Glycine receptors and glycinergic synaptic transmission in the deep cerebellar nuclei of the rat: a patch-clamp study

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ABSTRACT
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 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), D-(-)-2-amino-5-phosphonovaleric acid (APV) and bicuculline, which antagonize AMPA-, 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 reversal potential of -62 mV and showed sensitivities to strychnine and tetrodotoxin. Thus, the present 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 central nervous system, primarily in the spinal cord and brain stem (Legendre 2001; Laube et al. 2002). 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 (Elster et al. 1998; Sergeeva and Haas 2001; Chattipakorn and McMahon 2002; Kilb et al. 2002; Mangin et al. 2002; Martin and Siggins 2002). 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; Rivera et al. 1999; Kilb et al. 2002; Laube et al. 2002). 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, namely, the nucleus medialis, nucleus interpositus and nucleus lateralis (Sastry et al. 1997; Sultan et al. 2002). Neurons in these 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 (Anchisi et al. 2001; Czubayko et al. 2001; Aizenman et al.
The interneurons are thought to colocalize both GABA and glycine as neurotransmitters, but little is known about their input and output connections (Chen and Hillman 1993; Baurle and Grusser-Cornehls 1997). 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 (Sastry et al. 1997; Aizenman et al. 1998; Ouardouz and Sastry 2000), indicating that they were exclusively GABAergic and that glycinergic synapses, if any, were difficult to detect in the DCN.

In the present 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, that 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 glycinergic 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 ages from day 2 to day 14) and revealed relevant properties of glycine receptors and glycinergic transmission in the DCN. A preliminary report has appeared elsewhere (Kawa 2002b).
METHODS

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 then 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 in thickness with a vibrating slicer (DSK-1000, Dosaka Co. Ltd, 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, ca 1 ml) was continuously perfused with oxygenated saline solution kept at room temperature (23-25 °C). Deep cerebellar nuclei (DCN) in the cerebellum were easily identified with a low-magnification video camera (KP-140, Hitachi, Japan) under transillumination observation. Using a video-capture board (GV-VCP2M, I.O. Data, 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, Germany) using a long-working-distance 40x 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 (with diameters larger than 20 µm) located in the nucleus interpositus. Hence, according to previously reported anatomical data (Anchis et al. 2001; Pedroarena and Schwarz, 2003), most of the recorded neurons were considered to be glutamatergic projection neurons. After each experiment, an image of the configuration of the recording system was taken using a low-magnification objective (5x) 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 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 (SONY PCM-501/ES, Japan). Data were digitalized at 10 kHz using a Digidata 1320A interface (Axon Instruments, CA, USA) and were also displayed on a pen recorder (R62N3, Rikadenki Co., 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 less than 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., Japan). In some experiments, glycinergic postsynaptic currents were evoked by electrical stimulation (voltage pulses of duration of 200 µs, intensity of 2 to 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.

Fig. 1 near here

**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 and 5% CO₂). The ‘internal’ (pipette) solution (referred to as Cs-methanesulphonate internal saline) consisted of (in mM): 134 Cs-methanesulphonate (CH₃O₃SCs), 6 CsCl, 10 EGTA, 2 MgCl₂, 10 Hepes and 2 Na₂-ATP (adjusted to pH 7.3 with CsOH). In the 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, Tokyo, Japan) controlled the magnetic valve of the ‘Y tube’ system.

To block GABA\(_A\) receptors, bicuculline methiodide (BCC, 10 \(\mu\)M; Sigma, USA) was added to the external solution, while to block glutamatergic amino-3-hydroxy-5-methyl-isoxazol-propionate (APMA) receptors and glutamatergic N-methyl-D-aspartate (NMDA) receptors, 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 5 \(\mu\)M; RBI, Germany) and D(-)-2-amino-5-phosphonovaleric acid (APV, 50 \(\mu\)M, Tocris, UK), respectively, were added to the external solution. In some experiments, the following agents were also added to the external perfusion solution: glycine (10-2000 \(\mu\)M), L-alanine (1 mM), L-serine (1 mM), taurine (1 mM) and GABA (10 \(\mu\)M). Strychnine (1 \(\mu\)M) and tetrodotoxin (TTX, 1 \(\mu\)M) 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 (USA) or Sigma (USA). 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, Japan) at a concentration of 5 mM and added to the perfusing saline at a final concentration of 5 \(\mu\)M 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, GA, USA), Origin version 6 (Microcal Software, MA, USA), Excel 2000 (Microsoft, WA, USA) and KaleidaGraph (Synergy Software, PA, USA). All data are expressed as means±S.D. unless otherwise indicated. An unpaired Student's \(t\) test and Welch’s 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 4-5 times with distilled water for several hours. During these processes, the chamber was shaken gently and continuously on a horizontal shaker (R-IImini, Taitec Co., Japan). The preparation was transferred to a plastic dish containing distilled water (Falcon, Primaria 35-mm-diameter tissue culture dish, Becton Dickinson, USA) and stained with Giemsa’s solution for 2-3 min (final dilution, 30-40 fold). Each slice was then 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 up-right microscope (AxioVision system, Carl Zeiss Co., Germany, objective x63), 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 deep cerebellar nuclei (DCN) of the rat can be divided into three major subdivisions, namely, 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 ages of P2-14, Fig. 1). At higher magnification under Nomarski optics (40x), 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 unless otherwise stated. During the application of glycine, the currents decayed slowly to a steady level, and the time courses of decay of currents in neurons at different postnatal ages were similar (ex, P4 and P11 in Fig. 2). All of the 49 DCN neurons studied (from P2 to P13) responded to glycine (100 μM) by generating outward currents. The glycine-induced currents could be repeatedly observed in the same neuron without noticeable deterioration under the present experimental conditions (i.e., application of 100 μM glycine for 10-20 s with an
interval of 5 min). Peak amplitudes of the observed currents in the 49 neurons ranged from 54 pA to 1110 pA, with mean±S.D. of 460±273 pA, and showed no obvious relation to postnatal age. In the right part of Fig. 2A, the neurons have been divided into four groups according to age, and whole-cell membrane capacity of the neuron (upper) and density of the glycine-induced current (as pA/pF; lower) are shown. The latter was obtained by normalizing the peak amplitude of the current induced by glycine (100 µM) to the whole-cell membrane capacity of the cell. There were no significant differences among the four groups of neurons in both of the figures (P<0.05).

Figure 2B shows the dose-response relation of glycine-induced membrane currents in DCN neurons. The external medium used was the standard external saline containing TTX (1 µM) but containing none of the antagonists for the neuronal receptors. When glycine at a concentration of 1 mM or higher was applied to the neuron, the interval of the application was doubled to 10 min to ensure recovery from desensitization. The representative traces shown on the left were obtained from a DCN neuron at postnatal day 6 (P6) under these conditions (holding potential, -40 mV). The dose-response relationship obtained from 4 neurons (at P5-9) after normalization of the peak amplitude of glycine-induced currents in each cell to the control value (obtained by 1 mM glycine as shown by double circles; average value, 1240 pA, n=4) is shown on the right. The dotted line overlapping the data is an estimated sigmoidal relation, having an apparent dissociation constant of 170 µM and a Hill coefficient of 1.6.

**Figure 3 near here**

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

To further characterize the glycine-induced currents in DCN neurons, the current-voltage (I-V) relationship and the reversal potential of the currents were determined. Figure 3A 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 $-62\pm2$ mV (mean$\pm$S.D., n=4). The reversal potential showed good agreement with the estimated $\mathrm{Cl}^-$ equilibrium potential (-67 mV) if we take into consideration the fact that the internal concentration of $\mathrm{Cl}^-$ (10 mM) tended to increase due to influx of the external saline (containing 136 mM $\mathrm{Cl}^-$) into the patch electrode during establishment of the whole-cell configuration. In four other neurons (at P4 to 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. 3A. The average of the reversal potential was $-62\pm3$ mV (mean$\pm$S.D., 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 $\mathrm{Cl}^-$. 

**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, upper left). $\beta$-Alanine may also activate glycine receptors but was not included in the present study due to presumed simultaneous activation of $\mathrm{GABA}_A$ receptors on the same neuron. The relative amplitude of agonist-induced currents to that of glycine (100 $\mu$M) in each cell was obtained and averaged (n=4). The results presented in Fig. 3B (upper right) show that glycine (100 $\mu$M) has the highest potency among the agonists examined followed by taurine (1 mM, relative potency of 97$\pm$20%), L-alanine (1 mM, relative potency of 46$\pm$11%) and L-serine (1 mM, relative potency of 23$\pm$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, lower). Representative traces in Fig. 3B (lower left) show that strychnine at 1 $\mu$M 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 (lowermost 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 (lower 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 up-taking 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.

**Figure 4 near here**

*Specificities of strychnine and bicuculline on glycine and GABA_A receptors in DCN neurons*

To determine the specificities of strychnine (a glycine receptor antagonist) and BCC (a GABA_A 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. 4A(a), 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 zero or 1 µM strychnine in addition to glycine (100 µM). The thick trace in the figure was obtained with zero strychnine, while 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 been small. Blocking effects of strychnine were evaluated at 22 s after the start of the first application (i.e., at the
right-side end of the trace displayed). For BCC, measurements were made using second-application saline containing either zero or 10 µM BCC in addition to glycine (100 µM), as shown in the traces on the right side of Fig. 4A(a). Results of similar experiments on GABA-induced currents are shown in Fig. 4A(b). The representative traces revealed that the currents induced by GABA (100 µM) had low sensitivity to strychnine (1 µM) but high sensitivity to BCC (10 µM). The lower graphs in Fig. 4A summarize the results obtained from 4 cells at P7-11. The graphs show that the remaining amounts of glycine-induced currents after blockage by strychnine and BCC were 8±4% (mean±S.D., n=4) and 93±5% (mean±S.D., n=4), respectively, while the amounts of GABA-induced currents after blockade by strychnine and BCC were 85±5% (mean±S.D., n=4) and 1±2% (mean±S.D., n=4), respectively. These results confirmed the specificities of strychnine and BCC on the glycine and GABA receptors, respectively, of the present preparation and thus provided a pharmacological basis for isolation of glycine currents in the following experiments. The specificities are consistent with those reported previously (Jonas et al. 1998; Sergeeva 1998; Russier et al. 2002; Turecek and Trussell 2002).

**Effects of picrotoxin on glycine receptors in DCN neurons**

Picrotoxin is a classical non-competitive GABA<sub>A</sub> 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 zero picrotoxin (thick trace) and those obtained with 10 µM picrotoxin (thin trace). The time course of the blocking effects of picrotoxin is shown in the right part of the figure. 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 six seconds after the start of picrotoxin application. The blocking of currents (in % measured at the steady level) was 45±8% (mean±S.D., n=4) when measured from DCN neurons at P2-3. This suggests that the glycine receptors expressed on DCN neurons at P2-3 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 P6-9). The mean frequency of spontaneous inward synaptic currents was 0.29±0.10 Hz (mean±S.D., 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 (mean±S.D., n=6 at P6-9). When 100 μM ACh was applied around the neuron from a ‘Y tube’, the frequency of the spontaneous outward synaptic currents transiently increased (Fig. 5A(a); A part of the trace is expanded in lower part of the figure.). In a total 10 DCN neurons studied (3 neurons at P5, 3 neurons at P7, 3 neurons at P8 and one neuron at P11), 6 neurons showed such facilitation of synaptic currents by 100 μM ACh (3 neurons at P5, one neuron at P7, one neuron at P8 and one neuron at P11). The ACh-induced synaptic currents were sensitive to strychnine (1 μM; Fig. 5A(b)), 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 5 μM CNQX, 50 μM APV and 10 μM BCC, which blocked glutamatergic APMA-receptors, glutamatergic NMDA-receptors and GABAA-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 glycinergic
postsynaptic currents in the DCN neuron, evoked by presynaptic depolarization by 20 mM K⁺. Forty DCN neurons (at ages from P2 to 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 (P2-5, P6-9 and P10-14; Fig. 5B lower). 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 inter-cell variation even among these 14 neurons, with a range of 9 to 108 events per 10 s (mean±S.D.: 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, lower right). From P2-5 to P6-9, the average value increased significantly (P<0.05). This significant increase may reflect development in the pre- or post-synaptic 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 development.

**Figure 6 near here**

**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 mV, at 23-25 °C), the time course of which can be approximated 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 synaptic currents on holding potential is shown in the left part 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 brainstem 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 less than 200 µm from the recorded neuron were primarily chosen for sites of stimulation. The intensity of stimulation used was a two-fold 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 (mean±S.D.; 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 to 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, seven of the 15 DCN neurons at P7-10 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 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
The present study showed that ionotropic strychnine-sensitive glycine receptors are expressed in deep cerebellar nuclei (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 (with diameters larger than 20 µm) 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 (Aizenmann et al. 2003; Pedroarena and Schwarz 2003). Involvement of small local interneurons (with diameters of 15 µm or less; cf. Aizenman et al. 2003) in the present study was unlikely, as was also judged from the value of whole-cell membrane capacity of the cells (30 pF or more; Fig. 2A). Thus, it is likely that glycinergic transmission is physiologically functional in principal DCN neurons (i.e., projection neurons) of the rat.

Properties of glycinergic transmission in DCN
The rate of detection of glycinergic 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 glycinergic synaptic currents in DCN neurons (ex., Pedroarena et al. 2001). The low detection rate presumably reflects lower prevalence of glycinergic synapses than GABAergic synapses on DCN neurons (Momiyama and Takahashi 1994; Aizenman et al. 1998; Ouardouz and Sastry 2000; Anchisi et al. 2001). The magnitude and prevalence of glycinergic 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, $\alpha_1$, $\alpha_2$ and $\beta$ subunit mRNAs were detected in all of the three subnuclei of DCN of the neonatal rat at postnatal day 7 (Sato et al. 1992). In another study, $\alpha_1$ and $\beta$ 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 $\beta$ subunit probe was seen in the whole cerebellum after embryonic day 19, but $\alpha_2$ hybridization signals were seen only in the DCN neurons during the period from postnatal day 0 to day 15 (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-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, there occurs a switch of receptor subunit expression from mostly $\alpha_2$ homomers at birth to $\alpha_1\beta$ heteromers at around 20 days after birth (Laube et al. 2002). A similar switch in glycine receptors was also found in brainstem motoneurons and in the substantia nigra (Singer et al. 1998; Mangin et al. 2002). 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 Cl⁻ channels (Legendre 2001; Mangin et al. 2002). For example, adult-type $\alpha_1\beta$ heteromeric glycine receptors are resistant to picrotoxin, while juvenile-type homomeric $\alpha_2$ receptors are sensitive to picrotoxin at a low concentration (10 $\mu$M or less). In the present preparation, picrotoxin-resistant glycine receptors, presumably the adult type, were already expressed on DCN neurons at P2-3 (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 as it is in the striatum (Sergeeva and Haas 2001).

**Localization of glycine receptors**

The results of this study indicate that the majority glycine receptors exist in the
extrasynaptic region of DCN neurons, as was 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 glycinergic 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; Tapia et al. 2000; Furuya et al. 2000; Mangin et al. 2002; Mori et al. 2002). 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 glycinergic 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 (Ramon et al. 1996; Baurle and Grusser-Cornehls 1997). Glycine transporters of neuron-specific type (GlyT2), a marker for glycinergic 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 the present 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 glycinergic Purkinje axons seems unlikely from results of previous studies (Ito 1984; Aizenman et al. 1998; 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 (Singer and Berger...
2000; Gao et al. 2001; Garcia-Alcocer et al. 2001; Turecek and Trussell 2002; Zhou et al. 2002; Sultan et al. 2002). Additionally, in the cerebellar cortex of the rat, pure and mixed glycinergic and GABAergic transmission has been proved electrophysiologically in Golgi cells (Dumoulin et al. 2001).

The roles of these glycine receptors and glycinergic transmission in the brain seem crucial for proper neural function and for regulated development of the brain (Legendre 2001; Dutschmann and Paton 2002; Zhou et al. 2002). In many immature neurons, glycine causes excitation instead of inhibition, which may trigger several Ca\(^{2+}\)-dependent phenomena essential for the developmental process, such as neuronal proliferation, migration and synaptic maturation (Kirsch and Betz 1998; Flint et al. 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 (Rivera et al. 1999; Mikawa et al. 2002; Eilers et al. 2001). 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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FIGURE LEGENDS

FIG 1. Preparation of slices of the cerebellum from the newborn rat.
A: parasagittal section of two serial slices (#1 and #2; 200 µm in thickness, at P9). The two 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 brainstem (at lower part of the slice) and a part of the inferior colliculus (on the 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 DCN are distinctively recognized in the lower part of the figure.
C: recording configuration from a DCN neuron (at P10), which was taken using a low-magnification objective (5x). This figure also shows a microelectrode used for electrical stimulation (upper part), a ‘Y tube’ used for local perfusion (from the left) and a whole-cell patch electrode (from the right).
D: recording configuration from another DCN neuron (at P2; presumed projection neuron having a somatic diameter of 22 µm) observed with Nomarski optics (Zeiss, Germany) using a long-working-distance 40x water-immersion objective.
FIG 2. Glycine-induced membrane currents in neurons of DCN.

A: glycine-induced membrane currents observed in DCN neurons. (Left), glycine (100 µM) was applied from a ‘Y tube’ over the period of time indicated at the top (open box). Two representative records obtained from neurons at P4 and P11 are shown (with age on each trace). External medium, standard external saline containing 1 µM TTX. Internal saline, Cs-methanesulphonate. Holding potential, -40 mV. (Right), developmental changes in the whole-cell membrane capacity (upper) and the current density amplitude of glycine-induced currents (lower). Neurons are classified into four groups according to the ages of animals (from P2 to P13). In each cell, the peak amplitude of the current evoked by 100 µM glycine was normalized to the whole-cell membrane capacity of the cell to obtain the current density amplitude. In both figures, mean values (filled circles), their S.D.s (bars) and numbers of neurons tested (in parentheses) are shown. No significant differences were found among these four groups (P<0.05) in both figures.

B: dose-response relationship of the glycine-induced membrane currents. (Left), sample current traces obtained from a neuron at P6. Various doses of glycine were applied from a ‘Y tube’; the dose of glycine (µM) is indicated on each trace. Other experimental conditions were the same as those in A. (Right), the dose-response relationship calculated from 4 neurons, in each of which the peak amplitude of glycine-induced currents was normalized to each control value induced by 1000 µM glycine (double circles; average value, 1240 pA, n=4; measured at -40 mV). The means and S.D.s are shown. The neurons were at P5 (one neuron), P6 (two neurons), and P9 (one neuron). The dotted line over the plots shows an estimated sigmoidal relation having an apparent dissociation constant of 170 µM and Hill coefficient of 1.6 (obtained by using the computer software program ‘Origin’).
FIG 3. I-V relationship of glycine-induced currents and pharmacological properties of glycine receptors.

A: I-V relationship of glycine-induced currents in DCN neurons. Left traces show sample record for measurements of reversal potential by using a saw-tooth voltage clamp. Two seconds after the start of application of glycine (50 µM, bar at the top), the holding potential of the neuron (at P2) was changed from –60 mV to –90 mV and then to –30 mV and then returned to –60 mV (using a saw-tooth voltage of -60±30 mV) over a period of 1 s. Large membrane currents were recorded (upper). When a standard external saline containing no glycine was applied, the same saw-tooth voltage clamp evoked only small currents in the cell (lower). The figure on the right shows the difference between the two current traces (i.e., upper trace minus lower trace), which was plotted against the membrane potential. The dotted line on the I-V relationship is an approximation fit by a constant field equation. External medium, standard external saline containing 1 µM TTX. Internal saline, Cs-methanesulphonate.

B, upper: effect of glycinergic agonists. (Left), sample current traces in a DCN neuron at P8 showing responses to glycinergic agonists, including glycine (100 µM), taurine (1 mM), L-alanine (1 mM) and L-serine (1 mM). Each agonist was applied from a ‘Y tube’ over a period of 12 s (open bar at the top). Holding potential, -40 mV. External medium, standard external saline. Internal saline, Cs-methanesulphonate. Holding potential, -40 mV. (Right), comparison of agonist-induced currents obtained from four DCN neurons at P7-8. Peak amplitudes of agonist-induced currents were measured in each neuron, as in the left traces. The mean and S.D. values after normalization of these amplitudes to the value induced by 100 µM glycine (dotted line) are shown by columns and bars, respectively (n=4).

Lower: suppressive effect of strychnine on agonist-induced currents. (Left), sample records in a DCN neuron at P8. The current induced by glycine (100 µM) before application of strychnine is shown at the top (grey trace). In the presence of strychnine, outward currents induced by glycine (100 µM), taurine (1 mM), L-alanine (1 mM), L-serine (1 mM) and glycine (1 mM) were suppressed, as shown in sample traces (second to sixth traces). Each agonist was applied from a ‘Y tube’ as was the case in the upper traces. Other experimental conditions are the same as those used to obtain the upper traces. (Right), a summary of results obtained from four DCN neurons at P7-8. In each neuron, the peak amplitudes of agonist-induced currents were normalized to the value for 100 µM glycine
(dotted line). The means (columns) and S.D.s (bars) are shown.
FIG 4. Blocking effects of strychnine, bicuculline and picrotoxin on glycine receptors in DCN neurons.

A: (a), sample records of glycine-induced currents in a DCN neuron at P11 showing the effects of strychnine (left) and BCC (right). During a period of 7-8 s after the start of application of glycine (100 µM), the saline containing glycine was changed to the second saline containing an antagonist (thin trace, 1 µM strychnine in the left figure or 10 µM BCC in the right figure) or no antagonist (thick trace; control) in addition to glycine (100 µM). Holding potential, -40 mV. External medium, standard external saline. Internal saline, Cs-methanesulphonate. (b), sample records of GABA-induced currents in the same neuron showing the effects of strychnine (1 µM, left) and BCC (10 µM, right). The same methods as those used in (a) were employed, except that GABA (10 µM) was applied instead of glycine. Lower figures show comparison of the blocking effects of strychnine and BCC on glycine-induced currents (left) and on GABA-induced currents (right). Blocking effects were evaluated as percent of reduction in current amplitudes (measured 22 s after the start of agonist application). Columns and bars represent means and S.D.s, respectively (n=4 at P7-11). The difference between the means in each figure was significant (P<0.05).

B: blocking effects of picrotoxin on glycine-induced current from a neuron at P3, studied with the same two-step application technique as that used in A(a). Traces shown are currents induced by glycine alone (100 µM, thick trace, control) or obtained in the presence of glycine and picrotoxin (100 µM and 10 µM, respectively, thin trace). The difference between the control and picrotoxin-suppressed currents is shown in the figure on the right. The difference was plotted after normalization to the control current and shows that the blocking effect reached a steady level six to eight seconds after picrotoxin application (i.e., about 40% in this neuron as a picrotoxin (PTX)-sensitive component).
FIG 5. Bicuculline-resistant and strychnine-sensitive synaptic currents induced by 20 mM K⁺ in DCN neurons.

A: bicuculline-resistant synaptic currents measured in a DCN neuron at P7. In these recordings, holding potential was kept at -10 mV to facilitate detection of the outward synaptic currents. (a), in the presence of bicuculline (10 µM), the frequency of the synaptic currents was generally low. When 100 µM ACh was applied around the neuron, the frequency of the spontaneous synaptic currents transiently increased. (b), ACh-induced spontaneous synaptic currents disappeared in the presence of strychnine (1 µM). Segments in (a) and (b) are shown in expanded form below. Internal saline, Cs-methanesulphonate. External saline, standard external saline containing bicuculline (10 µM).

B: (a), in the presence of CNQX (5 µM), APV (50 µM) and bicuculline (10 µM), which antagonize AMPA-, NMDA- and GABA_A-receptors, respectively, nerve terminals were depolarized by 20 mM K⁺-saline applied from a ‘Y tube’. Large inward currents and overlapping synaptic currents were induced in a DCN neuron at P7. (b), in the presence of 1 µM strychnine, the 20 mM K⁺-induced synaptic currents of the same neuron disappeared. Segments in (a) and (b) are shown in expanded form below. Scale bars apply to both (a) and (b). Holding potential, -40 mV. External saline, standard external saline containing three antagonists. Internal saline, Cs-methanesulphonate. (Lower left), rates of detection of strychnine-sensitive synaptic currents induced by 20 mM K⁺. Detection rates were plotted with open circles after the neurons had been classified into three groups according to their ages. Each of the numbers in parentheses is the number of cells in which currents were detected (>5 pQ per 10s) over the number of cells tested. (Lower right), time integrals of the strychnine-sensitive synaptic currents during application of 20 mM K⁺ (from 5 to 15 s after the start of application; pQ per 10s). Data were obtained using software programs (Mini Analysis and Excel 2000). The figure shows the means and their S.E.M.s (bars) of the current integrals (i.e., charges) in three groups of DCN neurons. Numbers of neurons tested are shown in parentheses.
FIG 6. Glycinergic synaptic currents evoked in DCN neurons.

A: (left), sample records of glycinergic synaptic currents evoked by electrical stimulation (duration of 200 μs, intensity of 2-10 V, interval of 4 s) in a DCN neuron at P7. The external standard saline contained CNQX (5 μM), APV (50 μM) and BCC (10 μM) to block AMPA-, NMDA- and GABA_A-receptors, respectively. Internal saline, Cs-methanesulphonate. Holding potential, -40 mV. An average of synaptic currents from 30 successive traces is shown. The half decay time was obtained as 29 ms by using the software program ‘Origin, version 6’. The plot shows the dependence of the half decay time on the holding potential. Mean and S.D.s (bars) from four cells at P7-8 are shown. The regression line (dotted line with a slope of 0.16 s/V) was also obtained by using ‘Origin’. (Right), the I-V relationship of glycinergic synaptic currents from a DCN neuron at P7. Recordings were made in standard external saline containing 5 μM CNQX, 50 μM APV and 10 μM BCC. Internal saline, Cs-methanesulphonate. Electrical stimulation of double pulses evoked synaptic currents in the neuron kept at various holding potentials. At each holding potential, 20 successive synaptic currents were obtained and averaged. The inset shows specimen records of the currents (average of first 10 traces). The holding potential (mV) is shown on each trace.

B: sensitivity of glycinergic synaptic currents to strychnine (left figures) and TTX (right figures) in a DCN neuron at P7. Electrical stimulation of double pulses was applied with an interval of 4 s. Other experimental conditions are the same as those in A. Glycinergic synaptic currents were almost completely suppressed in the presence of 1 μM strychnine or 1 μM TTX, but they recovered after washing with control external saline (containing 5 μM CNQX, 50 μM APV and 10 μM BCC but no strychnine). Means (open circles) and S.D.s (bars) from four cells at P7-8 are shown in the lower figure. The average of glycinergic synaptic currents was calculated from 10 successive traces evoked in each cell. Their means (open circles) and S.D.s (bars) were plotted against experimental conditions (n=4).
Fig. 2

A. Glycine 100 µM

- P4: 200 pA for 2 s
- P11: 200 pA for 2 s

B. Dose of glycine (µM)

- 10
- 20
- 100
- 200
- 1000
- 2000

- Normalized amplitude vs. Dose of glycine (µM)
  - n=4
  - $K_d=170$ µM
  - Hill = 1.6

- Capacitance vs. Postnatal age (days)
  - P2-4, 5-7, 8-10, 11-13

- Current amp. (pA/pF) vs. Postnatal age (days)
  - P2-4, 5-7, 8-10, 11-13

Capacitance (pF)

- 10
- 20
- 40

- n=4

- Postnatal age (days)
  - P2-4, 5-7, 8-10, 11-13

- Current amp. (pA/pF)
  - P2-4, 5-7, 8-10, 11-13

- Glycine

- 2 s
- 200 pA
**A**

![Graph showing membrane potential and induced currents](image)

- **Glycine 50 µM**
  - Membrane potential (mV): -70, -30, -50, 0, 400
  - Amplitude of induced currents (pA): 200 pA

**B**

- **Gly 100 µM**
- **Gly 100 µM**, **L-Ala 1mM**, **L-Ser 1mM**, **Tau 1mM**
  - Normalized amplitude: 0, 0.5, 1.0

- **Gly 100 µM**, **Ala 1mM**, **Ser 1mM**
  - Normalized amplitude: 0, 0.5, 1.0

- **Gly 100 µM**, **L-Ala 1mM**, **L-Ser 1mM**, **Gly 1mM**
  - Normalized amplitude: 0, 0.5, 1.0
A  

a  

Glycine (100 µM)  

+Str 1 µM  

+BCC 10 µM  

2 s  

200 pA  

b  

GABA (10 µM)  

+Str 1 µM  

+BCC 10 µM  

2 s  

100 pA  

B  

Glycine (100 µM)  

+Picrotoxin 10 µM  

2 s  

50 pA  

Fig. 4  

Ratio of control response (%)  

Glycine (100 µM)  

(n=4)  

GABA (10 µM)  

(n=4)  

Current amplitude (%) control  

Time (s)  

PTX-sensitive component
Fig. 5

A

Holding potential, -10 mV

In: strychnine 1 µM

B

In: CNQX 5 µM, APV 50 µM, BCC 10 µM

Total; 40 neurons

Positive neurons (%)
Fig. 6

A

![Graph showing synaptic current decay time vs. membrane potential](image)

B

![Graph showing synaptic currents before and after washes](image)

**Synaptic Currents**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
<th>Current (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Stry</td>
<td>15 min</td>
<td></td>
</tr>
<tr>
<td>Before TTX</td>
<td>6 min</td>
<td></td>
</tr>
</tbody>
</table>

Before Stry 1 μM

Before TTX 1 μM