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The Dihydropyridine Nitrendipine Modulates *N*-Methyl-D-Aspartate Receptor Channel Function in Mammalian Neurons

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SUMMARY

Nitrendipine and other dihydropyridine voltage-sensitive calcium channel (VSCC) antagonists have been demonstrated to possess anticonvulsant and neuroprotectant activity in a variety of model systems. Likewise, antagonists of the *N*-methyl-D-aspartate (NMDA) glutamate receptor subtype act as potent anticonvulsant and neuroprotective agents. Both VSCC and NMDA antagonists exert their effects by inhibiting the neuronal influx of calcium associated with activation of VSCCs or the NMDA receptor, respectively. Although results that provide evidence for cross-reactivity between compounds acting at dihydropyridine-sensitive VSCCs and the NMDA receptor-channel complex have been reported, direct modulation of NMDA receptor function by dihydropyridines has not been demonstrated. In the present investigation, we report that nanomolar concentrations of nitrendipine reduced NMDA/glycine-evoked calcium flux and single-channel current in mouse cerebellar granule cell cultures. As measured with the calcium-specific probe indo-1, nitrendipine (0.1–10 μM) attenuated inward calcium flux evoked by bath application of NMDA (100 μM) and glycine (100 μM), in a concentration-dependent (IC_{50}, 0.56 ± 0.21 μM; 95% confidence interval, 0.19–1.3 μM) and reversible manner. Over a similar concentration range (0.01–100 μM), nitrendipine also inhibited the binding of [^{3}H]MK-801 to mouse cortical and hippocampal membranes (IC_{50}, 0.56 ± 0.12 μM; 95% confidence interval, 0.37–0.84 μM). Finally, nitrendipine concentration- and voltage-dependently reduced the frequency of NMDA (10 μM)- and glycine (1 μM)-evoked single-channel openings and bursts recorded from excised outside-out patches of mouse cerebellar granule cells. These results indicate that nitrendipine suppresses NMDA/glycine-mediated calcium influx by a rapid and direct interaction with the NMDA receptor-channel complex. Furthermore, these results suggest that the interaction of nitrendipine with the NMDA receptor-channel complex is not tissue specific and probably does not require participation of calcium-dependent second messenger systems. Together, the data presented here support the novel hypothesis that nitrendipine may exhibit anticonvulsant and neuroprotectant activity via the combined ability to modulate both NMDA-associated ion channels and L-type VSCCs.

The NMDA-preferring glutamate receptor plays a major role in physiological events such as learning and memory and pathological events such as seizures, epileptogenesis, and ischemia. Predictably, NMDA antagonists have been shown to possess anticonvulsant, antiepileptic, and neuroprotective activity and to impair formation of long term potentiation and memory (1–3). One way in which NMDA antagonists exert these effects is by attenuating the increase in $[Ca^{2+}]_i$ that is associated with glutamate/glycine-mediated activation of the NMDA receptor.

The NMDA receptor is permeable to calcium, potassium, and sodium ions via an associated channel that is distinct from conventional voltage-sensitive ion channels. Influx of sodium through the NMDA channel leads to membrane depolarization and subsequent activation of voltage-sensitive channels (4). Activation of the NMDA receptor thereby promotes inward calcium flux directly through a ligand-gated channel and indirectly through VSCCs. Thus, drugs that block the NMDA receptor (e.g., MK-801) would be expected to attenuate calcium influx through the NMDA receptor channel. By preventing the NMDA receptor-mediated membrane depolarization, NMDA antagonists would also be expected to attenuate calcium influx through VSCCs by reducing VSCC activation. Conversely, VSCC antagonists would be expected to maintain the magnesium ion voltage-dependent blockade of NMDA-gated calcium influx by reducing membrane depolarization resulting from VSCC activation. Hence, NMDA receptor-mediated inward calcium flux may be attenuated directly by NMDA antagonists and indirectly by VSCC antagonists.

The DHPs are a class of compounds that either potentiate (i.e., Bay-K8644) or inhibit (e.g., nitrendipine, nimodipine, and

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; $[Ca^{2+}]_i$, intracellular calcium concentration; VSCC, voltage-sensitive calcium channel; DHP, 1,4-dihydropyridine; KRB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HBSS, HEPES-buffered salt solution.

nifedipine) calcium influx mediated by the high-threshold L-type VSCC (1). DHPs have been shown to act as anticonvulsants (5–9) and to prevent glutamate-induced neurotoxicity (10, 11). Although DHP binding affinities have been shown to correlate well with the ability to diminish calcium influx (12), DHP binding affinities have not been shown to correlate with anticonvulsant potency (7). Therefore, direct evidence that the anticonvulsant activity of the DHPs is due to antagonism of VSCCs is lacking. Thus, the specific mechanism responsible for the anticonvulsant action of the DHPs remains unclear.

Studies conducted in the present investigation demonstrate that the DHP nitrendipine inhibits NMDA/glycine-evoked calcium influx and reduces NMDA/glycine-evoked average single-channel current in patches of primary mouse cerebellar granule cell cultures. In addition, the results presented in this report provide evidence that nitrendipine inhibits [^{3}H]MK-801 binding to isolated mouse cortical and hippocampal membranes. The findings presented here may be important for understanding the anticonvulsant and neuroprotectant mechanisms of action of nitrendipine, and possibly other DHPs. Preliminary results have been reported (13, 14).

Materials and Methods

Tissue culture. Cerebellar granule cells were cultured from 8-day-old Swiss Webster mouse pups according to the methods of Parks *et al.* (15). Briefly, eight dissected cerebella were placed in KRB (containing, in mM, 114 NaCl, 25 $NaHCO_3$, 11.7 glucose, 5 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, and 1.2 KH_2PO_4, pH 7.4) supplemented with 3 mg/ml BSA. Tissue was chopped with scalpels and incubated at 37° for 15 min with 10 ml of KRB containing 3.5 units/ml trypsin. Trypsin inhibitor (83 μg/ml) and DNase (1200 units/ml) in 10 ml of KRB were added and cells were pelleted by centrifugation at 200 × *g* for 45 sec. Cells were resuspended by trituration with a Pasteur pipette in KRB supplemented with trypsin inhibitor (415 μg/ml) and DNase (600,000 units/ml) and were pelleted again. Cells were resuspended, pelleted, and resuspended a third time in KRB alone. A gradient was then formed by the slow addition of 4% BSA to the bottom of the tube. The 4% BSA and suspended debris were removed and cells were pelleted at 100 × *g* for 6 min. The final pellet was resuspended in modified Eagle's basal medium (GIBCO) containing 2 mM glutamine, 25 mM KCl, 10% fetal calf serum (Hyclone), and 1% penicillin/streptomycin. Cells were counted using a hemocytometer and plated onto 25-mm Aclar coverslips (7.5×10^5 cells/coverslip). Cultures were treated with the antimitotic agent cytosine β-D-arabinofuranoside (final concentration, 10 μM) 20–24 hr after plating and were maintained in an environment of 95% humidity and 5% CO_2.

Calcium measurements. Individual coverslips with granule cells (7–9 days in culture) were rinsed in HBSS (containing, in mM, 135 NaCl, 5.8 Na-HEPES, 5.5 glucose, 4.2 HEPES, 4.2 KCl, 1.5 $CaCl_2$, 0.34 Na_2HPO_4, and 0.44 K_2HPO_4, pH 7.4) for 5 min and then loaded with the calcium-sensitive fluorescent probe indo-1/acetoxymethyl ester (5 μM) for 20 min (37°). Coverslips were placed in a flow-through cell chamber attached to the stage of a Nikon Diaphot microscope and were superfused (1 ml/min; total chamber volume, 0.75 ml) with warmed (37°) HBSS. The dead time for solution to bath contact was approximately 7 sec. Experiments were conducted according to the methods of White *et al.* (16), with instrumentation described previously (17). Briefly, a field of representative cells (>50 cells/field) was selected from each coverslip and corrected for background fluorescence. The $[Ca^{2+}]_i$ was estimated from the indo-1 emission ratio (410 nm/480 nm) as described by White *et al.* (16) and the formula originally described by Grynkiewicz *et al.* (18).

After several seconds of base-line $[Ca^{2+}]_i$ were recorded, calcium influx was evoked by perfusion for 3 min with HBSS containing 100 μM NMDA and 100 μM glycine (NMDA/glycine-HBSS). Cells were washed for at least 5 min after each 3-min application of NMDA/glycine-HBSS. This wash time between agonist applications was sufficient to ensure that subsequent responses returned to initial control values. After four to six control responses per culture were obtained with NMDA/glycine-HBSS alone, the effect of MK-801 and nitrendipine on NMDA/glycine-evoked calcium influx was examined. In these studies, cells were exposed to NMDA/glycine-HBSS for 1 min to obtain a stable level of calcium influx. After 1 min of NMDA/glycine-HBSS alone, the perfusate was switched such that the cells were exposed for an additional 2 min to NMDA/glycine-HBSS containing MK-801 or nitrendipine. The $[Ca^{2+}]_i$ obtained after 2 min of antagonist application was expressed as percentage of the $[Ca^{2+}]_i$ recorded immediately before antagonist application (1-min control). Using this protocol, each coverslip served as its own control. To assess the reversibility of the nitrendipine effect on NMDA-mediated influx, cells previously exposed to 1 μM nitrendipine were perfused for 5 min with HBSS. After this wash period, cells were perfused with NMDA/glycine HBSS for an additional 3 min.

To examine whether treatment with NMDA/glycine-HBSS produced a significant release of calcium from intracellular stores, cells were perfused for 2 min with HBSS that contained no added calcium (nominally calcium-free HBSS). After several seconds of base-line $[Ca^{2+}]_i$ were recorded, the cells were perfused for 3 min with nominally calcium-free HBSS containing 100 μM NMDA and 100 μM glycine.

[^{3}H]MK-801 binding assay. Membrane fractions were prepared from the cerebral cortices and hippocampi of adult CF1 mice according to the methods of Ransom and Stec (19) and were stored frozen. Individual membranes were thawed, suspended in ice-cold assay buffer (5 mM Tris·HCl, pH 7.4), and washed three times to remove excess glutamate. Membranes were incubated for 1 hr with [^{3}H]MK-801 (2 nM), glutamate (100 μM), glycine (30 μM), and various concentrations of nitrendipine. Nonspecific binding was determined with 100 μM MK-801. Binding was terminated by rapid filtration of the membranes on Whatman GF/B filters (conditioned in 0.05% polyethylenimine for 2 hr). Radioactivity was measured by scintillation counting.

Patch-clamp electrophysiology. External solution consisted of (in mM) 142 NaCl, 1.5 KCl, 1 $CaCl_2$, 10 Na-HEPES, 10 glucose, and 20 sucrose, pH 7.4, with 10 μM picrotoxin and 200 nM strychnine to block possible γ-aminobutyric acid and glycine chloride currents, respectively. Internal pipette solution consisted of (in mM) 153 CsCl, 10 Cs-HEPES, and 5 EGTA, pH 7.4. Therefore, sodium ions were the primary charge carriers for NMDA receptor currents. All experiments were conducted on granule cells (7–10 days in culture). Borosilicate glass microelectrodes (4–8 MΩ) were constructed and excised outside-out patch-clamp recordings were obtained using techniques described previously (20, 21). Recordings were obtained with a List Medical Instruments EPC-7 amplifier and digitized [at 20 kHz using Axotape (Axon Instruments) with a 2-kHz eight-pole Bessel filter interposed]. System dead time was 90 μsec and system rise time (10–90%) was 170 μsec. Patch membranes were voltage-clamped at −75 or +50 mV and experiments were performed at room temperature (22–24°). Inward currents were evoked by coapplication (30 sec/application, at 1–1.5-min intervals) of NMDA (10 μM) and glycine (1 μM), with or without nitrendipine (0.1–1 μM), to the patch using blunt-tipped microperfusion pipettes that were positioned within 30 μm of the patch. A low concentration of NMDA (10 μM) was chosen such that little desensitization occurred during repeated applications. Patches were exposed to the control NMDA/glycine solution and then to one or more concentrations of nitrendipine before spontaneous patch rupture. To assess recovery of responses, the control concentration of NMDA/glycine was applied to the patch after applications of the NMDA/glycine solutions, which contained 0.3 or 1 μM nitrendipine. Patch data were accepted for analysis if three or fewer simultaneous multiple openings were observed during the first control application of NMDA/glycine.

Single-channel data were analyzed by computer. Data were pooled from multiple patches. All-points amplitude histograms (Fetchan; Axon Instruments) and visual inspection of individual patch recordings re-

vealed two main current amplitudes at each holding potential. The amplitude histograms were curve fitted with Gaussian functions (pSTAT; Axon Instruments). A locally written program was used to detect independent channel openings to both main current amplitudes using the 50% threshold-crossing method (20, 21). Openings were accepted if they were longer than twice the system rise time and had amplitudes that were within 1 SD of the two main channel amplitudes determined from the Gaussian curve fits. Closings were accepted if they were longer than twice the system dead time and fell below the 50% threshold of the channel amplitude.

The two main channel amplitudes were analyzed together in the temporal analysis of open, closed, and burst durations. Temporal analysis and curve fitting were performed using locally written programs. Bursts were defined as groups of openings separated by a critical closed time, which was determined by the following method. Closed duration histograms were obtained using a square-root logarithm method (21, 22). Closed duration histograms used a 300-μsec lower limit and were binned at 10 bins/decade. Histograms were fit with a least mean squares method (21, 23). The number of exponentials present was determined by increasing the number of exponentials fitted until the fit was no longer greatly improved, when the maximum log-likelihood difference fell below 2 (21, 24). Using a modification of the equal-proportions method, the critical closed time (60 msec; see Results) was chosen between the third and fourth shortest time constants found in the closed time histogram fits. This empirical determination of bursts allowed for capturing of relatively longer duration bursts in the control and drug applications. Average current (total current evoked averaged over application duration), open, closed, and burst durations, and open and burst frequencies were compared for each treatment condition.

Solutions. Solutions were prepared from stock solutions on the day of the experiment. Nitrendipine (Miles, Inc.) was dissolved in 95% ethanol and diluted with buffer such that the final concentration of ethanol was <0.01%. Equimolar concentrations of ethanol were added to all imaging and binding solutions that did not contain nitrendipine, to control for the potential influence of ethanol on the NMDA receptor complex. To minimize experimental variability associated with degradation of nitrendipine, drug-containing solutions were protected from light at all times and were replaced within 3 hr after preparation. MK-801 (Research Biochemicals Inc), NMDA, and glycine were dissolved in distilled water and diluted with HBSS or the patch-clamp external salt solution. All other compounds were obtained from Sigma Chemical Co. and were dissolved in distilled water.

Statistics for imaging and [^{3}H]MK-801 binding studies. The mean, standard error, and statistical significance between control and treatment groups were calculated using the programs published in the *Manual of Pharmacological Calculations with Computer Programs* (25). The mean inhibitory concentrations (IC_{50}) and 95% confidence intervals were calculated by means of a computer program that is based on inverse prediction using a fitted single linear regression model (26).

Results

Co-perfusion of NMDA and glycine (100 μM each, for 3 min) evoked a 372 ± 29% (n = 50) increase in calcium flux into mouse cerebellar granule cells (calcium transient), which was 94 ± 2% (n = 10) dependent on the presence of extracellular calcium (data not shown). A typical NMDA/glycine-evoked transient (Fig. 1A) was characterized by a rapid increase of $[Ca^{2+}]_i$, which stabilized within 1 min and persisted for as long as the agonist was present (up to 5 min, the maximum time tested). The average basal $[Ca^{2+}]_i$ was 118 ± 6 nM (n = 50).

The noncompetitive NMDA antagonist MK-801 was used to verify that the calcium influx observed during the NMDA/glycine-evoked transient was mediated by NMDA receptors. MK-801 prepared with NMDA/glycine-HBSS was applied to the cells after 1 min of NMDA/glycine-HBSS alone (Fig. 1B). MK-801 (10 nM) attenuated 94 ± 0.5% of the NMDA/glycine-evoked calcium influx (n = 5).

The DHP nitrendipine (1 μM) also significantly attenuated (30 ± 7% of control at 3 min, n = 11) the NMDA/glycine-evoked transient (Fig. 1C). This effect of nitrendipine was reversible (90 ± 9% of control at 3 min, n = 5) after a 5-min wash with HBSS (Fig. 1D). The rate of rise of $[Ca^{2+}]_i$ associated with the NMDA/glycine-evoked transient after exposure to nitrendipine was notably slower (Fig. 1D) than the rate of rise of $[Ca^{2+}]_i$ before exposure to drug (Fig. 1, A-C). Concentrations of nitrendipine greater than 0.01 μM inhibited NMDA/glycine-evoked transients (IC_{50}, 0.56 μM; 95% confidence interval, 0.19–1.3 μM) (Fig. 2). Notably, 100 μM nitrendipine was less effective than 10 μM nitrendipine in attenuating the NMDA/glycine-mediated calcium influx (Fig. 2).

The ability of nitrendipine to interact with the NMDA receptor complex was examined further using an [^{3}H]MK-801 binding assay (Fig. 3). This binding assay was conducted with mouse cortical and hippocampal membranes to determine whether the interaction of nitrendipine with the NMDA receptor could be generalized to brain regions other than cerebellum. Nitrendipine inhibited specific [^{3}H]MK-801 binding to cortical and hippocampal membranes (Fig. 3) at concentrations similar to those that attenuated NMDA/glycine-evoked calcium influx (IC_{50}, 0.56 μM; 95% confidence interval, 0.37–0.84 μM). This assay was not conducted with cerebellar membranes, due to the low density of high affininty [^{3}H]MK-801 binding sites in the cerebellum.

Whether the inhibition of NMDA/glycine-evoked inward calcium flux and [^{3}H]MK-801 binding observed with nitrendipine was due to a direct or an indirect effect on the NMDA receptor-channel complex remained unclear. Electrophysiological experiments were carried out to identify a functional interaction between nitrendipine and the NMDA receptor. Initial experiments with whole-cell recordings of the small granule cells revealed that NMDA/glycine-evoked currents were difficult to maintain and were of rather small amplitude, precluding accurate quantitative studies. Qualitatively, whole-cell currents (n = 3 cells) were reduced by 1 μM nitrendipine in a fashion similar to that found in the calcium-imaging studies (data not shown). Excised outside-out patch-clamp techniques were used to directly observe NMDA/glycine-sensitive receptor channels. Thus, in comparison with whole-cell recordings, indirect involvement of synaptic connectivity was eliminated. Effects of calcium-dependent second messenger systems were minimized, in that calcium ions were buffered (by exposing the internal membrane surface to EGTA). Patches obtained from cultured cerebellar granule cells possessed small numbers (one to three) of detectable NMDA receptor channels. When individual patches were exposed to different concentrations of NMDA/glycine, evoked currents increased with increasing concentrations of NMDA (10–100 μM), coapplied with a saturating concentration of glycine (1 μM). A low concentration (10 μM) of NMDA coapplied with 1 μM glycine produced stationary responses. Hence, inward single-channel currents were evoked by exposing a patch to 10 μM NMDA and 1 μM glycine (Fig. 4A). These evoked currents were reduced by 1 μM MK-801 (data not shown). From the amplitude histograms, two main current amplitudes were detected at each holding potential (−75 mV and +50 mV) and corresponded to two main con-

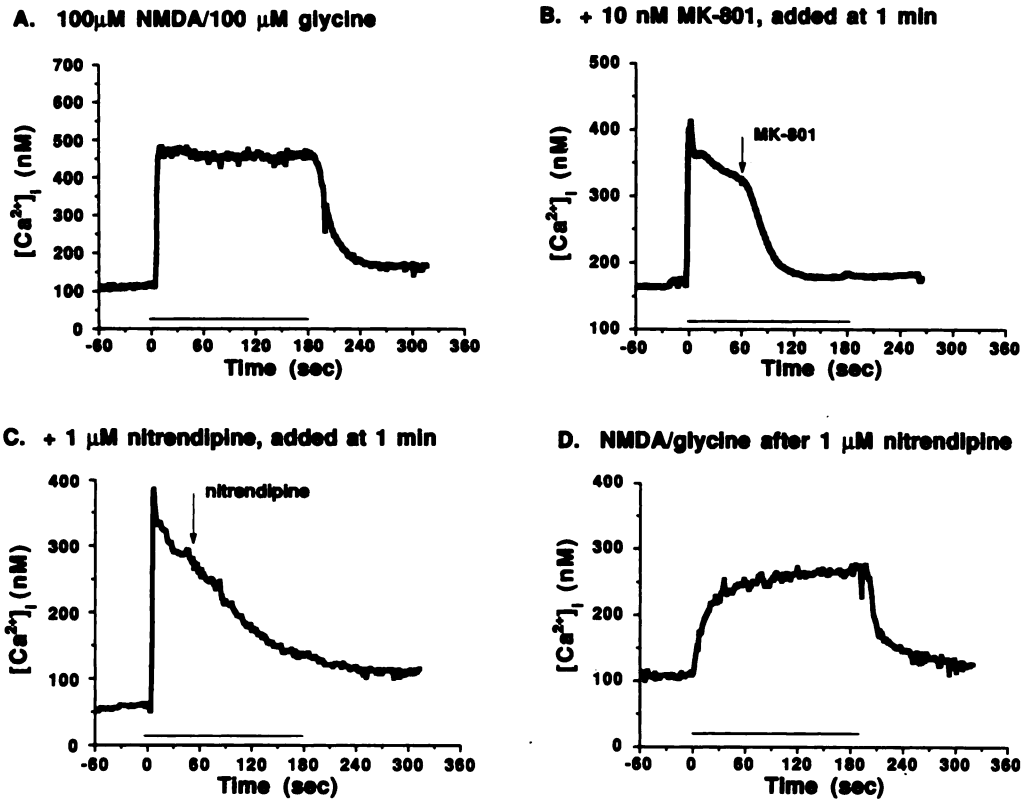

Fig. 1. Representative traces from the calcium ion imaging studies with primary cultures of mouse cerebellar granule cells. The $[Ca^{2+}]_i$ was measured from a population of indo-1-loaded granule cells by microspectrofluorimetry. A, After 1 min of base-line recording in HBSS (time zero), cells were exposed to NMDA/glycine-HBSS for 3 min. B, The NMDA/glycine-evoked calcium transient was blocked (6 ± 0.5% of control, $n = 5$) by the addition of 10 nM MK-801 (added at 1 min). C, Likewise, 1 μM nitrendipine (added at 1 min) significantly attenuated the NMDA-induced Ca^{2+} influx (30 ± 7% of control, $n = 11$). D, After a 5-min wash with HBSS, the effect of 1 μM nitrendipine on $[Ca^{2+}]_i$ was reversible (90 ± 9% of control, $n = 5$). *Line below each trace*, time during which the cells were exposed to NMDA/glycine-HBSS; *downward arrow* (B and C), time at which MK-801 (B) or nitrendipine (C) was added to the perfusate. *Traces* in C and D were taken from the same coverslip.

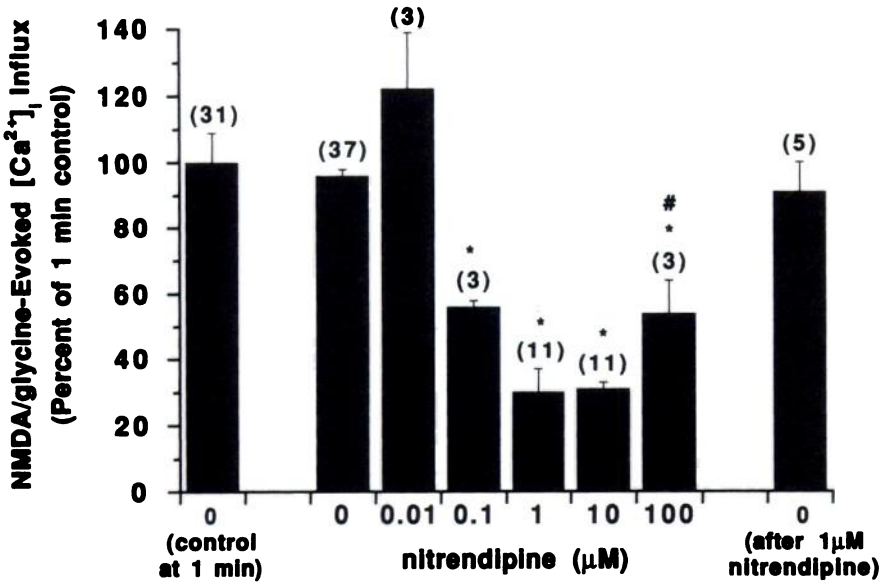

Fig. 2. Reduction of NMDA/glycine-evoked calcium flux into mouse cerebellar granule cells by nitrendipine was concentration dependent between 0.1 and 10 μM. Nitrendipine was less effective against the NMDA/glycine-induced $[Ca^{2+}]_i$ transients at a high concentration (100 μM) than at lower concentrations (1 and 10 μM). Results represent the mean $[Ca^{2+}]_i$ observed after 2 min of perfusion with NMDA/glycine-HBSS that contained a single concentration (0.01–100 μM) of nitrendipine (see Fig. 1C for representative experiment) and are expressed as a percentage of $[Ca^{2+}]_i$ observed after an initial 1-min exposure to NMDA/glycine-HBSS. *Leftmost bar*, control value, i.e., the mean ± standard error of $[Ca^{2+}]_i$ observed after an initial 1-min exposure to NMDA/glycine-HBSS; *rightmost bar*, results showing the reversibility of the nitrendipine (1 μM) effect after a 5-min wash with HBSS. Each *bar* represents data collected from individual coverslips (n is shown in parentheses above each bar), which were obtained from three separate cultures. The IC_{50} (calculated with the results obtained with 0.01–10 μM nitrendipine) was 0.56 ± 0.23 μM. *, Significantly different from control at $p < 0.01$; #, significantly different from 10 μM at $p < 0.05$.

ductances, of approximately 33 and 45 pS. In these studies, approximately 85% of the current was due to the 33-pS conductance.

Temporal kinetic analysis was performed on both main conductances. To determine a critical closed time for use in the quantitative analysis of bursts, closed duration histograms (data not shown) were fitted best with five exponentials. For NMDA/glycine control data at −75 mV, closed duration time constants were 0.56, 2.7, 30.5, 121, and 558 msec. Using a modification of the equal-proportions method (21) the critical closed time of 60 msec was selected (between the third and fourth shortest time constants). A similar value was found for the NMDA/glycine control data at +50 mV.

Nitrendipine (0.1, 0.3, and 1 μM, coapplied with NMDA and glycine) produced a concentration-dependent reduction in the frequency of NMDA/glycine-evoked single-channel currents recorded from patches held at −75 mV (Fig. 4A). Thus, opening frequency was reduced to 65, 44, and 15% of NMDA/glycine control at 0.1, 0.3, and 1 μM nitrendipine, respectively (Fig. 5B). This effect of nitrendipine on NMDA/glycine-evoked currents was partially reversible 3–7 min after exposure to nitrendipine (Fig. 4A). Nitrendipine (1 μM) did not produce a visually

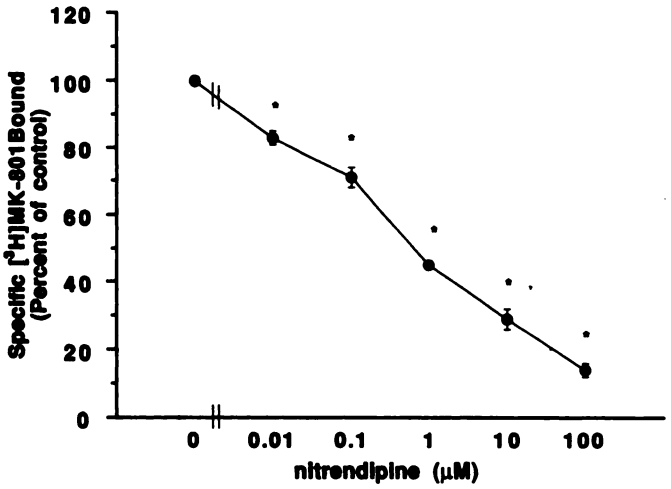

Fig. 3. Nitrendipine concentration-dependently reduced [^{3}H]MK-801 binding to purified membranes obtained from the cortex and hippocampus of adult mouse brains. Binding assays were conducted in the presence of glutamate (100 μM) and glycine (30 μM). Results were expressed as the mean ± standard error of the percentage of specific [^{3}H]MK-801 bound in the absence of nitrendipine. The calculated IC_{50} was 0.56 ± 0.12 μM (eight separate determinations from two experiments). *, Significantly different from control at $p < 0.01$.

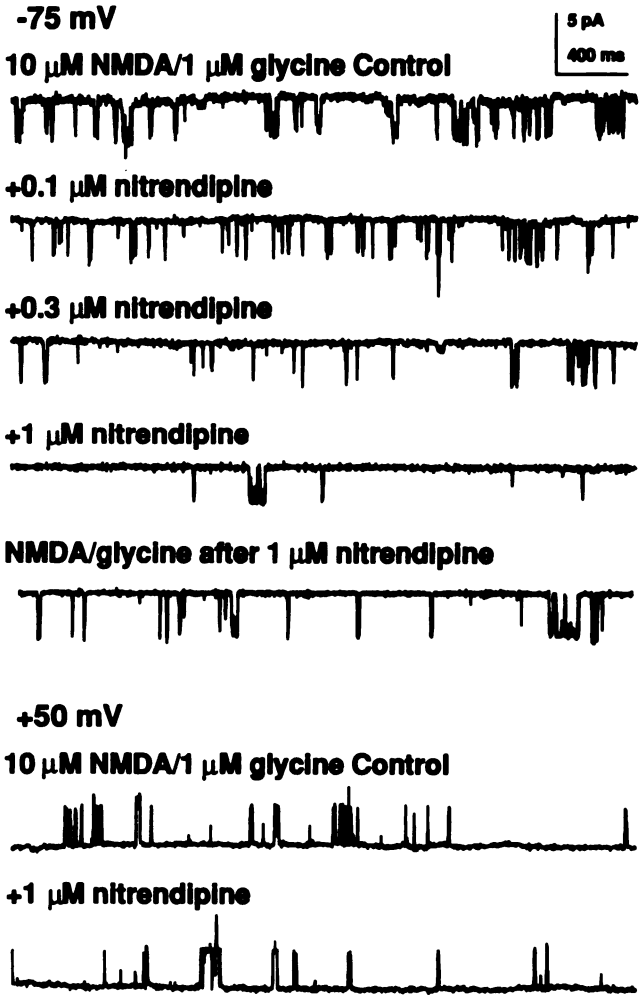

Fig. 4. Nitrendipine reduced the frequency of NMDA/glycine-evoked single-channel activity in excised outside-out patches of mouse cerebellar granule cells in primary culture. Short segments (3.3 sec) of channel activity taken from patches held at either −75 mV or +50 mV are shown and are representative of overall channel activity during NMDA (10 μM) and glycine (1 μM) coapplication (30 sec/application). A, The activity of NMDA/glycine-evoked channel openings recorded from patches held at −75 mV was reduced (compared with the NMDA/glycine control) by nitrendipine in a concentration-dependent manner. NMDA/glycine-evoked channel activity recovered 3–7 min after exposure to nitrendipine. B, The activity of NMDA/glycine-evoked channel openings recorded from patches held at +50 mV was not changed in the presence of 1 μM nitrendipine. A, The *upper three tracings* were obtained from a single patch, whereas the *lower two tracings* were taken from a second patch; B, *tracings* were taken from a third patch.

apparent effect on NMDA/glycine-evoked single-channel current activity at a holding potential of +50 mV (Fig. 4B).

Temporal stationarity of the NMDA receptor current responses was assessed by dividing applications into three consecutive 10-sec epochs. There were no significant differences or trends in the single-channel properties (average current, percentage of time open, mean open and burst durations, and opening and burst frequencies) during the 30-sec applications for NMDA/glycine control or in the presence of nitrendipine (data not shown). In successive applications of NMDA/glycine ($n = 3$ patches) there were no significant differences in the single-channel properties. Thus, single-channel properties were stationary during the application, and progressive desensitization with successive applications was minimal.

Quantitative analysis of the single-channel data revealed that nitrendipine concentration-dependently reduced average current and mean burst duration without affecting mean open duration (Fig. 5A). Nitrendipine also reduced the open and burst frequencies of the NMDA/glycine-sensitive channels recorded from patches held at −75 mV (Fig. 5B). In contrast, nitrendipine (1 μM) did not affect single-channel activity [i.e., average current, duration of openings and bursts (Fig. 5A), and frequency of openings and bursts (Fig. 5B)] recorded from patches held at +50 mV, compared with control NMDA/glycine activity (Fig. 5). In addition, the current amplitudes of the two large NMDA/glycine-evoked channels were not affected by nitrendipine at −75 mV (NMDA/glycine controls, 2.6 ± 0.2 and 3.5 ± 0.4 pA; with 1 μM nitrendipine, 2.7 ± 0.4 and 3.6 ± 0.4 pA) or at +50 mV (NMDA/glycine controls, 1.9 ± 0.2 and 2.6 ± 0.3 pA; with 1 μM nitrendipine, 1.8 ± 0.3 and 2.3 ± 0.2 pA).

Discussion

In the present investigation, it was demonstrated that the DHP VSCC antagonist nitrendipine inhibited NMDA/glycine-mediated calcium flux into cerebellar granule cells in a concentration-dependent and reversible manner. At similar concentrations, nitrendipine also inhibited [^{3}H]MK-801 binding to mouse cortical and hippocampal membranes. In addition, nitrendipine concentration-dependently and reversibly reduced the frequency of NMDA/glycine-evoked openings and bursts recorded from outside-out patches of mouse cerebellar granule cells that were held at −75 mV. Conversely, nitrendipine had little effect on NMDA/glycine-evoked channels when the patches were held at +50 mV. These data support the novel hypothesis that nitrendipine interacts directly and rapidly with the NMDA receptor-channel complex.

Primary cultures of cerebellar granule cells were used in the present investigation because they are an excellent model for the study of the effects of VSCC antagonists on the NMDA

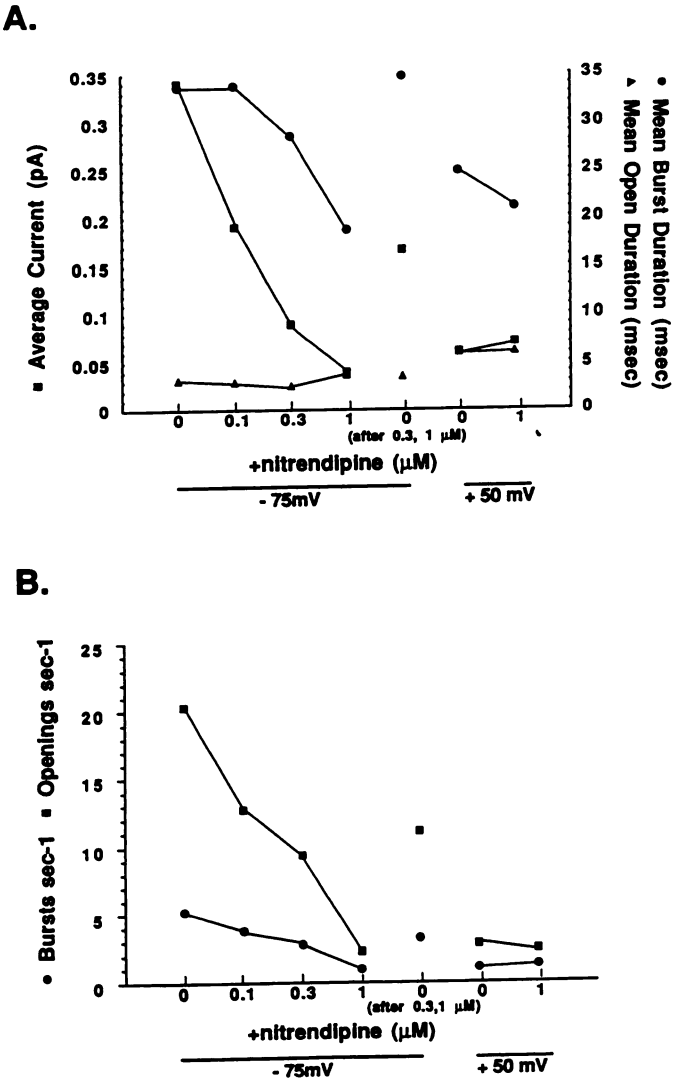

Fig. 5. Nitrendipine altered single-channel properties of NMDA receptor currents. In patches held at −75 mV, average currents and mean burst durations (A) as well as frequency of openings and bursts (B), were reduced by nitrendipine in a concentration-dependent manner. However, mean open durations did not change in the presence of nitrendipine. The properties of NMDA/glycine-evoked currents (−75 mV) recovered to control levels 3–7 min after exposure to either 0.3 or 1 μM nitrendipine. There were 5547 openings in the control NMDA/glycine current group (11 patches, 11 applications), 2653 openings with 0.1 μM nitrendipine (four patches, seven applications), 1122 openings with 0.3 μM nitrendipine (two patches, four applications), 942 openings with 1 μM nitrendipine (seven patches, 14 applications), and 3971 openings with NMDA/glycine after exposure to nitrendipine (seven patches, 12 applications). In recordings obtained from patches held at +50 mV, nitrendipine did not affect the open properties of NMDA/glycine-activated channels. Average current, mean burst durations, and mean open durations did not change in the presence of nitrendipine (A). In addition, neither frequency of bursts nor frequency of openings was affected by nitrendipine (B). There were 420 openings in the control NMDA/glycine current group (three patches, five applications) and 633 openings with 1 μM nitrendipine (four patches, nine applications). *Lines* were drawn to connect the *points* within each holding potential for illustrative purposes.

receptor. First, cerebellar granule cells are glutamatergic cells that possess functional NMDA receptors and all other glutamate receptor subtypes currently identified (15, 27). Second, the NMDA/glycine-evoked calcium transients in this study are highly dependent on the presence of external calcium and, thus, the transients are not greatly dependent on release of calcium from intracellular calcium stores. Third, although not demonstrated for our system, it has been reported by others that cultured rat cerebellar granule cells do not possess significant numbers of VSCCs (28–30). Therefore, the indirect modulation of NMDA receptors via L-type VSCCs would be minimal in this cell type. Together, these characteristics of cerebellar granule cells make them an appropriate and relatively isolated model to study the effects of nitrendipine on NMDA receptors.

Previous investigations have suggested that the cerebellar NMDA receptor displays different pharmacological and binding properties and may be composed of different subunits, compared with striatal, cortical, or thalamic NMDA receptors (31–33). For example, the K_d for [^{3}H]MK-801 binding to cerebellum was reported to be markedly higher than that for cortex (31). In addition, quinolinate was found to bind more potently to forebrain than to cerebellum (32). Our finding that nitrendipine inhibited [^{3}H]MK-801 binding to adult mouse cortical and hippocampal membranes at concentrations similar to those that inhibited NMDA/glycine-evoked calcium influx and single-channel currents of cultured cerebellar cells suggested that there are similarities in NMDA receptor pharmacology between these two cell preparations. Therefore, these results support the hypothesis that the effect of nitrendipine on the NMDA receptor-channel complex is not tissue specific or an artifact of tissue culture.

The observations reported in this investigation are of particular interest because they indicate a novel interaction between nitrendipine and the NMDA receptor-channel complex. Results that provide evidence for cross-reactivity between compounds acting at DHP-sensitive VSCCs and the NMDA receptor have been reported. For example, the polyamines spermine and spermidine, which are believed to modulate NMDA receptor function, have been demonstrated to inhibit [^{3}H]nitrendipine binding to rat brain membranes (34). Phencyclidine, a noncompetitive NMDA antagonist that shares the MK-801 binding site, has been shown to enhance [^{3}H]nitrendipine binding to mouse brain membranes at concentrations less than 10 μM but to inhibit [^{3}H]nitrendipine binding at concentrations higher than 10 μM. In addition, the DHP antagonist nifedipine has been demonstrated to inhibit the motor hyperactivity associated with administration of phencyclidine (35).

Other investigations, however, have failed to demonstrate an interaction between DHPs and the NMDA receptor-channel complex. For example, a high concentration of nitrendipine (100 μM) was reported to have no effect on NMDA/glycine-evoked currents of *Xenopus* oocytes that were injected with guinea pig forebrain mRNA (36). Whether this discrepancy is the result of a species difference or of the experimental paradigm utilized is not known. One possible explanation is that the ability of nitrendipine to inhibit NMDA/glycine-evoked currents is dependent upon an unknown component that was not expressed in the *Xenopus* oocytes described by this study. In another study, NMDA/glycine-evoked calcium influx of rat cerebellar granule cells was not significantly affected by a high concentration (10 μM) of nifedipine (15). Because the only significant difference between that study and the present investigation is the specific DHP used, it may be that the ability of nitrendipine to interact with the NMDA receptor-channel complex is not a characteristic common to all DHPs. This conclusion is supported in part by the observations of Moron

et al. (7), wherein nitrendipine and nimodipine displayed potent anticonvulsant activity against pentylenetetrazol-evoked seizures, whereas nifedipine and nicardipine were devoid of such anticonvulsant activity. Investigations designed to address the possible heterogeneity among the DHPs are ongoing in our laboratory.

In the imaging studies, the finding that nitrendipine was less effective at attenuating NMDA/glycine-evoked calcium influx at 100 μM than at 10 μM was unexpected and remains unexplained. This biphasic pattern within the imaging results was not observed in the results from either the binding or electrophysiology experiments. However, the calcium influx studies, unlike the binding and electrophysiology studies, were conducted with intact cultures that were synaptically active and presumably possessed fully functional second messenger systems. This difference in experimental preparations is important because high concentrations (≥10 μM) of the DHPs have been demonstrated to exhibit nonselective effects such as inhibiting potassium channels and blocking dopamine release (11, 37). The observation that nitrendipine produced a monophasic concentration-response profile when tested with outside-out patches and with isolated membranes supports the possibility that the inconsistency within the results of the imaging studies was probably due to nonselective effects, which currently remain undefined. Because nitrendipine does not autofluoresce at wavelengths detected by the microfluorimetry system used here, this effect of nitrendipine was probably not due to an experimental artifact (data not shown).

The finding that nitrendipine inhibited [^{3}H]MK-801 binding suggested that it may directly modulate the NMDA receptor-channel complex. Support for such a direct interaction was provided by the results obtained from the electrophysiology experiments, wherein nitrendipine rapidly reduced average NMDA/glycine-evoked single-channel current in a concentration-dependent manner when patches were hyperpolarized. The percentage of reduction of average current (11% of control at 1 μM nitrendipine) may be explained by the corresponding decrease in frequency of openings (12% of control at 1 μM nitrendipine) and of bursts (18% of control at 1 μM nitrendipine).

Although detailed examination of the functional interaction of nitrendipine with the NMDA receptor was beyond the scope of this study, these initial observations allow comparison with other agents that reduce NMDA/glycine-evoked current. Similar to that of magnesium, the effect of nitrendipine on single-channel currents was strongly voltage dependent, which suggests that nitrendipine may be acting as an open channel blocker. However, in contrast to magnesium, whose association with the channel is relatively fast and in the presence of which open duration is reduced (38), mean open duration was not changed in the presence of nitrendipine (at either holding potential tested); thus, if nitrendipine was acting as an open channel blocker, the association of nitrendipine with the open channel must be relatively slow. Notably, magnesium does not act purely as a simple open channel blocker; magnesium paradoxically reduces NMDA channel burst durations, particularly at high concentrations (39). Nitrendipine also reduced burst durations at the higher concentrations tested. Nitrendipine did not affect the amplitude or conductance of single NMDA/glycine-sensitive receptor channels, indicating that nitrendipine did not act in a manner similar to that of zinc (40, 41). Rather, nitrendipine altered a process resulting in reduction of the frequency of channel openings. Such processes include those involved in agonist binding, desensitization, or perhaps a "trapped" open channel block mechanism, wherein the channel can close with the blocker molecule inside the channel. Possible sites of interaction include the agonist binding sites, the MK-801 site, and the strychnine-insensitive glycine site. The stationarity of the NMDA receptor current responses during constant application of nitrendipine indicates that nitrendipine did not reduce NMDA receptor currents by inducing a progressive desensitization process. Also, patches exposed to nitrendipine exhibited slow recovery of currents, much like that reported for the use-dependent blocker MK-801 (42). Specifically, the inhibitory effects of MK-801 and nitrendipine on both NMDA/glycine-evoked currents and calcium influx required several minutes to return to control levels. A nonspecific effect resulting from membrane destabilization due to the lipophilic nature of nitrendipine also can be considered as a potential mechanism (43). However, neither the kinetics of the interaction of nitrendipine with the NMDA receptor complex nor the precise mechanisms responsible for the reduction in NMDA/glycine-evoked current have been clearly established, and they remain to be elucidated.

In conclusion, our present findings indicate that nanomolar concentrations of nitrendipine attenuate NMDA/glycine-evoked currents by a direct mechanism. It is thereby proposed that the anticonvulsant and neuroprotectant mechanisms of action of nitrendipine reflect a combined ability to modulate both DHP-sensitive VSCCs and NMDA receptors. As such, nitrendipine appears to represent a combined NMDA/VSCC antagonist. Further studies will be required to determine 1) the specific mechanism of the interaction of nitrendipine with the NMDA receptor-channel complex, 2) whether these effects of nitrendipine are universal for all DHP compounds or specific for only some, and 3) the potential clinical benefits of such an interaction.

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References

1. Bertolino, M., and R. R. Llinas. The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. *Annu. Rev. Pharmacol. Toxicol.* **32**:399–421 (1992).
2. Dingledine, R., C. J. McBain, and J. O. McNamara. Excitatory amino acid receptors in epilepsy. *Trends Pharmacol. Sci.* **11**:334–338 (1990).
3. Bashir, Z. I., S. Alford, S. N. Davies, A. D. Randall, and G. L. Collingridge. Long-term potentiation of NMDA receptor mediated synaptic transmission in the hippocampus. *Nature (Lond.)* **349**:156–158 (1991).
4. Courtney, M. J., J. J. Lambert, and D. G. Nicholls. The interactions between plasma membrane depolarization and glutamate receptor activation in the regulation of cytoplasmic free calcium in cultured cerebellar granule cells. *J. Neurosci.* **10**:3873–3879 (1990).
5. Dolin, S. J., T. L. Patch, M. Rabbani, R. J. Siarey, A. R. Bowhay, and H. J. Little. Nitrendipine decreases benzodiazepine withdrawal seizures but not the development of benzodiazepine tolerance or withdrawal signs. *Br. J. Pharmacol.* **101**:691–697 (1990).
6. Vezzani, A., H. Q. Wu, M. A. Stasi, P. Angelico, and R. Samanin. Effect of various calcium channel blockers on three different models of limbic seizures in rats. *Neuropharmacology* **27**:451–458 (1988).
7. Moron, M. A., C. W. Stevens, and T. L. Yaksh. The antiseizure activity of dihydropyridine calcium channel antagonists in the conscious rat. *J. Pharmacol. Exp. Ther.* **252**:1150–1155 (1990).
8. Dolin, S. J., A. B. Hunter, M. J. Halsey, and H. J. Little. Anticonvulsant profile of the dihydropyridine calcium channel antagonists, nitrendipine and nimodipine. *Eur. J. Pharmacol.* **152**:19–27 (1988).
9. De Sarro, G. B., B. S. Meldrum, and G. Nistico. Anticonvulsant effects of some calcium entry blockers in DBA/2 mice. *Br. J. Pharmacol.* **93**:247–256 (1988).

10. Pizzi, M., M. Ribola, A. Valerio, M. Memo, and P. Spano. Various calcium entry blockers prevent glutamate-induced neurotoxicity. *Eur. J. Pharmacol.* **209**:169–173 (1991).
11. Sucher, N. J., S. Z. Lei, and S. A. Lipton. Calcium channel antagonists attenuate NMDA receptor-mediated neurotoxicity of retinal ganglion cells in culture. *Brain Res.* **297**:297–302 (1991).
12. Carboni, E., and W. J. Wojcik. Dihydropyridine binding sites regulate calcium influx through specific voltage-sensitive calcium channels in cerebellar granule cells. *J. Neurochem.* **50**:1279–1286 (1988).
13. Skeen, G. A., and H. S. White. NMDA-induced calcium transients are attenuated by voltage-sensitive calcium channel antagonists. *Soc. Neurosci. Abstr.* **17**:432 (1992).
14. Skeen, G. A., and H. S. White. Voltage-sensitive calcium channel antagonists attenuate NMDA-evoked calcium influx. *Seizure* **1**:3P/17 (1992).
15. Parks, T. N., L. D. Artman, N. Alasti, and E. F. Nemeth. Modulation of *N*-methyl-D-aspartate receptor-mediated increases in cytosolic calcium in cultured rat cerebellar granule cells. *Brain Res.* **552**:13–22 (1991).
16. White, H. S., G. A. Skeen, and J. A. Edwards. Pharmacological regulation of astrocytic calcium channels: implications for the treatment of seizure disorders, in *Neuronal-Astrocytic Interactions: Implications for Normal and Pathological CNS Function* (A. Yu, L. Hertz, M. Norenberg, E. Eykova, and S. Waxman, eds.), Vol. 96. Elsevier Scientific Publishing Co., Amsterdam, 77–87 (1992).
17. Peeters, G. A., V. Hlady, J. H. B. Bridge, and W. H. Barry. Simultaneous measurement of calcium transients and motion in cultured heart cells. *Am. J. Physiol.* **253**:H1400-H1408 (1987).
18. Grynkiewicz, G., M. Poenie, and R. Y. Tsein. A new generation of calcium indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
19. Ransom, R. W., and N. L. Stec. Cooperative modulation of [^{3}H]MK-801 binding to the NMDA receptor-ion complex by L-glutamate, glycine, and polyamines. *J. Neurochem.* **51**:830–836 (1988).
20. Macdonald, R. L., C. J. Rogers, and R. E. Twyman. Kinetic properties of the $GABA_A$ receptor main-conductance state of mouse spinal cord neurones in culture. *J. Physiol. (Lond.)* **410**:479–499 (1989).
21. Twyman, R. E., and R. L. Macdonald. Neurosteroid modulation of $GABA_A$ receptor single channel kinetics. *J. Physiol. (Lond.)* **456**:215–245 (1992).
22. Sigworth, F. J., and S. M. Sine. Data transformations for improved display and fitting of single channel dwell time histograms. *Biophys. J.* **52**:1047–1054 (1987).
23. Twyman, R. E., R. M. Green, and R. L. Macdonald. Kinetics of open channel block of $GABA_A$ receptor channels by penicillin. *J. Physiol. (Lond.)* **445**:97–128 (1992).
24. McManus, O. B., and K. L. Magleby. Kinetic time constants independent of previous single-channel activity suggest Markov gating for a large conductance calcium-activated K-channel. *J. Gen. Physiol.* **94**:1037–1070 (1989).
25. Tallarida, R. J., and R. B. Murray. *Manual of Pharmacological Calculations with Computer Programs*, Ed. 2. Springer-Verlag, New York (1986).
26. Snedecor, G. W., and W. G. Cochran. *Statistical Methods*, Ed. 6. The Iowa State University Press, Ames, Iowa (1967).
27. McCaslin, P. P., and W. W. Morgan. Cultured cerebellar cells as an *in vitro* model of excitatory amino acid receptor function. *Brain Res.* **417**:380–384 (1987).
28. Cull-Candy, S. G., C. G. Marshall, and D. Ogden. Voltage-activated membrane currents in rat cerebellar granule neurones. *J. Physiol. (Lond.)* **414**:179–199 (1989).
29. Galdzicki, Z., F. Lin, O. Moran, A. Novelli, G. Puia, and M. Sciancalepore. Development of voltage-dependent ionic currents in rat cerebellar granule cells grown in primary culture. *Int. J. Neurosci.* **56**:192–200 (1991).
30. Jalonen, T., S. Johansson, I. Holopainen, S. S. Oja, and P. Arhem. Single-channel and whole-cell currents in rat cerebellar granule cells. *Brain Res.* **535**:33–38 (1990).
31. Ebert, B., E. H. F. Wong, and P. Krogsgaard-Larsen. Identification of a novel NMDA receptor in rat cerebellum. *Eur. J. Pharmacol.* **208**:49–52 (1991).
32. Monaghan, D. T., and J. A. Beaton. Quinolinate differentiates between forebrain and cerebellar NMDA receptors. *Eur. J. Pharmacol.* **194**:123–125 (1991).
33. Stern, P., P. Behe, R. Schoepfer, and D. Colquhoun. Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. *Proc. R. Soc. Lond. B Biol. Sci.* **250**:271–277 (1992).
34. Schoemaker, H. Polyamines allosterically modulate [^{3}H]nitrendipine binding to the voltage-sensitive calcium channel in rat brain. *Eur. J. Pharmacol.* **225**:167–169 (1992).
35. O'Neill, S. K., and G. T. Bolger. Phencyclidine and MK-801: a behavioral and neurochemical comparison of their interactions with dihydropyridine calcium antagonists. *Brain Res. Bull.* **22**:611–616 (1989).
36. Kaneko, S., and M. Satoh. The use of *Xenopus* oocytes in a molecular pharmacological study of NMDA receptors, in *NMDA Receptor Related Agents: Biochemistry, Pharmacology and Behavior* (T. Kameyama, T. Nabeshima, and E. F. Domino, eds.). NPP Books, Ann Arbor, MI, 189–201 (1992).
37. Chaudieu, I., R. Alonso, H. Mount, R. Quirion, and P. Boksa. Effects of L- and N-type Ca^{2+} channel antagonists on excitatory amino acid-evoked dopamine release. *Eur. J. Pharmacol.* **220**:203–209 (1992).
38. Asher, P., and L. Nowak. The role of divalent cations in the *N*-methyl-D-aspartate responses of mouse central neurons in culture. *J. Physiol. (Lond.)* **399**:247–266 (1988).
39. Nowak, L., P. Bregestovski, and P. Ascher. Magnesium gates glutamate-activated channels in mouse central neurons. *Nature (Lond.)* **307**:462–465 (1984).
40. Legendre, P., and G. L. Westbrook. The inhibition of single *N*-methyl-D-aspartate-activated channels by zinc ions in cultured neurons. *J. Physiol. (Lond.)* **429**:429–449 (1990).
41. Mayer, M. L., L. Vyklicky, Jr., and G. L. Westbrook. Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurons. *J. Physiol. (Lond.)* **415**:329–350 (1989).
42. Huettner, J. E., and B. P. Bean. Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. USA* **85**:1307–1311 (1988).
43. Reynolds, I. J., and E. A. Rush. Role of lipid solubility in the interaction of drugs within the *N*-methyl-D-aspartate receptor. *Synapse* **5**:71–76 (1990).

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