

θ , a novel γ -aminobutyric acid type A receptor subunit

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ABSTRACT γ -Aminobutyric acid type A (GABA-A) receptors are a major mediator of inhibitory neurotransmission in the mammalian central nervous system, and the site of action of a number of clinically important drugs. These receptors exist as a family of subtypes with distinct temporal and spatial patterns of expression and distinct properties that presumably underlie a precise role for each subtype. The newest member of this gene family is the θ subunit. The deduced polypeptide sequence is 627 amino acids long and has highest sequence identity (50.5%) with the $\beta 1$ subunit. Within the rat striatum, this subunit coassembles with $\alpha 2$, $\beta 1$, and $\gamma 1$, suggesting that γ -aminobutyric acid type A receptors consisting of arrangements other than $\alpha\beta + \gamma$, δ , or ϵ do exist. Expression of $\alpha 2\beta 1\gamma 1\theta$ in transfected mammalian cells leads to the formation of receptors with a 4-fold decrease in the affinity for γ -aminobutyric acid compared with $\alpha 2\beta 1\gamma 1$. This subunit has a unique distribution, with studies so far suggesting significant expression within monoaminergic neurons of both human and monkey brain.

In the mammalian central nervous system, inhibitory neurotransmission is mediated primarily by the γ -aminobutyric acid (GABA), which acts on GABA type A (GABA-A) receptors, ligand-gated ion channels acting over a rapid time frame. Over the past 10 years, it has become clear that a family of GABA-A receptor subtypes exists, generated through the coassembly of polypeptides selected from $\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , ϵ , and π to form what is most likely a pentameric macromolecule (1–6). The subunits show distinct patterns of temporal and spatial expression, so that GABA-A receptor subtypes have a defined localization presumably reflecting their physiological role (7–11).

In this article, we report the identification and characterization of a further member of the GABA-A receptor gene family that we have termed theta (θ).

MATERIALS AND METHODS

Cloning of θ Subunit cDNA. Full-length cDNA was cloned starting from sequence information in GenBank entry U47334. PCR was performed under standard conditions, on human whole-brain cDNA (CLONTECH) with oligonucleotide primers specific to the 5' and 3' ends of the U47334 sequence. A single PCR product of approximately 1,600 bp was obtained. The 5' and 3' ends of the coding region corresponding to full-length U47334 sequence were obtained by 5' and 3' anchored PCR using human brain Marathon cDNA (CLONTECH). Full-length cDNA (GenBank accession no. AF144648) was generated by PCR using a primer derived from sequences surrounding the initiating methionine incorporating a consensus Kozak sequence (12), and a primer based on the 3' untranslated region of the anchored PCR product. The PCR product (1,958 bp) was sequenced completely on both strands

by primer walking by using dye terminator chemistry and an Applied Biosystems model 373A sequencer.

Epitope-tagged θ subunit was constructed that contained nucleotides –224 to +99 (i.e., the 5' untranslated region, the signal peptide, 6 amino acids of the mature protein) of bovine GABA-A receptor $\alpha 1$ cDNA, a sequence encoding the c-myc epitope tag (EQKLISEEDL), a cloning site encoding the amino acids Asn-Ser-Gly, and DNA encoding amino acids 22–627 of the GABA-A receptor θ gene product. c-myc-tagged human GABA-A receptor $\beta 2$ was generated in the same way to contain amino acids 38–474 of the subunit.

c-Myc Cell Surface ELISA. Briefly, HEK 293 cells were seeded at 1×10^5 cells per well in a 24-well tissue culture plate. After 24 h, each well was transiently transfected with a total of 1 μ g of DNA by using calcium phosphate precipitation (13). Two days after transfection, the cells were aspirated, and aspirated cells were incubated at room temperature with 2 ml of PBS containing 4% (wt/vol) skimmed milk powder (4% MPBS) for 30 min. The cells were then aspirated and incubated at room temperature with 500 μ l of 4% MPBS containing the anti-c-myc monoclonal antibody 9E10 (American Type Culture Collection product CRL-1729) for 1 h. The cells were aspirated and washed once with 2 ml of 4% MPBS, and then incubated as before with 500 μ l of 4% MPBS containing horseradish peroxidase-conjugated anti-mouse IgG (Promega) for 1 h. The cells were aspirated and washed three times with 4% MPBS, three times with PBS, and the reaction product was developed with 500 μ l of K-Blue substrate (Neogen, Lexington, KY). Aliquots of 200 μ l were transferred to a 96-well ELISA plate, and the A_{620} was determined.

Generation of Polyclonal Sera to the θ Subunit. Antibodies to the human GABA-A receptor θ subunit were generated by using a glutathione S-transferase (GST) fusion protein consisting of residues 353–595 of the putative cytoplasmic loop region of the θ subunit. DNA encoding this region was cloned into the bacterial expression vector pGEX-2T (Pharmacia), transformed into *Escherichia coli* DH10B cells (Life Technologies), and expression and purification of the fusion protein was carried out by using the Pharmacia protocols. The purified GST fusion protein was used to immunize rabbits for the subsequent generation of antiserum.

Localization of the θ Subunit mRNA in Monkey Brain by *in Situ* Hybridization. The sequences used for the two antisense oligonucleotides (equivalent to nucleotides 1,035–1,079 and 1,532–1,576 of human θ cDNA sequence) showed no significant homology to any other nucleotide sequence in GenBank as of September 1996. Oligonucleotides were purified by preparative polyacrylamide gel electrophoresis and 3' end labeled with deoxyadenosine 5'-[[³⁵S]thio]triphosphate as described (4) to give specific radioactivities of 1.2 – 2.3×10^9 cpm/ μ g. Monkey brains were removed and fresh-frozen in 1-cm blocks. Sections (12 μ m)

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Abbreviations: GABA, γ -aminobutyric acid; GABA-A receptor, GABA type A receptor; TH, tyrosine hydroxylase; EC_n, effective concentration, n%; TM, transmembrane.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF144648).

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were taken, fixed for *in situ* hybridization, and processed as described (4, 13). Autoradiographs were analyzed by using a MCID computerized image analysis system (Image Research, Ontario, Canada).

Localization of θ Subunit in Monkey and Human Brain by Immunocytochemistry. Sections from monkey brains were prepared as described (4). To enhance the immunoreactivity, sections were subjected to antigen retrieval techniques, as described by Shi *et al.* (14). Sections were then incubated overnight at +4°C in the anti- θ -subunit rabbit polyclonal antibody diluted 1:1000 in blocking buffer (5% normal goat serum in PBS). Immunoreactivity was visualized with the Vector elite system (Vector Laboratories). Sections were counterstained in Gill's hematoxylin (Menarini, U.K.), dehydrated, and mounted. Postmortem human brainstems (fixed in 10% formalin) were processed in an identical manner. Antibody specificity was determined by preabsorption of the antibody with its cognate peptide, resulting in an loss of the observed immunoreactivity (data not shown). Sections used for immunofluorescent colocalization of θ subunit and tyrosine hydroxylase (TH) were pretreated in the same manner; θ subunit immunoreactivity was detected by using first a biotinylated anti-rabbit immunoglobulin antiserum diluted 1:200 (Vector Laboratories) followed by FITC-conjugated streptavidin (Sigma). The second rabbit polyclonal serum, anti-TH, was again visualized by using biotinylated anti-rabbit immunoglobulin antiserum, reacted with Cy3-conjugated streptavidin (Sigma). Sections were counterstained with Hoescht 33258 (0.5 μ g/ml). To avoid any cross-reactivity of the detection systems, sections were placed in boiling distilled water for 5 min before the application of the second primary antibody and its subsequent detection.

Immunoprecipitation of GABA-A Receptors from Rat Brain. Receptors were solubilized from rat brain, and immunoprecipitation experiments performed as described (15). The production of antiserum to the θ subunit is described above, and antisera to other GABA-A receptor subunits have been described (15). Briefly, protein-A agarose beads were first coated with antibody by incubating 50 μ l of antiserum with 100 μ l of packed protein A-agarose in a total volume of 1 ml of Tris-buffered saline (TBS) for 1 h at room temperature. The antibody-protein-A complex was then washed three times in TBS and bound to the receptor by overnight incubation at 4°C with 0.5 ml of detergent-solubilized membranes (0.1 M KCl/5 mM MgCl₂/1% Triton X-100/0.5% deoxycholate/1 mM PMSF/0.1 M Tris-HCl, pH 8.2). The beads were then washed three times in TBS/0.1% Tween 20 and resuspended in 10 mM KH₂PO₄/100 mM KCl, pH 7.4.

Control immunoprecipitation was determined as radioligand binding sites immunoprecipitated by the same amount of antiserum raised against an irrelevant antigen (5-HT₃ receptor) and was typically less than 50 specific dpm. Where coprecipitations were carried out, 50 μ l of each subunit specific antiserum was separately incubated with 100 μ l of protein beads, washed, and then combined before the addition of soluble receptor.

Binding of [³H]muscimol (8 nM) was carried out on beads and supernatants in 10 mM KH₂PO₄/100 mM KCl, pH 7.4, in a total volume of 0.5 ml. After incubation at 4°C for 1 h, the reaction was terminated by filtration through Whatman GF/C filters, followed by three 1-ml washing with 10 mM KH₂PO₄/100 mM KCl, pH 7.4, and radioactivity was measured by scintillation counting. Nonspecific binding was determined with 100 μ M GABA.

Expression of GABA-A Receptors in *Xenopus* Oocytes and Transiently Transfected HEK 293 Cells. Oocytes from adult female *Xenopus laevis* were isolated and injected with different combinations of subunit cDNAs (20 ng/ μ l), and electrophysiological analyses were performed as described (4). In all experiments, drugs were applied in the perfusate until the peak of the response was observed. Allosteric modulators were

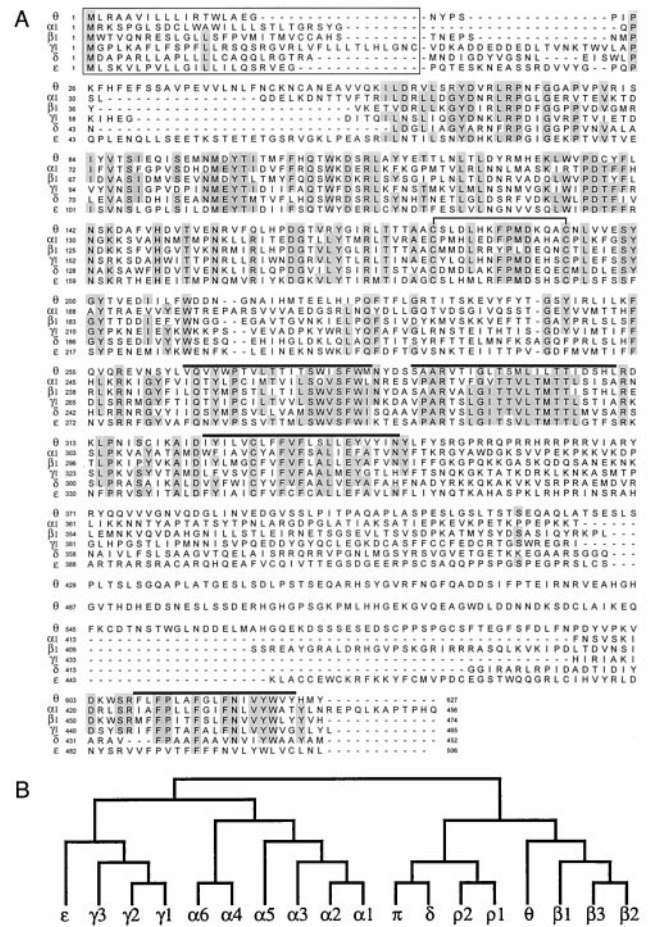


FIG. 1. Human GABA-A receptor θ subunit. (A) Alignment of the deduced amino acid sequences of the human GABA-A receptor α_1 (18), β_1 (18), γ_1 (19), δ (unpublished results), ϵ (4), and θ subunits. Numbering of amino acids is given by assigning the initiating methionine as 1. Positions where amino acid residues are conserved in four or more sequences are shaded. Putative signal peptides (20) are boxed, putative transmembrane domains are overlined, and the two cysteine residues conserved in the ligand-gated ion channel family are joined by a solid line. (B) Dendrogram of the deduced amino acid sequences of the GABA-A receptor (and p1-p2 of GABA-C) family. The analysis was performed with CLUSTALW (GCG).

preapplied for 40 sec before coapplication of GABA at an effective concentration, 50% (EC₂₀), concentration.

Experiments were performed on HEK 293 cells transiently transfected with human cDNA combinations $\alpha_2\beta_1\gamma_1$, and $\alpha_2\beta_1\gamma_1\theta$ (6 μ g of cDNA total per coverslip) by using calcium phosphate precipitation (13). Electrophysiological analyses were performed as described (4). GABA was applied from a double-barrel pipette assembly approximately 300 μ m in diameter positioned ~300 μ m from the cell. Solution equilibration times were on the order of 15 msec. Increasing GABA concentrations were applied for 2-sec pulses with a 30-sec interval between applications. Noncumulative concentration-response curves to GABA were constructed. Curves were fitted by using a nonlinear least square-fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$, where x is the drug concentration, EC₅₀ is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. EC₅₀ values were analyzed by using an unpaired Student's *t* test. Allosteric potentiation of GABA receptors was measured relative to a GABA EC₂₀ and antagonists were investigated by using a GABA EC₅₀, each determined for each cell to account for differences in GABA affinity. Receptor desensitiza-

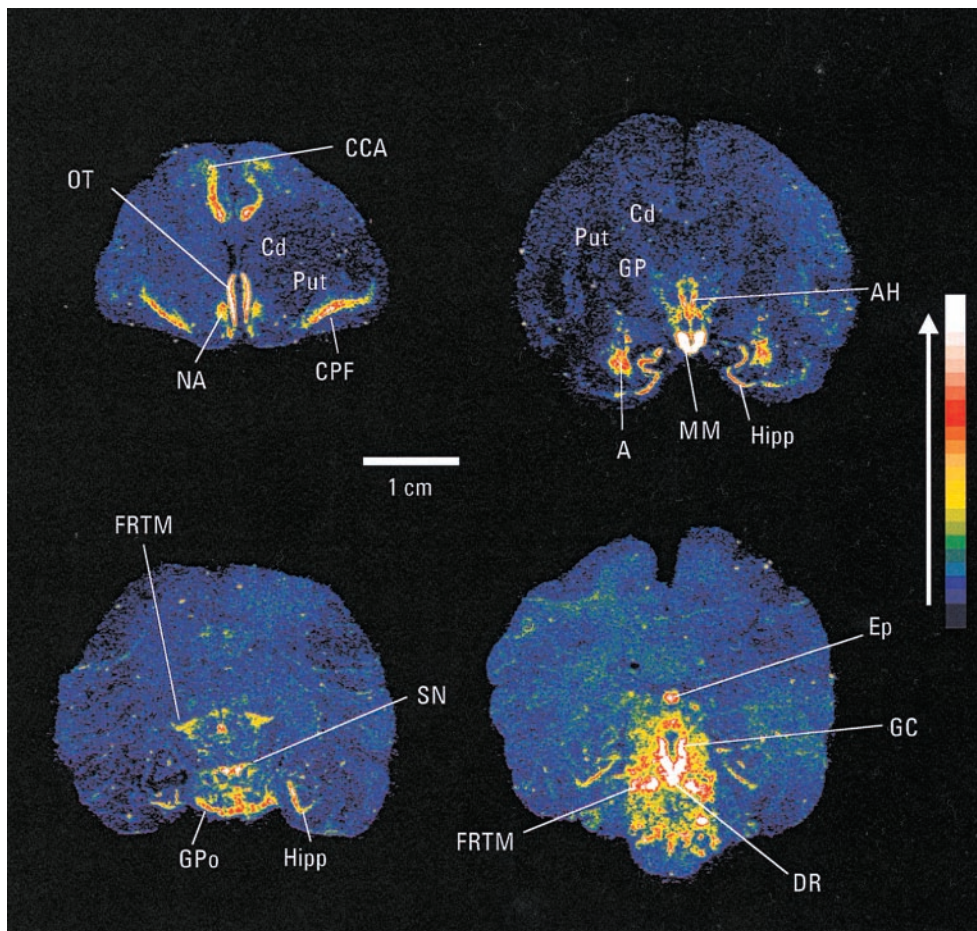


FIG. 2. *In situ* hybridization autoradiograms of monkey brain sections showing expression of θ subunit mRNA. The signal intensity is indicated, with white representing the strongest signal. CCA, anterior cingulate cortex; OT, olfactory tract; NA, nucleus accumbens; Cd, caudate; Put, putamen; CPF, piriform cortex; GP, globus pallidus; AH, anterior hypothalamus; Hipp, hippocampus; MM, mammillary body; A, amygdala; FRTM, reticular formation; GPO, pons; DR, dorsal raphe nucleus; GC, central grey; Ep, pineal gland.

tion was measured by applying a 10-sec application of a maximum GABA concentration and recording peak to steady-state ratio at the end of the 10-sec pulse.

RESULTS

U47334 Encodes a Partial Sequence of a GABA-A Receptor Subunit. Searching GenBank nucleotide database with the coding region of GABA-A receptor subunit identified the sequence U47334, submitted as an exon-trapped sequence from chromosome Xq28 annotated as "similar to chicken γ aminobutyric acid receptor beta4 subunit" (16). This is clustered with the GABA-A receptor ϵ and $\alpha 3$ genes (17). Full-length cDNA had a deduced ORF of 627 amino acids (Fig. 1A) that has all of the predicted features characteristic of ligand gated ion channel subunits: signal peptide, two cysteines separated by 13 amino acids, and four putative transmembrane (TM) domains, the third and fourth separated by a large cytoplasmic loop. There are two putative N-glycosylation sites, Asn-122 and Asn-317. Fig. 1A also shows an alignment of the θ subunit with other members of the gene family. The regions of significant sequence homology are the extracellular domain and the putative TM domains. The putative cytoplasmic loop between TM3 and TM4 shows no homology with other proteins, no obvious structural features, and no convincing consensus phosphorylation sites. Fig. 1b shows that θ is most similar to the β subunits. However, it exhibits only 50.5% sequence identity with $\beta 1$, whereas the amino acid sequence identity of $\beta 1$, $\beta 2$, and $\beta 3$ is around 80%, suggesting that it is not a β subunit. Additionally, it did not substitute functionally for a β subunit (see below). As such, it is more appropriately classified as a new subunit class, rather than $\beta 4$.

Localization of the θ Subunit in Monkey and Human Brain. The localization of the θ subunit in squirrel monkey brain was determined by *in situ* hybridization and by immunocytochem-

istry. θ mRNA had a discrete distribution (Fig. 2). Cortical expression was restricted to the cingulate and piriform regions. Significant expression was present in hypothalamic regions including the anterior hypothalamus and the mammillary body. Relatively strong expression of θ mRNA was present in the amygdala, and expression was also observed within the hippocampus. Very strong expression was seen within the substantia nigra, although interestingly the caudate putamen showed no significant labeling. Regions of the hindbrain, including the pons, dorsal raphe, and the central grey, were also strongly labeled. There was no significant hybridization signal in the cerebellum (data not shown).

Immunoreactivity for the θ subunit was detected in both monkey and human brain with a polyclonal serum raised against recombinant polypeptide of the putative cytoplasmic region of the θ subunit (Fig. 3). Confirmatory data (not shown) has also been generated with a polyclonal antiserum raised against a synthetic peptide corresponding to amino acids 492–510 of the θ subunit. The data shown for monkey brain are derived from a single animal. The same overall pattern of distribution for θ subunit immunoreactivity has also been observed in brain tissue from a second squirrel monkey brain and also from two cynomolgus monkey brains.

Immunolabeling of neurons in various monkey hypothalamic regions and cortical pyramidal neurons, particularly the deeper layers, was observed. Significant labeling of the monkey brainstem was found, including neurons in the substantia nigra pars compacta (Fig. 3C), and associated neurites of the pars reticulata, ventral and lateral tegmental areas, the locus coeruleus, reticular pontine nuclei, and the dorsal raphe. A pattern of labeling consistent with terminals within the monkey caudate putamen was observed with only occasional neuronal soma. TH-positive fibers, which constitute nigra-striatal projections, show a characteristic pattern as they pass through the globus pallidus; this

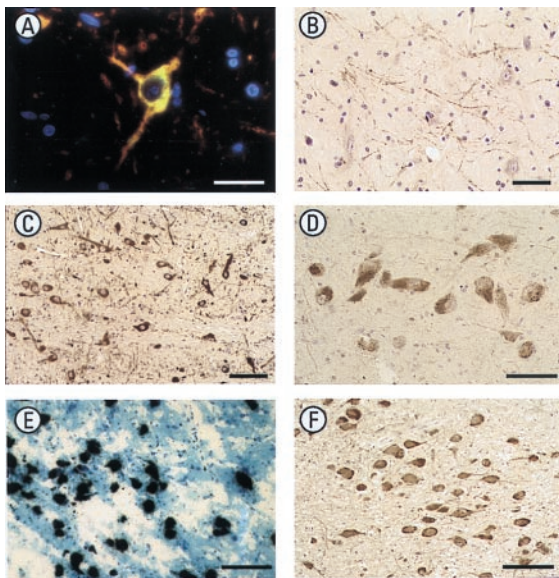


FIG. 3. Immunohistochemical localization of the GABA-A receptor θ subunit in monkey and human brain. (A) The expression of immunoreactivity (green) for the GABA-A receptor θ subunit in the dopaminergic neurons of the periventricular hypothalamus of monkey brain was confirmed by colocalization with TH immunoreactivity (red). Colocalization is observed as yellow/orange. Nuclei are counterstained with Hoechst 33258 (blue). (Bar = 30 μm .) (B) θ subunit immunoreactive processes in the monkey globus pallidus are similar in pattern to those demonstrated by TH, further suggesting expression by nigra-striatal projection neurons. (Bar = 50 μm .) (C) θ subunit immunoreactivity is expressed by the neurons of the primate substantia nigra pars compacta, suggesting expression in dopaminergic neurons. (Bar = 10 μm .) (D) θ subunit immunoreactivity in melanized neurons of the human substantia nigra pars compacta, again suggesting expression in dopaminergic neurons. (Bar = 50 μm .) (E) High-resolution autoradiography *in situ* hybridization experiments show expression of θ subunit mRNA as silver grains over melanized neurons of the human locus coeruleus. (Bar = 100 μm .) (F) θ subunit immunoreactivity in human locus coeruleus melanized neurons (probably noradrenergic). (Bar = 100 μm .)

pattern is mimicked by θ subunit immunoreactivity (Fig. 3B). This apparent expression of the θ subunit by TH containing neurons and processes was confirmed with combination immunofluorescence. For example, colocalization of θ subunit immunoreactivity and TH immunoreactivity was observed in dopaminergic neurons of the monkey periventricular hypothalamus (Fig. 3A). The expression of the θ subunit was further substantiated in sections of postmortem human brainstem; expression was observed in both the putative dopaminergic neurons of the substantia nigra pars compacta (Fig. 3D) and putative noradrenergic neurons of

Table 1. Immunoprecipitation of [^3H]muscimol binding from rat striatum by using combinations of antisera

Antiserum	% [^3H]muscimol binding sites immunoprecipitated		Theoretical sum if subunits present in different
	+control IgG	+ θ	
$\alpha 1$	40 \pm 3	56 \pm 5	57
$\alpha 2$	21 \pm 4	17 \pm 2	38
$\alpha 3$	10 \pm 2	24 \pm 5	27
$\beta 1$	28 \pm 4	34 \pm 4	47
$\gamma 1$	22 \pm 4	23 \pm 1	39
$\gamma 2$	35 \pm 5	58 \pm 3	52
θ	17 \pm 1		

Data shown are the mean \pm SEM of three determinations. No significant immunoprecipitation from striatum was observed if $\alpha 6$, δ , or ϵ subunit antiserum was used. The presence of very small amounts of receptors containing $\alpha 4$, $\alpha 5$, and $\gamma 3$ subunits was detected with the subunit-specific antisera but the amounts were too low for accurate quantitation by this approach.

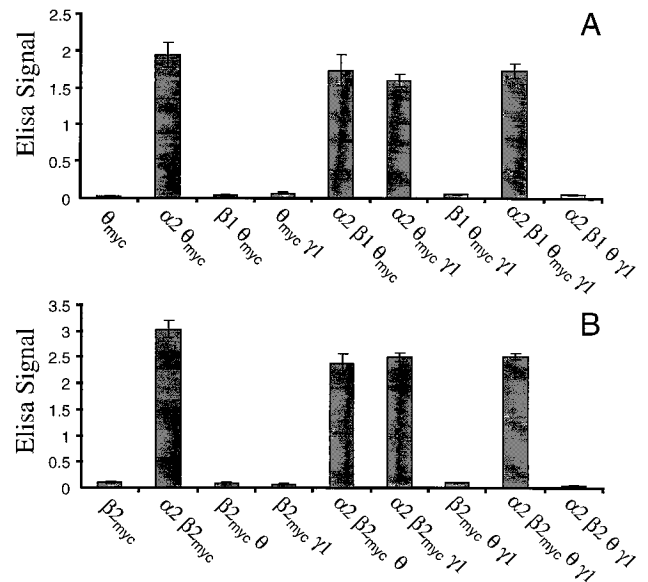


FIG. 4. Coassembly and cell surface expression of θ with other GABA-A receptor subunits. c-myc epitope tagged θ (A) or c-myc-epitope-tagged $\beta 2$ (B) was transiently transfected into HEK 293 cells, either alone or with other GABA-A receptor subunits. Cell surface expression of θ -myc or $\beta 2$ -myc was measured by a whole cell surface ELISA assay. The ELISA signal (A_{620}) is proportional to the amount of θ -myc or $\beta 2$ -myc at the cell surface. Background signal from mock-transfected control wells has been subtracted, and values are the mean \pm SEM of triplicate determinations.

the locus coeruleus, by using high-resolution *in situ* hybridization autoradiography (Fig. 3E) and immunolabeling (Fig. 3F).

Subunit Composition of Native θ Containing GABA-A Receptors. Polyclonal rabbit antisera raised against the putative cytoplasmic loop domain of the θ subunit were used to characterize native GABA-A receptors in rat brain that contain this subunit. Immunoprecipitation of [^3H]muscimol binding sites (muscimol is a high-affinity ligand for the GABA binding site of GABA-A receptors) from solubilized rat brain membranes was used to quantitate θ -containing receptors. The assumption in this experiment is that θ -containing receptors have a high-affinity [^3H]muscimol binding site, and this turned out to be the case. The striatum had the highest level of θ -subunit-containing receptors (22 \pm 4% of all [^3H]muscimol binding sites in striatum, 16 \pm 2% of binding sites in hypothalamus, 6 \pm 4% in hippocampus, and no significant binding in cortex and cerebellum). Receptors immunoprecipitated with the θ subunit antiserum did not bind benzodiazepine site ligands ([^3H]Ro 15-1788, [^3H]Ro 15-4513, [^3H]flunitrazepam, or [^3H]CGS-8216) with affinities high enough to be detected in a radioligand binding assay (data not shown). Assembly of the θ subunit with other GABA-A receptor subunits was investigated by quantitative co-precipitation of solubilized receptors with combinations of subunit-specific antisera. As shown in Table 1, the θ subunit can coassemble with $\alpha 2$, $\beta 1$, and $\gamma 1$. Because, within the detection limits of the approach, all θ immunoprecipitated receptors appear to contain $\gamma 1$ and $\beta 1$ subunits (theoretical sum of binding sites immunoprecipitated is equal to the highest antiserum individually), the preferred combination *in vivo* (at least in the striatum) is $\alpha 2\beta 1\gamma 1\theta$. Within the detection levels of this experimental approach, in rat striatum, there was no significant coassembly of the θ subunit with $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\gamma 2$, $\gamma 3$, δ , or ϵ . However, the possibility that there are small populations of receptors present where θ is coassembled with these subunits cannot be discounted.

Assembly of θ -Containing Receptors *In Vitro*. To investigate whether $\alpha\beta\gamma\theta$ subunits can coassemble *in vitro*, we have used a cell surface ELISA assay with intact transfected cells and myc-epitope-tagged θ subunit. The rationale behind this experiment is

Table 2. Activity of modulatory agents at $\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 1\theta\gamma 1$ GABA-A receptors

Drug	$\alpha 2\beta 1\gamma 1$ receptor		$\alpha 2\beta 1\theta\gamma 1$ receptor	
	EC ₅₀ /IC ₅₀	Maximum modulation, %	EC ₅₀ /IC ₅₀	Maximum modulation, %
Flunitrazepam	414 ± 167 nM	35.1 ± 4.6	245 ± 59 nM	35.4 ± 4.0
5 α -Pregnan-3 α -ol-20-one	9.9 ± 1.9 nM	158 ± 34	14.0 ± 2.3 nM	189 ± 16
Picrotoxin	3.5 ± 1.7 μ M		2.2 ± 0.7 μ M	
Zinc	11.4 ± 2.8 μ M		23.9 ± 12.1 μ M	

Values are determinations from six to nine oocytes and are not significantly different between subunit combinations.

as follows. Because θ does not get transported to the cell surface when expressed alone (Fig. 4), then if it appears at the cell surface when coexpressed with other subunits, there must be subunit coassembly. This assay does not determine if the subunits are "correctly" assembled or if they form functional receptors. Data in Fig. 4A are from experiments using $\alpha 2$, $\beta 1$, $\gamma 1$, and θ subunits because as discussed above these appear to be the preferred subunits for coassembly *in vivo*. Experiments have also been performed with $\alpha 1$, $\beta 1$, $\gamma 2$ and θ , with the same findings (data not shown). From Fig. 4B, it can be seen that when expressed alone, θ -myc is not expressed to a significant level at the cell surface. Most GABA-A receptor subunits behave this way, the main exceptions being $\beta 1$ and $\beta 3$ (11). When θ -myc is coexpressed with $\alpha 2$, it appears at the cell surface. Coexpression of an α subunit with a β subunit would also result in assembly and cell surface expression (e.g., see ref. 11). When coexpressed with $\beta 1$ and $\gamma 1$, individually or together, there is no surface expression of θ -myc. Thus, in terms of assembly and transportation to the cell surface (but not formation of functional receptors, see below), θ can substitute for a β subunit but cannot substitute for an α subunit. When coexpressed with $\alpha 2\beta 1$, $\alpha 2\gamma 1$, or $\alpha 2\beta 1\gamma 1$, θ -myc is expressed on the cell surface. However, from these experiments, it is not possible to determine whether these are simply $\alpha 2\theta$ -myc combinations or coassemblies containing all the expressed subunits. In addition, we do not know whether the $\alpha 2\gamma 1$ combination is able to get to the cell surface. Parallel experiments were also performed with c-myc-tagged $\beta 2$ (Fig. 4B). Like θ , $\beta 2$ does not get to the cell surface unless expressed with other subunits (ref. 11 and Fig. 4B). The same conclusions as above are obtained when $\beta 2$ -myc is expressed with other GABA-A receptor subunits. Additionally data in Fig. 4B indicate that the levels of cell surface expression of the various θ subunit-containing combinations are similar to that of $\alpha 2\beta 2$. Because the latter gives robust whole cell currents (data not shown), this suggests that the absence of detectable GABA gated channels for the various dimeric and

trimeric θ -containing receptors (see below) is not due to low levels of expression at the cell surface.

To determine the requirements for formation of functional θ -containing receptors, various subunit combinations (θ , $\beta 1\theta$, $\beta 1\gamma 2\theta$, $\alpha 1\theta$, $\alpha 1\gamma 2\theta$, $\alpha 1\beta 1\theta$, and/or $\alpha 1\beta 1\gamma 2\theta$) were expressed in *Xenopus* oocytes, and their electrophysiological response to 3 mM GABA was determined. The only subunit combination that lead to the expression of functional receptors was $\alpha 1\beta 1\gamma 2\theta$ (data not shown). In addition to testing for responses to GABA, all cells were exposed to 1 mM pentobarbital and 100 μ M picrotoxin to preclude the formation of channels gated by pentobarbital but not GABA or the formation of constitutively open channels. Thus although as discussed above $\alpha\theta$, $\alpha\beta\theta$, (and perhaps $\alpha\theta\gamma$) coassemble and are expressed on the cell surface, they do not form functional receptors. It is interesting that expression of $\alpha 1\beta 1\theta$ does not lead to the formation of functional receptors, but $\alpha 1\beta 1$ forms robust ion channels. This suggests that although θ is coassembling with $\alpha 1\beta 1$, an ion channel is not formed unless the full compliment of $\alpha\beta$ and γ are coexpressed. Furthermore, θ is preventing $\alpha 1$ and $\beta 1$ from forming a functional GABA gated channel.

The studies described above investigating the structure of native θ -containing receptors suggested an $\alpha\beta\gamma\theta$ subunit composition, and thus electrophysiological studies that focused on this subunit arrangement, using both $\alpha 1\beta 1\gamma 2\theta$ and $\alpha 2\beta 1\gamma 1\theta$ combinations were done. GABA concentration response curves were compared for $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 1\gamma 2\theta$ receptor combinations expressed in *Xenopus* oocytes. In this study, addition of the θ subunit produced a small but statistically significant increase in GABA EC₅₀ [24.9 ± 4.1 μ M for $\alpha 1\beta 1\gamma 2$ ($n = 8$) and 41.0 ± 4.1 μ M for $\alpha 1\beta 1\gamma 2\theta$ ($n = 5$); $P < 0.05$]. These experiments were repeated with whole-cell patch-clamp methods on HEK 293 cells expressing the same subunit combinations, and a similar increase in GABA EC₅₀ [5.5 ± 0.9 μ M for $\alpha 1\beta 1\gamma 2$ ($n = 10$) to 13.6 ± 3.0 μ M for $\alpha 1\beta 1\gamma 2\theta$ ($n = 10$), $P < 0.05$] was observed (Fig. 5a),

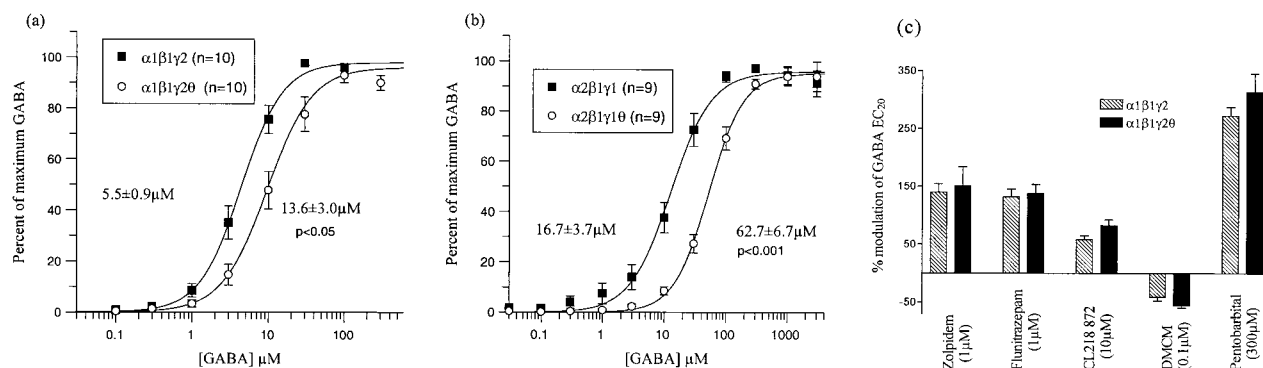


Fig. 5. Functional effects of θ expressed with other GABA-A receptor subunits. $\alpha 1\beta 1\gamma 2$ (a) and $\alpha 2\beta 1\gamma 1$ (b) GABA-A subunit cDNAs were transiently transfected both with (○) and without (■) the θ subunit cDNA into HEK 293 cells. Whole-cell patch-clamp techniques were used to measure currents in response to GABA after receptor expression. Concentration–response curves to GABA are shown normalized to a maximum response. Data are the mean ± SEM of the number of cells indicated. Values are the mean EC₅₀ ± SEM calculated from fits to individual cells. (c) Modulation of the GABA EC₂₀ response of $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 1\gamma 2\theta$ expressed in oocytes by a maximum concentration of benzodiazepine ligands, flunitrazepam, zolpidem, CL218 872, and DMCN, and the barbiturate pentobarbital. Data are the mean ± SEM from at least four oocytes.

suggesting that θ was incorporated into the $\alpha 1\beta 1\gamma 2$ combination as a fourth subunit. There were no marked differences in the desensitization kinetics of the θ -containing receptors expressed in HEK 293 cells, with peak to steady-state ratios of 2.2 ± 0.2 for $\alpha 1\beta 1\gamma 2$ and 2.1 ± 0.2 for $\alpha 1\beta 1\gamma 2\theta$ receptors. A number of different allosteric modulators were applied to $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 1\gamma 2\theta$ receptors to determine other possible differences in pharmacology. The benzodiazepine ligands flunitrazepam, DMCM, CL218,872, and zolpidem, as well as the barbiturate pentobarbital, showed identical degrees of modulation to that observed at $\alpha 1\beta 1\gamma 2$ (Fig. 5c), suggesting that θ incorporation does not affect benzodiazepine or barbiturate modulation. Because studies on the composition of native θ -containing receptors had suggested that the most likely subunits that assemble *in vivo* (at least in the rat striatum) with θ are $\alpha 2$, $\beta 1$, and $\gamma 1$, further experiments focused on $\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 1\gamma 1\theta$ subunit combinations expressed in HEK 293 cells. GABA concentration–response curves revealed a similar 4-fold increase in GABA EC₅₀ on addition of the θ subunit (Fig. 5b). These data demonstrate the coassembly of θ with α , β , and γ subunits to form a quaternary subunit combination. The pharmacology of $\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 1\gamma 1\theta$ receptors was also investigated. No difference in EC₅₀ or maximum modulation by flunitrazepam or the steroid 5 α -pregnan-3 α -ol-20-one was observed between $\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 1\gamma 1\theta$, and no differences in the IC₅₀ for the antagonists picrotoxin or zinc were seen (Table 2).

DISCUSSION

The GABA-A receptor gene family is now increased by the identification of a new member that we have termed θ . Immunoprecipitation data indicates that in the rat striatum θ -containing GABA-A receptors may represent around 20% of total receptors. For comparison, this is approximately the proportion of $\alpha 5$ -containing GABA-A receptors in the rat hippocampus (15). Quantitative immunoprecipitation experiments using subunit-specific antiserum suggest that in the rat striatum θ can coassemble with $\alpha 2$, $\beta 1$, and $\gamma 1$. Immunocytochemical studies have demonstrated the relatively abundant striatal expression of $\alpha 2$ immunoreactivity (9), in good agreement with this study. The coassembly of four types of subunit is also supported by the *in vitro* expression experiments that demonstrated that the only combination of θ -containing receptors that coassembled to form a functional receptor with distinct properties (i.e., a change in GABA EC₅₀) is $\alpha\beta\gamma\theta$. It is generally thought that the prototypic structure of GABA-A receptors is $\alpha\beta$ plus a γ , δ , or ϵ subunit (2). It has been reported that native receptors containing both γ and δ subunits do exist (21), although others find that γ and δ subunits are present in different receptor subtypes (22, 23). Thus the $\alpha\beta\gamma\theta$ subunit arrangement is unexpected. It is interesting to speculate on the stoichiometry of such a receptor. $\alpha\beta\gamma$ receptors are thought to have the stoichiometry $\alpha_2\beta_2\gamma_1$ (24, 25), although $\alpha_2\beta_1\gamma_2$ is also possible (26). Because two α subunits per receptor is a common theme, one could propose $\alpha_2\beta_1\gamma_1\theta_1$.

The coassembly in the rat striatum of θ with $\alpha 2$ and $\gamma 1$ is in accord with previous data indicating that $\alpha 2$ and $\gamma 1$ coassemble (27–29), although it has been reported that, in membranes from whole rat brain, $\gamma 1$ can also coassemble with $\alpha 1$ (28, 29). Native $\gamma 1$ -containing receptors have been reported to have a “low-affinity” [³H]flunitrazepam binding site ($K_d = 40$ –70 nM; refs. 28 and 29), with somewhat lower affinity still for other benzodiazepine site ligands. We have been unable to confirm the binding of [³H]flunitrazepam at receptors precipitated with either $\gamma 1$ or θ subunit antiserum.

It is interesting that θ does not form functional receptors when coexpressed with an α subunit or an α and β subunit, although there is no *a priori* reason to expect that it would. Perhaps more intriguing is that coexpression of θ with α and β subunit inhibits the formation of functional channels, further

suggesting that this is not a physiological combination. The subunit combination proposed here is $\alpha\beta\gamma\theta$. When expressed in a recombinant system (transfected mammalian cells or oocytes), this subunit combination has up to a 4-fold decrease in affinity for GABA compared with the $\alpha\beta\gamma$ combination. As discussed above, so far no other functional differences can be ascribed to the presence of the θ subunit.

θ -containing receptors have a striking colocalization with TH, although θ does not always colocalize with TH. This was seen in regions such as the substantia nigra pars compacta and caudate putamen, the periventricular hypothalamus and the locus coeruleus. Immunoreactivity for $\alpha 2$ and $\alpha 3$ has also been reported to be present in the substantia nigra and the striatum of the rat (9, 30). Indeed, Fritschy *et al.* (30) have reported that $\alpha 3$, but not $\alpha 1$, immunoreactivity is localized to TH positive cells in the substantia nigra pars compacta and the locus coeruleus. Because we have found no clear evidence for a significant population of receptors containing both $\alpha 3$ and θ , it could be concluded that within these TH-positive neurons, these two subunits are segregated into different receptor subtypes. That a given neuron can express multiple receptor subtypes sequestered to different subcellular domains has been documented in hippocampal pyramidal cells (31) and cerebellar granule cells (32). Localization of θ mRNA and immunoreactivity in the locus coeruleus, a region rich in TH-positive noradrenergic neurons, is in good agreement with the study by Luque *et al.* (33) describing the expression of $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 3$, and $\gamma 1$ mRNAs in these cells.

θ coassembles with $\gamma 1$, and these receptors have a benzodiazepine modulatory site. Although the affinity of $\gamma 1$ -containing receptors for existing benzodiazepine site ligands is generally low (28, 29, 34), in the future it may be possible to develop a new generation of compounds with selectivity and efficacy at this receptor subtype.

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