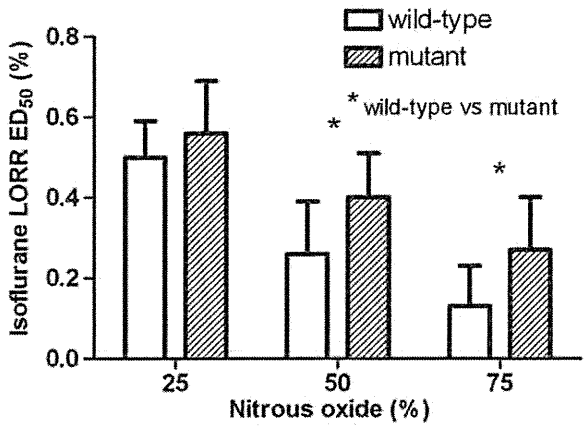

Fig. 3. Effects of various concentrations of nitrous oxide on isoflurane LORR ED_{50} values in wild-type and mutant mice. Values are means ± S.D. (n=10–12 per group). *$P < 0.05$, mutant vs wild-type, Bonferroni's test following 2-way ANOVA. LORR ED_{50}=the 50% effective dose for the loss of righting reflex.

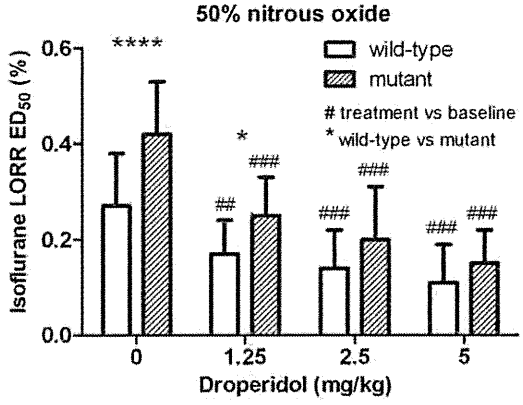

Fig. 4. Effects of 50% nitrous oxide on isoflurane LORR ED_{50} values in wild-type and mutant mice pretreated with various doses of droperidol. Values are means ± S.D. (n=15–20 per group). ##$P < 0.01$; ###$P < 0.001$, treatment vs corresponding baseline, Dunnett's test following 1-way ANOVA. *$P < 0.05$; ****$P < 0.0001$, mutant vs wild-type, Bonferroni's test following 2-way ANOVA. LORR ED_{50}=the 50% effective dose for the loss of righting reflex.

dose-dependent decrease in LORR ED_{50} values in both groups of mice compared with their respective baseline controls ($P < 0.0001$, 1-way ANOVA, for both experimental groups). The administration of droperidol also dose-dependently reduced and eliminated the differences in isoflurane LORR ED_{50} values in the presence of nitrous oxide between the two genotypes ($P < 0.05$, wild-type versus mutant, for 1.25 mg/kg droperidol; $P > 0.05$, wild-type versus mutant, for both 2.5 and 5 mg/kg droperidol, Bonferroni's test following 2-way ANOVA).

3.3.3. *Pretreatment with ketanserin*

Since serotonergic status also can influence general anesthesia (Dringenberg, 2000), we had to exclude the possibility that excessive serotonergic activity affected the results in mutant animals. For this purpose, we repeated the experiment with 50% nitrous oxide after pretreatment of both groups of mice with three increasing doses of the 5-HT $_{2A}$ receptor antagonist ketanserin. As shown in Fig. 5, in contrast to wild-type controls, even the lowest dose (2.5 mg/kg) of ketanserin could significantly reduce isoflurane LORR ED_{50} values in the presence of nitrous oxide compared with baseline in mutant mice, which demonstrated their greater susceptibility to the effect of ketanserin (P=0.002, and $P < 0.0001$, 1-way ANOVA, in wild-type and

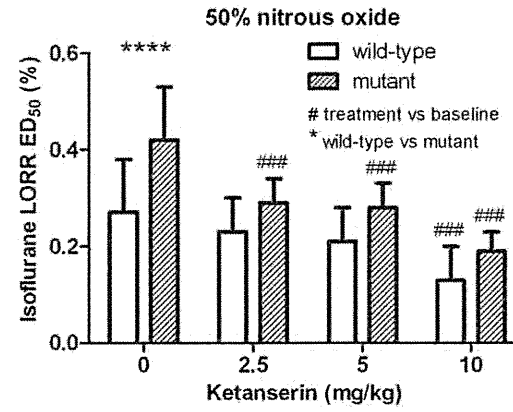

Fig. 5. Effects of 50% nitrous oxide on isoflurane LORR ED_{50} values in wild-type and mutant mice pretreated with various doses of ketanserin. Values are means ± S.D. (n=10–16 per group). ###$P < 0.001$, treatment vs corresponding baseline, Dunnett's test following 1-way ANOVA. ****$P < 0.0001$, mutant vs wild-type, Bonferroni's test following 2-way ANOVA. LORR ED_{50}=the 50% effective dose for the loss of righting reflex.

mutant mice, respectively). Furthermore, in the presence of ketanserin the ability of nitrous oxide to reduce isoflurane LORR ED_{50} values did not significantly differ between the two genotypes ($P > 0.05$, wild-type versus mutant, at all three doses of ketanserin, Bonferroni's test after 2-way ANOVA).

Thus, larger isoflurane LORR ED_{50} values in the presence of nitrous oxide in mutant animals are probably caused by augmented monoaminergic function secondary to the knockout and not by the impaired NMDA receptor function. This suggests that NMDA receptor GluN2A subunits do not play a critical role in mediating the LORR produced by nitrous oxide.

4. Discussion

The finding of similar sevoflurane and isoflurane LORR ED_{50} values in mutant and wild-type mice suggests that NMDA receptors are not critically involved in the hypnotic action of these halogenated anesthetics. This would be consistent with the respective mild and moderate abilities of sevoflurane and isoflurane to inhibit NMDA receptors in vitro (Yamakura and Harris, 2000). On the other hand, the finding of the compromised ability of nitrous oxide to potentiate the hypnotic effect of isoflurane in mutant mice suggests upon first consideration that NMDA receptors may mediate LORR by nitrous oxide. This would be consistent with the ability of nitrous oxide to potently inhibit NMDA receptors in vitro (Yamakura and Harris, 2000).

In this regard it should be noted that we (Petrenko et al., 2004), as well as other investigators (Sato et al., 2004), previously found that mutant mice were resistant to the hypnotic effect of ketamine, an intravenous anesthetic that also potently inhibits NMDA receptors. However, we were cautious of the possible secondary nature of these findings and therefore did not proceed, like other investigators (Sato et al., 2005), to conclude that NMDA receptors are directly involved in the anesthetic action of nitrous oxide. Rather, we showed that the results obtained in GluN2A mutant mice can be affected by changes occurring in these animals secondary to genetic manipulation (Petrenko et al., 2010). In more detail, these animals were reported to have enhanced NMDA-stimulated dopamine release from the striatum and intensified dopamine and serotonin metabolism in the frontal cortex and striatum indicating a hyperfunction of brain monoaminergic systems, accompanying the NMDA receptor dysfunction (Miyamoto et al., 2001).

Thus, in the present study we had to exclude the possibility that these changes in brain neurotransmission were not the cause of the observed reduced sensitivity to nitrous oxide. In fact, facilitation of dopamine and serotonin activity, being one of the mechanisms of action of psychostimulant drugs, can lead to enhanced alertness, wakefulness and locomotion (White et al., 1996). It is of note that GluN2A mutant mice exhibit hyperlocomotion in a novel environment which can be attenuated by dopamine receptor antagonists at doses that have no effect in wild-type mice (Miyamoto et al., 2001). Furthermore, drugs like amphetamines and dopamine agonists can produce an analeptic effect, shortening the duration of LORR induced by general anesthetics (Chemali et al., 2012; Horita and Carino, 1991; Horita et al., 1994; Solt et al., 2011). Such observations imply that the reduced isoflurane LORR ED_{50}-sparing effect of nitrous oxide in mutant animals could have resulted from dopaminergic hyperfunction. Therefore, to exclude the dopaminergic origin of the reduced isoflurane LORR ED_{50}-sparing effect of nitrous oxide after knockout we had to investigate the ability of the D_2 receptor antagonist droperidol to mitigate the difference between the two genotypes in isoflurane LORR ED_{50}values in the presence of nitrous oxide. We found that droperidol has an ability to restore the potentiating effect of nitrous oxide on isoflurane LORR ED_{50} in mutants to levels similar to those in wild-type controls.

The increased brain metabolism of serotonin in mutant mice is a result of serotonergic activation which accompanies excessive dopaminergic activity and is also likely to be responsible for behavioral changes observed after knockout (Geyer, 1996). As with the effect of droperidol, we found that in the mutant mice pharmacological antagonism of 5-HT $_{2A}$ receptors by ketanserin could also restore the potentiating effect of nitrous oxide and abolish the differences between the two genotypes in the isoflurane LORR ED_{50}-sparing effect of this gaseous anesthetic. Together, these normalizing effects of droperidol and ketanserin indicate that the monoaminergic hyperfunction and not the impaired NMDA receptor functioning was the cause of the reduced isoflurane LORR ED_{50}-sparing ability of nitrous oxide in the mutant animals.

Together with GluN2A subunits, GluN2B subunits represent another principal GluN2 subunit type widely expressed in the adult brain (Watanabe et al., 1993) that is likely to take on the role of the missing GluN2A subunits after knockout. As we previously demonstrated on the heteromeric NMDA receptor channels, butyrophenones such as haloperidol and droperidol can selectively inhibit NMDA receptors containing GluN2B subunits (Yamakura et al., 1998). Considering the fact that the NMDA receptors of the mutant animals would be primarily GluN2B-containing, they should be more susceptible to the inhibitory effect of droperidol. Consequently, if NMDA receptors were indeed the principle mediators of LORR by nitrous oxide, mutant mice would demonstrate significantly lower LORR ED_{50} values compared with wild-type controls after pretreatment with droperidol. Since such was not the case, this provides additional support for the secondary origin of the reduced hypnotic effect of nitrous oxide in mutant animals.

It should be noted that mutant animals also exhibit reduced NMDA-stimulated gamma-aminobutyric acid (GABA) release from striatal slices indicating an impaired GABAergic neuron activation in that brain area after GluN2A subunit knockout (Miyamoto et al., 2001). Although it has not been specifically examined, the latter GABAergic defect might not be limited to the striatum, but also be present in other brain areas, including the tuberomammillary nucleus (TMN). TMN is a brain area known to be critically involved in mediating the hypnotic effect of anesthetics preferentially targeting $GABA_A$ receptors (Nelson et al., 2002). Therefore, impaired GABAergic function in TMN could explain the resistance to GABAergic agents such as pentobarbital or propofol previously shown by us (Petrenko et al., 2004) and other investigators (Sato et al., 2005). Relative to the results of the present study, although nitrous oxide also potentiates $GABA_A$ receptors, this potentiation is minimal (Yamakura and Harris, 2000). Therefore, it is unlikely that impaired GABAergic signaling was a significant contributor to the observed resistance to nitrous oxide in mutant animals.

Finally, our present results demonstrating similar isoflurane LORR ED_{50} values in two genotypes differ from the findings of our previous study where we demonstrated greater isoflurane MAC values (i.e., a resistance to the immobilizing action of isoflurane) in mutant mice (Petrenko et al., 2010). Although we have no definitive explanation for this apparent discrepancy, one potential explanation may stem from the fact that immobility is realized through mechanisms and targets within the CNS that are different from hypnosis (Alkire and Miller, 2005; Sonner et al., 2003). Also, in mice, immobility occurs at isoflurane concentrations approximately 2-fold higher than those required for LORR (Petrenko et al., 2010). Multiple receptors, receptor system and neuronal circuits could be differently affected by different concentrations of anesthetics (Yamakura et al., 2001), sometimes making the net effect of anesthetics very difficult to predict (Joo et al., 1999; Pearce, 1999). This may be especially relevant in cases like ours when the anesthetic is applied in a situation with abnormalities already present in several neurotransmitter systems in the CNS.

5. Conclusions

Our findings suggest that NMDA receptors are not critically involved in mediating the LORR produced by conventionally-used inhalational anesthetics, such as isoflurane and nitrous oxide. The

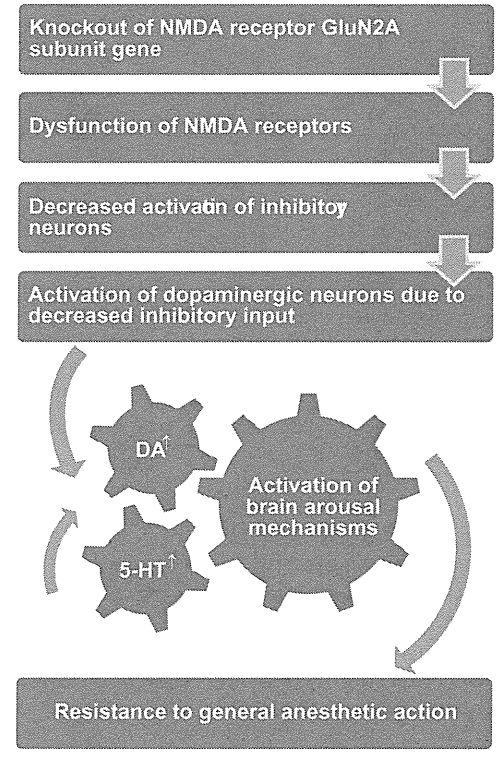

Fig. 6. Schematic diagram illustrating a suggested chain of events leading to resistance to general anesthetics after the NMDA receptor GluRε1 subunit gene knockout, based on previous reports (Miyamoto et al., 2001; Petrenko et al., 2004) and the results of this study. DA: dopamine; 5-HT: 5-hydroxytryptamine (serotonin); ↑: an increase in brain concentration.

observed resistance to nitrous oxide in mutant mice is likely to be of secondary monoaminergic origin (Fig. 6). Since the monoamines dopamine and serotonin are intimately involved in the mechanisms of wakefulness (Watson et al., 2010), our data from mutant mice also suggest that any condition or pharmacological manipulation resulting in increased monoaminergic tone can oppose the effects of general anesthetics. Furthermore, our results show that before reaching any definitive conclusions based on the results obtained in global knockouts, the possibility of changes ensuing secondary to the genetic manipulation and affecting the results should be carefully considered and excluded when possible.

Acknowledgments

The authors thank Yukio Sato for excellent technical assistance and Rie Natsume for expert assistance with animal care. This study was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) No. 23791689 to A.B.P.

References

Alkire, M.T., Hudetz, A.G., Tononi, G., 2008. Consciousness and anesthesia. Science 322, 876–880.

Alkire, M.T., Miller, J., 2005. General anesthesia and the neural correlates of consciousness. Prog. Brain. Res. 150, 229–244.

Chemali, J.J., Van Dort, C.J., Brown, E.N., Solt, K., 2012. Active emergence from propofol general anesthesia is induced by methylphenidate. Anesthesiology 116, 998–1005.

Dringenberg, H.C., 2000. Serotonergic receptor antagonists alter responses to general anaesthetics in rats. Br. J. Anaesth. 85, 904–906.

Eger 2nd, E.I., Raines, D.E., Shafer, S.L., Hemmings Jr., H.C., Sonner, J.M., 2008. Is a new paradigm needed to explain how inhaled anesthetics produce immobility? Anesth. Analg. 107, 832–848.

Geyer, M.A., 1996. Serotonergic functions in arousal and motor activity. Behav. Brain. Res. 73, 31–35.

Horita, A., Carino, M.A., 1991. D-1 agonist, SKF 38393, but not a D-2 agonist, produces a cholinergically mediated analeptic effect in rabbits. Pharmacol. Biochem. Behav. 39, 449–452.

Horita, A., Carino, M.A., Ukai, Y., 1994. The analeptic effect of methamphetamine in pentobarbital-narcotized rats is mediated via a dopaminergic-cholinergic mechanism. J. Pharmacol. Exp. Ther. 268, 311–318.

Irifune, M., Kikuchi, N., Saida, T., Takarada, T., Shimizu, Y., Endo, C., Morita, K., Dohi, T., Sato, T., Kawahara, M., 2007. Riluzole, a glutamate release inhibitor, induces loss of righting reflex, antinociception, and immobility in response to noxious stimulation in mice. Anesth. Analg. 104, 1415–1421.

Joo, D.T., Xiong, Z., MacDonald, J.F., Jia, Z., Roder, J., Sonner, J., Orser, B.A., 1999. Blockade of glutamate receptors and barbiturate anesthesia: increased sensitivity to pentobarbital-induced anesthesia despite reduced inhibition of AMPA receptors in GluR2 null mutant mice. Anesthesiology 91, 1329–1341.

Miyamoto, Y., Yamada, K., Noda, Y., Mori, H., Mishina, M., Nabeshima, T., 2001. Hyperfunction of dopaminergic and serotonergic neuronal systems in mice lacking the NMDA receptor epsilon1 subunit. J. Neurosci. 21, 750–757.

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

Mori, H., Mishina, M., 2003. Roles of diverse glutamate receptors in brain functions elucidated by subunit-specific and region-specific gene targeting. Life Sci. 74, 329–336.

Nelson, L.E., Guo, T.Z., Lu, J., Saper, C.B., Franks, N.P., Maze, M., 2002. The sedative component of anesthesia is mediated by GABA(A) receptors in an endogenous sleep pathway. Nat. Neurosci. 5, 979–984.

Pearce, R.A., 1999. Barbiturates and the brain. Anesthesiology 91, 1190–1192.

Petrenko, A.B., Yamakura, T., Askalany, A.R., Kohno, T., Sakimura, K., Baba, H., 2006. Effects of ketamine on acute somatic nociception in wild-type and *N*-methyl-D-aspartate (NMDA) receptor epsilon1 subunit knockout mice. Neuropharmacology 50, 741–747.

Petrenko, A.B., Yamakura, T., Baba, H., Sakimura, K., 2003. Unaltered pain-related behavior in mice lacking NMDA receptor GluRepsilon 1 subunit. Neurosci. Res. 46, 199–204.

Petrenko, A.B., Yamakura, T., Fujiwara, N., Askalany, A.R., Baba, H., Sakimura, K., 2004. Reduced sensitivity to ketamine and pentobarbital in mice lacking the *N*-methyl-D-aspartate receptor GluRepsilon1 subunit. Anesth. Analg. 99, 1136–1140.

Petrenko, A.B., Yamakura, T., Kohno, T., Sakimura, K., Baba, H., 2010. Reduced immobilizing properties of isoflurane and nitrous oxide in mutant mice lacking the *N*-methyl-D-aspartate receptor GluR(epsilon)1 subunit are caused by the secondary effects of gene knockout. Anesth. Analg. 110, 461–465.

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 epsilon 1 subunit. Nature 373, 151–155.

Sato, Y., Kobayashi, E., Hakamata, Y., Kobahashi, M., Wainai, T., Murayama, T., Mishina, M., Seo, N., 2004. Chronopharmacological studies of ketamine in normal and NMDA epsilon1 receptor knockout mice. Br. J. Anaesth. 92, 859–864.

Sato, Y., Kobayashi, E., Murayama, T., Mishina, M., Seo, N., 2005. Effect of *N*-methyl-D-aspartate receptor epsilon1 subunit gene disruption of the action of general anesthetic drugs in mice. Anesthesiology 102, 557–561.

Solt, K., Cotten, J.F., Cimenser, A., Wong, K.F., Chemali, J.J., Brown, E.N., 2011. Methylphenidate actively induces emergence from general anesthesia. Anesthesiology 115, 791–803.

Solt, K., Eger 2nd, E.I., Raines, D.E., 2006. Differential modulation of human *N*-methyl-D-aspartate receptors by structurally diverse general anesthetics. Anesth. Analg. 102, 1407–1411.

Sonner, J.M., Antognini, J.F., Dutton, R.C., Flood, P., Gray, A.T., Harris, R.A., Homanics, G.E., Kendig, J., Orser, B., Raines, D.E., Rampil, I.J., Trudell, J., Vissel, B., Eger 2nd, E.I., 2003. Inhaled anesthetics and immobility: mechanisms, mysteries, and minimum alveolar anesthetic concentration. Anesth. Analg. 97, 718–740.

Vahle-Hinz, C., Detsch, O., Siemers, M., Kochs, E., 2007. Contributions of GABAergic and glutamatergic mechanisms to isoflurane-induced suppression of thalamic somatosensory information transfer. Exp. Brain. Res. 176, 159–172.

Watanabe, M., Inoue, Y., Sakimura, K., Mishina, M., 1993. Distinct spatio-temporal distributions of the NMDA receptor channel subunit mRNAs in the brain. Ann. N.Y. Acad. Sci. 707, 463–466.

Watson, C.J., Baghdoyan, H.A., Lydic, R., 2010. Neuropharmacology of sleep and wakefulness. Sleep Med. Clin. 5, 513–528.

White, S.R., Obradovic, T., Imel, K.M., Wheaton, M.J., 1996. The effects of methylenedioxymethamphetamine (MDMA, "Ecstasy") on monoaminergic neurotransmission in the central nervous system. Prog. Neurobiol. 49, 455–479.

Yamakura, T., Bertaccini, E., Trudell, J.R., Harris, R.A., 2001. Anesthetics and ion channels: molecular models and sites of action. Annu. Rev. Pharmacol. Toxicol. 41, 23–51.

Yamakura, T., Harris, R.A., 2000. Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels. Comparison with isoflurane and ethanol. Anesthesiology 93, 1095–1101.

Yamakura, T., Sakimura, K., Mishina, M., Shimoji, K., 1998. Sensitivity of the *N*-methyl-D-aspartate receptor channel to butyrophenones is dependent on the epsilon2 subunit. Neuropharmacology 37, 709–717.

Kohno et al. Molecular Pain 2012, 8:45
http://www.molecularpain.com/content/8/1/45

MOLECULAR PAIN

RESEARCH Open Access

Milnacipran inhibits glutamatergic *N*-Methyl-D-Aspartate receptor activity in Spinal Dorsal Horn Neurons

Tatsuro Kohno[1,4*], Masafumi Kimura[2], Mika Sasaki[3], Hideaki Obata[2,4], Fumimasa Amaya[3,4] and Shigeru Saito[2]

Abstract

Background: Antidepressants, which are widely used for treatment of chronic pain, are thought to have antinociceptive effects by blockade of serotonin and noradrenaline reuptake. However, these drugs also interact with various receptors such as excitatory glutamatergic receptors. Thermal hyperalgesia was induced by intrathecal injection of NMDA in rats. Paw withdrawal latency was measured after intrathecal injection of antidepressants. The effects of antidepressants on the NMDA and AMPA-induced responses were examined in lamina II neurons of rat spinal cord slices using the whole-cell patch-clamp technique. The effects of milnacipran followed by application of NMDA on pERK activation were also investigated in the spinal cord.

Results: Intrathecal injection of milnacipran (0.1 μmol), but not citalopram (0.1 μmol) and desipramine (0.1 μmol), followed by intrathecal injection of NMDA (1 μg) suppressed thermal hyperalgesia. Milnacipran (100 μM) reduced the amplitude of NMDA (56 ± 3 %, 64 ± 5 % of control)-, but not AMPA (98 ± 5 %, 97 ± 5 % of control)-mediated currents induced by exogenous application and dorsal root stimulation, respectively. Citalopram (100 μM) and desipramine (30 μM) had no effect on the amplitude of exogenous NMDA-induced currents. The number of pERK-positive neurons in the group treated with milnacipran (100 μM), but not citalopram (100 μM) or desipramine (30 μM), followed by NMDA (100 μM) was significantly lower compared with the NMDA-alone group.

Conclusions: The antinociceptive effect of milnacipran may be dependent on the drug's direct modulation of NMDA receptors in the superficial dorsal horn. Furthermore, in addition to inhibiting the reuptake of monoamines, glutamate NMDA receptors are also important for analgesia induced by milnacipran.

Keywords: Antidepressants, *N*-methyl-D-aspartate (NMDA) Receptor, Spinal Analgesia

Background

It is well established that antidepressants have antinociceptive effects; because of this, they are widely used for treatment of chronic pain [1]. In particular, tricyclic antidepressants (TCAs) have long been the mainstay of treatment for neuropathic pain, which is due to lesion or dysfunction of the peripheral or central nervous system. Antidepressants have the unique ability to inhibit the presynaptic reuptake of monoamines, serotonin (5-HT), and noradrenaline (NA) at the neuronal terminals [2], and this activity can produce antinociceptive effects. Recently, more selective monoamine reuptake inhibitors, such as 5-HT and NA reuptake inhibitors (SNRIs) and selective 5-HT reuptake inhibitors (SSRIs), have been introduced and are clinically used to treat neuropathic pain [1]. However, the underlying mechanisms of these drugs may be more complex than simply the blockade of 5-HT and NA reuptake.

In fact, TCAs could also interact with various receptors including *N*-methyl-D-aspartate (NMDA) receptors to produce nociceptive effects. NMDA glutamate receptors are one of the major receptor channel types mediating rapid excitatory neurotransmission in the central nervous system, and they also play an important role in central sensitization regarding long-term pain [3,4].

* Correspondence: kohno-t@umin.net
[1]Department of Anesthesiology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi, Chuo ku Niigata 951-8510, Japan
[4]Pain Mechanism Research Group, 757 Asahimachi, Chuo ku Niigata 951-8510, Japan
Full list of author information is available at the end of the article

Some previous studies have suggested that there are interactions in nociceptive transmission between TCAs and NMDA receptors in the spinal cord [5]. However, there have been no studies demonstrating that the antinociceptive effects of antidepressants are attributable to the inhibition of NMDA receptors in the spinal cord at the cellular level.

The spinal cord is an important site of action of antidepressant-mediated antinociception [6]. The brainstem-spinal descending 5-HT and NA systems suppress nociceptive signals from primary afferent neurons to the spinal dorsal horn. Concurrently, the superficial dorsal horn preferentially receives nociceptive primary afferent fibers. Thus, the spinal dorsal horn is thought to play an important role in modulating nociceptive transmission from the periphery [7,8] as well as in regulating the antinociceptive effect of antidepressants.

The purpose of the current study was to test the hypothesis that 3 different antidepressants, milnacipran as a SNRI, citalopram as a SSRI, and desipramine as a TCA (a preferential noradrenergic reuptake inhibitor) have a direct antagonistic effect on NMDA and AMPA-mediated responses in the spinal dorsal horn.

Methods

Surgical Preparation

The study was approved by the Animal Care and Use Committee of the Gunma University School of Medicine (Maebashi, Japan). Male rats (250–270 g) were used in all experiments. Animals were housed under a 12-h light–dark cycle with food and water ad libitum. For intrathecal administration, a sterilized 32-gauge polyethylene catheter (ReCathCo, Allison Park, PA) connected to an 8.5-cm Tygon external tubing (Saint-Gobain Performance Plastics, Akron, OH) was inserted under isoflurane anesthesia, as previously described [9]. The catheter was passed caudally 8.0 cm from the cisterna magnum to the lumbar enlargement. The animals were allowed to recover for 1 week before being used experimentally. Only animals without evidence of neurologic dysfunction after catheter insertion were used for all studies.

Testing Procedures

The thermal nociceptive threshold was measured with a device (Plantar Test®, IITC Inc. Life Science, Woodland Hills, CA) using a method similar to that reported previously [10]. Rats were placed in individual plastic boxes (10 × 20 × 24 cm) on the glass surface of the testing apparatus, which was maintained at 30 °C during all testing, and were allowed to acclimate for 30 min. Paw withdrawal latency (PWL) was determined using an intense light focused on the hind paw, as previously described [10]. Light intensity was adjusted so that baseline latency was between 9 and 11 s in all animals. A cutoff of 20 s was selected to avoid tissue damage during periods of analgesia, but no animals reached this cutoff point.

The thermal hyperalgesic state was induced by intrathecal injection of 1 μg NMDA. The dose was selected according to a previous study [11]. This experiment is most specific to evaluate the effect of antidepressants on NMDA-mediated responses in the spinal cord. PWL was measured three times in the right or left foot in the middle of the footpad. These three observations were averaged for each animal. PWLs were measured before and after intrathecal injection of milnacipran, citalopram, desipramine, or saline (six animals in each group), and NMDA was injected intrathecally 15 min after the injection (time 0). PWLs were measured at 0, 30, 60, 90, and 120 min after intrathecal injection of NMDA.

Drugs and Their Administration

The agents administered in this study were milnacipran, citalopram, desipramine, and NMDA. Each antidepressant or saline was administered intrathecally 15 min prior to NMDA injection. Drugs were administered intrathecally in a volume of 5 μl, followed by an injection of 10 μl of saline to flush the catheter. All drugs were dissolved in normal saline. The doses of milnacipran were selected according to the previous studies [12,13]. The maximum dose of milnacipran (0.1 μmol) was used for desipramine and citalopram injections because the doses to produce analgesia in these 3 drugs are almost same [14,15].

In Vitro Patch-Clamp Recordings

This portion of the study was approved by the Animal Care and Use Committee at Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan). Male rats (150–200 g) were anesthetized with urethane (1.5 g/kg, i.p.). A dorsal laminectomy was performed, and the lumbosacral segment of the spinal cord with ventral and dorsal roots attached was removed [16,17]. The rats were then immediately killed by exsanguination, and the spinal cords were placed in pre-oxygenated ice-cold Krebs solution. After the arachnoid membrane was removed, the spinal cord was placed in an agar block and mounted on a metal stage. A transverse slice (500-μm thick) with an attached dorsal root was cut on a DTK-1500 Microslicer (Dosaka, Kyoto, Japan) and placed on a nylon mesh in the recording chamber. The slice was perfused continuously with Krebs solution (10 ml/min) equilibrated with a 95 % O_2 and 5 % CO_2 gas mixture at 36°C. The Krebs solution contained (in mM): NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and D-glucose, 11.5. Whole-cell patch-clamp recordings were made from lamina II neurons in voltage-clamp mode using patch pipette electrodes having a resistance of 10 MΩ. The patch pipette solution contained (in mM): Cs-sulfate, 110; $CaCl_2$, 0.5; $MgCl_2$, 2; EGTA, 5; HEPES, 5; tetraethylammonium

(TEA), 5; and ATP-Mg salt, 5. Signals were amplified using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) and were low-pass filtered with a 2-kHz cutoff and digitized at 5 kHz. Data were collected and analyzed using pClamp 10.0 software (Molecular Devices). All experiments were performed in voltage-clamp mode at a holding potential of −40 mV for recording exogenous NMDA current and −70 mV for recording exogenous AMPA current. To test the synaptic response, NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) were observed by dorsal root electrical stimulation at +40 mV in the presence of an AMPA/kainate receptor antagonist (6-cyano-7-nitroquinoxaline-2, 3-dione, CNQX; 10 μM), a $GABA_A$ receptor antagonist (bicuculline; 20 μM), and a glycine receptor antagonist (strychnine; 1 μM). AMPA receptor-mediated EPSCs were observed by dorsal root stimulation at −70 mV. Evoked EPSCs that displayed a constant latency and lack of failures with high frequency stimulation (20 Hz) were classified as monosynaptic. Drugs were applied by superfusion without alteration of the perfusion rate and temperature. NMDA (100 μM) and AMPA (10 μM) were applied to slices for 30 s, and each antidepressant was applied to slices for 3 min. Peak NMDA and AMPA currents were measured before and after each treatment and expressed as (posttreatment/pretreatment) × 100 (as percentages).

Immunohistochemistry

The spinal cord slices (700 μm) from male rats (150–250 g) were perfused with Krebs solution for at least 3 h before application of drugs. Milnacipran (100 μM) was applied for 10 min before NMDA stimulation (100 μM for 5 min) and was present during NMDA stimulation. After drug treatment, the slices were fixed in 4 % paraformaldehyde for 60 min, replaced with sucrose overnight, cut in a cryostat at a thickness of 16 μm, and mounted on slide glass. Phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) was visualized by indirect immunohistochemistry [18]. Sections were incubated with rabbit anti-pERK1/2 antibody (Cell Signaling Technology, Danvers, MA; 1:1000) for 2 days at 4 °C. The sections were incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA; 1:400) for 4 h at room temperature. Signals were visualized with Vectastain ABC systems (Vector Laboratories) following the manufacturer's instruction.

Signals were analyzed under a microscope-digital camera system (Nikon, Tokyo, Japan). Experimenters who were unaware of the experimental protocol counted cells in a blinded manner. At least five nonadjacent sections were randomly selected for cell count. The number of pERK1/2-positive neurons in the superficial dorsal horn from each of five sections was averaged for each animal.

Preparation of Drugs

The drugs used in this study were milnacipran, citalopram, desipramine (provided by Asahi Kasei Corporation, Osaka, Japan), NMDA, AMPA, CNQX, bicuculline, strychnine, WAY100635 maleate salt (Sigma-Aldrich, St. Louis, MO) and tetrodotoxin (TTX), yohimbine hydrochloride (Wako, Osaka, Japan). Citalopram and bicuculline were first dissolved in dimethyl sulphoxide (DMSO) at 1000 times the concentrations to be used. The other drugs were first dissolved in distilled water at 1000 times the concentrations to be used, and then these drugs were diluted to the final concentration in Krebs solution immediately before use.

Statistical Analysis

Data are expressed as means ± SEM. Statistical significance was determined as $P < 0.05$ using either the Student's paired *t*-test or a two-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls post hoc test for multiple comparisons.

Results

Milnacipran Suppresses NMDA-Induced Thermal Hyperalgesia

Intrathecal injection of saline or each antidepressant alone did not alter PWL (data not shown). Intrathecal injection of 1 μg NMDA 30 min after saline injection produced thermal hyperalgesia 30 min after the NMDA injection compared with the pre-value (9.87 ± 0.89 to 6.13 ± 0.89 s, $P < 0.01$). Mechanical allodynia was not induced by intrathecal injection of NMDA. Although the hyperalgesia induced by intrathecal administration of NMDA is a rapid and transient in a tail-flick test [19], the reduction in PWL continued up to 120 min after NMDA injection in the present study (saline group, Figure 1). Intrathecal injection of milnacipran followed by intrathecal injection of NMDA suppressed thermal hyperalgesia in a dose-dependent manner at doses from 0.01 to 0.1 μmol ($P < 0.05$ by two-way ANOVA, Figure. 1A). In contrast, citalopram (0.1 μmol) did not inhibit thermal hyperalgesia during the testing period ($P = 0.25$ by two-way ANOVA; Figure 1B). Similarly, desipramine (0.1 μmol) did not suppress NMDA-induced thermal hyperalgesia ($P = 0.62$ by two-way ANOVA, Figure 1C).

Milnacipran Inhibits NMDA, but not AMPA Receptor-Mediated Responses in Dorsal Horn Neurons

To study the effects of milnacipran on excitatory synaptic transmission, whole-cell patch-clamp recordings were made from rat lamina II neurons. Milnacipran did not alter the level of holding current required to maintain neurons at −40 mV and −70 mV, respectively. Milnacipran had no effect on the amplitude (102 ± 5 % of control, n = 4, $P = 0.81$) and frequency (97 ± 6 % of

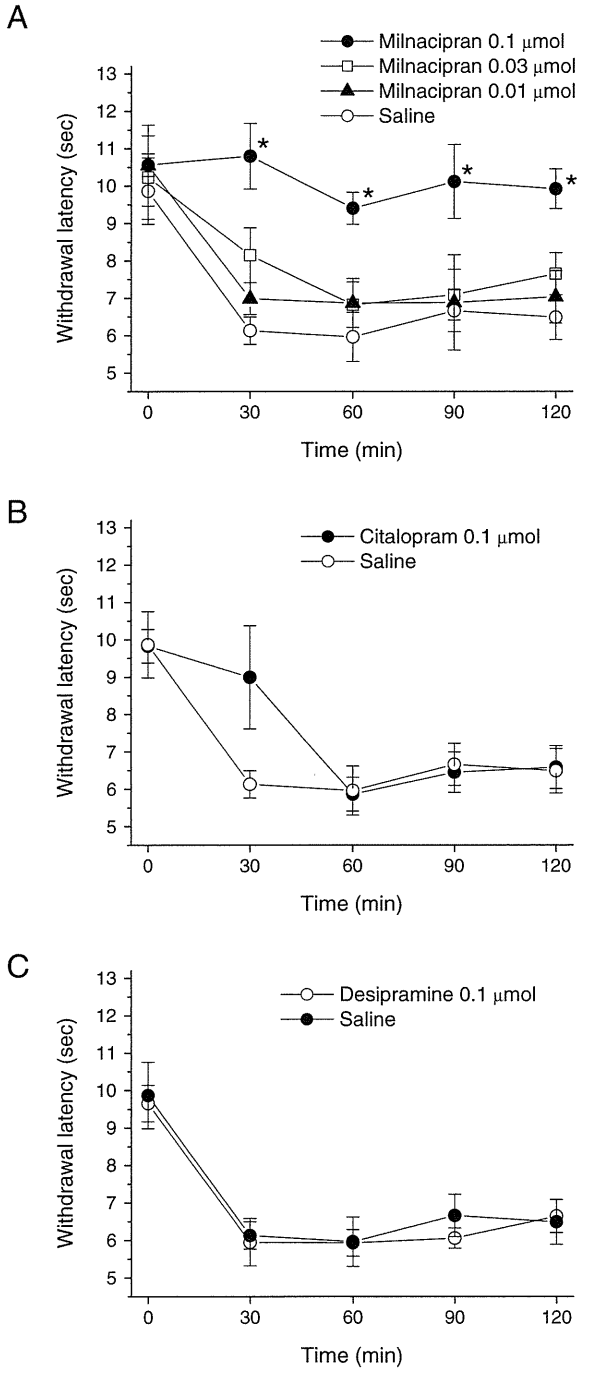

Figure 1 Milnacipran suppresses NMDA-induced thermal hyperalgesia (B) or desipramine + NMDA (C) on the paw withdrawal latency against thermal nociceptive stimuli. Thermal hyperalgesia was induced by intrathecal injection of NMDA. Saline or each drug was injected 15 min before NMDA. Paw withdrawal latencies are expressed as mean ± SEM for six rats in each group. * $P < 0.05$ compared with saline-treated group at each time point by a Student-Newman-Keuls post hoc after two-way ANOVA.

control, n = 4, $P = 0.62$) of spontaneous EPSCs at −70 mV. Exogenous application of NMDA (100 μM, 30 s, at −40 mV) elicited an inward current in neurons (Figure 2A, C), reflecting the activation of NMDA receptors. To confirm that NMDA-induced currents were postsynaptic phenomena, we examined the currents in the presence of TTX to remove any possible

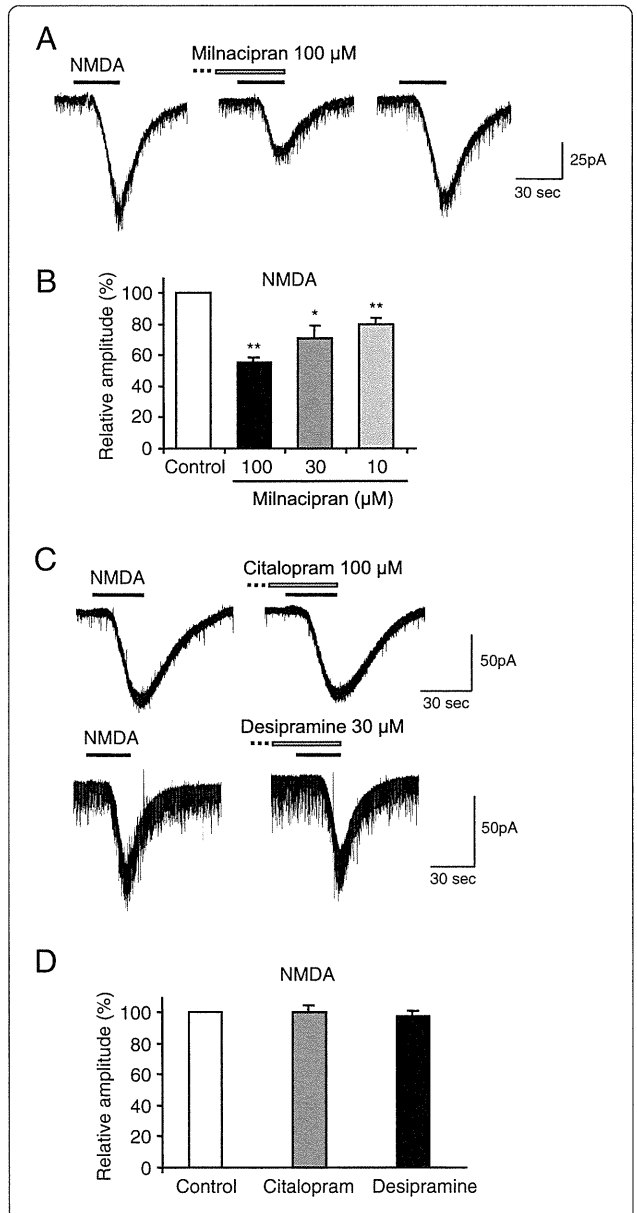

Figure 2 (A) Milnacipran inhibits the response to exogenous NMDA. Milnacipran reversibly inhibited NMDA-induced currents. In this figure and subsequent figures, the horizontal bars above the chart recordings indicate the duration of drug superfusion. **(B)** The relative amplitudes were shown in the presence of milnacipran. ** $P < 0.01$, * $P < 0.05$ **(C)** Neither citalopram nor desipramine affected the amplitudes of NMDA-induced currents. **(D)** Comparison of the NMDA-induced currents in control and in the presence of citalopram or desipramine.

influence of the NMDA receptors on presynaptic neurons. Since TTX (0.5 μM) did not affect the amplitudes of NMDA-induced currents (103 ± 4 % of control, n = 5; $P = 0.84$; data not shown), the currents were exclusively postsynaptic. Therefore, the following experiments were done in the absence of TTX. Pre-application of milnacipran (100 μM) for 3 min reduced the amplitudes of NMDA-induced currents to 56 ± 3 % (n = 10, $P < 0.01$; Fig. 2A, B) of the control values. These effects of milnacipran were reversible, and the amplitudes of currents recovered to the control values within 5–10 min (Figure 2A). At a lower concentration of 10 or 30 μM, milnacipran also decreased NMDA-induced currents (80 ± 4 % of the control, n = 5, $P < 0.01$; 71 ± 8 % of the control, n = 6, $P < 0.05$, respectively; Figure 2B). To elucidate whether the observed effects were specific for milnacipran, we examined other antidepressants; i.e., citalopram and desipramine. Pre-application of citalopram (100 μM) for 3 min failed to reduce the amplitudes of NMDA-induced currents (100 ± 5 % of control, n = 5, $P = 0.96$; Figure 2C, D). Pre-application of desipramine (30 μM) for 3 min also failed to reduce the amplitudes of NMDA-induced currents (98 ± 4 % of control, n = 5, $P = 0.57$; Figure 2C, D). In contrast, milnacipran (100 μM) had no effect on the amplitudes of exogenously applied AMPA (10 μM, 30 s, at −70 mV)-induced currents (98 ± 5 % of control, n = 7, $P = 0.63$; Figure 3A). These results suggest that milnacipran, but not the other antidepressants, inhibited NMDA, but not AMPA receptor-mediated responses in lamina II neurons.

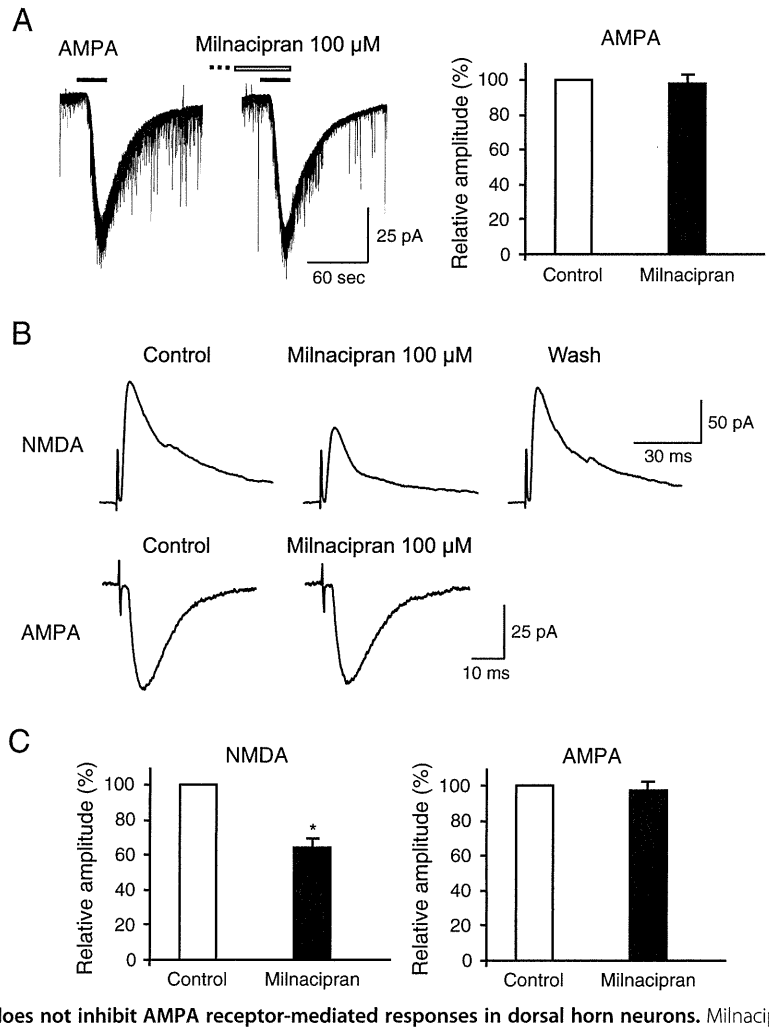

Figure 3 (A) Milnacipran does not inhibit AMPA receptor-mediated responses in dorsal horn neurons. Milnacipran did not inhibit AMPA-induced currents. **(B)** Representative traces of dorsal root stimulation evoked monosynaptic NMDA- and AMPA-mediated EPSCs. NMDA-mediated EPSCs were recorded at +40 mV. AMPA-mediated EPSCs were recorded at −70 mV. Milnacipran reversibly decreased the amplitudes of NMDA-, but not AMPA-mediated EPSCs. **(C)** Comparison of the NMDA- and AMPA-mediated EPSCs in control and in the presence of milnacipran. * $P < 0.01$.

We next tested the effects of milnacipran on the amplitudes of dorsal root stimulation evoked EPSCs. Milnacipran (100 μM) inhibited the amplitudes of monosynaptic NMDA-mediated EPSCs to 64 ± 5 % (n = 4, $P < 0.01$; Figure 3B, C) of the control values. In contrast, milnacipran (100 μM) did not inhibit the amplitudes of monosynaptic AMPA receptor-mediated EPSCs (97 ± 5 % of control, n = 5, $P = 0.62$; Figure 3B, C). To exclude the possibility that milnacipran inhibited the amplitudes of NMDA-mediated EPSCs as a result of the blockade of 5-HT and NA reuptake, we examined the NMDA-mediated EPSCs in the presence of 5-HT_{1A} receptor antagonist, WAY100635 (10 μM), and α2 receptor antagonist, yohimbine (1 μM). The concentrations of WAY100635 and yohimbine at the concentrations used here are sufficiently high to block the 5-HT_{1A} [20] and α2 receptors [21], respectively. However, milnacipran also inhibited the amplitudes of NMDA-mediated EPSCs to 69 ± 8 % (n = 3, $P < 0.01$) of the control values.

Effects of Antidepressants on ERK Activation in the Spinal Cord

The slice preparation offers a reliable condition under which to study pERK expression [22,23]. In addition, multiple slices can be prepared from each spinal cord segment, and milnacipran can be applied in a known condition *in vitro*. The slices were perfused for more than 3 h before stimulation to reduce possible pERK background caused by slice preparation [22,23]. There were very few pERK-positive neurons noted in control spinal cord slices (Figure 4).

In the present research, pERK-positive neurons were occasionally observed in the control dorsal horn. Bath application of NMDA (100 μM) for 5 min produced activation of pERK in the superficial dorsal horn neurons. Distribution of NMDA-evoked pERK expression was similar to that of previous observations [22]. The number of pERK-positive neurons significantly increased in the NMDA-treated group ($P < 0.01$ vs. control, Figure 4). The number of pERK-positive neurons in the group treated with milnacipran (100 μM), but not with citalopram (100 μM) or desipramine (30 μM) followed by NMDA was significantly lower compared with the NMDA-alone group ($P < 0.05$, Figure 4).

Discussion

We demonstrated that intrathecal administration of milnacipran, but not citalopram or desipramine mediated an inhibition of NMDA-induced thermal hyperalgesia. Moreover, we documented the inhibition of NMDA-mediated currents by milnacipran, but not by citalopram or desipramine in spinal lamina II. We also demonstrated that activation of pERK induced by NMDA was significantly suppressed by milnacipran in dorsal horn neurons. Taken together, these findings indicate that milnacipran has a direct antinociceptive effect in the spinal cord through its modulation of NMDA receptors.

Some investigators have reported interactions between TCAs and NMDA receptors in nociceptive transmission. Eisenach and Gebhart [5] reported that intrathecal administration of amitriptyline reversed thermal hyperalgesia via NMDA receptor antagonism in a rat model of inflammation. Kawamata et al. [24] also using the rat model of inflammation, reported that intrathecally injected desipramine produced analgesia unrelated to NA reuptake inhibition. These reports suggest that TCAs exert a direct inhibitory effect on NMDA receptors to produce analgesia in the spinal cord. TCAs may

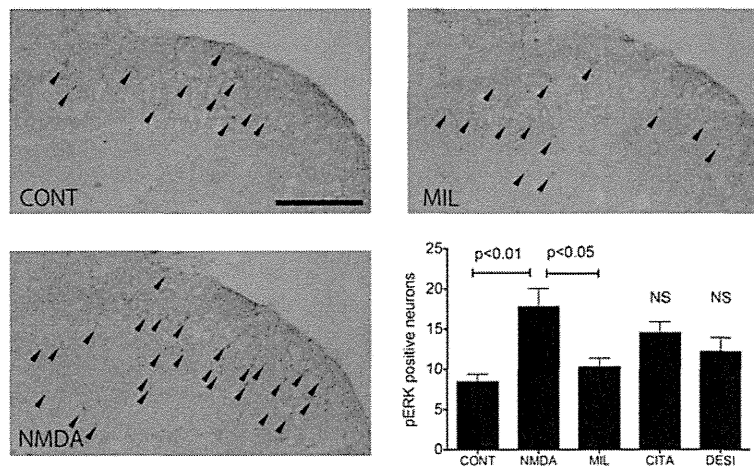

Figure 4 Effects of antidepressants on ERK activation in the spinal cord. ERK activation was significantly suppressed by simultaneous treatment with milnacipran compared with the NMDA-alone group, but not with citalopram or desipramine. Arrow indicates the pERK-positive neurons. NS = not significant.

not only inhibit 5-HT and NA reuptake, but could also interact with various receptors. It has been shown that TCAs block sodium channels [25,26] as well as voltage-dependent calcium channels [27,28] and that TCAs inhibit adenosine reuptake [29]. Further, most TCAs have affinity for opioid [30], NA, 5-HT, histamine, and muscarinic acetylcholine receptors [31]. Therefore, these various mechanisms of action of TCAs might contribute to antinociceptive effects in some kinds of chronic pain models.

Desipramine did not suppress NMDA-induced thermal hyperalgesia in the present study. However, Hwang and Wilcox [6] reported that desipramine was antinociceptive in three nociceptive tests, tail-flick test, intrathecal substance P-induced behavioral test and intradermal hypertonic saline-induced behavioral test. Moreover, they indicated that the analgesic effect by desipramine probably involves blockade of monoamine reuptake. This discrepancy between our result and that of previous study is likely to be due to different underlying mechanisms of desipramine.

Although there is some evidence that TCAs block NMDA receptor-mediated responses, the site of action is controversial. Based on radioligand binding studies, Reynolds and Miller [32] have suggested that TCAs act at the Zn^{2+} recognition site on the NMDA receptor. Sills and Loo [33] have reported that TCAs bind with higher affinity to the phencyclidine binding site on the NMDA receptor. Moreover, Sernagor et al. [34] have reported that desipramine blocked NMDA-induced currents in hippocampal neurons by acting on the open channel. In contrast to TCAs, milnacipran has no relevant affinity for any other receptors, including α-adrenergic, 5-HT, histamine, muscarinic acetylcholine, opioid, or NMDA receptors [35]. However, Shuto et al. [36] reported that milnacipran (IC50 = 6.3 μM), is a class of noncompetitive NMDA receptor antagonist, although the binding affinity of milnacipran for the NMDA receptor is not strong. Although there is no evidence that milnacipran binds to the NMDA receptor, it is assumed that it may act at the recognition site for Zn^{2+} or at the phencyclidine binding site of the NMDA receptor. Further study is required to clarify this point.

Milnacipran inhibits the presynaptic reuptake of monoamines, 5-HT and NA with an IC50 of 100 to 200 nM, respectively in the brain [37]. However, in this study, milnacipran has the antagonistic effect on NMDA-mediated responses in the spinal cord at a concentration of 10–100 μM. Some previous studies [32,36,38] indicate that TCAs also inhibits the NMDA receptors in the brain at a concentration of 10–100 μM. Therefore, it is likely that the concentration of inhibiting the NMDA receptors by milnacipran is higher than that of inhibiting reuptake of the monoamines.

In the present study, milnacipran reduced the amplitudes of exogenously applied NMDA-induced currents in lamina II neurons. Moreover, milnacipran inhibited the amplitudes of dorsal root stimulation evoked NMDA-mediated EPSCs. There are no differences in the degree of depression by milnacipran between NMDA induced-current and dorsal root stimulation evoked NMDA-mediated EPSCs. These results suggest that synaptic and extra synaptic NMDA receptors in dorsal horn neurons are similarly modulated by milnacipran. Moreover, milnacipran inhibited the amplitudes of NMDA-mediated EPSCs in the presence of 5-HT antagonist and α2 receptor antagonist. Therefore, it is unlikely that an antagonistic effect of milnacipran on NMDA receptors is mediated by 5-HT or NA receptors.

We observed that the highest dose of intrathecal milnacipran completely reversed thermal hyperalgesia induced by NMDA. In contrast, desipramine and citalopram did not produce any inhibitory effect, although the concentrations of desipramine and citalopram in this study are sufficiently high to inhibit reuptake of 5-HT or NA, respectively. These results suggest that increases in 5-HT or NA alone in the spinal cord have no effect on NMDA-induced thermal hyperalgesia. The antagonistic action of milnacipran for NMDA receptors may produce additional effects for some types of chronic pain. Previous studies demonstrated that intrathecal administration of milnacipran produced antiallodynic effects in rats with peripheral nerve injury [13,39,40], and that the effect was not completely reversed by an α2 receptor antagonist or a 5-HT receptor antagonist [13]. Therefore, it is conceivable that an antagonistic action for NMDA receptors contributes to the antiallodynic effect of milnacipran. Further studies are required to clarify the molecular mechanisms underlying the inhibitory effect of milnacipran on NMDA-mediated responses in dorsal horn neurons. In addition, to elucidate whether the observed effects were specific for milnacipran, further investigations using another SNRI are necessary to resolve this question.

ERK activation is detected in the spinal dorsal horn neurons after stimulation of nociceptive primary afferents and contributes to the development of central sensitization [22]. Activation of the NMDA receptor is partly involved in ERK induction following nociceptive stimulation [22]. Analgesic drugs such as local anesthetics [41], opioids, or cannabinoids [42] inhibit ERK induction in the spinal cord. In the present study, we demonstrated that milnacipran inhibited ERK induction following application of NMDA in the spinal cord. This result is consistent with our behavioral data showing that milnacipran attenuated thermal hyperalgesia following intrathecal NMDA injection. Our electrophysiological data clearly indicate that the direct inhibitory effect of milnacipran on NMDA-mediated current underlies these phenomena.

We show in the present study that milnacipran has an antagonistic effect of NMDA receptors in the spinal cord when administered intrathecally. However, it is not clear whether milnacipran has the similar effect in other central and peripheral tissue such as brain and skin. Moreover, NMDA receptor antagonists such as ketamine and phencyclidine have psychotomimetic or anti-depressant properties in humans when administered systemically. There have been no studies demonstrating that milnacipran has these similar properties in humans. Therefore, further study is required to clarify this point.

NMDA glutamate receptors are one of the major receptor channel types mediating rapid excitatory neurotransmission in the central nervous system. This receptor is composed of subunits from at least two families, NR1 and NR2. The NR1 subunit is essential for the function of NMDA receptors and is ubiquitously expressed in most neurons. The functional properties of NMDA receptors are determined by the NR2 subunit composition (NR2A–2D). Previous reports have demonstrated that desipramine inhibited NMDA-evoked responses in hippocampal neurons [32,43], but not in the present study. The respective NR2 subunits show different expression patterns in various regions of the brain and spinal cord. Whereas NR2A and NR2B subunits are prominent in the hippocampus [44], these subunits are not identified in spinal dorsal horn neurons [45]. This different composition of NMDA receptors may underlie the variability among tissues in the effects of desipramine on NMDA receptor-responses.

NMDA glutamate receptors also play a key role in central sensitization in chronic pain [3,4]. The pursuit of an NMDA receptor antagonist for the relief of chronic pain dates from the late 1980s when it was shown that NMDA antagonists inhibit the "wind-up" response [46,47]. The central sensitization that occurs in the spinal dorsal horn is held to be an important event in the pathway leading to neuropathic pain. For this reason, ketamine is currently widely applied in the treatment of neuropathic pain and for some kinds of chronic pain, including fibromyalgia [48]. Although there is little evidence that NMDA receptor antagonism is involved in the antinociceptive effect of milnacipran, milnacipran has been widely used in patients with fibromyalgia and has provided them with pain relief [49,50]. These effects of milnacipran for chronic pain have been previously discussed from the perspective of one aspect: balanced inhibition of the reuptake of 5-HT and NA. However, in the present study, our data suggest that milnacipran not only inhibits 5-HT and NA reuptake, but also acts as an NMDA receptor antagonist. Milnacipran may have the potential to prevent or suppress chronic pain related to central sensitization, especially when injected into intrathecal or epidural space.

Conclusions

In conclusion, the present study suggests that milnacipran, but not citalopram and desipramine, inhibits NMDA-induced glutamatergic transmission in rat dorsal horn neurons. These results suggest that not only is inhibition of 5-HT and NA reuptake an important factor for the analgesia induced by milnacipran in the spinal cord, but that inhibition of glutamate NMDA receptors is critical as well.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by a Grant-In-Aid for Scientific Research (grant number 20390414, 09 F09359, TK; 20591823, HO; 20591841, FA) from the Ministry of Education, Culture, Sports, Science and Technology and by a Grant-In-Aid for Scientific Research from Ministry of Health, Labour and Welfare, Tokyo, Japan.

Author details

[1]Department of Anesthesiology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi, Chuo ku Niigata 951-8510, Japan. [2]Department of Anesthesiology, Gunma University Graduate School of Medicine. [3]Department of Anesthesiology, Kyoto Prefectural University of Medicine. [4]Pain Mechanism Research Group.

Authors' contributions

MK and HO performed the animal surgery and behavioral test. TK carried out *in vitro* patch-clamp recordings. MS and FA conducted immunohistochemistry. SS performed the statistical analysis. TK, HO and FA drafted the manuscript. All authors read and approved the final manuscript.

Received: 2 February 2012 Accepted: 19 June 2012
Published: 19 June 2012

References

1. Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, *et al*: **Pharmacologic management of neuropathic pain: evidence-based recommendations.** *Pain* 2007, **132**:237–251.
2. Sindrup SH, Otto M, Finnerup NB, Jensen TS: **Antidepressants in the treatment of neuropathic pain.** *Basic Clin Pharmacol Toxicol* 2005, **96**:399–409.
3. Ji RR, Kohno T, Moore KA, Woolf CJ: **Central sensitization and LTP: do pain and memory share similar mechanisms?** *Trends Neurosci* 2003, **26**:696–705.
4. South SM, Kohno T, Kaspar BK, Hegarty D, Vissel B, Drake CT, Ohata M, Jenab S, Sailer AW, Malkmus S, *et al*: **A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain.** *J Neurosci* 2003, **23**:5031–5040.
5. Eisenach JC, Gebhart GF: **Intrathecal amitriptyline acts as an N-methyl-D -aspartate receptor antagonist in the presence of inflammatory hyperalgesia in rats.** *Anesthesiology* 1995, **83**:1046–1054.
6. Hwang AS, Wilcox GL: **Analgesic properties of intrathecally administered heterocyclic antidepressants.** *Pain* 1987, **28**:343–355.
7. Kumazawa T, Perl ER: **Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: indications of their place in dorsal horn functional organization.** *J Comp Neurol* 1978, **177**:417–434.
8. Yoshimura M, Nishi S: **Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents.** *Neuroscience* 1993, **53**:519–526.
9. Yaksh TL, Rudy TA: **Chronic catheterization of the spinal subarachnoid space.** *Physiol Behav* 1976, **17**:1031–1036.
10. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: **A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia.** *Pain* 1988, **32**:77–88.
11. Malmberg AB, Yaksh TL: **Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition.** *Science* 1992, **257**:1276–1279.

12. Obata H, Kimura M, Nakajima K, Tobe M, Nishikawa K, Saito S: **Monoamine-dependent, opioid-independent antihypersensitivity effects of intrathecally administered milnacipran, a serotonin noradrenaline reuptake inhibitor, in a postoperative pain model in rats.** *J Pharmacol Exp Ther* 2010, **334**:1059–1065.
13. Obata H, Saito S, Koizuka S, Nishikawa K, Goto F: **The monoamine-mediated antiallodynic effects of intrathecally administered milnacipran, a serotonin noradrenaline reuptake inhibitor, in a rat model of neuropathic pain.** *Anesth Analg* 2005, **100**:1406–1410. table of contents.
14. Otsuka N, Kiuchi Y, Yokogawa F, Masuda Y, Oguchi K, Hosoyamada A: **Antinociceptive efficacy of antidepressants: assessment of five antidepressants and four monoamine receptors in rats.** *J Anesth* 2001, **15**:154–158.
15. Yokogawa F, Kiuchi Y, Ishikawa Y, Otsuka N, Masuda Y, Oguchi K, Hosoyamada A: **An investigation of monoamine receptors involved in antinociceptive effects of antidepressants.** *Anesth Analg* 2002, **95**:163–168. table of contents.
16. Kohno T, Wakai A, Ataka T, Ikoma M, Yamakura T, Baba H: **Actions of midazolam on excitatory transmission in dorsal horn neurons of adult rat spinal cord.** *Anesthesiology* 2006, **104**:338–343.
17. Kohno T, Wang H, Amaya F, Brenner GJ, Cheng JK, Ji RR, Woolf CJ: **Bradykinin enhances AMPA and NMDA receptor activity in spinal cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity.** *J Neurosci* 2008, **28**:4533–4540.
18. Amaya F, Samad TA, Barrett L, Broom DC, Woolf CJ: **Periganglionic inflammation elicits a distally radiating pain hypersensitivity by promoting COX-2 induction in the dorsal root ganglion.** *Pain* 2009, **142**:59–67.
19. Meller ST, Dykstra C, Gebhart GF: **Acute thermal hyperalgesia in the rat is produced by activation of N-methyl-D-aspartate receptors and protein kinase C and production of nitric oxide.** *Neuroscience* 1996, **71**:327–335.
20. Ito A, Kumamoto E, Takeda M, Shibata K, Sagai H, Yoshimura M: **Mechanisms for ovariectomy-induced hyperalgesia and its relief by calcitonin: participation of 5-HT1A-like receptor on C-afferent terminals in substantia gelatinosa of the rat spinal cord.** *J Neurosci* 2000, **20**:6302–6308.
21. Kawasaki Y, Kumamoto E, Furue H, Yoshimura M: **Alpha 2 adrenoceptor-mediated presynaptic inhibition of primary afferent glutamatergic transmission in rat substantia gelatinosa neurons.** *Anesthesiology* 2003, **98**:682–689.
22. Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, Ji RR: **Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization.** *J Neurosci* 2004, **24**:8310–8321.
23. Ji RR, Baba H, Brenner GJ, Woolf CJ: **Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity.** *Nat Neurosci* 1999, **2**:1114–1119.
24. Kawamata T, Omote K, Kawamata M, Namiki A: **Analgesic effect of intrathecal desipramine on carrageenan-induced thermal hyperalgesia in the rat.** *Br J Anaesth* 1999, **83**:449–452.
25. Pancrazio JJ, Kamatchi GL, Roscoe AK, Lynch C 3rd: **Inhibition of neuronal Na + channels by antidepressant drugs.** *J Pharmacol Exp Ther* 1998, **284**:208–214.
26. Dick IE, Brochu RM, Purohit Y, Kaczorowski GJ, Martin WJ, Priest BT: **Sodium channel blockade may contribute to the analgesic efficacy of antidepressants.** *J Pain* 2007, **8**:315–324.
27. Lavoie PA, Beauchamp G, Elie R: **Tricyclic antidepressants inhibit voltage-dependent calcium channels and Na(+)-Ca2+ exchange in rat brain cortex synaptosomes.** *Can J Physiol Pharmacol* 1990, **68**:1414–1418.
28. Shimizu M, Nishida A, Yamawaki S: **Antidepressants inhibit spontaneous oscillations of intracellular Ca2+ concentration in rat cortical cultured neurons.** *Neurosci Lett* 1992, **146**:101–104.
29. Phillis JW, Wu PH: **The effect of various centrally active drugs on adenosine uptake by the central nervous system.** *Comp Biochem Physiol C* 1982, **72**:179–187.
30. Isenberg KE, Cicero TJ: **Possible involvement of opiate receptors in the pharmacological profiles of antidepressant compounds.** *Eur J Pharmacol* 1984, **103**:57–63.
31. Hall H, Ogren SO: **Effects of antidepressant drugs on different receptors in the brain.** *Eur J Pharmacol* 1981, **70**:393–407.
32. Reynolds IJ, Miller RJ: **Tricyclic antidepressants block N-methyl-D-aspartate receptors: similarities to the action of zinc.** *Br J Pharmacol* 1988, **95**:95–102.
33. Sills MA, Loo PS: **Tricyclic antidepressants and dextromethorphan bind with higher affinity to the phencyclidine receptor in the absence of magnesium and L-glutamate.** *Mol Pharmacol* 1989, **36**:160–165.
34. Sernagor E, Kuhn D, Vyklicky L Jr, Mayer ML: **Open channel block of NMDA receptor responses evoked by tricyclic antidepressants.** *Neuron* 1989, **2**:1221–1227.
35. Mochizuki D, Tsujita R, Yamada S, Kawasaki K, Otsuka Y, Hashimoto S, Hattori T, Kitamura Y, Miki N: **Neurochemical and behavioural characterization of milnacipran, a serotonin and noradrenaline reuptake inhibitor in rats.** *Psychopharmacology (Berl)* 2002, **162**:323–332.
36. Shuto S, Takada H, Mochizuki D, Tsujita R, Hase Y, Ono S, Shibuya N, Matsuda A: **(+/–)-(Z)-2-(aminomethyl)-1-phenylcyclopropanecarboxamide derivatives as a new prototype of NMDA receptor antagonists.** *J Med Chem* 1995, **38**:2964–2968.
37. Moret C, Charveron M, Finberg JP, Couzinier JP, Briley M: **Biochemical profile of midalcipran (F 2207), 1-phenyl-1-diethyl-aminocarbonyl-2-aminomethyl-cyclopropane (Z) hydrochloride, a potential fourth generation antidepressant drug.** *Neuropharmacology* 1985, **24**:1211–1219.
38. Watanabe Y, Saito H, Abe K: **Tricyclic antidepressants block NMDA receptor-mediated synaptic responses and induction of long-term potentiation in rat hippocampal slices.** *Neuropharmacology* 1993, **32**:479–486.
39. Shin SW, Eisenach JC: **Peripheral nerve injury sensitizes the response to visceral distension but not its inhibition by the antidepressant milnacipran.** *Anesthesiology* 2004, **100**:671–675.
40. King T, Rao S, Vanderah T, Chen Q, Vardanyan A, Porreca F: **Differential blockade of nerve injury-induced shift in weight bearing and thermal and tactile hypersensitivity by milnacipran.** *J Pain* 2006, **7**:513–520.
41. Yanagidate F, Strichartz GR: **Bupivacaine inhibits activation of neuronal spinal extracellular receptor-activated kinase through selective effects on ionotropic receptors.** *Anesthesiology* 2006, **104**:805–814.
42. Kawasaki Y, Kohno T, Ji RR: **Different effects of opioid and cannabinoid receptor agonists on C-fiber-induced extracellular signal-regulated kinase activation in dorsal horn neurons in normal and spinal nerve-ligated rats.** *J Pharmacol Exp Ther* 2006, **316**:601–607.
43. White G, Lovinger DM, Peoples RW, Weight FF: **Inhibition of N-methyl-D -aspartate activated ion current by desmethylimipramine.** *Brain Res* 1990, **537**:337–339.
44. Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH: **Developmental and regional expression in the rat brain and functional properties of four NMDA receptors.** *Neuron* 1994, **12**:529–540.
45. Tolle TR, Berthele A, Zieglgansberger W, Seeburg PH, Wisden W: **The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray.** *J Neurosci* 1993, **13**:5009–5028.
46. Davies SN, Lodge D: **Evidence for involvement of N-methylaspartate receptors in 'wind-up' of class 2 neurones in the dorsal horn of the rat.** *Brain Res* 1987, **424**:402–406.
47. Dickenson AH, Sullivan AF: **Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation.** *Neuropharmacology* 1987, **26**:1235–1238.
48. Domino EF: **Taming the ketamine tiger. 1965.** *Anesthesiology* 2010, **113**:678–684.
49. Gendreau RM, Thorn MD, Gendreau JF, Kranzler JD, Ribeiro S, Gracely RH, Williams DA, Mease PJ, McLean SA, Clauw DJ: **Efficacy of milnacipran in patients with fibromyalgia.** *J Rheumatol* 2005, **32**:1975–1985.
50. Mease PJ, Clauw DJ, Gendreau RM, Rao SG, Kranzler J, Chen W, Palmer RH: **The efficacy and safety of milnacipran for treatment of fibromyalgia. a randomized, double-blind, placebo-controlled trial.** *J Rheumatol* 2009, **36**:398–409.

doi:10.1186/1744-8069-8-45
Cite this article as: Kohno *et al.*: Milnacipran inhibits glutamatergic *N*-Methyl-D-Aspartate receptor activity in Spinal Dorsal Horn Neurons. *Molecular Pain* 2012 **8**:45.

Neuroscience Letters 503 (2011) 152–156

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Low barometric pressure aggravates neuropathic pain in guinea pigs

Jun Sato[a,*], Yuya Itano[a], Megumi Funakubo[a], Hiroyuki Mizoguchi[a], Mariko Itoh[a,b], Rarami Mori[a,b]

[a] Futuristic Environmental Simulation Center, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan
[b] Technical Center of Nagoya University, Nagoya 464-8601, Japan

ARTICLE INFO

Article history:
Received 20 July 2011
Received in revised form 11 August 2011
Accepted 17 August 2011

Keywords:
Barometric pressure
Neuropathic pain
Spinal nerve injury
Guinea pig

ABSTRACT

Several clinical studies have demonstrated a consistent relationship between changes in meteorological factors, particularly barometric pressure, and pain intensity in subjects with chronic pain. We have previously demonstrated that exposure to artificially low barometric pressure (LP) intensifies pain-related behaviors in rats with neuropathic pain. In the present study, guinea pigs with unilateral L5 spinal nerve ligation (SNL) were placed in a pressure-controlled chamber and subjected to LP of 10 or 27 hPa below the ambient pressure. The SNL surgery led to increased hindpaw withdrawal frequencies to 34-, 59-, and 239-mN von Frey filaments (VFFs). When the SNL animals were subjected to both LP exposures consecutively, the hindpaw withdrawal frequencies further increased; the effect was most significant when the animals were exposed to LP 27 hPa below ambient pressure. In contrast, no change was seen in a group of sham-operated control animals. These results indicate that fluctuations in LP within the range of natural weather patterns can potentiate neuropathic pain in guinea pigs.

Many studies have demonstrated that patients with chronic pain resulting from conditions such as rheumatoid arthritis, migraine, and neuropathic pain often complain that their condition is aggravated by weather changes [6,7,9,12]. With regard to meteorological factors that may influence pain, a variety of potential contributing factors have been identified, e.g., barometric pressure, humidity, rain, and temperature [6,7,9,12]. In particular, barometric pressure has long been suspected to contribute to changes in chronic pain. For example, the occurrence of migraine attacks was tracked by 77 patients over 2 years, and it was discovered that 13% were sensitive to changes in atmospheric pressure [16].

In a previous study, we demonstrated that artificially lowering barometric pressure (27 hPa decrease over 8 min) augmented pain-related behaviors of rats rendered neuropathic [18,20] or adjuvant inflamed [17]. More recently, we demonstrated that rates of decompression of $\geq$5 hPa/h and $\geq$10 hPa/h and magnitudes of decompression $\geq$5 hPa and $\geq$10 hPa augmented pain-related behaviors in rats with spinal nerve ligation (SNL) and chronic constriction injury to the sciatic nerve (CCI), respectively [5]. It has been also demonstrated a notable absence of a stimulus–response relationship; that is, the smallest barometric pressure decrease (5 hPa at 5 hPa/h) induced almost the full effect, whereas a more intense pressure decrease (e.g., 10 hPa at 5 hPa/h) induced no further increase in pain-related behaviors in rats [5]. These results indicate that low barometric pressure (LP) exposure that falls within the range of natural weather patterns augments neuropathic pain in rats. We also found that when rats with inner ear lesions in addition to SNL or CCI were exposed to LP, they showed no increase in pain-related behaviors, suggesting that the barometric sensing mechanism influencing how nociceptive behaviors are modulated by LP in rats is located in the inner ear [3].

Our previous research thus raises the possibility that similar mechanisms might contribute to the aggravation of chronic pain in humans during weather changes. However, no concrete evidence exists for LP effects in other species. Therefore the present study aims to demonstrate whether LP-induced aggravation of chronic pain occurs in species other than the rat. We chose the guinea pig as our experimental animal as its hearing and vestibular systems are more similar to those of humans than the vestibular systems of rats, making it an animal model suitable for conducting inner ear experiments [1].

All experiments were conducted with the approval of the Animal Care Committee of Nagoya University and were in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions in Japan. Thirty-two male Hartley guinea pigs with a mean body weight of 250 g (Japan SLC, Hamamatsu, Japan), were used. The animals were housed 2 per cage at a constant temperature (24 °C) with a 12-h light/dark cycle; they had free access to food and water. We attest that all efforts were made to minimize the number of animals used and their suffering.

All surgical procedures were performed under surgically clean conditions and sodium pentobarbital anesthesia (50–60 mg/kg, i.p.). The left L5 spinal nerve was tightly ligated with silk thread,

* Corresponding author. Tel.: +81 52 789 3929; fax: +81 52 789 3999.
E-mail address: jun@riem.nagoya-u.ac.jp (J. Sato).

0304-3940/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2011.08.030

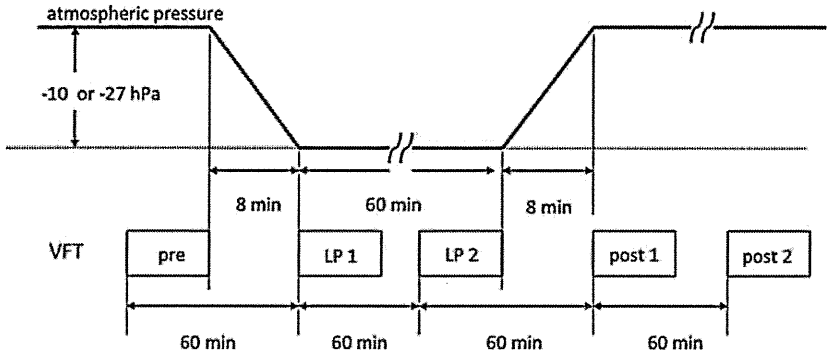

Fig. 1. Time schedule of low barometric pressure (LP) exposures. Behavioral tests (von Frey test; VFT) were carried out 5 times on the day of LP; just before exposure (pre), twice at the lowest pressure [just after (LP 1), and 60 min (LP 2) after reaching the pre-set LP level], and just after (post 1) and 60 min (post 2) after exposure.

according to the method described by Kim and Chung [10], except that only the L5 spinal nerve was ligated in this experiment. The controls were sham-operated guinea pigs, in which the left L5 spinal nerve was exposed but not ligated.

All behavioral tests were performed as blinded comparative studies. Each animal was individually placed beneath an inverted transparent plastic cage (11 cm × 17 cm × 11 cm) with a wire mesh bottom. Pain behaviors induced by mechanical stimulation were measured using homemade von Frey filaments (VFFs, diameter: 0.5 mm, bending forces 34, 59, and 239 mN). Each VFF was applied 10 times (once every 2–3 s) to the plantar surface of the nerve-injured hindpaw, and the number of foot withdrawals was counted. Stimulation of normal human skin with weak (34 and 59 mN) and then the stronger (239 mN) VFFs elicits a sensation of pressure and painful pricking, respectively. A significant increase in the frequency of hindpaw withdrawals in response to these mechanical stimuli was interpreted as punctate hyperalgesia [22,23].

We examined the effects of LP exposure on the SNL-induced pain behaviors in a pressure-controlled chamber, which had been developed and used in our previous studies [5,13]. The barometric pressure of the pressure-controlled chamber was lowered to 10 hPa or 27 hPa below the atmospheric pressure (Fig. 1), which simulates the typical change in pressure observed when a typhoon passes through Japan. This pressure drop was accomplished over 8 min. The pressure was maintained at this level for 60 min and then returned to atmospheric pressure over 8 min. The temperature and humidity were maintained at constant levels (22 ± 2 °C, 50 ± 10%). Behavioral tests were conducted 5 times: just before exposure (pre), twice at the lowered pressure [just after (LP 1) and 60 min (LP 2) after reaching the pre-set low-pressure level], and immediately after (post 1) and 60 min (post 2) after being returned to atmospheric pressure (Fig. 1).

All values are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by mixed-design two-way repeated measures analysis of variance (ANOVA) for multi-group comparisons, or by one-way repeated measures ANOVA, as appropriate. Tukey's test was used for post hoc comparisons when the F value was significant ($p < 0.05$). Kruskal–Wallis and Neuman–Keuls post hoc tests were used to compare the values of experimental groups in the LP-exposure test. Differences were considered statistically significant at $p < 0.05$.

Initial experiments were conducted to obtain a baseline and time course for pain-related behaviors in sham-operated and SNL animals without exposure to LP. Fig. 2 shows the time course of changes in withdrawal frequencies of the left hindpaw of SNL ($n = 9$) and sham-operated ($n = 7$) animals to 34-, 59-, and 239-mN VFFs. Mixed-design two-way repeated measures ANOVA revealed significant effects of both surgery and number of days post surgery on the number of hindpaw lifts [34 mN, surgery: $F_{(1,14)} = 44.5$, day: $F_{(10,140)} = 8.3$, surgery × day: $F_{(10,140)} = 5.1$, $p < 0.001$ for all; 59 mN, surgery: $F_{(1,14)} = 24.8$, day: $F_{(10,140)} = 3.2$, surgery × day: $F_{(10,140)} = 4.8$, $p < 0.001$ for all; 239 mN, surgery: $F_{(1,14)} = 16.1$ ($p < 0.005$), day: $F_{(10,140)} = 3.2$ ($p < 0.001$),

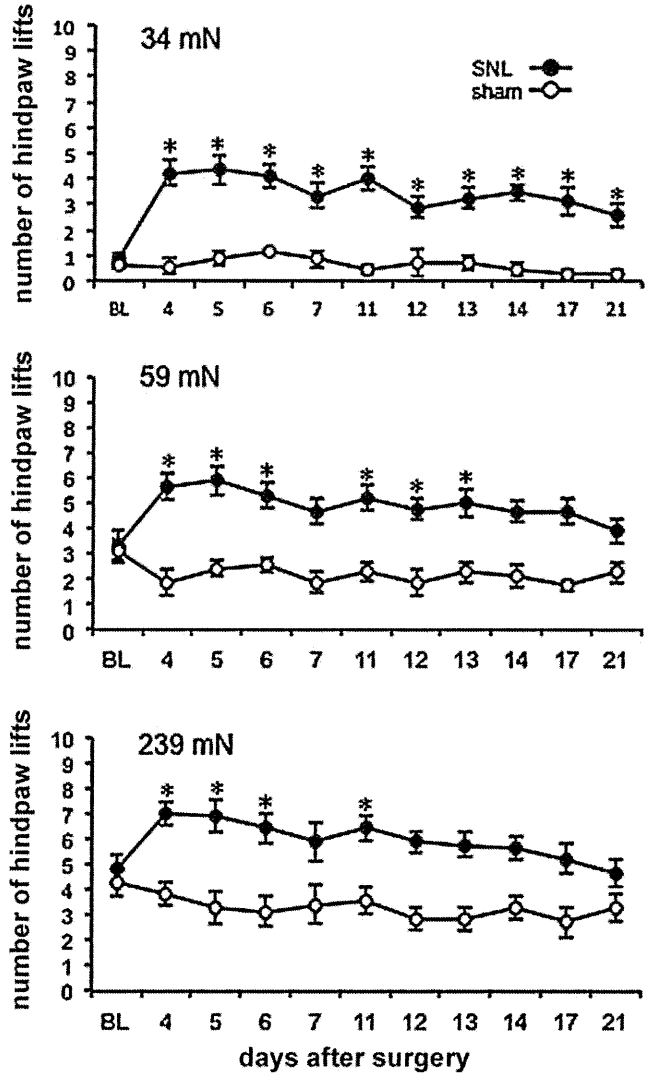

Fig. 2. Time course of changes in punctate hyperalgesia following SNL surgery. The number of hindpaw lifts of SNL ($n = 9$) and sham-operated ($n = 7$) guinea pigs in response to stimulation with 34-, 59-, and 6-mN von Frey filaments (VFFs) are shown (mean ± SEM). The horizontal axis indicates the measurement time points (BL: baseline). *$p < 0.05$ compared with associated baseline values (Tukey's post hoc test).

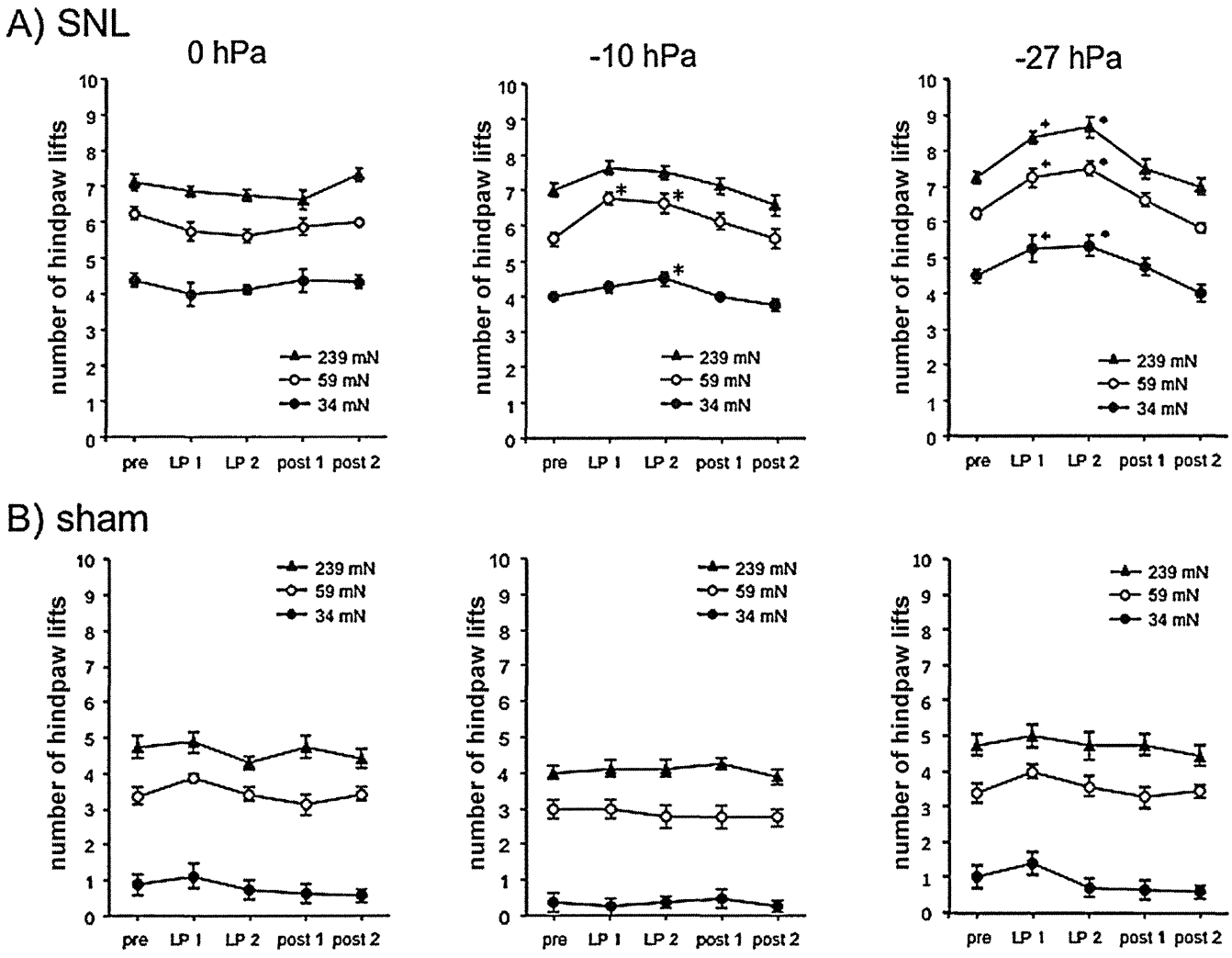

Fig. 3. LP exposure augmented punctate hyperalgesia in SNL guinea pigs. The number of hindpaw lifts in response to repeated mechanical stimuli with VFFs (34, 59, and 239 mN) are plotted against measurement time points. LP value is shown at the top of each graph. The period of measurement is expressed as *pre* for the pre-exposure control period, *LP 1, 2* for the first and second measurements during LP exposure, respectively, and *post 1, 2* for the first and second measurements during the post-exposure period, respectively. See Fig. 1 for the precise timing of measurements. In SNL animals (A), LP exposures to both 10 hPa and 27 hPa increased the number of hindpaw lifts in response to the 3 VFFs. In sham-operated animals (B), LP exposures did not induce any significant change in the number of hindpaw lifts. $^{*}p < 0.05$, compared with each *pre* value (repeated measures ANOVA followed by Tukey's multiple comparison test).

surgery × day: $F_{(10,140)} = 2.7$ ($p < 0.005$)], suggesting that SNL, but not sham surgery, significantly changed the number of hindpaw lifts. The post hoc analysis with Tukey's test revealed that significant increases in the withdrawal frequency to the 3 VFFs appeared as early as 4 days after SNL injury and persisted for 1–2 weeks. In contrast, no significant increases in the withdrawal frequency to the 3 VFFs were observed in the sham-operated control animals.

The time trial experiments showed that the punctate hyperalgesia in the hindpaw persisted for at least 11 days after SNL surgery (Fig. 2A); therefore, we next examined the effects of LP exposure on punctate hyperalgesia in SNL animals 7–11 days after SNL or sham surgery. Fig. 3A shows the changes in nociceptive behavior induced by LP exposure by 10 hPa or 27 hPa below atmospheric pressure in SNL animals ($n = 8$). One-way repeated measures ANOVA followed by Tukey's multiple comparison test revealed that the SNL animals showed a significant increase in the number of hindpaw lifts in response to stimulation by the 3 VFFs immediately after reaching both LP levels (LP 1). This increase persisted for the second measurement during the LP exposures taken 60 min later (LP 2). The increased hindpaw lift response returned to its pre-LP baseline levels shortly after the chamber pressure was restored to atmospheric levels (post 1). There was no significant change in pain-related behaviors when animals were placed in the chamber without a pressure change (0 hPa). In sham-operated animals ($n = 8$), LP exposure to either 10 hPa or 27 hPa below atmospheric pressure did not induce any significant change in the number of hindpaw lifts (Fig. 3B).

Fig. 3 shows that the increase in the number of hindpaw lifts was greater in magnitude when animals were exposed to a greater drop in pressure (27 hPa). This tendency is further highlighted in Fig. 4, which compares the potentiating effects on hindpaw withdrawal responses of 10-hPa and 27-hPa LP exposures. Kruskal–Wallis and Neuman–Keuls post hoc tests confirmed that the increases in the number of hindpaw lifts in response to the 3 VFFs were significantly larger when animals were exposed to LP of 27 hPa than that of 10 hPa.

This is the first study to investigate the effects of lowering barometric pressure on punctate hyperalgesia in guinea pigs. The present results demonstrate that LP of both 27 hPa and 10 hPa significantly aggravate hindpaw withdrawal responses; LP of 27 hPa has a significantly larger effect. This latter observation is unexpected, as our recent observations have indicated a notable absence

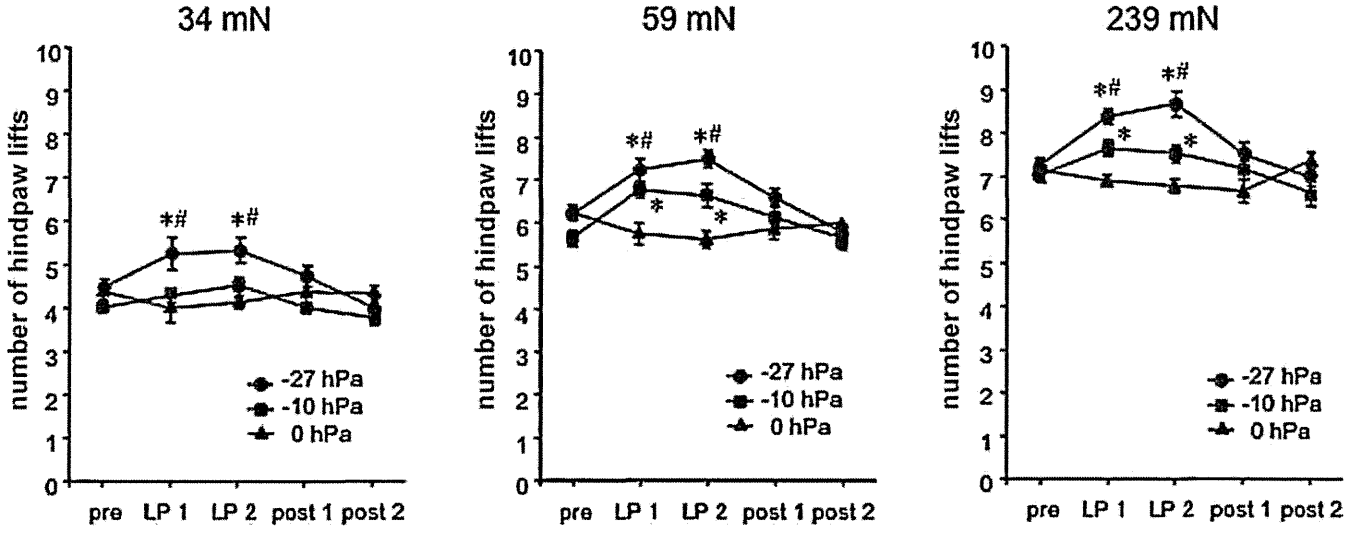

Fig. 4. Comparison of the augmenting effect on the hindpaw withdrawal responses between 10 hPa and 27 hPa LP exposures. The number of hindpaw lifts in response to repeated mechanical stimulation with VFFs (34, 59, and 239 mN) are plotted against time point measurements. VFF value is shown at the top of each graph. $^{*}p < 0.05$, compared with each *0 hPa* value, $^{\#}p < 0.05$, compared with that of *−10 hPa* exposure (Kruskal–Wallis test followed by Neuman–Keuls post hoc test).

of a stimulus–response relationship; that is, the smallest barometric pressure decrease (5 hPa at 5 hPa/h) induced almost the full effect, whereas a more intense pressure decrease (e.g., 10 hPa at 5 hPa/h) induced no further increase in pain-related behaviors in rats [5]. This difference between rats and guinea pigs may reflect physiological differences in either the barometric pressure-sensing system or efferent system, possibly including the sympathetic nervous system and/or hormonal system.

Previously, we showed that LP exposure intensifies pain-related behaviors in CCI rats via inner ear mechanisms, and inner ear activation plays a critical role in sensing changes in barometric pressure [3]. Similar mechanisms are likely to be involved in guinea pigs. It has been demonstrated that hyperbaric exposure significantly suppressed the development of endolymphatic hydrops following obliteration of the endolymphatic sac in guinea pigs [2]. This suggests that changes in atmospheric pressure easily influence inner ear fluid pressure in guinea pigs. It has been reported that vestibular activities in guinea pigs were altered by changes in middle ear pressure, although the study used a much more rapid pressure change (100 mmH_2O/s) than we did in the present experiments [21]. We also observed, although preliminarily, that some neurons in the rat vestibular nucleus responded to LP by 40 hPa within 8 min [4]. Such a pressure decrease temporarily renders pressure in the middle ear positive relative to atmospheric pressure, that of the perilymph negative relative to the middle ear, and that of the endolymph positive relative to the perilymph, thus producing a transient pressure difference between perilymph and endolymph. The authors suggest that this pressure differential between the perilymph and endolymph induced vestibular nerve activity. For the moment, the exact location and mechanism of pressure sensing remain unknown. This would present an interesting avenue for further investigation.

How did LP aggravate punctate hyperalgesia in the guinea pigs? Since LP-induced aggravation of nociceptive behaviors was observed only in animals with chronic pain, and not in normal animals, some mechanism involved in punctate hyperalgesia might be influenced by inner ear activities. One possible mechanism by which LP could aggravate chronic pain is through hormonal changes. This theory is based on reports that neurons in vestibular nuclei project to the hypothalamus, thereby potentially modulating levels of hormones such as adrenaline [8,11]. These circulating hormones could activate peripheral nociceptive fibers in the injured nerve, resulting in increased pain. It has been confirmed that cutaneous nociceptive fibers become responsive to adrenaline and noradrenaline after nerve injury [14,19]. The second possibility is that inner ear activation by LP leads to increased sympathetic nerve activity via the autonomic centers in the brain stem [15]. Sympathetic nerve volleys activate and sensitize nociceptive afferents via sympatho-nociceptor interactions that develop after nerve injury [19]. We cannot, however, rule out the possibility that LP stimulates non-neuronal peripheral tissues directly, thereby activating other processes.

In conclusion, exposure to pressure changes that fall within the range of natural weather patterns potentiates neuropathic pain responses in guinea pigs as well as rats. The mechanism by which this occurs requires further investigation, but may involve inner ear and sympathetic nerve pathways. This avenue of research is potentially important in elucidating mechanisms by which humans experience altered pain sensation in response to meteorological changes.

Conflict of interest statement

None of the authors have any conflicts of interest to declare.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (Nos. 20390415, 23590710) from the Japan Society for the Promotion of Science, and a Health Labour Sciences Research Grant (H23-ITAMI-SHITEI-005). We thank Dr. K. Mizumura for supporting this study.

References

[1] A.A.S. Albuquerque, M. Rossato, J.A.A. Oliveira, M.A. Hyppolito, Understanding the anatomy of ears from guinea pigs and rats and its use in basic otologic research, Braz. J. Otorhinolaryngol. 75 (2009) 43–49.
[2] F.-L. Chi, Q. Liang, Z.-M. Wang, Effects of hyperbaric therapy on function and morphology of guinea pig cochlea with endolymphatic hydrops, Otol. Neurotol. 25 (2004) 553–558.
[3] M. Funakubo, J. Sato, T. Honda, K. Mizumura, The inner ear is involved in the aggravation of nociceptive behavior induced by lowering barometric pressure of nerve injured rats, Eur. J. Pain 14 (2010) 32–39.
[4] M. Funakubo, J. Sato, K. Mizumura, Search for barometric pressure-sensitive neurons in the vestibular nuclei, J. Physiol. Sci. 57 (2007) S194.

[5] M. Funakubo, J. Sato, K. Obata, K. Mizumura, The rate and magnitude of atmospheric pressure change that aggravate pain-related behavior of nerve injured rats, Int. J. Biometeorol. 55 (2011) 319–326.
[6] D. Guedi, A. Weinberger, Effect of weather conditions on rheumatic patients, Ann. Rheum. Dis. 49 (1990) 158–159.
[7] N.H. Hendler, R.N. Jamison, C.H. Morrison, J.K. Piper, Z. Kahn, The relationship of diagnoses and weather sensitivity in chronic pain patients, J. Neuromuscloskeletal. Syst. 3 (1995) 10–15.
[8] A. Inokuchi, F. Liu, T. Uemura, Effects of stimulation of the vestibular nuclei on posterior hypothalamic neuron activity in guinea pigs, Eur. Arch. Otorhinolaryngol. 251 (1994) S23–S26.
[9] R.N. Jamison, K.O. Anderson, M.A. Slater, Weather changes and pain: perceived influence of local climate on pain complaint in chronic pain patient, Pain 61 (1995) 309–315.
[10] S.H. Kim, J.M. Chung, An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat, Pain 50 (1992) 355–363.
[11] B. Markia, Z.I. Kovács, M. Palkovits, Projections from the vestibular nuclei to the hypothalamic paraventricular nucleus: morphological evidence for the existence of a vestibular stress pathway in the rat brain, Brain Struct. Funct. 213 (2008) 239–245.
[12] S.W. Mitchell, The relations of pain to weather, Am. J. Med. Sci. 146 (1877) 305–329.
[13] H. Mizoguchi, K. Fukaya, R. Mori, M. Itoh, M. Funakubo, J. Sato, Lowering barometric pressure aggravates depression-like behavior in rats, Behav. Brain Res. 218 (2011) 190–193.
[14] K.D. O'Halloran, E.R. Perl, Effects of partial nerve injury on the responses of C-fiber polymodal nociceptors to adrenergic agonists, Brain Res. 759 (1997) 233–240.
[15] J.D. Porter, C.D. Balaban, Connections between the vestibular nuclei and brain stem regions that mediate autonomic function in the rat, J. Vestib. Res. 7 (1997) 63–76.
[16] P.B. Prince, A.M. Rapoport, F.D. Sheftell, S.J. Tepper, M.E. Bigal, The effect of weather on headache, Headache 44 (2004) 596–602.
[17] J. Sato, M. Aoyama, M. Yamazaki, S. Okumura, K. Takahashi, M. Funakubo, K. Mizumura, Artificially produced meteorological changes aggravate pain in adjuvant-induced arthritic rats, Neurosci. Lett. 354 (2004) 46–49.
[18] J. Sato, H. Morimae, Y. Seino, T. Kobayashi, N. Suzuki, K. Mizumura, Lowering barometric pressure aggravates mechanical allodynia and hyperalgesia in a rat model of neuropathic pain, Neurosci. Lett. 266 (1999) 21–24.
[19] J. Sato, E.R. Perl, Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury, Science 29 (1991) 1608–1610.
[20] J. Sato, K. Takanari, S. Omura, K. Mizumura, Effects of lowering barometric pressure on guarding behavior, heart rate and blood pressure in a rat model of neuropathic pain, Neurosci. Lett. 299 (2001) 17–20.
[21] M. Suzuki, H. Kitano, Y. Yazawa, K. Kitajima, Involvement of round and oval windows in the vestibular response to pressure changes in the middle ear of guinea pigs, Acta Otolaryngol. 118 (1998) 712–716.
[22] L.E. Ta, R.A. Dionne, J.R. Fricton, J.S. Hodges, K.C. Kajander, SYM-2081 a kainate receptor antagonist reduces allodynia and hyperalgesia in a freeze injury model of neuropathic pain, Brain Res. 858 (2000) 106–120.
[23] S. Zhou, L. Bonasera, S.M. Carlton, Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats, Neuroreport 7 (1996) 895–900.

Takiguchi *et al. Molecular Pain* 2012, **8**:31
http://www.molecularpain.com/content/8/1/31

MOLECULAR PAIN

RESEARCH Open Access

Distinct degree of radiculopathy at different levels of peripheral nerve injury

Noboru Takiguchi[1], Munehito Yoshida[1], Wataru Taniguchi[1], Hiroshi Hashizume[1], Hiroshi Yamada[1], Nobuyuki Miyazaki[1], Naoko Nishio[1] and Terumasa Nakatsuka[2*]

Abstract

Background: Lumbar radiculopathy is a common clinical problem, characterized by dorsal root ganglion (DRG) injury and neural hyperactivity causing intense pain. However, the mechanisms involved in DRG injury have not been fully elucidated. Furthermore, little is known about the degree of radiculopathy at the various levels of nerve injury. The purpose of this study is to compare the degree of radiculopathy injury at the DRG and radiculopathy injury proximal or distal to the DRG.

Results: The lumbar radiculopathy rat model was created by ligating the L5 nerve root 2 mm proximal to the DRG or 2 mm distal to the DRG with 6.0 silk. We examined the degree of the radiculopathy using different points of mechanical sensitivity, immunohistochemistry and *in vivo* patch-clamp recordings, 7 days after surgery. The rats injured distal to the DRG were more sensitive than those rats injured proximal to the DRG in the behavioral study. The number of activated microglia in laminas I–II of the L5 segmental level was significantly increased in rats injured distal to the DRG when compared with rats injured proximal to the DRG. The amplitudes and frequencies of EPSC in the rats injured distal to the DRG were higher than those injured proximal to the DRG. The results indicated that there is a different degree of radiculopathy at the distal level of nerve injury.

Conclusions: Our study examined the degree of radiculopathy at different levels of nerve injury. Severe radiculopathy occurred in rats injured distal to the DRG when compared with rats injured proximal to the DRG. This finding helps to correctly diagnose a radiculopathy.

Keywords: Radiculopathy, Microglia, Patch-clamp

Background

There are many patients who suffer from radiculopathy, characterized by spontaneous pain, weakness and numbness in the buttock, leg, and foot and difficulty in controlling specific muscles. Radiculopathy can occur in any part of the spine, most commonly in the lower back (lumbar radiculopathy) and neck (cervical radiculopathy) and not in the middle of the spine (thoracic radiculopathy). Radiculopathy is caused by compression or irritation of the spinal nerves. This can be due to mechanical compression of the nerve by a disc herniation or thickening of surrounding ligaments. Other causes of radiculopathy include diabetes, which can decrease the normal blood flow to the spinal nerves. Inflammation from trauma can also lead to radiculopathy from direct irritation of the nerves.

Radiculopathy is thought to be caused by a series of changes in the sensory processing system, functional reorganization of sensory transmission and development of neural plasticity, in both the peripheral and central nervous systems. Basic research has tended to focus on nerve injury preventing spinal cord neurons from receiving sensory information and relaying it to the brain. The superficial dorsal horn, especially the substantia gelatinosa (SG; lamina horn), plays an important role in modulating nociceptive transmission [1]. In previous studies, whole cell patch-clamp techniques have been adapted to SG neurons in a spinal cord slice with an attached dorsal root to investigate synaptic responses to peripheral nerve stimulation [2,3]. These studies revealed that SG neurons exhibit a variety of excitatory synaptic responses, however it remains

* Correspondence: nakatsuka@kansai.ac.jp
[2]Pain Research Center, Kansai University of Health Sciences, 2-11-1 Wakaba Kumatori Sennan, Osaka 590-0482, Japan
Full list of author information is available at the end of the article

to be settled what kinds of stimulation applied to the skin elicit these responses.

Wind-up is a progressive, frequency-dependent facilitation of neuronal responses induced by repetitive electrical stimulation of afferent C-fibers [4]. Long-term potentiation (LTP) of excitatory synaptic transmission is a long-lasting enhancement in signal transmission between two neurons that results from stimulating them synchronously. It is one of several phenomena underlying synaptic plasticity, the ability of chemical synapses to change their strength. LTP is synaptic plasticity not only in the peripheral nervous system and brain, but also in the spinal cord [5]. The cellular mechanisms of central sensitization and its relationship to the hypersensitivity and hyperalgesia of radiculopathy are still not fully elucidated. Previous research has used *in vivo* patch-clamp techniques to analyze excitatory synaptic responses evoked by cutaneous mechanical stimuli [6,7]. An *in vivo* preparation of a rat spinal cord was used to investigate the superficial dorsal horn neuron response to naturally applied noxious cutaneous stimuli, offering a more comprehensive study of nociceptive processing in the rats superficial dorsal horn.

Nerve injury produces the activation of not only neurons but also glial cells in the central nervous system (CNS) [8-10]. Glial cells make up over 70% of the total cell population in the CNS and are classified into astrocytes, oligodendrocytes, and microglia. Microglia activation following nerve injury is significantly increased compared with oligodendrocytes and astrocytes. Microgliosis (accumulation of activated microglia) around degenerative neurons is a common pathological feature of various neurological disorders including radiculopathy. Microglia exhibit a common, long-term response to a wide range of stimuli that threaten physiological homeostasis. This response includes changes in morphology, gene expression, function and number. Peripheral nerve injury leads to dramatic activation of microglia within the spinal dorsal horn [11]. Microglia activation in the spinal cord progresses through a hypertrophic morphology, with thickened and retracted processes and an increase in cell number. These criteria are immunohistochemical markers for assessing the activation state of microglia *in vivo* and among them, the change in cell number is the most prominent event [12]. Peripheral nerve injury increases the number of dorsal horn microglia by two to four fold [13-17].

Lumbar disc herniation occurs mostly in the spinal canal, because of injury proximal to the dorsal root ganglion (DRG). However, sometimes we face a specific type of radiculopathy, in which the percentage of lumbar disc herniation in the far lateral zone was 4.4–11.7% [18-20]. But the spinal nerve mechanisms around the DRG seem to be obscure, with little known about the degrees of radiculopathy injured at the DRG and proximal or distal to the DRG. Radiculopathy is an important and largely unresolved medical problem that requires further research into the etiological factors, to determine the correct diagnosis.

The purpose of this study was to examine the degrees of radiculopathy following injury at the DRG and proximal or distal to the DRG using different points of mechanical sensitivity, immunohistochemistry and *in vivo* patch-clamp recordings.

Results

Mechanical sensitivity

We counted the numbers of withdrawal reflexes in response to a sequential series of 10 tactile stimulations to the plantar surface of the ipsilateral (nerve root injured) hind paw using a 10 g von Frey filament. As rats rarely responded to the mechanical stimuli prior to surgery, the elevated behavioral responses evident after surgery were defined as allodynia. Behavioral tests were performed 7 days (7.8 ± 2.1 days, 220.5 ± 21.6 g) after surgery.

The number of withdrawal reflexes was 0.3 ± 0.2 times for group A (n = 10), 1.1 ± 0.5 times for group B (n = 10), 3.9 ± 0.5 times for group C (n = 10), 7.9 ± 0.5 times for group D (n = 10) and 6.2 ± 0.3 times for group E (n = 10). The number of withdrawal reflexes in the sham group (B) was not significantly increased compared with that in the normal group (A). While the rats in the nerve injury groups (C, D, and E) were significantly more sensitive compared with the rats in the sham groups (Student's *t* test, $P < 0.001$). The rats in group D were the most hypersensitive in the operated group, followed by groups E and C ($P < 0.001$) [Figure 1B].

Immunohistochemistry

We used a polyclonal antiserum directed against the ionized calcium-binding adapter molecule 1 (Iba1) to investigate whether microglia was activated and increased in the spinal dorsal horn 7 days after the surgery (208.4 ± 21.7 g) [Figure 2A]. We counted the number of microglia in laminas I–II of the L5 segment.

The number of activated microglia evident was 77.2 ± 2.5 for group A (n = 5), 80.0 ± 2.1 for group B (n = 5), 202.4 ± 8.0 for group C (n = 5), 354.6 ± 8.1 for group D (n = 5) and 292.1 ± 6.6 for group E (n = 5). There was no significant difference between groups A and B. The number of activated microglia was significantly increased in the nerve injury groups compared with the sham group ($P < 0.001$). The activated microglia seen in the nerve injury groups (C, D, E) developed at the superficial dorsal horn. The number of activated microglia was most significant in group D, followed by groups E and C ($P < 0.001$) [Figure 2B].

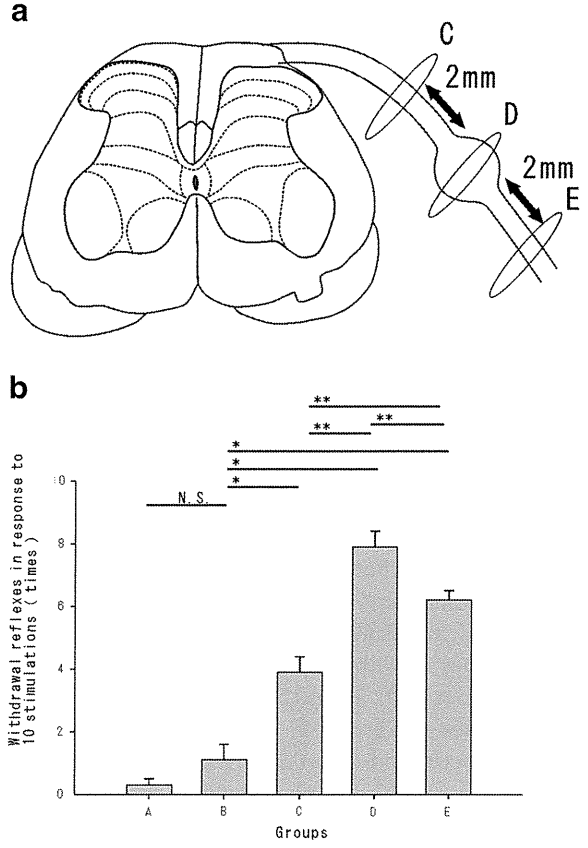

Figure 1 *A.* **Schematic diagram of the radiculopathy rat model from the L5 nerve to the spinal cord: The L5 nerve was ligated at 2 mm proximal to the DRG (group C), at the DRG (group D), at 2 mm distal to the DRG (group E) by a microscope at 5× magnification.** *B.* Withdrawal reflexes to 10 stimulations of a 10 g von Frey filament: The withdrawal threshold was a significantly different for nerve injury distal to the DRG compared with injury proximal to the DRG. There were no significant differences in the number of withdrawal reflexes between normal group A and the sham group B. The nerve injury groups (C, D, E) were more sensitive than the sham group (*$P < 0.001$), with significant differences between groups C, D and E (**$P < 0.001$).

In vivo patch-clamp recordings

Rats (218.4 ± 23.5 g n = 45) developed mechanical hypersensitivity approximately 7 days (7.6 ± 2.4 days) after surgery. A rat spinal cord preparation could be maintained in a stable condition for over 12 hours, which was equivalent to previous patch-clamp experiments using an artificial ventilator. Whole-cell patch-clamp recordings were performed in 50 SG neurons from the L5 segmental level of the spinal cord. All SG neurons were recorded at a holding potential (V_H) of −70 mV, where no inhibitory postsynaptic currents (IPSC) were observed [Figure 3A].

The average frequencies of excitatory postsynaptic currents (EPSC) were recorded in laminas I–II of the L5 segmental area. The frequencies of EPSC were 9.0 ± 1.4 Hz for group A (n = 10), 9.4 ± 1.3 Hz for Group B (n = 10), 14.6 ± 1.7 Hz for Group C (n = 10), 19.2 ± 2.0 Hz for Group D (n = 10), 17.3 ± 2.1 Hz for Group E (n = 10). The frequencies of EPSC in the nerve injury groups (C, D, E) were significantly increased compared with the sham group ($P < 0.001$). Interestingly, there were significant differences in the frequencies of EPSC between groups C, D and E ($P < 0.001$ and $P < 0.05$, respectively) [Figure 3B].

The amplitudes of EPSC were 12.3 ± 0.6 pA for group A (n = 10), 14.4 ± 1.0 pA for group B (n = 10), 19.8 ± 1.6 pA for group C (n = 10), 37.7 ± 2.2 pA for group D (n = 10) and 28.3 ± 2.4 pA for group E (n = 10). No significant difference in mean amplitudes of EPSC was observed between groups A and B. There were significant differences between groups B and C ($P < 0.05$), B and D ($P < 0.001$) and B and E ($P < 0.001$). Finally, there were significantly differences in mean amplitudes in C, D and E ($P < 0.001$) [Figure 3C].

Discussion

Several studies have investigated different types and degrees of nerve root or spinal nerve injuries [11,21]. Lesions close to the DRG produce more apoptosis when compared with lesions more distal to the DRG [22]. However, there was no report to compare the degrees of radiculopathy injured proximal or distal to the DRG. We investigated the degrees of radiculopathy following nerve injury via nerve ligation at an equal distance (2 mm proximal or distal) to the DRG. The degrees of radiculopathy were assessed using different points of mechanical sensitivity, immunohistochemistry and *in vivo* patch-clamp recordings.

Mechanical allodynia was observed at the plantar surface of the ipsilateral (nerve root injury site) hind paw at 7 days after surgery. The withdrawal threshold to von Frey stimulation in the nerve injury groups decreased compared with the sham model rats. Our behavioral data were consistent with the previous studies that showed development of maximum allodynia to occur at around 7 days post-injury in a rat model of nerve injury [23,24]. Interestingly, there was a significant difference in withdrawal threshold between the proximal and distal nerve injury to the DRG in this study.

Resting microglia (in the normal state) act as sensors for stimuli that threaten physiological homeostasis, including CNS trauma, ischemia, infection and neurodegeneration. Once activated by these stimuli, microglia undergo a common, series of progressive changes in morphology, function and number [13,25], which have been implicated in inducing central sensitization of spinal neurons [26]. ATP [27], substance P [28] and glutamate [29] are released in high amounts during central sensitization and participate in its induction and activate microglia [13,25], so that the sensitization of dorsal horn neurons may also stimulate microglia, which become further activated, establishing a