Differential and Transient Expression of GABA_A Receptor α -Subunit mRNAs in the Developing Rat CNS

M. O. Poulter,¹ J. L. Barker,¹ A.-M. O'Carroll,² S. J. Lolait,² and L. C. Mahan²

Laboratories of ¹Neurophysiology, NINDS, and ²Cell Biology, NIMH, NIH, Bethesda, Maryland 20892

The expression of mRNAs coding for α_1 , α_2 , α_3 , α_5 , and α_6 subunits of the GABA_A neurotransmitter receptor was followed during the development of the rat CNS by *in situ* hybridization histochemistry. Expression of these subunit mRNAs in tissue sections of embryonic day 15 and 17 (E15, E17) whole rat and in brain at ages >E17 to adult were varied, transient, and region specific. Subunit mRNAs first detected at E15 were those coding for the α_2 and α_3 subunits. At E17, α_2 , α_3 , and α_5 mRNAs were present in abundance in numerous areas in the CNS, with lower but significant amounts of α_6 being present in the cortical neuroepithelial layers. However, α_6 subunit mRNA expression in the cortex declined until little or no α_6 mRNA was detected at E19. α_1 subunit mRNA first appeared at E19 in the cortex, followed by expression in the hippocampus by postnatal 5 (PN5). Particularly high expression of α_2 and α_5 subunit mRNAs was detected throughout the developing CNS, but they were most abundant in the olfactory bulb neurons. The high levels of α_2 and α_5 subunit mRNAs began to decline around PN5 to the amounts observed in adult. These results demonstrate that numerous GABA_A receptor α -subunits are expressed before birth in a region- and age-specific manner. This complex and varied expression supports the hypothesis that GABA may play a role in cellular and synaptic differentiation.

The A-type receptors for the neurotransmitter GABA have been shown to be composed of a number of protein subunits designated α , β , γ , δ , and ρ (Burt and Kamatchi, 1991). *In situ* hybridization of cDNA probes to mRNA coding for these subunits has revealed a wide and varied distribution in the adult CNS in number of species (Olsen and Tobin, 1990). From these results, it is now clear that the number of possible subunit combinations that may form GABA_A receptors may theoretically range from several hundred to as many as several thousand. The relevance of this heterogeneity with regard to the action of GABA at the level of resolution of a single channel, single cell, or neural circuit has not been elucidated. However, it is likely that the dynamics of GABA-mediated neurotransmission may be considerably more complex than our present understanding of GABA ion channel function indicates.

Presently, the α -subunit appears to exist in the largest number of variants. Six different subunit cDNAs have been isolated from a number of species (α_1 – α_6 ; Schofield et al., 1987; Levitan et al.,

1988b; Khrestchatsky et al., 1989; Lolait et al., 1989a; Stephenson et al., 1989; Ymer et al., 1989; Kato, 1990; Luddens et al., 1990; Pritchett and Seeburg, 1990; α_4 : Wisden et al., 1991). It is generally accepted that the α -subunit possesses the benzodiazepine binding site, and each subtype has variable selectivity for different drugs within this class (Guidotti et al., 1990). In addition, different α -subunits may play a role in determining the efficacy of GABA's action (Levitan et al., 1988b; Sigel et al., 1990; Puia et al., 1991). Thus, in the adult CNS, it would appear that α -subunits physiologically (and pharmacologically) modulate the action of GABA at its receptor.

Beyond its role as a neurotransmitter, another action of GABA (as well as other neurotransmitters) that has been suggested is that it may play a trophic role before birth (Wolff, 1981; Lauder et al., 1986; Redburn and Schousboe, 1987). A number of biochemical studies have demonstrated the presence of GABA and its receptors in embryonic CNS (Meier et al., 1991). More importantly, perhaps, responses to exogenously applied GABA in embryonic hippocampal and spinal cord neurons has been recorded *in vitro* (Fizman et al., 1990; Mandler et al., 1990). Most recently, Northern analyses of some α -subunit mRNAs (α_1 , α_2 , $\alpha_{5(4)}$ [this article uses the nomenclature of Malherbe et al. (1990), who have called this sequence α_5 , whereas MacLennan et al. (1991) called this sequence α_4]) of whole embryonic and early postnatal brain tissues have shown that their transcription varies during late embryogenesis and early postnatal life (MacLennan et al., 1991). These observations imply that the expression of GABA_A receptor subunits may play a developmental role in the CNS by "tailoring" the action of GABA.

In light of these two aspects of the physiological action GABA, this study was carried out with the intent to describe the region-specific ontogenic expression of the five rat α -subunit sequences that have been reported to date (cloning of a rat α_4 subunit was reported after submission of this study; α_1 : Lolait et al., 1989a; α_2 : Khrestchatsky et al., 1991; α_3 and α_5 : Khrestchatsky et al., 1989; Malherbe et al., 1990; α_6 : Luddens et al., 1990). In combination with a recently completed study on the ontogenic expression of mRNAs for the β_1 – β_3 , γ_2 , and δ subunits (Poulter et al., 1991b; M. O. Poulter, J. L. Barker, A.-M. O'Carroll, S. J. Lolait, and L. C. Mahan, unpublished observations) in our laboratories, it is hoped that this knowledge will serve as a foundation for the following: (1) it may allow investigators to correlate the properties of the GABA_A receptor chloride channel to the known subunits combinations *in vivo*, and (2) it may further our understanding of how GABA may contribute as a neuromediator to cellular differentiation and synaptogenesis in the CNS.

These data have been reported in a preliminary form (Poulter et al., 1991a).

Received Oct. 2, 1991; revised Feb. 3, 1992; accepted Mar. 6, 1992.

Correspondence should be addressed to Dr. Michael Owen Poulter, Laboratory of Neurophysiology, NIH–NINDS, Building 36, Room 2C02, 9000 Rockville Pike, Bethesda, MD 20892.

Copyright © 1992 Society for Neuroscience 0270-6474/92/122888-13\$05.00/0

Figure 1. Expression of GABA α -subunit mRNA in E15 rat embryos. *SpC*, spinal cord; *Md*, medulla; *cb*, cerebellum ne; *Ph*, posterior hypothalamic area; *ath*, anterior thalamus; *itth*, intermediate thalamus; *bti*, basal telencephalic plate interm; *hi*, hippocampal ne; *oc*, olfactory cortex ne; *cx*, cortical ne. Arrows A–D correspond to Figure 2A–D, respectively.

Materials and Methods

Probe sequences. Oligodeoxynucleotide probes homologous to mRNA sequence coding for the amino acids in the putative cytoplasmic domains of the α -subunits (except the α_2 probe, which is homologous to the signal sequence) were synthesized using an Applied Biosystems 380A DNA synthesizer (courtesy Dr. Michael Brownstein LCB, NIMH). Sequences to which the probes were complementary are given below along with the source for the sequence:

α_1 (Lolait et al., 1989a):
5' CAA GAA AAG CCA AAG AAA GTG AAG GAT CCT CTC ATT
AAG AAA AAC AAC 3'

α_2 (Khrestchatsky et al., 1991):
5' GTA TGG TTT CCG CTG CTT GTT CTC CTG GTG TGG AAC
CCA GCC AGG TTG 3'

α_3 (Malherbe et al., 1990):
5' ATC TCC AAG GCT GCT GCT GCT CCC AGT GCT TCT TCA
ACT CCA ACA GTG 3'

α_4 (Khrestchatsky et al., 1989; Malherbe et al., 1990):
5' GAA CGT GAA CTC ATA CTA AAT AAG TCA ACA AAT GCT
TTT ACA ACT GGG 3'

α_6 (Luddens et al., 1990):
5' GGT AGC AAA GTC AAA AAC AAC TGA ATC ACT CGA AGC
TGA GAT TGT TGT 3'

In situ hybridization histochemistry. All tissues used in this study (obtained from timed-pregnant Sprague–Dawley rats killed by CO_2 asphyxiation or, in the case of postnatal and adult rats, by decapitation) were fresh frozen on dry ice and stored at -80°C until sectioning. The plane of section for embryonic day 15 and 17 (E15, E17) whole embryos was parasagittal. E19, E20, and postnatal day 5 (PN5) whole rat heads were sectioned horizontally. PN3 and adult rat brains were sectioned parasagittally.

Sections (12 mm thickness) were thaw mounted onto twice gelatin-coated slides and subsequently stored at -80°C until hybridization. Oligonucleotide probes were tailed using ^{35}S -ATP and deoxynucleotidyl transferase to a specific activity of 3500–5000 Ci/mmol (Lolait et al., 1989b). Fixed and dehydrated sections were incubated overnight (~ 18 hr) with ^{35}S -labeled probes in hybridization solution at a concentration of $1\text{--}2 \times 10^6$ dpm/45 ml as previously described (Young et al., 1986).

Sections were then washed four times for 15 min each in 55°C $1\times$ standard saline solution (SSC) followed by two washes in $1\times$ SSC for 1 hr at room temperature. Sections were rinsed in distilled water, dried, and then exposed for 3–4 weeks to Hyperfilm β max (Amersham). Signals detected above background on autoradiographic film were confirmed as being specific to cell bodies (density of graining more than twice background over cells vs intracellular space) by viewing NTB-2 emulsion-dipped slides (10–12 week exposure) counterstained with either toluidine blue or thionine. All observations reported in this study are those that were reproduced in a minimum of three different experimental occasions using different animals. On each occasion, sections of embryonic, postnatal, and adult rat (E12, E15, E17, E19, E20, E21, PN1, PN3, PN5, and adult) were sequentially fixed, incubated with hybridization fluid containing the appropriately labeled probe, and washed as a set (>200 slides at a time and including sections for hybridization with β , γ , and δ mRNAs; Poulter et al., 1991b; accompanying manuscript). Sections illustrated in Figures 1, 2A,B, 3, 5, 8, and 9 were processed together. Illustrations in Figures 2C,D, 4, 6, and 7 were processed on a different occasion.

The probes used in this study were determined to be selective based on the following criteria: (1) labeling tissues with sense probes (α_1 and α_2) showed no detectable cell-specific labeling; (2) identical patterns of expression were observed with three different probes to a single subunit on separate occasions and most importantly as a final control; (3) each probe yielded labeling patterns in adult coronal (not shown) and parasagittal sections (see Fig. 9; α_1 : Lolait et al., 1989a; α_1 , α_3 , α_6 : Luddens and Wisden, 1991; α_3 : Malherbe et al., 1990; α_6 : Luddens et al., 1990). Corroborative evidence that these probes do not cross-react is revealed by the different distribution and amount of labeling demonstrated by each probe.

For this study, we have defined the neuroanatomy of the embryonic rat based on information obtained from whole-body sagittal sections of E14 rat in Paxinos et al. (1991). Since there are rapid changes in the neuroanatomy occurring during development, the naming of the brain regions offered for E15 and E17 sections may be only general or only tentatively identified. No attempt has been made to define exhaustively all the neuroanatomy depicted in each embryonic slice. For E19 and PN5 sections, areas were labeled by extrapolation to the anatomical description the adult rat brain as defined in Paxinos and Watson (1986).

Table 1. Summary of α Subunit mRNAs Expression

	Subunit				
	α_1	α_2	α_3	α_5	α_6
E15					
Cortical ne	—	+	—	+/-	—
Hippocampal ne	—	+	—	+/-	—
Cerebellar ne	—	—	—	+/-	—
Superior colliculus ne	+	—	—	+/-	+/-
Olfactory ne	—	+++	—	+/-	+/-
Thalamic areas	—	++	+	+/-	+/-
Medulla	—	+	+	+/-	+/-
Basal telencephalic plate	—	—	—	+/-	+/-
Pons	+	+	+	+/-	—
Spinal cord					
Ventral	—	+/-	+	+/-	+/-
Dorsal	—	—	+	+/-	+/-
E17					
Cortical ne	—	++	—	+	+
Hippocampal ne	—	++	—	+	—
Cerebellar ne	+	+	—	+	—
Superior colliculus ne	+	+	+	+	—
Olfactory ne	—	+++	+	—	—
Thalamic areas	+	++	++	+	+
Medulla	+	+	++	+	—
Trigeminal ganglia	+	+	++	+	+
Basal telencephalic plate	—	+	+	+	—
Pons	+	+	+	+	—
Spinal cord					
Ventral	+	+	++	+	—
Dorsal	—	+	++	—	—
E19					
Cortex					
Parietal	+	+++	++	++	~
Entorhinal					
Layer I	++	+++	+	+	—
Layer II	+	~	++	+	—
Olfactory ne	+	+++	+	++	—
Hippocampus					
CA1	~	+++	+	+++	—
CA3	—	+++	—	+++	—
Dentate gyrus	—	+	+	++	—
Thalamic nuclei					
Ventral	—	+/-	++	+++	—
Dorsal	—	+++	++	+++	—
Superior colliculus	+	+	++	+++	—
Caudate putamen	—	+	+	++	—
Cerebellar ne	+	+	++	++	—
Olfactory ne	+	+++	+	++	—
Spinal cord	—	+	+	+	—
PN5					
Cortex					
Parietal	+	+	+	++	—
Entorhinal	++	+	+/-	+++	—
Hippocampus	+	++	+/-	+++	—
Dentate gyrus	+	+	+/-	+++	—
Thalamic nuclei					
Ventroposteromedial	—	+	+	++	—
Ventroposterolateral	—	+	+	+	—
Dorsal lateral geniculate	—	++	+	++	—

Table 1. Continued

	Subunit				
	α_1	α_2	α_3	α_5	α_6
Tenia tecta	+	+	—	+++	—
Caudate putamen	+	+	—	++	—
Cerebellum	+	—	—	+	+
Superior colliculus	++	+	+	++	—
Olfactory bulb cell layers					
Glomerular	—	+++	—	—	—
Mitral	++	+	—	—	—
Internal granule	—	—	—	+++	—
Spinal cord	—	+	+	+	—
Adult					
Cortex					
Parietal	+	++	++	+	—
Entorhinal	+	++	+	+/-	—
Hippocampus	+	++	+/-	++	—
Dentate gyrus	+	+	+/-	+++	—
Thalamus	++	+	+	—	—
Caudate putamen	—	++	—	+	—
Cerebellum	++	+	—	—	+++
Superior colliculus	++	++	+	++	—
Inferior colliculus	+	++	+	—	—
Spinal cord	+	++	++	+	—

—, not detectable above background; +/-, at level of detection; +, detectable; ++, intense; +++, very intense.

Results

Tissue sections of E12 and E13 rat embryos did not show definitive cell-specific labeling for any subunits (data not shown). It was not until E15 that significant cell-specific expression of mRNAs was detected, those belonging to the α_2 and α_3 subunits, as well as low levels of α_5 and α_6 mRNAs (see Table 1; Figs. 1, 2). In contrast to the diffuse expression of the other mRNAs, α_2 mRNA was discretely localized to a number of tissue regions. Cells of the olfactory cortex neuroepithelium (ne) and anterior thalamic region (Figs. 1; 2*A,B*) were rich in α_2 mRNA. Also, lower expression was observed in the interior thalamic region. There were also clusters of α_2 mRNA-positive cells observed immediately adjacent to the spinal cord and medulla (Fig. 2*C*). These are likely to be dorsal root ganglion cells. mRNA coding for the α_3 subunit was abundant in the spinal cord and medulla (Fig. 2*D*); however, little mRNA was detected in higher regions of the CNS (e.g., the cortical ne and thalamic regions).

The patterns of expression observed in E15 sections were also evident at E17; however, the clusters of α_2 mRNA-positive cells associated with the medulla and cervical spinal cord were not detected. It is not clear whether this loss of signal represents migration of the α_2 -positive cells or that the plane of section for the E17 embryos failed to include these cells. Expression of α_2 subunit mRNA was pronounced in thalamic and olfactory areas, while α_3 mRNA remained localized to more caudal brain areas (Figs. 3, 4). α_5 mRNA was detected in most CNS areas; however, this expression was not diffuse within the tissue. Rather, its distribution was often punctate as evidenced by the cellular distribution observed on the ventral surface of the basal telencephalic plate (Fig. 4*F*). Cellular expression of α_6 mRNA was most prominent in the cortices, whereas no signal was detectable in the cerebellar ne. This distribution is in sharp contrast to that

observed in the adult, where the expression of α_6 subunit mRNA is almost completely localized to the cerebellar tissue (see Fig. 9; Luddens et al., 1990).

After E17, mRNAs for all five subunits were expressed to some degree throughout the CNS (Table 1). However, most immediately striking was the abundance of α_5 mRNA in E19 brain sections. Particularly strong expression was seen in the hippocampus and thalamus. In contrast to the punctate distribution seen at E17, α_5 mRNA was diffusely distributed throughout a particular tissue region at E19. Expression was greater in anterior regions of the brain than in those more posterior. Thus, in the anterior caudate-putamen there were moderate levels of α_5 mRNA whereas only relatively low expression was observed in the posterior caudate and entorhinal cortex. α_2 mRNAs were observed in entorhinal cortex in the outer layer (labeled I in Fig. 5), whereas α_3 mRNAs was observed only in the inner layer (labeled II in Fig. 5). α_1 mRNA was in both layers. The expression of α_1 and α_3 subunit mRNAs decreased in more anterior regions of the cortices, in contrast to the abundant expression of α_2 mRNAs in the parietal cortex. Finally, at the tip of the developing olfactory bulb, a band of cells was intensely labeled by α_2 and α_5 subunit mRNA probes (Fig. 5). There was also moderate and diffuse expression of α_2 , α_3 , and α_5 mRNAs in horizontal sections of spinal cord (data not shown).

Horizontal sectioning of E20 brain above the hippocampus revealed a similar distribution of α_1 , α_2 , α_3 , and α_5 mRNAs in cortical regions (Fig. 6). Again, the most striking results were those obtained with the α_2 and α_5 probes. There was intense labeling in the outer cell layers of the parietal cortex for α_2 mRNA, in contrast to α_5 mRNA, which was found throughout the cortical cell layers. Labeling for α_2 mRNA declined in more posterior regions of the parietal cortex; however, there was some expression in the occipital cortex. In contrast to the distribution

Figure 2. High-magnification photographs of α_2 and α_3 mRNA-positive cells at E15. *A* and *B*, Bright-field photos of olfactory neurons (*A*) and anterior thalamic cells (*B*) positive for α_2 mRNA. *C*, Dark-field photo α_2 -positive cell cluster adjacent to medulla. *D*, Spinal cord neurons are positive for α_3 mRNA. *A–D* correspond to arrows *A–D*, respectively, in Figure 1. Scale bars: *A*, 10 μ m for *A* and *B*; *C*, 50 μ m for *C* and *D*.

observed in the entorhinal cortex of the E19 embryo, α_1 and α_3 mRNAs were not localized to any particular cell layer in the higher cortices. Also evident in the low-magnification photo of the α_2 section (Fig. 6) was the intense labeling at the inner edge of the occipital cortex, which was tentatively identified as from the cell bodies of the superior colliculus. This plane of sectioning also revealed the layer-specific expression of α_1 , α_2 , α_3 , and α_5 subunit mRNAs in the olfactory bulb. Thus, α_1 mRNA was highly expressed in the mitral cell layer while α_2 mRNA was present in the outer glomerular cell layer. α_3 mRNA was present in the outer cells of the granule cell layer, whereas α_5 mRNA predominated throughout the internal granule cell region.

Beginning at E19 and continuing through to the early postnatal period (PN5), several patterns of subunit mRNA expression were evident. There was transient, moderately high expression of α_2 and α_3 mRNA at E19 in areas where they are expressed in adult (e.g., thalamus; Fig. 5), which then declined. This pattern differs from the expression of α_5 subunit mRNA, which was high in most brain regions, some of which showed

little or no expression in adult (e.g., compare thalamic areas in Figs. 5, 7, and 8, α_5 , to those in Fig. 9). Another example of how mRNA expression varied during this time is exemplified by the distribution of α_1 mRNA. It was present in a number cortical regions and olfactory neuron layers as early as E19 at levels comparable to those seen in adult, yet only barely detectable levels were observed as late as PN3 in the hippocampus, an area where they are present in abundance in adult (compare Figs. 7, 9). α_6 mRNA first detected at E15 was not observed at all after E17. These differing complex profiles of expression suggest that many factors may regulate α -subunit gene transcription or mRNA turnover during development.

Finally, Figure 9 shows the distribution and relative abundance of the α -subunit mRNAs in sagittal sections of adult brain. Most noticeably, the amount of α_5 subunit mRNA declined to levels that are similar in magnitude to those of the other subunits, lending credence to the observation that α_5 is preferentially expressed in large amounts early in development. All distributions are similar to those reported in other studies (α_1 ;

Figure 3. Expression of GABA α -subunit mRNA in E17 rat embryos. *SpC*, spinal cord; *Md*, medulla; *cb*, cerebellum ne; *sc*, superior colliculus ne; *tg*, trigeminal ganglia; *Th*, thalamic area; *bti*, basal telencephalic plate interm.; *hi*, hippocampal ne; *oc*, olfactory cortex ne; *cx*, cortex ne. Arrows A–F correspond to Figure 4A–F, respectively.

Lolait et al., 1989b; α_1 , α_3 , α_6 : Luddens and Wisden, 1991; α_5 : Malherbe et al., 1990; α_6 : Luddens et al., 1990), indicating that our oligonucleotide probes would appear to be reliable markers of the ontogenetic expression of α -subunit mRNAs.

Discussion

This study has revealed the complex, transient, and region-specific expression of all GABA_A receptor α -subunit mRNAs thus far reported in rat CNS. (Preliminary data obtained by our laboratory indicate that the α_4 subunit mRNA is expressed at very low levels during embryogenesis; moderate levels do not appear until PN3–PN5, primarily being located in the olfactory bulb glomerular cell layers, parietal cortex, ventroposterolateral and ventroposteromedial thalamic nuclei, and caudate-putamen.) Certain α -subunits mRNAs are evident at mid embryogenesis before classical well-differentiated forms of synaptic transmission have been established. The early appearance and transient nature of some, but not all, of the α -subunit mRNAs increases the speculation that the activity of GABA_A receptor/chloride channels somehow contribute to the development of CNS. A corollary to this hypothesis would be that the nature of this presumably GABAergic signal would be controlled by the properties that the α -subunits confer to the embryonic GABA_A receptor ion channel complex.

Most of what is known about the influence different α -subunits have on GABA_A receptor properties has been elucidated from the expression of cloned subunits in various combinations in *Xenopus* oocytes and other non-neuronal cells. In general, these expression systems have shown that the potency of GABA varies with the α -subunit used to "construct" the receptor (Levitan et al., 1988a,b; Malherbe et al., 1990; Sigel et al., 1990; Puia et al., 1990). Two generalizations can be made about receptors expressed in this manner: (1) α_5 -containing receptors confer the highest sensitivity to GABA ($EC_{50} = 10 \mu M$; Mal-

herbe et al., 1990; Sigel et al., 1990), and (2) despite the varying affinity for different benzodiazepines depending on the α -subunit, the modulability still depends on some allosteric action with both β - and γ -subunits. The latter conclusion is based on data demonstrated in Sigel et al. (1990), who showed that the potentiation of the GABA response by diazepam was 30% in oocytes expressing $\alpha_5\beta_1\gamma_2$ and 136% in those oocytes injected with $\alpha_5\beta_2\gamma_2$ mRNAs. One discrepancy between the early work done in oocytes and other non-neuronal cells and GABA responses recorded from nerve cells was the variability in Hill coefficients obtained. Most combinations of subunits produced Hill coefficients significantly less than 2, the value that is usually calculated from data obtained from neurons (Mohler et al., 1990). Hill coefficients of 1 were obtained in oocytes expressing a single α -subunit in combination with β - and γ -subunits (Levitan et al., 1988a; Lolait et al., 1989b; Malherbe et al., 1990; Sigel et al., 1990). However, dose–response curves obtained from oocytes expressing receptors comprised two different α -subunits (along with β - and γ -subunits) or receptors expressed from whole brain mRNA have Hill coefficients that were close to 2 (Mohler et al., 1990; Sigel et al., 1990). Thus, is it evident that the α -subunit may also confer some degree of cooperativity to the action of GABA. However, this information was gleaned from non-neuronal cells, in which the receptor is expressed in an environment that may not be comparable to the receptor expressed in neuronal membranes. Examples of uncertainties that might arise that may significantly alter the function of the receptor are the nature of posttranslational modification (phosphorylation or glycosylation), as well as the lipid environment in which the receptor operates (for review, see Sigel, 1990). Therefore, while it is evident that α -subunits have profound effects on GABA-mediated responses *in vitro*, one should cautiously and not simplistically ascribe unique functional attributes to a single subunit or even to a class of subunit in receptors expressed *in vivo*.

Figure 4. Dark-field photos of the three highly expressed α -subunits at E17. Dark-field photos of olfactory cortex ne show expression of α_2 mRNA in the outer layer (*A*), α_3 mRNA throughout this region (*E*), and little α_3 mRNA (*C*). There was intense diffuse labeling of α_2 and α_3 mRNAs in thalamic regions (*B* and *D*, respectively), which contrasts to the apparently regular punctate distribution of α_5 mRNA in basal telencephalic plate interm (*F*). Arrows in *D* indicates the location of the cortical ne lacking α_3 mRNA; *cx*, cortex ne; *Th*, thalamic area. *A–F* correspond to arrows *A–F* in Figure 3. Scale bar, 50 μ m.

Figure 5. Horizontal sections of whole rat head at E19 showing the distribution of all α -mRNAs. *cb*, cerebellum; *Sc*, superior colliculus; *Th*, thalamic area; *hi*, hippocampus; *m*, olfactory mitral ne; *Par*, parietal cortex; *Ent*, entorhinal cortex; *I*, layer I; *II*, layer II; *dg*, dentate gyrus; *CPu*, caudate-putamen.

At this time, the most complete information that exists about the differences between α -subunits is the relative binding affinity they have for various benzodiazepine-type ligands. For example, Luddens et al. (1990) have shown that receptors composed of an α_6 subunit (along with β_2 , γ_2) does not bind diazepam or flunitrazepam with high affinity, in contrast to a receptor composed of $\alpha_1\beta_2\gamma_2$. However, α_6 does bind the negative modulator Ro15-4513 with high affinity. The detection of a high-affinity site for the drug Ro15-4513, which has been reported to antagonize the potentiating and GABA-mimetic effects of ethanol (EtOH; Suzdak et al., 1986; Ticku and Kulnarn, 1988), has led to speculation this α -subunit may at least partially mediate EtOH's behavioral effects (Luddens et al., 1990). Thus, in this

case the potentiation of GABA by EtOH, at least pharmacologically, may be determined by the presence or absence of different α -subunits.

Our results have shown that α_2 or α_5 mRNAs and to lesser extent α_3 were the most abundant of the transiently expressed subunit mRNAs in many regions of the CNS. Each peaked in expression in a region-specific manner by E19 and decreased thereafter to the levels seen in adult brain (including a complete disappearance in some regions). The expression profile reported in this study extends the recent data reported by MacLennan et al. (1991), who demonstrated, using Northern analysis of whole rat brain, that α_2 and α_4 (α_5 by our nomenclature) mRNAs were expressed in larger amounts during development than in adult.

Figure 6. Differential distribution of highly expressed α -subunit mRNAs in E20 cortex and olfactory bulb cell layers. α_1 and α_3 mRNAs predominate in the mitral cell layer, α_2 predominates in the glomerular cell layer, and α_5 mRNA predominates in the granule cells and is virtually absent from all other areas. Arrows indicate areas where dark-field photographs were taken. In cortical layers α_2 mRNA predominated in the outer layers of the parietal cortex, in contrast to the diffuse expression of α_3 and α_5 mRNAs. In the occipital cortex there was intense expression of α_5 mRNA and moderate expression of all others. Also note the intense expression of α_2 mRNA in the inner edge of the occipital cortex. Scale bar, 50 μ m.

However, they found little α_1 subunit mRNA before birth, whereas we have shown detectable amounts in some regions (see E17–E19 and E20, Figs. 3, 5, 6). This discrepancy would arise from the fact that α_1 is only expressed in low levels and only in a few tissue regions. Therefore, their detection method might not have been sensitive enough to pick up this diffuse expression. Support for this notion is found in Gambarana et al. (1990), who demonstrated low levels of α_1 receptors in E18 rat tissues using *in situ* hybridization.

Receptors containing any one of the α_2 , α_3 , or α_5 subunits have been correlated with the type II benzodiazepine binding site. This binding site has been distinguished from the type I site by having only a low affinity for the benzodiazepine analog CI 218 872 (Squires et al., 1979; Pritchett et al., 1989). An added complication has been reported by Pritchett and Seeburg (1990), who have shown that this classification could be further refined based on the data showing that α_5 -containing receptors have a higher affinity for CI 218 872 than receptors containing either

the α_2 or α_3 subunits (so that the rank order of affinity for CI 218 872 is $\alpha_1 > \alpha_5 > \alpha_2 = \alpha_3$). The ontogenic profile of type I and type II binding sites in homogenized neonatal mouse cortex and cerebellum has demonstrated that the affinity of CI 218 872 (based on displacement flunitrazepam from its high-affinity site) increased from the time of birth to a steady state 5 weeks after birth (Garrett and Tabakoff, 1985). These results imply that type II receptors predominate at birth but subsequently decline. This could not be explained by an alteration in affinity since Scatchard analyses showed an increase in high-affinity sites for CI 218 872 and a decrease in low-affinity sites and therefore an increase type I receptors after birth. Both the data reported in this study and those reported by MacLennan et al. (1991) correlate well with the predominance of type II receptors (α_2 , α_3 , and α_5 mRNA subunit expression) at birth and subsequent increased expression of type I binding site (associated with α_1 mRNA subunit; Pritchett et al., 1989) thereafter.

The patterns of expression of α -subunits reported here and

Figure 7. Expression of α -subunit mRNAs in PN3 hippocampal area. Sagittal sections revealing the hippocampal region show differential expression of α -subunits. Noteworthy is the low expression of α_1 (A) and α_3 (B) mRNAs in contrast to the high expression of α_2 (C) and α_5 (D) mRNAs. *Th*, thalamus; *dg*, dentate gyrus; *cx*, cortex.

the functional properties observed with different combinations of cloned α -subunits prompt the question of whether different subunit combinations of the GABA_A receptor confer attributes to embryonic neurons that may be important for the development of the CNS. This question is particularly relevant since classically described synaptic functions are in the process of differentiation and any activation of these receptors could only arise from any ambient GABA (i.e., GABA not released by synaptic transmission) in the extracellular space. Immunocytochemical evidence for the presence of GABA and glutamic acid decarboxylase has been reported throughout mid to late embryogenesis (Fuji et al., 1985; Lauder et al., 1986), and so it is possible that these receptors are coming in contact with significant amounts of GABA in the extracellular space. Additionally, some steroidal compounds may act on their own on embryonic GABA receptors or potentiate the action of the any

ambient GABA present (cf. Karavolas and Hodges, 1990). Numerous studies have demonstrated that GABA can act as neurotrophic agent (for review, see Meier et al., 1991). GABA also has been shown to alter receptor subtype expression in cultured cerebellar neurons. For example, the absence of GABA in the growth media of cultured cerebellar neurons allowed type I binding sites to predominate, whereas addition of GABA coincided with formation of type II binding sites (Meier et al., 1983, 1984). Therefore, based on these data, one might hypothesize that low concentrations of GABA in embryonic cerebrospinal fluid acting on embryonic GABA_A receptors, having high affinity for GABA, could be a signal for inducing gene expression important for differentiation and synaptogenesis (cf. Fiszman et al., 1990; EC₅₀ of GABA in E17–E19 hippocampal cells = 100 nM).

Electrophysiological studies have shown that the polarity of this potential neurotrophic signal produced by GABA in em-

Figure 8. Horizontal sections of whole rat head at PN5 showing distribution of all α -mRNAs. *cb*, cerebellum; *sc*, superior colliculus; *Th*, thalamic area; *hi*, hippocampus; *dg*, dentate gyrus; *ob*, olfactory bulb; *cx*, cortex ne; *CPu*, caudate-putamen. Positive thalamic nuclei labeled: *vpm*, ventroposteromedial nucleus; *vpl*, ventroposterolateral nucleus; *dlG*, dorsal lateral geniculate nucleus; *tt*, tenia tecta.

bryonic and early postnatal neurons is different than those typically reported in the adult nervous system. Numerous studies have shown that the GABA may depolarize and trigger action potentials in neonatal neurons in a number of species (Dunwiddle, 1981; Schwartzkroin, 1982; Ben-Ari et al., 1989; Fiszman et al., 1990; Mandler et al., 1990). Ben-Ari et al. (1989) have demonstrated a bicuculline-sensitive depolarizing potential in neonatal rat hippocampus that reversed at -57 mV or -25 mV depending on whether potassium acetate or potassium chloride intracellular microelectrodes were used. The shift in the reversal potential upon loading the cells with chloride ions (using the potassium chloride electrode) implies that the potential was at least partially mediated by chloride ions. Therefore, the ionic selectivity appears not to be different for embryonic GABA_A channels compared to those expressed in adult, but the reversal potential for chloride ions would appear to be more

positive in neonatal neurons. The ionic flux of chloride against its concentration gradient is not unprecedented since this is the mechanism by which primary afferent depolarization (a GABA-mediated synaptic event) occurs in adult spinal cord (Padjen and Hashiguchi, 1983). However, it may not be efflux of chloride ions that directly mediates any ontogenic modulation. Walton et al. (1991) have shown using fura-2 calcium indicator dye that in E15 spinal cord cells GABA produces a bicuculline-sensitive increase in internal calcium. Therefore, the Cl^- depolarization by GABA may merely trigger an influx of calcium into the neuron (through voltage-dependent calcium channels) that then may induce processes important for development and subunit expression. However, it is hard to imagine that a transient rise in the calcium would be the only signal responsible for all the neurotrophic effects and phenotypic expression of the GABA α -subunits, and other signals may be produced that may be

Figure 9. Sagittal sections of adult rat brain showing distribution of all α -mRNAs. cx, cortex; hi, hippocampus; Th, thalamus; cb, cerebellum; Md, medulla; CPu, caudate-putamen; sc, superior colliculus; ob, olfactory bulb.

important, such as changes in pH (Kaila and Voipoi, 1990).

In summary, this study has demonstrated the variable expression of GABA_A receptor α -subunit mRNA during ontogeny. *In vitro* functional assays would predict that the phenotype of the resulting embryonic GABA_A receptors would have a high sensitivity to GABA and low affinity for positive modulators (i.e., type II benzodiazepine binding site). This expression pattern and that of the other GABA_A subunits (Poulter et al., 1991b) provide a map of neuronal subpopulations expressing limited subunit combinations that should be useful for correlating GABA_A receptor physiology and pharmacology in native membrane. Preliminary studies from our laboratory have correlated significantly different GABA-mediated responses to embryonic neurons expressing different combinations of GABA subunit mRNAs (Poulter et al., 1991c). The significance of the expression pattern, with regard to ontogeny, implies that these embryonic GABA_A receptors composed of different α -subunits (particularly α_5) are important for synaptogenesis. However, the nature by which they may contribute to synaptogenesis is a matter for further investigation.

References

- Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa J-L (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol (Lond)* 416:303–325.
- Burt DR, Kamatchi GL (1991) GABA_A receptor subtypes: from pharmacology to molecular biology. *FASEB J* 5:2916–2923.
- Dunwiddie TV (1981) Age related differences in the *in vitro* rat hippocampus: development of inhibition and the effects of hypoxia. *Dev Neurosci* 4:165–175.
- Fizman ML, Novotny EA, Lange DG, Barker JL (1990) Embryonic and early postnatal cells respond to nanomolar concentrations of muscimol. *Dev. Brain Res* 53:186–193.
- Fuji K, Senba E, Fujii S, Nomura I, Wu JY, Ueba Y, Tohyama M (1985) Distribution ontogeny and projections of cholecystokinin-8, vasoactive intestinal polypeptide and aminobutyrate neurons in the rat spinal cord: an immunocytochemical analysis. *Neuroscience* 4: 881–894.
- Gambarana C, Pittman R, Siegel RE (1990) Developmental expression of the GABA_A receptor α_1 subunit mRNA in the rat brain. *J Neurobiol* 21:1169–1179.
- Garrett K, Tabakoff B (1985) The development of type I and type II benzodiazepine receptors in the mouse cortex and cerebellum. *Pharmacol Biochem Behav* 22:985–992.
- Guidotti A, Antonacci MD, Giusti P, Massotti M, Memo M, Schlichting JL (1990) The differences in the pharmacological profiles of various benzodiazepine recognition site ligands may be associated with the GABA_A receptor structural diversity. In: *GABA and benzodiazepine receptor subtypes* (Biggio G, Costa E, eds), pp 73–87. New York: Raven.
- Kaila K, Voipoi J (1990) GABA-activated bicarbonate conductance: influence on E_{GABA} and postsynaptic pH regulation. In: *Chloride channels and carriers in nerve muscle and glial cells* (Alvarez-Leefmans FJ, Russell JM, eds), pp 331–352. London: Plenum.
- Karavolas HJ, Hodges DR (1990) Neuroendocrine metabolism of progesterone and progestins. In: *Steroids and neuronal activity* (Chadwick D, Widdows K, eds), pp 22–55. New York: Wiley.
- Kato K (1990) Novel GABA_A receptor α -subunit is expressed only in cerebellar granule cells. *J Mol Biol* 214:619–624.
- Khrestchatsky M, MacLennan JA, Chang M-Y, Yu W, Jackson Brecha N, Sternini C, Olsen RW, Tobin AJ (1989) A novel α -subunit in the rat brain GABA_A receptors. *Neuron* 3:745–753.
- Khrestchatsky M, MacLennan JA, Tillakaratne NJK, Chiang M-Y, To-

- bin AJ (1991) Sequence regional distribution of the mRNA encoding the α_2 polypeptide of rat γ -aminobutyric acid_A receptors. *J Neurochem* 56:1717–1722.
- Lauder JM, Han VKM, Henderson P, Verdoorn T, Towle AC (1986) Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study. *Neuroscience* 19:465–493.
- Levitan ES, Balir LAC, Dionne VE, Barnard EA (1988a) Biophysical and pharmacological properties of cloned GABA_A receptor subunits expressed in *Xenopus* oocytes. *Neuron* 1:773–781.
- Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Köhler M, Fujita N, Rodriguez H, Stephenson FA, Darlison MG, Barnard EA, Seeburg PH (1988b) Structural and functional basis for GABA_A receptor heterogeneity. *Nature* 335:76–79.
- Lolait SJ, O'Carroll A-M, Kusano K, Muller J-M, Brownstein MJ, Mahan LC (1989a) Cloning and expression of a novel GABA_A receptor. *FEBS Lett* 246:145–148.
- Lolait SJ, O'Carroll A-M, Kusano K, Mahan LC (1989b) Pharmacological characterization and region-specific expression in brain of the β_2 and β_3 subunits of the rat GABA_A receptor. *FEBS Lett* 258:17–21.
- Luddens H, Wisden W (1991) Function and pharmacology of multiple GABA_A receptor subunits. *Trends Pharmacol Sci* 12:49–51.
- Luddens H, Pritchett DB, Köhler M, Killisch K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar GABA_A receptor selective for a behavioural alcohol antagonist. *Nature* 346:648–651.
- MacLennan AJ, Brecha N, Khrestchatsky M, Sternini C, Tillakaratne NJK, Chiang M-Y, Anderson K, Lai M, Tobin AJ (1991) Independent cellular and ontogenic expression of mRNAs encoding three α polypeptides of the rat GABA_A receptor. *Neuroscience* 43:369–380.
- Malherbe P, Sigel E, Baur R, Richards JG, Möhler H (1990) Functional expression and sites of transcription of a novel α -subunit GABA_A receptor in rat brain. *FEBS Lett* 260:261–265.
- Mandler R, Schaffner AE, Novotny EA, Lange GD, Smith SV, Barker JL (1990) Electrical and chemical excitability appear one week before birth in the embryonic rat spinal cord. *Dev Brain Res* 522:46–54.
- Meier E, Drejer J, Schousboe A (1983) Trophic actions of GABA on the development of physiologically active GABA receptors. In: *Glutamine and GABA in the central nervous system* (Hertz L, Kvamme E, McGeer EG, Schousboe A, eds), pp 509–516. New York: Liss.
- Meier E, Drejer J, Schousboe A (1984) GABA induces functionally active low-affinity GABA receptors on cultured cerebellar granule cells. *J Neurochem* 43:1737–1744.
- Meier E, Hertz L, Schousboe A (1991) Neurotransmitters as developmental signals. *Neurochem Int* 19:1–15.
- Möhler H, Malherbe A, Draguhn E, Sigel E, Sequier JM, Persohn E, Richards JG (1990) GABA_A-receptor subunits: functional expression and gene localization. In: *GABA and benzodiazepine receptor subtypes* (Biggio G, Costa E, eds), pp 23–34. New York: Raven.
- Olsen RW, Tobin AJ (1990) Molecular biology of GABA_A receptors. *FASEB J* 4:1469–1480.
- Padjen AL, Hashiguchi T (1983) Primary afferent depolarization in frog spinal cord is associated with an increase in membrane conductance. *Can J Physiol Pharmacol* 61:626–631.
- Paxinos G, Watson C (1986) *The rat brain in stereotaxic coordinates*. Orlando, FL: Academic.
- Paxinos G, Törk I, Tecott L, Valentino KL (1991) *Atlas of the developing rat brain*. San Diego: Academic.
- Puia G, Vicini S, Seeburg PH, Costa E (1991) Influence of recombinant GABA_A receptor subunit composition on the action of allosteric modulators of GABA-gated Cl⁻ currents. *Mol Pharmacol* 39:691–696.
- Poulter MO, Barker JL, O'Carroll A-M, Mahan LC, Lolait SJ (1991a) An *in situ* hybridization study of GABA_A receptor α -subunit mRNA expression in the developing rat embryo. *IBRO World Congress of Neuroscience Abstract* 3:133.
- Poulter MO, Barker JL, O'Carroll A-M, Mahan LC, Lolait SJ (1991b) An *in situ* hybridization study of GABA_A receptor β and γ_2 subunit mRNA expression in the developing rat embryo. *IBRO World Congress of Neuroscience Abstract* 3:133.
- Poulter MO, O'Carroll A-M, Mahan LC, Lolait SJ, Barker JL (1991c) Correlation of GABA_A receptor mRNA subunit expression and single channel kinetics in the developing rat central nervous system. *Soc Neurosci Abstr* 17:77.
- Pritchett DB, Seeburg PH (1990) γ -Aminobutyric acid_A receptor α_3 subunit creates novel type II benzodiazepine receptor pharmacology. *J Neurochem* 54:1802–1804.
- Pritchett DB, Luddens H, Seeburg PH (1989) Type I and type II GABA_A-benzodiazepine receptors produced in transfected cells. *Science* 245:1389–1392.
- Redburn DA, Schousboe A, eds (1987) Neurotrophic activity of GABA during development. *Neurol Neurobiol* 32:1–277.
- Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH, Barnard EA (1987) Sequence and functional expression of the GABA_A receptor shows ligand-gated receptor super family. *Nature* 328:221–227.
- Schwartzkroin PA (1982) Development of rabbit hippocampus physiology. *Dev Brain Res* 2:469–486.
- Sigel E (1990) Use of *Xenopus* oocytes for the functional expression of plasma membrane proteins. *J Membr Biol* 117:201–221.
- Sigel E, Baur R, Gerhard T, Möhler H, Malherbe P (1990) The effect of subunit composition of rat brain GABA_A receptors on channel function. *Neuron* 5:703–711.
- Squires RF, Benson DJ, Braestrup C, Coupet J, Klepner CA, Myers V, Beer B (1979) Some properties of brain specific benzodiazepine receptors: new evidence for multiple receptors. *Pharmacol Biochem Behav* 10:825–830.
- Stephenson FA, Duggan MJ, Casalotti SO (1989) Identification of the α_3 in the GABA_A receptor purified from bovine brain. *FEBS Lett* 243:358–362.
- Suzdak PD, Glowa JR, Crawley JN, Schwartz RD, Skolnick P, Paul SM (1986) A selective imidazobenzodiazepine antagonist for ethanol in the rat. *Science* 234:1243–1247.
- Ticku MK, Kularn RK (1988) Molecular interactions of ethanol with the GABAergic system and the potential of Ro15-4513 as an ethanol antagonist. *Pharmacol Biochem Behav* 30:501–510.
- Walton MK, Schaffner AE, Barker JL (1991) GABA_A receptor activation causes elevation of intracellular calcium in embryonic rat spinal cord cells. *Soc Neurosci Abstr* 17:749.
- Wisden W, Herb A, Weiland H, Keinänen K, Luddens H, Seeburg PH (1991) Cloning, pharmacological characteristics and expression pattern of the rat GABA_A receptor α_4 subunit. *FEBS Lett* 289:227–230.
- Wolff JR (1981) Evidence for a dual role of GABA as a synaptic transmitter and promoter of synaptogenesis. In: *Amino acid transmitters* (De Feudis FV, Mandel PM, eds), pp 459–465. New York: Raven.
- Ymer S, Draguhn A, Köhler M, Schofield PR, Seeburg PH (1989) Sequence and expression of a novel GABA_A receptor α -subunit. *FEBS Lett* 258:119–122.
- Young WS III, Bonner TI, Brann MR (1986) Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. *Proc Natl Acad Sci USA* 83:9827–9831.