

Fig. 3. Ca<sup>2+</sup> response in nicotine-stimulated microglia. Fura-2-loaded cells were stimulated with 1 mM nicotine in Hank's buffer. The traces shown are representative of mean increase in [Ca<sup>2+</sup>]<sub>i</sub> of 30 cells in each culture. The cells were stimulated with 1 mM nicotine (A) or stimulated with 1 mM nicotine in the presence of 10 nM MLA (B) or 10 nM α-bungarotoxin (C), selective inhibitors of α7 receptor, in the absence of extracellular Ca<sup>2+</sup> (D), in the presence of 50 μM BAPTA-AM, an intracellular Ca<sup>2+</sup> chelator (E); 5 μM U73122, an inhibitor of PLC (F); 100 nM xestospongins C, an inhibitor of IP<sub>3</sub> receptors (G); or 1 μM thapsigargin, an inhibitor of the endoplasmic reticulum Ca<sup>2+</sup>-ATPase (H). Similar results were obtained in at least three independent experiments.

was markedly inhibited by 50 μM BAPTA-AM, an intracellular Ca<sup>2+</sup> chelator, and 5 μM U73122, a selective inhibitor of PLC (Fig. 3E,F). In addition, nicotine-induced Ca<sup>2+</sup> increase was also abolished by 100 nM xestospongins C, an IP<sub>3</sub> receptor antagonist, and by the store depletion with 1 μM thapsigargin, a Ca<sup>2+</sup>-ATPase inhibitor (Fig. 3G,H). All these results suggest that, unlike neuronal α7 nAChRs as typical ion channels, microglial α7 nAChRs might link to the activation of PLC and the release of Ca<sup>2+</sup> from IP<sub>3</sub>-sensitive intracellular Ca<sup>2+</sup> stores.

To explore further the properties of α7 nAChRs in microglia, we tried to detect nicotine-elicited currents by a whole-cell patch clamp technique. In our previous studies (Matsubayashi et al., 1997, 1998, 2004), this technique successfully detected nicotine-induced currents via α7 nAChRs in neurons from hippocampus and substantia nigra of rats. Interestingly, in microglia, no nicotine-induced currents were observed (Fig. 4A), although ATP-stimulated currents were detected in all the cells tested (Fig. 4B). These results suggest again that α7 nAChRs may function like metabotropic receptors rather than ligand-gated ion channels in microglia.

Furthermore, to investigate whether such novel signaling generated through α7 nAChRs may be involved in the modulation of TNF release, we examined the effects of xestospongins C, a blocker of IP<sub>3</sub> receptors, on nicotine-induced suppression of LPS-stimulated TNF release. Xestospongins C (100 nM) completely abolished the suppression of LPS-induced TNF release by nicotine in microglia (Fig. 5). Therefore, the Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores stimulated via α7 AChRs may play

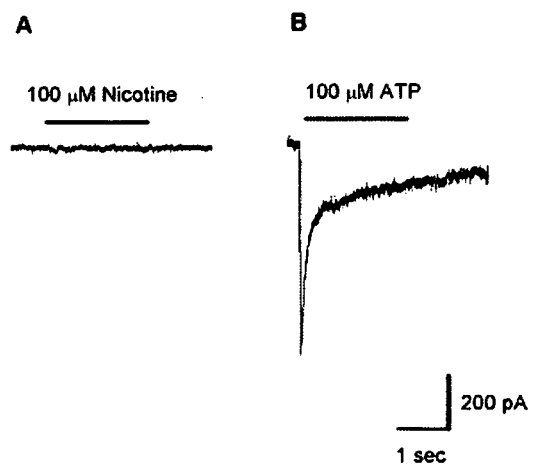


Fig. 4. Lack of nicotine-induced currents of rat microglia. A: Nicotine (100 μM) was delivered to microglia, and the whole-cell currents were recorded. B: ATP (100 μM) was applied to the same cells after nicotine stimulation. Similar results were obtained in at least 20 cells.

an important role in the modulation of LPS-induced TNF release.

### Nicotine Selectively Inhibits LPS-Induced Activation of JNK and p38 MAPK, but Not ERK, and Modulates a Posttranscriptional Step of TNF Synthesis

To investigate the mechanism underlying the modulation of LPS-induced TNF release via α7 AChRs, we

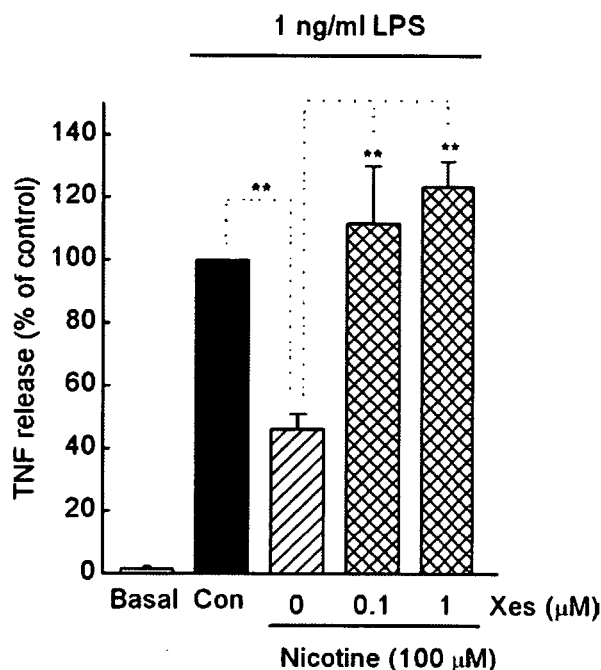


Fig. 5. Effects of xestospongins C, a blocker of IP<sub>3</sub> receptor, on nicotine-induced inhibition of TNF release in LPS-stimulated microglia. The microglia were treated with 0.1 μM or 1 μM xestospongins C for 30 min and treated with nicotine for another 10 min. Then, the cells were stimulated with 1 ng/ml LPS for 3 hr. The released TNF was measured by ELISA. Values are expressed as mean ± SEM of percentage of release compared with LPS alone from three independent experiments. Value of 100% for the release of TNF in LPS-stimulated microglia was 23.2 ± 4.0 ng/10<sup>6</sup> cells. \*\**P* < 0.01 compared with the control or nicotine-treated control as indicated with dotted lines (ANOVA with Dunnett's posttest).

examined whether nicotine could regulate LPS-induced MAP kinase activation, because all three members of the MAPK family (ERK, JNK, and p38) are shown to be involved in LPS-induced TNF production (Bhat et al., 1998; Lee et al., 2000). We also confirmed that all inhibitors, 10 μM U0126 (which inhibits MEK), 30 μM SP600125 (which inhibits JNK), and 15 μM SB203580 (which inhibits p38) significantly suppressed the release of TNF (Fig. 6A). In the case of TNF mRNA expression, U0126 caused a marked reduction, whereas neither SP600125 nor SB203580 did so (Fig. 6B). These results suggest that, although three kinases play critical roles in TNF release, they appear to have different modes; ERK acts to regulate transcription of the TNF gene, and p38 and JNK act posttranscriptionally. Nicotine was capable of selectively inhibiting the LPS-induced activation of JNK and p38 but appeared to have little effect on the activation of ERK (Fig. 6D). Nicotine itself did not cause the activation of any MAP kinases (data not shown). In accordance with these results, nicotine did not change LPS-induced TNF mRNA expression levels (Fig. 6C). Therefore, nicotine may modulate the release of TNF through regulating the posttranscriptional steps implicating JNK and p38 in LPS-stimulated microglia.

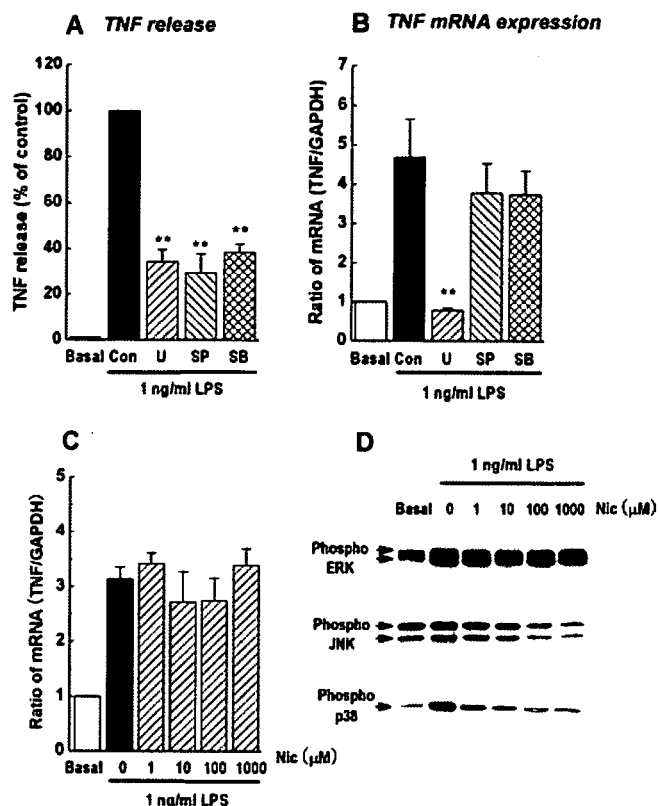


Fig. 6. Effects of U0126, SP600125, and SB203580 on LPS-induced TNF release and mRNA expression (A,B) and effects of nicotine on LPS-induced TNF mRNA expression (C) and on LPS-induced activation of ERK, JNK, and p38 MAP kinase (D) in microglia. A: The cells were treated with 10 μM U0126 (U), 30 μM SP600125 (SP), or 15 μM SB203580 (SB) for 15 min and stimulated with 1 ng/ml LPS for 3 hr. The released TNF were measured by ELISA. Values are expressed as mean ± SEM of percentage of release compared with LPS alone from three independent experiments. Values of 100% for the release of TNF in LPS-stimulated microglia were 12.5 ± 2.6 ng/10<sup>6</sup> cells. \*\**P* < 0.01 vs. control (ANOVA with Dunnett's posttest). B: The cells were treated with 10 μM U0126 (U), 30 μM SP600125 (SP), or 15 μM SB203580 (SB) for 15 min and stimulated with 1 ng/ml LPS for 1 hr. The expression of TNF mRNA was quantified by real-time RT-PCR. Values are shown as the ratio of TNF mRNA vs. GAPDH mRNA. Data are expressed as mean ± SEM of ratio of expression compared with LPS alone from three independent experiments. \*\**P* < 0.01 vs. control (ANOVA with Dunnett's posttest). C: The cells were treated with nicotine for 10 min and stimulated with 1 ng/ml LPS for 1 hr. The expression of TNF mRNA was quantified by real-time RT-PCR. Values are shown as the ratio of TNF mRNA vs. GAPDH mRNA. Data are expressed as mean ± SEM of ratio of expression compared with LPS alone from three independent experiments. D: The cells were treated with nicotine for 10 min and stimulated with 1 ng/ml LPS for 30 min. The phosphorylated (active) ERK, JNK, and p38 were detected by Western blotting with antibodies that recognize phosphorylated enzymes. The levels of each total MAP kinase were confirmed to be identical for each lane. Similar results were obtained in at least three independent experiments.

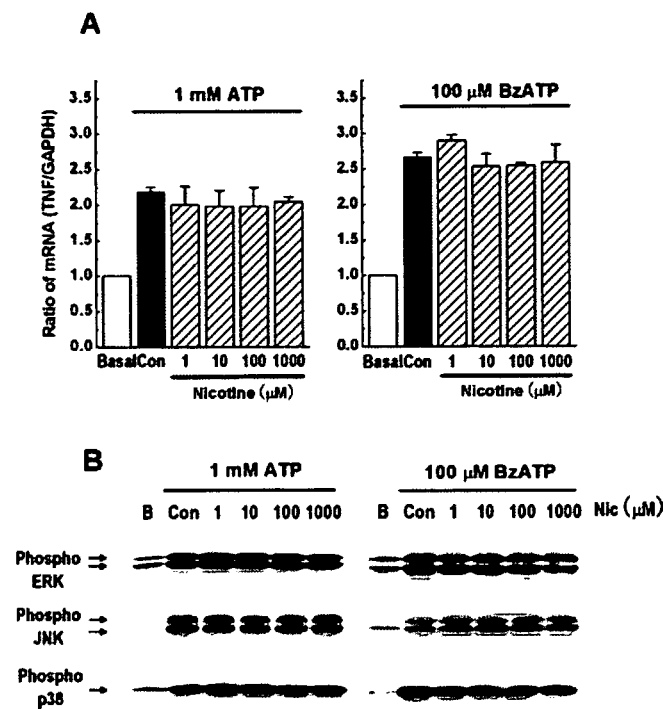


Fig. 7. Effects of nicotine on ATP- or BzATP-induced TNF mRNA expression and ATP- or BzATP-induced activation of ERK, JNK, and p38 MAP kinase in microglia. **A:** The cells were treated with nicotine for 10 min and stimulated with 1 mM ATP or 100 μM BzATP for 1 hr. The expression of TNF mRNA was quantified by real-time RT-PCR. Values are shown as the ratio of TNF mRNA vs. GAPDH mRNA. Data are expressed as mean ± SEM of ratio of expression compared with ATP or BzATP alone from three independent experiments. **B:** The cells were treated with nicotine for 10 min and stimulated with 1 mM ATP or 100 μM BzATP for 10 min. The phosphorylated (active) ERK, JNK, and p38 were detected by Western blotting with antibodies that recognize phosphorylated enzymes. The levels of each total MAP kinase were confirmed to be identical for each lane. Similar results were obtained in at least three independent experiments.

### Nicotine Does Not Affect the Activation of Any MAP Kinases in ATP- or BzATP-Stimulated Microglia

We previously reported the critical roles of each MAPK in ATP-stimulated TNF production; both ERK and JNK are involved in the regulation of TNF mRNA expression, and p38 is involved in the nucleocytoplasmic transport of TNF mRNA (Suzuki et al., 2004). Unlike LPS stimulation, nicotine did not affect the activation of any MAP kinases in ATP- or BzATP-stimulated microglia (Fig. 7B). Furthermore, nicotine did not change the mRNA expression of TNF after 1 hr stimulation with 1 mM ATP or 100 μM BzATP (Fig. 7A). These results suggest that nicotine enhances P2X<sub>7</sub> receptor-mediated TNF release, possibly via a posttranscriptional event, but not MAPK modification.

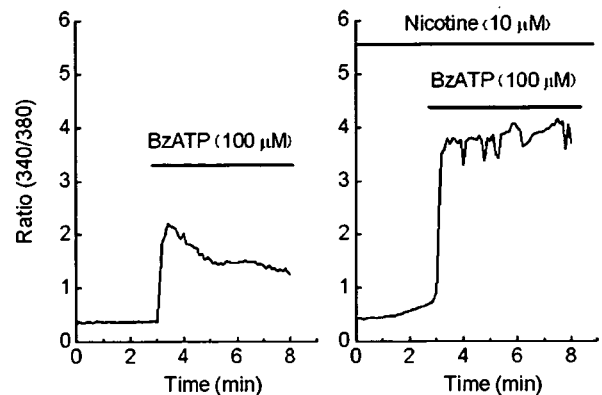


Fig. 8. Nicotine enhances BzATP-induced Ca<sup>2+</sup> signal in microglia. Fura-2-loaded cells were treated with 10 μM nicotine for 10 min and stimulated with 100 μM BzATP. The traces shown are representative of mean increase in intracellular Ca<sup>2+</sup> concentrations of 30 cells in each culture. Similar results were obtained in six independent experiments.

### Nicotine Enhances P2X<sub>7</sub> Receptor-Mediated Ca<sup>2+</sup> Influx in Microglia

To explore the mechanism underlying the enhancement of TNF release by nicotine in P2X<sub>7</sub> receptor-activated microglia, we examined the effect of nicotine on Ca<sup>2+</sup> response mediated by P2X<sub>7</sub> receptor agonist BzATP (Fig. 8). The most effective concentration (10 μM) of nicotine for increasing ATP-induced TNF release (Fig. 1B,C) was used to treat the cells. This low concentration of nicotine no longer elicited detectable Ca<sup>2+</sup> response by itself but after 10 minutes significantly enhanced Ca<sup>2+</sup> increase in response to BzATP. Therefore, nicotine may increase TNF release by enhancing P2X<sub>7</sub> receptor-mediated Ca<sup>2+</sup> influx.

### DISCUSSION

In this study, we present the following experimental evidence regarding the action of nicotine on microglial cell function. First, in rat brain primary cultured microglia, nicotine modulates the release of TNF, a multifunctional cytokine via α7 nAChRs, in a different manner depending on the nature of the stimulants; namely, negatively for LPS stimulation and positively for ATP or BzATP stimulation. Second, microglial α7 nAChR may not function as ligand-gated ion channels but instead couple to the activation of PLC and Ca<sup>2+</sup> release from IP<sub>3</sub>-sensitive intracellular Ca<sup>2+</sup> stores. This novel signaling is likely involved in nicotine modulation of LPS stimulation. Third, nicotine-induced suppression of TNF release seems to occur at a posttranscriptional step by interfering with the activation of JNK and p38. Finally, nicotine enhanced protective TNF release through enhancing P2X<sub>7</sub> receptor function, without affecting MAP kinase activation.

Upon ischemia and trauma, microglia secrete excessive amounts of TNF and induce inflammatory destruction in the brain (Barone et al., 1997). Most neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are also associated with neuronal inflammation and

overactivation of microglia (Akiyama et al., 2000; Gao et al., 2003; Liu and Hong, 2003). Therefore, the regulation of microglia cell activation should be very important issues in the treatment of such diseases. On the other hand, increasing evidence suggests that microglia also play a pivotal role in neuroprotection and that TNF itself is a neurotrophic mediator (Cheng et al., 1994; Gary et al., 1998; Nakajima and Kohsaka, 2004). In fact, we previously demonstrated that microglia that are activated via P2X<sub>7</sub> receptors secrete TNF, which in turn rescues the neurons from glutamate-induced cell death (Suzuki et al., 2004). The amounts of TNF released by LPS-stimulated microglia are much higher than the levels that occur from ATP or BzATP-stimulated microglia. Thus, the quantity of TNF released may reflect the state of the microglia with different functions, harmful or protective. Interestingly, the present results show that nicotine exerts opposite effects on TNF release in response to LPS and ATP/BzATP; namely, it significantly inhibited massive TNF release, whereas it enhanced protective TNF release. Therefore, nicotine may regulate the release of TNF toward neuroprotection by suppressing inflammatory TNF (via TLR4) and by strengthening neuroprotective TNF (via P2X<sub>7</sub> receptors).

These modulatory effects of nicotine have been proved to occur through the activation of  $\alpha 7$  AChRs in microglia. There are a number of reports describing the contribution of nAChRs to brain functions, including learning and memory (MacDermot et al., 1999), antinociception (Marubio et al., 1999), and neuroprotection (Messi et al., 1997; Laudénbach et al., 2002). Until recently, the common view was that  $\alpha 7$  nAChRs were expressed mainly in neurons, but increasing evidence suggests that this type of nAChR is also expressed in non-neuronal cells and is implicated in the regulation of cell function and Ca<sup>2+</sup> signalling (Buisson and Bertrand, 2002). In keratinocytes, for example, exposure to nicotine causes a reduction in cell migration (Zia et al., 2000), and, in astrocytes, nicotine modulates calcium signaling via calcium-induced calcium release (CICR; Sharma and Vijayaraghavan, 2001). More recently, it was revealed that  $\alpha 7$  nAChRs are also expressed in mouse peritoneal macrophages and mouse brain microglia (Wang et al., 2003, 2004; Shytle et al., 2004). In this study, we also confirmed the expression of functional  $\alpha 7$  nAChRs in rat primary cultured microglia, but they showed the unexpected properties.

Neuronal  $\alpha 7$  nAChRs are typical ligand-gated ion channels; however, microglial  $\alpha 7$  nAChRs seem to be different from those of their neuronal counterparts, which have a high permeability to Ca<sup>2+</sup>. First, in microglia, nicotine induced an increase in intracellular Ca<sup>2+</sup> concentrations even in the absence of extracellular Ca<sup>2+</sup>. Second, nicotine-induced Ca<sup>2+</sup> response was suppressed by chelating intracellular Ca<sup>2+</sup> (with BAPTA-AM), by inhibiting PLC (with U73122) as well as by blocking the IP<sub>3</sub> receptor (with xestospongine C). In addition, the electrophysiological studies showed that currents could not be elicited by nicotine stimulation, although ATP-induced currents were detected in the same cells tested. These results sug-

gest that microglial  $\alpha 7$  nAChRs may not function as a ligand-gated ion channel but may be instead coupled to the activation of PLC and the mobilization of Ca<sup>2+</sup> from IP<sub>3</sub>-sensitive intracellular Ca<sup>2+</sup> stores. Further studies will be required to clarify whether  $\alpha 7$  nAChRs could activate PLC in the same manner as G protein-coupled receptors, as previously reported as a nonclassical nicotine receptor in frog pituitary melanotrophs (Garnier et al., 1994), or via another mechanism. Recently, Severance et al. (2004) reported the finding that  $\alpha 7$  nAChRs have two isoforms, which may help to explain the multiplicity of  $\alpha 7$  nAChRs. Such a new typed  $\alpha 7$  nAChR isoform might be implicated in the diversity of signaling generated by nicotine.

All three members of MAP kinase family, ERK, JNK and p38, play key roles in the production of cytokines, including TNF (Bhat et al., 1998; Lee et al., 2000), but their roles are different. In LPS-stimulated microglia, as shown here, JNK and p38 mediate posttranscriptional steps of TNF production, whereas ERK is involved in the transcriptional regulation. Our results demonstrate that nicotine inhibited LPS-induced activation (phosphorylation) of JNK and p38, but not ERK, suggesting that nicotine may interfere with the posttranscriptional step of TNF synthesis by inhibiting the activation of JNK and p38. Consistently with this result, nicotine did not change TNF mRNA expression.

In the case of ATP stimulation, as we reported previously (Suzuki et al., 2004), all three MAP kinases play similar critical roles in TNF production; JNK and ERK regulate the transcription, and p38 is involved in the regulation of posttranscriptional steps, especially the export of TNF mRNA from nucleus to cytoplasm. Nicotine did not affect the activation of ERK, JNK, or p38 in ATP-stimulated microglia. Insofar as nicotine enhanced TNF release without promoting further mRNA expression, for at least 1 hour after stimulation,  $\alpha 7$  nAChRs may stimulate the posttranscriptional process of TNF synthesis through an MAP kinase-independent pathway. To investigate further the mechanism underlying nicotine-induced increase in TNF release in P2X<sub>7</sub> receptor-activated-stimulated microglia, we measured BzATP-elicited Ca<sup>2+</sup> signal after treating the cells with nicotine for 10 min at a concentration of 10  $\mu$ M, which showed the maximal increasing effect on TNF release. This low concentration of nicotine did not induce detectable Ca<sup>2+</sup> increase by itself but substantially increased the Ca<sup>2+</sup> responses elicited by BzATP. We previously confirmed that the BzATP-induced increase in intracellular Ca<sup>2+</sup> levels is due to Ca<sup>2+</sup> influx through P2X<sub>7</sub> receptors, so nicotine may modulate P2X<sub>7</sub> receptor functioning by increasing its channel activities. P2X<sub>7</sub> receptor-mediated MAP kinase activation is independent of extracellular Ca<sup>2+</sup> (Hide et al., 2000), in line with the present results showing that nicotine increased Ca<sup>2+</sup> signal without affecting MAP kinase activation. The mechanism of interaction between  $\alpha 7$  nAChR and P2X<sub>7</sub> receptors remains undefined.

Interestingly, macrophage  $\alpha 7$  nAChRs play a critical role in the cholinergic antiinflammatory pathway, which

has been revealed after electrical stimulation of vagus nerve (Borovikova et al., 2000; Wang et al., 2003). Moreover, a similar antiinflammatory role of microglial  $\alpha 7$  nAChRs in the mouse brain has been proposed (Shytle et al., 2004). Nicotine is a major component in tobacco, and it has been recognized that the incidence of neurodegenerative diseases such as Parkinson's disease is less in smokers, suggesting a neuroprotective role of nicotine (Picciotto and Zoli, 2002). Actually, it was shown that nicotine protects cortical neurons through neuronal  $\alpha 7$  nAChRs, which transduce signals to the PI3K/Akt pathway in a cascade (Kihara et al., 2001). Our present results as well as the report by Shytle et al. (2004) may give an additional explanation for nicotine-induced neuroprotection from the view of the regulation of neuroinflammation. In addition, our data provide other evidence for a possible protective role of nicotine, in that nicotine may be capable of enhancing ATP-mediated neuroprotective TNF release by microglia.

In conclusion, we have provided new evidence for the existence of nonconventional  $\alpha 7$  nAChRs in rat microglia. Although neuronal  $\alpha 7$  nAChRs belong to ligand-gated ion channels, microglial  $\alpha 7$  nAChRs may be coupled to the activation of PLC and evoke  $Ca^{2+}$  release from  $IP_3$ -sensitive  $Ca^{2+}$  stores. Such unique receptors should play an important role in neuroprotection, because the activation of these receptors is capable of modulating the stage of microglia from the overactive inflammatory type to a protective type. Therefore, it is conceivable that nicotinic receptor agonists that target  $\alpha 7$  nAChRs in microglia may have significant therapeutic potential in neuroinflammatory diseases in the brain.

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# NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain

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## Abstract

Neurons in the latero-capsular part of the central nucleus of the amygdala (CeA), a region now called the “nociceptive amygdala”, receive predominantly nociceptive information from the dorsal horn through afferent pathways relayed at the nucleus parabrachialis (PB). Excitatory synaptic transmission between the PB afferents and these neurons is reported to become potentiated within a few hours of the establishment of arthritic or visceral pain, making it a possible mechanism linking chronic pain and unpleasant negative emotional experiences. However, it remains unknown whether such synaptic potentiation is consolidated or becomes adaptively extinct in the longer-lasting form of chronic pain, such as neuropathic pain, an as yet serious and frequent type of pain of important clinical concern. To address this issue, we recorded postsynaptic currents in CeA neurons evoked by PB tract stimulation in acute brain slices from young rats with neuropathic pain, as evaluated by tactile allodynic responses, following unilateral spinal nerve ligation made 1 week earlier. CeA neurons contralateral to the nerve ligation showed significantly larger-amplitude postsynaptic currents than those in the ipsilateral CeA and sham- and non-operated groups. The degree of synaptic potentiation, as compared between two sides, was positively correlated to that of tactile allodynia responses. In addition, blockade of NMDA receptors did not affect this potentiation. We conclude that potentiation of the PB–CeA synapse is consolidated in long-lasting neuropathic pain but that this potentiation results from a molecular mechanism distinct from that in arthritic and visceral pain.

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**Keywords:** Neuropathic pain; Amygdala; Excitatory postsynaptic currents; Nucleus parabrachialis; Tactile allodynia; Emotion; NMDA receptor

## 1. Introduction

Persistent chronic pain has a strong emotional component associated with negative affection (Price, 2002; McWilliams et al., 2003; Rhudy and Meagher, 2003). The amygdala, a key player in emotional responses to various sensory stimuli (Davis, 1998; LeDoux, 2000; Zald, 2003), is one of the principle structures linking pain sensation and emotional response as it receives

pain-related information via the spino-parabrachio-amygdaloid pain pathway (Gauriau and Bernard, 2002). The nociceptive information arising from the superficial layers of the dorsal horn crosses the midline and forms synapses in the pontine parabrachial area (PB). From this area, an ascending projection reaches the central nucleus of the amygdala (CeA), in particular the latero-capsular part of the CeA, now called the “nociceptive amygdala” because of its abundance of nociceptive neurons (Bernard et al., 1992).

The amygdala is also associated with the synaptic plasticity underlying fear and painful memories in animals and humans (Maren, 2005; Phelps and LeDoux,

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2005). Such plasticity also occurs with nociceptive inputs. For example, using acute-onset models of the arthritis and visceral pain models of the rat, Neugebauer and colleagues (2003; 2004) demonstrated that excitatory synaptic transmission and neuronal excitability in CeA are potentiated due to augmented NMDA receptor functions in brain slice preparations prepared 6–8 h after the generation of the pain models.

Another frequently observed form of chronic pain in human patients is neuropathic pain, resulting from various etiologies including cancer, diabetes-linked neuropathy and peripheral nerve injury (Russo and Brose, 1998; Woolf and Mannion, 1999; Scholz and Woolf, 2002). For these patients, the pain sensation is long lasting and seriously affects quality of life, including its emotional aspects, against which drug-based therapeutic strategy has yet to be established. Despite recent demonstrations that hyperexcitability of neurons in the dorsal horn of the spinal cord underlies neuropathic pain (Coull et al., 2003; Tsuda et al., 2003; Inoue et al., 2004; Coull et al., 2005), how neuropathic pain affects the emotional responses in the brain network remains unknown.

To address this issue, we examined whether synaptic transmission in the CeA is affected in an animal model of neuropathic pain, for the following reasons. First, in animal models, neuropathic pain can be quantitatively evaluated by measuring touch-induced allodynia, a form of hypersensitivity to tactile stimulation. Second, because the hypersensitivity to tactile stimuli is long lasting in animal models, it allows analysis over several days of the functional synaptic changes following a sustained increase in pain information reaching the amygdala, in turn allowing the examination of any distinct mechanisms underlying the plastic changes in the late phase of chronic pain, as occurs in hippocampal late-phase long-term potentiation (LTP) which involves molecular mechanisms distinct from early phase LTP (Nayak et al., 1998; Soderling and Derkach, 2000). Here, we report that the synaptic transmission mediated by non-NMDA receptors is potentiated in the CeA with high correlation to allodynia establishment in chronic (>6 days) pain models. A part of this work was previously presented in abstract form (Ikeda et al., 2005).

## 2. Methods

### 2.1. Neuropathic pain model

The manipulation of the animals was approved by the Animal Experiment Committee at the Jikei University School of Medicine and conformed to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Japan (1998) and to the guidelines of the International Association for

the Study of Pain (Zimmermann, 1983). Young Wistar rats of either sex aged 20–26 days postnatal were used in this study. They were no older than 33 days postnatal at the time of electrophysiological recordings and we observed no systematic difference between the results from the male and female rats. The data from both sexes are therefore pooled in this study. Rats were anesthetized initially with diethyl ether and mounted on a surgical platform in a prone position with the limbs fixed. Anesthesia was then maintained with sevoflurane or isoflurane (1.5–2% in 100% O<sub>2</sub>). A longitudinal incision (about 0.5 cm in length) was made at the midline of the lower lumbar using a sterile surgical blade. The left paraspinal muscles were isolated and the left L6 transverse process was exposed. Under a dissecting microscope, the left L6 transverse process covering the L4 and L5 spinal nerves was carefully removed. The left L5 spinal nerve was isolated and tightly ligated with 6–0 silk threads in the “ligation” group ( $n=39$ ), ligation was not performed in the “sham-operated” group ( $n=25$ ), and no surgical procedure was carried out in the “non-operated” group ( $n=13$ ). After surgery, the muscles were sutured in layers, the skin was closed using nylon thread (4–0), and anesthesia discontinued. Animals fully recovered from anesthesia within 1 h and showed no signs of distress as evidenced by the monitoring of breathing patterns, grooming behavior and locomotion in the cage. The rats were housed in cages filled with soft cushion-like flooring in a temperature- (around 25 °C) and humidity- (around 50%) controlled room. Water and food were available ad libitum and there was no observed difference in water and food consumption between the groups. Body weight measured at post-operational day 6–7 did not differ significantly between the ligation, sham-operated and non-operated groups ( $P=0.386$ ; ANOVA), at  $75.6 \pm 2.7$  g (mean  $\pm$  SEM;  $n=34$ ),  $79.3 \pm 2.0$  g ( $n=25$ ) and  $81.2 \pm 5.2$  g ( $n=13$ ), respectively. Behavior was monitored regularly and no aberrant symptoms arising from excessive nociception were observed except for a mild deformity of the lesioned paw (Kim and Chung, 1992), because of which, the von Frey filament test described below could not be conducted entirely in a blind manner. Every 1–2 days, rats were individually moved to a cage for measurement of the paw withdrawal threshold (see below), during which special care was taken to avoid noxious stimulation of the hindlimb. No aberrant responses were observed during interactions with the investigator.

### 2.2. von Frey filament test

The paw withdrawal threshold to mechanical stimuli was evaluated by well-trained experimenters according to the previously reported method (Tsuda et al., 2003). Mechanical stimuli were applied using von Frey filaments of different rigidities (0.4–15.0 g). Each rat was placed on a metal mesh floor (25 cm  $\times$  25 cm) and a von Frey filament was applied manually from beneath. The 50% threshold was estimated by the up and down method (Chaplan et al., 1994). Care was taken to reduce the number of trials to avoid unnecessary pain sensation. The tests were performed immediately before the operation and repeated every 1–2 days for all rats until the final test immediately before decapitation (Fig. 1).



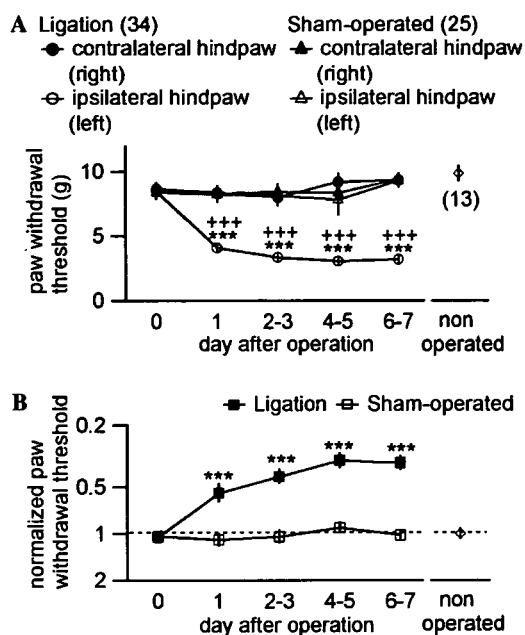


Fig. 1. Side-specific tactile allodynia after unilateral spinal nerve ligation. (A) Summary of the time-course of paw withdrawal threshold in the ligation, sham- and non-operated groups. Open and filled circles indicate responses in the hindpaws ipsilateral (left) and contralateral (right) to the ligation, respectively. Open and filled triangles show the responses in the hindpaws ipsilateral (left) and contralateral (right) to the sham-operation, respectively. Diamond shows the value from the non-operated group. Mean  $\pm$  SEM.  $***P < 0.001$  vs. sham- and non-operated groups (ANOVA).  $+++P < 0.001$  vs. values before operation (measured at post-operational day 0; ANOVA). Numbers in parentheses indicate the number of rats. (B) Time course of the paw withdrawal threshold (value in the hindpaw ipsilateral to the ligation (left) is normalized by that in the hindpaw contralateral to the ligation (right) measured in the same rat). Filled and open squares indicate values from the ligation and sham-operated groups, respectively. Diamond shows that value from the non-operated group. Mean  $\pm$  SEM.  $***P < 0.001$  vs. sham- and non-operated groups (Mann-Whitney *U* test).

### 2.3. Preparation of transverse brain slices

Immediately after the final von Frey filament test on post-operational day 6–7, the rats were decapitated under ketamine anesthesia (100–150 mg/kg, i.p.). In a subset of experiments ( $n = 5$ ), rats were anesthetized with isoflurane (2–3% in 100%  $O_2$ ) before decapitation to analyze the influence of ketamine anesthesia. We obtained both ipsilateral (left) and contralateral (right) coronal brain slices to the operation side (left) from the ligation group (69 unilateral slices from 34 rats), sham-operated group (51 unilateral slices from 25 rats) and non-operated group (33 unilateral slices from 13 rats) according to previously described procedures (Shigetomi and Kato, 2004; Ikeda and Kato, 2005). Briefly, the brain was dissected out and cut at the midline. The dissected hemisphere was secured on the cutting stage of a vibrating blade slicer (DSK-1000, Dosaka EM) with the rostral end upwards. Coronal slices of 400- $\mu$ m thickness containing the amygdala were cut in the ice-cold cutting artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 3 KCl, 0.1  $CaCl_2$ , 5 or 3  $MgCl_2$ , 1.25  $NaH_2PO_4$ , 10 D-glucose, 0.4 L-ascorbic acid and 25  $NaHCO_3$  (pH 7.4 bubbled with 95%  $O_2$  + 5%  $CO_2$ ; osmolarity,

$\sim 310$  mOsm/kg). The slices were first incubated in a holding chamber with a constant flow of standard ACSF, of which the concentrations of  $CaCl_2$  and  $MgCl_2$  were 2 and 1.3 mM, respectively, at 37  $^\circ C$  for 30–45 min. The slices were kept at room temperature (20–25  $^\circ C$ ) in the same chamber until electrophysiological recording. Each slice was transferred to a recording chamber ( $\sim 0.4$ -ml volume) and fixed with nylon grids to a platinum frame. The slice was submerged in and continuously superfused at a rate of 1–2 ml/min with standard ACSF. To isolate excitatory synaptic inputs, 100  $\mu M$  picrotoxin (Sigma) and 1  $\mu M$  strychnine HCl (Sigma) were dissolved in ACSF and bath-applied.

### 2.4. Patch-clamp recordings and cell visualization

Central amygdala (CeA) neurons were visually identified under an upright microscope (BX-50WI, Olympus) with infrared differential interference contrast (IR-DIC) optics. The IR-DIC images were captured using a chilled CCD camera (C5985-02, Hamamatsu Photonics) and stored digitally on a computer. The whole-cell transmembrane current was recorded from neurons in the left and right CeA (i.e., ipsilateral and contralateral, respectively, to the ligation). The patch-clamp electrodes were made from borosilicate glass pipettes (1B120F-4; World Precision Instruments). The composition of the internal solution was (in mM) 120 potassium gluconate, 6 NaCl, 1  $CaCl_2$ , 2  $MgCl_2$ , 2 ATPMg, 0.5 GTPNa, 12 phosphocreatine  $Na_2$ , 5 EGTA and 10 HEPES hemisodium (pH 7.2 as adjusted with KOH; osmolarity,  $\sim 310$  mOsm/kg). The tip resistance of the electrode was 3–6 M $\Omega$ . The evoked excitatory postsynaptic currents (eEPSCs) were recorded at a holding potential of  $-70$  mV. The membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments), low-pass filtered at 2 kHz and sampled at 4 kHz with a PowerLab interface (ADInstruments). To analyze the type of glutamate receptors involved, either 10  $\mu M$  6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma; a non-NMDA receptor antagonist) or 50  $\mu M$  D(-)-2-amino-5-phosphonopentanoic acid (APV; Sigma; a NMDA receptor antagonist) was dissolved in ACSF and bath-applied for more than 10 min. All recordings were made at room temperature (20–25  $^\circ C$ ). The order of recordings from the right and left amygdala was randomized to avoid side-dependent differences due to changes in the viability of neurons during the time from slice preparation to recording. All compounds except those noted above were purchased from Sigma or Nacalai Tesque (Kyoto, Japan).

### 2.5. Afferent pathway stimulation

To activate action potential-dependent glutamate release from the afferent fibers arising from the PB and basolateral amygdala (BLA), under microscopic control we carefully located the stimulating electrode on the fiber tract dorsomedial to the CeA (PB tract) and in the BLA near the borderline to the CeA; “PB stimulation” and “BLA stimulation”, respectively. To calculate the paired-pulse ratio of EPSCs as a measure of changes in presynaptic release properties (McKernan and Shinnick-Gallagher, 1997), double pulses with an inter-stimulus interval of 50 ms were delivered. The paired-pulse ratio (PPR) was calculated as the second EPSC amplitude (EPSC2) normalized by the first EPSC amplitude (EPSC1).

PB–CeA pathways showed paired-pulse facilitation (i.e., PPR > 1) in all neurons in a similar manner as that of a previous report (Neugebauer et al., 2003). Stimulation intensity was fixed at a sub-maximal value without spike current generation in the experiments examining the effects of glutamate receptor antagonists and in those for PPR measurement.

### 2.6. Data and statistical analysis

The recorded membrane current was analyzed off-line by an Igor Pro 5 (WaveMetrics, OR, USA) following procedures written by F.K. Peak amplitude was measured on the basis of the averaged waveform of eEPSCs (eight consecutive trials). Values are expressed as mean values  $\pm$  standard error of the mean (SEM). Differences in the values were compared using one-way analysis of variance (ANOVA) followed by a post hoc test (Dunnett), the paired *t*-test and Mann–Whitney *U* test. Differences with a probability (*P*) less than 0.05 were considered significant.

## 3. Results

### 3.1. Side-specific establishment of tactile allodynia after unilateral spinal nerve ligation

A total of 72 rats were divided into the ligation ( $n = 34$ ), sham-operated ( $n = 25$ ) and non-operated ( $n = 13$ ) groups. The paw withdrawal threshold was measured using von Frey filaments on post-operational day 0 in the ligation and sham-operated groups and immediately before the decapitation in the non-operated groups. The paw withdrawal threshold of the left hindlimb ipsilateral to the ligation decreased significantly at post-operational day 1 and remained decreased until post-operational day 6–7 (open circles in Fig. 1A). Such a decrease was not observed in the contralateral (right) hindlimb in the ligation group (filled circles in Fig. 1A) or in the bilateral hindlimbs in sham-operated rats (open and filled triangles in Fig. 1A), in which the paw withdrawal threshold gave consistently similar values to those in the non-operated group (diamond in Fig. 1A). These results indicate that animals that underwent spinal nerve ligation developed stable tactile allodynia within 1 week of operation, which is in good accordance with the results described by Kim and Chung (1992) who developed this neuropathic pain model. In

addition, as there was no significant change in the paw withdrawal threshold in the right hindlimb contralateral to ligation on post-operational day 6–7 (filled circles in Fig. 1A), it is likely that the establishment of allodynia using this operative procedure is limited to the ipsilateral side to the ligation (i.e., the left side in the present experiments), at least within 1 week of operation in the rats at the ages used (see Discussion in Kim and Chung, 1992). Using these characteristics, we evaluated the degree of allodynia establishment in each rat, which varied from animal to animal, by normalizing the paw withdrawal threshold of the left hindlimb by that of the right hindlimb (ipsilateral/contralateral). Fig. 1B indicates the time-course of this “normalized paw withdrawal threshold” after operation in the different groups. The normalized paw withdrawal threshold did not change significantly from post-operational day 0–7 in the sham-operated group (open squares in Fig. 1B) and it was not significantly different from that in the non-operated group, but it significantly decreased in the ligation group to a mean value of  $0.35 \pm 0.04$  on post-operational day 6–7 (filled squares in Fig. 1B). This result indicates that this normalized paw withdrawal threshold is a good index for the degree of side-specific tactile allodynia in each animal following unilateral nerve ligation.

### 3.2. Side-specific increase in the amplitude of EPSC evoked by PB–CeA pathway stimulation

Immediately following the final measurement of the paw withdrawal threshold on post-operational day 6–7, brain slices containing the amygdala were prepared for electrophysiological recordings. We recorded a total of 228 visually identified neurons in the capsular part of the CeA (Table 1). There was no apparent morphological difference in the amygdala nuclei or in the neurons as observed with IR-DIC optics between either side in the ligation, sham- and non-operated animals. There were also no differences between the groups in the ease with which good quality whole-cell recordings could be obtained from neurons. No significant difference was observed in the resting membrane potential or whole-cell capacitance between neurons from the right and left CeA in the ligation, sham- and non-operated groups

Table 1  
Membrane properties of the CeA neurons recorded

	Ligation Right CeA ( $n = 53$ )	Left CeA ( $n = 52$ )	Sham-operated Right CeA ( $n = 43$ )	Left CeA ( $n = 36$ )	Non-operated ( $n = 44$ )
Resting potential (mV)	$-63.6 \pm 0.9$	$-61.7 \pm 0.8$	$-62.5 \pm 0.8$	$-64.2 \pm 1.0$	$-61.8 \pm 1.0$
Input resistance (M $\Omega$ )	$147.8 \pm 8.7$	$154.5 \pm 8.0$	$169.4 \pm 9.7$	$160.9 \pm 9.7$	$166.4 \pm 6.5$
Cell capacitance (pF)	$17.7 \pm 0.8$	$16.9 \pm 0.8$	$15.7 \pm 0.9$	$16.1 \pm 1.0$	$14.5 \pm 0.7$
Action potential threshold (mV)	$-42.8 \pm 0.7^*$	$-41.1 \pm 0.8$	$-39.9 \pm 0.8$	$-39.4 \pm 0.9$	$-37.3 \pm 1.0$

Values were measured immediately following establishment of the whole-cell mode. Mean  $\pm$  SEM. \**P* < 0.05 vs. values in sham- and non-operated rats (ANOVA).

(Table 1). In contrast, the threshold membrane potential for action potential generation as measured by injecting depolarizing current under the current-clamp mode was significantly more hyperpolarized for neurons from the ligation group than from the sham- and non-operated groups. These data suggest that, as in the case of arthritic pain, some of the membrane properties of the CeA neurons are altered in the neuropathic pain model, but in a manner less manifest than in the arthritic pain models, which is accompanied by changes in resting membrane potential and input resistance (Neugebauer et al., 2003).

In the arthritic and visceral pain models, excitatory synaptic transmission at PB–CeA synapses is potentiated when measured 6–8 h after treatment (Neugebauer et al., 2003; Han and Neugebauer, 2004). We examined whether such plastic changes in CeA synaptic transmission occur in the longer-term neuropathic pain models. First, we stimulated the PB tract and recorded eEPSCs. When the stimulation intensity was gradually increased, PB tract stimulation gave rise to eEPSCs of larger amplitude with lower intensity in the right CeA (thick traces) than in the left CeA (thin traces) in the ligation group (Fig. 2A top). eEPSC amplitude in the right CeA of the ligation group was also significantly larger than that in the bilateral CeA in sham-operated rats (Fig. 2A bottom). In a large portion of slices, the larger amplitude of eEPSCs in the right CeA of the ligation group resulted in non-clamped spike-like fast inward current (thick trace in Fig. 2A top) at intensities of 500–1000  $\mu\text{A}$ , whereas the neurons in the left CeA of the ligation group and in the bilateral CeA in sham-operated rats did not generate such spike-like currents with stimulation intensities lower than 1000  $\mu\text{A}$  (see below for the stimulation intensity for action potential generation under the current-clamp mode; Fig. 3). Fig. 2B indicates the relation between intensity of the PB tract stimulation and eEPSC amplitude. From 100–600  $\mu\text{A}$ , eEPSC amplitude in the right CeA neurons (filled circles in Fig. 2B) was significantly larger than that in the neurons belonging to other groups. There was no significant difference between neurons in the left CeA in the ligation group (open circles) and those in bilateral CeA in the sham- and non-operated groups (triangles and diamonds, respectively) at any stimulation intensity in the range of 50–4000  $\mu\text{A}$  (Fig. 2B). These results strongly suggest that the potentiation of the PB–CeA transmission in the ligation group occurs in a manner highly restricted to the side receiving nociceptive information from the ligated side (i.e., the contralateral right CeA in the present experiments). We then evaluated the degree of synaptic potentiation in each animal by normalizing the eEPSC amplitude in the right CeA by that in the left CeA recorded from slices from the same rat. If several neurons ( $n \leq 4$ ) were recorded from one side of a slice, their mean eEPSC value was used. This right/left

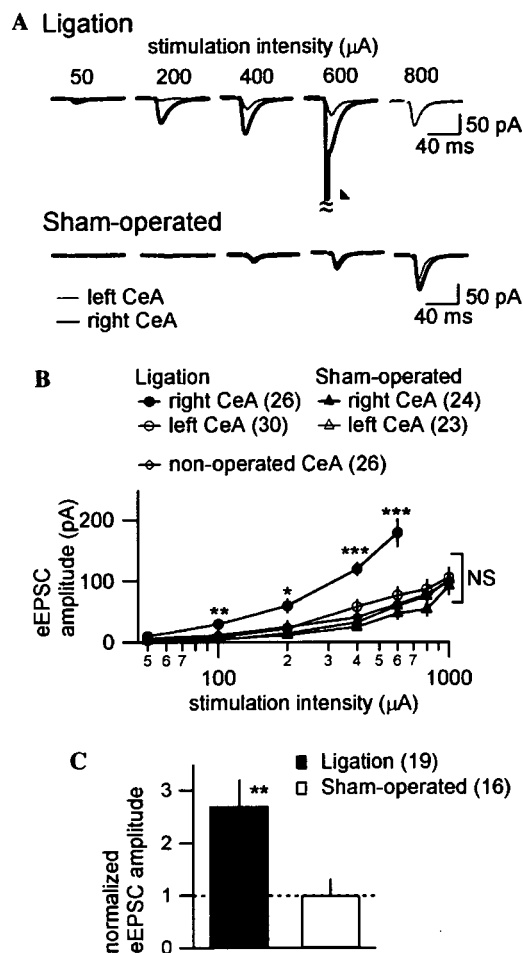


Fig. 2. EPSC evoked by PB tract stimulation in the CeA in neuropathic pain models. (A) Averaged waveforms of EPSCs evoked by PB tract stimulation at different intensities (average of eight consecutive responses). Top and bottom panels are recordings from neurons in the ligation and sham-operated groups, respectively. Thick and thin traces are recordings from neurons in the right and left CeA, respectively. (B) Relationship between stimulation intensity and eEPSC amplitude. Circles, triangles and diamonds indicate values from neurons in the ligation, sham-operated and non-operated groups, respectively. Mean  $\pm$  SEM. Open and filled symbols indicate values from neurons in the left and right CeA, respectively. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared to the left CeA in the ligation group, and to bilateral CeA in the sham- and non-operated groups (ANOVA). NS, not significantly different between groups (ANOVA). Numbers in parentheses indicate the number of neurons. (C) Summary of the normalized eEPSC amplitude (eEPSC amplitude in the right CeA normalized by that in the left CeA from the same rat) in the ligation (filled bar) and sham-operated (open bar) groups. eEPSCs were evoked by 400- $\mu\text{A}$  stimulation. \*\* $P < 0.01$  vs. sham-operated group (Mann–Whitney  $U$  test). Numbers in parentheses indicate the number of rats.

ratio of eEPSC amplitude (“normalized eEPSC amplitude”) was significantly larger in the ligation group than in the sham-operated group (Fig. 2C). In addition, this ratio was  $\sim 1$  in the sham-operated group (Fig. 2C), suggesting that the significant and marked difference in eEPSC amplitudes between right and left CeA in the

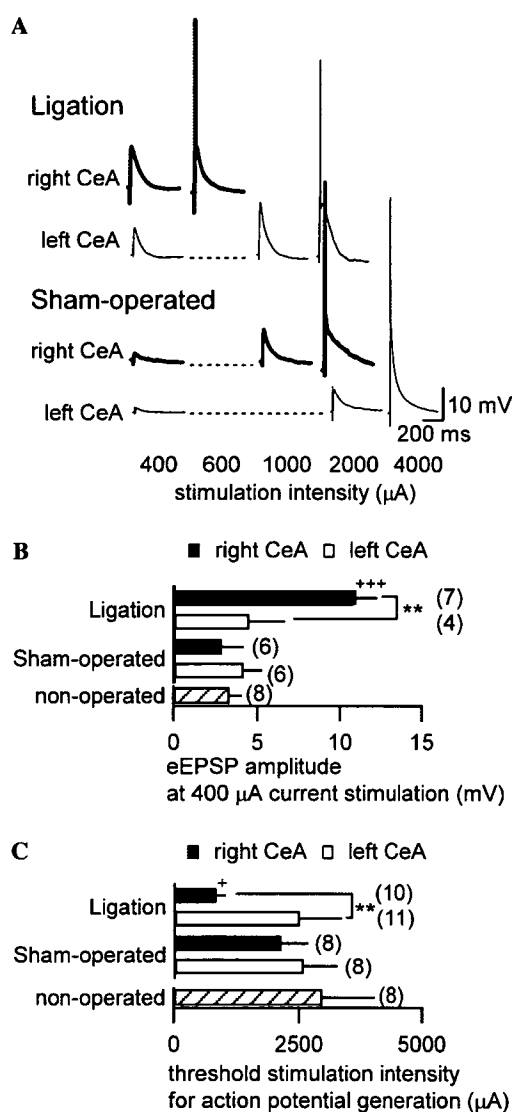


Fig. 3. EPSP and action potential generation by PB tract stimulation in the CeA in neuropathic pain models. (A) Averaged waveforms of EPSPs evoked by PB tract stimulation at different intensities (average of eight consecutive responses). Top and bottom panels are recordings from the ligation and sham-operated groups, respectively. Thick and thin traces show the membrane potential recording from the right and left CeA, respectively, from the same rat. (B) The summary of EPSP amplitude evoked by PB tract stimulation at 400- $\mu$ A stimulation. Open and filled bars indicate values from neurons in the left and right CeA, respectively. Hatched bar indicates the value from bilateral neurons in non-operated rats. \*\* $P < 0.01$  vs. left CeA in the ligation group; \*\*\* $P < 0.001$  compared to other groups (ANOVA). Numbers in parentheses indicate the number of neurons. (C) Summary of the threshold stimulation intensity for action potential generation in CeA neurons. Open and filled bars indicate values from neurons in the left and right CeA, respectively. Hatched bar indicates the value from bilateral neurons in non-operated rats. \*\* $P < 0.01$  compared to the left CeA in the ligation group; <sup>+</sup> $P < 0.05$  compared to other groups (Mann-Whitney  $U$  test). Numbers in parentheses indicate the number of neurons.

ligation group does not primarily result from the asymmetric nature of the PB–CeA connection or from the asymmetry inherent in the recording and stimulation

systems. Rather, it is likely that the synaptic potentiation in CeA was highly dependent on the side relative to the nociceptive input.

Does such synaptic potentiation in the contralateral (right) CeA in the ligation group affect the synaptic excitability of the CeA neurons? To directly address this issue, we recorded the membrane potential of the neurons under the current-clamp mode. When the intensity of the PB tract stimulation was increased, neurons in the right CeA in the ligation group generated action potential at a lower intensity than those in the left CeA as well as in those in sham-operated rats (Fig. 3A). In accordance with the above eEPSC data, the amplitude of evoked EPSP (eEPSP) was significantly larger in the right CeA in the ligation group than in the other groups (Fig. 3B), which resulted in a significantly lower minimum stimulation intensity required to make the post-synaptic neurons fire in the right CeA neurons (Fig. 3C). These results strongly indicate that such potentiated excitatory synaptic transmission facilitates the PB–CeA input to excite the neurons postsynaptic to the CeA neurons in a manner specific to the side receiving increased nociceptive information.

### 3.3. Increase in the amplitude of EPSC evoked by BLA–CeA pathway stimulation was less side-specific

The CeA neurons also receive strong excitatory inputs from the BLA, which transmits polymodal integrated sensory information originating in higher structures such as the thalamus and cortex (Shi and Davis, 1999; Sah et al., 2003; Pare et al., 2004). The striking difference between these reported models is that both PB–CeA and BLA–CeA transmissions are enhanced in the arthritis pain model, whereas BLA–CeA transmission is unaffected in the visceral pain model (Han and Neugebauer, 2004). It is therefore conceivable that enhancement of BLA–CeA transmission depends on the type of pain.

Stimulation of the ventrolateral pathway arising from the BLA resulted in eEPSC in CeA neurons. The BLA stimulation evoked EPSCs of larger amplitude in the neurons in the right CeA (Fig. 4A top, thick traces) than those in the left CeA (thin traces). In addition, in the majority of the left CeA neurons, the eEPSC amplitude was larger than that recorded in sham-operated rats (Fig. 4A top and bottom). Fig. 4B indicates the summary data of the amplitude of BLA–CeA eEPSC in response to increasing stimulation intensity. The amplitude of BLA–CeA eEPSC in the right CeA neurons in the ligation group (filled circles in Fig. 4B) was significantly larger than that in the left CeA neurons in the ligation group than that in the sham- and non-operated groups at 100–600  $\mu$ A stimulation. However, unlike in PB–CeA transmission, the amplitude of BLA–CeA eEPSC in the left CeA neurons in the ligation group

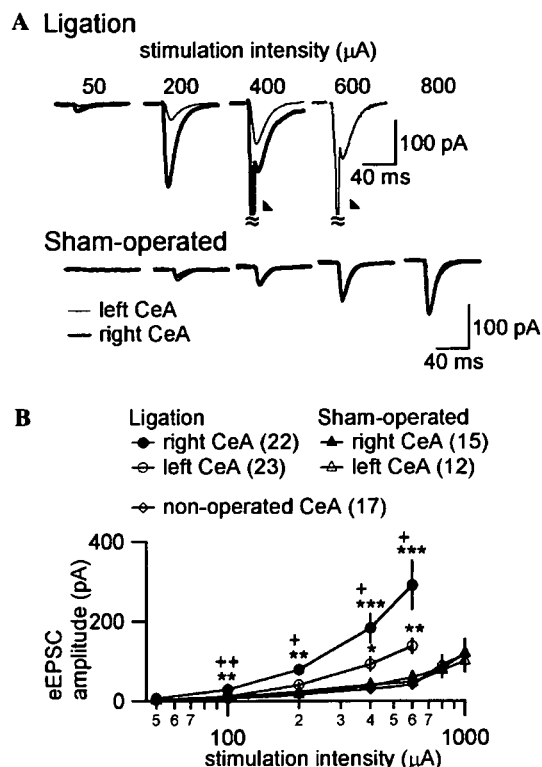


Fig. 4. EPSC evoked by BLA stimulation in the CeA in neuropathic pain models. (A) Averaged waveforms of EPSCs evoked by BLA stimulation at different intensities (average of eight consecutive responses). Top and bottom panels are recordings from neurons in the ligation and sham-operated groups, respectively. Thick and thin traces are recordings from neurons in the right and left CeA, respectively. (B) Relationship between stimulation intensity and eEPSC amplitude. Circles, triangles and diamonds indicate values from neurons in the ligation, sham-operated and non-operated groups, respectively. Mean  $\pm$  SEM. Open and filled symbols indicate values from neurons in the left and right CeA, respectively. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. sham- and non-operated groups; + $P < 0.05$ ; ++ $P < 0.01$  vs. left CeA in the ligation group (ANOVA). Numbers in parentheses indicate the number of neurons.

(open circles in Fig. 4B) was also significantly larger than in the sham- and non-operated rats at intensities larger than 400  $\mu\text{A}$  (triangles and diamonds, respectively, in Fig. 4B). These results indicate that, despite the difference in degree, BLA–CeA transmission is enhanced in both CeAs contra and ipsilateral to the ligation in the neuropathic pain models, suggesting that synaptic potentiation in the BLA–CeA pathway is less side-specific than that in the PB–CeA pathway. As with the PB–CeA synapse, the increased BLA–CeA eEPSC in the ligation group also resulted in eEPSP of larger amplitude that generated postsynaptic firing at significantly lower stimulation intensity than in the sham- and non-operated groups (data not shown). Because the eEPSC amplitude in response to BLA stimulation was increased in both sides of the CeA, the contralateral/ipsilateral ratio (“normalized eEPSC amplitude”) would not represent the degree of side-

specific potentiation in each rat and was therefore not estimated for the BLA–CeA transmission.

### 3.4. Increase in evoked EPSC amplitude at PB–CeA was positively correlated with the establishment of tactile allodynia

The above results indicate that in animal models of neuropathic pain, both the allodynic responses in vivo and synaptic transmission in the CeA in vitro are increased in a side-specific manner. There are, however, considerable rat-to-rat variations in these phenomena. If these two types of post-operative changes are mutually related, behavioral and electrophysiological changes in each rat would be expected to be correlated. We examined this hypothesis by analyzing the correlation between changes in eEPSC amplitude and changes in paw withdrawal threshold (Fig. 5). The values of normalized eEPSC amplitude by PB tract stimulation (eEPSC amplitude in the right CeA normalized by that in the left CeA, as plotted in Fig. 2C) at the highest stimulus intensity not evoking the spike currents in the slices from a rat were plotted against those obtained for the normalized paw withdrawal threshold (as plotted in Fig. 1B) at post-operational day 6–7 in the same rat (Fig. 5). The correlation coefficient between these variables was 0.75 ( $P < 0.001$ ;  $n = 26$ ; Spearman’s rank correlation), indicating that the increase in PB–CeA synaptic transmission in the right CeA contralateral to the ligation is positively correlated to the side-dependent increase in allodynic responses in the neuropathic pain models. In contrast, the correlation coefficient between the normalized eEPSC amplitude induced by BLA stimulation and the normalized paw withdrawal threshold in

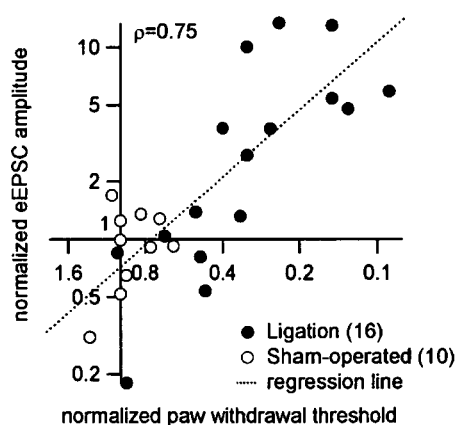


Fig. 5. Correlation between tactile allodynia and synaptic potentiation. A correlogram between the degree of tactile allodynia established and that of the PB–CeA transmission potentiation. Abscissa, the normalized paw withdrawal threshold (see Fig. 1); ordinate, the normalized eEPSC amplitude (see Fig. 2). Open and filled circles are values from the sham-operated ( $n = 16$ ) and ligation ( $n = 10$ ) groups, respectively. Dotted line indicates the linear regression line estimated by curve fitting ( $\log[y] = 1.19\log[x] - 0.16$ ).

each rat was much smaller and not significant ( $\rho = 0.40$ ;  $P = 0.06$ ;  $n = 23$ ).

### 3.5. Blockade of NMDA receptors did not affect the potentiated PB-CeA transmission in the neuropathic pain models

We further analyzed the mechanisms underlying this activity-dependent side-specific potentiation of PB-CeA transmission in the neuropathic pain models. The potentiation of PB-CeA transmission measured 6–8 h after establishment of acute arthritis in the rat is due to an increased NMDA receptor-mediated postsynaptic component, which is otherwise blocked by  $Mg^{2+}$  but becomes manifest even at a membrane potential of  $-60$  mV in arthritis models (Bird et al., 2005). In their report, eEPSC was not abolished by CNQX, a non-NMDA receptor antagonist, in the arthritis CeA, unlike in the control CeA, and the synaptic potentiation was not observed after application of APV, a NMDA receptor antagonist, in the amygdala slices. Therefore, we examined whether a similar mechanism involving NMDA receptor-mediated transmission underlies the synaptic potentiation in the CeA of long-term neuropathic pain models. Two sets of evidence clearly indicate that this synaptic potentiation does not result from enhanced NMDA receptor function. First, with a holding potential of  $-70$  mV, CNQX ( $10 \mu\text{M}$ ) almost completely abolished PB-CeA eEPSC (Fig. 6A) in both sides of the CeA in the ligation and sham-operated groups. Only the stimulation artifact (Fig. 6A) and a noise-level component remained in the presence of CNQX in all groups (Fig. 6A2). This result is in contrast to the results of Bird et al. (2005) who reported that in the arthritic rats, a significant amount of PB-CeA EPSC remained in the presence of CNQX ( $30 \mu\text{M}$ ). Second, application of APV ( $50 \mu\text{M}$ ;  $\sim 10$  min) did not significantly affect the amplitude of PB-CeA EPSC with  $400 \mu\text{A}$  stimulation in both sides of the CeA in the ligation group (Fig. 6B1 and 2). The EPSC amplitude of the neurons from the right CeA was still markedly larger than that from the left CeA even in the presence of APV (Fig. 6B), indicating that the marked right-left difference in EPSC amplitude primarily resulted from the increased non-NMDA receptor component in the right CeA. It is unlikely that the negligible contribution of the NMDA receptor-mediated component resulted from the ketamine anesthesia used before decapitation, because very similar responses to APV ( $50 \mu\text{M}$ ) were observed in five rats with strong allodynic responses being anesthetized with isoflurane prior to decapitation at post-operational day 6–7 (Fig. 6B). Our results strongly argue against the major involvement of an augmented NMDA receptor current in PB-CeA synaptic potentiation in neuropathic pain, unlike in the arthritis pain models. Rather, it is likely that the potentiation

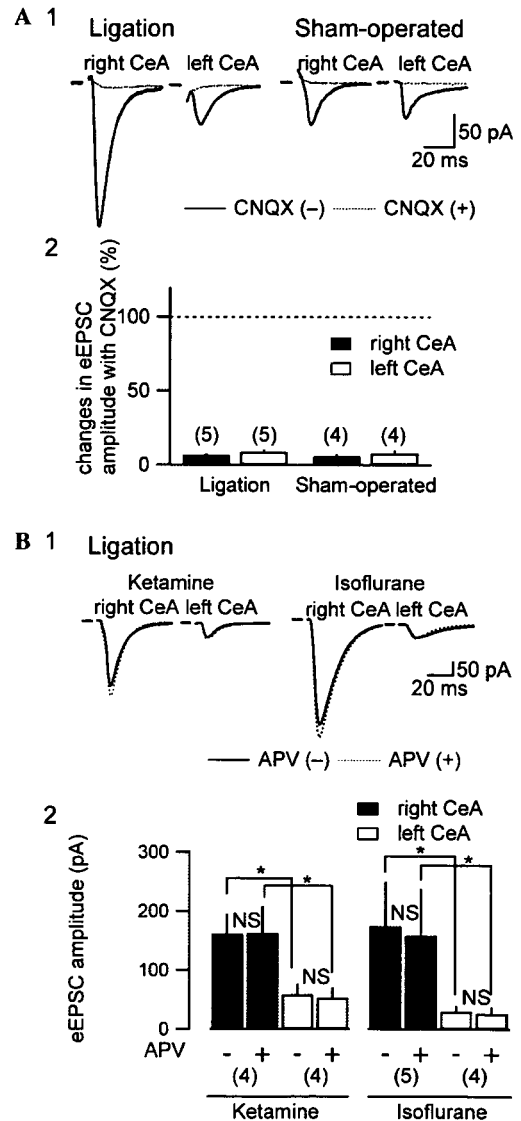


Fig. 6. NMDA receptor-independent synaptic plasticity in PB-CeA transmission in the rat model of neuropathic pain. (A1) Averaged waveforms of EPSCs evoked by PB tract stimulation in the absence (solid curves) and presence (broken curves) of CNQX ( $10 \mu\text{M}$ ) (average of eight consecutive responses). Left and right panels show membrane currents recorded from neurons in bilateral CeAs in the ligation and sham-operated groups, respectively. (A2) Summary of the effect of CNQX on eEPSC amplitude. Filled and open bars indicate changes in eEPSC amplitude in the right and left CeA, respectively. Mean  $\pm$  SEM. Numbers in parentheses indicate the number of neurons. (B1) Averaged waveforms of EPSCs evoked by PB tract stimulation in the absence (solid line) and presence (broken line) of APV ( $50 \mu\text{M}$ ) in the ligation group (average of eight consecutive responses). Left and right panels are the data from CeA neurons from two rats in which ketamine and isoflurane anesthesia, respectively, were used before decapitation. (B2) Summary of the effect of APV on eEPSC amplitude of the neurons in the right (filled bars) and left (open bars) CeA recorded before addition of APV (-) and in the presence of APV (+). Ketamine and isoflurane indicate the data from the rats in which ketamine and isoflurane were used before decapitation. Mean  $\pm$  SEM. NS, not significantly different between eEPSC amplitudes in the absence and presence of APV (paired  $t$ -test). \* $P < 0.05$ , Significantly different between the right and left CeA. Numbers in parentheses indicate the number of neurons.

of PB–CeA transmission in the neuropathic pain models involves enhanced non-NMDA receptor-mediated transmission.

### 3.6. Potentiation of PB–CeA synaptic transmission does not involve changes in the presynaptic release properties

The above results suggest that increased EPSC amplitude in the right CeA in the ligation group might result from postsynaptic alterations in the non-NMDA receptor-mediated transmission by increased nociceptive afferents from the PB. However, it is also possible that the properties of glutamate release from the presynaptic termini of the PB tract might play a role. To examine whether the potentiated transmission involves changes in the release properties, we analyzed the short-term plasticity of PB–CeA transmission, which is reported to show a strong short-term facilitation (Neugebauer

et al., 2003). The PB tract was stimulated twice with an interval of 50 ms (Fig. 7A) in the right and left CeA in the ligation (left in Fig. 7A) and sham-operated (right in Fig. 7A) groups. Short-term facilitation of a similar intensity was observed in both sides and in both groups (Fig. 7A bottom). The PPR (EPSC2/EPSC1) did not significantly differ between either side or between the two groups (Fig. 7B). It is therefore unlikely that changes in the presynaptic release properties play a principal role in the pain-related plastic changes in PB–CeA transmission.

## 4. Discussion

### 4.1. Particularities of CeA synaptic potentiation in neuropathic pain models

Using the spinal nerve ligation model of neuropathic pain (Kim and Chung, 1992) and electrophysiological evaluation of the synaptic transmission in the CeA, here we present the following novel findings. (1) In animal models of long-lasting chronic neuropathic pain, excitatory synaptic transmission at the PB–CeA synapses is potentiated such that the neurons in the latero-capsular part of the CeA fire with a weaker PB input. (2) This potentiation occurs predominantly in the PB–CeA synapses contralateral to the peripheral neuropathic pain. (3) The degree of synaptic potentiation, as compared between the two sides, was positively correlated to the degree of enhanced tactile allodynia in the hindlimb with neuropathic pain. (4) This potentiation, unlike in the semi-chronic arthritis pain model, does not involve potentiation of the NMDA receptor-mediated component of the postsynaptic current but instead involves enhancement of the postsynaptic non-NMDA receptor-mediated component. (5) The BLA–CeA transmission was also potentiated in these chronic neuropathic models but in a less side-dependent manner. Together these results point to the pivotal role of the long-lasting potentiation of synapses in the “nociceptive amygdala” in chronic pain-related emotional behaviors.

The potentiation of PB–CeA synaptic transmission in the neuropathic pain model is in good accordance with that in the arthritis and visceral pain models as pioneered by Neugebauer and colleagues (Neugebauer et al., 2004). However, the present results advance our knowledge along several lines and we discuss these issues below.

### 4.2. Positive correlation between tactile allodynia and synaptic potentiation

First, taking advantage of this model which allows the degree of chronic neuropathic pain to be quantitatively evaluated by measuring the tactile allodynia threshold in each side of the hindlimbs, we demonstrated for the first time, a positive correlation

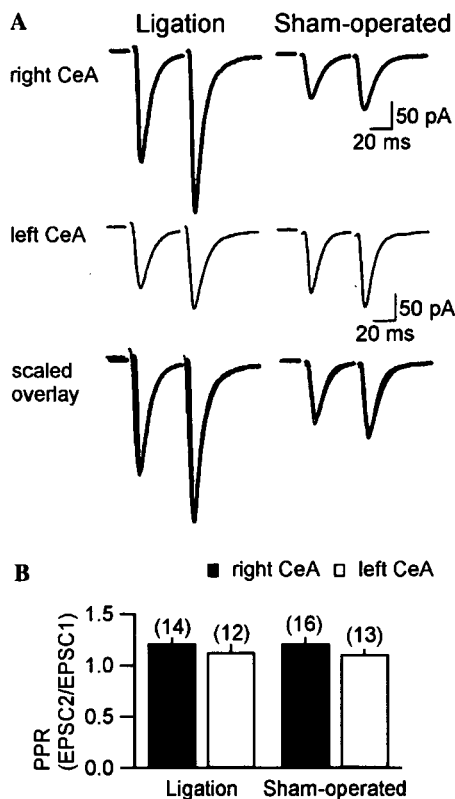


Fig. 7. Responses to paired-pulse stimulation of the PB tract of CeA neurons. (A) Averaged waveforms ( $n=8$ ) of EPSCs evoked by a paired stimulation of the PB tract at 50-ms intervals. Left and right panels show the membrane currents recorded from CeA neurons in the ligation and sham-operated groups, respectively. Top, typical recordings from neurons in the right CeA; middle, those in the left CeA; bottom, overlaid display of the responses in the right and left CeA scaled to the peak. Note the ratio of the second peak amplitude to the first peak amplitude is almost the same for groups and sides. (B) Summary of the paired-pulse ratio (the amplitude of the second eEPSC (EPSC2) normalized by the first eEPSC (EPSC1)). No significant difference between the groups was detected ( $P=0.566$ ; ANOVA). Numbers in parentheses indicate the number of neurons.

between allodynia establishment and synaptic potentiation. Such quantitative evaluation of tactile allodynia has been successfully used in the evaluation of neuropathic pain (Chapman et al., 1998) and is known to reflect sensitively the effects of drugs acting on the spinal nociceptive systems (Tsuda et al., 2003). In addition, the laterality-specific nature of this spinal cord–PB–CeA projection (see Gauriau and Bernard, 2002) enabled direct comparison between the painful and intact sides.

This positive correlation indicates that the synaptic potentiation of PB–CeA transmission depends on the intensity of nociceptive information of spinal origin. The tactile allodynia results from aberrant activation of nociceptive neurons in the spinal cord by tactile sensation that normally does not evoke nociception (Woolf and Mannion, 1999). It might therefore be possible that, in the neuropathic rats, simple touches of the hindpaw of the ligated side on the floor evoked allodynic nociception and activated the spino-parabrachio-amygdaloid pathways more frequently. It is thus likely that the increased sustained nociceptive signals from the spinal cord carried to the CeA via the PB triggered the side-specific synaptic potentiation in the CeA. The mechanism underlying this activity-dependent plasticity is an interesting subject for future study.

#### 4.3. NMDA receptor-independent potentiation

The second particularity of the present results is the absence of NMDA receptor potentiation. This is in a marked contrast to the synaptic potentiation of PB–CeA synapses in semi-chronic arthritis pain models, which was occluded by NMDA receptor blockade in slice preparations (Bird et al., 2005). In the present chronic neuropathic pain model, potentiation was almost entirely dependent on the enhanced non-NMDA receptor-mediated transmission as evidenced by complete suppression with CNQX and the lack of a significant effect of APV on eEPSC amplitude in the ligation group. The marked difference in eEPSC amplitudes between the right and left CeA even in the presence of APV, which did not significantly affect the eEPSC amplitude, both in rats anesthetized with ketamine and with isoflurane before decapitation, indicates that enhancement of the NMDA component plays a limited, if any, role in the potentiated transmission in the chronic neuropathic pain model, despite some differences in recording conditions between their study and ours. This characteristic is reminiscent of late-phase LTP in the hippocampal CA1 in which *de novo* insertions of the AMPA receptors at the postsynaptic membrane give rise to potentiated synaptic transmission in an activity-dependent manner (Nayak et al., 1998). The most obvious difference between our study and that by Bird et al. (2005) is the duration after the pro-nociceptive manipulations (a few hours vs. about 1 week). It is therefore

likely that the differences in the NMDA receptor contribution between these distinct models result from time-dependent alterations in the molecular mechanism underlying synaptic potentiation. A possible schema to explain such time-dependent alteration is as follows: (i) increased afferent activity from the PB due to increased nociception induces early phase synaptic potentiation through a mechanism that is common to the early onset synaptic potentiation in arthritic pain models involving CGRP receptor activation and NMDA receptor phosphorylation (Bird et al., 2005; Han et al., 2005) and/or through an unidentified mechanism specific to neuropathic pain, and (ii) sustained early phase potentiation for several days in neuropathic pain leads to late-phase potentiation that involves a switch from potentiated NMDA receptor function to enhanced non-NMDA receptor-mediated transmission, thus consolidating synaptic plasticity. This interpretation is consistent with the view that different molecular mechanisms participate in distinct steps of long-term potentiation in the central synapses, including those involved in early and late hippocampal LTP (Soderling and Derkach, 2000). This finding is of particular importance in the development of novel therapeutic approaches against neuropathic pain and related emotional disorders at various stages of chronic pain in human patients.

#### 4.4. Distinct pathway-dependence of CeA synaptic potentiation in neuropathic pain

In the present study, unilateral neuropathic pain potentiated contralateral PB–CeA transmission and, to a lesser extent, bilateral BLA–CeA transmission. While visceral pain predominantly potentiates PB–CeA transmission, arthritic pain potentiates both the PB–CeA and BLA–CeA pathways (Neugebauer et al., 2003), indicating that the pain-related synaptic plasticity in the CeA depends both on the type of pain and on the pathway. CeA receives inputs from other amygdala nuclei and from the thalamus and cortex via the BLA (Pare et al., 2004) as well as from the spino-PB pathway via the PB tract (Bernard and Besson, 1990; Jasmin et al., 1997); the former carries highly processed polymodal sensory information and the latter carries nociception-specific information (Gauriau and Bernard, 2002; Neugebauer et al., 2004). It is conceivable that the difference in the pattern of synaptic potentiation in the CeA between different models of pain stems principally from the differences in the site and pathway at which the nociceptive information is generated and sent to the brain.

Unlike in arthritis pain induced by kaolin and carrageenan injection to the knee joint (Neugebauer et al., 2003) and in visceral colitis pain induced by intracolonic zymosan injection (Han and Neugebauer, 2004; Yu et al., 2005), in neuropathic pain as used in this study, nociception occurs through aberrant activation of noci-



ceptive neurons in the dorsal horn (Woolf et al., 1992; Tsuda et al., 2003; Inoue et al., 2004; Coull et al., 2005) and does not arise from the site of perceived pain (in the present case, the hindpaw). It is therefore likely that the sensory and nociceptive components of the allodynic sensation are dissociated and processed by specific pathways in neuropathic pain models, resulting in the specific patterns of synaptic potentiation in the CeA neurons.

Another issue to be noted in the present study is that ligation was made in the left spinal nerve and synaptic potentiation was observed in the right amygdala. In vivo and in vitro studies to date have reported no lateral specificity in the spino-brachio-amygdaloid pathways (Gauriau and Bernard, 2002; Neugebauer et al., 2003, 2004). However, several studies in the rat demonstrated that the right amygdala plays a greater role than the left amygdala in the acquisition and expression of certain forms of emotional memories (Baker and Kim, 2004; Scicli et al., 2004; Adamec et al., 2005; LaLumiere and McGaugh, 2005), in a similar manner to those found in humans and cats. Therefore an interesting topic for future research is to investigate whether such long-lasting potentiation of PB–CeA transmission occurs specifically in the right amygdala, which might have a greater impact on the emotional responses of the animal.

The amygdala is composed of several anatomically and functionally distinct nuclei (Sah et al., 2003). The CeA serves as the output nucleus for major amygdala functions. Localized lesion experiments have demonstrated that the roles played by the CeA and BLA are distinct in chemical somatic and visceral pains (Tanimoto et al., 2003), appetitive emotional learning (Parkinson et al., 2000), fear-conditioned behavior (Killcross et al., 1997) and Pavlovian second-order conditioning (Hatfield et al., 1996). The behavioral consequence of the distinct activation patterns of the PB–CeA and BLA–CeA pathways in the neuropathic pain models was not addressed in this study, however, identification of the downstream effects of such differential potentiation of these pathways will lead to the development of efficient therapeutic strategies against intractable chronic pain accompanied by emotional problems in human patients.

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# Involvement of P2X<sub>4</sub> and P2Y<sub>12</sub> Receptors in ATP-Induced Microglial Chemotaxis

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## KEY WORDS

microglia; ATP; chemotaxis; P2Y<sub>12</sub>; P2X<sub>4</sub>

## ABSTRACT

We previously reported that extracellular ATP induces membrane ruffling and chemotaxis of microglia and suggested that their induction is mediated by the Gi/o-protein coupled P2Y<sub>12</sub> receptor (P2Y<sub>12</sub>R). Here we report discovering that the P2X<sub>4</sub> receptor (P2X<sub>4</sub>R) is also involved in ATP-induced microglial chemotaxis. To understand the intracellular signaling pathway downstream of P2Y<sub>12</sub>R that underlies microglial chemotaxis, we examined the effect of two phosphatidylinositol 3'-kinase (PI3K) inhibitors, wortmannin, and LY294002, on chemotaxis in a Dunn chemotaxis chamber. The PI3K inhibitors significantly suppressed chemotaxis without affecting ATP-induced membrane ruffling. ATP stimulation increased Akt phosphorylation in the microglia, and the increase was reduced by the PI3K inhibitors and a P2Y<sub>12</sub>R antagonist. These results indicate that P2Y<sub>12</sub>R-mediated activation of the PI3K pathway is required for microglial chemotaxis in response to ATP. We also found that the Akt phosphorylation was reduced when extracellular calcium was chelated, suggesting that ionotropic P2X receptors are involved in microglial chemotaxis by affecting the PI3K pathway. We therefore tested the effect of various P2X<sub>4</sub>R antagonists on the chemotaxis, and the results showed that pharmacological blockade of P2X<sub>4</sub>R significantly inhibited it. Knockdown of the P2X<sub>4</sub> receptor in microglia by RNA interference through the lentivirus vector system also suppressed the microglial chemotaxis. These results indicate that P2X<sub>4</sub>R as well as P2Y<sub>12</sub>R is involved in ATP-induced microglial chemotaxis. © 2007 Wiley-Liss, Inc.

## INTRODUCTION

Microglia are the immune effector cells that participate in tissue repair, amplification of inflammatory responses, and neuronal degeneration in the central nervous system (CNS) (Kreutzberg, 1996; Streit, 2002). They are present in the form of ramified cells under normal conditions, but in response to pathological stimuli microglia rapidly transform into a motile amoeboid form and migrate toward lesion sites, where they secrete a variety of substances and clear cell debris (Moran and Graeber, 2004; Nakajima and Kohsaka, 2005; Stence et al., 2001). Thus, microglial migration plays a crucial role in the

amelioration of a damaged CNS; however, the intracellular signals underlying microglial cell migration are poorly understood.

Extracellular ATP is known to play a role as a neurotransmitter or neuromodulator in the CNS (Illes and Alexandre Ribeiro, 2004), and it regulates various physiological functions of microglia (Inoue, 2002). ATP receptors are classified into two families: the ionotropic P2X receptor (P2XR) family and the GTP-binding (G-) protein coupled P2Y receptor (P2YR) family (Ralevic and Burnstock, 1998), and microglia have been reported to possess functional ATP receptors, including P2X<sub>4</sub>R, P2X<sub>7</sub>R, and P2Y<sub>12</sub>R (Cavaliere et al., 2003; James and Butt, 2002; Sasaki et al., 2003; Tsuda et al., 2003). Davalos et al. (2005) and Nimmerjahn et al. (2005) recently reported that processes of ramified microglia extended toward a confocal laser injury, where ATP is likely to be released by damaged tissue and surrounding astrocytes. These observations suggest that ATP is a primary molecule in the induction of the change in microglial morphology.

We have also previously reported that ATP-induced microglial membrane ruffling and chemotaxis are mediated by Gi/o-protein coupled P2Y<sub>12</sub>R (Honda et al., 2001; Sasaki et al., 2003); however, the intracellular signaling pathway downstream of P2Y<sub>12</sub>R following ATP stimulation is not fully understood. Several recent articles have revealed that P2Y<sub>12</sub>R stimulation results in activation of the phosphatidylinositol 3'-kinase (PI3K) pathway in some cells (Czajkowski et al., 2004; Soulet et al., 2004; Van Kolen and Slegers, 2004). Although PI3K is known to be a crucial enzyme in the regulation of chemotaxis by monocytes and macrophages (Procko and McColl, 2005; Ridley, 2001; Van Haastert and Devreotes, 2004), whether the PI3K pathway participates in ATP-induced microglial chemotaxis remained unclear.

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In this study we demonstrated that activation of the PI3K pathway is required for ATP-induced microglial chemotaxis and found that the PI3K/Akt activation was suppressed when extracellular Ca<sup>2+</sup> was chelated. ATP stimulates P2XRs in microglia and causes an increase in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) by inducing an extracellular Ca<sup>2+</sup> influx (Inoue et al., 1998; Tsuda et al., 2003). Therefore, to clarify involvement of P2XRs in microglial chemotaxis we also examined the effect of antagonists and RNA interference (RNAi) with P2XRs on microglial chemotaxis, and the results demonstrated that P2X<sub>4</sub>R is involved in ATP-induced chemotaxis.

## MATERIALS AND METHODS

### Isolation of Microglia

Microglia were obtained from primary cell cultures of neonatal Wistar rat cerebral cortex as described previously (Nakajima et al., 1992). In brief, mixed glial cultures were maintained for 12–23 days in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA). Microglia were prepared as floating cells by gentle shaking and allowed to attach to appropriate dishes or glasses.

### Membrane Ruffling

Microglia attached to glass coverslips were incubated for 4 h in DMEM without FCS and stimulated with 50 μM ATP (Yamasashyoyu, Chiba, Japan) for 5 min at 37°C. The cells were then fixed with 3.7% formaldehyde for 10 min, permeabilized for 5 min with PBS containing 0.1% Triton X-100, and stained for 1 h with 2 U/mL Texas Red-conjugated phalloidin (Invitrogen) diluted in PBS containing 1% BSA. The cells were mounted in PermaFluor (Thermo Fisher Scientific, Waltham, MA) and examined under a fluorescence microscope AX70 (Olympus, Tokyo, Japan). To quantify membrane ruffles, cells were stained with 1 μg/mL anti-Iba1 polyclonal antibody (Imai et al., 1996) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:1,000, Invitrogen) and then incubated with 2 U/mL Alexa Fluor 647-conjugated phalloidin (Invitrogen). The F-actin content of cells positive for Iba1 was quantified as the integral intensity of Alexa Fluor 647 fluorescence with a laser scanning cytometer (LSC2, CompuCyte, Cambridge, MA). The mean fluorescent intensity of the cells pretreated with each inhibitor was calculated from the data obtained from 1,000 cells. Increases in membrane ruffles are reported as ratios of the mean fluorescent intensity of the ATP-stimulated cells to that of the unstimulated cells. The effect of the inhibitors was assessed by preincubating cells with wortmannin (Sigma, St. Louis, MO) (100 nM) for 20 min, LY294002 (Wako, Osaka Japan) (50 μM) for 20 min, AR-C69931MX (AstraZeneca, UK) (1 μM) for 10 min, 2,3-*O*-(2,4,6-trinitrophenyl) adenosine 5-triphosphate (TNP-ATP) (Invitrogen) (100 μM) for 5 min, pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid

(PPADS) (Sigma) (300 μM) for 5 min, or Brilliant blue G (BBG) (Nacalai Tesque, Kyoto, Japan) (1 μM) for 5 min, and then stimulating them with ATP.

### Chemotaxis Assay

Dunn chemotaxis chambers (Weber Scientific International, Teddington, UK) were used to perform the chemotaxis assays according to the method described previously (Honda et al., 2001; Webb et al., 1996). In brief, microglia attached to square coverslips were incubated for 4 h in DMEM without FCS. Each coverslip was then inverted onto a chamber and the medium in the outer well was replaced with DMEM containing 50 μM ATP. The chamber was placed on the stage of a microscope (ECLIPSE TE300; Nikon, Tokyo Japan), and cell images were collected every 5 min for 1 h with a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and imaging software (fishPPC; Hamamatsu Photonics). Time-lapse video images were used to calculate the final position of cells relative to their starting position, and the distance each cell migrated was measured by plotting the positions of the cell nucleus on a computer display with software (Image-Pro Plus; Media Cybernetics, MD). The distance and direction moved are shown as *x* and *y* coordinates on scatter diagrams in which the *x*-axis is parallel to the outer ring and the position of the outer well is above the *y*-axis.

### Akt Activation

Microglia were incubated for 4 h in DMEM without FCS and then stimulated with 50 μM ATP or 100 ng/mL recombinant murine macrophage-colony stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN) for 5 min at 37°C, and lysed with SDS sample buffer. Proteins were separated by 10% SDS-PAGE and transferred onto an Immobilon P membrane (Millipore, MA). The membrane was incubated for 1 h at room temperature with a blocking solution containing 25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20 (TTBS), and 5% (v/v) nonfat dry milk, and then incubated overnight at 4°C with mouse monoclonal anti-phospho-Akt (Ser473) antibody (diluted 1:1,000, Cell Signaling Technology, Beverly, MA) or rabbit polyclonal Akt antibody (diluted 1:1,000, Cell Signaling Technology). The membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (diluted 1:1,000, GE Healthcare, Little Chalfont, UK) or HRP-conjugated donkey anti-rabbit IgG (diluted 1:1,000, GE Healthcare), and phosphorylated Akt and total Akt were detected with an ECL Western blotting detection system (GE Healthcare). The Akt phosphorylation level was quantified by densitometry with NIH image software. Before stimulating the cells in the calcium-depleted experiment, they were incubated for 30 min in a balanced salt solution composed of 20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1.2 mM MgCl<sub>2</sub>, and 10 mM glucose in the presence of 1.2 mM Ca<sup>2+</sup> (BSS) or 1 mM ethy-