Glycine Receptors and Glycinergic Synaptic Transmission in the Deep Cerebellar Nuclei of the Rat: A Patch-Clamp Study

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Kawa, Kazuyoshi. Glycine receptors and glycinergic synaptic transmission in the deep cerebellar nuclei of the rat: a patch-clamp study. J Neurophysiol 90: 3490–3500, 2003; 10.1152/jn.00447.2003. To clarify possible glycinergic transmission in the cerebellum, principal neurons in deep cerebellar nuclei (DCN) of sliced cerebella (200 μm in thickness) from rats (aged 2–14 days) were studied using whole cell patch-clamp techniques. When glycine (100 μM) was applied to the DCN neurons from a “Y tube,” large outward currents were induced (average peak amplitude of about 600 pA at −40 mV). The currents were blocked by strychnine (1 μM) and showed a reversal potential of −62 mV, which was approximately the estimated Cl− equilibrium potential. The dose-response relation of the currents showed an apparent dissociation constant of 170 μM for glycine and Hill coefficient of 1.6. In the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), N-methyl-D-aspartate (NMDA), and GABA A receptors, respectively, postsynaptic currents sensitive to strychnine (1 μM) were induced in DCN neurons by external perfusion of 20 mM K+ saline. Electrical stimulation of surrounding tissues in DCN evoked definite inhibitory postsynaptic currents (IPSCs) in these neurons. The IPSCs had a reversal potential of −62 mV and showed sensitivities to strychnine and tetrodotoxin. Thus this study has revealed that strychnine-sensitive glycine receptors are expressed in neurons of the DCN of rats and that glycinergic transmission mediated by these receptors is functional in these neurons from stages immediately after birth. The glycinergic innervations are presumably supplied by small interneurons located in the DCN.

INTRODUCTION

Glycine is a major inhibitory transmitter in the CNS, primarily in the spinal cord and brain stem (Laube et al. 2002; Legendre 2001). Recent molecular studies have shown that glycine receptors are expressed more extensively than previously thought, particularly in the higher brain of mammals, including the cerebral cortex, hippocampus, midbrain, and cerebellum (Chattipakorn and McMahon 2002; Elster et al. 1998; Kilb et al. 2002; Mangin et al. 2002; Martin and Siggins 2002; Sergeeva and Haas 2001). Characteristics of glycine receptors in these areas are regulated distinctly in terms of subunit compositions and their amounts as well as in terms of functional plasticity during or after development (Mangin et al. 2002). At an early stage of development, glycine and its agonists cause excitation rather than inhibition of receptive neurons because the concentration of Cl− in immature neurons is higher than that in developed neurons (Ehrlich et al. 1999; Kilb et al. 2002; Laube et al. 2002; Rivera et al. 1999). Such excitatory action of glycine mediated by subsynaptic or extrasynaptic glycine receptors may play an essential role in cellular and functional maturation of neurons (Flint and Kriegstein 1998; Legendre 2001). In the cerebellum, cellular immunoreactivity for glycine and the presence of transcripts for the neural glycine transporter (namely, GlyT2 subtype) have been described (Chen and Hillman 1993; Zafra et al. 1995). Both of them are considered essential for glycinergic transmission. Rampon et al. (1996) found that the immunoreactivity for glycine was positive both in neurons of deep cerebellar nuclei and in cerebellar Lugaro cells, one type of cortical inhibitory interneurons. Also, the mRNA transcripts for GlyT2 were detected in these cells in the cerebellum (Luque et al. 1995). Furthermore, electrical stimulation of Lugaro cells was found to evoke glycinergic synaptic currents in postsynaptic Golgi cells, the observation of which has confirmed functional glycinergic transmission in the cerebellum (Dumoulin et al. 2001).

The deep cerebellar nuclei of rodents can be divided into three subdivisions according to their location: the nucleus medialis, nucleus interpositus, and nucleus lateralis (Sastry et al. 1997; Sultan et al. 2002). Neurons in these deep cerebellar nuclei (DCN) receive input from three major extra-nuclear sources: excitatory mossy and climbing fibers and inhibitory Purkinje cell axons (Ito 1984; Sastry et al. 1997; Telgkamp and Raman 2002). The neurons in the DCN consist of three types: large glutamatergic projection neurons, smaller GABAergic projection neurons, and local interneurons (Aizenman et al. 2001; Anchisi et al. 2001; Czubayko et al. 2001; Pedroarena and Schwarz 2003). The interneurons are thought to colocalize both GABA and glycine as neurotransmitters, but little is known about their input and output connections (Baurle and Grusser-Cornelius 1997; Chen and Hillman 1993). Neither the synaptic currents in DCN neurons mediated by glycine nor the presence or properties of ionotropic glycine receptors on DCN neurons have been investigated in detail. Rather, spontaneous or evoked inhibitory synaptic currents (IPSCs) recorded in DCN neurons have been found to be completely abolished by the addition of bicuculline (Aizenman et al. 1998; Ouardouz

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and Sastry 2000; Sastry et al. 1997), indicating that they were exclusively GABAergic and that glycineric synapses, if any, were difficult to detect in the DCN.

In this study, the possible existence and functional properties of glycine receptors in DCN neurons were re-examined using the slice-patch technique and a rapid drug application system, the “Y tube” system, which had been modified for use in brain slice preparations (Kawa 2002a). To avoid possible regional differences among the DCN neurons, slices containing the nucleus interpositus were used, and neurons in the DCN with larger diameters were selected for whole cell recordings. The aims of this study were to determine whether functional ionotropic glycine receptors are expressed on DCN neurons and whether glycineric postsynaptic currents can be evoked by high K⁺ or electrical stimulation of presynaptic elements. The results of this study confirmed the above-stated speculation to be true in the rats studied (postnatal age, day 2–14) and revealed relevant properties of glycine receptors and glycineric transmission in the DCN. A preliminary report has appeared elsewhere (Kawa 2002b).

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Slice preparation

All experiments were carried out in accordance with the Guiding Principles of the Physiological Society of Japan. The procedures for preparing and preserving thin slices from a rat brain and for cleaning cells in the slices for patch-clamp recordings have been described in detail elsewhere (Kawa 2002a). Briefly, newborn male and female rats (Wister strain) on postnatal days 2–14 (P2–P14) were killed by decapitation after ether anesthesia, and the cerebellum was quickly dissected out of each rat and immersed for a few minutes in ice-cold bicarbonate-buffered saline. The tissue was cut sagittally into thin slices of 200 μm thickness with a vibrating slicer (DSK-1000, Dosaka, Kyoto, Japan). Each slice was transferred to a storage chamber containing oxygenated (95% O₂-5% CO₂) normal saline at 30°C. The cerebellar slices were then transferred to a recording chamber placed on the stage of a Zeiss Axioskop upright microscope. This chamber (volume, ~1 ml) was continuously perfused with oxygenated saline solution kept at room temperature (23–25°C). DCN in the cerebellum were easily identified with a low-magnification video camera (KP-140, Hitachi, Tokyo, Japan) under transillumination observation. Using a video-capture board (GV-VC72M, I.O. Data, Kanazawa, Japan), images of the cerebellar nuclei and mesh lines covering the cerebellar slice were saved as photo files and referred to for further identification of the neurons (Fig. 1A). When observed with Nomarski optics (Zeiss, Obercochen, Germany) using a long-working-distance 40× water-immersion objective, the location of neurons for recording with a whole cell patch clamp could be identified clearly (Fig. 1D). Whole cell recordings were made from large DCN neurons (>20 μm diam) located in the nucleus interpositus. Hence, according to previously reported anatomical data (Anchisi et al. 2001; Pedroarena and Schwarz 2003), most of the recorded neurons were considered to be glutamateric projection neurons. After each experiment, an image of the configuration of the recording system was taken using a low-magnification objective (5×) and saved as a photo file (Fig. 1C).

Electrical recordings

Whole cell current recordings were made from neurons in the DCN using patch pipettes with open resistances of 1.5–2 MΩ and an EPC-7 patch clamp amplifier with a 3-kHz internal filter (List Electronics, Darmstadt, Germany). The procedure used has been described in

FIG. 1. Preparation of slices of the cerebellum from the newborn rat. A: parasagittal section of 2 serial slices (1 and 2; 200 μm in thickness, at P9). The 2 slices were placed on the base of the recording chamber and fixed with a mesh (horizontal lines). Under transparent illumination, the cerebellar cortex and deep cerebellar nuclei (whitish mass in the center) were clearly recognized. A part of the brain stem (at bottom of the slice) and a part of the inferior colliculus (right) are also visible in each of the slices. B: a parasagittal slice of the cerebellum (at P5) stained with Giemsa’s solution. Neurons of the deep cerebellar nuclei (DCN) are distinctively recognized in the bottom of the figure. C: recording configuration from a DCN neuron (at P10), which was taken using a low-magnification objective (5×). This figure also shows a microelectrode used for electrical stimulation (top), a “Y tube” used for local perfusion (left), and a whole cell patch electrode (right). D: recording configuration from another DCN neuron (at P2; presumed projection neuron having a somatic diameter of 22 μm) observed with Nomarski optics using a long-working-distance 40× water-immersion objective.
detail previously (Kawa 2002a). Briefly, currents and membrane potentials were monitored with an oscilloscope and were recorded on videotape using a PCM/VCR recording device (PCM-501/ES, Sony). Data were digitalized at 10 kHz using a Digitada 1320A interface (Axon Instruments) and were also displayed on a pen recorder (R62N3, Rikadenki, Tokyo, Japan). Patch pipettes were pulled from a 1.5-mm capillary glass in two stages using a vertical pipette puller (PC-10, Narishige, Tokyo, Japan) and filled with intracellular solution. During whole cell recordings, the access resistance of the electrode was occasionally monitored by applying rectangular voltage pulses (2 mV, 100 ms) and by measuring the capacitative current amplitude. The access resistance usually remained in the range of 2–5 MΩ; the error in holding membrane potential caused by this was <1 mV. If recording conditions such as cell input resistance or access resistance changed or became unstable, the experiment was stopped. The liquid junction potentials (9.6 mV; inside negative) were determined and corrected by previously described procedures (Kawa 2002a). In some neurons, the I-V relationship of the glycine-induced currents was obtained using a saw-tooth voltage clamp (−60 ± 30 mV in 1 s), the voltage wave for which was generated by a function generator (FG-12113, NF Circuit Designing Block Co., Yokohama, Japan). In some experiments, glycinergic postsynaptic currents were evoked by electrical stimulation (voltage pulses of duration of 200 µs, intensity of 2–10 V, at 0.25 Hz) delivered to sites within the DCN through a glass micropipette filled with standard external saline (tip resistance, 20–50 MΩ; Fig. 1C). To confirm the effectiveness of stimulation, double-pulse stimulation with a train interval of 50 ms was also used.

Solutions and drugs

The external standard saline used for slicing and incubation contained (in mM) 125 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, and 25 glucose (pH 7.4 when bubbled with 95% O₂-5% CO₂). The “intertet” (pipette) solution (referred to as Cs-methanesulphonate internal saline) consisted of (in mM) 134 Cs-methanesulphonate (CH₃O₂SCs), 6 CsCl, 10 EGTA, 2 MgCl₂, 10 HEPES, and 2 Na₂-ATP (adjusted to pH 7.3 with CsOH). In this high-potassium external solution (containing 20 mM KCl), NaCl was replaced with equimolar KCl. The estimated equilibrium potential for Cl⁻ ions under the present experimental conditions is ~67 mV. Unless otherwise stated, membrane currents and synaptic currents in DCN neurons were recorded at the holding potential of −40 mV, because excitatory and inhibitory synaptic currents at this potential appeared distinctly as inward and outward currents, respectively. Drugs were applied using a rapid application technique, the “Y tube” method, usually for 10–20 s with an interval of 5 min (Kawa 2002a). A programmable pulse generator (SEN-3201, Nihon-Koden) controlled the magnetic valve of the “Y tube” system.

To block GABA_α receptors, bicuculline methiodide (BCC, 10 µM; Sigma) was added to the external solution, while to block glutamatergic amino-3-hydroxy-5-methyl-isoxazol-propionate (AMPA) receptors and glutamatergic N-methyl-D-aspartate (NMDA) receptors, 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 5 µM; RBI) and d-(-)-2-amino-5-phosphonovaleric acid (APV, 50 µM, Tocris), respectively, were added to the external solution. In some experiments, the following agents were also added to the external perfusion solution: glycine (10–2,000 µM), l-alanine (1 mM), L-serine (1 mM), taurine (1 mM), and GABA (10 µM). Strycline (1 µM) and TTX (1 µM) were also added when necessary to the external saline for blocking ionotropic glycine receptors and voltage-dependent Na⁺ channels, respectively. These drugs were obtained from RBI or Sigma. The drugs were dissolved in solution and kept frozen in aliquots, and they were thawed just before each experiment. CNQX was dissolved in dimethylsulfoxide (Wako) at a concentration of 5 mM and added to the perfusing saline at a final concentration of 5 µM in the dark.

Data analysis

The following software programs were used for analyses of amplitude distribution, time course of the synaptic currents, dose-response relations for the agonist, and electric charges carried by synaptic currents: Mini Analysis (Synaptosoft), Origin version 6 (Microcal Software), Excel 2000 (Microsoft), and KaleidaGraph (Synergy Software). All data are expressed as mean ± SD unless otherwise indicated. An unpaired Student’s t-test and Welch’s t-test were used to evaluate the statistical significance of differences between two groups being compared. For significant changes in the ratios, a test for the F distribution was used.

Histological study

After whole cell recordings, each slice was transferred to a small chamber and was fixed overnight with 10% formalin in a phosphate-buffered saline (PBS) and then gently rinsed four to five times with distilled water for several hours. During these processes, the chamber was shaken gently and continuously on a horizontal shaker (R-Inini, Taitec Co.). The preparation was transferred to a plastic dish containing distilled water (Falcon, Primaria 35-mm-diameter tissue culture dish, Becton Dickinson) and stained with Giemsa’s solution for 2–3 min (final dilution, 30–40-fold). Each slice was rinsed in a plastic dish containing distilled water and transferred to a glass plate. After having been half-dried, each slice was embedded in synthetic mounting medium (Aquatex, Merk, Germany). Each slice was viewed under an upright microscope (AxioVision system, Carl Zeiss; objective 63×), and photographs of the DCN neurons and the sites of recording were taken using an attached digital camera (Fig. 1B).

RESULTS

Identification of DCN neurons in the cerebellar slice

The DCN of the rat can be divided into three major subdivisions, nucleus medialis, nucleus interpositus, and nucleus lateralis, according to their locations. In parasagittal sections of the cerebellum under transparent illumination, deep cerebellar nuclei were clearly recognized as a pale mass (at P2–14, Fig. 1). At higher magnification under Nomarski optics (40×), the location of the recorded neurons in the DCN was readily confirmed by referring to grid lines covering the slice. In the present experiments, most of the whole cell recordings were made from large neurons in the nucleus interpositus (Fig. 1D). Boundaries between the DCN subnuclei were not always clear in slices from rats at P7 or younger. Recordings from neurons in the nucleus interpositus were attempted by carefully selecting an appropriate parasagittal slice in the incubation chamber. The location of the neurons was also checked by staining the slice with Giemsa’s solution after recording (Fig. 1B). Most of the neurons recorded were presumably projection neurons in the nucleus, having a glutamatergic nature as judged from their morphology (namely, large oval soma with diameter of more than 20 µm and multiple dendritic shafts). More detailed analyses of these neurons and other smaller neurons using intracellular staining are needed to determine the intricate makeup of the neural circuit during the early postnatal period.

Glycine-induced membrane currents in DCN neurons

When glycine (100 µM) was applied from a “Y tube” to a neuron in the DCN, large outward currents were induced in the cell at the holding potential of −40 mV (Fig. 2A). Glycine was applied from a “Y tube” for 10–20 s with an interval of 5 min

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The glycine-induced currents could be repeatedly observed in the same neuron without noticeable decay at holding potentials. Previous studies (22) reported similar results with the glycine-induced currents in the cerebellar nuclei. In our study, the glycine-induced currents were normalized to the control value (obtained by 1 mM glycine as shown by double circles; average value, 1.240 pA; n = 4) is shown on the right. The dotted line connecting the data are an estimated sigmoidal relation, having an apparent dissociation constant of 170 μM and a Hill coefficient of 1.6.

I-V relationship of glycine-induced membrane currents in DCN neurons

To further characterize the glycine-induced currents in DCN neurons, the I-V relationship and the reversal potential of the currents were determined. Figure 2A shows the I-V relationship of glycine-induced currents obtained using a saw-tooth voltage clamp (voltage wave, −60 ± 30 mV over a period of 1 s). In this experiment, glycine at a low concentration (50 μM) was used to avoid desensitization of the glycine receptor. Furthermore, TTX was added to the external saline to block generation of voltage-dependent Na+ currents. The representative I-V relationship showed mild outward rectification, which could be approximated by a constant field equation (dotted line on the I-V relationship) and thus may be due to the intrinsic property of Cl− ions permeating through the open channel of the receptor. The reversal potential obtained using a saw-tooth voltage clamp was −62 ± 2 mV (n = 4). The reversal potential showed good agreement with the estimated Cl− equilibrium potential (−67 mV) if we take into consideration the fact that the internal concentration of Cl− (10 mM) tended to increase due to influx of the external saline (containing 136 mM Cl−) into the patch electrode during establishment of the whole cell configuration. In four other neurons (at P4–P9), the I-V relationship was obtained by measuring peaks of glycine-induced currents at various holding potentials (ranging from −50 to −70 mV). As the holding potential shifted to more negative values from −60 mV, the peak of glycine-induced currents gradually decreased and became inward at holding potentials below −64 mV. The I-V relationship of the currents showed mild outward rectification like in Fig. 2A. The average of the reversal potential was −62 ± 3 mV (n = 4), when measured after interpolation to the voltage axis. These results strongly indicate that currents induced by glycine in DCN neurons are carried predominantly by Cl−.
DCN neurons, currents induced by 1 mM of each substance were recorded as a primary measure. Each agonist was applied only for 12–15 s with an interval of 5 min or more (Fig. 3B, top left). β-Alanine can also activate glycine receptors, but it was not included in this study due to presumed simultaneous activation of GABA<sub>A</sub> receptors on the same neuron. The relative amplitude of agonist-induced currents to that of glycine (100 μM) in each cell was obtained and averaged (n = 4). The results presented in Fig. 3B (top right) show that glycine (100 μM) has the highest potency among the agonists examined followed by taurine (1 mM, relative potency of 97 ± 20%), L-alanine (1 mM, relative potency of 46 ± 11%), and L-serine (1 mM, relative potency of 23 ± 8%).

Strychnine is thought to be one of the specific blockers for ionotropic glycine receptors. In DCN neurons, the blocking effects of strychnine on whole cell currents induced by glycine or its agonists were examined (Fig. 3B, bottom). Representative traces in Fig. 3B (bottom left) show that strychnine at 1 μM suppressed the whole cell currents completely when induced by glycine (100 μM), taurine (1 mM), L-alanine (1 mM), or L-serine (1 mM). When the concentration of glycine was increased by 10-fold, definite, but still small, outward currents were detected in the presence of 1 μM strychnine (bottommost trace, glycine at 1 mM). It is likely that a high concentration of glycine can attenuate the blocking effect of strychnine since it is a competitive antagonist. Current amplitudes thus obtained are plotted in Fig. 3B (bottom right) after normalization to the control amplitude (which was obtained by 100 μM glycine).

Interestingly, the currents induced by L-alanine (1 mM) or L-serine (1 mM) were very small but seemed inward. One possible explanation is that transporters capable of uptaking these amino acids may be electrogenic and induce inward currents under the experimental conditions. Alternatively, these amino acids might bind to some sites of ionotropic or metabotropic receptors on the neuron and activate inward currents or inhibit steady outward currents of the cell. Due to the small amplitudes (<10 pA), further analyses are needed.

**Specificities of strychnine and bicuculline on glycine and GABA<sub>A</sub> receptors in DCN neurons**

To determine the specificities of strychnine (a glycine receptor antagonist) and BCC (a GABA<sub>A</sub> receptor antagonist), their effects on the currents induced by glycine or by GABA were examined in the same DCN neuron. The technique used was two-step application with a “Y tube.” In the experiment for which traces are shown on the left in Fig. 4Aa, glycine (100 μM) was applied to a DCN neuron from a “Y tube.” Between 7 and 8 s after the start of application, the first-application saline containing glycine (100 μM) was changed to the second-application saline that contained either 0 or 1 μM strychnine in addition to glycine (100 μM). The thick trace in the figure was obtained with 0 μM strychnine, whereas the thin trace was obtained with 1 μM strychnine. The abrupt reduction in current traces at the time of switching of the external solution (i.e., between 7 and 8 s after the start of application) was due to change in flow of the solutions. As shown by control glycine- or GABA-induced current traces (thick traces in Fig. 4A), the current amplitude after the switching recovered rapidly to the original level. Thus the effect of change in flow of the external solution on the following analyses, if any, was thought to have

**Pharmacological properties of glycine receptors in DCN neurons**

Glycine receptors are also activated by substances other than glycine, such as taurine, L-alanine, and L-serine. To examine the potencies of these substances on the glycine receptors of DCN neurons, currents induced by 1 mM of each substance were recorded as a primary measure. Each agonist was applied only for 12–15 s with an interval of 5 min or more (Fig. 3B, top left). β-Alanine can also activate glycine receptors, but it was not included in this study due to presumed simultaneous activation of GABA<sub>A</sub> receptors on the same neuron. The relative amplitude of agonist-induced currents to that of glycine (100 μM) in each cell was obtained and averaged (n = 4). The results presented in Fig. 3B (top right) show that glycine (100 μM) has the highest potency among the agonists examined followed by taurine (1 mM, relative potency of 97 ± 20%), L-alanine (1 mM, relative potency of 46 ± 11%), and L-serine (1 mM, relative potency of 23 ± 8%).

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Effects of picrotoxin on glycine receptors in DCN neurons

Picrotoxin is a classical noncompetitive GABAA receptor antagonist, but it also blocks homomeric glycine receptors consisting solely of α subunits at a low dose (Mangin et al. 2002). The effects of picrotoxin on glycine receptors of DCN neurons were studied by a two-step application technique using a “Y tube.” Representative traces shown in the left of Fig. 4B are currents obtained with 0 μM picrotoxin (thick trace) and those obtained with 10 μM picrotoxin (thin trace). The time course of the blocking effects of picrotoxin is shown on the right. The difference between the two current traces was calculated and normalized to the value of the control trace (i.e., thick trace). The difference shows that the blocking effect reached a steady level at about 6 s after the start of picrotoxin application. The blocking of currents (in % measured at the steady level) was 45 ± 8% (n = 4) when measured from DCN neurons at P2–P3. This suggests that the glycine receptors expressed on DCN neurons at P2–P3 are heterogeneous in their subunit composition, and about 45% may be homomeric receptors, consisting solely of α subunits and being sensitive to picrotoxin, while 55% of the receptors have presumably formed heteromers consisting of α and β subunits and thus show resistance to picrotoxin.

ACh- and high K+ -induced synaptic currents observed in DCN neurons

Figure 5 shows that glycinergic synaptic transmission is actually present in DCN neurons. After blocking GABAergic synaptic currents by BCC (10 μM), the frequency of spontaneous synaptic currents, either inward or outward, was generally low in DCN neurons (measured over a period of 20–60 s from each neuron at P6–P9). The mean frequency of spontaneous inward synaptic currents was 0.29 ± 0.02 Hz (n = 6). To facilitate detection of the outward synaptic currents shown in Fig. 5A, the holding potential of the neuron during recording was kept at −10 mV. Most recordings show that no events or only one event occurred during a period of 20 s. Accordingly, the mean frequency of the spontaneous outward synaptic currents was only 0.03 ± 0.02 Hz (n = 6 at P6–P9). When 100 μM ACh was applied around the neuron from a “Y tube,” the
neurons showed such facilitation of synaptic currents by 100 
µM ACh (3 neurons at P5, 1 neuron at P7, 1 neuron at P8, and 1 neuron at P11). The ACh-induced synaptic currents were 
sensitive to strychnine (1 µM; Fig. 5Ab), thus indicating a 
glycinergic nature (n = 3). The facilitatory effect of ACh is 
thought to be due to the presence of nicotinic receptors in 
presynaptic glycinergic neurons (cf. Kawa 2002a). Thus it is 
likely that glycinergic neurons or fibers are actually present in 
cerebellar nuclei of the rat and that they make synapses on the 
DCN neurons.

Next, as shown in Fig. 5B, whole cell membrane currents 
were recorded in the presence of 50 µM CNQX, 50 µM APV, 
and 10 µM BCC, which blocked glutamatergic APMA recep-
tors, glutamatergic NMDA receptors, and GABA A receptors, 
respectively. When external saline containing 20 mM K + 
was applied around the DCN, whole cell currents of the neuron 
showed slow but large inward deflection (Fig. 5B). Many 
spike-like outward currents overlapping the slow inward 
currents were observed. The spike-like currents seemed to consist of 
summated synaptic currents as judged from their amplitudes and 
time courses. These spike-like currents disappeared in the 
presence of strychnine (1 µM). It is thus likely that the spike-
like outward currents are glycineric postsynaptic currents in 
the DCN neuron, evoked by presynaptic depolarization by 20 
mM K + . Forty DCN neurons (P2–P14) were challenged with 
high K + application, and definite spike-like currents were 
detected from 14 neurons. No remarkable differences between 
rates of detection were found in three groups of DCN neurons 
at different postnatal ages (P2–P5, P6–P9, and P10–P14; Fig. 
5B, bottom). For more qualitative determination, the time 
integral of the spike-like postsynaptic currents (i.e., electric charges carried by these synaptic currents) was obtained using 
the software programs Mini Analysis and Excel 2000. From 5 
to 15 s after the start of K + application, synaptic currents 
evoked in each neuron having a peak amplitude of more than 
5 pA (above the noise level during K + application) and half-
width of 6–30 ms were detected. The total number of synaptic 
events thus measured showed considerable intercell variation 
even among these 14 neurons, with a range of 9–108 events 
per 10 s (37 ± 30 events per 10 s). Average electric charges 
were calculated by using the software programs after cells had 
been divided into three groups of different postnatal ages (Fig. 
5B, bottom right). From P2–P5 to P6–P9, the average value 
increased significantly (P < 0.05). This significant increase 
may reflect development in the pre- or postsynaptic elements 
during this period or it may reflect an increased sensitivity of 
the presynaptic terminals to 20 mM K + , possibly due to a 
hyperpolarizing shift of the resting potential during develop-
ment.

Glycinergic synaptic currents evoked in DCN neurons

In the presence of three specific antagonists for blocking 
AMPA, NMDA, and GABA A receptors (5 µM CNQX, 50 µM 
APV and 10 µM BCC, respectively), electrical stimulation 
could still evoke postsynaptic currents in the DCN neurons. 
As can be seen in a representative current trace shown in Fig. 
6A, the synaptic currents rose to a peak in a period of 1.5–2.5 ms 
and then decayed to the resting level in about 80 ms (at −40 
µV, at 23–25°C), the time course of which can be approxi-
mated by a single exponential curve. When the neuron was
hyperpolarized, the decay time course became faster, while it became slower when the neuron was depolarized. The dependence of half-decay time of the synthetic currents on holding potential is shown on the left of Fig. 6A. The slope of the regression line (dotted line) is 0.16 s/V. Similar voltage dependence has been observed in glycine receptors of rat auditory brain stem neurons (Kungel and Friauf 1997). In subsequent experiments, double-pulse electrical stimulation was usually applied (duration of 200 μs, intensity of 2–10 V, pulse interval of 50 ms, train interval of 4 s). This is because more synaptic currents could be observed during a given time, thus enabling faster estimation of the threshold of stimulus intensity. In the present study, only the first responses evoked by double pulse-stimulation were analyzed. Small-sized neurons (with diameters of 12 μm or less) at a distance of <200 μm from the recorded neuron were primarily chosen for sites of stimulation. The intensity of stimulation used was a twofold value of the threshold intensity for evoking minimal glycinergic synaptic currents, and it was kept constant during the recording. If the threshold intensity exceeded 5 V, a search for a new site for stimulation in the DCN was made. To characterize the glycinergic synaptic currents thus evoked, the I-V relationship was obtained by measuring the synaptic currents at various levels of holding potential (+20 to −90 mV). The I-V relationship showed mild outward rectification (Fig. 6A, right) like that of the glycine-induced whole cell currents. By an interpolation to the voltage axis, the reversal potential of the synaptic currents was determined to be −62 ± 2 mV (n = 4), which is consistent with those of glycine-induced membrane currents. Furthermore, the synaptic currents evoked by electrical stimuli were completely blocked by strychnine (1 μM) but recovered slowly after washing in external saline containing 5 μM CNQX, 50 μM APV, and 10 μM BCC (Fig. 6B, left). In the presence of TTX (1 μM), these synaptic currents were also reversibly blocked (Fig. 6B, right), indicating that the glycinergic transmission depended on generation of action potentials in the slice. The slow time course of recovery from the blocking by strychnine, compared with that of TTX, may reflect the low rate of unbinding of strychnine at the glycine receptor. In the present study, reversal of strychnine blockage was examined, when necessary, after washing with control saline for more than 40 min. Thus it seems reasonable to conclude that the synaptic currents observed in the DCN neurons are mediated by activation of ionotropic glycine receptors. In this series of experiments, 7 of the 15 DCN neurons at P7–P10 studied showed glycinergic IPSCs evoked by electrical stimuli. Electrical stimuli (duration of 200 μs; maximum intensity, 5 V) for each neuron were applied to four or less surrounding sites. The rate of detection of glycinergic inhibitory postsynaptic currents (IPSCs) might have been higher if a more rigorous search for stimulation sites had been carried out. Detailed morphology of these neurons and changes in detection rate during postnatal development remain to be studied.

**DISCUSSION**

This study showed that ionotropic strychnine-sensitive glycine receptors are expressed in DCN of the rat (Figs. 3 and 4). When ACh, high K+, or electrical stimulation was applied to the DCN, fast glycinergic synaptic currents were evoked in the
neurons of DCN (Figs. 5 and 6). There are three types of neurons in the DCN: large glutamatergic projection neurons, middle-sized GABAergic projection neurons, and small local interneurons. Judging from their sizes, most of the neurons (>20 μm diam) recorded are thought to have been excitatory projection neurons, and some may have been GABAergic projection neurons. These two types of projection neurons were often regarded as the same group (Anchisi et al. 2001; Czubayko et al. 2001), but they seem to be distinguishable due to the smaller sizes of GABAergic projection neurons than those of glutamatergic projection neurons (Aizenman et al. 2003; Pedroarena and Schwarz 2003). Involvement of small local interneurons (±15 μm diam; cf. Aizenman et al. 2003) in this study was unlikely; this was also judged from the value of whole cell membrane capacity of the cells (±30 pF; Fig. 2A). Thus it is likely that glycineric transmission is physiologically functional in principal DCN neurons (i.e., projection neurons) of the rat.

Properties of glycineric transmission in DCN

The rate of detection of glycineric synaptic currents in DCN neurons by using high K+ saline was only about 35% (Fig. 5B). This low detection rate may explain the failure in previous studies to detect glycineric synaptic currents in DCN neurons (e.g., Pedroarena et al. 2001). The low detection rate presumably reflects lower prevalence of glycineric synapses than GABAergic synapses on DCN neurons (Aizenman et al. 1998; Anchisi et al. 2001; Momiyama and Takahashi 1994; Ouardouz and Sastry 2000). The magnitude and prevalence of glycineric synaptic currents may be influenced by their developmental stages (Gao et al. 2001; Russier et al. 2002) and also by the amount of specific glycine transporters expressed (Luque et al. 1995; Zafra et al. 1995). In this regard, there is an interesting possibility that glycine might be co-released with GABA from existent “GABAergic” terminals such as those of GABAergic interneurons and generate synaptic currents with differed time courses (Gao et al. 2001; Russier et al. 2002).

Molecular subtypes of glycine receptors

Previous studies have shown that mRNAs or subunit molecules of glycine receptors are present in the DCN of the rat. For example, α1, α2, and β subunit mRNAs were detected in all of the three subnuclei of DCN of the neonatal rat at P7 (Sato et al. 1992). In another study, α1 and β subunits of the glycine receptor mRNA were detected in the lateral nucleus of DCN of adult rats (Malosio et al. 1991). Intense labeling with a β subunit probe was seen in the whole cerebellum after embryonic day 19, but α2 hybridization signals were seen only in the DCN neurons during the period from P0–P15 (Malosio et al. 1991). Morphological studies on the development of DCN neurons in the rat have suggested that maturation of the deep cerebellar nuclei appear to be at an advanced stage by the time of birth (Altman and Bayer 1978; Ito 1984; Sastry et al. 1997). These results, however, should be interpreted carefully because it has been shown that massive GABAergic synapses are formed on DCN neurons during the postnatal period from P1 to P15 (Garin and Escher 2001) and that the metabolic state of DCN neurons also undergoes dramatic changes after birth (Console-Bram et al. 1996).

In the developing spinal cord, a switch occurs of receptor subunit expression from mostly α2 homomers at birth to α1β heteromers at around 20 days after birth (Laube et al. 2002). A similar switch in glycine receptors was also found in brain stem motoneurons and in the substantia nigra (Mangin et al. 2002; Singer et al. 1998). In contrast, it has been reported that there is no evidence of a molecular switch in midbrain neurons (Garcia-Alcocer et al. 2001). It is notable that subtypes of glycine receptors show different sensitivities to picrotoxin, a blocker of agonist-gated CI− channels (Legendre 2001; Mangin et al. 2002). For example, adult-type α1β heteromeric glycine receptors are resistant to picrotoxin, whereas juvenile-type homomeric α2 receptors are sensitive to picrotoxin at a low concentration (±10 μM). In the present preparation, picrotoxin-resistant glycine receptors, presumably the adult type, were already expressed on DCN neurons at P2–P3 (Fig. 4B), which is consistent with the results of an in situ hybridization study (Sato et al. 1992). How this fraction of glycine receptors changes during maturation is a new concern in DCN and the striatum (Sergeeva and Haas 2001).

Localization of glycine receptors

The results of this study indicate that the majority of glycine receptors exist in the extrasynaptic region of DCN neurons, as judged from a comparison of the amplitudes of glycine-induced whole cell currents (range, 300–600 pA at 100 μM glycine; cf., Figs. 2A and 4A) and those of synaptically evoked glycineric currents (range, 10–40 pA; Figs. 5B and 6B). Glycine receptors at extrasynaptic sites may also act as taurine receptors, which modulate the resting membrane currents, or as trophic receptors during development (Flint et al. 1998; Furuya et al. 2000; Mangin et al. 2002; Mori et al. 2002; Tapia et al. 2000). It has been shown that DCN neurons in adult rat cerebella expressed α1 and α2 subunit mRNAs both in somata and dendrites, while neurons of the cerebellar cortex expressed these mRNAs only in somata (Racca et al. 1998). It has also been revealed that anchoring of glycine receptors at synaptic sites is under refined metabolic control and regulated by specific interaction between molecules of the receptor and the cytoskeleton (Kneussel and Betz 2000; Legendre 2001). These observations suggest that there are intricate mechanisms for controlling glycine receptor expression and localization during and after development.

Origin of glycineric innervation and its functional roles

Based on results of histochemical observation, some types of neurons in the DCN, particularly local interneurons, are believed to contain GABA, glycine, or both as neurotransmitters (Baurle and Grusser-Cornehls 1997; Rampon et al. 1996). Glycine transporters of neuron-specific type (GlyT2), a marker for glycineric terminals, have also been detected on neurons of the DCN (Luque et al. 1995; Zafra et al. 1995). The presence of glycine in local interneurons has been confirmed by electron microscopy (Chen and Hillman 1993). Thus in this study, the most probable origin of presynaptic elements containing and releasing glycine is neurons in the DCN, particularly local interneurons. This speculation is consistent with the results obtained using electrical stimuli (delivered to a small neuron in the DCN; Fig. 6). The possibility of glycineric Purkinje axons
seems unlikely from results of previous studies (Aizenman et al. 1998; Ito 1984; Ouardouz and Sastry 2000; Telgkamp and Raman 2002). Therefore the most probable source of glycine as a neurotransmitter on DCN neurons is interneurons of the DCN, which may release pure glycine or glycine and GABA simultaneously. It is relevant in this regard to note the phenomenon of co-localization of GABA and glycine in the same terminal and changes in the neurotransmitter from GABA to glycine or vice versa at certain synapses during development or under some diseased conditions can occur (Gao et al. 2001; Garcia-Alcocer et al. 2001; Singer and Berger 2000; Sultan et al. 2002; Turecek and Trussell 2002; Zhou et al. 2002). Additionally, in the cerebellar cortex of the rat, pure and mixed glycineergic and GABAergic transmission has been proved electrophysiologically in Golgi cells (Dumoulin et al. 2001).

The roles of these glycine receptors and glycineergic transmission in the brain seem crucial for proper neural function and for regulated development of the brain (Dutschmann and Paton 2002; Legendre 2001; Zhou et al. 2002). In many immature neurons, glycine causes excitation instead of inhibition, which may trigger several Ca2+-dependent phenomena essential for the developmental process, such as neuronal proliferation, migration, and synaptic maturation (Flint et al. 1998; Kirsch and Betz 1998; Legendre 2001). The molecular mechanism underlying the shift from an excitatory nature to an inhibitory nature is thought to be the development of a K+/Cl− co-transporter that extrudes intracellular Cl− and promotes fast hyperpolarizing postsynaptic inhibition in the brain (Eilers et al. 2001; Mikawa et al. 2002; Rivera et al. 1999). When and how such a shift occurs in DCN neurons, including those at the embryonic stage, are issues for a future study. The results of this study should contribute to elucidation of the functional roles of glycine, which has so far been thought to be a minor neurotransmitter in the cerebellum but may be essential under physiological and pathological conditions in developing as well as in matured nervous systems.

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DISCLOSURES

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