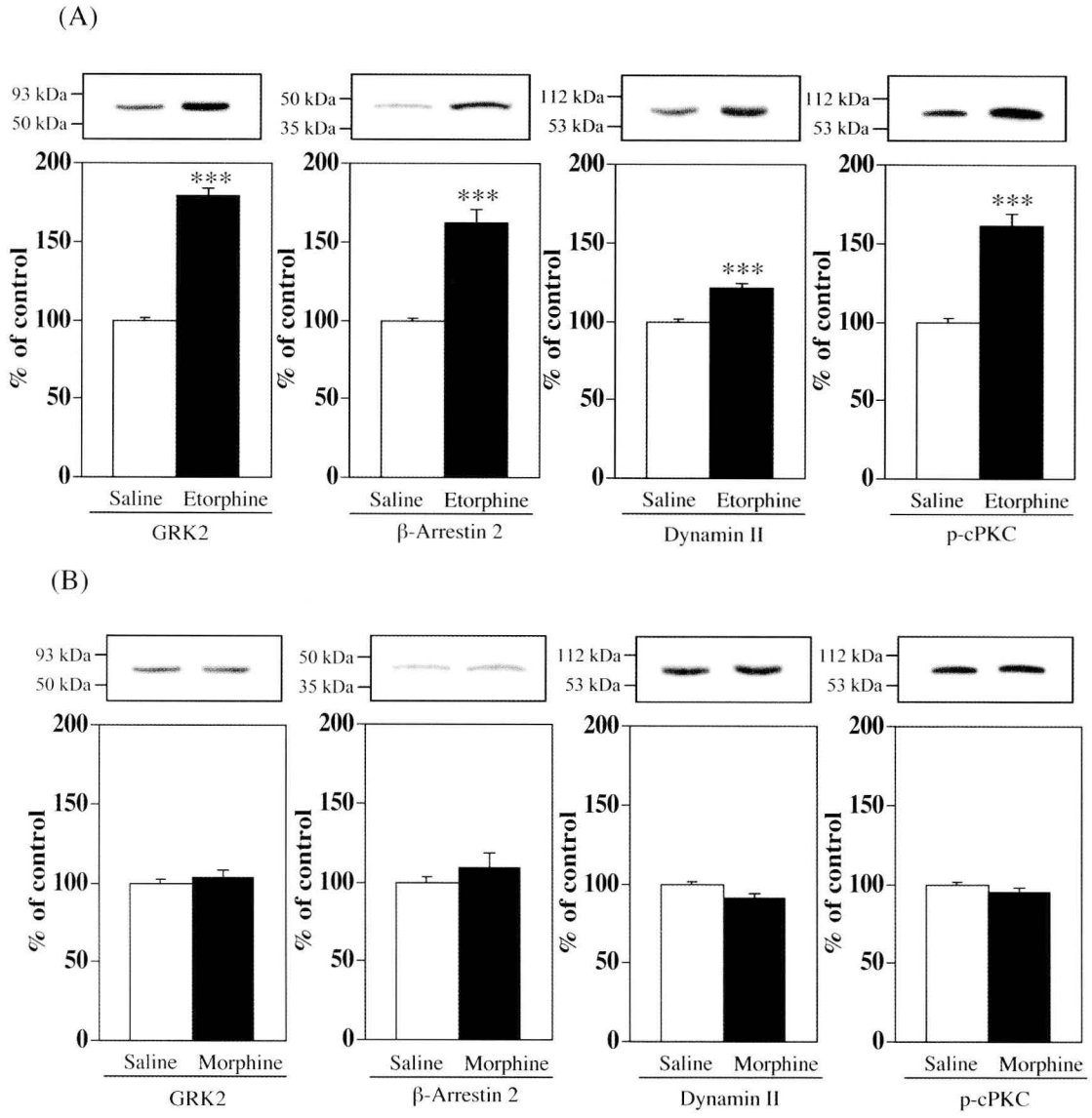


Fig. 2 Change in protein levels of membrane-bound GRK 2, dynamin-II, -arrestin 2 and p-cPKC in the whole spinal cord by repeated treatment with etorphine (A) or morphine (B). Groups of mice were injected with saline, etorphine (10 μg/kg, s.c.) or morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. The membranous fractions were prepared at 24 hr after the last injection. *Upper*: Representative Western blots of GRK 2, -arrestin 2, dynamin-II and p-cPKC. *Lower*: Changes in the immunoreactivities for GRK 2, -arrestin 2, dynamin-II and p-cPKC in membranous fraction of whole spinal cords obtained from saline-, etorphine- or morphine-treated mice. Each column represents the mean with S.E.M. of 3 independent samples. *** $p < 0.001$ vs. saline-treated mice.

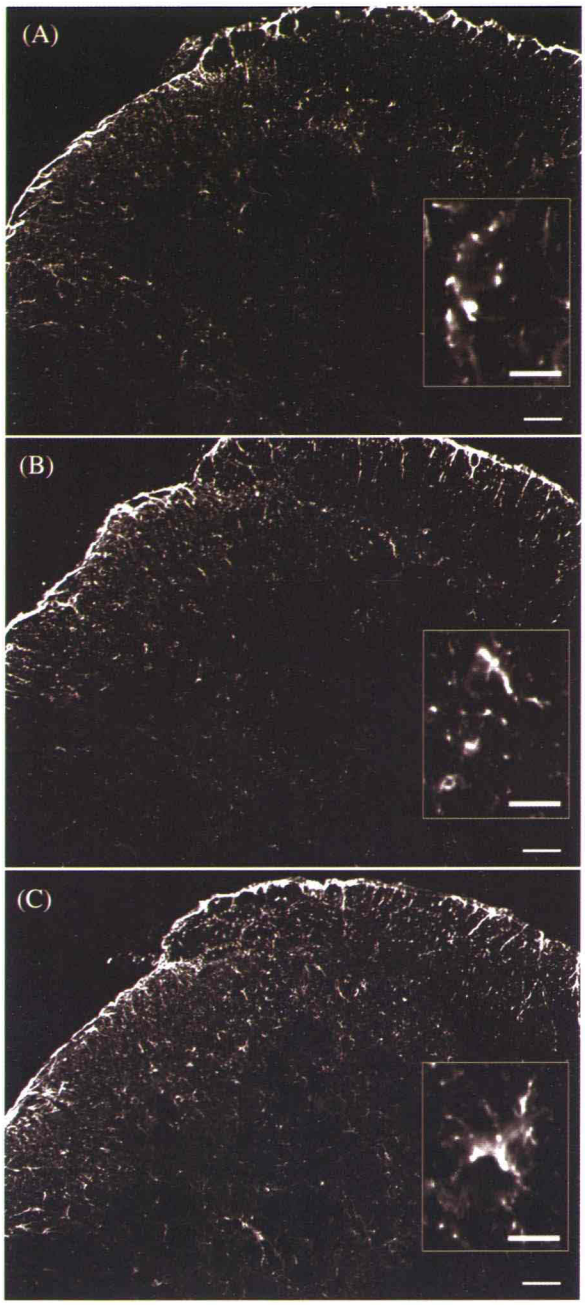


Fig. 3 Change in GFAP-IR in the L5 lumbar spinal dorsal horn of the mouse spinal cord by repeated injection of etorphine or morphine. Mice were repeatedly injected with saline, etorphine or morphine once a day for 7 consecutive days. Twenty-four hr after repeated treatment, the samples were prepared. In mice treated repeatedly with morphine (C), the level of GFAP-IR was increased with morphological differentiation as compared to that in saline-treated mice (A). In etorphine-treated mice (B), GFAP-IR was not changed as compared to that in saline-treated mice (A). Three independent samples were performed in this study. Scale bars, 50 μm (A, B, C), 10 μm (inset).

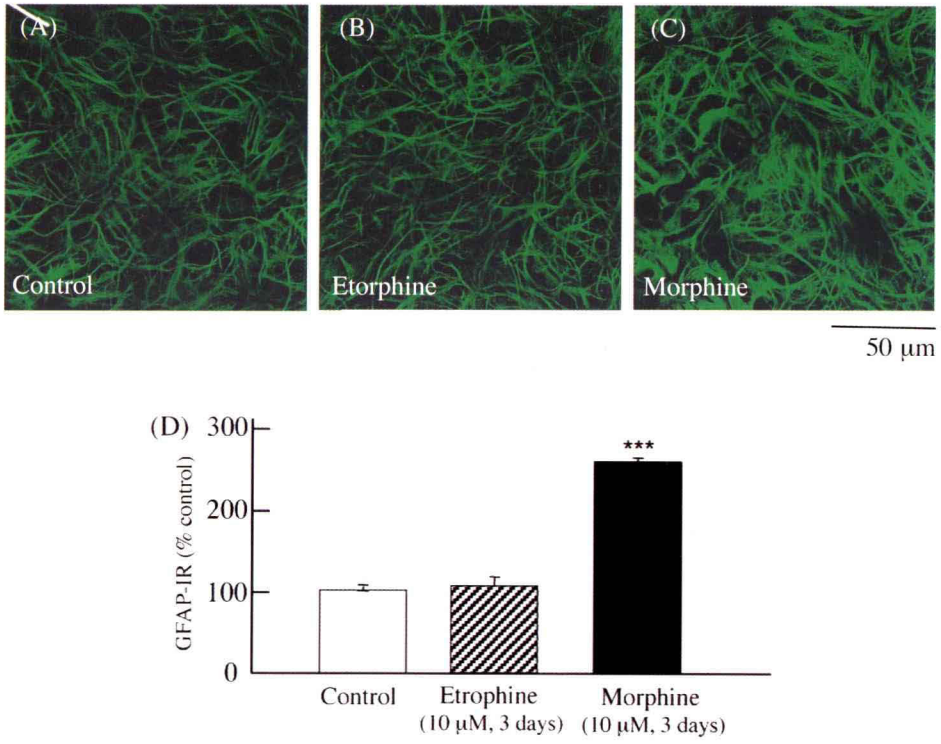


Fig. 4 Change in GFAP-IR in spinal neuron/glia cocultures following *in vitro* treatment with etorphine or morphine. Spinal neuron/glia cocultures were incubated with normal medium, etorphine (10 μM) or morphine (10 μM) for 3 days. The density of GFAP-IR of each image was measured using an NIH image. The levels of GFAP-IR on etorphine- and morphine-treated cells are expressed as a percent increase (mean with S.E.M.) with respect to that on control cells. *** $p < 0.001$ vs. control cells.

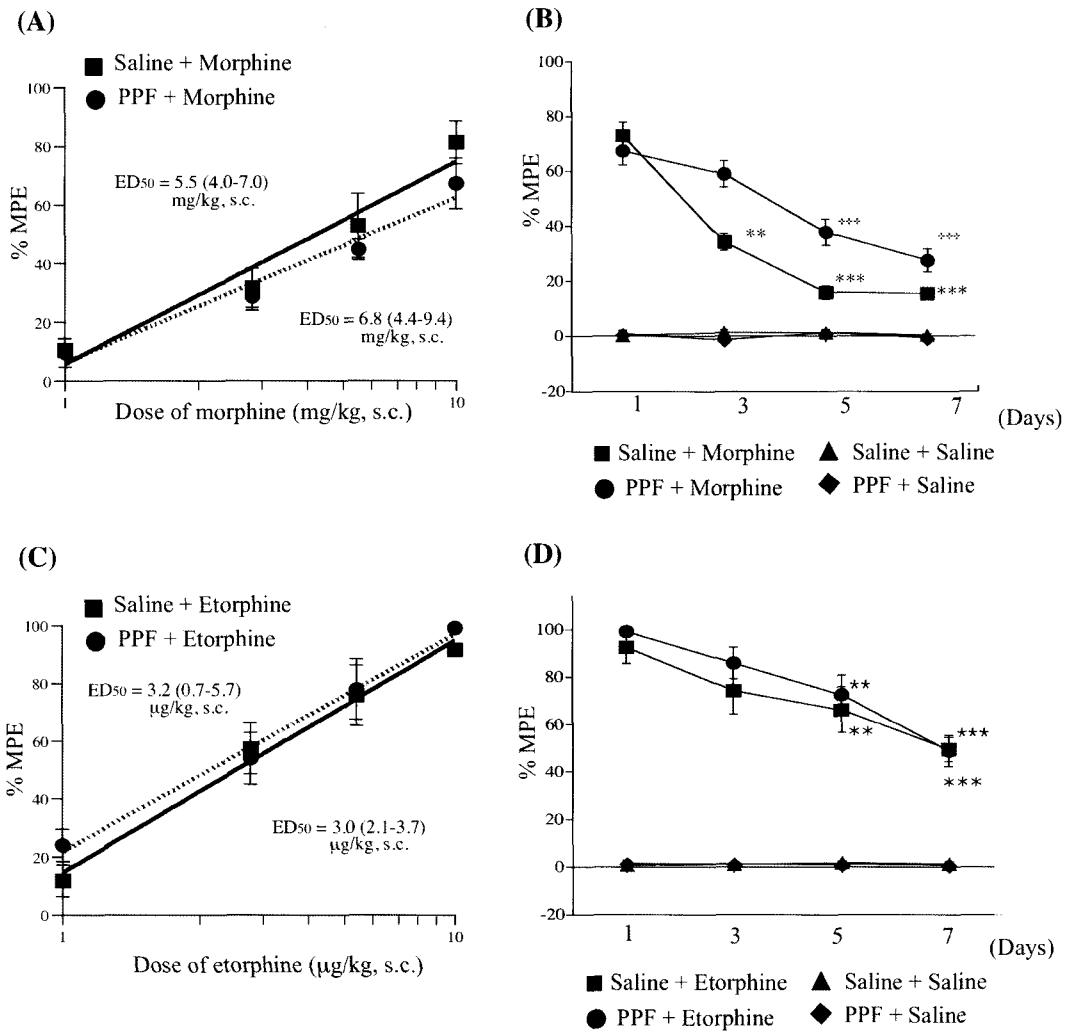


Fig. 5 Effect of pretreatment with propentofylline (PPF) on the dose-response curves for the antinociceptive effects of morphine or etorphine (A, C) and the development of tolerance to morphine- or etorphine-induced antinociception (B, D). (A, C) Groups of mice were pretreated with saline or PPF (5.0 mg/kg, i.p.) 30 min before an injection of morphine (1.0-10 mg/kg, s.c., A) or etorphine (1.0-10 g/kg, s.c., C). (B, D) Groups of mice were injected with saline, morphine (10 mg/kg, s.c.) or etorphine (10 g/kg, s.c.) once a day for 7 consecutive days. PPF (5.0 mg/kg, i.p.) was administered 30 min before every drug treatment. The data represent the mean with S.E.M. Each group used 6-12 mice. **p<0.01 and ***p<0.001 vs. the first day of saline-pretreated morphine group. ***p<0.001 vs. the first day of PPF-pretreated morphine group. **p<0.01 and ***p<0.001 vs. the first day of saline-pretreated etorphine group. p<0.01 and p<0.001 vs. the first day of PPF-pretreated etorphine group. p<0.01, $F_{1,29}$ = 10.6, PPF-pretreated morphine group vs. saline-pretreated morphine group.

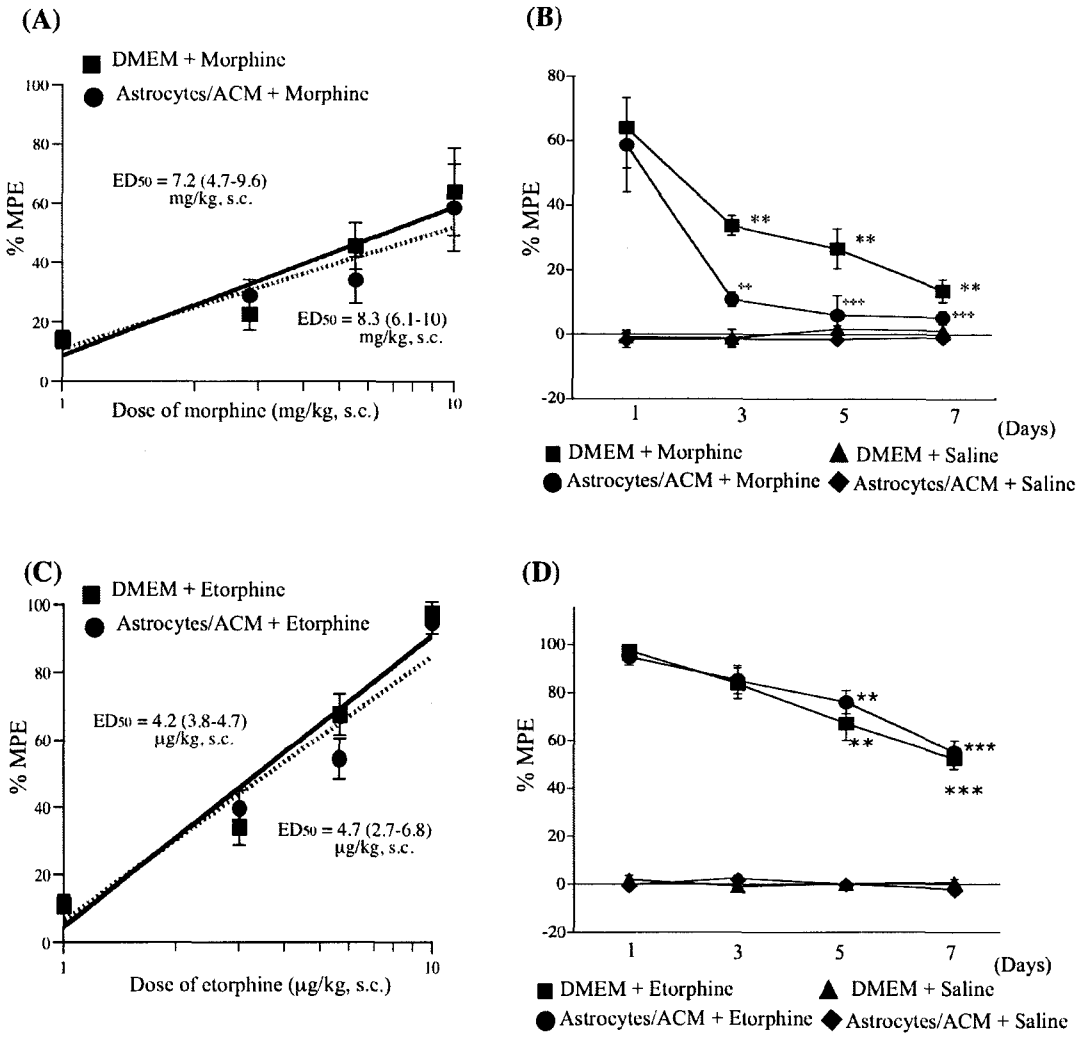


Fig. 6 Effect of a single i.t. injection of cultured spinal cord astrocytes with astrocyte-conditioned medium (astrocytes/ACM) mixture on the dose-response curves for the antinociceptive effects of morphine or etorphine (A, C) and the development of tolerance to morphine- or etorphine-induced antinociception (B, D). (A, C) Groups of mice were pretreated i.t. with DMEM or astrocytes/ACM 24 hr before an injection of morphine (1.0-10 mg/kg, s.c., A) or etorphine (1.0-10 μg/kg, s.c., C). (B, D) Groups of mice were injected with saline, morphine (10 mg/kg, s.c.) or etorphine (10 μg/kg, s.c.) once a day for 7 consecutive days. DMEM or astrocytes/ACM mixture was administered 24 hr before first drug treatment. The data represent the mean with S.E.M. Each group used 6-15 mice. **$p<0.01$ vs. the first day of DMEM-pretreated morphine group. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. the first day of astrocytes/ACM-pretreated morphine group. **$p<0.01$ and ***$p<0.001$ vs. the first day of DMEM-pretreated etorphine group. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. the first day of astrocytes/ACM-pretreated etorphine group. $p<0.05$, $F_{1,11} = 7.93$, astrocytes/ACM-pretreated morphine group vs. DMEM-pretreated morphine group.

Discussion

Following the binding of the agonist to μ-opioid receptors, the receptor signals *via* activation of heterotrimeric G proteins of the Gi/o family. With continued exposure to agonist, μ-opioid receptors are rapidly phosphorylated by GRK, and this phosphorylation stimulates the binding of arrestins to the receptor. The μ-opioid receptor/arrestin complex is then recruited to a constitutive pathway that utilized clathrin-coated pits to endocytose a wide variety of cell surface proteins in a dynamin-dependent manner [25-27]. In the present study, repeated injection of etorphine and morphine produced a significant inhibition of the spinal antinociceptive effect produced by DAMGO, indicating the μ-opioid receptor desensitization in the spinal cord. Here, I demonstrated that repeated *in vivo* treatment with etorphine, but not morphine, caused a significant increase in all protein levels of GRK 2, dynamin II and β-arrestin 2 in membranes of the mouse spinal cord compared to those in saline-treated mice. In support of the role of these trafficking proteins in μ-opioid receptor regulation, chronic *in vivo* treatment with the opioid antagonist induces up-regulation of μ-opioid receptors associated with a reduction in GRK 2 and dynamin II [69]. Furthermore, it has been reported that chronic treatment with etorphine produces a significant increase in protein levels of dynamin II, but not GRK 2 [61,62,70]. The down-regulation of immunoreactive μ-opioid receptor assessed by Western blots and the decrease of ^{3}[H]DAMGO binding in the spinal cord are observed following continuous s.c. infusion of etorphine [70]. Although there are some of differences between these reports and current data, the

discrepancy seems to be caused by the differences of etorphine dosing protocol and/or sample preparation protocol. Taken together, these findings strongly suggest that the etorphine-induced μ-opioid receptor desensitization may result from the GRK 2/dynamin II/β-arrestin 2-dependent phosphorylation of μ-opioid receptors.

Many GPCRs are phosphorylated by PKC. Like other GPCRs, μ-opioid receptor contains PKC phosphorylation sites on the third intracellular loop and the carboxyl terminus [71,72], which are important for the desensitization. Here I demonstrated that mice tolerant to etorphine exhibited a significant increase in activities of membrane-bound cPKC in the spinal cord. Several studies have pointed out that PKC can modify the functional state of GRKs and arrestin providing a novel level of cross-talk in signal transduction [73-75]. In addition, it has been reported that functional μ-opioid receptors can be protected from degradation by phosphorylation and subsequently μ-opioid receptor-mediated antinociception is enhanced in PKCγ-deficient mice [76]. These results indicate that PKC in the spinal cord is implicated in the development of spinal antinociceptive tolerance to μ-opioid receptor agonists in mice. Consistent with these findings, the increase in the membranous PKC activity due to repeated treatment with etorphine may be associated with the phosphorylation of μ-opioid receptor and several trafficking-associated proteins.

Like etorphine, repeated *in vivo* treatment with morphine produced a significant attenuation of the DAMGO-induced antinociception. However, this treatment showed no change in protein levels of membranous fraction of GRK 2, dynamin II, β-arrestin 2 and p-cPKC in the spinal cord, which is consistent with previous reports [61,62]. These

findings suggest that the desensitization of μ-opioid receptors following repeated morphine treatment may not be associated with the functional changed in the receptor trafficking proteins.

Recent morphological inspection that astrocytes enwrap synaptic terminals indicates that astrocytes can influence neuronal activity and synaptic function by secreted neuromodulators [63]. In contrast, activated neurons can also promote the differentiation of astrocytes, implying the existence of the functional interaction between neurons and astrocyte [77]. Here I found that the level of GFAP in the mouse spinal cord was clearly increased by chronic *in vivo* and *in vitro* treatment with morphine, whereas this phenomenon was not observed by chronic etorphine treatment. Furthermore, pretreatment with the glial-modulating agent, PPF, suppressed the development of the antinociceptive tolerance to morphine. In addition, a single i.t. injection of astrocytes/ACM mixture significantly enhanced the development of tolerance to morphine-induced antinociception. Interestingly, these agents failed to affect the development of tolerance induced by etorphine. Consistent with the present data, Raghavendra et al. [67] and Tawfik et al. [78] demonstrated that repeated treatment with morphine increases the glial activation and enhances proinflammatory cytokine levels, including interleukin-1β (IL-1β), IL-6 and tumor necrosis factor α (TNF α), associated with the expression of morphine tolerance. In addition, Shavit et al. [79] have shown that the antinociceptive response induced by morphine can be curtailed by proinflammatory cytokine production due to chronic morphine treatment, which leads to a shift in the nociceptive equilibrium toward pain rather than analgesia. Taken

together, these findings support the idea that activated astrocytes following chronic morphine treatment may release several cytokines. These released cytokines may alter the nociceptive threshold. Thus, this phenomenon could explain the mechanism of the suppression of morphine-induced antinociception following chronic morphine treatment, which is called analgesic tolerance to morphine.

Several lines of evidence suggest that spinal cord glial cells create exaggerated pain state [78-81]. Activated glial cells have been known to release a variety of neuroactive substances, including arachidonic acid, prostaglandins, excitatory amino acids and nerve growth factors to increase the excitatory synaptic transmission [78-81]. Although the molecular mechanism underlying the present phenomenon, which only morphine affects GFAP, is still unclear, one possibility is that lower efficacy drugs, like morphine, engage another pathways on account of differences in several respects (e.g., receptor selectivity and kinetics).

In conclusion, the present data provide direct evidence for the distinct mechanisms between etorphine and morphine on the development of tolerance to antinociception. These findings raise the possibility that the increased astroglia response due to chronic morphine treatment may result in the morphine-specific receptor modulating profile, which could be related to the down-regulation of μ-opioid receptor function without receptor internalization and may actively participate in the development of morphine tolerance and the induction of neuronal plasticity.

Chapter 2

Neuronal protein kinase Cγ-dependent proliferation and hypertrophy of spinal cord astrocytes following repeated *in vivo* administration of morphine

Introduction

In the central nervous system, astrocytes form an intimately connected network with neurons and their processes often closely enwrap synapses. The critical role of these cells in metabolic and trophic support to neurons, ion buffering and clearance of neurotransmitters is well established [43]. Recent accumulating evidence suggests that astrocytes are active partners of neurons in additional and more complex functions. Astrocytes express a repertoire of neurotransmitter receptors mirroring that of neighboring synapses [44-46]. Such receptors are stimulated during synaptic activity and start calcium signaling into the astrocyte network. Recent evidence indicates that intracellular waves of calcium in astrocytes represent the start of backsignaling to neurons, as they trigger release of chemical transmitter (i.e. glutamate, IL, interferon and chemokines) [47,48]. In Chapter 1, I stated that repeated treatment with morphine increases the astrocytic activation in the dorsal horn of the spinal cord, which is related to the development of tolerance to morphine-induced antinociception.

Protein kinase C (PKC) is an integral part of the cell signaling machinery [28-30]. Biochemical and molecular cloning analysis have revealed that PKC comprises a large family with multiple isoforms exhibiting individual characteristics and distinct patterns of tissue distribution [31,32]. It has been demonstrated that PKC inhibitors attenuate the development of tolerance to morphine-induced antinociception [82]. Furthermore, chronic *in vivo* treatment with a selective μ-opioid receptor agonist [*D*-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) results in the increase of the membrane-bound

PKC γ isoform in the spinal cord of mice [82]. These findings indicate that activated PKC is involved in the development of opioid tolerance. However, none or little is known about the direct communication between PKC and astroglial response under the condition with repeated *in vivo* administration of morphine. Therefore, the present study was undertaken to investigate the role of neuronal PKCγ in the activation of spinal astrocytes by repeated *in vivo* treatment with morphine using the transgenic mice with GFAP promoter-controlled enhanced green fluorescent protein (EGFP) expression and PKCγ knockout mice.

Materials and Methods

Animals

The PKCγ knockout mice (The Jackson Laboratory, Bar Harbor, ME, USA), which were C57BL/6j and 129Sv mixed genetic backgrounds as described previously [8], their wild-type mice, transgenic mice which express EGFP under the control of the mouse GFAP promoter (GFAP/EGFP transgenic mice) [84,85], and male ICR mice (Tokyo Laboratory Animals Science Co., Ltd, Tokyo, Japan) were used in the present study. Animals were housed in a room maintained at 23 ± 1 °C with an alternating 12 hr light-dark cycle. Food and water were available *ad libitum* during the experimental period.

Immunohistochemistry

Mice were repeatedly injected with morphine (10 mg/kg, s.c.) or saline (10 mL/kg, s.c.) once a day for 7 days. The procedure for the sample preparation was performed following the method described in Chapter 1.

The spinal cord sections were blocked in 10 % normal horse serum (NHS) in 0.01 M PBS for 1 hr at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10 % NHS [1:500 PKCγ (Santa Cruz Biotechnology, Inc., Santa Cruz), 1:500 microtubule associated protein 2a/b (MAP2a/b, Chemicon International, Inc., Temecula, CA, USA), 1:500 neuronal nuclei (NeuN, Chemicon International, Inc.) and 1:100 GFAP (Chemicon International, Inc.)] and was incubated for 48 hr at 4 °C. The antibodies were then rinsed and incubated with each secondary antibodies conjugated

Alexa 488 and Alexa 546 for 2 hr at room temperature. Since the staining intensity might vary between experiments, control sections were included in each run of staining.

The slides were then cover-slipped with PermaFluor Aqueous mounting medium (Immunon™; ThermoShandon, Pittsburgh, PA, USA). Fluorescence of both EGFP and the immunolabelings was detected using the light microscope (Olympus AX-70; Olympus, Tokyo, Japan) and photographed with a digital camera (Polaroid PDMCII/OL; Olympus). The density of PKCγ 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 (IR) 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 superficial laminae of dorsal horn of the spinal cord area from morphine-treated mice. The area and density of pixels within the threshold value representing IR 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 superficial laminae of dorsal horn of the spinal cord area from saline-treated mice and the integrated density of pixels within the same threshold was calculated again.

Statistical analysis

All data are expressed as mean ± S.E.M. Differences in integrated immunoreactive density on the dorsal horn in the spinal cord of morphine-treated mice vs. that of saline-treated mice was tested with Student's *t*-test.

Results

Increase in the immnoreactivity for neuronal specific γ isoform of PKC (PKCγ-IR) by repeated treatment with morphine in the superficial dorsal horn of the spinal cord of ICR mice

Repeated s.c. treatment with morphine once a day for 7 consecutive days produced a time-dependent inhibition of the morphine-induced antinociceptive action (data not shown). Twenty-four hr after the last injection, the immnoreactivity for PKCγ (PKCγ-IR) in the spinal cord was observed by immunohistochemical analysis. The PKCγ-IR was highly restricted in the inner part of laminae II (laminae IIi) in the dorsal horn of saline-treated ICR mice (Fig. 1A). Using semi-quantitative analysis, repeated s.c. treatment with morphine produced a significant increase in the level of PKCγ-IR in the dorsal horn of the spinal cord (129.1 ± 1.0 % of control, $p < 0.001$ vs. saline-treated ICR mice, Fig. 1B, C). It should be noted that the increased PKCγ-IR was extended to the outer part of laminae II (laminae IIo) as well as laminae IIi. Furthermore, the increased PKCγ-IR was highly co-localized with both the neuron-specific nuclear protein marker NeuN (Fig. 1D) and the dendritic protein marker MAP2a/b (Fig. 1E) in the superficial layer of the dorsal horn.

Change in GFAP-IR in the dorsal horn of the spinal cord of ICR mice by repeated treatment with morphine

In ICR mice treated chronically with morphine, the level of GFAP-IR was elevated

mostly in gray matter and partly in white matter of the dorsal horn of the spinal cord (Fig. 2B-i), without changing in the ventral horn of the spinal cord (Fig. 2C, D). It was apparent that each individual astrocyte labeled by GFAP was hypertrophied with an enlarged cell body (Fig. 2B-ii). In contrast, the level of GFAP-IR in the dorsal horn of the spinal cord was not affected by a single injection of morphine (Fig. 2F, G).

No apparent co-localization of PKCγ-IR with GFAP-IR in the dorsal horn of the spinal cord of ICR mice by repeated treatment with morphine

Double-labeling experiments showed that the increased PKCγ-IR was expressed in non-glial cells in the dorsal horn of the spinal cord of morphine-treated ICR mice, as shown by no apparent co-localization with GFAP-IR (Fig. 2E). The hypertrophied GFAP-IR by repeated morphine treatment was adjacent to the increased PKCγ-IR.

Implication of morphological change in astrocytes by repeated treatment with morphine in GFAP/EGFP transgenic mice

The change in GFAP by repeated treatment with morphine using GFAP/EGFP transgenic mice is shown in Fig. 3. In these mice, astrocytes were discerned by their green fluorescence caused by GFAP promote-driven EGFP expression. The pale and fibriform EGFP-expressing cells were found in the superficial layer of saline-treated transgenic mice (Fig. 3A-i, A-ii). In morphine-treated transgenic mice, the extensively bright and branched EGFP-expressing cells were observed mostly in gray matter (Fig.3B-i) and partly in white matter (Fig. 3B-ii) in the dorsal horn of the spinal

cord. In order to chemically identify the branched EGFP-expressing cells as reactive astrocytes, immunofluorescent studies using specific antibody to GFAP were performed. Almost all of the EGFP-expressing cells were found to express GFAP (Fig. 3C-i, C-ii).

Implication of activated PKC isoform in astroglial proliferation and hypertrophy in the superficial layers of the spinal cord following repeated treatment with morphine

In the wild-type mice, PKC -IR was dramatically increased by repeated s.c. treatment with morphine (Fig. 4B , 127.4 ± 2.0 % of control, $p<0.001$ vs. wild-type mice treated with saline, Fig. 4A). In saline- and morphine-treated mice lacking PKC gene, PKC -IR was abolished in the dorsal horn of the spinal cord (Fig. 4C, D). In wild-type mice treated repeatedly with morphine, the level of GFAP-IR was markedly increased and each individual astrocyte labeled by GFAP was hypertrophied in the laminae I and laminae II of the spinal cord (Fig. 4F), as compared to that found in wild-type mice treated with saline (Fig. 4E). In PKC knockout mice treated repeatedly with morphine, the level of GFAP-IR was not changed (Fig. 4H), as compared to that observed in PKC knockout mice treated with saline (Fig. 4G).

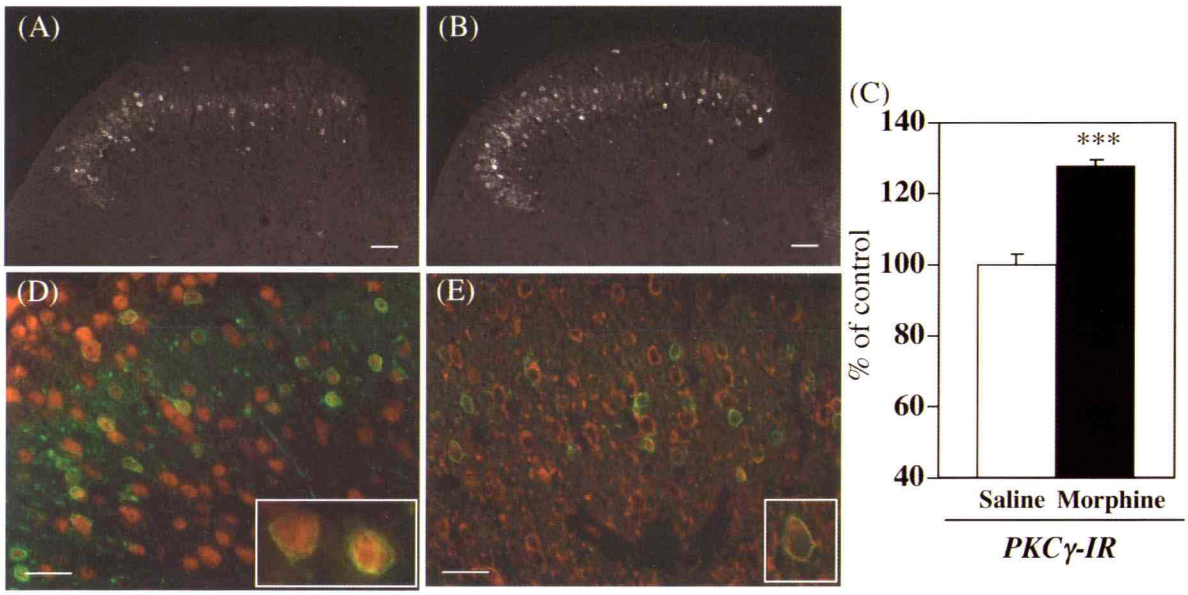


Fig. 1 (A, B) Increase in the level of PKC -IR in the superficial layers of ICR mouse spinal cord after repeated treatment with morphine. Mice were repeatedly injected with saline (A) or morphine (10 mg/kg, s.c.; B) once a day for 7 consecutive days. Twenty-four hr after the last treatment, mice were perfused with saline followed by freshly prepared 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). (C) Semi-quantitative analysis of PKC -IR in the superficial layers of the spinal cord from mice treated repeatedly with morphine was performed by NIH image (*** $p < 0.001$ vs. saline-treated mice). Each column represents the mean with S.E.M. of three independent samples. (D, E) The increased PKC -IR (green) in the superficial layers of the spinal cord of morphine-treated mice was co-localized with NeuN (red; D) and MAP2a/b (red; E). Scale bars: (A, B) = 50 μm, (D, E) = 25 μm.

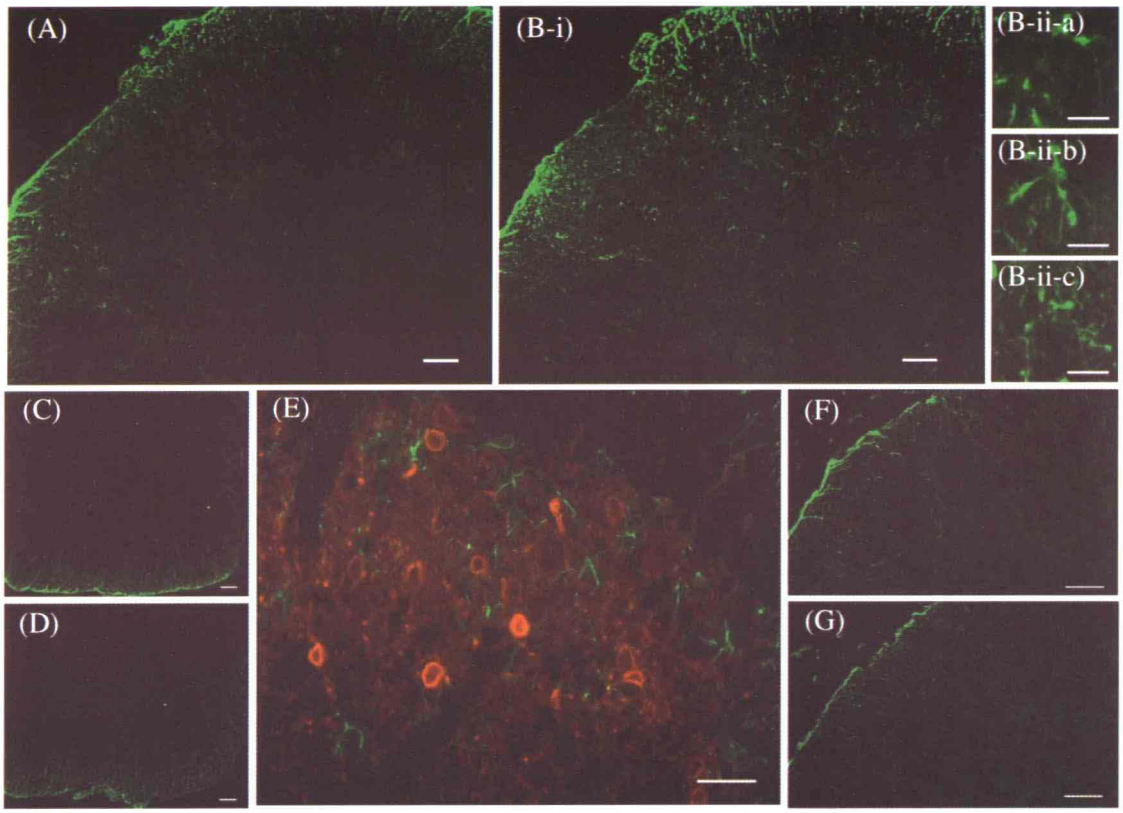


Fig. 2 (A, B) GFAP-IR in the dorsal horn of the spinal cord of ICR mice was dramatically increased with morphologic differentiation by repeated morphine treatment (B-i, B-ii; high magnification) as compared to saline treatment (A), without changing in the ventral horn of the spinal cord (C; saline, D; morphine). (E) The red labeled for PKC and the green labeled for GFAP are no apparent co-localization in the superficial layers of the spinal cord of morphine-treated mice. (F, G) GFAP-IR in the dorsal horn of the spinal cord of ICR mice was not changed by a single injection of morphine. The spinal cord slices were prepared at 30 min after s.c. injection of (F) saline or (G) morphine. Three independent samples were performed in this study. Scale bars: (A, B-i, C, D, F, G) = 50 μm, (B-ii) = 10 μm, (E) = 25 μm.

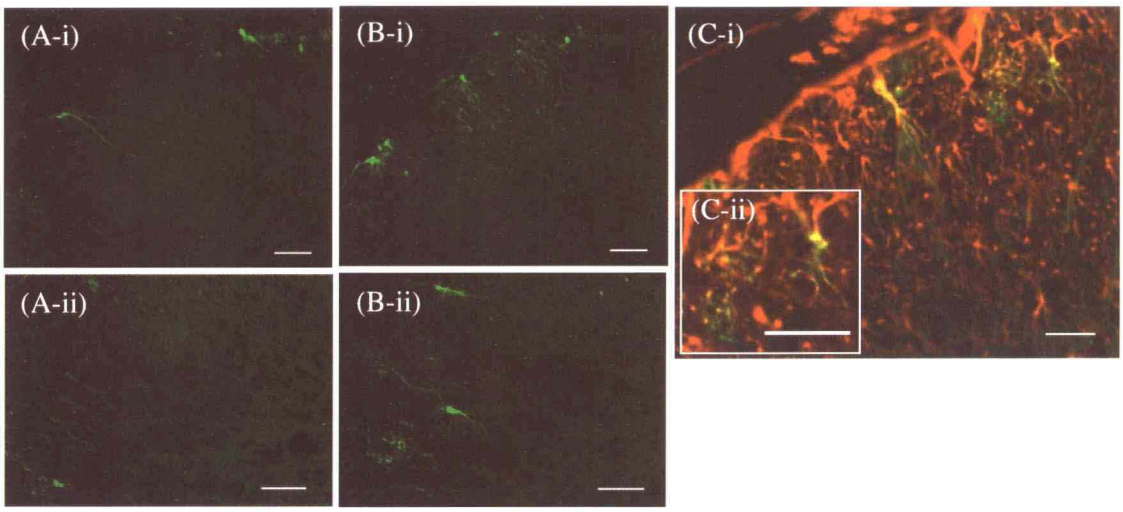


Fig. 3 (A, B) The extensively bright and branched EGFP-expressing cells were observed in both gray matter (B-i) and white matter (B-ii) in the dorsal horn of the spinal cord in morphine-treated EGFP/GFAP-transgenic mice as compared to saline-treated mice (A-i; gray matter, A-ii; white matter). Mice were repeatedly injected with morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. Twenty-four hr after the last treatment, the samples were prepared. (C) The enhanced EGFP (green) in the superficial layers of the spinal cord of morphine-treated mice was co-localized with GFAP-IR (red). Two independent samples were performed in this study. Scale bars: (A, B) = 50 μm, (C-i, C-ii) = 25 μm.

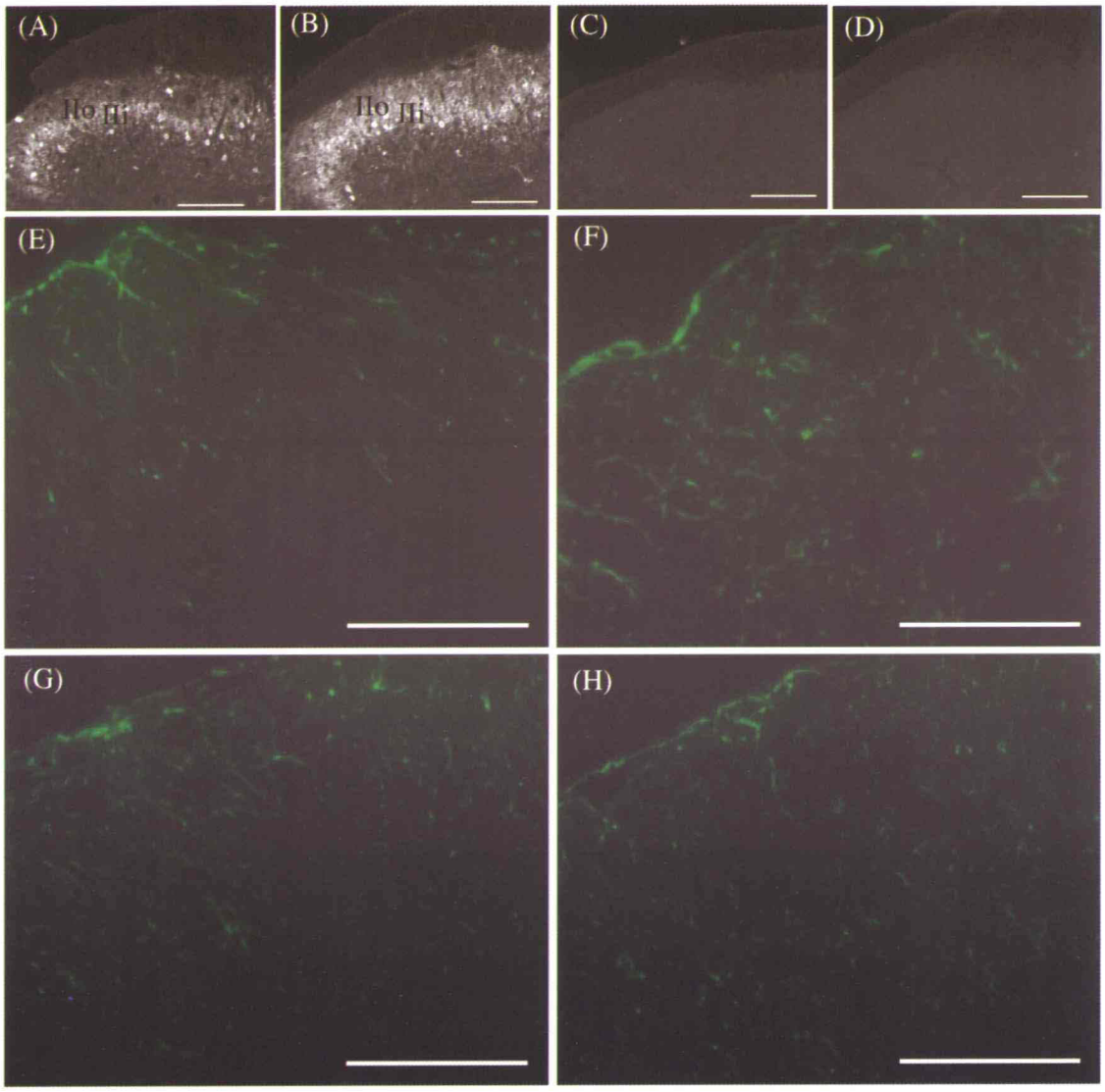


Fig. 4 Implication of activated PKC isoform in astroglial proliferation and hypertrophy in the superficial layers of the spinal cord following chronic *in vivo* treatment with morphine. Mice were repeatedly injected with morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. Twenty-four hr after the last treatment, the samples were prepared. (A) The PKC -IR was highly restricted in the inner part of laminae II (IIi) in the dorsal horn of saline-treated wild-type mice. Repeated s.c. treatment with morphine produced a significant increase in the level of PKC -IR in the dorsal horn (B) as compared to saline-treated mice (A). It should be noted that the increased PKC -IR was extended to the outer part of laminae II (IIo) as well as laminae IIi. In saline- (C) and morphine-treated (D) PKC knockout mice, PKC -IR is completely absent in the dorsal horn of the spinal cord. In wild-type mice treated repeatedly with morphine (F), the level of GFAP-IR was dramatically increased with morphological differentiation as compared to saline-treated wild-type mice (E). In morphine-treated PKC knockout mice (H), GFAP-IR was not changed as compared to saline-treated PKC knockout mice (G). Three independent samples were performed in this study. Scale bars: 50 μm.

Discussion

The neuron-specific distribution of PKC seems to be the most unique characteristics of this isotype. Abundant expression of PKC in the hippocampal pyramidal cells and cerebellar Purkinje cells has implicated it in the modulation of synaptic plasticity, including long term potentiation (LTP) and long term depression (LTD) [83,86]. PKC is also abundant in the dorsal horn of the spinal cord and has been suggested to be important for the sensory signal processing including pain, and the interaction with the opioidergic system. [39-41,87]. Previously, it has been demonstrated that the activation of μ-opioid receptors in the spinal cord induces prolonged PKC translocation [88] and that the inhibition of PKC prevents the development of antinociceptive tolerance to μ-opioid receptor agonists [82]. Furthermore, in PKC deficient mice, functional μ-opioid receptors can be protected from degradation by phosphorylation and subsequently μ-opioid receptor-mediated antinociception is enhanced [83]. In the present study, I found that repeated *in vivo* treatment with morphine produced a significant increase in neuron-specific PKC -IR with its expanding distribution in the dorsal horn of the spinal cord. These results indicate that the activation of PKC may be critical for the expression of morphine-induced antinociceptive tolerance.

The synaptic astrocytes have been shown to integrate synaptic transmission by responding to the signaling molecules through the extracellular space [44-46]. In the present study, repeated treatment with morphine induced astroglial proliferation as characterized by the increase in GFAP-IR levels, and astroglial hypertrophy as detected

by a stellate morphology of GFAP-IR in the dorsal horn of the spinal cord. Furthermore, the present molecular approach using GFAP/EGFP transgenic mice provides direct evidence for the increase in astroglial proliferation following repeated *in vivo* treatment with morphine in the dorsal horn of the spinal cord. Collectively, these findings strongly support the idea that mice tolerant to morphine exhibit the production of reactive astrocytes in the dorsal horn of the spinal cord.

The key approach for the present study was to investigate the influence of the PKCγ gene deletion in the astroglial response following repeated *in vivo* treatment with morphine in the dorsal horn of the mouse spinal cord. The almost complete failure to induce the astroglial proliferation and hypertrophy following repeated treatment with morphine was observed by PKCγ gene deletion. Although the exact mechanism is unclear at this time, a hypothesis would be advanced that the release of transmitters including glutamate and adenosine 5'-triphosphate (ATP), neuromodulators such as brain-derived neurotrophic factor (BDNF) and prostaglandins (PGs), and other signaling molecules from neurons through the activation of neuronal PKCγ following repeated administration of morphine may be responsible for the activation of spinal astrocytes.

In conclusion, the present data indicate that repeated *in vivo* treatment with morphine induces astroglial hypertrophy and proliferation associated with activating neuronal PKCγ in the mouse spinal cord. These findings suggest that PKCγ isoform is likely to be one of the most important factors to modulate the communication between neurons and glial cells. Such findings raise the fascinating possibility that the increased astroglial response is involved in the development of opioid tolerance and the induction

of neuronal plasticity associated with the sustained activation of neuronal PKCγ isoform.

Chapter 3

Involvement of spinal metabotropic glutamate receptor 5 in the development of tolerance to morphine-induced antinociception

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian CNS, the actions of which are regulated by ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) [49,50]. mGluRs have been identified and classified into three groups according to their sequence homology, signal transduction pathways and pharmacological selectivity: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8). Of these mGluRs, group I mGluRs are predominately located postsynaptic neurons where they couple to G_q proteins to activate phospholipase C (PLC). PLC catalyzes the production of diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol (1,4,5)-triphosphate (IP_3), which activates IP_3 receptor to release of Ca^{2+} from intracellular stores [51-53].

In the spinal cord, glutamate mediates the transmission of sensory information. Recent behavioral and electrophysiological evidences have shown that administration of selective mGluR1 and mGluR5 agonists enhances behavioral responses to noxious stimulation and induces activity in dorsal horn neurons [89,90]. Expression of mGluR5 is predominantly found in the soma and dendrites of superficial dorsal horn neurons and sparsely found in the astrocytes [91-93]. Recent observations have revealed that glial mGluRs can be involved in the interaction between glial cells and neurons in physiological as well as pathological conditions [94,95].

The administration of morphine into the spinal cord produces a powerful

antinociception. It is well known that prolonged exposure to morphine results in tolerance to morphine-induced antinociception. Furthermore, as described in Chapter 2, I found that repeated *in vivo* treatment with morphine produced a significant increase in neuronal PKC in the dorsal horn of the mouse spinal cord. It has been documented that systemic and brain injections of mGluR5 antagonists significantly attenuate the development of tolerance to morphine-induced antinociception [96,97]. However, the specific contribution of mGluR5 in the spinal cord to the suppression of tolerance to morphine-induced antinociceotion remains unclear. The aim of the present chapter was then to investigate whether the spinal mGluR5 could contribute to the development of tolerance to morphine-induced antinociception in mice.

Materials and Methods

Animals

Male ICR mice weighing about 25 g (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) at the beginning of the experiments were used in the present study. Animals were housed in a room maintained at 23 ± 1 ˚C with an alternating 12 hr light-dark cycle. Food and water were available *ad libitum* during the experimental period.

Drugs

Morphine hydrochloride (Sankyo, Tokyo, Japan) and methyl-6-(phenylethynyl)-pyridine hydrochloride (MPEP, Sigma-Aldrich, MO, USA) were dissolved in 0.9 % sterile. 3,5-Dihydroxyphenylglycine (DHPG) was purchased from Tocris (MO, USA).

Intrathecal injection procedure

Intrathecal (i.t.) injection was performed following the method described in Chapter 1.

Assessment of antinociception

The morphine-induced antinociceptive response was evaluated by recording the latency to paw licking or tapping in the hot-plate test (55 ± 0.5 ˚C, Muromachi Kikai Co., LTD., Tokyo). To prevent tissue damage, we established a 30 sec cut-off time. The test was performed 30 min after morphine treatment. Each animal served as its

own control, and the latency to response was measured both before and after drug administration. To investigate the development of antinociceptive tolerance following repeated treatment with morphine, mice were repeatedly injected with morphine (10 mg/kg, s.c.) or saline (10 ml/kg, s.c.) once a day for 7 consecutive days. In the combination study, MPEP (10 mg/kg, s.c. or 1 nmol/mouse, i.t.) was administered 30 min before s.c. treatment with morphine. Antinociception was calculated as percentage of the maximum possible effect (% MPE) according to the following formula; % MPE = (test latency – pre-drug latency)/ (cut-off time – pre-drug latency) x 100. Antinociceptive response represents as the mean % MPE ± SEM.

Western blotting

The procedure for Western blotting was performed following the method described in Chapter 1.

The membrane was incubated with primary antibody diluted in TBS [1:50,000 mGluR5 (Upstate, VA, USA)] containing 5 % nonfat dried milk overnight at 4 °C.

Immunohistochemistry

Immunohistochemistry was performed following the method described in Chapter 2.

Mice were repeatedly injected with morphine (10 or 20 mg/kg, s.c.) or saline once a day for 7 days. The primary antibodies were diluted in 0.01 M PBS containing 10 % NGS [1:3000 mGluR5 (Upstate), 1:320 microtubule associated protein 2a/b (MAP2a/b, Chemicon International, Inc.), 1:500 neuronal nuclei (NeuN, Chemicon International,

Inc.) and 1:400 GFAP (Chemicon International, Inc.), 1:800 S100β (Sigma-Aldrich)] and incubated for 48 hr at 4 °C. Fluorescence immunolabeling was observed with a light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus) or a confocal microscope (Radiance 2000, Bio-Rad Laboratories, CA, USA). The density of mGluR5 labeling was measured with a computer-assisted imaging analysis system (NIH Image) described in Chapter 2.

In vitro receptor binding assay

For the membrane preparation, the spinal cords of mice were quickly removed 24 hr after repeated morphine (10 mg/kg, s.c.) injection and rapidly transferred to a tube filled with ice-cold buffer. The membrane homogenate was prepared as described previously [98]. Briefly, the dissected tissue was homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 1 mM EGTA. The homogenate was centrifuged at 1,000 x g for 10 min at 4 °C and the supernatant was ultracentrifuged at 48,000 x g for 20 min at 4 °C. The pellet was suspended in ice-cold assay buffer containing 50 mM HEPES (pH 7.4) and 2 mM $MgCl_2$ followed by centrifugation at 48,000 x g for 20 min at 4 °C. The resultant pellet was resuspended in ice-cold assay buffer and stored at -80 °C until used.

Saturation binding experiments were performed in triplicate with increasing concentrations of [^{3}H]MPEP (0.2-100 nM). The binding assay was carried out by incubation for 2 hr, and non-specific binding was determined in the presence of 10 μM MPEP. The binding was terminated by rapid filtration through glass fiber filters

(Unifilter-96 GF/C plate; ParkinElmer Life Sciences, MA, USA) presoaked with 0.3 % polyethyleneimine using a 96-well plate cell harvester. Filters were washed three times with ice-cold assay buffer. After the addition of scintillant, the radioactivity was determined by liquid scintillation spectrometry (TopCount; Packerd Instruments, CT, USA). Protein concentrations were measured by Bicinchoninate (BCA) Compatible protein Assay kit (Pierce, Rockford, IL USA) using bovine serum albumin as the standard. The binding curves were fitted using the GraphPad prism 4.0 program (Graphpad Software, CA, USA).

Confocal Ca^{2+} imaging

The procedure for the tissue processing was performed according to the method described in Chapter 2. On day 8, the cells were treated with morphine (10 μM) and MPEP (10 μM) for 3 days.

Cells were loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Inc., 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 Laboratories). 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: $Rs = F/F_0$).

Cultured spinal neurons were perfused with DHPG (10-100 μM) for 30 sec at 5

mL/min at room temperature in cultured spinal cord neurons followed by the superfusion of balanced salt saline (BSS, pH 7.4) containing 150 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 10 mM D-glucose.

Statistical analysis

The data are presented as the mean ± SEM. The statistical significance of differences between the groups was assessed with Student's *t*-test or one-way ANOVA followed by Bonferroni/Dunnett test.

Results

Suppression of the development of tolerance to morphine-induced antinociception by pretreatment with MPEP

The effect of pretreatment s.c. and i.t. with the selective mGluR5 antagonist MPEP on the development of tolerance to morphine-induced antinociception is shown in Fig.1. In both s.c. and i.t. saline-pretreated mice, the s.c. injection of morphine produced about 70 % antinociceptive effect on the first day. However, the antinociception was significantly decreased during consecutive exposure to morphine and was clearly reduced over 7 days, indicating the development of tolerance to morphine-induced antinociception ($p<0.01$ and $p<0.001$ vs. the first day of saline-pretreated morphine group, Fig. 1A, B). The development of tolerance to morphine-induced antinociception was significantly suppressed by both s.c. and i.t. pre-injection with MPEP (Fig. 1A: s.c. MPEP-pretreated morphine group vs. s.c. saline-pretreated morphine group, $F_{1,18} = 6.67$, $p<0.05$; Fig. 1B: i.t. MPEP-pretreated morphine group vs. i.t. saline-pretreated morphine group, $F_{1,15} = 16.23$, $p<0.01$).

Increase in mGluR5 density performed by [^{3}H]MPEP binding to the mouse spinal cord membrane preparations obtained from mice repeatedly treated with morphine

I next examined the change in density of mGluR5 by monitoring the binding of [^{3}H]MPEP to membranes of the mouse spinal cord following repeated treatment with

morphine. Figures 2A and 2B show the saturation curves and Scatchard plots of [^{3}H]MPEP binding in spinal cord membranes from morphine- and saline-treated mice. The B_{max} value of [^{3}H]MPEP in the membrane preparation from the spinal cord was significantly increased in morphine-treated mice as compared to saline-treated mice ($p<0.05$). There was no significant different in the K_d values for [^{3}H]MPEP between saline-treated and morphine-treated mice (Fig. 2B).

Increase in levels of mGluR5 by repeated treatment with morphine in the superficial dorsal horn of the mouse spinal cord

The change in protein levels of membrane-bound mGluR5 following repeated treatment with morphine in the mouse spinal cord detected by Western blotting is shown in Fig. 3. The spinal cord membrane was prepared at 24 hr after the last injection of saline and morphine. Repeated s.c. treatment with morphine produced a significant increase in protein levels of mGluR5 in membranes of the mouse spinal cord as compared to that found in saline-treated mice (159.9 ± 4.5 % of increase, $p<0.001$ vs. saline-treated mice, Fig. 3).

Twenty-four hr after the last repeated injection of morphine, the IR for mGluR5 in the spinal cord was observed by immunohistochemical analysis. In saline-treated mice, mGluR5-IR was strongly distributed in lamina I and II, progressively decreasing density in lamina III, IV and V (Fig. 4A). Furthermore, mGluR5-IR was found in the network of lateral spinal nucleus (LSN) neurites, located in the dorsolateral funiculus. The most intense mGluR5-IR appeared to be concentrated in inner part of lamina II (lamina

IIi). In high-magnification image of the heavily immunoreactive lamina IIi, it was apparent that the neuropil contained granular immunolabelings were observed surrounding neuronal somata (Fig. 4B). Using semi-quantitative analysis, repeated s.c. treatment with morphine produced a significant increase in the level of mGluR5-IR in the dorsal horn of the spinal cord in a dose-dependent manner (10 mg/kg: 168.9 ± 10.6 % of control; 20 mg/kg: 192.9 ± 1.8 % of control, $p < 0.001$ vs. saline-treated mice, Fig. 4C, D, E, F). It should be mentioned that the increased mGluR5-IR by morphine was extended to the outer part of laminae II and laminae I as well as laminae IIi. Furthermore, double-labeling experiments showed that the neuron-specific nuclear protein marker NeuN-IR (red) in the dorsal horn of the spinal cord was surrounded by mGluR5-IR (green, Fig. 5A, B). In addition, mGluR5-IR (green) was apparent colocalization with the dendritic protein marker MAP2a/b-IR (red) in the spinal cord of morphine-treated mice (Fig. 5D). In mice treated chronically with morphine, GFAP-IR (red) in the dorsal horn of the mouse spinal cord was increased with morphologic differentiation (Fig. 6A), which was sparsely co-localized with mGluR5-IR (green, Fig. 6B). In order to further investigate whether mGluR5 could be expressed on the reactive astrocytes more clearly, mGluR5-IR was analyzed at high magnification by using the optical-sectioning capabilities of confocal microscopy. As a result, the increased mGluR5-IR by morphine was hardly any co-localization with either GFAP-IR (Fig. 6C-i, C-ii) or the marker of the cell body for astrocytes, S100β-IR (Fig. 6C-iii).

Enhancement of DHPG-evoked increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in morphine-treated spinal cord neuron/glia cocultures

I next investigated the change in neuronal activity evaluated by monitoring the $[Ca^{2+}]i$ evoked by DHPG in morphine-treated neuron/glia cocultures. DHPG (10-100 μM) produced a transient increase in the $[Ca^{2+}]i$ in cultured spinal cord neurons (Fig. 7). The Ca^{2+} responses induced by DHPG in neurons were dose-dependently enhanced by 3 days of treatment with morphine (10 μM: $p< 0.01$ vs. control cells, 100 μM: $p< 0.001$ vs. control cells, Fig. 7). These effects were blocked by 3 days of treatment with MPEP (10 μM).

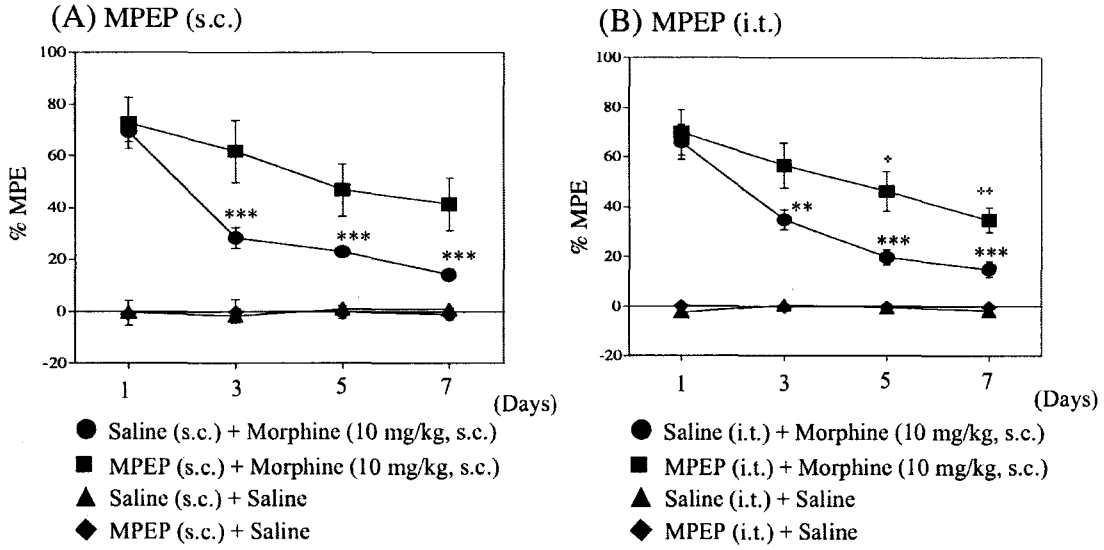


Fig. 1 Effect of pretreatment with MPEP on the development of tolerance to morphine-induced antinociception. Mice were repeatedly injected with morphine (10 mg/kg, s.c.) or saline once a day for 7 consecutive days. MPEP (A: 10 mg/kg, s.c., B: 1 nmol/mouse, i.t.) was administered 30 min before every morphine treatment. Each point represents % antinociception at 30 min after saline or morphine injection. In both s.c. and i.t. saline-pretreated mice, the antinociception induced by morphine was significantly decreased during consecutive exposure to morphine (**$p<0.01$ and ***$p<0.001$ vs. the first day of saline-pretreated morphine group, *$p<0.05$ and **$p<0.01$ vs. the first day of MPEP-pretreated morphine group, Fig. 1A and 1B). The development of tolerance to morphine-induced antinociception was significantly inhibited by both s.c. and i.t. injection with MPEP (Fig. 1A: s.c. MPEP-pretreated morphine group vs. s.c. saline-pretreated morphine group, $F_{1,18} = 6.67$, $p<0.05$; Fig. 1B: i.t. MPEP-pretreated morphine group vs. i.t. saline-pretreated morphine group, $F_{1,15} = 16.23$, $p<0.01$). Each group used 10-12 mice.

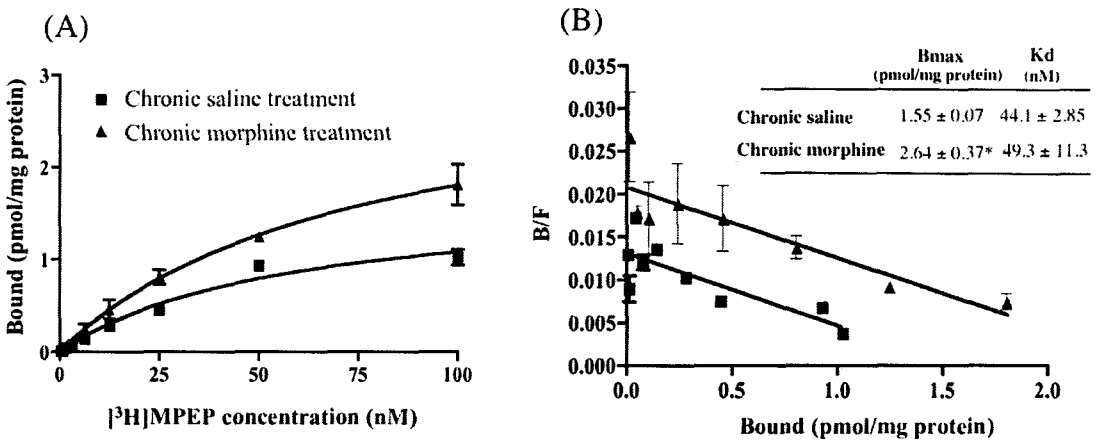


Fig. 2 Saturation curves (A) and Scatchard plots (B) for the specific binding of [^{3}H]MPEP to spinal cord membranes from chronic saline- and morphine-treated mice. Groups of mice were treated with morphine (10 mg/kg, s.c.) and saline once a day for 7 consecutive days. Twenty-four hour after the last injection, the membrane fractions were prepared. [^{3}H]MPEP binding assay was carried out in a range from 0.2 to 100 nM. The specific binding was defined as the difference in binding observed in the absence and presence of 10 μM unlabeled MPEP. The data represent the mean ± S.E.M. from three separate experiments performed in triplicate. *P<0.05 vs. chronic saline-treated mice.

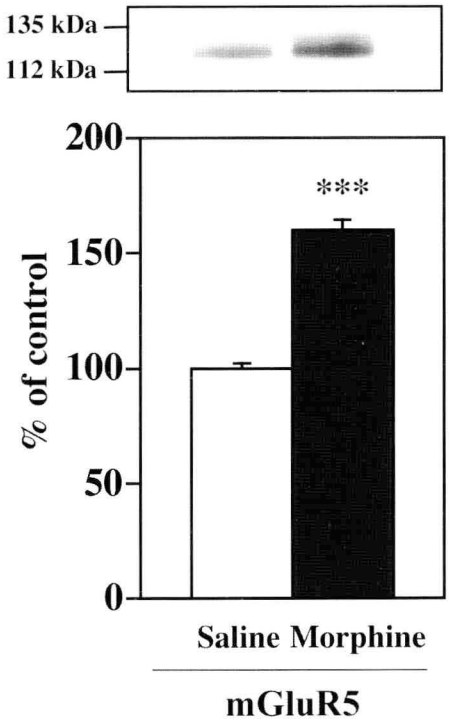


Fig. 3 Influence of levels of mGluR5 in membranes of the mouse spinal cord after repeated morphine treatment. *Upper*: Representative Western blot of mGluR5. *Lower*: Changes in immunoreactivity for the mGluR5 in membranes of spinal cords obtained from saline- or morphine-treated mice. Mice were repeatedly injected with saline or morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. The membrane fraction was prepared at 24 hr after the last injection. Each column represents the mean ± S.E.M. of 3 independent samples. *** $p < 0.001$ vs. saline-treated mice.

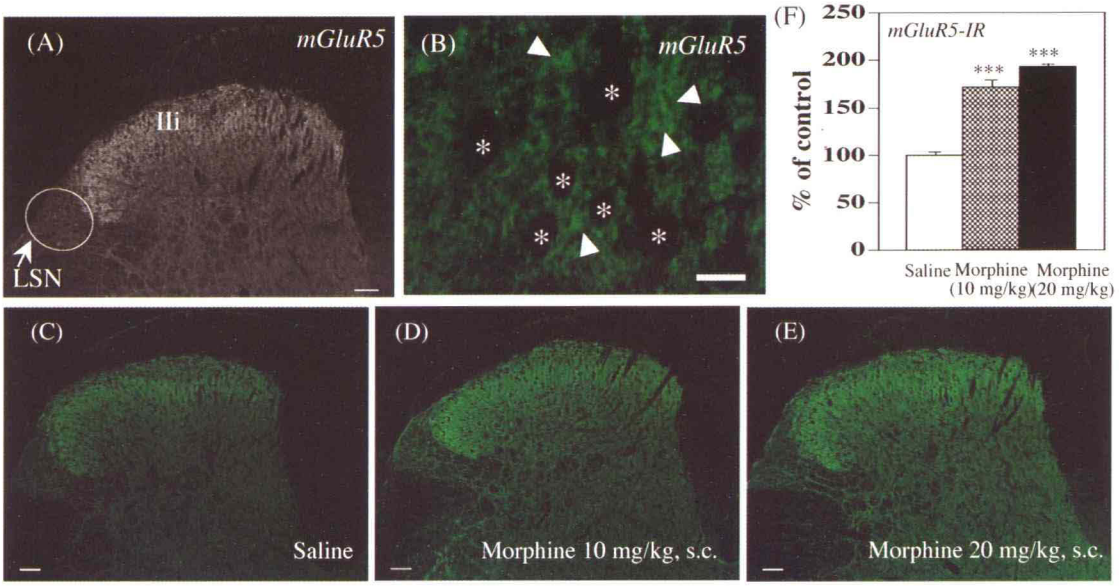


Fig. 4 (A) Distribution of mGluR5-IR in the mouse spinal cord. mGluR5-IR was distributed in inner part of lamina II (IIi) and in the lateral spinal nucleus (LSN; arrow). (B) High magnification of the heavily immunoreactive lamina II. The neuropil contained granular-like mGluR5-IR (arrowhead) and many neuronal somata (astarisk). (C, D, E) Increase in levels of mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine in a dose-dependent manner. (F) Semi-quantitative analysis of mGluR5-IR was performed using NIH image (10 mg/kg: 168.9 ± 10.6 % of control ***$p < 0.001$ vs. saline-treated mice; 20 mg/kg: 192.9 ± 1.8 % of control ***$p < 0.001$ vs. saline-treated mice). Each column represents the mean ± S.E.M. of three independent samples. Scale bars: 50 μm.

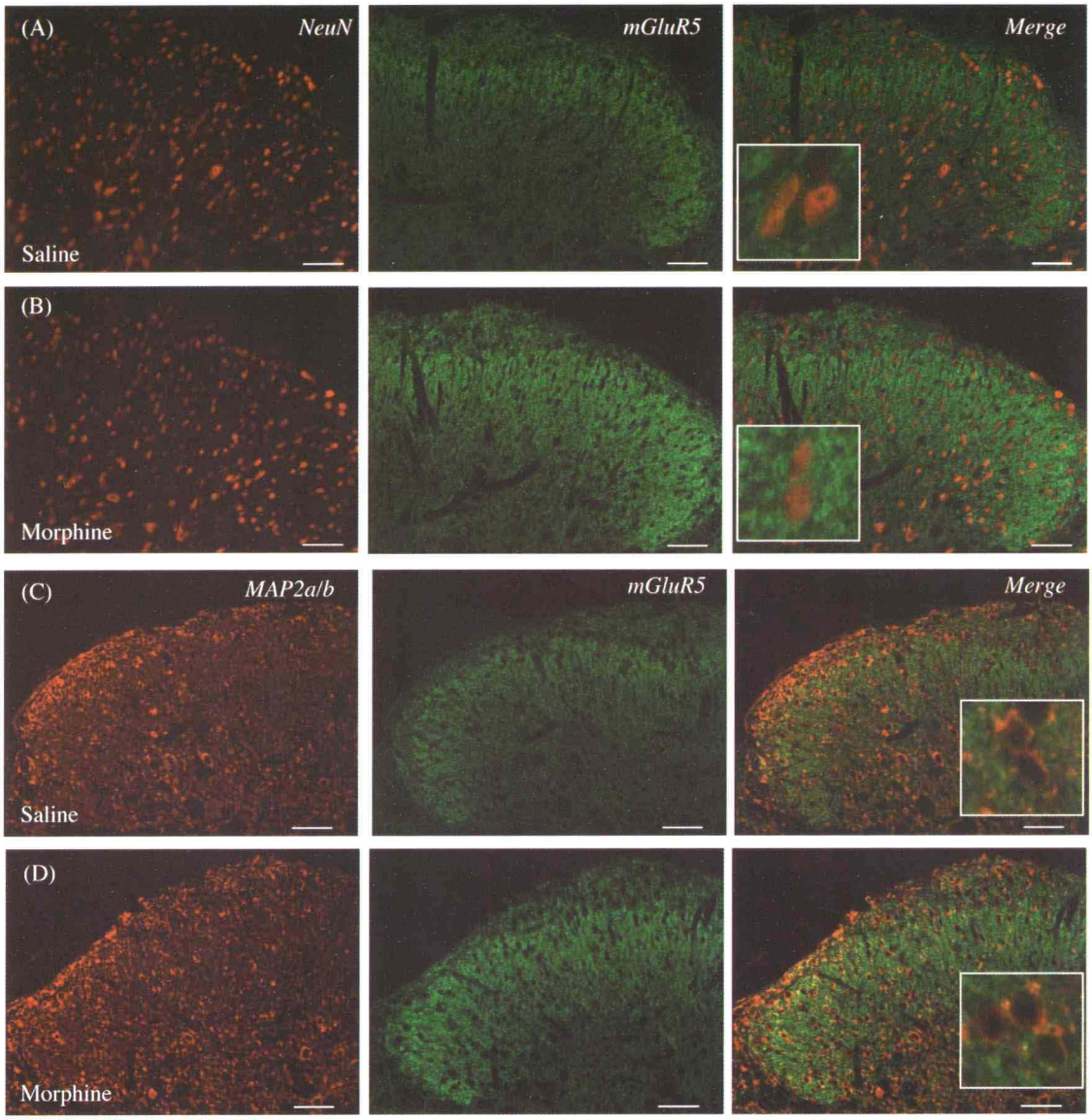


Fig. 5 Localization of the increased mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine. (A, B) NeuN-IR (red) in the dorsal horn of the spinal cord was surrounded by mGluR5-IR (green). (C, D) The increased mGluR5-IR (green) was more apparent colocalization with MAP2a/b-IR (red) in the spinal cord of morphine-treated mice, as compared to saline-treated mice. Scale bars: 50 μm.

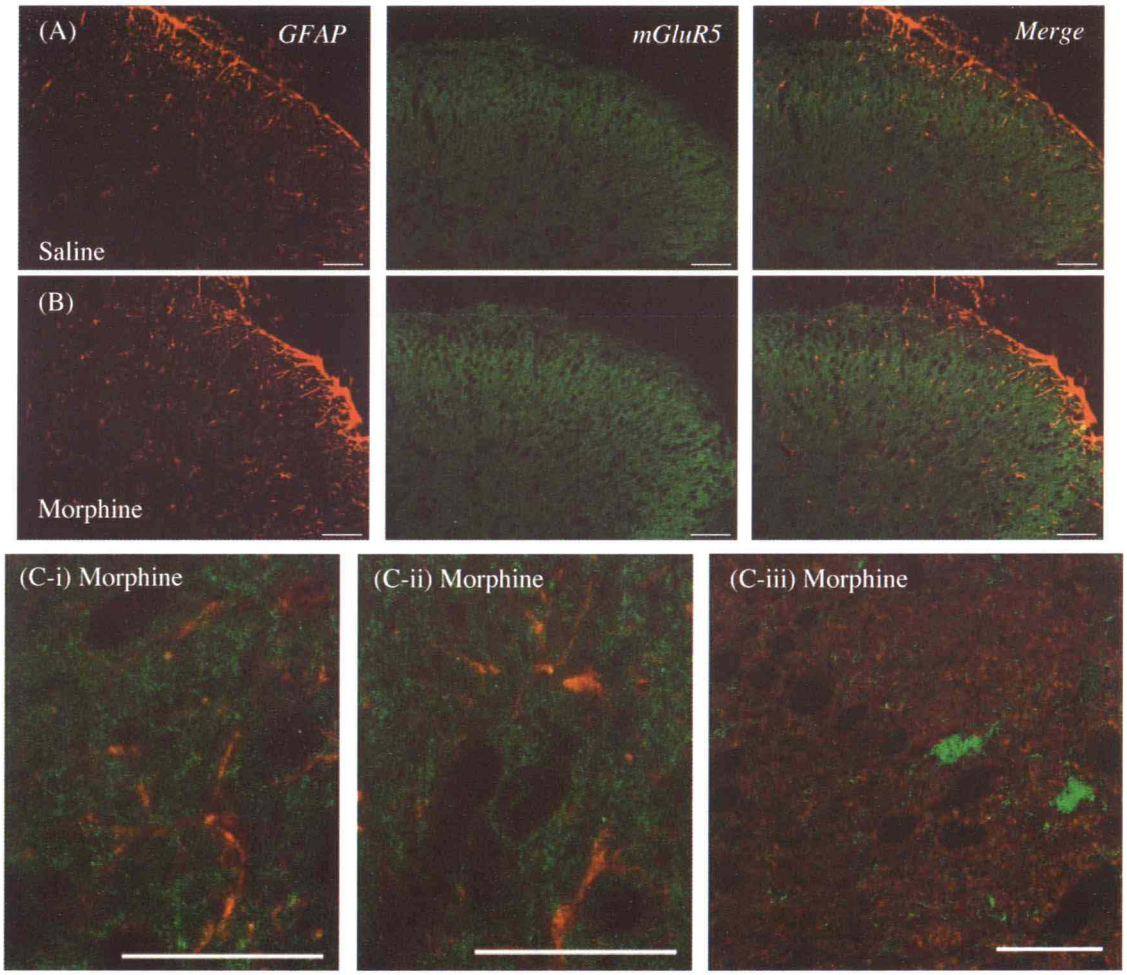


Fig. 6 Localization of the increased mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine. GFAP-IR (red) in the dorsal horn of the mouse spinal cord was increased with morphologic differentiation after repeated morphine treatment (B), which was sparsely co-localized with the increased mGluR5-IR (green). High-magnification image of the superficial laminae of the dorsal horn analyzed by confocal microscope. The red labeled for GFAP and the green labeled for mGluR5 (C-i, C-ii) or the green labeled for S100 and the red labeled for mGluR5 (C-iii) are no apparent co-localization in the superficial layers of the spinal cord of morphine-treated mice. Scale bars: (A, B) = 50 μm, (C) = 10 μm.

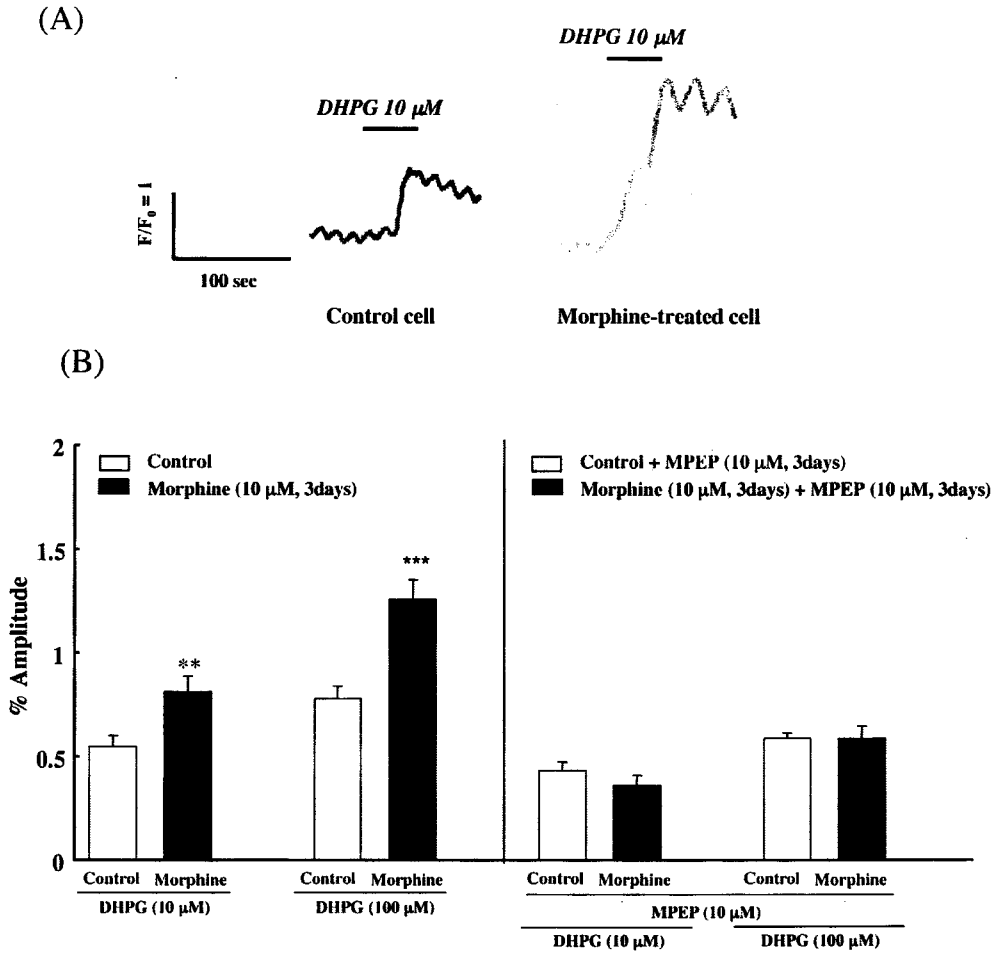


Fig. 7 Changes in Ca^{2+} response to DHPG in the spinal cord neuron following repeated treatment with morphine. (A) Traces show the DHPG (10 μM)-evoked increase in the intracellular Ca^{2+} concentration in control and morphine (10 μM)-treated spinal cord neurons. (B) The response of Ca^{2+} to DHPG in control and morphine-treated spinal cord neurons are summarized. The response of Ca^{2+} to DHPG (10-100 μM) in spinal cord neurons was dose-dependently enhanced by 3 days treatment with morphine (10 μM: **$p < 0.01$ vs. control cells, 100 μM: ***$p < 0.001$ vs. control cells). These effects were blocked by 3 days of treatment with MPEP (10 μM). Data represent the mean ± S.E.M. of 35-40 cells from 3 separate observations.

Discussion

The key finding in the present study was that repeated *in vivo* treatment with morphine produced a dose-dependent increase in mGluR5-IR in the superficial dorsal horn of the spinal cord. Double-immunofluorescence analysis revealed that the increased mGluR5 was predominantly localized in the neuropil and the surface of neural membrane of laminae I-III neurons following repeated treatment with morphine. Furthermore, I found using receptor binding assay that mice tolerant to morphine exhibited a marked increase in the B_{max} value of [^{3}H]MPEP, a selective radioligand for mGluR5, without changing in the K_d value in the spinal cord. Although it has been reported that s.c. or i.c.v. administration of the selective mGluR5 antagonist MPEP prevents the development of the antinociceptive tolerance to morphine [96,97], I found here for the first time that i.t. pretreatment with MPEP significantly inhibited the development of tolerance to morphine-induced antinociception.

Activation of mGluR5 results in PLC-catalyzed PI hydrolysis, which leads to the release of Ca^{2+} from intracellular sources and stimulation of PKC [99]. Previously, it has been reported that PKC inhibitors attenuated the development of tolerance to morphine's actions [83]. In addition, repeated *in vivo* treatment with morphine produced a significant increase in neuron-specific PKCγ-IR located in the laminae IIi with its expanding distribution in the dorsal horn of the spinal cord described in Chapter 2. Taken together, these findings support the idea that the increased number of membrane-bound mGluR5 following repeated treatment with morphine may lead to a long-lasting

activation of neuronal PKC in the dorsal horn of the spinal cord, which is responsible for the development of tolerance to morphine-induced antinociception.

Considering the increase in mGluR5 by repeated morphine treatment, one wonder whether this increase is due to the increased expression of functional receptors located on the membrane. Thus, I next investigated whether the function of mGluR5 in cultured spinal cord neurons could be enhanced following chronic treatment with morphine as evaluated by monitoring the intracellular Ca^{2+} concentration induced by the group I mGluR agonist DHPG. In the present study, the increased intracellular Ca^{2+} concentration induced by DHPG in cultured spinal cord neurons was potently enhanced by chronic *in vitro* exposure to morphine. This effect was blocked by treatment with MPEP. It is, therefore, likely that chronic treatment with morphine leads to the functional up-regulation of group I mGluR including mGluR5, which may result in the enhancement of neuronal activity and synaptic transmission in the spinal cord.

In Chapters 1 and 2, I stated that mice tolerant to morphine exhibited astroglial hypertrophy and proliferation associated with activating neuronal PKC in the dorsal horn of the spinal cord. In support of these findings, it has been reported that quantitative change in levels of GFAP can be observed in the rat spinal cord after chronic administration of morphine [66,67]. Accumulating evidences indicate that activation of glial mGluR5 leads to the release of glutamate through a SNARE-dependent exocytotic mechanism to modulate neuronal excitability and synaptic functions [100,101]. In the present study, the increased IR for mGluR5 was expressed in nonglial cells in the dorsal horn of the spinal cord of morphine-treated mice, as shown

by no apparent co-localization with either GFAP-IR or S100β-IR. These findings suggest that the increased mGluR5 located in the spinal cord neuron is implicated in the development of tolerance to morphine-induced antinociception.

Here I found that repeated treatment with morphine produced a dose-dependent increase in mGluR5-IR with its expanding distribution in the dorsal horn of the spinal cord. Although the data suggest that the increased expression of neuronal mGluR5 following repeated treatment with morphine may be responsible for the development of tolerance to morphine-induced antinociception, the molecular mechanisms underlying this phenomenon is unclear. It has been documented that most mGluR5-IR is observed in the dendritic shafts, spike-like structures and cell body of the postsynaptic regions [91]. One speculation related to the present results is that repeated stimulation of μ-opioid receptor promotes the new protein synthesis of mGluR5 or suppresses the protein degradation of mGluR5 associated with receptor internalization to increase the excitatory synaptic transmission in opposition to excessive activation of inhibitory neurons in the dorsal horn. It should be noted in our preliminary data that MPEP-treated mice failed to exhibit the increased level of mGluR5 in the spinal dorsal horn following repeated morphine treatment (data not shown). Furthermore, mice tolerant to morphine exhibited the significant increase in protein levels of the vesicular glutamate transporter 1 and the synaptic vesicle-specific small G protein Rab3A, but not vesicular glutamate transporter 2 and vesicular GABA transporter. In addition, we found that high K^+-induced glutamate release in spinal neuron/glia co-cultures was largely increased following chronic morphine treatment compared with that of the

control (data not shown). These data support the idea that repeated stimulation of μ-opioid receptors may initially cause the long-lasting suppression of the release of glutamate, and in turn may increase the number of functional mGluR5 associated with the new production of mGluR5 or the suppression of the degradation of mGluR5 through its related intracellular signaling pathway to enhance the glutamate synaptic transmission. This phenomenon would be, at least in part, responsible for the suppression of the morphine-induced antinociception, which could be eventually called tolerance to spinal antinociception induced by morphine.

In conclusion, the present study indicates that repeated *in vivo* treatment with morphine induces the increase in the functional mGluR5s in the mouse spinal cord, which contributes to the development of tolerance to morphine-induced antinociception.

General Conclusion

The above findings lead to the following conclusions:

In Chapter 1:

Repeated s.c. treatment with etorphine, but not morphine, produced a significant increase in protein levels of GRK 2, dynamin II, β-arrestin 2 and p-cPKC in membranes of the mouse spinal cord, suggesting that the etorphine-induced μ-opioid receptor desensitization may result from GRK 2/dynamin II/β-arrestin2-dependent phosphorylation of μ-opioid receptors. Unlike etorphine, morphine failed to change the levels of these trafficking proteins. Furthermore, I found that the level of GFAP in the mouse spinal cord was clearly increased by chronic *in vivo* and *in vitro* treatment with morphine, whereas no such effect was noted by etorphine. In consistent with these results, intrathecal pretreatment with the glial-modulating agent propentofylline suppressed the development of tolerance to morphine-induced antinociception. In addition, intrathecal injection of astrocytes/ACM mixture, which were obtained from cultured astrocytes of the newborn mouse spinal cord, aggravated the development of tolerance to morphine. In contrast, these agents failed to affect the development of tolerance induced by etorphine. These findings provide direct evidence for the distinct mechanisms between etorphine and morphine on the development of tolerance to spinal antinociception. These findings raise the possibility that the increased astroglia response due to chronic morphine treatment may result in the morphine-specific

receptor modulating profile, which could be related to the down-regulation of μ-opioid receptor function without receptor internalization and may actively participate in the development of morphine tolerance and the induction of neuronal plasticity.

In Chapter 2:

Repeated treatment with morphine caused a significant increase in PKCγ-IR with expanding distribution in the dorsal horn of the spinal cord associated with the development of tolerance to morphine-induced antinociception. The PKCγ-IR was exclusively co-localized with neuron specific markers, NeuN and MAP2a/b. Furthermore, mice tolerant to morphine exhibited a dramatic increase of reactive astrocytes in the dorsal horn of the spinal cord by repeated treatment with morphine, as characterized by the increase and morphological changes in GFAP-positive cells. In addition, the transgenic mice that express an EGFP under the control of the mouse GFAP promoter displayed the enhanced levels of EGFP expression by repeated treatment with morphine. Under these conditions, mice lacking the PKCγ gene failed to show any changes in astroglial hypertrophy and proliferation by repeated treatment with morphine. These findings suggest that the sustained activation of neuronal PKCγ due to chronic treatment with morphine is implicated in the increased levels of reactive astrocytes in the dorsal horn of the spinal cord, which may be involved in the development of morphine tolerance and the induction of neuronal plasticity.

In Chapter 3:

The development of tolerance to morphine-induced antinociception caused by repeated treatment with morphine was suppressed by repeated i.t. or s.c. treatment with the selective mGluR5 antagonist MPEP. Furthermore, the density and protein level of mGluR5 in membranous fraction of the mouse spinal cord was significantly increased by repeated treatment with morphine. In addition, repeated treatment with morphine produced a dose-dependent increase in mGluR5-IR in the superficial dorsal horn of the spinal cord. Double-labeling experiments showed that the increased mGluR5 was predominantly expressed in the neurons and sparsely expressed in the processes of astrocytes following repeated treatment with morphine. In consist with these results, the increased intracellular Ca^{2+} concentration induced by the selective group I mGluR agonist, DHPG, in cultured spinal cord neurons was potently enhanced by 3 days in vitro treatment with morphine. These findings support the idea that the increased mGluR5 in neurons following repeated treatment with morphine leads to the enhanced neuronal excitability and synaptic transmission in the dorsal horn of the spinal cord, and in turn suppresses the morphine-induced antinociception in mice.

Working hypothesis

In the dorsal horn of the spinal cord, presynaptic sensory neurons receive and transmit nociceptive information in the periphery to the spinal cord. Postsynaptic second-order neuron receives and transmits nociceptive information to the brain. Synaptic astrocytes surround neurons and maintain the environment. Here, I describe my hypothesis concerning the change in spinal function following chronic morphine

treatment (See Fig. A). Activation of μ-opioid receptors located on presynaptic sensory neurons induces the inhibition of glutamate release. In the morphine-tolerant state, repeated stimulation of μ-opioid receptors causes the prolonged suppression of the release of glutamate, which results in an increase in the number of mGluR5 in postsynaptic second-order neurons. Consequently, the mGluR5-dependent increase in the intracellular Ca^{2+} concentration and the activation of PKCγ can be potentially enhanced. The release of transmitters including glutamate, ATP and other signaling molecules from neurons through the activation of PKCγ induces astroglial proliferation and hypertrophy. The activated astrocytes release a variety of neuroactive substances, which may result in a shift in the balance of excitatory input and alter the nociceptive threshold. This phenomenon could explain the mechanism of the suppression of morphine-induced antinociception following chronic morphine treatment, which is called analgesic tolerance to morphine.

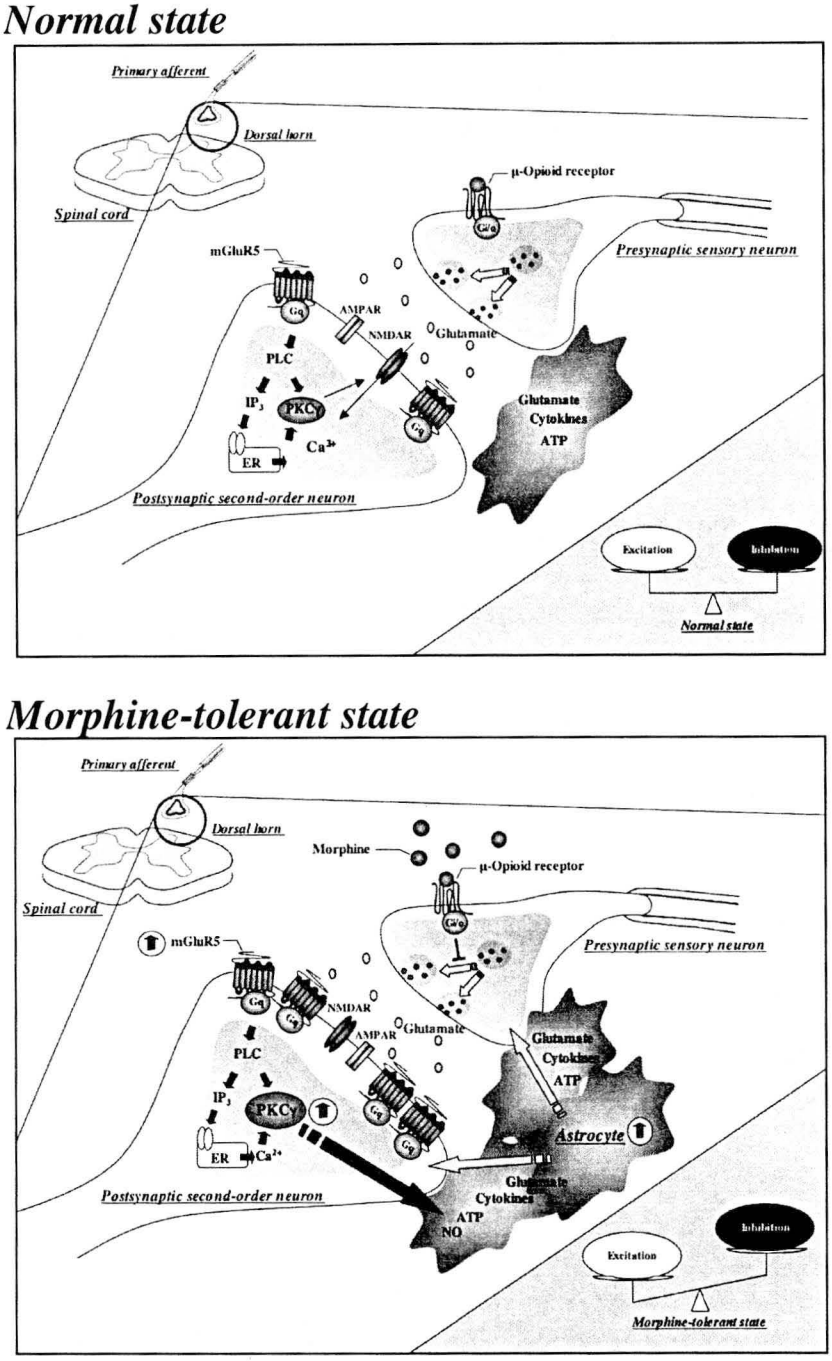


Fig. A Simplified schema of molecular mechanism of the development of tolerance to morphine-induced antinociception following repeated treatment with morphine in the dorsal horn of the spinal cord. See the detail in "Working hypothesis".

List of Publications

This dissertation is based on the following original publications:

1, Minoru Narita, Masami Suzuki, Michiko Narita, Keiichi Niikura, Atsushi Nakamura, Mayumi Miyatake, Yoshinori Yajima and Tsutomu Suzuki: μ-Opioid receptor internalization-dependent and -independent mechanisms of the development of tolerance to μ-opioid receptor agonists: Comparison between etorphine and morphine. Neuroscience (in press): Chapter 1

2, Minoru Narita, Masami Suzuki, Michiko Narita, Yoshinori Yajima, Ryusuke Suzuki, Seiji Shioda and Tsutomu Suzuki: Neuronal protein kinase Cγ-dependent proliferation and hypertrophy of spinal cord astrocytes following repeated *in vivo* administration of morphine. Eur J Neurosci 19, 479-484 (2004): Chapter 2

3, Minoru Narita, Masami Suzuki, Michiko Narita, Keiichi Niikura, Atsushi Nakamura, Mayumi Miyatake, Takeshi Aoki, Yoshinori Yajima and Tsutomu Suzuki: Involvement of spinal metabotropic glutamate receptor 5 in the development of tolerance to morphine-induced antinociception. J Neurochem, 94, 1297-1305 (2005): Chapter 3

Acknowledgements

This research will never be materialized without the help of the following people and organization:

First, I would like to express my gratitude and appreciation to Professor Tsutomu Suzuki (Department of Toxicology, School of Pharmacy and Pharmaceutical Science, Hoshi University) and Associate Professor Minoru Narita (Department of Toxicology, School of Pharmacy and Pharmaceutical Science, Hoshi University) for their helpful guidance in my research work and preparing this dissertation, and for giving a chance of this research work.

I would like to thank Ms. Michiko Narita for her excellent technical assistance, helpful guidance and valuable advice in my research work.

I would like to thank Dr. Seiji Shioda (Professor, Department of Anatomy, Showa University School of Medicine) for his great technical suggestions.

I wish to thank Dr. Yoshinori Yajima, Dr. Takeshi Aoki, Mr. Keiichi Niikura, Mr. Atsushi Nakamura, Ms. Mayumi Miyatake, Ms. Megumi Asato, Ms. Naoko Kuzumaki and Dr. Junaidi Khotib for their great technical assistance in my research work.

Also, I wish to thank Mr. Hideaki Kato, Mr. Satoshi Imai, Ms. Miho Soma and Ms. Masumi Ioka for their stimulating discussions and kindly guidance in my research work. Further, I wish to thank Ms. Mayumi Negishi, Mr. Kan Miyoshi, Ms. Sayaka Enomoto, Ms. Ayumi Ozeki and Mr. Yuuki Takigawa for their technical assistance in my research work.

Finally, I would like to express my gratitude to my parents, grandmother, brothers and friends for their assistance in my life.

References

1) Lord J, Waterfield A, Hughes J and Kosterlitz H. Endogenous opioid peptides: Multiple agonists and receptors. *Nature* **267**, 495-499 (1977).

2) Chang K. and Cuatrescasas P. Multiple opiate receptors. Enkephalins and morphine binding to receptors of different specificity. *J Biol Chem* **254**, 2610-2618 (1979).

3) Paterson S, Robson L and Kosterlitz H. Classification of opioid receptors. *Br Med Bull* **39**, 31-36 (1983).

4) Evans CJ, Keith DEJr, Morrison H, Magendzo K and Edwards RH. Cloning of a delta opioid receptor by functional expression. *Science* **258**, 1952-1955 (1992).

5) Kieffer BL, Befort K, Gaveriaux-Ruff C and Hirth CG. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U S A* **89**, 12048-12052 (1992).

6) Chen Y, Mestek A, Liu J, Hurley JA and Yu L. Molecular cloning and functional expression of a μ-opioid receptor from rat brain. *Mol Pharmacol* **44**, 8-12 (1993).

7) Wang JB, Imai Y, Eppler CM, Gregor P, Spivak CE and Uhl GR. μ Opiate receptor: cDNA cloning and expression. *Proc Natl Acad Sci U S A* **90**, 10230-10234 (1993).

8) Meng F, Xie GX, Thompson RC, Mansour A, Goldstein A, Watson SJ and Akil H. Cloning and pharmacological characterization of a rat κ opioid receptor. *Proc Natl Acad Sci U S A* **90**, 9954-9958 (1993).

9) Li S, Zhu J, Chen C, Chen YW, Deriel JK, Ashby B and Liu-Chen LY. Molecular cloning and expression of a rat κ opioid receptor. *Biochem J* **295**, 629-633 (1993).

10) Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, Low M and Grandy DK. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a μ, δ or κ opioid receptor type. *FEBS Lett* **347**, 284-288 (1994).

11) Chen Y, Fan Y, Liu J, Mestek A, Tian M, Kozak CA and Yu L. Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. *FEBS Lett* **347**, 279-283 (1994).

12) Childers SR. Opioid receptor-coupled second messengers. *Life Sci* **48**, 1991-2003 (1991).

13) Christie MJ and North RA. Agonist at μ-opioid, M_2-muscarinic and $GABA_B$-receptors increase the same potassium conductance in rat lateral parabrachial neurons. *Br J Pharmacol* **95**, 896-902 (1988).

14) Rhim H and Miller RJ. Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. *J Neurosci* **14**, 7608-7615 (1994).

15) Murthy K and Makhlouf G. Opioid μ, δ, and κ receptor-induced activation of phospholipase C-β3 and inhibition of adenylyl cyclase is mediated by G_{i2} and G_o in smooth muscle. *Mol Pharmacol* **50**, 870-877 (1996).

16) Kramer HK and Simon EJ. Role of protein kinase C (PKC) in agonist-induced μ-opioid receptor down-regulation: I. PKC translocation to the membrane of SH-SY5Y neuroblastoma cells is induced by μ-opioid agonist. *J Neurochem* **72**, 585-593 (1999).

17) Fukuda K, Kato S, Morikawa H, Shoda T and Mori K. Functional coupling of the δ-,

μ- and κ-opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. *J Neurochem* **67**,1309-1316 (1996).

18) Ventafridda V and De Conno F. Organizing pain control and rehabilitation service in a cancer centre. *Int Rehabil Med* **3**, 149-154 (1981).

19) Bohm SK, Grady EF and Bunnet NW. Regulatory mechanisms that modulate signaling by G-protein-coupled receptors. *Biochem J* **322**, 1-18 (1997).

20) Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ and Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci* **27**, 107-144 (2004).

21) Lefkowitz RJ. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* **273**, 18677-18680 (1998).

22) Claing A, Laporte SA, Caron MG and Lefkowitz RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol* **66**, 61-79 (2002).

23) Wang JM, Ali H, Richardson R, Snyderman R and Oppenheim JJ. Opiates transdeactivate chemokine receptors: δ and μ opiate receptor-mediated heterologous desensitization. *J Exp Med* **188**, 317-325 (1998).

24) Koeing JA and Edwarson JM. Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sci* **18**, 276-287 (1997).

25) Wang HL. A cluster of Ser/Thr residues at the C-terminus of μ-opioid receptor is required for G protein-coupled receptor kinase 2-mediated desensitization.

Neuropharmacology **39**, 353-363 (2000).

26) Kovoor A, Celver JP, Wu A and Chavkin C. Agonist induced homologous desensitization of μ-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. *Mol Pharmacol* **54**, 704-711 (1998).

27) Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY and Caron MG. Role for G protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. *Proc Natl Acad Sci U S A* **95**, 7157-7162 (1998).

28) Nishizuka Y. The molecular heterogeneity of protein kinase C and implications for cellular regulation. *Nature* **334**, 661-665 (1988).

29) Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-614 (1992).

30) Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* **9**, 484-496 (1995).

31) Newton AC. Protein kinase C: Structure, function, and regulation. *J Biol Chem* **270**, 28495-28498 (1995).

32) Tanaka C and Nishizuka Y. The protein kinase C family for neuronal signaling. *Annu Rev Neurosci* **17**, 551-567 (1994).

33) Asaoka Y, Kikkawa U, Sekiguchi K, Shearman MS, Kosaka Y, Nakano Y, Satoh T and Nishizuka Y. Activation of a brain-specific protein kinase C subspecies in the presence of phosphatidylethanol. *FEBS Lett* **231**, 221-224 (1988).

34) Ito A, Saito N, Hirata M, Kose A, Tsujino T, Yoshihara C, Ogita K, Kishimoto A, Nishizuka Y and Tanaka C. Immunocytochemical localization of the alpha

subspecies of protein kinase C in rat brain. *Proc Natl Acad Sci U S A* **87**, 3195-3199 (1990).

35) Hua XY, Chen P and Yaksh TL. Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. *Neurosci Lett* **276**, 99-102 (1999).

36) Munro FE, Fleetwood-Walker SM and Mitchell R. Evidence for a role of protein kinase C in the sustained activation of rat dorsal horn neurons evoked by cutaneous mustard oil application. *Neurosci Lett* **170**, 199-202 (1994).

37) Mao J, Price DD, Mayer DJ and Hayes RL. Pain-related increases in spinal cord membrane-bound protein kinase C following peripheral nerve injury. *Brain Res* **588**, 144-149 (1992).

38) Coderre TJ. Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. *Neurosci Lett* **140**, 181-184 (1992).

39) Zeitz KP, Malmberg AB, Gilbert H and Basbaum AI. Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC γ mutant mice. *Pain* **94**, 245-253 (2001).

40) Ohsawa M, Narita M, Mizoguchi H, Cheng E and Tseng LF. Reduced hyperalgesia induced by nerve injury, but not by inflammation in mice lacking protein kinase C γ isoform. *Eur J Pharmacol* **429**, 157-160 (2001).

41) Malmberg AB, Chen C, Tonegawa S and Basbaum AI. Preserved acute pain and reduced neuropathic pain in mice lacking PKCγ. *Science* **278**, 279-283 (1997).

42) Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S and Hydon PG. Glutamate-

mediated astrocyte-neuron signaling. *Nature* **369**, 744-747 (1994).

43) Araque A, Parpura V, Sanzgiri RP and Hydon PG. Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci* **22**, 208-215 (1999).

44) Barres BA, Chun LL and Corey DP. Glial and neuronal forms of the voltage-dependent sodium channel: characteristics and cell-type distribution. *Neuron* **2**, 1375-1388 (1989).

45) Barres BA, Chun LL and Corey DP. Ion channels in vertebrate glia. *Annu Rev Neurosci* **13**, 441-474 (1990).

46) Verkhratsky A. and Steinhauser C. Ion channels in glial cells. *Brain Res Rev* **32**, 380-412 (2000).

47) Bezzie P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T and Volterra A. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* **391**, 281-285 (1998).

48) Hansson E and Ronnback L. Astrocytes in neurotransmission. A review. *Cell Mol Biol* **36**, 487-496 (1990).

49) Dingledine R, Borges K, Bowie D and Traynelis SF. The glutamate receptor ion channels. *Pharmacol Rev* **51**, 7-61 (1999).

50) Sugiyama H, Ito I and Hirono C. A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature* **325**, 531-533 (1987).

51) Conn PJ and Pin JP. Pharmacology and functions of metabotropic glutamate receptors. *Ann Rev Pharmacol Toxicol* **37**, 205-237 (1997).

52) Schoepp DD. Unveiling the functions of presynaptic metabotropic glutamate

receptors in the central nervous system. *J Pharmacol Exp Ther* **299**, 12-20 (2001).

53) Cartmell J and Schoepp DD. Regulation of neurotransmitter release by metabotropic glutamate receptors. *J Neurochem* **75**, 889-907 (2000).

54) Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG and Lefkowitz RJ. Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* **333**, 370-373 (1988).

55) Pippig S, Andexinger S and Lohse MJ. Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**, 666-676 (1995).

56) von Zastrow M, Svingos A, Haberstock-Debic H and Evans C. Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr Opin Neurobiol* **13**, 348-353 (2003).

57) Burford NT, Tolbert LM and Sadee N. Specific G protein activation and μ-opioid receptor internalization caused by morphine, DAMGO and endomorphin I. *Eur J Pharmacol* **342**, 123-126 (1998).

58) Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ and von Zastrow M. Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **271**, 19021-19024 (1996).

59) Keith DE, Anton B, Murray S, Zaki PA, Chu PC, Lissin DV, Agius GM, Stewart PL, Evans CJ and von Zastrow M. μ-Opioid receptor internalization: Opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**, 377-384 (1998).

60) Whistler JL and von Zastrow M. Dissociation of functional roles of dynamin in

receptor-mediated endocytosis and mitogenic signal transduction. *J Biol Chem* **274**, 24575-24578 (1999).

61) Patel CN, Rajashekara V, Patel K, Purohit V and Yoburn BC. Chronic opioid antagonist treatment selectively regulates trafficking and signaling proteins in mouse spinal cord. *Synapse* **50**, 67-76 (2003).

62) Zhang Q, Purohit V and Yoburn BC. Continuous opioid agonist treatment dose-dependently regulates μ-opioid receptors and dynamin-2 in mouse spinal cord. *Synapse* **56**, 123-128 (2005).

63) Fields RD and Atevens-Graham B. New insights into neuron-glia communication. *Science* **298**, 556-562 (2002).

64) Norton WT, Aquino DA, Hozumi I, Chiu FC and Brosnan CF. Quantitative aspects of reactive gliosis. *Neurochem Res* **17**, 877-885 (1992).

65) Meller ST, Dykstra C, Grzybycki D, Murphy S and Gebhart GF. The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. *Neuropharmacology* **33**, 1471-1478 (1994).

66) Song P and Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res* **39**, 281-286 (2001).

67) Raghavendra V, Rutkowski MD and DeLeo JA. The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J Neurosci* **22**, 9980-9998 (2002).

68) Hylden JL and Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* **67**, 313-316 (1980).

69) Yoburn BC, Purohit V, Patel K and Zhang Q. Opioid agonist and antagonist treatment differentially regulates immunoreactive μ-opioid receptors and dynamin-2 in vivo. *Eur J Pharmacol* **498**, 87-96 (2004).

70) Patel MB, Patel CN, Rajashekara V and Yoburn BC. Opioid agonists differentially regulate μ-opioid receptors and trafficking proteins in vivo. *Mol Pharmacol* **62**, 1464-1470 (2002).

71) Capeyrou R, Riond J, Corbani M, Lepage JF, Bertin B and Emorine LJ. Agonist-induced signaling and trafficking of the μ-opioid receptor: role of serine and threonine residues in the third cytoplasmic loop and C-terminal domain. *FEBS Lett* **415**, 200-205 (1997).

72) Kouhen ER, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY and Loh HH. Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates μ-opioid receptor internalization. *J Biol Chem* **276**, 12774-12780 (2001).

73) Berrada K, Plesnicher CL, Luo X and Thibonnier M. Dynamic interaction of human vasopressin/oxytocin receptor subtypes with G protein-coupled receptor kinases and protein kinase C after agonist stimulation. *J Biol Chem* **275**, 27229-27237 (2000).

74) Premont RT, Inglese J and Lefkowitz RJ. Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J* **9**, 175-182 (1995).

75) Ji S, Liu X, Li S, Shen L, Li F, Wang J, Han J and Yao L. PH domain of G protein-coupled receptor kinase-2 binds to protein kinase C (PKC) and negatively regulates activity of PKC kinase. *Front Biosci* **8**, A34-39 (2003).

76) Narita M, Mizoguchi H, Suzuki T, Narita M, Dun NJ, Imai S, Yajima Y, Nagase H, Suzuki T and Tseng LF. Enhanced μ-opioid responses in the spinal cord of mice lacking protein kinase Cγ isoform. *J Biol Chem* **276**, 15409-15414 (2001).

77) Haydon PG. Glia: listening and talking to the synapse. *Nat Rev Neurosci* **2**, 185-193 (2001).

78) Tawfik VL, LaCroix-Fralish ML, Nutile-McMenemy N and DeLeo JA. Transcriptional and translational regulation of glial activation by morphine in a rodent model of neuropathic pain. *J Pharmacol Exp Ther* **13**, 1239-1247 (2005).

79) Shavit Y, Wolf G, Goshen I, Livshits D and Yirmiya R. Interleukin-1 antagonizes morphine analgesia and underlies morphine tolerance. *Pain* **115**, 50-59 (2005).

80) Svensson CI, Hua XY, Protter AA, Powell HC and Yaksh TL. Spinal p38 MAP kinase is necessary for NMDA-induced spinal PGE(2) release and thermal hyperalgesia. *Neuroreport* **14**, 1153-1157 (2003).

81) Krenz NR and Weaver LC. Nerve growth factor in glia and inflammatory cells of the injured rat spinal cord. *J Neurochem* **74**, 730-739 (2000).

82) Narita M, Narita M, Mizoguchi H and Tseng LF. Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered μ-opioid receptor agonist in the mouse. *Eur J Pharmacol* **280**, R1-R3 (1995).

83) Abeliovich A, Chen C, Goda Y, Sliva AJ, Stevens CF and Tonegawa S. Modified hippocampal long-term potentiation in PKCγ-mutant mice. *Cell* **75**, 1253-1262 (1993).

84) Miura M, Tamura T and Mikoshiba K. Cell-specific expression of the mouse glial fibrillary acidic protein gene: identification of the cis- and trans-acting promoter elements for astrocyte-specific expression. *J Neurochem* **55**, 1180-1188 (1990).

85) Suzuki R, Sato A, Nakajo S, Ikenaka K, Kikuyama S and Shioda S. Expression of the receptor for pituitary adenylate cyclase-activating polypeptide (PAC_1-R) in the reactive astrocytes. *Mol Brain Res* **115**, 10-20 (2003).

86) Bliss TVP and Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31-39 (1993).

87) Martin WJ, Malmberg AB and Basbaum AI. PKCγ contributes a subset of the NMDA-dependent spinal circuits that underlie injury-induced persistent pain. *J Neurosci* **21**, 5321-5327 (2001).

88) Narita M, Mizoguchi H, Narita M, Dun NJ, Nagase H, Suzuki T and Tseng LF. Involvement of spinal protein kinase Cγ in the attenuation of opioid μ-receptor-mediated G-protein activation after chronic intrathecal administration of [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin. *J Neurosci* **21**, 3715-3720 (2001).

89) Fisher K and Coderre TJ. Comparison of nociceptive effects produced by intrathecal administration of mGluR agonists. *Neuroreport* **7**, 2743-2747 (1996).

90) Neugebauer V, Chen PS and Willis WD. Role of metabotropic glutamate receptor subtype mGluR1 in brief nociception and central sensitization of primate STT cells. *J Neurophysiol* **82**, 272-282 (1999).

91) Alvarez FJ, Villalba RM, Carr PA, Grandes P and Somohano PM. Differential distribution of metabotropic glutamate receptors 1a, 1b, and 5 in the rat spinal cord. *J*

Comp Neurol **422**, 464-487 (2000).

92) Gallo V and Ghiani CA. Glutamate receptors in glia: new cells, new inputs and new functions. *Trends Pharmacol Sci* **21**, 252-258 (2000).

93) Tao YX, Li YQ, Zhao ZQ and Johns RA. Synaptic relationship of the neurons containing a metabotropic glutamate receptor, MGluR5, with nociceptive primary afferent and GABAergic terminals in rat spinal superficial laminae. *Brain Res* **875**, 138-143 (2000).

94) Aronica E, van Vliet EA, Mayboroda OA, Troost D, da Silva FH and Gorter JA. Upregulation of metabotropic glutamate receptor subtype mGluR3 and mGluR5 in reactive astrocytes in a rat model of mesial temporal lobe epilepsy. *Eur J Neurosci* **12**, 2333-2344 (2000).

95) Peavy RD, Sorensen SD and Conn PJ. Differential regulation of metabotropic glutamate receptor 5-mediated phosphoinositide hydrolysis and extracellular signal-regulated kinase responses by protein kinase C in cultured astrocytes. *J Neurochem* **83**, 110-118 (2002).

96) Kozela E, Pilc A and Popik P. Inhibitory effects of MPEP, an mGluR5 antagonist, and memantine, an *N*-methyl-D-aspartate receptor antagonist, on morphine antinociceptive tolerance in mice. *Psychopharmacology* **165**, 245-251 (2003).

97) Smith FL, Smith PA, Dewey WL and Javed RR. Effects of mGlu1 and mGlu5 metabotropic glutamate antagonists to reverse morphine tolerance in mice. *Eur J Pharmacol* **492**, 137-142 (2004).

98) Aoki T, Narita M, Shibasaki M and Suzuki T. Metabotropic glutamate receptor 5

localized in the limbic forebrain is critical for the development of morphine-induced rewarding effect in mice. *Eur J Neurosci* **20**, 1633-1638 (2004).

99) Kawabata S, Kohara A, Tsutsumi R, Itahana H, Hayashibe S, Yamaguchi T and Okada M. Diversity of calcium signaling by metabotropic glutamate receptors. *J Biol Chem* **273**, 17381-17385 (1998).

100) Bezzi P, Gundersen V, Galbete JL, Seifert G, Steinhauser C, Pilati E and Volterra A. Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. *Nat Neurosci* **7**, 613-620 (2004).

101) Montana V, Ni Y, Sunjara V, Hua X and Parpura V. Vesicular glutamate transporter-dependent glutamate release from astrocytes. *J Neurosci* **24**, 2633-2642 (2004).