Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 503 (2004) 71–75

www.elsevier.com/locate/ejphar

Characterization of *N*-methyl-D-aspartate receptor subunits responsible for postoperative pain

Wataru Nishimura[a], Tadatoshi Muratani[a], Shinichi Tatsumi[a], Kenji Sakimura[b], Masayoshi Mishina[c], Toshiaki Minami[a,*], Seiji Ito[d]

[a]*Department of Anesthesiology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki 569-8686, Japan*
[b]*Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan*
[c]*Department of Molecular Neurobiology and Pharmacology, School of Medicine, University of Tokyo, Tokyo 113-0033, Japan*
[d]*Department of Medical Chemistry, Kansai Medical University, Moriguchi 570-8506, Japan*

Received 2 September 2004; accepted 14 September 2004
Available online 6 October 2004

Abstract

N-methyl-D-aspartate (NMDA) receptors have been suggested to be critical for the development of central sensitization, which may amplify postoperative pain. NMDA receptors are formed by GluRζ (NR1) with any one of four GluRε1–4 (NR2A–D) subunits. To clarify the involvement of NMDA receptors in postoperative pain, we examined the effect of the GluRε2-selective antagonist (1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenyl piperidino)-1-propanol (CP-101,606) on postoperative pain caused by plantar incision. We also applied the postoperative pain model to GluRε1 and GluRε4 knockout mice. CP-101,606 administered intrathecally 30 min prior to incision significantly increased mechanical withdrawal thresholds 2 h and 1–3 days after surgery and reduced postoperative pain dose-dependently. Neither GluRε1 nor GluRε4 knockout mice showed a difference in withdrawal thresholds as compared with wild-type mice. Pretreatment with CP-101,606 did not produce an additive analgesic effect in the mice. These results demonstrate that GluRε2-containing NMDA receptors are involved in postoperative pain and that CP-101,606 may be effective in reducing it.

Keywords: Postoperative pain; CP-101,606; NMDA receptor; (Knockout mouse)

1. Introduction

A common cause of persistent pain and hyperalgesia in humans is postoperative pain. Tissue damage associated with surgery initiates a local change in the sensitivity of nociceptors and also triggers central sensitization, an increase in the excitability of neurons in the spinal cord, which may contribute to the severity of postoperative pain. A model of postoperative pain involving a brief surgical incision at the plantar hind paw has been developed in rats (Brennan et al., 1996) and mice (Pogatzki and Raja, 2003). This model is distinguished by persistent quantifiable mechanical hyperalgesia lasting several days, the time course of which has similarities to patient's pain after surgery. There is abundant evidence that glutamatergic transmission at *N*-methyl-D-aspartate (NMDA) receptors is one mechanism for an increase in synaptic strength and induction of central sensitization. Many studies, including ours, have demonstrated that nociception and hyperalgesia are mediated by NMDA receptors with the antagonists (Meller and Gebhart, 1993; Woolf and Salter, 2000; Ito et al., 2001; Minami et al., 2001). However, the NMDA receptor antagonists (+)-5-methyl-10,11-dihydro-5*H*-dibenzyl[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) and D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5) showed no effect on postoperative pain in the rat incision model (Zahn and Brennan, 1998; Pogatzki et al., 2000, 2003). Motor impairment was observed with these

* Corresponding author. Tel.: +81 72 683 1221; fax: +81 72 684 6547.
E-mail address: ane022@poh.osaka-med.ac.jp (T. Minami).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.09.033

antagonists at antinociceptive doses, which might prohibit precise evaluation of the analgesic effect by these NMDA receptor antagonists. The NMDA receptor is composed of GluRζ (NR1) and GluRε (NR2) subunits, and the molecular diversity of the GluRε (GluRε1–GluRε4) subunit family is considered to underline the functional heterogeneity of the NMDA receptor channel (Mori and Mishina, 1995). A GluRε2-selective NMDA receptor antagonist (1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenyl piperidino)-1-propanol (CP-101,606), a derivative of ifenprodil (Boeckman and Aizenman, 1996), is antinociceptive in preclinical pain models and exhibits a much lower side-effect profile compared with other NMDA receptor antagonists (Boyce et al., 1999; Chizh et al., 2001). Whether NMDA receptors are involved in postoperative pain and, if involved, which GluRε subunit(s) of NMDA receptors are responsible for it remain unclear due to the side effects of nonselective NMDA receptor antagonists and lack of subunit-specific antagonists except for CP-101,606. In the present study, we tried to address these questions by use of mice lacking NMDA receptor GluRε1 or GluRε4 subunit (GluRε1$^{-/-}$ or GluRε4$^{-/-}$) (Ikeda et al., 1995; Sakimura et al., 1995). Inasmuch as GluRε2 subunit-deficient mice showed no suckling response and died shortly after birth (Kutsuwada et al., 1996), here, we used CP-101,606 instead of GluRε2$^{-/-}$ mice.

2. Materials and methods

2.1. Animals

GluRε1$^{-/-}$ or GluRε4$^{-/-}$ mice were obtained by gene targeting technique (Ikeda et al., 1995; Sakimura et al., 1995). Male C57BL/6 (wild type) mice and mutant mice weighing 25–30 g were used for experiments. The animals were housed in a group (n=6) before surgery and individually after operation in a 12-h light/darkness cycle, a constant temperature of 22±2 °C, and 60±10% humidity. They were allowed free access to food and water over the experimental period. Each animal was used for one experiment and killed at the end of the experiment. All animals were conformed to the regulations of the Animal Care Committee of Osaka Medical College and conducted in accordance with the guidelines of the ethics committee of the International Association for the Study of Pain (Zimmermann, 1983).

2.2. Surgical procedures

All mice were anesthetized with an intraperitoneal administration of pentobarbital (60 mg/kg). The postoperative pain model was prepared in mice with skin, fascia, and muscle incision essentially according to the procedure reported by Brennan et al. (1996). Briefly, a 3-mm longitudinal incision was made with a number 11 blade through skin and fascia of the plantar aspect of the foot. The plantaris muscle was elevated and incised longitudinally. After gentle pressure for hemostasis, the skin was apposed with two sutures of 5-0 nylon thread. After surgery, the incisions were checked daily, and animals with any sign of wound infection were excluded from the study.

2.3. Assessment of mechanical hyperalgesia

For behavioral assessment of mechanical hyperalgesia, mice were placed individually on an elevated metal grid covered with a clear plastic cage. Withdrawal responses to punctuate mechanical stimulation were determined using calibrated von Frey filaments. The site of stimulation was in the plantar aspect of the foot adjacent to the wound. Each filament was presented perpendicularly against the paw, with sufficient force to cause slight bending and held 2–3 s. The filaments were applied once to the paw at an increasing force until the mouse withdrew its hind limb followed by a decreasing force to the initial filament. A cutoff pressure of 15.1 g was set to avoid tissue damage. The animals were tested before and 2 h, 1 day, 3 days, and 5 days after surgery. The area under the curve (AUC) values were obtained by calculating the area between the zero line and the curve for the time course of the withdrawal threshold from 0 to 5 days after surgery. For intrathecal (i.t.) drug administration, a 27-gauge stainless steel needle attached to a microsyringe was inserted between the L5 and L6 vertebrae, as described previously (Minami et al., 2001). A drug in saline (5 μl) was injected slowly into the subarachnoid space of anesthetized mice 30 min before operation.

2.4. Rota-rod performance test

To determine the effect of CP-101,606 on motor coordination, animals were first trained on the morning before the test to remain for 300 s on a Rota-rod Treadmill for mice MK-500 (Muromachi Kikai, Tokyo, Japan) revolving at 6 rpm. Animal received 1 μg CP-101,606 or saline (i.t.) were placed on a Rota-rod at a speed of 20 rpm, and the time for which the animals were able to remain on the Rota-rod was recorded up to a cutoff time of 5 min.

2.5. Drug

CP-101,606, kindly donated by Pfizer (New London, CT), was dissolved in sterile saline on the day of experiments and kept on ice until used.

2.6. Statistics

The results are expressed as mean±S.E.M. The data were compared using nonparametric analyses. Wilcoxon

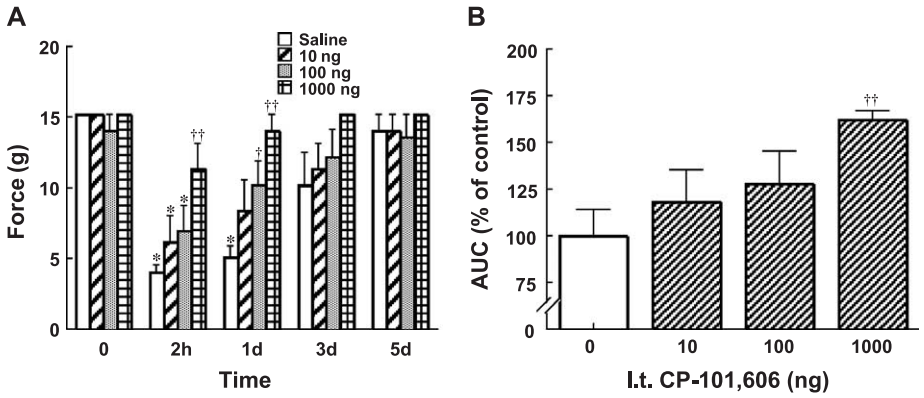

Fig. 1. Dose dependency of CP-101,606 for the analgesic effect on postoperative pain. (A) Indicated doses of CP-101,606 or saline were i.t. injected 30 min prior to surgery. In each group, six mice were tested by von Frey filaments before and at indicated times after surgery, as described under Materials and methods. Data are expressed as mean with S.E.M. *P<0.05 vs. preincision values by Wilcoxon signed rank test. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 vs. saline by Mann–Whitney rank–sum test. (B) The magnitude of the analgesic effect by i.t. CP-101,606 on postoperative pain was presented as the AUC values over 5 days after surgery, as described under Materials and methods. $^{\dagger\dagger}P$<0.01 compared with the saline-injected group (Mann–Whitney rank–sum test).

signed rank test and Mann–Whitney rank–sum test were used for within-group and between-group comparisons, respectively. P<0.05 was considered significant.

3. Results

3.1. Effect of GluRε2-selective NMDA receptor antagonist on postoperative pain

Throughout the experimental period, the mice moved freely except for impaired weight bearing on the area of the incision and have access to food and water ad libitum.

In saline-treated mice, mechanical withdrawal thresholds to mechanical stimulation by von Frey filaments decreased from 15.1 g before surgery to 4.25 g at 2 h after surgery and gradually returned to the preincisional level over 5 days (Fig. 1A). The time course of mechanical hyperalgesia in mice was quite similar to that observed in rats with the same incisional model (Brennan et al., 1996) and mice (Pogatzki and Raja, 2003). We examined the effect of the GluRε2-selective NMDA receptor antagonist CP-101,606 on postoperative pain in this model. When administered i.t. 30 min prior to surgery, CP-101,606-treated mice showed greater withdrawal thresholds at 2 h (P<0.01), day 1 (P<0.01), and day 3 (P<0.05), compared with the control, and returned to the preincisional level by postoperative day 5 (Fig. 1A). The AUC values of the groups increased by i.t. administration of CP-101,606 in a dose-dependent manner and the AUC value at 1 μg was significantly larger than that of the saline-injected group (Fig. 1B). CP-101,606 at 1 μg did not show any detectable effect in the Rota-rod test for 2 h after i.t. injection (Fig. 2). Motor impairment was not observed over the experimental period at doses employed.

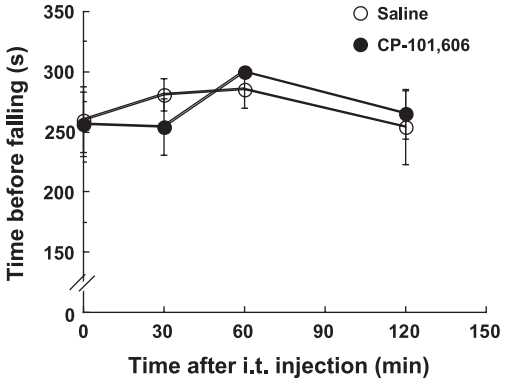

Fig. 2. Effect of CP-101,606 on Rota-rod performance test. The time of which the mice were able to remain on the Rota-rod was recorded up to a cutoff time of 5 min before and after i.t. injection of 1 μg CP-101,606 or saline. Data are expressed as mean±S.E.M. (n=6).

3.2. Postoperative pain in GluRε1 and GluRε4 knockout mice

To further clarify which subunit(s) of NMDA receptor are involved in postoperative pain, we applied the incisional pain model to GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice. Fig. 3 shows

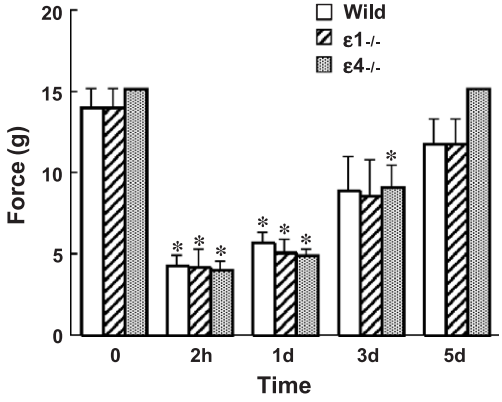

Fig. 3. Foot withdrawal thresholds in plantar incision model in wild-type, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. In each group, six mice were tested by von Frey filaments before and at indicated times after surgery, as described under Materials and methods. Data are expressed as mean with S.E.M. *P<0.05 vs. preincision values by Wilcoxon signed rank test.

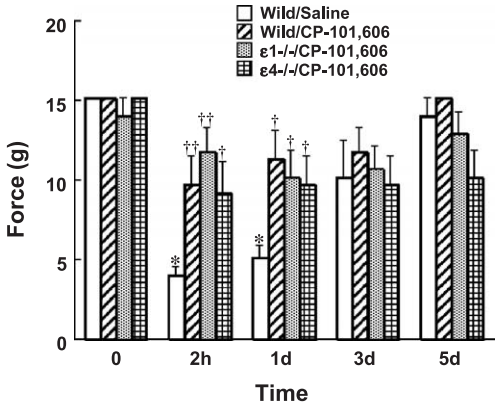

Fig. 4. Effect of GluRε2-selective antagonist CP-101,606 on foot withdrawal thresholds in wild-type, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. CP-101,606 (1 μg) was i.t. injected in wild-type, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice 30 min prior to surgery. Details are described in the legend for Fig. 1. *P<0.05 vs. preincision values by Wilcoxon signed rank test. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 vs. the saline-injected wild-type mice by Mann–Whitney rank–sum test.

time courses of withdrawal thresholds to mechanical stimuli in wild-type, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice after surgery. In all groups, withdrawal thresholds decreased significantly at 2 h and 1 day after surgery. The withdrawal threshold remained significantly low on postoperative day 3 and returned to the preincisional level by day 5 after operation. There was no difference among groups.

To clarify whether NMDA receptors composing different GluRε subunits played different roles in incisional pain at the period of surgery and the postoperative period, we examined the effect of CP-101,606 in wild-type, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice. The withdrawal threshold at 2 h after surgery was markedly increased in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice by 30-min pretreatment with 1 μg CP-101,606, and there was no significant difference among wild-type, GluRε1$^{-/-}$, and GluRε4$^{-/-}$ mice (Fig. 4). Similarly, there was no difference in the time courses of withdrawal thresholds on postoperative day 1 to day 5 among them. Pretreatment with CP-101,606 produced no significant additive or synergistic effect on the withdrawal threshold in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice.

4. Discussion

Glutamate is contained in primary afferent fibers and in the dorsal horn (Battaglia and Rustioni, 1988; Valtschanoff et al., 1994), and many studies have demonstrated that spinal glutamate is critical for nociception and hyperalgesia. In fact, glutamate release was increased in the extracellular fluid in the dorsal horn of the spinal cord immediately after a plantar incision (Zahn et al., 2002). Because the release was blocked by hind paw denervation or tetrodotoxin treatment, plantar incision resulted in segmental increase in glutamate concentration in the spinal cord that was driven by input from primary afferent fibers from the site of injury and required axonal conduction within the spinal cord (Zahn et al., 2002). It was previously reported that, while postoperative pain was blocked by spinal non-NMDA receptor antagonists, i.e., α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite, (Zahn et al., 1998; Pogatzki et al., 2003), the behavioral responses were not affected by spinal administration of NMDA receptor antagonists (Zahn and Brennan, 1998). Secondary hyperalgesia, an exaggerated response to stimuli applied remote from an injury, is a result of central sensitization. Intrathecal NMDA receptor antagonists influenced secondary hyperalgesia but only at doses that produced motor impairment and caused some behavioral activation (Pogatzki et al., 2003). These side effects may affect the withdrawal threshold, but low doses devoid of side effects did not affect pain behaviors. CP-101,606, a GluRε2-selective antagonist, was previously shown to inhibit wind-up of a spinal nociceptive reflex, inflammatory pain induced by carrageenan and other algogens, and neuropathic pain and exhibited a good therapeutic window with no motor side effects (Taniguchi et al., 1997; Boyce et al., 1999; Chizh et al., 2001). The present study demonstrates that i.t. administration of CP-101,606 prior to incision increased withdrawal thresholds to mechanical stimuli 2 h and 1–3 days after surgery without motor impairment (Figs. 1, and 2). On the other hand, we have failed to demonstrate any change of the withdrawal threshold after operation in GluRε1$^{-/-}$ and GluRε4$^{-/-}$ mice (Fig. 3). Similarly, the pretreatment of these knockout mice with CP-101,606 showed the time courses of postoperative pain similar to that of wild-type mice (Fig. 4). The present study demonstrates that NMDA receptors are involved in postoperative pain through GluRε2-containing NMDA receptors in this incision model and that the effect of CP-101,606 administered prior to incision continues into the postoperative period and, in this way, can alleviate peripheral and central sensitization, which amplifies postoperative pain. Consistent with previous reports, GluRε1 and GluRε4 subunits of NMDA receptors appeared not to play an important role in postoperative pain. The difference in the effects of NMDA receptor antagonists on postoperative pain between the present study and previous reports of Brennan's group may be due to the use of GluRε2-selective NMDA receptor antagonist and nonsubunit selective ones.

In situ hybridization histochemistry of mouse spinal cord revealed that, while GluRε1 mRNA signals were found in all regions of the gray matter except for lamina II, GluRε2 subunit mRNA signals were restricted to the lamina II (Watanabe et al., 1994). The expression of GluRε4 mRNA was low, and that of GluRε3 mRNA was undetectable. The restricted expression of GuRε2 in the substantia gelatinosa was also shown by immunocytochemical studies (Boyce et al., 1999). Such distinct distribution of GluRε subunits in the spinal cord supports our findings that NMDA receptors containing GluRε2 subunits play a critical role in processes leading to central sensitization and to persistent postoperative pain, independent of ones containing GluRε1 subunits.

Sensitization is a continuous phenomenon, largely dependent on the magnitude and duration of the nociceptive stimulus. The way to prevent sensitization might be to block any pain originating from the surgical wound from the time of incision until final wound healing and rehabilitation. However, conventional NMDA receptor antagonists induce unacceptable side effects at analgesic doses, which prohibits their widespread use. The present study demonstrates that pretreatment of CP-101,606 shows a long-lasting analgesic activity in the postoperative pain model, as reported previously in other preclinical models (Chizh et al., 2001) and in patients with neuropathic pain (Sang et al., 2003). CP-101,606, allowing the use of a larger dose to achieve sufficient and GluRε2-selective antagonistic action at the NMDA receptor with the rapid onset required in acute pain states with negligible side effects at analgesic doses, may provide a better alternative than NMDA receptor antagonists currently employed in clinics.

Acknowledgements

This work was supported in part by grants from the programs Grants-in-Aid for Scientific Research on Priority Areas and Creative Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid for Scientific Research (B) and (C) from Japan Society for the Promotion of Science, and by grants from the Science Research Promotion Fund of the Japan Private School Promotion Foundation.

References

Battaglia, G., Rustioni, A., 1988. Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. J. Comp. Neurol. 277, 302–312.

Boeckman, F.A., Aizenman, E., 1996. Pharmacological properties of acquired excitotoxicity in Chinese hamster ovary cells transfected with *N*-methyl-D-aspartate receptor subunits. J. Pharmacol. Exp. Ther. 279, 515–523.

Boyce, S., Wyatt, A., Webb, J.K., O′ Donnell, R., Mason, G., Rigby, M., Sirinathsinghji, D., Hill, R.G., Rupniak, N.M.J., 1999. Selective NMDA NR2B antagonists induce antinociception without motor dysfunction: correlation with restricted localisation of NR2B subunit in dorsal horn. Neuropharmacology 38, 611–623.

Brennan, T.J., Vandermeulen, E.P., Gebhart, G.F., 1996. Characterization of a rat model of incisional pain. Pain 64, 493–501.

Chizh, B.A., Headley, P.M., Tzschentke, T.M., 2001. NMDA receptor antagonists as analgesics: focus on the NR2B subtype. Trends Pharmacol. Sci. 22, 636–642.

Ikeda, K., Araki, K., Takayama, C., Inoue, Y., Yagi, T., Aizawa, S., Mishina, M., 1995. Reduced spontaneous activity of mice defective in the ε4 subunit of the NMDA receptor channel. Mol. Brain Res. 33, 61–71.

Ito, S., Okuda-Ashitaka, E., Minami, T., 2001. Central and peripheral roles of prostaglandins in pain and their interactions with novel neuropeptides nociceptin and nocistatin. Neurosci. Res. 41, 299–322.

Kutsuwada, T., Sakimura, K., Manabe, T., Takayama, C., Katakura, N., Kushiya, E., Natsume, R., Watanabe, M., Inoue, Y., Yagi, T., Aizawa, S., Arakawa, M., Takahashi, T., Nakamura, Y., Mori, H., Mishina, M., 1996. Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor ε2 subunit mutant mice. Neuron 16, 333–344.

Meller, S.T., Gebhart, G.F., 1993. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 52, 127–136.

Minami, T., Matsumura, S., Okuda-Ashitaka, E., Shimamoto, K., Sakimura, K., Mishina, M., Mori, H., Ito, S., 2001. Characterization of the glutamatergic system for induction and maintenance of allodynia. Brain Res. 895, 178–185.

Mori, M., Mishina, M., 1995. Structure and function of the NMDA channel. Neuropharmacology 34, 1219–1237.

Pogatzki, E.M., Raja, S.N., 2003. A mouse model of incisional pain. Anesthesiology 99, 1023–1027.

Pogatzki, E.M., Zahn, P.K., Brennan, T.J., 2000. Effect of pretreatment with intrathecal excitatory amino acid receptor antagonists on the development of pain behavior caused by plantar incision. Anesthesiology 93, 489–496.

Pogatzki, E.M., Niemeier, J.S., Sorkin, L.S., Brennan, T.J., 2003. Spinal glutamate receptor antagonists differentiate primary and secondary mechanical hyperalgesia caused by incision. Pain 105, 97–107.

Sakimura, K., Kutsuwada, T., Ito, I., Manabe, T., Takayama, C., Kushiya, E., Yagi, T., Aizawa, S., Inoue, Y., Sugiyama, H., Mishina, M., 1995. Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor ε1 subunit. Nature 373, 151–155.

Sang, C.N., Weaver, J.J., Jinga, L., Wouden, J., Saltarelli, M., 2003. The NR2B subunit-selective NMDA receptor antagonist, CP-101,606, reduces spontaneous pain intensity in patients with central and peripheral neuropathic pain. Soc. Neurosci. Abstr., 814.9.

Taniguchi, K., Shinjo, K., Mizutani, M., Shimada, K., Ishikawa, T., Menniti, F.S., Nagahisa, A., 1997. Antinociceptive activity of CP-101,606, an NMDA receptor NR2B subunit antagonist. Br. J. Pharmacol. 122, 809–812.

Valtschanoff, J.G., Phend, K.D., Bernardi, P.S., Weinberg, R.J., Rustioni, A., 1994. Amino acid immunocytochemistry of primary afferent terminals in the rat dorsal horn. J. Comp. Neurol. 346, 237–252.

Watanabe, M., Mishina, M., Inoue, Y., 1994. Distinct spatiotemporal distributions of the *N*-methyl-D-aspartate receptor channel subunit mRNAs in the mouse cervical cord. J. Comp. Neurol. 345, 314–319.

Woolf, C.J., Salter, M.W., 2000. Neuronal plasticity: increasing the gain in pain. Science 288, 1765–1768.

Zahn, P.K., Brennan, T.J., 1998. Lack of effect of intrathecally administered *N*-methyl-D-aspartate receptor antagonists in a rat model for postoperative pain. Anesthesiology 88, 143–156.

Zahn, P.K., Umali, E., Brennan, T.J., 1998. Intrathecal non-NMDA excitatory amino acid antagnonists inhibit pain behaviors in a rat model of postoperative pain. Pain 74, 213–223.

Zahn, P.K., Sluka, K.A., Brennan, T.J., 2002. Excitatory amino acid release in the spinal cord caused by plantar incision in the rat. Pain 100, 65–76.

Zimmermann, M., 1983. Ethical guidelines for investigation of experimental pain in conscious animals. Pain 16, 109–110.