Donepezil reverses intermittent stress-induced generalized chronic pain syndrome in mice

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Running title page

a) **Running title**: Cholinomimetics for the treatment of fibromyalgia

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d) **List of abbreviations**: aCSF, artificial cerebrospinal fluid; ICS, intermittent cold stress; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.t., intrathecal; SEM, standard error of the mean

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Abstract

Treatment for fibromyalgia is an unmet medical need. To develop novel therapies for the treatment of fibromyalgia, we explored pain therapeutic actions of existing pharmaceuticals, which inhibit the somatic symptoms frequently observed in fibromyalgia patients. The present study first examined the therapeutic actions of pilocarpine, which inhibits dry-eye and dry-mouth symptoms, using an experimental fibromyalgia-like chronic pain model produced by intermittent cold stress (ICS) in mice. A single intraperitoneal (i.p.) and intracerebroventricular (i.c.v.), but not intrathecal, pilocarpine administration attenuated ICS-induced thermal hyperalgesia and mechanical allodynia, and this action was abolished by muscarinic antagonist pirenzepine (i.c.v.). Treatment with 1-10 μg/kg donepezil (i.p.), which can easily penetrate into the brain, also showed similar therapeutic effects. Importantly, we found that both of pilocarpine and donepezil produced anti-hyperalgesic effects via supraspinal action. Furthermore, repeated donepezil treatments completely cured the ICS-induced hyperalgesia and allodynia even after the cessation of drug treatments. Acute and chronic treatments of these cholinomimetics had no effects on the nociceptive threshold in control animals. In contrast, the lack of morphine (i.c.v.) analgesia initially observed in the ICS model remained in ICS model mice treated with long-term donepezil. Collectively, these findings suggest that stimulation of the muscarinic cholinergic system effectively inhibits some mechanisms underlying chronic pain in the ICS model, but not the lack of descending pain inhibitory mechanisms, which is driven by central morphine.
Introduction

Acute pain is physiologically significant and serves as a danger alarm to the body, while chronic pain exhibits a disease nature and should be prevented (Costigan et al., 2009). Chronic pain is often accompanied by emotional disorders that lead to different types of pain states (Seymour and Dolan, 2013), implying the presence of a vicious cycle of pain (Costigan et al., 2009). Neuropathic pain, a representative chronic pain, occurs following damage of pain pathways from the periphery to the brain (Costigan et al., 2009), and is clearly distinguished from nociceptive pain in terms of its nature, which is characterized by hyperalgesia and allodynia (Baron, 2006). Since the establishment of animal models for neuropathic pain in the late 1980s (Bennett and Xie, 1988; Ossipov and Porreca, 2013), extensive studies of neuronal plasticity, based on physiological and anatomical characterization (Basbaum et al., 2009; Devor, 2013), and molecular biological studies, based on identification of key molecules, have contributed to the better understanding and research development of therapeutics (Ueda, 2006; Ueda, 2008; Costigan et al., 2009; Kuner, 2010; Hill, 2013).

Central pain, which is closely related to the emotional disturbance, has been poorly characterized, because it is unclear which parts of the pain-regulatory system are disordered. Fibromyalgia, an intractable generalized pain syndrome, is known to comprise an approximately 2% population ratio in developed countries (Russell, 2013; Clauw, 2014). Limited etiology information of fibromyalgia, a representative central pain, delays the reasonable diagnosis and therapy (Russell, 2013; Clauw, 2014). Basic studies using animal models, which mimic symptoms or pharmacotherapeutical sensitivities in fibromyalgia patients, would be essential for advancing diagnosis or
therapy. To establish animal models of fibromyalgia, Levine’s group has used subdiaphragmatic vagotomy (Khasar et al., 2003), while Sluka’s group has used repeated intramuscular injections of acidic saline (Sluka et al., 2001). There are also several unique models induced by reserpine treatment (Nagakura et al., 2009) and intermittent sound stress (Khasar et al., 2009). These animal models share some pathophysiological or behavioral features with fibromyalgia patients (DeSantana et al., 2013). Additionally, we have reported another fibromyalgia-like pain model caused by intermittent cold stress (ICS) (Nishiyori and Ueda, 2008). This model has shown characteristics of a generalized chronic pain phenotype and shares female-predominant sex differences and pharmacotherapeutic features with fibromyalgia patients. Specifically, ICS-induced pain is sensitive to gabapentin and antidepressants (Nishiyori and Ueda, 2008; Nishiyori et al., 2011), but not to morphine (Nishiyori et al., 2010), which is consistent with the clinical evidence of fibromyalgia patients (Clauw, 2014).

Currently, the therapy for chronic pain, such as neuropathic pain and fibromyalgia, has predominantly included the use of existing analgesics and analgesic adjuvants (Arnold, 2009; Toelle and Backonja 2013). The rationale for using analgesic adjuvants, despite a poor understanding of mechanisms underlying fibromyalgia, is based on the remedy of somatic symptoms. However, because of insufficient therapeutic actions and adverse effects, the therapeutic strategies have not been established (Smith et al., 2011). According to the new guidelines approved by the American College of Rheumatology (Russell, 2013; Clauw, 2014), the criteria for fibromyalgia include severity of somatic symptoms as well as widespread pain. In line with the current situation for chronic pain control, we focused on the muscarinic agonist pilocarpine, which inhibits dry-eye and dry-mouth, which are often observed in fibromyalgia patients (Bennett, 2005). Here, we
report that cholinomimetics, such as pilocarpine and donepezil, a remedy for Alzheimer disease, have significant therapeutic effects against ICS-induced, fibromyalgia-like generalized pain syndrome.
Materials and Methods

**Animals.** Male C57BL/6J mice (TEXAM Corporation, Nagasaki, Japan), weighing 20-25 g, were used for the present study. The mice were housed in a room with a temperature of 22 ± 3°C with free access to a standard laboratory diet and tap water. All procedures used in this work were approved by the Nagasaki University Animal Care Committee, and complied with the fundamental guidelines for the proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Drug treatments.** Pilocarpine was purchased from Wako (Osaka, Japan), while donepezil, atropine, and pirenzepine were obtained from Sigma-Aldrich (St. Louis, MO, USA). These drugs were dissolved in physiological saline for intraperitoneal (i.p.) injection or in artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3.8 mM KCl, 1.2 mM KH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, pH 7.4) for intrathecal (i.t.) and intracerebroventricular (i.c.v.) injection. Pirenzepine was injected 10 min prior to pilocarpine injection, while atropine was administrated 10 min prior to donepezil injection. Morphine hydrochloride (Takeda Chemical Industries, Osaka, Japan) was dissolved in aCSF. The i.t. injection was given into the space between spinal L5 and L6 segments, according to the method described by Hylden and Wilcox (Hylden and Wilcox, 1980).

**Intermittent cold stress exposure.** Mice were exposed to ICS, as previously described (Nishiyori and Ueda, 2008). Briefly, mice were placed in a cold room at 4°C overnight (from 4:30 pm to 10:00 am), followed by ICS with alternating environmental
temperatures between 24°C and 4°C every 30 min from 10:00 am to 4:30 pm. These procedures were repeated twice. On day 3, the mice were returned to and adapted to a room temperature of 24°C for 1 h before the nociception tests. We designated day 3 following the onset of stress exposure as day 1 post-stress exposure (P1). Mice in the control group were maintained at 24°C for all 3 days (from 4:30 pm on day 1 to 10:00 am on day 3). During the stress period, two mice were kept in each cage (12 × 15 × 10.5 cm), with free access to food and agar in place of fluid.

**Nociception tests.** In the thermal paw withdrawal tests, the nociception threshold was determined by the latency of paw withdrawal upon a thermal stimulus (Uchida et al., 2010). Non-anesthetized animals were placed in Plexiglas cages on top of a glass sheet, and an adaptation period of 1 h was allowed. The thermal stimulator (IITC Inc., Woodland Hills, CA, USA) was positioned under the glass sheet, and the focus of the projection bulb was aimed directly at the middle of the plantar surface of the animal. A mirror attached to the stimulator permitted visualization of the plantar surface. A cut-off time of 20 s was set to avoid tissue damage. The mechanical paw pressure test was performed as previously described (Uchida et al., 2010). Briefly, mice were placed in a Plexiglas chamber on a 6 × 6 mm wire mesh-grid floor and allowed to acclimatize for a period of 1 h. A mechanical stimulus was then delivered to the middle of the plantar surface of the right hindpaw using a Transducer Indicator (Model 1601; IITC Inc.). The pressure needed to induce a flexor response was defined as the pain threshold. A cutoff pressure of 20 g was set to avoid tissue damage. In experiments using mechanical and thermal tests, the thresholds were determined from three repeated challenges at 10-min intervals, and the response averages were evaluated. An electrical, stimulation-induced paw withdrawal test was performed as previously described (Uchida et al., 2010).
Briefly, electrodes (Neurotron, Baltimore, MD, USA) were fastened to the plantar surfaces and insteps of mice. Transcutaneous nerve stimuli with three sine-wave pulses (5, 250, and 2000 Hz) were applied using a Neurometer® CPT/C (Neurotron). The minimum intensity (μA) at which each mouse withdrew its paw was defined as the current stimulus threshold.

**Statistical analysis.** Statistical analyses were performed using the Dunnett’s test or one-way analysis of variance with the Tukey-Kramer multiple comparison *post-hoc* test. The criterion of significance was set at $p < 0.05$. All results are expressed as means ± standard error of the mean (SEM).
Results

Systemic injection of the muscarinic agonist pilocarpine reverses hyperalgesia in ICS-exposed mice. As shown in Fig. 1A, paw withdrawal latency against thermal stimuli was approximately 10 s in naive animals. Previously, we have shown that ICS markedly reduces the thermal pain threshold to the level of roughly 6 s at day 1 (P1) post-stress exposure (Nishiyori et al., 2010). Thermal hyperalgesia lasts for at least 15 days, whereas constant cold stress decreases the threshold to 6 s at P1, and hyperalgesia disappears at P5 (Nishiyori and Ueda, 2008; Nishiyori et al., 2010). Therefore, we adopted a P5 time point for the ICS-induced chronic pain mechanism studies.

The i.p. injection of pilocarpine at 1 mg/kg markedly increased the threshold to the level of roughly 13 s at 30 min in ICS-treated mice, while it did not significantly affect the threshold of control naive mice (Fig. 1A; 0 min, $F_3 = 157.2$; 10 min, $F_3 = 34.5$; 20 min, $F_3 = 71.5$; 30 min, $F_3 = 12.2$; 40 min, $F_3 = 13.4$; 50 min, $F_3 = 14.2$; 60 min, $F_3 = 33.4$; **$p < 0.01$, ##$p < 0.01$). Because pilocarpine-induced salivation was only observed at doses greater than 10 mg/kg, the anti-hyperalgesic effects were likely to be independent of distraction because of salivation. The anti-hyperalgesic actions of pilocarpine at 20 min were dose dependent in the range of 0.1-1.0 mg/kg (Fig. 1B; $F_5 = 27.4$, **$p < 0.01$, #$p < 0.05$, ##$p < 0.01$). In addition, ICS-induced mechanical allodynia was also significantly inhibited by pilocarpine (1 mg/kg, i.p.) treatment (Fig. 1C; 0 min, $F_3 = 170.9$; 10 min, $F_3 = 16.4$; 20 min, $F_3 = 9.4$; 30 min, $F_3 = 11.4$; 40 min, $F_3 = 9.3$; 50 min, $F_3 = 11.7$; 60 min, $F_3 = 26.4$; **$p < 0.01$, #$p < 0.05$, ##$p < 0.01$).

Pilocarpine produces anti-hyperalgesia via supraspinal actions. To study the site of therapeutic actions of pilocarpine, this compound was i.c.v. or i.t. administered in...
ICS-treated mice. The i.c.v. pilocarpine injection (1 μg) rapidly reversed the ICS-induced hyperalgesia with a peak time of 10 min, and this effect completely disappeared at 40 min (Fig. 2A; 0 min, $F_3 = 92.2$; 10 min, $F_3 = 14.7$; 20 min, $F_3 = 17.3$; 30 min, $F_3 = 29.3$; 40 min, $F_3 = 23.0$; 50 min, $F_3 = 30.9$; 60 min, $F_3 = 42.6$; **$p < 0.01$, $^#p < 0.05$, **$p < 0.01$). In contrast, the i.t. pilocarpine injection (1 μg) showed no effect in the ICS-exposed mice for 60 min (Fig. 2B; 0 min, $F_3 = 39.8$; 10 min, $F_3 = 5.8$; 20 min, $F_3 = 17.9$; 30 min, $F_3 = 22.7$; 40 min, $F_3 = 13.5$; 50 min, $F_3 = 13.6$; 60 min, $F_3 = 11.7$; *$p < 0.05$, **$p < 0.01$). The i.c.v. or i.t. pilocarpine injection had no effects on basal pain thresholds in control mice (Fig. 2, A and B). The therapeutic effects of pilocarpine (i.c.v.) were dose dependent in the range of 0.1-1 μg, while no significant effect was observed by the i.t. injection at 3 μg (Fig. 2C; i.c.v., $F_5 = 12.5$; i.t. at P5, $F_3 = 5.8$; i.t. at P7, $F_3 = 12.2$; *$p < 0.05$, **$p < 0.01$, $^#p < 0.05$, **$p < 0.01$), suggesting that pilocarpine produced anti-hyperalgesic effects via activation of supra-spinal targets. Indeed, we found that the pilocarpine (1 mg/kg, i.p.)-induced anti-hyperalgesic effect was markedly blocked by i.c.v. injection of pirenzepine (1 μg), an antagonist of muscarinic acetylcholine receptor (Fig. 2D; $F_3 = 26.6$; **$p < 0.01$, **$p < 0.01$).

Donepezil reverses ICS-induced pain via supra-spinal actions. The above findings prompted us to investigate whether similar beneficial effects were observed with donepezil, which readily penetrates into the brain (Banks, 2012). When doses of donepezil as low as 10 μg/kg were i.p. administered to ICS-treated mice, anti-hyperalgesic actions of this compound were observed with peak effects at 90-120 min post-injection (Fig. 3A; 0 h, $F_3 = 120.4$; 0.5 h, $F_3 = 18.8$; 1 h, $F_3 = 15.4$; 1.5 h, $F_3 = 14.7$; 2 h, $F_3 = 12.4$; 2.5 h, $F_3 = 13.8$; 3 h, $F_3 = 16.0$; **$p < 0.01$, $^#p < 0.05$, **$p < 0.01$).
Some, although not significant, anti-hyperalgesic effects still remained even at 180 min (Fig. 3A). There was a dose-dependent range of 1-100 μg/kg, with an ED50 as low as 2 μg/kg (Fig. 3B; $F_0 = 9.4$; *$^0p < 0.01$, **$^p < 0.01$). Similar results were also observed in the mechanical nociception test (Fig. 3C; 0 h, $F_3 = 49.0$; 0.5 h, $F_3 = 7.2$; 1 h, $F_3 = 12.0$; 1.5 h, $F_3 = 11.0$; 2 h, $F_3 = 5.0$; 2.5 h, $F_3 = 13.8$; 3 h, $F_3 = 8.9$; *$p < 0.05$, **$^p < 0.01$, *$^p < 0.05$, **$^p < 0.01$). Similar to the actions of pilocarpine, an i.c.v. injection of donepezil at a dose of 10 μg completely reversed the thermal hyperalgesia with a peak time of 60-90 min (Fig. 3D; 0 h, $F_3 = 105.1$; 0.5 h, $F_3 = 11.3$; 1 h, $F_3 = 14.1$; 1.5 h, $F_3 = 13.4$; 2 h, $F_3 = 18.2$; 2.5 h, $F_3 = 19.2$; 3 h, $F_3 = 27.5$; **$p < 0.01$, **$^p < 0.01$). Significant anti-hyperalgesia effects still remained at 180 min after injection. However, as shown in Fig. 3, E and F, there was no change with an i.t. injection of donepezil at 10 μg (Fig. 3E; 0 h, $F_3 = 32.7$; 0.5 h, $F_3 = 8.9$; 1 h, $F_3 = 24.7$; 1.5 h, $F_3 = 15.3$; 2 h, $F_3 = 18.4$; 2.5 h, $F_3 = 15.8$; 3 h, $F_3 = 18.5$; *$p < 0.05$, **$^p < 0.01$). According to the dose-dependent effects (Fig. 3F; i.c.v. at P5, $F_5 = 8.3$; i.c.v. at P7, $F_3 = 10.6$; i.t. at P5, $F_3 = 15.3$; i.t. at P7, $F_3 = 17.8$; **$p < 0.01$, **$^p < 0.01$), the calculated ED50 (i.c.v.) of donepezil was 1 μg. In all experiments above, no significant effects of donepezil, either through systemic or central routes, were observed in control mice. To observe muscarinic antagonism, we tested the basal effects on nociceptive threshold with 100 ng (i.c.v.) atropine sulfate in normal mice. Because this antagonist had no effect on thermal nociception in control mice (Fig. 3G; 0 h, $F_4 = 70.0$; 0.5 h, $F_4 = 8.7$; 1 h, $F_4 = 6.4$; 1.5 h, $F_4 = 12.4$; 2 h, $F_4 = 10.6$; 2.5 h, $F_4 = 25.1$; 3 h, $F_4 = 8.8$; *$p < 0.05$, **$^p < 0.01$, *$^p < 0.05$, †$p < 0.05$), we examined the blockade of the anti-hyperalgesia effect of donepezil (i.p.) by i.c.v. injection of atropine. As shown in Fig. 3, G and H, atropine (i.c.v.) at 10 and 100 ng dose-dependently blocked the donepezil effect. The ID50 of atropine (i.c.v.) was...
approximately 10 ng (Fig. 3H; $F_5 = 8.2$; $p < 0.05$, $p < 0.05$, $p < 0.05$).

**Complete cure of chronic pain by repeated treatments with donepezil.** Based on the long-lasting effects of donepezil by systemic or i.c.v. injection, we examined the possibility of accumulative donepezil effects. In the present paradigm, donepezil was administered at a dose of 10 $\mu$g/kg (i.p.) once daily from P5 to P10. As seen in Fig. 4A (0 h at P5, $F_3 = 120.4$; 0.5 h at P5, $F_3 = 18.8$; 1 h at P5, $F_3 = 15.4$; 1.5 h at P5, $F_3 = 14.7$; 2 h at P5, $F_3 = 12.4$; 2.5 h at P5, $F_3 = 13.8$; 3 h at P5, $F_3 = 16.0$; 0 h at P7, $F_3 = 88.5$; 0.5 h at P7, $F_3 = 17.6$; 1 h at P7, $F_3 = 17.4$; 1.5 h at P7, $F_3 = 9.7$; 2 h at P7, $F_3 = 18.3$; 2.5 h at P7, $F_3 = 9.6$; 3 h at P7, $F_3 = 15.0$; 0 h at P9, $F_3 = 32.7$; 0.5 h at P9, $F_3 = 13.4$; 1 h at P9, $F_3 = 19.6$; 1.5 h at P9, $F_3 = 11.1$; 2 h at P9, $F_3 = 10.3$; 2.5 h at P9, $F_3 = 14.2$; 3 h at P9, $F_3 = 22.3$; $**p < 0.01$, $^p < 0.05$, $^{##}p < 0.01$), donepezil had no acute effects at day P5, P7, or P9 in control mice. In addition, the basal threshold at day P11 was not affected by previous donepezil administrations in control mice (Fig. 4B; P1, $F_3 = 72.3$; P5, $F_3 = 63.2$; P6, $F_3 = 45.4$; P7, $F_3 = 88.1$; P8, $F_3 = 37.8$; P9, $F_3 = 36.6$; P10, $F_3 = 32.1$; P11, $F_3 = 47.7$; P12, $F_3 = 11.3$; P14, $F_3 = 15.6$; P16, $F_3 = 19.0$; P18, $F_3 = 70.0$; $**p < 0.01$, $^p < 0.05$, $^{##}p < 0.01$). In experiments using ICS mice, however, the basal nociceptive threshold at P7 was significantly greater than the basal threshold on P5 (Fig. 4B). The acute anti-hyperalgesia effects of donepezil were still observed, but the maximum threshold never exceeded the naive threshold (Fig. 4A). This fact resulted in some decrease in the acute anti-hyperalgesia effects, which were evaluated by area under the curve (Fig. 4C; $F_3 = 32.2$; $**p < 0.01$). Similar results were also observed with donepezil injection at P9 (Fig. 4C). In addition, the basal threshold at P11 reached approximately 10 s, which corresponds to the threshold of naive mice (Fig. 4B).

When the basal thresholds prior to donepezil injection were plotted, they gradually
increased along with daily injection of donepezil (six times from P5 to P10), as shown in Figure 4B. The basal thresholds from P11 to P18, even after cessation of donepezil treatments, were similar to the naive level (Fig. 4B). Repeated treatments with donepezil showed no significant changes in the basal threshold of control mice (Fig. 4B). When mechanical allodynia effects were evaluated instead of thermal hyperalgesia, the basal thresholds of ICS-treated mice at P1 through P18 were significantly less than the control (Fig. 4D; P1, $F_3 = 47.7$; P12, $F_3 = 13.8$; P18, $F_3 = 22.9$; **$p < 0.01$, ##$p < 0.01$). Repeated treatments with donepezil completely reversed mechanical allodynia at P12 and P18 in ICS-treated mice, but these treatments had no effect on basal mechanical threshold in control mice (Fig. 4D). When paw withdrawal behaviors induced by differential frequencies of electrical stimulation were evaluated, there were significant decreases in the nociceptive threshold to electrical stimuli of 250 Hz and 2000 Hz, but not 5 Hz (Fig. 4E; 250-Hz at P13, $F_3 = 7.1$; 250-Hz at P18, $F_3 = 18.0$; 2000-Hz at P12, $F_3 = 7.8$; 2000-Hz at P19, $F_3 = 11.7$; *$p < 0.05$, **$p < 0.01$, #$p < 0.05$, ##$p < 0.01$). This type of hypersensitivity to 250 Hz or 2000 Hz electrical stimuli was also completely reversed with repeated donepezil treatments at P12-13 and P18-19 (Fig. 4E).

**No influence on the lack of morphine analgesia by repeated donepezil treatments.** We have successfully demonstrated that no significant analgesia is observed by subcutaneous or i.c.v. injection of morphine in ICS-treated mice (Nishiyori et al., 2010). As shown in Fig. 5, A and B, analgesic activity of morphine (i.c.v.) remained poor even after the basal nociceptive threshold in ICS-treated mice completely recovered following repeated donepezil treatments (Fig. 5A; control-vehicle-morphine, $F_6 = 6.3$; control-donepezil-morphine, $F_6 = 4.5$; *$p < 0.05$). Figure 5B demonstrated
quantitative evidence that repeated donepezil treatments did not affect the
dose-dependent (0.03-0.3 nmol, i.c.v.) morphine analgesia in control mice or the lack of
morphine analgesia in ICS-treated mice (Fig. 5B; control-vehicle-morphine, $F_3 = 10.2$;
control-donepezil-morphine, $F_3 = 9.7$; *$p < 0.05$, **$p < 0.01$).
Discussion

In the present study, we used an ICS model to replicate a unique, generalized, chronic pain syndrome, which includes fibromyalgia. Our previous study has shown that ICS triggers chronic and bilateral thermal hyperalgesia, as well as mechanical allodynia (Nishiyori and Ueda, 2008). ICS-induced hyperalgesia and allodynia is specific for intermittent stress, because pain hypersensitivity against thermal and mechanical stimuli can be observed for only 1 day after constant cold stress (Nishiyori and Ueda, 2008; Nishiyori et al., 2010). Although there is no significant difference in the basal pain threshold between male and female mice, significant female-predominant sex differences in mechanical allodynia can be observed (Nishiyori and Ueda, 2008). Compared with other experimental fibromyalgia-like pain models, the ICS model appears to have several advantages in terms of pharmacotherapeutic responses, or their description in terms of similarity to clinical evidence.

First, the gabapentin dose required for the suppression of ICS-induced mechanical allodynia is 10 times less than that for the partial sciatic nerve ligation-induced neuropathic pain (Nishiyori and Ueda, 2008). Interestingly, i.c.v. injection of gabapentin as low as 3 μg completely inhibits ICS-induced allodynia for 2 days, and significant anti-allodynia effects last at least for 4 days, whereas i.c.v. injection of gabapentin has no significant action on neuropathic pain (Nishiyori and Ueda, 2008). Similar differences between ICS and injury models can be observed in the analgesic action of morphine. The analgesic effects of systemically administered morphine is 10 times less potent in nerve injury-induced neuropathic pain model mice, but is completely lost in the ICS model (Nishiyori et al., 2010). Clinical evidence has also shown that opioids are
ineffective in suppressing pain in fibromyalgia patients (Clauw, 2014). One of the underlying mechanisms could be attributed to reduced availability of opioid receptors in the brain (Harris et al., 2007), possibly owing to excess amounts of endogenous opioids in the CSF of fibromyalgia patients (Baraniuk et al., 2004). The reduced sensitivity to morphine in the ICS model may be related to functional deficits in the descending serotonergic activity (Ohara et al., 1991; Omiya et al., 2000; Nishiyori et al., 2010), which is consistent with biochemical evidence in clinical samples (Clauw, 2009).

Our initial intention was to use various existing drugs that have some indications for fibromyalgia patients. Pilocarpine is the representative drug used to treat dry-eye and dry-mouth, which are often observed in fibromyalgia patients (Bennett, 2005). Systemic injection of pilocarpine reversed thermal hyperalgesia and mechanical allodynia after ICS exposure. Analysis of its dose dependency revealed that pilocarpine caused not only reversal of ICS-induced hyperalgesia and allodynia, but also some ‘analgesic’ activity in the ICS model. Although details remain to be determined, we speculate the involvements of up-regulation of muscarinic receptors in pilocarpine and donepezil actions from the following findings; (1) as pirenzepine or atropine (i.c.v.) had no hyperalgesic action in naive animals, the muscarinic activation by tonic release of ACh unlikely plays pain inhibitory roles in the brain. (2) The findings that pilocarpine (i.c.v.) showed ‘analgesic’ actions over the reversal of abnormal pain in the ICS model mice, but it had only less significant effects in naive mice, suggested that up-regulated muscarinic receptor or related down-stream mechanisms may underlie the analgesic activity and blockade of abnormal pain in the ICS model mice. The identification of muscarinic receptor subtype would be the next important subject. Pilocarpine-induced inhibition of abnormal pain and pirenzepine-induced antagonism were observed when it
was administered by i.c.v. injection, but not by i.t. injection, which suggests that major sites of muscarinic receptor-mediated actions are in the brain.

To further assess the role of central cholinergic systems in fibromyalgia pain, we used donepezil, which has a high uptake into the brain (De Vos et al., 2000). Donepezil showed potent beneficial actions in the ICS model. It should be noted that the dose required for maximum inhibition of abnormal hyperalgesia and allodynia was 10 μg/kg (i.p.), which was several hundred-times less than that reported to treat dementia in animal models (Saxena et al., 2008). Similar to pilocarpine, donepezil inhibited ICS-induced thermal hyperalgesia only by i.c.v. injection, and the action by systemic donepezil was antagonized by i.c.v. atropine and pirenzepine. Considering that nicotinic acetylcholine receptor is also known to participate in the pain regulation (Jones and Dunlop, 2007), further studies are required to elucidate whether donepezil could inhibit ICS-induced hyperalgesia and allodynia via central nicotinic acetylcholine receptors.

On the other hand, it should be noted that pilocarpine-treatment reversed the hyperalgesia and exceeds the normal pain threshold, while donepezil-treatment only reversed it. Although mechanisms underlying different actions of pilocarpine and donepezil remain elusive, it may be interesting to compare the pharmacological mechanisms of both compounds. Because donepezil inhibits degradation of released acetylcholine, the action is presumably confined in the synaptic area, while pilocarpine may have more potential targets, including extrasynaptic receptors, which could be expressed only under pathological situations.

Of importance, systemic or i.c.v. donepezil inhibited ICS-induced hyperalgesia and allodynia for more than 3 h. As shown in Figure 4B, the basal threshold prior to the donepezil treatment at P5 after ICS was approximately 6 s, while it increased to
approximately 8 s 2 days later, at P7. As expected, repeated daily treatments with donepezil gradually increased the threshold to the level of normal mice. The most important finding was observed in the complete amelioration of chronic pain, even after the cessation of donepezil treatments, implying that pain memory in fibromyalgia might disappear after repeated treatments with donepezil. Likewise, a complete amelioration of chronic pain in the ICS model has also been observed when antidepressants, such as milnacipran, amitriptyline, mianserin, and paroxetine, are repeatedly administered by i.t. injection (Nishiyori et al., 2011). Thus, chronic pain in this model may be maintained by vicious cycles, which include the attenuation of a spinally descending, pain inhibitory system and enhancement within pain matrices in the brain. In other words, pain memory may disappear once these vicious cycles are completely lost.

The present study showed that donepezil inhibited ICS-induced fibromyalgia-like pain via supraspinal mechanisms, while it blocks neuropathic pain via spinal mechanisms (Kimura et al., 2013). Such different pharmacotherapeutical actions against neuropathic pain and ICS-induced fibromyalgia-like pain can be observed in gabapentinoid actions, as described above. We have shown that gabapentin blocks ICS-induced fibromyalgia-like pain via supraspinal mechanisms, while it inhibits nerve injury-induced neuropathic pain via spinal mechanisms (Nishiyori and Ueda, 2008). Consistently, a recent clinical neuroimaging study has shown that pregabalin inhibits neuronal connectivity in the brain regions of fibromyalgia patients (Harris et al., 2013). Therefore, neuropathic pain and fibromyalgia-like pain may show the difference in the site of donepezil actions, possibly owing to their distinct molecular basis.

Finally, a complete amelioration of using extremely low doses of donepezil would be ideal. However, at the same time, the present successful therapeutic actions of
donepezil may be a result of simple experimental animal models. In addition, because the majority of fibromyalgia patients may have more complicated causes, some additional drugs might be required to treat their symptoms.

In conclusion, we successfully demonstrated that cholinomimetics, such as pilocarpine and donepezil, inhibited generalized pain syndrome in ICS-exposed mice, which share some pathophysiological and pharmacotherapeutic features with fibromyalgia patients. Repeated treatments with donepezil completely ameliorated the chronic pain, although the lack of morphine analgesia remained even after complete amelioration of pain. As the current criteria of fibromyalgia (Wolfe et al., 2010) uses pain-related somatic symptoms as well as muscle pain, future investigation is necessary to evaluate some more different assessments, such as muscle pain using Randall-Selitto test, according to the report in rats using similar paradigm of ICS/repeated cold stress (Nasu et al., 2010) as well as known tail-flick and hot plate test.
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Authorship Contributions

Participated in research design: Mukae, Uchida and Ueda
Conducted experiments: Mukae
Performed data analysis: Mukae and Uchida
Wrote or contributed to the writing of the manuscript: Uchida and Ueda
References


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Footnotes

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Figure legends

**Fig. 1.** Inhibition of ICS-induced hyperalgesia and allodynia by systemic pilocarpine injection. Thermal and mechanical paw withdrawal tests were performed at P5 after ICS exposure. (A) Time course of thermal paw withdrawal latencies (PWL, in seconds) after pilocarpine (1 mg/kg, i.p.) injection. (B) Dose-dependent effects of pilocarpine injection on thermal pain thresholds in control mice and ICS-treated mice, assessed at 20 min after pilocarpine (i.p.) treatment. (C) Time course of mechanical paw withdrawal latencies (PWT, in grams) after pilocarpine (1 mg/kg, i.p.) injection. Data represent means ± SEM from experiments using 3-14 mice. **p < 0.01 vs. vehicle-treated control mice; #p < 0.05, ##p < 0.01 vs. vehicle-treated ICS mice.

**Fig. 2.** Anti-hyperalgesic effects of pilocarpine via supra-spinal action. (A and B) Time course of thermal paw withdrawal latencies (PWL, in seconds) after i.c.v. (A) and i.t. (B) injection of pilocarpine (1 μg) at P5 after ICS. *p < 0.05, **p < 0.01 vs. vehicle-treated control mice; #p < 0.05, ##p < 0.01 vs. vehicle-treated ICS mice. (C) Dose-dependent effects of i.c.v. and i.t. injection of pilocarpine on thermal pain thresholds in control mice and ICS-treated mice at P5, assessed at 10 min after pilocarpine (i.c.v. and i.t.) treatment. In experiments with i.t. injection, mice treated with pilocarpine (1 μg) at P5 were used to evaluate effects of pilocarpine (3 μg) at P7. *p < 0.05, **p < 0.01 vs. vehicle-treated control mice; ##p < 0.01 vs. pilocarpine-treated ICS mice. (D) Effect of i.c.v. injection of pirenzepine (1 μg) on pilocarpine (1 mg/kg, i.p.)-induced anti-hyperalgesic action in ICS-treated mice, assessed at 20 min after pilocarpine injection. **p < 0.01 vs. vehicle-treated ICS mice; ##p < 0.01 vs.
pilocarpine-treated ICS mice. Data represent means ± SEM from experiments using 3-6 mice.

**Fig. 3.** Anti-hyperalgesic effects of donepezil via supraspinal action. (A) Time course of thermal paw withdrawal latencies (PWL, in seconds) after donepezil (10 μg/kg, i.p.) injection at P5 post-ICS. (B) Dose-dependent effects of donepezil (i.p.) injection on thermal pain thresholds in control and ICS-treated mice, assessed at 2 h after donepezil treatment. (C) Time course of mechanical paw withdrawal latencies (PWT, in grams) after donepezil (10 μg/kg, i.p.) injection. (D and E) Time course of thermal pain threshold after i.c.v. (D) and i.t. (E) injection of donepezil (10 μg). (F) Dose-dependent effects of donepezil (i.c.v. and i.t.) injection on thermal pain thresholds in control and ICS-treated mice, assessed at 1.5 h after donepezil (i.c.v. and i.t.) treatment. Mice treated with donepezil (10 μg) at P5 were used to evaluate effects of donepezil (100 μg) at P7. *p < 0.05, **p < 0.01 vs. vehicle-treated control mice; #p < 0.05, ##p < 0.01 vs. vehicle-treated ICS mice. (G) Effects of atropine (100 ng, i.c.v.) on basal pain threshold in control mice and anti-hyperalgesic action of donepezil (10 μg/kg, i.p.) in ICS-treated mice. (H) Dose-dependent inhibitory action of atropine (i.c.v.) on donepezil-induced anti-hyperalgesic effect, assessed at 2 h after donepezil (i.p.) injection. *p < 0.05, **p < 0.01 vs. vehicle-treated control mice; #p < 0.05 vs. vehicle-treated ICS mice; †p < 0.05 vs. donepezil-treated ICS mice. Data represent means ± SEM from experiments using three to six mice.
Fig. 4. Recovery of ICS-induced hyperalgesia and allodynia by repeated treatments with donepezil. Donepezil (10 μg/kg, i.p.) or vehicle was treated once daily from P5 to P10 after ICS. (A and B) Time course of thermal paw-withdrawal latencies (PWL, in seconds) (A) and basal thermal pain threshold (assessed just before the daily injection of vehicle or donepezil) (B) after donepezil injection in control and ICS-treated mice. *p < 0.05, **p < 0.01 vs. vehicle-treated control mice; #p < 0.05, ##p < 0.01 vs. vehicle-treated ICS mice. (C) Comparison of analgesic actions of donepezil at P5, P7, and P9 by area under the curve (AUC) in ICS-treated mice. **p < 0.01 vs. vehicle-treated ICS mice at P5. (D) Time course of basal mechanical paw withdrawal latencies (PWT, in grams) after donepezil injections in control and ICS-treated mice. (E) Effects of donepezil treatments on paw withdrawal thresholds against 5, 250, and 2000 Hz of electrical stimuli (μA) after ICS exposure. *p < 0.05, **p < 0.01 vs. vehicle-treated control mouse; #p < 0.05, ##p < 0.01 vs. vehicle-treated ICS mice. Data represent means ± SEM from experiments using three to six mice.

Fig. 5. Failure to improve lack of morphine analgesia in ICS-treated mice by donepezil treatments. Donepezil (10 μg/kg, i.p.) or vehicle was treated once daily from P5 to P10 after ICS. (A) Time course of morphine (0.3 nmol, i.c.v.) analgesia in ICS model mice and control mice repeatedly treated with vehicle or donepezil (10 μg/kg, i.p.). *p < 0.05 vs. corresponding 0 min. (B) Effects of repeated treatments with donepezil on dose-dependent analgesic action of morphine (i.c.v.) in control and ICS-treated mice. Mice treated with aCSF at P11 were used to evaluate effects of morphine (0.03 nmol) at P12, morphine (0.1 nmol) at P13, and then morphine (0.3 nmol) at P14. *p < 0.05, **p < 0.01 vs. control-vehicle-aCSF-treated mice. Data represent means ± SEM from
experiments using four mice.
Figure 1

A

B

C

Cont
ICS

Pilocarpine (mg/kg, i.p.)

0 1.0

0 0.1 0.3 1.0

Cont-vehicle
Cont-pilocarpine (1 mg/kg, i.p.)
ICS-vehicle
ICS-pilocarpine (1 mg/kg, i.p.)

PWL (sec)

PWT (g)

Time after injection (min)

Time after injection (min)
Figure 5

A

Donepezil (10 μg/kg, i.p.)
Morphine (0.3 nmol, i.c.v.)

- ○ Cont-vehicle-morphine
- ■ Cont-donepezil-morphine
- □ ICS-vehicle-morphine
- ■ ICS-donepezil-morphine

PWL (sec)

Time after injection (min)

B

Cont

ICS

Veh DPZ Veh DPZ

AUC

Morphine (nmol, i.c.v.)