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Molecular and electrophysiological characterization of a allelic variant of the rat $\alpha6$ $GABA_A$ receptor subunit

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A 1.45 kb DNA sequence encoding the rat $\alpha6$ $GABA_A$ receptor subunit (nucleotides 33–1483) was cloned from a Sprague–Dawley rat brain cDNA library by PCR amplification. Dideoxy sequencing of two individual clones revealed that the nucleotide sequence differed at only one basepair ($T^{480} \rightarrow G$) from that published previously. This difference altered the deduced amino acid sequence, producing a conservative amino acid substitution ($His^{121} \rightarrow Gln$). A Gln residue is present at the same location in the bovine $\alpha6$ subunit. Restriction endonuclease analysis of the total PCR product demonstrated that this variant of the rat $\alpha6$ subunit was the only allele found in this particular rat brain library; the original allele was not present. These results were further verified by RNAse protection assays performed with RNA isolated from individual rat cerebella. $\alpha6$, $\beta1$, and $\gamma2S$ subunits were transiently expressed in L929 cells for electrophysiological analysis. Whole-cell recordings obtained from the cells demonstrated that $GABA_A$ receptor channels with the expected GABA and benzodiazepine pharmacology were produced. Excised outside out single channel recordings from the same cells revealed that GABA elicited brief duration openings to a 33 pS main conductance level and to at least one smaller (approximately 21 pS) subconductance level. Thus this allelic variant of rat $\alpha6$ subunit could assemble with other subunits to form a functional $GABA_A$ receptor channel with similar properties to the original allelic form.

The $GABA_A$ receptor, the major receptor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA), is a member of the ligand-gated ion channel supergene family. Molecular cloning studies from rat, bovine, and human have identified multiple subunit families (α, β, γ, δ, and ρ) and various isoforms within each subunit family have been isolated[1]. The properties and drug sensitivity of the different subpopulations of $GABA_A$ receptors in vivo are most likely determined by cell-specific expression and assembly of various receptor subunits[5].

To date, the best correlation of cell-specific expression of a receptor subunit with its pharmacology has been with the $\alpha6$ subunit isoform. This subunit was first cloned from rat and bovine brain and subsequently shown to be expressed only in cerebellar granule cells and to possess unique pharmacology at the benzodiazepine-site[9,12]. Unlike other subunit isoforms which are highly conserved at the amino acid level across various species (> 98% conserved)[16], the $\alpha6$ and also the $\alpha4$ isoforms only share approximately 90% amino acid identity between the rat and bovine sequences[12,23]. However, the potential protein kinase phosphorylation sites and transmembrane domains are highly conserved within either the $\alpha6$ and $\alpha4$ subunits between the two species. Interestingly, these two isoforms possess similar benzodiazepine pharmacology when co-expressed with rat $\beta2$ and $\gamma2$ subunits[5].

Allelic variants within any species of the $GABA_A$ receptor family have only been described for the human $\beta1$ and rat $\alpha1$ subunit cDNAs[6,21]. In the human $\beta1$ subunit variant, a conservative amino acid change was found at position 10 ($Ser^{10} \rightarrow Pro$). No functional effects, if any, of the amino acid change were demonstrated. However, expression of the rat $\alpha1$ variant ($Phe^{64} \rightarrow Leu$) with $\beta2$ and $\gamma2$ subunits produced a 200- or 60-fold decrease in affinity for GABA and GABA-site antagonists, respectively. Similarly, Betz et

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al.[11] had previously cloned an isoform of the glycine receptor rat $\alpha2$ subunit that encoded a receptor protein insensitive to the antagonist strychnine, yet was $>99\%$ homologous at the amino acid level to the human $\alpha2$ subunit. The single amino acid change that accounted for the difference in pharmacology was Glu^{167} (human, sensitive) $\rightarrow$ Gly (rat, insensitive). Subsequently, they cloned a rat allelic variant that possessed a Glu residue at position 167 and was sensitive to antagonism by strychnine[10]. These results suggested that allelic variants of the $GABA_A$ and glycine receptors exist and that they may alter the function of the receptor channel.

In the process of PCR cloning the rat $\alpha6$ subunit, we discovered a variant form that possessed a different amino acid at position 121 (Gln) than that published previously (His)[12]. Interestingly, this amino acid substitution (Gln) was found in the bovine homolog. We thus undertook this series of studies to determine if the clone was a true allelic variant of the rat $\alpha6$ subunit and to characterize the whole-cell and single-channel electrophysiological properties of $GABA_A$ receptor channels containing this subunit.

The rat $\alpha6$ subunit cDNA was amplified from a 14 to 16-day-old male/female Sprague–Dawley rat whole brain cDNA UniZAP library (Stratagene, La Jolla, CA). The 5′ amplification primer was based upon the published sequence[12] and extended from nucleotides 33–51 (GTC/GGATCC/GTGACCTGGCATTTCAGTG, GTC/*Bam*HI linker/amplification primer). The 3′ amplification primer corresponded to nucleotides 1464–1483 (GTC/GGATCC/CTGTAAGCGAGGAAAATGG, GTC/*Bam*HI/amplification primer). PCR was performed with *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in 100 μl of standard buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTP) containing 1–5 μl of cDNA library and 1 μM primers. The cDNA library was boiled for 5 min and quickly cooled on ice prior to initializing the reaction. PCR products were obtained after 30 cycles of amplification with protocol A: 60 s, 94°C; 120 s, 55°C; 180 s, 72°C or alternatively with protocol B: 60 s, 94°C; 120 s, 72°C. The PCR products were purified after electrophoresis through a 1% agarose gel using a Geneclean kit (Bio101, La Jolla, CA) and an aliquot was ligated into the vector pCR1000 (Invitrogen, San Diego, CA), according to the manufacturers' instructions, to form the vector pCRr$\alpha6$. Dideoxy sequencing was performed on individual clones of the vector containing the amplified fragment using either standard forward or reverse M13 primers or rat $\alpha6$ specific primers using a Sequenase 2.0 kit with [α^{35}S]dATP (United States Biochemicals, Cleveland, OH), according to the manufacturer's instructions. Restriction enzyme digests were carried out at 37°C for 1 h in a 10 μl volume with 200 ng of purified, amplified DNA, 10 units of the specified enzyme (Promega, Madison, WI), and the resultant fragments were resolved on an ethidium bromide-stained 1% agarose gel.

Total RNA was isolated from the cerebella of female rats (150–200 g, Harlan Sprague–Dawley Inc. Indianapolis, IN), using the guanidinium isothiocyanate procedure[4]. For the preparation of probe, the vector pCRr$\alpha6\Delta$ was created by digestion of pCRr$\alpha6$ with *Hin*dIII (multiple cloning site) and *Ppu*MI (bp 656), followed by ligation of the blunted ends. Probe was synthesized using T7 polymerase and [^{32}P]UTP, after digestion of the construct with *Eco*RV. This produced an antisense probe which spanned the site of the base pair change (bp 243–656). Full-length radiolabelled probe was purified from a 8% polyacrylamide/8 M urea gel using the crush-soak method[20]. Total RNA (50 μg) was hybridized with 50000 cpm of probe and RNAse protection assays were performed as described previously[2].

For expression studies of the rat $\alpha6$ subunit, the amplified sequence was released from pCRr$\alpha6$ by digestion with *Bam*HI, purified as above from an agarose gel, and subsequently subcloned into the *Bgl*II site of the expression vector pCMVNeo[8] to form the construct pCMVr$\alpha6$. Similar pCMVNeo-based expression constructs for the rat $\beta1$ and human $\gamma2S$ subunits are described elsewhere (T.P. Angelotti, M.D. Uhler and R.L. Macdonald, manuscript submitted). L929 cells (ATCC CCL 1) were transfected using the modified calcium phosphate technique[3] with equal parts by weight of $\alpha6$, $\beta1$, and $\gamma2S$ vector constructs. Positively transfected cells were identified by fluorescent staining for a co-expressed marker gene (T.P. Angelotti, M.D. Uhler and R.L. Macdonald, manuscript submitted). Membrane currents were recorded from cells 48 h after transfection. Recording of membrane currents and analysis were performed using solutions and equipment described previously for mouse spinal cord $GABA_A$ receptor channels[13]. Amplitude histograms were constructed using the software IPROC (Axon Instruments, Burligame, CA). Only openings with durations longer than four times the system rise time (rise time = 130 μs) were analyzed to obtain true amplitude measurements. Drug solutions were made immediately prior to use in either water (GABA) or DMSO (diazepam (DZ), methyl-4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate (DMCM)) and diluted into external recording solution to the indicated final concentrations.

Amplification of a rat whole brain cDNA library with primers specific for the $\alpha6$ subunit produced a 1.45 kb DNA fragment corresponding to the complete open reading frame (data not shown). After subcloning this fragment, an individual bacterial colony harboring the plasmid was picked and grown. Sequence analysis of the purified plasmid revealed a single nucleotide change at position 480 (T → G) that converted the encoded amino acid from His^{121} to Gln (CAT → CAG) (Fig. 1A, arrow). This finding was intriguing since the bovine homolog of the $\alpha6$ subunit also encoded a Gln at amino acid residue 121[12]. This residue was positioned to the amino terminal side of the third putative N-linked glycosylation site. To verify the authenticity of this change, a second PCR amplification was carried out using the same primers but a slightly different protocol (second protocol listed above). Again, the amplified fragment was subcloned and an individual bacterial colony was grown. Sequencing of purified plasmid DNA again revealed the same single nucleotide change at position 480 (Fig. 1B). No other deviations from the published rat $\alpha6$ sequence were noted in the amplified DNA (data not shown).

It was possible, but highly unlikely, that two independent clones obtained from the two different PCR reactions possessed the same base pair change due to the result of *Taq*-induced error at a region of high mutability[7]. To rule out this possibility, the original samples of amplified DNA prior to subcloning were analyzed by restriction endonuclease digestion. If the

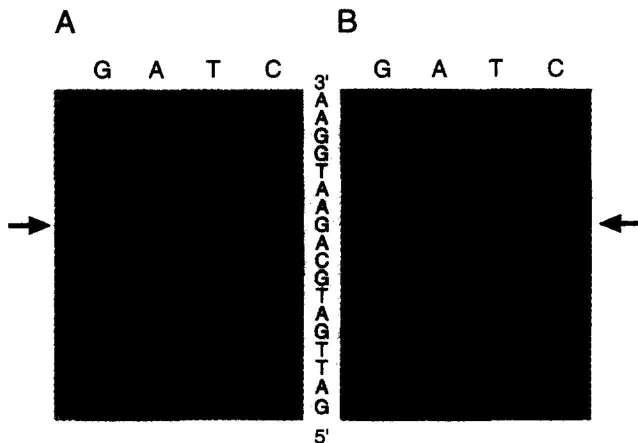

Fig. 1. Autoradiogram of dideoxy sequencing analysis of two independent cDNA clones (A, B) encoding the rat $\alpha6$ subunit. Each clone was obtained by independent PCR amplification with subunit specific probes under different reaction conditions (as described in the text), followed by ligation into the vector pCR1000. One individual clone from each reaction was then chosen for sequencing. The arrow denotes the position of the nucleotide change (T^{480} → G) from the previously published rat sequence[12]. This nucleotide change converted the encoded amino acid from His^{121} to Gln (CAT → CAG). Sequencing by the dideoxy-chain termination method was performed using an internal primer (5′-TTCCGACAGACATGGAC-3′).

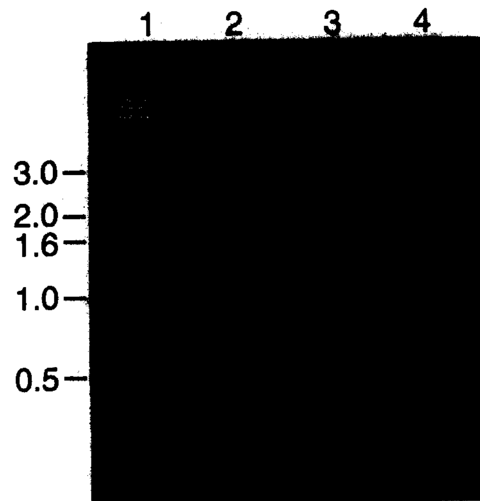

Fig. 2. Restriction enzyme analysis of the total amplified PCR product encoding the rat $\alpha6$ subunit. The original amplified DNA product from the second PCR reaction (Fig. 1B) was purified and digested with either *Nsi*I (lane 2), *Nco*I (lane 3), or not digested (lane 4). The digestion products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lane 1 contained a 1 kb ladder. Molecular weights are denoted on the left.

nucleotide change at base pair 480 was due to a *Taq*-induced error, then this should represent some subpopulation of the total amplified DNA, that was just fortuitously cloned. Even if this error occurred during the first round of amplification and only one template copy was present in the reaction, the resulting population of amplified DNA should contain a minimum of 50% wildtype sequences. The wildtype sequence surrounding nucleotide 480 contained a recognition sequence for cleavage by the restriction endonuclease *Nsi*I (5′-ATGCAT-3′) and the basepair change removed it (5′-ATGCAG-3′). *Nsi*I digestion of the total amplified DNA from the second PCR reaction produced no fragments (Fig. 2, lane 2), except for the original, uncut DNA (Fig. 2, lane 4). The DNA could be digested however with *Nco*I to produce the expected fragments of approximately 450 and 950 bp (Fig. 2, lane 3) proving that the DNA was suitable for cleavage. Digestion of the total amplified DNA from the first PCR reaction produced similar results (data not shown). These results suggest that the amplified DNA with the base pair/amino acid change represented a form of the $\alpha6$ subunit cDNA found in the original library.

To demonstrate that this form of the $\alpha6$ subunit did not arise from the amplification of a rare, single, mutated cDNA template present in the library, RNAse protection assays were performed on total RNA isolated from single cerebella from different rat strains (Fig. 3). The antisense probe (bp 243–656) was designed to span the basepair change (bp 480). The

presence of a G at bp 480 of the mRNA would cause full-length protection of the probe and would produce a band of 413 bp. Alternatively, the presence of the original basepair (T) in the mRNA, would yield two fragments of 176 and 237 bp. Analysis of five different rat strains, including Sprague–Dawley, demonstrated that the probe was fully protected (413 bp). The faint, smaller fragments represent degradation of the probe during the assay. Note that no consistent products were found at the two smaller expected sizes. This result was consistent with the presence of an authentic basepair change at position 480.

Co-expression of this variant of the rat $\alpha6$ subunit in L929 cells along with the rat $\beta1$ and human $\gamma2S$ subunits produced functional $GABA_A$ receptors (Fig. 4A). Thus this change in amino acid did not effect the assembly of the ion channel. It has been shown by both binding and whole-cell recording that expression of the $\alpha6$ subunit with β and γ subunits produced receptors which lacked diazepam (DZ) sensitivity and had a much weaker response to the inverse agonist methyl-4-ethyl-6,7-dimethoxy-A-carboline-3-carboxylate (DMCM)[9,12]. The co-application of GABA with DZ to

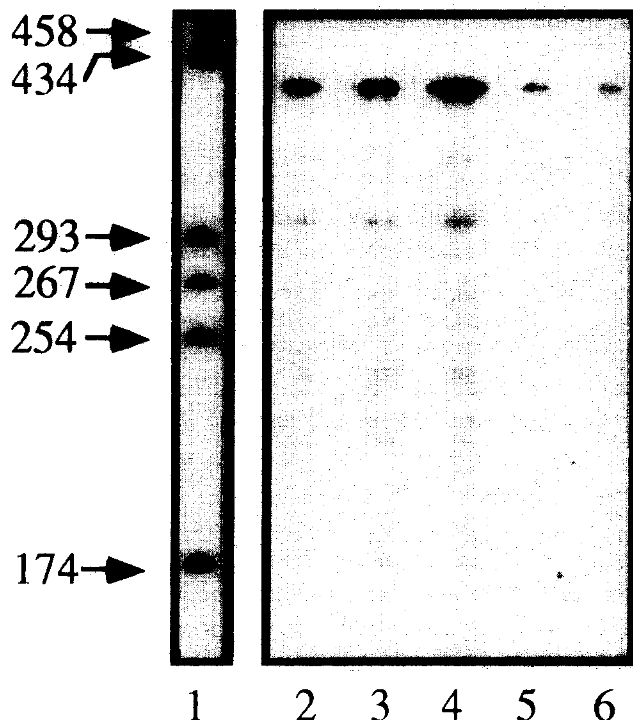

Fig. 3. RNAse protection analysis of $\alpha6$ subunit expression. The presence of the $\alpha6$ subunit allele was examined in five different rat strains using an antisense probe which spanned the variant basepair (480). Total cerebellar RNA isolated from individual rats was hybridized with [^{32}P]UTP labelled antisense probe as described in the text. The protected fragments were resolved on an 8% polyacrylamide/8 M urea gel. Lane 1, molecular weight ladder; lane 2, Buffalo rat RNA; lane 3, Fisher rat RNA; lane 4, Long–Evans rat RNA; lane 5, Sprague–Dawley rat RNA; lane 6, Wistar rat RNA. See text for discussion.

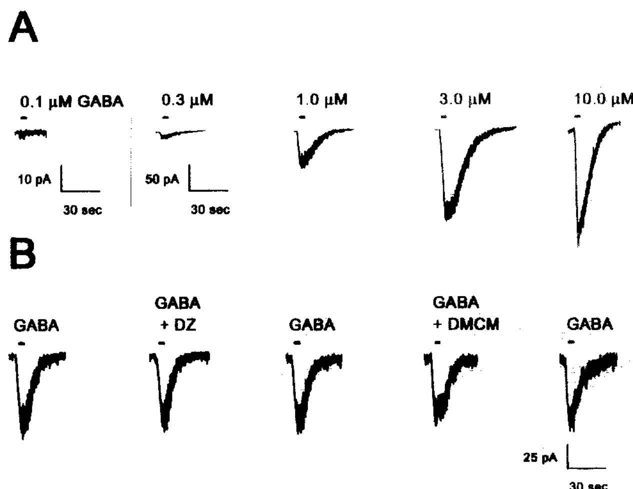

Fig. 4. Heterologously expressed rat $\alpha6$/rat $\beta1$/human $\gamma2S$ subunits in L929 cells studied using the whole-cell patch clamp configuration. A: representative whole-cell currents elicited by increasing concentrations of GABA (3 s application) applied to an individual cell voltage clamped at -75 mV. B: whole-cell currents elicited by successive applications of GABA alone (1 μM) and GABA+DZ (1 μM) or GABA+DMCM (1 μM) to a cell held at -75 mV. The results are representative of those seen with three different cells.

receptors containing the variant $\alpha6$ subunit similarly demonstrated no enhancement or reduction of current (Fig. 4B). The inverse agonist DMCM when co-applied with GABA produced a slight diminution of current (approximately 10%) (Fig. 4B). Thus the unique pharmacology described for the original form of the $\alpha6$ subunit was also produced upon expression of this alternate form of the subunit.

Application of GABA to outside-out patches excised from L929 cells expressing the variant $\alpha6$, rat $\beta1$, and human $\gamma2S$ subunits elicited single-channel currents (Fig. 5A, B). These currents were of brief duration and consisted predominantly of openings to a 33.6 ± 1.1 pS conductance level (2.52 ± 0.08 pA at -75 mV, $n = 3$ patches) with less frequent openings to at least one smaller subconductance level of approximately 18–23 pS (1.4–1.7 pA) (Fig. 5C). Though no single channel recordings of $GABA_A$ receptors containing the original rat $\alpha6$ allele have been published, comparison to recordings of receptors composed of $\alpha1\beta2\gamma2$ subunits suggests that these currents were similar in amplitude, but not identical[22]. These currents were also larger in amplitude than receptor currents composed of only α and β subunits[15,22].

Various alleles of genes that encode ligand-gated ion channels[10,21], voltage-gated ion channels[19], and anion channels[18] have been reported. In many instances, these allelic variants produce a mutant form of the protein with an altered function and phenotype. It is also possible that "non-mutant" alleles are present which code for conservative[6] or non-conservative amino

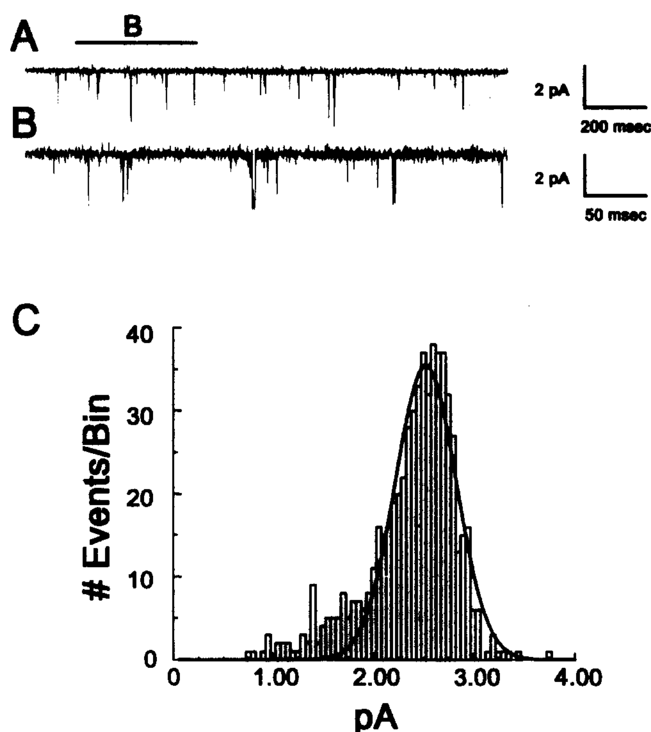

Fig. 5. Single-channel analysis of $GABA_A$ receptors composed of rat $\alpha6$/rat $\beta1$/human $\gamma2S$ subunits expressed in L929 cells. A, B: representative tracings of GABA (1 μM) evoked single channel openings in outside-out patches voltage clamped at -75 mV. The higher resolution trace (B) gave a more accurate representation of the true amplitudes of the open channels. C: amplitude histogram of single-channel currents superimposed with the best fit Gaussian curve representing the main conductance state of 33.6 ± 1.1 pS. Note the rarer, smaller conductance openings that were not able to be fit with a Gaussian curve (see text for further details about the amplitude selection criteria).

acid changes that do not effect the protein's function. Amplification of the $GABA_A$ receptor $\alpha6$ subunit cDNA from a Sprague–Dawley rat brain-derived library revealed that this species of rat possessed a variant form of the subunit with a conservative amino acid change ($His^{121} \rightarrow Gln$) when compared to the previously published sequence (Fig. 1). This change appeared not to be due to a cloning artifact and is the only form of the subunit found in this rat strain (Figs. 2 and 3). Since the remaining 1450 bp of amplified DNA did not have any other nucleotide changes, and it is doubtful that two such homologous genes developed during evolution, we propose that this sequence is a true allelic variant of the rat $\alpha6$ subunit. The specific rat strain used to create the cerebellar cDNA library from which Lüddens et al. cloned the original allele was not described[12], hence a comparison of the lineages of the rat strains cannot be made. Since many strains of rats are outbred (e.g. Wistar, Long Evans, and Sprague–Dawley), it is possible that the presence of these two alleles may exist within the same strain. Consistent with this hypothesis could be the rat $\alpha1$ subunit allelic variant identified by Sigel et al.[21]. The variant form of the subunit was originally cloned from a neonatal Wistar rat, but it was not found in any other Wistar rat brains.

The conservative amino acid change did not drastically effect functional expression of the subunit with rat $\beta1$ and human $\gamma2S$ subunits. Functional receptors were produced which were sensitive to GABA, not sensitive to diazepam, and slightly sensitive to DMCM inhibition (Fig. 3). This was in agreement with a previous study of the pharmacology of $\alpha6$ subunit containing receptors[9]. Though this previous study co-expressed the $\alpha6$ subunit with rat $\beta2$ and rat $\gamma2$ subunits, the homology between the human and rat $\gamma2$ subunit isoforms[16] and the lack of noticeable effect of switching the $\beta1$ and $\beta2$ subunits in previous studies of benzodiazepine modulation[17] suggested that any effect would be minimal. Single-channel recordings demonstrated that GABA elicited brief openings to one main- and at least one sub-conductance level (Fig. 4) in a manner similar to $GABA_A$ receptors found in vivo[14]. Thus, this form of the rat $\alpha6$ subunit had similar basic pharmacological and electrophysiological properties as the original subunit. More importantly, however, was the demonstration that $GABA_A$ receptor channels composed of $\alpha6\beta1\gamma2S$ subunits had a main conductance level of 33–34 pS, unlike $GABA_A$ receptor channels composed of $\alpha1\beta1\gamma2S$, $\alpha3\beta1\gamma2S$, and $\alpha5\beta1\gamma2S$ subunits, which have main conductance levels of 27–30 pS (T.P. Angelotti, T. Ryan-Jastrow, M.D. Uhler and R.L. Macdonald, manuscript submitted, and unpublished observations). Further studies will be needed to determine if any other receptor properties were modified by this allelic variation of the rat $\alpha6$ subunit.

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