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INTRATHECAL ADMINISTRATION OF ATP PRODUCES LONG-LASTING ALLODYNIA IN RATS: DIFFERENTIAL MECHANISMS IN THE PHASE OF THE INDUCTION AND MAINTENANCE

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Abstract—Several lines of evidence suggest that extracellular ATP plays a role in pain signaling through the activation of ionotropic P2X-receptors, especially homomeric $P2X_3$- and heteromeric $P2X_{2/3}$-receptors on capsaicin-sensitive and -insensitive primary afferent neurons, respectively, at peripheral and spinal sites. We investigated the mechanisms of the induction and maintenance of mechanical allodynia produced by a single intrathecal (i.t.) administration of ATP in rats. We found that i.t. administration of ATP and the P2X-receptor agonist α,β-methylene-ATP produced tactile allodynia which lasted more than 1 week. The i.t. ATP- and α,β-methylene-ATP-produced long-lasting allodynia remained in neonatal capsaicin-treated adult rats. I.t. administration of a $P2X_3$/$P2X_{2/3}$-receptor selective antagonist completely prevented the induction (co-administration on day 0) and partially attenuated the early phase (day 1 post-ATP administration), but not the late phase (day 7 post-ATP administration) of maintenance of allodynia. The *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 completely prevented the induction phase, but not the early and late phases of maintenance of allodynia. Immunohistochemical and immunoblotting studies for microglial and astrocytic markers revealed that i.t. ATP administration caused spinal microglial activation within 1 day, and astrocytic activation which peaked at 1–3 days after ATP administration. Furthermore, minocycline, a microglial inhibitor, attenuated the induction but not the early and late phases of maintenance, while fluorocitrate, a glial metabolic inhibitor, attenuated the induction and the early phase but not the late phase of maintenance. Taken together, these results suggest that the activation of P2X-receptors, most likely spinal $P2X_{2/3}$-receptors on capsaicin-insensitive primary afferent neurons, triggers the induction of long-lasting allodynia through NMDA receptors, and the induction and early maintenance phase, but not the late phase, is mediated through the functions of spinal glial cells.

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Abbreviations: ANOVA, analysis of variance; EGTA, ethyleneglycol-bis-(β-aminoethyl)*N*,*N*,*N*′,*N*′-tetraacetic acid; GFAP, glial fibrillary acidic protein; i.t., intrathecal; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; SDS, sodium dodecyl sulfate; TRPV1, transient receptor potential channel, vanilloid subfamily member 1.

Key words: ATP, intrathecal, central sensitization, glutamate, astrocyte, microglia.

A growing body of evidence suggests that extracellular ATP, an endogenous fast-acting neurotransmitter, plays a key role in peripheral and spinal pain signaling (Burnstock, 2006). ATP is known to depolarize primary afferent neurons as well as spinal dorsal horn neurons through the activation of P2-purinoceptors (Jahr and Jessell, 1983; Krishtal et al., 1988). To date at least seven ionotropic P2X- and eight metabotropic P2Y-subtypes have been cloned, most expressed on primary afferent neurons or spinal dorsal horn neurons. Electrophysiological, immunohistochemical and behavioral studies have shown that some of them, especially homomeric $P2X_3$- and heteromeric $P2X_{2/3}$-receptors, on capsaicin-sensitive and -insensitive primary afferent neurons, respectively, are involved in pain signaling (for review, see Chizh and Illes, 2001; North, 2002). ATP and P2X-receptor agonists elicited at least two types of currents: rapidly desensitizing, capsaicin-sensitive current through homomeric $P2X_3$ receptors which mediate transient nociceptive responses, and slowly desensitizing, capsaicin-insensitive current through heteromeric $P2X_{2/3}$ receptors which mediate sustained nociceptive responses (Lewis et al., 1995; Li et al., 1999; Ueno et al., 1999; Cockayne et al., 2005). It was reported that activation of P2X-receptors elicited the release of glutamate from the central terminals of primary afferent neurons (Gu and MacDermott, 1997; Nakatsuka and Gu, 2001). Systemic, intraplantar and intrathecal (i.t.) administration of P2-purinoceptor antagonists including a selective $P2X_3$/$P2X_{2/3}$-receptor antagonist decreased various nociceptive behaviors, inflammatory hyperalgesia and neuropathic pain (Chen et al., 2005; Jarvis et al., 2002; McGaraughty et al., 2003; Tsuda et al., 1999b). On the other hand, exogenous administration of ATP and P2X-receptor agonists into the hind paw caused short-lasting nocifensive behaviors and thermal hyperalgesia (<15 min) (Bland-Ward and Humphrey, 1997; Wismer et al., 2003), and relatively long-lasting mechanical allodynia (<2 h) (Tsuda et al., 2000) in rodents. The former was mediated through $P2X_3$-receptors on the peripheral terminals of capsaicin-sensitive primary afferent neurons, while the later was through $P2X_{2/3}$-receptors on capsaicin-insensitive primary afferent neurons (Tsuda et al., 2000). We and other groups reported that i.t. administration of ATP and P2X-receptor agonists also produced short-lasting hyperalgesia (<15 min) (Tsuda et al., 1999a), as well as allodynia which lasted for at least 90 min

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(Fukuhara et al., 2000; Okada et al., 2002). However, neither the duration period nor the mechanisms underlying the induction and maintenance of allodynia have been investigated.

Recent accumulating data suggest that spinal glial cells contribute to the induction and maintenance of pathological pain (for review see Marchand and Perretti, 2005; Watkins and Maier, 2003; Wieseler-Frank et al., 2004). Early studies indicated that both astrocytes and microglia in the spinal cord were activated in diverse models of pathological pain (Garrison et al., 1991, 1994; Coyle, 1998; Sweitzer et al., 1999). Blocking the activation and function of spinal glial cells prevented and/or reversed hyperalgesia and allodynia (Meller et al., 1994; Milligan et al., 2001, 2003; Raghavendra et al., 2003). It was shown that spinal nerve ligation activated mitogen-activated protein kinases sequentially in spinal microglia and astrocytes which contributed to allodynia (Zhuang et al., 2005, 2006). Tsuda et al. (2003) reported that the increased expression of $P2X_4$-receptors induced by nerve injury or ATP stimulation in the spinal microglia produced allodynia.

Here, we report that i.t. administration of ATP and the P2X-receptor agonist α,β-methylene-ATP produced long-lasting allodynia which lasted over 1 week in rats. Furthermore, we examined whether the induction and maintenance of long-lasting allodynia are capsaicin-sensitive or insensitive, and which P2X-receptor subtypes are involved. Involvement of spinal *N*-methyl-D-aspartate (NMDA) receptors in the induction and maintenance of long-lasting allodynia was also examined. To elucidate the role of spinal glial cells, we investigated microglial and astrocytic activation following i.t. ATP administration and the effects of two glial inhibitors, minocycline and fluorocitrate, on the induction and maintenance phases of allodynia.

EXPERIMENTAL PROCEDURES

The present study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee and the guidelines of the Japanese Pharmacological Society, and was in complete compliance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used, and limit experimentation to what was necessary to produce reliable scientific information.

Animals

Male Sprague–Dawley rats initially weighing 180–220 g were used. They were kept at a constant ambient temperature of 24±1 °C under a 12-h light/dark cycle, and were provided free access to food and water. The rats were individually housed in plastic cages with woodchip bedding for at least 1 day before surgery.

Materials

ATP, the P2X-receptor agonist α,β-methylene-ATP, the non-competitive NMDA receptor antagonist MK-801, the microglial inhibitor minocycline, and the selective $P2X_3$/$P2X_{2/3}$-antagonist, A-317491 (Sigma, St. Louis, MO, USA) were freshly dissolved in phosphate-buffered saline (PBS) each day. The glial metabolic inhibitor fluorocitrate (Sigma) was dissolved initially in 2 M HCl and then diluted in PBS (pH 6.0). The vehicle for fluorocitrate was 0.3% 2 M HCl in PBS, pH 6.0.

Neonatal capsaicin treatment

Neonatal Sprague–Dawley rats were injected s.c. with 50 mg/kg capsaicin (Nacalai Tesque, Kyoto, Japan) dissolved in 10% absolute alcohol, 10% Tween-20 and 80% saline or vehicle on postnatal day 2. The capsaicin-treated animals were used at 6–7 weeks of age after assessing efficiency of the treatment by application of capsaicin solution (0.01%) to the cornea. Only animals that responded with two or only a few eye wipes were used in the study.

I.t. administration of drugs

I.t. injection of drugs was performed according to the method of Satoh et al. (1983). Briefly, at least 1 day before the i.t. administration, the skin of the back was incised along the spinous processes at the L2–L5 level under ether anesthesia; and then the resulting wound was treated with local anesthetic, 2% lidocaine jelly, and sutured. On the day of the experiment after the extraction of the suture in the absence of anesthesia, the drug or vehicle was intrathecally administered in a volume of 10 μl to the conscious animal through an acute lumbar puncture between L4 and L5 using a 25-gauge stainless steel needle attached to a glass microsyringe via polypropylene tube. A quick flick of the rat's tail confirmed the accurate i.t. position of the needle.

Behavioral experiments

All behavioral experiments were performed between 13:00 and 17:00 h.

von Frey filament test

Tactile allodynia was measured using calibrated von Frey filaments (North Coast Medical Inc., Morgan Hill, CA, USA), as previously described (Okada et al., 2002). Briefly, for testing, animals were individually placed on a wire mesh floor and acclimatized to the environment for at least 30 min. Cages were mounted in a position that allowed the experimenter access to the bottom of the cage. After acclimatization, the tactile stimulus was applied to the middle plantar surface of the right paw by placing the von Frey filament (0.60 g) perpendicular to the surface of the paw. The von Frey filament was held in this position with enough force to cause a slight bend. The response of animals was graded with a score of 0=no response; 1=moderate effort to avoid the probe, such as licking the stimulated paw, and transient vocalization; and 2=vigorous effort to escape the stimulus, such as jumping, shaking the paw, biting at the probe or the stimulated paw, and frequent and sustained vocalization in response to the probe. One trial involved 10 applications of filaments every 3 or 4 s, each of which was scored as 0, 1 or 2. The trial was evaluated based on a total score of 0–20 at culmination. Soon after measuring the control score, drugs were administered intrathecally and the allodynic score was measured at the times indicated.

Capsaicin test

Capsaicin test was performed according to the method of Sakurada et al. (1992) with slight modifications. The neonatal capsaicin- or vehicle-treated adult rats (6–7 weeks) were individually placed in Plexiglas cages, and acclimatized to the environment for at least 30 min. The animals were injected with capsaicin solution (2 μg/100 μl) dissolved in 10% absolute alcohol, 10% Tween-20, and 80% saline in the plantar surface of the left hind paw in a volume of 100 μl using a 26-gauge needle fitted to a Hamilton microsyringe. The rats were immediately put back into the cages, and the time during which the animals showed lifting, licking or flinching of the hind paw was measured for a period of 10 min.

Western blot analysis

After i.t. administration of ATP, the animals were rapidly killed by decapitation. The L4–L6 lumbar spinal cord was rapidly removed, immediately frozen in liquid nitrogen and stored at −80 °C until use. The segments were homogenized with a polytron homogenizer and sonicated in ice-cold 20 mM Tris buffer (pH 7.0) containing 2 mM EGTA, 1% Triton X-100, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail (Sigma), and the protein concentrations were determined. Western blots were conducted as previously described with slight modifications (Ozawa et al., 2004). Aliquots of protein (2 μg) were diluted with an equal volume of sample buffer (124 mM Tris–HCl (pH 7.5), 4% sodium dodecyl sulfate (SDS), 10% glycerol, 4% 2-mercaptoethanol and 0.02% Bromophenol Blue), subjected to SDS–poly-acrylamide gel electrophoresis (12% acrylamide gels), and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Blots were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween-20 for 1 h, and then incubated overnight at 4 °C with anti-OX-42 (microglial marker, 1:2000, Serotec, Ltd., Oxford, UK) or anti-glial fibrillary acidic protein (GFAP) (astrocytic marker, 1:50,000, Sigma). After washing, the blots were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. The immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL, USA) and visualized by exposure to X-ray film. The membranes were stripped with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and reblotted with anti-actin (1:30,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the quantification of Western signals, the densities of specific OX-42, GFAP and actin bands were measured with a computer-assisted imaging analysis system (NIH Image). Levels of OX-42 and GFAP were normalized against the corresponding actin level as a control for sample loading. The value obtained for the naïve control rat served as the control (100%), and the results were presented as the mean of the percentage±S.E.M. of the control.

Immunohistochemistry

The animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and perfused transcardially through the ascending aorta with 0.1 M PBS (pH 7.4), immediately followed by 4% paraformaldehyde in 0.1 M PBS. The L4–L6 lumbar spinal cord was removed, post-fixed in the same fixative for 3–4 h, cryoprotected with 15% sucrose in 0.1 M PBS overnight at 4 °C, and then frozen in liquid nitrogen. Coronal sections (30 μm) were prepared using a cryostat and collected in PBS at 4 °C to be processed immunohistochemically as free-floating sections. The sections were gently washed three times (10 min each) in PBS, and then permeabilized and blocked at room temperature for 1 h in 4% normal goat serum in PBS containing 0.1% Triton X-100. For immunofluorescence imaging of the spinal transient receptor potential channel, vanilloid subfamily member 1 (TRPV1), sections were then incubated overnight at 4 °C with primary polyclonal guinea-pig anti-TRPV1 antibody (1:1000, Neuromics, Bloomington, MN, USA) in PBS containing 0.5% Triton X-100 and 4% normal goat serum. The sections were washed three times in PBS, and incubated for 1 h at room temperature with Alexa Fluoro 488-labeled goat anti-guinea-pig IgG antibody (1:200; Molecular Probes, Inc., Eugene, OR, USA) in PBS with 0.5% Triton X-100 and 4% normal goat serum. For immunofluorescence imaging of the marker proteins for astrocyte and microglia, spinal sections were incubated overnight at 4 °C with anti-GFAP (1:400) or anti-OX-42 (1:400), followed by Alexa Fluoro 568-labeled goat anti-mouse IgG antibody (1:200; Molecular Probes) for 1 h at room temperature. Immunofluorescence was visualized on a Nikon fluorescence microscope, and images were captured with a CCD Spot camera.

Partial sciatic nerve ligation

For a positive control of spinal glial activation the sciatic nerve was partially ligated, as previously described (Okada et al., 2002). Briefly, under diethylether anesthesia, the right sciatic nerve was exposed, and the 1/3–1/2 dorsal section of the nerve was ligated tightly using 7–0 silk suture just distal to the point at which the posterior biceps semitendinosus nerve branches off the common sciatic nerve. The wound was closed by suturing the muscle and skin layers. Two days after the sciatic nerve ligation, the development of allodynia was assessed using the von Frey filament test just before sacrificing the animals. Samples were used for immunohistochemistry staining of OX-42 and GFAP.

Statistical analysis

In the von Frey filament test, the statistical significance was calculated using two-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. In the capsaicin test, the statistical significance was calculated using Student's *t*-test. Western blot data were analyzed by one-way ANOVA. Differences with $P<0.05$ were considered significant.

RESULTS

I.t. administration of ATP and P2X-receptor agonist produced long-lasting allodynia

The effects of i.t. administration of ATP and α,β-methylene-ATP on tactile allodynia were examined (Fig. 1). I.t. administration of PBS did not affect the allodynic score until 4–5 weeks post administration. However, i.t. administration of ATP (30, 100, and 300 nmol) significantly increased allodynic scores in a dose-dependent manner ($F_{3,423}=129.2$, $P<0.001$). Allodynia appeared within 5 min, reached a plateau between 15 and 30 min, and was sustained for 7 days postadministration. Furthermore, significant allodynia produced by i.t. administration of 100 nmol ATP lasted for 3 to 4 weeks ($F_{2,153}=63.1$, $P<0.001$). Similarly, i.t. administration of α,β-methylene-ATP (10 and 30 nmol) produced significant allodynia for 7 days ($F_{2,192}=174.6$, $P<0.001$).

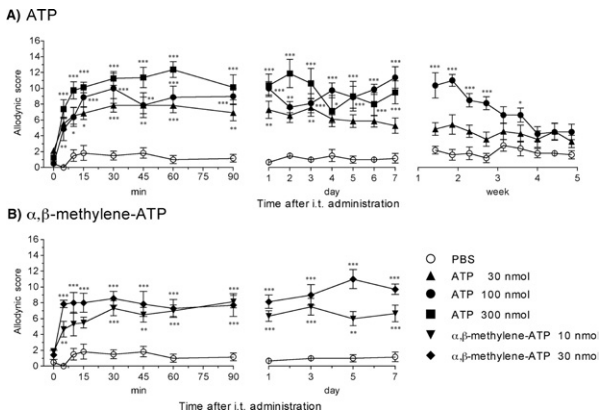

Fig. 1. I.t. administration of ATP (A) and the P2X-receptor agonist α,β-methylene-ATP (B) produced long-lasting allodynia in rats. ATP (30, 100 and 300 nmol), α,β-methylene-ATP (10 and 30 nmol), or PBS was administered intrathecally at time 0, and allodynia in response to a tactile stimuli was evaluated in the von Frey filament test at the times indicated. Values are presented as the mean±S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with the PBS-treated group ($n=6–8$).

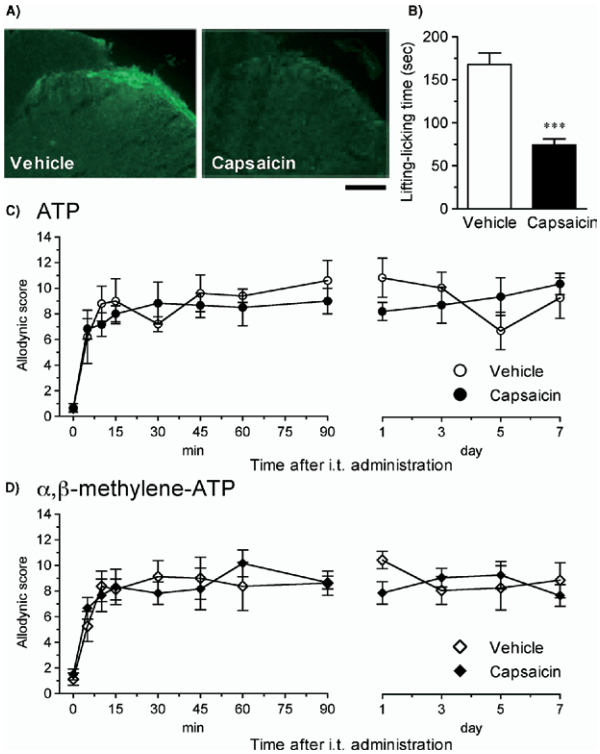

Fig. 2. Effect of neonatal capsaicin-pretreatment on i.t. ATP- and α,β-methylene-ATP-produced long-lasting allodynia. (A) Immunofluorescence for TRPV1 (green) in the spinal cord was observed in the superficial dorsal horn of the neonatal vehicle-treated adult rats, but not in the neonatal capsaicin-treated adult rats. Scale bar=200 μm. (B) Capsaicin test. Neonatal vehicle- or capsaicin-pretreated adult rats were injected with capsaicin in the hind paw. The duration of time that neonatal capsaicin-pretreated rats showed lifting, licking or flinching of the hind paw was shorter than that of neonatal vehicle-treated rats. The values are presented as the mean±S.E.M. *** $P<0.001$ ($n=14$–16). (C, D) von Frey filament test. Long-lasting allodynia produced by i.t. administration of ATP (C) and α,β-methylene-ATP (D) was not affected by the neonatal capsaicin-pretreatment. Values are presented as the mean±S.E.M. ($n=5$–8). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Effect of neonatal capsaicin-pretreatment

The effect of neonatal capsaicin-pretreatment on i.t. ATP- and α,β-methylene-ATP-produced long-lasting allodynia was examined (Fig. 2). Immunofluorescence for TRPV1 which is a marker of capsaicin-sensitive neurons, was observed in the superficial dorsal horn of the spinal cord of the neonatal vehicle-pretreated adult rats, but was absent in the neonatal capsaicin-pretreated adult rats (Fig. 2A). In the capsaicin test, the neonatal vehicle-treated rats showed intraplantar capsaicin-evoked nocifensive behaviors, such as lifting, licking or flinching of the hind paw. The duration time of the nocifensive behaviors in the neonatal capsaicin-pretreated rats was significantly less than that in the neonatal vehicle-pretreated rats ($P<0.001$, Fig. 2B). These results indicate that the neonatal capsaicin-pretreatment destroyed the capsaicin-sensitive primary afferent neurons (Jancsó et al., 1977). However, i.t. administration of ATP and α,β-methylene-ATP produced similar patterns of long-lasting allodynia, which lasted for 7 days, even in the neonatal capsaicin-pretreated animals (Fig. 2C, D). There were no significant differences between vehicle- and capsaicin-pretreated groups ($F_{1,108}=0.44$, $P=0.51$ and $F_{1,128}=0.02$, $P=0.89$, respectively).

Effect of $P2X_3/P2X_{2/3}$-receptor selective antagonist, A-317491

A-317491 is a novel, non-nucleotide antagonist selective for $P2X_3/P2X_{2/3}$-receptors (Jarvis et al., 2002). The effect of co-administration of A-317491 and ATP on the induction of long-lasting allodynia was examined (Fig. 3A). I.t. administration of ATP (100 nmol) alone produced significant long-lasting allodynia, while A-317491 (30 nmol) alone had no effect. I.t. co-administration of ATP and A-317491

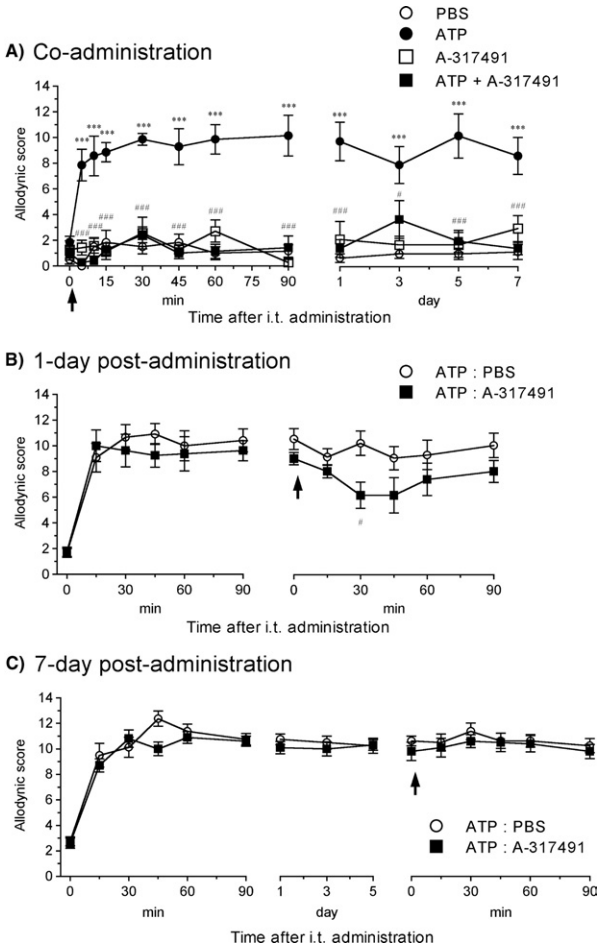

Fig. 3. Effect of the $P2X_3/P2X_{2/3}$-receptor selective antagonist, A-317491, on i.t. ATP-produced long-lasting allodynia. (A) A-317491 (30 nmol) or PBS was co-administered intrathecally with or without ATP (100 nmol). (B, C) ATP (100 nmol) alone was administered intrathecally, and allodynia was produced. One day (B) or 7 days (C) after the i.t. ATP administration, A-317491 (30 nmol) or PBS was administered intrathecally, and the allodynia was evaluated at the times indicated. Values are presented as the mean±S.E.M. Arrows indicate the time of i.t. administration of A-317491 or PBS. *** $P<0.001$ compared with the PBS-treated group. # $P<0.05$, ### $P<0.001$ compared with the ATP alone or ATP: PBS-treated group ($n=6$–12).

(30 nmol) significantly prevented the ATP-produced allodynia. There was a significant difference among drug treatment groups ($F_{3,276}=188.0$, $P<0.001$). Similarly, co-administration of the P2-receptor antagonists suramin and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) significantly prevented the induction of long-lasting allodynia (data not shown). Next, the effect of post-ATP administration of A-317491 on the development of long-lasting allodynia was examined. On day 0, ATP (100 nmol) alone was administered intrathecally to two test groups: one for subsequent PBS administration, and one for subsequent A-317491 administration. One day post-ATP administration, i.t. administration of A-317491 (30 nmol) partially but significantly attenuated the development of allodynia, exerting a maximum effect between 30 and 45 min, and dissipating by 60 min ($F_{1,108}=16.1$, $P<0.001$). A significant effect was observed in the A-317491 group at 30 min, as compared with the PBS group (Fig. 3B). Similarly, 1 day post-ATP administration PPADS partially but significantly attenuated the development of allodynia (data not shown). However, i.t. administration of A-317491 7 days post-ATP administration had no effect on the development of allodynia ($F_{1,144}=1.77$, $P=0.19$, Fig. 3C).

Effect of the NMDA receptor antagonist, MK-801

I.t. co-administration of MK-801 (10 nmol) and ATP significantly prevented the induction of long-lasting allodynia, while MK-801 alone had no effect on the allodynic score (Fig. 4A). There was a significant difference among the drug treatment groups ($F_{3,240}=197.1$, $P<0.001$). However, i.t. administration of MK-801 (10 nmol) alone had no effect on the development of allodynia at 1 or 7 days post-ATP administration ($F_{1,96}=1.83$, $P=0.18$ and $F_{1,66}=0.46$, $P=0.50$, respectively, Fig. 4B, C).

Activation of spinal glial cells following i.t. ATP administration

To examine whether the spinal glial cells were activated in each phase of the long-lasting allodynia, changes in microglial and astrocytic activation were evaluated based on cellular morphology and intensity of immunoreactivity of OX-42, a microglial activation marker, and GFAP, an astrocytic activation marker, following i.t. ATP administration.

In the L4–L5 lumbar dorsal horn of naïve animals, low levels of OX-42 immunostaining were observed. Spinal microglia stained with OX-42 showed features of a resting state; they had extensive, thinly branched processes and were well spaced. In the neuropathic pain model animals 2 days following partial ligation of the sciatic nerve, a procedure as a positive control, microglia on the ipsilateral side were heavily stained and exhibited short, thick processes and enlarged cell bodies, characteristic of an activated state. Such changes were not observed on the contralateral side. Following i.t. administration of ATP (100 nmol) microglial morphology changed from a resting state to an activated state and greater OX-42 staining intensity was observed, although the statistic value by one-way ANOVA for Western blot analysis did not reach significance ($F_{6.21}=1.33$, $P=0.29$). Post-ATP

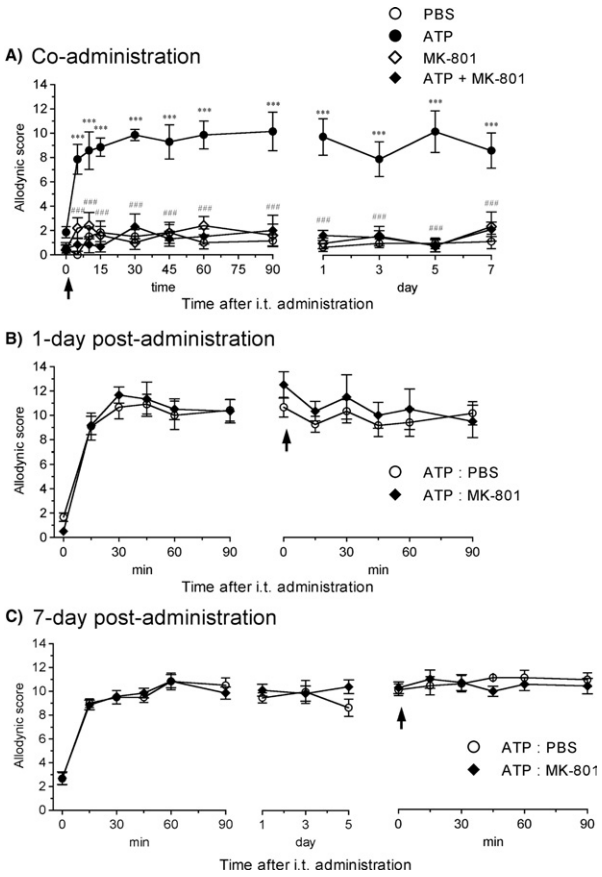

Fig. 4. Effect of the NMDA receptor antagonist, MK-801, on i.t. ATP-produced long-lasting allodynia. (A) MK-801 (10 nmol) or PBS was co-administered intrathecally with or without ATP (100 nmol). (B, C) ATP (100 nmol) alone was administered intrathecally, and allodynia was produced. One day (B) or 7 days (C) after the i.t. ATP administration, MK-801 (10 nmol) or PBS was administered intrathecally, and the allodynia was evaluated at the times indicated. Values are presented as the mean±S.E.M. Arrows indicate the time of i.t. administration of MK-801 or PBS. *** $P<0.001$ compared with the PBS-treated group. ### $P<0.001$ compared with the ATP-treated group ($n=5$–12).

administration, the OX-42 immunoreactivity began to increase at 1 h, and peaked by day 1; microglial activation was only mild to moderate when compared with that following sciatic nerve ligation. The changes in OX-42 immunoreactivity, indicative of spinal microglial activation, gradually reversed at 3 and 7 days after i.t. ATP administration (Fig. 5).

In the naïve animal dorsal horn, the GFAP immunostaining was low, and the stained astrocytes had extensive processes and were well spaced, showing no overt sign of astrocytic activation. In the neuropathic pain model animals, spinal astrocytes on the ipsilateral side were intensely stained and appeared to have an altered shape, showing an activated state of the astrocyte, while such changes were not observed on the contralateral side. Following i.t. ATP administration the spinal astrocytes appeared to be in a moderately activated state; the GFAP immunoreactivity began to increase by day 1, and peaked after 3 days, although the statistic value by one-way ANOVA for Western blot analysis did not reach signifi-

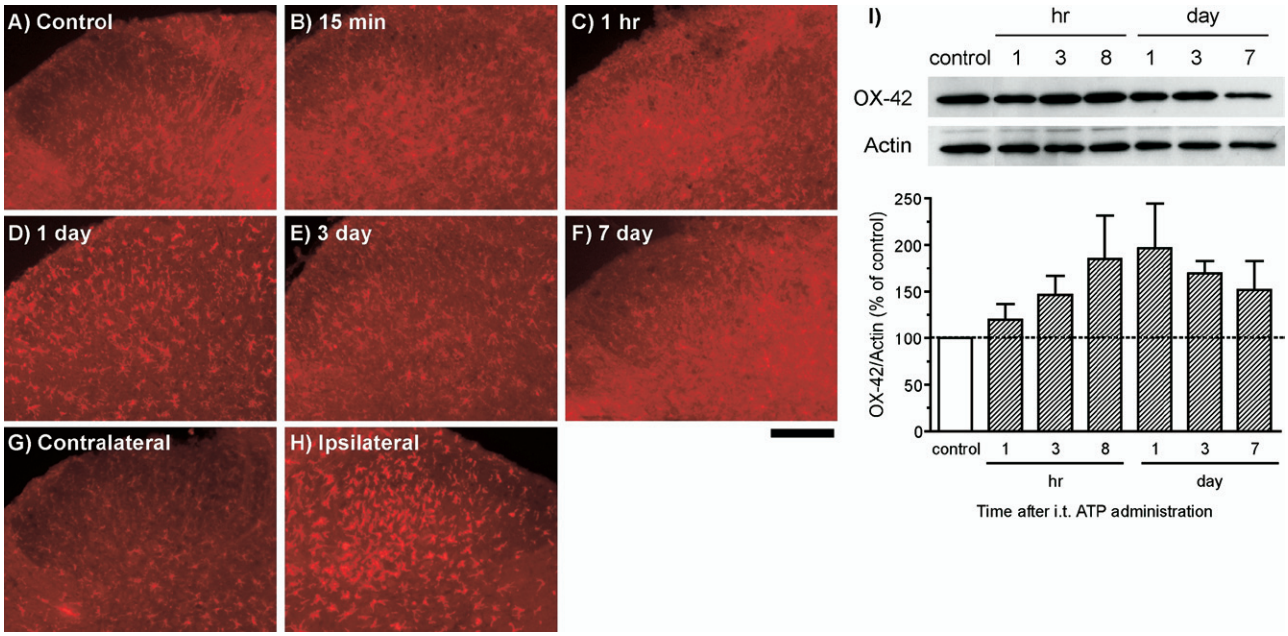

Fig. 5. OX-42 immunostaining, a microglial activation marker, in the L4–L5 lumbar dorsal horn of the spinal cord following i.t. ATP administration. Representative photomicrographs from the spinal cord sections of non-treated control animals (A), the animals administered intrathecally with ATP (100 nmol) at the times indicated (B–F), and the contralateral (G) and ipsilateral (H) sides of the neuropathic pain model animals 2 days following partial sciatic nerve ligation. Scale bar=200 μm. (I) Western blot analysis of OX-42 immunoreactivity. Actin was used as a control for sample loading. The upper panel shows a representative blot. The densities of specific OX-42 and actin bands were measured, and OX-42 levels were normalized against the corresponding actin levels. In the lower panel, the value obtained for control rats served as the control (100%), and the results are presented as the mean of the percentage±S.E.M. of four animals. The change in OX-42 immunoreactivity indicative of spinal microglial activation peaked 1 day after and gradually reversed at 3 and 7 days after the i.t. administration, although the microglial activation was less than that following sciatic nerve ligation.

cance ($F_{6.21}$=1.59, P=0.20). The astrocytic activation was mild to moderate, when compared with that following sciatic nerve ligation (Fig. 6).

Effect of glial inhibitors, minocycline and fluorocitrate

Two glial inhibitors were examined for their effect on each phase of i.t. ATP-produced long-lasting allodynia: minocycline which selectively disrupts the activation of microglia without affecting neurons and astrocytes (Tikka et al., 2001) and fluorocitrate which disrupts the function of astrocytes as well as microglia by inhibiting the glia-specific enzyme aconitase of the Krebs energy cycle (Hassel et al., 1992) (Figs. 7 and 8).

I.t. co-administration of minocycline (200 nmol) and ATP significantly attenuated the induction of the i.t. ATP-produced long-lasting allodynia ($F_{1,234}$=117.2, P<0.001). The significant inhibitory effect lasted for 3 days, and declined to the control level 7 days post administration. On the other hand, i.t. administration of minocycline (200 nmol) 1 day post-ATP administration had little effect on the development of allodynia, although there was a significant difference between the vehicle- and minocycline-administered groups ($F_{1,105}$=8.31, P<0.01). A significant inhibitory effect was observed at 15 min, although an unexpected increase of the allodynic score was observed in the vehicle-administered group at that time point. Similarly, i.t. administration of minocycline 7 days after ATP administration had no effect on the development of allodynia ($F_{1,105}$=0.38, P=0.54).

I.t. co-administration of fluorocitrate (1 nmol) and ATP significantly attenuated the induction of the i.t. ATP-produced long-lasting allodynia ($F_{1,234}$=226.4, P<0.001). The significant inhibitory effect lasted for 7 days. One day post-ATP administration i.t. administration of fluorocitrate (1 nmol) significantly reversed the development of allodynia ($F_{1,105}$=43.9, P<0.001). A significant inhibitory effect was observed at 30 min, sustained for 120 min, and then declined to the control level 1 day post-ATP administration. I.t. post-administration of fluorocitrate 7 days after ATP administration had no effect on the development of allodynia ($F_{1,140}$=0.24, P=0.63).

DISCUSSION

In this study we found that the activation of P2X-receptors, most likely spinal $P2X_{2/3}$-receptors on capsaicin-insensitive primary afferent neurons, produced long-lasting allodynia, which lasted, at least, over 1 week. Furthermore, the present data show possible mechanisms underlying the induction and maintenance of long-lasting allodynia.

Spinal P2X-receptor activation triggered the long-lasting allodynia

I.t. administered ATP produced allodynia which lasted for several weeks, and similar long-lasting allodynia, which

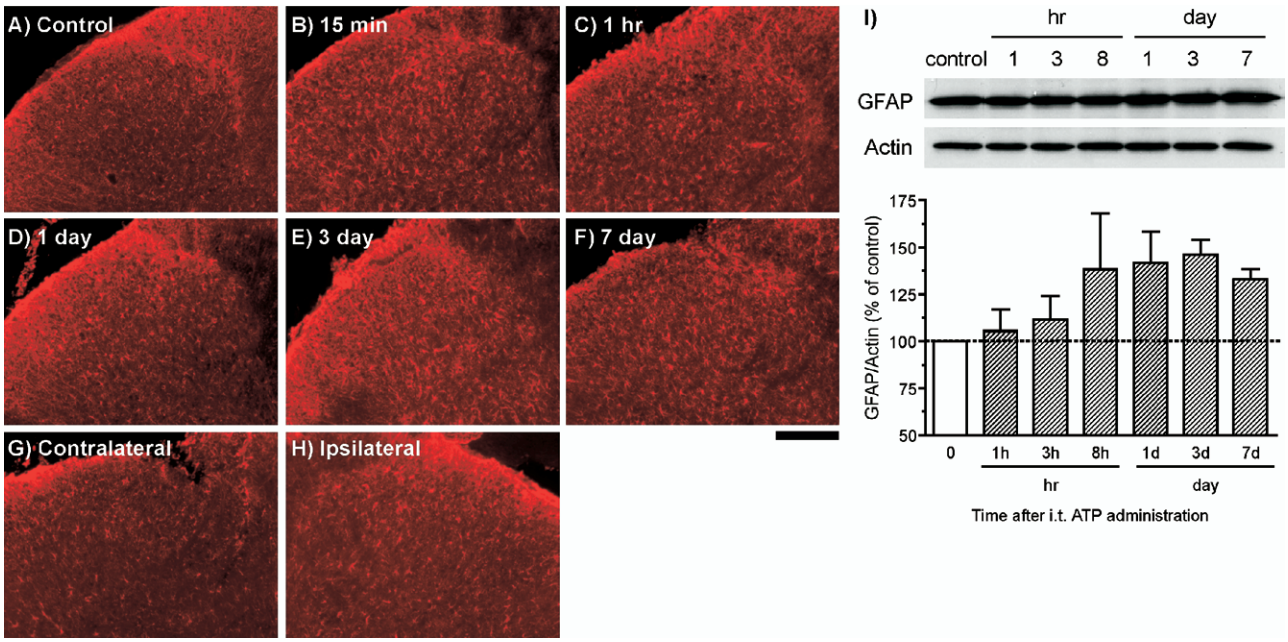

Fig. 6. GFAP immunostaining, an astrocytic activation marker, in the L4–L5 lumbar dorsal horn of the spinal cord following i.t. ATP administration. Representative photomicrographs from the spinal cord sections of non-treated control animals (A), the animals administered intrathecally with ATP (100 nmol) at the times indicated (B–F), and the contralateral (G) and ipsilateral (H) sides of the neuropathic pain model animals 2 days following partial sciatic nerve ligation. Scale bar=200 μm. (I) Western blot analysis of GFAP immunoreactivity. Actin was used as a control for sample loading. The upper panel shows a representative blot. The densities of specific GFAP and actin bands were measured, and GFAP levels were normalized against the corresponding actin levels. In the lower panel, the value obtained for control rats served as the control (100%), and the results are presented as the mean of the percentage±S.E.M. of four animals. The change in GFAP immunoreactivity indicative of spinal astrocytic activation peaked 1–3 days after the i.t. administration, although the astrocytic activation was less than that following sciatic nerve ligation.

lasted over 1 week, was produced by α,β-methylene-ATP administration. Because extracellular ATP is rapidly hydrolyzed by ecto-nucleotidases (Zimmermann, 1996), we postulate that the transient activation of spinal P2X-receptors triggers the long-lasting allodynia. I.t. administration of ATP and P2X-receptor agonists was reported to produce short-lasting hyperalgesia which dissipated within 15 min (Morita et al., 2004; Okada et al., 2002; Tsuda et al., 1999a). The difference between the sustained duration of hyperalgesia and allodynia clearly suggests a difference in their mechanisms. In the present study, we confirmed that neonatal capsaicin-pretreatment successfully destroyed capsaicin-sensitive neurons, i.e. small-diameter C-afferent neurons, using TRPV1-immunofluorescence and the capsaicin test (Jancsó et al., 1977). Both i.t. ATP and α,β-methylene-ATP produced long-lasting allodynia that was not affected by capsaicin pretreatment. These findings suggest that the induction as well as the maintenance of the long-lasting allodynia is not likely to be mediated through capsaicin-sensitive primary afferent neurons. It was reported that the short-lasting hyperalgesia produced by i.t. α,β-methylene-ATP was not observed in neonatal capsaicin-pretreated animals (Morita et al., 2004). In addition, Tsuda et al. (2000) reported that intraplantar α,β-methylene-ATP produced 'peripheral' allodynia that dissipated within 2 h and was also capsaicin-insensitive. These findings suggest that the induction of spinal and peripheral allodynia is mediated through the same pathway, i.e. capsaicin-insensitive primary afferent neurons, while the mechanisms for the transition from induction to long-lasting maintenance of spinal allodynia may be different from that of peripheral allodynia.

Involvement of spinal $P2X_{2/3}$-receptors

I.t. α,β-methylene-ATP which is selective for homomeric $P2X_1$- and $P2X_3$-receptors and several functional heteromeric P2X-receptors including $P2X_{2/3}$ (Lewis et al., 1995; North, 2002) produced long-lasting allodynia. Furthermore, A-317491, the selective $P2X_3$/$P2X_{2/3}$-receptor antagonist, completely blocked the induction of i.t. ATP-produced long-lasting allodynia. It was reported that $P2X_2$- and $P2X_3$-receptors are located at the central primary afferent terminals rather than at spinal dorsal horn neurons and glial cells (Vulchanova et al., 1997). Furthermore, the P2X-receptor subtype on the central terminals of capsaicin-insensitive primary afferent neurons is thought to be the $P2X_{2/3}$-receptor (North, 2002). Taken together, these data suggest that the activation of spinal pre-synaptic $P2X_{2/3}$-receptors on capsaicin-insensitive primary afferent neurons triggers long-lasting allodynia. However, it is possible that i.t. administered ATP and α,β-methylene-ATP could diffusely reach and activate P2X-receptors in the dorsal root ganglion.

I.t. administration of A-317491, as well as PPADS, partially but significantly attenuated the development of long-lasting allodynia 1 day after i.t. administration of ATP. It was reported that i.t. A-317491 had anti-hyperalgesic

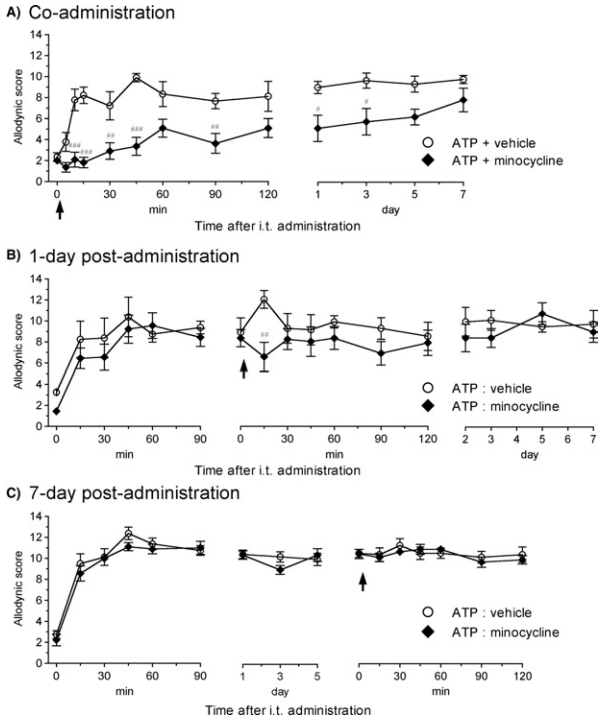

Fig. 7. Effect of minocycline, an inhibitor of microglial activation, on i.t. ATP-produced long-lasting allodynia. (A) Minocycline (200 nmol) or vehicle was co-administered intrathecally with ATP (100 nmol). (B, C) ATP (100 nmol) alone was administered intrathecally, and allodynia was produced. One day (B) or 7 days (C) after the i.t. ATP administration, minocycline (200 nmol) or vehicle was administered intrathecally, and the allodynia was evaluated at the times indicated. Values are presented as the mean±S.E.M. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with the ATP+vehicle, or the ATP: vehicle-treated group (n=8–11).

and anti-allodynic effects in several inflammatory and neuropathic pain models (McGaraughty et al., 2003). Following peripheral inflammation and nerve injury, $P2X_3$-immunoreactivity in the spinal dorsal horn was increased (Novakovic et al., 1999; Xu and Huang, 2002). Chen et al. (2005) reported that sensitivity and trafficking to the cell membrane of $P2X_3$-receptors were enhanced following spared nerve injury. However, our data show that administration of A-317491 7 days post-ATP administration had no effect on the development of long-lasting allodynia. Taken together, these findings suggest that the enhanced function of $P2X_{2/3}$-receptors may play a role in generating long-lasting allodynia in the early phase of maintenance, but not in the late phase. However, i.t. ATP could potentially stimulate many P2X- and P2Y-receptor subtypes expressed on primary afferent and spinal dorsal horn neurons and glial cells. Recent studies showed that $P2X_4$- and $P2X_7$-receptors in spinal microglia were implicated in neuropathic pain (Tsuda et al., 2003; Honore et al., 2006). In addition, $P2Y_{1,2,4}$-receptors have been shown to modulate spinal pain signaling (Burnstock, 2006). The possibility that these P2-purinoceptors may contribute to the induction and/or maintenance of long-lasting allodynia cannot be overlooked, however our data show that the specific activation of $P2X_{2/3}$-receptors is necessary for triggering induction.

Involvement of spinal NMDA receptors

Consistent with a previous finding (Fukuhara et al., 2000), i.t. ATP-produced long-lasting allodynia was completely prevented by the co-administration of MK-801 which suggest that spinal NMDA receptors play an essential role in the induction of long-lasting allodynia. Spinal glutamate has been shown to produce hyperalgesia and allodynia and to further contribute to spinal neural plasticity, i.e. central sensitization via NMDA receptors (Baranauskas and Nistri, 1998). Experiments reported here show that i.t. administration of MK-801 post-ATP administration did not affect the development of long-lasting allodynia which suggests that spinal NMDA receptors play little role in the maintenance of allodynia. Corroborating data showed that i.t. dynorphin A-produced long-lasting allodynia was prevented by pretreatment, but not post-treatment, with MK-801 (Vanderah et al., 1996). Furthermore, our unpublished observations showed that i.t. administration of the group I metabotropic glutamate receptor antagonist had no effect on the development of allodynia, although this does not completely rule out the involvement of spinal glutamate via

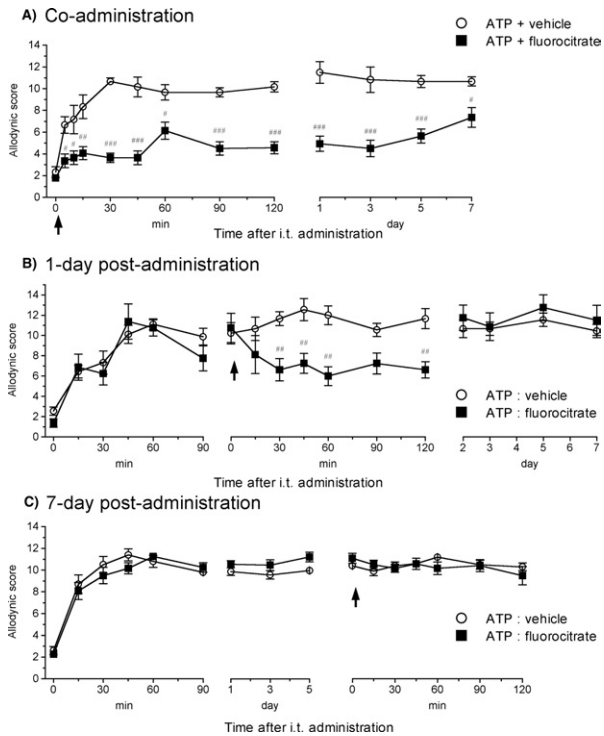

Fig. 8. Effect of fluorocitrate, a glial metabolic inhibitor, on i.t. ATP-produced long-lasting allodynia. (A) Fluorocitrate (1 nmol) or vehicle was co-administered intrathecally with ATP (100 nmol). (B, C) ATP (100 nmol) alone was administered intrathecally, and allodynia was produced. One day (B) or 7 days (C) after the i.t. ATP administration, fluorocitrate (1 nmol) or vehicle was administered intrathecally, and the allodynia was evaluated at the times indicated. Values are presented as the mean±S.E.M. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with the ATP+vehicle, or the ATP: vehicle-treated group (n=6–14).

other AMPA/kainate and metabotropic glutamate receptors.

Differential roles of spinal glial cells in the phase of the long-lasting allodynia

Early microglial activation, and subsequent, slightly delayed astrocytic activation were observed in our studies. This temporal pattern appears to correspond to the same pattern observed following peripheral nerve injury (Tanga et al., 2004; Zhang and De Koninck, 2006). However, ATP-induced glial activation was weak and rapid, when compared with the nerve injury-induced glial activation (Zhang and De Koninck, 2006). It was noted that the temporal profile of i.t. ATP-induced glial activation did not correlate with the duration of the long-lasting allodynia. Furthermore, the results of minocycline administration suggest that spinal microglia are implicated in the induction of i.t. ATP produced long-lasting allodynia, while it played little role in the early and late phases of maintenance. These data correspond to previous findings in neuropathic and inflammatory pain models (Ledeboer et al., 2005; Raghavendra et al., 2003). Fluorocitrate was reported to attenuate both the induction and maintenance of allodynia and hyperalgesia induced by peripheral nerve injury and inflammation (Clark et al., 2007; Meller et al., 1994; Milligan et al., 2003; Watkins et al., 1997). The data presented here also showed that fluorocitrate attenuated the induction, and reversed the early phase of developed allodynia, while it failed to reverse the late phase of developed allodynia. Taken together with findings that show fluorocitrate disrupts the function of astrocytes as well as microglia (Hassel et al., 1992), these results suggest that spinal astrocytes play a role in the early, but not the late phase of maintenance, and perhaps in the induction of the allodynia. This interpretation is partially consistent with a recent suggestion that early microglial activation contributes to the initial induction of allodynia and precipitates, in turn, the subsequent and sustained astrocytic activation, which is implicated in the maintenance of the persistent pain state (Marchand and Perretti, 2005; Tanga et al., 2004; Watkins and Maier, 2003; Wieseler-Frank et al., 2004; Zhang and De Koninck, 2006; Zhuang et al., 2005, 2006). However, fluorocitrate has diverse effects not only on astrocytes. The lack of specific inhibitor for astrocytic function makes it difficult to clearly elucidate the roles of spinal astrocytes. In contrast, fluorocitrate was reported to reverse the developed neuropathic pain even 50 days after peripheral nerve injury (Clark et al., 2007). In most neuropathic pain models, the nerve is continuously injured by chronic ligation or transection for an extended period of time. It is conceivable that the signaling of tissue damage and peripheral nerve injury is chronically transmitted to the spinal cord, which may cause sustained spinal glial activation. In our i.t. ATP-produced long-lasting allodynia model, the transient stimulation of spinal $P2X_{2/3}$-receptor may trigger the generation of a sustained pain state, which lasts throughout the late phase of maintenance, without tissue damage and nerve injury. To elucidate the mechanisms underlying the late maintenance phase of allodynia, i.e. central sensitization, further investigation is needed.

CONCLUSION

In conclusion, these data suggest that the activation of P2X-receptors, most likely spinal $P2X_{2/3}$-receptors on capsaicin-insensitive primary afferent neurons triggers the induction of long-lasting allodynia through NMDA receptors. Furthermore, the induction and early maintenance phase of long-lasting allodynia is differentially mediated through the functions of spinal microglia and astrocytes, while mediation of the late phase is not. The i.t. ATP-produced long-lasting allodynia methodology is a useful model to stimulate chronic pain without tissue damage or peripheral nerve injury, and a tool to elucidate the mechanisms underlying the process of chronic pain development.

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