Modulatory effects of serotonin on GABAergic synaptic transmission and membrane properties in the deep cerebellar nuclei

Running Title: Serotonergic modulation in the DCN

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ABSTRACT

Cerebellar outputs from the deep cerebellar nuclei (DCN) are critical for generating and controlling movement. DCN neuronal activity is primarily controlled by GABAergic inhibitory transmission by Purkinje cells in the cerebellar cortex, and is also modulated by nerve inputs originating from other brain regions within and outside the cerebellum. In this study, we examined the modulatory effects of serotonin (5-HT) on GABAergic synapses in the DCN. 5-HT decreased the amplitude of stimulation-evoked inhibitory postsynaptic currents (eIPSCs) in DCN neurons, and this effect was abolished by a 5-HT\textsubscript{1B} antagonist, SB 224289. The decrease in IPSC amplitude was associated with an increased paired-pulse ratio of the IPSC. 5-HT also decreased the frequency of miniature IPSCs without altering the amplitude. These data suggest that 5-HT presynaptically inhibited GABA release. Furthermore, 5-HT elicited a slow inward current in DCN neurons. Pharmacological studies revealed that 5-HT activated the 5-HT\textsubscript{5} receptor, which is positively coupled to G-protein, and elicited the slow inward current through enhancement of hyperpolarization-activated cation channel activation. Finally, we examined the effects of 5-HT on the spike generation that accompanies repetitive stimulation of inhibitory synapses. 5-HT increased the spontaneous firing rate in DCN neurons due to depolarization. Increase in the 5-HT-induced tonic firing relatively decreased the contrast difference from the rebound depolarization-induced firing. However, the inhibitory transmission-induced silencing of DCN firing remained during the conditioning stimulus. These results suggest that 5-HT plays a regulatory role in spike generation and contributes to the gain control of inhibitory GABAergic synapses in DCN neurons.

Keywords:
Cerebellum; serotonin (5-HT); modulation; synaptic transmission
INTRODUCTION

Several studies have demonstrated that fibers from the cerebellar cortex and nuclei control and fine tune the motor functions through various neural networks (Eccles, 1973; Ito, 2000), including the brain stem, midbrain, and thalamus. Among these neural networks, a large part of the final output from the cerebellum is generated by neurons from the deep cerebellar nuclei (DCN). DCN neurons are primarily controlled via GABA\(_A\)-receptor-mediated inhibitory input from Purkinje cells (PCs) in the cerebellar cortex (Mouginot & Gahwiler, 1995; Teune et al., 1998; Aizenman et al., 1998; Gauck & Jaeger, 2000; Anchisi et al., 2001). PC synapses constitute more than 70% of the total synapses on DCN neurons, and almost all somatic synapses of DCN neurons are inhibitory (De Zeeuw & Berrebi, 1995). Therefore, it has been proposed that PC inhibitory inputs are important for controlling the rate and temporal precision of DCN spiking (Gauck & Jaeger, 2000; Shin et al., 2007). Moreover, both the synaptic modulation and plasticity of PC inhibitory inputs are thought to be important regulatory mechanisms for DCN activity. For instance, in terms of synaptic plasticity in DCN inhibitory synapses, both LTP and LTD can be induced, depending on changes to postsynaptic intracellular Ca\(^{2+}\) concentration (Morishita & Sastry, 1996; Ouardouz & Sastry, 2000). On the contrary, however, few studies have focused on the modulatory effects of monoamines, such as 5-hydroxytryptamine (5-HT, serotonin) and noradrenaline in the DCN (Di Mauro et al., 2003). The study by Di Mauro et al. focused on the modulatory effects of monoamines on the neuronal firing in each deep nucleus. Microiontophoretic application of 5-HT showed a decrease in the firing rate in the medial nucleus and complicated (inhibitory, excitatory, and biphasic) effects on the interpositus and the lateral nuclei. However, receptor subtypes and ionic mechanisms underlying these serotonergic modulations of DCN neurons have not been clarified.

5-HT exerts its actions via 14 classes of receptors (Millan et al., 2008). With the
exception of 5-HT₃ receptors, which are cation-permeable ionotropic receptors, all other 5-HT receptors are of the seven transmembrane-spanning G-protein-coupled types. All of these receptors are present, but differentially distributed, in the central nervous system, including the frontal cortex, hippocampus, amygdala, striatum, hypothalamus, and dorsal horn (Millan, 2006; Bockaert et al., 2006). Through the actions of these multiple subtypes of receptors, 5-HT plays a key role in CNS functions such as mood, cognition, sleep, pain, motor function, and endocrine secretion. The serotonergic fibers are the third largest population of fibers that input to the cerebellum after mossy fibers and climbing fibers, and these fibers innervate and affect all regions of the cerebellar circuitry. Within the cerebellar nuclei, there is a dense uniform plexus of serotonergic fibers (Kerr & Bishop, 1991; Kitzman & Bishop, 1994), and all cerebellar nuclei receive 5-HT afferents from the dorsal raphe nuclei. Although radioligand binding and immunohistochemical studies have revealed the presence of various 5-HT receptor subtypes, including 5-HT₁B, 5-HT₂A, and 5-HT₅A, in the DCN region (Pazos & Palacios, 1985; Sari et al., 1999; Geurts et al., 2002), details of the mechanisms underlying 5-HT receptor-mediated synaptic modulations have not been fully elucidated. Therefore, clarification of the role of 5-HT-induced modulation of the GABAergic synapse, as well as the membrane properties of DCN neurons, might provide a better understanding of the functional significance of the cerebellar nuclei.

Results from the present paper demonstrate the following: 1) exogenous 5-HT decreased the stimulation-evoked IPSCs in DCN neurons in a dose-dependent manner; 2) the presynaptic 5-HT₁B receptors were responsible for decreasing GABA release from the PC axon terminals; 3) slow inward currents were elicited in DCN neurons by 5-HT; 4) the 5-HT₅ receptor coupled to G-protein was possibly involved in the inward currents by 5-HT induced enhancement of Iₖ, channel activation at the DCN neuronal membranes. These results suggest
that 5-HT release to the DCN might play a regulatory role in spike generation and contribute to
the gain control of inhibitory GABAergic synapses in DCN neurons.

MATERIALS AND METHODS

Preparation

Experiments were performed using thin cerebellar slices prepared from Wistar rats (Saitow et al.,
2005), aged 11-15 days, in compliance with a protocol approved by the Ethics Review of
Nippon Medical School (approval number: H19-046). Animals of both sexes were deeply
anesthetized with halothane inhalation (approximately, 2% in air, v/v), and the brains were
rapidly removed. Parasagittal slices of 250-μm thickness were cut using a vibratome (VT1000S,
Leica, Germany) at 4°C in Na+-deficient saline that contained the following: 299.2 mM sucrose,
3.4 mM KCl, 0.3 mM CaCl₂, 3.0 mM MgCl₂, 10 mM HEPES, 0.6 mM NaH₂PO₄, and 10 mM
glucose. This solution appeared to prevent tissue damage due to excessive excitation during
tissue slicing. The slices were maintained in a submerge chamber for more than 1 h in artificial
cerebrospinal fluid (ACSF) that contained the following: 138.6 mM NaCl, 3.4 mM KCl, 2.5
mM CaCl₂, 1.0 mM MgCl₂, 21.0 mM NaHCO₃, 0.6 mM NaH₂PO₄, and 10 mM glucose. ACSF
was maintained at a pH of 7.4 by bubbling 95% O₂/5% CO₂ gas through it. Unless otherwise
specified, slices were superfused with ACSF, in which 6-cyano-7-nitroquinoxaline-2,3-dione
(CNQX), D-2-amino-5-phosphonopentanoic acid (D-APV), and strychnine were added at 10
μM, 40 μM, and 2 μM concentrations, respectively, to eliminate both glutamatergic excitatory
and glycnergic inhibitory synaptic responses.

Patch-clamp recordings

Individual slices were transferred to a recording chamber attached to a microscope stage
(BX50WI, Olympus, Japan) and continuously perfused with oxygenated ACSF at a flow rate of
1.5 ml/min and temperature of 25–26°C. Patch electrodes were used for whole-cell voltage-clamp recordings from DCN neurons with a resistance of 2–3.5 MΩ when filled with an internal solution that contained the following: 150 mM K-methanesulphonate, 5 mM KCl, 0.1 mM K-EGTA, 5 mM Na-HEPES, 3 mM Mg-ATP, and 0.4 mM Na-GTP (pH 7.4). During recording of miniature IPSCs in DCN neurons, as shown in Fig. 2C, CsCl based-high concentration of Cl\(^-\) internal solution (150 mM CsCl, 5 mM KCl, 0.1 mM Cs-EGTA, 5 mM Cs-HEPES, 3 mM Mg-ATP, and 0.4 mM Na-GTP [pH 7.4]) was used. DCN neurons were visually identified under Nomarski optics using a water-immersion objective (63×, N.A., 0.90, Olympus, Japan). Membrane currents and potentials were recorded using a patch-clamp amplifier (EPC-9, HEKA Elektronik, Lambrecht/Pfalz, Germany). The series resistance, which was monitored throughout the experiment, was in the range of 10–20 MΩ and was not compensated. Data were discarded if this value changed by more than 30%. In the present study, recordings were made from large neurons with somatic diameter larger than 25 µm at the interpositus nuclei (Fig. 1A), most of which are thought to correspond to the glutamatergic projection neurons of the cerebellar nuclei (De Zeeuw and Berrebi, 1995; Aizenman et al., 2003). Although a small number of GABAergic inhibitory projection neurons might have been included in the data, as reported recently (Uusisaari et al., 2007), the possibility seems low because the half-height widths of action potentials (Fig. 7B, 0.37 ± 0.04 ms, n = 8) were narrower than the value of GABAergic neurons (approximately 1.5 ms) reported by Uusisaari et al. (2007). Data were analyzed using PULSEFIT (HEKA Elektronik) and Kyplot (Kyence, Tokyo, Japan) software. Miniature synaptic currents were visually inspected, and the peak amplitudes were determined for analysis using the software Mini analysis (Synaptosoft, Decatur, USA). Data were also continuously stored on a videotape recorder after digitizing with a PCM data recorder (NF Electronic Instruments, Japan). All signals were filtered at 2 kHz and sampled at 5 kHz. Membrane
currents were recorded at a holding potential of \(-50\) mV unless otherwise noted. The reasons for setting the holding potential at \(-50\) mV were that we could stably maintain the membrane current and constantly record robust IPSCs under such conditions. IPSCs were evoked every 20 s by stimulation (10–30 V and 100–150 µs) via glass microelectrodes (tip diameter of 1–2 µm) filled with ACSF and placed in the white matter surrounding the DCN. The amplitude ratio of the second to first IPSCs evoked by paired-pulse protocol with an inter-stimulus interval of 50 ms was defined as the paired-pulse ratio. In this case, the amplitude of the second IPSC was measured from the value right before the second stimulation to the peak of second IPSC. Evoked IPSCs were completely abolished by bicuculline (10 µM) or gabazine (5µM), indicating that they were mediated by GABA\(_A\) receptor activation. In the experiment on membrane potential recording (current-clamp), the resting membrane potential was determined by averaging the membrane potential except for firing phases. Activation curves (Fig. 5) of hyperpolarization-activated cation channel (H channel)-mediated currents (I\(_h\)) were constructed from tail current analysis and normalized to the maximal current under control condition. The activation curves for I\(_h\) were fitted by Boltzmann functions, with \(I/I_{\text{max}} = 1/[1 + \exp \{(V_m - V_{0.5})/k\}]\), where \(V_m\) is the membrane potential during the initial voltage step, \(V_{0.5}\) is the membrane potential at which I\(_h\) is half-activated, and \(k\) is the slope factor.

**Chemicals**

Chemicals used in this study were obtained from the following sources: serotonin hydrochloride (5-HT), bicuculline, forskolin, gabazine, (R)-(+)\()-8\)-hydroxy-2-(di-n-propylamino)tertraline (8-OH-DPAT) hydrobromide, nifedipine, GTP\(\gamma\)S, and GDP\(\beta\)S from Sigma (St. Louis, USA); 5-carboxamidotryptamine maleate (5-CT), cyanopindolol hemifumarate, m-chlorophenylbiguanide (mCPBG), CNQX, CP93129 dihydrochloride, D-APV, SB 224289 hydrochloride, SB 269970 hydrochloride, zacopride hydrochloride, and ZD7288 from Tocris...
Bioscience (Bristol, UK); tetrodotoxin (TTX) and nickel chloride from Wako Pure Chemicals (Osaka, Japan); ω-conotoxin GVIA (ω-CgTX) and ω-agatoxin IVA (ω-AgTX) were obtained from Peptide Institute (Osaka, Japan).

Statistics

Numerical data were reported as mean ± SEM and n represented the number of independent experiments. Statistical differences were evaluated using Student’s paired t-test, unless otherwise noted. For multiple comparisons between experimental groups and the comparison between control and treated groups, we performed Tukey-Kramer multiple comparison test and Dunnett multiple comparison test, respectively. To compare the difference in the cumulative curves in Fig. 2D and 2E, Kolmogorov-Smirnov (K-S) test was applied.

RESULTS

Effects of 5-HT on GABAergic transmission and membrane properties in DCN neurons

Stimulation via glass microelectrodes placed in the white matter surrounding the DCN held at a membrane potential (V_m) of –50 mV produced outward synaptic currents in DCN principal neurons (Fig. 1A). These synaptic responses (eIPSCs) were almost completely abolished by the application of a GABA_A receptor antagonist, bicuculline (10 µM) or gabazine (5 µM), but not by the application of a glycine receptor antagonist, strychnine (2 µM), which suggests that the eIPSCs were produced by GABA_A receptor activation. Under control conditions, the rise time (10–90%) and decay time constants of the eIPSCs were 2.6 ± 0.1 ms and 25.1 ± 2.0 ms, respectively (n = 62). Bath application of 5-HT produced distinguishable actions of GABA_A receptor-mediated transmission to DCN neurons (Fig. 1C), and also inwardly shifted the membrane current, as illustrated in Fig. 1C (middle) and 1E. Initially, bath application of 5-HT
at a concentration of 5 µM significantly decreased the eIPSC amplitude in all tested DCN neurons (see Fig. 1B and 1C) by 59.0 ± 6.8% of the IPSC amplitude recorded prior to 5-HT application (baseline). However, 5-HT caused no significant changes in IPSC kinetics—the rise times (10–90%) of the eIPSCs prior to and after 5-HT application were 2.6 ± 0.1 and 2.5 ± 0.1 ms, respectively (n = 14, p > 0.7, paired t-test). eIPSC amplitude was maximally depressed within 5 min after the initiation of 5-HT application. The inhibitory effect of the eIPSCs was recovered within 20 min after washing out the 5-HT-containing ACSF (Fig. 1B; n = 13). The effect of 5-HT (0.2–20 µM) on depressing eIPSC amplitude was dose dependent (Fig. 1D). The maximal inhibitory effect was approximately 49% of the baseline, and the IC50 value was 0.51 µM. Secondly, small inward currents (20–150 pA, 78.6 ± 11.1 pA, n = 28) were observed during 5-HT application (5 µM: Vh = –50 mV). On the basis of these results, the mechanisms of the two types of modulatory effect induced by 5-HT were investigated in DCN neurons.

**Locus of the 5-HT-induced inhibitory effects on GABAergic transmission**

In order to determine whether the site of action of the eIPSC inhibitory effect was presynaptic or postsynaptic, changes in the paired-pulse ratio were measured prior to and during 5-HT-induced inhibition of eIPSCs. In the control medium, paired-pulse stimulation, with an inter-stimulus interval of 50 ms, produced IPSCs with a mean paired-pulse ratio (PPR) of 0.68 ± 0.04 (n = 14). These values are consistent with those obtained in previous studies (Pedroarena & Schwarz, 2003; Sausbier et al., 2004). The PPR increased to 0.81 ± 0.04 during 5-HT-induced inhibition of GABAergic transmission (Fig. 2A; p < 0.001, paired t-test, n = 14). eIPSCs were also measured in order to estimate the fractional change of CV−2, where CV is the coefficient of variation (Faber & Korn, 1991). As shown in Fig. 2B, most of the data points were distributed in the section below the identity line (dotted line); that is, the magnitude of decrease in the ratio of CV−2 (control/5-HT treatment) was greater than that in the mean IPSC amplitude, and this
difference was statistically significant (p < 0.001, paired t-test, n = 13). Therefore, these results suggest that 5-HT presynaptically inhibited GABAergic transmission.

In order to further confirm these conclusions, miniature IPSCs (mIPSCs) in DCN neurons were measured in the presence of TTX (0.5 µM), and the effects of 5-HT on the mIPSCs were observed (Fig. 2C). In the present study, we used an internal solution containing a high concentration of Cl⁻ (see Materials and Methods) and recorded mIPSCs at a holding potential of −60 mV; these conditions provide a sufficient driving force for Cl⁻ currents and enable the detection of a large inhibitory current response. In the presence of TTX, the mean frequency of mIPSC was 24.6 ± 4.6 Hz (n = 11). Subsequently, perfusion of 5-HT (5 µM) significantly decreased the mIPSC frequency to 20.0 ± 4.0 Hz (Fig. 2D and 2F; 76.6 ± 3.6% of control; p < 0.005 with paired t-test, n = 11). Conversely, the mIPSC amplitude was not affected by 5-HT (Fig. 2E and 2G; −42.0 ± 2.7 and −40.2 ± 3.0 pA before and after 5-HT application, respectively; p = 0.21 with paired t-test, n = 11). Therefore, it can be concluded that the 5-HT-induced inhibitory action of IPSC was mediated by a presynaptic mechanism(s).

**5-HT₁B receptor is involved in the 5-HT-induced depression**

Next, we sought to determine the 5-HT receptor subtype mediating IPSC depression in DCN neurons. Figure 4A and 4B show that a 5-HT₁/5/7 receptor agonist, 2 µM 5-CT, and a 5-HT₁B receptor selective agonist, CP93129 (2 µM), exhibit similar inhibitory effects on the eIPSC amplitude [69.0 ± 2.7% (Fig. 3A; n = 9) and 60.3 ± 9.6% (Fig. 3B; n = 9) of the baseline, respectively]. Conversely, a 5-HT₁A receptor selective agonist, 8-OH-DPAT (10 µM), did not have significant effects, despite the relatively high concentration (Fig. 3C; n = 12). The 5-HT-induced inhibitory effect on the eIPSC amplitude in DCN neurons was markedly suppressed by pretreatment with a 5-HT₁B receptor antagonist, SB 224289 (Fig. 3D)—in the presence of 5 µM SB 224289, the 5-HT-induced decreased eIPSCs amplitude was 97.3 ± 1.0% of the baseline (Fig.
Moreover, another 5-HT$_{1A/1B}$ receptor antagonist, cyanopindolol, also suppressed the inhibitory effect of 5-HT (data not shown, 96.0 ± 2.7%, p < 0.05 vs. control, unpaired t-test, n = 9). These pharmacological data indicate that the 5-HT-induced depression of GABA release was mediated through the activation of 5-HT$_{1B}$ receptors.

**5-HT-induced inhibitory effects are not associated with inhibition of presynaptic Ca$^{2+}$ channels and GIRK channel activation**

It has been demonstrated that N- and P/Q-type voltage-operated Ca$^{2+}$ channels (VOCCs) are involved in neurotransmitter release in mammalian central synapses, and also in the inhibition of voltage-dependent Ca$^{2+}$ currents by various transmitters such as 5-HT, GABA, and adenosine. Therefore, in the experiments illustrated in Fig. 4A and 4B, the 5-HT-mediated inhibitory effect of GABAergic transmission was tested under the inhibition of specific Ca$^{2+}$ channels.

Specifically, ω-AgTX (0.2 µM) and ω-CgTX (1 µM) were used as P/Q- and N-type VOCC blockers, respectively. As shown in Fig. 4D, ω-CgTX did not significantly depress the IPSC amplitude (97.4 ± 6.2% of control values at t = 15 min in Fig. 4B; p > 0.7, n = 7), whereas ω-AgTX depressed the IPSC amplitude to 32.6 ± 6.3% of the control value (t = 15 min in Fig. 4A; n = 7). These results suggest that Ca$^{2+}$ influx through P/Q-type VOCCs is mainly responsible for GABA release from presynaptic terminals. In the presence of ω-AgTX, the inhibitory effect of (5 µM) on IPSC amplitude remained unaltered, depressing the IPSC amplitude to 61.8 ± 8.6% of the baseline level immediately prior to the 5-HT application (Fig. 4A and 4E; n = 7).

Similarly, in the presence of ω-CgTX, 5-HT depressed the IPSC amplitude to 63.0 ± 6.0% of the baseline value (Fig. 4B and 4E; n = 7), which was not significantly different from the magnitude of inhibitory action in the presence of ω-AgTX (p > 0.7, Tukey-Kramer multiple comparison test). Taken together, these results suggest that neither P/Q- nor N-type VOCCs are involved in the mechanism underlying the inhibitory effects of 5-HT on the IPSC amplitude in DCN.
neurons. The fact that 5-HT₁B receptors are coupled to G<sub>i/o</sub>-type G-proteins and negatively regulate intracellular adenylyl cyclase (Sari, 2004) suggests another possibility for the mechanism of the 5-HT-induced inhibition; namely, that 5-HT may cause hyperpolarization at the presynaptic terminals via activation of G-protein-coupled inward rectifier K (GIRK) channels. If this is the case, activation of GIRK channel-mediated hyperpolarization of the synaptic terminals might cause a decrease in the frequency of mIPSCs and/or elevation of the threshold for generating action potentials. Therefore, as shown in Fig. 4C, we applied 200 µM Ba<sup>2+</sup> in order to block the GIRK channels (Seeger & Alzheimer, 2001) before 5-HT application. However, the 5-HT-induced inhibition of eIPSC amplitude in DCN neurons remained unchanged (62.4 ± 5.2% of the baseline, n = 8), which suggests that GIRK channel activation in PC axon terminals does not contribute to the reduction of the eIPSC amplitude (Fig. 4E; p > 0.8 vs. control condition, Tukey-Kramer multiple comparison test). Taken together, these findings show that the depression of VOCCs or activation of GIRK channels at PC axon terminals is not associated with the downstream mechanisms of 5-HT₁B receptor activation.

**5-HT-induced inward current is associated with the G-protein-coupled signal pathway and hyperpolarization-activated cation channels**

As mentioned earlier, the exogenous application of 5-HT not only inhibited GABA release but also elicited inward currents in DCN neurons with large diameters (Fig. 1C and 1E). Since the functional 5-HT receptor subtypes of DCN neurons are largely unknown, pharmacological examinations were performed in order to identify the responsible receptor subtypes and the downstream signaling pathway in DCN neurons. Membrane currents were elicited by bath application of 5 µM 5-HT and were recorded at a holding potential of −60 mV in the presence of 0.5 µM TTX. When ionotropic GABA, glycine, and glutamate receptor antagonists (5 µM gabazine, 2 µM strychnine, 40 µM D-APV, and 10 µM CNQX) were used instead of TTX
perfusion, 5-HT-induced inward currents were similarly maintained (−124.7 ± 18.9 pA, p = 0.84, n = 4, Dunnett multiple comparison test). Therefore, the 5-HT-induced currents were not due to the activation of these ionotropic receptor channels in the recorded DCN neurons. The 5-HT-induced current was then tested for potential involvement in the activation of the ionotropic 5-HT₁ receptor. As shown in Fig. 5A (bottom trace), a selective 5-HT₁ receptor agonist, m-chlorophenylbiguanide (mCPBG, 5 µM), did not alter the membrane current of DCN neurons (+3.7 ± 3.9 pA, n = 6). These results indicated that the 5-HT-induced excitatory effects were not the result of activated 5-HT₁ ionotropic receptors. Conversely, as shown in Fig. 5A (middle trace), 5-CT (5 µM), which has been used as a tool to define the 5-HT₃ and 5-HT₇ receptors as well as 5-HT₁ receptors (Yamada et al., 1998; Prins et al., 2001; Monro et al., 2005), mimicked the 5-HT effect on the membrane currents (−107.7 ± 16.8 pA, n = 11). In fact, slow postsynaptic inward currents were also observed on 5-CT application when the inhibitory effects of 5-CT on the IPSC amplitude were examined (Fig. 3A). However, a 5-HT₁B receptor agonist, CP93129, evoked an outward rather than an inward current (data not shown) when we recorded the eIPSCs in DCN neurons. Moreover, we were unable to detect an 8-OH-DPAT (5-HT₁A receptor agonist)-induced membrane current (Fig. 6Ba; −6.1 ± 3.5 pA, n = 3). It is, therefore, unlikely that the 5-HT₁A/B receptors were involved in the slow inward current generation. Next, the receptor subtype responsible for the 5-HT-induced inward current was investigated by using a selective 5-HT₇ receptor antagonist, SB 269970 (Mahe et al., 2004; Monro et al., 2005). However, SB 269970 (5–10 µM) produced no significant effect on the 5-HT-induced inward current (Fig. 6A and 6Bb; −93.9 ± 12.1 pA, n = 6). Taken together with the results described above, it is likely that the 5-HT₃ receptor is involved in producing the inward current in DCN principal neurons. However, this conjecture could not be verified, since at present there are no specific 5-HT₃ receptor agonists or antagonists available.
It is well known that DCN neurons possess voltage-dependent conductance, including low-threshold, voltage-gated (T-type) Ca\(^{2+}\) channels, \(I_h\), and a persistent Na\(^+\) conductance (Llinas & Muhlethaler, 1988; Aizenman & Linden, 1999; Raman et al., 2000; Molineux et al., 2006; Pugh and Raman, 2006; Uusisaari et al., 2007). Among these, it has been reported that both Na\(^+\) and Ca\(^{2+}\) channel-mediated persistent currents are facilitated by 5-HT in the rat spinal cord, and that these channels are blocked by TTX and nimodipine, respectively (Harvey et al., 2006; Li et al., 2007). In order to rule out the possibility that these persistent cationic currents are involved in the 5-HT-induced inward currents in DCN neurons, the effects of a mixture of an L-type Ca\(^{2+}\) channel blocker, nifedipine (20 µM), and TTX were examined. The 5-HT-induced inward currents remained (–89.8 ± 15.2 pA, \(p > 0.8\), \(n = 4\), Dunnett multiple comparison test) despite the presence of these channel blockers. Moreover, the addition of 200 µM Ni\(^{2+}\) to block T-type Ca\(^{2+}\) channels did not affect the 5-HT-induced inward current amplitude (Fig. S1; –102.0 ± 19.2 pA, \(p = 0.77\), \(n = 4\), Dunnett multiple comparison test). The I-V relationship of the 5-HT-induced current was next determined in order to identify the ion(s) responsible for the inward current. The I-V relationships were explored by applying a voltage ramp between –120 and –10 mV before and during the 5-HT application (Fig. 5B) and were subsequently subtracted to yield the voltage dependency of the 5-HT-evoked current (Fig. 5C). The mean value of the reversal potential was –31.6 ± 3.3 mV (Fig. 5C; \(n = 13\)), suggesting that the responses were mediated by non-selective cationic channels in DCN neurons. Taken together, these results suggest that 5-HT increased the cationic conductance that was susceptible to the activation at hyperpolarized potentials in DCN neurons. Therefore, a possible candidate for mediating the 5-HT-evoked current could be the hyperpolarized-activated cationic channels (H channel). This conjecture is supported by the observation that activated H channels are responsible for 5-HT-induced depolarization in spinal motoneurons (Larkman et al., 1995) and monoaminergic control of
action potential firings in thalamic neurons (Pape & McCormick, 1989). Next, we sought to
determine whether 5-HT application would activate H channels and elicit a current (Ih) in DCN
neurons. Accordingly, we examined whether modulation of Ih is involved in the 5-HT-induced
inward current. Application of 5-HT (5 µM) caused a marked enhancement of the Ih current (Fig.
5D). Analysis of tail currents following hyperpolarizing voltage steps revealed that the increase
in current magnitude mediated by 5-HT was due to a rightward shift of the Ih activation curve
(Fig. 5Ea). 5-HT shifted the half-activation voltage $V_{0.5}$ by 9.4 mV (Fig. 5E; n = 6). These data
suggest that 5-HT increased the Ih, and that this activation of Ih contributed partly to the 5-HT-
induced inward currents in DCN neurons. Next, we examined the effects of blocking Ih on the 5-
HT-induced inward currents. As shown in Fig. 5F, a brief hyperpolarizing voltage command
(from −60 to −90 mV for 2000 ms) to the DCN neurons produced a slow inward current (Fig.
5Fa; black lines) that decreased in amplitude in a time-dependent manner upon application of an
H channel blocker, ZD7288 (20 µM). The action onset of this compound was slow,
approximately 2 min, to exert a discernible blockade, with steady Ih suppression occurring after
10 min. These observations are consistent with the blocking effects of the drug observed in our
previous studies (Saitow & Konishi, 2000). Following confirmation that ZD7288 completely
suppressed Ih (Fig. 5Fa; gray lines), 5-HT was applied and the 5-HT-evoked currents were
monitored. As shown in trace b of Fig. 5F, 5-HT failed to produce an obvious inward current
(see also Fig. 6A; −16.0 ± 5.3 pA; p < 0.001, Dunnett multiple comparison test, n = 11).
Although the blocking effect of ZD7288 on T-type Ca$^{2+}$ channels has been recently reported
(Sanchez-Alonso et al., 2008), T-type Ca$^{2+}$ channel blockade with Ni$^{2+}$ did not affect the 5-HT-
induced inward currents, as described above. Moreover, another blocker, Cs$^{+}$ (1 mM), was
tested in order to confirm the involvement of the Ih. We found that Cs$^{+}$ also significantly
suppressed the 5-HT-induced inward currents (Fig. 6A and 6Bc; −34.2 ± 8.1 pA, p < 0.001,
Dunnett multiple comparison test, $n = 15$). These observations suggest that ZD7288-sensitive H channels contributed predominantly to the 5-HT-induced inward current. Further support for this notion was provided by the data shown in Fig. 6A and 6B. The experiments were performed in order to determine whether the 5-HT-induced current response was dependent on G-protein-dependent processes; this is because H channels are known to be activated by an intracellular cyclic nucleotide increase coupled to G-protein signaling. If this is the case, the addition of a non-hydrolysable stimulatory GTP analogue, GTP$\gamma$S (500 µM), to the pipette solution should constitutively activate the intracellular signal pathway in question and block subsequent 5-HT-induced responses. GTP$\gamma$S decreased the 5-HT-induced current response to approximately 30% of the control level (Fig. 6A and 6B; $-30.3 \pm 6.63$ pA, $p < 0.001$ vs. control condition, Dunnett multiple comparison test, $n = 15$). Thus, it is suggested that 5-HT-induced inward current generation is occluded by irreversible activation of G-protein signaling. Next, we tested the inhibition of G-protein-dependent processes by GDP$\beta$S, a competitive inhibitor of GTP binding. Infusion of 1 mM GDP$\beta$S via a pipette significantly suppressed the 5-HT-induced current response by blocking the G-protein-dependent pathway. The average value of the decrease in amplitude was approximately 50% (Fig. 6A and 6B; $-49.9 \pm 9.8$ pA, $p < 0.001$ vs. control condition, Dunnett multiple comparison test, $n = 24$). Moreover, since it is well known that $I_h$ is positively modulated by intracellular cyclic nucleotides (Santoro et al., 1998), we examined the effect of an adenylyl cyclase activator, forskolin, on the hyperpolarization-activated current in DCN neurons. Forskolin largely mimicked the action of 5-HT application (Fig. 6A and 6B; $-87.4 \pm 13.4$ pA, $p = 0.56$ vs. control condition, Dunnett multiple comparison test, $n = 5$), whereas an inactive analogue, dideoxy-forskolin, failed to elicit the inward current (data not shown, $n = 2$). Taken together, these results suggest that G-protein-mediated 5-HT signaling might increase intracellular cyclic AMP levels, resulting in enhanced $I_h$ activation and
subsequent induction of the inward current in DCN neurons.

Effects of 5-HT on spontaneous and rebound depolarization-induced action potential firing in DCN neurons

5-HT exhibited an inhibitory effect on GABAergic transmission and an excitatory effect (generating an inward current) on DCN neurons. These results suggest that 5-HT contributes to the disinhibition of DCN neurons. It has, however, been demonstrated that DCN neurons display postinhibitory rebound firing after membrane hyperpolarization because PCs provide powerful inhibitory synaptic influence on DCN neurons (Aizenman & Linden, 1999). Therefore, we examined the physiological role of 5-HT in the spontaneous and rebound depolarization-induced firing of DCN neurons. After obtaining the whole-cell configuration, we recorded the membrane potential in current-clamp mode, and the rebound firing was induced by stimulation-evoked GABAergic IPSP (25 Hz for 500 ms) (Fig. 7A). The resting membrane potential was –55.3 ± 4.1 mV (n = 10), and the action potential frequency was 2.6 ± 0.5 Hz (n = 8, range from 0.9 to 5.2 Hz). Figure 7B shows the representative waveform (Fig. 7Ba) and basic properties (Fig. 7Bb) of the spontaneous action potential. Since there were no significant differences between control and 5-HT application (Half-height width: p = 0.92; AP threshold: p = 0.68, n = 8, respectively), these results indicated that 5-HT did not alter the properties of the action potential of DCN neurons directly. Rebound firings after the conditioning stimulus were classified into two time-windows, referred to as the early (for 1000 ms after conditioning) and late (for 2500 ms after the early phase) phase, respectively (Fig. 7A). In the absence or a low concentration (0.2 µM) of 5-HT, postinhibitory rebound firing was clearly observed at the early and late phases and distinguishable from the basal firing (Fig. 7A and 7C in the control; Tonic: 2.4 ± 0.61 Hz; Early: 7.3 ± 0.9 Hz, p < 0.001 vs. Tonic; Late: 3.9 ± 0.4 Hz, p < 0.001 vs. Tonic, paired t-test, n = 7). After washing out 5-HT, a GABA_A receptor antagonist, gabazine (GBZ, 5
µM), was applied in most experiments in order to confirm that rebound firing was driven by inhibitory transmission accompanied by membrane hyperpolarization. As shown in the traces (GBZ) in Fig. 7A and b and 7C, GBZ significantly suppressed the rebound firings (Fig. 7C; Tonic: 2.8 ± 0.77 Hz; Early: 3.8 ± 0.84 Hz, p = 0.16 vs. Tonic; Late: 2.9 ± 0.66 Hz, p = 0.47 vs. Tonic, paired t-test, n = 6–7), although the firing frequency slightly increased during the conditioning stimulation. This increase in firing frequency during the conditioning stimulation could be attributable to the activation of certain metabotropic receptors, such as glutamate receptors (Zhang and Linden, 2006), which elicit weak depolarization. Moreover, GBZ itself did not increase the firing frequency during the resting level (Fig. 7C; control: 2.4 ± 0.61 Hz, GBZ: 2.8 ± 0.77 Hz, p = 0.75, unpaired t-test, n = 6–7), which suggests that, in contrast to PCs, tonic inhibition by activated GABA<sub>A</sub> receptors might be less effective in DCN neurons (Hausser & Clark, 1997). At 5-HT concentrations greater than 0.5 µM, the firing frequency increased, and eventually there was no difference in the frequency of action potentials between before and after stimulation of GABAergic synapses when the applied 5-HT concentration was more than 2 µM. (“5-HT” in Fig. 7A and 7C; Tonic (5 µM): 7.3 ± 1.16 Hz; Early (5 µM): 8.1 ± 1.3 Hz, p = 0.25 vs. Tonic; Late (5 µM): 7.1 ± 1.04 Hz, p = 0.52 vs. Tonic, paired t-test, n = 7). Application of 5 µM 5-HT significantly reduced the IPSPs recorded in DCN neurons (right panel of Fig. 7A and 7Ac; p < 0.05, n = 8); however, IPSP-induced silencing of DCN firing remained during the conditioning stimulus (middle panel in Fig. 7A and 7Ab). In comparison with the inhibitory action of 5-HT on IPSC amplitude as shown in Fig. 1 (inhibited to ~60% of the baseline), it appeared that the inhibitory action of 5-HT on the amplitude of IPSP was less effective (inhibited to 75% of the baseline). A possible explanation for this observation is that 5-HT slightly depolarized the membrane potential of DCN neurons (4.6 ± 0.76 mV, n = 8) under the current-clamp condition, and that this depolarization counteracted the inhibitory action of 5-HT.
on GABAergic transmission as a result of an increase in the electromotive force for GABA-mediated IPSP in DCN neurons. Therefore, these results suggest that 5-HT increased the spontaneous spiking activity of DCN neurons and decreased the relative effect of rebound depolarization-induced firing, rather than by the presynaptic suppression of the inhibitory transmission from the cerebellar cortex.

**DISCUSSION**

This study demonstrates two types of 5-HT receptor-mediated modulation that elicit varying excitability regulation in DCN principal neurons. The decreased GABAergic synaptic current in DCN neurons following 5-HT application was mediated by presynaptic 5-HT$_{1B}$ receptor activation (Fig. 8A). Conversely, 5-HT-evoked excitatory effects on the slow inward current and depolarization in DCN neurons were induced by a postsynaptic mechanism (Fig. 8B). These results therefore suggest that 5-HT plays regulatory roles in the control of both membrane excitability and inhibitory GABAergic transmission in the DCN, thereby promoting the voluntary activity of DCN neurons.

**Presynaptic depression of GABA release in DCN inhibitory synapses**

In the present study, stimulation-evoked GABA-mediated IPSC was pharmacologically isolated from EPSC (Anchisi et al., 2001; Zhang & Linden, 2006) and glycine-mediated IPSC (Kawa, 2003) through the application of CNQX, APV, and strychnine. Under these conditions, 5-HT produced a significantly decreased IPSC amplitude in DCN neurons, although preliminary findings revealed that 5-HT also depressed (probably mossy fiber-mediated) EPSC amplitude in DCN neurons (unpublished observation). The present results indicated that 5-HT$_{1B}$ receptors were responsible for the presynaptic inhibition of IPSC amplitude in DCN neurons (Figs. 1–3). These findings are consistent with the predicted localization of 5-HT$_{1B}$ receptors in the
cerebellum (Sari, 2004); that is, the detection of high density staining of 5-HT_{1B} receptor mRNAs in the cell bodies of PCs (Voigt et al., 1991; Maroteaux et al., 1992). 5-HT_{1B} receptor immunoreactivity has also been detected in the DCN that receive projections from the PCs (Boschert et al., 1994). However, when we observed the stimulation-evoked IPSCs in DCN neurons (Fig. 3B), a 5-HT_{1B} receptor agonist, CP93129, postsynaptically evoked an outward rather than an inward current. This was most likely due to the modulation of GIRK channels through the activation of the 5-HT_{1B} receptors.

Since 5-HT_{1B} receptors are coupled to G_{i/o} protein, which negatively regulates adenylyl cyclase, the present study assessed the involvement of certain ion channels in the inhibitory effects following 5-HT_{1B} receptor activation at the presynaptic terminals (Fig. 4). It has been reported that 5-HT inhibits VOCCs in mammalian CNS synapses (Chen & Regehr, 2003; Mizutani et al., 2006); however, blocking of both N- and P/Q-type VOCCs did not alter the 5-HT-induced inhibitory effect (Fig. 4E). Similarly, blocking GIRK channels did not change the inhibitory effect of 5-HT (Fig. 4E). Further, 5-HT_{1B} receptor activation decreased the frequency, but not the amplitude, of mIPSCs in DCN neurons (Fig. 2C–2G). These findings are consistent with a previous report on GABAergic transmission in the suprachiasmatic nuclei (Bramley et al., 2005). However, both the subcellular mechanisms and the molecular target(s) of 5-HT_{1B} receptor activation remain unknown. Further studies are required in order to determine, for example, whether protein kinase(s) are involved in these inhibitory effects of the DCN inhibitory synapses.

**Postsynaptic 5-HT-induced inward currents of DCN principal neurons**

5-HT presumably activated the 5-HT_{5} receptors, which are positively coupled to G-proteins and elicit the slow inward current or depolarization through H channels in DCN neuronal membranes. The pharmacological results suggest that 5-HT_{5} receptors are likely to be of the 5-
HT subtype, although the lack of selective pharmacological tools precludes a more precise determination of the receptor subtype. Furthermore, the conclusions of the present study are supported by results from previous studies; 5-HT$_{5A}$ mRNA and protein immunoreactivity were observed in the DCN (Pasqualetti et al., 1998; Geurts et al., 2002). In addition, the present study demonstrated the ionic mechanisms of the 5-HT-induced inward current caused by activation of H channels. This activation is generally required for an increase in intracellular cyclic nucleotides such as cyclic AMP (cAMP) and cGMP (Ludwig et al., 1998; Santoro et al., 1998; Saitow et al., 2005). According to pharmacological assays, 5-HT$_{5A}$ receptor activation has exhibited inhibitory effects in cAMP formation in 5-HT$_{5A}$ receptor-transfected HEK 293 cells (Francken et al., 1998) and C6 glioma cells (Carson et al., 1996; Thomas et al., 2004). On the other hand, a recent study has proposed that 5-HT$_{5A}$ receptors are coupled with subcellular multiple signaling cascades, including the formation of inositol trisphosphate (IP$_3$) (Noda et al., 2003). Further, another modulation of I$_h$ that has been proposed (Pan, 2003) concerns the enhancement of the maximum I$_h$ amplitude via mobilization of intracellular calcium released from IP$_3$-sensitive calcium stores. These possibilities, which are depicted in Fig. 8B, remain to be elucidated in DCN neurons.

**Physiological and pathological implications of 5HT-modulated DCN neuron activity**

5-HT increased the spike rate of DCN neurons at rest and decreased the impact of rebound firing after repetitive inhibitory inputs (Fig. 7). It has been previously reported that the spike rate of DCN neurons can be controlled in a linear manner by the rate of inhibitory inputs (Gauck & Jaeger, 2000; Shin & De Schutter, 2006; Shin et al., 2007). Namely, both the pattern (synchronicity) and the extent of inhibitory PC input from the cerebellar cortex influence the rate and temporal precision of DCN spiking. In addition, the rebound firing, which relies on the strength and duration of hyperpolarization, is thought to be a key phenomenon for generating
the cerebellar timing signal (Aizenman & Linden, 1999; Koekkoek et al., 2003). In the present experiments, the impact of rebound firing was impaired at relatively high 5-HT concentrations because the background spike rate significantly increased with membrane depolarization. Thus, this suggests that qualitative and/or quantitative mechanisms of cerebellar timing signals are perturbed by excessive 5-HT levels. Abnormal serotonin levels may, for example, occur in the case of overdosing serotonin reuptake inhibitors, which could cause the disturbance of motor coordination pertinent to cerebellar function. It is well known that almost all drugs that increase 5-HT levels exert the negative side effect of ataxia. Taken together, the present results may also provide insight into the important neuropathological aspects of 5-HT modulation in the DCN.
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**FIGURE LEGENDS**

**Figure 1.** 5-HT-induced decrease in the amplitude of electrical stimulation evoked-IPSC (eIPSC) in a dose-dependent manner. **A:** Reconstitution of a DCN neuron filled with AlexaFluor 488 (50 µM) during patch-clamp recording. The calibration bar indicates 50 µm. **B:** Time course of the eIPSC amplitude changes. 5-HT (5 µM) applied during the period indicated by a horizontal bar decreased the eIPSC amplitude. Each point represents the mean ± SEM obtained from 13 independent experiments. **C:** Averaged traces of 8 consecutive IPSCs recorded before 5-HT administration (a), during 5-HT administration (b), and after it was washed out (c); these 3 periods are indicated in B. The holding potential of DCN neurons was maintained at –50 mV. It should be noted that, as shown in (b), inward shift of the resting current was observed during 5-HT administration. **D:** Dose-dependent 5-HT-induced depression of eIPSC amplitude. Data are expressed as the least-mean square fit to a dose-dependence curve with Hill coefficient. The fitted curve revealed an IC_{50} of 0.51 µM, y\text{max} of 49.3% of the control, and Hill coefficient (n) value of 1.0 (n = 6–28). Each point represents mean ± SEM. The numbers adjacent to each point indicate the number of independent experiments. **E:** Effects of 5-HT on the resting membrane current in DCN neurons. 5-HT (5 µM) was applied by superfusion during the period indicated by the horizontal bar. Periodic stimulus artifacts for the eIPSC recording were digitally removed.

**Figure 2.** Exploring the locus of the 5-HT-induced inhibitory effects on GABAergic IPSC amplitude. **A:** 5-HT increased the paired-pulse ratio during the application of 5-HT (PPR, n = 14). **Left graph:** An example of the averaged traces of IPSCs (8 sweeps) evoked by paired-pulse stimulation before (top, thin trace) and during the application of 5-HT (middle, thick line). For comparison, these traces were scaled to the amplitude of the first IPSC and superimposed over each other (bottom). **Right graph:** The paired-pulse protocol was applied with a 50-ms interval. PPRs were determined before (Control, open column) and 5 min after applying 5-µM 5-HT (5-HT, grey column). The PPR significantly increased on 5-HT application (p < 0.001, paired t test). Each column represents the mean ± SEM. **B:** Variance analysis of eIPSC in DCN neurons. The ratio of CV^{-2} was plotted against the ratio of the mean IPSC amplitude (n = 13). Both CV^{-2} and the mean IPSC amplitude were determined by using 10 sweeps before and during the application of 5-µM 5-HT. The filled circle represents mean ± SEM. **C–G:** Effects of 5-HT on mIPSC in DCN neurons. The mIPSCs were recorded in the presence of 0.5-µM TTX, which was used to block spontaneous action potentials. **C:** Representative traces of mIPSC obtained before (a) and 5 min after the application of 5-µM 5-HT (b). **D & E:** Cumulative curves for the inter-event
intervals (D) and amplitude (E) of mIPSCs recorded in a DCN neuron before and during the application of 5-µM 5-HT. mIPSCs recorded from the same neuron shown in C were counted for a constant period (120 s) before (617 events, 47.6 ± 1.0 pA) and after 5-HT application (403 events, 46.8 ± 1.4 pA). The inter-event curve was significantly changed on 5-HT application (p < 0.001, K-S test) in D. On the other hand, 5-HT did not significantly alter the amplitude of the mIPSCs (p = 0.47, K-S test) in E. F & G: Pooled data for the effects of 5-HT on the frequency (F) and amplitude (G) of mIPSC. 5-HT decreased the frequency (p < 0.005, paired t test, n = 11) but not the amplitude (p = 0.21, paired t test, n = 11) of mIPSC. Both the frequency and amplitude of mIPSCs were determined by 2-min recordings.

**Figure 3.** Effects of 5-HT receptor agonists and a 5-HT₁B receptor antagonist on the 5-HT-induced inhibitory effect of eIPSCs in DCN neurons. A–C: Responses shown are the average of 8 consecutive sweeps recorded before and during 5-carboxamidotryptamine (5-CT), CP93129, and (R+)8-OH-DPAT application, respectively (left panel). The traces a and b were obtained at the time points indicated in the graphs in the right panel. The graphs displaying the time course of the effects of the 5-HT₁ receptor agonist 5-CT (A, n = 9), 5-HT₁B agonist CP93129 (B, n = 9), and 5-HT₁A agonist (R+)8-OH-DPAT (C, n = 12) on the eIPSC amplitude. Application of 2-µM 5-CT and CP93129 showed similar inhibitory effects on IPSC amplitude; however, 10-µM (R+)8-OH-DPAT had no effect (right panel). D: The responses shown are the average of 8 consecutive sweeps recorded before and during 5-HT application in the presence of the 5-HT₁B antagonist SB 224289 (left panel). The traces a and b were obtained at the time points indicated in the graph in the right panel. The effects of 5-HT (5 µM) on eIPSC amplitude in the presence of SB 224289 (5 µM, grey circles, n = 12). The open circles indicate the time course of eIPSC amplitude changes in the absence of SB 224289 (Control, n = 13) for comparison. Each point represents the mean ± SEM.

**Figure 4.** Effects of VOCCs and GIRK channel blockers on eIPSC amplitude and 5-HT-induced depression of eIPSC amplitude. A & B: Effects of ω-AgTx (0.2 µM) and ω-CgTx (1 µM) on eIPSC amplitude and 5-HT-induced inhibitory action of this amplitude (A & B, n = 7). Both blockers were added to standard ACSF and were perfused for 10 min as indicated by the thin bar. 5-HT was applied 5 min after washing out the blockers (at t = 15 min). The inset in A shows altered IPSC amplitude during application of 5-µM 5-HT. eIPSC amplitude was expressed as a percentage of the amplitude determined 3 min before 5-HT application. C: Effects of the GIRK channel blocker Ba²⁺ (200 µM) on eIPSC amplitude and the 5-HT-induced inhibitory action of this amplitude (n = 8). Ba²⁺ and 5-HT were applied as indicated by the thin bar (for 15 min) and
thick bar (for 5 min), respectively. **D:** Pooled data on the effects of VOCCs and GIRK channel blockers on eIPSC amplitude. For experiments with ω-AgTx and ω-CgTx, the grey (n = 7) and black (n = 7) columns depict the altered amplitudes obtained before and 15 min after the application of each toxin. In the results for Ba²⁺ application, the hatched column (n = 8) indicates changes in eIPSC amplitude before and 5 min after the application of Ba²⁺. Only the application of ω-AgTx largely suppressed the basal amplitude of eIPSCs (p < 0.001, Tukey-Kramer multiple comparison test). The vertical lines on the columns indicate SEM. **E:** Pooled data on the effects of VOCCs and GIRK channel blockers on 5-HT-induced inhibitory action. The eIPSC amplitude recorded before 5-HT application was regarded as 100%. On an average, there was no significant difference in inhibitory action in the absence and presence of ion channel blockers (Control, 62.6 ± 4.8%; CgTx, 63.0 ± 6.0%; AgTx, 61.9 ± 8.6%; at least p > 0.7, Tukey-Kramer multiple comparison test). The vertical lines on the columns indicate SEM.

**Figure 5.** Effects of 5-HT on membrane currents in DCN neurons. **A:** Representative traces for inward current induction in DCN neurons after the application of 5-HT (5 µM, top trace), 5-CT (5 µM, middle trace) and m-chlorophenylbiguanide (mCPBG, 5 µM, bottom trace). 5-HT and 5-CT induced an inward current; however, the 5-HT3 agonist mCPBG did not elicit any membrane current response. All the agonists were perfused for 5 min as indicated by the bar. **B:** As illustrated in the inset, the I-V relationship determined before (a, grey trace) and after 5-HT application (b, black trace) by a constant voltage-ramp command between –110 and 0 mV at a rate of 10 mV·s⁻¹. The 5-HT-induced current was obtained by subtracting current (a) from (b). **C:** Pooled data on the I-V relationship in DCN neurons induced by 5-HT. 5-HT-evoked currents were normalized by the current value at –110 mV and plotted to –10 mV at every 10-mV step. Each point represents the mean ± SEM (n = 13). **D:** Effects of 5-HT on Iᵦ. (a) An overlay of Iᵦ activation was observed during the voltage step from –60 mV to –90 mV in the control condition (thin trace) and during 5-HT application (thick trace). (b) The Iᵦ amplitude was enhanced by 5-HT. The components of Iᵦ were aligned on instantaneous currents in (a). **E:** Tail current analysis for determination of the Iᵦ activation curve. (a) Sample traces of current responses produced in response to the voltage-clamp protocol. The membrane potential of a DCN neuron was held at –50 mV, stepped to a series of test potentials, and then poststepped to –140 mV (inset). (b) 5-HT increased the Iᵦ amplitude by shifting the activation curve by 9.4 mV towards positive voltages (p < 0.01, paired t test, n = 6) without changing the slope factor: k is equal to 11.37 and 11.38 before and after 5-HT application, respectively (p = 0.71, paired t test, n = 6). The open circles represent the normalized amplitudes of Iᵦ determined by the tail currents in the control ACSF, and the closed circles represent the normalized amplitudes of Iᵦ determined by the tail currents in the
presence of 5-µM 5-HT. \textbf{F}: Effects of ZD7288 on the 5-HT-induced inward currents of DCN neurons. \textit{(a)} Inhibition of I_h of a DCN neuron by ZD7288. Current responses recorded during a voltage step (from −60 to −90 mV for 2000 ms) in the absence of ZD7288 (Control, black traces) and approximately 10 min after its application (20-µM ZD7288, grey traces). \textit{(b)} Effects of ZD7288 on the 5-HT-induced inward currents of DCN neurons. Following the inhibitory effect of ZD7288, DCN neurons were superfused with 5-µM 5-HT. In this case, 5-HT elicited only 25 pA of inward current. Note that the statistical results obtained for the application of ZD7288 are shown in Fig. 6.

\textbf{Figure 6}. Summary of the 5-HT-induced current responses under various conditions. \textit{A}: The steady-state inward currents evoked at a holding potential of −60 mV on either application of 5-HT in the presence of individual drugs applied by direct dialysis with the recording electrode or isolated bath application of Cs\(^+\) (1 mM), ZD7288 (20 µM), and SB269970 (10 µM). The H channel activation that accompanied the G protein-dependent signaling pathway and the intracellular cAMP level were involved in generating an inward current response. Both GTP\(\gamma\)S (500 µM) and GDP\(\beta\)S (1 mM) were dialyzed via a recording pipette for more than 20 min after performing whole-cell recording. 5-HT (5 µM) was perfused by bath application for 5 min. Compared with the control condition, the treatment for blocking both the G-protein-dependent pathway and H channel activity significantly inhibited the amplitude of 5-HT-induced responses (GTP\(\gamma\)S, \(p = 2.5 \times 10^{-6}\) and \(n = 15\); GDP\(\beta\)S, \(p = 9.1 \times 10^{-6}\) and \(n = 24\); Cs, \(p = 2.7 \times 10^{-6}\) and \(n = 15\); ZD7288, \(p = 1.9 \times 10^{-6}\) and \(n = 11\)). Treatment with a 5-HT\(_7\) receptor antagonist, SB 269970 (5–10 µM), did not suppress the inward current-eliciting effect of 5-HT (\(p = 0.81, n = 6\)). Moreover, the adenylyl cyclase activator forskolin (20 µM) elicited an inward current that was, on average, comparable to the amplitude in the control condition (\(p = 0.56, n = 5\)). Conversely, a selective 5-HT\(_3\) receptor agonist, m-chlorophenylbiguanide (mCPBG, 5 µM), did not affect the membrane current of DCN neurons (\(p = 1.3 \times 10^{-5}\), \(n = 6\)). Statistical tests were performed with the Dunnett multiple comparison test to compare these results with those obtained with the control condition (\(n = 36\)). The circles indicate the amplitude of 5-HT-induced inward current for individual recordings. Bars indicate mean ± SEM values for each experimental series. ***(indicates \(p < 0.001\) versus the control group. \textit{B}: Representative traces of the effects of the 5-HT receptor agonists under various conditions and the application of an adenylyl cyclase activator. Membrane currents were recorded at a holding potential of −60 mV in the presence of 0.5-µM TTX. \textit{(a)} 5-HT\(_{1A,7}\) receptor agonist 8-OH-DPAT (10 µM). \textit{(b)} Effects of 5-HT in the presence of the 5-HT\(_7\) receptor antagonist SB 269970 (10 µM). \textit{(c)} Effects of 5-HT in the presence of 1-mM Cs ions. \textit{(d)} Effects of dialysis of GTP\(\gamma\)S (500 µM) via infusion with the recording pipette on the
5-HT-induced response. (e) Effects of dialysis of GDPβS (1 mM) via infusion with the recording pipette infusion on the 5-HT-induced response. (f) Effects of the adenylyl cyclase activator forskolin (20 μM). All the drugs were applied for 5 min, as indicated by the shaded area.

**Figure 7.** Increased spontaneous firing and decreased rebound depolarization-induced firing by 5-HT application in DCN neurons. **A:** (a) Representative membrane potential and firing before and after repetitive stimulation of GABAergic synapses (25 Hz for 500 ms) in DCN neurons subjected to the control condition (**Control, upper 5 traces**), and during the application of 5-μM 5-HT (**5-HT, middle 5 traces**) and gabazine (**GBZ, bottom 3 traces**) (left panel). The upper rectangles indicate the time windows for the calculation of instantaneous action potential frequencies as shown in (c). We set 3 time windows that covered the prestimulus (**Tonic, 2500 ms**) and poststimulus periods in the early phase (**Early, 1000 ms**) and late phase (**Late, 2500 ms**). In the traces for **Control** and **5-HT**, the duration of time for which the action potentials were absent corresponded to the time duration for which repetitive stimuli (for 500 ms) were applied. The IPSPs recorded during the stimulus train are shown at a higher amplitude and time resolution (right panel). The averaged traces for the 5 membrane potentials evoked by the stimulus train are superimposed in 2 conditions—control and during 5-HT application. Note that the stimulus artifacts in these traces were digitally removed. (b) Raster plots of the above 3 conditions. Each letter corresponds to the data shown in (a). (c) Bar graph showing the average change in the first IPSP amplitude in the control and during the application of 5-μM 5-HT (**p < 0.05, paired t test, n = 8**). **B:** Waveforms of spontaneous action potentials. (a) Representative peak-aligned averages (20 sweeps) of action potentials under the 2 different conditions. (b) The bar graphs illustrate the half-height width (upper, **p = 0.92, paired t test, n = 8**) and the action potential threshold (bottom, **p = 0.68, paired t test, n = 8**) before (control) and during the application of 5-HT (5 μM). **C:** Effects of 5-HT on firing frequency and rebound discharge in DCN neurons. Instantaneous frequencies of the action potentials were calculated at each time window as described in **A** (**Tonic, open circles; Early, filled circles; Late, squares; n = 7**). In particular, the background firing frequencies (open circles) were significantly increased (versus the control, **p < 0.01** at 2 μM and **p < 0.005** at 5 μM), and the effect of rebound depolarization-induced firing was attenuated with increasing 5-HT concentrations. Asterisks indicate the significance level compared with the tonic frequency of each 5-HT concentration. ****, and * indicate **p < 0.001**, **p < 0.005**, and **p < 0.01**, respectively. Each point represents the mean ± SEM.

**Figure 8.** Schematic drawing of the site of action of 5-HT and the expected signal pathways of activation. **A:** 5-HT-induced presynaptic inhibitory effects on GABA release were due to the
activation of 5-HT_{1B} receptors. **B:** The 5-HT-induced inward current was probably due to the activation of 5-HT_{5} receptors on the membrane of DCN neurons. The dotted line showed 2 possible signal pathways for the activation of the H channel after activation of the 5-HT_{5} receptor. *(a)* Activation of H channels by the elevation in the intracellular cAMP level. *(b)* Coupling with IP_{3} formation after 5-HT_{5} receptor activation (see text).
A. Example traces showing control, 5-HT, and scaled conditions.

B. Paired-pulse ratio graph with ratios for control and 5-HT conditions.

C. a) Inter-event intervals cumulative distribution for control and 5-HT.
b) Cumulative distribution of mIPSC amplitudes.

D. mIPSC frequency graph showing control and 5-HT conditions.

E. mIPSC amplitude graph showing control and 5-HT conditions.
A

2 μM 5-CT

B

2 μM CP93129

C

10 μM (R+)8-OH-DPAT

D

5 μM 5-HT

Control
**Figure A**

- Graph showing the effects of 5 μM 5-HT on IPSC amplitude.
- Time in minutes (0-30).
- IPSC amplitude in percentage (%).

**Figure B**

- Graph showing the effects of 1 μM 5-HT on IPSC amplitude.
- Time in minutes (0-30).
- IPSC amplitude in percentage (%).

**Figure C**

- Graph showing the effects of 5 μM 5-HT and 0.2 μM 5-HT (ω-AgTx) on IPSC amplitude.
- Time in minutes (0-30).
- IPSC amplitude in percentage (%).

**Figure D**

- Graph showing the effects of 5 μM 5-HT and 200 μM 5-HT (Ba^2+ AgTx) on IPSC amplitude.
- Time in minutes (0-30).
- IPSC amplitude in percentage (%).

**Figure E**

- Bar graph showing the comparison of 5-HT-induced depression among different conditions:
  - Basal
  - Control
  - Ctx
  - Agtx
  - Ba^2+
- Percentage depression (%).