$GABA_A$ Receptor ϵ and θ Subunits Display Unusual Structural Variation between Species and Are Enriched in the Rat Locus Ceruleus

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Previously, $GABA_A$ receptor ϵ and θ subunits have been identified only in human. Here, we describe properties of the ϵ and θ subunit genes from mouse and rat that reveal an unusually high level of divergence from their human homologs. In addition to a low level of amino acid sequence conservation (~70%), the rodent ϵ subunit cDNAs encode a unique Pro/Glx motif of ~400 residues within the N-terminal extracellular domain of the subunits. Transcripts of the rat ϵ subunit were detected in brain and heart, whereas the mouse θ subunit mRNA was detectable in brain, lung, and spleen by Northern blot analysis. *In situ* hybridization revealed a particularly strong signal for both subunit mRNAs in rat locus ceruleus in which expression was detectable from the first postnatal day. Lower levels of coexpression were also detected in other brainstem nuclei and in the hypothalamus. However, the expression pattern of θ subunit mRNA was more widespread than that of ϵ subunit, being found also in the cerebral cortex of rat pups. In contrast to primate brain, neither subunit was expressed in the hippocampus or substantia nigra. The results indicate that $GABA_A$ receptor ϵ and θ subunits are evolving at a much faster rate than other known $GABA_A$ receptor subunits and that their expression patterns and functional properties may differ significantly between species.

Key words: rat $GABA_A$ receptor subunits; subunit sequence variation; brain regional localization; locus ceruleus; hypothalamus; subunit coexpression

Eighteen vertebrate $GABA_A$ receptor subunits have been categorized within seven families on the basis of sequence similarity (α1–α6, β1–β4, γ1–γ4, δ, ϵ, θ, and π) (Barnard et al., 1998; Bonnert et al., 1999). The β4 and γ4 subunits have been identified only in chick, whereas the most recently discovered subunits (ϵ and θ) have been described only in human. Notably, the human ϵ and θ subunits share greatest sequence similarity with the chick γ4 and β4 subunits, respectively (45–50% amino acid identity). Although the sequences of orthologous $GABA_A$ receptor subunits are generally well conserved between human and chick (83–98%), it is possible that the human ϵ and θ subunits are orthologs of the chick γ4 and β4 subunits, which have diverged to an unusually large extent. The proposal that the ϵ and θ subunit genes have mutated at a relatively rapid rate is supported by their chromosomal locations. The clustering of θ, α3, and ϵ subunit genes on human chromosome Xq28 (Levin et al., 1996; Wilke et al., 1997; Bonnert et al., 1999) indicates a common ancestry with the three $\beta/\alpha/\gamma$ subunit gene clusters in the human genome (Greger et al., 1995; McLean et al., 1995; Kostrzewa et al., 1996; Russek, 1999). However, whereas the α3 subunit has retained strong similarity to other members of the α subunit family, the θ and ϵ subunits have diverged significantly from their ancestral β- and γ-like subunit sequences.

If the human ϵ and θ subunits are orthologs of the chick γ4 and β4 subunits, respectively, the human genes have clearly diverged from their common ancestors to a much greater extent than the chick genes. This raises the possibility that the ϵ and θ subunits can confer properties to human $GABA_A$ receptors that are absent from chick. To better understand the divergence of ϵ and θ subunit orthologs and to examine their unique properties in different species, we have identified genes that encode homologs of ϵ and θ subunits in rodents. Recently, several reports have begun to describe expression patterns of rodent ϵ and θ subunits, using probes that were derived from homologous human subunits (Brooks-Kayal et al., 1998; Bonnert et al., 1999; Tobet et al., 1999). However, the rodent subunits described here display an unusually high level of sequence divergence from their human homologs. It is therefore essential to define the expression patterns of ϵ and θ subunits in rat brain using probes that are derived from rat cDNAs. Here, the brain regional expression patterns of ϵ and θ subunit mRNAs were studied by *in situ* hybridization (ISH) using rat subunit-specific oligonucleotide probes. The data suggest significantly different expression patterns for the subunits between rat and primate brain. Interestingly, a particularly strong enrichment of both subunits was observed in the locus ceruleus of adult and developing rats.

MATERIALS AND METHODS

Isolation of rodent ϵ subunit cDNAs. Primers were designed from exon 6 of the human ϵ subunit gene (see below) for amplification of any homologous exons from rat genomic DNA. The primers were nucleotides 695–718 (sense) and 758–781 (antisense) of GenBank accession number U66661. Amplification at 95°C for 45 sec, 50°C for 60 sec, and 72°C for 2 min was performed for 30 cycles using the XL-PCR system (Perkin-Elmer, Norwalk, CT). A single reaction product was detected, purified from an agarose gel, and sequenced directly. Oligonucleotide primers were designed from the rat exon sequence to amplify the 5′ and 3′ flanking sequences from a rat brain cDNA library, using the Marathon system (Clontech, Palo Alto, CA). Amplification at 95°C for 45 sec, 60°C for 60 sec, and 72°C for 2 min was performed for 35 cycles using the XL-PCR system. Reaction products were purified from agarose gels and sequenced directly. Primers that flank the complete open reading frame of the rat subunit were then used to amplify a contiguous cDNA from the same library. The primers were 5′-tctagagtcgacGTCGTGCCAGGCAC-

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CGCTGAGATG and 5′-actagtctgcagGGTGATTGCCCCATGAGCTACCAG. Primers were also designed from the rat subunit cDNA sequence for amplification of homologous cDNA fragments from a mouse brain cDNA library (Clontech). Fragments of mouse cDNA that span the entire open reading frame of the homologous mouse subunit were purified from agarose gels and sequenced directly. Primers that flank the open reading frame were then used to amplify a contiguous cDNA from the same library. The primers were 5′-tctagagtcgacATGTTGCCTAAAGTTCTCCTGATG and 5′-actagtctgcagCTGGAGCCTACAGGTTAAGGCAAA. All cloned products were sequenced over their entire length to ensure that no mutations had been introduced.

Isolation of human and mouse ϵ subunit genes. Two libraries of human genomic DNA, cloned in λDASH II and λFIX (Stratagene, La Jolla, CA), were screened at high stringency (Kirkness et al., 1991) with two ^{32}P-labeled fragments of the human ϵ subunit cDNA (nucleotides 20–328 and 695–1329 of GenBank accession number U66661). Sixteen hybridizing clones were obtained from $\sim 2 \times 10^6$ plaques. Overlapping inserts were determined by restriction fragment mapping and Southern blotting. Exons and flanking introns were sequenced from templates of purified λ DNA using the Dye Terminator Cycle Sequencing system (PE Biosystems, Foster City, CA). The sequences of three genomic fragments that contain all of the exons have been deposited with GenBank under accession numbers U92281, U92282, and U92283. A library of mouse genomic DNA (strain 129SVJ), cloned in λ FIX II (Stratagene), was probed at moderate stringency [1× SSC (0.15 M NaCl and 0.0015 M Na-citrate) and 0.1% SDS, 50°C] with ^{32}P-labeled fragments of the human and rat ϵ subunit cDNAs (nucleotides 695–1329 of GenBank accession number U66661, and nucleotides 1–278 and 1611–3000 of GenBank accession number AF189262). Seventeen hybridizing clones were obtained from $\sim 1 \times 10^6$ plaques. Overlapping inserts were determined by restriction fragment mapping and Southern blotting. Exons and flanking introns were sequenced from templates of purified λ DNA, and the sequences of five genomic fragments that contain all of the exons have been deposited with GenBank under accession numbers AF189264–AF189268.

Isolation of human, mouse, and rat θ subunit cDNAs. The amino acid sequence of the chick $GABA_A$ receptor β4 subunit (GenBank accession number X56647) was used to search the nr and est databases of GenBank using the TBLASTN algorithm. Homologous peptide fragments were identified within the six-frame translations of GenBank accession numbers U47334 (a trapped exon from human chromosome Xq28) and W15780 (an expressed sequence tag from a fetal mouse cDNA library). Oligonucleotide primers were designed from these sequences to amplify 5′ and 3′ flanking sequences from human and mouse brain cDNA libraries using the Marathon system. Amplification at 95°C for 45 sec, 60°C for 60 sec, and 72°C for 2 min was performed for 35 cycles using the XL-PCR system. Reaction products were purified from agarose gels and sequenced directly. Sequences that flank the complete open reading frames of the human and mouse subunits were then used to amplify contiguous cDNAs from the same libraries. For the human θ subunit cDNA (GenBank accession number AF189259), the primers contained nucleotides 1–24 (sense) and 1930–1953 (antisense). For the mouse θ subunit cDNA (GenBank accession number AF189260), the primers contained nucleotides 11–34 (sense) and 1940–1963 (antisense). A fragment of the rat θ subunit cDNA (GenBank accession number AF189261) was amplified from a rat brain cDNA library (Clontech) using primers derived from the mouse θ subunit cDNA sequence. The primers were nucleotides 1394–1417 (sense) and 1940–1963 (antisense), and amplification at 95°C for 45 sec, 55°C for 60 sec, and 72°C for 2 min was performed for 35 cycles using the XL-PCR system. The reaction product was purified from an agarose gel and sequenced directly.

Northern blot analysis. Samples of ~ 2 μg of poly(A^+) RNA (Clontech) were electrophoresed on a 1.2% formaldehyde agarose gel, transferred to nylon membranes, and hybridized with ^{32}P-labeled fragments of the human θ subunit cDNA (nucleotides 730–1953) or mouse θ subunit cDNA (nucleotides 1602–1963). The blots were washed at 60°C in 0.1× SSC and 0.1% SDS before exposure. The blots were reprobed with ^{32}P-labeled fragments of the glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA (nucleotides 789–1140) (Tokunaga et al., 1987).

In situ *hybridization.* Male Sprague Dawley rats ($n = 22$; University of Turku, Turku, Finland) at four different ages [postnatal day 0 (P0), P6, P12, and adult] were killed, and brains were removed and frozen on dry ice. P0 rat heads were frozen as a whole. Fourteen micrometer thick sections were cut on a cryostat (Microm HM 500 OM; Microm Laborgeräte GMbH, Walldorf, Germany), mounted onto poly-L-lysine-coated slides, and dried at room temperature (RT) for 1–2 hr. Sections were fixed in 4% paraformaldehyde, washed in PBS for 5 min, dehydrated in 70% ethanol for 5 min, and stored in 95% ethanol at 4°C until used in hybridization.

ISH for detection of $GABA_A$ receptor subunit transcripts and localization of locus ceruleus was done using the protocol described by Wisden and Morris (1994). In detail, 36- to 45-bases-long antisense oligonucleotide probes were synthesized (Institute of Biotechnology, University of Helsinki, Helsinki, Finland) complementary to rat cDNA sequences. Tyrosine hydroxylase (TH) has been known to be heavily expressed in the locus ceruleus (Pickel et al., 1975), and a TH probe complementary to nucleotides 867–911 of rat cDNA (GenBank accession number M10244) was created to locate this nucleus in the rat brain. Rat $GABA_A$ receptor ϵ subunit has been shown to have two different splice variants (Whiting et al., 1997). Three different probes were thus created to detect ϵ subunit transcripts in the rat brain. They were complementary to (1) the boundary between exons 6 and 7 [functional variant (Whiting et al., 1997); nucleotides 2240–2275 of GenBank accession number AF189262], (2) exon 6 (both variants; nucleotides 2212–2256 of GenBank accession number AF189262), and (3) exon 9 (both variants; nucleotides 2616–2660 of GenBank accession number AF189262). Two probes against different positions [nucleotides 41–85 and 86–130 (probe 2; see Figs. 5–7) of GenBank accession number AF189261] of rat $GABA_A$ receptor θ subunit mRNA were created to establish the expression pattern of this novel subunit.

Probes were [α-^{33}P] (NEN, Boston, MA) 3′ end-labeled with terminal transferase (Boehringer Mannheim, Mannheim, Germany) using 1:15–1:60 molar ratio of probe and radioactive nucleotide according to the labeling properties of different probes. Unincorporated nucleotides were separated by Bio-Spin 6 chromatography columns (Bio-Rad, Los Angeles, CA), and labeling efficiency was determined with a scintillation counter. One hundred microliters of hybridization buffer (50% formamide, 10% dextran sulfate, and 4× SSC) containing diluted probe (0.06 fmol/μl, 290–1100 dpm/μl) was applied to each slide and hybridized under parafilm coverslips overnight at 42°C. Sections were then washed in 1× SSC at RT for 10 min, 1× SSC at 55°C for 30 min, and finally through 3 min washing steps at RT as follows: 1× SSC, 0.1× SSC, 70% ethanol, and 95% ethanol. Sections were then air-dried and exposed to Biomax MR films (Eastman Kodak, Rochester, NY) with ^{14}C standards for 3–12 weeks. Specificity of probes was determined with 100× excess of nonradioactively labeled probes. Images from representative films were produced by scanning the films using an HP ScanJet 4c/T scanner and HP DeskScan II program (Hewlett-Packard, Palo Alto, CA) and Adobe PhotoShop (version 3.0; Adobe Systems, Mountain View, CA). For anatomical localization, some of the serial slides were stained in 0.1% thionin (Sigma, St. Louis, MO) solution, washed in 70, 95, and 100% ethanol, air-dried, and mounted with Permount (Fisher, Pittsburgh, PA) and glass coverslips.

RESULTS

A fragment of the human ϵ subunit cDNA (Davies et al., 1997) was used to isolate homologous cDNAs from rat and mouse brain (see Materials and Methods). The proteins that are encoded by these cDNAs are highly unusual (Fig. 1). Although they display all of the features that are characteristic of $GABA_A$ receptor subunits and are most similar to the human ϵ subunit, they each contain a large insertion near the N terminus of the protein (Fig. 1*B*). This additional sequence (483 amino acid residues in the rat subunit) is composed primarily of Pro/Glu and Pro/Gln tandem repeats. However, in contrast to the peptide sequence, the encoding cDNA sequence contains few repetitive elements. It is therefore unlikely that this sequence has arisen from recent expansion of unstable repetitive units, as occurs in various genetic disorders (Reddy and Housman, 1997). The repetitive Pro/Glx element follows a predicted signal sequence (Nielsen et al., 1997) and is therefore expected to be located extracellularly.

Notably, even if the repetitive Pro/Glx insertion is ignored, the rodent ϵ subunits display only 68% amino acid identity with the human ϵ subunit. This value is significantly less than that observed for rodent orthologs of other human $GABA_A$ receptor subunits (90–100%). This observation raised the possibility that the rodent subunits are a novel subtype of the ϵ subunit class and that the true orthologs of the human ϵ subunit had not been identified. However,

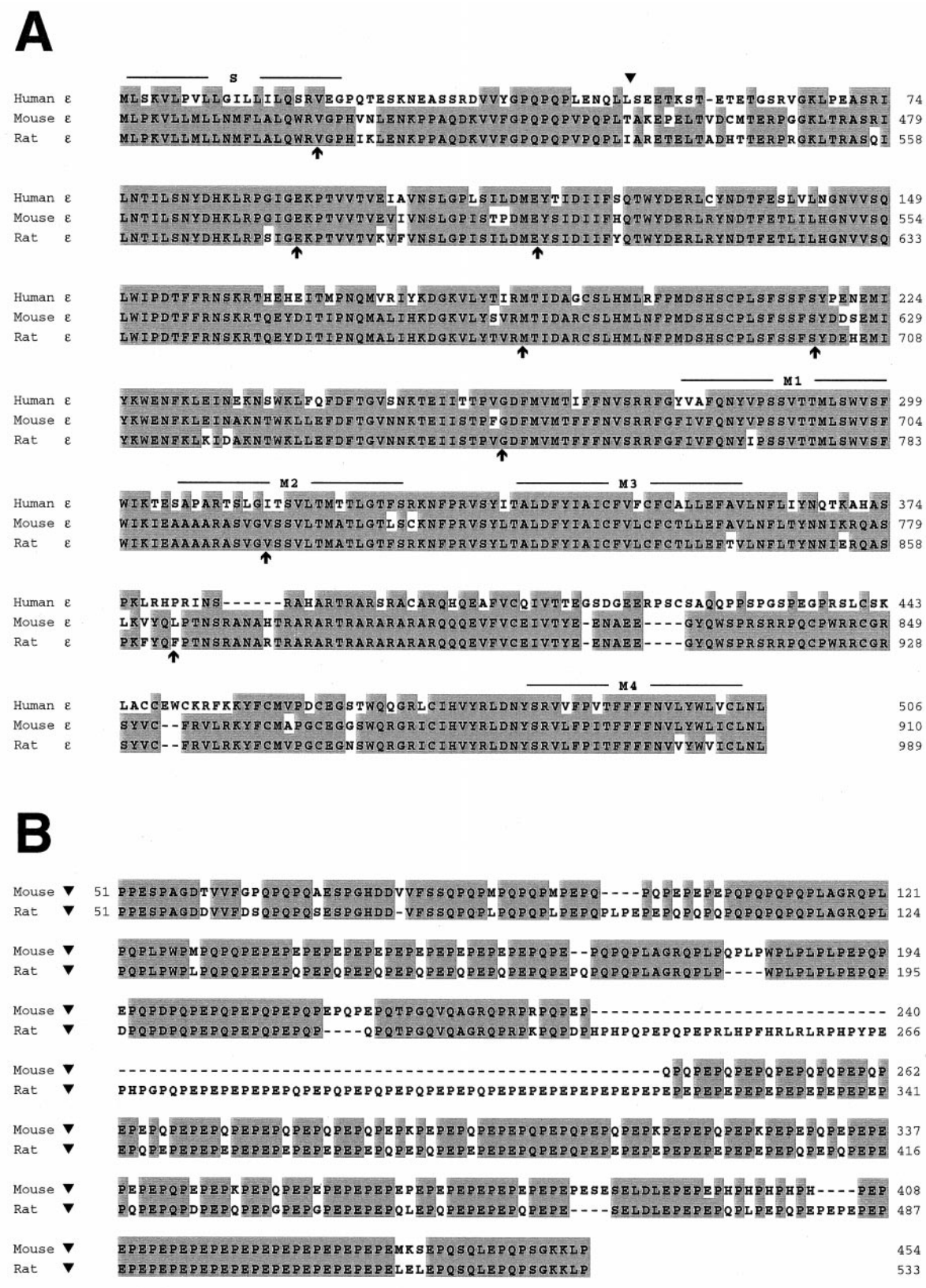

Figure 1. Alignment of amino acid sequences for human, mouse, and rat $GABA_A$ receptor ϵ subunit homologs. *A*, The translated human, mouse, and rat ϵ subunit cDNAs were aligned after editing of the rodent sequences to remove a peptide fragment that is absent from the human sequence (see *B*). The normal location of this peptide fragment within the rodent sequences (after residue 50) is indicated (▼). Conserved residues are *shaded*, and the putative signal sequence (*S*) and transmembrane domains (*M1–M4*) are highlighted by *lines* above the corresponding sequences. Segments of the human and mouse subunits that are encoded by distinct exons are indicated by the locations of exon termini (↑). *B*, Alignment of the rodent-specific peptide fragments that were edited from *A*. Residues that are conserved between the rat and mouse sequences are *shaded*.

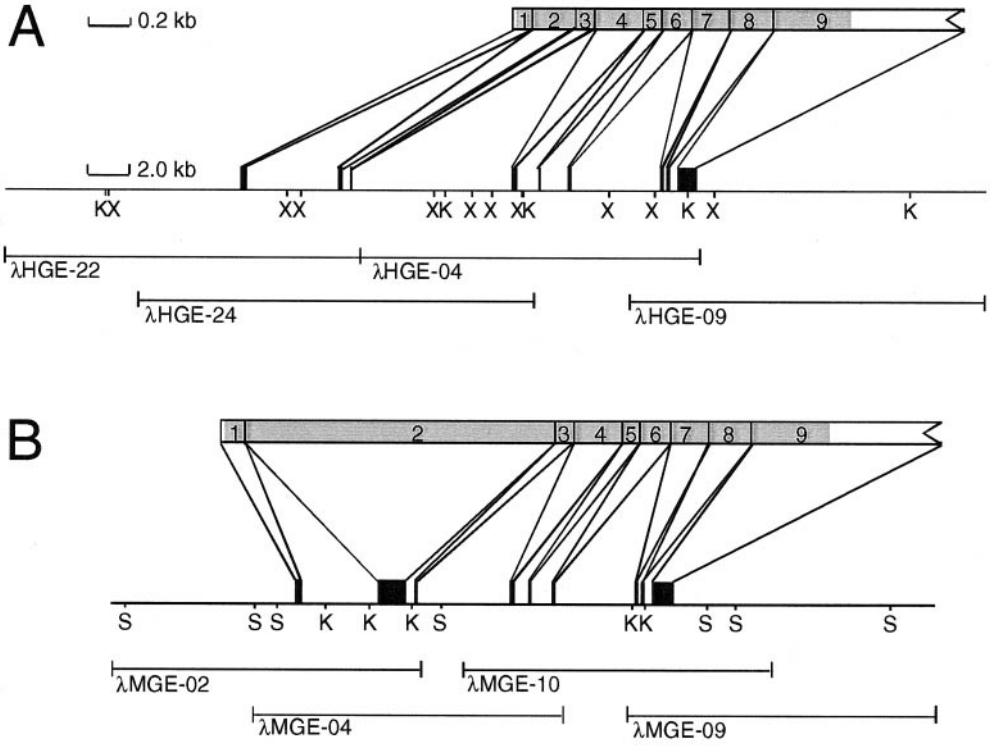

Figure 2. Gene structures of human and mouse $GABA_A$ receptor ϵ subunit homologs. *A* and *B* represent the human and mouse ϵ subunit genes, respectively. For each *panel*, the *top* illustration represents the subunit mRNA. The protein-coding regions are *shaded*. Segments of the mRNA that are encoded by distinct exons are represented by *numbered rectangles* that are joined to the corresponding genomic sequence below. The *bottom* portion of each *panel* illustrates cloned fragments of genomic DNA.

screening of mouse genomic DNA libraries with the human ϵ subunit cDNA at moderate stringency detected only the known mouse ϵ subunit gene (see below). It was therefore concluded that there are no ϵ subunit subtypes within the mouse genome that are more similar to the human ϵ subunit than that which was identified by cDNA cloning. In common with previous Northern blot analyses of human ϵ subunit mRNA (Garrett et al., 1997; Whiting et al., 1997; E. F. Kirkness, unpublished observations), transcripts of the rat ϵ subunit (~7.2 kb) were relatively abundant in heart but were not detectable in samples of whole brain, liver, kidney, or skeletal muscle (data not shown).

It was of interest to determine whether the unusual sequence of the rodent cDNAs is derived from a distinct exon and whether such an exon also exists within the human ϵ subunit gene. For this reason, the complete human and mouse ϵ subunit genes were cloned and partially sequenced. The structure and sequences of the human ϵ subunit gene (Fig. 2*A*) are essentially identical to those derived from an independent GenBank accession (number U82696). However, this structure conflicts with that reported by Wilke et al. (1997) in which intron 3 and a fragment of intron 6 were not identified. Their conclusion that the structure of the ϵ subunit gene is different from all related subunit genes (Wilke et al., 1997) is not supported by this study. The structures of the human and mouse genes are identical (Fig. 2), and an additional exon cannot account for the unusual extra sequence of the mouse cDNA. It was conceivable that the repetitive Pro/Glx segments of the rodent subunits were derived from the use of an alternative 3′ intron splice site between exons 1 and 2. However, the additional sequence of the mouse cDNA is not homologous to any region of intron 1 of the human gene. Furthermore, after PCR amplification of rat brain cDNA libraries with primers from exon 1 (sense) and exon 3 (antisense), all products were found to contain the complete exon 2 sequence. It is therefore concluded that the additional sequence of the rodent subunit genes is located within exon 2 and is not derived from a distinct exon or from the use of alternative splice sites.

Human and mouse θ subunit cDNAs were first identified by searching GenBank for sequences that are homologous to the chick $GABA_A$ receptor β4 subunit. The identified sequence fragments were used to clone longer cDNAs from human and mouse brain cDNA libraries (see Materials and Methods). These cDNAs contain the complete open reading frames of the human and mouse θ subunits (Fig. 3). The mouse cDNA sequence was also used to isolate a fragment of the rat θ subunit cDNA for *in situ* hybridization studies (see below). The human θ subunit cDNA encodes a polypeptide of 632 amino acid residues that is most closely related to the chick β4 subunit (56% amino acid identity in extracellular and transmembrane domains). After the completion of this work, Bonnert et al. (1999) reported a human cDNA sequence that is essentially identical to that described in Fig. 3. However, the cDNA described here encodes five additional amino acid residues at the N terminus of the subunit. The first Met codon is flanked by a consensus sequence for initiation of translation (Kozak, 1991), which is absent from that proposed previously (Met_6; Bonnert et al., 1999). The only other difference between the two sequences is at Ile_{478} at which the sequence of Bonnert et al. (1999) encodes a Phe residue. The human and mouse θ subunits share only 76% amino acid identity. Consequently, in common with the ϵ subunit homologs, there was concern that the human and mouse cDNAs are not orthologous but represent distinct subtypes of a θ subunit family. However, Southern blot analysis of mouse genomic DNA with the human θ subunit cDNA detected only the known mouse θ subunit gene (data not shown). Northern blot analysis of θ subunit mRNA expression in human and mouse tissues indicated predominant expression in the brain (Fig. 4). Transcripts of the θ subunit are relatively large, with human brain displaying at least two mRNA species of 7.3 and 8.0 kb. In mouse, a single transcript of 7.6 kb was detectable in brain, spleen, and lung.

Brain regional distribution of ϵ and θ subunit transcripts revealed a common site of strong expression in the adult rat, i.e., locus ceruleus in the brainstem (Fig. 5). The ϵ subunit mRNA,

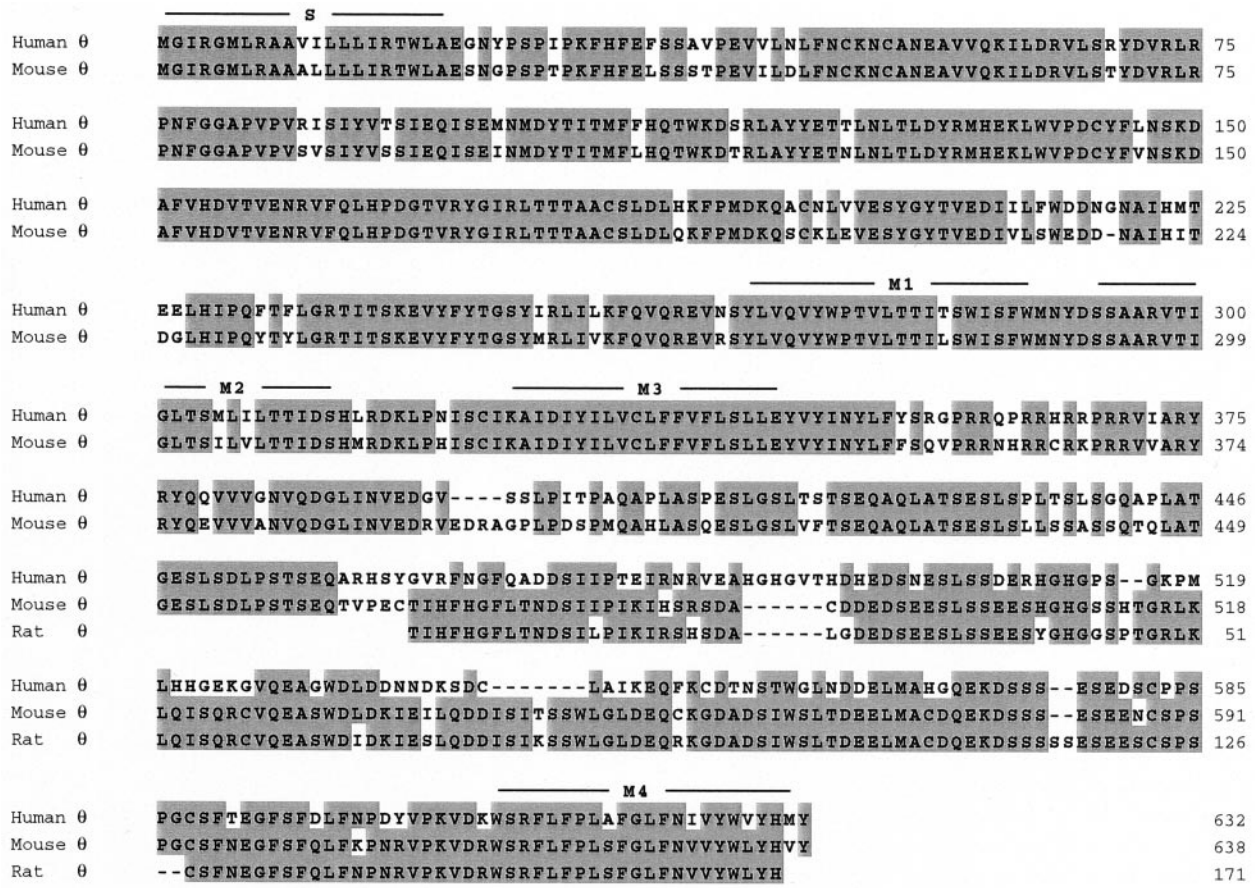

Figure 3. Alignment of amino acid sequences for human, mouse, and rat $GABA_A$ receptor θ subunit homologs. The amino acid sequences of θ subunit homologs from human (full-length), mouse (full-length), and rat (partial) were translated from cloned cDNAs. Conserved residues are *shaded*, and the putative signal sequence (*S*) and transmembrane domains (*M1–M4*) are highlighted by *lines* above the corresponding sequences.

visualized with three different oligonucleotide probes with identical results, was also lightly expressed in the hypothalamus but not in the cerebral cortex or hippocampus (Fig. 6). Signals from all probes were consistent with the expression of the long, functionally active splice variant of ϵ subunit mRNA (Whiting et al., 1997). The θ subunit mRNA, visualized with two different probes with identical results, was also detectable in the hypothalamus and some thalamic nuclei and was nonexistent in the hippocampus and cerebral cortex (Fig. 6). No significant amount of ϵ and θ subunit mRNA was found in the substantia nigra or caudate putamen (data not shown).

During postnatal development of the rat brain, both ϵ and θ subunit transcripts were detectable at birth in the locus ceruleus (Fig. 7). There was also weaker expression in the brainstem, including the dorsal raphe nuclei, for both subunits. The expression of θ subunit mRNA appeared slightly more widespread, being also detectable in the inner layer of the cerebral cortex, especially at the postnatal day 6, and in the basal forebrain nuclei, such as the bed nucleus of stria terminalis. No clear signals for either ϵ and θ subunit transcripts were detected in the hippocampal regions, basal ganglia, and most of the cerebral cortex at postnatal days 0–12.

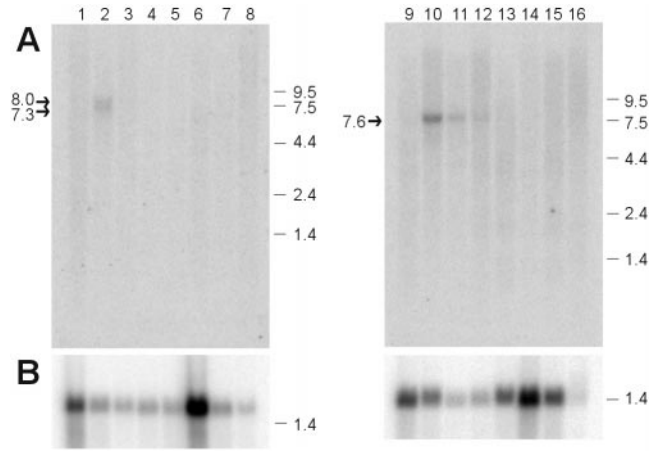

Figure 4. Distribution of $GABA_A$ receptor θ subunit mRNA in human and mouse tissues. *A*, Poly(A^+) RNA from human tissues (*lanes 1–8*) or mouse tissues (*lanes 9–16*) was hybridized with ^{32}P-labeled fragments of the human and mouse θ subunit cDNA, respectively. The mRNA was derived from heart (*lane 1*), whole brain (*2*), placenta (*3*), lung (*4*), liver (*5*), skeletal muscle (*6*), kidney (*7*), pancreas (*8*), heart (*9*), whole brain (*10*), spleen (*11*), lung (*12*), liver (*13*), skeletal muscle (*14*), kidney (*15*), and testes (*16*). *B*, The same blots were reprobed with a ^{32}P-labeled fragment of the human GAPD cDNA.

DISCUSSION

The cloning of rodent ϵ and θ subunit cDNAs has revealed an unusually large degree of sequence divergence from their human orthologs. Rodent ϵ subunits also exhibit an unusual insertion of

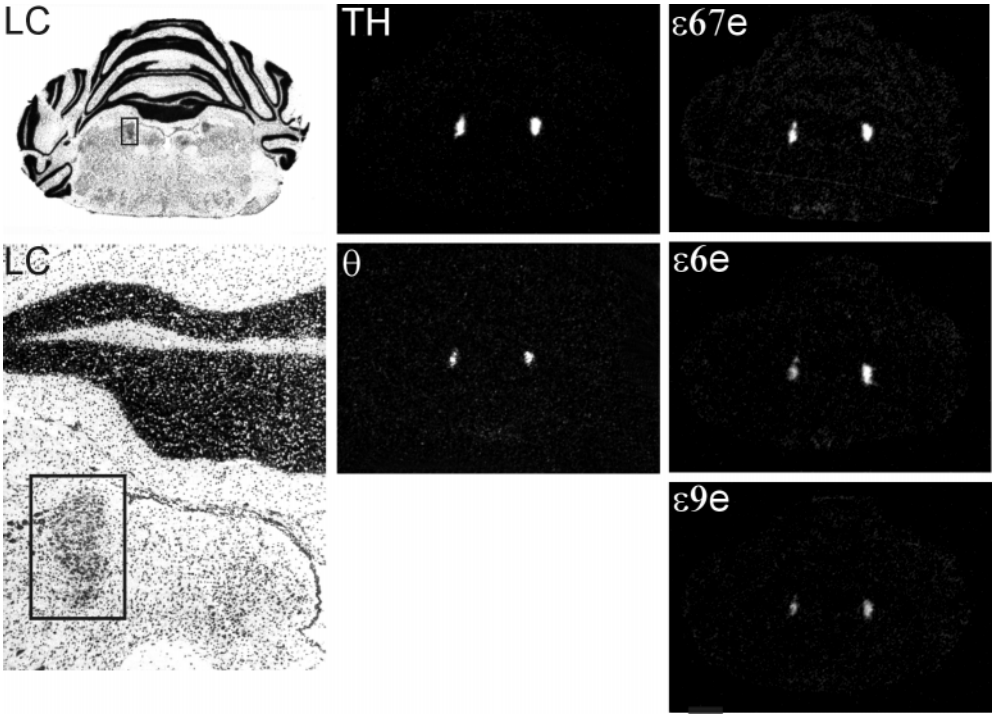

Figure 5. Localization of $GABA_A$ receptor ε and θ subunit mRNAs in the rat locus ceruleus. The subunits were localized by *in situ* hybridization using ^{33}P-labeled specific oligonucleotide probes. Images from serial frontal sections are shown. *LC*, Thionin staining of the section probed with tyrosine hydroxylase; locus ceruleus in the *box*. *TH*, Tyrosine hydroxylase probe. *θ*, θ subunit probe 2. *ε67e*, ε subunit probe against exon border 6–7. *ε6e*, ε subunit probe against exon 6. *ε9e*, ε subunit probe against exon 9. Hundred-fold excess of cold probes abolished the hybridization to the background level in each case, indicating specificity of the signals (data not shown).

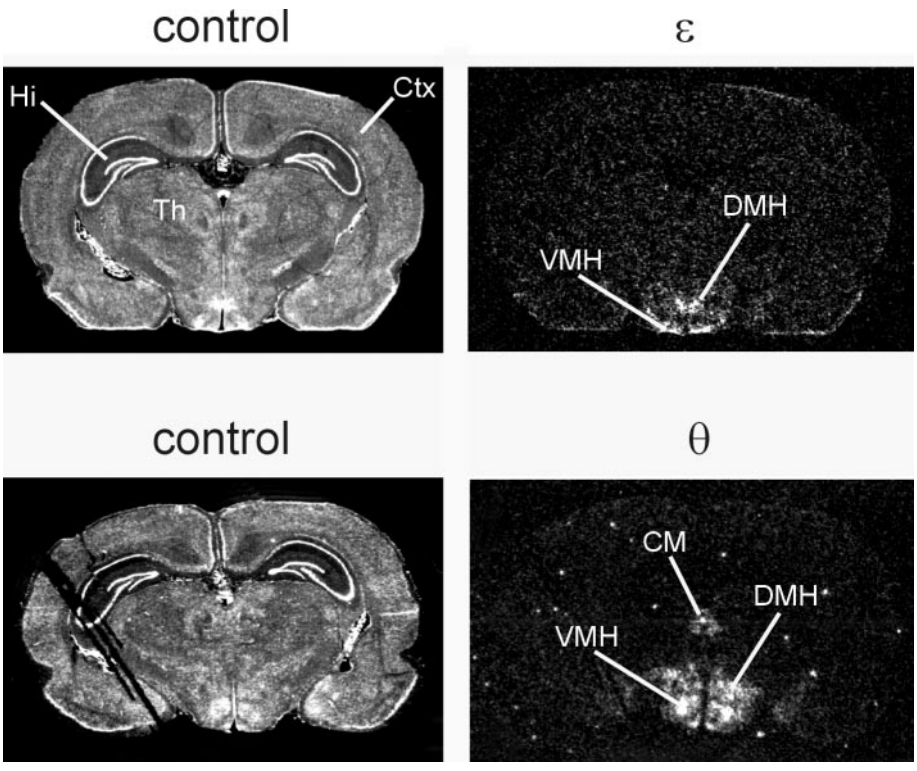

Figure 6. Localization of $GABA_A$ receptor ε and θ subunit mRNAs in the adult rat hypothalamus. Images by *in situ* hybridization of ε and θ subunit transcripts were done using ^{33}P-labeled specific oligonucleotide probes *ε67e* and probe 2, respectively. *Control*, Thionin staining; *CM*, centromedial thalamic nucleus; *Ctx*, cerebral cortex; *DMH*, dorsomedial hypothalamus; *Hi*, hippocampus; *Th*, thalamus; *VMH*, ventromedial hypothalamus.

repetitive amino acid sequence within the large extracellular domain. There are few examples of such long repetitive Pro/Glx motifs in the databases of known protein sequences. Among eukaryotic genes, these include a microtubule-associated protein (Goedert et al., 1996), a putative transmembrane transporter (Lafreniere et al., 1994), and a repressor of apoptosis (Koseki et al., 1998). The function of the Pro/Glx motifs in these proteins is uncertain. For the putative transmembrane transporter, the Pro/Glu repeat has been classified as a PEST domain (Lafreniere et al., 1994), predicting a role in targeting the protein for rapid proteolysis (Rechsteiner and Rogers, 1996). To date, we have been unable to demonstrate unique functional properties that can be attributed to the rat ε subunit when expressed with known $GABA_A$ receptor subunits in human embryonic kidney 293 cells. It is possible that the nascent polypeptide must undergo post-translational processing before assembly within functional receptors, and this will be pursued by probing native and recombinant subunits with subunit-specific antibodies.

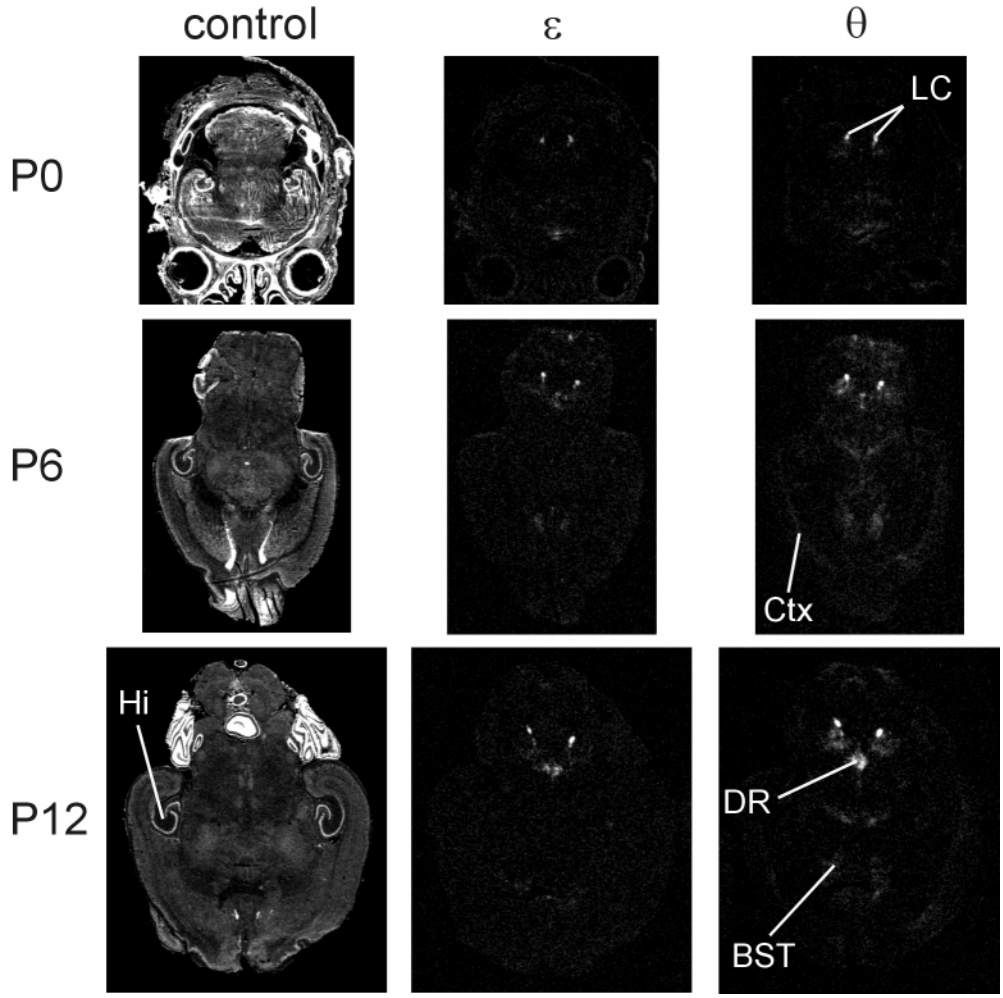

Figure 7. Expression of $GABA_A$ receptor ϵ and θ subunit mRNAs in the rat brain during postnatal development. Serial horizontal sections for *in situ* hybridization of ϵ (probe *ϵ67e*) and θ (probe 2) are shown. *Control*, Thionin staining; *LC*, locus ceruleus; *Ctx*, cerebral cortex; *DR*, raphe dorsalis; *BST*, bed nucleus of stria terminalis; *Hi*, hippocampus.

For both the ϵ and θ subunits, the sequences of rodent and human homologs suggest that these subunits are evolving at a much faster rate than other known $GABA_A$ receptor subunits. Differences in the rates of mutation have been noted previously for other gene families. For example, the genes encoding α-fetoprotein and serum albumin likely arose from a gene duplication event (Kioussis et al., 1981) but have mutated at significantly different rates since the divergence of rodents and human (Minghetti et al., 1985). The biological function that is acquired by each new gene family member appears to impose unique structural constraints. This explanation suggests that the structural requirements for function of ϵ and θ subunits are more flexible than for other known $GABA_A$ receptor subunits. Consequently, they have evolved under less constraint and can tolerate more mutations. It is possible that their biological functions can be maintained by fewer conserved residues or that their functions in rodents and human have diverged. The large insertion within the rodent ϵ subunits is clearly suggestive of at least some distinctive properties, and it will be important to define how the structural variation might distinguish neuronal activity in rodents and human.

Owing to the sequence variation between rodent and human ϵ subunits, many probes for the human subunit are unlikely to cross-react with the appropriate rodent subunit. An antiserum that recognizes a peptide of the human subunit (residues 409–427) (Whiting et al., 1997) has been used for immunolocalization (Tobet et al., 1999) and immunoprecipitation (Bonnert et al., 1999) of rodent ϵ subunits. However, the human peptide shares only ~30% sequence identity with either the rat or mouse ϵ subunit peptides described here, and the data derived from the use of this antiserum in rodents are therefore questionable. In addition, a fragment of the human θ subunit has been used to generate an antiserum for immunoprecipitation of rodent θ subunits (Bonnert et al., 1999), although this peptide shares only 63% amino acid identity with the mouse θ subunit described here.

The localized expression of these subunits is consistent with the fact that all $GABA_A$ receptor subunit genes have distinct expression patterns in the brain (Wisden et al., 1992). However, there is evidence that some clustered genes exhibit colocalized expression, such as the β2, α1, and γ2 subunit genes on mouse chromosome 11 that very likely form the major $GABA_A$ receptor subtype in the brain (McKernan and Whiting, 1996). Our data support this idea by demonstrating that the clustered θ, α3, and ϵ subunit genes also show colocalized expression. Regions in which the ϵ and θ subunits are coexpressed are known to contain α3 subunit mRNA (Wisden et al., 1992). Although the α3 subunit is also expressed in other brain regions, it is predicted to assemble with ϵ and θ subunits in discrete brain regions, such as the locus ceruleus.

Our localization data differ from previously reported patterns for ϵ and θ subunit expression in primate brain (Whiting et al., 1997; Bonnert et al., 1999). There are clear differences in the hippocampus and substantia nigra, which show expression of ϵ and θ subunits in the monkey but not in the rat. Furthermore, the θ subunit peptide has been reported to be strongly expressed in the rat striatum (Bonnert et al., 1999). This is inconsistent with the lack of detectable mRNA expression by *in situ* hybridization in the caudate putamen or substantia nigra pars compacta. Therefore, the

existence of a native $\alpha2\beta1\gamma1\theta$ subunit combination, suggested to be responsible for ~20% of [^{3}H]muscimol binding in the rat striatum (Bonnert et al., 1999), should be reexamined for the reasons concerning species specificity of the antisera (see above).

The most interesting localization for both ϵ and θ subunits was the bilateral noradrenergic locus ceruleus, which has been implicated with a number of behavioral and physiological processes, e.g., anxiety, panic, attention, and drug withdrawal syndromes. This nucleus has been "lacking" a normal repertoire of $GABA_A$ receptor subunits to form functional $GABA_A$ receptors (Luque et al., 1994a). The absence of $\gamma2$ subunit expression (Luque et al., 1994b) is likely to explain why benzodiazepines are often inefficient in reducing evoked neuronal activity in the locus ceruleus (Simson and Weiss, 1989; Beck and Loh, 1990). It is suggested that $\alpha3\beta\theta\epsilon$ receptors form a receptor population with unique locus ceruleus-enriched expression pattern and benzodiazepine insensitivity, which might be exploited to find subtype-selective nonbenzodiazepine site compounds for the treatment of stress and anxiety. The θ subunit has been localized in the human locus ceruleus by immunohistochemistry (Bonnert et al., 1999), but no information is available for the ϵ subunit. Furthermore, double or triple immunohistochemical technique should be used to directly demonstrate the colocalization of ϵ and θ subunits with α and β subunits (Fritschy et al., 1992; Waldvogel et al., 1999). Because both ϵ and θ subunit mRNA was expressed already at birth, ϵ and θ subunit-containing receptors may be involved in selective trophic action of GABA on monoaminergic neurons (Liu et al., 1997).

In summary, our results provide the rational basis for examining the possible pharmacological modulation of discrete brain nuclei in the brainstem and hypothalamus by targeting ϵ and θ subunit-containing $GABA_A$ receptors. Furthermore, the low sequence conservation and distinctive expression patterns of these X chromosome-clustered genes indicate that their functional properties may differ significantly between human and rodents.

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