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Multiple $GABA_A$ receptor α subunit mRNAs revealed by developmental and regional expression in rat, chicken and human brain

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$GABA_A$ receptor α subunit transcripts were detected by Northern analysis of rat, chicken and human brain mRNA using a series of ^{32}P-labelled antisense RNA probes derived from human α_1 subunit cDNAs. These α subunit mRNAs differ in their distribution among various brain regions in the rat and at least one species is detected primarily in fetal brain. $GABA_A$ receptor α_1 subunit probes encoding the putative extracellular domain detect at least five α subunit transcripts in rat brain, whereas probes encoding the putative intracellular domain detect only two mRNAs. These data suggest the presence in brain of multiple $GABA_A$ receptor α subunits having homologous extracellular domains and whose expression is regionally and developmentally regulated. These α subunit transcripts may encode proteins that comprise $GABA_A$ isoreceptors differing in their pharmacological and physiological properties.

$GABA_A$/benzodiazepine receptor; Multiple α subunit mRNA; Isoreceptor; Regional expression; Developmental expression; (Rat, Chicken, Human, Brain)

1. INTRODUCTION

The $GABA_A$/benzodiazepine receptor is an oligomeric protein complex comprised of homologous but unique membrane-spanning subunits (α, β, γ) which form an integral chloride ion channel [1,2]. Recognition sites for a number of important psychoactive drugs including anesthetic barbiturates, benzodiazepine receptor agonists, antagonists, inverse agonists, naturally occurring pregnane steroids and neuropeptides exist on one or more of these subunits (cf. [2] for review) and serve to modulate GABA-activated Cl^- ion conductance allosterically [3]. Several lines of evidence suggest that the benzodiazepine and GABA recognition sites reside on the α and β subunits respectively [2–4]. Early pharmacological data suggested the presence of $GABA_A$/benzodiazepine receptor heterogeneity among varoius brain regions [2] and more recently molecular cloning studies have confirmed in several species [4,5] the presence of multiple cDNAs for both the α and β subunits. Coexpression of these subunits in various combinations yield $GABA_A$ receptors with unique gating properties and affinities for GABA [6,7], suggesting a molecular basis for the apparent receptor heterogeneity. Little is known, however, about the differential expression of these isoreceptors in various brain regions. Recently, we have isolated human $GABA_A$ receptor α_1 subunit cDNAs [8] which are highly homologous to those of bovine and rat. Using radiolabelled cRNAs to study the developmental and regional expression of α subunit gene(s) in rat brain [8], we now report: (1) the presence of multiple $GABA_A$ receptor α subunit transcripts in rat, chicken and human brain, (2) the differential developmental expression of these transcripts in rat and chick brain and (3) the apparent homology of these mRNAs to sequences encoding the putative extracellular, but not intracellular, domains of the deduced α subunit protein. These data provide further evidence in several species for multiple $GABA_A$ isoreceptors which are regionally distributed in brain.

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2. MATERIALS AND METHODS

A full-length $GABA_A$ receptor α_1 subunit cDNA was isolated from a human cerebellum cDNA library constructed in λgt11 using oligodeoxynucleotides derived from the bovine $GABA_A$ receptor α_1 subunit [8]. The nucleotide sequence of this human α_1 subunit cDNA clone is nearly identical (>96% and 98% homologous) to the bovine and rat α_1 subunit cDNAs respectively [4,5,8]; but less than 50% homologous to the human or rat β_1, β_2 or β_3 subunit cDNAs [8–10]. Rat cDNAs for the $GABA_A$ receptor α_1, β_2 and β_3 subunits were isolated from a rat cerebral cortex library constructed in the pcDV1 plasmid vector using a 699 bp nick-translated human cDNA probe [5,11]. Following linearization, labelled antisense RNA probes were prepared from these cDNAs using T7 RNA polymerase in the presence of [^{32}P]α-CTP (Amersham) as previously described [8].

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Several α_1 subunit cRNA probes corresponding to the putative extracellular, membrane-spanning, and intracellular domains of the α_1 subunit, as suggested by hydropathy plots of the deduced amino acid sequence [4] were synthesized (fig.1).

Total RNA was extracted according to the method of Chirgwin et al. [12]. Briefly, tissues were homogenized in 4 M guanidine thiocyanate and the RNA purified by centrifugation over a 5.7 M CsCl cushion, followed by phenol, chloroform/isoamyl alcohol (49:1) extractions and ethanol precipitation [8]. Polyadenylated mRNA (poly A^+) was isolated using oligodT-cellulose affinity chromatography as previously described [13]. Poly A^+ mRNA (0.5–2 μg) from various brain regions was denatured at 68°C for 10 min in formamide/formaldehyde and applied to a 1% agarose formaldehyde gel. Following electrophoretic separation, the RNA was transferred to nitrocellulose membranes (BRL BA 85) and heated for 2 h at 80°C. The membranes were prehybridized for at least 1 h in a solution containing 50% formamide, 50 mM $NaHPO_4$ (pH 6.5), 5 × SSC (standard saline citrate), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.05% Ficoll, 0.05% PVP, and 200 μg/ml salmon sperm DNA. [^{32}P]CTP-labelled cRNA probes (5 × 10^5 cpm/ml) were added (cf. legend fig.1) and the filters hybridized in the same solution for 16 h at 55°C. Filters were then washed twice in 0.1 × SSC and 0.1% SDS at room temperature for 15 min, once at 55°C for 15 min, and then air-dried and exposed to X-ray film at −80°C (Kodak XAR-5) using intensifying screens. Exposure times were adjusted in preliminary experiments to give autoradiograms within the linear range of densitometric measurements. Optical density measurements were standardized using Kodak calibration step tablet 809ST601 using a light box (Northern Light Precision Illuminator) and camera (model 890, Imaging Research Inc.) and the Image 1.15 MacIntosh II software program [14].

In vitro transcription of the linearized α_1, β_2 and β_3 subunit cDNA

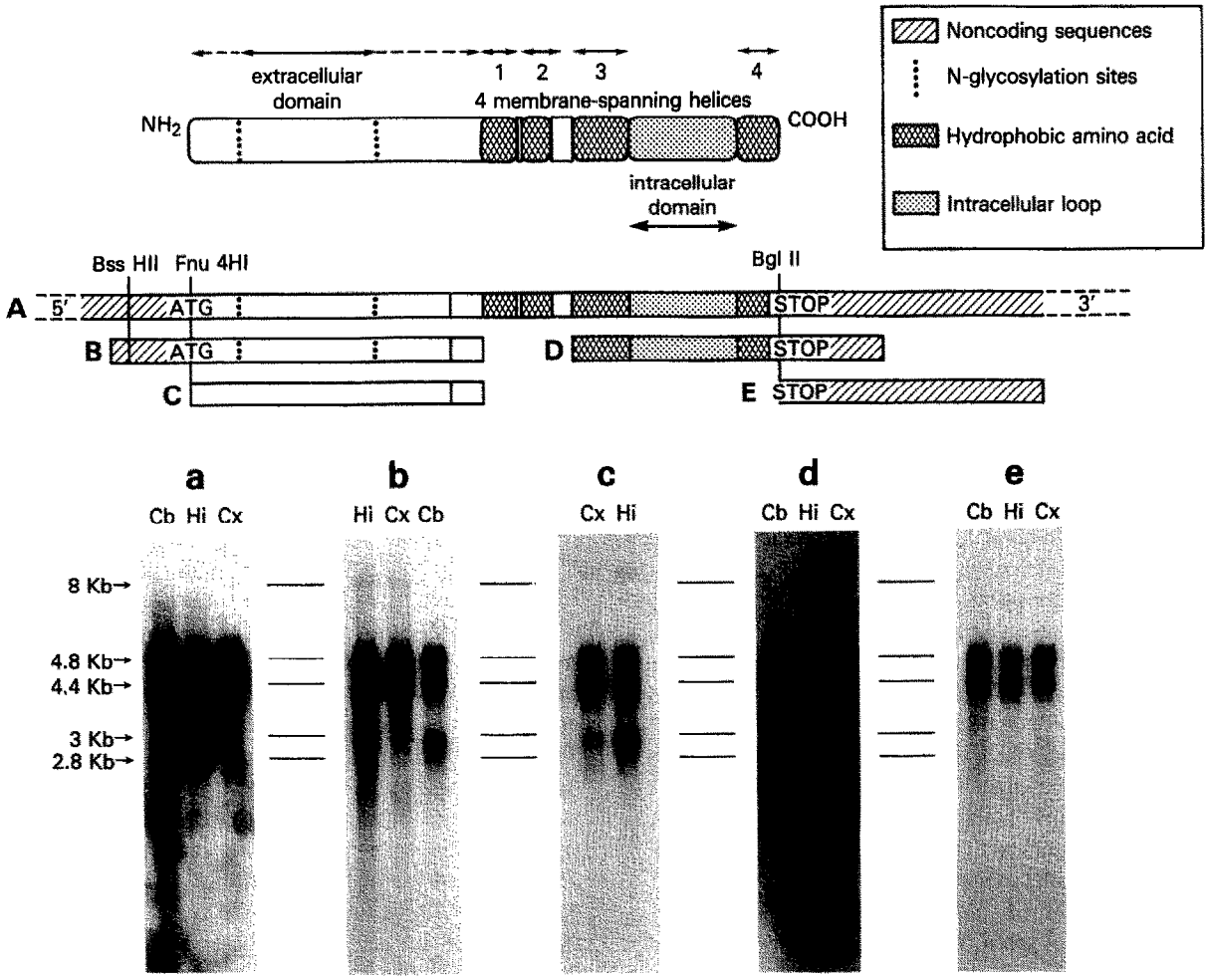

Fig.1. Northern analysis showing differential expression of $GABA_A$ receptor α subunit mRNAs in the rat cerebellum, cerebral cortex and hippocampus. Brain tissue from adult male Sprague–Dawley rats (150–300 g) was quickly dissected on ice, frozen on dry ice, and stored at −80°C. Total RNA was extracted as described [12] and poly A^+ RNA was further purified by oligodT-cellulose affinity chromatography [13]. One μg of poly A^+ RNA from both the hippocampus and cerebral cortex and 0.5 μg from the cerebellum were separated by electrophoresis as described in the text. Following transfer to nitrocellulose membranes the blots were hybridized to a series of [^{32}P]CTP-labelled cRNA probes, corresponding to the different putative functional domains of the α subunit [4]. Probe A contains the entire coding sequence as well as 220 bp and 607 bp of 5′ and 3′ untranslated sequences respectively. Probe B corresponds to nucleotides encoding the putative extracellular portion of the α subunit and includes 150 bp of the 5′ untranslated sequence. Probe C corresponds to nucleotides encoding only the putative extracellular portion of the α subunit. Probe D corresponds to nucleotides encoding the intracellular domain and carboxyterminal portion of the protein and includes 300 bp of the 3′ untranslated sequence. Probe E contains 600 nucleotides of the 3′ untranslated sequence. Following linearization, [^{32}P]CTP-labelled antisense RNA probes were prepared using T7 polymerase (see text). The hybridization patterns using probes A–E are shown in a–e. Exposure times for each autoradiogram ranged from 16 to 24 h. Numbers at the left represent the size of each transcript in kb and were determined using standard DNA and RNA markers.

templates using SP6 polymerase, yielded relatively large amounts of sense RNA (30 $\mu g/\mu g$ DNA template; cf. legend fig.3). Cross-hybridization of the α_1 subunit cRNA probe with β_2 and β_3 sense RNA and vice versa was carried out using dot blots (nitrocellulose) (fig.3) to confirm the specificity of the α_1 subunit probes.

3. RESULTS AND DISCUSSION

Using ^{32}P-labelled antisense RNA probes spanning the $GABA_A$ α_1 subunit coding sequence (fig.1), at least five α subunit transcripts are detected in various regions of the rat brain. Using the longest probe available (A), which contains the entire coding sequence, as well as 220 bp and 607 bp in the 5′ and 3′ untranslated regions respectively, hybridization carried out at high stringency reveals transcripts of 2.8 kb, 3.0 kb, 4.4 kb, 4.8 kb and 8.0 kb (fig.1). All rat and human brain regions examined contain the 4.4 and 4.8 kb mRNA species, whereas the 2.8 kb and 3.0 kb mRNAs were most abundant in the cerebellum and hippocampus, respectively (figs 1, 2 and data not shown). The 8.0 kb species is clearly detected in most brain regions with longer exposure times (figs 1 and 4) and, therefore, may be of lower abundance; although less efficient transfer of larger mRNAs may in part contribute to these results. It is clear, however, that the 3.0 kb species is more abundant in the rat hippocampus than the cerebral cortex and that the 2.8 kb species is detected predominantly in the rat and human cerebellum (figs 1A–C,2,3A).

To determine whether the observed pattern of hybridization of the α_1 subunit cRNA probes to brain RNA was due to cross-hybridization to β subunit transcripts, we synthesized sense RNAs from α_1, β_2, β_3 rat cDNA templates (cf. section 2, legend fig.3). These sense RNAs were blotted on nitrocellulose membranes at concentrations of 25–500 ng and hybridized with either the ^{32}P-labelled human α_1 or rat β_3 subunit cRNA probe (fig.3B,C). Even following long exposure times (64 h), no hybridization of the human α_1 subunit cRNA probe to either the synthetic β_2 or β_3 RNA transcripts was observed; despite strong hybridization to the rat α_1 subunit synthetic RNA (fig.3B). The full-length rat β_3 subunit cRNA probe weakly cross-hybridized to full-length β_2 and α_1 sense RNAs, but only at the highest concentration of RNA (fig.3C). The amount of α_1, β_2, and β_3 RNA used in these experiments is substantially greater than the levels that would be present in our Northern blots of brain mRNA, strongly suggesting that the α_1 subunit probe(s) does not cross-hybridize with β subunit transcripts under the conditions employed. Given the failure of our α subunit probe to hybridize to β subunit mRNAs (despite considerable sequence homology), and the high stringency of our Northern analyses, it is likely that the observed transcripts (figs 1–4) are closely related α subunit mRNAs.

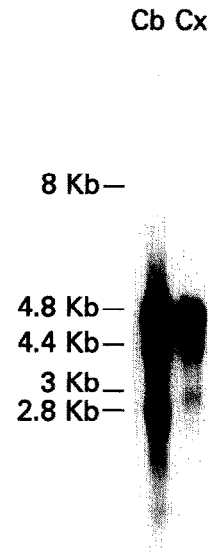

Fig.2. Multiple $GABA_A$ receptor α subunit transcripts in human cerebral and cerebellar cortex. Poly A^+ RNA was prepared from human cerebral cortex and cerebellar cortex (see section 2 and legend fig.1) obtained at autopsy from a patient without central nervous system pathology. The postmortem interval was <24 h and the brain tissue was rapidly frozen and stored at −80°C. Northern analysis was performed as described (cf. text and legend fig.1) using 10 μg of poly A^+ RNA [12] from both the cerebellar and cerebral cortex. Probe C (cf. fig.1) was used to detect α subunit transcripts. Note the presence of a 2.8 kb transcript present in the cerebellar but not cerebral cortex.

To test the hypothesis that the multiple α subunit transcripts detected by Northern analyses (fig.1A) encode homologous, but unique, α subunit mRNAs, we hybridized mRNA from the same brain regions with probes corresponding to either the putative intracellular or extracellular domains of the α_1 subunit. These experiments demonstrate that the five α subunit mRNAs detected by Northern analysis differ primarily in nucleotide sequences which encode the putative intracellular domain or loop, whereas probes containing sequences encoding the extracellular domain detect all 5 transcripts (fig.1A–E). Since the 4.4 kb and 4.8 kb mRNAs are detected by each probe, including those that only contain sequences complementary to the 3′ untranslated region of the α_1 subunit cDNA (fig.1E), it appears that these species are highly conserved, and may result from alternate splicing of a single gene or the presence of multiple polyadenylation sites. These data suggest that the five α subunit transcripts encode subunit proteins with homologous extracellular, but unique intracellular, domains.

Since earlier work had suggested functional changes in $GABA_A$/benzodiazepine receptors in the rat brain during development [15], we compared the expression of the α subunit transcripts in fetal rat and chick brain

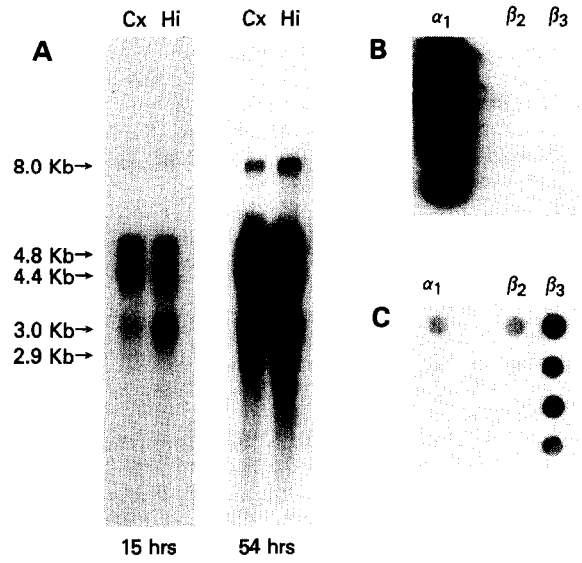

Fig.3. (A) Demonstration of a low abundance $GABA_A$ receptor α subunit transcript in rat cerebral cortex and hippocampus. Northern analysis of rat cerebral cortical and hippocampal poly A^+ RNA (1 μg) was carried out as described in the text and fig.1 using probe C. Autoradiograms obtained after long exposures (54 h) revealed the presence of an 8.0 kb transcript which was more abundant in the hippocampus and hypothalamus (data not shown) than in the cerebral cortex. (B) Attempts to hybridize the α subunit cRNA probe (probe C) to full-length sense β_2 and β_3 subunit RNAs (25 ng, 50 ng, 100 ng and 500 ng; from bottom of nitrocellulose filter to top) revealed no hybridization of probe C with β subunit sequences, despite strong hybridization to sense α subunit RNA. (C) Cross-hybridization of the [^{32}P]CTP-labelled β_3 subunit probe to full length sense α_1, β_2 and β_3 RNAs reveal strong hybridization to β_3 RNA but only weak hybridization to the α_1 and β_2 RNAs (only at 500 ng RNA). In these experiments full length sense RNAs were synthesized from their corresponding cDNA templates as described in the text. Sense RNAs on nitrocellulose membranes were hybridized to the [^{32}P]CTP cRNA probes for 16 h. Filters were washed and exposed to X-ray film with intensifying screens as described in the text. Autoradiograms were obtained after 24 h exposure.

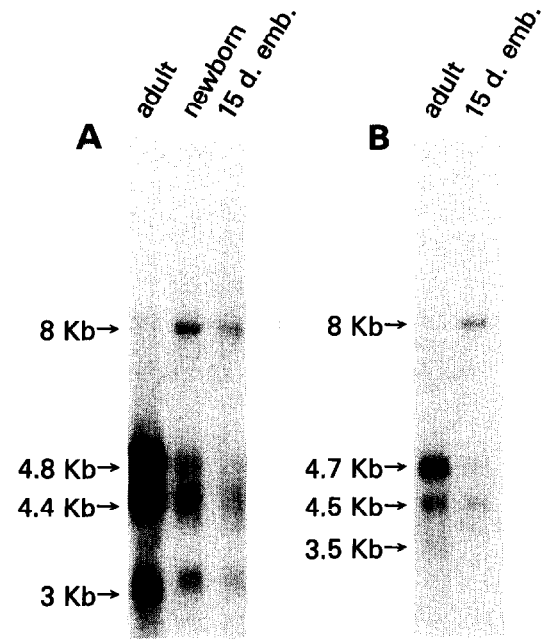

Fig.4. Developmental expression of $GABA_A$ receptor α subunit transcripts in rat (A) and chick (B) brain. Poly A^+ RNA was prepared from whole embryonic brain (15 day), newborn (rat) or adult rat and chicken brain as described in the text. Northern analysis using 3 μg of poly A^+ RNA and the [^{32}P]CTP-labelled cRNA probe (probe C, fig.1) was performed as described in the text. Autoradiograms were obtained after 16–24 h exposure and densitometric readings were made as described in the text. In embryonic brain the 8.0 kb α subunit mRNA was approximately 8-fold more abundant than in adult brain, whereas the 4.4 kb and 4.8 kb transcripts (rat) or the 4.5 kb and 4.7 kb transcript (chick) were more than 50-fold and 15-fold, respectively, more abundant than in embryonic brain. In rat and chicken the levels of the 3 kb and 3.5 kb α subunit mRNA species also increased during brain development and were more than 40-fold and 4-fold, respectively, more abundant in adult than in embryonic brain. In newborn rat brain the 8.0 kb mRNA is approximately 13-fold more abundant than in the adult brain, whereas the 4.4 kb and 4.8 kb mRNAs are approximately 20-fold less abundant than in adult brain.

(fig.4). Messenger RNA was prepared from whole adult and fetal (15 day) rat and chicken brain, as well as newborn rat brain. Hybridization of poly A^+ mRNA from these tissues with the [^{32}P]labelled α_1 subunit cRNA probe (probe C, fig.1) revealed a distinct developmental pattern of α subunit mRNA expression. The relative concentration of the 8.0 kb mRNA, compared to the 4.4 kb and 4.8 kb species (rat) or 4.5 and 4.7 kb species (chick), was much greater in fetal and newborn than adult brain (fig.4). Moreover, the 3.0 kb, 4.4 kb and 4.8 kb species increase in abundance during the last week of embryogenesis and following birth and reach their maximum levels in adult brain (fig.4 and unpublished data). It appears, therefore, that in both rat and chick brain the 8.0 kb α subunit transcript may represent a predominantly embryonic form. Whether this 8.0 kb species is derived from a different gene, or represents a precursor of the smaller species, and (or) has a different rate of turnover during development remains to be determined. Further work will also be required to determine whether this transcript encodes an embryonic α_1 subunit or isoreceptor which accounts for the previously reported pharmacological and biochemical properties of embryonic $GABA_A$/benzodiazepine receptors [15].

Our data describing the regional and developmental expression of multiple $GABA_A$ receptor α subunit transcripts extends recent reports on the isolation of multiple α subunit cDNAs [4–6] and strongly suggest the presence of $GABA_A$/benzodiazepine isoreceptors in brain which are regionally distributed and developmentally regulated. It is possible that these isoreceptors account for the pharmacological differences observed among the various drugs known to interact with these receptors (e.g., the benzodiazepines). Since it is generally accepted that the benzodiazepine recognition site resides on the α subunit [2–4] it is tempting to speculate that the 2.8 kb and 3.0 kb α subunit transcripts present

in the cerebellum and hippocampus respectively (figs 1 and 2) may represent types I and II benzodiazepine receptors previously delineated on the basis of pharmacological and biochemical data [16]. Work on the effects of acute or chronic drug administration or exposure on the expression of these $GABA_A$ receptor α subunit transcripts is in progress and should further address these questions.

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