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A novel γ subunit of the $GABA_A$ receptor identified using the polymerase chain reaction

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We have utilized a polymerase chain reaction (PCR) strategy to identify a novel subunit, γ3, of the $GABA_A$ receptor. The γ3 cDNA encodes a mature protein of 450 amino acids that contains structural features typically conserved among subunits of the $GABA_A$ receptor family. The γ3 subunit shares approximately 66% sequence identity with the γ2 subunit but only 38% and 29% with α1 and β1 subunits, respectively. Localization of the γ3 mRNA indicates that it is widely distributed throughout the mouse brain in a pattern similar to that observed for mRNAs encoding the γ2 subunits.

$GABA_A$ receptor; Ligand-gated ion channel; Polymerase chain reaction

1. INTRODUCTION

The γ-aminobutyric acid (GABA)/benzodiazepine receptor complex is the site of action of a number of important pharmacological agents including benzodiazepines, barbiturates, some convulsants, and possibly ethanol (reviewed in [1–3]). Molecular cloning of a number of subunits of the $GABA_A$ receptor has revealed it to be a member of a ligand-gated ion channel superfamily which includes the nicotinic acetylcholine and glycine receptors [4–13]. Based on sequence relatedness several distinct subunit types of the $GABA_A$ receptor have been identified (α, β, γ, and δ), and show 20–40% amino acid sequence identity with each other [14]. A particular subunit type can be comprised of one to several members, which typically exhibits 60–80% identity to other members within that class.

In order to explore the possibility that additional molecular heterogeneity exists within this receptor complex we developed a strategy using the polymerase chain reaction (PCR)[15] to identify novel members of the $GABA_A$ receptor family. One of the cDNAs obtained from this approach appeared to be most closely related to the γ subunit type but was clearly distinct from both the γ1 [16] and γ2 [8] subunits, the only previously reported members of this class. Therefore, this subunit (named γ3) appears to be a new member of the γ subunit type. A preliminary desription of the identification of the γ3 subunit has been reported [22] and we here describe its complete sequence and regional localization.

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2. MATERIALS AND METHODS

2.1. *DNA synthesis*

Poly A+ mRNA was obtained from the brains of adult male Swiss Webster mice using the Fasttrack kit (Invitrogen, San Diego, CA) according to the protocol specified by the supplier. For cDNA synthesis mRNA (5 μg) was converted to first strand cDNA using the Amersham (Arlington Heights, IL) cDNA synthesis kit as directed by the manufacturer. The cDNA was phenol extracted and ethanol precipitated prior to use for PCR.

2.2. *PCR amplification*

PCR amplification of cDNA was performed using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) as directed by the supplier using a Perkin-Elmer Cetus DNA thermal cycler. Typically, 5 μl (1/200) of the cDNA reaction was used together with degenerate primers derived from the 2nd membrane spanning region (200 pmols) and 4th membrane spanning region (300 pmols). These regions are highly conserved between different $GABA_A$ receptor subunits. Conditions for the first 5 PCR cycles were 1 min at 94°C, 2 min at 37°C and 3 min at 72°C. This was followed by 25 cycles of 1 min at 94°C, 2 min at 48°C and 3 min at 72°C. Finally a 7 min incubation at 72°C was carried out to extend incomplete products. PCR products were digested with *Not*I and *Hin*dIII, preparatively electrophoresed in 1% agarose, and amplified DNA of the expected size (~450–600 bp) recovered using glassmilk (Geneclean) as described by the supplier (BIO101, La Jolla, CA). The DNA was cloned into *Not*I- and *Hin*dIII-digested Bluescript plasmid ('Stratagene'; La Jolla, CA) and resulting individual recombinant (white) colonies selected for DNA sequence analysis. For both one or four base DNA sequencing the dideoxy method [17] was used together with the modified T_7 polymerase (Sequenase; US Biochemical, Cleveland, OH) as directed by the supplier.

2.3. *Isolation and sequencing of γ3 cDNAs*

A 1.2 kb cDNA corresponding to the carboxy region of the mouse γ3 subunit was isolated by screening a BALB/C mouse brain cDNA library (generously provided by Y. Citri) using the gel-purified insert of clone MG54 as probe. To obtain the remaining upstream region 2 sequential PCR amplifications were performed using the same library. The first reaction used an insert-specific primer (MGγ3-2: 5'-CATGGTTAGCAGCGTGGTGAT-3') and a primer derived from the λgt11 vector (11E: 5'-CGGGCAGACATGGCCTGCCCGGTT-3') together with 5 μl (~10^7 pfu) of the library stock. PCR con-

ditions were as follows: 40 cycles consisting of 94°C for 1 min, 45°C for 2 min and 72°C for 3 min. This was then followed by a 72°C final extension for 7 min. One μl of a 10-fold dilution of the PCR reaction was used for a 2nd PCR reaction with the same vector primer and a 2nd insert-specific primer (MGγ3-4: 5'-GAGAAGCTTTGGGGTA-GCCATAGCTAGAGA-3') located slightly upstream of the first primer. The 2nd primer contained a *Hin*dIII site near the 5' end to allow cloning of the amplified material. PCR products greater than 500 bp were gel purified, digested with *Eco*RI and *Hin*dIII, and ligated into pBluescript (Stratagene). A colony containing the largest insert was identified and the DNA sequence of the insert (~650 bp) determined as described above. The original 1.2 kb cDNA and the upstream 650 bp cDNA, as predicted, contained 220 bp of overlapping sequence that was identical. Sequence alignment and analysis of the 2 overlapping cDNAs and alignment of the γ3 subunit with other $GABA_A$ subunits were carried out using PC Gene sequence analysis programs (Intelligenetics).

2.4. *Hybridization histochemistry*

Brains from C57 mice were removed rapidly, dipped in 2-methylbutane at −25°C to preserve morphology, and then frozen on dry ice. Ten micron parasagittal sections were cut on a cryostat and mounted onto gelatin-coated slides. All sections were stored at −80°C until use for hybridization histochemistry.

Tissue processing and in situ hybridization histochemistry were performed as previously described [18]. The γ2 oligonucleotide probe (40-mer) had the sequence 5'-GGTTGCTGATCTGGGACGAATATC-AATGGTAGGGGCAGGG-3'; this sequence is complementary to a region in the putative cytoplasmic loop between transmembrane domains 3 and 4 and recognizes both the γ2S and γ2L variants of the subunit (Sikela et al., submitted). The γ3 probe (40-mer) had the sequence 5'-CGCGATCATCACAGGTGGTGGGGGCCTCATGTC-CAGTAGA-3' and was also complementary to a region in the cytoplasmic loop. Both probes were derived from mouse cDNA sequences. When used for Northern blot analysis, brain mRNAs of 2.2 and 1.7 kb hybridized with the γ3 probe. Probes were labeled on the 3' end using terminal deoxynucleotidyl transferase (Bethesda Research Labs) and [^{35}S]deoxyadenosine 5'-(α-thio)triphosphate (NEN) as previously described [18].

3. RESULTS AND DISCUSSION

Comparison of the deduced amino acid sequences of the $GABA_A$ receptor cDNAs with those of the acetylcholine and glycine receptors reveals a common evolutionary origin [14]. Among the features conserved between these proteins are 4 proposed membrane spanning regions (MSRs) which occur at similar positions in subunits within this receptor superfamily [14](Fig. 1A). In addition, the size and, to varying degrees, the sequence of each MSR is conserved between different subunits. Differences between subunits are, to a large extent, found in the region thought to represent the large cytoplasmic loop between the 3rd and 4th MSRs [14]. We have used this information to develop a PCR strategy designed to identify novel members of the $GABA_A$ receptor family. Specific PCR primers were synthesized that corresponded to the conserved regions of the 2nd and 4th MSRs of several known $GABA_A$ receptor subunits (Fig. 1B). These primers were used for PCR amplification with total mouse brain cDNA. Amplified DNA of the predicted size was then isolated (Fig. 1C) and subcloned. Fifty-five individual recombinants were picked and analyzed by single base DNA sequencing to quickly categorize the clones. Eight different categories of clones were identified and one member of each was then sequenced using conventional four-base sequencing. Each of these classes either corresponded to known $GABA_A$ subunits (α, β, or γ) or contained amino acid sequences typically found in conserved regions of $GABA_A$ subunits.

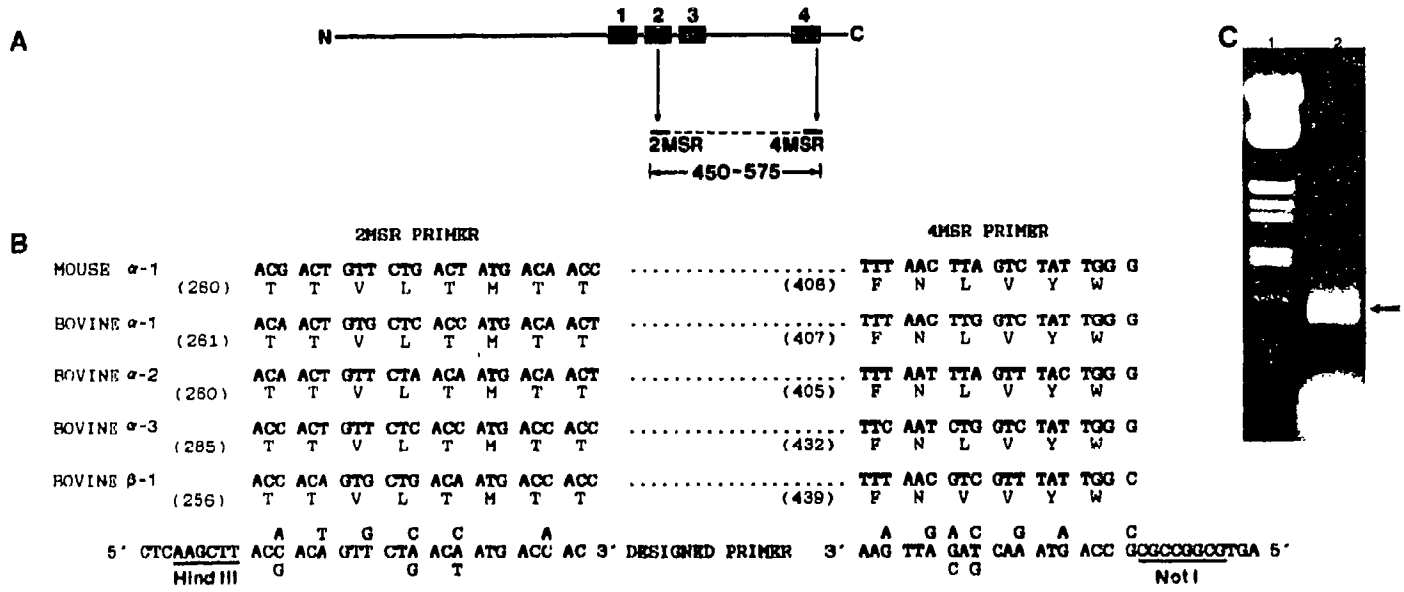

Fig. 1. (A) Conserved features of subunits of the $GABA_A$ receptor. Four proposed MSRs are boxed. Based on previously reported subunit sequences, the predicted size range of PCR products that would be expected to encode $GABA_A$ receptor subunits is indicated. (B) Primer design. The PCR primers were derived from conserved amino acid sequences from the 2nd and 4th proposed MSRs of the $GABA_A$ receptor. Sequences used were from the mouse α1 subunit [19] and bovine α1, α2, α3, and β1 subunits [4,5]. (C) Gel electrophoresis of PCR products. Lane 1, λ/*Hin*dIII/*Eco*RI DNA (size marker); lane 2, 20 μl (1/5) of the PCR reaction. Arrow denotes PCR-amplified DNA of the expected size (~450-600 bp).

Alignment of the deduced amino acid sequence of one cDNA, MG54, with the corresponding regions of several known $GABA_A$ subunits indicated that it was more closely related to the two γ subunits than to other subunits, and therefore was provisionally named $\gamma 3$. Isolation and sequencing of overlapping cDNAs encoding the complete $\gamma 3$ subunit (Fig. 2) indicates that the mature protein is 450 amino acids in length ($M_r = 52\,507$ Da) and that several structural motifs commonly associated with $GABA_A$ receptor subunits are present [14]. These include 4 proposed membrane spanning regions (MSRs) (Fig. 3), a large cytoplasmic

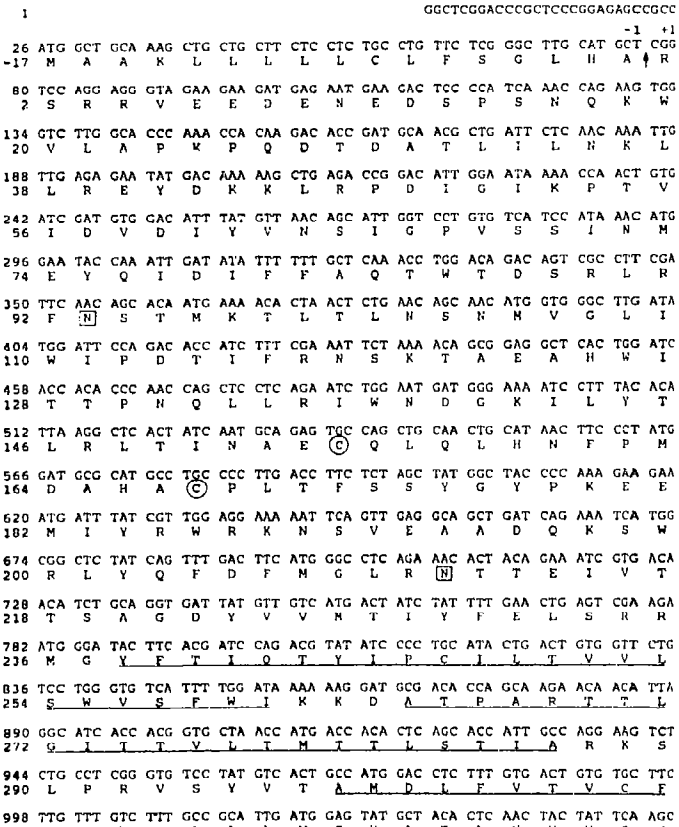

Fig. 2. cDNA and deduced amino acid sequence of the mouse $\gamma 3$ subunit. Putative signal sequence cleavage site is indicated by a vertical arrow. Sites for *N*-linked glycosylation are indicated by boxes. The 2 cysteines thought to form a disulfide bridge found in ligand-gated ion channel receptors are circled. Four putative membrane spanning regions are underlined.

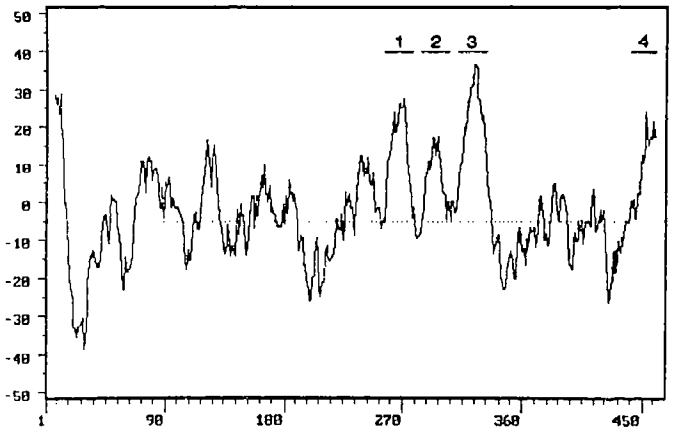

Fig. 3. Hydropathy profile of the $\gamma 3$ subunit. A hydropathy profile of the $\gamma 3$ subunit was generated using the Soap subprogram in PC Gene with a window of 13 amino acids. Overlined regions indicate the 4 putative membrane spanning regions.

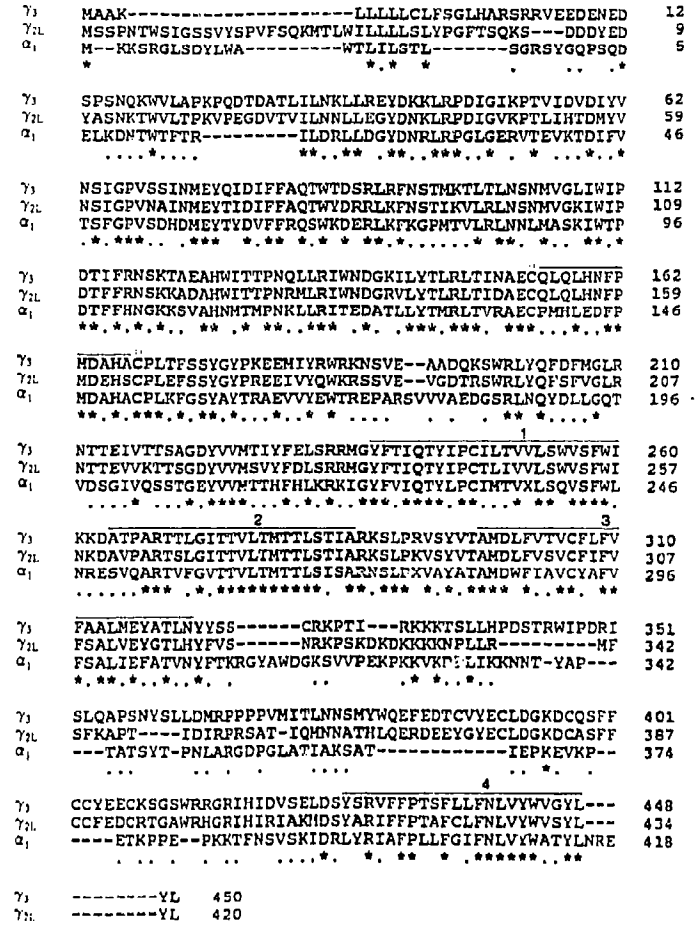

Fig. 4. Alignment of $\gamma 3$, $\gamma 2L$, and $\alpha 1$ subunits. The deduced amino acid sequences of the mouse $\gamma 3$, $\gamma 2L$ [21] and $\alpha 1$ [19] subunits were aligned using the Clustal program of PC Gene. The 2 cysteines involved in the putative disulfide bridge are denoted by circles and proposed transmembrane regions are overlined and numbered. Asterisks indicate identity of the 3 sequences and dots indicate positions moderately conserved among the 3 sequences.

loop region between the 3MSR and 4MSR, a putative signal sequence, and 2 cysteines in the predicted extracellular domain that are thought to form a disulfide bridge. Additional conserved features of the $\gamma 3$ sequence include 2 potential asparagine-linked glycosylation sites, a proline in the middle of the first MSR, and a high percentage of serine and threonine residues in the 2nd MSR. The indicated start ATG and its flanking sequences are identical to the 8 amino acid Kozak consensus sequence for translational start codons [20] and therefore this ATG very likely represents the start of translation. Finally, the $\gamma 3$ subunit appears to be slightly more distantly related to the $\gamma 1$ and $\gamma 2$ subunits than they are to each other [16] and contains a larger cytoplasmic loop region than is found in either the $\alpha 1$ or $\gamma 2L$ subunits (Fig. 4).

Results of in situ hybridization histochemistry indicate that the mRNA encoding the $\gamma 3$ subunit is widely distributed throughout the mouse brain (Fig. 5A). Signals of greatest intensity were observed over the granule cell layer of the cerebellum, the mitral cell layer of the olfactory bulb, and the hippocampus. In the latter region, the mRNA was localized in the pyramidal cell layer of CA1, 2, and 3 and the granule cell layer of the dentate gyrus. Signals of uniform and lower intensity were observed over the cortex, thalamus, inferior and superior colliculi, caudate putamen, septum, hypothalamus, brainstem, and islands of Calleja. Significant levels of hybridization were not observed after hybridization with a sense probe (not shown), supporting the specificity of the signal. While the localization of the $\gamma 3$ subunit mRNA is very similar to the pattern observed following hybridization with the $\gamma 2$ subunit probe (Fig. 5B), the expression of the $\gamma 3$ subunit mRNA is more uniform.

Differences in the expression of the $\gamma 3$ and $\gamma 2$ subunit mRNAs were more apparent when the sections were observed at higher magnification following autoradiography (Fig. 6). In the cerebellum, differences in hybridization were particularly apparent in the Purkinje cell layer. In this region very few grains were detected over cells following hybridization with the $\gamma 3$ subunit probe. In contrast, a higher grain density was observed with the $\gamma 2$ subunit probe. Moreover, cells positive for the $\gamma 3$ subunit mRNA could not be detected in the deep cerebellar nuclei, a region in which the $\gamma 2$ subunit is abundant (not shown).

While the functional characteristics of the $\gamma 3$ subunit are not yet known, another member of the γ subunit class, $\gamma 2$, has been shown to be critical for potentiation of the GABA response by benzodiazepines [8] and ethanol [23]. Therefore, it is possible that the $\gamma 3$ subunit may also confer important functional properties on the $GABA_A$ receptor. While testing of this possibility must await additional experiments, the identification of the $\gamma 3$ subunit further increases the molecular heterogenei-

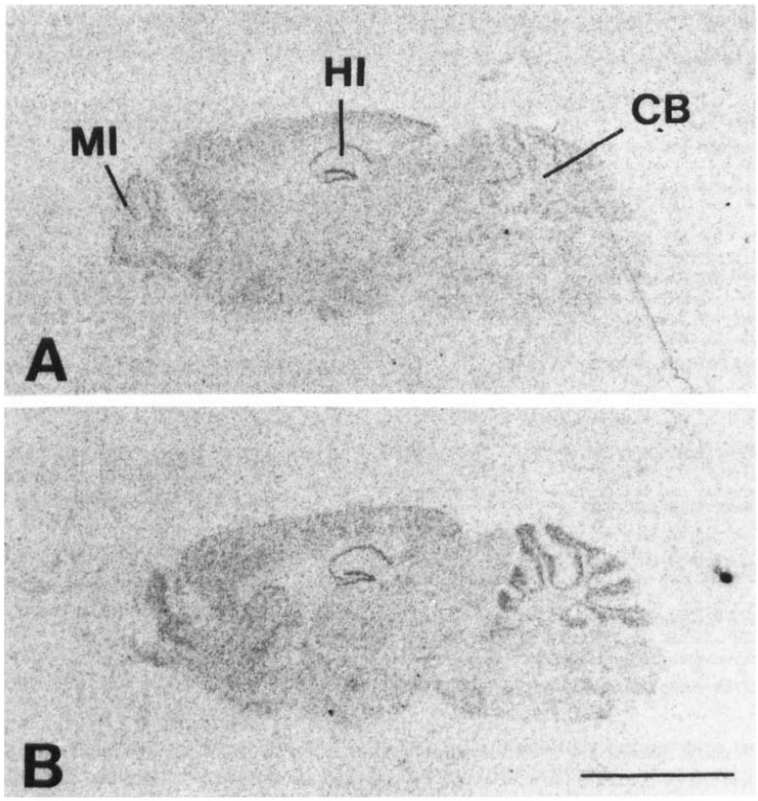

Fig. 5. Regional distribution of mRNAs encoding γ subunits of the $GABA_A$ receptor complex in the mouse brain. Sections were hybridized with probes specific for the $\gamma 3$ (A) and $\gamma 2$ (B) subunit mRNAs. Following hybridization, the sections were placed against Kodak X-AR film and exposed 4 weeks for autoradiography. Bar = 5 mm.

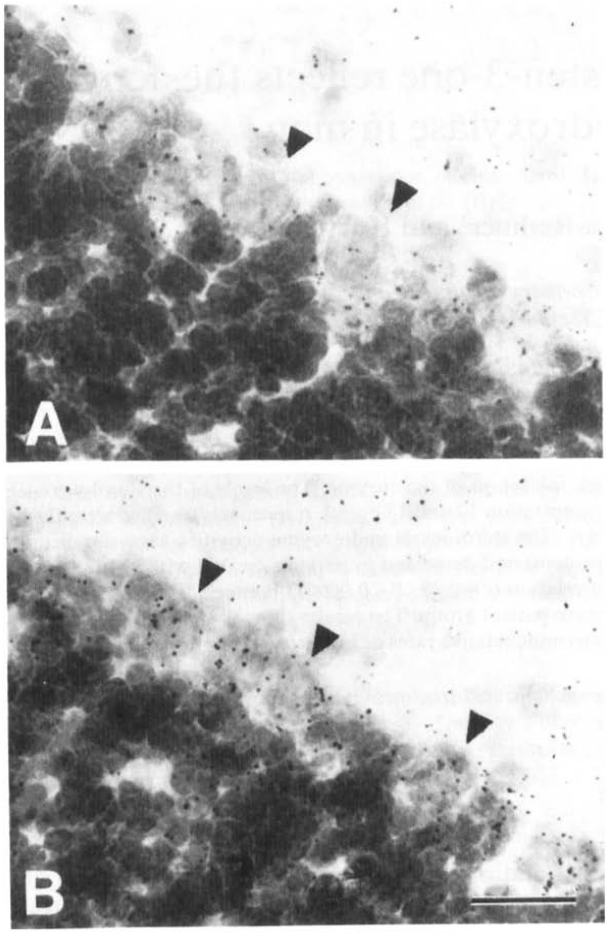

Fig. 6. Localization of $GABA_A$ receptor subunit mRNAs in the cerebellar cortex. Phase contrast views of sections hybridized with the $\gamma 3$ (A) and $\gamma 2$ (B) subunit probes. The sections were processed for liquid emulsion autoradiography and exposed 7 weeks. Arrowheads indicate Purkinje neurons. Bar = 50 μm.

ty and potential functional diversity of this already complex receptor/ion channel.

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