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NMDA-Mediated Modulation of γ-Aminobutyric Acid Type A Receptor Function in Cerebellar Granule Neurons

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$GABA_A$ receptors are ligand-gated Cl^- ion channels with multiple clinically relevant drug-recognition sites. We have previously shown that stimulation of *N*-methyl-D-aspartic acid (NMDA)-specific glutamate receptors quantitatively alters selected $GABA_A$ receptor subunit mRNAs and proteins in primary cultures of rat cerebellar granule neurons. We used whole-cell recordings of GABA-elicited Cl^- currents and flunitrazepam binding experiments in granule cell cultures maintained in low K^+ (12.5 mM), cells maintained in low K^+ and treated with a single dose of NMDA (10 μM), and cell cultures maintained in depolarizing concentrations of K^+ (25 mM). The EC_{50} obtained from the dose–response curves for GABA in eliciting a maximal response was comparable in neurons maintained in high K^+ or in low K^+ and treated with a single dose of NMDA, but that it increased significantly in cells maintained in low K^+. The potentiation of GABA-gated Cl^- currents by flunitrazepam increased significantly, while the negative allosteric modulator methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxlyate (DMCM) was significantly more effective in cultures either maintained in high K^+ or treated with NMDA. This was coincident with a twofold increase in the B_{max} associated with flunitrazepam binding. To further characterize the receptor assemblies present in the depolarization and NMDA induced paradigms, the Zn^{2+}-induced inhibition of GABA-gated Cl^- currents was reduced as was the inhibition mediated by furosemide. Our data indicate that $GABA_A$ receptor assemblies alter their composition in response to excitatory afferent receptor stimulation.

[Key words: $GABA_A$ receptor, patch clamp, Cl^- currents, neuronal cells* in vitro*, development, NMDA receptor, benzodiazepine receptor, and Zn^{2+}]

Molecular cloning studies have demonstrated the existence of a large gene family for the mammalian γ-aminobutyric acid type A ($GABA_A$) receptor, including six α, four β, three γ, one δ, and two ρ subunits (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; MacDonald and Olsen, 1994). A number of studies demonstrate that the receptor subunit mRNAs and proteins are differentially expressed in different cell populations in distinct anatomical CNS structures and during different developmental stages as well (Gambarana et al., 1990; Zhang et al., 1991; Baude et al., 1992; Fritschy et al., 1992; Laurie et al., 1992a,b; Poulter et al., 1992; Wisden et al., 1992; Thompson and Stephenson, 1994; Zheng et al., 1994). $GABA_A$ receptors are hetero-pentameric protein complexes (Nayeem et al., 1994), although the composition and subunit stoichiometry of native $GABA_A$ receptors are still unknown (Angelotti et al., 1993; Perez-Velazquez and Angelides, 1993). Data obtained thus far suggest that α subunits determine ligand recognition while γ subunits appear to be an absolute requirement for allosteric modulation (Pritchett et al., 1989a; Puia et al., 1992; Ebert et al., 1994; Mihi et al., 1994; Saxena and MacDonald, 1994). However, Zn^{2+}-induced inhibition of GABA-gated currents decreases with an increase in γ_2 subunits in $GABA_A$ receptor subunit assemblies (Draguhn et al., 1990; Smart et al., 1991), while furosemide has been recently reported to be a specific inhibitor of α6 containing receptor complexes (Korpi et al., 1995).

The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptor, appears to play a crucial role in neuronal plasticity and the development of the CNS (Balázs et al., 1988a,b, 1992; Nakanishi, 1992). K^+-induced depolarization or NMDA treatment promotes the survival of cultured cerebellar granule cells *in vitro* and increases the amount of functional NMDA receptors present (Gallo et al., 1987; Balázs et al., 1988a,b, 1992; Van der Valk et al., 1991; Bessho et al., 1994; Resink et al., 1995). NMDA receptors are highly permeable to both Na^+ and Ca^{2+}, so that the effect of NMDA receptor stimulation on neuronal differentiation may be due to the resulting changes that occur in intracellular Ca^{2+} concentrations (Balázs et al., 1992; Nakanishi, 1992; Bessho et al., 1994).

Our previous studies suggest that afferent synaptic signalling through the NMDA selective glutamate receptor increases the transcription rates, mRNA contents, and immunoreactivities of selected $GABA_A$ receptor subunits (Memo et al., 1989; Harris et al., 1994, 1995). Therefore, in this study, cerebellar granule cells were maintained *in vitro* under conditions of high K^+ (25 mM KCl)-induced depolarization, low K^+ (12.5 mM KCl)-induced depolarization, and following a single treatment of cultures maintained in the lower K^+ conditions with NMDA. We performed whole-cell patch-clamp recordings (Hamill et al., 1981), from which we observed changes in the sensitivity of $GABA_A$ receptors to GABA (EC_{50}), the responsiveness to allosteric modulators, the numbers of ^{3}H-flunitrazepam binding sites,

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and in the Zn^{2+}-induced and furosemide-mediated inhibition of GABA-activated Cl^- currents. Collectively, our data suggest that excitatory amino acid receptor stimulation alters the $GABA_A$ receptor assemblies that form, which exhibit different electrophysiological and pharmacological properties.

Materials and Methods

Cell cultures. Primary cultures of rat cerebellar granule neurons were prepared from 8-d-old Sprague–Dawley rat cerebella as previously described (Gallo et al., 1982). Briefly, cells were dispersed with trypsin (0.25 mg/ml) (Sigma Chemical Co., St. Louis, MO) and plated at a density of 0.8–1 × 10^6 on 35 mm Nunc dishes, coated with poly-L-lysine (10 μmg/ml; Sigma). Cells were cultured in basal Eagle's medium (GIBCO, BRL) supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 μg/ml gentamycin (GIBCO, BRL), and maintained at 37°C in 6% CO_2. Cytosine arabinoside (10 μM) was added to all cultures 18–24 hr after plating to inhibit glial proliferation. The final concentration of KCl in the culture medium was adjusted to either 12.5 mM (low K^+) or 25 mM (high K^+). On day 4 after plating, some of the cultures maintained in 12.5 mM KCl were treated with a single addition of 10 μM NMDA (low K^+ + NMDA) or 5 μM L-glutamate (low K^+ + glu). In a set of experiments, 1 μM dibenzocycloheptene imine (MK-801) was added to cell cultures 30 min before glutamate treatment (low K^+ + glu + MK-801). These granule cell culture conditions represent the experimental paradigms used throughout the studies described below.

^{3}H-flunitrazepam binding. ^{3}H-flunitrazepam binding was performed directly on cerebellar granule neurons in culture as previously described (Gallo et al., 1985; Aronica et al., 1993). After washing cultures once with Locke's solution, 1 to 50 nM ^{3}H-flunitrazepam (Sp. activity—83 Ci/mmol) (NEN) in Locke's solution was added to multiple 35 mm diameter culture dishes in the absence (three dishes/point) or presence (two dishes/point) of nonradiolabeled diazepam (1 μM). The cultures were incubated with ^{3}H-flunitrazepam ± diazepam for 30 min at 25°C. After incubation, the dishes were washed with three quick successions of Locke's solution (removed by aspiration) to effectively remove nonbound ligands. The granule neurons were then solubilized with 1 ml of 0.1 *N* NaOH. This solubilized material (100 μl) was used for protein determination (Biorad protein assay), and 700 μl was added to 3 ml of scintillation cocktail and counted on a Beta counter. Nonspecific binding to Locke's-washed, poly-D-lysine-coated dishes that had contained medium supplemented with serum (but no cells) was determined to be about 10–20% of the total and was subtracted from each sample. Specific binding was obtained by subtracting radioligand bound in the presence of diazepam from total binding. Free ^{3}H-flunitrazepam was calculated by subtracting bound counts from total counts.

Electrophysiological studies. Cultured granule cells on the fifth day *in vitro* (5 DIV) were voltage clamped at −50 mV in the whole-cell configuration using the patch-clamp technique (Hamill et al., 1981) on the stage of an inverted microscope (Nikon) at room temperature. The recording pipette contained (in mM) 145 CsCl, 2 $MgCl_2$, 11 ethylene glycol bis (β-aminoethlether)-N, N, N′, N′-tetraacetic acid (EGTA), 2 NaATP, and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) at pH 7.2 with CsOH. Cells were bathed in (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, and 5 HEPES at pH 7.2 with NaOH. Osmolarity was adjusted to 325 mOsms with sucrose. The culture dish in the recording chamber (< 500 μl total volume) was continuously perfused (5 ml/min) to prevent the accumulation of drugs.

Drug application. All drugs were dissolved in bath solution containing dimethylsulfoxide at a maximal final concentration of 0.01%, which failed per se to modify GABA responses (data not shown). GABA was applied directly by a gravity-fed Y-tubing delivery system (Murase et al., 1989) placed within 100 μm of the recorded cell. Drug application had fast onset (< 5 msec) and achieved a completely local perfusion of the recorded cell. In all experiments, DMCM or $ZnCl_2$ was coapplied with GABA. However, the application of flunitrazepam required a short period of preperfusion before coapplication with GABA.

Data acquisition and analysis. Currents were monitored with a patch amplifier (EPC-7; List Electronics, Darmstadt, Germany), filtered at 1.5 KHz (eight-pole low-pass Bessel; Frequency Devices, Haverhill, MA), and digitized in an IBM-PC computer with the software Axotape 2 (Axon Instrument, Foster City, CA) for off-line analysis. After normalization, fitting of the dose–response relationship was performed using the logistic equation

$$\% I_{max} = 100/I_{max}\{1 + (EC_{50}/[GABA]^{nh})\},$$

where I_{max} is the maximal Cl^- current, elicited by GABA, EC_{50} is the GABA concentration eliciting the half-maximal response, and n_h is the Hill coefficient. Results are expressed as mean ± SEM. Origin (MicroCal Software, Northampton, MA) was used for figure preparation and statistical analysis using ANOVA with a $P < 0.05$ and a paired t test with $P < 0.01$. The Bonferroni correction was applied for multiple group comparisons.

Results

^{3}H-Flunitrazepam binding is increased after treatment of granule cell cultures maintained in low KCl treated with NMDA and in cultures maintained in high KCl

In vitro binding of ^{3}H-flunitrazepam directly on intact cells from primary cerebellar granule neuron cultures demonstrated a significantly increased number of binding sites (B_{max}) in neurons maintained in low KCl conditions treated with NMDA for 36 hr and in high KCl conditioned neurons as compared to nontreated low KCl-maintained granule neurons. There were almost twice as many flunitrazepam binding sites in neurons from these two paradigms as in the nontreated cultures maintained in low K^+. However, the apparent K_D values for each of the three paradigms were not significantly different, as indicated below. Figure 1 shows a representative experiment with the binding curves (Fig. 1, top) and the corresponding Scatchard plots (Fig. 1, bottom) for ^{3}H-flunitrazepam binding in the three-culture paradigms. Similar results were observed in two other independent sets of experiments, and mean K_D and B_{max} values were determined. B_{max} (low K^+: 59 ± 7 fmol/mg protein, low K^+ + NMDA: 115 ± 12* fmol/mg protein, high K^+: 100 ± 7*), and KD (low K^+: 1.9 ± 0.4, low K^+ + NMDA: 1.5 ± 0.3, high K^+: 1.5 ± 0.2) values were calculated from Scatchard analysis of three independent experiments, each performed in triplicate. [*Statistically significant differences ($p < 0.05$) from the low K^+ culture group were calculated using ANOVA and the Newman Keuls test.]

Regulation of the $GABA_A$ receptor sensitivity to GABA by K^+-induced depolarization and NMDA treatment

To examine functional modifications of $GABA_A$ receptors after K^+-induced depolarization and NMDA treatment, the voltage-clamp technique was used to monitor GABA-gated currents in each experimental paradigm. The rapid application of increasing concentrations of GABA to voltage-clamped granule neurons, followed each time by a recovery period of at least 2 min, was used to examine inward currents when symmetrical Cl^- concentrations were present in the intracellular recording pipette solution and in the extracellular bath solution. As shown in Figure 2*A*, in each culture paradigm the application of GABA elicited inward currents that were characterized by a rapidly desensitizing peak followed by a slower steady-state component. The peak amplitude at individual GABA concentrations was measured and normalized to the maximal GABA current using the logistic equation described in Materials and Methods. The results from these experiments (Fig. 2*B*, Top) indicated a significant difference between the GABA-gated currents in each culture condition with respect to the sensitivity to GABA as determined by the average EC_{50} values (Table 1). Of particular interest was the observation that NMDA treatment of cultures maintained in low K^+ yielded the same effect as did K^+-induced depolarization on GABA receptor sensitivity (Fig. 1, Table 1). The distribution of EC_{50} values from individual granule cells is shown in Figure 2*B*, bottom. Both K^+-induced depolarization and NMDA treatment failed to change the maximal current amplitude elicited by

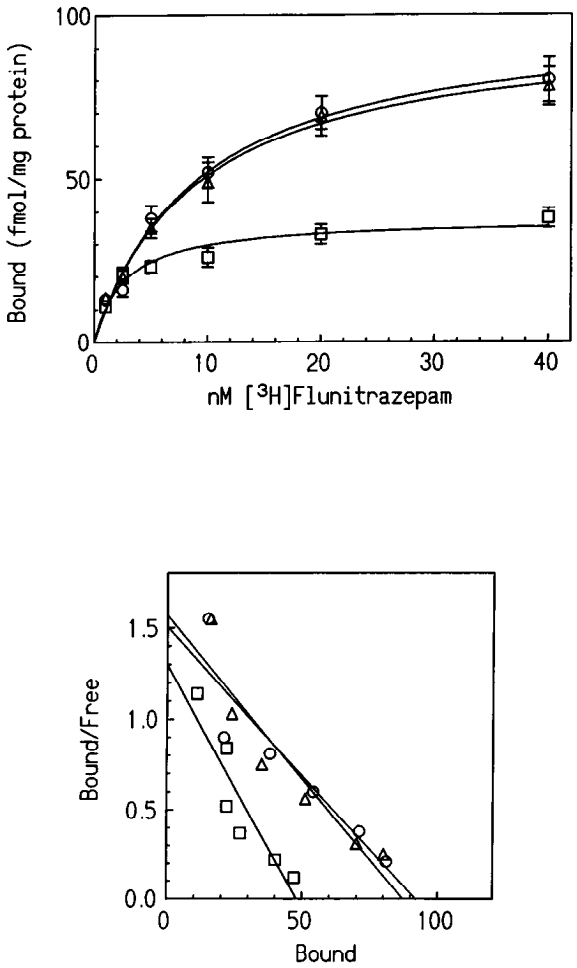

Figure 1. ^{3}H-Flunitrazepam saturation curve (*top*) and Scatchard plot (*bottomB*) for binding experiment of primary cultures of cerebellar granule neurons. These curves represent the mean of triplicate values (± SEM) from one of three independent experiments from cells maintained in low KCl conditions (□), low KCl plus NMDA (10 μM—36 hr) (○), and high KCl conditions (△). The *in situ* binding experiments were performed as described in Materials and Methods. Nonspecific binding was determined using 1 μM diazepam. No significant differences in estimated K_D values were found between the means for the three groups.

GABA, as compared to the low K^+-maintained cultures (Fig. 2*B*, inset). In a separate set of experiments, we normalized the maximal response to the cell capacitance (typically 3 to 5 pF), and no statistically significant differences were observed between the different experimental paradigms (n = 12 cells per culture condition). In addition, in 10 granule neurons cultured in high K^+-containing media, we measured the reversal potential of the peak Cl currents elicited by the application of GABA (3 μM) to be 2.5 ± 2.1 mV (mean ± SD). In 10 additional granule neurons cultured in low K^+, the reversal potential of the peak chloride current was not significantly different, being 3.1 ± 5.7 mV (mean ± SD).

Changes in allosteric modulation of $GABA_A$ receptors after K^+-induced depolarization and NMDA treatment

GABA, binding to its recognition site on $GABA_A$ receptors, opens an ion channel and mediates Cl^- influx, the extent of which can be altered by both positive and negative allosteric modulators (Burt and Kamatchi, 1991; MacDonald and Olsen, 1994) However, the potentiation and/or reduction by allosteric modulators is determined by the subunit composition of the the $GABA_A$ receptor populations that are present (Pritchett et al., 1989a,b; Sigel et al., 1990; Verdoorn et al., 1990; Puia et al., 1991, 1992; Saxena and Macdonald, 1994). To examine whether the altered expression of distinct functional $GABA_A$ receptor subunits after K^+-induced depolarization and/or NMDA treatment changes the allosteric modulation of $GABA_A$ receptors grown in each culture condition, the BZD flunitrazepam was briefly preapplied, and potentiation of GABA induced Cl^- currents was monitored. To test the degree of potentiation by flunitrazepam, the peak current was elicited using a GABA concentration that was about 20% of the EC_{50} value obtained from the respective GABA dose response curve without the BZD coapplication (Table 1). Figure 3*A* shows individual current traces at a single concentration of GABA with and without flunitrazepam in each experimental paradigm. Flunitrazepam was then briefly preapplied in increasing concentrations (0.01, 0.1, 1, and 10 μM) with a constant concentration of GABA (Fig. 3*C*). Application of flunitrazepam required a bath perfusion for a few seconds prior to the application of GABA to exert its maximal effect (White, 1992). As shown in Figure 3*C*, for $GABA_A$ receptors cultured in both high K^+ and in cultures maintained in low K^+ and treated with NMDA, the GABA-induced currents were potentiated by flunitrazepam in a dose-dependent manner, with a maximal potentiation value of 136 ± 17.9% and 137 ± 31.9%, respectively. However, flunitrazepam was much less efficacious in potentiating GABA responses from receptors recorded from granule cells cultured in low K^+ containing media (15.2 ± 6.3%) (Fig. 3*C*).

In a separate set of experiments, the β-carboline DMCM was used to examine the negative modulation of GABA-gated Cl^- currents in each experimental group. Figure 3*B* shows individual currents recorded with and without the addition of DMCM (1 μM) at the indicated GABA concentrations, while Figure 3*D* shows the corresponding dose–response curves obtained at a single GABA concentration with increasing concentrations of DMCM. DMCM markedly decreased GABA-activated Cl^- currents in granule neurons cultured in high K^+ and in those treated with NMDA (Fig. 3*B,D*). In contrast, the $GABA_A$ receptors present in granule cells grown in low K^+-containing media showed a much lowered sensitivity to DMCM (same figures). Figure 3*D* shows the dose-dependent negative allosteric modulation of GABA-gated currents by DMCM in cultured granule neurons, but the efficacies of DMCM (10 μm) to inhibit GABA-gated Cl^- currents in granule neurons cultured in low K^+, compared with those obtained from neurons cultured in high K^+ or following NMDA treatment, were significantly different (48 ± 4% for high K^+, 45 ± 8% for NMDA treatment, and 14 ± 3% for low K^+).

Modulation of $GABA_A$ receptor currents by Zn^{2+} in cerebellar granule cells cultured in high K^+, low K^+, and low K^+ + NMDA

To further distinguish the $GABA_A$ receptor subtypes present in granule cells cultured in high K^+, low K^+, and in low K^+ + NMDA, we studied the effect of Zn^{2+}-induced inhibition of GABA-gated currents, which is also dependent on the subunit composition of the $GABA_A$ receptor (Draguhn et al., 1990; Saxena and MacDonald, 1994; White and Gurley, 1995). It has been reported that Zn^{2+} reduces GABA-gated currents in recombinantly expressed receptor assemblies lacking a γ subunit, but that it has less of an effect on $GABA_A$ receptors containing a γ subunit, depending on the α subunit present (Smart et al., 1991; White and Gurley, 1995). Figure 4*A* shows superimposed cur-

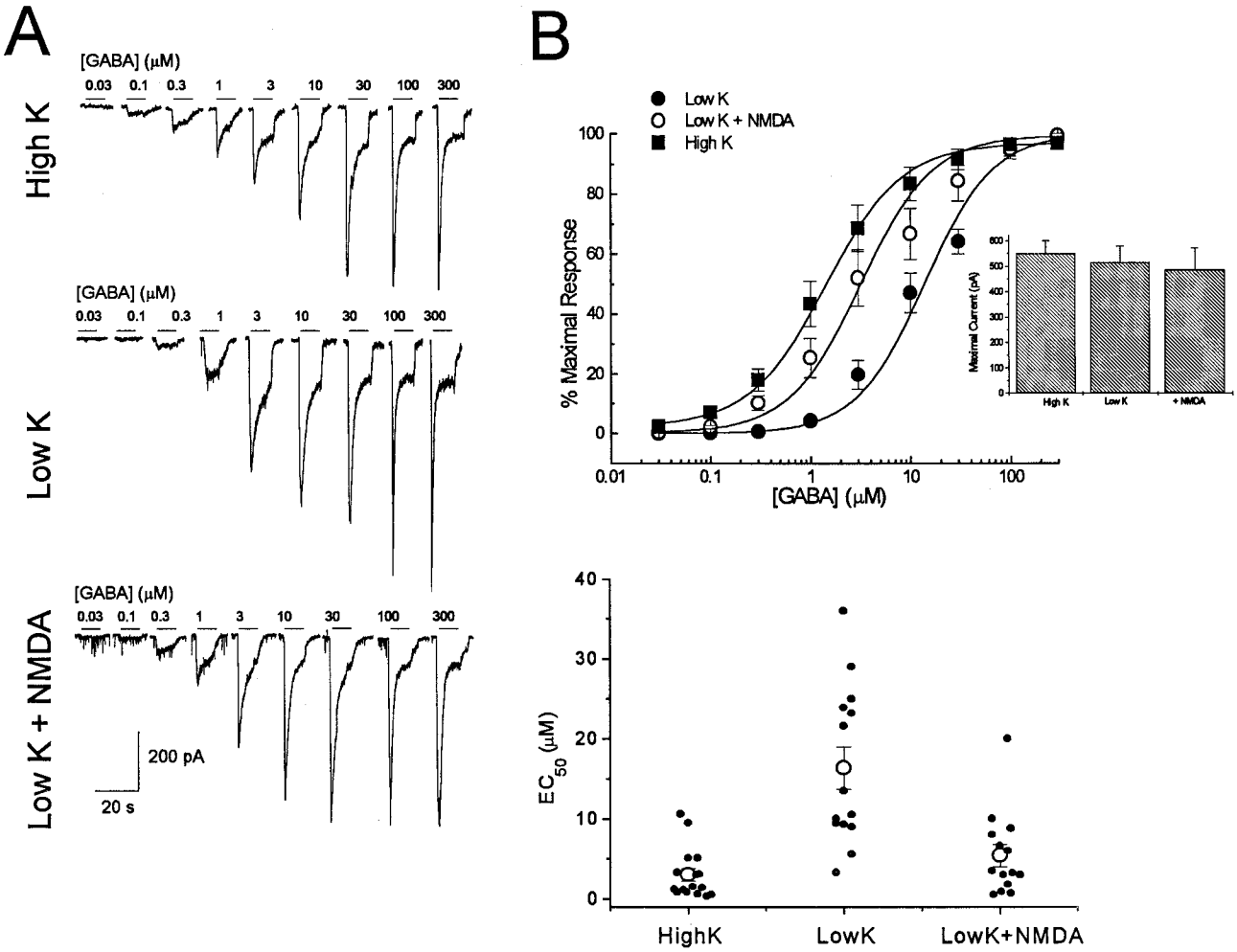

Figure 2. Whole-cell membrane current recordings from the cultured cerebellar granule cells. *A*, Current traces recorded from granule cells elicited by increasing GABA concentrations. GABA was applied using a Y-tubing system for the duration indicated by the *bars*. Granule cells were prepared from postnatal day 8 rats and were maintained and treated as described in the text. GABA-induced currents were recorded from granule cells voltage clamped at a membrane potential of −50 mV. The calibration bar applies to all cells shown. *B*, (*Top*) Normalized GABA dose–response curves obtained from granule cells cultured in high K (*filled squares*), low K (*filled circles*), and low K + NMDA (*open circles*). Peak currents recorded from individual cells during the application of GABA were normalized to the maximal GABA response recorded for a given cell by using the equation given in experimental procedures. Each point represents the mean ± SEM of the normalized current at each GABA concentration recorded from at least 14 cells. The corresponding average EC_{50} values and Hill coefficients (*nh*) are listed in Table 1. The maximal Cl^- current recorded in each condition failed to differ significantly (*inset*). (*Bottom*) EC_{50} values derived from normalized GABA dose responses in individual granule neurons (*filled circles*) and their averages in each culture condition (*empty circles*).

Table 1. GABA responses obtained from neurons maintained in the different culture conditions

Culture condition	EC_{50} (μM)	n_h	*N*
High K^+	1.4 ± 0.08	0.9	16
Low K^+	13.8 ± 1.2	1.0	14
Low K^+ + NMDA	3.5 ± 0.3	0.85	14
Low K^+ + Glu	3.2 ± 0.2	1.0	12
Low K^+ + Glu + MK801	13.4 ± 0.7	1.0	13

Granule cells were cultured in a medium with the final KCl concentration adjusted to either 12.5 mM (low K^+) or 25 mM (high K^+). On day 4 after plating, some of cultures maintained in low K were treated with a single addition of 10 μM NMDA (low K^+ + NMDA), or 5 μM glutamate (low K^+ + Glu), and one group of cultured cells was pretreated with MK801 (1 μM) before the addition of glutamate (low K^+ + Glu + MK801). Values are the mean ± SEM. A significant difference at most concentrations among different culture conditions was determined using one-way ANOVA ($p < 0.05$) followed by a paired *t* test.

rent traces at a constant GABA concentration and at two different concentrations of Zn^{2+}. Our results showed that Zn^{2+} produced an inhibition of GABA-gated currents in a concentration-dependent manner (Fig. 4*A*,*B*). NMDA treatment and K^+-induced depolarization shifted the dose–response curve of the Zn^{2+}-induced inhibition to the right (Fig. 4*B*) and therefore increased the IC_{50} estimated from the corresponding inhibition curve (Table 2). The GABA-gated currents in the presence of Zn^{2+} (300 μM) were 30.6% and 31.4% of the currents evoked by 10 μM GABA in cells cultured in high K^+ and low K^+ + NMDA, respectively. These results were significantly decreased from those obtained from cells cultured in low K^+ (16.9%) (Fig. 4*C*).

Effect of furosemide on GABA-gated Cl^- currents in cerebellar granule cells cultured in high K^+, low K^+, and low K^+ + NMDA

To obtain additional information related to changes in the composition and assembly of $GABA_A$ receptors after high K^+-in-

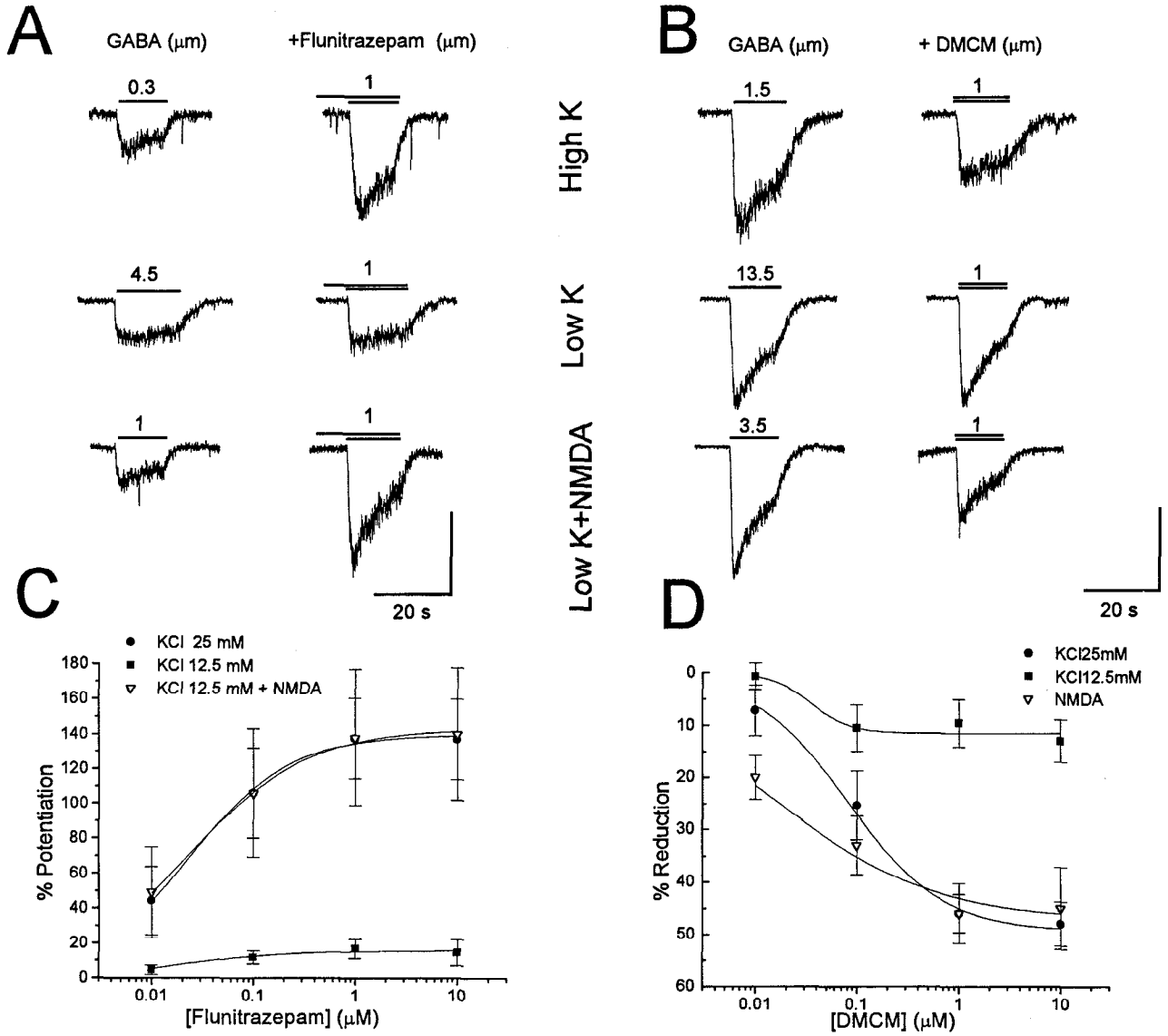

Figure 3. Modulation of GABA-gated Cl^- channels by flunitrazepam and DMCM. *A* and *B*, Current traces recorded from cultured granule cells voltage clamped at a holding potential of −50 mV. Each pair of currents consists of the control response evoked by a GABA concentration as determined by the EC_{20} value reported in Table 1 for that particular culture condition, and the response evoked by the coapplication of GABA and flunitrazepam (1 μM, *left*). The inhibiton of GABA evoked Cl^- currents by the negative modulator DMCM (1 μm, *right*) was determined at a GABA concentration coinciding with the EC_{50} value as indicated in Table 1. The duration of drug application is indicated by the *bars*. Vertical calibrations are 80 pA (*left panel*) and 130 pA (*right panel*). *C* and *D*, Concentration dependence of the response to flunitrazepam (*left*) and DMCM (*right*). Experiments were carried out for the indicated concentrations of flunitrazepam or DMCM in granule cells cultured in high K^+ (*filled circles*), low K^+ (*filled squares*), and low K^+ treated with the addition of 10 μM NMDA (*open triangles*). Potentiation was calculated by comparing current levels induced by coapplication of GABA and flunitrazepam or DMCM. Each point represents the mean ± SEM of the variation from control of at least 10 cells. The GABA concentrations used for each paradigm are as indicated above and represent the appropriate EC_{20} value for the flunitrazepam dose–response curve, while for the DMCM at the appropriate EC_{50} values were used as determined for each experimental paradigm.

duced depolarization and NMDA treatment, we examined the effect of furosemide on GABA-activated Cl^- currents. This compound, at low μM concentration, is a selective noncompetitive inhibitor of the GABA-activated currents in transfected HEK-293 cells (W. J. Zhu and S. Vicini, unpublished data) or in *Xenopus* oocytes (Korpi et al., 1995) expressing α6β2γ2 $GABA_A$ receptor combinations, but it requires much higher concentrations to inhibit recombinant α1β2γ2 subunit containing receptor assemblies. GABA-activated channel currents were examined in cerebellar granule cells cultured in high K^+, low K^+, and low K^+ + NMDA with or without the addition two concentrations of furosemide. Currents evoked by GABA (1 μM) alone were used as the control values for each paradigm, and percent inhibition was calculated when currents elicited by coapplication of GABA and furosemide were expressed relative to controls (i.e., no furosemide added). The values recorded from cells maintained in high KCl were 94 ± 2.1% SEM of control (at 30 μM furosemide) and 84 ± 2.8% SEM (100 μM furosemide). Those obtained from granule cells maintianed in low KCl were 78 ± 3.4% SEM of control (30 μM furosemide), and 66 ± 4.5% SEM of control (100 μM furosemide). Finally, those cells maintained in low KCl and treated with NMDA on the 4 d DIV, were 90 ± 3.2% of control (30 μM furosemide) and 82 ± 2.8% of control (100 μM furosemide). The concentration-

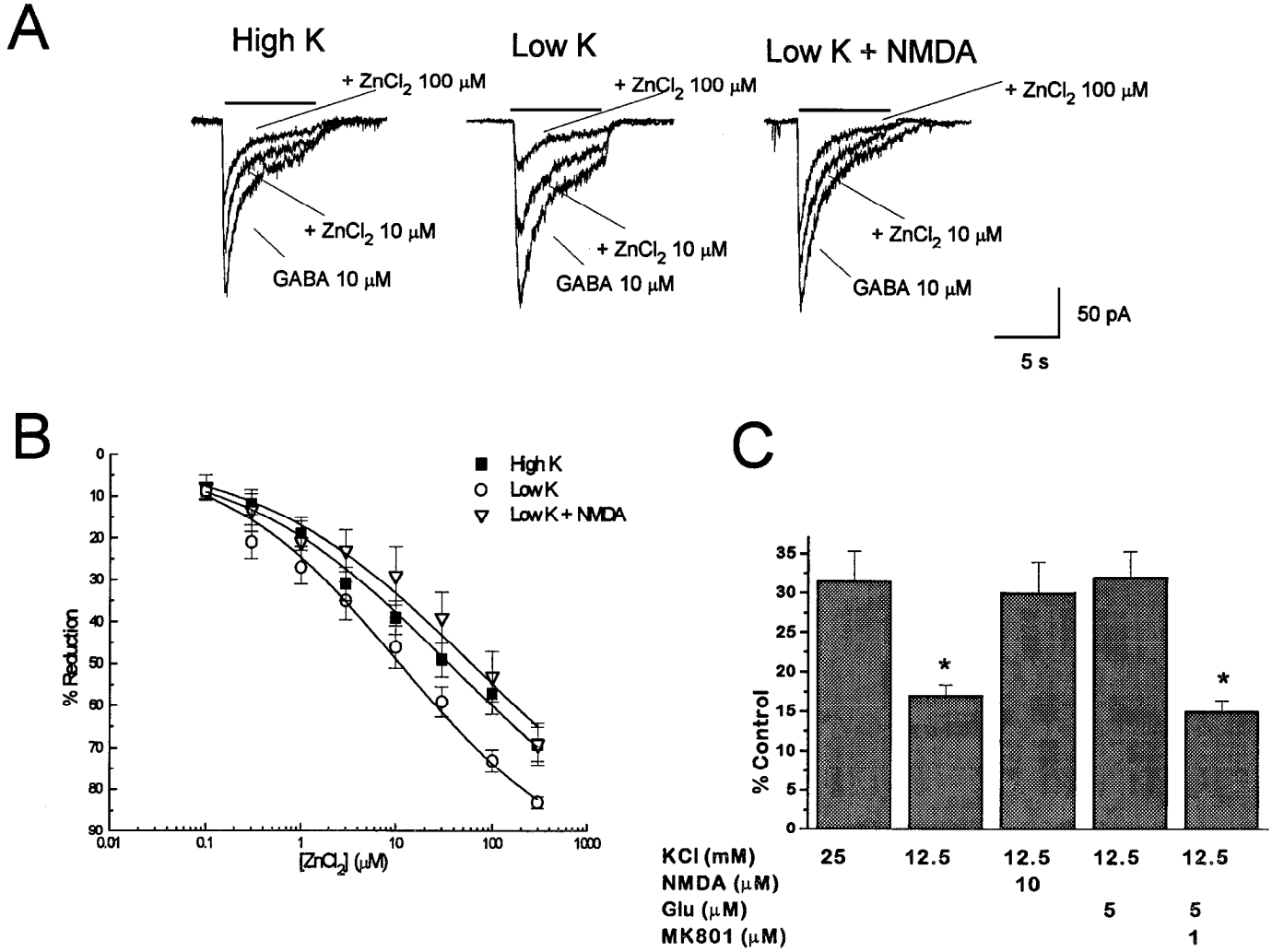

Figure 4. Modulation of GABA-activated currents by Zn^{2+} in cultured granule cells. *A*, Superimposed traces showing the inhibition of GABA-induced Cl^- currents by $ZnCl_2$ in granule neurons cultured in high K and low K in the absence and presence of NMDA (10 μM). Cl^- currents were induced with GABA (10 μM) in the absence or presence of $ZnCl_2$ at the indicated concentration. The duration of drug applications is indicated by the *bars*. *B*, Dose–response curves for $ZnCl_2$ inhibition of Cl^- currents with granule cells cultured in high K^+, low K^+, and low K^+ with the addition of NMDA (10 μM) on day 4 *in vitro*. On the following day, cells were voltage clamped at −50 mV, and GABA-gated currents were recorded. In each culture condition, current levels recorded in the presence of 10 μM GABA were used as a control value (100%). The percentage inhibition of Cl^- currents produced by Zn^{2+} at the indicated concentrations were plotted and fitted to a logistic equation (see Experimental Procedures). The corresponding IC_{50} values and Hill coefficients (n_h) calculated from these data are presented in Table 2. The data represent mean ± SEM of at least 12 cells. *C*, The inhibitory effect of Zn^{2+} (at one concentration) on the GABA-gated currents in granule cells cultured in high or low KCl in the absence and presence of the indicated compounds is expressed as the percentage of control. NMDA, glutamate, and MK-801 were added to the media at the indicated concentrations on day 4 *in vitro*. Whole-cell GABA-gated currents were recorded at a membrance potential of −50 mV by the application of GABA (10 μM) either with or without coapplication of $ZnCl_2$ (300 μM). In each culture condition, current levels recorded in the presence of 10 μM GABA were used as a control value (100%). Data are expressed as mean ± SEM. *Denotes significant difference by using one-way ANOVA ($p < 0.05$) of the percentage of control in each culture condition compared to the 25 mM KCl group.

dependent furosemide inhibition of GABA-gated currents was significantly greater statistically in neurons cultured in low K^+-containing media than in cells cultured either in high K^+-induced depolarization conditions or 24 hr following NMDA treatment ($n = 15$ cells at each concentration; $p < 0.05$, paired t test). In contrast, no significant difference was observed in the inhibition of GABA-gated currents between cells cultured in high K^+ or in low K^+ + NMDA.

In a separate set of experiments, these data were repeated on cells maintained in low KCl at 10 μM GABA, keeping the concentrations of furosemide as indicated above. No significant differences were observed at the higher concentrations of GABA used with respect to the percentage inhibition observed at either furosemide concentration shown (data not shown). These data are consistent with the reported noncompetitive nature of furosemide-mediated inhibition that is substrate concentration independent.

Inhibition of the effect of glutamate treatment on $GABA_A$ receptors by MK-801

To demonstrate that the changes in the sensitivity to GABA, allosteric modulation, and Zn^{2+}-induced inhibition of GABA-gated currents were mediated by the activation of NMDA-selective glutamate receptors and were not secondary to a generalized depolarization, cerebellar granule cells cultured in low K^+ were treated with the excitatory amino acid glutamate (5 μM) on day

Table 2. Inhibition of GABA-gated Cl^- currents in cultured granule cells by $ZnCl_2$

Culture condition	IC_{50} (μM)	n_h	*N*
High K^+	36 ± 3.7	0.4	19
Low K^+	11 ± 1.2	0.4	16
Low K^+ + NMDA	61 ± 13	0.4	12
Low K^+ + Glu	67 ± 4.9	0.5	12
Low K^+ + Glu + MK801	22 ± 2.3	0.6	11

Granule cells were cultured in a medium with the final KCl concentration adjusted to either low K^+ or high K^+. On day 4 after plating, some of cultures maintained in low K^+ were treated with a single addition of 10 μM NMDA (low K^+ + NMDA), or 5 μM glutamate (low K^+ + Glu), and one group of cultured cells was pretreated with MK801 (1 μM) before the addition of glutamate (low K^+ + Glu + MK801). IC_{50} values were derived from inhibition curves with $ZnCl_2$. Values are mean ± SEM. A significant difference at most concentrations among different cultured conditions was noted by using one-way ANOVA ($p < 0.05$) followed by a paired *t* test.

4 *in vitro*. As shown in Figures 5*A, B, C,* and 4*C*, the addition of glutamate produced the same pharmacological effect on the electrophysiological properties of $GABA_A$ receptors as did the treatment with cultures maintained in low K^+ + NMDA. The respective EC_{50}s from the GABA dose response curve (Fig. 5*A*) are shown in Table 1, and IC_{50}s from the Zn^{2+} inhibition curves (Fig. 5*C*) are in Table 2. The percent potentiation of GABA-elicited Cl^- currents by flunitrazepam is comparable to the curve obtained from cultures maintained in high K^+ or in low K^+ + NMDA (Fig. 3*C*). In separate experiments performed in parallel, cerebellar granule cells cultured in low K^+ were pretreated with MK-801 (1 μM), a noncompetitive antagonist of NMDA-selective glutamate receptors, 30 min before the addition of glutamate on the fourth DIV. Figures 4*C* and 5 show that MK-801 antagonized the effect of the glutamate treatment on the electrophysiological properties of the $GABA_A$ receptor, decreasing the receptor sensitivity to GABA (Fig. 5*A*), reducing the potentiation of $GABA_A$ receptor by flunitrazepam (Fig. 5*B*), and increasing the sensitivity to Zn^{2+}-induced inhibition (Fig. 5*C*). The EC_{50} from the GABA dose–response curve (Fig. 5*A*) and IC_{50} from the Zn^{2+}-induced inhibition curve (Fig. 5*C*) in the presence of MK-801 are shown in Tables 1 and 2 (respectively). These values are significantly different from those obtained from the dose–response curves from granule cells maintained in low K^+ and treated with either glutamate or NMDA. In contrast, the maximal Cl^- currents recorded from each condition (i.e., glutamate either with or without MK-801) failed to show statistically significant differences (Fig. 5*A*, inset).

Discussion

In vitro, immature cerebellar granule cells have survival requirements that can be met by chronic membrane depolarization and/or persistent NMDA receptor stimulation (Gallo et al., 1987; Balázs et al., 1988a,, 1992). In the work presented here, we demonstrate alterations in the pharmacology of $GABA_A$ receptors after K^+-induced depolarization and NMDA treatment in cerebellar granule cells in culture. Using whole-cell patch-clamp recordings, we have shown that both treatments decreased the EC_{50} for GABA, enhanced the amount of potentiation induced by benzodiazepine (BZD) agonists, and enhanced the reduction mediated by β-carbolines (DMCM) of GABA-gated Cl^- currents of $GABA_A$ receptors in cultured granule cells. At the same time, both Zn^{2+}- and furosemide-induced inhibition of GABA activated currents was decreased relative to the observed inhi-

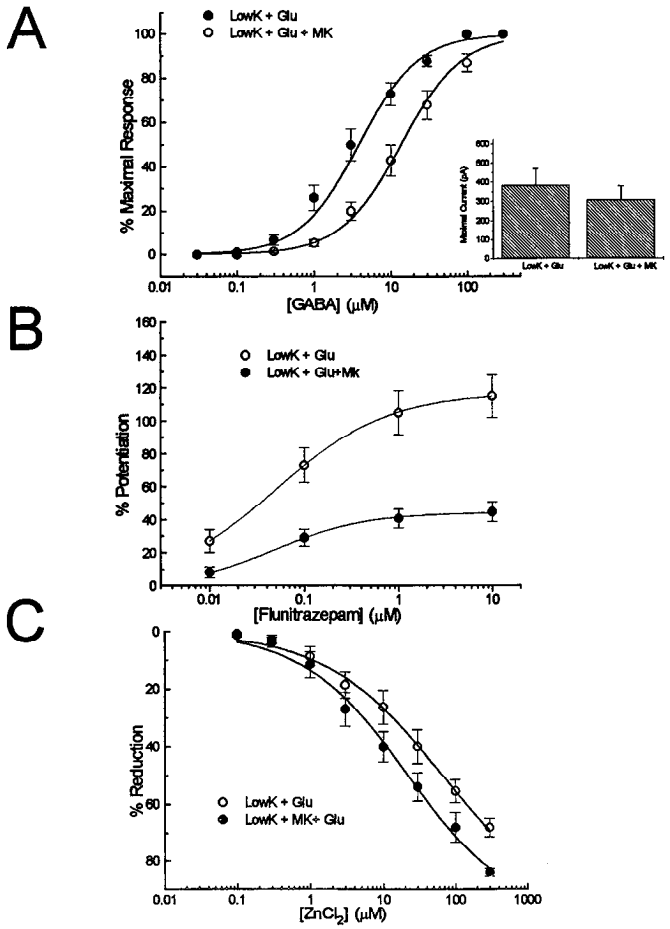

Figure 5. Effect of glutamate and MK-801 treatments on $GABA_A$ receptors in granule cells. *A,* GABA dose–response curves derived from granule cells cultured in low K^+ in the presence of glutamate (5 μm) or glutamate + MK-801 (1 μm). Glutamate and MK-801 were added to the cultures on day 4 *in vitro*. After 24 hr, the cells were clamped at a membrane potential of −50 mV to record GABA-gated Cl^- currents. Each point represents the mean ± SEM of the normalized peak currents at each GABA concentration in 12 cells. The corresponding EC_{50} values and Hill coefficients calculated from these data are given in Table 1. The addition of glutamate (5 μM) to the culture media shifted the GABA dose–response curve towards the left and produced an increase in GABA potency that was attenuated by the MK-801 pretreatment. The maximal Cl^- current recorded in each condition failed to differ significantly (*inset*). *B,* Concentration dependence of flunitrazepam-induced potentiation of GABA-activated Cl^- currents. On day 4 *in vitro*, one group of granule cells cultured in low K^+ was treated with 5 μM glutamate (*open circles*), and another was pretreated with 1 μM MK-801 (*filled circles*) prior to the addition of glutamate. On the following day, cells were voltage clamped at a membrane potential of −50 mV to record GABA-gated currents. Potentiation was calculated by comparing peak currents induced by the coapplication of GABA and flunitrazepam with GABA application alone. Each point represents the mean ± SEM derived from the potentiation as compared to control in 12 cells. *C,* Dose–response curves for $ZnCl_2$ inhibition of Cl^- currents with granule cells cultured in low K^+ (*filled triangles*) and low K^+ with the addition of 5 μM glutamate (*filled circles*) or glu + MK-801 (1 μM, *open circles*) on day 4 *in vitro*. Cells were voltage clamped at −50 mV to record GABA-gated currents. In each culture condition, current levels recorded in the presence of 10 μM GABA were used as the control value (100%). The percent inhibition of Cl^- currents produced by Zn^{2+} at the indicated concentrations were fit using the equation given in Experimental Procedures. The corresponding IC_{50} values and Hill coefficients (n_h) calculated from these data are given in Table 2. Data represent the mean ± SEM from the inhibition of control GABA-evoked currents in 11 cells.

bition obtained from whole-cell recordings of granule cells maintained in low K^+. In addition, glutamate treatment produced a similar pharmacological profile of $GABA_A$ receptors as did NMDA treatment, and this effect was blocked by the noncompetitive NMDA receptor antagonist MK-801. This finding is consistent with the effects of NMDA and glutamate occurring through the stimulation of selective NMDA receptors. This is in contrast to a generalized glutamate and/or NMDA-induced depolarization, with subsequent release of other transmitter/trophic factors acting at their respective receptors.

In vivo, in situ hybridization studies have shown that cerebellar granule neurons contain a number of $GABA_A$ receptor subunit mRNAs, including the mRNAs encoding the $\alpha 1$, $\alpha 6$, $\beta 2$, $\gamma 2$, and δ subunits, and to a lesser extent, the $\alpha 4$, $\beta 1$, and $\gamma 3$ subunits (Laurie et al., 1992a; Farrant and Cull-Candy, 1993). Quantitative reverse transcription polymerase chain reaction (RT-PCR) studies have shown that cerebellar granule cells *in vitro* express each of the receptor subunit mRNAs, albeit at different absolute levels (Bovolin et al., 1992). From immunoprecipitation studies, it seems that the most abundant $GABA_A$ receptor subunit combinations may be the $\alpha 6\gamma 2$, $\alpha 1\gamma 2$, $\alpha 2\gamma 1$, and $\alpha 6\delta$ containing isoforms with a small population of $\alpha 3\gamma 2$ or $\alpha 1\alpha 3\gamma 2$ (McKernan et al., 1991; Qurik et al., 1994). The $\beta 2$ and $\beta 3$ subunits are highly expressed in the cerebellum, whereas the $\beta 1$ subunit is less abundant and therefore less likely to contribute to many cerebellar $GABA_A$ receptor populations (McKernan et al., 1991; Laurie et al., 1992; Qurik et al., 1994).

A number of studies have established that the subunit composition and assembly of ligand-gated ion channels determines the physiological and pharmacological properties of the receptor subtypes (Levitan et al., 1988; Pritchett et al., 1989b; Sigel et al., 1990; Verdoorn et al., 1990; Puia et al., 1991, 1992, 1994; Saxena and MacDonald, 1994; Dučić et al., 1995). At the same time, a variety of studies have shown that posttranslational modifications can alter the pharmacological profile of $GABA_A$ receptor subtypes (Gyenes et al., 1994; Krishek et al., 1994; Lin et al., 1994). Our present results indicate that K^+-induced depolarization and NMDA treatment decreased the EC_{50} of GABA dose responses that occurred in parallel with an increase in the abundance of the $\alpha 1$ and $\alpha 5$ receptor subunit mRNAs and of their corresponding receptor subunit immunoreactivities following both treatments, as previously reported (Memo et al., 1991; Harris et al., 1994, 1995). Taken together, the data suggest the possibility that a superabundance of selected α subunits may form $GABA_A$ receptor populations with a higher affinity for GABA.

Data have demonstrated that the EC_{50} of $GABA_A$ receptors for GABA is much lower in receptors that contain $\alpha 6$ receptor subunits than receptor subtypes coassembled with subunits that contribute to benzodiazepine sensitive combinations (Kleingoor et al., 1993). Our previous studies have shown that the amount of the $\alpha 6$ receptor subunit mRNA remains constant in each granule cell preparation paradigm used in the present studies (Harris et al., 1994). Provided that the amount of the receptor subunit also does not change, the observed increase in $GABA_A$ receptor sensitivity cannot be explained by an increased proportion of $\alpha 6$ subunit containing receptor assemblies following NMDA or glutamate treatment. This possibility is consistent with the data that demonstrate that furosemide, reported to be a noncompetitive inhibitor selective for $\alpha 6$ receptor subunit containing assemblies (Korpi et al., 1995), exhibits a greater inhibition in granule cells maintained in either low K^+ or low K^+ cultures treated with glutamate in the presence of MK-801. One possibility is that the amount of available $\gamma 2$ receptor subunits is limited and that the assemblies that are subsequently formed are determined by a competition between available α subunits present. This is supported by the data that show no difference in the maximal current elicited by GABA in each of the experimental paradigms, indicating no change in the total numbers of receptors present. These data also support the hypothesis that distinct structural assemblies of $GABA_A$ receptors, rather than protein kinase C (PKC) phosphorylation, underlie the observed pharmacological changes. In fact, all of the reports of pharmacological modifications induced by PKC phosphorylation indicate consistent changes in the observed maximal response (Gyenes et al., 1994; Krishek et al., 1994; Lin et al., 1994). Recombinant receptor expression studies have shown that when two distinct α subunits are coexpressed in the same receptor complex, the EC_{50} is an intermediate value of the individual EC_{50}s without changing the Hill coefficient (Verdoorn, 1994). Our data show no change in the Hill coefficient in the different culture paradigms, indicating that the relative abundances of the α subunits present may be responsible for modulating the EC_{50}s in each experimental group.

It has been shown that combinations of α, β, and γ subunits are required to confer high-affinity benzodiazepine binding to recombinant receptors, and that different α subunits produce receptors that have different BZD pharmacology (Pritchett 1989a,b; Puia et al., 1991, 1992; Wafford et al., 1993). The $\alpha 6$ subunit, found exclusively in cerebellar granule cells, does not contribute to BZD-sensitive receptor subtypes (Kato, 1990; Laurie et al., 1992a; Farrant and Cull-Candy, 1993; Kleingoor et al., 1993). The lack of BZD responsiveness is more prominent in neurons maintained in low K^+, and under these conditions it has previously been shown that the absolute amounts and, hence, relative proportions of $\alpha 6$ mRNA to $\alpha 1$ mRNA are significantly higher in RNA isolated from these cultures (Harris et al., 1994). Additionally, the selective presence of the δ subunit mRNA in cerebellar granule cells *in vitro* (Bovolin et al., 1992) may underlie some of the distinct pharmacological regulation by BZDs (Saxena and MacDonald, 1994) in the distinct culture conditions. The observed changes in pharmacological properties of $GABA_A$ receptors suggests that NMDA treatment and K^+-induced depolarization lead to the formation of functional $GABA_A$ receptor isoforms with altered pharmacological and electrophysiological properties. Although it remains a possibility that multiple mechanisms may be operative in eliciting the observed electrophysiological changes, the pharmacological profiles observed suggest that the mRNA and protein changes are at least, in part, responsible for the present results.

In addition, concomitant with the maturation of $GABA_A$ receptors maintained *in vitro* in high K^+ (beyond 7 to 10 DIV), it has been shown that the expression of the $GABA_A$ receptor $\alpha 6$ subunit mRNA increases, which reduces the ratio of $GABA_A$ receptor containing $\alpha 1$ subunits in the population of native receptors (Mathews et al., 1994; Zheng et al., 1994). The effect of this change in subunit proportions in $GABA_A$ receptor assemblies results in a pharmacological profile similar to that obtained from recombinant $\alpha 6\beta\gamma 2$ $GABA_A$ receptors, i.e., there are increases in BZD-insensitive ^3H Ro 15-4513 binding, reduced potentiation for BZD agonists, and increased sensitivities to GABA (Mathews et al., 1994; Thompson et al., 1994; Zheng et al., 1994). The lack of sensitivity of $GABA_A$ receptors for BZD agonists in cerebellar granule cells maintained in low K^+ con-

ditions is in agreement with the decreased flunitrazepam binding data presented here.

In eukaryotic expression systems, the γ subunit appears to be an absolute requirement for allosteric modulation (Pritchett et al., 1989a; Verdoorn et al., 1990; Puia et al., 1991, 1992). On the other hand, GABA receptors reconstituted from combinations of α, β, and/or δ subunits are potently blocked by Zn^{2+}, while the presence of the γ subunit in any combination with α, β, and/or δ subunits leads to the formation of $GABA_A$ receptors that are differentially sensitive to Zn^{2+} (Draguhn et al., 1990; Smart et al., 1991; Saxena and MacDonald, 1994), depending on the specific α subunit present (White and Gurley, 1995). Our data indicate that K^+-induced depolarization and NMDA treatment results in a higher proportion of receptor subtypes sensitive to BZDs and insensitive to the effects of Zn^{2+} suggesting an increase in the proportion of α1γ containing receptor subtypes.

Finally, our results suggest that excitatory afferent synaptic signaling may establish activity-dependent $GABA_A$ receptor plasticity as an autoregulatory mechanism to protect overexcitation of the stimulated neuron and thus may play a role in preventing seizure activity. Experimental data have shown that divalent metal ions, contained in nerve terminals, are released into the extracellular space during neuronal activity (Assaf and Chung, 1984; Xie and Smart, 1992). Our results demonstrate that NMDA treatment modified $GABA_A$ receptor sensitivity to Zn^{2+}-induced inhibition of GABA-gated currents, especially in the presence of Zn^{2+} when the latter is present at high concentrations. These concentrations may be reached during neuronal activity, suggesting that NMDA selective receptor stimulation regulates inhibitory synaptic transmission secondary to the modulation of $GABA_A$ receptor expression.

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