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α6 and γ2 Subunit Antisense Oligodeoxynucleotides Alter γ-Aminobutyric Acid Receptor Pharmacology in Cerebellar Granule Neurons

WEI JIAN ZHU, JIAN FENG WANG, STEFANO VICINI, and DENNIS R. GRAYSON

Department of Psychiatry, Medical College of Pennsylvania and Hahnemann University, Neuroscience Research Center, Pittsburgh, Pennsylvania 15212 (D.R.G.), and Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, D.C. 20007 (W.J.Z., J.F.W., S.V.)

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SUMMARY

To characterize the role of the α6 subunit in γ-aminobutyric acid (GABA) receptors in cerebellar granule cells, primary cerebellar cultures were treated with antisense oligodeoxynucleotides (ODNs) complementary to and overlapping the initial codon of the α6 subunit cDNA. The specific reduction in the expression of the α6 receptor subunit protein after a 48-hr antisense ODN treatment was assessed with the use of immunoblot assays. Sister cultures were treated in parallel with mismatched (scrambled) ODNs. Inhibition of GABA-gated currents by furosemide, a selective inhibitor of $GABA_A$ receptors containing α6 subunits, was attenuated after the α6 antisense treatment. Furosemide was tested in parallel in transfected cells expressing various combinations of the α1 and α6 subunits, which showed that the relative abundance of these subunit mRNAs determines the extent of furosemide-induced inhibition of GABA-gated currents. Compared with control or mismatched ODN-treated cell cultures, treatment of granule neurons with α6 antisense ODNs caused a decrease in GABA-induced maximal current density and increased the half-maximal concentration derived from GABA dose-response curves. Furthermore, the depletion of α6 subunits from cerebellar granule cells enhanced flunitrazepam-induced potentiation of GABA-activated currents. In contrast, γ2 antisense ODN treatments of cell cultures increased the receptor sensitivity to GABA and potently decreased the response to flunitrazepam. Our results show that α6 and γ2 subunit expression can be blocked with the use of synthetic ODNs and that these subunits are crucial determinants of the pharmacological properties of native $GABA_A$ receptors in cerebellar granule cells.

$GABA_A$ receptors, which mediate fast inhibitory neurotransmission at central and peripheral synapses, are heterooligomeric proteins (1–4). The $GABA_A$ receptor/ion channel complex is activated by GABA and modulated by clinically important drugs, including steroids, barbiturates, ethanol, anesthetics, and BZDs (1–5). Each subunit of the $GABA_A$ receptor has four transmembrane domains, a large extracellular domain with several putative glycosylation sites, and an intracellular loop between the third and fourth transmembrane domains with the drug-recognition sites located in various parts of the pentameric structure (2, 3).

An extensive heterogeneity of $GABA_A$ receptor subunits has been identified through molecular cloning, and the receptor consists of five classes of subunits (α, β, γ, δ, and ρ), most of which contain several isoforms (α1–6, β1–3, γ1–3, and ρ1–2) (1–5). Immunocytochemical studies indicate that the α1, α6, β2/3, γ1, γ2, and δ subunits are coexpressed in cerebellar granule neurons (6). In the developing cerebellum, α1 subunit mRNAs have been detected as early as postnatal day 0, whereas the α6 and δ subunit mRNAs are detected only after postnatal days 6 and 12, respectively (7, 8). However, a parallel increase in the levels of the α1 and α6 subunit mRNAs has been reported between postnatal days 14 and 21, well after cerebellar granule cell migration, with a peak for both subunits at postnatal day 21 and the largest increases occurring during postnatal days 7–14 (9, 10).

Combinations of the α1, α2, α3, α6, γ1, γ2S, γ2L, and δ $GABA_A$ receptor subunits have been demonstrated through quantitative immunopurification and Western blot studies of membranes prepared from adult cerebellum (11–13) as well as with immunocytochemical techniques (14). These studies suggest a large diversity in the possible subunit combinations that can be assembled to form specific $GABA_A$ receptor

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ABBREVIATIONS: GABA, γ-aminobutyric acid; BZD, benzodiazepine; ODN, oligodeoxynucleotide; aODN, antisense oligodeoxynucleotide; mODN, mismatched oligodeoxynucleotide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DIV, day(s) *in vitro*; NMDA, *N*-methyl-D-aspartate; IL, interleukin.

subtypes in cerebellum or cerebellar granule cells. cDNA expression studies show that functional $GABA_A$ receptors are formed from one or combinations of two or three distinct subunits (1–5). However, the biophysical and pharmacological properties of the ion channel depend on the specific combinations of subunits that form the $GABA_A$ receptor (1–5). For example, although results of photolabeling experiments indicate that BZD recognition sites are localized on the extracellular domain of α subunits (3, 5), data obtained from transient transfection studies demonstrate that the presence of the $\gamma 2$ subunit in the receptor complex is required for the modulatory effect of these compounds (15). Further studies have demonstrated that $GABA_A$ receptors expressing $\alpha 1\beta 2\gamma 2$ subunits bind flunitrazepam with high affinity, whereas those expressing the $\alpha 6\beta 2\gamma 2$ combinations do not, suggesting that the specific α subunit present determines the BZD binding affinity (1–3, 5).

In the native cellular environment, the physiological and pharmacological properties of $GABA_A$ receptors may result from various functional $GABA_A$ receptor subtypes having unique properties. Several reports have demonstrated that $GABA_A$ receptor subunit mRNAs are differentially expressed in distinct cell populations in various anatomic central nervous system structures at different developmental stages (6–10). The change in the ratio of various receptor subunit mRNAs could lead to a switch in the $GABA_A$ receptor subtype assembly and to a change in the functional and pharmacological properties of the receptors (16). In fact, it has recently been found that a variety of distinct manipulations, such as NMDA treatment, K^+-induced depolarization, chronic BZD and/or GABA treatments, and development *in vitro*, selectively regulate the expression of $GABA_A$ receptor subunits (17–21). The $\alpha 6$ subunit was first cloned from mouse (22) and rat (23) brain and was shown to be expressed only in cerebellar granule cells. $\alpha 6$-containing $GABA_A$ receptors transiently expressed *in vitro* possess unique pharmacological properties at the GABA and BZD sites (5, 18, 23–25). Therefore, our present study was designed to determine whether a change in $\alpha 6$ expression alters the pharmacological profile of native $GABA_A$ receptors in cerebellar neurons by modulating the composition and assembly of receptor subtypes. We used aODN strategies to interfere with the information flow from mRNA to protein in a specific manner to determine the functional contribution of $\alpha 6$ subunits to native $GABA_A$ receptors. For comparison, we also report the effects of antisense knockdown of the $\gamma 2$ receptor subunit on GABA function in cerebellar granule cells. Because $GABA_A$ receptors exhibit an increased sensitivity to GABA and reduced BZD-mediated potentiation with maturation of the receptors *in vitro* (18, 19), cell cultures at different stages were treated with $\alpha 6$ and $\gamma 2$ aODNs.

Materials and Methods

aODNs. Phosphorothioate ODNs (Bio-Synthesis, Lewisville, TX) were used in our experiments. The $\alpha 6$ subunit aODN had the following sequence: 5′ CAG CAT CCT ATG GTT CAC 3′, which corresponds to the reverse complement of nucleotides −12 to +6 (23). The mismatched (scrambled) control (mODN) was treated with the same concentration of the following antisense sequence: 5′ GTC TCA TAC GCT CAG ATC 3′.

The $\gamma 2$ subunit aODN had the following sequence: 5′ ACT CAT CGC TTT TTT TTC 3′, which corresponds to the reverse complement of nucleotides −12 to +6 (26). The mismatched control group was treated with the same concentration of the following scrambled antisense sequence: 5′ TTC TCT TTC TCT TAG ATC 3′.

Cell cultures. Primary cultures of rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rat cerebella. Cells were dispersed with trypsin (0.25 mg/ml; Sigma Chemical Co., St. Louis, MO), plated at a density of $0.8–1 \times 10^6$ onto 35-mm Nunc dishes coated with poly-L-lysine (10 μg/ml; Sigma). Cells were cultured in basal Eagle's medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 μg/ml gentamycin (GIBCO-BRL) and maintained at 37° in 6% CO_2. Cytosine arabinoside (10 μM; Sigma) 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 25 mM. aODNs were added to the culture media twice (at 48 and 24 hr) before whole-cell recordings at a final concentration of 5 μM (27, 28). Previous work has demonstrated that *in vitro* maturation of $GABA_A$ receptors increases the sensitivity of their response to GABA and decreases the BZD potentiation of GABA-gated currents (18, 19). Based on these studies, cerebellar granular cells at 7 or 14 DIV were used in the experiments to assay the effects of aODNs on the $\gamma 2$ and $\alpha 6$ subunits, respectively. Although we do not know the mechanism of ODN uptake, we can speculate that ODNs are incorporated intracellularly through endogenous endocytotic processes or through cell surface carrier proteins (29).

cDNA transient transfection studies. HEK 293 cells (No. CRL1573; American Type Culture Collection, Rockville, MD) were grown in minimal essential medium (GIBCO-BRL) and supplemented with 10% fetal bovine serum, 100 units/ml penicillin (GIBCO-BRL), and 100 units/ml streptomycin (GIBCO-BRL) in a 6% CO_2 incubator. Exponentially growing cells were dispersed with trypsin, seeded at 2×10^5 cells/35-mm dish in 1.5 ml of culture medium, and plated onto 12-mm glass cover slips (Fisher Scientific, Pittsburg, PA). Rat $\alpha 1$, $\beta 2$, and $\gamma 2$ $GABA_A$ receptor subunit cDNAs singly subcloned into the expression vector pCDM8 (Invitrogen) and the $\alpha 6$ subunit cloned into the pCIS2 expression vector were provided by Dr. Peter Seeburg (Center for Molecular Biology, University of Heidelberg, Germany). HEK 293 cells were transfected according to the calcium phosphate precipitation method (30) with various combinations of pCDM8$\alpha 1$, pCIS2$\alpha 6$, pCDM8$\beta 2$, pCDM8$\gamma 2$, and pRSV.IL-2R (generous gift of Dr. Anna Riegel, Department of Pharmacology, Georgetown University, Washington, D.C.) containing the IL-2 receptor cDNA (31). The expression of cDNAs cloned into the pCDM8 and pCIS2 vectors is under the control of the same promoter/enhancer system (cytomegalovirus promoter). The following plasmid combinations were mixed: $\alpha 1/\beta 2/\gamma 2$/IL-2, $\alpha 6/\beta 2/\gamma 2$/IL-2, and $\alpha 1/\alpha 6/\beta 2/\gamma 2$/IL-2 (1 μg/construct), and the coprecipitates were added to the culture dish containing 1.5 ml of minimal essential medium for 12–16 hr at 37° under 3% CO_2. The media was removed, and the cells were rinsed twice with culture media and finally incubated in the same media for 24 hr at 37° under 6% CO_2. Finally, the cells were incubated with Dynabead magnetic particles (Dynal, Lake Success, NY) complexed with anti-human interleukin 2 receptor monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY) in the extracellular solution for 20 min at 37°. Coverlips were mounted onto a recording chamber, and the transfected cells were readily identified by the presence of the beads for the electrophysiological studies.

Membrane preparation and Western blot analysis. Cultured granule cells (at 7 and 14 DIV) were harvested in ice-cold phosphate-buffered saline and collected through centrifugation for 10 min at $1000 \times g$, followed by homogenization in 10 mM Tris-acetate, pH 7.4, 5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at $15{,}000 \times g$ for 15 min at 4° to obtain the crude membrane fraction. Pellets were resuspended in buffer containing 50 mM Tris-acetate, pH 7.4, and 5 mM EGTA and stored at −70°. Crude membrane preparations (2–16 μg/protein) from cultured cerebellar granule cells were separated on 5% stacking/10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitro-

cellulose membranes. Blots were blocked with Tris-buffered saline/Tween-20 (0.1%) containing 5% nonfat dry milk for 2 hr at room temperature, followed by an incubation with affinity-purified antibodies raised in rabbits (23) directed against the α6 $GABA_A$ receptor subunit (1:500 dilution; a kind gift of Dr. Peter Seeburg) or against the α1 $GABA_A$ receptor subunit (1:500 dilution; a kind gift of Dr. Joseph H. Neale, Department of Biology, Georgetown University, Washington, D.C.) or with antiserum raised in guinea pig (14) directed against the γ2 $GABA_A$ receptor subunit (1:500 dilution; a kind gift of Dr. Jean Marc Fritschy, University of Zurich, Switzerland). After several washes with Tris-buffered saline/Tween-20 (0.1%), the peroxidase-labeled secondary antibody (1:2000) and Western blot chemiluminescence reagent (Pierce, Rockford, IL) were used to visualize reacted bands.

Electrophysiological studies. Cultured granule cells or isolated HEK 293-transfected cells were voltage-clamped at −50 mV in the whole-cell configuration using the patch-clamp technique (32) on the stage of an inverted microscope at room temperature. The recording pipette contained 145 mM CsCl, 5 mM $MgCl_2$, 11 mM EGTA, 5 mM Na-ATP, 0.2 mM GTP, and 10 mM HEPES, pH 7.2, with CsOH. Cells were bathed in 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM HEPES, pH 7.2. Osmolarity was adjusted to 325 mOsM with sucrose. The culture dish in the recording chamber (<500 μl total volume) was continuously perfused (5 ml/min) to prevent accumulation of drugs. All drugs dissolved in bath solution contained dimethylsulfoxide at a maximal final concentration of 0.01%, which by itself failed to modify GABA responses. GABA was applied directly through a gravity-fed Y-tubing delivery system placed within 100 μm of the recorded cell (21). Bath perfusion for the 2 min preceding coapplication with GABA was required to observe full potentiation of GABA responses by flunitrazepam. 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 with the use of an IBM-PC computer with Axotape 2 software (Axon Instrument, Foster City, CA) for off-line analysis. After normalization, fitting of the dose-response relationship was performed with the following logistic equation: $I_{max} = 100/I_{max} \cdot \{1 + (EC_{50}/[GABA]^{n_H})\}$, 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 ± standard error. Origin (MicroCal Software, Northampton, MA) was used for figure preparation and statistical analysis using analysis of variance with $p < 0.05$ and a paired t test with $p < 0.01$ to indicate statistical significance. The Bonferroni correction was applied for multiple-group comparison.

Results

Specific inhibition of α6 subunit protein synthesis by aODNs. α6 and γ2 aODNs, complementary to and overlapping the start codon of the corresponding cDNA sequences, were added to cerebellar granule cell cultures 48 and 24 hr (5 μM/addition) before the isolation of membrane proteins (27, 28). The inhibition of α6 and γ2 subunit protein expression in antisense-treated cultures was evaluated through Western blot analysis with membrane proteins from rat liver as a negative control. As shown in Fig. 1, the intensity of the α6 immunoreactive band from cell cultures treated with α6 aODNs (Fig. 1A) and of the γ2 immunoreactive band from cell cultures treated with γ2 aODNs (Fig. 1B) was reduced >60% compared with control (nontreated cultures). To examine whether ODN treatment caused a nonspecific reduction in the synthesis of $GABA_A$ receptor proteins, we tested the α6 and γ2 subunit protein levels in the respective scrambled antisense (mODN)-treated cell cultures. mODN treatment had no effect on the level of α6 subunit or γ2

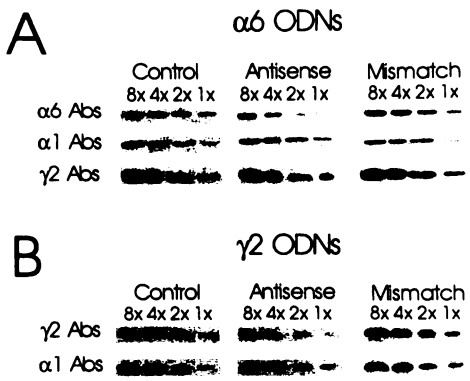

Fig. 1. aODNs knockdown the expression of specific $GABA_A$ receptor subunits in cerebellar granule cells. A, Western blot analysis of α6, α1, and γ2 $GABA_A$ receptor subunit protein levels in cerebellar granule cells treated for 48 hr with α6 subunit- (A) and γ2 subunit- (B) specific ODNs. Crude membrane fractions from granule cells were isolated, and increasing amounts of protein (1× corresponds to 2 μg of protein) were added to sodium dodecyl sulfate-polyacrylamide gels for electrophoresis. After being transferred to nitrocellulose and blocked with 0.1% Tween-20/Tris-buffered saline, subunit-specific antibodies (*Abs*) against the α6, α1, and γ2 subunits were incubated with the blots. The peroxidase-labeled secondary antibody and Western blot chemiluminescence reagent were used to visualize reacted bands.

subunit protein after a 48-hr exposure to the mODN (Fig. 1, A and B). In parallel, we tested the α1 subunit protein levels in membranes isolated from cell cultures treated with both sets of aODNs and mODNs. There was no detectable reduction in the levels of the α1 subunit protein after any of these treatments (Fig. 1, A and B). Finally, we examined γ2 subunit protein levels after a 48-hr exposure of cells to α6 ODNs (Fig. 1A). There was no significant reduction in γ2 subunit protein levels after treatment of cultures with the α6 ODNs.

Effect of α6 aODNs on furosemide-induced inhibition of GABA-activated Cl^- currents. Electrophysiological studies have shown that furosemide selectively antagonizes GABA-induced currents of recombinant receptors containing the $\alpha6\beta2\gamma2$ but not the $\alpha1\beta2\gamma2$ subunit combinations in *Xenopus* oocytes, suggesting that this drug is a subunit-specific $GABA_A$ receptor antagonist (33). To examine the consequence of reducing α6 receptor subunits in the assembly and composition of $GABA_A$ receptors during cerebellar granule cell maturation, we investigated the pharmacological properties of furosemide in granule cells after α6 aODN treatment at DIV 14. The knockdown of α6 subunits abolished the furosemide-induced inhibition of GABA-gated currents in a concentration-dependent manner (Fig. 2, A and B). Fig. 2A shows representative individual traces recorded from granule neurons in the three different paradigms with 30 μM furosemide. Fig. 2B shows the resulting histograms obtained from the recordings of multiple cells at both 30 and 100 μM furosemide. Cells treated with mODNs show the same extent of inhibition by furosemide as do age-matched nontreated granule cells (Fig. 2, A and B). The data indicate that the depletion of α6 subunits in a given cell altered the ratio of α1- to α6-containing-$GABA_A$ receptor/channel complexes. Below, we present results obtained from recombinant $GABA_A$ receptor expression studies that confirm this interpretation and allow a better understanding of the effects of furosemide in antisense-treated neurons.

Response to GABA after α6 aODN treatment. Recombinant $GABA_A$ receptor studies have demonstrated that the

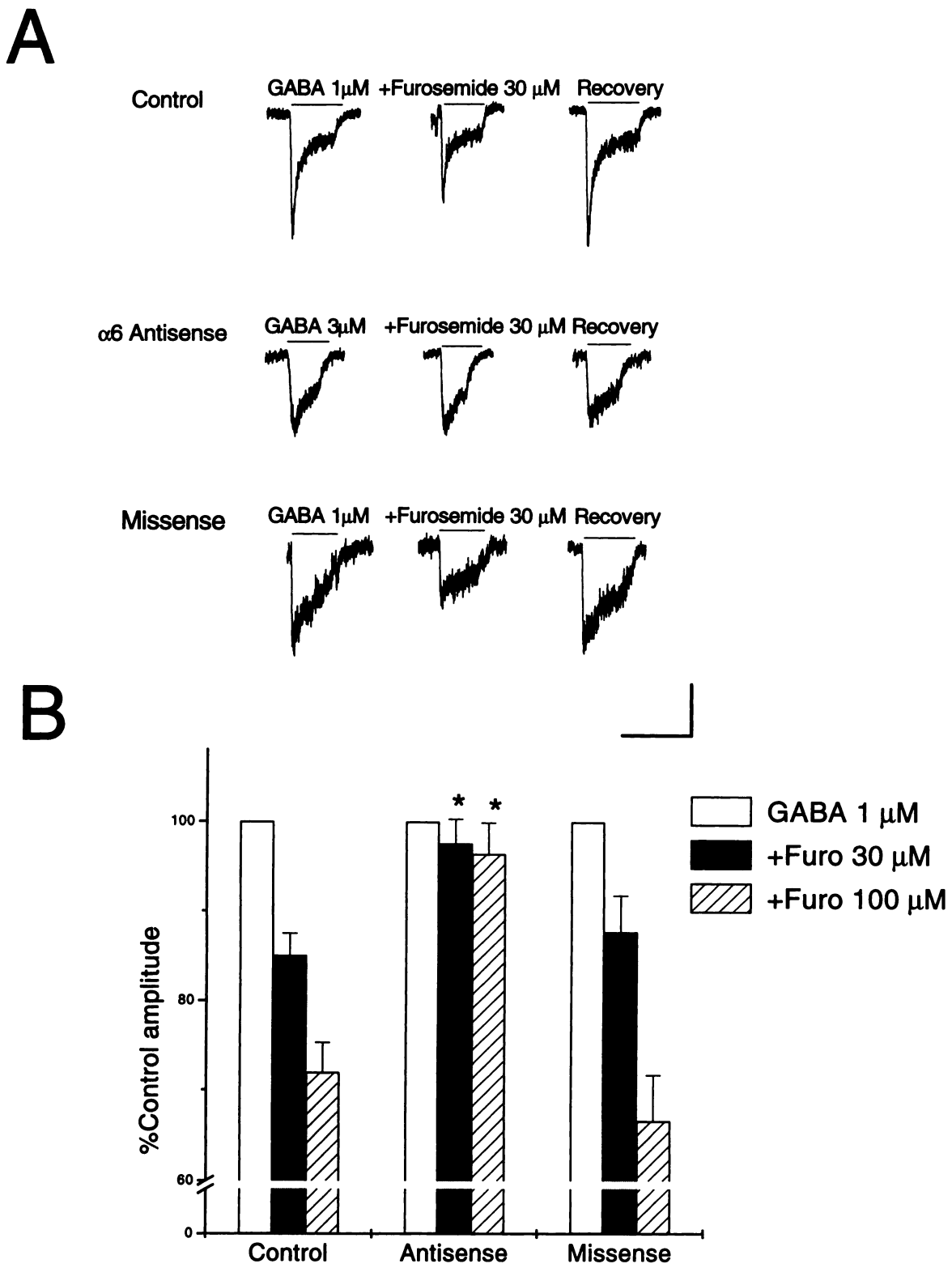

Fig. 2. Effect of α6 aODNs on the furosemide-induced inhibition of GABA-gated currents in cerebellar granule cells. A, Current traces recorded from cultured (14 DIV) granule cells (*control*), α6 aODN-treated granule cells (*α6 antisense*), and mODN-treated granule cells (*missense*) voltage-clamped at −50 mV. Each group includes a control GABA response and a GABA response in the presence of furosemide (30 μM), followed by recovery. *Calibration bars*, 20 sec and 100 pA. B, Histograms summarizing results of furosemide (*Furo*)-mediated inhibition of GABA-gated currents. Cultured granule cells were treated with α6 aODNs (*α6 antisense*) or mODNs (*missense*) 48 hr before the experiment; untreated cells were considered controls. At 14 DIV, GABA-gated currents were recorded at a holding potential of −50 mV. Percent inhibition of GABA responses by furosemide is compared with that of controls in ≥15 cells in each group from three separate experiments. Data are expressed as the mean ± standard error. ★, Significant difference in percent inhibition by the presence of furosemide compared with control and mODN-treated cultures ($p < 0.01$, one-way analysis of variance).

receptors containing α6 subunits show that the highest affinity to GABA compared with receptors containing other α subunits (5, 24, 25). Theoretically, whole-cell currents recorded from individual cells could be a summation of single-channel currents from subtypes with distinct combinations of $GABA_A$ receptor subunits, each of which has a distinct affinity, leading to dose-response relationships with low Hill coefficients and/or with intermediate EC_{50} values (34). To verify the effect of knocking down the α6 subunit on GABA dose responses in granule cells, we studied membrane currents activated by rapid application of GABA (0.03–300 μM) to α6 aODN-treated cells. In Fig. 3A, trace recordings from cultures (DIV 14) of control (nontreated), aODN-treated, and mODN-treated cells show a dose-dependent increase in the peak whole-cell current amplitude, followed by a dose-dependent increase in desensitization rate. However, cells treated with α6 aODNs show an increased half-maximal GABA concentration compared with control and α6 mODN-treated cultures (Fig. 3B). A comparison of whole-cell responses of cells in control, α6 aODN-treated, and mODN-treated cultures suggests that the receptors assembled after α6 aODN treatment had distinct pharmacological properties (Table 1). The large shift to the right of the GABA dose-response curve in α6 aODN-treated cells was not accompanied by a change in the Hill slope. However, the maximal response elicited by GABA normalized by the cell capacitance was significantly reduced by the knockdown of α6 subunits in the primary cell cultures (Table 1).

TABLE 1

Effect of α6 aODNs on GABA responses in the cultured cerebellar granule cells

Granule cells were treated with α6 aODNs or mODNs, and time-matched untreated granule cells were considered controls. Recordings were performed on DIV 14. GABA concentrations eliciting a half-maximal response (EC_{50}) were determined at a holding potential of −50 mV from normalized GABA dose-response curves obtained from control granule cells and after ODN treatment. Peak currents recorded from individual cells during the application of GABA were normalized to the maximal GABA response recorded for a given cell. EC_{50} values and Hill coefficients (n_H) were obtained from the logistic fitting of the averaged dose response with the equation given in Experimental Procedures. Maximal current (I_{max}) in each cell was divided by the cell capacitance estimated by the responses to short voltage transients given at the beginning of each recording. Values are mean ± standard error.

	EC_{50}	n_H	I_{max}	No. of experiments
	μM		pA/pF	
Control	0.9 ± 0.1	1.2	216 ± 21	7
α6 aODNs	7.1 ± 1.2[a]	1.3	70 ± 11[a]	10
mODNs	0.8 ± 0.1	1.1	176 ± 27	10

[a] Statistically significant difference compared with both control and mODNs by one-way analysis of variance ($p < 0.01$) followed by paired t test.

Allosteric modulation of GABA-gated currents after knockdown of α6 subunits. GABA-gated currents generated by activation of the $GABA_A$ receptor/ion channel complex have been shown to be allosterically modulated by BZDs. Recombinant expression studies of $GABA_A$ receptors with cDNAs selective for specific subunits show that the allosteric modulation of $GABA_A$ receptors is dependent on the presence of specific receptor subunits (5). In our study, we used aODNs

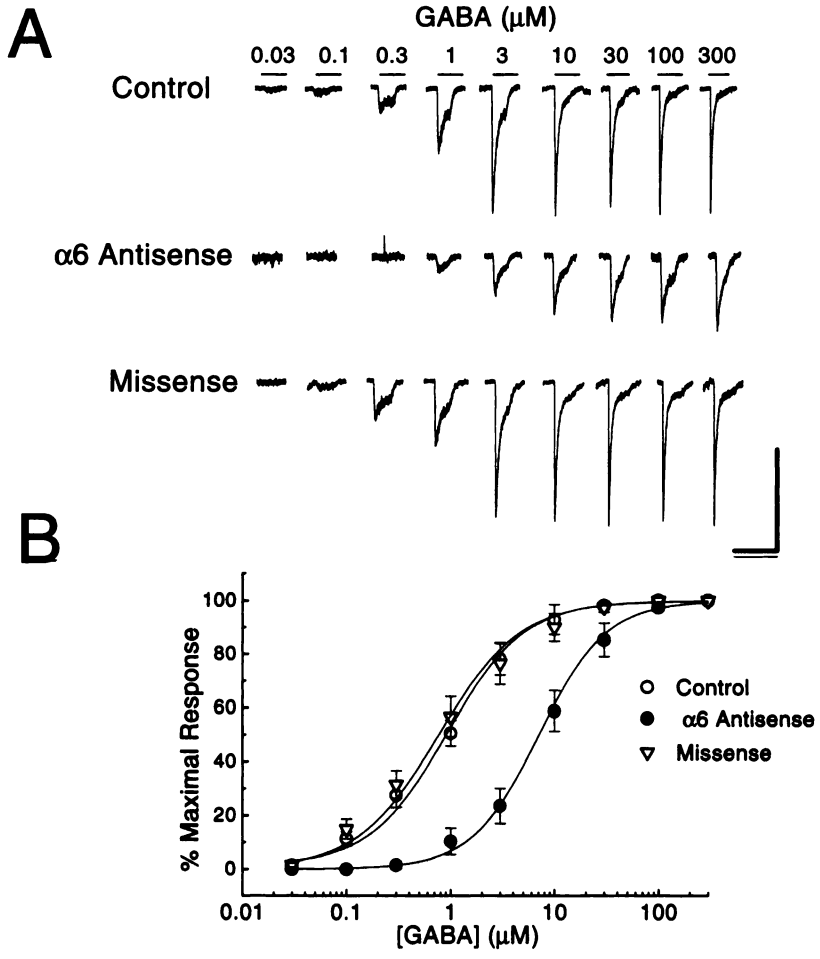

Fig. 3. Effect of α6 aODNs on GABA sensitivity of $GABA_A$ receptors in the cerebellar granule cell. A, Current traces recorded from granule cells elicited by increased GABA concentrations. GABA was applied with a Y-tubing system for the duration indicated (*bars*). Control granule cells were cultured for 14 days. At 12 DIV, one group of cultures was treated with α6 aODNs (*α6 antisense*; 5 μM final concentration) for 48 hr, and another group was treated at the same time with mODNs (*missense*; 5 μM final concentration). GABA-induced currents were recorded from cells voltage-clamped at a membrane potential of −50 mV. *Calibration bars*, 20 sec and 500 pA. B, Normalized GABA dose-response curves recorded from control (seven experiments) and α6 aODN- (10 experiments) and mODN- (10 experiments) treated cerebellar granule cells. *Points*, the mean ± standard error of the normalized currents obtained at each GABA concentration in each individual cell after normalization to the maximal GABA response recorded in that cell. Dose-response relations are shown superimposed on the logistic fit obtained with the equation given in Experimental Procedures. The corresponding average EC_{50} values and Hill coefficients are given in Table 1.

to selectively reduce α6 subunit levels in primary cerebellar granule neurons so we could examine changes in BZD modulation of the $GABA_A$ receptor/ion channel complex. Cerebellar granular cells at 14 DIV were used for this experiment because at this maturation stage, the cultures show a vastly reduced sensitivity to BZDs (18, 19). GABA was applied alone and in the presence of flunitrazepam (Fig. 4A). In control cultures, $GABA_A$ receptors in cerebellar granule cells at 14 DIV had a markedly reduced sensitivity to the BZD flunitrazepam compared with responses at earlier DIVs (18, 19). No significant differences in the degree of enhancement of GABA currents by flunitrazepam were observed between the control and mODN-treated cells. However, flunitrazepam produced a concentration-dependent increase in GABA-acti-

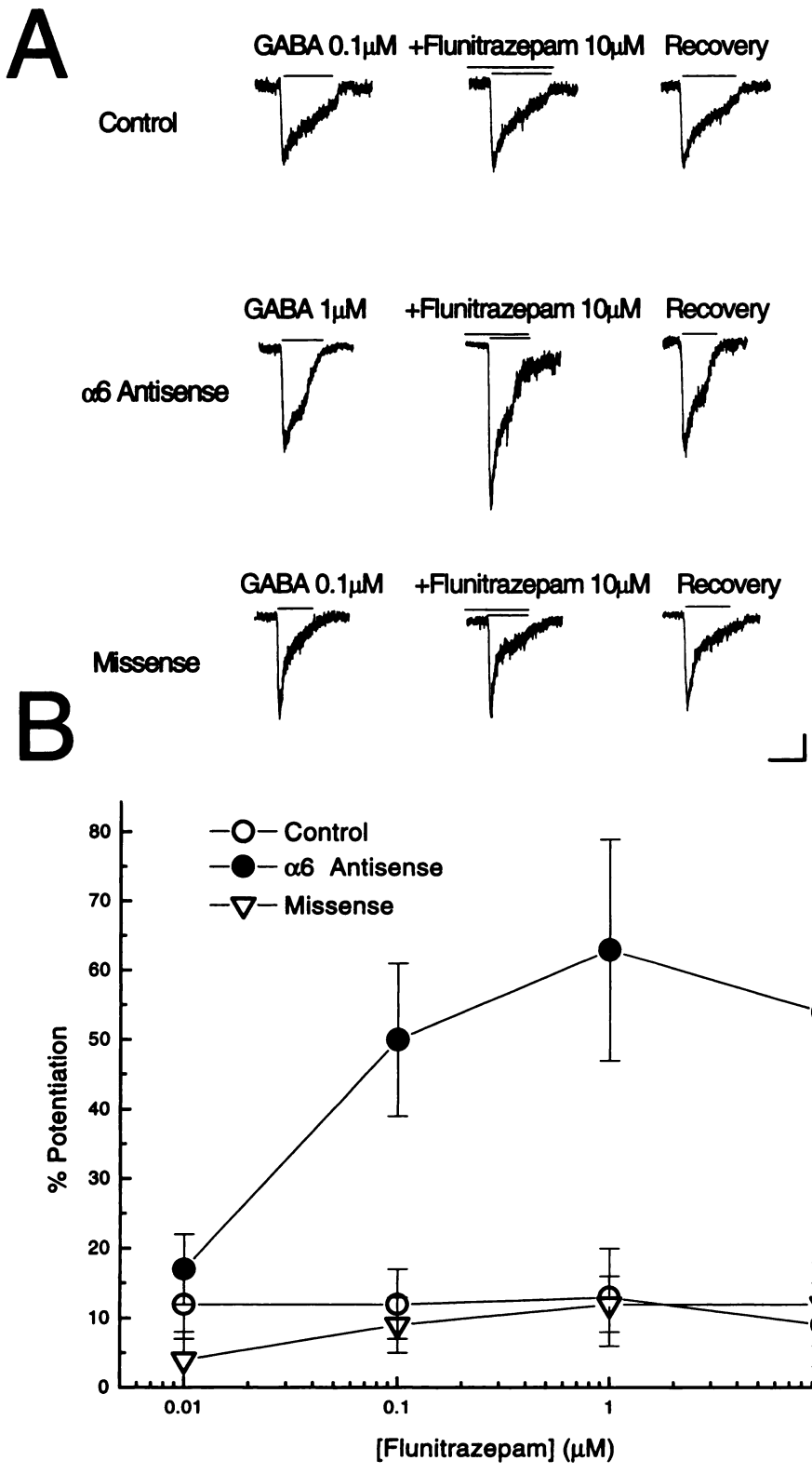

Fig. 4. α6 aODNs increase the potentiation of GABA-gated Cl^- channels by flunitrazepam. A, Current traces recorded from cultured granule cells voltage-clamped at a holding potential of −50 mV. Each group of currents consists of the control response evoked by a GABA concentration close to the EC_{20} for that experimental condition (Fig. 3), the GABA response in the presence of flunitrazepam (10 μM), and recovery. *Bars*, duration of drug application; *horizontal and vertical scale bars*, 10 sec and 40 pA, respectively. B, In cerebellar granule cells at 14 DIV, α6 antisense treatment confers on $GABA_A$ receptors responsiveness to increasing flunitrazepam concentrations. Experiments were carried out for the indicated concentrations of flunitrazepam in control and α6 aODN- and mODN-treated cultures on DIV 14. Potentiation was calculated by comparing peak current induced by coapplication of GABA and flunitrazepam with that induced by control GABA application. *Points*, mean ± standard error of the potentiation relative to control (no flunitrazepam) in ≥10 cells from three separate experiments.

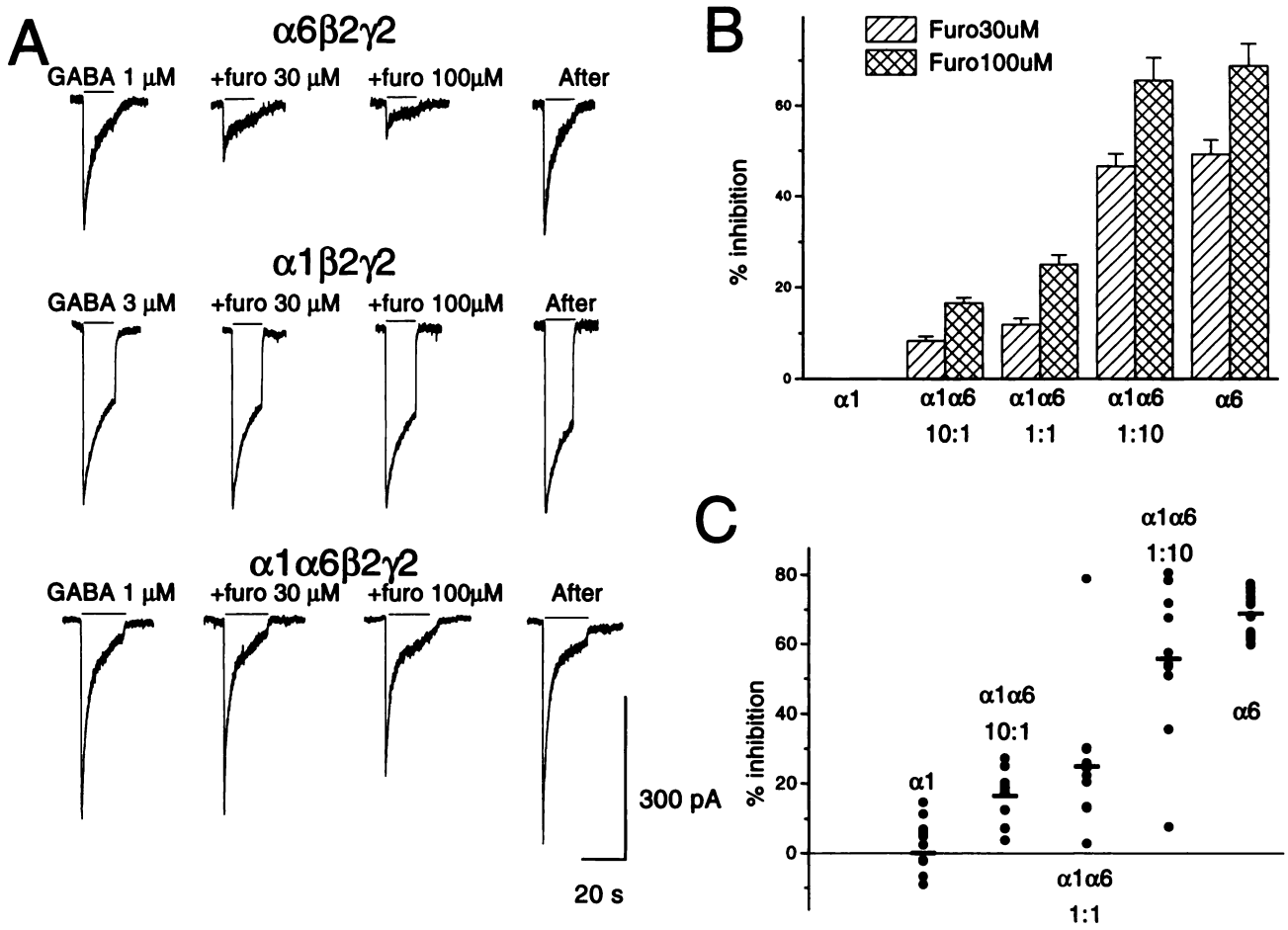

Fig. 5. Furosemide inhibits GABA-activated currents in transfected HEK 293 cells expressing GABA$_A$ receptors with distinct combinations of α1, α6, β2, and γ2 subunits. A, Examples of currents evoked with the Y-tubing application of GABA and GABA combined with furosemide (*furo*), followed by washout. B and C, Average percent inhibition of the GABA-activated current produced by 30 and 100 μM furosemide (*Furo*) for various combinations of subunits transfected. B, Average ± standard error from ≥10 cells in each experimental condition. *, Statistical significance with respect to the α1 transfection ($p < 0.05$, ANOVA followed by paired *t* test). C, Percent inhibition of GABA-activated currents produced by 100 μM furosemide for each transfected cell.

vated currents in cell cultures treated with α6-specific ODNs (Fig. 4B). This change in allosteric modulator sensitivity after knockdown of α6 subunit levels demonstrates an altered pharmacology of the GABA$_A$ receptors, which may be the consequence of a change in the GABA$_A$ receptor subunit assemblies.

Furosemide differentially inhibits recombinantly expressed GABA$_A$ receptors containing either the α1 or α6 subunit in combination with the β2 and γ2 subunits. Cerebellar granule cells express both α1 and α6 subunits, and the ratio of expression of these subunits is modulated by age, K^+-induced depolarization, and chronic drug treatment (17–21). To complement our studies on the effects of furosemide in aODN-treated cells, we further investigated the effect of this compound on recombinant GABA$_A$ receptors obtained through cotransfection of α1- and α6-containing expression vectors. We compared the furosemide-induced inhibition of GABA-activated Cl^- currents in transfected mammalian HEK 293 cells expressing receptors containing the α1, α6, or α1/α6 subunits plus both the β2 and γ2 subunits. We applied GABA (10 μM) alone or combined with 30 or 100 μM furosemide to transfected cells with the different subunit combinations. Representative current traces elicited by 1 μM GABA are shown for the various combinations tested (Fig. 5A). Also shown are recordings elicited by GABA in the presence of 30 and 100 μM furosemide followed by GABA alone. Although furosemide was ineffective at inhibiting receptors containing α1β2γ2 subunits, it was very effective in

TABLE 2

Effect of γ2 aODNs on GABA responses in the cultured cerebellar granule cells

Granule cells were treated with γ2 aODNs or mODNs, and untreated granule cells were considered controls. Recordings were performed on DIV 7. GABA concentrations eliciting a half-maximal response (EC_{50}) were determined at a holding potential of −50 mV from normalized GABA dose-response curves obtained from control granule cells and after ODN treatment. Peak currents recorded from individual cells during the application of GABA were normalized to the maximal GABA response recorded for a given cell. EC_{50} values and Hill coefficients (n_H) were obtained from the logistic fitting of the averaged dose response with the equation given in Experimental Procedures. Maximal current (I_{max}) in each cell was divided by the cell capacitance. Values are mean ± standard error.

	EC_{50}	n_H	I_{max}	No. of experiments
	μM		pA/pF	
Control	1.7 ± 0.4	1.4	196 ± 27	10
γ2 aODNs	0.3 ± 0.1[a]	1.3	76 ± 12[a]	13
mODNs	1.5 ± 0.3	1.5	204 ± 25	9

[a] Statistically significant difference compared with both control and mODNs by one-way analysis of variance ($p < 0.01$) followed by paired *t* test.

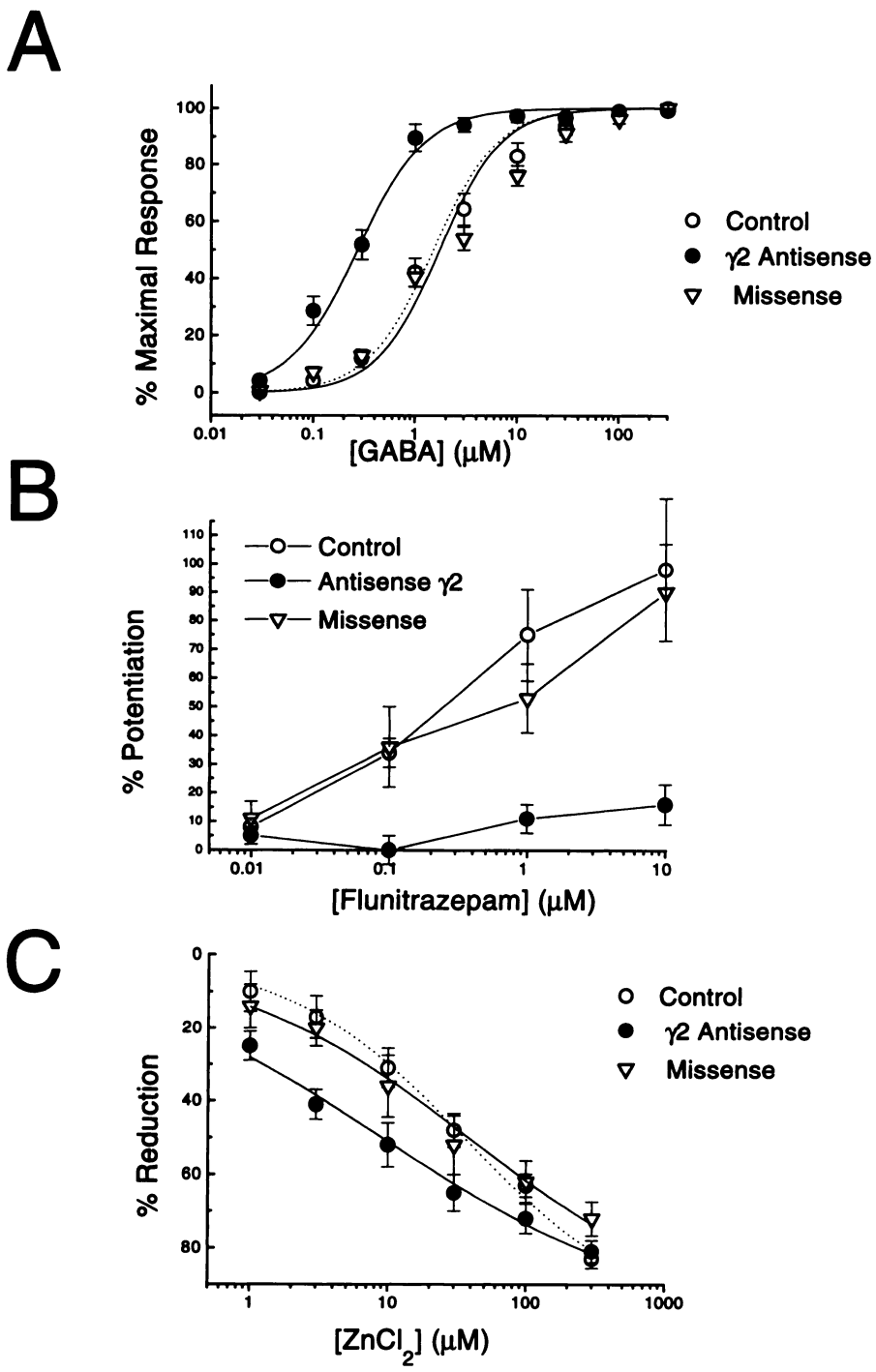

Fig. 6. Effect of γ2 subunit aODNs on the pharmacological profiles of $GABA_A$ receptors in cerebellar granule cells. On 5 DIV, one group of cultures was treated with γ2 aODNs (*γ2 antisense*; 5 μM final concentration) for 48 hr; at the same time, another group was treated with mODNs (*missense*; 5 μM final concentration). Untreated cells were used as controls. At 7 DIV, GABA-induced currents were recorded from granule cells voltage-clamped at a membrane potential of −50 mV. A, Normalized GABA dose-response curves obtained from control (10 cells) and γ2 aODN- (13 cells) and mODN- (nine cells) treated cultures. Peak currents recorded from individual cells during the application of GABA were normalized to the maximal GABA response recorded. *Points*, mean ± standard error of the normalized current at each GABA concentration. The corresponding average EC_{50} values and Hill coefficients (Table 2) were derived from the superimposed logistic curve obtained by fitting the data to the equation given in Experimental Procedures. B, Effects of γ2 aODNs on the responsiveness of granule cell $GABA_A$ receptors to flunitrazepam. At 5 DIV, granule cells were treated for 48 hr with γ2 aODNs and mODNs (5 μM). GABA-gated currents were produced by the application of GABA alone or GABA plus flunitrazepam in voltage-clamped granule neurons at −50 mV. The percent potentiation was evaluated by comparing current levels induced by coapplication of GABA and flunitrazepam with that induced by GABA application alone. *Points*, mean ± standard error of the percent potentiation at each flunitrazepam concentration in ≥10 cells. C, Effects of γ2 aODNs on the responsiveness of granule cell $GABA_A$ receptors to inhibition by $ZnCl_2$. At 5 DIV, granule cells were treated with γ2 aODNs or mODNs (5 μM) for 48 hr. Cl^- currents were evoked by GABA in the absence or presence of the indicated $ZnCl_2$ concentrations at a holding potential of −50 mV. Percent inhibition produced by $ZnCl_2$ was evaluated in comparison with whole-cell Cl^- currents induced by GABA alone and plotted as a function of the $ZnCl_2$ concentration, as indicated. The corresponding IC_{50} and Hill coefficient from this data are shown in Table 3. Data represent the mean ± standard error.

inhibiting receptors containing $\alpha6\beta2\gamma2$ subunits and differentially effective on those cells containing a combination of $\alpha1$ and $\alpha6$ subunits (Fig. 5B). The relative ratios indicate the relative abundance of $\alpha1$ and $\alpha6$ cDNA plasmids (Fig. 5B). By transfecting cDNA plasmids expressing the $\alpha1$ and $\alpha6$ subunits in defined ratios (1:1, 1:10, and 10:1), we observed that an increase in the $\alpha6$ subunit plasmid enhanced the reduction of the GABA-gated current by furosemide (Fig. 5B). When the inhibition of GABA-gated currents was investigated across the five groups of transfected cells that we studied (Fig. 5C), it became clear that with few exceptions, most cells within each group showed a similar degree of inhibition by furosemide.

Pharmacological profiles of $GABA_A$ receptors were altered by $\gamma2$ aODN treatment. Whole-cell recordings from cerebellar granule cell cultures at 7 DIV were performed at a holding potential of −50 mV to assess the functional consequences of knockdown of $GABA_A$ receptor $\gamma2$ subunits. The normalized maximal response induced by rapid application of increasing GABA concentrations in cells treated with $\gamma2$ aODNs was much lower than that measured in control and mODN-treated cells (Table 2). Furthermore, as shown in Fig. 6A, $\gamma2$ aODN treatment of cell cultures shifted the GABA dose-response curve to the left and significantly reduced the EC_{50}. The EC_{50} values from control, $\gamma2$ aODNs, and mODNs are shown in Table 2. No statistically significant differences were detected in the Hill coefficients in the three culture paradigms (Table 2). The decreased EC_{50} value implies that receptors assembled from $GABA_A$ receptor subunits after knockdown of the $\gamma2$ subunit have a higher affinity for GABA. To characterize the BZD-recognition site of $GABA_A$ receptors, granule cell cultures at 7 DIV were treated with $\gamma2$ aODNs and mODNs, and flunitrazepam potentiation of GABA responses was assessed with whole-cell patch-clamp recordings (Fig. 6B). mODN treatment did not affect the ability of flunitrazepam to enhance the GABA-induced currents (Fig. 6B). In contrast, GABA responses from cell cultures treated with $\gamma2$ aODNs were insensitive to potentiation by flunitrazepam (Fig. 6B), suggesting a loss of diazepam-sensitive components in the $GABA_A$ receptors after knocking down the expression of $\gamma2$ subunits. *In vitro* transfection studies have demonstrated that $GABA_A$ receptors containing $\gamma2$ subunits are insensitive to Zn^{2+}-induced inhibition of GABA-gated currents (35). Although we observed a low IC_{50} value for Zn^{2+} inhibition in control cultures (Table 3), $GABA_A$ receptors assembled after knockdown of $\gamma2$ subunit levels showed an increased sensitivity to Zn^{2+}-induced inhibition in cerebellar granule cells (Fig. 6C), an additional indication that the $\gamma2$ subunit modifies the Zn^{2+}-recognition site of $GABA_A$ receptors in both transfected cells and cerebellar granule neurons (Table 3). This increased sensitivity to Zn^{2+}-mediated inhibition of GABA-gated currents was not accompanied by a statistically significant difference in Hill coefficient (Table 3).

TABLE 3

Inhibition of GABA-gated Cl^- currents in cultured granule cells by $ZnCl_2$ after $\gamma2$ subunit knockdown

Granule cells were treated with $\gamma2$ aODNs or mODNs, and untreated granule cells were considered controls. At DIV 7, Cl^- currents were induced with GABA (10 μm) in the absence or presence of $ZnCl_2$ at increasing concentration. Dose-response curves of the relative percent inhibition of Cl^- currents produced by $ZnCl_2$ in control and ODN-treated granule cells were plotted and fit to a logistic equation (see Experimental Procedures). The corresponding IC_{50} values and Hill coefficients (n_H) were calculated. Values are mean ± standard error.

	IC_{50}	n_H	No. of experiments
	μM		
Control	36 ± 3.2	0.67	9
$\gamma2$ aODNs	9.5 ± 1.0[a]	0.42	13
mODNs	38 ± 3.7	0.49	8

[a] Statistically significant difference compared with both control and mODNs by one-way analysis of variance ($p < 0.01$) followed by paired t test.

Discussion

Transient transfection studies with various subunit combinations have succeeded in reconstituting the pharmacological profiles of $GABA_A$ receptors and allowed an understanding of the specific roles of cloned subunits in heterologous cells (1–5). However, the functional and pharmacological properties of native $GABA_A$ receptors in relation to their subunit composition remain poorly understood (1–5). In the current study, we attempted to dissect the contribution of individual subunits to the pharmacological properties of native $GABA_A$ receptors in cerebellar granule cells and to extend results from *in vitro* expression studies of recombinant $GABA_A$ receptors to native receptors.

We used aODNs to manipulate mRNA or protein levels and showed with both immunoblotting and the electrophysiological evaluation of the pharmacological properties of $GABA_A$ receptors that a 48-hr antisense treatment effectively reduced $GABA_A$ receptor $\alpha6$ and $\gamma2$ subunit levels. This inhibition was specific, as demonstrated by the lack of effect on $\alpha1$ subunit expression by Western blot analysis, and it was not reproduced by mODNs, suggesting that aODNs may be a powerful tool for dissecting the role of specific subunits with respect to their contribution to the function of ligand-gated ion channel/receptor complexes. The effectiveness and the relative extent of subunit down-regulation mediated by the aODN treatments led us to speculate that the turnover rate of GABA receptors in these cells must be faster than our treatment time.

The relative abundance of the $\alpha1$ and $\alpha6$ subunit expression determines the sensitivity to furosemide in the granule cell as well in transfected HEK 293 cells. Transfection studies have demonstrated that expression of the $\alpha1$ and $\alpha6$ subunits confers a specific and distinct effect on GABA- and BZD-binding sites (5, 24, 25). Therefore, the subtype and the ratio of receptors expressed in the cell may determine the pharmacology of native $GABA_A$ receptors. We took advantage of the recently demonstrated selective inhibition of $\alpha6\beta2\gamma2$ subunit-containing $GABA_A$ receptors expressed in *Xenopus* oocytes (33) to investigate the effect of furosemide on GABA-activated currents in granule neurons treated for 48 hr with $\alpha6$ subunit-specific aODNs. The results of these studies showed that knockdown of $\alpha6$ subunit levels significantly reduced the ability of furosemide to inhibit GABA-gated currents in cerebellar granule cells, suggesting that the assembly of furosemide-sensitive $GABA_A$ receptor subtypes is decreased after a reduction in $\alpha6$ subunit protein.

With the goal of better understanding the effects of furosemide in antisense-treated cells, we further investigated the effect of this compound on recombinant $GABA_A$ receptors obtained through transfection of $\alpha1$ or $\alpha6$ mRNAs together with $\beta2\gamma2$ subunit mRNAs. Our data support previously published results (33) demonstrating that furosemide is an

inhibitor selective for α6-containing receptors but also that the coexpression of α1α6 subunits with β2γ2 subunits reduces the inhibitory effect of furosemide. Our results suggest that the relative abundance of α1 and α6 transfection plasmids influences the degree of inhibition of $GABA_A$ receptors by furosemide. We have also demonstrated that a small amount of α6 subunit plasmid cotransfected with the α1, β2, and γ2 subunit plasmids is sufficient to confer furosemide sensitivity to $GABA_A$ receptors in most transfected cells. Therefore, because furosemide sensitivity of GABA currents in cerebellar granule cells is totally abolished on antisense treatment, we propose that the ODNs block the formation of α6 subunit-containing receptor subtypes. Interpretation of the results with recombinant receptors is complex in terms of actual receptor composition and stoichiometry. In the adult rat cerebellum, the presence of multiple subunits (α1, α6, β2/3, γ1, γ2, and δ) of $GABA_A$ receptors and their combination has been demonstrated (6, 7, 11–13, 17). Currently, the relative proportions of these combinations that exist in developing granule neurons in primary culture are not known, although evidence is growing that receptors containing the α1 and α6 subunits can be formed (12). Our data with recombinant receptors do not allow us to infer the subunit stoichiometry of the receptors formed before and after ODN treatments. Rather, our intent was to establish that the relative ratio of α1 to α6 subunit plasmids determines the sensitivity to furosemide of $GABA_A$ receptors formed in the presence of mixed populations of subunits, which supports the proposal that the relative abundance of mRNAs for distinct subunits contributes to the assembled subtype of both native and recombinant receptors and their pharmacological profiles.

Knockdown of α6 or γ2 alters the receptor sensitivity to GABA. Recombinant $GABA_A$ receptor expression studies demonstrate that receptors containing the α6 subunit have a low EC_{50} value for GABA and that receptors containing γ2 subunits have higher EC_{50} values (24, 25, 36, 37). We therefore investigated the effects of α6 or γ2 ODN knockdown on GABA dose responses from antisense-treated cerebellar granule cells. As expected, the knockdown of α6 subunits from cerebellar granule cells significantly increased the EC_{50} and shifted the GABA dose-response curve to the right. In contrast, a reduced EC_{50} and a left-shifted dose response for GABA were observed in the cerebellar granule cells in which the γ2 subunit aODNs were used. Our data could be explained by a mass action of receptor assembly (36). This implies that after knockdown of α6 subunits, receptors containing α1 subunits with lower affinity to GABA are proportionally increased, and that after knockdown of γ2 subunits, $GABA_A$ receptors lacking this subunit and exhibiting a higher sensitivity to GABA are assembled. As shown by our data, both α6 and γ2 subunit knockdown reduced GABA-activated maximal currents normalized by the individual cell membrane capacitance (GABA current density). Because differences in the biophysical properties of individual Cl^- channels could only in part account for this result, the reduction in GABA current density may very likely be due to a decrease in the number of functional $GABA_A$ receptors after α6 or γ2 aODN treatment. Previous recombinant expression studies have shown that depletion of α6 or γ2 from α6β2γ2 or α1β1γ2 receptor assemblies markedly reduces GABA-gated currents in $GABA_A$ receptors (36, 38). The observed shift in GABA dose responses after antisense treatment therefore indicates that both the α6 and γ2 subunits confer unique properties to the neurotransmitter binding domain and that a change in the levels of either the α6 or γ2 subunit modifies the receptor sensitivity to the agonist in the native receptor assembly.

Knockdown of α6 or γ2 subunit modifies allosteric modulation of $GABA_A$ receptor. The $GABA_A$ receptor complex has many relevant allosteric regulatory sites that modify GABA-gated channel function, such as those for BZDs and Zn^{2+}. Photolabeling studies have demonstrated that BZD allosteric modulatory sites are located between residues 59 and 148 of the α1 subunit (3). In transfection studies, two BZD receptor types can be mimicked by the coexpression of cDNAs encoding an α1, α2, α3, or α5 variant; any β variant (βx); and the γ2 subunit (5). Receptors containing the α1βxγ2 subunit combinations display BZD type I pharmacology and have a high affinity for BZDs, whereas α2βxγ2, α3βxγ2, and α5βxγ2 combinations exhibit properties of BZD type II receptors, which have a lower affinity for certain BZDs (5). Also, BZD-insensitive sites that have virtually no affinity to classic BZD agonists such as flunitrazepam are also found in the central nervous system, presumably corresponding to receptors containing α4 or α6 subunits coexpressed with βx and γ2 or δ (5). During maturation *in vitro*, $GABA_A$ receptors from cerebellar granule cells show an increased sensitivity to GABA and lose BZD-induced allosteric modulation (18, 19). These changes have been demonstrated to be related to an increased expression of α6 subunits (rather than to a decrease in the expression of γ2 subunits) with the *in vitro* maturation of $GABA_A$ receptors (18, 19). Cerebellar granular cells at 14 DIV were nearly completely insensitive to flunitrazepam, but after a 48-hr treatment with α6 aODNs, flunitrazepam showed an increased ability to enhance GABA-gated currents, implying that knockdown of α6 subunits favors the formation of BZD-sensitive $GABA_A$ receptor subtypes.

The BZD sensitivity of $GABA_A$ receptors is not solely determined by the coexpression of α subunit variants but rather depends on the proper interaction of α with γ subunits (1, 3, 5). This result underlies our findings showing that knockdown of γ2 subunits from cerebellar granule cells at 7 DIV, a time at which these receptors are sensitive to BZDs, produced a loss in the response to flunitrazepam. We believe that these data indicate that γ2 subunits are essential in these neurons for the assembly of BZD-sensitive $GABA_A$ receptors. Knockdown of γ2 subunit levels increased the extent of Zn^{2+}-induced inhibition of GABA-gated currents. This result again parallels the findings obtained from studies with recombinant receptors that demonstrate that $GABA_A$ receptors formed with γ2 subunits are much less sensitive to Zn^{2+}-induced inhibition (35). Although an increased Zn^{2+} sensitivity was observed (Fig. 6C), the effect was modest compared with what might have been expected based on previously published studies. A possible reason for this discrepancy is related to the results of White and Gurley (39) and Saxena *et al.* (40), which show that the distinct Zn^{2+} sensitivities of recombinantly expressed $GABA_A$ receptors are dependent not only on the presence of the γ2 subunit but also on the α subunit variant coexpressed. In particular, the unexpectedly low IC_{50} for Zn^{2+} inhibition that we observed in control cultures is likely accounted for by the increased Zn^{2+} sensitivity observed when the α6 subunit is present (40). The data presented here complement and extend transfection

studies by identifying important consequences of native $GABA_A$ receptor subunit knockdown, which may result in the change of receptor subtype expression, followed by a change in pharmacological profiles of $GABA_A$ receptors in cerebellar granule neurons.

In conclusion, by suppressing the information flow from mRNA to protein in a highly specific manner, the use of aODNs to knockdown the $\alpha 6$ and $\gamma 2$ subunit protein levels leads to subsequent changes in pharmacological properties. This experimental approach may be a useful molecular tool with which to dissect the contribution of specific subunits in other ligand-gated channel/receptor complexes.

Our previous work shows that development, K^+-induced depolarization, and NMDA treatment selectively regulate $GABA_A$ receptor subunit expression, which is accompanied by the modification of the pharmacological properties of the receptor (17, 19–21). Our findings further support the notion that these changes are at least in part based on the selective regulation of specific subunit expression.

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Send reprint requests to: Dr. Dennis R. Grayson, Department of Psychiatry, Medical College of Pennsylvania and Hahnemann University, Neuroscience Research Center, 320 East North Avenue, Pittsburgh, PA 15210. E-mail: grayson@asri.edu.