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Site-Directed Mutagenesis of *N*-Linked Glycosylation Sites on the γ-Aminobutyric Acid Type A Receptor α1 Subunit

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SUMMARY

Oligonucleotide-directed mutagenesis was used to mutate the two potential sites for *N*-linked glycosylation on the rat γ-aminobutyric acid $(GABA)_A$ receptor α1 subunit. Wild-type (WT) or mutant α1 subunits [asparagine to glutamine substitutions at position 10 ($\alpha1Q^{10}$), 110 ($\alpha1Q^{110}$), or both 10 and 110 ($\alpha1Q^{10/110}$)] were coexpressed with β1 and γ2 subunits in *Xenopus* oocytes. Removal of either one or both potential sites for *N*-linked glycosylation resulted in expression, in *Xenopus* oocytes, of functional $GABA_A$ receptors with pharmacological properties similar to those observed for the WT receptor. WT and mutant α1 subunits were co-transfected with β1 and γ2 subunits in human embryonic kidney 293 cells. WT and mutant α1 subunits expressed in 293 cells were photoaffinity labeled with [^{3}H]flunitrazepam. Co-transfection of α1WT, $\alpha1Q^{10}$, or $\alpha1Q^{110}$ subunits in combination with β1 and γ2 $GABA_A$ receptor subunits resulted in the labeling of single bands, with approximate molecular masses of 54, 49, and 50 kDa, respectively. The decrease in molecular mass for both the $\alpha1Q^{10}$ and $\alpha1Q^{110}$ mutants suggests that both consensus sequences for *N*-linked glycosylation are used in 293 cells. Low levels of [^{3}H]flunitrazepam binding prevented visualization of the $\alpha1Q^{10/110}$ double mutant. The 293 cells transfected with either the $\alpha1Q^{10}$ or $\alpha1Q^{110}$ mutant in combination with β1 and γ2 subunits expressed significantly lower levels of [^{3}H]Ro15–1788 binding, relative to WT levels. In addition, [^{3}H]Ro15–1788 binding was undetectable in 293 cells expressing the $\alpha1Q^{10/110}$ double mutant. When transfected 293 cells were grown at 30°, [^{3}H]Ro15–1788 binding to $\alpha1Q^{10}$ and $\alpha1Q^{110}$ $GABA_A$ receptors was restored to levels comparable to that for WT receptors. [^{3}H]Ro15–1788 binding to $\alpha1Q^{10/110}$ was not reliably detected at 30°. Similar results were observed using [^{3}H]muscimol. These data suggest that intracellular processing and transport of the glycosylation-deficient $GABA_A$ receptor α1 subunit is temperature sensitive. Furthermore, the observed differences between the two expression systems may be accounted for by the typically lower temperature used for maintaining microinjected *Xenopus* oocytes. Thus, although glycosylation is not an absolute requirement for $GABA_A$ receptor expression, it has a profound effect on the processing of at least the α1 receptor and its subsequent assembly into a mature receptor.

GABA is the primary inhibitory neurotransmitter in the mammalian central nervous system. The binding of GABA to its receptor (the $GABA_A$ receptor) results in the opening of a chloride-selective ion channel (1, 2). The actions of GABA are potentiated by benzodiazepines, barbiturates, and alcohol (3) and can be antagonized by the convulsants picrotoxin and bicuculline (4). Five subunit families, i.e., α, β, γ, δ, and ρ, have been identified by cDNA cloning (2). Functional homomeric ion channels have been observed in *Xenopus* oocytes microinjected with $GABA_A$ receptor α or β subunits (5, 6) and in mammalian cells transfected with cDNA coding for a single subunit (7). However, greatly enhanced currents are observed upon coexpression of both the α and β subunits (6). Furthermore, the presence of α, β, and γ subunits is necessary for expression of receptors exhibiting the properties of native $GABA_A$ receptors (8, 9).

The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily and is an integral membrane glycoprotein. Although the subunits each contain consensus sequences for *N*-linked glycosylation (Asn-X-Ser/Thr) (1, 10), the role of glycosylation in expression of the $GABA_A$ receptor has not been studied. *N*-Linked glycosylation has been demonstrated to play a critical role in the expression of other ligand-gated ion channels, most notably the nicotinic AChR. In mouse muscle cells (11, 12) or oocytes expressing *Torpedo* AChRs (13, 14), inhibition of AChR glycosylation reduced cell surface receptor expression. Similarly, mutagenesis of potential *N*-linked glycosylation sites on AChR subunits decreased the expression of receptors on the cell surface (15–17). Although the specific role of the carbohydrate moieties in functional expression of the AChR

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; CFTR, cystic fibrosis transmembrane regulator; WT, wild-type; AChR, acetylcholine receptor.

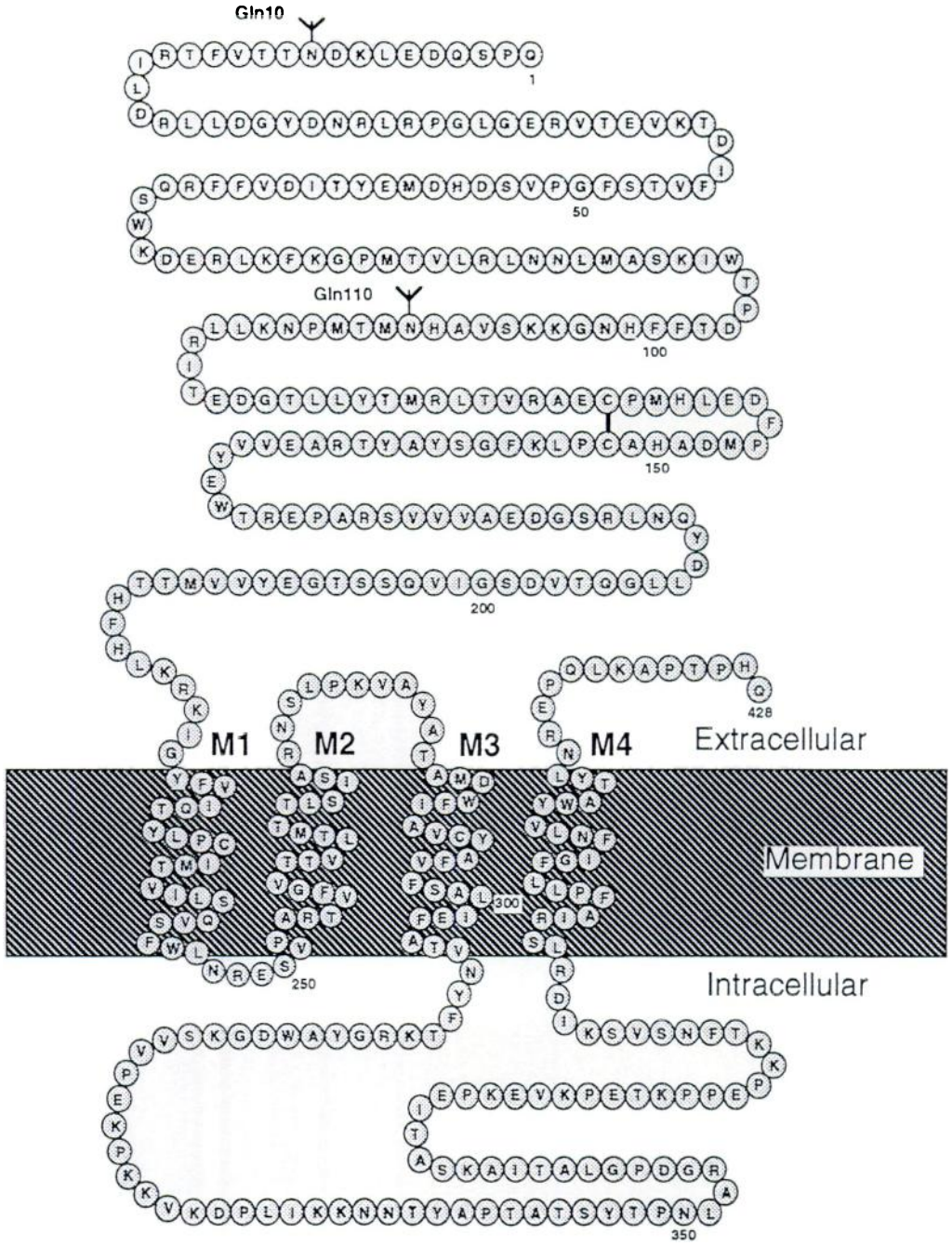

Fig. 1. Predicted secondary structure of the rat GABA$_A$ receptor α1 subunit. Shown are the putative four transmembrane domains (*M1–M4*) and the extracellular amino and carboxyl termini. Putative *N*-linked glycosylation sites were changed from asparagine to glutamine by site-directed mutagenesis (*Gln10* and *Gln110*). Three α1 subunit glycosylation mutants are described in this report and are referred to as α1Q^{10}, α1Q^{110}, and the double mutant α1Q$^{10/110}$.

remains controversial, increasing evidence suggests that these domains confer stability, preventing rapid proteolysis and thus allowing subunit assembly and transport to the cell surface (16, 17). However, glycosylation is not an absolute requirement for AChR subunit assembly, because nonglycosylated subunits are capable of assembling into heteromeric complexes (14, 16, 17).

The present investigation sought to determine the role of glycosylation of the α1 subunit in expression of the $\alpha1\beta1\gamma2$ GABA$_A$ receptor. Coexpression of these three subunits has been reported in several brain regions, including the cerebral cortex, olfactory bulb, hippocampus, and cerebellum (18), suggesting that this hetero-oligomeric combination exists in native brain. We now report that the effect of removal of the two potential glycosylation sites on the α1 subunits depends upon the particular expression system.

Experimental Procedures

Materials

Tissue culture reagents were from GIBCO-BRL (Gaithersburg, MD). Human embryonic kidney 293 cells (CRL 1573) were from the American Type Culture Collection (Rockville, MD). [^{3}H]Ro15–1788, [^{3}H]muscimol, and [^{3}H]flunitrazepam were from DuPont/New England Nuclear (Boston, MA). Clonazepam, pentobarbitol, and flunitrazepam were from Sigma Chemical Co. (St. Louis, MO).

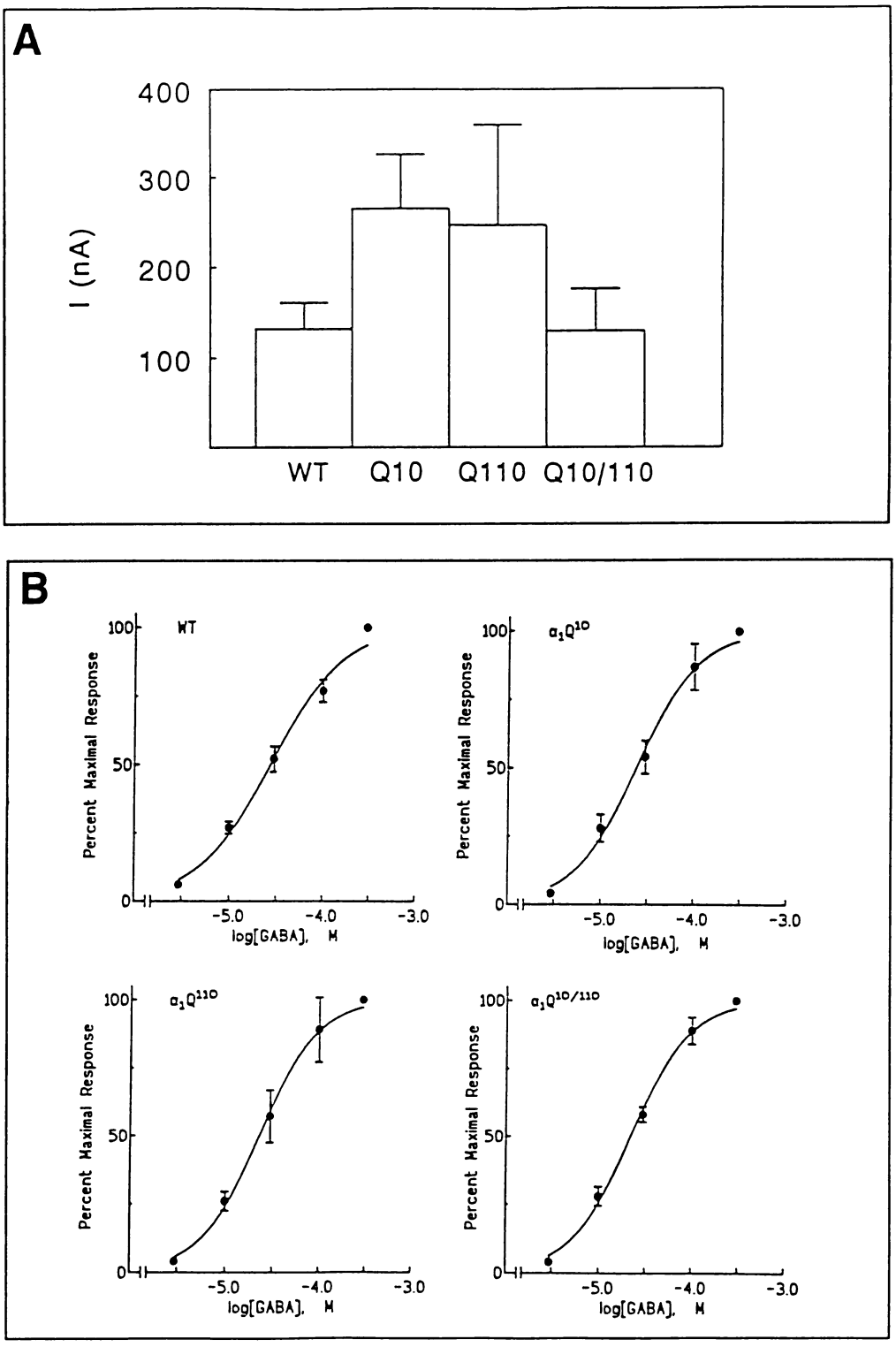

Fig. 2. Expression of WT and asparagine to glutamine mutant α1 subunits in *Xenopus* oocytes. A, Whole-cell current responses to bath application of 10 μM GABA in oocytes expressing WT or mutant $GABA_A$ receptors. Shown are the current responses (mean ± standard error) from four separate experiments, each with three to seven oocytes. Current responses of the three $GABA_A$ receptor α1 subunit mutants were not significantly different from WT control responses, by Student's *t* test. B, Activation of WT and $\alpha1Q^{10}$, $\alpha1Q^{110}$, and $\alpha1Q^{10/110}$ mutant $GABA_A$ receptors in *Xenopus* oocytes by GABA. Dose-response curves generated for GABA activation of WT and mutant $GABA_A$ receptors were fit according to the equation $I = I_{max}/[1 + (EC_{50}/A)^{\gamma}]$, as described in Results. Data were standardized to the maximal current response observed with 300 μM GABA. Each *point* represents the mean ± standard error of three to six oocytes taken from the same frog. See text for EC_{50} values and Hill coefficients.

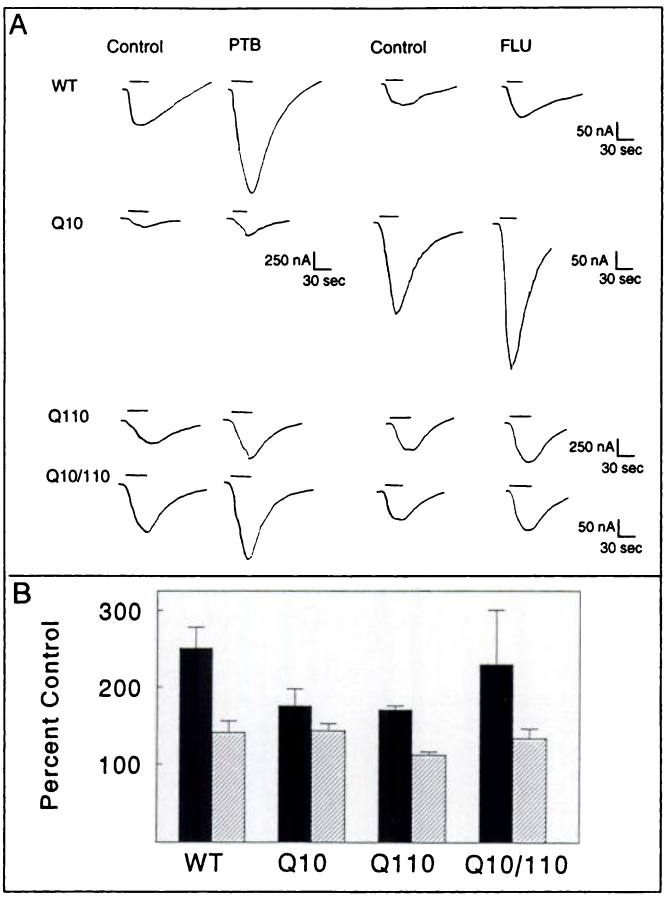

Fig. 3. Pentobarbitol and flunitrazepam potentiation of currents elicited by 10 μM GABA in *Xenopus* oocytes expressing WT or mutant GABA$_A$ receptors. A, Current traces from individual oocytes expressing the indicated α1 subunit in combination with WT β1 and γ2 subunits. Traces were obtained before (*Control*) and after application of 25 μM pentobarbitol (*PTB*) or 1 μM flunitrazepam (*FLU*). *Bars over current traces*, drug applications. B, Potentiation of GABA-elicited currents by pentobarbitol and flunitrazepam in oocytes expressing WT or α1Q^{10}, α1Q^{110}, or α1Q$^{10/110}$ mutant GABA$_A$ receptors. Data are expressed as the percentage of control response (response to 10 μM GABA) in the presence of 25 μM pentobarbitol (■) or 1 μM flunitrazepam (▨). Data are presented as the mean ± standard error for three to six oocytes taken from the same frog. Potentiation of GABA responses by pentobarbitol or flunitrazepam in oocytes expressing the three GABA$_A$ receptor α1 subunit mutants was not significantly different from WT control values, by Student's *t* test.

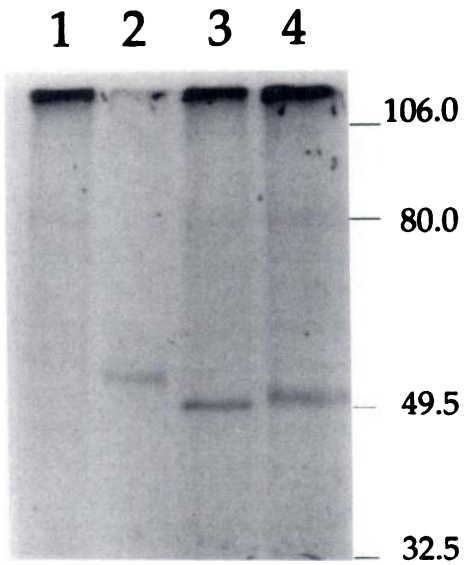

Fig. 4. [^{3}H]Fluntitrazepam photoaffinity labeling and gel electrophoresis of WT and asparagine to glutamine mutant α1 subunits expressed in 293 cells. Membranes were prepared from cells transfected with the following GABA$_A$ receptor subunit combinations: β1γ1 (*lane 1*), α1β1γ2 (*lane 2*), α1Q^{10}β1γ2 (*lane 3*), and α1Q^{110}β1γ2 (*lane 4*). Photoaffinity labeling was performed as described in Experimental Procedures, using 0.2 pmol of [^{3}H]Ro15–1788 binding sites (*lanes 2-4*) or 250 μg of protein (*lane 1*). Of the labeled membranes, 10% were electrophoresed on SDS-polyacrylamide gels and bands were visualized by fluorography. Films were exposed at −70° for 10 days.

GABA$_A$ Receptor Subunit cDNAs

Full length rat GABA$_A$ receptor α1 and β1 subunit cDNAs inserted into pBluescript (Stratagene, San Diego, CA) were the generous gift of Dr. Allan Tobin (University of California, Los Angeles). To isolate rat γ2 cDNA, a cDNA fragment was amplified from rat brain by polymerase chain reaction, using oligonucleotide primers based on the published γ2 subunit sequence (8). The resulting probe (complementary to nucleotides 471–728) was hybridized to a λgt11 rat cerebellar cDNA library (Clontech, Palo Alto, CA), in 50% formamide, 5× SSPE (Saline-sodium phosphate-EDTA buffer, 5× = 0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 5× Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, at 42° for 18 hr. Filters were washed in 2× standard saline citrate (2× = 0.3 M NaCl, 0.1 M sodium citrate)/0.1% SDS at 65°, followed by 0.2× standard saline citrate/0.1% SDS at 65° for 30 min. Two positive clones were isolated. Restriction analysis revealed that approximately 40 base pairs were missing from the 5′ end of the isolated γ2 subunit cDNA, including part of the coding sequence. A 2.1-kilobase *Eco*RI fragment of the partial γ2 clone was ligated into the *Eco*RI site of pVZ1 and then digested with *Eco*RI and *Cla*I (approximately 17 base pairs downstream from the stop codon) to yield a 1.3-kilobase cDNA, which was cloned into the corresponding sites in pGEM7Z(+) (Promega, Madison, WI). Reverse transcription-polymerase chain reaction was performed to amplify a cDNA fragment extending from nucleotide −43 to nucleotide +229 of the published γ2 sequence, including the initiation codon. The resulting cDNA was ligated to the partial γ2 sequence in pGEM7Z(+). The presence of a full length γ2L subunit cDNA was confirmed by sequencing. Full length GABA$_A$ receptor cDNAs were subcloned into the eukaryotic expression vector pCDM8 (Invitrogen, San Diego, CA).

In Vitro Transcription

Plasmid cDNAs were linearized by digestion with *Xho*I (α1 and β1) or *Bam*HI (γ2) and were transcribed *in vitro* using T3 (α1 and β1) or T7 (γ2L) RNA polymerase, essentially as described (19).

Site-Directed Mutagenesis

The entire coding region of the rat α1 subunit cDNA was subcloned into the *Eco*RI site of pSELECT (Promega). Asn^{10} and Asn^{110} were changed to Gln^{10} and Gln^{110} using the following mutant oligonucleotides: Asn^{10} to Gln^{10}, 5′-CGTGAAGACAGTGGTCTGGTCCTTAA-GTTCATC-3′; Asn^{110} to Gln^{110}, 5′-TGGGCATGGTCATCTGGTGG-GCCACAGAC-3′. Oligonucleotides were 5′-phosphorylated with T4 polynucleotide kinase and annealed to single-stranded DNA. The mutant strand was synthesized using T4 DNA polymerase and ligated before two rounds of transformation into the appropriate bacterial hosts. Transformants were screened by direct sequencing. Mutagenesis reactions were performed to generate single α1 subunit mutants in which one of the two potential sites for *N*-linked glycosylation was removed or to generate a double mutant, completely lacking both potential sites for *N*-linked glycosylation.

Translation in *Xenopus* Oocytes

Oocytes were removed from mature *Xenopus laevis* (Xenopus One, Ann Arbor, MI) and were dissociated with 2 mg/ml collagenase (type

TABLE 1

[^{3}H]Ro15-1788 binding to WT and α1 subunit mutant GABA$_A$ expressed in 293 cells

Data are presented as means ± standard errors and were derived from saturation binding studies performed in duplicate or triplicate, with the indicated number of transfections (n).

Receptor	37°		30°	
	B_{max}	K_d	B_{max}	K_d
	fmol/mg of protein	nM	fmol/mg of protein	nM
WT ($\alpha_1\beta_1\gamma_2$)	1220 ± 89 (n = 3)	0.78 ± 0.25	1053 ± 174 (n = 3)	1.19 ± 0.33
Q10 ($\alpha_1^{Q10}\beta_1\gamma_2$)	338 ± 105[a] (n = 2)	1.15 ± 0.25	851 ± 1 (n = 2)	1.56 ± 0.36
Q110 ($\alpha^{Q110}\beta_1\gamma_2$)	254 ± 43[a] (n = 2)	1.13 ± 0.63	850 ± 56 (n = 2)	0.90 ± 0.24
Q10/110 ($\alpha_1^{Q10/110}\beta_1\gamma_2$)	ND[b]	ND	ND	ND

[a] Significantly different ($p < 0.05$) from WT values at the corresponding temperature.
[b] ND, specific binding was undetectable for this mutant receptor, and therefore B_{max} and K_d values could not be determined.

IA; Sigma) in Ca^{2+}-free OR-2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6). The remaining follicle layer was removed manually using micro-fine dissecting forceps. Isolated stage V and stage VI oocytes were maintained in ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 2.5 mM sodium pyruvate, 1000 units/ml penicillin, and 0.1 mg/ml streptomycin. GABA$_A$ receptor subunit RNAs were dissolved in sterile distilled H_2O and mixed in equimolar stoichiometry. Fifty nanoliters (0.5 ng/nl) of the final RNA mixture were microinjected into the oocyte cytoplasm as described previously (20). Oocytes were incubated in complete ND-96 solution at 19° for 24–72 hr before electrophysiological assay.

Electrophysiology

Electrophysiological responses to bath application of GABA or other GABA-related ligands were measured using a standard two-microelectrode voltage clamp (Axoclamp 2A; Axon Instruments, Foster City, CA). Electrodes were filled with 3 M KCl and had resistances of 0.6–3.0 MΩ. The recording chamber was continuously perfused with ND-96. Whole-cell current elicited by bath application of GABA was determined at a holding potential of −60 mV. Dose-response curves for GABA activation of oocyte-expressed receptors were fit (GraphPAD; ISI Software, San Diego, CA) according to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$, where I is the current response, I_{max} is the current response elicited at maximal GABA concentration, A is the GABA concentration, n is the Hill coefficient, and EC_{50} is the GABA concentration producing a half-maximal response. Flunitrazepam was dissolved in ethanol and diluted in ND-96 to a final ethanol concentration of 0.01%. This concentration of ethanol had no effect on GABA-elicited current responses in oocytes (data not shown). Only healthy oocytes with a minimum resting potential of −30 mV were used.

Expression in Mammalian Cells

Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified CO_2 atmosphere at 37°. For temperature-effect studies, cells were grown under identical conditions except that the temperature was maintained at 30°. Subconfluent 293 cells were co-transfected with plasmid cDNAs encoding the rat WT or mutant α1 in combination with β1 and γ2 GABA$_A$ subunits (12 μg of plasmid DNA/subunit), as described (21). Cells were co-transfected with pRSV-T and pCMV/β-gal, which express simian virus 40 T antigen and β-galactosidase, respectively.

Membrane Preparation

The 293 human embryonic kidney cells were harvested 72 hr after transfection by scraping into buffer containing 10 mM potassium phosphate, pH 7.4, and 100 mM KCl. An aliquot of cells was removed for β-galactosidase assay (Promega) and the remainder was homogenized with a Brinkmann Polytron PT3000 homogenizer, set at top speed, for 15 sec. A crude membrane pellet was obtained by centrifugation of the homogenate at 30,000 × g for 30 min at 4°. The membrane pellet was resuspended in the appropriate assay buffer at a protein concentration of 2–4 mg/ml and was used immediately or frozen at −70°. Protein concentrations were normalized to β-galactosidase activity to control for differences in transfection efficiencies.

Radioligand Binding

[^{3}H]Ro15–1788. Cell membranes were suspended in assay buffer (10 mM potassium phosphate, pH 7.4, 100 mM KCl). Saturation binding of [^{3}H]Ro15–1788 (70–87 Ci/mmol) to the GABA$_A$ receptor was performed in an assay volume of 400 μl with increasing concentrations of [^{3}H]Ro15–1788 (0.1–10 nM), in the presence or absence of 10 μM clonazepam to define nonspecific binding. Samples were incubated for 60 min at 4°, filtered over Whatman GF/C filters, and washed with 10 ml of ice-cold assay buffer.

[^{3}H]Muscimol. Cell membranes were suspended in TEN buffer (40 mM Tris·HCl, 1 mM EDTA, 15 mM NaCl, pH 7.5). Binding of 20 nM [^{3}H]muscimol (19.5 Ci/mmol) to the GABA$_A$ receptor was performed in the absence or presence of 1 mM GABA, in a total volume of 200 μl. Samples were incubated for 30 min at 0°, diluted with 5 ml of ice-cold TEN buffer, and filtered over GF/C filters. Filters were washed twice with 5 ml of ice-cold TEN buffer. Data were analyzed by computerized curve fitting using GraphPAD INPLOT software (ISI Software).

Photoaffinity Labeling

Cell membranes (60–250 μg of protein) were photolabeled with [^{3}H] flunitrazepam as described (22). Briefly, cell membranes were incubated with [^{3}H]flunitrazepam (50 nM), in the presence or absence of 1 μM clonazepam, for 60 min at 0–4°. Membranes were exposed to an UV light source (340 nm, Foto/prep I; Fotodyne, Hartland, WI) at a distance of 10 cm for 15 min at 0–4°. Flunitrazepam (1 μM) was added and incubation was continued for 30 min on ice. Membranes were then pelleted (30,000 × g for 30 min) and electrophoresed on 12% SDS-polyacrylamide gels (23). Labeled bands were visualized by fluorography.

Results

Role of *N*-linked glycosylation in expression of GABA$_A$ receptors in *Xenopus* oocytes. Analysis of the published α1 subunit cDNA sequence revealed two potential sites for *N*-linked glycosylation (Fig. 1). Using site-directed mutagenesis, asparagine residues were replaced with glutamine at position 10 (α1Q^{10} mutant), at position 110 (α1Q^{110} mutant), or at both positions (α1Q$^{10/110}$ double mutant).

RNA encoding either WT or mutant α1 subunits (Q^{10}, Q^{110}, or Q$^{10/110}$), in combination with β1 and γ2 subunits, was microinjected into the cytoplasm of *Xenopus* oocytes. Functional expression of WT or mutant GABA$_A$ receptors was assayed electrophysiologically. As shown in Fig. 2A, removal of either or both potential glycosylation site(s) had no effect on func-

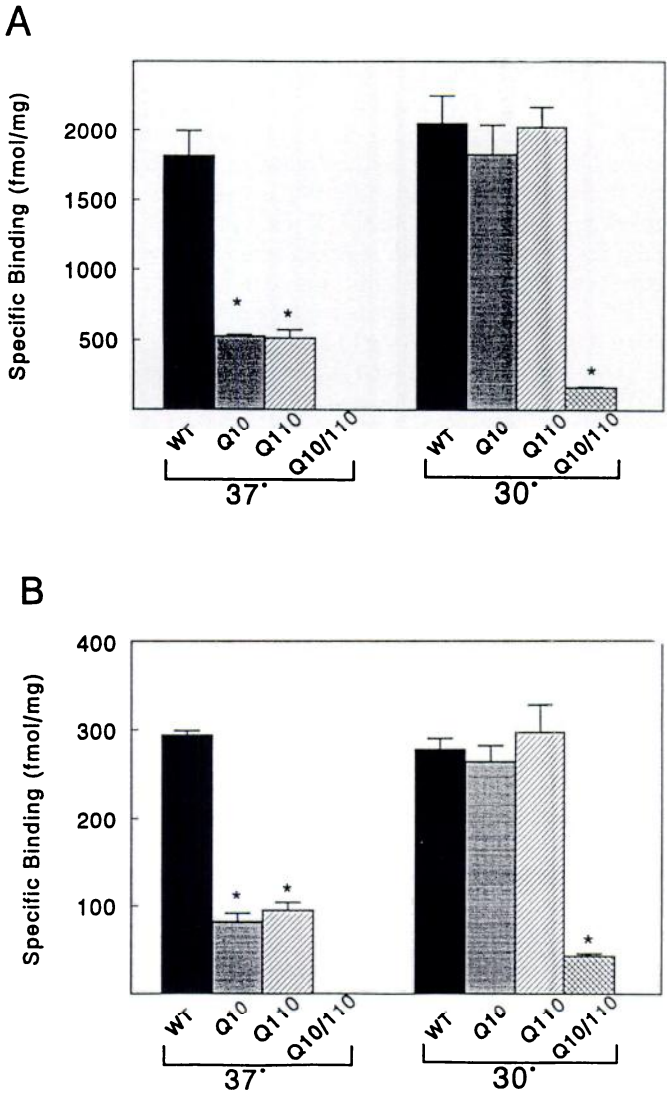

Fig. 5. Radioligand binding to WT and asparagine to glutamine α1 subunit mutant GABA$_A$ receptors expressed in 293 cells. A, Specific binding of [^{3}H]Ro15–1788 to WT and asparagine to glutamine α1 subunit mutant GABA$_A$ receptors expressed in human embryonic kidney 293 cells grown at 37° or 30°. Data are expressed as fmol/mg of protein, determined from the binding of 1 nM [^{3}H]Ro15–1788 to membranes from cells expressing WT, α1Q^{10}, α1Q^{110}, or α1Q$^{10/110}$ GABA$_A$ receptors. Shown are the means ± standard errors of values assayed in duplicate or triplicate, with two or three separate transfections. *, Significant difference ($p < 0.05$, analysis of variance), compared with WT GABA$_A$ receptor binding. B, Comparison of [^{3}H]muscimol binding to WT and asparagine to glutamine α1 subunit mutant GABA$_A$ receptors expressed in human embryonic kidney 293 cells grown at 37° or 30°. Data are expressed as fmol/mg of protein, determined from the binding of 20 nM [^{3}H]muscimol to membranes from cells expressing WT, α1Q^{10}, α1Q^{110}, or α1Q$^{10/110}$ GABA$_A$ receptors. Shown are the means ± standard errors of values assayed in duplicate or triplicate, with two or three separate transfections. *, Significant difference ($p < 0.05$, analysis of variance), compared with WT GABA$_A$ receptor binding.

tional expression of GABA$_A$ receptors in *Xenopus* oocytes. WT and mutant GABA$_A$ receptors expressed in oocytes displayed similar EC$_{50}$ values and similar Hill coefficients for GABA (Fig. 2B). EC$_{50}$ values were 27.4 μM, 24.1 μM, 26.9 μM, and 21.6 μM for the α1WT, α1Q^{10}, α1Q^{110}, and α1Q$^{10/110}$ GABA$_A$ receptors, respectively. Hill coefficients were derived from the limiting slope of the log-log plot (data not shown) and were calculated to be 1.1, 1.3, 1.4, and 1.4 for the α1WT, α1Q^{10}, α1Q^{110}, and α1Q$^{10/110}$ GABA$_A$ receptors, respectively. These values are in good agreement with other reports on expression of GABA$_A$ receptors in *Xenopus* oocytes (18).

WT and mutant α1 GABA$_A$ receptors were similarly potentiated by the barbiturate pentobarbitol and the benzodiazepine flunitrazepam. Fig. 3A shows representative current traces from individual oocytes expressing the β1 and γ2 GABA$_A$ receptor subunits in combination with the indicated α1 subunit. As demonstrated previously (6, 18), potentiation of WT receptors by pentobarbitol was greater than that by flunitrazepam (Fig. 3B).

Role of *N*-linked glycosylation in expression of GABA$_A$ receptors in cultured mammalian cells. *N*-Linked glycosylation of the α1 subunit and inhibition of glycosylation by asparagine to glutamine mutation at positions 10 and 100 were demonstrated by photoaffinity labeling of WT and mutant α subunits with [^{3}H]flunitrazepam (24, 25). The 293 cells transfected with only β1 and γ2 subunits were not labeled by [^{3}H]flunitrazepam (Fig. 4, *lane 1*). In contrast, co-transfection of β1 and γ2 subunits with α1WT, α1Q^{10}, or α1Q^{110} subunit resulted in the labeling of a single band on SDS-polyacrylamide gels, with approximate sizes of 54, 49, and 50 kDa, respectively (Fig. 4, *lanes 2-4*). The smaller size of the mutant α1 subunits is consistent with the use of both consensus sites for *N*-linked glycosylation (Asn^{10} and Asn^{110}) in 293 cells. Preincubation of transfected cell membranes with 1 μM clonazepam blocked labeling of WT and mutant α1 subunits by [^{3}H]flunitrazepam (data not shown). Cells expressing the α1Q$^{10/110}$ double mutant did not bind detectable amounts of [^{3}H]flunitrazepam and, consequently, the molecular mass of the double mutant could not be assessed by this method. However, based on the reduction in size of the α1Q^{10} and α1Q^{110} mutants (5 kDa and 4 kDa, respectively), the α1Q$^{10/110}$ double mutant should have a molecular mass of approximately 45 kDa. This is consistent with a previous report that endoglycosidase F treatment of purified GABA$_A$ receptor preparations reduces the size of the predominant α subunit species (α1) from approximately 53 kDa to approximately 44 kDa (26).

Membranes from 293 cells expressing the WT α1 subunit or one of the three mutant α1 subunits, in combination with the β1 and γ2 subunits, were assayed for [^{3}H]Ro15–1788 binding. A significant reduction in the specific binding of [^{3}H]Ro15–1788 to both α1Q^{10} and α1Q^{110} GABA$_A$ receptor mutants was observed. Scatchard analysis of [^{3}H]Ro15–1788 binding revealed that removal of either one of the two potential glycosylation sites had no effect on the affinity of the radioligand for the GABA$_A$ receptor (Table 1). However, a significant reduction in the B_{max} for both α1Q^{10} and α1Q^{110} GABA$_A$ receptor mutants was observed (Fig. 5A; Table 1). Interestingly, no binding was detected in mammalian cells expressing the double mutant α1Q$^{10/110}$, in which both potential glycosylation sites had been mutated.

The marked reduction in receptor density observed in 293 cells expressing mutant GABA$_A$ receptors was in contrast to the results observed in *Xenopus* oocytes. Differences in the intracellular processing and transport of glycosylated and nonglycosylated GABA$_A$ receptor subunits may contribute to the observed differences in expression between the two systems studied. Recently, Denning *et al.* (27) have observed that maturation of the mutant CFTRΔ508 is temperature dependent.

This protein, a glycosylation-deficient mutant, is expressed in *Xenopus* oocytes and Sf9 insect cells, which are typically grown at lower temperatures, but is not expressed in mammalian cells grown at higher temperatures. We tested the hypothesis that the asparagine to glutamine mutant α1 subunits are temperature sensitive by comparing the expression of mutant $GABA_A$ receptors with that of α1WT receptors in 293 cells grown at either 37° or 30°. If 293 cell expression of mutant $GABA_A$ receptors is temperature dependent, then incubation of transfected cells at lower temperatures would be expected to result in an increase in mutant receptor density, relative to the density of mutant receptors expressed in cells grown at 37°. Growth of transfected cells at 30° restored [^{3}H]Ro15–1788 binding to the $\alpha1Q^{10}$ and $\alpha1Q^{110}$ mutants to WT values (Fig. 5A; Table 1). In addition, temperature had no effect on the affinity of [^{3}H]Ro15–1788 for WT or mutant receptors. Temperature-sensitive binding to $\alpha1Q^{10/110}$ $GABA_A$ receptors expressed in 293 cells was only inconsistently observed, and the expression of these receptors was largely undetectable at lower growth temperatures.

The ability of [^{3}H]muscimol to bind to the WT and mutant $GABA_A$ receptors expressed in 293 cells was also examined (Fig. 5B). Due to the relatively low specific activity and low binding affinity of [^{3}H]muscimol, it was impractical to perform Scatchard analysis for this ligand with all membrane preparations. However, at a single ligand concentration, $GABA_A$ receptors containing mutant α1 subunits showed lower levels of binding, compared with WT controls (Fig. 5B). Similar to results with [^{3}H]Ro15–1788 binding, incubation of cells at 30° restored [^{3}H]muscimol binding to the $\alpha1Q^{10}$ and $\alpha1Q^{110}$ $GABA_A$ receptor mutants to control levels, with smaller effects on binding to the double mutant receptor (Fig. 5B).

Discussion

N-Linked glycosylation is an important post-translational modification that may affect the structure, stability, and intracellular transport of proteins (for review, see Refs. 28). The signals directing the addition of oligosaccharide residues to a growing polypeptide chain lie within the primary sequence of the protein itself. We report here the effects of removing the potential sites of *N*-linked glycosylation from the $GABA_A$ receptor α1 subunit. The decrease in molecular mass for both the $\alpha1Q^{10}$ and $\alpha1Q^{110}$ mutants suggests that both consensus sequences for *N*-linked glycosylation are used in 293 cells. The data suggest that inhibition of glycosylation of the α1 subunit does not affect the ability of the oocyte-expressed $GABA_A$ receptor to assemble into a functional, ligand-binding receptor complex. In contrast, expression of $GABA_A$ receptors in mammalian cells appears to require proper glycosylation of at least the α1 subunit. The observation that the effects of mutation of $GABA_A$ receptor α1 subunit glycosylation sites depend on the expression system used may, in fact, reflect differences in culture temperature. The ability of *Xenopus* oocytes to express functional mutant receptors may be due to the lower culture temperature used for oocytes (19°) versus mammalian cells (37°). Growth of mammalian cells expressing mutant $GABA_A$ receptors at 30° restored [^{3}H]Ro15–1788 binding to control levels. Attempts to culture oocytes at the higher temperatures used with mammalian cells were unsuccessful, due to the compromised health and reduced viability of the oocytes at these higher temperatures.[2] Interestingly, although no radioligand binding was observed with 293 cells expressing the $\alpha1Q^{110/110}$ double mutant at 37°, this receptor was inconsistently detected at 30° (see Fig. 5). In addition, functional expression of the $\alpha1Q^{110/110}$ $GABA_A$ receptor in oocytes suggests that it also assembles into a ligand-binding complex. The possibility that oocytes do not glycosylate the WT α1 subunit, although not ruled out entirely, is unlikely. A wide range of glycoproteins (29), including the AChR, a ligand-gated ion channel (13, 17), have been reported to be glycosylated in oocytes. Furthermore, in 293 cells the two consensus sequences for *N*-linked glycosylation appear to be used (see Fig. 4).

Temperature has been shown to affect the processing of the CFTR glycoprotein, a chloride channel that is mutated in cystic fibrosis (27, 30–32). The most commonly occurring mutant in cystic fibrosis (CFTRΔ508) exists primarily as a nonglycosylated or core-glycosylated protein (27, 33). CFTRΔ508 is expressed as a functional chloride channel in *Xenopus* oocytes (32) and Sf9 insect cells (34) but not in mammalian cells (31, 32) cultured at higher temperatures. Interestingly, the glycosylation pattern of CFTRΔ508 was shifted at temperatures below 30°, resulting in the expression of fully functional, mutant proteins in mammalian 3T3 cells.

Temperature-sensitive processing of glycosylation-deficient $GABA_A$ receptors is likely to occur at some step in the biosynthetic pathway between the synthesis of the nascent protein and the insertion of the fully assembled oligomeric complex into the cell membrane, as has been demonstrated for other glycoproteins (35, 36). For example, whereas the nonglycosylated vesicular stomatitis virus G protein does not reach the cell membrane at 37° (37–40), it is transported at lower temperatures (35). At 37°, the nonglycosylated vesicular stomatitis virus G proteins form intermolecular disulfide bonds, which create high molecular mass aggregates that are retained in the endoplasmic reticulum (30). At higher temperatures, aberrant protein folding prevents the proper processing of the mutant glycoprotein (see also Ref. 41). Thus, glycosylation may serve as a marker for protein processing. In the absence of proper glycosylation, appropriate protein folding may not occur. The results of this report support these conclusions by suggesting that the absence of sugar residues prevents or slows the proper folding of the $GABA_A$ receptor α1 subunit, resulting in a decrease in expression of a ligand-binding receptor complex. However, when the temperature is lowered, the mature conformation is achieved more rapidly or more efficiently.

Temperature-dependent expression of glycosylation-deficient $GABA_A$ receptors may be a unique property of the $GABA_A$ receptor, not shared by other members of the ligand-gated ion channel superfamily. Assembled nonglycosylated nicotinic AChR subunits appear to accumulate intracellularly in *Xenopus* oocytes (14). Mutagenesis of potential glycosylation sites on the α subunit of the nicotinic AChR, followed by expression of mutant and WT AChRs in transfected mammalian cells, also results in a decrease in the levels of expression of the receptor (16). Indeed, although the nonglycosylated α subunit does assemble with other subunits, it is degraded more rapidly than the WT α subunit (16, 17). Thus, in *Xenopus* oocytes and transfected mammalian cells the nonglycosylated AChR α sub-

[2] A. L. Buller, G. A. Hastings, E. F. Kirkness, and C. M. Fraser, unpublished observations.

unit behaves similarly, showing altered intracellular processing, relative to that of the WT receptor.

The results of the present investigation demonstrate the critical importance of the particular heterologous system used for expression of foreign proteins. For example, *Xenopus* oocytes do not properly glycosylate the *Torpedo* AChR (13), the *Electrophorus* Na^+ channel (42), or human chorionic gonadotropin (43). In addition, whereas *Torpedo* AChR subunits expressed in *Xenopus* oocytes assemble into fully functional receptors, *Torpedo* AChR subunits expressed in mammalian fibroblasts assemble only at 26° (44). This effect appears to be due to the improper folding of at least the α subunit of the *Torpedo* AChR. The dependence on temperature for proper folding and subsequent assembly of the *Torpedo* AChR may reflect the physiological temperature at which these biosynthetic processes occur naturally in *Torpedo*. These results, together with the data in this report, demonstrate the caution that must be used in interpreting data on protein expression in heterologous systems.

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References

1. Olson, R. W., and A. J. Tobin. Molecular biology of GABA$_A$ receptors. *FASEB J.* **4**:1469–1480 (1990).
2. Burt, D. R., and G. L. Kamatchi. GABA$_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–1922 (1991).
3. Doble, A., and L. Martin. Multiple benzodiazepine receptors: no reason for anxiety. *Trends Pharmacol. Sci.* **13**:76–81 (1992).
4. Sieghart, W. GABA$_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug-binding sites. *Trends Pharmacol. Sci.* **13**:446–450 (1992).
5. Blair, L. A. C., E. S. Levitan, J. Marshall, V. E. Dionne, and E. A. Barnard. Single subunits of the GABA$_A$ receptor form ion channels with properties of the native receptor. *Science (Washington D. C.)* **242**:577–579 (1988).
6. Sigel, E., R. Baur, G. Trube, H. Möhler, and P. Malherbe. The effect of subunit composition of rat brain GABA$_A$ receptors on channel function. *Neuron* **5**:703–711 (1990).
7. Pritchett, D. B., H. Sontheimer, C. M. Gorman, H. Kettenmann, P. H. Seeburg, and P. R. Schofield. Transient expression shows ligand gating and allosteric potentiation of GABA$_A$ receptor subunits. *Science (Washington D. C.)* **242**:1306–1308 (1988).
8. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, P. Kettenmann, R. Schofield, and P. H. Seeburg. Importance of a novel GABA$_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
9. Pritchett, D. B., H. Luddens, and P. H. Seeburg. Type I and Type II GABA$_A$ benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
10. Kornfeld, R., and S. Kornfeld. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**:631–664 (1985).
11. Merlie, J. P., R. Sebbane, S. Tzartos, and J. Lindstrom. Inhibition of glycosylation with tunicamycin blocks assembly of newly synthesized acetylcholine receptor subunits in muscle cells. *J. Biol. Chem.* **257**:2694–2701 (1982).
12. Prives, J., and D. Bar-Sagi. Effect of tunicamycin, an inhibitor of protein glycosylation, on the biological properties of acetylcholine receptors in cultured muscle cells. *J. Biol. Chem.* **258**:1775–1780 (1983).
13. Buller, A. L., and M. M. White. Altered patterns of *N*-linked glycosylation of the *Torpedo* acetylcholine receptor expressed in *Xenopus* oocytes. *J. Membr. Biol.* **115**:179–189 (1990).
14. Sumikawa, K., and R. Miledi. Assembly and *N*-glycosylation of all AChR receptor subunits are required for their efficient insertion in plasma membranes. *Mol. Brain Res.* **5**:183–192 (1989).
15. Mishina, M., T. Tobimatsu, K. Imoto, K. Tanaka, Y. Fujita, K. Fukuda, M. Kurasaki, H. Takahashi, Y. Morimoto, T. Hirose, S. Inayama, T. Takahashi, M. Kuno, and S. Numa. Location of functional regions of acetylcholine receptor α subunit by site-directed mutagenesis. *Nature (Lond.)* **313**:364–369 (1985).
16. Blount, P., and J. P. Merlie. Mutational analysis of muscle nicotinic acetylcholine receptor assembly. *J. Cell Biol.* **111**:2613–2622 (1990).
17. Gehle, V. M., and K. Sumikawa. Site-directed mutagenesis of the conserved *N*-glycosylation site on the nicotinic acetylcholine receptor subunits. *Mol. Brain Res.* **11**:17–25 (1991).
18. Malherbe, P., E. Sigel, R. Baur, E. Persohn, J. G. Richards, and H. Mohler. Functional characteristics and sites of gene expression of the α1,β1,γ2-isoform of the rat GABA$_A$ receptor. *J. Neurosci.* **10**:2330–2337 (1990).
19. Buller, A. L., and M. M. White. Control of *Torpedo* acetylcholine receptor biosynthesis in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **85**:8717–8721 (1988).
20. White, M. M., K. M. Mayne, H. A. Lester, and N. Davidson. Mouse-*Torpedo* hybrid acetylcholine receptors: functional homology does not equal sequence homology. *Proc. Natl. Acad. Sci. USA* **82**:4852–4856 (1985).
21. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
22. Mohler, H., M. K. Battersby, and J. G. Richards. Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc. Natl. Acad. Sci. USA* **77**:1666–1670 (1980).
23. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
24. Kirkness, E. F., and A. J. Turner. Antibodies directed against a nonapeptide sequence of the γ-aminobutyrate (GABA)/benzodiazepine receptor α-subunit. *Biochem. J.* **256**:291–294 (1988).
25. Fuchs, K., H. Mohler, and W. Sieghart. Various proteins from rat brain, specifically and irreversibly labelled by [^{3}H]flunitrazepam, are distinct α-subunits from the GABA-benzodiazepine receptor complex. *Neurosci. Lett.* **90**:314–319 (1988).
26. Mamalaki, C., F. A. Stephenson, and E. A. Barnard. The GABA$_A$/benzodiazepine receptor is a heterotetramer of homologous α and β subunits. *EMBO J.* **6**:561–565 (1987).
27. Denning, G. M., M. P. Anderson, J. F. Amara, J. Marshall, A. E. Smith, and M. J. Welsh. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature (Lond.)* **358**:761–764 (1992).
28. Olden, K., J. B. Parent, and S. J. White. Carbohydrate moieties of glycoproteins: a re-evaluation of their function. *Biochim. Biophys. Acta* **650**:209–232 (1982).
29. Colman, A. Translation of eukaryotic messenger RNA in *Xenopus* oocytes, in *Transcription and Translation: A Practical Approach* (B. D. Hames and S. J. Higgins, eds.). IRL, Oxford, UK, 271–302 (1984).
30. Rich, D. P., M. P. Anderson, R. J. Gregory, S. H. Cheng, S. Paul, D. M. Jefferson, J. D. McCann, K. W. Klinger, A. E. Smith, and M. J. Welsh. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature (Lond.)* **347**:358–363 (1990).
31. Anderson, M. P., R. J. Gregory, S. Thompson, D. W. Souza, S. Paul, R. C. Mulligan, A. E. Smith, and M. J. Welsh. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science (Washington D. C.)* **253**:202–205 (1991).
32. Anderson, M. P., D. P. Rich, R. J. Gregory, A. E. Smith, and M. J. Welsh. Generation of cAMP-activated chloride currents by expression of CFTR. *Science (Washington D. C.)* **251**:679–682 (1991).
33. Drumm, M. L., D. J. Wilkinson, L. S. Smit, R. T. Worrell, T. V. Strong, R. A. Frizzell, D. C. Dawson, and F. S. Collins. Chloride conductance expressed by Δ508 and other mutant CFTRs in oocytes. *Science (Washington D. C.)* **254**:1797–1799 (1991).
34. Bear, C. E., T. J. Jensen, and J. R. Riordan. Functional capacity of the major mutant form of the cystic fibrosis transmembrane conductance regulator. *Biophys. J.* **61**:A127 (1992).
35. Machamer, C. E., and J. K. Rose. Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.* **263**:5955–5960 (1988).
36. Ljunggren, H.-G., N. J. Stam, C. Ohlén, J. J. Neefjes, P. Höglund, M.-T. Heemels, J. Bastin, T. N. M. Schumacher, A. Townsend, K. Kärre, and H. L. Ploegh. Empty MHC class I molecules come out in the cold. *Nature (Lond.)* **346**:476–480 (1990).
37. Gibson, R., R. Leavitt, S. Kornfeld, and S. Schlesinger. Synthesis and infectivity of vesicular stomatitis virus containing nonglycosylated G protein. *Cell* **13**:761–769 (1978).
38. Gibson, R., S. Schlesinger, and S. Kornfeld. The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperature. *J. Biol. Chem.* **254**:3600–3607 (1979).
39. Morrison, T. G., C. O. McQuain, and D. Simpson. Assembly of viral membranes: maturation of the vesicular stomatitis virus glycoprotein in the presence of tunicamycin. *J. Virol.* **28**:368–374 (1978).
40. Machamer, C. E., R. Z. Florkiewicz, and J. K. Rose. A single *N*-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. *Mol. Cell. Biol.* **5**:3074–3083 (1985).
41. Hurtley, S. M., and A. Helenius. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Biochem.* **5**:277–307 (1989).
42. Thornhill, W. B., and S. R. Levinson. Biosynthesis of electroplax sodium channels in *Electrophorous* electrocytes and *Xenopus* oocytes. *Biochemistry* **26**:4381–4388 (1987).
43. Mous, J., B. Peeters, and W. Rombauts. Synthesis and core-glycosylation of the α subunit of human chorionic gonadotropin in *Xenopus* oocytes. *FEBS Lett.* **122**:105–108 (1980).
44. Paulson, H. L., and T. Claudio. Temperature-sensitive expression of all-*Torpedo* and *Torpedo*-rat hybrid AChR in mammalian muscle cells. *J. Cell Biol.* **110**:1705–1717 (1990).

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