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Quantitative Changes in α1 and α5 γ-Aminobutyric Acid Type A Receptor Subunit mRNAs and Proteins after a Single Treatment of Cerebellar Granule Neurons with *N*-Methyl-D-aspartate

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SUMMARY

It was previously demonstrated that daily administration of *N*-methyl-D-aspartate (NMDA) to primary cultures of cerebellar granule neurons for 5 days *in vitro* mediates an increase in the relative content of mRNAs encoding selected subunits of the γ-aminobutyric acid $(GABA)_A$ receptor. This analysis was extended using a competitive polymerase chain reaction assay with internal standards to quantitate changes that occur in the absolute amounts of selected $GABA_A$ receptor subunit mRNAs in cerebellar granule neurons *in vitro* after the single administration of nontoxic doses of either NMDA or glutamate. For these studies, we focused on the α1, α5, and α6 receptor subunit mRNAs and examined their absolute contents in cultures maintained in low KCl (12.5 mM), maintained in low KCl and treated with NMDA (10 μM) for 24 hr, or maintained in high KCl (25 mM). The absolute amounts of each mRNA varied in these paradigms; whereas the α1 and α5 receptor subunit mRNAs increased in response to NMDA-selective glutamate receptor stimulation, the α6 receptor subunit mRNA did not. The time course of the α1 and α5 mRNA increases, dose dependence, and effects of glutamate in the presence or absence of MK-801 were also analyzed. Treatment of cultures maintained in 12.5 mM KCl with 5 μM glutamate resulted in comparable changes in the α1 and α5 receptor subunit mRNA contents, and a somewhat smaller increase in the α6 mRNA content was observed. Using corresponding $GABA_A$ receptor subunit-specific antibodies, it was shown that the observed mRNA changes are accompanied by increased expression of α1- and α5-like receptor subunit immunoreactivity. Collectively, these data demonstrate that signal transduction mechanisms triggered by NMDA-selective glutamate receptor stimulation differentially modulate the levels of selected $GABA_A$ receptor subunit mRNAs and the corresponding proteins they encode.

The $GABA_A$ receptor is a hetero-oligomeric integral membrane protein that defines an anion-selective channel, allowing the influx of Cl^- in response to binding of the neurotransmitter GABA to specific recognition sites located on this ionotropic receptor. Based on their amino acid sequence identity, the receptor subunits have been classified into various subfamilies, and in the rat the following subunits have been identified: α1–6, β1–3, γ1, γ2S, γ2L, γ3, δ, ρ1, and ρ2 (1). Although the stoichiometry of the $GABA_A$ receptor has not directly been defined, by analogy to direct structural studies performed on the nicotinic acetylcholine receptors the structurally distinct subunits are thought to assemble into pentameric complexes (2). Using transient expression assays with receptors assembled from various recombinant $GABA_A$ receptor subunit cDNAs, the subunit composition of a given $GABA_A$ receptor assembly has been shown to impart a differential sensitivity of the receptor complex to GABA and to various allosteric modulators, such as benzodiazepines and β-carbolines, that act to positively or negatively modulate the receptor response to GABA (3–5). This implies that the structural composition of the receptor assembly confers a specific efficacy and potency to the receptor response to GABA. Thus, the coordinated temporal and spatial expression of genes encoding the multiple $GABA_A$ receptor subunits defines the operational flexibility of any given GABAergic synaptic link.

Primary cultures of cerebellar granule neurons from postnatal day 8 rats express a continuous developmental change in the content of mRNAs encoding selected subunits of $GABA_A$ receptors (6, 7), various ionotropic NMDA-selective and non-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); DIV, day(s) *in vitro*; NMDA, *N*-methyl-D-aspartate; MK-801, dibenzocycloheptenеimine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; DAB, diaminobenzamidine; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ANOVA, analysis of variance.

NMDA-selective glutamate receptors,[1] and metabotropic glutamate receptors (8–10). Thus, these cultures provide a useful model to study the changes in neurotransmitter receptor gene expression after receptor stimulation. These neuronal cultures require depolarizing concentrations of KCl (25 mM or greater) for long term survival and differentiation (11–13). That is, granule cell cultures plated in the presence of lower KCl concentrations (12.5 mM or lower) differentiate more slowly and begin to degenerate at shorter times in culture (11–13). These same cultures maintained in the presence of lower KCl concentrations can be treated with glutamate or the synthetic excitatory amino acid agonist NMDA, both of which have a trophic effect on the granule neurons (14). This effect appears to be both time and dose dependent, with lower doses of NMDA (500 nM to 50 μM) or glutamate (500 nM to 10 μM) promoting this trophic effect and higher doses (50 μM or greater of either compound) causing excitotoxicity (15). Persistent depolarization of the granule neurons, brought about by 25 mM or higher concentrations of KCl, has been shown to cause a time-dependent release of amino acids, including glutamate, into the culture medium (16–18). The endogenously released glutamate, alone or together with other released trophic factors such as the brain-derived neurotrophic factor (19), may be responsible for the increased survival and differentiation of these neurons. An increased calcium influx through ligand- and/or voltage-gated channels appears to be a determinant for these survival-promoting effects of the glutamate- and/or potassium-induced depolarization (11).

The expression of the mRNAs encoding various $GABA_A$ receptor subunits has been studied extensively both during development and after pharmacological manipulation of animals *in vivo* and various neuronal cultures *in vitro*. For example, several $GABA_A$ receptor subunit mRNAs have been shown to increase continuously during cerebellar granule neuron maturation *in vitro* (6). Homologous receptor stimulation of neuronal culture systems with GABA (20–21) or the specific $GABA_A$ receptor agonist muscimol (22) decreases the content of selected $GABA_A$ receptor subunit mRNAs. Other studies using cerebellar granule neuron cultures, though, suggest that GABA receptor stimulation increases the mRNAs for selected α $GABA_A$ receptor subunits (23), although the concentrations of GABA necessary to produce this result are unusually high (500 μM). *In vivo*, the chronic administration of diazepam is associated with regional variations in the α1 receptor subunit mRNA content (24), whereas both flurazepam (25) and ethanol (26) produce differential effects on the levels of individual α receptor subunit mRNAs. Collectively, these reports illustrate the neuronal-type specific plasticity of neurons, in terms of their ability to alter $GABA_A$ receptor subunit expression due to a variety of epigenetic events associated with changes in the balance between excitatory and inhibitory afferent input.

Although considerable work has focused on the regulation of $GABA_A$ receptor subunit mRNAs in response to ligands interacting with recognition sites associated with the $GABA_A$ receptor complex, much less is known with respect to the regulation that occurs after stimulation of excitatory amino acid receptors. Earlier work from our laboratory has shown that the amounts of various $GABA_A$ receptor subunit mRNAs increase to different extents after chronic treatment of granule neurons (maintained in the presence of 12.5 mM KCl) with NMDA (27, 28). For example, it was shown using semiquantitative reverse transcription-PCR that the α1 and α5 receptor subunit mRNAs increased 6- and 10-fold, respectively, after 5 days of daily treatment with 10 μM NMDA, compared with nontreated, time-matched, control cultures. In contrast, other receptor subunit mRNAs, such as β3 and δ, were found not to change under these conditions. In cerebellar granule neurons maintained in the presence of high KCl concentrations (25 mM) and persistently treated with the NMDA-selective glutamate receptor antagonists MK-801 or 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, the relative abundances of the α1, α5, β1, β2, and γ2 mRNAs decreased, whereas the β3 and δ mRNAs remained unchanged. The unique sensitivity of each receptor subunit mRNA content to change after persistent stimulation by NMDA of NMDA-selective glutamate receptors probably reflects differential regulation of the corresponding genes by the expression of nuclear third messengers regulated by the transduction events that are operative after excitatory receptor stimulation.

This report details selective changes in the content of several of the $GABA_A$ receptor subunit mRNAs and the amount of subunit immunoreactivity expressed in primary cultures of rat cerebellar granule neurons maintained under different culture conditions. Using a competitive PCR technique developed in our laboratory (6, 7, 29), absolute amounts of the α1, α5, and α6 $GABA_A$ receptor subunit mRNAs were measured in cerebellar granule neurons maintained *in vitro* in the presence of 12.5 mM KCl or 25 mM KCl and after stimulation of NMDA-selective glutamate receptors present in cultures maintained in the presence of 12.5 mM KCl with a single dose of NMDA (10 μM) or glutamate (5 μM). The effect of glutamate and NMDA on α1 and α5 subunit immunoreactivity was assessed qualitatively by immunocytochemistry and Western analysis and quantitatively by ELISA using subunit-specific polyclonal antibodies. The plasticity of $GABA_A$ receptor subunit mRNAs and proteins observed after stimulation of NMDA-selective glutamate receptors may allow neurons to maintain membrane potential signaling flexibility by increasing their capacity to respond to the afferent excitatory input by increasing their potential inhibitory tone. In this way, interneuronal signaling may be maintained in neuronal circuits that are consistently stimulated.

Materials and Methods

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co.

Neuronal cultures and RNA isolation. Primary cultures of rat cerebellar granule neurons were prepared as described previously (16). In brief, cerebella from 8-day-old Sprague-Dawley rats were removed, and cells were dissociated with trypsin and by trituration. Cells were then plated on poly-D-lysine-coated 10-cm diameter dishes (Nunc) for RNA and protein isolation or 3.5-cm diameter dishes for immunocytochemistry. The cells were cultured in basal Eagle's medium (GIBCO BRL) supplemented with 10% bovine calf serum, 2 mM glutamine, and 50 μg/ml gentamicin. The final concentration of KCl was adjusted to either 12.5 mM (low KCl) or 25 mM (high KCl). Cells were plated at a density of 2×10^7 cells/10-cm dish or 3×10^6 cells/3.5-cm dish and were maintained at 37° in 6% CO_2. Cytosine arabinoside (final concentration, 10 μM) was added to all cultures 18–24 hr after plating, to inhibit glial proliferation.

On day 4 after plating, cultures maintained in 12.5 mM KCl were

[1] D. R. Grayson and B. T. Harris, unpublished observations.

treated with a single addition of NMDA (0.1–25 μM for dose-response studies and 10 μM for other studies) or glutamate (final concentration, 5 μM). Cells were lysed at subsequent time points using 5 M guanidium isothiocyanate (GIBCO BRL), and total RNA was prepared by gradient ultracentrifugation over a CsCl cushion (30).

Viability of the cultures was assessed by treating cultures in the various paradigms with 500 μg/ml MTT [in PBS (17 mM KH_2PO_4, 50 mM Na_2HPO_4, 1.5 M NaCl, pH 7.2)], which is reduced in live cells to a colored formazan product detectable at 570 nm (31). Cell death was determined by decreased absorbance at 570 nm, relative to time-matched and untreated controls, using the assay described above. Significant cell death (30% or greater decrease in A_{570}) was observed with doses of NMDA that were greater than or equal to 50 μM or with glutamate doses greater than or equal to 10 μM. All experiments reported here were performed with subtoxic doses of ligands, and no significant cell death was detected. Also, viability was routinely monitored by fluorescence microscopy after addition of propidium iodide (4.6 μg/ml) and fluorescein diacetate (15 μg/ml) (15) (data not shown).

Quantitative receptor subunit RNA analysis by competitive PCR. Absolute amounts of selected receptor subunit mRNAs were determined using a competitive PCR assay with internal standard cRNAs, as described previously (6, 7, 29). The construction of the internal standards used in this study has also been reported previously (6, 32). The following modification was introduced to avoid ethanol precipitation after reverse transcription, with respect to the measurements described in this study. After reverse transcription of the experimental group RNA and subunit-specific internal standard cRNA, we adjusted the reaction to the recommended buffer and initiated the PCR directly. The PCR assay mixture contained cDNA, 25 mM Tris·HCl, pH 9.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 μM subunit-specific primer pairs, 200 μM concentrations each of dATP, dTTP, dGTP, and dCTP (Pharmacia), with trace amounts of [^{32}P]dCTP (Amersham), and 2.5 units of Hot Tub Polymerase (Amersham), in a 100-μl volume. Cycle parameters were identical to those described previously (6). The amount of specific receptor subunit mRNA is reported as attomoles/microgram of total RNA for each subunit studied in the given culture and/or treatment conditions.

Antisera production and purification. Subunit-specific antibodies were produced against the α1 and α5 subunits of the $GABA_A$ receptor as described in detail elsewhere (33) and briefly below. Peptides corresponding to unique carboxyl-terminal sequences of the α1 (amino acids 416–428, NREPQLKAPTPHQ) (34) and α5 (amino acids 421–433, NREPVIKGATSPK) (34) subunits were synthesized with solid-phase technology using a Beckman 990 peptide synthesizer, as described by the manufacturer. Sequences of the synthesized peptides were confirmed using a Beckman 890M protein sequencer. To increase the efficiency of coupling to carrier molecules and affinity column linker arms, peptides were synthesized with an additional cysteine residue at the amino terminus. Before coupling, disulfide bonds were reduced and the reduced peptides were coupled, using MBS, to either bovine serum albumin (α1) or keyhole limpet hemocyanin (α5), as described by the manufacturer. Noncoupled MBS was removed by chromatography using a PD-10 column (Bio-Rad), and fractions containing the MBS-peptide conjugates were monitored by measurement of the absorbance at 260 nm.

Equal volumes of the conjugated peptide (500 μg of peptide) and Freund's complete adjuvant were emulsified and injected subcutaneously, at a minimum of five sites, into New Zealand white rabbits (Buckshire Farms). Rabbits were boosted three times, at 40-day intervals, and then bled 7–10 days after the last injection. Antisera (diluted 1/10 in PBS) were purified on an affinity column (Affigel 401) to which the reduced peptide had been coupled. Antibodies were eluted with 6 M guanidine-HCl and dialyzed against PBS, pH 7.4. Purified antibodies were stored with 0.02% sodium azide at 4°. The anti-α6 polyclonal antibody (a generous gift from Dr. Peter Seeburg, University of Heidelberg, Heidelberg, Germany), prepared against the carboxyl-terminal dodecapeptide (amino acids 423–434) (35) of the corresponding receptor subunit, was used for immunocytochemical experiments. Because only limited amounts of this antibody were available, it was not possible to perform a semiquantitative analysis of the data and for this reason the immunocytochemical studies have not been included.

Immunocytochemistry. Standard methods for immunocytochemistry using a Vectastain Elite ABC kit, as recommended by the manufacturer (Vector Labs), were used. Briefly, cells from different experimental conditions were washed with PBS (Biofluids) and were fixed in 4% paraformaldehyde/PBS, pH 7.2, for 15 min. The fixed cells were again washed in cold PBS (three 5-min washes were performed after each subsequent incubation) and then incubated overnight at 4° with the primary antibody. The cells were then incubated for 4 hr at 4° with biotinylated secondary antibody, followed by 30 min at room temperature with the preformed avidin-biotinylated horseradish peroxidase macromolecular complex. DAB and H_2O_2 were used to initiate the peroxidase reaction, yielding a reddish-brown pigment, the characteristic index of positive immunoreactivity.

ELISA. Cerebellar granule neuron membranes were prepared as described previously (36). Granule neuron P2-enriched membranes were obtained on day 5 from nontreated 12.5 mM KCl cultures, NMDA-treated 12.5 mM KCl cultures, 25 mM KCl cultures, and rat liver tissue (a control tissue that has not been reported to express $GABA_A$ receptor subunits). Cerebellar granule neurons harvested from five or more 10-cm culture dishes were washed with PBS on ice and then scraped and pooled in PBS. All subsequent steps were carried out at 4°. After a 5-min centrifugation at 500 × *g*, neurons were resuspended in buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM benzamidine HCl, 0.3 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.1 μg/ml aprotinin. Cells were homogenized with a Tissuemizer (Tekmar) and centrifuged again at 500 × *g* for 5 min. The supernatant was subsequently centrifuged at 27,000 × *g* for 20 min. The pellet obtained was resuspended in the buffer indicated above, without sucrose or phenylmethylsulfonyl fluoride, and the 27,000 × *g* centrifugation was repeated twice. The crude membrane fraction was resuspended in PBS, and total protein was estimated using a dye-binding protein assay (Bio-Rad), following the manufacturer's recommended protocol. Aliquots (5 μg, determined empirically to give the most consistent results) of these crude membrane proteins were diluted in 100 μl of PBS and applied to multiple wells of a 96-well ELISA plate (Maxisorb; Nunc). To generate a standard curve for α1 and α5 immunoreactivity, serial dilutions (0.05–10 ng) of the respective antigen peptides were also incubated on the same plates with the experimental cell membrane proteins. The synthesized peptides corresponded to α1 amino acid residues 417–429 and α5 amino acid residues 421–433 (36). Nonspecific sites were blocked with PBS/Tween 0.05%. Direct ELISA was performed with empirically determined concentrations of primary and secondary antibodies, with 1/250 and 1/1000, respectively, yielding optimal results. The secondary antibodies (Kirkegaard and Perry Laboratories, Inc.) were linked to horseradish peroxidase, which reacted with ABTS (Bio-Rad) to give a soluble colored product detectable at 410 nm (MR600 microplate reader; Dynatech Labs).

Western blot analysis. Crude cerebellar membranes were prepared, as for ELISA (see above), from 12.5 mM KCl cultures, 12.5 mM KCl cultures plus NMDA (10 μM, 48 hr), 25 mM KCl cultures, and liver as a control (postnatal day 8 pups). For each group, 20 μg were applied to a 5% stacking/10% separating polyacrylamide gel. One-dimensional SDS-polyacrylamide gel electrophoresis was performed, followed by transfer to nitrocellulose (Schleicher and Schuell). Blots were blocked with PBS/Tween 0.5% for at least 2 hr at room temperature, followed by primary antibody incubation overnight at 4°. After several washes with PBS/Tween 0.1%, the blots were incubated with secondary antibody-horseradish peroxidase for 1–2 hr at room temperature. They were washed again and reacted with a DAB/NiCl solution.

To detect the small amounts of subunits expressed in transfected HEK 293 cells, an enhanced chemiluminescence system (Amersham) was employed, following the manufacturer's recommended protocol.

Essentially, the protocol was the same as described above. However, instead of visualization of the reaction with DAB/H_2O_2, the blots were incubated for 1 min in the enhanced chemiluminescence solution, wrapped immediately in cellophane, and exposed briefly to X-ray film (10–60 sec). Processed autoradiographs were then aligned with standard molecular weight markers.

Statistical analysis. One-tailed ANOVA, followed by the Newman-Keuls test, was used to define significant differences among the various groups studied. The p values are shown in the figure and table legends. A minimum of three values from three separate experiments were always used, and often several more experiments were performed to confirm results.

Results

Characterization of the absolute amounts of various $GABA_A$ receptor subunit mRNAs in selected cerebellar granule neuron culture paradigms. In a previous study, relative changes in the amounts of selected $GABA_A$ receptor subunit mRNAs expressed in cerebellar granule cell cultures maintained in high KCl (25 mM) and treated chronically with MK-801 and in cultures maintained in low KCl (12.5 mM) and treated daily with NMDA for 5 DIV were determined (27). To extend this analysis we determined the absolute amounts of the α1, α5, and α6 receptor subunit mRNAs in low KCl (12.5 mM) cultures, in low KCl (12.5 mM) cultures treated with a single dose of NMDA (10 μM) for 24 hr, and in high KCl (25 mM) cultures. The α1 and α5 receptor subunit mRNAs were examined because they had been shown previously to be the most sensitive to change after protracted NMDA treatment (27). The α6 receptor subunit mRNA, which had not been previously examined, was included in this study because of its high level of expression in cerebellar granule cells (7, 34). The 24-hr NMDA treatment paradigm was chosen based on preliminary Northern analysis, which showed significant increases for the α1 and α5 mRNAs, relative to time-matched controls.[2] Granule cells were prepared and plated in medium containing either low or high KCl. On DIV 4, a single dose of NMDA (10 μM final) was added to one set of cultures maintained in low KCl, and RNA was harvested 24 hr later from treated and nontreated groups. The results of these analyses are presented in Table 1. Each RNA value reflects the mean ± standard error of three or more separate measurements made on three separately treated or nontreated cultures prepared from different litters. The data indicate, in absolute amounts, that these three receptor subunit mRNAs differed considerably in their abundance and, furthermore, that a single NMDA treatment of granule cell cultures maintained in low KCl increased significantly the mRNA content of both the α1 and α5 $GABA_A$ receptor subunits, whereas the α6 receptor subunit mRNA content was not statistically changed by this treatment within a 24-hr time frame. Moreover, daily treatment of granule cell cultures maintained in 25 mM KCl with 1 μM MK-801 from DIV 2 to DIV 7 had no effect on the absolute amounts of α6 mRNA (200 ± 15 amol/μg of total RNA). We have previously reported that this same treatment results in a 72% decrease in the relative amount of α1 receptor subunit mRNA (27). The data in Table 1 also show the changes in mRNA content that this treatment induced, with respect to cultures maintained in high KCl for the same length of time (i.e., 5 DIV).

TABLE 1

Absolute amounts of $GABA_A$ receptor subunit mRNAs in primary cultures of postnatal rat cerebellar granule neurons measured by quantitative PCR

Cultures were prepared from postnatal day 8 rats and were maintained for 5 days in either 12.5 mM KCl or 25 mM KCl. On day 4, one group of 12.5 mM KCl-maintained cultures was treated with NMDA (10 μM final concentration) for 24 hr. On day 5, all cultures were harvested for RNA isolation. Reverse transcription and quantitative PCR (see Materials and Methods) were then carried out for the subunit of interest. Results represent mean ± standard error of absolute values for three or more independent culture groups and PCR experiments.

Subunit	Amount		
	12.5 mM KCl	12.5 mM KCl + NMDA (24 hr)	25 mM KCl
	amol/μg of total RNA		
α1	290 ± 20[a]	740 ± 90[a]	520 ± 85[a]
α5	22 ± 2.5[b]	56 ± 3.3[b]	90 ± 1.5[b]
α6	170 ± 11	170 ± 15	190 ± 12

[a] Significant difference, $p < 0.05$, in mRNA content for that particular subunit, compared with the other two culture paradigms, by ANOVA and Newman-Keuls analysis.
[b] $p < 0.01$.

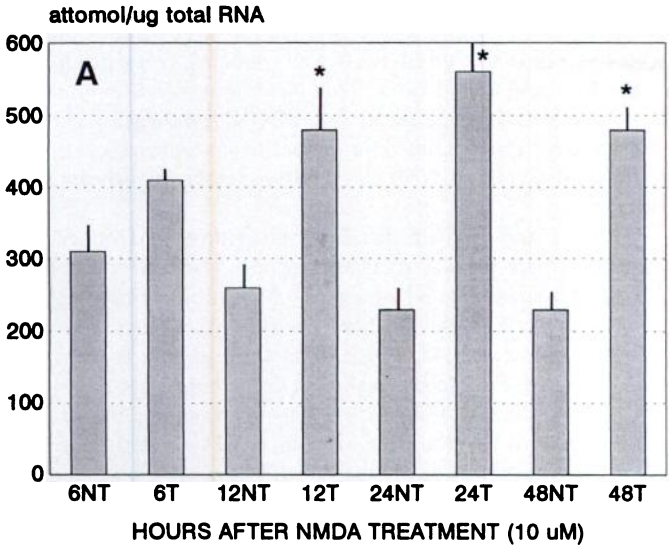
Temporal induction of the α1 and α5 $GABA_A$ receptor subunit mRNAs after a single addition of NMDA. To characterize the time course of the α1 and α5 $GABA_A$ receptor subunit mRNA content increase, cerebellar granule neurons were prepared and plated in medium containing 12.5 mM KCl. On DIV 4, the cultures were treated with a single dose of NMDA (10 μM) and RNA was harvested from the treated cultures and untreated time-matched controls at selected times after NMDA addition. Competitive PCR with internal standards was used to determine the absolute amounts (in attomoles/microgram of total RNA) of the α1 (Fig. 1A) and α5 (Fig. 1B) receptor subunit mRNAs in RNA obtained for each culture and at each time point examined. Similar time courses of induction by NMDA were observed for the α1 and α5 subunit mRNAs, with statistically significant differences between the NMDA-treated low KCl cultures and control nontreated cultures by 12 hr for the α1 mRNA and 6 hr for the α5 mRNA. Absolute amounts of each receptor subunit mRNA were maximal by 24 hr after the single addition of NMDA and did not begin to decline until 48 hr after treatment. These data extend our initial Northern analysis, which demonstrated that the relative amounts of mRNAs encoding the α1 and α5 subunits of the $GABA_A$ receptor increased approximately 2.5- and 4-fold, respectively, after addition of 10 μM NMDA to low KCl (12.5 mM) cultures for 24 hr.[3]

Dose dependence of NMDA-mediated increases in α1 and α5 $GABA_A$ receptor subunit mRNA content. To determine the dose dependence of the observed increases in α1 and α5 mRNA content, we treated granule cell cultures maintained in low KCl with increasing doses of NMDA and analyzed the corresponding mRNA content 24 hr after each single treatment. As shown in Fig. 2, the content of each mRNA increased in a dose-dependent manner. A comparison of the accumulation profiles obtained for the α1 (Fig. 2A) and α5 (Fig. 2B) mRNA contents indicates that the α5 mRNA dose-response increment was sharper than that of α1. Each mRNA showed a comparable dose dependence, although the α1 mRNA increased with an EC_{50} of 2 μM, whereas the α5 mRNA increased with an EC_{50} of 5 μM.

Because of the excitotoxic potential of excessive NMDA

[2] B. T. Harris, unpublished observations.

[3] B. T. Harris, unpublished observations.



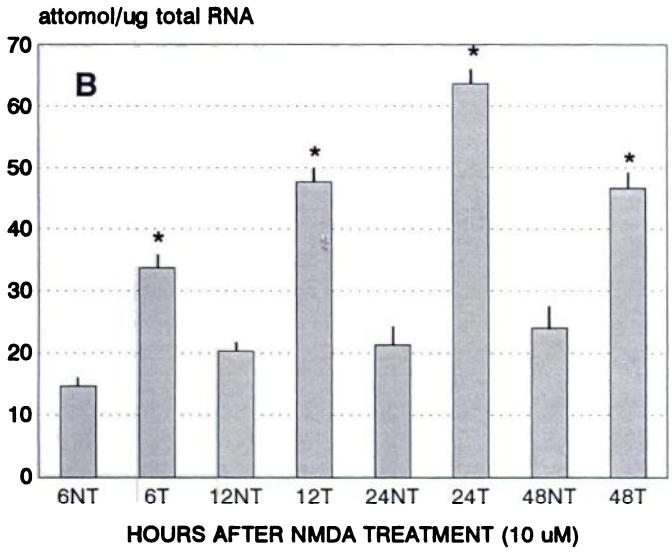


Fig. 1. Time course of GABA$_A$ receptor α1 (A) and α5 (B) subunit mRNA increases after stimulation by NMDA. Rat cerebellar granule neuron cultures maintained in 12.5 mM KCl-containing medium were treated on DIV 4 with NMDA (10 μM final concentration) added directly to the medium. At 6, 12, 24, and 48 hr, cells were harvested and pooled for RNA isolation, from a minimum of four 10-cm dishes/point from NMDA-treated (*T*) and time-matched nontreated (*NT*) cultures. RNA from the various groups was reverse transcribed, followed by competitive PCR using subunit-specific primers and internal standards for α1 or α5. Values represent the mean ± standard error (amol of subunit mRNA/μg of total RNA) from three or more independent experiments. Significant differences ($p < 0.01$) between NMDA-treated and corresponding nontreated cultures were observed for all times points, with the exception of the 6-hr point for α1 (ANOVA and Newman-Keuls analysis). *Asterisks* indicate statistically signifcant differences.

receptor stimulation of these neurons, cultures treated in parallel with each dose of NMDA were examined for cell death 24 hr after the treatment (as described in Materials and Methods). No significant excitotoxicity was evident 24 hr after addition of each dose used in this study (data not shown). Doses of 50 μM and higher were found to cause significant excitotoxicity and were therefore not included.

Inhibition by MK-801 of glutamate-mediated increases in the α1 and α5 receptor subunit mRNAs. To further demonstrate that GABA$_A$ receptor subunit increase is mediated by stimulation of NMDA-selective glutamate receptors, cerebellar granule cell cultures maintained in low KCl (12.5 mM) were treated with the endogenous excitatory amino acid glutamate. Similarly to the NMDA-induced changes in GABA$_A$ receptor subunit mRNA levels, increases in the absolute amounts of the α1 (Fig. 3A) and α5 (Fig. 3B) subunit mRNAs were detected 24 hr after a single addition of glutamate (5 μM). No excitotoxicity was observed at this dose of glutamate. As also shown in Fig. 3, A and B, MK-801 at 1 μM had no effect alone but, when added 30 min before the glutamate addition, it was able to attenuate the increases in α1 and α5 receptor subunit mRNA content by glutamate. These data further support the selectivity of the NMDA-selective glutamate receptor in the α1 and α5 GABA$_A$ receptor subunit mRNA increases. Fig. 3C shows that, unlike NMDA (Table 1), a single treatment with glutamate resulted in a statistically significant

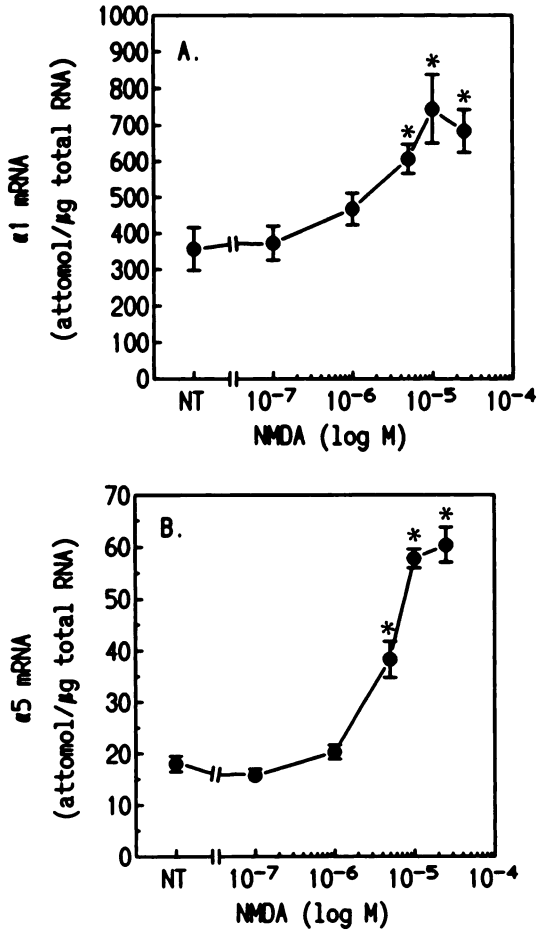


Fig. 2. Dose-response relationship for NMDA treatment (24 hr) of cerebellar granule neurons and increases in absolute amounts of GABA$_A$ receptor α1 (A) and α5 (B) subunit mRNAs. Cultures maintained in 12.5 mM KCl-containing medium were treated with NMDA for 24 hr on DIV 4, after which total RNA was harvested from the neurons for reverse transcription and competitive PCR. Values represent the mean ± standard error (amol of subunit mRNA/μg of total RNA) for three or more independent experiments. The EC$_{50}$ for the α1 mRNA increase produced by NMDA is 2 μM, whereas that for the α5 mRNA increase is approximately 5 μM. Significant differences (*) from nontreated (*NT*) amounts of α1 and α5 subunit mRNAs were observed after treatment of cultures with 5, 10, or 25 μM NMDA ($p < 0.05$), as assessed by ANOVA and Newman-Keuls test.

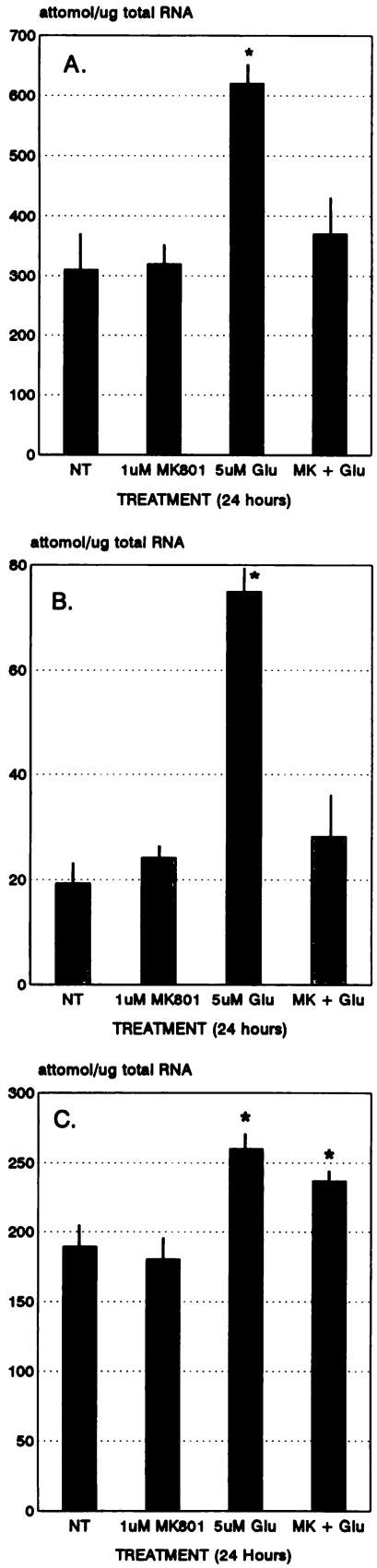
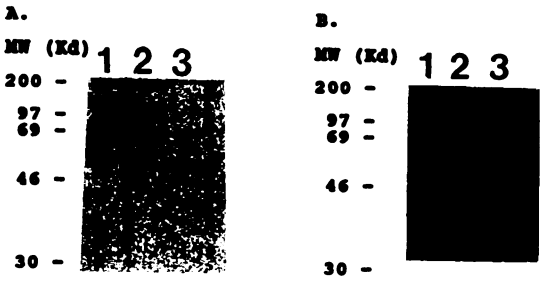


Fig. 3. Effect of glutamate and/or MK-801 on $GABA_A$ receptor $\alpha 1$, $\alpha 5$, and $\alpha 6$ subunit mRNA amounts in cerebellar granule neurons. Primary cultures of cerebellar granule neurons were maintained in 12.5 mM KCl-containing medium for 4 days. On day 4 several of the culture dishes were treated with MK-801 (1 μM final concentration). Thirty minutes later a subset of these cultures and another set of untreated cultures were treated with glutamate (5 μM final concentration). All four groups of cultures were then harvested 24 hr later for RNA isolation, followed by reverse transcription and competitive PCR with subunit-specific primers and internal standards for $\alpha 1$ (A), $\alpha 5$ (B), and $\alpha 6$ (C). Values represent the mean ± standard error (amol of subunit mRNA/μg of total RNA) for three independent experiments. Significant differences ($p < 0.01$) were noted for the glutamate-treated groups for $\alpha 1$ and $\alpha 5$ subunit mRNAs, as determined by ANOVA and Newman-Keuls analysis. For $\alpha 6$, the glutamate-treated (*Glu*) group and the MK-801/glutamate-treated (*MK + Glu*) group were significantly different (*asterisks*, $p < 0.05$) from non-treated (*NT*) and MK-801-only groups but were not significantly different from each other.

increase in the content of $\alpha 6$ $GABA_A$ receptor subunit mRNA, although the observed increase was smaller in magnitude than that of $\alpha 1$ and $\alpha 5$ mRNAs. This effect was not attenuated by pretreatment of the cultures with MK-801, indicating the possibility that signal transduction pathways operating through the activation of non-NMDA-selective glutamate receptors may be involved.

Anti-$\alpha 1$ and anti-$\alpha 5$ antibody characterization. Several strategies were used to characterize the specificity and cross-reactivity of the anti-$\alpha 1$ and anti-$\alpha 5$ peptide affinity-purified polyclonal antibodies. Synthetic peptides (0.5–5 μg) were spotted onto nitrocellulose, which was subsequently treated with 0.05% Tween/PBS to block nonspecific binding sites. Primary antibodies (diluted 1/50 to 1/5000) in blocking buffer were incubated with the dot blots at 4° for 12–14 hr. After the blots were washed, they were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. Immunoreactivity was visualized with 0.01% DAB/0.01% H_2O_2, as described in Materials and Methods. Using this approach, no cross-reactivity was observed between the affinity-purified antibodies and the corresponding peptide antigens (33) (data not shown). Using a similar approach, the primary affinity-purified antibodies were preadsorbed with either the $\alpha 1$ or $\alpha 5$ peptide before incubation with the dot blots. Preincubation of the $\alpha 1$ peptide with the anti-$\alpha 1$ affinity-purified antibody blocked the observed immunoreactivity, whereas preincubation with the $\alpha 5$ peptide antigen did not (33). Conversely, preincubation of the $\alpha 5$ peptide antigen with the anti-$\alpha 5$ purified antibody similarly blocked the observed immunoreactivity, whereas preincubation with the $\alpha 1$ peptide antigen did not (33). Similar results were obtained by using this approach immunocytochemically and by using the semiquantitative ELISA (data not shown). The anti-$\alpha 1$ affinity-purified antibodies recognized a 51-kDa band of partially purified receptor in Western analysis of membranes prepared from multiple central nervous system regions (cortex, hippocampus, and cerebellum), whereas the anti-$\alpha 5$ affinity-purified antibody recognized a 56-kDa band in membranes prepared from rat hippocampus (33).

Finally, HEK 293 cells transiently transfected with the $\alpha 5/\beta 1/\gamma 2$, $\alpha 1/\beta 1/\gamma 2$, and $\alpha 6/\beta 1/\gamma 2$ receptor subunit expression vectors were tested for immuno-cross-reactivity by Western analysis (Fig. 4, *top*) and immunocytochemistry (Fig. 4, *bottom*) with the anti-$\alpha 1$ and anti-$\alpha 5$ affinity-purified antibodies. As shown in Fig. 4, *top*, A, membranes prepared from 293 cells transfected with the $\alpha 5/\beta 1/\gamma 2$ combination showed a single immunoreactive band in Western analysis (Fig. 4, *top*, A, *lane 1*) using the anti-$\alpha 5$ affinity-purified antibody. Membranes prepared from either the $\alpha 1/\beta 1/\gamma 2$ (Fig. 4, *top*, A, *lane 2*) or $\alpha 6/\beta 1/\gamma 2$ (Fig. 4, *top*, A, *lane 3*) expression vector combinations



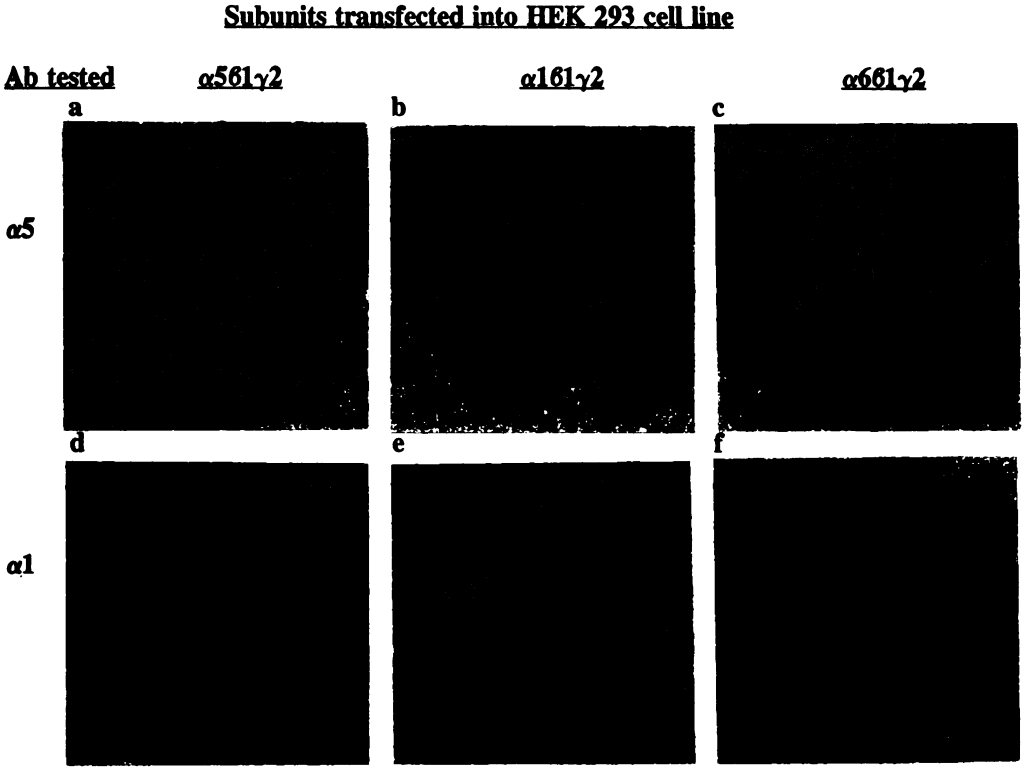


Fig. 4. Specificity of the anti-α1 and anti-α5 affinity-purified antibodies, as determined by Western analysis (*top*) and immunocytochemistry (*bottom*) of HEK 293 cells transiently transfected with expression vectors encoding various GABA$_A$ receptor subunit combinations. HEK 293 cells were transfected using the calcium/phosphate co-precipitation technique, as previously described for these receptor subunit combinations (4). *Top*, for Western analysis, 10 μg of each plasmid combination were used to transfect 10-cm dishes and crude membranes were prepared as described in Materials and Methods. Ten micrograms of each membrane preparation were applied to individual lanes of the same SDS-polyacrylamide gel. After blotting to nitrocellulose, the mebrane was cut and the two sections were incubated individually with each affinity-purified antibody. *Lanes 1* (A and B), membranes isolated from 293 cells transfected with the $\alpha5/\beta1/\gamma2$ combination; *lanes 2*, membranes isolated from cells transfected with the $\alpha1/\beta1/\gamma2$ combination; *lanes 3*, membranes isolated from cells transfected with the $\alpha6/\beta1/\gamma2$ combination. A, Incubation with the anti-α5 affinity-purified antibody; B, incubation with the anti-α1 affinity-purified antibody. *Bottom*, representative immunocytochemical fields of cells transfected with these same receptor subunit combinations in parallel on 3.5-cm dishes (3 μg of each plasmid were used for the transfection). a and d, Cells transfected with the $\alpha5/\beta1/\gamma2$ combination; b and e, cells transfected with the $\alpha1/\beta1/\gamma2$ receptor subunit combination; c and f, cells transfected with the $\alpha6/\beta1/\gamma2$ combination; a, b, and c, incubation with the anti-α5 affinity-purified antibody; d, e, and f, incubation with the anti-α1 affinity-purified antibody. Immunoreactivity was visualized as described in Materials and Methods.

did not cross-react with the anti-α5 affinity-purified antibody. Similarly, membranes prepared from 293 cells transfected with the $\alpha1/\beta1/\gamma2$ receptor subunit combinations showed a single immunoreactive band (Fig. 4, *top*, B, *lane 2*) with the anti-α1 affinity-purified antibody. Membranes prepared from 293 cells transfected with either the $\alpha5/\beta1/\gamma2$ (Fig. 4, *top*, B, *lane 1*) or $\alpha6/\beta1/\gamma2$ receptor subunit combinations showed no antibody cross-reactivity. Similar results were obtained when these cells were tested immunocytochemically (Fig. 4, *bottom*). That is, approximately 25–30% of the cells transfected with the $\alpha5/\beta1/\gamma2$ subunit combinations were immunpositive with the anti-α5 affinity-purified peptide antibody, whereas no immunoreactivity was observed with the $\alpha1/\beta1/\gamma2$ combination of receptor subunits (or with the $\alpha6/\beta1/\gamma2$ combination) using the anti-α5 affinity-purified antibody (Fig. 4, *bottom*, a, b, and c, respectively). Cells transfected with the $\alpha1/\beta1/\gamma2$ receptor subunit combinations showed positive immunoreactivity with the α1 affinity-purified antibody, whereas no cross-reactivity was observed with cells transfected with the $\alpha5/\beta1/\gamma$ or $\alpha6/\beta1/\gamma2$ receptor subunit combinations and treated with the anti-α1 affinity-purified antibody (Fig. 4, *bottom*, e, d, and f, respectively).

Evidence that changes in the α1 and α5 GABA$_A$ receptor subunit immunoreactivity parallel the observed mRNA changes in various granule cell cultures. Fig. 5 shows photomicrographs of α1 (Fig. 5, A-C) and α5 (Fig. 5, D-

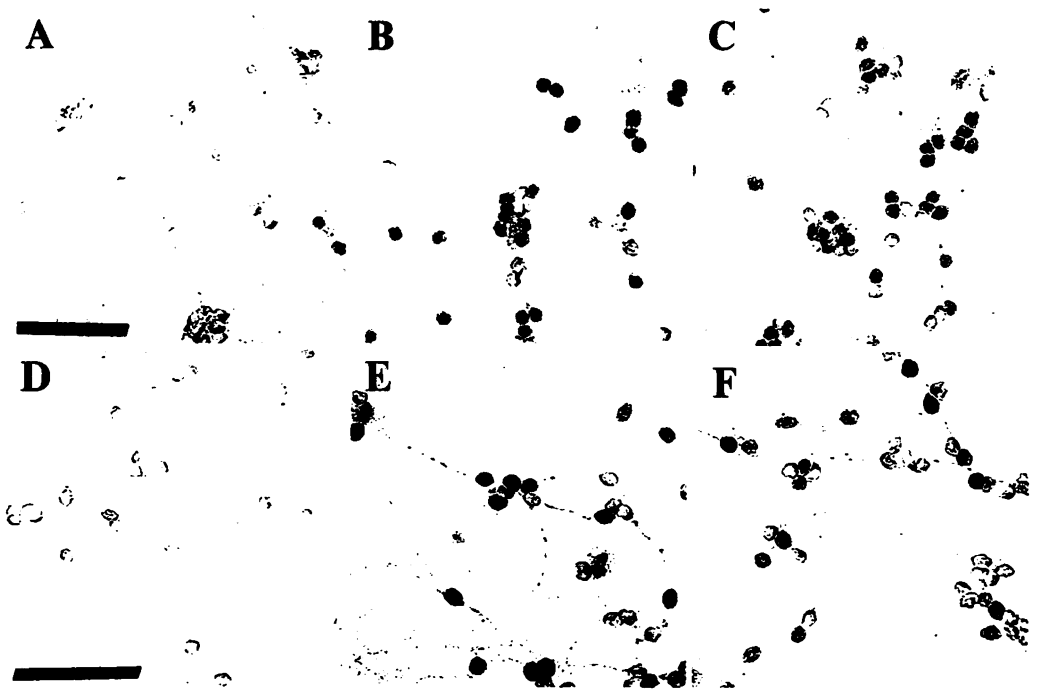


Fig. 5. Immunocytochemistry of $GABA_A$ receptor subunit-like immunoreactivity in primary cultures of cerebellar granule neurons. $GABA_A$ receptor $\alpha 1$ and $\alpha 5$ subunit-like immunoreactivity was assessed under a number of different culture conditions. Shown are representative fields for the two subunits in cultures maintained for 5 days in 12.5 mM KCl (A, $\alpha 1$; D, $\alpha 5$), 12.5 mM KCl plus 10 μM NMDA for 36 hr (B, $\alpha 1$; E, $\alpha 5$), or 25 mM KCl (C, $\alpha 1$; F, $\alpha 5$). Qualitatively similar results were obtained in three or more independent experiments. *Scale bar*, 5 μm. Details for the immunocytochemistry are described in Materials and Methods.

F) immunoreactivity in several of the culture paradigms. Qualitative differences in immunocytochemistry were evident in the NMDA-treated and high KCl cultures, compared with untreated 12.5 mM KCl cultures, for both the $\alpha 1$ and $\alpha 5$ receptor subunits. In contrast, the pattern of $\alpha 6$ immunocytochemistry appeared to be similar in the three paradigms examined (data not shown). Many, but not all, of the cells in the NMDA and high KCl groups appeared to be immunoreactive with the $\alpha 1$ and $\alpha 5$ subunit-specific antibodies. In contrast, only a small percentage of neurons appeared to react positively in the low KCl cultures. Cerebellar granule cells maintained in low KCl and treated with 5 μM glutamate for 24 hr demonstrated increases in $\alpha 1$ (Fig. 6A) and $\alpha 5$ (Fig. 6C) immunostaining comparable to those observed in the NMDA-treated cultures (Fig. 5). Similarly, the group pretreated with MK-801 before glutamate treatment showed a lower intensity of $\alpha 1$ (Fig. 6B) and $\alpha 5$ (Fig. 6D) immunoreactivity and fewer positively stained neurons, appearing similar to the pattern obtained with the nontreated cultures maintained in 12.5 mM KCl. Positive $\alpha 1$ and $\alpha 5$ immunoreactivity appeared to be present in both the soma and neuronal processes. These photomicrographs show representative fields from a single experiment. Three immunocytochemical experiments from three separate and independently prepared cultures yielded comparable results.

The controls for the specificity of the granule cell immunocytochemistry were performed either by omitting primary antibodies or by preabsorbing the primary antibodies with the $\alpha 1$ and $\alpha 5$ peptides against which the polyclonal antibodies were directed (data not shown). Also, when the alternative peptide was preabsorbed with the antibody, we observed the same level of immunoreactivity as shown in Fig. 6, indicating the subunit selectivity of these antibodies in the granule neurons. Dilutions

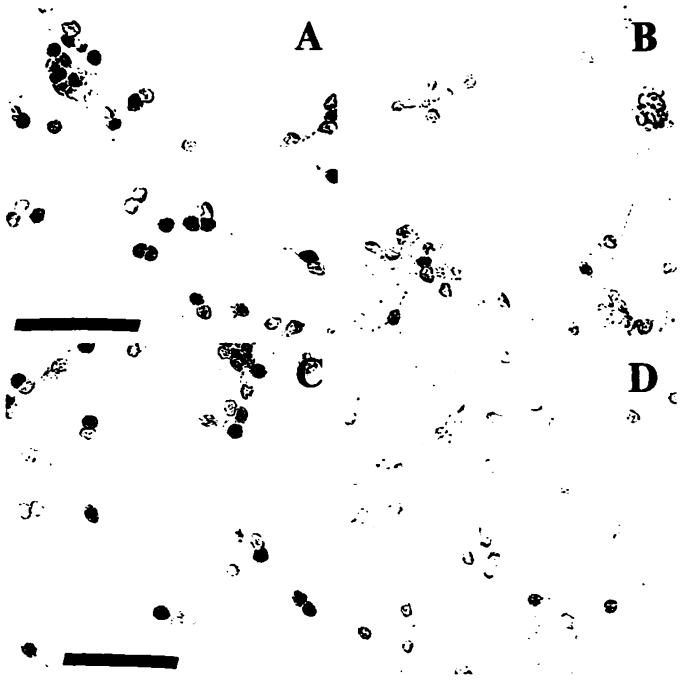


Fig. 6. Immunocytochemistry of the $\alpha 1$ (A and B) and $\alpha 5$ (C and D) $GABA_A$ receptor subunit immunoreactivity in primary cultures of cerebellar granule neurons treated with a single dose of glutamate and with the same dose of glutamate in the presence of MK-801. Cultures maintained in 12.5 mM KCl and treated with glutamate (5 μM, for 48 hr) (A and C) appeared similar to those treated with NMDA (see Fig. 4), whereas cultures pretreated with MK-801 (1 μM, 30 min before addition of glutamate) (B and D) appeared similar to cultures maintained in 12.5 mM KCl (Fig. 4). Qualitatively comparable results were obtained in three or more independent experiments. *Scale bar*, 5 μm.

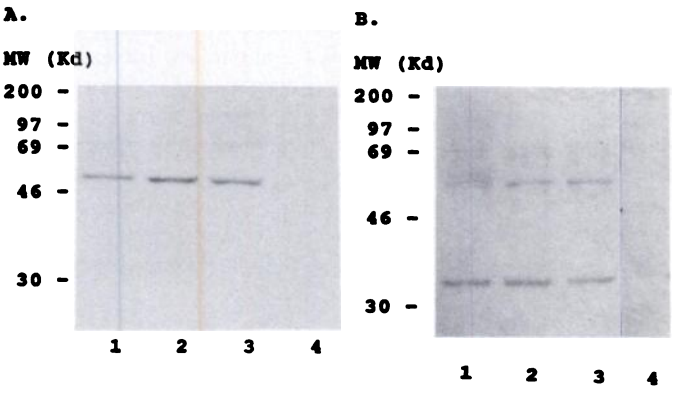


Fig. 7. Western blot analysis of α1 (A) and α5 (B) GABA$_A$ receptor subunit-like immunoreactivity in membranes from primary cultures of cerebellar granule neurons. Cultures were maintained under previously described conditions, and 10 dishes were pooled from each group and harvested for membrane protein preparation (see Materials and Methods). Twenty micrograms of membrane protein were used for SDS-polyacrylamide gel electrophoresis. After electrophoresis, transfer to nitrocellulose, and blocking with 0.5% Tween/PBS, subunit-specific affinity-purified antibodies were incubated with the blots. Secondary antibodies linked to horseradish peroxidase were then incubated with the blots, and bands were visualized by reaction with DAB/NiCl. *Lanes 1*, 12.5 mM KCl-maintained cultures; *lanes 2*, 12.5 mM KCl-maintained cultures plus 10 μM NMDA (48 hr); *lanes 3*, 25 mM KCl-maintained cultures; *lanes 4*, rat liver membrane proteins (control).

of primary antibody were tested that ranged from 1/100 to 1/1000, with optimal results being obtained at 1/500 (as shown in Fig. 5).

Western blot analysis (Fig. 7) also revealed a qualitative increase in membrane immunoreactivity for the NMDA-treated and high KCl cultured granule neurons, compared with non-treated low KCl-maintained neurons. Immunoreactive proteins at approximately 51 kDa and 55 kDa were observed, corresponding to the previously reported sizes for the α1 and α5 GABA$_A$ receptor subunits, respectively (37). An additional positive band of roughly equal intensity in each paradigm was also observed at approximately 34 kDa in the α5 receptor subunit blot. Whether this protein represents a breakdown product of the α5 receptor subunit or an unrelated cross-reacting species is unclear at this time. The latter possibility seems more plausible, because the 55-kDa species changed in intensity in the three paradigms tested, whereas the 34-kDa protein did not. Although this cross-reacting antigen complicates estimates of the absolute amounts of α5 immunoreactivity, the relative differences between paradigms were still readily apparent.

To obtain a semiquantitative analysis of the subunit protein levels in the multiple culture paradigms, we used a direct ELISA approach. Fig. 8 illustrates results obtained from representative experiments using serial dilutions of the α1 (Fig. 8A) and α5 (Fig. 8B) peptides against which the antibodies were raised, showing the corresponding absorbance values obtained after the secondary antibody reaction was developed (see Materials and Methods). Standard curves were generated with the diluted peptide antigens, so that the immunoreactivity of various cerebellar granule neuron membrane preparations could be compared. For example, in Fig. 8, *arrows* indicate points along the curve that correspond to the immunoreactivity of 5 μg of membrane protein from neuronal cultures in 12.5 mM KCl, 12.5 mM KCl plus NMDA (10 μM) for 24 hr, and 25 mM KCl. Table 2 summarizes results obtained from nontreated 12.5 mM KCl

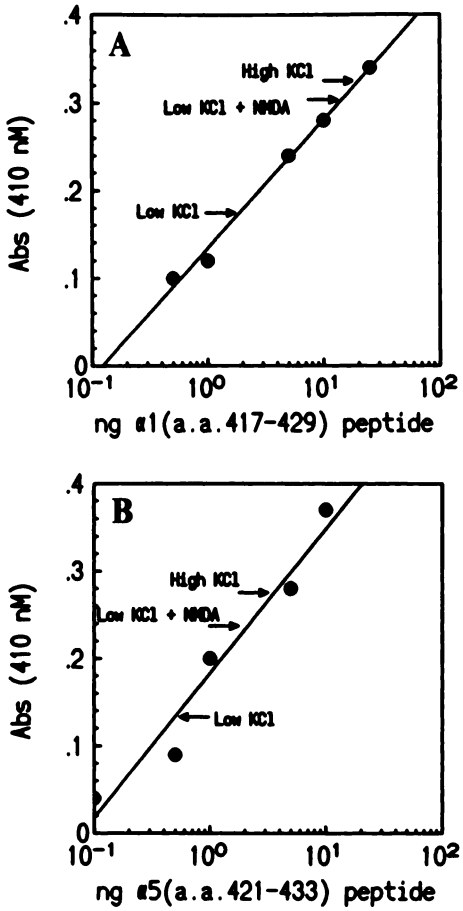


Fig. 8. Representative calibration curves for GABA$_A$ receptor α1 (A) and α5 (B) peptide antigen immunoreactivity using ELISA. Synthetic peptides corresponding to unique portions in the carboxyl terminus of the rat α1 (amino acids 416–428) and α5 (amino acids 421–433) subunits were diluted in PBS at various dilutions (0.1–25 ng) and adsorbed to a 96-well immunoplate. On the same plate, 5 μg of total membrane protein from various culture groups were also adsorbed. Wells were washed and blocked with PBS/0.1% Tween and then incubated with polyclonal antibodies directed against either the α1 or α5 synthetic peptides. Secondary antibodies linked to horseradish peroxidase were added, followed by incubation with ABTS, which produced a green chromogen detectable at 410 nm. *Arrows and labels*, representative absorbance values for several of the experimental groups in that particular experiment.

cultures, 12.5 mM KCl cultures treated with 10 μM NMDA for 24, 36, or 48 hr, and 25 mM KCl cultures. Because of inherent assumptions with respect to differences in epitope accessibility between the antigen peptides and membrane-bound proteins, the data are expressed in arbitrary units, where 1 arbitrary unit corresponds to the absorbance elicited by 1 ng of either the α1 or α5 synthetic peptide. The α1-like immunoreactivity increased up to 7-fold by 48 hr of NMDA (10 μM) treatment of the low KCl cultures, reaching levels similar to those found in cultures maintained in 25 mM KCl. Similarly, the α5 receptor subunit also increased 7-fold after 48-hr NMDA treatment. Interestingly, the ratio of α1:α5-like immunoreactivity remained fairly constant in each paradigm, ranging from 6- to 8-fold for each of the culture conditions.

Discussion

This report extends a previous study (27) by quantitatively examining the changes in GABA$_A$ receptor subunit and mRNA

TABLE 2

Effect of NMDA and KCl on $GABA_A$ receptor α1 and α5 subunit-like immunoreactivity in membranes from primary cultures of rat cerebellar granule neurons

Primary cultures were prepared and treated as described in Table 1 with the addition of 36- and 48-hr NMDA-treated single treatments with 10 μM NMDA on day 4) groups. Crude membranes were prepared as described previously (35) and a direct ELISA was performed using polyclonal antibodies directed against unique portions of carboxyl-terminal α1 or α5 subunit peptides. A standard curve was made (see Fig. 8) using the subunit-specific antigen that generated the particular antibody. Immunoreactivity of the experimental membrane was then compared with this standard curve, and the relative increases above the 12.5 mM KCl group are represented. Each value represents the mean ± standard error for three to six experiments. Mean values for each culture condition group were significantly different ($p < 0.01$) from each other for that particular subunit, with the exception of the NMDA (48 hr) and 25 mM KCl culture groups, as determined by ANOVA and Newman-Keuls analysis.

	Immunoreactivity				
	12.5 mM KCl	12.5 mM KCl + NMDA (24 hr)	12.5 mM KCl + NMDA (36 hr)	12.5 mM KCl + NMDA (48 hr)	25 mM KCl
	arbitrary units				
α1	1 ± 0.1	2.7 ± 0.2	4.3 ± 0.7	7.3 ± 1.0	6.8 ± 1.0
α5	1 ± 0.1	2.5 ± 0.4	4.2 ± 0.6	7.2 ± 1.0	8.5 ± 0.8

content that occur after excitatory amino acid receptor stimulation. To obtain absolute mRNA values, we have utilized a competitive PCR approach (6, 7, 29). The data consistently demonstrate a statistically significantly increased content of both the α1 and α5 receptor subunit mRNAs in cerebellar granule cells maintained in 12.5 mM KCl and treated once with NMDA. The observed increases were dependent on the dose of NMDA, they persisted for approximately 48 hr after the single administration of the excitatory amino acid receptor agonist, and comparable increases occurred after a single addition of a nonexcitotoxic dose of glutamate. The addition of NMDA to the low KCl granule cell cultures increased the content of these two mRNAs to levels comparable to those present in cultures maintained in high KCl. The values reported here in the high KCl-conditioned cultures are consistent with those reported previously (7).

The increased mRNA levels were accompanied by increased α1 and α5 $GABA_A$ receptor subunit immunoreactivity with affinity-purified subunit-specific antibodies. This was demonstrated by several different experimental techniques, including immunocytochemistry, Western analysis, and semiquantitative ELISA. The Western analysis of granule neuron membranes with the anti-α5 receptor subunit antibody showed a lower molecular weight cross-reacting species, but the relative increases in α5 immunoreactivity were readily apparent with all of the techniques used. Although this complicates the interpretation of absolute amounts of α5 receptor subunit amounts in the various paradigms examined, the relative increases observed were not affected. Of particular relevance to this potential artifact is the finding that the levels of this cross-reacting antigen did not change after NMDA receptor stimulation and were constant in granule neurons maintained in either 12.5 mM KCl or 25 mM KCl. This suggests the possibility that this cross-reacting epitope is accessible only under denaturing conditions, as are used during Western blot analysis. This possibility is supported by both the immunocytochemical and ElISA data, in which the relative increases in α5 immunoreactivity were readily evident. This would not be as apparent if a cross-reacting antigen significantly contributed to the background levels of immunoreactivity.

Collectively, these data suggest that, in response to NMDA-selective glutamate receptor stimulation, cerebellar granule neurons maintained in low KCl respond by increasing the proportion of $GABA_A$ receptor subtypes that contain α1 and α5 receptor subunits. This probably occurs through the formation of additional receptor subunit assemblies, generating additional populations of receptor subtypes present in the cerebellar granule cell cultures. This is further supported by preliminary data showing increased [^{3}H]flunitrazepam binding (2-fold increase in B_{max}) 48 hr after a single treatment of granule neurons maintained in 12.5 mM KCl.[4]

The α6 subunit mRNA levels were not examined in the previous study. This subunit is of particular interest because it is expressed at high levels in cerebellar granule neurons *in vivo* (34) and *in vitro* (7). Moreover, $GABA_A$ receptor subunit assemblies containing the α6 subunit display differential sensitivity to selected benzodiazepines and low efficacy but high affinity responsiveness to GABA (4, 34). Of particular interest in the context of the current report is the lack of responsiveness of the α6 $GABA_A$ receptor subunit mRNA content to signal transduction mechanisms that are operative after NMDA-selective glutamate receptor stimulation. Although a small, but statistically significant, increase in α6 mRNA content was observed after the addition of glutamate to the low KCl-conditioned cultures, this response was less robust than those of the α1 and α5 receptor subunit mRNAs with the doses used in this study. *In vivo*, the postnatal developmental expression of the α1 and α6 subunit mRNAs coincides in rat cerebellar granule cells (32). Analysis by *in situ* hybridization histochemistry revealed that the induction of these two $GABA_A$ receptor subunit mRNAs in granule neurons corresponds temporally to the completion of granule cell migration to the internal granule cell layer during the second postnatal week (32). It is during this time that the granule neurons form functional synapses and receive glutamatergic input from mossy fiber afferents and GABA-ergic input from Golgi type 1 neurons found in this area (38). These synaptic events are likely to be associated with the subsequent maturation of these neurons and the changes in receptor expression that occur. Our results suggest the possibility that AMPA/kainate and/or metabotropic glutamate receptor stimulation may be, in part, a determinant responsible for regulating the expression of the α6 receptor subunit gene. However, they do not exclude the possibility that other signal transduction events may also be necessary.

The mechanism by which NMDA or glutamate receptor stimulation regulates the $GABA_A$ receptor subunit expression is currently not known. Glutamate acting at each class of glutamate receptors (NMDA, AMPA/kainate, and metabotropic glutamate receptors) can increase intracellular calcium either directly through the opening of cation-selective channels or indirectly via depolarization and activation of voltage-sensitive Ca^{2+} channels or through second messengers that activate release of Ca^{2+} from internal endoplasmic reticular stores (for review, see Ref. 39). Intracellular calcium plays a major role in neuronal physiology but also is thought to be a key mediator of the toxic events after excessive glutamate receptor stimulation (for review, see Ref. 40). Dihydropyridine and related agonists of the voltage-activated calcium channels have been shown to increase intracellular Ca^{2+} in cerebellar granule cells in culture (41). These same ligands have been found to promote

[4] B. T. Harris, unpublished observations.

survival of granule neurons maintained in low KCl, and treatment of high KCl (25 mM) cultures with dihydropyridine antagonists results in cell death (11). How calcium influx promotes survival and differentiation remains unknown but may involve the activation and translocation of protein kinases as an initial component of this process. Additionally, how increases in intracellular calcium are coupled to the changes in GABA$_A$ receptor subunit expression that are observed after excitatory amino acid stimulation and/or after changes in the level of depolarization is also currently not known, but these changes are likely to occur through the same mechanism. However, the observed increases in α1 and α5 mRNA content that occur after NMDA treatment appear not to be simply coupled to the survival-promoting effects of increased intracellular Ca^{2+}, because voltage-dependent Ca^{2+} channel agonists that promote granule cell survival in the presence of MK-801 fail to increase the content of these two mRNAs to the same extent as does treatment with NMDA.[5]

It was previously established that several members of the cellular immediate-early gene family (specifically c-*fos*, c-*jun*, *zif*/268, and *nur*77) are expressed in a temporally distinct fashion in PC12 pheochromocytoma cells treated with nerve growth factor and *in vivo* after convulsions (reviewed in Ref. 42). In cerebellar granule neurons *in vitro*, these mRNAs are coordinately induced after treatment of the cultures with NMDA or glutamate (43–45). One or more of these transcriptional regulatory proteins may interact directly with regulatory elements within the α1 and α5 GABA$_A$ receptor subunit promoters to increase transcriptional activity. The differential sensitivity of each GABA$_A$ receptor subunit mRNA to induction by second messengers generated after NMDA-selective glutamate receptor stimulation suggests a complex regulation of the corresponding gene family. Recent reports indicate that some of the GABA$_A$ receptor subunit genes have been localized in clusters on different chromosomes in both mice (46) and humans (47, 48). However, the human α1 and α5 receptor subunit genes exist on different chromosomes (47–49). This implies similar arrays of transcriptional elements involved in controlling the coordinate expression of these two receptor subunit genes in response to glutamate receptor stimulation. Moreover, the different temporal patterns of expression observed with the α1 and α5 GABA$_A$ receptor subunit mRNAs *in vivo* in the cerebellum (6) suggest that the responsiveness to glutamate may be limited to distinct developmental stages that are gene specific. An alternative mechanism is that excitatory amino acid receptor stimulation alters the half-life of the GABA$_A$ subunit mRNAs by inhibiting RNA-degrading protein(s) and/or by stabilizing the receptor subunit mRNA molecules. Although transcription serves a primary role in the control of gene expression, subsequent post-transcriptional events also contribute to regulating the final levels of gene products. Currently, we are investigating these mechanisms by using nuclear run-on assays to directly measure the rate of new α1, α5, and α6 receptor subunit mRNA synthesis in cerebellar granule neurons in each experimental paradigm.

Our hypothesis is that neurons continuously adjust their transcriptional programs in response to excitatory afferent synaptic signaling, to modify neurotransmitter receptor expression and to establish activity-dependent neuronal circuits. In this way neurons select information based on patterns of afferent stimuli so as to refine their function. One component of this process may be an increase in the expression of selective subtypes of inhibitory receptors in either newly formed or already established synaptic contacts, as a means of increasing the potential inhibitory tone in the stimulated neuron to promote a balance between excitation and inhibition. Altered receptor expression may also provide a compensatory mechanism after pathological insult from glutamate excitotoxicity. Neurons not irreversibly damaged by the insult may increase the number or alter the assembly of inhibitory GABA$_A$ receptors as a mechanism to increase their potential inhibitory tone, to counterbalance the level of depolarization mediated by excessive excitatory amino acid receptor stimulation.

[5] B. T. Harris, unpublished observations.

Acknowledgments

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