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Original

NMDA NR2B Subunit Antagonist May Attenuate Mechanical Allodynia by Increasing the Release of Enkephalin in the Spinal Dorsal Horn

Shunsaku Kobayashi, Shiho Tanaka and Eiko Kato

Department of Physiology & Biological Information, Dokkyo Medical University
Kitakobayashi 880, Mibu, Tochigi 321-0293, JAPAN

SUMMARY

Background : Neurons containing enkephalin (ENK) are distributed at high concentrations in the superficial dorsal horn (SDH) of the spinal cord, where they play an important role in the modulation of nociceptive information. In addition to ENK, the SDH exhibits high expression levels of the NR2B subunit of the *N*-methyl-D-aspartate (NMDA) receptor and large-conductance calcium-activated potassium (BK) channels. In the present behavioral experiments, we investigated the effects of the BK channel antagonist charybdotoxin (CTX) and the NR2B subunit antagonist ifenprodil (IFN) on a nociceptive behavior in peripheral-nerve-injured mice.

Methods : The experiments were performed in 6- to 8-week-old male ICR mice. Partial sciatic nerve ligation (PSL) was performed as described previously (Seltzer model). Mechanical allodynia was assessed by stimulation with von Frey filaments. On postsurgical day 7, the effects of CTX and IFN on mechanical allodynia were analyzed. Additionally, to test the possibility that the actions of CTX and IFN are mediated by an altered release of ENK, we investigated the effect of the selective μ- and δ-opioid receptor antagonists naloxone (NAL) and naltrindole (NTL), respectively. CTX (1 pmol/10 μL per mouse), IFN (50 nmol/10 μL per mouse), and saline as a control (10 μL per mouse) were intrathecally injected. Additionally, NAL (5 mg/kg) and NTL (5 mg/kg) were intraperitoneally administered. All behavioral experiments were performed in a double-blind fashion.

Results : PSL significantly increased the occurrence of the withdrawal reflex to von Frey stimuli, indicating the development of mechanical allodynia. CTX significantly reduced the occurrence of the withdrawal reflex compared to the saline group. Additionally, both NAL and NTL attenuated the analgesic effect of CTX. Intrathecal IFN significantly reduced the occurrence of the withdrawal reflex, which was also reduced by NAL and NTL.

Conclusion : The obtained behavioral observations suggest that the NMDA receptors and BK channels might inhibit the release of ENK in the SDH, which is speculated to be involved in the chronic pain state induced by peripheral nerve injury.

Key words : NMDA receptor, BK channel, enkephalinergic neuron, neuropathic pain, spinal dorsal horn

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Reprint requests to : Eiko Kato, Ph.D.
Department of Physiology and Biological Information, Dokkyo Medical University, Kitakobayashi 880, Mibu, Tochigi 321-0293, Japan

INTRODUCTION

Enkephalins (ENKs) are pentapeptides (Tyr-Gly-Gly-Phe-Met or Tyr-Gly-Gly-Phe-Leu) that are derived from the precursor preproenkephalin. ENKs are involved in many physiological functions, including modulation of nociceptive perception, regulation of memory, emotional condition, food and liquid consumption, and regulation of the immunological response[1].

ENK-containing neurons are widely distributed in the central nervous system. One of these regions includes the superficial dorsal horn (SDH) of the spinal cord[2~4]. Here, ENK-containing neurons play an important role in regulating the transmission of nociceptive information[1,5,6].

Several other groups of investigators and we have generated a transgenic mouse using an artificial bacterial chromosome, which expresses the green fluorescent protein (GFP) under the control of the preproenkephalin promoter[7~9]. Using such ENK-GFP transgenic mice, we have electrophysiologically characterized the ENK-containing SDH neuron[7]. However, it remains unclear how the activity of the ENK-containing neuron is regulated in the SDH.

In addition to ENK, the SDH contains a high concentration of *N*-methyl-D-aspartate (NMDA) subtype glutamate receptors[10~12]. These receptors are mostly involved in the modulation of nociceptive transmission in the SDH. It has been reported that the NR2B subunit of NMDA receptors is upregulated, which is associated with the development of neuropathic pain[10,11]. Furthermore, calcium-activated large-conductance potassium (BK) channels exhibit high-density distribution in the SDH and are involved in the nociceptive modulation[13~16].

Recently, Song and Marvizón measured the internalization of μ-opioid receptors and suggested that the Ca^{2+} entering through activated NMDA receptors opens BK channels, resulting in the hyperpolarization of ENK-containing neurons, and thus, leading to a decrease in ENK release in the SDH[17]. Furthermore, electrophysiological investigation has suggested that NMDA receptors interact with BK channels, which leads to hyperpolarization of neurons in several regions of the central nervous system, including the olfactory bulb[18] and hippocampus[19]. As such, the present behavioral experiments investigate whether NMDA receptors, interacting with BK channels, are involved in the regulation of ENK release in the SDH.

Our results show that intrathecal (i.t.) administration of a BK channel antagonist or an NMDA receptor antagonist has an antinociceptive effect, which is blocked by intraperitoneal (i.p.) administration of opiate receptor antagonists. These findings provide clues to understand further how the activity of ENK-containing neurons in the spinal dorsal horn is regulated. Additionally, the findings reveal information regarding the pathophysiological mechanisms of the development of neuropathic pain induced by peripheral nerve injury.

METHODS

Animals

Experiments were performed on 6- to 8-week-old male ICR mice. The mice were maintained at controlled room temperature under a 12 h/12 h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committees at Dokkyo Medical University. The animals were treated in accordance with the National Institutes of Health guidelines on animal care and the International Association for the Study of Pain[20].

Neuropathic pain model

The sciatic nerve was partially ligated of the mice while anesthetized with sevoflurane as previously described (partial sciatic nerve ligation [PSL], Seltzer model)[21]. In sham-operated control mice, the sciatic nerve was exposed but not ligated.

Behavioral assessment of mechanical allodynia

To assess nociceptive behavior associated with sciatic nerve ligation, we measured the frequency of withdrawal responses to 10 repetitive stimuli with von Frey filaments (Stoeling, USA) using 0.16 g of force.

Behavioral assessments were performed one day prior to the ligation surgery. On postsurgical day 7, the frequency of withdrawal responses significantly increased, indicating the development of mechanical allodynia induced by peripheral nerve injury. On this day, the effects of the BK channel antagonist charyb-

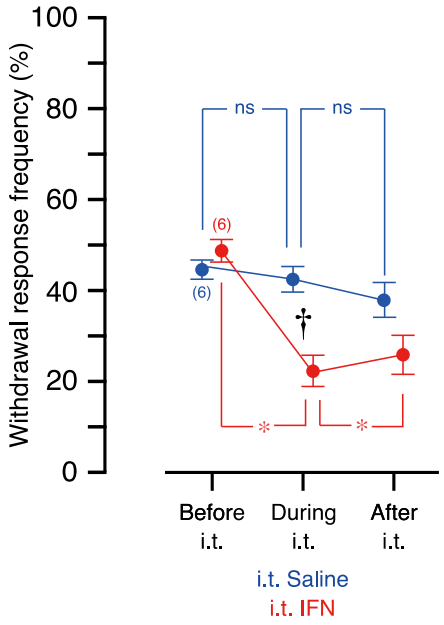

Figure 1 Attenuation of mechanical allodynia by intrathecal IFN
The vertical axis indicates the frequency of hindlimb-withdrawal response to von Frey stimuli.
Values in parentheses represent the number of observations in each group.
"†" indicates a significant difference between the IFN and saline group, with $p < 0.05$.
"*" indicates a significant difference compared to before and during the i.t. injection, with $p < 0.05$.
"ns" indicates no significant difference.

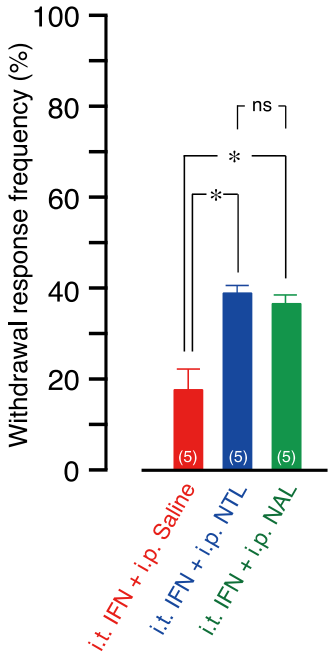

Figure 2 Reversal of the analgesic effect of intrathecal IFN by intraperitoneal administration of NTL (blue bar) and NAL (green bar)
The vertical axis indicates the frequency of hindlimb-withdrawal response to von Frey stimuli.
"*" indicates a significant difference between the three treatment groups, with $p < 0.05$.
"ns" indicates no significant difference.

dotoxin (CTX) and the NR2B antagonist ifenprodil (IFN) on nociceptive behavior were analyzed.

Chemicals used

The following chemicals were injected intrathecally according to the modified method originally described by Hylden *et al.* (1980)[22] : CTX, a specific antagonist to BK channels (1 pmol/10 μL per mouse)[13] ; IFN, a specific antagonist to the NR2B subtype of NMDA receptors (50 nmol/10 μL per mouse)[23,24] ; and saline as a control (10 μL per mouse).

Withdrawal tests were performed 15 minutes after i.t. administration of IFN or CTX when the effects of the drugs were maximal (the label 'During i.t.' on the horizontal axis in Figure 1 and 3). Recovery from IFN or CTX was evaluated 90 minutes after i.t. administration of the drugs (the label 'After i.t.' on the horizontal axis in Figure 1 and 3).

To also address the possibility that the actions of CTX and IFN are mediated by an alteration in the release of ENK, we investigated the effect of the selective δ-opioid receptor antagonist naltrindole (NTL, 5 mg/kg body weight) and the μ-opioid receptor antagonist naloxone (NAL, 5 mg/kg body weight), which were both injected intraperitoneally.

All behavioral experiments were performed in a double-blind setting.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed by conducting a two-way repeated measurements analysis of variance (ANOVA), with one between-group "antagonist" factor (antagonist/saline) and one within-subject "time" factor (before/during/after). When the antagonist-by-time interaction was significant, additional within-group effects were determined by a test for simple main effects. One-way ANOVA with Tukey's post hoc com-

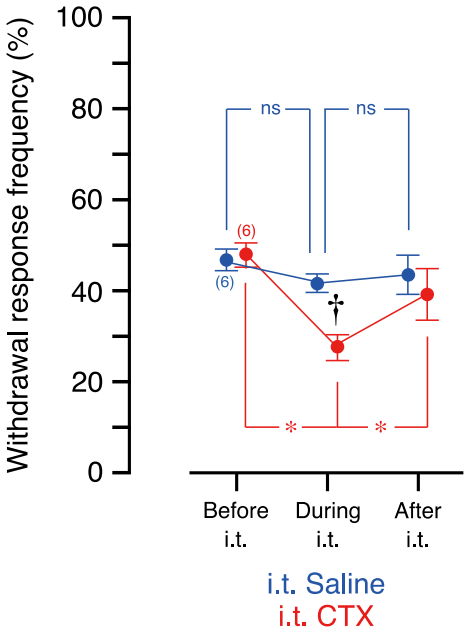

Figure 3 Attenuation of mechanical allodynia by intrathecal CTX
The vertical axis indicates the frequency of hindlimb-withdrawal response to von Frey stimuli.
"†" indicates a significant difference between the CTX and saline group, with $p < 0.05$.
"*" indicates a significant difference compared to before i.t. injection, with $p < 0.05$.
"ns" indicates no significant difference.

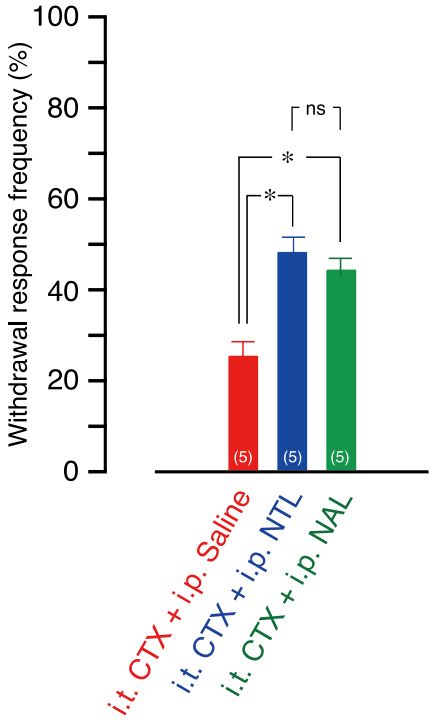

Figure 4 Reversal of the analgesic effect of intrathecal CTX by intraperitoneal administration of NTL (blue bar) and NAL (green bar)
The vertical axis indicates the frequency of hindlimb-withdrawal response to von Frey stimuli.
"*" indicates a significant difference between the three treatment groups, with $p < 0.05$.
"ns" indicates no significant difference.

parisons among three to four treatment groups was also used when appropriate. Comparisons with a difference of $p < 0.05$ were considered statistically significant.

RESULTS

Attenuation of mechanical allodynia by i.t. IFN and its reversal by i.p. NAL and NTL

The i.t. administration of IFN ameliorated mechanical allodynia reversibly, as shown in Figure 1. To evaluate the possibility that the analgesic action of IFN was mediated by an increased release of ENK, the effect of i.p. administration of the selective δ-opioid receptor antagonist NTL and the selective μ-opioid receptor antagonist NAL were investigated. Here, Figure 2 shows that the analgesic effect of i.t. IFN was reduced by i.p. injection of NAL (green bar) and NTL (blue bar).

Attenuation of mechanical allodynia by i.t. CTX and its reversal by i.p. NAL and NTL

The i.t. administration of CTX ameliorated mechanical allodynia reversibly, as shown in Figure 3. The effect of i.p. NTL and i.p. NAL on i.t. CTX was investigated to address the possibility that the analgesic action of CTX was mediated by an increased release of ENK. Here, the results showed that the analgesic action of i.t. CTX was reduced by i.p. NAL (green bar in Figure 4) and i.p. NTL (blue bar in Figure 4).

The i.t. coadministration of CTX and IFN did not show any synergic antinociceptive effect.

Previous studies have shown that BK channels in neurons in various regions of the central nervous system are activated by Ca^{2+} influx through NMDA receptors, which then leads to the hyperpolarization of neurons[18,19]. To determine if i.t. IFN-induced analgesia is mediated by inhibition of BK channels in

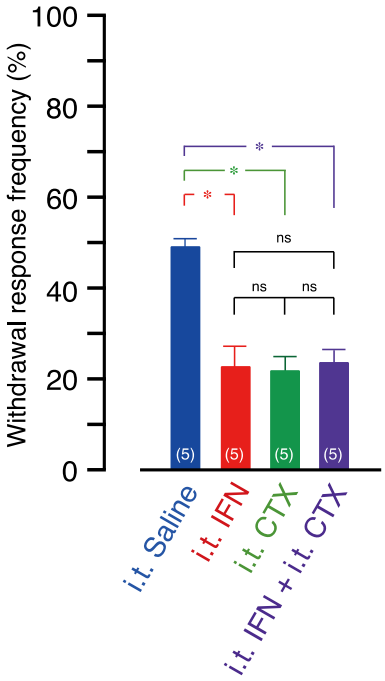

Figure 5 No synergy between the analgesic effects of intrathecal CTX and IFN
The vertical axis indicates the frequency of hindlimb-withdrawal response to von Frey stimuli.
"*" indicates a significant difference between the four treatment groups, with $p < 0.05$.
Blue bar : i.t. saline group. Purple bar : i.t. coadministration of the CTX and IFN group. Green bar : i.t. CTX only group. Red bar : i.t. IFN only group.
"ns" indicates no significant difference.

ENK-containing neurons in the SDH, we investigated whether i.t. coadministration of CTX and IFN exhibits any synergic antinociceptive actions. The results showed that simultaneous i.t. administration of CTX and IFN attenuated mechanical allodynia (purple bar in Figure 5) to the same extent as CTX and IFN alone (green and red, respectively, in Figure 5).

DISCUSSION

Analgesic effect of i.t. IFN and its involvement of ENK release

The present experiments showed that i.t. injection of the NR2B antagonist IFN attenuated mechanical allodynia induced by peripheral nerve injury and that this action was blocked by i.p. administration of the opioid receptor antagonists NAL and NTL.

The antinociceptive action of the i.t. NR2B antagonist has previously been reported[23,24]. For example, Kim *et al.* reported that i.t. administration of IFN (100, 200, 500, and 1000 nmol) increased the paw withdrawal threshold in a neuropathic model mouse[23].

Peripheral nerve injury upregulates the NR2B subunit of NMDA receptors in the SDH[10,11], resulting in an increased excitability of the spinal interneurons, which in turn underlies the mechanisms for the neuropathic pain state[10~12]. Here, several studies have suggested that the i.t. NR2B antagonist suppresses the excitability of the SDH neural circuit, and therefore, exerts analgesic actions[23,24].

In addition to this analgesic mechanism of the NR2B antagonist, the present observations seem to indicate that at least a part of the analgesic action of the i.t. NR2B antagonist might lead to an increase in ENK release and the activation of opioid receptors in the SDH.

Analgesic effect of i.t. CTX and its involvement of ENK release

The i.t. injection of the BK channel blocker CTX was observed to exert inhibitory actions on mechanical allodynia. Moreover, this action was blocked by i.p. administration of the opioid receptor antagonists NAL and NTL.

Along the same lines as our present observations, i.t. BK channel blocker has been reported to significantly inhibit tactile allodynia induced by nerve injury[13]. However, in a study by Chen *et al.*, it was reported that the activation of BK channels reversed allodynia and hyperalgesia caused by nerve injury[14]. Although the exact reason for this discrepancy remains currently unclear, the BK channels in the SDH do seem to play an important role in the modulation of nociceptive information.

BK channels assume various roles in the central nervous system[15]. For example, it was shown that the activation of BK channels in the spinal cord inhibits the release of the inhibitory neurotransmitter glycine from spinal interneurons[25]. Hence, it is reasonable to speculate that a BK channel blocker, such as CTX, would enhance the release of the inhibitory neurotransmitter ENK from spinal ENK-containing interneurons.

Possible interaction of the NR2B subunit NMDA receptors with the BK channels in the SDH

The blocking of BK channels by i.t. CTX negated the effects of i.t. IFN in the present experiments. Furthermore, the analgesic action of both i.t. IFN and i.t. CTX might involve an increased release of ENK in the SDH.

Based on these findings, it is speculated that IFN inhibits NMDA receptors, and thus, reduces Ca^{2+} influx through the NMDA receptor, which then results in the inhibition of the BK channels, leading to depolarization of ENK neurons. Depolarization of ENK neurons results in an increase of ENK release in the SDH, which might underlie the mechanism of the presently observed antinociceptive actions of IFN and CTX.

The coupling between the NMDA receptors and the BK channels in neurons has been found in various regions of the central nervous system, including the olfactory bulb[18] and the hippocampus[19]. For example, neurons in the olfactory bulb hyperpolarize in response to the activation of NMDA receptors[18]. This hyperpolarization is mediated by the BK channel opening caused by the influx of Ca^{2+} through activated NMDA receptors.

Although it is not yet clear whether the coupling between NMDA receptors and BK channels exists in ENK-containing neurons in the SDH, the present behavioral results might indicate that this is the case. This speculation is supported by a study of Song *et al*., where they reported that the μ-opioid receptor internalization was inhibited by the activation of NMDA receptors in rat spinal cord slices — in which the internalization of μ-opioid receptors reflects the release of opioids — and that the inhibition was blocked by a BK channel blocker[17].

Conclusions

The present findings of this study strongly suggest that Ca^{2+} influx through NMDA receptors opens the BK channels, causing the hyperpolarization of inhibitory interneurons in the SDH. In turn, this causes a decrease in release of inhibitory transmitters such as ENK. In support to these findings, the NR2B subunit of NMDA receptors has been shown to be upregulated in the neuropathic pain state[10,11]. NMDA-receptor-mediated hyperpolarization of inhibitory interneurons in the SDH might contribute to the hyperalgesic actions of spinal NMDA receptors. Finally, further investigation is required to clarify whether immediate coupling between NMDA receptors and BK channels exists in inhibitory interneurons in the SDH.

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Conflict of interest

The authors state that they have no conflict of interest.

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