

# Pharmacological characterization and region-specific expression in brain of the $\beta 2$ - and $\beta 3$ -subunits of the rat GABA<sub>A</sub> receptor

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The cDNA for a third  $\beta$ -subunit of the rat GABA<sub>A</sub> receptor has been cloned using another  $\beta$ -subunit, which we had previously cloned [(1989) FEBS Lett. 246, 145–148], as a probe. The ~8-kb cDNA for this  $\beta$ -subunit (termed  $\beta 2$ ) encodes a protein of 474 amino acid residues that shares ~80% sequence identity with the rat and bovine  $\beta 1$ - and  $\beta 3$ -subunits. Coexpression of the cloned  $\beta$ -subunit cDNA with the  $\alpha 1$ -subunit cDNA of the rat GABA<sub>A</sub> receptor in *Xenopus* oocytes produced a functional receptor and Cl<sup>−</sup> channel with pharmacological characteristics of a GABA<sub>A</sub> receptor. In contrast to interchanging  $\alpha$ -subunits [(1988) Nature 335, 76–79], exchange of  $\beta 2$ - or  $\beta 3$ -subunits in an  $\alpha 1/\beta$  receptor complex did not markedly alter the pharmacological properties of expressed receptors. In situ hybridization histochemistry with synthetic subunit-specific oligodeoxynucleotide probes revealed a region-specific expression of  $\alpha 1$ -,  $\beta 2$ - and  $\beta 3$ -subunit mRNAs in the rat central nervous system. These observations provide an additional molecular basis for the functional heterogeneity in the GABA<sub>A</sub> receptor complex.

Receptor subtype; cDNA cloning; Expression; Hybridization histochemistry, in situ; (*Xenopus* oocyte)

## 1. INTRODUCTION

The  $\gamma$ -aminobutyric acid (GABA<sub>A</sub>) receptor is a ligand-gated Cl<sup>−</sup> channel through which GABA and the anxiolytic benzodiazepines mediate many of their effects (for a review, see [1]). Both benzodiazepines and barbiturates potentiate the increase in neuronal hyperpolarization underlying the inhibition mediated by GABA in the CNS. Biochemical evidence indicates that the binding sites for benzodiazepines and muscimol (a GABA<sub>A</sub> agonist) reside in two major proteins, the  $\alpha$ - and  $\beta$ -subunits, respectively, of the purified GABA<sub>A</sub> receptor complex [2–5]. Recently, the primary structures of the bovine GABA<sub>A</sub> receptor  $\alpha$ - and  $\beta$ -subunit have been deduced from cloned complementary DNAs [6,7]. To date, bovine cDNAs encoding three different  $\alpha$ -subunit subtypes (designated 1–3) have been isolated [7], providing evidence for the concept of GABA<sub>A</sub> receptor heterogeneity. Coexpression of any  $\alpha$ -subunit with a single bovine  $\beta$ -subunit in mammalian cells or *Xenopus* oocytes results in GABA<sub>A</sub> receptors which display many of the pharmacological properties of their neural counterparts, including potentiation by barbiturates. Furthermore, the three  $\alpha$ -subunits are differentially expressed in brain and produce, when expressed with the bovine  $\beta$ -subunit in vitro, receptor subtypes which can be distinguished by their apparent sensitivity to GABA [7–10].

We have previously described the cDNAs encoding the  $\alpha 1$ - and a  $\beta$ -subunit of the rat GABA<sub>A</sub> receptor [11]. Based on amino acid sequence identity, our rat  $\beta$ -subunit and the bovine  $\beta$ -subunit were not equivalent. In the same study, we also reported the isolation of a clone for a third  $\beta$ -subunit. More recently, cDNAs encoding the rat equivalent of the bovine  $\beta 1$ -subunit and a third rat  $\beta$ -subunit have been identified [12]. To keep the nomenclature for these multiple  $\beta$ -subunits clear, we have termed our previously published  $\beta$ -subunit (clone  $\beta 2a$ ; [11]),  $\beta 3$ , as was subsequently described in [12]. Here we report the pharmacological characterization of the third  $\beta$ -subunit, termed  $\beta 2$  to be consistent with the terminology of Ymer et al. [12]. In this report we show that, while the rat  $\beta 2$ - and  $\beta 3$ -subunits are differentially expressed in the brain, the receptor complexes formed when each of these  $\beta$ -subunits is coexpressed with the rat  $\alpha 1$ -subunit in *Xenopus* oocytes do not differ in their sensitivity to GABA.

## 2. MATERIALS AND METHODS

### 2.1. Isolation and sequencing of cDNA clones

An adult rat cortex cDNA library constructed in the pcDV1 plasmid vector was used [11]. Recombinants (~7 × 10<sup>6</sup>) were screened by Southern blot analysis [13] at moderate stringency (hybridizing and washing in 3 × SSC at 60°C) using as a probe a <sup>32</sup>P-labeled 1.5-kb *NruI*-*HpaI* fragment from the rat GABA<sub>A</sub>  $\beta 3$ -subunit clone, RC $\beta 2a$  [11]. Four clones distinct from previously described  $\beta$ -subunits [6,11] were isolated, one of which, an ~8-kb clone (RC $\beta 5e$ ), was determined to be full-length by restriction and sequence analysis. Overlapping restriction fragments were isolated, subcloned into M13, and sequenced in both directions by the Sanger chain-termination method [14] us-

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ing the modified T7 polymerase [15] (Sequenase; US Biochemical Corp., Cleveland, OH).

### 2.2. Expression of rat GABA<sub>A</sub> receptor subunit mRNA in *Xenopus* oocytes

Preparation of pGEM-7Zf(+) plasmid vectors (Promega, Madison, WI) for in vitro RNA transcription of the  $\alpha 1$ - and  $\beta 2$ -subunit cDNAs of the rat GABA<sub>A</sub> receptor has been previously described [11]. The entire coding sequence for the  $\beta 2$ -subunit cDNA (clone RC $\beta 5e$ ) contained within a 1.85-kb *Bam*HI-*Xba*I fragment was similarly subcloned into pGEM-7Zf(+). Since there were no convenient restriction sites in the 78-bp 5'-untranslated sequence of this cDNA, the *Bam*HI site in the intron of the SV40 promoter region of the pcDV1 vector (101-bp upstream from the *Pst*I site which normally defines the 5' end of a cDNA) was used; the *Xba*I site is 307-bp downstream from the stop codon in the 3'-untranslated sequence of the cDNA. Capped RNA transcripts were obtained from linearized templates using SP6 polymerase and 7mG(5')ppp(5')G according to the manufacturer's instructions. Transcripts were mixed as indicated at a final concentration of 250–300 ng/ml and ~50 nl was injected into each oocyte (defolliculated). Oocytes were maintained in Modified Barth's solution, pH 7.4 (MBS) [16] containing penicillin and streptomycin at 18°C and assayed for GABA-induced Cl<sup>-</sup> current at 72–96 h. All currents were recorded at a voltage-clamped potential of -60 mV. Drugs were applied by bath perfusion over 2 min to ensure saturating responses. Antagonists or potentiating drugs were applied at the indicated concentrations 1.5–2 min prior to the addition of GABA. To avoid the prolonged desensitization observed after applications of high doses of GABA, oocytes were perfused with MBS for 20 min between applications. In general, individual oocytes were carried through each series of drug applications as indicated. Data shown are representative of those obtained with 2–3 oocytes using different batches of synthetic subunit-specific mRNAs. EC<sub>50</sub>s in dose-response experiments were determined from pooled, normalized data by non-linear least-squares regression analysis according to Marquardt algorithms using a commercially available data analysis software (GraphPAD InPlot, San Diego, CA).

### 2.3. Probes

Oligodeoxynucleotide probes were synthesized on an Applied Biosystems 380A DNA Synthesizer. For each rat GABA<sub>A</sub> receptor subunit two 48-bp probes complementary to mRNA sequences encoding segments of the putative signal peptides and cytoplasmic domains ([11] and this report) were prepared. The probes were complementary to sequences encoding amino acids -3 to 13 ( $\alpha 1a$ ) and 323–338 ( $\alpha 1b$ ), -23 to -8 ( $\beta 2a$ ) and 325–340 ( $\beta 2b$ ), and -23 to -8 ( $\beta 3a$ ) and 325–340 ( $\beta 3b$ ) of the rat  $\alpha 1$ -,  $\beta 2$ - and  $\beta 3$ -subunits, respectively. As a control for non-specific labeling, two synthetic message sense probes, exactly complementary to the  $\alpha 1b$  and  $\beta 2b$ -subunit antisense probes, were also prepared. Probes were labeled on their 3'-ends with deoxyadenosine triphosphate in the  $\alpha$  position with <sup>35</sup>S (1300 Ci/mmol, New England Nuclear) for in situ hybridization histochemistry using terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) to a specific activity between 3500–5000 Ci/mmol.

### 2.4. In situ hybridization histochemistry

Serial 12- $\mu$ m sections of adult, male rat brains were cut on a cryostat microtome at -18°C, thaw-mounted onto twice-gelatin-coated slides and stored at -80°C until hybridization. The overall procedure for in situ hybridization histochemistry has been outlined in detail [17]. <sup>35</sup>S-Labeled probe (1–2  $\times$  10<sup>6</sup> dpm in 45  $\mu$ l hybridization buffer containing 4  $\times$  SSC, 50% formamide, 1  $\times$  Denhardt's, 10% dextran sulfate, 250  $\mu$ g/ml yeast tRNA, 500  $\mu$ g/ml herring sperm DNA and 0.1 M dithiothreitol) was applied to the sections for 18 h at 37°C. After hybridization the sections were washed 4  $\times$  15 min at 55°C in 1  $\times$  SSC followed by 2  $\times$  1 h washes at room temperature, rinsed quickly in distilled water, dried and exposed to Hyperfilm- $\beta$ max (Amersham) for 1 week at room temperature.

## 3. RESULTS AND DISCUSSION

The nucleotide and deduced amino acid sequence of clone RC $\beta 5e$  (data not shown) are identical to the recently published rat  $\beta 2$ -GABA<sub>A</sub> receptor subunit [12]. The predicted amino acid sequence shows 80% identity with the bovine [6], rat [11,12] and human [19] GABA<sub>A</sub> receptor  $\beta 1$ - and  $\beta 3$ -subunits, but only 30–40% amino acid identity with the GABA<sub>A</sub> receptor  $\alpha 1$ –3-subunits [7,11]. Sequence similarity between the  $\beta$ -subunits is highest in the 4 hydrophobic putative membrane-spanning domains, M1–M4. In contrast, the extreme N-terminal signal peptides and the sequence between M3 and M4 are quite divergent. For example, of the 97 amino acid differences found between the rat  $\beta 2$ - and  $\beta 3$ -subunit sequences, 55 (57%) occur in the M3–M4 loop.

Injection of synthetic rat  $\alpha 1$  mRNA in combination with either synthetic rat  $\beta$ -subunit mRNA produced functional Cl<sup>-</sup> channel complexes in the oocyte membrane (fig.1A). Response to the addition of GABA was immediate and sustained at low doses (generally <EC<sub>50</sub>); however, at higher doses both subunit combinations exhibited marked desensitization during agonist perfusions. Injection of  $\beta 2$ -subunit mRNA alone did not produce a detectable GABA response; this was also true of rat  $\alpha 1$ - and  $\beta 3$ -subunit transcripts [11]. The response to GABA in oocytes expressing  $\alpha 1/\beta 2$ - or  $\alpha 1/\beta 3$ -subunits was markedly attenuated in the presence of the GABA<sub>A</sub> antagonist, bicuculline (fig.1A). Similar results (data not shown) were observed in the presence of picrotoxin (5  $\mu$ M) which specifically blocks Cl<sup>-</sup> conductance through the GABA<sub>A</sub> receptor complex. Barbiturates, such as sodium pentobarbital, have been shown to enhance the actions of GABA by increasing mean channel opening time [19]. Pentobarbital (10  $\mu$ M) potentiated GABA-stimulated Cl<sup>-</sup> conductance approximately 5–6-fold in oocytes expressing  $\alpha 1/\beta 2$  or  $\alpha 1/\beta 3$  (fig.1A). This concentration of pentobarbital alone caused little (<10 nA) or no Cl<sup>-</sup> conductance. Higher concentrations of pentobarbital (25–50  $\mu$ M) directly stimulated Cl<sup>-</sup> conductance (data not shown), as has also been shown for cultured spinal neurons [20] and cloned bovine GABA<sub>A</sub> receptor subunits [7,8]. In oocytes with either subunit combination the threshold of sensitivity for potentiation was ~1  $\mu$ M pentobarbital which caused a 1.5–2-fold increase in conductance (data not shown). In contrast, no significant enhancement of the GABA response was observed with diazepam (10–50  $\mu$ M; data not shown).  $\alpha 1/\beta 2$  or  $\alpha 1/\beta 3$  heteromeric receptors displayed an identical rank order of agonist-stimulated Cl<sup>-</sup> conductance as previously demonstrated for native CNS GABA<sub>A</sub> receptors [21]. At equivalent doses, muscimol was more potent than GABA in increasing Cl<sup>-</sup> current with either  $\beta$ -subunit tested (fig.1B). Isoguvacine, an additional GABA<sub>A</sub> agonist, was less potent than GABA while the

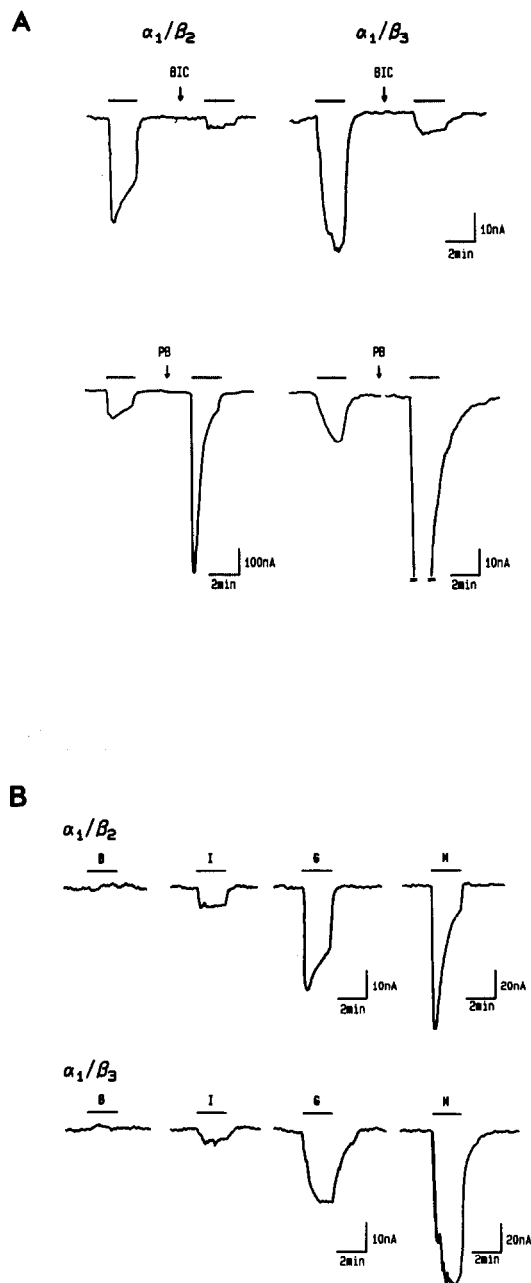


Fig.1. (A) Expression of cloned GABA<sub>A</sub> receptors in *Xenopus* oocytes injected with either  $\alpha 1/\beta 2$  (left) or  $\alpha 1/\beta 3$  (right) synthetic mRNAs. Response to GABA (solid bars) was measured at a voltage-clamped membrane potential of  $-60$  mV. Concentrations of GABA in the bath perfusion ( $1.0 \mu\text{M}$  for  $\alpha 1/\beta 2$  and  $0.5 \mu\text{M}$  for  $\alpha 1/\beta 3$ ) were chosen to generate equivalent amplitudes of  $\text{Cl}^-$  current based upon the slight differences in sensitivity to GABA. For blockade of response to GABA,  $5 \mu\text{M}$  bicuculline methobromide (top) was applied 1.5–2 min prior to the addition of GABA. For potentiation of response to GABA (bottom),  $10 \mu\text{M}$  pentobarbital was similarly perfused prior to the addition of GABA. (B) Agonist-stimulated increase in  $\text{Cl}^-$  current in cloned GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. Oocytes expressing either  $\alpha 1/\beta 2$ - (top) or  $\alpha 1/\beta 3$ - (bottom) subunits were perfused for 2 min (solid bar) in the presence of the GABA<sub>B</sub> agonist, baclofen (B), GABA (G), or the GABA<sub>A</sub>-selective agonist isoguvacine (I) and muscimol (M). The concentrations of agonists applied were  $1.0 \mu\text{M}$  and  $0.5 \mu\text{M}$  for  $\alpha 1/\beta 2$  and  $\alpha 1/\beta 3$ , respectively.

GABA<sub>B</sub> agonist, baclofen, was without effect.  $\alpha 1/\beta 2$ - and  $\alpha 1/\beta 3$ -subunit combinations displayed similar sensitivities to GABA (fig.2). The  $\text{EC}_{50}$ s for GABA-stimulated increases in  $\text{Cl}^-$  conductance in oocytes expressing  $\alpha 1/\beta 2$  and  $\alpha 1/\beta 3$  were  $5.8 \mu\text{M}$  ( $n=3$ ) and  $4.3 \mu\text{M}$  ( $n=4$ ), respectively; Hill coefficients for both  $\alpha/\beta$  combinations were identical, slope =  $1.1 \pm 0.1$ . Thus, while the  $\beta 2$ - and  $\beta 3$ -subunit cDNAs encode proteins capable of forming GABA<sub>A</sub> receptor complexes with the  $\alpha 1$ -subunit, interchanging the  $\beta$ -subunits does not produce distinguishable differences in the pharmacological properties of these complexes when expressed in oocytes.

To compare the regional cellular distribution of the mRNAs encoding the  $\alpha 1$ -,  $\beta 2$ - and  $\beta 3$ -subunits in the CNS, in situ hybridization histochemistry on sections of rat brain was performed using oligodeoxynucleotide probes specific for each subunit. No specific hybridization was observed in myelinated regions such as the corpus callosum; in addition, no labeling above background was detected using message sense probes as controls (data not shown). Our findings concur with, and extend those of others [9,24,25] in that mRNA encoding GABA<sub>A</sub> receptor subunits is detectable in brain regions that also contain benzodiazepine and muscimol binding sites.  $\alpha 1$  mRNA was prominently expressed throughout the CNS, including neurons of the olfactory bulb, anterior olfactory nuclei, islands of Calleja, neocortex, globus pallidus, CA1–3 hippocampal pyramidal cells, granule layer of the dentate gyrus, thalamic nuclei, inferior colliculus, geniculate nuclei, amygdala, pontine nuclei, cerebellum (granular, Purkinje and molecular cell layers) and cerebellar nuclei. Fig.3a illustrates labeling of a representative section through the parietal cortex, hippocampal formation and thalamic nuclei. In the cortex, a slight laminar distribution was evident;  $\alpha 1$  mRNA was more prominent in laminae IV and VI. With few exceptions, the distribution of  $\beta 2$  mRNA (fig.3c,e) closely matched that of  $\alpha 1$  transcripts.

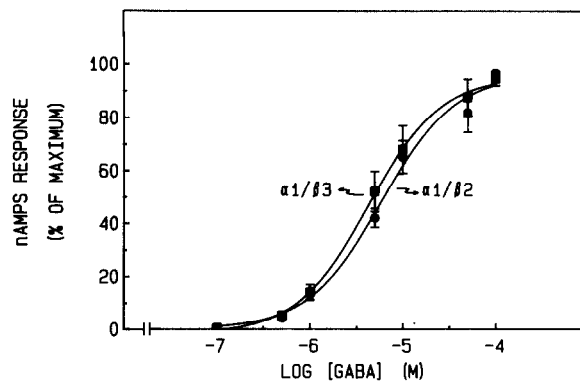


Fig.2. Dose-response relationship for the activation of cloned GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. Oocytes expressing either  $\alpha 1/\beta 2$ - (●) or  $\alpha 1/\beta 3$ - (■) subunits were perfused in the indicated concentrations of GABA for 2 min. Maximum current amplitude varied between 400–1300 nA.

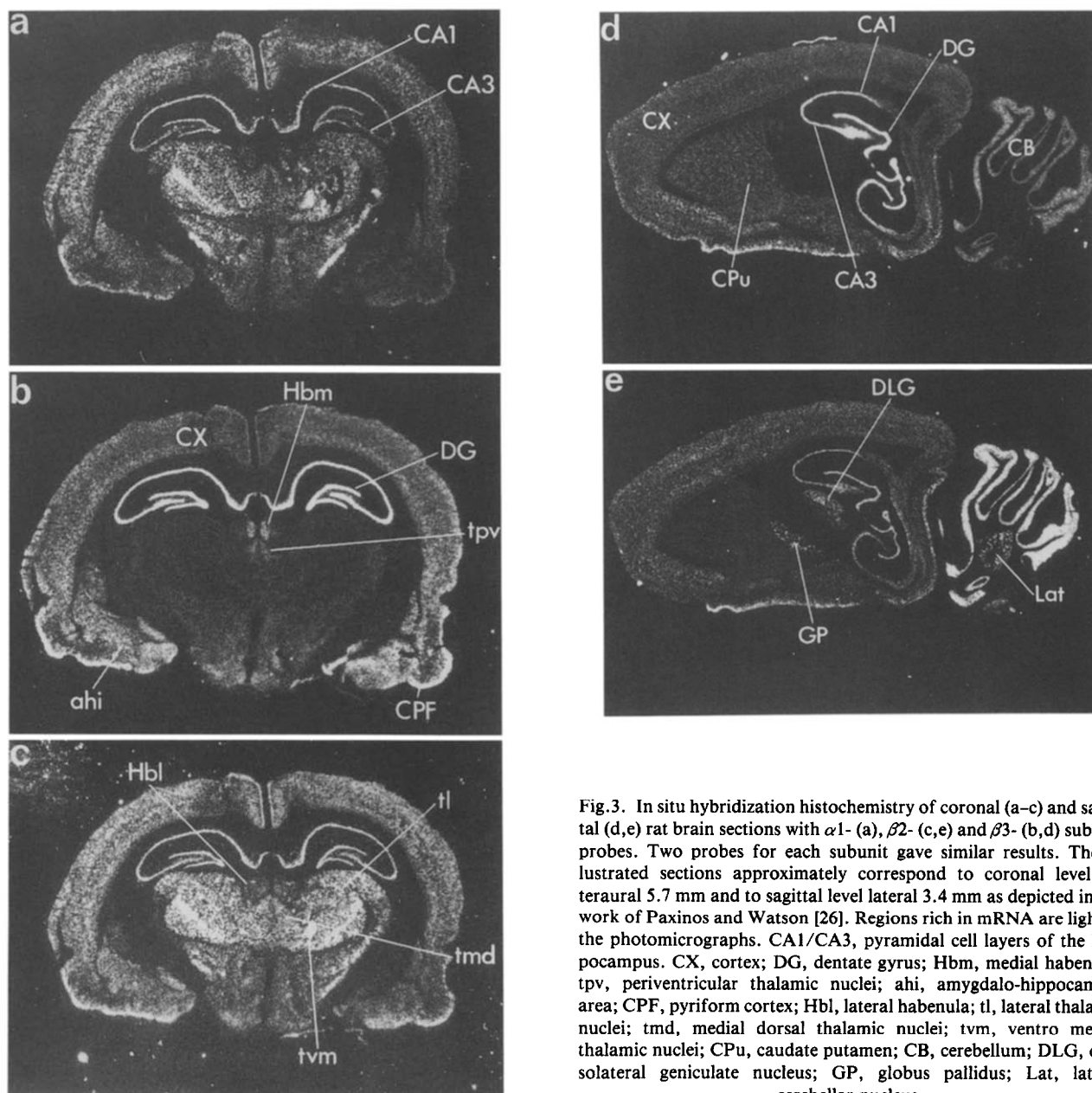


Fig.3. In situ hybridization histochemistry of coronal (a-c) and sagittal (d,e) rat brain sections with  $\alpha 1$ - (a),  $\beta 2$ - (c,e) and  $\beta 3$ - (b,d) subunit probes. Two probes for each subunit gave similar results. The illustrated sections approximately correspond to coronal level interaural 5.7 mm and to sagittal level lateral 3.4 mm as depicted in the work of Paxinos and Watson [26]. Regions rich in mRNA are light in the photomicrographs. CA1/CA3, pyramidal cell layers of the hippocampus. CX, cortex; DG, dentate gyrus; Hbm, medial habenula; tpv, periventricular thalamic nuclei; ahi, amygdalo-hippocampal area; CPF, pyriform cortex; Hbl, lateral habenula; tl, lateral thalamic nuclei; tmd, medial dorsal thalamic nuclei; tvn, ventro medial thalamic nuclei; CPu, caudate putamen; CB, cerebellum; DLG, dorsolateral geniculate nucleus; GP, globus pallidus; Lat, lateral cerebellar nucleus.

The most conspicuous  $\beta 3$  mRNA levels were observed in the cortex, caudate, hippocampal formation, medial habenula, periventricular thalamic nucleus, pyriform cortex, amygdalo-hippocampal area and cerebellum (fig.3b,d). Cortical labeling appeared more pronounced in the superficial and deeper layers. Notable differences in the distribution of  $\beta 2$  and  $\beta 3$  transcripts were apparent. For example, while  $\beta 2$  mRNA was expressed in the geniculate nucleus, globus pallidus, most thalamic nuclei and lateral cerebellar nuclei,  $\beta 3$  mRNA was virtually undetectable in these areas. Thus, both  $\alpha$ - [9] and  $\beta$ -subunit subtypes appear to be heterogeneously expressed in the CNS.

GABA<sub>A</sub> receptor complexes formed upon coexpression of  $\alpha$ - and  $\beta$ -subunits possess many, but not all, of the functional properties of the native receptor ([7,8,11] and this report). Most noticeably, they do not demonstrate Hill coefficients close to 2.0 and they appear to lack the potentiation of response to GABA by benzodiazepines. Our data suggest that the GABA-sensitivity of the  $\alpha/\beta$  complex appears to be conferred by the  $\alpha$ - [7] and not the  $\beta$ -subunit. The insensitivity to benzodiazepines may have been resolved by the recent cloning and expression of the cDNA for an additional GABA<sub>A</sub> receptor subunit, termed  $\gamma 2$  [22]. Cotransfection of  $\gamma 2$ -,  $\alpha 1$ - and  $\beta 1$ -subunit cDNAs into mammalian

cells produce GABA<sub>A</sub> receptors which display high-affinity binding for central benzodiazepine receptor ligands.

GABA<sub>A</sub> receptor complexes with an  $\alpha/\beta$  configuration may still be functionally relevant, since the binding sites for GABA<sub>A</sub> agonists and benzodiazepines are not always associated [23], and not all GABA<sub>A</sub> receptors are benzodiazepine-sensitive [19]. Our results with in situ hybridization histochemistry begin to address the heterogeneous distribution of  $\alpha$ - and  $\beta$ -subunits in the CNS. This regional specificity may reflect underlying functional variations such as dictating neuronal responsiveness to GABAergic input. Further studies are underway to determine the cellular colocalization of individual subunits that comprise functional GABA<sub>A</sub> receptor complexes. The use of subunit-specific probes at the in situ and cellular level opens the way for studies on the regulation of GABA<sub>A</sub> receptor gene expression to assess receptor synthesis in normal and pathological conditions.

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