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Mechanisms for Ovariectomy-Induced Hyperalgesia and Its Relief by Calcitonin: Participation of 5-HT_{1A}-Like Receptor on C-Afferent Terminals in Substantia Gelatinosa of the Rat Spinal Cord

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Chronic treatment with calcitonin in osteoporotic patients alleviates the pain associated with this condition by an unknown mechanism. In ovariectomized rats that develop osteoporosis and hyperalgesia, we examined whether a functional change in serotonergic systems in the spinal dorsal horn was involved, using whole-cell recordings from substantia gelatinosa neurons in spinal cord slices and [^{3}H]8-hydroxy-2(di-*n*-propylamino) tetralin ([^{3}H]8-OH-DPAT) binding. Hyperalgesia could be attributed to the elimination of presynaptic inhibition by 5-HT of glutamatergic primary C-afferent terminals and an associated decrease in the density of [^{3}H]8-OH-DPAT binding sites whose receptors are neither 5-HT_{1A}- nor 5-HT_7-subtype. These changes in serotonergic systems were restored after chronic treatment with calcitonin. Reversal of 5-HT receptor changes by calcitonin treatment may provide an explanation for its analgesic actions in patients.

Key words: serotonin; substantia gelatinosa; EPSC; spinal cord slice; patch-clamp; ovariectomy; hyperalgesia; calcitonin; plasticity

Calcitonin, a polypeptide hormone secreted from the parafollicular C cells of the mammalian thyroid gland (Munson, 1976; Potts and Aurbach, 1976), is widely used clinically to improve bone mass in osteoporosis (Chesnutt et al., 1981; Gruber et al., 1984; Orimo et al., 1996) and also to relieve pain accompanying it. In the clinical treatment for pain, repeated injections of calcitonin to the periphery are required for ~1 month (Franceschini et al., 1983; Gennari and Agnusdei, 1988; Pontiroli et al., 1991). Almost all of the experimental studies conducted until now have examined only the acute anti-nociceptive effects of calcitonin (Pecile et al., 1975; Yamamoto et al., 1979; Clementi et al., 1984; Spampinato et al., 1984; Guidobono et al., 1985, 1986; Maeda et al., 1995). One exception is the report that repeated systemic (subcutaneous) injections of calcitonin result in maintained anti-nociception in formalin-induced hyperalgesia in rats (Umeno et al., 1996). However, the mechanisms underlying this action and the analgesic effect of calcitonin in osteoporotic patients remain unresolved.

It has been demonstrated recently that ovariectomized (OVX) rats with osteoporosis exhibit hyperalgesia (using the tail-withdrawal test), which is alleviated by repetitive subcutaneous injections of elcatonin (eCT) ([Asu^{1,7}]eel calcitonin) in a dose-dependent manner (Shibata et al., 1998); this antinociception is significant only after 3–4 weeks of treatment. Because this result is very similar to the clinical effect of calcitonin in patients, this rat model seems appropriate for studying the antinociceptive mechanisms of eCT. In this model, eCT-induced anti-nociception was completely inhibited by the intraperitoneally injection of *p*-chlorophenylalanine, an inhibitor of serotonin (5-HT) biosynthesis (Shibata et al., 1998). This observation is consistent with the fact that descending inhibitory serotonergic systems from the raphe nuclei in the brainstem contribute to pain modulation in the spinal cord (Yaksh, 1979; Zemlan et al., 1980; Xu et al., 1994).

Much evidence has suggested that the substantia gelatinosa (SG) (lamina II) of the spinal dorsal horn plays an important role in the modulation of nociceptive transmission from the periphery to the CNS (Kumazawa and Perl, 1978; Light et al., 1979; Cervero and Iggo, 1980; Fitzgerald, 1981; Brown, 1982). Fine myelinated Aδ-afferent and unmyelinated C-afferent fibers, many of which carry nociceptive information, terminate preferentially in the SG (Kumazawa and Perl, 1978; Light and Perl, 1979; Sugiura et al., 1986, 1989; Yoshimura and Jessell, 1989, 1990). From the action of *p*-chlorophenylalanine mentioned above, it seems possible that OVX rats exhibit an alteration in the 5-HT receptors normally involved in modulating nociceptive transmission to the SG. The abolition of hyperalgesia by chronic treatment with eCT might then be attributable to a reversal of 5-HT receptor plasticity. To investigate this possibility, the effects of 5-HT on excitatory transmission to SG neurons were examined in spinal cord slices obtained from sham-operated (Sham), OVX, or eCT-treated OVX (OVX + eCT) rats using the blind whole-cell patch-clamp technique. We also studied whether or not [^{3}H]8-hydroxy-2(di-*n*-propylamino) tetralin ([^{3}H]8-OH-DPAT) binding sites in the spinal cord differ in number between these three groups of rats.

MATERIALS AND METHODS

Animals. Female Sprague Dawley rats (7-week-old) were either ovariectomized bilaterally or sham-operated under anesthesia with ether. In the latter group, the ovaries were exteriorized but not removed. At 3 weeks after the operation, eCT (Asahi Chemical Industry Co., Shizuoka, Japan) or vehicle was administered for 4 weeks (20 $U \cdot k^{-1} \cdot gm^{-1} \cdot d^{-1}$, s.c.; 5 times a week) to OVX rats that demonstrated hyperalgesia; Sham rats were injected with vehicle for the same period, as reported previously (Shibata et al., 1998). All of the experimental procedures involving rats have been approved by the Saga Medical School Animal Use and Care Committee.

Slice preparations and electrophysiological recordings. The methods used for obtaining spinal cord slices from Sham, OVX, and eCT-treated OVX rats were similar to those described previously (Yoshimura and Nishi, 1993). Either 500-μm-thick transverse slices in which all of the dorsal roots were cut or 650-μm-thick slices that retained an attached L4 or L5 dorsal root were made from the rats. The slice was superfused at a rate of 15–20 ml/min with Krebs' solution equilibrated with 95% O_2 and 5% CO_2 and maintained at 36 ± 1°C. The Krebs' solution contained (in mM): NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.

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Table 1. Electrophysiological properties of SG neurons in spinal cord slices obtained from Sham, OVX, and OVX + eCT rats

			mEPSCs		
	RMP (mV)	Input resistance (MΩ)	Frequency (Hz)	Amplitude (pA)	HDT (msec)
Sham	−66 ± 1	990 ± 218	16 ± 1	8.7 ± 0.5	3.9 ± 0.1
	(n = 22)	(n = 23)	(n = 64)	(n = 64)	(n = 64)
OVX	−66 ± 1	719 ± 83	15 ± 1	8.3 ± 0.5	3.9 ± 0.1
	(n = 19)	(n = 17)	(n = 68)	(n = 68)	(n = 68)
OVX + eCT	−64 ± 1	1012 ± 101	13 ± 1	9.1 ± 0.5	3.9 ± 0.1
	(n = 27)	(n = 28)	(n = 60)	(n = 60)	(n = 60)
p by ANOVA	0.40	0.38	0.22	0.60	0.79

RMP, Resting membrane potential; HDT, half-decay time. RMPs and input resistances were determined using a K-gluconate patch-pipette solution; mEPSCs were measured at −70 mV.

Blind whole-cell voltage-clamp recordings were made from SG neurons, as described previously (Yoshimura and Nishi, 1993; Yajiri et al., 1997; Kohno et al., 1999). The patch pipette was filled with a solution having the composition of either (in mM): Cs_2SO_4 110, tetraethylammonium (TEA)-Cl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, Mg-ATP 5, and GDP-β-S 1; or K-gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, and Mg-ATP 5. It had a resistance of 8–15 MΩ. The GDP-β-S and K^+ channel blockers (Cs^+ and TEA) in the former solution were added to inhibit any postsynaptic effect of 5-HT resulting from the activation of G-proteins and to block activation of K^+ channels by a postsynaptic effect, respectively. Signals were acquired with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Data were low-pass filtered at 5 kHz, digitized at 333 kHz with an analog-to-digital converter, stored, and analyzed with a personal computer using pCLAMP version 6.0 and AxoGraph version 3.5 (Axon Instruments). Input resistance was determined in a potential range of −90 to −50 mV. The holding potential (V_H) used was −70 mV at which glycine- and GABA-mediated synaptic currents were invisible (Yoshimura and Nishi, 1993). Stimuli (duration, 100 μsec) to elicit EPSCs were given to the dorsal root at a frequency of 0.2 Hz via a suction electrode; the intensities used were 1.2–1.5 times the threshold required to elicit an EPSC in the most excitable Aδ- or C-afferent fibers. Aδ-fiber- or C-fiber-evoked EPSCs (eEPSCs) were distinguished on the basis of the conduction velocity of afferent fibers (C, <0.8 m/sec; Aδ, 2–8 m/sec) and stimulus threshold (C, 160–420 μA; Aδ, 10–40 μA), as described previously (Nakatsuka et al., 1999). The Aδ or C responses, respectively, were considered as monosynaptic in origin when the latency remained constant during stimulation at 20 Hz (Nakatsuka et al., 1999) or when failures did not occur during stimulation at 1 Hz; these criteria were based on intracellular recordings of antidromic action potentials from dorsal root ganglion neurons (Nakatsuka et al., 2000). Neurons from which recordings were made were identified as SG neurons under a binocular microscope in which the SG could be easily distinguished as a colorless band located in the superficial dorsal horn (Yajiri et al., 1997). Because the border between laminae I and II and also that between laminae II and III were not determined with certainty, the patch electrode was inserted at the center of SG under visual control.

The drugs used were 5-HT creatine sulfate (Sigma, St. Louis, MO), 8-OH-DPAT (Research Biochemicals, Natick, MA), WAY100635 (synthesized at Asahi Chemical Industry Co.), tetrodotoxin (TTX) (Wako, Osaka, Japan), and CNQX (Tocris Cookson, St. Louis, MO); they were applied by superfusion in which a change in solution in the recording chamber was completed within 20 sec.

[^{3}H]8-OH-DPAT binding assay. Rats were killed by decapitation, and the lumbar spinal cord and all of the hippocampus were rapidly removed. The respective tissues from two or three rats were pooled, homogenized in 10 vol of ice-cold 10% sucrose, and centrifuged at 1000 × g for 10 min. The supernatant was removed and further centrifuged at 31,000 × g for 20 min. The pellet was homogenized in 10 vol of ice-cold 50 mM Tris-HCl, pH 7.4, and centrifuged at 31,000 × g for 20 min. The pellet was homogenized in the same buffer and incubated at 37°C for 10 min to remove endogenous 5-HT. The suspension was then centrifuged as above, and the final pellet was resuspended in the same buffer and stored at −80°C until use.

The membrane suspensions were melted rapidly and were added to ice-cold binding buffer (50 mM Tris-HCl, pH 7.4, 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbic acid). The mixtures were centrifuged at 40,000 × g for 15 min, and the pellet was suspended in the binding buffer. Membrane solutions including the human 5-HT_{1A} receptor or the rat 5-HT_7 receptor were purchased from BioSignal (Montreal, Canada). Each of the membrane solutions was incubated in duplicate or triplicate with 10 nM [^{3}H]8-OH-DPAT (NEN, Boston, MA) at 30°C for 30 min (0.5 ml of total volume per tube). Nonspecific binding was defined with 10 μM unlabeled 5-HT. The binding reaction was terminated by rapid filtration under vacuum through 0.3% polyethyleneimine presoaked GF/C filters. The filters were washed three times with 3 ml of the binding buffer. Radioactivity was measured with a liquid scintillation counter (TRI-CARB 2300TR; Packard, Meriden, CT). Protein concentration was determined by the DC protein assay kit (Lowly's method; Bio-Rad, Tokyo, Japan). Displacement curves were analyzed by using a nonlinear regression analysis program, LIGAND (Munson and Rodbard, 1980).

Statistical analysis. All results are presented as mean ± SEM. Statistical significance was determined as $p < 0.05$ using t test (unless otherwise noted), Kolmogorov–Smirnov test, or ANOVA followed by Scheffe's test. In all cases, n refers to the number of neurons studied.

RESULTS

Data presented in this study were obtained from SG neurons of Sham, OVX, and OVX + eCT rats (n = 109, 119, and 88, respectively). Whole-cell patch-clamp recordings could be obtained from slices maintained *in vitro* for >12 hr, and stable recordings were made from individual SG neurons for up to 4 hr. As shown in Table 1, resting membrane potential and input resistance did not differ between SG neurons in the three groups.

Postsynaptic effects of 5-HT

When examined using a K-gluconate patch-pipette solution, 40 μM 5-HT superfused for 30 sec induced in SG neurons of Sham rats either outward or inward currents with peak amplitudes of 19.0 ± 3.1 (n = 13) and 12.6 ± 2.2 (n = 9) pA, respectively, at −70 mV (data not shown). SG neurons of OVX rats exhibited similar responses; the amplitudes of outward and inward currents were, respectively, 20.3 ± 4.8 (n = 16) and 13.5 ± 2.1 (n = 4) pA, values not significantly different from those of Sham rats (each $p > 0.05$). These slow 5-HT currents were not examined further because of this lack of difference.

5-HT receptors of various subtypes (Boess and Martin, 1994) are expressed within the spinal cord (Hamon et al., 1989; Marlier et al., 1991); in particular, binding sites for 8-OH-DPAT, an agonist specific to 5-HT_{1A} and 5-HT_7 receptors (Boess and Martin, 1994), are localized in the superficial dorsal horn (laminae I and II) (Marlier et al., 1991). Furthermore, 8-OH-DPAT applied to the spinal cord is known to modulate nociceptive transmission (Xu et al., 1994; Gjerstad et al., 1996). When examined in SG neurons, 10 μM 8-OH-DPAT evoked an outward but not an inward current (n = 4), which was completely blocked by the selective 5-HT_{1A} antagonist WAY100635 (10 μM; n = 4) (according to binding studies, this drug has a dissociation constant for 5-HT_{1A} of 0.8 nM, a value less than that for 5-HT_7 by >74-fold or those for other subtypes of 5-HT_1, 5-HT_{2A}, and 5-HT_3 by >500-fold; see Forster et al., 1995, and Gozlan et al., 1995), suggesting the expression of 5-HT_{1A} receptors in postsynaptic neurons. These postsynaptic responses had disappeared later than 5 min after the establishment of the whole-cell configuration with a patch-pipette solution containing GDP-β-S, Cs^+, and TEA, suggesting the involvement of G-protein-coupled K^+ channels. Subsequent results were obtained after this time when there were no postsynaptic currents induced by 5-HT.

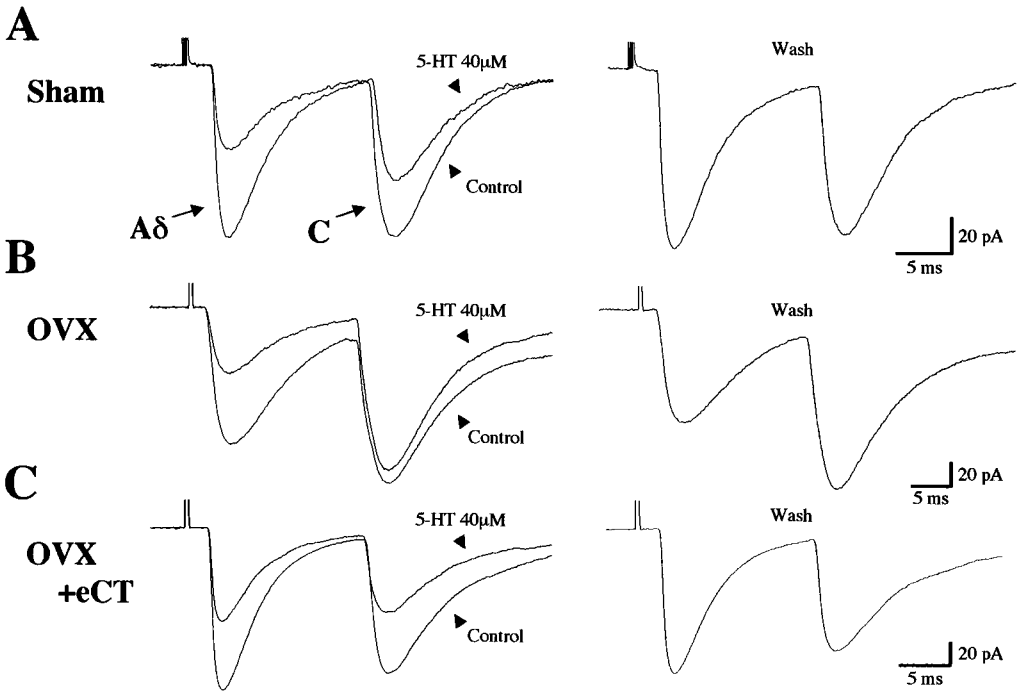

Figure 1. Effects of 5-HT on eEPSCs in SG neurons. *A–C*, The effects of 5-HT (40 μM) superfused for 1 min in Sham, OVX, and OVX + eCT rats, respectively. The stimulus intensities used were 210 (*A*), 210 (*B*), and 270 μA (*C*), values large enough to activate both Aδ- and C-fibers. Averages of 10 and 5 eEPSCs, respectively, in control solution and after 1 min in 5-HT are superimposed on the *left*. Shown on the *right* are averages of 10 eEPSCs at 4 min after washout of 5-HT. Note that 5-HT inhibited both Aδ- and C-derived eEPSCs in the Sham rat but depressed only Aδ-fiber eEPSCs in the OVX rat; results for the OVX + eCT rat were similar to the Sham rat. V_H of −70 mV.

Effects of 5-HT on primary afferent-evoked EPSCs

We tested whether 5-HT affects responses evoked in SG neurons by dorsal root stimulation and whether its effect on evoked release differs between the Sham, OVX, and OVX + eCT groups. In 73% of SG neurons tested, stimulating the dorsal root elicited monosynaptic EPSCs that were caused by the activation of Aδ- and/or C-afferent fibers (see Materials and Methods); these were blocked by the non-NMDA receptor antagonist CNQX (10 μM), as reported previously (Yoshimura and Jessell, 1990; Nakatsuka et al., 1999; Yang et al., 1999). Figure 1 demonstrates the effects of superfusing 40 μM 5-HT for 1 min on Aδ- and C-fiber eEPSCs. In Sham rats, both eEPSCs were reversibly reduced in amplitude by 5-HT [to 61 ± 8 (n = 12) and 61 ± 9% (n = 11), respectively, of control for Aδ- and C-fiber eEPSCs] (Fig. 1*A*). In OVX rats, on the other hand, Aδ-fiber eEPSCs were inhibited by 5-HT to 64 ± 5% (n = 22) of control, as in the Sham group, whereas C-fiber eEPSCs were unaffected (Fig. 1*B*). This difference did not occur after treatment of OVX rats with eCT. In the latter case, 5-HT depressed both eEPSCs, as in the Sham group (Fig. 1*C*). These effects are summarized in Figure 2. The degree of inhibition of the Aδ-fiber eEPSCs by 5-HT was almost the same in all three groups (Fig. 2, *left panel*), whereas inhibition of C-fiber eEPSCs was significantly less in the OVX group than in the Sham and OVX + eCT groups ($p < 0.0001$, ANOVA; Sham vs OVX, $p = 0.0003$; OVX vs OVX + eCT, $p = 0.0012$, Scheffe's test) (Fig. 2, *right panel*).

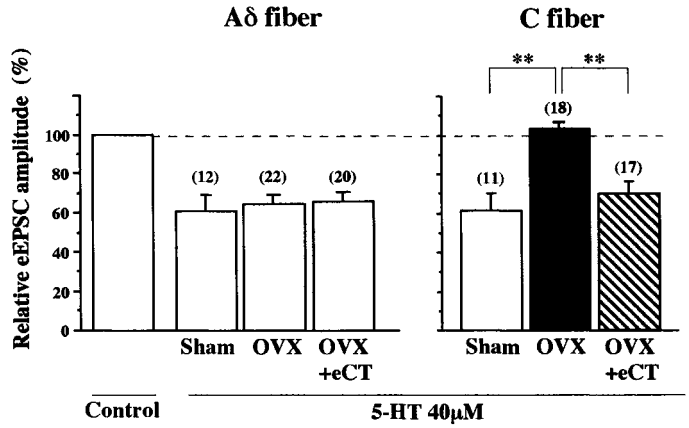

Figure 2. Relative peak amplitude of eEPSCs in the presence of 5-HT to that in the control. The *left* and *right panels* exhibit the effects (expressed in percentage) of 5-HT (40 μM) on Aδ- and C-fiber eEPSCs, respectively. Their amplitudes in control solution and after 1 min in 5-HT were determined from averages of 10 and 5 eEPSCs, respectively. The relative Aδ-fiber eEPSC amplitudes were similar in Sham, OVX, and OVX + eCT rats. In contrast, the relative C-fiber eEPSC amplitude was higher in OVX rats than in the other two groups (**$p < 0.0001$). The *number* of neurons examined is shown in *parentheses*.

Effects of 5-HT on miniature EPSCs

In the presence of 0.5 μM TTX, all SG neurons examined exhibited miniature EPSCs (mEPSCs), which were completely blocked by CNQX (10 μM). The frequency, amplitude, and half-decay time of mEPSCs did not differ in magnitude between SG neurons in the three groups (Table 1).

In ~50% of SG neurons (n = 64) examined of Sham rats, superfusion with 40 μM 5-HT for 1 min produced inhibition followed by facilitation of mEPSCs (Fig. 3*A*). The action of 5-HT was analyzed over two periods of 1 min starting 0.5 and 2.5 min after the onset of 5-HT application (termed 1st and 2nd phase, respectively). Figure 3, *B* and *C*, demonstrates the cumulative distributions of the interevent interval and amplitude of mEPSCs, respectively. The frequency of mEPSCs in the 1st and 2nd phases was decreased and increased, respectively, by 5-HT (Fig. 3*B*), whereas the cumulative probability of mEPSC amplitude was unaltered (Fig. 3*C*), suggesting a presynaptic action. Furthermore, the kinetics of non-NMDA receptor channel appeared unaffected judging from the lack of difference in the decay phases of mEPSCs during the 1st and 2nd phases relative to control (Fig. 3*D*). The effect of 5-HT on mEPSC frequency was variable from neuron to neuron; this frequency was either reduced or unchanged during the 1st

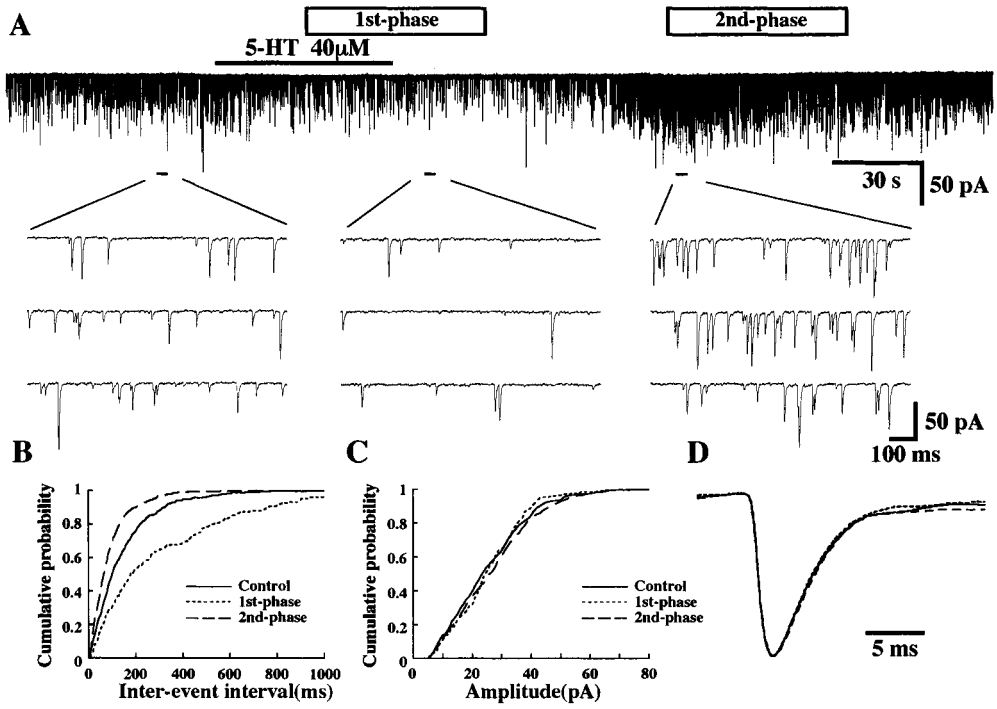

Figure 3. Effects of 5-HT on mEPSCs in SG neurons of Sham rats. *A*, Continuous chart recording of mEPSCs in control solution and under the action of 5-HT (40 μM) superfused for 1 min (*top record*). The periods of time indicated by *two open column bars* (each of which has a duration of 1 min) were defined as 1st and 2nd phase (which started 0.5 and 2.5 min after the beginning of 5-HT perfusion, respectively). *Three bottom records*, Consecutive traces of mEPSCs in the control (*left*), 1st phase (*middle*), and 2nd phase (*right*) for a period indicated by a *bar* shown *below* the chart recording, which are shown in an expanded scale in time. mEPSC facilitation after 5-HT perfusion lasted for >150 sec (only part is shown). *B*, *C*, Cumulative probabilities of the interevent interval (*B*) and amplitude of mEPSCs (*C*), in the control (*continuous line*), 1st phase (*dotted line*), and 2nd phase (*dashed line*), which were, respectively, obtained by analyzing 414, 191, and 670 mEPSC events, each of which occurred for 1 min. The difference in interevent interval was significant between the control and 1st phase and also between the control and 2nd phase (each $p < 0.0001$, Kolmogorov–Smirnov test); on the contrary, there was no difference in amplitude between the groups. *D*, Averaged mEPSCs in the 1st and 2nd phase (*dotted* and *dashed lines*, respectively), which were normalized in amplitude to that in the control (*continuous line*). Data in *A–D* were obtained from the same neuron; V_H of −70 mV.

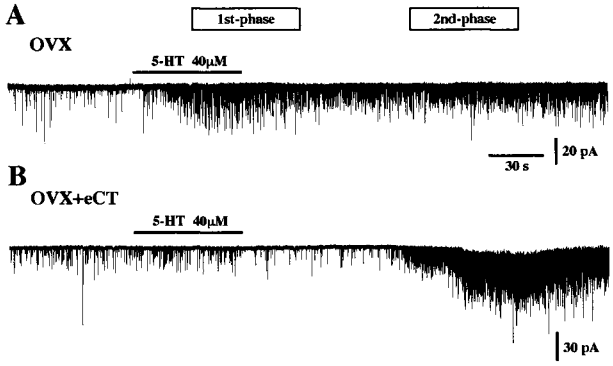

Figure 4. Effects of 5-HT on mEPSCs in SG neurons of OVX and OVX + eCT rats. *A*, *B*, Continuous chart recordings of mEPSCs in the OVX and OVX + eCT rat, respectively, in which 5-HT (40 μM) was superfused for 1 min. Note that the mEPSC inhibition in the 1st phase seen in Figure 3*A* was absent in *A* but was present in *B*; mEPSC facilitation in the 2nd phase was seen in both *A* and *B*. V_H of −70 mV.

phase (see Fig. 5*A*, *top panel*) but was facilitated in the 2nd phase (see Fig. 5*B*, *bottom panel*). These effects were the same in the absence of 0.5 μM TTX ($n = 3$), supporting the idea that 5-HT modulates transmitter release without any involvement of spontaneously active interneurons. Together, these results indicate that 5-HT initially inhibits and then facilitates the release of quanta, as reported previously for dorsal horn neurons in spinal cord slices of neonatal rats (Hori et al., 1996). The variability in the actions of 5-HT on the frequency of mEPSC may be attributable to the fact that the SG contains different classes of neurons (for review, see Willis and Coggeshall, 1991) and that mEPSCs originate from terminals of not only primary afferent fibers but also of interneurons innervating SG neurons.

In slices from OVX rats, ~60% of SG neurons ($n = 68$) examined responded to 5-HT by facilitation of mEPSCs with no preceding inhibition (Fig. 4*A*). This result indicates that the reduction in mEPSC frequency observed in Sham rats is eliminated after ovariectomy. This change in the action of 5-HT observed in OVX rats did not occur after chronic treatment of OVX rats with eCT (Fig. 4*B*), so that the action of 5-HT was restored to that observed in Sham rats by the eCT treatment. In both OVX and OVX + eCT rats, as in Sham rats, the actions of 5-HT were attributable to changes in the frequency but not the amplitude of mEPSCs. Figure 5*A* summarizes the 5-HT-induced changes in frequency in the 1st phase in the three groups. The frequency of mEPSC was inhibited by >25% in 12 of 64 cells in the Sham group (Fig. 5*A*, *top panel*). In the OVX group, however, the frequency of mEPSC was facilitated by >35% in 21 of 68 cells; the remaining cells did not exhibit a change (of >35%) in the frequency (Fig. 5*A*, *middle panel*). In slices from the OVX + eCT rats, 7 of 60 cells exhibited inhibition (of >25%) in the 1st phase (Fig. 5*A*, *bottom panel*), which was similar to the result in Sham rats. The percentage of cells showing inhibition of >25% was similar between the Sham (19%) and OVX + eCT groups (12%); these values were quite distinct from that (0%) in the OVX group. Furthermore, the average of the relative mEPSC frequency in the 1st phase to that in the control was significantly greater in the OVX than in the other two groups ($p < 0.0001$, ANOVA; $p < 0.0001$ for each of Sham vs OVX and OVX vs OVX + eCT, Scheffe's test) (Fig. 5*B*, *top panel*), whereas that in the 2nd phase did not differ between the three groups ($p > 0.05$, ANOVA) (Fig. 5*B*, *bottom panel*) Thus, the increase in mEPSC frequency after ovariectomy was hardly present in the rats that had been treated with eCT, whereas their facilitation during the 2nd phase was similar in all groups. This implies that the facilitation was not simply an effect of ovariectomy. It seems that facilitation of mEPSC frequency normally occurs at the same time as inhibition of mEPSC frequency during the 1st phase and that ovariectomy is followed by downregulation of the inhibitory action unveiling facilitation during the 1st phase.

When examined in a neuron in which mEPSC frequency was increased by 5-HT, primary afferent eEPSCs were never enhanced in amplitude by 5-HT ($n = 20$). Therefore, the facilitation of mEPSC frequency may be explained by 5-HT acting on the terminals of interneurons innervating SG neurons but not on terminals of primary afferent fibers. Alternatively, it is likely that spontaneous and evoked release are affected differently by 5-HT, because each of the releases in the SG is suggested to be mediated by different types of Ca^{2+} channels (Bao et al., 1998).

With respect to primary afferent eEPSCs, the inhibition of C-fiber but not Aδ-fiber eEPSCs by 5-HT was mimicked by 10 μM 8-OH-DPAT (amplitude, 51.4 ± 23.5% of control; $n = 3$), and this

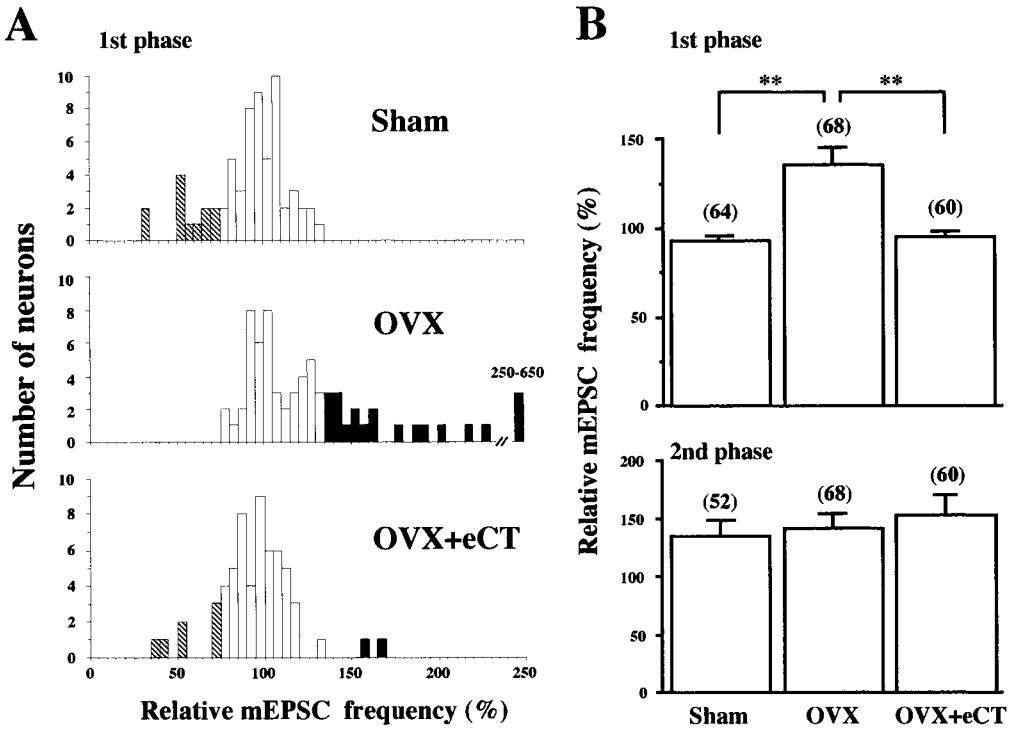

Figure 5. Effects of 5-HT on mEPSC frequency in SG neurons of Sham, OVX, and OVX + eCT rats. *A*, Histograms of the numbers of neurons that were plotted against the frequency of mEPSC in the 1st phase relative to that in the control (100%) in which 5-HT (40 μM) was superfused for 1 min; they were obtained in Sham (*top*), OVX (*middle*), and OVX + eCT (*bottom*) rats. *Hatched* and *closed bars* show the numbers of neurons exhibiting a decrease (of >25%) and increase (of >35%) in mEPSC frequency, respectively. *B*, Average frequencies of mEPSCs in the 1st (*top*) and 2nd (*bottom*) phase relative to that in the control (100%) in Sham, OVX, and OVX + eCT rats. The *number* of neurons examined is shown in *parentheses*. There was a significant facilitation of mEPSC frequency by 5-HT in the 1st phase in OVX but not in Sham rats; this enhancement was not seen after treatment with eCT (**$p < 0.0001$). There was no difference in the increase in mEPSC frequency in the 2nd phase between Sham, OVX, and OVX + eCT rats.

inhibitory action was not blocked by 10 μM WAY100635 [when examined in the same neuron ($n = 2$), 22.7, 22.0% and 22.0, 15.9% of control in the presence and absence of the antagonist, respectively], indicating that the 8-OH-DPAT action is mediated by a 5-HT receptor that is unlikely to be sensitive to WAY100635. The pharmacology of 8-OH-DPAT binding was investigated further by measuring the expression of 5-HT receptors, especially in the spinal cord.

Density of [^{3}H]8-OH-DPAT binding sites

The *curves* in Figure 6*A* demonstrate the displacement by WAY100635 of 10 nM [^{3}H]8-OH-DPAT binding to membranes prepared from spinal cord and from hippocampus in Sham rats, and also to membranes of Chinese hamster ovary (CHO) cells expressing the human 5-HT$_{1A}$ receptor and of Sf9 cells expressing the rat 5-HT$_7$ receptor. [^{3}H]8-OH-DPAT binding to spinal cord membranes was partially inhibited by WAY100635, and this inhibition was not completed, even at a high concentration (10 μM); the dose dependency was not monophasic (Fig. 6*A*). On the other hand, WAY100635 reduced [^{3}H]8-OH-DPAT binding to hippocampal membranes in a monophasic manner, and this was completed at 1 μM. In addition, [^{3}H]8-OH-DPAT bindings to the cloned 5-HT$_{1A}$ and 5-HT$_7$ receptors were reduced in a monophasic manner by WAY100635, and these were completed at 10 μM. Thus, the bind-

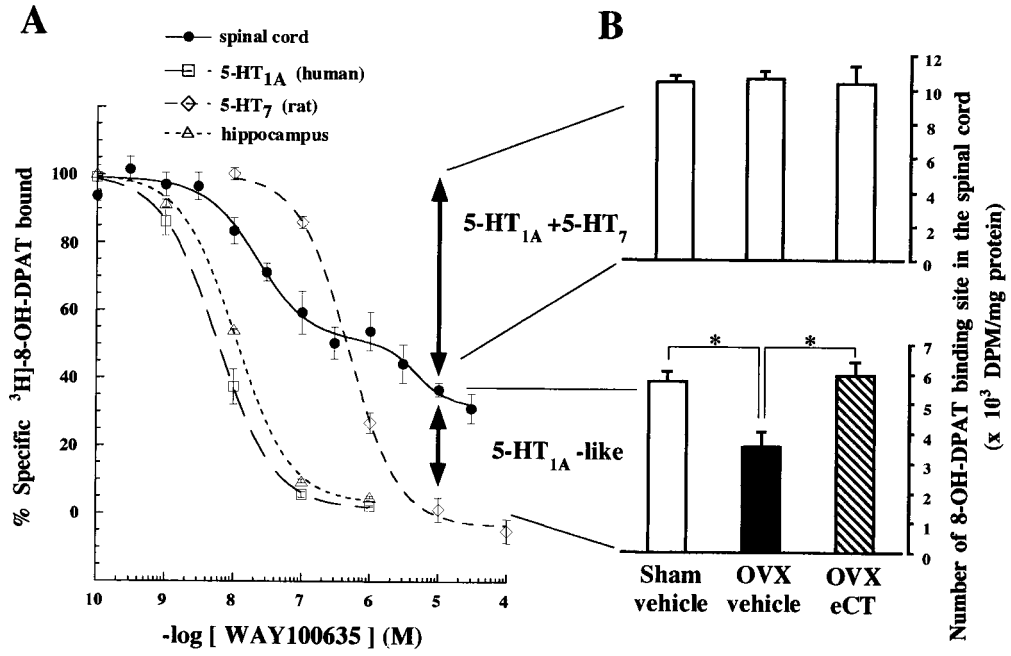

Figure 6. Analysis of [^{3}H]8-OH-DPAT binding in various systems expressing 5-HT receptors. *A*, Inhibition of [^{3}H]8-OH-DPAT binding by WAY100635. [^{3}H]8-OH-DPAT binding to spinal cord (●), hippocampal membranes (△), membranes of CHO cells expressing the human 5-HT$_{1A}$ receptor (□), and membranes of Sf9 cells expressing the rat 5-HT$_7$ receptor (◇) relative to that in the control (100%) is plotted against the logarithm of WAY100635 concentration. Note that 10 μM WAY100635 completely blocked [^{3}H]8-OH-DPAT binding to either 5-HT receptors in hippocampal membranes, the cloned 5-HT$_{1A}$ or 5-HT$_7$ receptor, but was unable to inhibit ~37% of the binding in spinal cord membranes, the component of which was named as 5-HT$_{1A}$-like. *B*, Number of either the 5-HT$_{1A}$ plus 5-HT$_7$ receptor (*top*) or 5-HT$_{1A}$-like receptor (*bottom*) in spinal cord membranes obtained from Sham, OVX, and OVX + eCT rats, estimated as [^{3}H]8-OH-DPAT binding sites. Note that either the ovariectomy or eCT treatment significantly changed the density of 5-HT$_{1A}$-like receptor (*$p < 0.05$) without affecting the density of 5-HT$_{1A}$ plus 5-HT$_7$ receptors ($n = 4$). Three rats were pooled to make one spinal or hippocampal membrane; each assay was performed in duplicate. Data from the spinal cord were obtained from four samples of membranes, whereas the others were obtained from three assays.

ing properties of spinal cord membranes were quite different from those of cloned 5-HT_{1A} and 5-HT_7 receptors (expressed on CHO and Sf9 cells, respectively), whereas binding to hippocampal membranes resembled that of the 5-HT_{1A} receptors. These results indicate that spinal cord membranes may be endowed not only with 5-HT_{1A} and 5-HT_7 receptors (the former of which appears to be expressed in postsynaptic SG neurons, as described above) but also with 5-HT_{1A}-like receptors that bind 8-OH-DPAT in a manner insensitive to WAY100635.

We examined whether specific binding of 10 nM [^{3}H]8-OH-DPAT to spinal cord and to hippocampal membranes is quantitatively changed after the development of hyperalgesia in OVX rats and its relief by eCT administration. The presence or absence of hyperalgesia was confirmed by the tail-withdrawal test, as described previously (Shibata et al., 1998). The density of 5-HT_{1A}-like receptors was estimated from the binding of 10 nM [^{3}H]8-OH-DPAT in the presence of 10 μM WAY100635 (when both 5-HT_{1A} and 5-HT_7 receptors would have been blocked). This 5-HT_{1A}-like receptor density was significantly lower in the OVX group than in the Sham or the OVX + eCT groups ($p < 0.014$, ANOVA; $p = 0.031$ for Sham vs OVX; $p = 0.028$ for OVX vs OVX + eCT, Scheffe's test) (Fig. 6*B*, *bottom panel*). On the other hand, the density of 5-HT_{1A} plus 5-HT_7 receptors, which was calculated by subtracting the density of 5-HT_{1A}-like receptor from the total specific activity of [^{3}H]8-OH-DPAT binding sites, was not altered by either the ovariectomy or the treatment with eCT ($p > 0.05$, ANOVA) (Fig. 6*B*, *top panel*). Consistent with this observation, the specific binding of [^{3}H]8-OH-DPAT to hippocampal membranes from Sham, OVX, and OVX + eCT rats was 93172 ± 1937, 96529 ± 3990, and 99190 ± 5175 dpm/mg protein ($n = 6$), respectively; these values were not different from each other ($p > 0.05$, ANOVA).

DISCUSSION

The present study demonstrates that SG neurons in OVX rats lack a presynaptic 5-HT-induced inhibition of excitatory glutamatergic transmission evoked monosynaptically in SG neurons by stimulating C-afferent fibers, which is restored after chronic treatment with eCT; there is no such effect after Aδ-afferent fiber stimulation. A similar loss of presynaptic inhibition by 5-HT was observed for spontaneous excitatory transmitter release. Inhibition of C-afferent eEPSCs was mimicked by 8-OH-DPAT but was not inhibited by WAY100635. This action of 8-OH-DPAT on C-fiber terminals is consistent with the observation using autoradiography that the number of [^{3}H]8-OH-DPAT binding sites in the rat spinal cord was decreased by 20–30% after either neonatal capsaicin treatment or dorsal rhizotomy, which is known to eliminate C-fibers (Daval et al., 1987). Furthermore, we revealed from binding studies that the binding of [^{3}H]8-OH-DPAT to spinal cord membranes, which is resistant to WAY100635, is reduced in OVX rats; this effect disappears when OVX rats are chronically treated with eCT. We showed previously that OVX rats exhibit hyperalgesia, which is alleviated by eCT administration (Shibata et al., 1998). Furthermore, we suggested in electrophysiological studies using rats with peripheral inflammation that changes in sensory inputs to SG neurons play a critical role in the development of hyperalgesia (Nakatsuka et al., 1999) (see also Baba et al., 1999). Altogether, the present results indicate that the hyperalgesia and eCT-induced antinociception may be attributed to changes in the number of 5-HT receptors involved in inhibition of the release of L-glutamate from primary afferent C-fiber terminals in the SG. This 5-HT receptor, by its resistance to WAY100635, appears to be a 5-HT_{1A}-like receptor that is neither the 5-HT_{1A}- nor the 5-HT_7-subtype. This idea appears consistent with the observation from studies using PCR that there are no mRNAs for the 5-HT_{1A} receptor in rat dorsal root ganglion neurons (Pierce et al., 1996; Chen et al., 1998). Although mRNAs for the 5-HT_7 receptor exist there, it is unlikely that this receptor is involved in the 5-HT-induced inhibition of transmitter release, because the 5-HT_7 receptor is positively coupled to adenylate cyclase, thus potentiating transmitter release (Boess and Martin, 1994). The change in the number of 5-HT receptors demonstrated here could underlie the analgesic effects of eCT in osteoporotic pain in humans.

Although a cellular mechanism for the alteration of 5-HT_{1A}-like receptor expression in C-afferent terminals has not been examined here, it is possible that this is regulated by glucocorticoids. It is well established that steroid hormones, such as glucocorticoids, regulate the expression of various genes by forming a complex with their receptors, followed by binding to a particular sequence in the promoter region of genes (Beato, 1989). Shimizu et al. (1996) have demonstrated that ovariectomy in rats results in a reduction in corticosterone levels in serum for 3 weeks. A decrease of glucocorticoid levels in OVX rats would be expected to reduce the amount of gene products, including 5-HT_{1A}-like receptors. Because it is known in humans that a single peripheral injection of calcitonin causes a rise in ACTH and subsequently cortisol levels in plasma for >2 hr (Laurian et al., 1986), it may be that repetitive treatment of OVX rats with eCT results in a recovery of glucocorticoid levels, leading to a resumption of synthesis of 5-HT_{1A}-like receptors. This hypothesis remains to be verified. In any event, calcitonin may be an exceptional analgesic that acts by altering the density of 5-HT_{1A}-like receptors in C-fiber terminals innervating SG neurons. Considering that the C-fibers convey predominantly diffuse and long-lasting pain sensations, the present results indicate that 5-HT_{1A}-like receptors may play an important role in controlling pain; identification of the 5-HT_{1A}-like receptor may accelerate the development of drugs that potentially affect nociceptive transmission.

REFERENCES

Baba H, Doubell TP, Woolf CJ (1999) Peripheral inflammation facilitates Aβ fiber-mediated synaptic input to the substantia gelatinosa of the adult rat spinal cord. J Neurosci 19:859–867.

Bao J, Li JJ, Perl ER (1998) Differences in Ca^{2+} channels governing generation of miniature and evoked excitatory synaptic currents in spinal laminae I and II. J Neurosci 18:8740–8750.

Beato M (1989) Gene regulation by steroid hormones. Cell 56:335–344.

Boess FG, Martin IL (1994) Molecular biology of 5-HT receptors. Neuropharmacology 33:275–317.

Brown AG (1982) The dorsal horn of the spinal cord. Q J Exp Physiol 67:193–212.

Cervero F, Iggo A (1980) The substantia gelatinosa of the spinal cord: a critical review. Brain 103:717–772.

Chen JJ, Vasko MR, Wu X, Staeva TP, Baez M, Zgombick JM, Nelson DL (1998) Multiple subtypes of serotonin receptors are expressed in rat sensory neurons in culture. J Pharmacol Exp Ther 287:1119–1127.

Chesnut III CH, Baylink DJ, Roos BA, Gruber HE, Ivey JL, Matthews M, Nelp WB, Sisom K (1981) Calcitonin and postmenopausal osteoporosis. In: Calcitonin 1980 (Pecile A, ed), pp 247–255. Amsterdam: Excerpta Medica.

Clementi G, Prato A, Conforto G, Scapagnini U (1984) Role of serotonin in the analgesic activity of calcitonin. Eur J Pharmacol 98:449–451.

Daval G, Vergé D, Basbaum AI, Bourgoin S, Hamon M (1987) Autoradiographic evidence of serotonin$_1$ binding sites on primary afferent fibres in the dorsal horn of the rat spinal cord. Neurosci Lett 83:71–76.

Fitzgerald M (1981) A study of the cutaneous afferent input to substantia gelatinosa. Neuroscience 6:2229–2237.

Forster EA, Cliffe IA, Bill DJ, Dover GM, Jones D, Reilly Y, Fletcher A (1995) A pharmacological profile of the selective silent 5-HT_{1A} receptor antagonist, WAY- 100635. Eur J Pharmacol 281:81–88.

Franceschini R, Bottaro P, Panopoulos C, Messina V (1983) Long-term treatment with salmon calcitonin in postmenopausal osteoporosis. Curr Ther Res 34:795–800.

Gennari C, Agnusdei D (1988) Calcitonin in bone pain management. Curr Ther Res 44:712–722.

Gjerstad J, Tjϕlsen A, Hole K (1996) The effect of 5-HT_{1A} receptor stimulation on nociceptive dorsal horn neurones in rats. Eur J Pharmacol 318:315–321.

Gozlan H, Thibault S, Laporte A-M, Lima L, Hamon M (1995) The selective 5-HT_{1A} antagonist radioligand [^{3}H]WAY 100635 labels both G-protein-coupled and free 5- HT_{1A} receptors in rat brain membranes. Eur J Pharmacol 288:173–186.

Gruber HE, Ivey JL, Baylink DJ, Matthews M, Nelp WB, Sisom K, Chesnut III CH (1984) Long-term calcitonin therapy in postmenopausal osteoporosis. Metabolism 33:295–303.

Guidobono F, Netti C, Sibilia V, Olgiati VR, Pecile A (1985) Role of catecholamines in calcitonin-induced analgesia. Pharmacology 31: 342–348.

Guidobono F, Netti C, Pagani F, Sibilia V, Pecile A (1986) Relationship of analgesia induced by centrally injected calcitonin to the CNS serotonergic system. Neuropeptides 8:259–271.

Hamon M, Gallissot MC, Menard F, Gozlan H, Bourgoin S, Vergé D

(1989) 5-HT_3 receptor binding sites are on capsaicin-sensitive fibres in the rat spinal cord. Eur J Pharmacol 164:315–322.
Hori Y, Endo K, Takahashi T (1996) Long-lasting synaptic facilitation induced by serotonin in superficial dorsal horn neurones of the rat spinal cord. J Physiol (Lond) 492:867–876.
Kohno T, Kumamoto E, Higashi H, Shimoji K, Yoshimura M (1999) Actions of opioids on excitatory and inhibitory transmission in substantia gelatinosa of adult rat spinal cord. J Physiol (Lond) 518:803–813.
Kumazawa T, Perl ER (1978) Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: indications of their place in dorsal horn functional organization. J Comp Neurol 177:417–434.
Laurian L, Oberman Z, Graf E, Gilad S, Hoerer E, Simantov R (1986) Calcitonin induced increase in ACTH, β-endorphin and cortisol secretion. Horm Metab Res 18: 268–271.
Light AR, Perl ER (1979) Reexamination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. J Comp Neurol 186:117–131.
Light AR, Trevino DL, Perl ER (1979) Morphological features of functionally defined neurons in the marginal zone and substantia gelatinosa of the spinal dorsal horn. J Comp Neurol 186:151–171.
Maeda Y, Yamada K, Hasegawa T, Nabeshima T (1995) Neuronal mechanism of the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior. Eur J Pharmacol 275:163–170.
Marlier L, Teilhac J-R, Cerruti C, Privat A (1991) Autoradiographic mapping of 5-HT_1, 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors in the rat spinal cord. Brain Res 550:15–23.
Munson PL (1976) Physiology and pharmacology of thyrocalcitonin. In: Handbook of physiology, Vol 7 (Aurbach GD, ed), pp 443–464. Washington, DC: American Physiological Society.
Munson PJ, Rodbard D (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239.
Nakatsuka T, Park J-S, Kumamoto E, Tamaki T, Yoshimura M (1999) Plastic changes in sensory inputs to rat substantia gelatinosa neurons following peripheral inflammation. Pain 82:39–47.
Nakatsuka T, Ataka T, Kumamoto E, Tamaki T, Yoshimura M (2000) Alteration in synaptic inputs through C afferent fibers to substantia gelatinosa neurons of the rat spinal dorsal horn during postnatal development. Neuroscience, in press.
Orimo H, Morii H, Inoue T, Yamamoto K, Minaguchi H, Ishii Y, Murota K, Fujimaki E, Watanabe R, Harata S, Honjo H, Fujita T (1996) Effect of elcatonin on involutional osteoporosis. J Bone Miner Metab 14:73–78.
Pecile A, Ferri S, Braga PC, Olgiati VR (1975) Effects of intracerebroventricular calcitonin in the conscious rabbit. Experientia 31:332–333.
Pierce PA, Xie G-X, Levine JD, Peroutka SJ (1996) 5-Hydroxytryptamine receptor subtype messenger RNAs in rat peripheral sensory and sympathetic ganglia: a polymerase chain reaction study. Neuroscience 70:553–559.
Pontiroli AE, Pajetta E, Calderara A, Alberetto M, Pozza G, Manganelli V, Resmini G, Tessari L, Maresca V (1991) Intranasal and intramuscular human calcitonin in female osteoporosis and in Paget's disease of bones: a pilot study. J Endocrinol Invest 14:47–51.
Potts Jr JT, Aurbach GD (1976) Chemistry of the calcitonins. In: Handbook of physiology, Vol 7 (Aurbach GD, ed), pp 423–430. Washington, DC: American Physiological Society.
Shibata K, Takeda M, Ito A, Takeda M, Sagai H (1998) Ovariectomy-induced hyperalgesia and antinociceptive effect of elcatonin, a synthetic eel calcitonin. Pharmacol Biochem Behav 60:371–376.
Shimizu H, Ohtani K, Kato Y, Tanaka Y, Mori M (1996) Withdrawal of estrogen increases hypothalamic neuropeptide Y (NPY) mRNA expression in ovariectomized obese rat. Neurosci Lett [Erratum (1997) 227:143] 204:81–84.
Spampinato S, Candeletti S, Cavicchini E, Romualdi P, Speroni E, Ferri S (1984) Antinociceptive activity of salmon calcitonin injected intrathecally in the rat. Neurosci Lett 45:135–139.
Sugiura Y, Lee CL, Perl ER (1986) Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. Science 234:358–361.
Sugiura Y, Terui N, Hosoya Y (1989) Difference in distribution of central terminals between visceral and somatic unmyelinated (C) primary afferent fibers. J Neurophysiol 62:834–840.
Umeno H, Nagasawa T, Yamazaki N, Kuraishi Y (1996) Antinociceptive effects of repeated systemic injections of calcitonin in formalin-induced hyperalgesic rats. Pharmacol Biochem Behav 55:151–156.
Willis Jr WD, Coggeshall RE (1991) Sensory mechanisms of the spinal cord. New York: Plenum.
Xu W, Qiu XC, Han JS (1994) Serotonin receptor subtypes in spinal antinociception in the rat. J Pharmacol Exp Ther 269:1182–1189.
Yajiri Y, Yoshimura M, Okamoto M, Takahashi H, Higashi H (1997) A novel slow excitatory postsynaptic current in substantia gelatinosa neurons of the rat spinal cord *in vitro*. Neuroscience 76:673–688.
Yaksh TL (1979) Direct evidence that spinal serotonin and noradrenaline terminals mediate the spinal antinociceptive effects of morphine in the periaqueductal gray. Brain Res 160:180–185.
Yamamoto M, Kumagai F, Tachikawa S, Maeno H (1979) Lack of effect of levallorphan on analgesia induced by intraventricular application of porcine calcitonin in mice. Eur J Pharmacol 55:211–213.
Yang K, Kumamoto E, Furue H, Li Y-Q, Yoshimura M (1999) Action of capsaicin on dorsal root-evoked synaptic transmission to substantia gelatinosa neurons in adult rat spinal cord slices. Brain Res 830:268–273.
Yoshimura M, Jessell TM (1989) Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons *in vitro*. J Neurophysiol 62:96–108.
Yoshimura M, Jessell T (1990) Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. J Physiol (Lond) 430:315–335.
Yoshimura M, Nishi S (1993) Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. Neuroscience 53:519–526.
Zemlan FP, Corrigan SA, Pfaff DW (1980) Noradrenergic and serotonergic mediation of spinal analgesia mechanisms. Eur J Pharmacol 61:111–124.