Modulatory Effects of Serotonin on GABAergic Synaptic Transmission and Membrane Properties in the Deep Cerebellar Nuclei

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Saitow F, Murano M, Suzuki H. Modulatory effects of serotonin on GABAergic synaptic transmission and membrane properties in the deep cerebellar nuclei. J Neurophysiol 101: 1361–1374, 2009. First published January 14, 2009; doi:10.1152/jn.90750.2008. Cerebellar outputs from the deep cerebellar nuclei (DCN) are critical for generating and controlling movement. DCN neuronal activity is primarily controlled via 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 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-HT1B 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 showed that 5-HT activated the 5-HT5 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 caused by 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.

INTRODUCTION

Several studies have shown 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 GABAergic receptor–mediated inhibitory input from Purkinje cells (PCs) in the cerebellar cortex (Aizenman et al. 1998; Anchisi et al. 2001; Gauck and Jaeger 2000; Mouginot and Gahwiler 1995; Teune et al. 1998). PC synapses constitute >70% of the total synapses on DCN neurons, and almost all somatic synapses of DCN neurons are inhibitory (De Zeeuw and Berrebi 1995). Therefore it has been proposed that PC inhibitory inputs are important for controlling the rate and temporal precision of DCN spiking (Gauck and 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 long-term potentiation (LTP) and long-term depression (LTD) can be induced, depending on changes to postsynaptic intracellular Ca2+ concentration (Morishita and Sastry 1996; Ouadrouz and Sastry 2000). On the contrary, however, few studies have focused on the modulatory effects of monoamines, such as 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 nucleus. 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 CNS, including the frontal cortex, hippocampus, amygdala, striatum, hypothalamus, and dorsal horn (Bockaert et al. 2006; Millan 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 and Bishop 1991; Kitzman and 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 (Geurts et al. 2002; Pazos and Palacios 1985; Sari et al. 1999), 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

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DCN neurons, might provide a better understanding of the functional significance of the cerebellar nuclei.

Results from this study show 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-HT3 receptor coupled to G protein was possibly involved in the inward currents by 5-HT induced enhancement of I\textsubscript{h} 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.

**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 H19-046). Animals of both sexes were deeply anesthetized with halothane inhalation (~2% in air, vol/vol), and the brains were rapidly removed. Parasagittal slices of 250 μm thickness were cut using a vibratome (VT1000S, Leica) at 4°C in Na\textsuperscript{+}-deficient saline that contained the following: 299.2 mM sucrose, 3.4 mM KCl, 0.3 mM CaCl\textsubscript{2}, 3.0 mM MgCl\textsubscript{2}, 10 mM HEPES, 0.6 mM NaH\textsubscript{2}PO\textsubscript{4}, and 10 mM glucose. This solution seemed to prevent tissue damage caused by excessive excitation during tissue slicing. The slices were maintained in a submerge chamber for >1 h in artificial cerebrospinal fluid (ACSF) that contained the following: 138.6 mM NaCl, 3.4 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.0 mM MgCl\textsubscript{2}, 21.0 mM NaHCO\textsubscript{3}, 0.6 mM NaH\textsubscript{2}PO\textsubscript{4}, and 10 mM glucose. ACSF was maintained at a pH of 7.4 by bubbling 95% O\textsubscript{2}–5% CO\textsubscript{2} 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, 40, and 2 μM concentrations, respectively, to eliminate both glutamatergic excitatory and glycinergic inhibitory synaptic responses.

**Patch-clamp recordings**

Individual slices were transferred to a recording chamber attached to a microscope stage (BX50WI, Olympus) 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-methanesulfonate, 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\textsuperscript{−} 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 (×62; NA, 0.90, Olympus). 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 >30%. In this study, recordings were made from large neurons with somatic diameter >25 μm at the interpositus nuclei (Fig. 1A), most of which are thought to correspond to the glutamatergic projection neurons of the cerebellar nuclei (Aizenman et al. 2003; De Zeeuw and Berrebi 1995). 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 (~1.5 ms) reported by Uusisaari et al. (2007). Data were analyzed using PULSEFIT (HEKA Elektronik) and Kypiot (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, GA). Data were also continuously stored on a videotape recorder after digitizing with a PCM data recorder (NF Electronic Instruments). 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 interstimulus interval of 50 ms was defined as the paired-pulse ratio (PPR). 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 (5 μM), indicating that they were mediated by GABA\textsubscript{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\textsubscript{h}) were constructed from tail current analysis and normalized to the maximal current under control condition. The activation curves for I\textsubscript{h} were fitted by Boltzmann functions, with \( \frac{I(I_{max})}{I(I + exp [(V_m - V_o)/k])] \), where \( V_m \) is the membrane potential during the initial voltage step, \( V_o \) 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: 5-HT, bicuculline, forskolin, gabazine, (R)(+)-8-hydroxy-2-(di-n-propylamino)tertraline (8-OH-DPAT) hydrobromide, nifedipine, GTP\textsubscript{S}, and GDP\textsubscript{BS} from Sigma (St. Louis, MO); 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); TTX and nickel chloride from Wako Pure Chemicals (Osaka, Japan); and o-conotoxin GVI\textsubscript{A} (o-CgTX) and o-agatoxin IVA (o-AgTX) were obtained from Peptide Institute (Osaka, Japan).

**Statistics**

Numerical data were reported as means ± SE, 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 Dunnnett multiple comparison test, respectively. To compare the difference in the cumulative curves in Fig. 2, D and E, a 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 ($V_h$) 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 before and during 5-HT application were 2.6 ± 0.1 and 2.5 ± 0.1 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 inward shifted the membrane current, as shown in Fig. 1, C (middle) and E. Initially, bath application of 5-HT at a concentration of 5 μM significantly decreased the eIPSC amplitude in all tested DCN neurons (see Fig. 1, B and C) by 59.0 ± 6.8% of the IPSC amplitude recorded before 5-HT application (baseline). However, 5-HT caused no significant changes in IPSC kinetics—the rise times (10–90%) of the eIPSCs before 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 ~49% of the baseline, and the IC_{50} value was 0.51 μM. Second, small inward currents (20–150 pA, 78.6 ± 11.1 pA, $n = 28$) were observed during 5-HT application (5 μM: $V_h = −50$ mV). On the basis of these results, the mechanisms of the two types of modulatory effect induced by 5-HT were studied in DCN neurons.

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

To determine whether the site of action of the eIPSC inhibitory effect was presynaptic or postsynaptic, changes in the PPR were measured before and during 5-HT–induced inhibition of eIPSCs. In the control medium, paired-pulse stimulation, with an interpulse interval of 50 ms, produced IPSCs with a mean PPR of 0.68 ± 0.04 ($n = 14$). These values are consistent with those obtained in previous studies (Pedroarena and 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 to estimate the fractional change of CV$^2$ (Faber and 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.

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 this study, we used an internal solution containing a high concentration of Cl− (see 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. 2, D and F; 76.6 ± 3.6% of control; P < 0.005 with paired r-test, n = 11). Conversely, the mIPSC amplitude was not affected by 5-HT (Fig. 2, E and G; −42.0 ± 2.7 and −40.2 ± 3.0 pA before and after 5-HT application, respectively; P = 0.21 with paired r-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 3, A and B, shows that a 5-HT1B 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. 3D; t = 4−6 min, n = 11). Moreover, another 5-HT1A/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 r-test, n = 9). These pharmacological data indicate that the 5-HT−induced depression of GABA release was mediated through the activation of 5-HT1B receptors.

5-HT−induced inhibitory effects are not associated with inhibition of presynaptic Ca2+ channels and G protein−coupled inward rectifier K channel activation

It has been shown that N- and P/Q-type voltage−operated Ca2+ channels and G proteins−coupled inward rectifier K channel activation in mammalian central synapses and also in the inhibition of voltage−dependent Ca2+ currents by various transmitters such as 5-HT, GABA, and adenosine. Therefore in the experiments shown in Fig. 4, A and B, the 5-HT−mediated inhibitory effect of GABAergic transmission was tested under the inhibition of specific Ca2+ channels. Specifically, α-AgTX (0.2 μM) and α-CgTX (1 μM) were used as P/Q- and N-type VOCC blockers, respectively. As shown in Fig. 4D, α-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 α-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 Ca2+ influx through P/Q-type VOCCs is mainly responsible for GABA release from presynaptic terminals. In the presence of α-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 before the 5-HT application (Fig. 4, A and E; n = 7). Similarly, in the presence of α-CgTX, 5-HT depressed the IPSC amplitude to 63.0 ± 6.0% of the baseline value (Fig. 4, B and E; n = 7), which was not significantly different from the magnitude of inhibitory action in the presence of α-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 Gαo−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 Ba2+ to block the GIRK channels (Seeger and 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. 1, C and E). Because the functional 5-HT receptor subtypes of DCN neurons are largely unknown, pharmacological examinations were performed 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 agonists (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 caused by the activation of these ionotropic receptor channels in the recorded DCN neurons. The 5-HT–induced current was tested for potential involvement in the activation of the ionotropic 5-HT₃ receptor. As shown in Fig. 5A (bottom trace), a selective 5-HT₃ 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-HT₃ 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-HT₁, and 5-HT₇ receptors as well as 5-HT₁ receptors (Monro et al. 2005; Prins et al. 2001; Yamada et al. 1998), 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. 6B; −6.1 ± 3.5 pA, n = 3). It is, therefore unlikely that the 5-HT₁B receptors were involved in the slow inward current generation. Next, the receptor subtype responsible for the 5-HT–induced inward current was studied 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. 6, A and 6B; −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, because at present there are no specific 5-HT₅ receptor agonists or antagonists available.

**Fig. 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). The traces a and b were obtained at the time points indicated in the graphs in the right panel. The graphs showing the time course of the effects of the 5-HT receptors agonist 5-CT (A, n = 9), 5-HT₁B agonist CP93129 (B, n = 9), and 5-HT₇ 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). D: 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). 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, gray circles, n = 12). The open circles indicate the time course of eIPSC amplitude changes in the absence of SB 22429 (Control, n = 13) for comparison. Each point represents the means ± SE.
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 (Aizenman and Linden 1999; Llinas and Muhlethaler 1988; Molinuevo et al. 2006; Pugh and Raman 2006; Raman et al. 2000; 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). 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 \(\mu\)M), 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 \(\mu\)M Ni\(^{2+}\) to block T-type Ca\(^{2+}\) channels did not affect the 5-HT–induced inward current amplitude (Supplementary Fig. S1; –102.0 ± 19.2 pA, \(P = 0.77, n = 4\), Dunnett multiple comparison test).\(^1\) The \(I-V\) relationship of the 5-HT–induced current was next determined 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 nonselective 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 can-

\(^1\) The online version of this article contains supplemental data.
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 and McCormick 1989). Next, we sought to determine whether 5-HT application would activate H channels and elicit a current ($I_h$) in DCN neurons. Accordingly, we examined whether modulation of $I_h$ is involved in the 5-HT–induced inward current. Application of 5-HT (5 μM) caused a marked enhancement of the $I_h$ current (Fig. 5D). Analysis of tail currents following hyperpolarizing voltage steps showed that the increase in current magnitude mediated by 5-HT was caused by a rightward shift of the $I_h$ activation curve (Fig. 5Eb). 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 $I_h$ and that this activation of $I_h$ contributed partly to the 5-HT–induced inward currents in DCN neurons. Next, we examined the effects of blocking $I_h$ on the 5-HT–induced inward currents. As shown in Fig. 5F, a brief hyperpolarizing voltage command (from −60 to −90 mV for 2,000 ms) to the DCN neurons produced a slow inward current (Fig. 5Fa, black lines) that decreased in amplitude in a time-dependent manner on application of an H...
channel blocker, ZD7288 (20 μM). The action onset of this compound was slow, ~2 min, to exert a discernible blockade, with steady \( I_h \) suppression occurring after 10 min. These observations are consistent with the blocking effects of the drug observed in our previous studies (Saitow and Konishi 2000). Following confirmation that ZD7288 completely suppressed \( I_h \) (Fig. 5A; 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; \(-16.0 \pm 5.3\) 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 to confirm the involvement of the \(I_h\). We found that Cs\(^{+}\) also significantly suppressed the 5-HT–induced inward currents (Fig. 6, A and Bc; \(-34.2 \pm 8.1\) pA, \(P < 0.001\), Dunnett multiple comparison test, \(n = 15\)). These observations suggest that ZD7288-sensitive H channels contributed predominantly
Moreover, because it is well known that the inhibition of G protein–dependent processes by GDP irreversible activation of G protein signaling. Next, we tested that 5-HT–induced inward current generation is occluded by a competitive inhibitor of GTP binding. Infusion of 1 mM GDPβS 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 ~50% (Fig. 6, A and B; −30.3 ± 6.63 pA, P < 0.001 versus 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 GDPβS, a competitive inhibitor of GTP binding. Infusion of 1 mM GDPβS 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 ~50% (Fig. 6, A and B; −49.9 ± 9.8 pA, P < 0.001 vs. control condition, Dunnett multiple comparison test, n = 24). Moreover, because it is well known that I_inactivation 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. 6, A and B; −87.4 ± 13.4 pA, P = 0.56 vs. control condition, Dunnett multiple comparison test, n = 5), whereas an inactive analog, 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_inactivation 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) in DCN neurons. These results suggest that 5-HT contributes to the disinhibition of DCN neurons. It has, however, been shown that DCN neurons display postinhibitory rebound firing after membrane hyperpolarization because PCVs provide powerful inhibitory synaptic influence on DCN neurons (Aizenman and 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. Because there were no significant differences between control and 5-HT application (half-height width: P = 0.92; action potential (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 1,000 ms after conditioning) and late (for 2,500 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. 7, A and C, 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 to confirm that rebound firing was driven by inhibitory transmission accompanied by membrane hyperpolarization. As shown in the traces (GBZ) in Fig. 7A, a and b, and C, 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 PCVs, tonic inhibition by activated GABA_A receptors might be less effective in DCN neurons (Hauser and Clark 1997). At 5-HT concentrations >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 GABAAergic synapses when the applied 5-HT concentration was >2 µM. (5-HT in Fig. 7, A and C; 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 IPSCs recorded in DCN neurons (Fig. 7A, a and c, right panel; P < 0.05, n = 8); however, IPSP-induced silencing of DCN firing remained during the conditioning stimulus (Fig. 7A, a and b, middle panel). In comparison with the inhibitory action of 5-HT on IPSC amplitude as shown in Fig. 1 (inhibited to ~60% of the baseline), it seemed 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 showed two types of 5-HT receptor–mediated modulation that elicit varying excitability regulation in DCN principal neurons. The decreased GABAAergic synaptic current in DCN neurons following 5-HT application was mediated by presynaptic 5-HT1B 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 GABAAergic transmission in the DCN, thereby promoting the voluntary activity of DCN neurons.

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

In this study, stimulation-evoked GABA-mediated IPSCs was pharmacologically isolated from EPSCs (Anchisi et al. 2001; Zhang and Linden 2006) and glycine-mediated IPSCs (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 showed that 5-HT also depressed (probably mossy fiber mediated) EPSC amplitude in DCN neurons (unpublished observations). These results indicated that 5-HT1B 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-HT1B receptors in the cerebellum (Sari 2004); that is, the detection of high-density staining of 5-HT1B Receptor mRNAs in the cell bodies of PCs (Maroteaux et al. 1992; Voigt et al. 1991). 5-HT1B 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-HT1B receptor agonist, CP93129, postsynaptically evoked an outward rather than an inward current. This was most likely caused by the modulation of GIRQ channels through the activation of the 5-HT1B receptors.

Because 5-HT1B receptors are coupled to G<sub>i</sub>, protein, which negatively regulates adenylyl cyclase, this study assessed the involvement of certain ion channels in the inhibitory effects following 5-HT1B receptor activation at the presynaptic terminals (Fig. 4). It has been reported that 5-HT inhibits VOCCs in mammalian CNS synapses (Chen and Regehr 2003; Mizutani et al. 2006); however, blocking of both N- and P/Q-type VOCCs in both current-clamp and voltage-clamp recordings showed no effect on IPSCs, indicating that 5-HT may not affect VOCCs in DCN neurons.

**FIG. 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 caused by the activation of 5-HT<sub>1B</sub> receptors. B: the 5-HT–induced inward current was probably caused by the activation of 5-HT<sub>5</sub> 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<sub>1B</sub> receptor. a: activation of H channels by the elevation in the intracellular cAMP level. b: coupling with IP<sub>3</sub> formation after 5-HT<sub>5</sub> receptor activation.
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). Furthermore, 5-HT$_{1B}$ receptor activation decreased the frequency, but not the amplitude, of mIPSCs in DCN neurons (Fig. 2, C–G). 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 needed 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 this study are supported by results from previous studies; 5-HT$_{5A}$ mRNA and protein immunoreactivity were observed in the DCN (Geurts et al. 2002; Pasqualetti et al. 1998). In addition, this study showed the ionic mechanisms of the 5-HT–induced inward current caused by activation of H channels. This activation is generally needed for an increase in intracellular cyclic nucleotides such as cAMP and cGMP (Ludwig et al. 1998; Saitow et al. 2005; Santoro et al. 1998).

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). Furthermore, 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 5-HT–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 and Jaeger 2000; Shin and 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 and Linden 1999; Koekkoek et al. 2003). In these 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, these results may also provide insight into the important neuropathological aspects of 5-HT modulation in the DCN.

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