Nonopioid and Neuropathy-Specific Analgesic Action of the Nootropic Drug Nefiracetam in Mice

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ABSTRACT
Nootropic drug nefiracetam and related compounds are used in diseases with learning and memory deficits. Recent studies have implicated relationships between learning, memory, and chronic pain. Thus, in the present report, we have studied the effects of nootropic drug nefiracetam on the thermal and mechanical hyperalgesia induced by partial sciatic nerve ligation or streptozotocin treatment in mice. In the thermal paw withdrawal test, p.o., s.c., i.t., and i.c.v. administration of nefiracetam dose dependently reversed the thermal hyperalgesia observed in nerve-injured mice. Nefiracetam (p.o. and i.t.) also significantly reversed the thermal hyperalgesia observed in streptozotocin-induced diabetic mice. In the paw pressure test, p.o. and i.t. administration of nefiracetam dose dependently reversed the mechanical hyperalgesia observed in both nerve-injured and diabetic mice. In contrast, nefiracetam had no effect in sham-operated or control nondiabetic mice in all paradigms. Among other pyrrolidine nootropics (p.o.), aniracetam produced significant analgesic effects. Other analogs also had some, but not significant, analgesic effects. Finally, nefiracetam (p.o.)-induced analgesia in injured mice was not affected by opioid antagonist naloxone (s.c., i.t., and i.c.v.) but was dose dependently inhibited by nicotinic antagonist mecamylamine (i.t. and i.c.v.). The analgesic effect of i.t. nefiracetam was also blocked by i.t. mecamylamine pretreatment. Together, these findings suggest that nefiracetam, a new member of the piracetam group of cognition enhancers, could be a good therapeutic tool against neuropathic pain. We also demonstrate that nefiracetam-induced analgesic action was nonopioid in nature and was due to stimulation of nicotinic cholinergic system at spinal and supraspinal levels.

Clinical management of neuropathic pain is currently an area of potential therapeutic need because of its unresponsiveness to most conventional therapeutic agents. Although the opioids have been used for centuries to cure many extremely painful conditions, there have been continuous debates over their efficacy in neuropathic pain (Ossipov et al., 1995; Bleeker et al., 2001). Moreover, the other unconventional therapeutic approaches such as use of certain antidepressants or anticonvulsants and use of topical capsaicin are associated with suboptimal efficacies and/or side effects (Bridges et al., 2001). Thus, there has been continuous search for novel drug molecules to alleviate this intractable pain. Nefiracetam, a cyclic derivative of γ-aminobutyric acid, has been developed as a nootropic or cognition-enhancing agent. It has been reported to increase learning and memory in various animal experiments (Sakurai et al., 1989; Nabeshima et al., 1991, 1994). Recently, it is undergoing preclinical and clinical trials as a cognition-enhancing drug in Alzheimer’s disease and poststroke dementia. On the other hand, learning and memory process has been postulated to be involved in the mechanisms of chronic pain (for review, see Flor, 2000). Thus, recent evidences support the view that chronic pain is a maladaptive learned phenomenon (Arnett, 1997; Kumazawa, 1998). Cognitive dysfunction has been described in various neuropathy states (Zaslavsky et al., 1995; Kuhajda et al., 1998). Severe memory dysfunction has also been reported in patients with neuropathy that occurred endemically in Cuba during 1991 to 1993 (Cubero et al., 1999).

Neuroplastic changes at both central and peripheral level after nerve injury have been hypothesized as a mechanism of neuropathic pain (Woolf and Salter, 2000; Woolf et al., 1999; Hokfelt et al., 1994), and the piracetam group of nootropics is believed to restore the neuronal plastic changes that occur after many traumatic conditions (Chepкова et al., 1995; Coq and Xerri, 1999). Neuronal plastic changes at both spinal and brain regions have also been indicated in opioid tolerance and dependence (Mayer et al., 1999), and very recently, it has been reported that chronic administration of nefiracetam attenuates the development of morphine dependence and tolerance in mice (Itoh et al., 2000).

Although the exact mechanism of nootropic action of nefiracetam is not known, it is believed to modulate the neuronal nicotinic receptors in the brain (Nishizaki et al., 2000;
Nomura and Nishizaki, 2000). Very recently, potentiation of α4β2 nicotinic receptor currents by nefiracetam was reported in a patch-clamp study with rat cortical neurons (Zhao et al., 2001). On the other hand, the neuronal nicotinic receptors, specifically the α4β2 subtype, located at spinal cord and brain are well known for their contribution to nicotinic antinociception in rodents (Khan et al., 1998; Marubio et al., 1999; Bitner et al., 2000). Thus, considering the above-mentioned facts, we investigated the effects of nootropic drug nefiracetam in partial sciatic nerve injury and streptozotocin (STZ)-induced diabetic models of neuropathic pain.

Materials and Methods

Animals. Male ddY mice weighing 25 to 30 g were used in all experiments. The mice were housed in a room maintained at 21 ± 2°C with free access to standard laboratory diet and tap water. All procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

Partial Ligation of Sciatic Nerve. Partial ligation of the sciatic nerve of the mice was performed under pentobarbital (50 mg/kg i.p.) anesthesia, following the methods of Malmeberg and Basbaum (1998). Briefly, the common sciatic nerve of right hind limb was exposed at high thigh level through a small incision. The nerve was carefully cleared off the surrounding connective tissues. A silk suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was held within the ligation. The wound was closed with a single muscle suture and antibiotic powder was dusted over the wound area after surgery. Sham operation was performed similarly except without touching the sciatic nerve. Immediately after surgery, the animals were kept in a soft bed cage with some food inside so that the animals could feed themselves without difficulty in standing. The wound healed within 1 to 2 days and the animals behaved normally. The mice were hyperalgesic to thermal and mechanical stimuli at 1st, 2nd, and 3rd week after nerve ligation (data not shown). Experiments were carried out at 7 days postligation.

Streptozotocin Treatment. Mice (~30 g) were injected intravenously in the tail with 200 mg/kg streptozotocin (Wako Pure Chemicals, Richmond, VA) prepared in saline adjusted to pH 4.5 in 0.1 N citrate buffer. Age-matched nondiabetic control mice were injected with the vehicle alone. Streptozotocin solutions were freshly prepared due to the limited stability of the compound. Animals were kept in a group of four per cage with special care of food and water supplement. The bed of the cage was changed every day. In a set of preliminary control experiments, serum glucose level was measured spectrophotometrically at 7, 14, and 21 days after streptozotocin treatment and was found to be consistent with a diabetic level (above 300 mg/dl) throughout the periods. The serum glucose level was measured by glucose oxidase method from blood samples obtained by tail vein pricking. The animals were found to develop both thermal and mechanical hyperalgesia at 1st, 2nd, and 3rd weeks after streptozotocin treatment (data not shown). Animals at 7 days poststreptozotocin treatment were used in this study.

Drug Administration. Nefiracetam, aniracetam, piracetam, oxiracetam, and levetiracetam were kindly provided by Daiichi Pharmaceutical Co. (Tokyo, Japan). Naloxone hydrochloride and mecamylamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in physiological saline except aniracetam. For aniracetam, the drug was dissolved in saline for 10-mg/kg solution, and for 30-mg/kg solution, a few drops of Tween 80 were added in the saline suspension. Intrathecal injections were performed free hand between L5 and L6 lumber space in unanesthetized mice according to the method of Hylden and Wilcox (1980). The injection was performed using a 30-gauge needle attached to a Hamilton glass microsyringe. The i.c.v. injections were carried out into the left lateral ventricle of mice. Injections were performed using a Hamilton microliter syringe fitted with a 26-gauge needle, according to the method of Haley and McCormick (1957). The site of injection was 2 mm caudal and 2 mm lateral to the bregma, and 3 mm in depth from the skull surface. The injection volume was 5 µl in both cases. Pernoral administration was performed with a clinical syringe connected to a 5-cm-long p.o. dispenser. Animals received p.o. and s.c. in a volume of 0.1 ml/10 g of body weight. In antagonism experiments, pretreatment with the antagonists was done 10 min before the agonist injection or administration.

 Thermal Paw Withdrawal Test. Antinociception or analgesia was measured from the latency to withdrawal evoked by exposing the right hind paw to a thermal stimulus (Hargreaves et al., 1988). Unanesthetized animals were placed in Plexiglas cages on top of a glass sheet, and an adaptation period of 1 h was allowed. The thermal stimulus (ITTC, Inc., Woodland Hills, CA) was positioned under the glass sheet to focus the retraction bulb exactly on the middle of plantar surface of the animals. A mirror attached to the stimulus permitted visualization of undersurface of the paw. A cutoff time of 15 s was set to prevent tissue damage.

Paw Pressure Test. Mice were placed into a Plexiglas chamber on a 6- × 6-mm wire mesh grid floor and were allowed to accommodate for a period of 1 h. The mechanical stimulus was then delivered onto the middle of the plantar surface of right hind paw using a Transducer Indicator (model 1601; ITTC, Inc.,). It has the advantage over conventional von Frey filaments in that it measures the paw withdrawal threshold at once and the result is shown digitally on a display screen. Moreover, the limitations of variability in filament’s strength and multiple stimulation of the paw for a single data point are overcome in this method. With this apparatus, a control response of 10 g was previously adjusted for naive mice. This control response in naive mice is higher than the usual control response with von Frey filaments due to the greater diameter of the tip of the transducer (0.8–0.9 mm compared with 0.1–0.2 mm in von Frey filaments) and consequent greater area of stimulation on the paw. In this experiment, a cutoff pressure of 15 g was set to avoid tissue damage.

Data Presentation. In the time course figures, the thermal latencies (s) or mechanical thresholds (g) were plotted against the time after drug administration (min). In the area under the time-response curve (AUC) figures, analgesia was evaluated by calculating the AUC obtained by plotting paw withdrawal latency or threshold (s/g) on the ordinate and time after drug administration (from 10 to 60 min at 10-min intervals) on the abscissa. The analgesic actions for different drugs were assessed as the percentage of maximal AUC, which represents the area under the curve for cutoff time/pressure and that value was 750 units [15 × (60 – 10)]. The data were analyzed using Student’s t test following comparisons with repeated measures analysis of variance and suitable post hoc analysis. The criterion of significance was set at p < 0.05. All results are expressed as the mean ± S.E.M.

Results

Reversal of Thermal Hyperalgesia in Nerve-Injured Mice after Administration of Nefiracetam through Various Routes. To examine analgesic effect of nefiracetam in neuropathic pain, administration of the drug was done through various conventional routes in partial sciatic nerve-injured mice, and the paw withdrawal latency to thermal stimuli was measured. Administration of nefiracetam by p.o., s.c., i.t., and i.c.v. routes dose-dependently reversed the thermal hyperalgesia observed in partial sciatic nerve-injured mice (Fig. 1, A–D). At the higher doses, analgesic effects persisted for more than an hour. In the systemic routes of administration (p.o. and s.c.), saturation in the analgesic effects was observed at higher doses (Fig. 1, A and B). On the
other hand, i.c.v. nefiracetam was more potent than i.t. nefiracetam to elicit analgesia (Fig. 1, C–E). The peak responses after i.c.v injection were also higher than the i.t. route (Fig. 1, C and D). The i.t. and i.c.v doses of nefiracetam to elicit an analgesic response equivalent to 40% of maximal AUC were 17.5 nmol (i.t.) and 2.2 nmol (i.c.v.), respectively (Fig. 1E). However, when administered or injected in sham-operated mice through all above-mentioned routes, nefiracetam (30 mg/kg p.o., 30 mg/kg s.c., 30 nmol i.t., and 30 nmol i.c.v.) did not produce any antinociceptive effect (Fig. 1F).

Reversal of Mechanical Hyperalgesia by Peroral and Intrathecal Nefiracetam in Injured Mice. Peroral administration of nefiracetam dose dependently reversed the mechanical hyperalgesia in nerve-injured mice from 3 to 30 mg/kg (Fig. 2A). The analgesic actions at doses of 10 and 30 mg/kg were almost of same level. Nefiracetam-induced analgesic action after p.o. administration persisted for more than an hour (Fig. 2A). However, nefiracetam, after 30-mg/kg p.o. administration, had no effect in sham-operated mice (Fig. 2B). Similarly, intrathecal (i.t.) injection of 1 and 10 nmol of nefiracetam dose dependently attenuated the nerve injury-induced mechanical hyperalgesia (Fig. 2C). As in the case with p.o. nefiracetam, i.t. nefiracetam (10 nmol) also had no effect in sham-operated mice (Fig. 2D).

Effect of Nefiracetam on Streptozotocin-Induced Thermal and Mechanical Hyperalgesia. To extend the analgesic action of nefiracetam in other neuropathy models, effect of the drug was examined in STZ-induced diabetic mice. As stated under Materials and Methods, STZ treatment in mice induced both thermal and mechanical hyperalgesia. Both the thermal and mechanical hyperalgesia observed after STZ treatment were dose dependently reversed by p.o. administration of nefiracetam (Fig. 3, A and C). Although the analgesic action of nefiracetam in thermal paw withdrawal test almost completely diminished by 40 min, the effects persisted for almost an hour in the paw pressure test (Fig. 3, A and C). Similar to the case with nerve injury model, nefiracetam did not produce any antinociceptive effect in control nondiabetic mice after p.o. administration at doses of 30 mg/kg with both thermal and mechanical nociceptive tests (Fig. 3, B and D).

Effects of Other Nefiracetam Analogs on Thermal and Mechanical Hyperalgesia in Injured Mice. We also studied the effects of other nefiracetam-like nootropics after p.o. administration in nerve-injured mice. As shown in Table...
Effects of nefiracetam and its analogs after p.o. administration are compared herein. Data are presented as the percentage of maximal AUC (see Materials and Methods for details). Each data point represents the mean ± S.E.M. of six separate experiments. *p < 0.05, statistically significant compared with the vehicle administration.

1, nefiracetam produced significant analgesic effects in both thermal and mechanical modalities at doses of 10 and 30 mg/kg (p.o.) compared with the vehicle. The analgesic effect of nefiracetam seems to be higher in mechanical modality than in thermal modality. Moreover, nefiracetam induced almost the same level of analgesia after 10- and 30-mg/kg p.o. administration. Next, we examined the effects of other nefiracetam analogs after p.o. administration at doses of 10 and 30 mg/kg in injured mice. At 10-mg/kg p.o. dose, the analogs had no analgesic effects in sham-operated mice at these doses compared with the vehicle (Table 1). We did not perform experiments with further higher doses of the analogs because our main objective was to evaluate the analgesic effects of nefiracetam. Moreover, as with nefiracetam, the analogs also had no analgesic effects in sham-operated mice at these doses (data not shown).

**Effects of Opioid and Nicotinic Receptor Antagonists on Nefiracetam-Induced Analgesia in Injured Mice.** In an attempt to identify the mechanism of nefiracetam-induced analgesia in injured mice, we first attempted to see whether the effects were through activation of opioidergic system. However, as shown in Fig. 4A, the nefiracetam-induced (30-mg/kg p.o.) analgesic effect was insensitive to opioid receptor antagonist naloxone pretreatment (1 mg/kg s.c.; 1 nmol i.c.v. or i.t.). The dose of naloxone was chosen in accordance with various previous studies in mice. On the other hand, the neuronal nicotinic receptor antagonist mecamylamine dose dependently inhibited the nefiracetam-induced (30-mg/kg p.o.) analgesic action after both i.c.v. and i.t. pretreatment (Fig. 4, B and C). Moreover, i.t. nefiracetam-induced (30 nmol) analgesia was also dose dependently

### TABLE 1

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Thermal Paw Withdrawal Test</th>
<th>Paw Pressure Test</th>
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<tr>
<td>Vehicle</td>
<td>31.23 ± 2.18</td>
<td>35.95 ± 4.99</td>
</tr>
<tr>
<td>Nefiracetam (3 mg/kg)</td>
<td>33.98 ± 2.40</td>
<td>50.08 ± 4.66#</td>
</tr>
<tr>
<td>Nefiracetam (10 mg/kg)</td>
<td>48.60 ± 3.46*</td>
<td>61.00 ± 7.94#</td>
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<td>Nefiracetam (30 mg/kg)</td>
<td>49.55 ± 5.83*</td>
<td>63.43 ± 6.48#</td>
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<tr>
<td>Aniracetam (10 mg/kg)</td>
<td>37.53 ± 1.94</td>
<td>43.45 ± 4.88</td>
</tr>
<tr>
<td>Aniracetam (30 mg/kg)</td>
<td>42.90 ± 2.17*</td>
<td>46.01 ± 1.90#</td>
</tr>
<tr>
<td>Oxiracetam (10 mg/kg)</td>
<td>30.17 ± 2.07</td>
<td>36.10 ± 6.31</td>
</tr>
<tr>
<td>Oxiracetam (30 mg/kg)</td>
<td>35.85 ± 1.02</td>
<td>37.91 ± 2.68</td>
</tr>
<tr>
<td>Piracetam (10 mg/kg)</td>
<td>32.40 ± 1.96</td>
<td>33.50 ± 2.27</td>
</tr>
<tr>
<td>Piracetam (30 mg/kg)</td>
<td>38.76 ± 1.65</td>
<td>38.05 ± 0.52</td>
</tr>
<tr>
<td>Levetiracetam (10 mg/kg)</td>
<td>33.19 ± 2.24</td>
<td>38.13 ± 7.99</td>
</tr>
<tr>
<td>Levetiracetam (30 mg/kg)</td>
<td>40.50 ± 0.65</td>
<td>41.85 ± 3.71</td>
</tr>
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</table>

* Statistically significant compared with vehicle administration in the thermal paw withdrawal test at p < 0.05.
# Statistically significant compared with vehicle administration in the paw pressure test at p < 0.05.
blocked by i.t. mecamylamine pretreatment (Fig. 4D), all suggesting that nefiracetam gave the analgesic action by acting on the nicotinic acetylcholine systems present at both supraspinal and spinal level.

**Discussion**

The recent demonstration that coadministration of nefiracetam with morphine attenuated the development of morphine tolerance and dependence in mice (Itoh et al., 2000) indicates a possible interaction of this compound with the nociceptive system. Moreover, considering the effects of nefiracetam-like nootropics in neurodegenerative diseases as well as their ability to restore neuroplastic changes after traumatic conditions (Nabeshima et al., 1991; Chepkova et al., 1995), they are expected to exert some effects on different neuropathic states that are also results of some neuronal plastic changes. Indeed, in our experiments administration of nefiracetam through different conventional routes dose dependently reversed the thermal and mechanical hyperalgesia observed in partial sciatic nerve-injured and streptozotocin-treated diabetic mice (Figs. 1–3). Interestingly, in all of the routes of administration nefiracetam did not produce any antinociceptive effects in sham-operated or in control nondiabetic mice (Figs. 1E, 2B and D, and 3B and D). Thus, the analgesic action of nefiracetam seems to be specific for neuropathic pain. This specificity of the drug should be of particular interest. Most of the available conventional and unconventional therapeutic approaches to treat neuropathic pain are subjected to various limitations. The opiates are reported to have suboptimal therapeutic efficacies in neuropathic pain (Ossipov et al., 1995; Bleeker et al., 2001). They are also well known to induce tolerance and dependence in addition to the other side effects such as gastrointestinal distress and respiratory depression. The unconventional therapeutic agents such as antidepressants, anticonvulsants, or topical capsaicin have various side effects. Thus, development of more efficacious and safer analgesics for neuropathic pain has been a long-time quest. Recently nicotinic acetylcholine receptor agonists have been reported to possess potent nonopioid analgesic actions (Bannon et al., 1998). However, their narrow therapeutic window made them unsuitable for possible clinical use. On the other hand, the nootropic drug nefiracetam has very wide therapeutic window (Oyaizu and Narahashi, 1999; Zhao et al., 2001). The exact mechanism of nootropic effect of nefiracetam is not known yet. Modulation of neuronal calcium channels and nicotinic acetylcholine receptors by nefiracetam has been extensively studied (Oyaizu and Narahashi, 1999; Nishizaki et al., 2000; Nomura and Nishizaki, 2000; Fujita et al., 2002). Recently, Zhao et al. (2001) reported a potent and selective stimulation of α4β2-type currents by nefiracetam in rat cortical neurons through Gs protein. Sakurai et al. (1998) recently reported a direct dose-dependent increase in the extracellular acetylcholine level in the frontal cortex of freely moving rats using a microdialysis technique after p.o. administration of nefiracetam. On the other hand, the nicotinic cholinergic systems located at both spinal and supraspinal levels are involved in nicotinic antinociception (Khan et al., 1998; Marubio et al., 1999; Bitten et al., 2000). Thus, the nicotinic cholinergic system seems to be an important target site of nefiracetam-induced analgesic action in our experiment. Indeed, the neuronal nicotinic receptor antagonist mecamylamine dose dependently blocked the nefiracetam-induced analgesia in nerve-injured mice (Fig. 4, B–D). Both spinal and supraspinal nicotinic receptors were found to be stimulated by nefiracetam (Fig. 4, B–D). Furthermore, to examine the possibility that nefiracetam may interact with opioidergic system to produce the analgesia, we performed antagonism experiments with opioid receptor antagonist, naloxone. However, the nefiracetam-induced analgesia could not be blocked by naloxone after pretreating systemically, intrathecally, or intracerebroventricularly (Fig. 4A). Thus, the analgesic action of nefiracetam seems to be through activation of nicotinic cholinergic system in the brain and spinal cord.

Another characteristic of the nefiracetam-induced actions was the apparent saturation of analgesic effects at higher doses as well as its lack of effect in non-neuropathic animals (Table 1; Figs. 1–3). The first one is consistent with previous reports where nefiracetam produced bell-shaped dose-response curve both in in vitro and in vivo experiments (Nabeshima et al., 1994; Oyaizu and Narahashi, 1999; Zhao et al., 2001). However, the absence of antinociceptive effects in control animals indicates that nefiracetam-induced activation of nicotinic cholinergic system under normal state was not sufficient to produce antinociception to acute thermal or mechanical stimuli. Alternatively, the endogenous nicotinic cholinergic system is maximally activated under normal state so that exogenous stimulation could not produce any further effects. Evidence for presence of such endogenous tonic nicotinic inhibitory control on nociception at spinal and supraspinal level has been reported elsewhere (Cordero-Erausquin and Changeux, 2001; Hama and Menzaghi, 2001). We speculate that this tonic inhibitory tone on nociceptive transmission at the spinal and supraspinal level is somehow reduced due to some changes in the neuronal network after injury or diabetes. Nefiracetam selectively stimulated this nicotinic cholinergic system whose inhibitory tone has been reduced after injury and thus gave its neuropathy-specific analgesic action. This neuropathy-specific effect of nefiracetam seems to be consistent with its effects in other neurodegenerative diseases. Finally, we observed the analgesic effects of other piracetam analogs in the nerve injury neuropathy model mice. As shown in Table 1, nefiracetam was more potent compared with the other analogs to alleviate both thermal and mechanical hyperalgesia. This apparently lower potency of the other analogs might be due to their differential modulation of the neuronal cholinergic system. Nefiracetam had been reported to facilitate hippocampal cholinergic neurotransmission by a mechanism independent of piracetam and aniracetam (Nomura and Nishizaki, 2000). Shiotani et al. (2000) also reported a similar differential modulation of the peripheral type benzodiazepine receptors for the anticonvulsant actions of the piracetam analogs. However, significant analgesic effects by the other analogs cannot be excluded at further higher doses. On the other hand, prevention of development of neuropathic pain by piracetam has been reported elsewhere (Danilova et al., 1996). Such differences might be due to use of different doses of the drug.
References


