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SPECIAL REPORT

Enhanced nociception by exogenous and endogenous substance P given into the spinal cord in mice lacking NR_2A/ε_1, an NMDA receptor subunit

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In capsaicin-pretreated mice, the nociceptive responses induced by intrathecally (i.t.) administered substance P (SP) were enhanced by N-methyl-D-aspartate (NMDA)-type receptor antagonists, dizocilpine (MK801) and D-2-amino-5-phosphonopentanoate (D-AP5) in a dose-dependent manner. Similar enhancement of SP-induced nociception was also observed in mice lacking the NMDA-type glutamate receptor NR2A/ε_1 subunit gene (GluR$\varepsilon_1{}^{-/-}$ mice). On the other hand, GluR$\varepsilon_1{}^{-/-}$ mice showed a marked enhancement of the peripheral nociceptive responses induced by intraplantar (i.pl.) injection of SP and bradykinin (BK). As the nociceptive responses to SP and BK (i.pl.) were both antagonized by CP-99994, an neurokinin$_1$ (NK_1) antagonist (i.t.), these results suggest that GluRε_1 receptor may play an inhibitory role in the downstream mechanisms of primary nociceptive SP neurones, possibly through activation of unidentified inhibitory neurones.
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Abbreviations: BK, Bradykinin; CP-99994, [(±)-(2S,3S)-3-(2-methoxybenzyl-amino)-2-phenylpiperine]; CP-100263, [(−)-(2R,3R)-3-(2-methoxybenzyl-amino)-2-phenylpiperine]; D-AP5, D-2-amino-5-phosphonopentanoate; i.pl., intraplantar; i.t., intrathecal(ly); MK-801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclo-hepten-5,10-imine; SBL, scratching, biting and licking; SP, substance P

Introduction It is well known that glutamate and substance P (SP) are putative pain neurotransmitters of nociceptive primary afferent neurones. Recent reports suggest that NMDA-type glutamate receptors not only mediate glutamate-induced pain signalling in the dorsal horn of spinal cord (Murray *et al*., 1991; Nishiyama *et al*., 1998), but enhance SP-release from primary afferent neurones (Liu *et al*., 1994; 1997). However, in several *in vivo* analgesic tests, NMDA receptor antagonists have little effects on pain perception (Okano *et al*., 1998; Inoue *et al*., 1998c). As glutamate would also play a neurotransmitter role in pain-suppressive large diameter afferent neurones (Coggeshall & Carlton, 1998), as has been hypothesized in the 'gate-control theory' (Melzack & Wall, 1965), the lack of change in pain perception by antagonists could be attributed to mutual blockade by such counteracting inputs. In order to understand the physiological role of NMDA receptor in pain regulation, either the use of specific stimulation or the design of experiments to avoid counteracting inputs should be performed. Here we report the unexpected enhancement of SP-induced nociception by intrathecally administered NMDA receptor antagonists in capsaicin-treated mice or in GluR$\varepsilon_1{}^{-/-}$ mice.

Methods Male ddY mice, wild-type (GluR $\varepsilon_1{}^{+/+}$) mice and mice lacking NMDA-type glutamate receptor NR2A/ε_1 subunit gene (GluR$\varepsilon_1{}^{-/-}$ mice) weighing 20–22 g were used. Both GluR$\varepsilon_1{}^{+/+}$ and GluR$\varepsilon_1{}^{-/-}$ mice with highly homogenous genetic background had been developed previously (Sakimura *et al*., 1995; Kiyama *et al*., 1998). These mice were kept in a room maintained at 21±2°C with free access to a standard laboratory diet and tap water. All experiments were performed in compliance with the relevant laws and institutional guidelines. All procedures throughout the present study were approved by Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). In some experiments, capsaicin was injected subcutaneously into the back of newborn (P4) ddY mice at a dose of 50 mg kg^{-1}. This treatment is known to cause a degeneration of small-diameter afferent sensory neurones (Hiura & Ishizuka 1989; Inoue *et al*., 1999). Here we used two different nociceptive tests in mice, one due to the central noxious stimulation (Hylden & Wilcox 1981; Inoue *et al*., 1999; Liu *et al*., 1997), and the other to peripheral noxious stimulation (Inoue *et al*., 1998a,b; Ueda, 1999). In the former test, SP (in 5 µl saline) was given intrathecally (i.t.) to mice, and the nociceptive responses, characterized as scratching, biting and licking (SBL) behaviours were observed. The nociceptive activity was expressed as total periods (s) showing nociceptive SBL behaviours for 20 min following i.t. injection, according to the protocol of Hylden & Wilcox, 1980. In the latter test, nociceptive flexor responses induced by intraplantar injection (2 µl) of nociceptive substances were evaluated in mice. As the intensity of flexor responses differs from mouse to another, we used the biggest spontaneous flexor response (in amplitude) occurring immediately after cannulation (maximal reflex) to normalize the test drug-induced responses. Therefore, the nociceptive activity was represented as the per cent of maximal reflex observed at the beginning of each experiment before drug administration. The following drugs were used: SP (Peptide Institute, Osaka, Japan), BK (Sigma, St. Louis, MO, U.S.A.), MK-801 (Research Biochemical), D-AP5 (Nacalai Tesque, Kyoto, Japan) and capsaicin (Nacalai Tesque, Kyoto, Japan).

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CP-99994 and CP-100263 were generously provided by Pfizer. SP, BK, MK-801, D-AP5, CP99994 or CP100263 were dissolved in physiological saline and capsaicin in 10% ethanol and 10% Tween 80 in physiological saline. The data was analysed using Student's *t*-test after multiple comparisons of ANOVA. The criterion of significance was set at $P<0.05$. All results are expressed as the mean ± s.e.mean.

Results As NMDA receptors might affect SP release from polymodal C-fibre neurones (Liu *et al*., 1994; 1997), we decided to pretreat neonatal ddY mice with capsaicin to degenerate afferent neurons containing SP as a major neurotransmitter. As reported elsewhere (Hiura & Ishizuka, 1989), we have confirmed the loss of SP-like immunoreactivity in the substantia gelatinosa of spinal cord of capsaicin-pretreated mice (Inoue *et al*., 1999). The SBL responses by SP, characterized by scratching the body, biting and licking the limbs (Hylden & Wilcox, 1980; 1981; Inoue *et al*., 1999; Liu *et al*., 1997) were slightly higher in capsaicin-pretreated mice (37.02 ± 2.33 s, $n=9$) than those in untreated ones (24.86 ± 4.70 s, $n=5$), whilst there was no significant change in the time-course of SP (30 pmol)-induced SBL responses between both mice, consistent with previous reports (Mantyh & Hunt, 1985). In capsaicin-pretreated ddY mice, intrathecally administered SP induced nociceptive SBL responses in a dose-dependent manner from 10 pmol (18.12 ± 7.93 s, $n=5$) to 100 pmol (94.65 ± 3.72 s, $n=5$). The nociceptive responses by SP at 30 pmol were markedly enhanced by pretreatment with MK-801, a non-competitive NMDA receptor antagonist, in a dose-dependent manner at doses between 10 to 100 pmol, compared to vehicle-pretreated mice (Figure 1a). Similarly, D-AP5, a competitive NMDA receptor antagonist, dose-dependently enhanced the SP-induced nociception at doses between 30 to 300 pmol (Figure 1a). However, MK-801 (100 pmol) or D-AP5 (300 pmol) itself did not show any significant differences in the behavioural responses (9.01 ± 3.12 s/20 min^{-1}, $n=5$, 13.84 ± 5.30 s/20 min^{-1}, $n=5$, respectively), compared to vehicle (8.25 ± 1.95 s/20 min^{-1}, $n=5$). Similar results were also obtained when GluR$\varepsilon_1{}^{-/-}$ mice were used. As shown in Figure 1b, SP (i.t.)-induced SBL responses in GluR$\varepsilon_1{}^{+/+}$ mice lasted for 18.45 ± 4.59 s/20 min^{-1} ($n=5$), which is slightly less than that in ddY mice (24.86 ± 4.70 s/20 min^{-1}, $n=5$). A marked enhancement of SBL responses was observed in GluR$\varepsilon_1{}^{-/-}$ mice (74.79 ± 7.88 s/20 min^{-1}, $n=6$).

We have previously reported that intraplantarly administered SP induced nociceptive flexor responses through stimulation of SP-containing polymodal C-fibres (Inoue *et al*., 1998a; Ueda, 1999). Here we used this peripheral nociception test to stimulate primary afferent SP neurones, and the assumed release of endogenous SP into the spinal cord (Inoue *et al*., 1998a,b). As shown in Figure 2a, SP (30 pmol, i.pl.)-induced flexor responses were abolished by intrathecally administered CP-99994 (100 pmol, i.t.), a selective NK_1 receptor antagonist, but not by CP-100263 (100 pmol, i.pl.), its inactive isomer. We have also reported that bradykinin (BK) is another peripheral stimulator of polymodal C-fibres (Inoue *et al*., 1997). As shown in Figure 2b, the nociceptive flexor responses induced by intraplantarly administered BK (2 pmol, i.pl.) were also abolished by CP-99994 (100 pmol, i.t.), but not by CP-100263 (100 pmol, i.t.). These findings suggest that peripheral stimulation by SP (or BK) cause a release of SP into the spinal cord to evoke nociceptive responses. Based on these findings, we examined the difference in sensitivities to SP or BK (i.pl.) between GluR$\varepsilon_1{}^{+/+}$ and GluR$\varepsilon_1{}^{-/-}$ mice. In wild-type GluR$\varepsilon_1{}^{+/+}$ mice, the SP-induced nociceptive flexor responses were dose-dependent in the ranges between 0.01 and 10 pmol (Figure 2c), and the average ND_{50}, nociception dose (± s.e.mean, n) showing 50% of maximal reflex was calculated to be 0.66 ± 0.15 pmol, i.pl. ($n=6$), which is equivalent to that in normal ddY mice (1.35 ± 0.34 pmol, $n=5$). In GluR$\varepsilon_1{}^{-/-}$ mice, however, the dose-dependency was markedly shifted to the left (Figure 2c), with an average ND_{50} of 3.31 ± 0.50 fmol, i.pl. ($n=5$). Similarly, the average ND_{50} of BK in GluR$\varepsilon_1{}^{+/+}$ mice was 0.72 ± 0.36 pmol, i.pl. ($n=5$), being equivalent to that in ddY mice (0.71 ± 0.09 pmol, $n=5$), and it

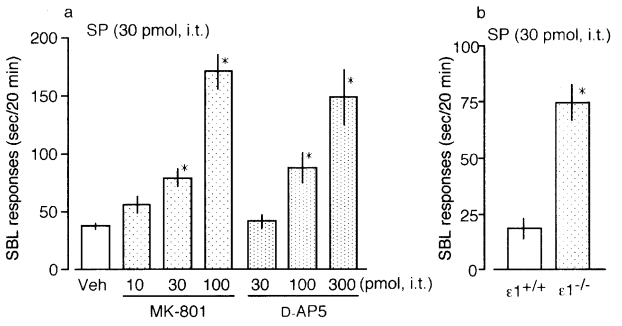

Figure 1 Enhancement of intrathecal application of SP-responses by glutamate receptor antagonist in capsaicin-pretreated mice, and in GluR$\varepsilon_1{}^{-/-}$ mice. (a) Enhancement of SP (i.t.)-induced SBL responses by MK-801, a non-competitive NMDA receptor antagonist or D-AP5, a competitive NMDA receptor antagonist in capsaicin-pretreatment mice. (b) Enhancement of SP (i.t.)-induced SBL responses in GluR$\varepsilon_1{}^{-/-}$ mice. Nociceptive activity was expressed as total period (s) showing nociceptive SBL behaviour for 20 min following i.t. injection of SP. The data were the mean ± s.e.mean from five or six separate experiments. *$P<0.05$, compared to vehicle-treatment.

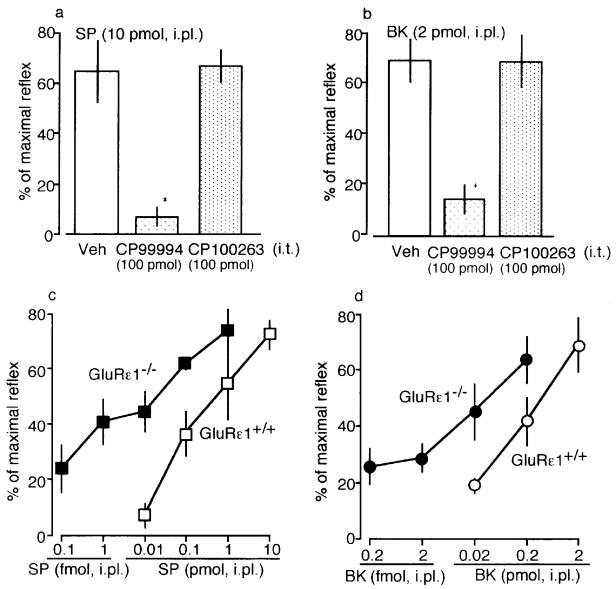

Figure 2 Enhancement of local application of BK- or SP-responses in GluR$\varepsilon_1{}^{-/-}$ mice. (a, b) Signal transduction in spinal cord with SP (or BK)-induced nociception. Blockade of SP (or BK)-induced nociceptive responses by the specific NK_1 receptor antagonist CP-99994 (100 pmol, i.t.), but not CP-100263 (100 pmol, i.t.), its inactive isomer. *$P<0.05$, compared to vehicle-treated mice. The data were analysed using Student's *t*-test following multiple comparisons of the analysis of variance (ANOVA). (c, d) Dose-responses curve for SP or BK (i.pl.)-induced nociceptive responses in GluR$\varepsilon_1{}^{-/-}$. SP- or BK-induced nociceptive activity was expressed as the ratio of maximal reflex in each mouse. Results represent the mean ± s.e.mean from five or six separate experiments.

was markedly reduced to 19.23 ± 7.77 fmol, i.pl. ($n=5$) in GluR$\varepsilon_1^{-/-}$ mice (Figure 2d).

Discussion We expected that nociceptive responses following stimulation of polymodal nociceptors would be reduced by NMDA antagonists, or in GluR$\varepsilon_1^{-/-}$ mice, since there are many reports that glutamate neurones and NMDA receptors play important roles in pain transmission (Murray *et al*., 1991; Liu *et al*., 1994; 1997). However the present observation demonstrates opposite changes in such treatments. The nociceptive responses induced by SP (i.t.) were enhanced by NMDA antagonists. In this experiment, we used capsaicin-treated mice to reduce the presynaptic contribution through primary afferent neurones. As these antagonists themselves have no effects on SBL responses, NMDA-antagonist-induced potentiation of SP-nociception can be attributed to the postsynaptic mechanism, being downstream of SP-responsive neurones. Similar results were also observed when GluR$\varepsilon_1^{-/-}$ mice were used, suggesting that endogenous glutamate is released from SP-responsive or downstream neurones to produce recurrent inhibition on such neurones through NMDA receptors. As it is unlikely that NMDA receptors have inhibitory actions by themselves, however, some unidentified inhibitory interneurons may be involved, as seen with the recurrent inhibitory Renshaw cells regulating motor neurone activity. On the other hand, peripheral stimulation of polymodal C-fibres by SP or BK was found to release SP and produced nociception in mice. Therefore, the present findings that SP- or BK-induced peripheral nociception was enhanced in GluR$\varepsilon_1^{-/-}$ mice would be further evidence for inhibitory NMDA receptor system downstream to the neurone receiving SP *in vivo* released from primary afferent neurones.

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