JPET Fast Forward. Published on June 23, 2004 as DOI: 10.1124/jpet.104.070300
JPET Fast Forward. Published on June 23, 2004 as DOI: 10.1124/jpet.104.070300
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I. Title Page

The Role of 5-HT_3 Receptors in the Vagal Afferent Activation-Induced Inhibition of C_1 Spinal Neurons Projected from Tooth-Pulp in the Rat

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II. Running Title Page

Running title; *Role of local 5-HT_3 receptors in tooth pain modulation*

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Number of text pages: **23**
Number of tables: **0**
Number of figures: **7**
Number of references: **40**

Number of words in the ***Abstract*, 223**; ***Introduction*, 625**; ***Discussion*, 1003**

Section: **Neuropharmacology**

ABBREVIATIONS: C_1, the first cervical dorsal horn; TP, tooth-pulp; VA, vagal afferent; NRM, nucleus raphe magnus; PAG, periaqueductal gray matter; spVo, trigeminal spinal nucleus oralis; spVc, trigeminal spinal nucleus caudalis

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ABSTRACT

To test the hypothesis that vagal afferent (VA) stimulation modulates the first cervical dorsal horn (C_1) neuron activity, which is projected by tooth-pulp (TP) afferent inputs, through the activation of a local GABAergic mechanism via 5-HT_3 receptors, we used the technique of microiontophoretic application of drugs. In pentobarbital-anesthetized rats, we recorded C_1 spinal neuron activity responding to TP stimulation. The TP stimulation-evoked C_1 spinal neuron excitation was inhibited by VA stimulation and this inhibition was significantly attenuated by iontophoretic application of the 5-HT_3 receptor antagonist ICS 205-930 (40 nA) or the $GABA_A$ receptor antagonist bicuculline (40 nA). In another series of experiments, we determined that 60 nA iontophoretic application of glutamate produced a maximal increase in the C_1 spinal neuron activity at a minimal current. In 53 of 65 neurons (81.5 %), VA conditioning stimulation (1.0 mA×0.1 ms, 50 Hz for 30 s) caused a significant inhibition (35.1 %) of the glutamate (60 nA) application-evoked C_1 spinal neuron excitation. Iontophoretic application of ICS 205-930 (40 nA) or bicuculline (40 nA) significantly attenuated the VA stimulation-induced inhibition of glutamate iontophoretic application (60 nA)-evoked C_1 spinal neuron excitation. These results suggest that VA stimulation-induced suppression of C_1 spinal neuron activity, responding to TP stimulation, involves 5-HT_3 receptor activation, possibly originating in the descending serotonergic inhibitory system, and post-synaptic modulation of inhibitory GABAergic neurons.

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The trigeminal spinal nucleus is considered to be closely associated with the perception and transmission of orofacial sensory information, including nociceptive signals from tooth-pulp (TP) (Strassman and Vos, 1993; Iwata et al., 1998). The trigeminal spinal nucleus is functionally and anatomically subdivided into three nuclei: oralis, interpolaris and caudalis (Sessle, 1987). The subnucleus caudalis (spVc) is thought to be analogous to the spinal dorsal horn (Hu et al., 1981), and the histological structures of the first cervical dorsal horn (C_1) have an analogy to the spVc. From these observations, it is possible to speculate that certain inputs from TP afferent fibers terminate in the C_1 segment of the spinal cord. This possibility was confirmed by evidence that C_1 spinal neurons responded to electrical stimulation of the TP (Matsumoto et al., 1999; Takeda et al., 1999; Tanimoto et al., 2002). Furthermore, we reported that both *N*-methyl-*D*-aspartate (NMDA) and non-NMDA receptors contribute to excitation of the C_1 spinal neuron activity evoked by TP stimulation (Takeda et al., 1999). These results led us to suggest that C_1 spinal neurons process nociceptive information, carried in the trigeminal nerve from the TP and this involves excitatory glutaminergic mechanisms.

Vagal afferent (VA) inputs play an important role in the regulation of autonomic function (circulation and respiration) as well as in the modification of nociception (Ren et al., 1989; Randich and Gebhart, 1992; Takeda et al., 2002). Bossut and Maixner (1996) demonstrated that VA stimulation could inhibit the responses of trigeminal and trigeminothalamic neurons to noxious orofacial stimulation. Furthermore, Takeda et al. (1998) demonstrated that VA stimulation inhibited nociceptive transmission in the trigeminal spinal nucleus oralis (spVo) related to the TP-evoked jaw-opening reflex and this inhibition is modulated by activation of the opioid system in rats. We have also demonstrated that VA stimulation produces inhibition of TP stimulation-evoked C_1 spinal neuron excitation through activation of serotonergic (5-HT_3) descending inhibition (Tanimoto et al., 2002). 5-HT_3 receptors are ligand gated cation channels, activation of which resulting in neuronal excitation (Derkach et al., 1989). Autoradiographic experiments showed that there is a dense band of 5-HT_3 receptors in the superficial dorsal horn where small-diameter primary afferent fibers terminate, and

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that the number of binding sites is greatly reduced by neonatal capsaicin application (Hamon et al., 1989) or after dorsal rhizotomy (Laporte et al., 1995). However 5-HT_3 binding sites are still present in the intrinsic central nervous system (CNS) following rhizotomy (Kilpatrick et al., 1987). Furthermore, 5-HT-mediated antinociception in the rat tail-flick test is mediated by 5-HT_3 receptors (Glaum et al., 1990). A behavioral study also shows the involvement of 5-HT_3 receptors in spinal antinociception and suggests the existence of 5-HT_3 receptors on intrinsic CNS neurons (Wilcox and Alhaider, 1990). Alhaider et al. (1991) have found that iontophoretic application of γ-amino-butyric-acid receptor type A ($GABA_A$) antagonists blocked the 5-HT_3 receptor agonist-induced inhibitory action involving the demonstration of post-synaptically serotonergic inhibitory effect on the lumber dorsal horn. This probably implies that the endogenously released 5-HT in the spinal cord can activate an inhibitory neuron, which in turn releases GABA. Because there is evidence demonstrating that electro-acupuncture decreases TP stimulation-evoked substance P release in the spVc and its decrease is blocked by intravenous administration of the 5-HT_3 receptor antagonist ICS 205-930 (Yonehara, 2001), it is needed to determine as to whether the trigeminal nociceptive information of C_1 spinal dorsal horn neurons occurs pre- or post-synaptically.

Based on the fact that the activation of 5-HT_3 receptors mediates VA stimulation evoked inhibition of the C_1 spinal neuron activity (Tanimoto et al., 2002), the present study was to investigate the hypothesis that there is a possible interaction between 5-HT_3 receptors and $GABA_A$ receptors on VA stimulation-induced inhibition in the C_1 spinal neuron excitation evoked by TP electrical stimulation or glutamate iontophoretic application.

Materials and Methods

The experiments were approved by the Animal Use and Care Committee of Nippon Dental University and were consistent with the ethical guidelines of the International Association for the Study of Pain. Efforts were made to minimize the number of animals used and their suffering.

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Animal preparation. The experiments were performed on 31 male Wistar rats (280-345 g). Each animal was initially anesthetized with sodium pentobarbital (45 mg/kg, i.p.), and anesthesia was maintained with additional doses of 2-3 mg/kg/h through a cannula in the jugular vein. The trachea was cannulated and body temperature of animals were maintained at 37.0± 0.5 ℃ with a radiant heater. Arterial blood pressure (ABP) was measured by means of a pressure transducer through a cannula inserted into the femoral artery. Adequate depth of anesthesia was checked by lack of response to paw pinching. All wound margins were repeatedly covered with 2 % lidocaine throughout the experiment.

Stimulus electrode of tooth-pulp and vagal afferent stimulation. The method of TP stimulation was similar to that in previous studies (Takeda et al., 1999; Tanimoto et al., 2002). In brief, bipolar stimulating electrodes made from silver wire (diameter 150 μm, enamel insulated except for the tip) were inserted into the pulp of upper incisors. To avoid spread of the stimulus current, electrodes were isolated from surrounding tissues with dental cement.

The right cervical vagus nerve was isolated from the connective tissues of the common carotid artery. The nerve was cut and its proximal portion placed across a pair of silver wires, with the cathode proximal. The stimulating electrodes were embedded in a cuff made from a polyethylene tube which was implanted in the muscles around the vagus nerve. The nerve was bathed in warm mineral oil to prevent it from drying.

Extracellular recordings C_1 spinal neuron activity. The rats were then placed in a stereotaxic apparatus, and a laminectomy was performed to expose the C_1 region of the spinal cord. The dura was cut, and the brain surface was covered with a warm liquid paraffin oil (37.0-37.5 ℃). The single unit activity of C_1 spinal neurons was recorded with a five-barreled glass micropipette filled with 2 %-pontanime sky blue in 0.5 M sodium acetate (tip resistance of 5-12 MΩ at 1 kHz), amplified with a preamplifier (DAM 80, WPI, USA) and monitored on an oscilloscope (VC-11, Nihon Kohden, Japan)

Somatic receptive fields of C_1 spinal neurons that responded to TP stimulation were examined by tactile stimulation with a small brush and by pinching the skin with forceps. Neurons that responded to TP stimulation only, which had no somatic receptive field, were classified as nociceptive specific (NS), and those excited by both brush and pinch were classified as having a wide-dynamic range (WDR). The responses of C_1 spinal neurons to somatic field stimulation

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were monitored on an oscilloscope and recorded on a thermal recorder. The conduction velocity for each neuron responding to TP stimulation was calculated by dividing the distance between the site of the ipsilateral TP and the C_1 region (about 45 mm) by the latency between the stimulus artifact and the first evoked spike. The values for conduction velocity were corrected for a 0.5-ms synaptic delay.

Microiontophoretic application of drugs. Five lateral barrels of the micropipette, one containing 160 mM-NaCl was used for autonomic current balancing to prevent the occurrence of tip polarization artifacts. The remaining barrels contained the following solutions: L-glutamate (100 mM in 160 mM-NaCl, pH 8.5) (169-11, Nacalai Tesque, Japan.), ICS 205-930, 3-Tropanyl-indole-3-carboxylate hydrochloride [endo-8-Methyl-8-azabicyclo [3.2.1] oct-3-ol Indol-3-yl- carboxylate hydrochloride] (5-HT_3 receptor antagonist, 10 mM in 160 mM-NaCl, pH 5.0) (89565-68-4, RBI, U.S.A.) and bicuculline, bicuculline methiodide ($GABA_A$ receptor antagonist, 5 mM in 160 mM-NaCl, pH 3.5) (485-49-4, RBI, U.S.A.). Ejection, retention and balancing currents were provided by constant current unit (DPI-25, DiaMedical, Japan.). Glutamate was ejected with anionic currents of 20-80 nA, and ICS 205-930 and bicuculline were ejected with cationic currents of 20-40 nA. Retaining currents of 10-30 nA were used. Before administration of glutamate, ICS 205-930 and bicuculline, vehicles (same volume and pH of saline) were administrated, and no changes in C_1 spinal neuron activity had been observed.

Experimental protocols and data analysis. VA conditioning parameters were similar to those reported by Tanimoto et al. (2002). In brief, the stimulus intensity was 1.0 mA, the duration was 0.1 ms and the frequency was 10 or 50 Hz for 30 s. Prior to test VA conditioning stimulation, we confirmed stimulus frequency-dependent effects (10 and 50 Hz) of VA stimulation on the C_1 spinal neuron activity. If the changes from control activity were more than 20 %, a given stimulus was considered effective. During the VA stimulation, changes in ABP were also monitored.

The TP stimulation (duration 0.1-0.18 ms, single pulse and stimulation frequency of 1 Hz)-evoked C_1 spinal neuron activity was recorded. TP stimulation-evoked responses were quantified on post-stimulus histograms (16 sweeps, bin width 1 ms) and we determined the spike number to one TP stimulation (s*pikes/stimulus*). Then we examined the TP stimulation

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intensity dependent-increase in C_1 spinal neuronal firing frequency. In the same neuron, the responses of C_1 spinal neuron activity to TP electrical stimulation before and after VA stimulation were compared. The inhibitory effect of VA stimulation on the C_1 spinal neuron activity was quantified by subtracting background discharges from evoked activities. The microiontophoretic applications of ICS 205-930 or bicuculline, we examined the effects of ICS 205-930 (20 and 40 nA) or bicuculline (20 and 40 nA), to enable determination of iontophoretic conditions (40 nA).

Initial studies were performed to determine optimum concentrations of iontophoretically-applied glutamate, ICS 205-930 and bicuculline. In belief, in order to assess the effectiveness of the iontophoretic application of glutamate on the firing frequencies of C_1 spinal neurons, we varied the intensity (20, 40, 60 and 80 nA, 5 s), enabling us to determine the iontophoretic conditions. Sixty nA iontophoretic application of glutamate showed a maximal increase in the C_1 spinal neuron activity at a minimal current. The responses of C_1 spinal neuron activity to glutamate application (60 nA, 5 s) were compared before and after VA conditioning stimulation as described above. Furthermore, we also examined the effects of ICS 205-930 (40 nA) or bicuculline (40 nA) iontophoretic application on VA conditioning stimulation-induced inhibition of glutamate application (60 nA)-evoked C1 spinal neuron excitation. Glutamate-evoked responses of C_1 spinal neuron activity to iontophoretic application of drugs were recorded on 3 times (one trial for 5 s and intervals 5 s) describing on the thermal recorder and the quantity was made through a spike counting system and expressed as for *impulses/s* (Imp/s).

The data were stored on Digital Audio Tape (DAT) for off-line analysis. Data are expressed as the mean±*SEM*. The statistical significance of the changes in responses of C_1 spinal neuron activity to TP stimulation or glutamate iontophoretic application by VA stimulation were calculated by using Student's paired t-test. A P value less than 0.05 was statistically significant. Changes in C_1 spinal neuron activity were expressed as the firing frequency (imp/s) pre- and post-drug application and assessed using Student's paired t test. A P value less than 0.05 was statistically significant.

Histology. At the end of the recording sessions, the rats were deeply anesthetized anyway use terminally with pentobarbital sodium. Then cathodal DC currents (30-50 μA for 15

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min) were passed through a glass micropipette. The animals were transcardially perfused with saline and 10% buffered formalin. Frozen coronal sections were cut into 40 μm sections and stained with haematoxylin-eosin stain, and then recording sites were identified from the blue spots and reconstruction of electrode tracks done using with micromanipulator readings (Paxinos and Watson, 1986).

Results

Unit sample. The ipsilateral TP stimulation excited a total of 65 C_1 spinal neurons. All neurons were located on the ipsilateral side of the stimulation. Fifty-three (81.5 %) lesion sites were found in laminae Ⅰ-Ⅲ, and 12 neurons (18.5 %) were located in laminae Ⅳ-Ⅴ. The number of spikes increased with increasing stimulus intensities. As the electrical stimulus intensity was increased, the C_1 spinal neuron activity was increased proportionally. The threshold of TP stimulation for activation of 65 C_1 spinal neurons was 0.90 ± 0.04 mA and the onset-latency in those neurons during TP stimulation was 9.1 ± 0.5 ms. The average value for conduction velocity was 6.1 ± 0.4 m/s. Sixty-two (95.4 %) C_1 spinal neurons were classified as the WDR neuron and the remaining 3 C_1 spinal neurons (4.6 %) had no excitatory somatic receptive fields, except for TP, and were identified by the NS neuron.

Effect of VA stimulation on the TP electrical stimulation-evoked C_1 spinal neuron activity before and after iontophoretic application of the 5-HT_3 receptor antagonist. Figure 1, A, B and C, show typical responses of C_1 spinal neuron activity to TP stimulation before and after VA stimulation. The TP-evoked C_1 spinal neuron excitation was inhibited by VA stimulation, and this inhibition was attenuated by iontophoretic application of 5-HT_3 receptor antagonist, ICS 205-930 (40 nA). The summarized results are shown in Fig. 1D. After VA stimulation the mean number of spikes in the 10 C_1 spinal neurons decreased to 44.2±5.2 % (7.1±0.4 v.s. 4.1±0.4 spikes/stimulus, n=10, p<0.05). The VA stimulation-induced C_1 spinal neuron inhibition was significantly attenuated by iontophoretic application (40 nA) of ICS 205-930 (7.9±4.9 %, n=10,

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$p<0.05$), but not by 20 nA-iontophoretic application (39.7±5.2, $n=10$). Similarly, as shown in Fig. 2D, TP stimulation-evoked C_1 spinal neuron activity was significantly suppressed by VA conditioning stimulation, and this suppression was significantly attenuated by 40 nA-bicuculline iontophoretic application (3.3±7.0 %, $n=7$, $p<0.05$), but not by 20 nA-iontophoretic application (25.3±7.8 %, $n=7$).

Effect of VA stimulation on glutamate iontophoretic application-evoked C_1 spinal neuron activity. As shown in Fig. 3, A and B, all of the C_1 spinal neurons, responding to electrical stimulation of the TP, were also activated by iontophoretic application of glutamate ($n=65$). The firing frequency of glutamate application-evoked spikes was current-dependently increased (20, 40, 60 and 80 nA). The iontophoretic application of glutamate stimulated C_1 spinal neuron activity in a current-dependent manner (Fig. 3C). Sixty nA iontophoretic application of glutamate resulted in a maximal increase in the C_1 spinal neuron activity at a minimal current.

Figure 4A show typical responses of C_1 spinal neuron activity to glutamate iontophoretic application (60 nA) before and after VA stimulation. The glutamate-evoked C_1 spinal neuron excitation was suppressed by VA stimulation. Of 65 units, 53 (81.5 %) were suppressed by VA stimulation. After VA stimulation the mean number of spikes of the 53 C_1 spinal neuron significantly decreased by 35.1 % (14.1± 0.7 *v.s.* 9.3±0.6, Imp/s, $n=53$, $p<0.01$) (Fig. 4B). After VA stimulation at 50 Hz the suppression of the glutamate (60 nA) application-evoked C_1 spinal neuron excitation became more prominent as compared to that at 10 Hz (34.2±1.2 % *v.s.* 21.4±0.9 %, $n=11$, $p<0.05$). The maximum suppressive effect of VA stimulation on C_1 spinal neuron activity was observed just after VA stimulation (0.05 min). One min after VA stimulation, suppression of C_1 spinal neuron activity still remained. The suppressive effect of VA stimulation lasted for over 60 s and was restored within 5 min. In 12 out of 53 C_1 spinal neurons, 4 were facilitated by VA stimulation and in 8 it had no effect. In 26 out of 65 neurons (44.6 %), spontaneous discharges of C_1 spinal neurons were activated by VA stimulation.

Effect of ICS 205-930 iontophoretic application on inhibition of glutamate-induced C_1 spinal neuron activity evoked by VA stimulation. To

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determine whether the 5-HT_3 receptor influences the VA stimulation-induced inhibition of C_1 spinal neuron activity, the 5-HT_3 receptor antagonist, ICS 205-930 was used at current of 40 nA, which was effective in experiments with TP electrical stimulation. In 25 of 29 C_1 spinal neurons, the VA stimulation-induced inhibition of the neuron activity was attenuated by iontophoretic pre-application of the 5-HT_3 receptor antagonist ICS 205-930. Typical examples are shown in Fig. 5, A and B. ICS 205-930 iontophoretic application (40 nA) markedly attenuated the inhibitory effect of glutamate-evoked C_1 neuron by VA stimulation. The blockade of C_1 spinal neuron activity following ICS 205-930 was recovered at the end of ICS 205-930 iontophoretic application. The results are summarized in Fig. 5C. The VA stimulation-induced C_1 spinal neuron activity of the glutamate iontophoretic application-evoked neuronal excitation was significantly attenuated after 0.05 min (8.9±0.7 *v.s.* 13.3±1.1 Imp/s, $p<0.05$, $n=25$) and 1 min (9.4± 0.9 *v.s.* 13.3±1.1 Imp/s, $p<0.05$, $n=25$) of local application of ICS 205-930. After stopping local application of ICS 205-930, such an effect was not observed.

The inhibition of glutamate-induced C_1 spinal neuron activity evoked by VA stimulation is reverted by bicuculline iontophoretic application. To determine whether $GABA_A$ receptors alter the VA stimulation-induced inhibition of C_1 spinal neuron activity, the $GABA_A$ receptor antagonist, bicuculline was used at current of 40 nA, which was effective in experiments used for TP electrical stimulation. In thirteen out of 14 neurons, inhibitory effects of C_1 spinal neuron activity by VA stimulation were also reverted by bicuculline application (40 nA) (Fig. 6, A and B). In the only one neuron, the application of bicuculline did not cause any significant change in the C_1 spinal neuron activity. As shown in Fig. 6C, bicuculline treatment significantly attenuated the VA stimulation-induced C_1 spinal neuron activity of the glutamate application-evoked neuronal excitation (after 0.05 min, 10.1±0.7 *v.s.* 14.6±0.9 Imp/s; after 1 min, 10.9±1.3 *v.s.* 14.6±1.0 Imp/s, $p<0.05$, $n=13$).

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Discussion

In the present study, we demonstrated that the effects of 5-HT_3 receptors on C_1 spinal neurons are involved in the VA stimulation-induced antinociceptive mechanisms in the C_1 spinal neuron activity, which may be post-synaptically mediated through activation of GABAergic ($GABA_A$ receptor) inhibitory interneurons.

Foreman (2000) reviewed the functional significance of the upper cervical dorsal horn for visceral pain. Concerning this, there are many reports that the C_1 spinal neuron plays an important role in both nociceptive transmission and VA projection in rats (Matsumoto et al., 1999; Qin et al., 2001; Tanimoto et al., 2002), cats (Thies and Foreman, 1981) and monkeys (Ammons et al., 1983; Chandler et al., 2000). Chandler et al. (1996) demonstrated that 90 % of the C_1 spinothalamic tract cells were excited by vagal stimulation and 80 % of these neurons also responded to algesic stimulation of the heart. These results lead us to suggest that the nociceptive information is primarily transmitted by VA via activation of some C_1 spinal neurons in the superficial laminae. This suggestion is at least in part supported by evidence that VA stimulation at 1.0 mA for 30 s facilitated spontaneous activity in 15 out of 65 C_1 spinal neurons responding to TP stimulation (Tanimoto et al., 2002). However, at higher intensities of VA stimulation it can inhibit neuronal activity when the recording sites are made from cells below the C_2 segments (Ammons et al., 1983). Thus, it is possible to speculate the idea that the facilitatory effect of VA stimulation on C_1 spinal neuron activity may be due to the activation of myelinated VA fibers, which has different effects on the brainstem (reticular formation, nucleus reticularis gigantocellularis and pontine reticular formation) (Zhuo and Gebhart, 1990; 1992), as compared to that seen at nonmyelinated VA fiber activation. Zhuo and Gebhart (1997) reported that descending inhibitory and facilitatory influences could be simultaneously engaged throughout the RVM, including nucleus raphe magnus, and that such influences are conveyed in different spinal funiculi. The NRM that is thought to be one of the endogenous pain control system may produce the biphasic effects on the TP-evoked C_1 spinal neuron activity. The circuitry of descending facilitation and inhibition influences originating from the NRM was not

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identified in this study. However, further studies are needed to elucidate their interaction. In the present study, approximately 83 % of 65 C_1 spinal neurons, responding to TP stimulation, were inhibited by VA stimulation. The magnitude of this inhibition was quite similar to that seen in a previous study (Tanimoto et al., 2002).

Serotonergic descending pain modification in the CNS is known to be a major pathway for the pain inhibition, which project to the trigeminal spinal nucleus and spinal dorsal horn. (Basbaum and Fields, 1984; Millan, 2002). There are observations indicating that spinally projecting serotonergic neurons modulate nociceptive transmission via individual 5-HT receptors from a brainstem nuclei source involving the nucleus raphe magnus (NRM) (Basbaum and Fields, 1984). Several 5-HT receptor subtypes have so far been identified in the CNS, for example 5-HT_1, 5-HT_2, 5-HT_3, 5-ht_4, 5-ht_5, 5-HT_6 and 5-HT_7 (Peroutka, 1988; Hoyer et al., 1994). Because there are no reports to demonstrate 5-HT_3 receptor protein and/or mRNA expression in the trigeminal ganglion (Lazarov, 2002; Morales et al., 2002), it is quite possible that descending serotonergic fiber terminals via a 5-HT_3 receptor project to spinal dorsal horn neurons (Morales et al., 1996a; 1998) or interneurons in the CNS (Todd and McKenzie 1989; Wilcox and Alhaider 1990). In nociceptive neurons, located in the lumbar spinal cord and which respond to iontophoretically applied NMDA receptor agonists, application of the 5-HT_3 receptor agonist, 2-methyl-5-HT, inhibits NMDA-induced neuronal excitation in a current-dependent manner (Alhaider et al., 1991). This inhibition is also blocked by the 5-HT_3 receptor antagonist zacopride or the $GABA_A$ receptor antagonists, bicuculline and picrotoxin (Alhaider et al., 1991). The results suggest a post synaptically action of nociceptive inhibition by GABAergic interneurons. The present study obtained evidence that an iontophoretic application of bicuculline blocked the inhibition of C_1 spinal neuron excitation produced by VA conditioning similar to the effect of ICS-205 930 application. This probably implies that 5-HT_3 receptors play a significant role in a descending serotonergic inhibitory pathway, particularly in which the afferent excitatory transmission is mediated through NMDA receptors. Based on evidence that NMDA receptors contribute to excitation of the C_1 spinal neuron activity during TP stimulation (Takeda et al., 1999), the results of this

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study suggest that the activation of the descending serotonergic pathway produced by VA stimulation may facilitate the release of 5-HT to stimulate 5-HT_3 receptors and this inhibitory effect occurs as a result of the 5-HT_3 receptor activation that facilitates the release of GABA from spinal GABAergic neurons (Todd and McKenzie, 1989). We also found that bicuculline application significantly blocked VA stimulation-induced inhibition of the C_1 spinal neurons similar to that of ICS 205-930 application. In fact, the quantitative analysis with in situ hybridization and immunocytochemistry indicated that more than 90 % of 5-HT_3 receptors expressing cells presented GABA in the neocortex and hippocampus (Morales et al., 1996b). Therefore, as shown in Fig. 7, we hypothesize that the application of ICS 205-930, one of the 5-HT_3 receptor antagonists, attenuates VA stimulation-induced inhibition of the C_1 spinal neuron activity projecting from TP, and that VA stimulation-induced antinociception is mediated by the activation of GABAergic (a $GABA_A$ receptor) interneurons.

In conclusion, the present study provided evidence that descending inhibition, which was produced by continuous stimulation of cervical VA inputs inhibited the excitatory response of C_1 spinal neuron activity to TP stimulation or glutamate application, and that these inhibitory effects were significantly attenuated by pretreatment with a 5-HT_3 receptor antagonist ICS 205-930 or a $GABA_A$ receptor antagonist bicuculline. These results suggest that VA stimulation-induced descending pain modulation of excitation of C_1 spinal neurons originating in the TP is mediated by the activation of 5-HT_3 receptor-mediated descending inhibitory pathways and its effect is post synaptic. The 5-HT_3 receptor-mediated descending pain modification of trigeminal antinociception may act on GABAergic inhibitory interneurons in the rat C_1 dorsal horn.

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Footnotes

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Legend for figures

Fig. 1. Effect of ICS 205-930 iontophoretic application on the VA stimulation-induced inhibition of TP electrical stimulation-evoked C_1 spinal neuron activity. Typical example of TP stimulation-evoked neuronal activity (Control) **(A)**, after VA stimulation **(B)** and after VA stimulation following ICS 205-930 iontophoretic application (40 nA) **(C)**. Left side is photograph (1 sweep/stimulus) and left side is post-stimulus histogram (16 times, 1 bin=1 ms). Arrows shows TP stimulation (3.5 mA×0.15 ms). Summary of effect of ICS 205-930 (20 and 40 nA) on the VA stimulation-induced inhibition of TP electrical stimulation **(D)**. Values show mean±*S.E.M.* **p*<0.05 (*n*=6) v.s. control; ***p*<0.05 (*n*=6) v.s. after VA stimulation.

Fig. 2. Effect of bicuculline iontophoretic application on the VA stimulation-induced inhibition of TP electrical stimulation-evoked C_1 spinal neuron activity. Typical example of TP stimulation-evoked neuronal activity (Control) **(A)**, after VA stimulation **(B)** and after VA stimulation following bicuculline iontophoretic application (40 nA) **(C)**. Left side is photograph (1 sweep/stimulus) and left side is post-stimulus histogram (16 times, 1 bin=1 ms). Arrows shows TP stimulation (3.5 mA×0.15 ms). Summary of effect of bicuculline (20 and 40 nA) on the VA stimulation-induced inhibition of TP electrical stimulation **(D)**. Values show mean±*S.E.M.* **p*<0.05 (*n*=6) v.s. control; ***p*<0.05 (*n*=6) v.s. after VA stimulation.

Fig. 3. Effects of glutamate iontophoretic application on the C_1 spinal neuron activity. Typical example of tooth-pulp (TP) electrical stimulation (2.3 mA×0.15 ms, single pulse)-evoked **(A)** and glutamate (20, 40, 60 and 80 nA, for 5 s)-evoked C_1 spinal neuron activity. Summary of effect of glutamate application (20, 40, 60 and 80 nA, for 5 s) on the C_1 spinal neuron activity responding to TP stimulation

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(C). (*p<0.05, 20 nA *v.s.* 40 nA, 40 nA *v.s.* 60 nA, 40 A *v.s.* 80nA, respectively, n=65. A, B and C are all same neuron.

Fig. 4. Effect of vagal afferent (VA) stimulation on the glutamate (60 nA)-evoked C_1 spinal neuron activity. Typical example of effect of VA stimulation (1.0 mA× 0.1 ms, 50 Hz for 30 s) **(A)** and Summary of time course changes of VA stimulation-induced inhibition on the glutamate-evoked C_1 spinal neuron activity. Vertical bars are the mean ± *S.E.M.* (Imp/s, n=53, *p<0.01 *v.s.* before VA stimulation).

Fig. 5. Effect of ICS 205-930 iontophoretic application on the vagal afferent (VA) stimulation-induced inhibition of the glutamate application-evoked C_1 spinal neuron activity. Effect of VA stimulation on the glutamate application (60 nA)-evoked C_1 spinal neuron activity before **(A)** and after **(B)** ICS 205-930 (40 nA) application. VA stimulation, 1 mA×0.1 ms, 50 Hz for 30 s. Summary of disinhibitory effect following ICS 205-930 application on the VA stimulation-induced inhibition of the glutamate iontophoretic application-evoked C_1 spinal neuron excitation at each time after VA stimulation **(C)**. Each column represents the mean±*S.E.M.* (Imp/s, n=25, *p<0.01, Control *v.s.* after ICS 205-930, respectively). Empty column, Control (before ICS 205-930 application, corresponding panel **A**); black column, after ICS 205-930 iontophoretic application (40 nA), corresponding panel **B**.

Fig. 6. Effect of bicuculline iontophoretic application on the vagal afferent (VA) stimulation-induced inhibition of the glutamate application-evoked C_1 spinal neuron activity. Effect of VA stimulation on the glutamate application (60 nA)-evoked C_1 spinal neuron activity before **(A)** and after **(B)** bicuculline (40 nA) application. VA stimulation, 1 mA × 0.1 ms, 50 Hz for 30 s. Summary of disinhibitory effect following bicuculline application on the VA stimulation-induced inhibition of the glutamate iontophoretic application-evoked

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C_1 spinal neuron excitation at each time after VA stimulation **(C)**. Each column represents the mean**±***S.E.M.* (Imp/s, n=25, *p<0.01, Control *v.s.* after bicuculline, respectively). Empty column, Control (before ICS 205-930 application, corresponding panel **A**); black column, after bicuculline iontophoretic application (40 nA), corresponding panel **B**.

Fig. 7. Schematic drawing showing hypothesized mechanisms of vagal afferent activation-induced 5-HT_3 receptor-mediated descending inhibition in the C_1 spinal neuron responding tooth-pulp stimulation. Hypothesis of serotonergic descending pathway via GABAergic interneuron ($GABA_A$ receptor).

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Fig 1

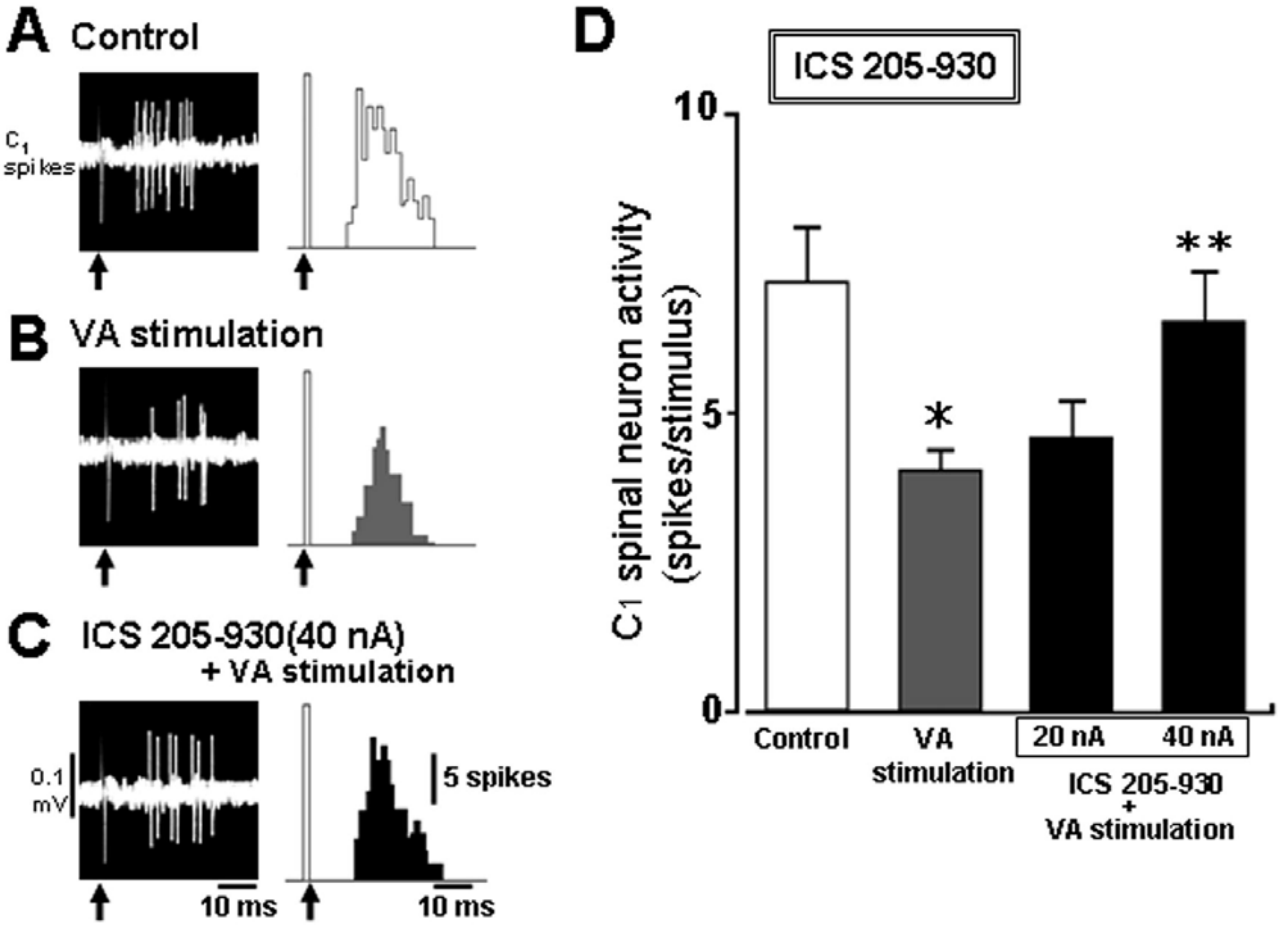

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Fig 2

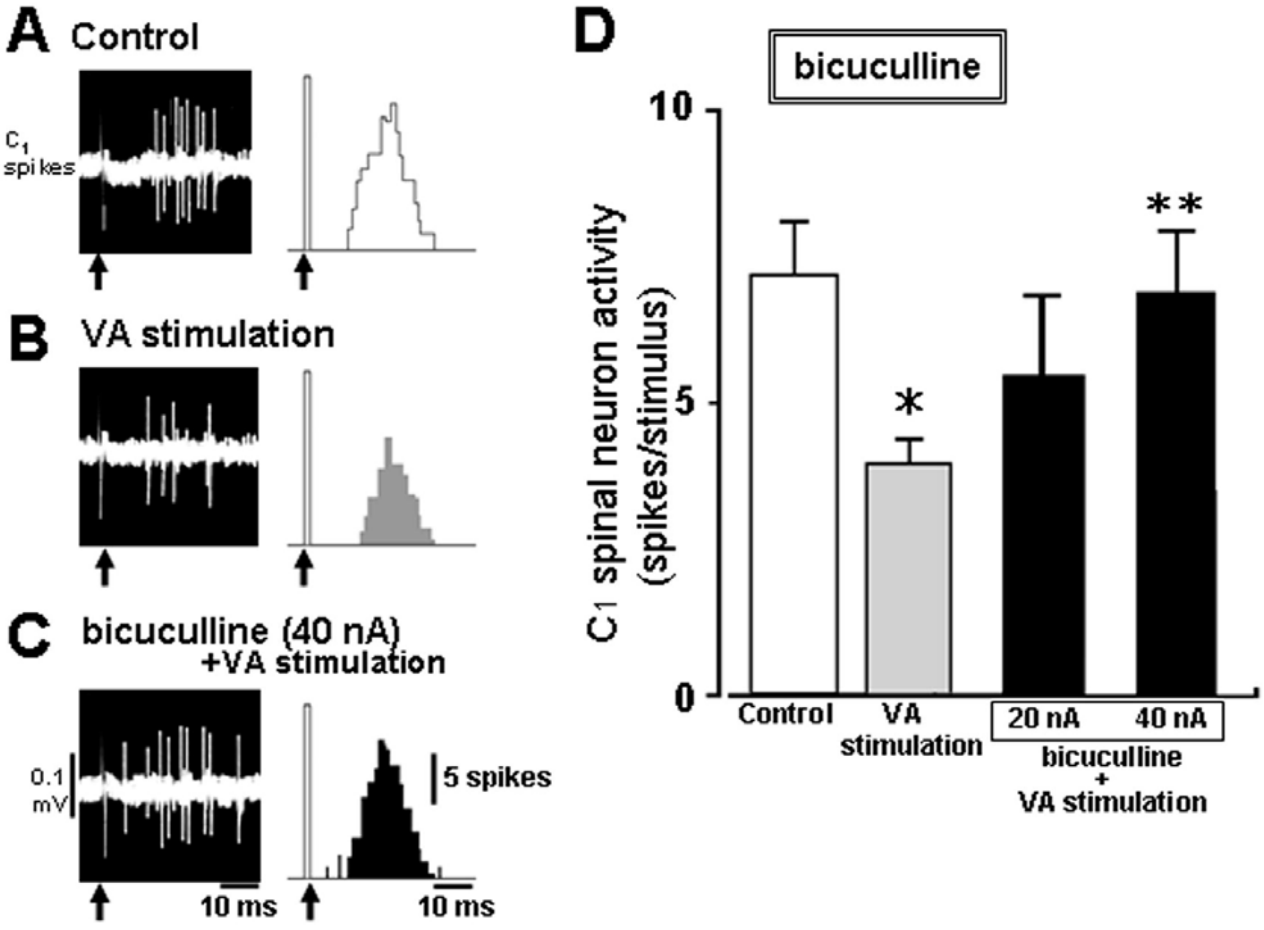

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Fig 3

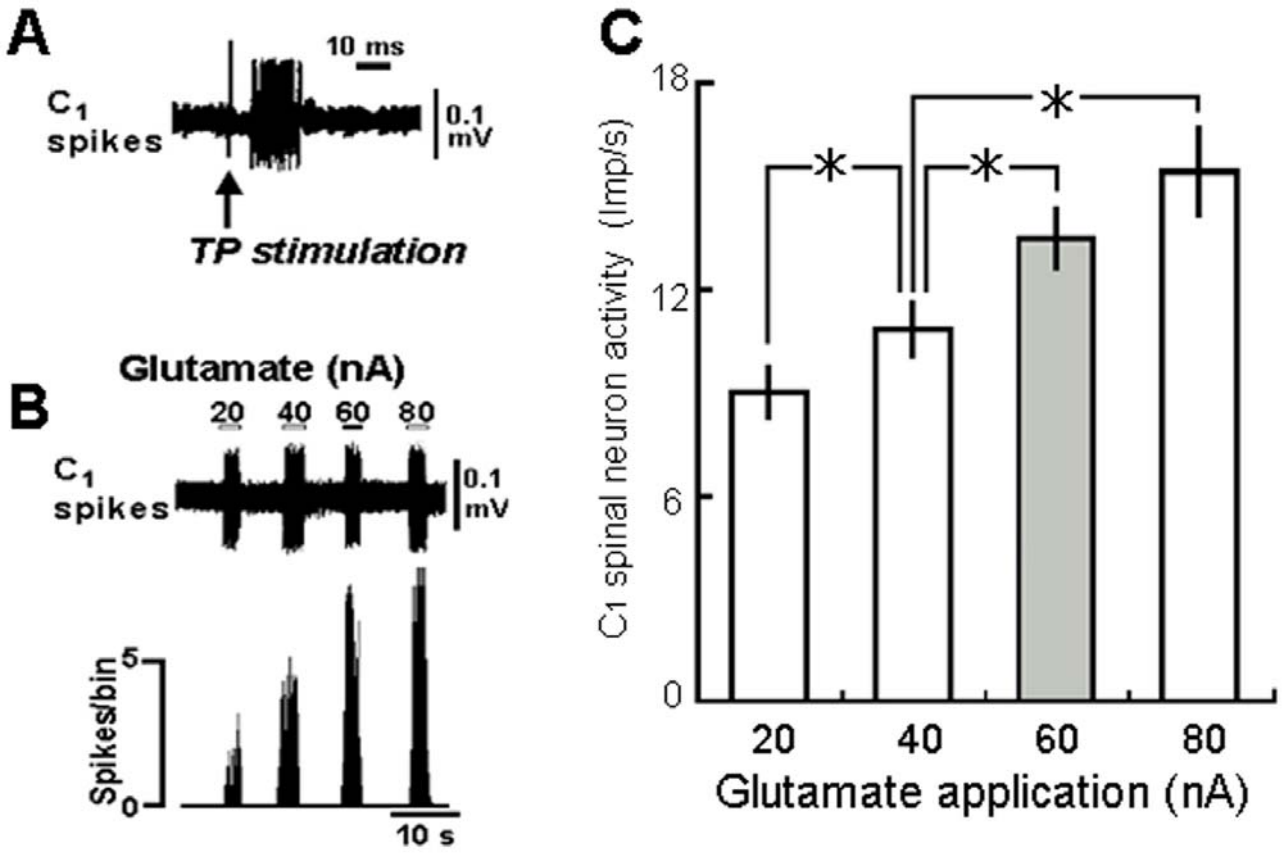

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Fig 4

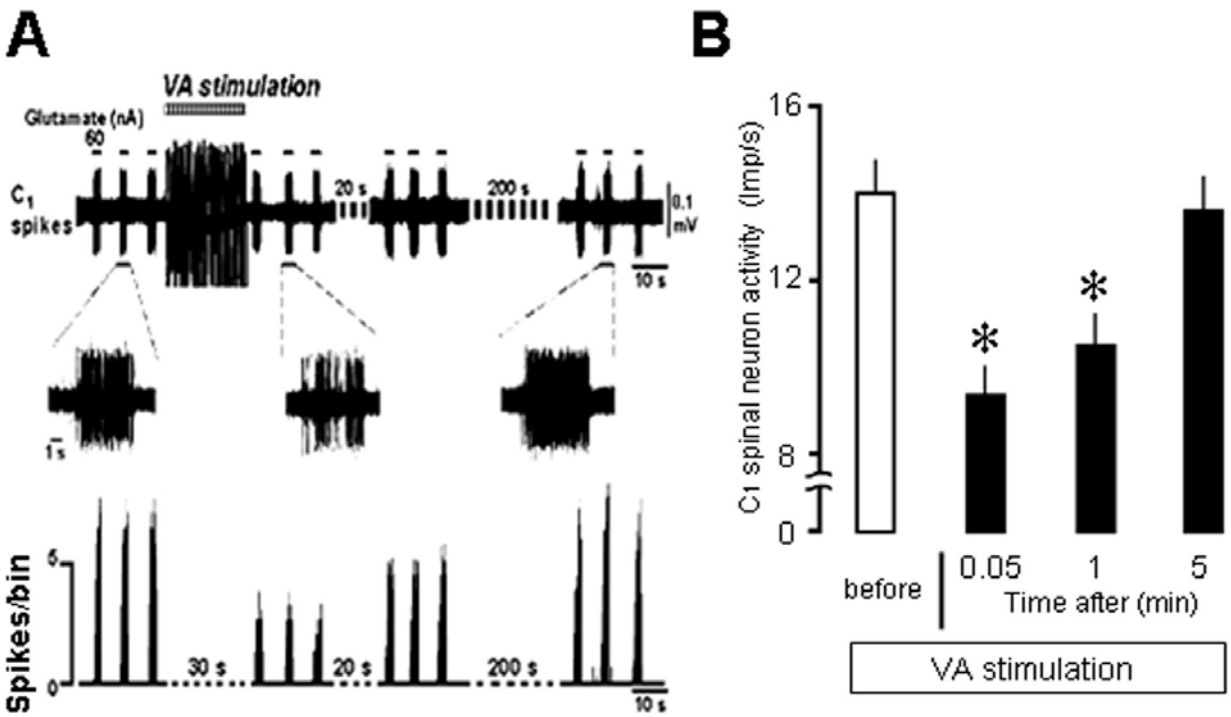

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Fig 5

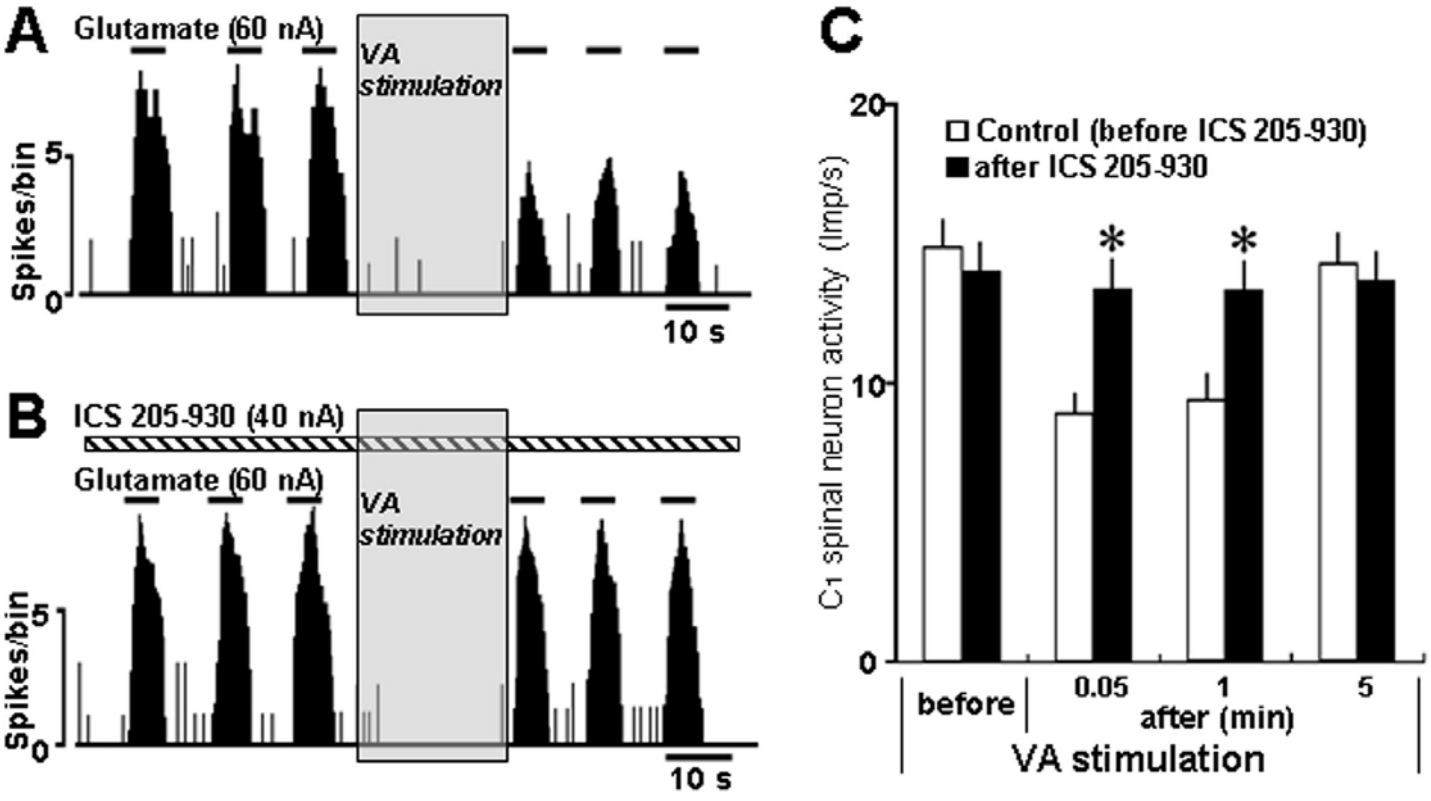

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Fig 6

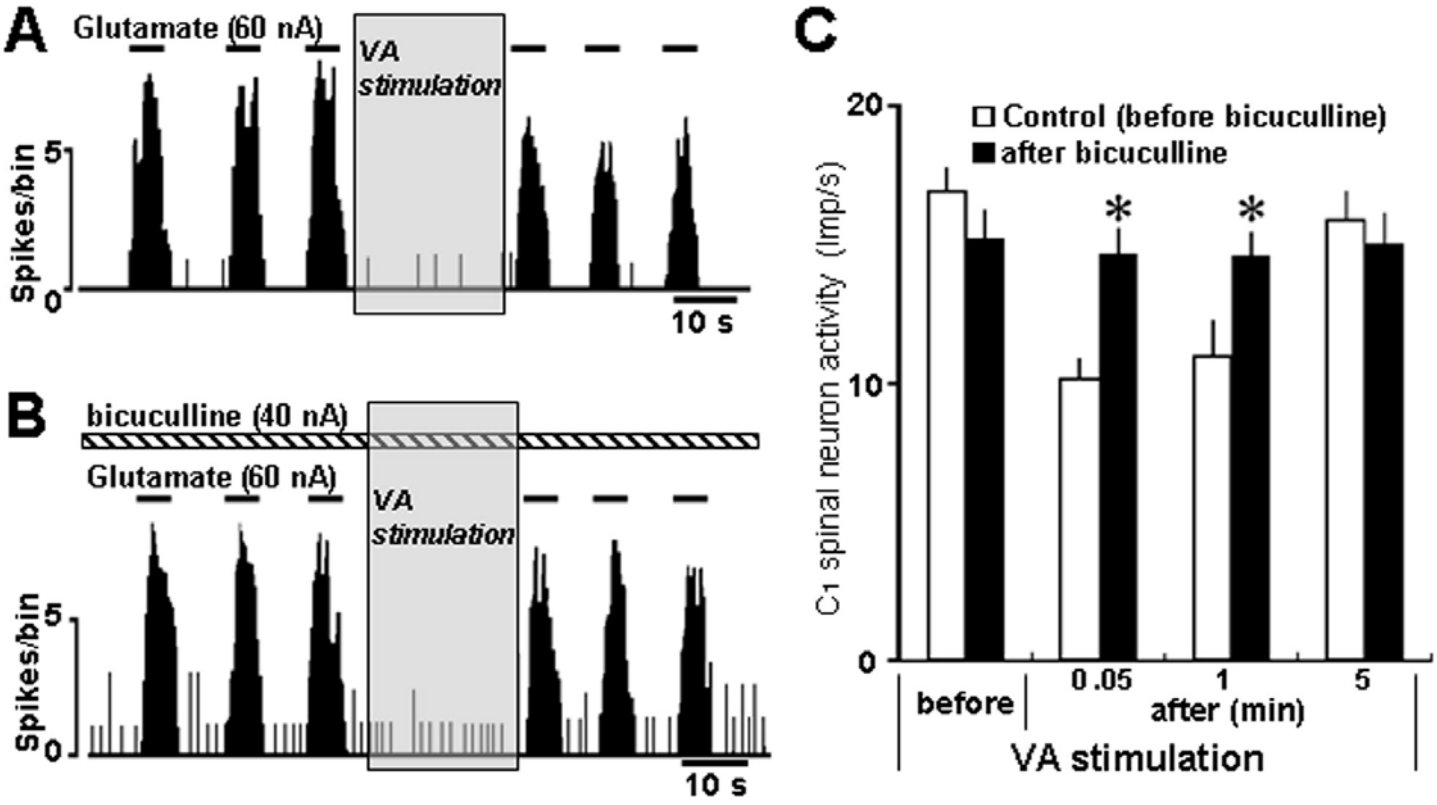

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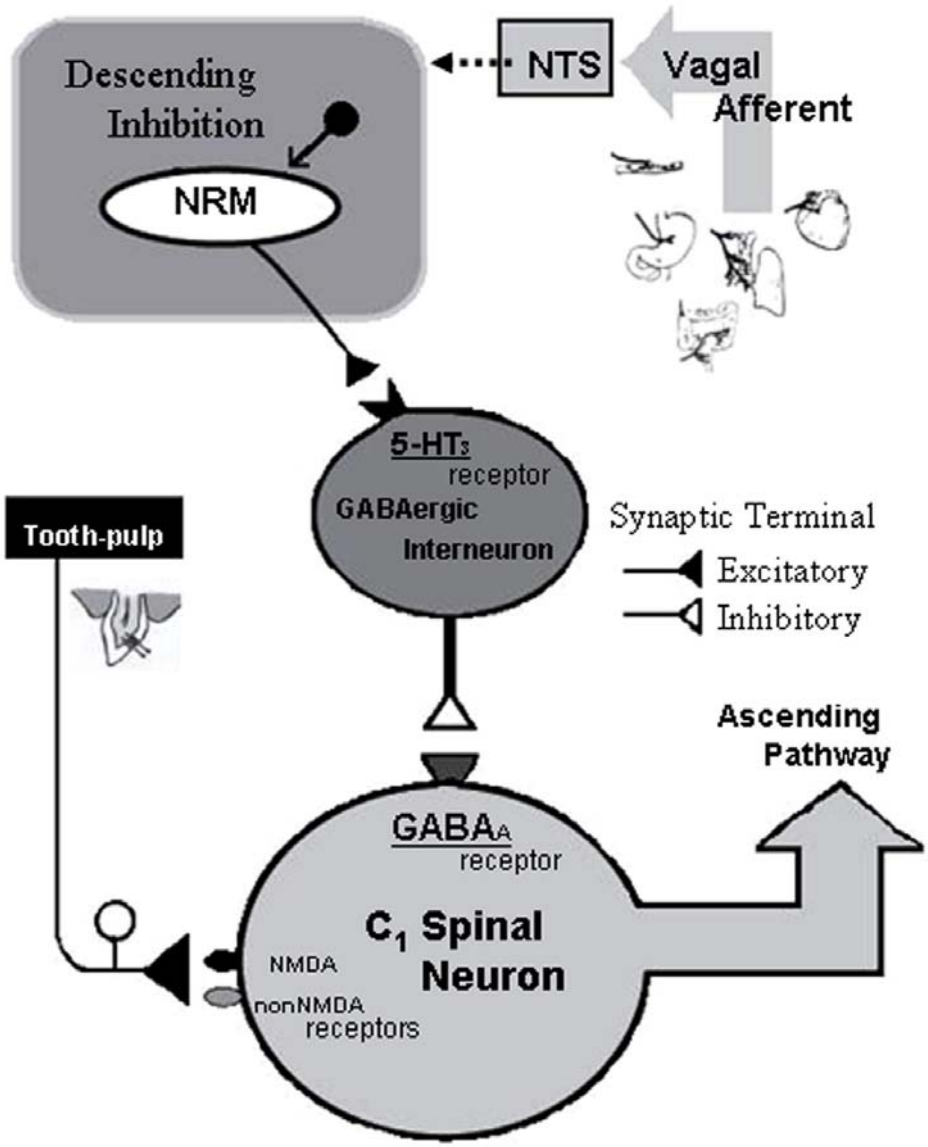
Fig 7



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