NMDA Receptor-Mediated Currents in Rat Cerebellar Granule and Unipolar Brush Cells

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Billups, Daniela, Ying-Bing Liu, Susanne Birnstiel, and N. Traverse Slater. NMDA receptor-mediated currents in rat cerebellar granule and unipolar brush cells. J Neurophysiol 87: 1948–1959, 2002; 10.1152/jn.00599.2001. The properties of N-methyl-D-aspartate (NMDA) receptor-mediated currents at the giant cerebellar mossy-fiber unipolar brush cell (UBC) synapse were compared with those of adjacent granule cells using patch-clamp recording methods in thin slices of rat cerebellar nodulus. In UBCs, NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) decayed as a single exponential whose time constant was independent of membrane potential. The EPSP was reduced in all cells by the NR1/NR2B-selective antagonist ifenprodil, and the Zn2+ chelator N,N,N′,N′-tetakis(2-pyridylmethyl)ethylenediamine (TPEN) produced a transient potentiation in 50% of cells. In contrast, the NMDA EPSC in granule cells decayed as a double exponential that dramatically switched to a slower rate at positive membrane potentials. The synaptic response in some granule cells also displayed a late second peak at positive potentials, and in others, activation of mossy fibers produced repetitive trains of EPSCs indicating they may be postsynaptic to the UBC network. Single-channel recordings of outside-out somatic patches from UBCs in magnesium-free solution revealed only high-conductance (50 pS) channels whose open time was increased with depolarization, but the opening frequency was decreased to yield a low (p_o = 0.0298), voltage-independent opening probability. Lowering extracellular calcium (2.5–0.25 mM) had no effects on channel gating, although an increase of single-channel conductance was observed at lower calcium concentrations. Taken together, the data support the notion that the NMDA receptor in UBCs may comprise both NR1/NR2A and NR1/NR2B receptors. Furthermore, the properties of the EPSC in these two classes of feedforward glutamatergic interneurons display fundamental differences that may relate to their roles in synaptic integration.

INTRODUCTION

The granule cells (GCs) of the cerebellum have long been considered to be the only excitatory interneurons of the cerebellar cortex, acting both to integrate afferent synaptic input from extrinsic mossy fibers and to serve as feedforward glutamatergic interneurons projecting to populations of GABAergic interneurons and Purkinje cells (Eccles et al. 1967; Ito 1984; Llinás 1984; Mugnaini 1972; Palay and Chan-Palay 1974). However, more recently a second class of neuron within the cerebellar granular layer has been identified, termed the unipolar brush cell (UBC), which also receives afferent synaptic input from extrinsic mossy fibers and whose axons are confined to the cerebellum (for reviews, see Diño et al. 2000a; Mugnaini et al. 1997; Slater et al. 1997, 2000). Both UBCs and GCs may share the same presynaptic extrinsic mossy fiber but form synapses with very different ultrastructure: mossy fiber-GC synapses are of small diameter, whereas the mossy fiber-UBC synapse contains areas of very extensive synaptic apposition with multiple release sites and a continuous distribution of postsynaptic ionotropic glutamate receptors (Floris et al. 1994; Jaarsma et al. 1995; Rossi et al. 1995). During development, the mossy fiber-UBC synapse progressively elaborates, and the number of very long synaptic contacts increase in number (Morin et al. 2001).

Like the mossy fiber-GC synapse, the “giant synapse” formed between mossy fibers and UBCs is glutamatergic, but the time course of the excitatory postsynaptic current (EPSC) is very much longer than that of adjacent GCs (Rossi et al. 1995; Slater et al. 1997). One hypothesis that has been advanced to explain the longer duration of the EPSC in UBCs is that following release, glutamate becomes entrapped within the tortuous three-dimensional space of the synaptic cleft, allowing for extensive re-binding of neurotransmitter with postsynaptic ionotropic receptors prior to the eventual diffusional escape of the glutamate molecules. In the case of the AMPA receptor-mediated component of the EPSC, this hypothesis was examined by studying the consequences of manipulation of release probability, receptor desensitization and transporter function, and it was concluded that glutamate could be trapped within the cleft for periods of >5 s following release (Kinney et al. 1997). As a result of this entrapment of glutamate, a near equilibrium concentration of glutamate is achieved, and the unusual biphasic time course of the AMPA receptor-mediated EPSC could be predicted from the steady-state dose–response curve for glutamate acting on AMPA receptors (Kinney et al. 1997). The prolonged EPSC that results in turn drives a repetitive spike train during the long-lasting depolarization that is supported by the presence of resurgent sodium currents in the cell (Mossadeghi and Slater 1998) similar to those previously described in cerebellar Purkinje cells (Raman and Bean 1997).

In cerebellar GCs, the time course of the NMDA receptor-mediated EPSC is determined primarily by the subunit composition of the receptor (Cathala et al. 2000; Ebralidze et al. 1996; Kadotani et al. 1996; Rumbaugh and Vicini 1999; Takahashi et al. 1996), and the presence of glial glutamate trans-
porters in the surrounding glomerular capsule (Overstreet et al. 1999). In UBCs, however, if the time course of the AMPA receptor-mediated EPSC is determined primarily by the slow diffusional escape of glutamate from the cleft, then it might be predicted that the time course of the NMDA receptor-mediated EPSC would be less sensitive to factors that govern the gating kinetics of individual receptor macromolecules, such as membrane potential and external cation concentration. These processes would be in a state of relative equilibrium, with the slow decay of the EPSC being determined primarily by the rate of diffusional escape. To better understand the mechanisms that regulate synaptic transmission at the giant mossy fiber-UBC synapse, we have examined the properties of the macroscopic NMDA receptor-mediated EPSC in whole-cell recordings, and compared these with those of adjacent GCs. We have also examined the voltage and calcium dependence of NMDA receptor-activated single-channels in outside-out membrane patches of UBC somas. The results support the notion that the time course of the NMDA receptor-mediated EPSC is independent of receptor gating.

A preliminary report of some of these data has been presented (Liu et al. 1998).

METHODS

Preparation of brain slices

The methods employed for the preparation of thin brain slices and patch-clamp recording of visually identified UBCs and GCs in thin cerebellar slices were similar to those previously employed by this laboratory (Ebralidze et al. 1996; Kinney et al. 1997; Overstreet et al. 1999; Rossi et al. 1995). Experiments were conducted on Sprague-Dawley rats of either sex, aged 8–20 days postnatal. Animals were anesthetized using isoflurane by inhalation and killed by decapitation using a guillotine while under general anesthesia. The brain was removed by dissection and placed in a chilled (0–5°C) extracellular solution of the following composition (mM): 126 NaCl, 3 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26 NaHCO₃, and 16 D-glucose (gassed with 95% O₂-5% CO₂, pH 7.4; osmolality 310 mosmol). Thin (150–250-μm thick) parasagittal slices of cerebellar vermis were cut using a vibrating tissue chopper (Vibratome). Slices were maintained at room temperature after the initial hour of incubation until needed for recording.

For recording, slices were transferred to a submersion chamber mounted on the stage of an upright microscope (Leitz Laborlux or Olympus BX50WI) and viewed with a ×40 water-immersion objective (Zeiss or Olympus) with Hoffman or Nomarski Optics. The slices were continuously perfused throughout the experiment with external medium at room temperature (22–24°C). All recordings were made from UBCs and GCs in the granular layer of the nodulus (lobule X). UBCs were identified in living slices by their larger soma diameter and greater whole cell capacitance than adjacent GCs, and the prolonged synaptic response to white matter stimulation.

Patch-clamp recording and synaptic stimulation in slices

Patch recording pipettes were fabricated from thick-walled borosilicate glass capillaries (resistance 3–10 MΩ when filled with internal solution) using a Flaming-Brown Model P-87 horizontal pipette puller (Sutter Instruments). In the majority of experiments, electrodes were filled with an internal solution containing (mM): 145 CH₂(O)SC₅, 10 QX-314, 2 MgCl₂, 5 K₂ATP, 0.5 EGTA, and 5 HEPES (pH = 7.2; osmolarity adjusted to 290 mosmol). Patch pipettes were mounted in the headstage input of a stage-mounted micromanipulator and positioned over the soma of the neuron by visual control. Transmembrane voltage and current were recorded using an Axopatch 200B amplifier (filtered at 10 kHz; –3 dB), stored on video tape (VR-10C, Instrutech), and played back off-line for analysis using pClamp (v.6.0.1) software (Axon Instruments). Conventional methods for whole cell recording and the preparation of excised outside-out membrane patches were employed (Edwards et al. 1989; Hamill et al. 1981). The reference electrode was connected to the bath by means of either a chlorided silver wire or a KCl-agar bridge.

Concentric bipolar tungsten stimulating electrodes (Rhodes) were placed in the white matter to activate mossy fiber (MF) inputs to UBCs and GCs. In all experiments, a stimulus of 100-μs duration was delivered every 15–25 s (0.04 Hz) and 5–25 EPSCs averaged. NMDA receptor-mediated synaptic currents were recorded in a nominally magnesium-free solution in the presence of bicuculline (10 μM), 5–10 μM glycine, and the competitive AMPA receptor antagonist CNQX (10 μM). For analysis of the synaptic current decay time constants 5–25 EPSCs were averaged and fit with a single or double exponential using the Simplex fitting method of Clampfit 6.

In many experiments Lucifer yellow (0.05%; K* salt; Molecular Probes) was included in the patch pipette to verify the identity of the recorded neuron as a UBC, based on the characteristic morphology of the cell (Berthé and Axelrad 1994; Mognaini and Floris 1994; Rossi et al. 1995). After the completion of whole cell experiments, the morphology of the recorded neuron was viewed using fluorescence attachments to the microscope.

Single-channel recording in excised patches

Single NMDA receptor-activated channels were recorded in excised, outside-out membrane patches from UBCs using conventional methods (Hamill et al. 1981). Patch pipettes were fire polished using a microforge and coated with silicone elastomer (Sylgard) resin 184 (Dow Corning) to within 100–500 μm of the tip. Currents were recorded with an Axopatch 200B amplifier (Axon Instruments), filtered on-line at 5 kHz (~3 dB, 4-pole Bessel filter), digitized at 100 kHz (Digidata 1200, Axon Instruments) and stored on video tape for post hoc analysis using an Instrutech VR10C interface. In the majority of experiments the mean single-channel conductance at each membrane potential was derived from the peak of Gaussian fits to the data, and the slope conductance determined from measurements at four or five different membrane potentials, after subtraction of liquid junction potentials. In all single-channel experiments, channel activity was elicited by bath application of 5 μM NMDA in the presence of 5–10 μM glycine. Before recording, each patch was exposed to glycine alone, and only patches, which did not respond to glycine were used. 5 min of data were obtained at each holding potential or external calcium concentration.

For analysis of the single-channel currents, the data were replayed from video tape onto the computer, continuously sampled at a rate of 15.15 kHz and low-pass filtered at 1 kHz. Events lists were created with Fetchan 6.3.1 and subsequently utilized for analysis of amplitude, mean open time, and closed time distributions using pStat 6.3.1 (pClamp 6.3.1, Axon Instruments). An “event” was counted as an opening if it passed a threshold set at 50% of the amplitude of fully resolved openings. Where double openings occurred, the period of the double opening and the open periods immediately adjacent to the double openings were ignored and not included in the events list. Such events represented <5% of the data. Dwell time histograms were created and fit with a single exponential using Simplex fitting methods. The goodness of the fits was evaluated visually. The decay time constant τ of the exponential was used as an estimate of the mean channel open-time, and plotted either versus calcium concentration or versus voltage. Only values of single-channel amplitude measured from openings of duration of at least two filter rise-time constants (677 μs) were used to create amplitude distribution histograms. These should include only openings that attain 98.8% of their original amplitude. Amplitude distribution histograms were fit with the sum of
one or two Gaussian components, the peak value of which was used to create the voltage-current and calcium-current concentration graphs. The open probability \( p_o \) of a channel was calculated as

\[
n \cdot p_o = t_o / t_i
\]

where \( t_o \) represents the time that the channel spent in the open state during the recording, \( t_i \) is the total duration of the recording, and \( n \) is the number of channels in the patch (assumed to be 1). The number of events per second \( E_i \) was calculated by counting the total number of events occurring during the recording \( E_{\text{tot}} \) in relation to the total recording time \( t_i \)

\[
E_i = E_{\text{tot}} / t_i
\]

**Application of drugs**

All drugs were dissolved in distilled water or dimethyl sulfoxide (DMSO) and diluted in external saline to their final concentrations prior to bath perfusion. The final concentration of DMSO was always <0.1% in saline. The following compounds were used: bicuculline methobromide (Sigma), N,N,N,N-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; Sigma), ifenprodil (Sigma), and N,N,N,N-tetrais(2-pyridylmethyl)ethylenediamine (TPEN; Sigma). Drugs were delivered to the bath by means of a peristaltic pump, which fed initially into a premixing chamber above the microscope in which further gassing with 95% O₂ - 5% CO₂ was performed.

All experiments were performed at room temperature (22–24°C). Data are expressed as means ± SE.

**RESULTS**

Experiments were conducted on >50 UBCs and GCs located in the granular layer of the nodulus (lobule X) in thin rat cerebellar slices. UBCs were distinguished from adjacent GCs by criteria previously described (Kinney et al. 1997; Rossi et al. 1995) that included their larger soma diameter (9–13 μm), greater whole cell capacitance, prolonged synaptic response to single mossy fiber stimuli, and post hoc visualization of the cell morphology by fluorescent illumination of the Lucifer yellow-filled cell following completion of the experiment. All experiments, unless otherwise noted, were performed in the presence of bicuculline (10 μM), glycine (5–10 μM), CNQX (10 μM), and nominally Mg²⁺-free extracellular solution at a holding potential of −70 mV, to pharmacologically isolate the NMDA receptor-mediated EPSC.

**NMDA receptor-mediated synaptic currents in UBCs**

NMDA receptor-mediated EPSCs in UBCs were studied over a wide range of holding potentials (−90 to +50 mV) and the peak current, 10–90% rise time and decay time constant (\( \tau \)) were examined. In the majority of cells studied to date, the time course of decay of NMDA receptor-mediated EPSCs in neurons is biphasic (e.g., Anchisi et al. 2001; D’Angelo et al. 1994, Ebralidze et al. 1996), and highly voltage dependent, slowing with increasing membrane depolarization (D’Angelo et al. 1994; Hestrin 1992; Keller et al. 1991; Konnerth et al. 1990). Surprisingly, in UBCs the time course of decay of the NMDA receptor-mediated EPSC in UBCs was well fit by a single exponential (Fig. 1). This is illustrated in Fig. 1A (top trace) where the residuals plot displays little systematic error, indicative of an essentially random deviation between the data and the fit of a single exponential. This was true for all UBCs examined over the full range of membrane potentials (Fig. 1B; \( n = 6 \)). The peak synaptic current in UBCs recorded in the presence of external magnesium has been shown to display marked rectification at membrane potentials more negative than −10 mV (Rossi et al. 1995). In the absence of external magnesium, the peak synaptic current displayed a roughly linear relation to membrane potential at potentials more positive than −50 mV (Fig. 1C), but some rectification could be observed at more negative membrane potentials (−60 to −90 mV), presumably due to channel block by residual magnesium in the tissue. As a result of this, chord conductance plots for the synaptic current displayed a hyperbolic form (Fig. 1E), reflective of the progressive block of the response with membrane.
The I-V relations for the total charge (area) showed a similar rectification to that of the peak current at hyperpolarized potentials (Fig. 1D). The total charge varied between -11.04 ± 3.36 pC at negative holding potentials to +15.05 ± 3.39 pC at positive potentials (+50 mV). When normalized to the values obtained at -50 mV, the I-V relations for both the peak current and the total charge were very similar (Fig. 1D), suggesting that the time course of the EPSC was not affected by voltage. This apparent lack of voltage dependence of the time course of the EPSC can be seen in individual records over a range of membrane potentials (Fig. 1B).

This lack of voltage dependence of the time course of the EPSC can be graphically illustrated by scaling synaptic currents obtained over a range of holding potentials as shown in Fig. 2A. Measurements of the fit of a single exponential to the decay of the EPSC showed no voltage dependence at hyperpolarized potentials (Fig. 2B) with a mean value of 295.69 ± 14.39 ms (n = 6). Similarly, measures of the 10–90% rise time (Fig. 2C) and decay (Fig. 2D) displayed no significant voltage dependence (Student’s t-test; P < 0.05). The mean rise time in five cells measured was 31.01 ± 1.14 ms (90 to -10 mV), the mean 10–90% decay time was 493.34 ± 29.54 ms. Values near the reversal potential were omitted due to inaccuracies in the measurement of these small currents. This lack of voltage dependence of both the rise time and decay of the EPSC is reminiscent of the time course of NMDA receptor-mediated EPSCs in medial vestibular nucleus neurons (Kinney et al. 1994), which also receive innervation from primary vestibular afferents.

Pharmacological properties of the NMDA receptor-mediated synaptic currents in UBCs

Previous studies have shown that the NMDA receptor-mediated EPSC in UBCs is blocked by the competitive NMDA receptor antagonist d-AP5 and external Mg2+ (Rossi et al. 1995), but no studies to date have examined the effects of NMDA subunit-specific antagonists or other agents. To determine the subunit composition of the NMDA receptor at mossy fiber-UBC synapses, we first examined the actions of ifenprodil, which preferentially blocks NMDA receptors containing the NR2B subunit (Williams 1993). In native cerebellar membranes, ifenprodil has also been demonstrated to block the NMDA response of migrating cerebellar GCs (Misra et al. 2000), which express only NR1-NR2B receptors (Akazawa et al. 1994; Monyer et al. 1994; Watanabe et al. 1992, 1994), and the NMDA receptor-mediated component of both evoked and spontaneous EPSCs on Golgi cells (Misra et al. 2000). In UBCs, the bath application of ifenprodil produced a reversible reduction of the peak of the NMDA receptor-mediated EPSC (Fig. 3, A and C). The application of 10 μM ifenprodil produced a 34.9 ± 8.8% reduction (n = 9; P < 0.01), while 30 μM ifenprodil produced a 56.4 ± 10.3% reduction in the EPSC (n = 5; P < 0.05). These effects were not associated with significant effects on holding current or input resistance, but a slowing of the 10–90% rise time was observed (control: 10.7 ± 1.9 ms; 10 μM ifenprodil: 15.4 ± 3.4 ms; n = 9; P < 0.05). While the degree of blockade produced by 10 μM ifenprodil was somewhat less than that reported for Golgi cells (63.7%) (Misra et al. 2000), the difference might be accounted for by a greater proportional expression of NR2A subunits in the NMDA receptors of UBCs.

The effects of the Zn2+ chelator TPEN (1–3 μM) were also tested on the NMDA receptor-mediated EPSC in UBCs. TPEN chelates trace amounts of Zn2+ that would otherwise produce a voltage-independent block of NR2A-containing NMDA receptors, thus inducing a potentiation of the response (Paoletti et al. 1997). In UBCs, bath application of 1 μM TPEN produced only a transient potentiation of the NMDA receptor-mediated EPSC in 3/6 UBCs examined (Fig. 3B) during the first 2–3 min after wash in of the drug (26.3 ± 6.3% potentiation in these 3 cells). In all cells (n = 6), a modest reduction of the peak (15.1 ± 2.4%) was observed after 5 min of application that was associated with a decrease of the time constant of decay of the EPSC (control: 161.9 ± 31.6 ms; 1 μM TPEN: 101.6 ± 23.4 ms; n = 6; P < 0.05). At 3 μM TPEN, a greater reduction of the peak of the EPSC was observed (21.8 ± 6.4%; n = 9; P < 0.05). At both concentrations of TPEN, a significant increase in input resistance was observed, but TPEN had no effect on the 10–90% rise time. The reduction of the peak amplitude, increased input resistance and increased rate of decay of the EPSC were presumably not related to Zn2+ chelation by TPEN, but represent some other (unknown) effect of the compound and were reminiscent of similar effects of TPEN on glycinergic currents (Suwa et al. 2001).

NMDA receptor-mediated synaptic currents in GCs

While there have been many studies of the basic properties of the NMDA receptor-mediated EPSC in GCs in normal rodent cerebellar slices (e.g., D’Angelo et al. 1994, 1995; Silver et al. 1992) and in studies of NMDA subunit expression with development and/or gene knockout (Cathala et al. 2000; Ebralidze et al. 1996; Kadotani et al. 1996; Rumbaugh and Vicini 1999; Takahashi et al. 1996), little comparable data are available from GCs in specific cerebellar regions. We therefore sought to obtain comparable data on the properties of the
NMDA receptor-mediated EPSC in GCs of the cerebellar nodulus that receive synaptic input from the same presynaptic sources as UBCs.

The time course of monosynaptic NMDA receptor-mediated EPSCs evoked by white matter stimulation was studied in 13 GCs in the nodulus of slices derived from rats aged P11–P20 at holding potentials between −90 and +50 mV. Examples of averaged synaptic currents are illustrated in Fig. 4A. The NMDA receptor-mediated EPSCs in nodulus GCs differed from those of adjacent UBCs in a number of fundamental ways. First, the time course of decay of the synaptic current was best fit as a double exponential in all cells examined (Fig. 4B) rather than as a single exponential process as in UBCs (Fig. 1, A and B). The I-V relations for the peak current in the absence of magnesium was linear with a reversal potential near 0 mV (Fig. 4C), and chord conductance plots (Fig. 4D) displayed no voltage dependence (mean = 557.83 ± 29.24 pS).

Thus synaptic NMDA receptors in adjacent GCs appear to be less sensitive to magnesium than those of UBCs (Fig. 1, C and D). While some GCs displayed large synaptic currents (Fig. 4A), in general the peak NMDA receptor-mediated EPSC amplitude was relatively small (−56.56 ± 12.38 pA at −90 mV to +26.62 ± 4.95 pA at +50 mV), suggesting that only a fairly small number of individual NMDA receptor-channels were simultaneously open during the peak of the EPSC (10–12 channels, assuming a single-channel conductance of 50 pS; see Fig. 8A). The I-V relations for the normalized peak current and total charge also differ from those of UBCs in that a significant departure between these measures was observed at positive membrane potentials (Fig. 4E). This nonlinearity of the I-V relations for the total synaptic charge would suggest that a significant voltage dependency of the time course of the EPSC exists in GCs. Significant rectification was also seen in the total charge at hyperpolarized potentials (Fig. 4E).

The superimposition of scaled synaptic responses recorded over a range of membrane potentials revealed a surprising finding: while the time course of the NMDA receptor-mediated EPSC from −10 to −70 mV appear nearly identical in their time course, the shape of the EPSC changes dramatically at depolarized potentials (Fig. 5A). At positive membrane potentials, the EPSC appeared to slow in the last phase of the rise time, and the decay phase was considerably slower. Exponential fits to the decay in five cells show that the change in the decay primarily arose from an increase in the slow time constant of decay (Fig. 5B; τslow = 254.81 ± 31.16 ms at negative potentials, and 457.81 ± 71.67 ms at positive potentials). τslow at both +30 and +50 mV differed significantly (P < 0.05, n = 5) from that at −70 mV. By contrast, the time constant of the fast component did not display any significant dependence on voltage (mean τfast for all potentials = 43.49 ± 2.56 ms, n = 5). The relative amplitude of the fast component [A1/(A1 + A2); where A2 is the amplitude of the fast component and A1 is the amplitude of the slow component] was slightly larger at negative potentials (e.g., −70 mV: 0.544 ± 0.056; +50 mV: 0.45 ± 0.093, n = 6), although no statistically significant trend was detected, in contrast to a previous report (D’Angelo et al. 1994). Measures of the 10–90% rise time also displayed some change with the polarity of membrane potential (Fig. 5C; −70 mV: 11.0 ± 1.48 ms; +50 mV: 14.39 ± 1.62 ms, n = 5), but this was without statistical significance. As would be expected, the 10–90% decay time of the EPSC also displayed a slowing with the sign of membrane polarity (Fig. 5D). The averaged 10–90% decay times for six cells were 215.03 ± 10.10 ms for negative potentials and 395.09 ± 36.58 ms for positive membrane potentials. Thus the decay time nearly doubles at positive membrane potentials, although no other voltage-dependent changes in time course were observed.

Atypical synaptic responses of GCs

In addition to the more commonly observed NMDA receptor-mediated EPSCs described in the preceding text, in some GCs (n = 3) the synaptic current displayed a late component that was evident at membrane potentials at or depolarized to −10 mV (Fig. 6). These late “humps” in the synaptic current at depolarized potentials were reminiscent of the late component of the AMPA receptor-mediated EPSC in UBCs (Kinney et al. 1997), but the origin of this phenomenon was not explored. Another type of response to stimulation of the white matter that could be observed in GCs was a long-lasting burst to single white matter stimuli, even when recorded in the
presence of 1.3 mM external magnesium (Fig. 7). Current- and voltage-clamp recordings of these bursts in response to white matter stimuli revealed a long train of excitatory synaptic responses. UBCs are concentrated in high numbers in the cerebellar nodulus, respond to white matter stimulation with a long-lasting EPSC and associated burst of spikes (Rossi et al. 1995; Slater et al. 1997, 2000), and provide glutamatergic innervation of GCs and second-order UBCs (Dino et al. 2000b; Nunzi et al. 2001). It appears likely, therefore, that the burst responses of GCs to white matter stimulation represent polysynaptic excitation mediated via feedforward drive from UBCs.

Properties of single NMDA receptor-channels in UBCs

While much is known regarding the single-channel properties of NMDA receptor-channels in cerebellar GCs, no studies to date have examined these in UBCs. Because the properties of the macroscopic NMDA receptor-mediated EPSCs in UBCs and GCs differed substantially, we sought to determine whether these differences might arise in part from the underlying single-channel behavior. While the electronic compactness of cerebellar GCs allows the visualization of single NMDA receptor-channel events during an EPSC (Fig. 8A) (Clark et al. 1997; Ebralidze et al. 1996; Silver et al. 1992), the larger cell membrane surface area of UBCs prevents the visualization of individual channels during the time course of an EPSC. To study single NMDA receptor-channels in UBCs, outside-out patches were pulled from the soma of UBCs in thin slices and superfused with a nominally magnesium-free saline containing 5–10 μM NMDA and 5 μM glycine at varying membrane potentials and external calcium concentrations.
It can be seen that both the amplitude and frequency of channel opening declined (Fig. 9D; range: 17.89 ± 1.42 s⁻¹ at −90 mV to 2.41 ± 0.71 s⁻¹ at +30 mV). The opposing effects of membrane potential on open time and frequency were such that no significant change in open probability (n.pₒ) was observed (Fig. 9E; n.pₒ = 0.0298 ± 0.0042, n = 6).

The effects of membrane potential on somatic NMDA channels were studied in six patches obtained from rats aged between P8 and P13 at membrane potentials ranging between −90 and +30 mV. An example of single NMDA channel activity in one patch at various membrane potentials is illustrated in Fig. 8B. It can be seen that both the amplitude and frequency of channel opening declined with depolarization (Rossi et al. 1995) and a concomitant fall in free calcium concentration in the synaptic cleft would be anticipated. To examine the potential effects of external calcium on the behavior of single NMDA channels, eight patches from rats aged between P10 and P15 were studied over a range of external calcium concentrations (0.25–2.5 mM) at a holding potential of −70 mV. The effects of varying external calcium concentration on single UBC NMDA channels for one patch is illustrated in Fig. 10A. At each calcium concentration, the amplitude histograms were well fit as a single Gaussian distribution, and the peak value was used to estimate the mean amplitude for all patches. The measurements of unitary conductance are shown in Fig. 10B, and examples of the amplitude frequency histograms in one patch at two different external calcium concentrations are illustrated in Fig. 10C. The data show an increase in single-channel conductance with decreasing external calcium with a net change of 21.23 ± 2.79 pS per

Voltage dependence of single NMDA receptor-channels in UBCs

The effects of membrane potential on somatic NMDA channels were studied in six patches obtained from rats aged between P8 and P13 at membrane potentials ranging between −90 and +30 mV. An example of single NMDA channel activity in one patch at various membrane potentials is illustrated in Fig. 8B. It can be seen that both the amplitude and frequency of channel opening declined with depolarization (Rossi et al. 1995) and a concomitant fall in free calcium concentration in the synaptic cleft would be anticipated. To examine the potential effects of external calcium on the behavior of single NMDA channels, eight patches from rats aged between P10 and P15 were studied over a range of external calcium concentrations (0.25–2.5 mM) at a holding potential of −70 mV. The effects of varying external calcium concentration on single UBC NMDA channels for one patch is illustrated in Fig. 10A. At each calcium concentration, the amplitude histograms were well fit as a single Gaussian distribution, and the peak value was used to estimate the mean amplitude for all patches. The measurements of unitary conductance are shown in Fig. 10B, and examples of the amplitude frequency histograms in one patch at two different external calcium concentrations are illustrated in Fig. 10C. The data show an increase in single-channel conductance with decreasing external calcium with a net change of 21.23 ± 2.79 pS per

Effects of external calcium concentration on single NMDA receptor-channels in UBCs

Synaptic activation of UBCs is associated with a prolonged depolarization (Rossi et al. 1995) and a concomitant fall in free calcium concentration in the synaptic cleft would be anticipated. To examine the potential effects of external calcium on the behavior of single NMDA channels, eight patches from rats aged between P10 and P15 were studied over a range of external calcium concentrations (0.25–2.5 mM) at a holding potential of −70 mV. The effects of varying external calcium concentration on single UBC NMDA channels for one patch is illustrated in Fig. 10A. At each calcium concentration, the amplitude histograms were well fit as a single Gaussian distribution, and the peak value was used to estimate the mean amplitude for all patches. The measurements of unitary conductance are shown in Fig. 10B, and examples of the amplitude frequency histograms in one patch at two different external calcium concentrations are illustrated in Fig. 10C. The data show an increase in single-channel conductance with decreasing external calcium with a net change of 21.23 ± 2.79 pS per

Voltage dependence of single NMDA receptor-channels in UBCs

The effects of membrane potential on somatic NMDA channels were studied in six patches obtained from rats aged between P8 and P13 at membrane potentials ranging between −90 and +30 mV. An example of single NMDA channel activity in one patch at various membrane potentials is illustrated in Fig. 8B. It can be seen that both the amplitude and frequency of channel opening declined with depolarization (Rossi et al. 1995) and a concomitant fall in free calcium concentration in the synaptic cleft would be anticipated. To examine the potential effects of external calcium on the behavior of single NMDA channels, eight patches from rats aged between P10 and P15 were studied over a range of external calcium concentrations (0.25–2.5 mM) at a holding potential of −70 mV. The effects of varying external calcium concentration on single UBC NMDA channels for one patch is illustrated in Fig. 10A. At each calcium concentration, the amplitude histograms were well fit as a single Gaussian distribution, and the peak value was used to estimate the mean amplitude for all patches. The measurements of unitary conductance are shown in Fig. 10B, and examples of the amplitude frequency histograms in one patch at two different external calcium concentrations are illustrated in Fig. 10C. The data show an increase in single-channel conductance with decreasing external calcium with a net change of 21.23 ± 2.79 pS per
10-fold change in external calcium concentration over the range studied (0.25–2.5 mM). A theoretical fit of the dependence of the single-channel conductance on external calcium concentration based on Jahr and Stevens (1993) is shown as a solid line (—) in Fig. 10B, according to the relation

\[ \gamma_i = \frac{G_o + n_o h}{1 + (n_o h g_o)} \]

where \( \gamma_i \) is the single-channel conductance, \( n_o \) is the extracellular calcium ion concentration (mM), and \( G_o, g_o, \) and \( h \) are constants as defined by Jahr and Stevens (1993) with values of 65 mV, 8 pS/mM, and 25 pS.

In contrast to the marked changes in open time and opening frequency observed with depolarization (Fig. 9), no effects of calcium were observed on the mean open time (mean 4.92 ± 0.31 ms; data not shown). Similarly, no effect of manipulation of external calcium was observed on the opening frequency (mean 4.68 ± 0.99 events/s; data not shown), nor the open probability \( (n_p = 0.0147 ± 0.0033; \) data not shown). No appearance of subconductance states was observed for any patch at any external calcium concentration.

**DISCUSSION**

**Time course of NMDA receptor-mediated synaptic currents**

At most glutamatergic synapses, the NMDA receptor-mediated EPSC decays with a biexponential decay rate that is highly sensitive to membrane potential, slowing with membrane depolarization (Anchisi et al. 2001; D’Angelo et al. 1994; Hestrin 1992; Keller et al. 1991; Konnerth et al. 1990). The biexponential time course of decay of the NMDA receptor-mediated EPSC at small-diameter synapses reflects in a large part the kinetic properties of the underlying NMDA channels. This decay rate does not represent an ensemble expression of the varying subunit stoichiometry of the receptors alone, for in neurons in which a single NR2 subunit is expressed following the selective knockout of other NR2 subunit genes, the EPSC also decays as a biexponential (Ebralidze et al. 1996; Kadotani et al. 1996; Takahashi et al. 1996). Furthermore, the rate of glutamate clearance at these synapses is too fast to account for the slow component of the decay (Clements 1996; Clements et al. 1992; Jones and Westbrook 1996). Thus, at many synapses the time constants of the decay of the EPSC likely result from the influence of the slow unbinding of glutamate on the temporal distribution of bursts and clusters of channel opening.

**FIG. 8.** Single NMDA receptor-gated channels in UBCs and granule cells. A: synaptic currents evoked by mossy fiber stimulation in a cerebellar granule cell. These neurons are electronically very compact and single NMDA channels can be seen in the synaptic response. B: single NMDA receptor-channels in an excised patch from a UBC soma recorded at varying membrane potentials in the absence of external magnesium. C: I-V relation of the single-channel current for 6 patches recorded in 5 μM NMDA and 5 μM glycine. Error bars are smaller than the symbols. D: amplitude-frequency histograms for single NMDA channels recorded at 2 membrane potentials with superimposed Gaussian fits. Data are derived from the patch illustrated in B.

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**FIG. 9.** The NMDA channel open time and frequency in UBCs is highly voltage dependent. A: the mean open time of single NMDA channels in UBC patches is highly voltage dependent, lengthening with membrane depolarization. Graph shows a semilogarithmic plot of the relation between open time and membrane potential for 6 patches (mean ± SE). B and C: dwell time histograms for the NMDA channel open time at −70 (B) and −30 mV (C). Lines show a single exponential fit to the data. D: the opening frequency appears to be highly voltage dependent. However, estimation of the apparent open probability \( n_p = O_t / O_t \) reveals no obvious voltage dependence (E). The explanation lies in the observation that, as the membrane is depolarized, channels open less frequently, but for longer durations, and the 2 opposing properties cancel one another out.
The monoexponential rate of decay of the EPSCs in UBCs. While this might be consistent with the view that the time course of the EPSC is determined by the rate of voltage dependence in itself is not conclusive evidence but may simply reflect underlying properties of the NMDA receptors such as a lack of voltage dependence of desensitization or glutamate unbinding. The monoexponential rate of decay of these receptors may simply reflect property of the NMDA receptor complex. Ifenprodil is a relatively specific antagonist of NMDA receptors containing the NR2B subunit in expression systems (Williams 1993) and also blocks NMDA receptor-mediated EPSCs in a variety of native cell types in a manner consistent with NR2B expression (Cathala et al. 2000; Ito et al. 2000; Kirson and Yaari 1996; Misra et al. 2000; Plant et al. 1997; Stocca and Vicini 1998). In UBCs, ifenprodil also produced a concentration-dependent block of the NMDA receptor-mediated EPSC (Fig. 3). By contrast, the Zn$^{2+}$ chelator TPEN, which potentiates NR1-NR2A receptors (Paoletti et al. 1997), did not reliably enhance the NMDA receptor-mediated EPSCs in UBCs. Thus, the high single-channel conductance, blockade by ifenprodil, and lack of consistent potentiation by TPEN would suggest that all NMDA receptor-mediated EPSCs in UBCs are mediated by receptors composed of NR1-NR2B subunits, and some UBCs may also express NR2A.

One caveat to this conclusion is that although no low-conductance channels were observed in somatic membrane, their presence at the synapse cannot be ruled out. However, in studies in which differences in subunit expression were found at synaptic and extrasynaptic sites, the full complement of NR2 subunits could be found at extrasynaptic sites, whereas some of these subunits were not expressed in the synapse (e.g., Misra et al. 2000; Momiyama 2000; Rumbaugh and Vicini 1999; Tovar and Westbrook 1999). It seems likely, therefore, that the absence of low-conductance channels in the somatic patches obtained from UBCs would suggest an absence of these channels in the synapse also.

A complication in the analysis of subunit composition by pharmacological means is the question of whether the UBCs sampled in this study represent a homogeneous population. Cerebellar UBCs are immunopositive for both calretinin (Diofoo et al. 1999; Floris et al. 1994) and mGluR1a (Jaarsma et al. 1998; Takacs et al. 1999, 2000), but double-labeling studies have revealed that antibodies to calretinin and mGluR1a label two distinct, nonoverlapping populations of UBCs in mouse cerebellar nodulus (Nunzi and Mugnaini 2001). While both cell classes display the same characteristic morphology, it is currently not known whether the expression of GluR subunits in the two groups is similar. The transient potentiation of EPSCs by 1 μM TPEN (Fig. 3A) may represent expression of
NR2A subunits in some UBCs that reflect one of these two classes.

**NMDC receptor-mediated EPSCs in GCs**

The NMDA receptor-mediated EPSCs in GCs displayed a very unusual switch in the time course of the EPSC at positive and negative membrane potentials. This behavior is difficult to explain on the basis of voltage-dependent changes in receptor gating, as no obvious membrane potential dependence was observed across a range of hyperpolarized potentials (Fig. 5, A and B). The switch in time course appeared to be dependent on the sign of membrane potential, and not its magnitude, and was reflected in the substantial deviation between the slope of the I-V relations for the peak current and total area (Fig. 4E). This phenomenon was not observed in UBCs. In a previous study of the voltage-dependence of NMDA EPSCs in GCs (D’Angelo et al. 1994), a similar difference in the decay time course at −40 and +40 mV was noted, although the kinetics of the EPSC was not systematically studied over a range of membrane potentials.

One possible explanation for this behavior in GCs may be that Ca\(^{2+}\)-sensitive desensitization plays a role in sculpting the time course of the EPSC in GCs but not UBCs. Ca\(^{2+}\)-sensitive desensitization is prominent in NR2A-containing receptors such as those in GCs but is not significant for NR2B-containing receptors (Krupp et al. 1996; Medina et al. 1995) as expressed in UBCs. Thus, at hyperpolarized potentials the entry of calcium through NMDA channels in GCs may promote Ca\(^{2+}\)-sensitive desensitization, whereas at positive membrane potentials this process is much reduced. However, this mechanism would not likely display a switch dependent on membrane polarity as observed here.

In nominally magnesium-free saline, the I-V relation of the peak of the EPSC in GCs was roughly linear (Fig. 4C) and the chord conductance was not sensitive to transmembrane voltage (Fig. 4D), whereas in UBCs prominent rectification of both the peak of the EPSC and chord conductance was observed at hyperpolarized potentials (Fig. 1, C and E). This difference may reflect both the greater sensitivity to magnesium of NR1/NR2B-containing receptors than those that contain NR2C (Kuner and Schoepfer 1996), as well as the difficulty of effectively washing magnesium out of the three-dimensionally tortuous mossy fiber-UBC synaptic cleft.

**NMDC receptor-mediated single-channel currents in UBCs**

In addition to providing information regarding the subunit composition of the NMDA receptors in UBCs (see preceding text), a strong effect of membrane potential and external calcium concentration on single-channel activity was observed (Figs. 9 and 10). The effect of membrane potential on mean open time may reflect channel block by trace magnesium contamination rather than a true voltage dependence of channel gating. In a previous study of NMDA receptor-channels in dissociated hippocampal neurons, a similar voltage dependence of the mean open time was observed in nominally magnesium-free solutions, but this effect was abolished when glutamate was applied in an EDTA-buffered external solution lacking divalent cations (Gibb and Colquhoun 1992). Such solutions, however, destabilize patches from UBCs too rapidly to obtain comparable data.

The increase in the NMDA single-channel conductance observed at lowered external calcium concentration (Fig. 10) is of interest because during synaptic transmission at a giant synapse, the restricted diffusional access of the cleft volume to extracellular space could allow for a significant decrement in cleft calcium concentration during transmission (e.g., Egelman and Montague 1999; King et al. 2001). Indeed, at the giant calyx of Held synapse in the auditory brain stem, a fall of cleft calcium concentration during synaptic transmission has been demonstrated due to calcium entry at pre- and postsynaptic sites (Borst and Sakmann 1999; Stanley 2000). In both the auditory pathways of the brain stem and the vestibular cerebellar regions (where UBCs are enriched), firing rates may be very high, and consequently cleft calcium levels will be lowered, reducing presynaptic release probability (King et al. 2001; Vassilev et al. 1997). In the case of UBCs, this lowered release probability would be countered to some extent by an enhancement of single-channel conductance.

**Functional significance**

UBCs and GCs are both classes of feedforward excitatory interneurons in the cerebellar granular layer that receive input both from extrinsic mossy fibers (Eccles et al. 1967; Ito 1984; Linás 1984; Mugnaini 1972; Palay and Chan-Palay 1974) and intrinsic mossy fibers that arise from a subset of first-order UBCs (Diño et al. 2000; Nunzi and Mugnaini 2000; Nunzi et al. 2001). A small number of first-order UBCs receive extrinsic mossy fiber input and their axons form a network within the granular layer whose terminals form miniature glomeruli which drive populations of second-order UBCs and GCs. Because mossy fiber firing rates in most cerebellar regions are high, the NMDA receptors at the synapses in this network will play a key role in the temporal summation of excitatory postsynaptic potentials (EPSPs). In GCs, firing will result from repetitive activity of a single mossy fiber to produce temporal summation (Overstreet et al. 1999) or spatial summation via the activation of several simultaneously active inputs (D’Angelo et al. 1995). In UBCs, a single mossy fiber action potential will evoke a prolonged burst of firing (Rossi et al. 1995) that will be transmitted to GCs to produce a temporally summating burst (Fig. 7A). At higher frequencies of extrinsic mossy fiber firing, however, the EPSPs in UBCs fuse to form a depolarization plateau (Slater et al. 1997). The presence of resurgent sodium currents in UBCs (Mossadeghi and Slater 1998) supports the repetitive firing of the UBC from this depolarized level. The NMDA receptors of UBCs thus play a major role in sculpting the firing pattern of the cell, and in turn regulating the activity of a large ensemble of granule cells that are postsynaptic to the UBC network (Nunzi et al. 2001).

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