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# Interactions Between Glutamate Receptors and TRPV1 Involved in Nociceptive Processing at Peripheral Endings of Primary Afferent Fibers

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## 1. Introduction

Glutamate (Glu) is a main excitatory neurotransmitter in the central nervous system. Concerning the existence of Glu in the small-diameter afferent fibers, their central (Westlund et al., 1989; Keast and Stephensen, 2000) and peripheral (Westlund et al., 1992; Keast and Stephensen, 2000) processes as well as dorsal root ganglion (DRG) cells (Battaglia and Rustioni, 1988; Keast and Stephensen, 2000) contain Glu. Recently, Glu has been shown to have a role in transduction of sensory input at the periphery (Carlton, 2001).

Electron microscope studies demonstrate that Glu receptors are transported from the DRG cell bodies into central and/or peripheral primary afferent terminals (Liu et al., 1994). The N-methyl-D-aspartic acid (NMDA),  $\alpha$ -amino-3-hydroxy-5methyl-4-isoxazole propionic acid (AMPA) and kainate receptors (NMDA/AMPA-kainate receptors) are localized on unmyelinated axons at the dermal-epidermal junction in the glabrous and hairy skin of the rat (Carlton et al., 1995; Coggeshall and Carlton, 1998), and in human hairy skin (Kinkelin et al., 2000). Approximately 20% of the fibers were immunostained in one of the receptor subtypes. As Sato et al. (1993) reported that virtually all DRG cells as well as their central (Laurie et al., 1995; Zou et al., 2002) and peripheral (Carlton et al., 1995) processes are positively labeled for the NMDA receptor, it is highly likely that two or more of the ionotropic Glu receptors are colocalized.

Behavioral evidence supports a role for peripheral Glu receptors in normal nociceptive transmission. Intraplantar injection of L-Glu into the hindpaw evokes hyperalgesia in rats (Follenfant and Nakamura-Craig, 1992; Carlton et al., 1995). Furthermore, intraplantar injection of the specific Glu receptors agonists NMDA, AMPA or kainate results in mechanical hyperalgesia and allodynia that can be blocked by appropriate antagonists (Zhou et al., 1996). Hyperalgesia is induced by binding the released glutamate to NMDA receptor (Leem et al., 2001; Du et al., 2003), group I mGluR (Bhave et al., 2001; Zhou et al., 2001; Hu et al., 2002; Walker et al., 2001; Lee et al., 2007), but not group II mGluR (Yang and Gereau IV, 2003).

In addition to these behavioral and anatomical data, Omote et al. (1998) showed that subcutaneous administration of inflammatory substances such as formalin induced the release of peripheral EAAs (Glu and aspartate) on the ipsilateral side. We have already reported that local application of capsaicin cream evoked a marked increase in Glu level in the s.c. perfusate. In addition, electrical stimulation of the sciatic nerve or noxious heat stimulation (50°C) also caused increase of Glu level in the s.c. space, and this capsaicin-evoked Glu release was significantly decreased by daily high-dose pretreatment with capsaicin for three consecutive days (Jin et al., 2006).

The capsaicin receptor, transient receptor potential vanilloid 1 (TRPV1), is located in a neurochemically heterogeneous population of small diameter primary afferent neurons (Tominaga et al., 1998). This receptor is sensitive to high temperature in the noxious range of 43°C to 50°C (Hardy, 1953; Beitel and Dubner, 1976; Caterina et al., 1997). Furthermore, repeated exposure to high-dose capsaicin selectively produces a prolonged influx of cations leading to desensitization of small-diameter sensory neurons to subsequent noxious stimulation (Yonehara et al., 1987; Lynn, 1990; Zhou et al., 1998; Caterina and Julius, 2001), while myelinated A $\beta$  fibers are insensitive to capsaicin (Jancso et al., 1977; Nagy et al., 1983; Michael and Priestly, 1999).

There is an evidence suggesting possibility that capsaicin-evoked pain responses might be regulated by peripheral GluRs. In this connection, Lam et al. (2005) demonstrated that peripheral NMDA receptor modulate jaw muscle electromyographic activity induced by capsaicin injection into the temporomandibular joint of rats.

This study, therefore, has been done to elucidate at large in what manner Glu receptors and Glu existing in the peripheral endings of small-diameter afferent fibers and their extracellular space, respectively, are involved in development and/or maintenance of nociception evoked by capsaicin. Additionally, in order to demonstrate a link between the increase of Glu levels in the extracellular space following noxious stimulation and pain behavior, the changes in thermal withdrawal latency and the expression of c-Fos protein in the dorsal horn were determined following subcutaneous (s.c.) injection of drugs associated with Glu receptors with/without capsaicin.

## 2. Materials and methods

All surgical and experimental procedures for animals were reviewed and approved by the Ohu University Intramural Animal Care and Use Committee and conformed to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

## 2.1 Experimental procedures

Adult male Sprague-Dawley rats weighing between 200-300 g (CLEA Japan, INC. Tokyo, Japan) were used in all experiments. Rats were on a 12 hrs light/dark cycle and received food and water ad libitum.

## 2.2 Release of Glu into the subcutaneous space

Animals were anesthetized with urethane (1 g/kg i.p.). A single loop catheter whose tip was covered with a 5000 molecular weight dialysis membrane (MS 0045, PSS® SELECT, Florida) was introduced into the s.c. space of the instep using a 2.2 mm outer diameter polyethylene tube as a guide. Ringer's solution was perfused at 15 µl/min through this catheter with a micro syringe pump (EP-60, Eicom, Kyoto, Japan) and perfusate was collected into the tubes placed in an ice bath at intervals of 20 min. The samples were kept at -80°C until analysis.

## 2.3 Amino acid analysis

Amino acids in the dialysate were analyzed by a high-performance liquid chromatography (HPLC) system for automated analysis of amino acids using o-phthalaldehyde derivatization and fluorescence detection. Amino acids were quantified by reverse-phase chromatography using a C<sub>18</sub> octadecylsilyl (ODS) silica-gel column (EICOMPAK SC-50DS 2.1 mm x 150 mm) with pre-column (EICOM PREPAKSET-AC 3 mm x 4 mm). An HPLC system (HTEC500, EICOM) attaching this column consists of a pump connected with a degasser, a sampling injector with a sample processor and a cool pump, a fluorescence HPLC monitor and a personal computer with the data processor (Power Chrom; EPC-500, EICOM). The mobile phase used for separation of amino acids was 100 mM, pH 6 phosphate buffer containing 30% methanol and 10 µM EDTA. The flow rate was 0.23 ml/min. Peak areas of unknown substances were compared to those of control compounds for quantitation.

To determine the effect of drugs on the level of Glu, the average amounts of Glu concentration in two 20-min fractions collected over periods of 40 min before and after local application of capsaicin cream were obtained and expressed as percentages of the control value before stimulation.

## 2.4 Drug administration

While the animals were inside the small cage, drugs were administered into the s.c. left hindpaw in a volume of 50 µl using a 100 µl Hamilton syringe (Reno, NV, USA) with a 30-gauge needle without any anesthesia. The needle was inserted into the plantar skin proximal to the midpoint of the hindpaw. Capsazepine (30mg/kg) was injected in the volume of 50 µl into the s.c. of the neck.

## 2.5 Behavioral assessments

The Plantar Test (model 7370; Ugo Basile, Verese, Italy) was used in accordance with previously described methods (Yonehara et al., 1997) to determine whether the rats were hyperalgesic. In brief, prior to testing, the animals were placed in a small cage on a glass

plate. They were not restrained and could move about and explore freely. Radiant heat was beamed onto the plantar surface of the hindpaw. The intensity of the beam was controlled and adjusted prior to the experiments, and the cutoff latency was set at 24 sec. The beam was applied to the test and control foot in turns and the latency of the withdrawal reflexes was recorded. The mean of the four responses was determined (Figs 4-8), and the ratio of the test foot latency divided by control foot latency, multiplied by 100, was calculated and termed the "percentage withdrawal latency" (Fig.3), at hourly intervals, from 1 hr before injection of the drugs to 6 h after the injection, except for 15 min after the injection

## 2.6 c-Fos immunohistochemistry

Two hours after the drug injection, animals were deeply anesthetized with sodium-pentobarbital and perfused transcardially with 100ml of 0.9% saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and the spinal cord was taken out, postfixed in the same fixative overnight at 4°C, and then immersed into 20% sucrose in 0.1M PB at 4°C until it sank. Serial transverse 60 µm thick sections at L4-6 were cut using a freezing microtome and collected in 0.02 M phosphate buffered saline (PBS). Sections were washed in PBS for 30 min and blocked with 1% normal goat serum for 30 min and then incubated in a rabbit antibody against c-Fos (1:7000 dilution; Santa Cruz Biotech, Santa Cruz, CA, USA) for 60 min in room temperature and then for 12 hrs at 4°C. After washing in PBS for 30 min, sections were incubated in biotinylated goat anti-rabbit antiserum, and washed in PBS for 30 min and then immunohistochemically stained for 60 min using avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA, USA). To visualize peroxidase activity, sections were immersed in 0.05% diaminobenzidine tetrahydrochloride, 0.1% ammonium nickel sulfate and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.2). Sections were washed in PBS for 30 min and then mounted on gelatin-coated slides, air-dried and coverslipped. The c-Fos-immunoreactive cells of 10 best-labeled sections were counted in the L5 spinal dorsal horn. In all these tests a double blind procedure was used to prevent the observers from knowing the experimental groups.

## 2.7 Drugs

The list of drugs and chemicals were as follows: as Glu receptors agonist, L-glutamic acid; selective NMDA receptor agonist, NMDA; AMPA receptor agonist,  $\alpha$ -amino-3-hydroxy-4-isoxazolepropionic acid (AMPA); selective group 1 mGlu receptor agonist, (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG); group II mGlu receptor agonist, (2S,1'S,2'S)-2-(carboxycyclopropyl) glycine (L-CCG-I); selective group III mGlu receptor agonist, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4). The following drugs were used for Glu receptors antagonists, selective non-competitive NMDA receptor antagonist, (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d] cyclo-hepten-5, 10-imine hydrogen maleate ((+)-MK-801 hydrogen maleate); competitive kainite/AMPA receptor antagonist, 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX); group 1 mGlu receptor selective non-competitive mGlu<sub>1</sub> receptor antagonist, 7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt); group 1 mGlu receptor mGlu5 subtype-selective antagonist, 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP); group II mGlu receptor antagonist, ((2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine

(MCCG); selective group III mGlu receptor antagonist, (RS)- $\alpha$ -methylserine-O-phosphate (MSOP). These compounds of Glu receptors were obtained from Tocris (Ballwin, MO, USA). 8-Methyl-N-vanillyl-6-noneamide (capsaicin) was obtained from Sigma Chemical Co. (USA). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

In accordance with the product material safety data sheets, L-glutamate acid, L-CCG-I and L-AP4 were diluted in NaOH; and MK801, NMDA, (S)-3, 5,-DHPG, MCCG and MSOP were diluted in water. CNQX, CPCCOEt and MPEP were diluted in dimethyl sulphoxide. The other drugs except for these were dissolved in saline. Capsaicin was prepared as a 10 mg/ml solution in saline containing 10% ethanol and 10% Tween 80. The pH of all solutions was adjusted to 7.4. Capsazepine was dissolved in dimethyl formamide and then diluted with saline. O-phthalaldehyde was dissolved in methanol and adjusted to 4 mM with 0.1 M, pH 9.5 carbonate buffer.

## 2.8 Statistical analysis

All data are shown as mean  $\pm$  S.E.M. In the study of Glu release, statistical analyses were performed using posthoc test of Fisher's protected least significant difference and  $P < 0.05$  was considered to be statistically significant. In the behavioral study, statistical analyses were performed with Dunnett's test for multiple comparison subsequent to analyses of variance. In the c-Fos immunohistochemical study, a Student's test was used to test significant differences of the c-Fos expression between the treatments.

## 2.9 Abbreviations

AMPA;  $\alpha$ -amino-3-hydroxy-4-isoxazole propionic acid, Cap+MK801; Capsaicin combined with MK801, Cap+CNQX; Capsaicin combined with CNQX, Cap+NBQX; Capsaicin combined with NBQX, Cap+CPCCOEt; Capsaicin combined with CPCCOEt, Cap+MCCG; Capsaicin combined with MCCG, Cap+MSOP; Capsaicin combined with MSOP, CNQX; 6-Cyano-7-nitroquinoxaline-2,3-dione disodium, CPCCOEt; 7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester, (S)-3,5-DHPG; (S)-3,5- dihydroxyphenylglycine, DRG; dorsal root ganglion, Glu, glutamate; L-CCG-I; (2S,1'S,2'S)-2-(carboxycyclopropyl) glycine, L-AP4; L-(+)-2-amino-4-phosphonobutyric acid, MCCG; (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl) glycine, mGluRs; metabotropic glutamate receptors, (+)-MK-801; (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d] cyclo-hepten-5, 10-imine hydrogen maleate, MPEP; 2-Methyl-6-(phenylethynyl) pyridine hydrochloride, MSOP; (RS)- $\alpha$ -methylserine-O-phosphate, NBQX; 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt, NMDA; N-methyl-D-aspartic acid.

## 3. Results

### 3.1 Basal Glu release

The concentration of Glu in the perfusate was initially high, but gradually decreased with time reaching a stable level after 2 hrs of perfusion, which was then maintained for at least 4.5 h. Glu was present at  $1.95 \pm 0.25 \mu\text{M}$  ( $n=10$ , S.E.M.) in the resting state which is defined here as the mean of the two 20-min fraction collected from 80 min after starting perfusion to 120 min (fraction 5~6 in control group in Fig.1).

3.2 Effects of capsazepine on capsaicin-evoked Glu release

The s.c. injection of capsaicin (3 mM) in the vicinity of the perfusion side evoked a significant increase in Glu release (Fig.1). The average concentration of the released Glu was  $4.86 \pm 0.48 \mu\text{M}/20 \text{ min}$  in 2 fractions collected after the injection of capsaicin. This augmentation of Glu release was last over 2 h. This effect was remarkably suppressed by preadministration of capsazepine (30 mg/kg, s.c.) 30 min before capsaicin injection (Fig.1). In the group of pretreatment with capsazepine, the average concentrations of the released Glu were  $2.25 \pm 0.4 \mu\text{M}/20 \text{ min}$  and  $2.36 \pm 0.31 \mu\text{M}/20 \text{ min}$  in 2 fractions collected after the injection of vehicle or capsaicin, respectively. S.c. injections of vehicle or capsazepine alone did not produce any significant changes in the levels of Glu in the perfusates.

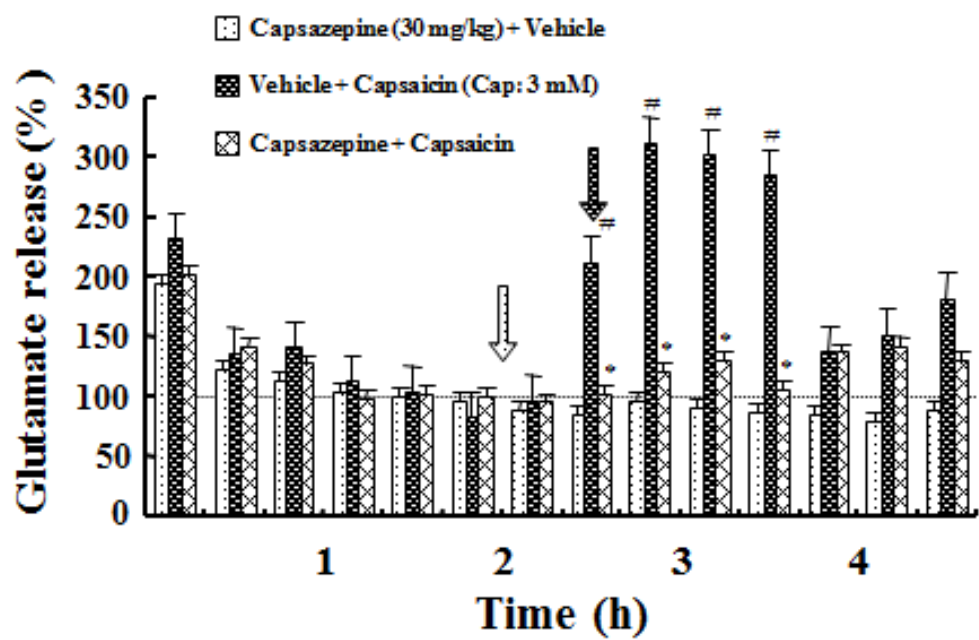


Fig. 1. Effect of capsazepine on the capsaicin-induced glutamate release. Capsazepine (s.c.) was injected subcutaneously into the neck 30 min before capsaicin treatment. Capsazepine (30 mg/kg) or vehicle for capsazepine, and capsaicin (3 mM) or vehicle for capsaicin were subcutaneously injected at the time indicated by the arrows, (□) and (▣), respectively. All data are presented as the mean  $\pm$  S.E.M. obtained from 10 animals. # $P < 0.05$  compare with the value prior to s.c. administration of capsaicin+vehicle. \* $P < 0.05$  compared with capsaicin+vehicle (for capsazepine) group at each time measured.

3.3 Effects of iGluRs antagonists injection on capsaicin-evoked Glu release

The combined injection of capsaicin with MK801(1 mM) (Cap + MK-801) or NBQX (5 mM) (Cap + NBQX) into the perfusion region showed far less Glu release than injection of capsaicin alone (Fig 2-A). The average concentration of the released Glu was  $1.20 \pm 0.1 \mu\text{M}/20 \text{ min}$  or  $1.70 \pm 0.1 \mu\text{M}/20 \text{ min}$  in 2 fractions collected after the co-injection of MK-801 or NBQX with capsaicin, respectively. These inhibitory effects of iGluRs antagonists sustained over 2.5 h.

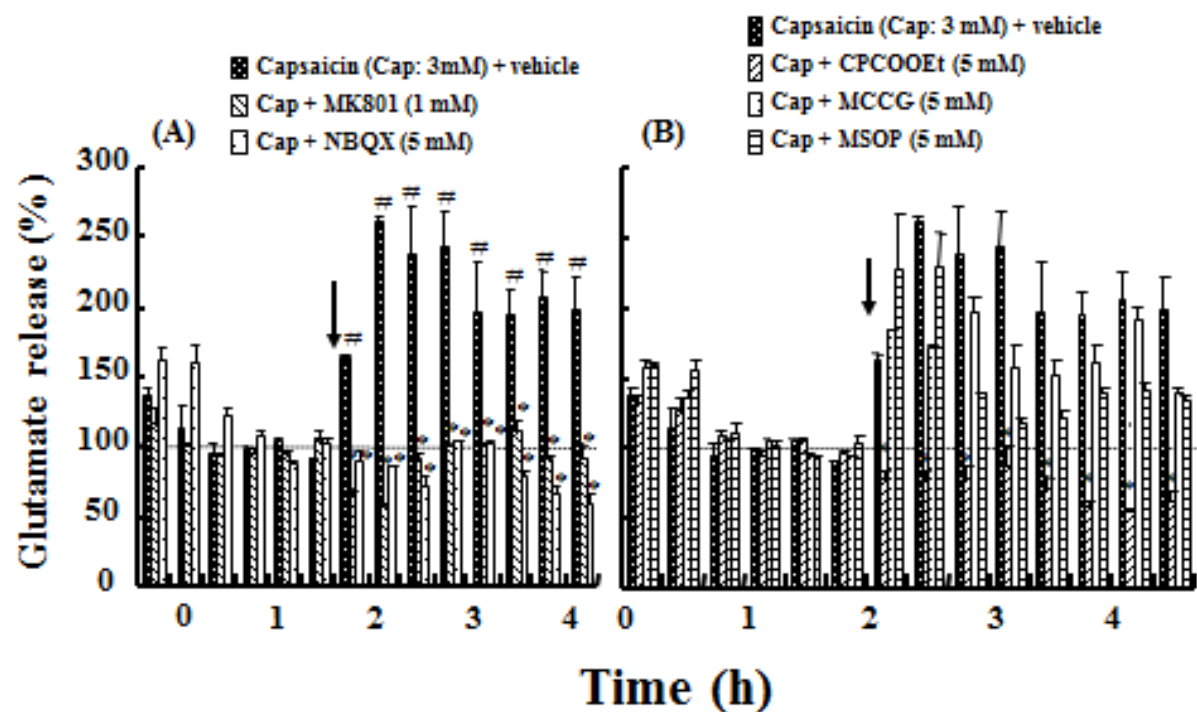


Fig. 2. Effect of the ionotropic (A) and metabotropic (B) glutamate receptor antagonists on the capsaicin-induced glutamate release. The glutamate receptor antagonists were subcutaneously injected together with capsaicin at the time indicated by the arrow. All data are presented as the mean  $\pm$  S.E.M. obtained from 10 animals.  $\#P<0.05$  compare with the value prior to s.c. administration of capsaicin+vehicle.  $*P<0.05$  compared with capsaicin+vehicle group at each time measured. MK801, selective non-competitive NMDA receptor antagonist; NBQX, competitive kainate/AMPA receptor antagonist; CPCOOEt, group 1 mGlu receptor selective non-competitive mGlu<sub>1</sub> receptor antagonist; MCCG, group II mGlu receptor antagonist; MSOP, selective group III mGlu receptor antagonist.

### 3.4 Effects of mGluRs antagonists injection on capsaicin-induced Glu release

At the doses employed, CPCCOEt (5 mM) (Cap + CPCCOEt) showed remarkable inhibition in capsaicin-evoked Glu release. The average concentration of the released Glu was  $1.46 \pm 0.1 \mu\text{M}$ / 20 min after the co-injection of CPCCOEt with capsaicin. S.c. combined injection of MCCG (5 mM) (Cap + MCCG) or MSOP (5 mM) (Cap + MSOP) with capsaicin did not show significant decrease in Glu release compared to capsaicin injection alone. The average concentration of the released Glu was  $3.68 \pm 0.38 \mu\text{M}$  / 20 min or  $4.31 \pm 0.60 \mu\text{M}$ / 20min in 2 fractions collected after the co-injection of MCCG or MSOP with capsaicin, respectively. (Fig. 2-B)

### 3.5 Effects of capsazepine on capsaicin-induced thermal hypersensitivity

The mean withdrawal latencies to stimulation with radiant heat at pre-injection were  $11.2 \pm 0.3$  s and  $11.2 \pm 0.3$  s (n=40) on the left and right side, respectively (Fig.3-A). The withdrawal

latency did not significantly change after injection of vehicle or low dose of capsaicin (0.6 mM). A quarter and one h after injection of capsaicin (3 mM and 6 mM), withdrawal latency to irradiation decreased to much shorter than that of vehicle injection, which was recorded at the same interval ( $P < 0.05$ ), and then recovered gradually to the level of vehicle injection by 4 h after injection of capsaicin. Pretreatment of capsazepine (30 mg/kg, s.c.) produced a marked inhibition against capsaicin-induced thermal hyperalgesia (Fig.3-B). We did not observed any signs of motor deficiency or other side effects for any of the doses of any drugs in all paradigms described here and below.

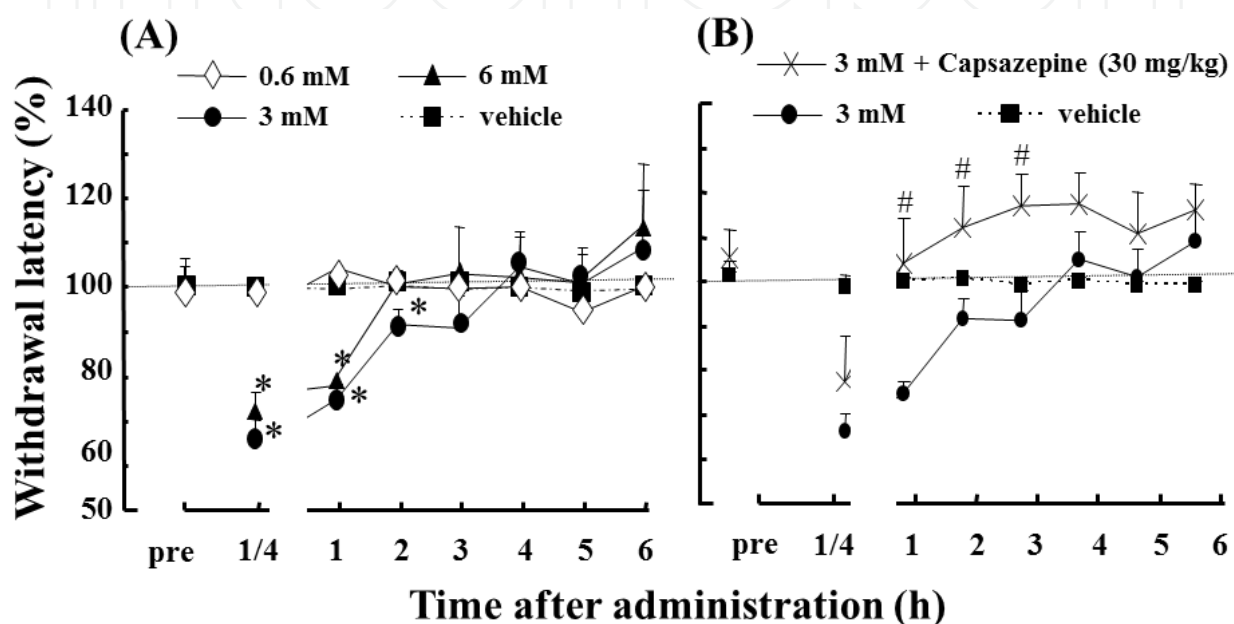


Fig. 3. Time course of withdrawal latencies in response to noxious heat stimulation after s.c. injection of capsaicin, and co-injection of capsazepine with capsaicin. The data for each group (10 animals) are presented as the means  $\pm$  S.E.M. The withdrawal latency per animal at respective time points was calculated as the average of the latencies obtained from 3 consecutive stimuli applied at intervals of 5 min. The value at time zero (pre) was obtained 1 h prior to s.c. injection of capsaicin. \* and #  $P < 0.05$  significantly different from vehicle-treated group and capsaicin-treated group (3 mM), respectively.

### 3.6 Thermal sensitivity after injection of iGluRs agonists

S.c. injections of Glu, NMDA or AMPA produced dose-dependent decreases in withdrawal latency on the ipsilateral side 15 min after s.c. injection, and lasted for a few hours (Fig. 4). S.c. injection of vehicle did not produced any changes in thermal-withdrawal latency.

### 3.7 Thermal sensitivity after injection of mGluRs agonists

S.c. injection of (s)-DHPG caused a dose-dependent decrease in withdrawal latencies on the ipsilateral side from 15 min to 6 h, but L-CCG-I and L-AP4 did not show any significant changes (Fig. 5).

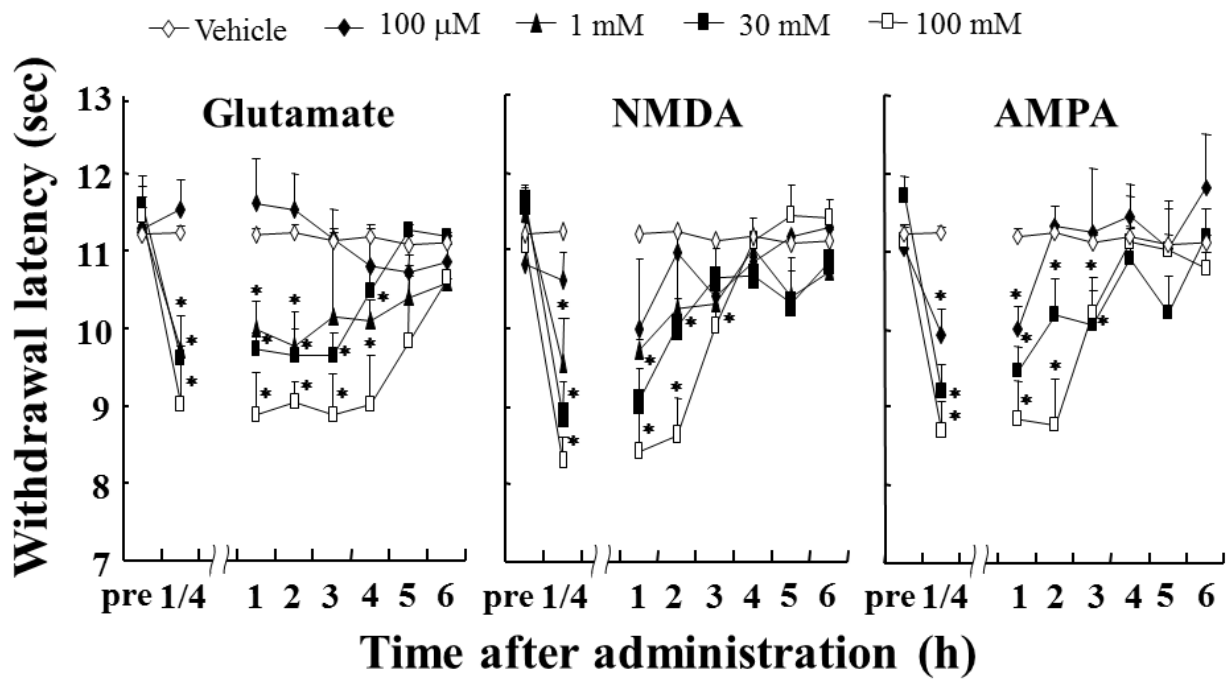


Fig. 4. Time course of withdrawal latencies in response to noxious heat stimulation after s.c. injection of various concentration of the ionotropic glutamate receptor agonists; glutamate, NMDA and AMPA. The data for each group (at least 10 animals) are presented as the means  $\pm$  S.E.M. \* $P < 0.05$  significantly different from vehicle-treated group.

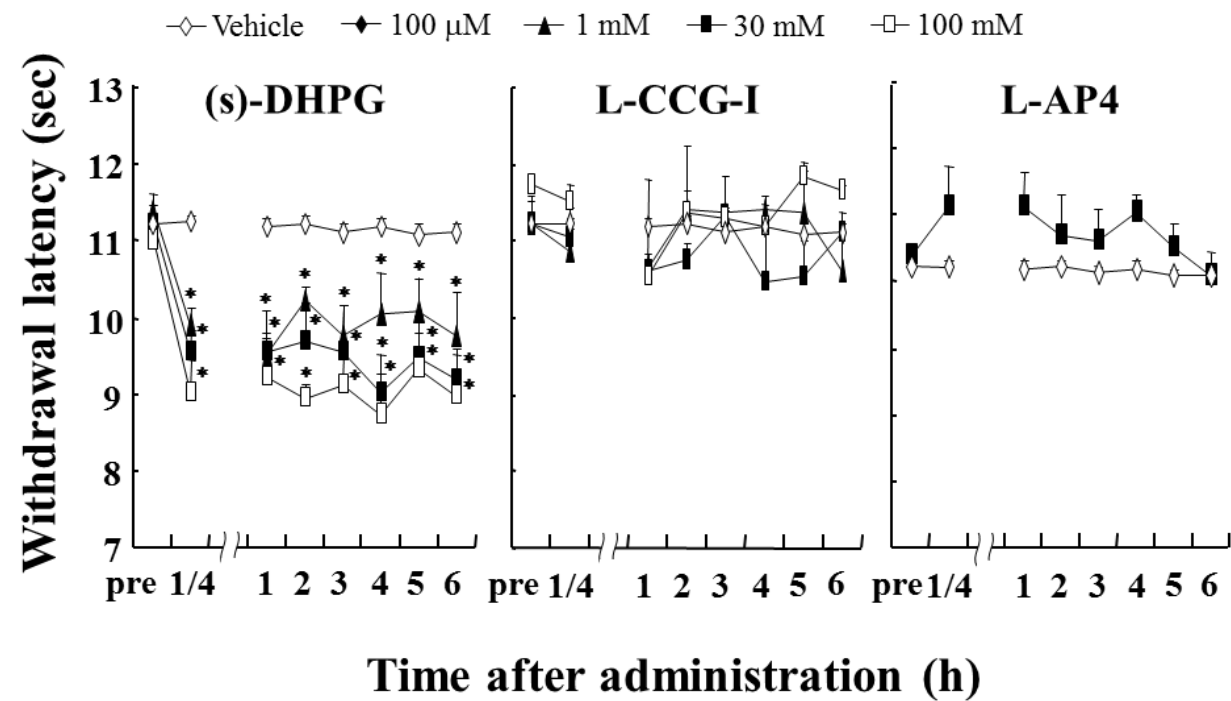


Fig. 5. Time course of withdrawal latencies in response to noxious heat stimulation after s.c. injection of various concentration of the metabotropic glutamate receptor agonists; (s)-DHPG, L-CCG-I and L-AP4. The data for each group (at least 10 animals) are presented as the means  $\pm$  S.E.M. \* $P < 0.05$  significantly different from vehicle-treated group.

3.8 Effect of iGluRs antagonists injection on capsaicin-induced thermal hypersensitivity

When MK801 or CNQX were injected together with capsaicin (Cap+MK801 or Cap+CNQX), a dose-dependent increase in withdrawal latency was observed. These analgesic effects of MK801 or CNQX on capsaicin-induced thermal hyperalgesia lasted for more than 6 h (Fig. 6). The single injection of MK801 or CNQX into the hindpaw did not show changes in withdrawal latencies compared to vehicle injection.

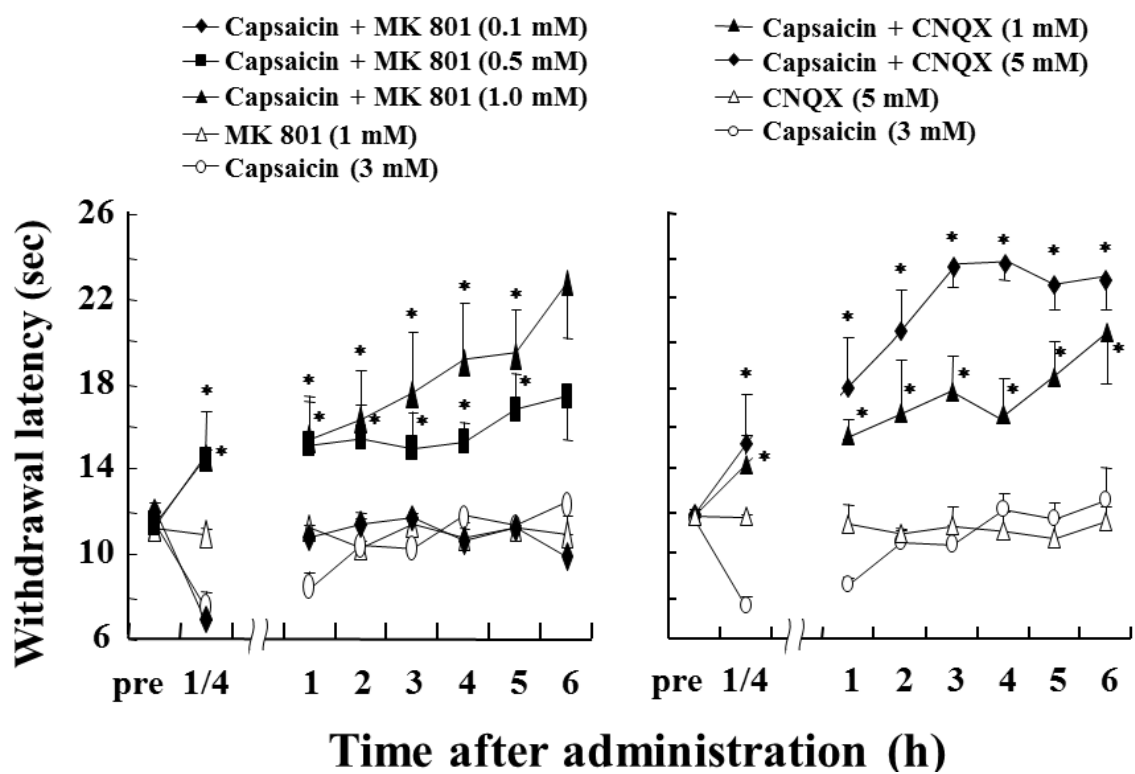


Fig. 6. Time course of withdrawal latencies in response to noxious heat stimulation after s.c. injection of capsaicin in combination with the ionotropic glutamate receptor antagonists; MK801 and CNQX. The data for each group (at least 10 animals) are presented as the means  $\pm$  S.E.M. \* $P < 0.05$  significantly different from capsaicin (3 mM)-treated group.

3.9 Effect of mGluRs antagonists injection on capsaicin-induced thermal hypersensitivity

Following s.c. injection of CPCCOEt (5 mM), MPEP (30 mM), MCCG (5 mM), and MSOP (5 mM) into hindpaw, there was no changes in withdrawal latencies compared to vehicle injection (Figs. 7 and 8). When CPCCOEt or MPEP were injected together with capsaicin (Cap+CPCCOEt or Cap+MPEP), withdrawal latencies showed a dose-dependent increase from 15 min to 2~3 h after the injection compared with when capsaicin was injected alone ( $P < 0.05$ ) (Fig. 7). The heat insensitivity evoked in ipsilateral side following Cap+CPCCOEt and Cap+MPEP injection continued for 5 h or more. S.c. injection of MCCG or MSOP combined with capsaicin did not show any significant changes in withdrawal latencies compared to capsaicin injection alone (Fig. 8).

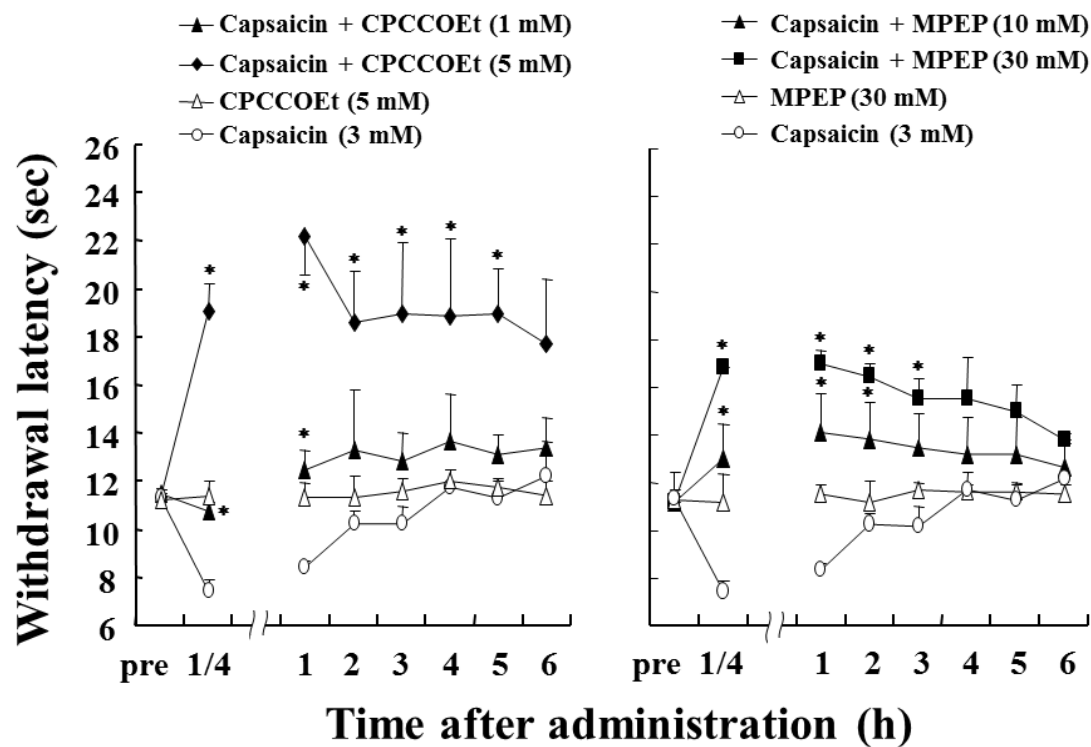


Fig. 7. Time course of withdrawal latencies in response to noxious heat stimulation after s.c. injection of capsaicin in combination with the metabotropic glutamate receptor antagonists; CPCCOEt and MPEP. The data for each group (at least 10 animals) are presented as the means  $\pm$  S.E.M. \* $P$  < 0.05 significantly different from capsaicin (3 mM)-treated group.

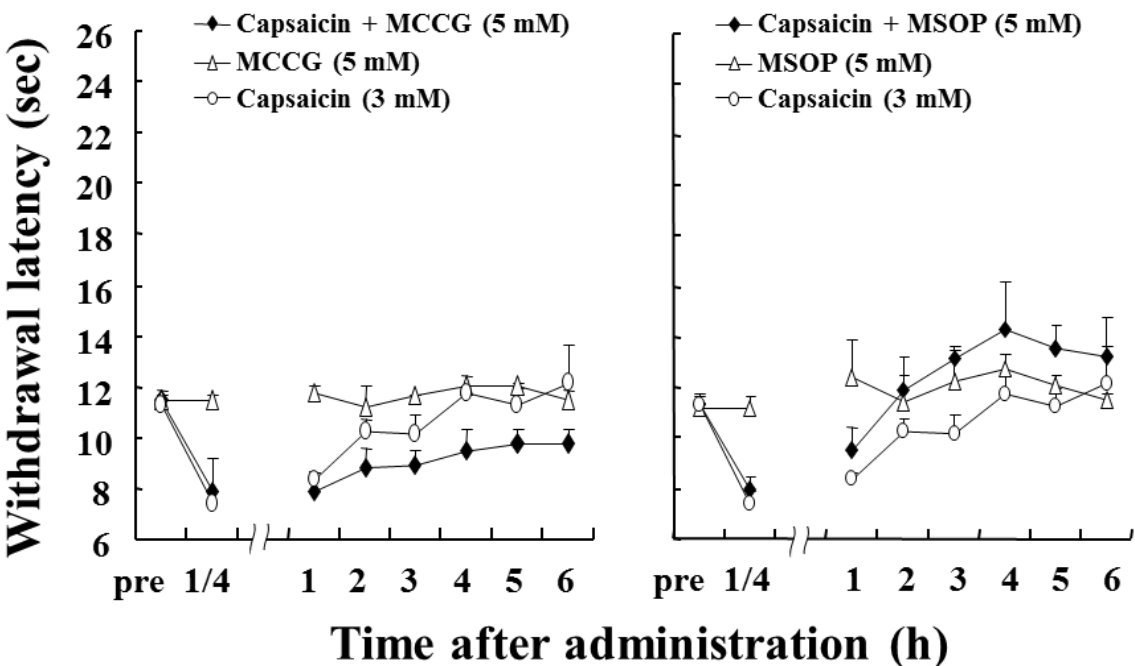


Fig. 8. Time course of withdrawal latencies in response to noxious heat stimulation after s.c. injection of capsaicin in combination with the metabotropic glutamate receptor antagonists; MCCG and MSOP. The data for each group (at least 10 animals) are presented as the means  $\pm$  S.E.M.

### 3.10 Basal c-Fos expression in dorsal horn after injection of vehicle, capsaicin and Glu into hindpaw

Immunoreactivity for c-Fos appeared gray-to-black and homogeneously labeled the oval or roundish nucleus of cells in spinal dorsal horn at L5 (Figs. 9-11). In all the experimental tests with injection of Glu, the maximum number of labeled cells occurred consistently in laminae I and II (I/II) of the spinal dorsal horn on the ipsilateral side (mean number  $\pm$  S.E.M.= $268 \pm 21$ ) (Figs. 9-11 and Table 1). Much smaller number of c-Fos immunopositive cells occurred in laminae III and IV (III/IV,  $30 \pm 7$ ). The capsaicin-induced c-Fos expression in laminae I/II ( $489 \pm 34$ ) and laminae III/IV ( $63 \pm 18$ ) on the ipsilateral side was greater than that with Glu (Figs. 9, 10 and Table 1). The numbers of c-Fos-immunopositive cells on the contralateral side was modest either with glutamate (I/II,  $16 \pm 7$ ; III/IV,  $12 \pm 6$ ) or capsaicin (I/II,  $44 \pm 13$ ; III/IV,  $20 \pm 9$ ). In animals administered with vehicle, c-Fos-immunopositive cells were rarely distributed either in laminae I/II ( $60 \pm 5$ ) or in laminae III/IV ( $22 \pm 8$ ) on the ipsilateral side or on the contralateral side (I/II,  $12 \pm 4$ ; III/IV,  $7 \pm 2$ ) (Table 1).

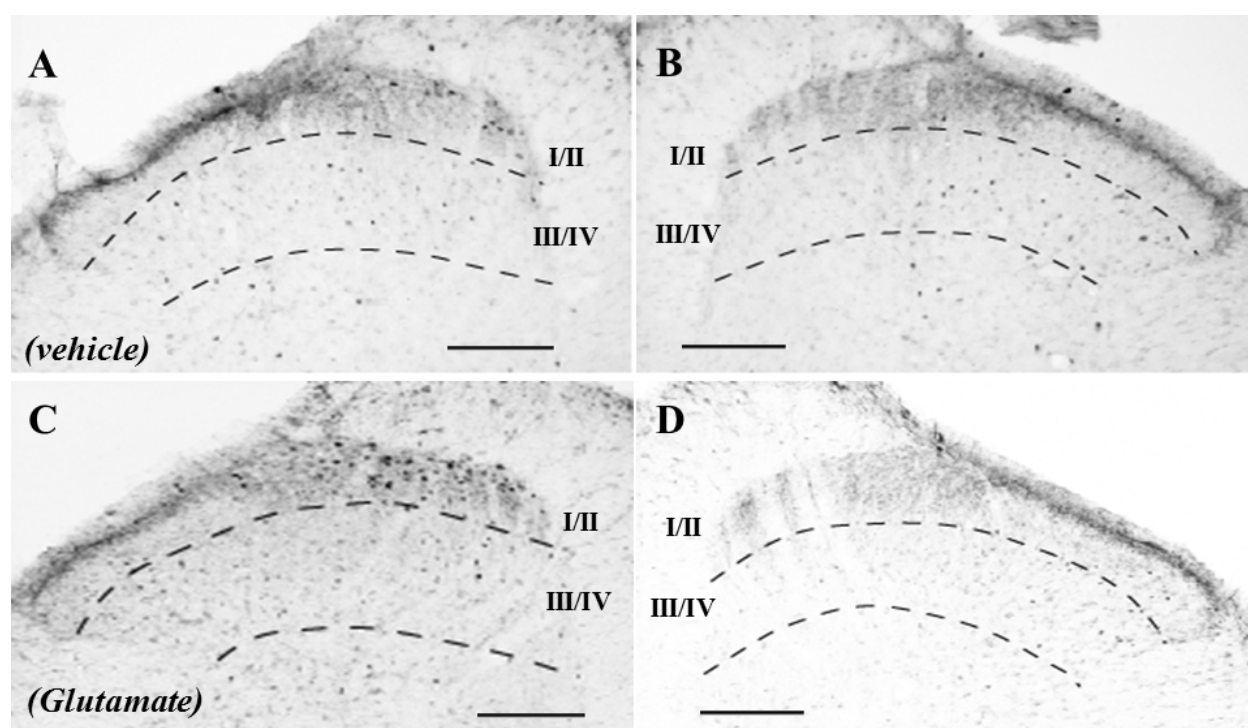


Fig. 9. Photomicrographs showing c-Fos-positive neurons in the dorsal horn of L5 2 h after hindpaw injection of vehicle and glutamate. A and C: ipsilateral side. B and D: contralateral side. Solid line indicates 100  $\mu$ m.

### 3.11 Effects of ionotropic Glu receptors antagonists injection on the capsaicin-induced c-Fos expression

Few c-Fos-immunopositive cells were found in laminae I/II and laminae III/IV of the ipsilateral dorsal horn after each single injection of ionotropic Glu receptors antagonists MK-801 (I/II,  $79 \pm 3$ ; III/IV,  $11 \pm 7$ ) and CNQX (I/II,  $70 \pm 8$ ; III/IV,  $7 \pm 3$ ) similar to vehicle

injection (I/II,  $60 \pm 5$ ; III/IV,  $22 \pm 8$ ). The numbers of capsaicin-induced c-Fos-immunopositive cells in laminae I/II ( $489 \pm 34$ ), but not in laminae III/IV ( $63 \pm 18$ ), were significantly decreased ( $P < 0.005$ ), when MK801 and CNQX were injected with capsaicin (Cap+MK801, I/II,  $227 \pm 32$ , III/IV,  $14 \pm 4$ ; Cap+CNQX, I/II,  $205 \pm 40$ , III/IV,  $11 \pm 7$ ) (Fig. 10 and Table 1). The numbers of capsaicin-induced c-Fos-immunopositive cells on the contralateral sides did not significantly change by any of drugs with/without capsaicin.

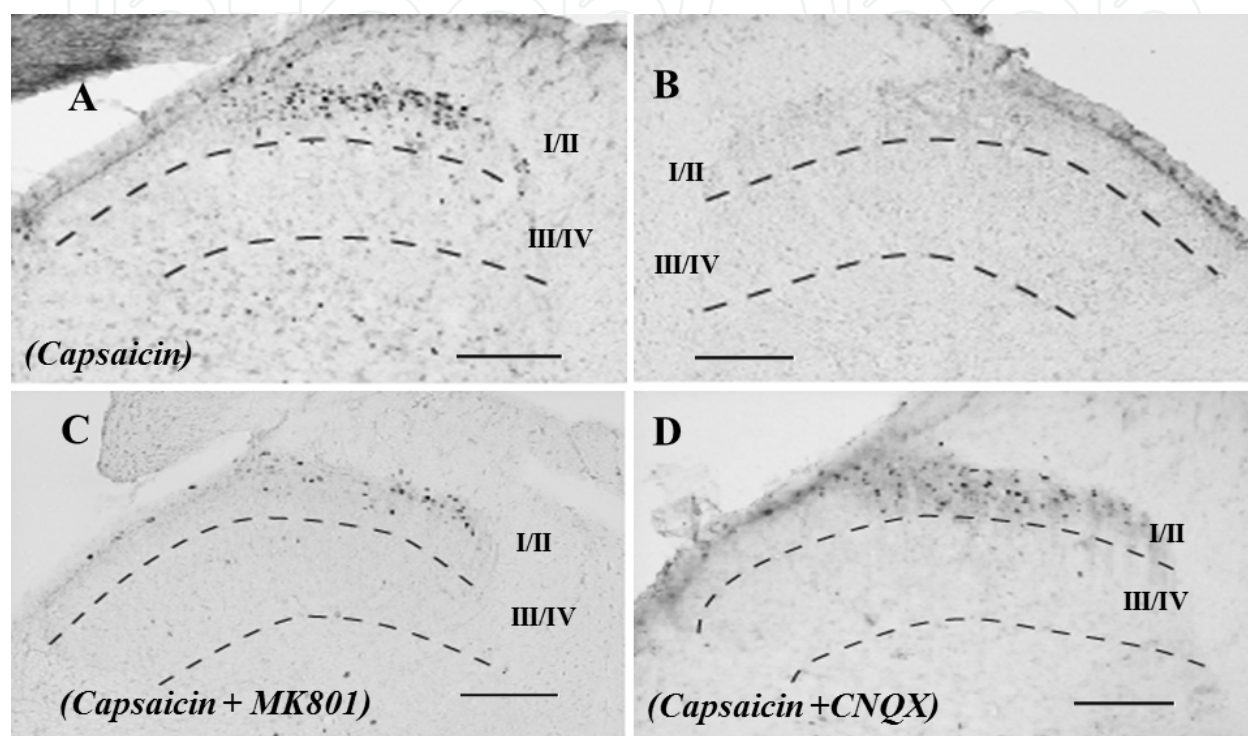


Fig. 10. Photomicrographs showing c-Fos-positive neurons in the dorsal horn of L5 2 h after hindpaw injection of capsaicin alone (A, B), combined with MK801 (C), and combined with MK801 CNQX (D). A, C and D: ipsilateral side. B: contralateral side. Solid line indicates 100  $\mu$  m.

### 3.12 Effects of metabotropic glu antagonists injection on the capsaicin-induced c-Fos expression

Few c-Fos-immunopositive cells in the ipsilateral laminae I/II and III/IV, and fewer cells in the contralateral sides, were observed with single injection of CPCCOEt (I/II,  $59 \pm 8$ , III/IV,  $1 \pm 1$ ), MCCG (I/II,  $63 \pm 10$ , III/IV,  $3 \pm 2$ ) and MSOP (I/II,  $66 \pm 16$ , III/IV,  $5 \pm 3$ ). Co-injection of CPCCOEt with capsaicin (Cap+CPCCOEt) significantly decreased the number of capsaicin-induced c-Fos-immunopositive cells in the ipsilateral laminae I/II ( $236 \pm 58$ ), but not in laminae III/IV ( $6 \pm 4$ ) and contralateral laminae I/II and III/IV. There was no significant change in the number of c-Fos-immunopositive cells in the ipsilateral laminae I/II, and III/IV by administration of MCCG combined with capsaicin (Cap+MCCG) or by administration of MSOP combined with capsaicin (Cap+MSOP; I/II,  $383 \pm 21$ , III/IV,  $22 \pm 3$ ) compared to single injection of capsaicin, respectively (Fig. 11 and Table 1).

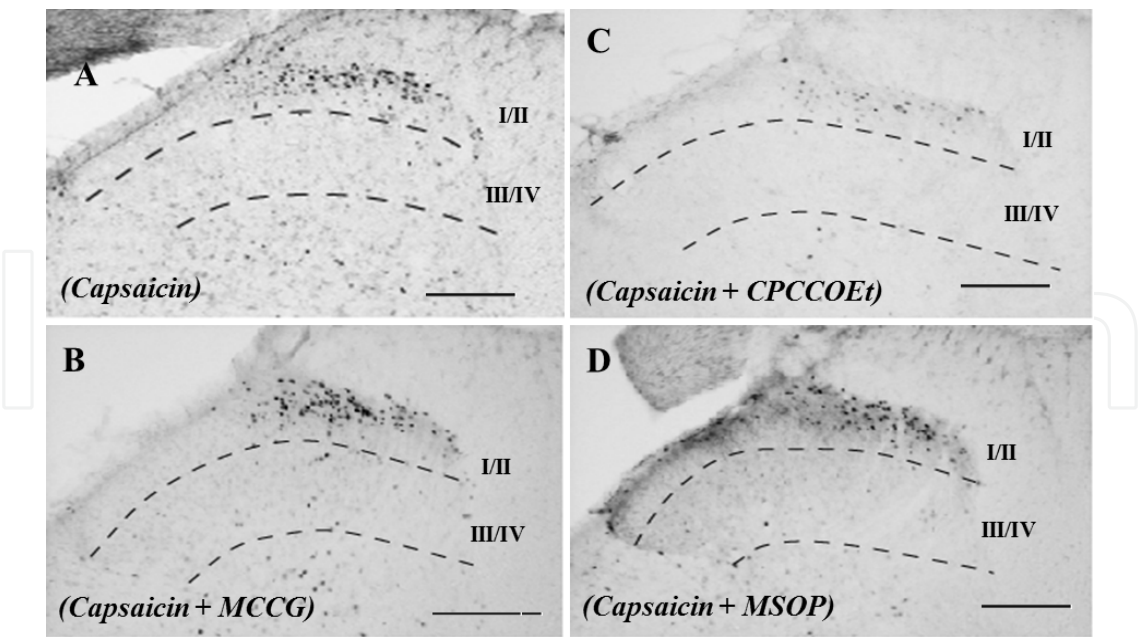


Fig. 11. Photomicrographs showing c-Fos-positive neurons in the dorsal horn of L5 2 h after hindpaw injection of capsaicin alone (A), combined with CPCCOEt (B), with MCCG (C), with MSOP (D). A, B, C and D: ipsilateral side. Solid line indicates 100  $\mu$  m .

Group	Ipsilateral		Contralateral	
	I/II-layer	III/IV-layer	I/II-layer	III/IV-layer
Vehicle	60 $\pm$ 5	22 $\pm$ 8	12 $\pm$ 4	7 $\pm$ 2
Capsaicin(Cap)	489 $\pm$ 34*	63 $\pm$ 18	44 $\pm$ 13	20 $\pm$ 9
Glutamate	283 $\pm$ 18*	36 $\pm$ 5	19 $\pm$ 8	12 $\pm$ 6
MK801	79 $\pm$ 3	11 $\pm$ 7	33 $\pm$ 12	9 $\pm$ 5
CNQX	70 $\pm$ 8	7 $\pm$ 3	14 $\pm$ 7	3 $\pm$ 2
CPCCOEt	59 $\pm$ 8	6 $\pm$ 3	10 $\pm$ 4	6 $\pm$ 2
MCCG	63 $\pm$ 10	5 $\pm$ 2	28 $\pm$ 11	5 $\pm$ 2
MSOP	66 $\pm$ 16	5 $\pm$ 3	9 $\pm$ 4	5 $\pm$ 3
Cap + MK801	227 $\pm$ 32 <sup>#</sup>	14 $\pm$ 4	8 $\pm$ 6	3 $\pm$ 2
Cap + CNQX	205 $\pm$ 40 <sup>#</sup>	11 $\pm$ 7	22 $\pm$ 12	3 $\pm$ 2
Cap + CPCCOEt	236 $\pm$ 58 <sup>#</sup>	17 $\pm$ 11	12 $\pm$ 7	4 $\pm$ 3
Cap + MCCG	560 $\pm$ 85	27 $\pm$ 10	24 $\pm$ 9	3 $\pm$ 1
Cap + MSOP	383 $\pm$ 21	22 $\pm$ 3	18 $\pm$ 13	4 $\pm$ 1

Table 1. Mean value of c-Fos-positive neurons in the dorsal horn of L5 2 h after s.c. injection of Glu receptors agonists and antagonists. The value in each group was represented mean  $\pm$  S.E.M. obtained from at least 10 animals, and the difference of the means was analyzed with the Student's t-test. \* Significant difference at P< 0.05 between vehicle and capsaicin, or glutamate-treated group. # Significant difference at P< 0.05 between capsaicin and capsaicin+MK801, or capsaicin+CNQX, or capsaicin+CPCCOEt-treated group

#### 4. Discussion

We confirmed a large release of Glu immediately after the introduction of the catheter, followed by a rapid decrease, like in our previous study (Yonehara et al., 1987; Yonehara et al., 1992; Yonehara et al., 1995). Insertion of the polyethylene tube into the s.c. space of the rat instep did not evoke any inflammatory responses such as extravasation (Yonehara et al., 1995). All these data suggest that the basal levels of Glu in the s.c. perfusate were caused by neither acute noxious stimulation nor inflammation.

Topical application of capsaicin cream to the instep evoked a marked increase in Glu level in the s.c. perfusate, similar to the results in our previous study (Jin et al., 2006). In addition, electrical stimulation of the sciatic nerve or noxious heat stimulation (50°C) also caused an increase of Glu level in the s.c. space, and this capsaicin-evoked Glu release was significantly decreased by daily high-dose pretreatment with capsaicin for three consecutive days (Jin et al., 2006).

The TRPV1 is located in a neurochemically heterogeneous population of small diameter primary afferent neurons (Tominaga et al., 1998). Furthermore, repeated exposure to high-dose capsaicin selectively produces a prolonged influx of cations leading to desensitization of small-diameter sensory neurons to subsequent noxious stimulation (Yonehara et al., 1987; Lynn, 1990; Zhou et al., 1998; Caterina and Julius, 2001), while myelinated A $\beta$  fibers are insensitive to capsaicin (Jancso et al., 1977; Nagy et al., 1983; Michael and Priestly, 1999). These findings and the present results suggest that the activation of capsaicin-sensitive afferent fibers by capsaicin causes release of Glu from the peripheral endings via activation of peripheral TRPV1, particularly from those of small-diameter fibers possibly through a mechanism such as the axon-reflex pathway, or autocrine and/or paracrine. It is reasonable to speculate that axon-reflex mechanism is involved in capsaicin-induced Glu release observed in Figs. 1 and 2, as only nociceptive afferent fibers have the axon-reflex mechanism which is localized on superficial tissues exposed to noxious influences (Celerander and Folkow, 1953).

Amount of capsaicin-induced Glu release was remarkably decreased by concomitant administration of ionotropic Glu receptors antagonists; MK801 and NBQX, and mGluR I antagonist; CPCCOEt in the hindpaw, but not by administration of group II and III mGluR antagonist; MCCG and MSOP. These results suggest that peripheral ionotropic Glu receptors and group I mGluR appear to play a role in mediating capsaicin-evoked increases in Glu release. The Glu release through the activation of TRPV1 could then further activate ionotropic Glu receptors and group I mGluR on the same neuronal terminal or adjacent neighboring peripheral terminals. In this connection, there were evidences supporting the co-localization of peripheral NMDA and TRPV1 receptors on the same primary afferent terminal (Lam et al., 2003; Lam et al., 2004).

Activation of peripheral Glu receptors could lead to enhance the Glu release in the peripheral tissues and might alter TRPV1 receptor responsiveness to reinforce nociceptive responses. As it is necessary to investigate the interaction between TRPV1 and glutamate receptors by using specific receptor antagonists of TRPV1 in detail, the mechanism to account for the antagonism of peripheral Glu receptors contributes to inhibit capsaicin-induced Glu release remains unanswered. However, it may be possible that glutamate receptors play a pivotal role for the activation of TRPV1 in the peripheral terminals. This

idea is supported by the results that the intraplantar injection of ionotropic Glu receptors and group I mGluR agonists evoked dose-dependent thermal hyperalgesia. Moreover, it is very interesting to note that injection of Glu receptors antagonists alone did not produce any changes on withdrawal latency, and intraplantar co-injection of ionotropic Glu receptors and group I mGluR antagonists with capsaicin not only antagonized capsaicin-induced hyperalgesia, but also resulted in remarkable longer withdrawal latency to heat irradiation.

Concerning the mechanism that ionotropic Glu receptors and mGluR antagonists produced remarkable analgesic action in the presence of capsaicin, there is evidence that capsaicin injected into the rat temporomandibular joint evoked a dose-dependent increase in jaw muscle electromyographic activity. This capsaicin-evoked increase in electromyographic activity was attenuated by ipsilateral injection of NMDA receptor antagonists into the temporomandibular joint (Lam et al. 2005). This finding and our present results indicate that the activation of peripheral Glu receptors, especially ionotropic Glu receptors and group I mGluR could be indispensable in the mechanisms whereby capsaicin evokes nociceptive responses.

The ionotropic, and metabotropic subunits of Glu receptors are present in DRG cell bodies and on unmyelinated fibers in the glabrous skin of the mammalian foot (Carlton et al., 1995; Bhawe et al., 2001; Carlton et al., 2001; Sato et al., 1993; Carlton et al., 2007). It is well established that the excitatory amino acids in the peripheral endings of small-diameter afferent fibers contribute to development and/or maintenance of pain in humans (Nordlind et al., 1993; Warncke et al., 1997) and in laboratory animals (Davidson et al., 1997; Cairns et al., 1998; Davidson et al., 1998). For example, peripherally applied NMDA and non-NMDA receptor antagonists attenuate or block nociceptive behaviors in several animal models of inflammation (Jackson et al., 1995; Lawand et al., 1997; Carlton et al., 1998).

In the present study, we examined the c-Fos expression in spinal cord dorsal horn following injection of drugs associated with glutamate receptors with/without capsaicin into the hindpaw. c-Fos is rapidly and transiently induced in cells of the spinal dorsal horn after noxious stimulation (Hunt et al., 1987, Strassman and Vos, 1993, Takemura et al., 2000), c-Fos has been widely used as a marker for analyzing nociceptive processing.

Our present data support the view that Glu receptors, in particular, ionotropic Glu receptors and group I mGluR existing in peripheral ending of capsaicin-sensitive afferent fibers play an important role on development and/or maintenance of pain following excitation of TRPV1. In addition, the formulation of the peripheral ionotropic Glu receptors and group I mGluR antagonists that do not cross the blood brain barrier may be of potential benefit by reducing peripheral nociceptive excitability, and therefore they could provide a new therapeutic target to pain control in the periphery.

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