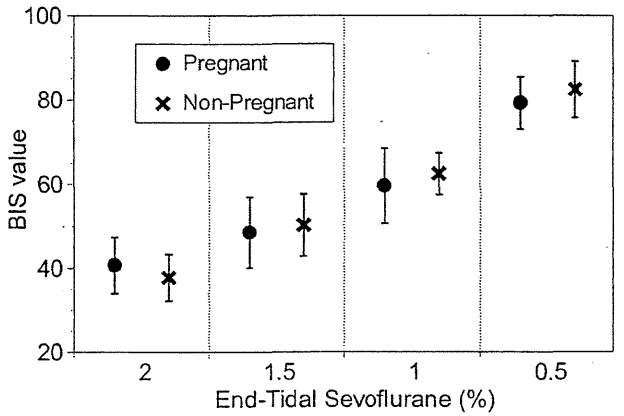

Fig. 5. Bispectral index (BIS) value at 2.0, 1.5, 1.0, and 0.5% sevoflurane concentrations in the pregnant and nonpregnant groups. Data are mean values with SD. There was no significant difference between groups.

Because sample size calculation was designed on the assumption of detecting 20% differences in mean value between two groups, we could have missed differences under 20%. In clinical studies, evaluating the difference often provides more information than statistical significance testing. Actually, the observed differences between electroencephalographic parameters in the two groups at each sevoflurane concentration were all less than the SD (table 4). Therefore, we believe that there are no clinically significant differences between the two groups. This consequently suggests that there is no important difference in the hypnotic effect of sevoflurane in pregnant and nonpregnant women.

Table 5. Results of Two-way Analysis of Variance

Parameter & Effect	NDF	DDF	*F*	*P* Value
SEF95				
Group	1	112	2.83	0.0953
Sevoflurane	3	112	49.17	< 0.0001
Group·Sevoflurane	3	112	1.16	0.3265
Amplitude				
Group	1	112	0.02	0.8777
Sevoflurane	3	112	88.71	< 0.0001
Group·Sevoflurane	3	112	0.61	0.6076
BIS				
Group	1	112	0.99	0.3224
Sevoflurane	3	112	195.89	< 0.0001
Group·Sevoflurane	3	112	1.25	0.2968
BIC-low				
Group	1	112	0.55	0.4593
Sevoflurane	3	112	23.4	< 0.0001
Group·Sevoflurane	3	112	1.42	0.2396
BIC-high				
Group	1	112	1.93	0.1672
Sevoflurane	3	112	41.7	< 0.0001
Group·Sevoflurane	3	112	0.29	0.8293

The group effect (pregnant/nonpregnant), sevoflurane concentration effect, and group·sevoflurane interaction for each electroencephalographic parameter.

BIS = bispectral index; DDF = denominator degrees of freedom; Group = pregnant *vs.* nonpregnant group; Group·Sevoflurane = interaction between group and sevoflurane; NDF = numerator degrees of freedom; pBIC-high = peak heights of bicoherence at 5–10 Hz; pBIC-low = peak heights of bicoherence at 3–5 Hz; SEF95 = 95% spectral edge frequency; Sevoflurane = sevoflurane concentration (0.5, 1.0, 1.5, and 2.0%).

Why did the electroencephalogram not indicate a difference in the hypnotic effect in the subjects in whom MAC was thought to be different? MAC represents the alveolar concentration of inhalational anesthetics that prevents 50% of subjects from moving in response to noxious stimuli.[6] Because such movement is considered an escape response from painful stimuli, MAC has been considered an indicator of the effect of anesthetics on the brain. However, as indicated in Introduction, animal studies have shown that MAC indicates the effect of anesthetics on the spinal cord,[7–9] whereas the electroencephalogram shows the anesthetic effect on the brain. Therefore, it is not surprising that hypnotic levels indicated by electroencephalogram are similar in pregnant and nonpregnant women, although MAC may differ between the two groups. The results of this study show that the decrease in MAC during pregnancy cannot validate the rationale that parturients require less volatile anesthetics.

Various factors affect the electroencephalographic waveforms during surgery, one being noxious stimuli. If analgesic dosage is insufficient, noxious stimuli can increase the frequency and decrease the amplitude of high-amplitude slow waves induced by anesthetics.[15] This phenomenon is called desynchronization,[15] in which consciousness likely returns by noxious stimuli reaching the brain. In this study, two types of operations (cesarean section in the pregnant group and gynecological surgery in the nonpregnant group) were performed. It remains possible that the intensity of noxious stimulus differed depending on the type of operation. One method to evaluate the intensity of the noxious stimulus is intraoperative hemodynamic changes. In our study, because both the preoperative and intraoperative heart rates were higher in the pregnant group, the hemodynamic values are not appropriate. Another method to evaluate the intensity of noxious stimuli is bicoherence, a parameter that indicates the hypnotic effect of anesthetics. Bicoherence disappears when a strong noxious stimulus is applied, because noxious stimuli induce an arousal electroencephalographic pattern.[16] It is restored or prevented by sufficient opioid analgesia.[16] In this study, bicoherence was similar in the pregnant and nonpregnant groups at 2 and 1.5% sevoflurane. We believe that fentanyl analgesia minimized the noxious stimuli, and the degree of noxious stimuli was equivalent in both groups.

Some drugs administered intraoperatively also affect the electroencephalographic waveforms. Thiopental is known to affect electroencephalogram; however, the duration is short. According to a previous report, nonpregnant patients recovered consciousness 330 ± 153 s after induction of 4 mg/kg thiopental.[17] At the time of return of consciousness, the BIS value was 81 ± 5.[17] Redistribution is the principal mechanism accounting for this early awakening. Approximately 10–15 min after 4–6 mg/kg thiopental administration, serum levels fall to 5 μg/ml, which is equivalent to 50% of awakening level in both parturients and nonpregnant patients.[18,19] Al-

though the dose requirement of thiopental in parturients is approximately 18% lower compared with nonpregnant women,[20] the blood concentration of thiopental at our study period (20–150 min after induction) is thought to be sufficiently low to affect the electroencephalogram. A muscle relaxant itself does not have an effect on electroencephalogram. However, electromyogram artifacts greatly change the electroencephalographic waveform. If an electromyogram is combined with the electroencephalogram, the electroencephalographic frequency increases, and it comes to show an arousal pattern. To prevent this, we administered muscle relaxant in all cases.

Some factors affect not only the electroencephalogram but also MAC. Drugs such as ephedrine increase MAC by increasing the catecholamine in the brain.[21] Such drugs are usually dose-related and require a very high dose.[22] The amount of ephedrine necessary to raise MAC by 50% has been reported to be as high as 0.04 mg/kg/min in a dog study.[21] Because the dosage of the ephedrine in our study was small (0.01 mg/kg, bolus), it was probably not enough to influence the MAC.

Hormonal changes associated with delivery also affect the MAC. According to previous human studies, the postpartum changes in MAC were as follows. During the first 1–12 h postpartum, the MAC of isoflurane was similar to the 0.775% measured in pregnant patients of 8–12 weeks' gestation.[3] MAC increased to 0.825% during the next 36 h, to reach normal values (1.125%) by 72 h postpartum.[23] Although MAC in the third trimester in parturients has not been examined because of ethical considerations, these results suggested that changes in MAC at the postpartum period are very slow. Therefore, we believe that rapid changes in MAC did not occur during our study period.

So how much volatile anesthetic should we administer during general anesthesia for cesarean section? It has been shown that the volatile anesthetic requirement for general anesthesia is lower than that required for prevention of body movements.[24] The results of this study showed that the BIS ranged from 40 to 60 in most patients in both groups at 1.5% sevoflurane concentration. By contrast, the BIS exceeded 60 in almost half of the patients in both groups at 1.0% sevoflurane concentration. The highest BIS value at 1.0% was 80. These results are almost the same as the BIS values for sevoflurane concentration determined by Katoh *et al.*[25] in nonpregnant patients and by Chin *et al.*[26] in pregnant patients.

Under the assumption that the BIS value for appropriate hypnosis during operation is less than 60,[10,27] approximately half of patients are insufficiently anesthetized at 1.0% sevoflurane concentration. Therefore, in cases without both electroencephalographic monitoring and nitrous oxide administration, at least 1.5% sevoflurane may be needed during maintenance of general anesthesia.

The dose requirements of inhalational anesthetics has been believed to be less for parturients because MAC is significantly decreased by pregnancy. To prevent undesired outcomes, such as neonatal suppression or uterine atony, parturients have routinely been administered volatile anesthetics at a lower concentration compared with nonpregnant women. However, our electroencephalographic study indicates that there is no difference between pregnant and nonpregnant women in the hypnotic effects of sevoflurane, despite the groups supposedly having different MAC values. These findings suggest that a decrease in MAC during pregnancy does not mean an enhanced volatile anesthetic effect on the brain. We believe that parturients should be given the same dose of anesthetics as nonpregnant women for prevention of intraoperative awareness. Thus, anesthesiologists should reconsider using MAC as an indicator of efficacy of volatile anesthetics.

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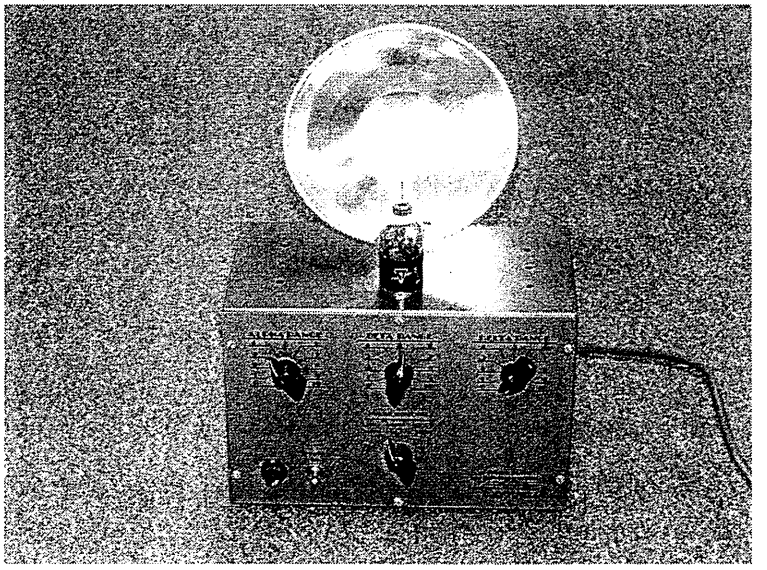
ANESTHESIOLOGY REFLECTIONS

The Schneider Brain Wave Synchronizer



After observing how some radar technicians had become "transfixed" by rhythmic flashing dots on their radar screens, inventor Sidney Schneider designed his Brain Wave Synchronizer (BWS) to hypnotize by visually stimulating subjects at frequencies mimicking those of their alpha, beta, or delta brainwaves. In 1959 Schneider and hypnotist-obstetrician William Kroger, M.D., published their use of the BWS in prenatal classes for thousands of women prior to its use as an "electronic aid for hypnotic induction" during labor and delivery. Four years later, Chicago anesthesiologist Max S. Sadove, M.D., published his work on how BWS-induced hypnosis could reduce anesthetic agent requirements during general anesthesia. By 1994 the BWS would be cited for causing epileptic seizures in a patient. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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5-HT2C receptor agonists attenuate pain-related behaviour in a rat model of trigeminal neuropathic pain

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ABSTRACT

Peripheral branches of the trigeminal nerve may be damaged during maxillofacial injury or surgical procedures and trigeminal trauma may induce severe pain that is very challenging to treat. Chronic constriction injury to the infraorbital nerve (ION-CCI) by loose ligatures has proven a useful model for some types of trigeminal neuropathic pain disorder. Using ION-CCI rats, we examined the antiallodynic effects of intrathecally administered agents which are selective for 5-HT2C receptors. Allodynia was evaluated by applying von Frey filaments to skin innervated by the injured ION. Dose-dependent antiallodynic effects followed administration of three 5-HT2C receptor agonists, 6-chloro-2-(1-piperazinyl)-pyrazine (MK212: 10, 30, and 100 μg); (S)-2-(chloro-5-fluoro-indol-l-yl)-1-methyamine fumarate (RO 60-0175: 10, 30, and 100 μg); (AaR)-8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-5(6H)-one (WAY-161503: 10, 30, and 100 μg). ED50 values for antiallodynic effects of MK212, RO 60-0175, and WAY-161503 were 39.62, 46.67, and 51.22 μg, respectively. Intrathecal administration of the 5-HT2C receptor antagonist, 8-[5-2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4,5]decane-2,4-dione (RS-102221: 30 μg) did not alter the mechanical threshold. Intrathecal pretreatment with RS-102221 (10 and 30 μg) reduced the antiallodynic effects of the highest dose of 5-HT2C agonists. These results indicated that, in this rat model, the 5-HT2C receptor plays a role in spinal inhibition of trigeminal neuropathic pain.

1. Introduction

Injury to the peripheral branches of the trigeminal nerve (TN) can arise from maxillofacial injuries, including surgical procedures. The incidence of post-traumatic TN disturbances ranges from 46% to 82% for facial fractures (Renzi et al., 2004; Schultze-Mosgau et al., 1999; Marchena et al., 1998; Jungell and Lindquist, 1987). This kind of peripheral nerve injury results in neuropathic pain. Clinical studies suggest that chronic pain syndrome occurs more frequently in the trigeminal system than at spinal levels (Sweet, 1984). Trigeminal neuropathic pain is very difficult to manage. So far, only antidepressants and anticonvulsants have been efficacious in treating trigeminal neuralgia, and as many as half of the patients become intolerant to these medications (Swerdlow, 1984).

Recently a rat model of trigeminal neuropathic pain produced by chronic constriction injury to the infraorbital nerve (ION-CCI) was developed (Kryzhanovski et al., 1992, 1993; Vos et al., 1994). In this animal model of trigeminal neuropathic pain, the behavioural abnormalities are difficult to treat, closely resembling the clinical disorders of human subjects suffering from trigeminal neuralgia (Idänpään-Heikkilä and Guilbaud, 1999; Christensen et al., 1999, 2001). Some studies have revealed that the pain syndrome caused by injury to the trigeminal nerve has distinctive pathophysiological mechanisms different to those occurring after lesion of other peripheral nerves (Latrémolière et al., 2008; Benoleil et al., 2001).

The neurotransmitter serotonin (5-HT) is important in modulating pain transmission in the spinal cord. In rat models, while the 5-HT1A receptor agonist, F13640, inhibits tactile allodynia in both ION-CCI and chronic constriction injury of the sciatic nerve (SN-CCI) (Deseure et al., 2002; Colpaert, 2002), 5-HT1B/1D receptor agonists, sumatriptan and zolmitritan, attenuate pain-related behaviour in ION-CCI but not SN-CCI (Kayser et al., 2002). In a rat model of spinal-nerve-ligation, 5-HT2C receptor agonists produce antiallodynic effects (Obata et al., 2004), but the antiallodynic effect mediated by 5-HT2C receptor agonists in the trigeminal neuropathic pain model remains unclear.

The present study evaluated, in ION-CCI rat model, the potential antiallodynic effects of intrathecal administration of 5-HT2C receptor agonists, MK212, RO 60-0175, and WAY-161503. In addition, the possible implication of the 5-HT2C receptor in these effects was assessed by investigating whether they could be prevented

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by intrathecal administration of the 5-HT2C receptor antagonist, RS-102221.

2. Methods

2.1. Animals

All surgical and experimental protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Osaka Medical College and performed according to the National Institutes of Health guidelines. We also conformed to the Guidelines of the International Association for the Study of Pain (Zimmermann, 1983). In particular, the duration of the experiments was kept as short as possible and as few as possible animals were used. Male Sprague–Dawley (SD) rats (body weight at time of surgery 180–220 g) were used. With free access to chow and tap water, the animals were housed at a temperature of 22 ± 2 °C in a 12-h light-dark cycle (lights on 08:00–20:00). Before surgery, the animals were allowed at least 1 week to habituate to the housing facilities.

2.2. Surgical procedure

Essentially according to the method described by Vos et al. (1994), injured rats received a unilateral chronic constriction injury to the right ION performed under direct control using an operation microscope. In brief, the animals were anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg i.p.). A midline scalp incision was made, exposing the skull and nasal bone. The edge of the orbit, formed by maxillary, frontal, lacrimal, and zygomatic bones, was dissected free. The ION was dissected free at its most rostral extent in the orbital cavity, just caudal to the infraorbital foramen. Two nylon (5–0) ligatures (2 mm apart) were loosely tied around the ION. To obtain the desired degree of constriction, a criterion formulated by Bennett and Xie (1988) was applied: the ligature reduced the diameter of the nerve by a just noticeable amount and retarded, but did not interrupt the epineural circulation through the superficial vasculature. The scalp incision was closed in layers using nylon sutures (5–0). After surgery, animals were housed with free access to food and water, and allowed to recover for at least 7 days. Tactile allodynia of the territory of the ligated nerve was confirmed at this time by measuring the mechanical response threshold in response to application of von Frey filaments. Only rats with hyper-responsiveness to mechanical stimulation were used. Then, an intrathecal catheter for upper cervical spinal injection of drugs was implanted to the rat anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg i.p.) (Yaksh and Rudy, 1976; Ahn et al., 1998). This polyethylene tube (PE10) was advanced 10 mm caudally through a tiny hole in the atlanto-occipital membrane and the dura. Rats with evidence of neuromuscular dysfunction were killed immediately. The other animals were allowed to recover 7 days before drug testing.

2.3. Behavioural analysis

All behavioural assessments were carried out in a quiet room, generally between 09:00 and 16:00. A series of von Frey filaments were used to determine pain hypersensitivity to mechanical stimulation. To observe behavioural response to the mechanical stimulation, rats were individually placed in a plastic cage (25 × 40 × 18 cm) with bedding. After the rats were accommodated for 1 h, von Frey filaments (bending force 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 15.0 g) were delivered to skin innervated by the injured ION near the center of vibrissal pad, on the hairy skin surrounding the mystacial vibrissae. Each von Frey filament was applied five times to the same region at approximately 1 s intervals. Upon application of a von Frey filament, head withdrawal and touching or scratching the facial regions were counted as positive pain responses as previously described (Piao et al., 2006). The response threshold was defined as the lowest filament force that produced at least three positive responses in five trials. If no pain response was elicited with the 15 g filament, the threshold was recorded as 15 g. Motor function was evaluated by righting reflex, stepping reflex, posture, and ambulation. The investigator was blinded to drug treatment for all studies.

2.4. Experimental protocol and drugs

The first series of experiments examined the time course of the antiallodynic effects and the dose–response effects of intrathecally administered the 5-HT2C receptor agonists. The 5-HT2C receptor agonists were 6-chloro-2-(1-piperazinyl)-pyrazine (MK212: 10, 30, and 100 μg), (S)-2-(chloro-5-fluoro-indol-l-yl)-1-methyamine fumarate (RO 60-0175: 10, 30, and 100 μg), and (AaR)-8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-5(6H)-one (WAY-161503: 10, 30, and 100 μg). The second series of experiments evaluated the time course of the mechanical threshold following intrathecal administration of the 5-HT2C receptor antagonist, 8-[5-2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4,5]decane-2,4-dione (RS-102221: 30 μg). The third series of experiments determined the effect of pretreatment with intrathecal administration of RS-102221 (10 or 30 μg), on the antiallodynic action of MK212, RO 60-1075, and WAY-161503. MK212, RO 60-0175, and WAY-161503 were dissolved in distilled water. RS-102221 was dissolved in 50% dimethylsulfoxide (DMSO). All drugs were purchased from Tocris Bioscience (Bristol UK). Drugs were delivered in 10 μl volume of solution followed by 10 μl of saline to flush the catheter. Throughout the study, after determining the baseline value, withdrawal thresholds were measured at 30, 60, 90, 120 min after drug injection. In the antagonist studies, the antagonist was administered first and the agonist was injected 10 min later.

2.5. Data analysis and statistics

Data were expressed as mean ± SEM. Time-course data are presented as withdrawal threshold. Percentage of the maximum possible effect (%MPE) was calculated using the following formula, %MPE = (postdrug threshold − predrug threshold)/(15 g − predrug threshold) × 100. The areas under the time-course curves (AUC) for the %MPE were then calculated from individual scores at each time point and divided by the maximum score that could be obtained over the 120 min observation period (% maximum possible AUC) .The respective ED50 values and 95% confidence intervals (CI) were calculated using linear regression. Data for the time course of dose–response effects of the agonists were analyzed using two-way analysis of variance, and statistical differences were resolved post hoc using the Tukey–Kramer multiple-comparison test. For antagonist studies, the % maximum possible AUC of agonists, and those observed in the presence of the antagonist were compared by an analysis of variance and statistical differences were resolved post hoc using the Tukey–Kramer multiple-comparison test. Statistical significance was set as $P < 0.05$.

3. Results

3.1. Antiallodynic effects of 5-HT2C receptor agonists

Intrathecal administration of each 5-HT2C receptor agonist, MK212, RO 60-0175, and WAY-161503, produced dose-dependent

antiallodynic effects. Fig. 1A–C shows the time course of the antiallodynic effects of each agonist. From 30 min to 120 min after injection, mechanical thresholds after treatment with 100 μg of each agonist were significantly higher than after treatment with 10 μg of each agonist. After treatment with 30 μg of MK212 or WAY-161503, 30 min after injection, mechanical thresholds were significantly higher than 30 min after treatment with 10 μg of either agonist. The peak effects of intrathecal administration of these agonists occurred 30 min after injection; the peak effect of RO 60-0175 lasted for up to 60 min. Fig. 2 shows the dose–response relation of the peak effect of each agonist. The ED50 (95% CI) values for the antiallodynic effect of MK212 was 39.62 μg (26.03–53.22 μg), RO 60-0175, 46.67 μg (31.31–62.03 μg), and WAY-161503, 51.22 μg (39.23–63.21 μg), respectively. Although

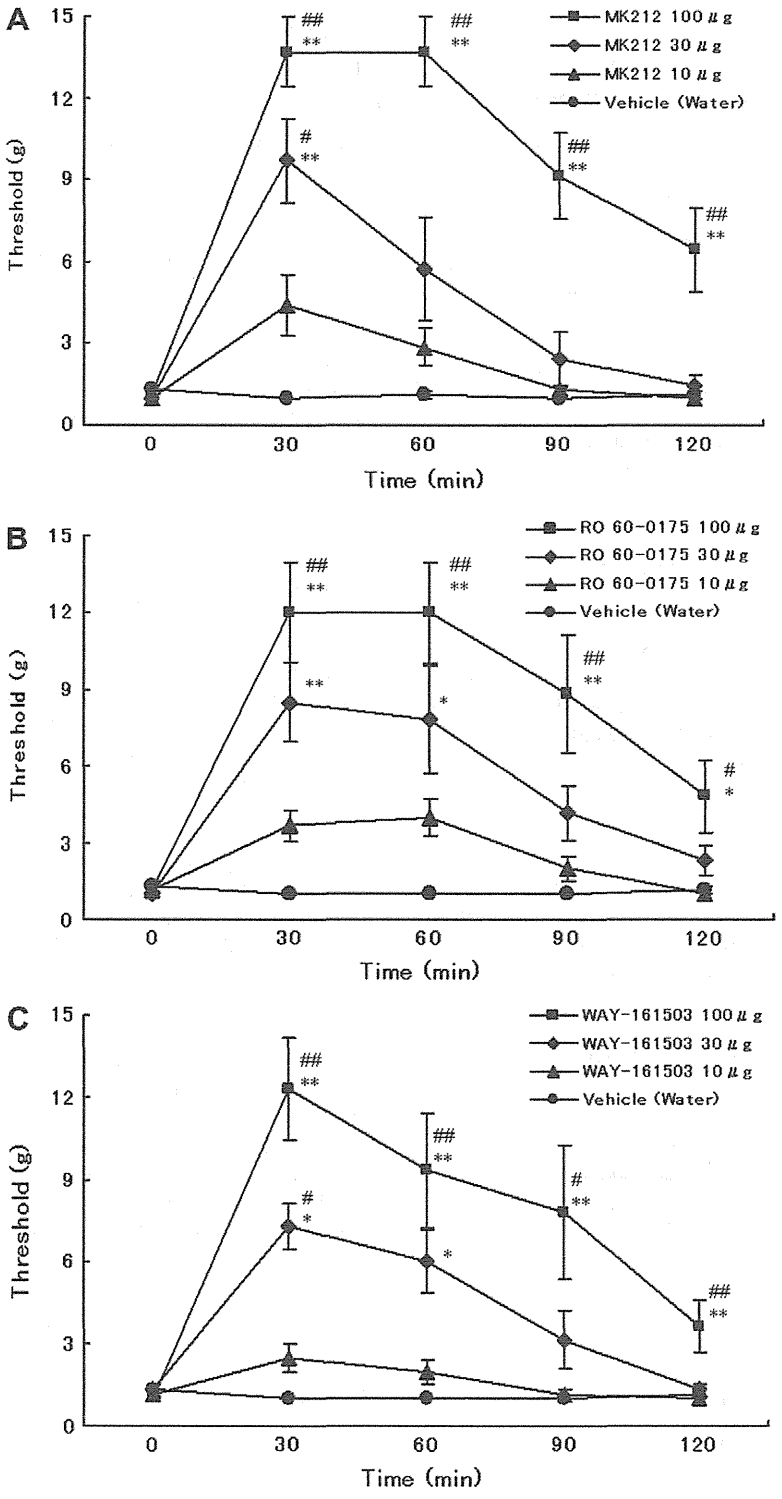

Fig. 1. In rats with infraorbital nerve ligation: time course of antiallodynic effects of intrathecally administered 5-HT2C receptor agonists: (A) MK212, (B) RO 60-0175, or (C) WAY-161503. Mechanical thresholds are expressed as mean ± SEM for six or seven rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle (water) group; $\#P < 0.05$ $\#\#P < 0.01$ compared with 10 μg-treated group.

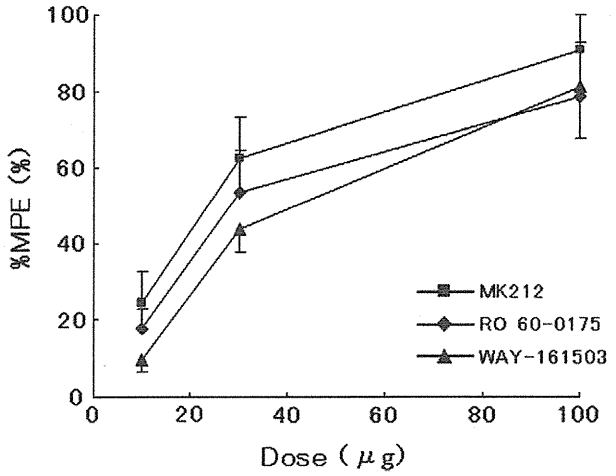

Fig. 2. In rats with infraorbital nerve ligation: dose–response curves plotting the peak effect of intrathecally administered 5-HT2C receptor agonists. The data are expressed as mean ± SEM of a percentage of the maximal possible effect (%MPE) for six or seven rats.

the ED50 values of these three agonists were similar, the value of MK212 was lower than that of RO 60-0175 and WAY-161503. Motor function assessed by righting reflex, stepping reflex, posture, and ambulation was normally preserved after intrathecal injection of all doses of these agonists.

3.2. Mechanical thresholds of the 5-HT2C receptor antagonist

Intrathecal administration of 30 μg of RS-102221 neither altered the mechanical threshold nor had any intrinsic effect. Intrathecally administered vehicle (50% DMSO) had no effect on the mechanical threshold. Fig. 3 shows the time courses for the mechanical threshold of RS-102221 and 50% DMSO.

3.3. Antagonist studies

Fig. 4A–C shows how intrathecal pretreatment with 5-HT2C receptor antagonist, RS-102221, or vehicle (50% DMSO) affected the antiallodynic action of MK212, RO 60-1075, and WAY-161503. The antiallodynic effect of the maximum dose of each 5-HT2C receptor agonist was statistically significantly reduced by administration of 10 and 30 μg of RS-102221. Although there were not significant differences among the reverse effects to these 5-HT2C receptor agonists by 30 μg of RS-102221, 10 μg of RS-102221 significantly less attenuated the antiallodynic effect of RO 60-0175 than WAY-161503 (Fig. 5A and B).

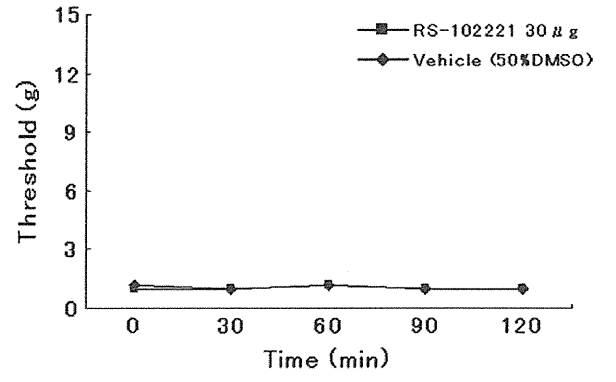

Fig. 3. In rats with infraorbital nerve ligation: mechanical thresholds after intrathecal administration of 5-HT2C receptor antagonist, RS-102221, or vehicle (50% DMSO). The data are expressed as mean ± SEM for six rats in each group.

4. Discussions

In this ION-CCI model in rats, intrathecal administration of the 5-HT2C receptor agonists MK212, RO 60-1075, and WAY-161503 produced antiallodynic effects. Although the ED50 values of these three agonists were similar, the MK212 value was lower than the other two agonists. Intrathecal pretreatment with the 5-HT2C receptor antagonist RS-102221 attenuated the effects of all three 5-HT2C receptor agonists. Although there were not significant differences among the reverse effects to these 5-HT2C receptor agonists by 30 μg of RS-102221, 10 μg of RS-102221 significantly less attenuated the antiallodynic effect of RO 60-0175 than WAY-161503. The difference between the three compounds is probably connected with the strength of 5-HT2C receptor affinity or propensity to bind to other receptors. It is known that MK212 has some affinity for 5-HT2A receptors (Conn and Sander-Bush, 1987). RO 60-0175 binds to other 5-HT receptor subtypes including 5-HT2A receptors, although the affinity to the 5-HT2C receptor is the most pronounced (Martin et al., 1998). WAY-161503 exhibits some affinity for 5-HT2B sites and weak affinity for 5-HT2A sites (Rosenzweig-Lipson et al., 2006). The effects of these 5-HT2C receptor agonists were markedly reduced by intrathecal pretreatment with RS-102221. RS-102221 is a selective antagonist that is 100 times more likely to bind to 5-HT2C receptors than other receptors (Bonhaus et al., 1997). These results suggest that spinal 5-HT2C receptors might play an inhibitory role in this trigeminal neuropathic pain model.

The neurotransmitter 5-HT plays important roles in modulating spinal nociceptive transmission. Activation of the descending serotonergic bulbospinal system modulates behavioural responses and dorsal horn neuron responses to noxious stimuli (Mayer et al., 1971; Zemlman et al., 1980). There is strong evidence that both the spinal cord and the trigeminal subnucleus caudalis (Vc) are the sites affected by 5HT released from fibers descending from the rostroventral medial medulla or administered intrathecally in experimental studies (Beitz, 1982; Clatworthy et al., 1988; Fields et al., 1991). Recently, converging lines of evidence have shown that the trigeminal subnucleus caudalis/upper cervical junction region (Vc/C2 region) plays a significant role in craniofacial nociceptive processing (Vos and Strassman, 1995; Piao et al., 2006; Latrémolière et al., 2008). 5-HT2C receptors are present in the dorsal horn of the spinal cord and the spinal nucleus of the trigeminal nerve (Sp5c) (Fonseca et al., 2001; Pompeiano et al., 1994; Molineaux et al., 1989). Although the effects of ION injury on the serotonergic descending inhibitory pathway has not been evaluated, a previous study found that administration of SSRI suppresses tactile allodynia in ION-CCI rats (Nakae et al., 2008b). Investigations of changes in 5-HT levels in mice with neuropathic pain have shown that 5-HT levels in the raphe magnus nucleus decrease in sciatic-nerve-ligated mice (Souvoravong et al., 2004) and that 5-HT concentration in the lumbar spinal cord decreased in mice with SN-CCI (Vogel et al., 2003). Thus, the antiallodynic effect of SSRI might be attributable to an increased extracellular concentration of 5-HT, which had decreased subsequent to nerve injury. Meanwhile, spinal 5-HT2C receptors mediate antinociception produced by 5-HT in the formalin test (Jeong et al., 2004), and, in a rat model of spinal-nerve-ligation, intrathecal administration of 5-HT2C receptor agonists produces antiallodynic effects (Obata et al., 2004). In the present study, intrathecal administration of 5-HT2C receptor agonists produced antiallodynic effects in a rat model of ION-CCI. Consequently, the descending inhibitory pathway mediated by 5-HT2C receptors probably produces the antiallodynic effects in rat ION-CCI model.

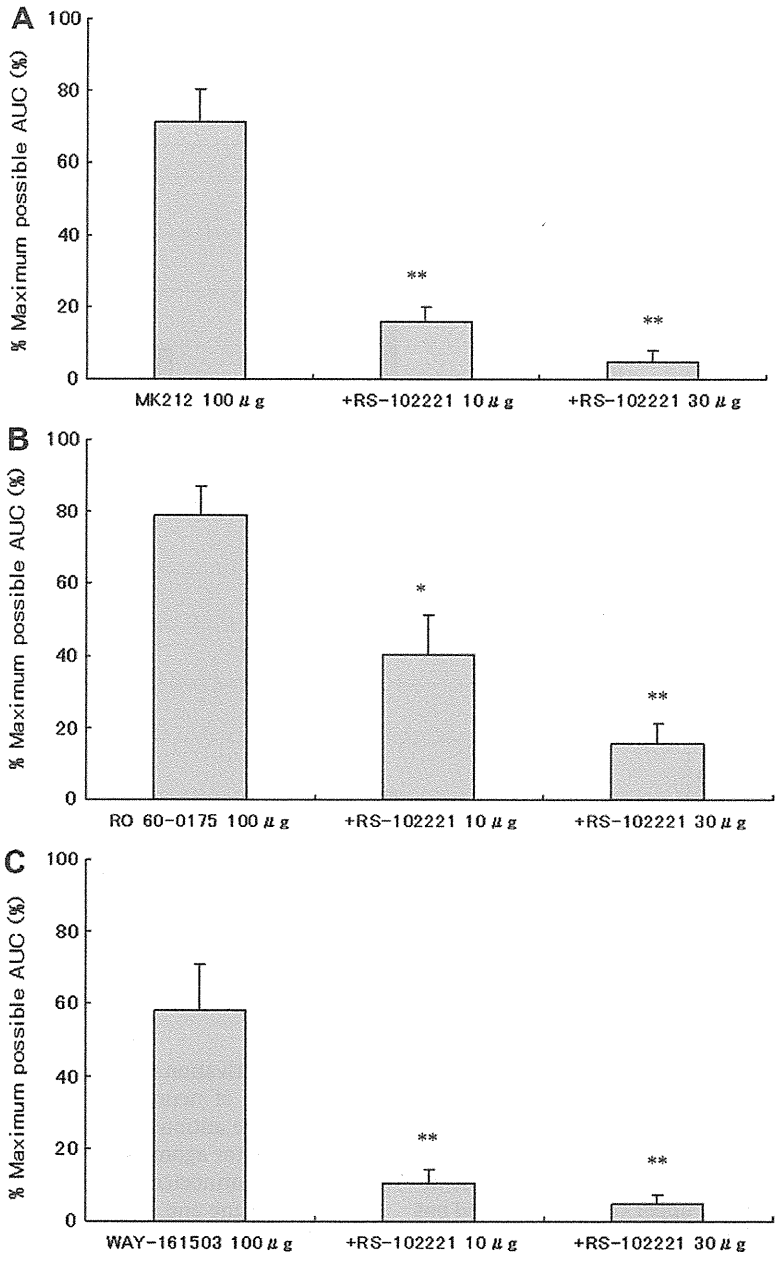

Fig. 4. In rats with infraorbital nerve ligation: effects of intrathecal pretreatment with 5-HT2C receptor antagonist, RS-102221, or vehicle (50% DMSO) on the antiallodynic effect of: (A) MK212, (B) RO 60-0175, or (C) WAY-161503. Percentages of maximal possible area under the curves (AUC) are shown in the presence of RS-102221 10 μg or 30 μg. The data are expressed as mean ± SEM for six rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the group treated with vehicle (50% DMSO) + each agonist.

The 5-HT2C receptor is a G-protein-coupled receptor whose pre-mRNA is a substrate for base modification that, via hydrolytic deamination of adenosines, yields inosines (Burns et al., 1997). Five adenosines can be converted to inosines at editing sites. In human and rodent brain, 32 possible mRNA variants are produced (Burns et al., 1997; Niswender et al., 2001). RNA editing produces receptor isoforms with varying degrees of basal activity. An in vitro study has shown that the INI isoform exhibits great basal activity, agonist affinity and potency, and that ISV and INV are slightly less active than INI; by contrast, the most statistically significant decreases in basal activity were observed with VNV and VSV isoforms (Herrick-Davis et al., 1999). In a previous study, we found that spinal-contusion-injury rats exhibit 5-HT2C receptor mRNA editing (Nakae et al., 2008a). Infraorbital nerve injury also affects the RNA editing efficiency of the 5-HT2C receptor in the cervical spinal cord. This RNA editing increases the expression of 5-HT2C mRNA isoforms that encode receptors which are more sensitive to 5-HT. In detail, ION-CCI rats had proportionally more of ISV and INV isoforms, and fewer of the VNV isoform at the upper cervical spine than naïve rats. Fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), produces antiallodynic effects and alters this editing (Nakae et al., 2008b). ION-CCI rats that received SSRI tended to show less sensitivity to pain, and the proportion of the isoforms in the ION-CCI rats returned to that of naïve rats (Nakae et al., 2008b). These findings suggest that the proportion of the isoforms which have high basal activity, agonist affinity and potency increase, while the proportion of the isoforms which have low basal activity, agonist affinity and potency decrease after infraorbital nerve injury, and the altered proportion is restored to the pre-nerve-injury state after the administration of SSRI. But it had been yet to be determined whether such a change of 5-HT2C-receptor mRNA editing might either inhibit or facilitate the pain-related

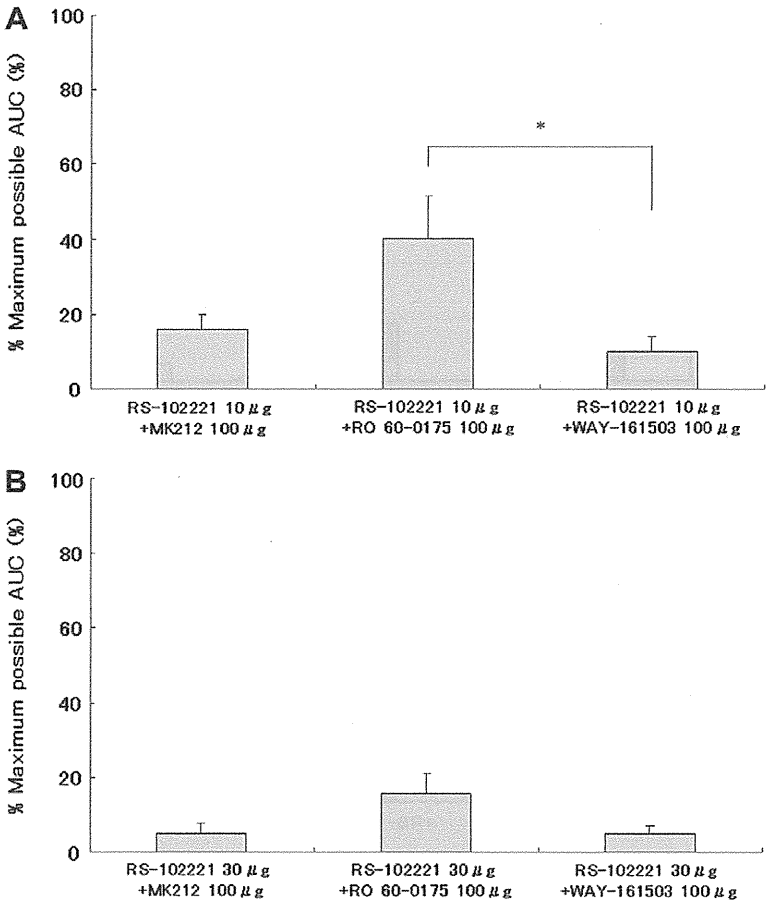

Fig. 5. In rats with infraorbital nerve ligation: effects of intrathecal pretreatment with the 5-HT2C receptor antagonist, RS-102221 10 μg (A), or 30 μg (B), on the antiallodynic effect of MK212, RO 60-0175, and WAY-161503. Percentages of the maximal possible area under the curves (AUC) are shown in the presence of RS-102221 10 μg or 30 μg. The data are expressed as mean ± SEM for six rats in each group. $^{*}P < 0.05$ compared with the group treated with RS-102221 10 μg + WAY-161503 100 μg.

behaviour in the trigeminal neuropathic pain model. In the present study, 5-HT2C receptor agonists attenuated pain-related behaviour in ION-CCI rats. So, modulation of 5-HT2C receptor mRNA editing could be an adaptive mechanism that functions in response to serotonergic dysfunction due to nerve injury. In this case, post-injury 5-HT2C receptor expression at the upper cervical spine did not change in ION-CCI rats: 5-HT2C receptor modulation changed the character, not the number (Nakae et al., 2008b). This mechanism may be unique to the 5-HT2C receptor.

Furthermore, serotonergic neurons in the central nervous system impinge on many other neurons and modulate their neurotransmitter release (Fink and Göthert, 2007). The serotonergic system may interact with other neurotransmitters in the modulation of nociception: for example, through an action at the 5HT3 receptor, 5-HT may evoke the release of γ-aminobutyric acid (GABA), which may, in turn, inhibit thermal nociceptive transmission (Alhaider et al., 1991). Operating through the 5-HT2 receptor, 5-HT acutely activates GABAergic interneurons in the prefrontal cortex (Abi-Saab et al., 1999). Selective activation of 5-HT2C receptors stimulates GABAergic function in the rat substantia nigra pars reticulate (Invernizzi et al., 2007). The above studies might support the idea that 5-HT2C receptor agonist mediates activation of the GABA interneurons in the Sp5c, as a result, lead to improve pain-related behaviour. While this may seem a likely mechanism for antiallodynic effects, GABA interneurons in the dorsal horn undergo degeneration in rats with neuropathy (Moore et al., 2002). WAY-161503, the selective 5-HT2C receptor agonist, inhibits 5-HT cell firing in the dorsal raphe nucleus via 5-HT2C receptor mediated activation of GABA neurons (Boothman et al., 2006). In a rat model of neuropathic pain, GABA receptor antagonists do not affect the antinociceptive effects of intrathecally administered 5-HT2 receptor agonist α-m-5-HT (Obata et al., 2002; Sasaki et al., 2003). The antinociceptive effect of 5-HT is associated with the release adenosine and noradrenaline from the spinal cord (Sawynok and Reid, 1996). Intrathecal administration of α2-adrenoceptor agonists mediate antiallodynic action (Yaksh et al., 1995). Intrathecal administration of α2-adrenoceptor antagonist, yohimbine, attenuates the antiallodynic effects of 5-HT2C receptor agonists (Obata et al., 2007). In the rat hot-plate test, antinociception produced by intrathecal administration of 5-HT2C receptor agonists, mCPP and TFMPP, depends on the release of endogenous noradrenaline (Sawynok and Reid, 1992, 1996). These findings suggest that the stimulation of 5-HT2C receptors in the spinal cord induces the release of noradrenaline. An antiallodynic effect of 5-HT2C receptor agonist might be produced by the activation of descending noradrenaline system. Meanwhile, mediated by cholinergic activity, 5-HT2 or 5-HT4 receptor agonists exert an antinociceptive effect (Ghelardini et al., 1996; Obata et al., 2002; Sasaki et al., 2003). Intrathecal administration of cholinomimetic agents and cholinesterase inhibitors produces antinociceptive effects via muscarinic receptors (Naguib and Yaksh, 1994; Yaksh et al., 1985). The muscarinic-receptor-antagonist, atropine, reduces the antiallodynic effects of an intrathecally administered 5-HT2C receptor agonist in spinal-nerve-ligation rats (Obata et al., 2007). So, intrathecal administration of 5-HT2C receptor agonists in this rat model of neuropathic pain might induce the release of acetylcholine, resulting

in antiallodynia via muscarinic receptors. Dopamine may be involved in the relief of pain (Coffeen et al., 2008; Lopez-Avila et al., 2004). 5-HT2C receptors in the medial prefrontal cortex facilitate cocaine-induced dopamine release in the rat nucleus acuumbens (Leggio et al., 2009). On the other hand, 5-HT inhibits central dopamine transmission via activation of 5-HT2C receptors mediated by GABA receptors (Matteo et al., 2001; Giorgetti et al., 2002; Giorgetti and Tecott, 2004; Chen and Rice, 2002; Westerink et al., 1996) RO 60-0175, selective 5-HT2C agonist, reduces cocaine-reinforced behaviour, alcohol- and nicotine-induced self administration and hyper-reactivity (Grottick et al., 2000). Our pharmacological procedures were performed by the intrathecal catheter implanted for the upper cervical cord just caudal the brain. So, we could not deny the possibility that agitated behaviour caused by painful stimuli were reduced by central dopaminergic effects. 5-HT2C receptors are considered to be involved in mediating negative feedback on central 5-HT neurons (Boothman et al., 2003, 2006; Sharp et al., 2007; Queree et al., 2009) by the selective 5-HT2C receptor agonist, WAY-161503(Boothman et al., 2006). As considered above, 5-HT2C receptor agonist generates an antiallodynic effect in neuropathic pain model not by direct action but by indirect effects involving the activation of other neurotransmitters.

In conclusion, the present study demonstrated that intrathecal administration of the 5-HT2C receptor agonists MK212, RO 60-1075, and WAY-161503 produced antiallodynic effects in ION-CCI rats. The effects of the 5-HT2C receptor agonists were attenuated by intrathecal pretreatment with the 5-HT2C receptor antagonist RS-102221. These results indicate that, in the ION-CCI rat model, 5-HT2C receptors play a role in spinal inhibition of trigeminal neuropathic pain.

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Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT_{2C} receptors

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Muscle paralysis after spinal cord injury is partly caused by a loss of brainstem-derived serotonin (5-HT), which normally maintains motoneuron excitability by regulating crucial persistent calcium currents. Here we examine how over time motoneurons compensate for lost 5-HT to regain excitability. We find that, months after a spinal transection in rats, changes in post-transcriptional editing of 5-HT_{2C} receptor mRNA lead to increased expression of 5-HT_{2C} receptor isoforms that are spontaneously active (constitutively active) without 5-HT. Such constitutive receptor activity restores large persistent calcium currents in motoneurons in the absence of 5-HT. We show that this helps motoneurons recover their ability to produce sustained muscle contractions and ultimately enables recovery of motor functions such as locomotion. However, without regulation from the brain, these sustained contractions can also cause debilitating muscle spasms. Accordingly, blocking constitutively active 5-HT_{2C} receptors with SB206553 or cyproheptadine, in both rats and humans, largely eliminates these calcium currents and muscle spasms, providing a new rationale for antispastic drug therapy.

Severe spinal cord injury (SCI) causes an immediate paralysis of muscles innervated by motoneurons directly caudal to the injury site. This results not only from a loss of supraspinal tracts that subserve voluntary initiation of movement (for example, corticospinal and reticulospinal tracts that use fast glutamatergic synaptic transmission[1,2]) but also from a loss of descending brainstem tracts that provide spinal motoneurons with their major source of neuromodulators, such as 5-HT (refs. 1,3–5). Normally, brainstem-derived 5-HT sets spinal motoneurons and interneurons into an excitable state, ready to respond to fast glutamate synaptic inputs and cause appropriate muscle contractions[2,4,6,7]. 5-HT does this by activating 5-HT_2 receptors that facilitate ionic currents intrinsic to the motoneurons, including voltage-gated persistent Ca^{2+} and Na^+ currents (termed persistent inward currents: PICs)[1,8–11]. These PICs are easily activated by brief synaptic inputs because of their unusually low threshold and, thus, serve a crucial role in amplifying and prolonging the action of synaptic inputs, ultimately enabling sustained muscle contractions[2,7,11–14]. Consequently, when SCI eliminates brainstem-derived 5-HT, motoneurons are left in an unexcitable state with small PICs[7,9,12,15], consistent with the paralysis, areflexia and spinal shock seen early after SCI[16–18]. The key role of brainstem-derived 5-HT is demonstrated by the repeated finding that motoneuron excitability (PICs) and associated motor functions (locomotion) can be regained shortly after SCI with exogenous application of 5-HT or selective agonists that activate 5-HT_2 receptors[7,9–11,19].

Remarkably, over the weeks after SCI (chronic injury), motoneurons spontaneously recover their excitability, with large PICs and associated sustained firing[12,20], despite the continued absence of brainstem-derived 5-HT. However, unlike before injury, the powerful depolarizing actions of PICs are difficult to terminate, because after injury motoneurons have weaker inhibitory inputs[21], especially from spinal interneurons that are normally regulated by descending tracts[12,17,22–26]. Thus, the PICs (especially Ca^{2+} PICs) can lead to excessive motoneuron activity that produces uncontrolled and debilitating muscle contractions (spasms, lasting many seconds), in both humans[27] and rats[12,17]. To make matters worse, these PICs and spasms are readily triggered by synaptic inputs arising from normally innocuous cutaneous stimulation or muscle stretch, because these synaptic inputs are enhanced after SCI[12,17,23,28–30].

A major question that remains is how motoneurons adapt so profoundly, recovering large PICs in the absence of brainstem-derived 5-HT. Here we consider the hypothesis that 5-HT_2 receptors on spinal motoneurons become constitutively active to compensate for lost brainstem 5-HT, ultimately helping to produce recovery of motoneuron excitability (PICs) and related motor functions such as locomotion. Constitutively active receptors spontaneously couple to

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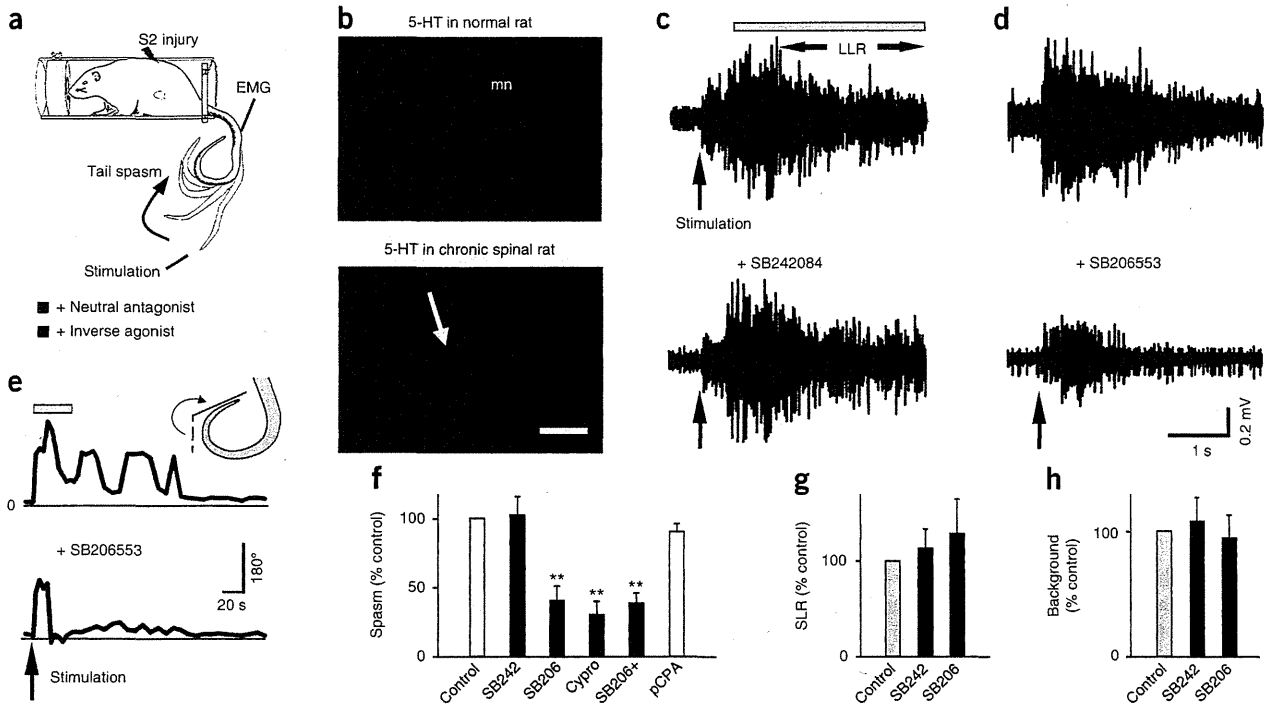

Figure 1 Constitutive 5-HT_2 receptor activity, but not residual 5-HT, causes spasms. (**a**) Schematic of tail spasm in an awake chronic spinal rat with S2 sacral transection. (**b**) Representative immunofluorescence images of 5-HT fibers (beaded) in the S4 ventral horn of normal rats (top; mn, motoneuron, $n = 5$ rats) and chronic spinal rats (bottom; the arrow indicates a residual fiber, $n = 5$; scale bar, 50 μm). (**c**,**d**) Spasms in chronic spinal rat evoked by cutaneous electrical stimulation of the tail (pulse three times the threshold (3×T)) and recorded with EMG (quantified during the length of time indicated by the bar, LLR) before and after blocking effects of residual 5-HT with i.t. injection of the neutral antagonist SB242084 (3 mM in 30 μl saline). (**d**) Lack of spasm (LLR) after blocking constitutive receptor activity with the inverse agonist SB206553 (i.t., 3 mM in 30 μl saline). (**e**) Tail flexion angle during spasms before and after SB206553 injection, quantified during the length of time indicated by the bar. (**f**) Group means of spasms (normalized to predrug control) with SB242084 (abbreviated SB242; LLR), SB206553 (SB206 for LLR EMG recording; and SB206+ for tail-angle spasms) and cyproheptadine (cypro; LLR; 10 mg per kg body weight, orally), and after depletion of residual 5-HT with para-chlorophenylalanine-methyl-ester (pCPA) (two 300 mg per kg body weight intraperitoneal injections over 48 h; tail-angle), with $n = 5$ rats per drug. (**g**,**h**) Normalized group means of SLR and background EMG with SB242084 and SB206553. $^{**}P < 0.01$ relative to predrug control, 100%. Error bars indicate s.e.m.

their Gq proteins and initiate intracellular signaling without being bound to 5-HT or any other ligand[31–37], a process well understood in isolated cell culture systems but not previously considered for motoneurons. The 5-HT_{2C} receptor is an ideal candidate for such constitutive activity because it has a number of native isoforms that have a high degree of constitutive activity in humans and rats (>50% active)[32,35]. Furthermore, expression of these constitutively active isoforms increases in the cortex after depletion of 5-HT[38], suggesting that a similar change may be possible after SCI. We thus examined whether recovery of motoneuron function after SCI depends on constitutive 5-HT receptor activity. We initially focused on showing that constitutive receptor activity causes spasms, because the emergence of spasms after SCI is an indirect measure of recovery of motoneuron and general motor function (albeit maladaptive) that is readily studied in rats and humans (motoneuron PICs cause spasms). After this, we evaluated how constitutive activity contributes to locomotor recovery. For studying spasms, we used a complete spinal transection model (chronic spinal rat, **Fig. 1**), which eliminates brainstem-derived 5-HT, thus minimizing the chance that receptors remain activated by 5-HT. Nevertheless, we still had to consider the role of other 5-HT sources, because even with a complete transection, some residual spinal 5-HT remains caudal to the injury[39], and motoneurons are extremely sensitive to small amounts of 5-HT after SCI[8,10].

RESULTS

Lack of contribution of residual 5-HT to spasms

Before injury, the spinal cord was densely innervated by 5-HT fibers along its whole length, particularly in the ventral horn (**Fig. 1b**). In contrast, after SCI in the chronic spinal rat, only a few short (43.3 ± 25.0 μm) fibers remained (**Fig. 1b**) with, on average, 18 ± 11 such fibers along the whole length of the spinal cord below the injury.

To examine whether the remaining 5-HT fibers in chronic spinal rats had any functional effect on spasms and associated 5-HT_2 receptors, we blocked the action of 5-HT with an intrathecal (i.t.) injection of the highly selective 5-HT_{2C} receptor antagonist SB242084 (**Fig. 1c**,**f**). This injection did not significantly change the tail muscle spasms recorded with EMG *in vivo* (**Fig. 1c**; evoked by cutaneous stimulation), indicating that the 5-HT_2 receptors were not activated by residual 5-HT (or other endogenous ligands). Notably, SB242084 is a neutral antagonist that blocks only the action of 5-HT (or other agonists) on the 5-HT_2 receptors, and does not inhibit constitutive receptor activity[31,34]. We also found that depleting residual 5-HT with pCPA[38] did not significantly influence spasms (**Fig. 1f**).

Spasms depend on constitutive 5-HT_2 receptor activity

We next examined whether the loss of 5-HT after injury was compensated for by constitutive activity in 5-HT_2 receptors by intrathecally injecting SB206553, which selectively binds 5-HT_{2C} receptors and potently

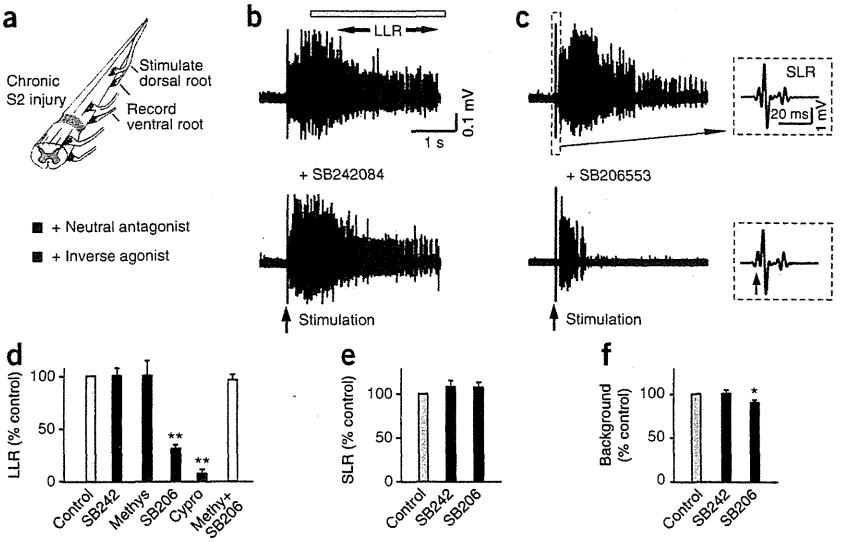

Figure 2 Constitutive 5-HT_2 receptor activity contributes to LLRs in the isolated spinal cord *in vitro*. (**a**) Whole sacrocaudal spinal cord below chronic S2 transection maintained *in vitro*. (**b**) Long-lasting reflex triggered by dorsal root stimulation (single pulse, 3×T) and recorded from the ventral roots (LLR, quantified during the length of time indicated by the horizontal bar; counterpart of spasms in **Figure 1**) before and after blocking effects of residual 5-HT with the neutral 5-HT_2 receptor antagonist SB242084 (3–5 μM). (**c**) Elimination of LLR, but not SLR, after blocking constitutive 5-HT_2 receptor activity with the inverse agonist SB206553 (3–5 μM). Inset, SLR (expanded time scale). (**d**) Group means of LLRs (normalized to predrug LLRs) with SB242084 (abbreviated SB242, $n = 11$), methysergide (Methys, 10 μM, neutral antagonist, $n = 12$), SB206553 (SB206, $n = 24$), cyproheptadine (Cypro, 20 μM; $n = 6$), and SB206553 after prior application of methysergide (30 μM; white bar; Methy+SB206; $n = 8$). (**e**,**f**) Normalized group means of the SLR and background ventral root activity with SB206553 and SB242084. $^*P < 0.05$, $^{**}P < 0.01$ relative to control, 100%. Error bars indicate s.e.m.

inhibits their constitutive activity (termed an inverse agonist[31,34,37]). This injection reduced the magnitude of the spasms recorded with either electromyography (EMG) (**Fig. 1d**,**f**) or tail kinematics (**Fig. 1e**,**f**) by well over 50%, whereas control saline injections had no effect. Likewise, oral application of the non-selective 5-HT_2 receptor inverse agonist cyproheptadine[33] significantly reduced spasms (**Fig. 1f**).

We next examined the whole spinal cord from chronic spinal rats (caudal to the injury) after it was removed and maintained *in vitro*, which eliminated possible peripheral or brain-derived 5-HT influences. We recorded long-lasting reflexes (LLRs) from the ventral roots in response to a brief stimulation of dorsal roots (**Fig. 2a–c**); these LLRs have previously been shown to underlie muscle spasms recorded *in vivo*[12,17]. The LLRs were not significantly affected by blocking the possible action of endogenous 5-HT with the 5-HT_{2C} receptor neutral antagonists SB242084 or methysergide[33] (**Fig. 2b**,**d**), even though these antagonists blocked the increase in LLRs induced by exogenous application of selective 5-HT_{2C} agonists (**Supplementary Fig. 1**). Furthermore, enhancing available residual endogenous 5-HT with either the 5-HT transport-blocker citalopram or 5-HT releaser fenfluramine did not significantly affect LLRs (**Supplementary Fig. 2**). In contrast, the LLRs were markedly inhibited by blocking constitutive 5-HT_2 receptor activity with inverse agonists (SB206553 and cyproheptadine; **Fig. 2c**,**d**). This inhibitory action of SB206553 was blocked by a prior application of methysergide (**Fig. 2d**), which competitively inhibits SB206553 binding to 5-HT_{2C} receptors[34].

The transient short latency reflexes (SLRs) evoked immediately after stimulation (**Fig. 2c**) were not affected by SB206553, both *in vitro* (**Fig. 2e**) and *in vivo* (**Fig. 1g**), and did not correlate with the LLRs (spasms; $r^2 = 0.10$)[29], consistent with a negligible modulation of SLRs by Ca^{2+} PICs and associated 5-HT_{2C} receptors. Also, the background activity before the LLRs had relatively little (**Fig. 2f**, *in vitro*) or no (**Fig. 1h**, *in vivo*) change with SB206553 treatment.

Constitutive 5-HT_2 receptor activity in motoneurons

Given that spasms result from persistent calcium currents (Ca^{2+} PICs) in motoneurons[12,27], we made intracellular recordings from motoneurons after SCI to investigate whether there were constitutively active 5-HT_{2C} receptors on motoneurons that regulate Ca^{2+} PICs (**Fig. 3**). As previously described, the large voltage-dependent Ca^{2+} PICs in motoneurons were readily observed in isolation as a sharp downward deflection in the current response during an increasing voltage ramp (**Fig. 3b**) after sodium currents and synaptic inputs were eliminated with tetrodotoxin[12]. Blocking constitutively active 5-HT_2 receptors with the inverse agonists SB206553 or cyproheptadine markedly decreased the magnitude of these Ca^{2+} PICs (**Fig. 3b**,**f**), whereas SB242084 had no effect on Ca^{2+} PICs (**Fig. 3c**,**f**). The portion of the Ca^{2+} PICs that resulted from constitutive 5-HT_2 receptor activity (SB206553-sensitive decrease) was 1.99 ± 0.42 nA, which was 42.9 ± 8.9% of the maximum possible Ca^{2+} PICs produced by activating all 5-HT_2 receptors (with 1 μM 5-HT). The small remaining Ca^{2+} PICs with inverse agonists in chronic spinal rats was similar to the small Ca^{2+} PICs observed acutely after spinal transection (**Fig. 3f**).

When we stimulated the dorsal roots during recording from a motoneuron at rest and in the absence of tetrodotoxin, the PIC produced a sustained depolarization (plateau)[12] that caused many seconds of repetitive firing (LLR; **Fig. 3d**). As expected, the LLR and plateau were eliminated by the inverse agonist SB206553 (**Fig. 3e**). The LLR and plateau were also eliminated by simply hyperpolarizing the motoneuron to prevent activation of the underlying voltage-dependent PIC (**Fig. 3d**)[12], although there remained a polysynaptic excitatory postsynaptic potential (EPSP) lasting about 0.5 s. The inverse agonists SB206553 and cyproheptadine had no effect on this EPSP (**Fig. 3e**,**g**).

Increase in constitutively active 5-HT_{2C} receptor isoform

The 5-HT_{2C} receptor RNA undergoes post-transcriptional editing at five sites (labeled A to E) that leads to numerous native receptor isoforms in rats and humans, by changing three amino acids on an intracellular loop of the receptor (isoforms are named after the amino acid sequences, such as INI, VSV and VNI, as depicted in **Fig. 4a**)[32–35,40]. Functionally, the unedited isoform (INI) shows a high degree of constitutive activity, whereas editing reduces this activity, producing isoforms with less constitutive activity, such as

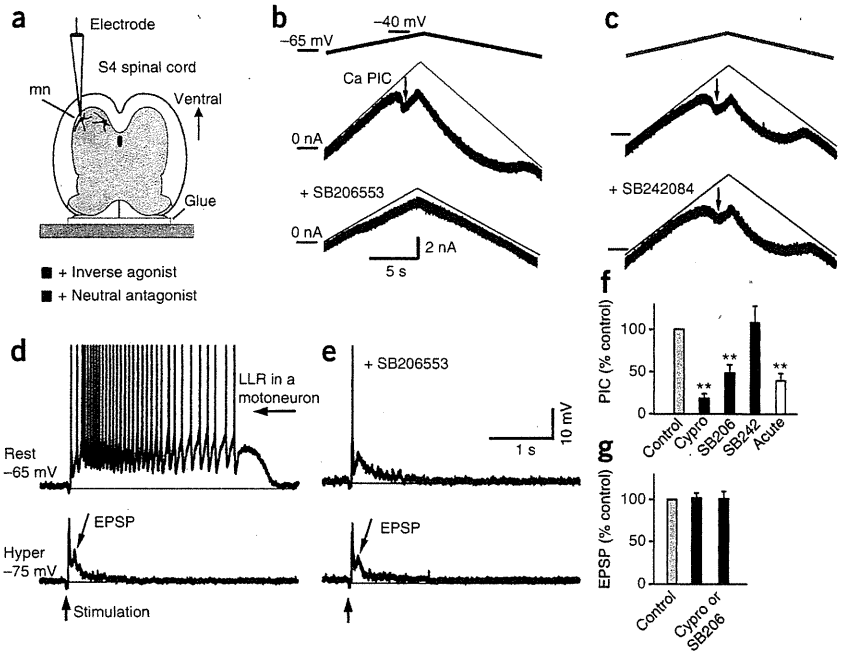

Figure 3 Constitutively active 5-HT$_2$ receptors on motoneurons contribute to Ca^{2+} PICs underlying spasms. (**a**,**b**) Intracellular recording from motoneuron (mn) in whole spinal cord, *in vitro*. (**b**) Top, Ca^{2+} PIC in motoneuron of chronic spinal rat, activated by slowly increasing the membrane potential under voltage-clamp in presence of 2 μM tetrodotoxin (TTX) and quantified at its initial peak, where it produced a downward deflection in the recorded current (thick black plot, at arrow, Ca^{2+} PIC) relative to the leak current (thin line). Bottom plot, small Ca^{2+} PIC after SB206553 application (5 μM). (**c**) Ca^{2+} PIC in another motoneuron (arrow), which is unaffected by SB242084 application (5 μM). (**d**) Top, PIC-mediated plateau and sustained firing (LLR) evoked by dorsal root stimulation (3×T; without TTX) in a motoneuron at rest (without injected current; top). Bottom, with a hyperpolarizing bias current to prevent PIC activation, the same stimulation only evoked a polysynaptic EPSP (lower plot). (**e**) Response of same motoneuron as in **d** to dorsal root stimulation after application of SB206553 (5 μM), at rest (top) and with a hyperpolarizing bias current (bottom). (**f**) Group means of Ca^{2+} PIC (normalized to predrug Ca^{2+} PIC in chronic spinal rats, control), with SB206553 (SB206; $n = 7$), cyproheptadine (cypro, 20 μM; $n = 16$) and SB242084 (SB242; $n = 5$) in chronic spinal rats and in acute spinal rats (white bar, no drugs, $n = 7$). (**g**) Normalized group means of EPSP amplitude (middle bar; control mean 4.4 mV) and duration (right bar, control 480 ms) with inverse agonists cyproheptadine or SB206553 (chronic). $^{**}P < 0.01$ relative to control, 100%. Error bars represent s.e.m.

VNI (with 51% of INI activity) and VSV (32% of INI)[36]. We thus compared 5-HT$_{2C}$ receptor mRNA levels from spinal cords of normal (unlesioned) and chronic spinal rats (below S2 injury level). The total amount of 5-HT$_{2C}$ mRNA did not change with SCI (**Fig. 4b**). However, there was a decrease in the amount of RNA editing at the A site (**Fig. 4c**). Corresponding to this, there was also a decrease in the relative proportion of the VNI receptor isoform and an increase in the relative proportion of the highly constitutively active INI isoform (**Fig. 4d**). The increase in INI isoform expression (400%) was similar to the increase in PIC with chronic injury (**Fig. 4e**).

We directly confirmed that the motoneurons of the sacral spinal cord had 5-HT$_{2C}$ receptors after SCI by immunolabeling (**Supplementary Fig. 3**). Furthermore, a large fraction of the 5-HT$_{2C}$ receptor labeling was inside the motoneurons (intracellular) in chronic spinal rats, and this receptor internalization was reduced by SB206553, consistent with the presence of constitutively active isoforms of the receptor on motoneurons, the hallmark of which is a high degree of activity-dependent internalization (INI isoform[34,37]; **Supplementary Figs. 3** and **4**).

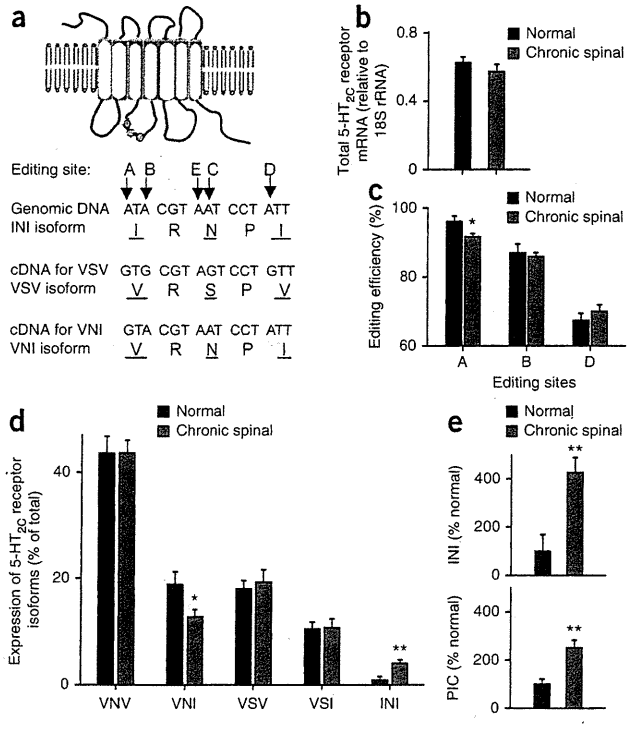

Figure 4 A highly constitutively active 5-HT$_{2C}$ receptor isoform is upregulated with injury. (**a**) Schematic showing 5-HT$_{2C}$ receptor with various isoforms produced by changing three amino acids on its intracellular loop (green; isoforms named by amino acid triplet). These three amino acids (underlined) are changed by post-transcriptional editing of RNA at five sites (A–E; adenosine editing), leading to various native receptor isoforms, of which the unedited isoform (INI) is most highly constitutively active. (**b**) Total 5-HT$_{2C}$ receptor mRNA (normalized to an internal control, 18S rRNA) in chronic spinal rats ($n = 6$) and normal uninjured rats ($n = 6$). (**c**) Proportion of 5-HT$_{2C}$ receptor mRNA with editing at sites A, B and D (editing efficiency) in chronic spinal and normal rats (C and E site editing efficiency < 30% and not changed, data not shown). (**d**) Distribution of 5-HT$_{2C}$ receptor isoform mRNA in the spinal cord of normal and chronic spinal rats (15 isoforms detected; the five most prevalent are shown). (**e**) Comparison of change in INI isoform expression (top) and Ca^{2+} PIC (bottom, recorded *in vitro*) after chronic spinal injury. $^{*}P < 0.05$, $^{**}P < 0.01$, significant change with injury. Error bars indicate s.e.m.

Antispastic action of inverse agonists in humans with SCI

In humans with SCI, we evoked leg muscle spasms with cutaneous stimulation of the foot while recording tibialis anterior muscle EMG

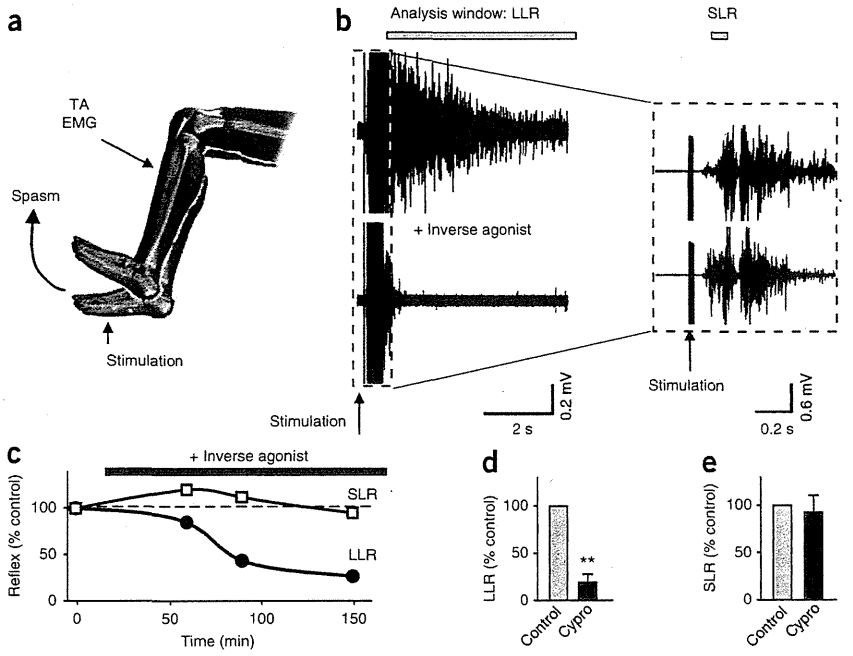

Figure 5 5-HT$_2$ receptor inverse agonist blocks spasms in spinal cord injured humans. (**a**) Leg spasm triggered by brief electrical stimulation of the medial arch of the foot (3–5×T). TA, tibialis anterior. (**b**) Spasm recorded with tibialis anterior muscle surface EMG and quantified over the time windows indicated (LLR and SLR), before and 2 h after blocking constitutively active 5-HT$_2$ receptors with cyproheptadine (8 mg administered orally). The inset on a different scale shows SLR. (**c**) Gradual reduction in the spasms (LLRs), but not SLRs, over time after inverse agonist application. (**d**,**e**) Normalized group means for LLRs (**d**) and SLRs (**e**) with cyproheptadine ($n = 7$ subjects). $^{**}P < 0.01$ relative to control, 100%. Error bars represent s.e.m.

(**Fig. 5**)[27]. Blocking constitutive 5-HT$_2$ receptor activity with oral administration of the inverse agonist cyproheptadine significantly decreased the muscle spasms (**Fig. 5b**,**d**). Furthermore, the effect was again selective to the long-lasting portion of the spasm (LLR, **Fig. 5b–d**), with no drug-induced change in the SLR (**Fig. 5b**,**e**). Spasms were equally reduced by cyproheptadine in subjects with varying impairment of motor function (B–D on the American Spinal Injury Association Impairment Scale, which ranges from A–E; **Supplementary Table 1**).

Dependence of walking on constitutively active 5-HT$_2$ receptors

To evaluate whether constitutive 5-HT receptor activity contributes to recovery of locomotion after partial SCI, we used a staggered hemisection injury model (**Fig. 6a**) that transects all descending 5-HT axons but spares enough propriospinal neurons that traverse the injury site to allow the rat to voluntarily initiate functional hindlimb locomotion[41]. Three weeks after this injury, rats regained good hindlimb locomotor ability, voluntarily initiating walking with near normal weight support, although they retained a deficit in forelimb-hindlimb coordination (with a BBB score[42] < 12; **Fig. 6b**). Blocking constitutively active 5-HT$_2$ receptors with the inverse agonist SB206553 (i.t.) dramatically reduced weight support (hindlimbs dragged; **Fig. 6c**) and overall locomotor ability (BBB score, **Fig. 6c**,**d**). In contrast, blocking possible action of residual 5-HT with the neutral antagonist SB242084 had no significant effect (**Fig. 6d**).

DISCUSSION

A loss of brainstem-derived 5-HT after SCI acutely reduces motoneuron excitability[6–9,15] and accordingly depresses all motor functions. Our results demonstrate a previously undescribed mechanism for how spinal motoneurons compensate for this lost 5-HT over the months after injury (chronic injury). Decreased editing at a single site on the 5-HT$_{2C}$ receptor RNA (A site) leads to increased expression of the constitutively active INI isoform of this receptor. Constitutive 5-HT$_2$ receptor activity in turn leads to large Ca^{2+} PICs in motoneurons, which ultimately enable motoneurons to recover their excitability, as evidenced by their sensitivity to inverse agonists. Because large PICs in motoneurons have been shown to have key roles in normal motor function in uninjured humans and animals[11], these results suggest that constitutive 5-HT receptor activity (with its associated PICs) is essential in recovery of motor function after SCI. Indeed, we show

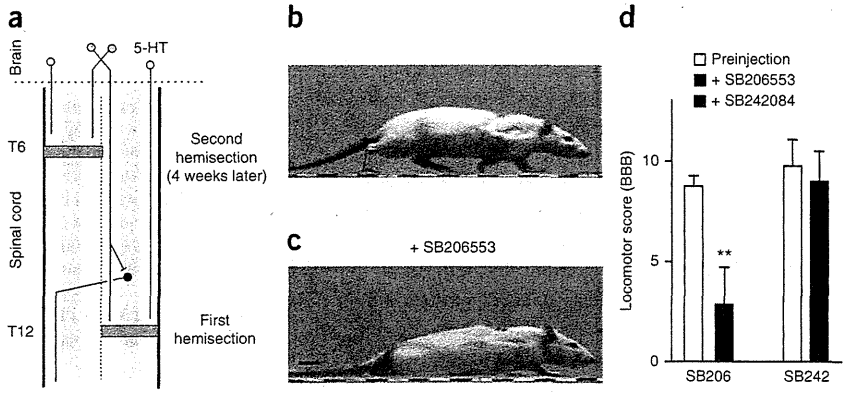

Figure 6 Spontaneous recovery of locomotion in staggered-hemisected rats depends on constitutively active 5-HT$_2$ receptors. (**a**) Schematic of staggered-hemisection SCI, which transects all descending axons from the brain, including 5-HT neurons (white circles), but leaves local propriospinal neurons (black) that transverse the injury and help relay descending signals for initiation of locomotion (gray)[41]. (**b**) Rat walking with good weight support and toe clearance three weeks after the staggered-hemisection (after second hemisection). (**c**) Same rat with little hindlimb weight support (just foot paddling motions), while the forelimbs dragged the hindquarters during walking after blocking constitutively active 5-HT$_2$ receptors with SB206553 (3 mM in 30 µl saline, i.t.; same dose as in **Fig. 1**). Scale bar, 2 cm. (**d**) Group means of BBB locomotor scores before and after SB206553 injection ($n = 8$) and control SB242084 injection (3 mM in 30 µl saline, i.t.; $n = 8$ rats). $^{**}P < 0.01$ relative to preinjection. Error bars represent s.e.m.

that constitutive 5-HT_2 receptor activity is crucial for spontaneous recovery of hindlimb locomotor function after partial SCI, because inverse agonists impair locomotion.

Given that inverse agonists inhibit conventional activation of 5-HT_2 receptors by 5-HT, as well as constitutive activity, their action alone is not definitive proof of constitutive activity, without ruling out the influence of endogenous residual 5-HT[31,34]. We thus ruled out residual 5-HT by showing a complete lack of effect of neutral antagonists, 5-HT depletion, *in vitro* spinal cord isolation, SERT blockers and 5-HT releasers after SCI in rats.

Our results also show that, without normal descending supraspinal control, these constitutively active 5-HT_2 receptors and associated PICs can, unfortunately, lead to uncontrolled motoneuron firing and associated muscle spasms (LLRs), which emerge over the weeks after injury[17]. However, blocking this constitutive receptor activity with inverse agonists decreases spasms in rats and humans with SCI, suggesting a new rationale for antispastic drug development, although care must be taken to use a dose that preserves some residual function. For example, the high dose of SB206553 used here to maximally block spasms in the transected rat also eliminates locomotion in the rat after partial SCI. In contrast, low doses of the broad-spectrum inverse agonist cyproheptadine have been shown to improve locomotion in humans[43], presumably by reducing the amplitude and incidence[44] of spasms that can interfere with stepping without completely eliminating PICs and muscle strength. The EPSPs that trigger spasms (and associated SLRs) are not affected by 5-HT_{2C} receptor inverse agonists, whereas they are inhibited by traditional antispastic drugs such as baclofen, because they are regulated by other receptors presynaptically[45,46]. Thus, inverse agonists provide an independent and complementary approach to traditional spasticity management[29,30,46].

Taken together, our pharmacological, mRNA and immunolabeling data suggest that the large PICs on motoneurons after SCI are facilitated by constitutive activity in 5-HT_{2C} type receptors on motoneurons (perhaps with additional involvement of 5-HT_{2B} receptors, because SB206553 blocks both 5-HT_{2B} and 5-HT_{2C} receptors[47–49]). 5-HT_{2C} receptors activate the intracellular phospholipase C (PLC) pathway that leads to inositol phosphate synthesis and mobilization of intracellular Ca^{2+} stores[50,51]. Constitutive 5-HT_{2C} receptor activity leads to a basal level of activity in this PLC pathway, which is inhibited by receptor blockade with inverse agonists such as SB206553 but not by neutral antagonists such as SB242084 (refs. 31,34,47,48). Our analogous results with SB206553 and SB242084 suggest that an intracellular PLC pathway in motoneurons may be tonically activated after SCI by constitutive activity, especially considering that motoneurons (PICs) are known to be regulated by PLC, inositol phosphate and intracellular Ca^{2+} concentrations[52–54].

The INI 5-HT_{2C} receptor isoform that we find upregulated in the spinal cord after chronic SCI shows substantial constitutive activity, with basal levels of inositol phosphate production approaching that achieved by 5-HT (fully active)[32,35]. Other isoforms show substantially less constitutive activity[32,36] and do not increase in expression with injury. However, these isoforms probably contribute to a basal level of constitutive receptor activity in the normal rat, which should persist acutely after injury, contributing to the small PICs measured *in vitro* in the acutely isolated spinal cord of normal rats[15]. Also, the increase in total 5-HT_{2C} receptor expression reported with severe chronic SCI[55] should increase the constitutive activity contributed from all isoforms. This might explain why the PIC that is produced by constitutive activity (SB206553-sensitive) after chronic SCI is about 40% of the maximum PIC that can be induced by activating all 5-HT_2 receptors, even though INI isoform represents only about 4% of all 5-HT_{2C} receptors after injury. This discrepancy might also be explained by the especially effective intracellular signaling capacity of INI receptor isoforms, producing many times more inositol phosphate than other isoform[56], and thus perhaps producing a disproportionately large PIC.

We do not know what initiates the remarkable adaptation in 5-HT_{2C} receptors that we see after SCI. Perhaps it is the loss of 5-HT itself[38]. Alternatively, the lack of motoneuron activity and associated intracellular calcium signaling may trigger the adaptation, as in synaptically isolated single neurons[57]. That is, motoneurons may require an optimal amount of activity, regardless of where it arises or what form it takes (spasms or walking), and activity-dependent tuning of constitutive activity in 5-HT receptors may help achieve such optimal activity. Perhaps this explains why intense locomotor training activity after SCI in humans not only improves walking but also reduces spastic muscle activity[58].

Our finding of constitutive 5-HT receptor activity opens up new possibilities for understanding spinal cord plasticity in disease and injury. Although the spinal cord is densely innervated by brainstem-derived 5-HT fibers, there are actually relatively few neurons in the brainstem that provide all of this innervation (<10,000; each neuron branches extensively)[4], leaving motoneurons and spinal functions vulnerable to injury or disease that affects activity in these few 5-HT neurons. Constitutive 5-HT_2 receptor activity provides a safeguard against such loss of 5-HT innervation of the spinal cord and probably even contributes to basal receptor activity in normal rats. With the loss of 5-HT after SCI, this constitutive activity increases dramatically, replacing the lost 5-HT–mediated activity.

In summary, we have demonstrated that substantial constitutive 5-HT_{2C} receptor activity emerges after SCI and contributes to recovery of motoneuron function, with both positive (walking) and negative (spasms) outcomes. This constitutive activity must work in concert with the many other factors that contribute to locomotion and spasticity[5,22,23,28–30,41,45,59].

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.C.M. performed the *in vitro* rat experiments, contributed to all other rat studies and co-wrote the paper. M.R., P.J.H., R.L., W.H., L.S., M.J.S., R.V., X.L. and K.F. contributed to the *in vivo* rat experiments. K.F., R.V., E.W.B., R.A. and C.J.H. contributed to immunolabeling experiments. K.F. co-wrote the paper and shared equally with D.J.B. in senior authorship (last author). A.N. and T.M. conducted mRNA analysis. J.D. and M.A.G. conducted the human experiments. D.J.B. performed *in vitro* and *in vivo* rat experiments, supervised or co-supervised all of the experiments and co-wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Spinal lesions. All rat use was approved by the University of Alberta Animal Care and Use Committee: Health Sciences. We completely transected spinal cords of adult female Sprague-Dawley rats (locally bred) at the S2 sacral spinal level and evaluated spasticity and motoneuron properties 6–12 weeks post-injury (chronic spinal state, see **Supplementary Methods**)[12,17]. Also, a separate group of female rats underwent a staggered hemisection[41], which, like a transection, removes most descending supraspinal axons below the injury (including 5-HT axons), but leaves intact some propriospinal neuron connections that enable the rat to voluntarily initiate walking, as detailed in the **Supplementary Methods**.

All human experiments were carried out with signed, informed consent of subjects and approved by the University of Alberta Health Research Ethics Board. Human subjects had chronic SCI with varied severity (**Supplementary Table 1**) and did not take their antispastic medications on the experiment day.

Spasms in awake chronic spinal rats. We evoked tail muscle spasms with brief electrical (3× afferent threshold (T)) or manual stimulation of the skin of the tail and recorded these spasms with tail muscle EMG and video kinematic analysis, as detailed in the **Supplementary Methods**. Briefly, EMG was rectified and averaged over 10–40 ms after stimulus (SLR) and 500–4,000 ms after stimulus (LLR), and tail flexion angle measured.

Spasms in humans with SCI. We evoked leg spasms with a brief electrical stimulation of the medial arch of the foot (3–5×T) and recorded surface EMG responses over the tibialis anterior (TA) muscle (**Fig. 5**)[27]. We computed the SLR and LLR by averaging EMG over the intervals 50–100 and 500–5,000 ms after stimulation, respectively, and then subtracting background EMG (see **Supplementary Methods**).

Ventral root and intracellular motoneuron recording in rats, *in vitro*. The whole spinal cord caudal to the S2 injury level was removed from chronic spinal rats and maintained *in vitro* for ventral root and intracellular motoneuron recordings[12,45], as described in the **Supplementary Methods**. Briefly, we stimulated a coccygeal dorsal root (Co1) with a single pulse (0.1 ms, 0.02 mA, 3×T), recorded the reflex response on the S4 and Co1 sacrocaudal ventral roots, and computed the mean SLR (over 10–40 ms after stimulation), LLR (500–4,000 ms after stimulation) and background activity (over 300 ms before stimulation). For intracellular recordings, sharp intracellular electrodes were advanced into motoneurons, and the Ca^{2+} PIC was measured under voltage-clamp. The Ca^{2+} PIC was quantified as the downward current deflection (**Fig. 3b**, thick black line, at arrow) recorded during a slow upward voltage ramp (**Fig. 3b**, top, gray), relative to the leak current (thin line), in tetrodotoxin. Characteristically, this Ca^{2+} PIC was activated at low voltages (-56.7 ± 6.0 mV), deactivated at even lower voltages (on downward ramp) and mediated by L-type calcium channels (nimodipine-sensitive), as previously reported[12,15].

Locomotor assessment after spinal cord injury in rats. Locomotion was evaluated 3 weeks after the staggered hemisection using the BBB score[42], as detailed in the **Supplementary Methods**.

Drugs and solutions. The drugs used were 5-HT, fenfluramine, SB242084, strychnine, para-chlorophenylalanine-methyl-ester (all Sigma-Aldrich), α-methyl-5-HT, citalopram, cyproheptadine, methysergide, MK212, nimodipine, SB206553 (all Tocris) and tetrodotoxin citrate (Alomone). *In vitro*, the artificial cerebrospinal fluid consisted of (in mM) 122 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 3 KCl, 1 $MgCl_2$ and 12 D-glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at 22–24 °C. Drugs were dissolved in the artificial cerebrospinal fluid. *In vivo*, drugs were administered via transcutaneous i.t. injection[60], intraperitoneal injection or oral gavage, and peak effects were reported (at 5–20 min after i.t. and 60 min after oral gavage). SB206553 was used at a dose that produced maximal effects (on spasms) both *in vivo* and *in vitro* (determined by titration), and SB242084 was used at the same dose, because SB206553 and SB242084 have similar binding affinity at 5-HT_{2C} receptors[49].

mRNA measurements. We extracted RNA from the whole spinal cord below the S2 injury level and from this synthesized and amplified cDNA (with RT-PCR) to quantify the mRNA. We quantified RNA editing and 5-HT_{2C} isoforms by sequencing the DNA of bacterial colonies grown from single bacteria cells transfected with DNA fragments synthesized and amplified from spinal cord cDNA (using 5-HT_{2C} receptor–related PCR primers; each colony adopts a single 5-HT_{2C} receptor isoform). We computed editing efficiency at each of five sites (A–E in **Fig. 4a**) as the proportion of colonies with editing at that site in their sequence. We computed the proportion of each 5-HT_{2C} receptor isoform in the spinal cord, from the number colonies with that isoform, relative to the total number of colonies. See further details in the **Supplementary Methods**.

Histology. Immunofluorescence labeling for 5-HT and 5-HT_{2C} receptors was performed as described in the **Supplementary Methods**.

Statistical analyses. Statistical comparisons were performed by a paired *t* test after verifying normality. Data are reported as means ± s.e.m.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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慢性疼痛に対する薬物治療を中心とした治療実態調査

―日本、米国、ドイツの比較―

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1. はじめに

痛みは日常診療でもっとも多く遭遇する訴えである。慢性疼痛保有率に関する疫学調査は各国で報告されており，欧州で成人の約19%，米国では成人の約9%であり，痛みによる経済的損失は欧州で年間340億ユーロ（約5兆円），米国で年間650億ドル（約8兆円）といわれている[1-3)]。米国では，2001～2010年を「痛みの10年（The Decade of Pain Control and Research）」とし，痛みを体温，脈拍，呼吸，血圧に次ぐ5番目のバイタルサインとして，患者の痛みの評価を診療の義務とし，国策として痛みの治療に取り組んでいる。最近，日本で実施された疫学調査（20-79歳を対象）によると，慢性疼痛を抱える患者数は，対象人口（20-79歳）の約23%，約2,200万人にのぼり，その約半数にあたる約1,000万人が通院治療を行っているものの，そのうち約40%，約390万人が既存治療で痛みが解消されていない，と報告されている[4)]。このことからも，いかに多くの患者が慢性疼痛で悩んでおり，かつ治療が困難で，多くの慢性疼痛患者に十分な治療がなされていないことがうかがい知れる。また，慢性疼痛は気分障害や不安障害との強い関連性や，脳の機能への影響，癌発生率の上昇など，精神身体機能に及ぼす影響が報告[5-9)]されていることからも，適切な治療が求められる。

慢性疼痛の発症機序，病態は複雑であり，その治療方法は多岐にわたる。治療方法としては薬物療法や理学療法，心理療法などの非侵襲的治療および神経ブロック，脊髄刺激法などの侵襲的治療があるが，これらの治療方法がどのように選択されているかなどの治療実態調査はこれまで報告がない。そこで，今回，日本と欧米の慢性疼痛に対する治療実態および，オピオイド鎮痛薬の使用実態を調査し，日本と欧米の違いを比較した。

2. 対象と方法

本アンケート調査は，外部調査機関（ニールセン・カンパニー株式会社）の協力のもと，パネル登録医師［内科，整形外科，麻酔科・ペインクリニック科（以下麻酔科）合計日本46,141名，米国41,950名，ドイツ5,750名］に本調査を行った。インターネット上で告知し，アクセスのあった非癌性慢性疼痛を診療している医師（日本234名，米国212名，ドイツ146名）に，主要5疾患［慢性腰痛（非特異性腰痛，椎間板ヘルニア，変形性腰椎症），関節痛（変形性関節症（OA），関節リウマチ（RA）），帯状疱疹後神経痛（PHN），複合性局所疼痛症候群（CRPS），線維筋痛症（FB）］を原因とする非癌性慢性疼痛に対する治療方針（薬物療法を中心に）および，直近に診療した3症例における治療実態について，インターネット上で質問票に回答する形式で実施した（調査期間：2008年8月29日～10月3日）（**表1**）。

3ヵ月にわたって日常生活に支障をきたす疼痛