Relief of Hypersensitivity after Nerve Injury from Systemic Donepezil Involves Spinal Cholinergic and γ-Aminobutyric Acid Mechanisms

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ABSTRACT

Background: Evoking spinal release of acetylcholine (ACh) produces antinociception in normal animals and reduces hypersensitivity after nerve injury, and some studies suggest that ACh-mediated analgesia relies on γ-aminobutyric acid (GABA)-ergic signaling in the spinal cord. In this study, the authors tested the spinal mechanisms underlying the anti-hypersensitivity effects of donepezil, a central nervous system–penetrating cholinesterase inhibitor, in a rat model of neuropathic pain.

Methods: Male Sprague-Dawley rats were anesthetized, and L5 spinal nerve ligation was performed unilaterally. Withdrawal threshold to a paw pressure test was measured before and after intraperitoneal administration of donepezil, with or without intrathecal antagonists for cholinergic and GAB-Aergic receptors. Microdialysis studies in the ipsilateral dorsal horn of the lumbar spinal cord were also performed to measure extracellular ACh and GABA.

Results: Donepezil increased the withdrawal threshold in spinal nerve ligation rats but not in normal rats. The anti-hypersensitivity effect of donepezil (1 mg/kg) in spinal nerve ligation rats was reduced by intrathecal pretreatment with atropine (30 μg), a muscarinic receptor antagonist; mecamylamine (100 μg), a nicotinic receptor antagonist; bicuculline (0.03 μg), a γ-aminobutyric acid receptor type A antagonist; and CGP 35348 (30 μg), a γ-aminobutyric acid receptor type B antagonist. ACh and GABA concentrations in the microdialysates from the spinal dorsal horn were increased after intraperitoneal donepezil treatment (1 mg/kg) in both normal and spinal nerve ligation rats.

Conclusions: Systemic administration of donepezil reduces hypersensitivity after nerve injury by increasing extracellular ACh concentration, which itself induces GABA release in the spinal cord. Activation of this spinal cholinergic–GABAergic interaction represents a promising treatment for neuropathic pain.

PERIPHERAL nerve injury can result in neuropathic pain that is not alleviated by conventional pain relievers. Currently, the most common drugs for treating neuropathic pain are antidepressants and calcium channel α2-δ ligands such as gabapentin and pregabalin.1 These drugs rely in part on spinal cholinergic mechanisms for analgesia,2,3 which suggests that cholinergic pathways may be critical in the therapeutic targeting of neuropathic pain.

Cholinesterase inhibitors such as physostigmine and neostigmine produce analgesia to acute pain in humans.4-6 A recent study demonstrated that the oral administration of donepezil, an approved cholinesterase inhibitor for the symptomatic treatment of Alzheimer dementia, produced an antihypersensitivity effect in rats after nerve injury but not in normal animals; this involved a spinal mechanism.7 Although the detailed mechanism is not fully understood, manipulations that increase acetylcholine (ACh) release in the spinal cord produce analgesia via both muscarinic and nicotinic receptors.8,9 Cholinergic signaling is an attractive target for analgesia because it may be involved in endogenous inhibition of pain10 and may be augmented under painful conditions.7,11

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In the current study, we tested whether the analgesic efficacy of donepezil was increased in the presence of chronic neuropathic injury compared with the normal state because of a greater effect on release of ACh concentration in the spinal cord. Although previous studies suggest that stimulation of spinal muscarinic and nicotinic ACh receptors increases \( \gamma \)-aminobutyric acid (GABA) release to inhibit pain transmission in the spinal cord,\(^{12-16} \) this has never been directly tested. We therefore tested the behavioral reliance of donepezil-mediated antihypersensitivity on activation of GABA receptors and whether donepezil-induced increases in ACh would increase GABA release in the spinal cord.

**Materials and Methods**

**Surgical Preparation**

The study was approved by the Animal Care and Use Committees of the Gunma University Graduate School of Medicine (Maebashi, Japan) and Wake Forest University School of Medicine (Winston-Salem, North Carolina). Male Sprague-Dawley rats (250 g) were used in all experiments. Microdialysis studies for GABA release were performed at Wake Forest University, and other experiments were performed at Gunma University. The animals were housed under a 12-h light–dark cycle, with food and water available ad libitum. Spinal nerve ligation (SNL) surgery was performed as previously described.\(^{17} \) The wound was closed, and the animals were allowed to recover for 2 weeks. Animals were singly housed after surgery for the remainder of the experiment. For behavioral studies, each rat was used two to three times at 4- to 5-day intervals. In total, 82 SNL rats were used.

**Behavioral Testing**

Withdrawal threshold to pressure applied to the hindpaw, expressed in grams, was measured using an analgesimeter (37215, Ugo Basile, Comerio, Italy) as previously described.\(^{18} \) The device applies increasing pressure to the hindpaw until the animal withdraws the paw. The pressure is immediately released, and the pressure at withdrawal measured in grams. A cutoff of 250 g was used to prevent potential tissue injury. The experimenter was blinded to the treatment group. All animals were trained for 3 days with this apparatus before baseline values were recorded.

**Drugs and Their Administration**

Drug testing was performed 2–3 weeks after nerve ligation. Rats received intraperitoneal donepezil (0.3, 0.6, and 1.0 mg/kg). Antagonist studies were performed using the muscarinic receptor antagonist, atropine (30 \( \mu \)g); the nicotinic receptor antagonist, mecamylamine (100 \( \mu \)g); the \( \gamma \)-aminobutyric acid type A receptor antagonist, biccuculline (0.01 and 0.03 \( \mu \)g); and the \( \gamma \)-aminobutyric acid type B receptor antagonist, CGP 35348 (10 and 30 \( \mu \)g). Saline or antagonist solution was administered intrathecally 15 min before intraperitoneal donepezil injection. The doses of the antagonists were selected according to previous studies\(^{7,8,19,20} \) and our preliminary studies. For intrathecal administration, drugs were dissolved in saline in a volume of 5 \( \mu l \) and injected in the L5–6 intervertebral space using a 30-gauge needle. Donepezil was a gift from Eisai Co. (Tokyo, Japan). Other drugs were purchased from Sigma Co. (St. Louis, MO).

**Microdialysis Studies**

**ACh Measurement.** Microdialysis studies were performed in normal rats and those 2–3 weeks after SNL surgery in a manner as previously described.\(^{21} \) Anesthesia was induced by intraperitoneal injection with urethane (1.2–1.5 g/kg) and maintained with 0.5% isoflurane in 100% oxygen through a nose cone. The left femoral vein was cannulated for transfusion of saline at a rate of 1 ml/h. For intraperitoneal injection of donepezil, a 24-gauge intravenous catheter was inserted into the intraperitoneal space. Rectal temperature was maintained at 37–38°C by a heating pad placed beneath the animal. The L3–L5 level of the spinal cord was exposed by a thoracolumbar laminectomy, and the rat was placed in a stereotaxic apparatus. The microdialysis probe (OD = 0.22 mm, ID = 0.20 mm, length = 2 mm, AI-8-02, Eicom Co., Kyoto, Japan) was inserted from just lateral to the dorsal root and advanced at a 30° angle to a depth of 2 mm using a micro-manipulator (model WR-88; Narishige, Tokyo, Japan). The microdialysis probe was perfused with Ringer’s solution (147 m\( \text{M} \) NaCl, 4 m\( \text{M} \) KCl, 2.3 m\( \text{M} \) CaCl\(_2\)) at a constant flow rate (1 \( \mu \)l/min) using a syringe pump (ESP-64, Eicom Co.). After 120 min of constant perfusion, 30-min perfusate fractions were collected into an auto injector (EAS-20, Eicom Co.) After two consecutive samples were collected to determine the basal ACh concentrations in the dialysate, saline (0.5 ml) or donepezil (1.0 mg/kg) was administered intrathecally through an indwelling catheter.

Samples (30 \( \mu \)l) were automatically injected and analyzed for the ACh concentration using high-performance liquid chromatography with electrochemical detection by an HTEC-500 analyzing system (Eicom Co.). The chromatographic conditions were as follows: the mobile phase consisted of 50 \( \text{mM} \) KHCO\(_3\), containing 300 mg/l sodium 1-decanesulphonate (pH = 8.5) and 50 mg/l ethylenediaminetetraacetic acid-2Na. The column was an EICOMPAC AC-GEL (2.0 mm \( \times \) 150 mm; Eicom Co.). A glassy carbon working electrode (WE-3G, Eicom Co.) was used with a flow rate of 0.15 ml/min. The detector voltage was 0.45 V, and the detector temperature was 33.0°C. The retention time for ACh was 12.4 min. The detection limit of the ACh assay in the current study was 10 fg per injection (information from Eicom Co.), and the interassay coefficient of variation at 29 ng (200 fmol) per injection was 12.3%.
GABA Measurement. Microdialysis studies were performed to measure spinal GABA release using the same condition for ACh microdialysis. Samples were kept at −80°C until GABA was measured. GABA content in the microdialysates was measured by high-pressure liquid chromatography with electrochemical detection by an HTEC-500 analyzing system (Eicom co.). GABA in the samples was derivatized with 2-mercaptoethanol and o-phthalaldehyde (4 mM) in 0.1 M carbonate buffer (pH = 9.5). The o-phthalaldehyde derivatives were then separated on the column (3.0 mm × 150 mm, SC-5ODS, EICOM) at 30°C, using a mobile phase that consisted of 50 mM phosphate buffer (pH = 2.8) and methanol (1:1 vol/vol) containing 5 mg/ml ethylenediaminetetraacetic acid-2Na at a flow rate of 0.5 ml/min. The limit of detection for GABA in the current study was 1.5 pg per injection (10 µl), and the interassay coefficient of variation at 100 pg per injection was 7.2%.

Rotarod Test. Sedation and motor coordination were tested using the accelerating rotarod (ENV577; Med Associates Inc., St. Albans, VT) in normal and SNL rats. The rats were required to walk against the motion of a rotating drum with the speed accelerating from 4 to 40 rpm over 300 s. The time on the rod from the start of acceleration until the animal fell from the drum onto the counter-trip plate was recorded before and 15 min after intraperitoneal administration of donepezil (1.0 mg/kg). A 300-s cutoff was used. One training period per day was performed for 2 days before the drug treatment, and the animals were acclimated to the device and habituated to handling to minimize stress during testing.

Statistics. The statistical analysis was conducted using SigmaPlot 12 (Systat Software Inc., San Jose, CA). Data were normally distributed (Shapiro–Wilk test) and are presented as the mean ± SD. Time-course data from the behavioral and microdialysis studies were analyzed using a two-way repeated-measures ANOVA. When significant differences were observed, Student–Newman–Keuls post hoc tests were performed for between-group comparisons and comparisons at each time point. Other data were analyzed using a paired or unpaired t test. A P value less than 0.05 was defined as statistically significant.

Results

Behavioral Studies

Intraperitoneal administration of donepezil (0.3, 0.6, and 1.0 mg/kg) produced antihypersensitivity effects in SNL rats (n = 6 in each group, P < 0.001 by two-way repeated-measures ANOVA, fig. 1A). Withdrawal thresholds were higher in groups treated with 0.6 and 1.0 mg/kg of donepezil compared with the saline-treated group (P < 0.05 by Student–Newman–Keuls post hoc test). The peak was observed 15 min after donepezil administration in both the 0.6 and 1.0 mg/kg groups, but the duration of the effect was longer in the 1.0 mg/kg group than in the 0.6 mg/kg group (120 min vs. 30 min) compared with the saline group (n = 6 in each group, P < 0.05 by Student–Newman–Keuls post hoc test). In contrast, withdrawal threshold in normal animals did not differ between saline and donepezil (1.0 mg/kg) groups (fig. 1B). On the basis of these results, we selected the donepezil dose of 1.0 mg/kg for the following experiments.

Intrathecal pretreatment with the muscarinic receptor antagonist atropine (30 µg) completely blocked the antihypersensitivity effect of donepezil (n = 6 in each group, P = 0.002 by Student–Newman–Keuls post hoc test after statistically significant interaction in two-way repeated-measures ANOVA; fig. 2A). The nicotinic receptor antagonist mecamylamine (100 µg) also inhibited the effect of donepezil (n = 6 in each group, P = 0.002 by Student–Newman–Keuls post hoc test after statistically significant interaction in two-way repeated-measures ANOVA; fig. 2B). Neither cholinergic antagonist alone affected the withdrawal threshold compared with saline.

Intrathecal pretreatment with the γ-aminobutyric acid type A receptor antagonist bicuculline (0.03 µg) and the γ-aminobutyric acid type B receptor antagonist CGP 35348...
Neither of which affected the withdrawal threshold when administered alone, attenuated the antihypersensitivity effect of donepezil in SNL rats (n = 6 in each group, P < 0.05 by Student–Neuman–Keuls post hoc test after statistically significant interaction in two-way repeated-measures ANOVA). A previous study showed that intrathecal administration of bicuculline and another γ-aminobutyric acid type B receptor antagonist phaclofen produced tactile allodynia and thermal hyperalgesia in normal rats.19 In the current study, however, intrathecal administration of bicuculline (0.03 μg) and CGP 35348 (30 μg) alone did not affect the withdrawal threshold in normal rats (the withdrawal threshold before and 30 min after bicuculline was 148.3 ± 9.3 and 147.5 ± 8.8 g, respectively, and before and 30 min after CGP 35348 were 141.7 ± 8.2 and 140.0 ± 16.7 g, respectively, n = 6 in each group).

The intraperitoneal administration of donepezil (1 mg/kg) did not affect the rotarod performance time in normal and SNL rats (the rotarod performance time before and 15 min after donepezil was 136.6 ± 33.1 and 141.3 ± 31.2 s in normal rats and 137.3 ± 51.4 and 135.0 ± 48.9 s in SNL rats, respectively, n = 6 in each group). No adverse behavioral effects, such as sedation or agitation, were observed with any of the treatments.

**Microdialysis Studies**

The baseline ACh concentrations in spinal cord dorsal horn microdialysates before drug injection did not differ between SNL (2.67 ± 0.40 pg/30 μl) and normal rats (3.05 ± 0.45 pg/30 μl). In the saline-treated normal and SNL rats (n = 6 in each group), neither of which affected the withdrawal threshold when administered alone, attenuated the antihypersensitivity effect of donepezil in SNL rats (n = 6 in each group, P < 0.05 by Student–Neuman–Keuls post hoc test after statistically significant interaction in two-way repeated-measures ANOVA). A previous study showed that intrathecal administration of bicuculline and another γ-aminobutyric acid type B receptor antagonist phaclofen produced tactile allodynia and thermal hyperalgesia in normal rats.19 In the current study, however, intrathecal administration of bicuculline (0.03 μg) and CGP 35348 (30 μg) alone did not affect the withdrawal threshold in normal rats (the withdrawal threshold before and 30 min after bicuculline was 148.3 ± 9.3 and 147.5 ± 8.8 g, respectively, and before and 30 min after CGP 35348 were 141.7 ± 8.2 and 140.0 ± 16.7 g, respectively, n = 6 in each group).
SNL rats, ACh concentrations in the dialysates did not change over time. In the donepezil-treated normal and SNL rats, ACh concentrations in the dialysates increased within 30 min, peaked at 1 h with approximately 1,800–2,200% of the baseline value, and remained increased for 2 h after injection compared with the saline-treated group (n = 6 in each group, \( P < 0.05 \) by Student–Neuman–Keuls post hoc test after statistically significant interaction in two-way repeated-measures ANOVA; fig. 4). The donepezil-induced ACh increase did not differ between normal and SNL rats.

Baseline GABA concentrations in spinal cord dorsal horn microdialysates before drug injection were lower in SNL rats (23.7 ± 3.3 pg/30 μl) than in normal rats (40.0 ± 4.5 pg/30 μl; an unpaired \( t \) test, \( P = 0.012 \)). Although a slight decrease in GABA concentrations in the dialysates was observed over time, the intraperitoneal injection of saline did not affect the GABA concentration compared with the baseline. In the donepezil-treated groups, GABA concentrations increased over time after drug injection and were significantly different from that after saline treatment in the SNL and normal rats (n = 6 in each group, \( P < 0.05 \) by Student–Neuman–Keuls post hoc test after statistically significant interaction in two-way repeated-measures ANOVA; fig. 5).

**Discussion**

In the current study, the intraperitoneal administration of donepezil produced a dose-dependent antihypersensitivity effect in SNL rats without inducing any adverse effects. Consistent with behavioral studies using cholinergic and GABAergic antagonists, direct measurements of ACh and GABA in the spinal dorsal horn with microdialysis revealed that donepezil increased the ACh and GABA concentrations. Although the analgesia provided by donepezil was specific for hypersensitivity after SNL, the increases in spinal ACh and GABA were similar in normal and SNL rats. Therefore, other mechanisms may be involved in the enhanced efficacy of donepezil for neuropathic pain.

Previous animal studies show that cholinesterase inhibitors produce analgesia by supraspinal, spinal, and peripheral mechanisms, and oral administration of donepezil has previously been shown to reduce hypersensitivity after SNL in rats via an action on spinal muscarinic receptors. Consistent with these findings, we showed that intrathecal administration of donepezil produced reduced hypersensitivity in a manner reversed by intrathecal pretreatment with atropine. Nicotinic receptors in the spinal cord have been shown to be involved in nociceptive transmission, and we confirmed in the current study a partial role for nicotinic receptors in the antihypersensitivity effect of donepezil. Consistent with our study, previous studies showed that intrathecal nicotinic receptor agonists increased withdrawal thresholds measured by paw pressure test in SNL animals. In contrast, a previous study demonstrated that antiallodynic effect from orally administered donepezil is not affected by mecamylamine in SNL rats. In normal animals, nicotinic receptors have no or little role on thermal antinociception from cholinesterase inhibition.
These results suggest that nicotinic receptors may contribute to only mechanical hyperalgesia in neuropathic pain. We hypothesized that donepezil would cause a greater release of ACh in the spinal cord in SNL rats than in normal rats because cholinergic analgesia is augmented in neuropathic pain, as indicated by the current behavioral study and others.\(^7\) In our microdialysis studies, however, the peak levels and the time course of the ACh increase after donepezil treatment were similar between normal and SNL rats. Furthermore, the basal levels of ACh were not significantly different between normal and SNL rats. Therefore, other mechanisms may contribute to the increased potency of donepezil against hypersensitivity after nerve injury. For example, inhibitory M2 muscarinic receptors have been reported to play an essential role in cholinergic analgesia,\(^25,30,31\) and a recent study demonstrated that peripheral nerve injury upregulates M2 muscarinic receptors in primary sensory afferent neurons.\(^32\) In addition, intrathecal administration of nicotinic receptor agonists inhibits hypersensitivity after nerve injury but does not produce analgesia in normal animals.\(^14,27\) These findings suggest that peripheral nerve injury enhances the efficacy of ACh action in the spinal cord and that this plasticity, rather than a change in ACh release in injury state, is used by donepezil to provide enhanced analgesia after nerve injury compared with the normal condition.

Extensive evidence indicates a close functional relationship between spinal cholinergic and GABAergic mechanisms.\(^33,34\) Stimulation of M2, M3, and M4 muscarinic receptors results in presynaptic GABA release in the spinal cord and γ-aminobutyric acid type B receptor–mediated analgesia.\(^13,16,35\) Stimulation of spinal nicotinic receptors has been reported to produce presynaptic GABA release and γ-aminobutyric acid type A receptor–mediated analgesia.\(^14,15,27,36\) However, no previous study measured GABA concentrations in the spinal cord after stimulation of cholinergic receptors. Our microdialysis experiments clearly demonstrate that intraperitoneal administration of donepezil increased GABA concentrations in the spinal cord. Furthermore, intrathecal administration of GABA receptor antagonists produced a dose-related suppression of donepezil-mediated antihypersensitivity at doses that themselves failed to alter withdrawal threshold in normal and SNL rats. Although we did not identify the specific cholinergic receptor that is involved in this activity, our data suggest that the systemic administration of donepezil increases extracellular ACh concentrations in the spinal cord to induce subsequent GABA release, and that GABA release is essential to the antihypersensitivity effect of donepezil after peripheral nerve injury.

Decreases in spinal GABA immunoreactivity and the GABA-synthesizing enzyme glutamic acid decarboxylase have been reported after nerve injury.\(^37-41\) Electrophysiologic studies confirmed that primary afferent-evoked inhibitory postsynaptic currents in the spinal dorsal horn were reduced after nerve injury.\(^40,42,43\) Although controversy exists with regard to the actual loss of GABAergic interneurons,\(^44-47\) the dysfunction of GABAergic inhibition in the spinal cord likely contributes to neuropathic pain,\(^48,49\) and several lines of evidence suggest that stimulation of the spinal GABAergic system produces analgesia in neuropathic pain.\(^19,50-52\) Consistent with these observations, our microdialysis study suggests that donepezil inhibited SNL-induced hypersensitivity by inducing spinal GABA release, despite the decrease of baseline GABA concentration in SNL rats compared with normal rats. Although the peak level and time course of the GABA increase after donepezil were similar between normal and SNL rats, donepezil may produce antihypersensitivity by activating inhibitory GABAergic neurons to re-establish the inhibitory tone that had been reduced by nerve injury.

Although donepezil may have some inhibitory effects for pain in patients using opiates,\(^53\) there is no previous study that reported relief of neuropathic pain in patients treated with donepezil. The doses of donepezil used in the current study were slightly greater than doses that were effective for cognitive responses in animals.\(^54,55\) Therefore, this discrepancy might be related to dosage. The spinal cholinergic system is heavily involved in α2-adrenoceptor–mediated analgesia for neuropathic pain. As such, activation of α2-adrenoceptors, which inhibits ACh release in the normal state, facilitates ACh release after nerve injury via a G-protein–mediated mechanism.\(^56\) Several antidepressants (e.g., tricyclic antidepressants and serotonin and noradrenaline reuptake inhibitors) and calcium channel α2-δ ligands (e.g., gabapentin and pregabalin) that are routinely used in the treatment of neuropathic pain rely on spinal α2-δ-adrenoceptors and cholinergic mechanisms for analgesia in neuropathic pain.\(^2,3\) The current study builds on previous observations to strengthen the rationale for clinical trials examining the benefit from add-on therapy to these agents with donepezil.\(^57\)

In summary, systemic administration of donepezil reduces hypersensitivity after peripheral nerve injury in rats by increasing the concentrations of ACh and GABA in the spinal dorsal horn. The greater effect of donepezil on behavior in the presence of nerve injury did not depend on increased interstitial concentrations of ACh in the spinal cord in the injured state. Rather, there was a dependence of the behavioral effect of donepezil on activation of GABA receptors in the spinal cord, and donepezil administration was associated with increased release of GABA in the spinal cord. These results further our understanding regarding the spinal mechanisms by which cholinesterase inhibitors produce analgesia in neuropathic states and strengthen the rationale for their clinical application alone and in combination with antidepressants and gabapentin-like compounds.

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Anesthesiology 2013; 118:173-80

178

Kimura et al.


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