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Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain

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The inhibitory glycine receptor (GlyR) is a ligand-gated ion channel which mediates post-synaptic inhibition in spinal cord and other regions of the vertebrate central nervous system. Previous biochemical and molecular cloning studies have indicated heterogeneity of GlyRs during development. Here, the distribution of GlyR subunit transcripts in rat brain and spinal cord was investigated by *in situ* hybridization using sequence-specific oligonucleotide probes. In adult animals, GlyR α1 subunit mRNA was abundant in spinal cord, but was also seen in a few brain areas, e.g. superior and inferior colliculi, whereas α2 transcripts were found in several brain regions including layer VI of the cerebral cortex and hippocampus. GlyR α3 subunit mRNA was expressed at low levels in cerebellum, olfactory bulb and hippocampus, while high amounts of β subunit transcripts were widely distributed throughout spinal cord and brain. During development, α2 mRNA accumulated already prenatally and decreased after birth, whereas α1 and α3 subunit transcripts appeared only in postnatal brain structures. Hybridization signals of β subunit mRNA were seen already at early embryonic stages and continuously increased to high levels in adult rats. These data reveal unexpected differences in the regional and developmental expression of GlyR subunit mRNAs and point to novel functions of GlyR proteins in the mammalian central nervous system.

Key words: glycine receptor/heterogeneity/*in situ* hybridization/mRNA distribution/rat brain

Introduction

Glycine is now widely recognized as a major inhibitory neurotransmitter in the vertebrate central nervous system (Aprison and Daly, 1978). Glycine-mediated inhibition of neuronal activity results from activation of the inhibitory glycine receptor (GlyR), a ligand-gated chloride channel in spinal cord and other regions of the central nervous system (reviewed in Betz and Becker, 1988; Langosch *et al.*, 1990). The GlyR has been purified from mammalian spinal cord and is thought to represent a pentamer composed of ligand-binding subunits of 48 kDa (α) and homologous polypeptides of 58 kDa (β) (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985; Langosch *et al.*, 1988; Betz, 1990a). The primary structures of these GlyR subunits (α1 and β) have been determined by cDNA sequencing and shown to share a common transmembrane topology and significant sequence homology with nicotinic acetylcholine and $GABA_A$ receptor proteins (Grenningloh *et al.*, 1987, 1990a). Moreover, recently α subunit variants (α2 and α3) have been identified which are thought to represent ligand-binding subunits of developmentally regulated neonatal and adult GlyR isoforms (Becker *et al.*, 1988; Akagi and Miledi, 1988; Grenningloh *et al.*, 1990b; Kuhse *et al.*, 1990a,b). Biochemical data (Becker *et al.*, 1988; Hoch *et al.*, 1989) and heterologous expression in *Xenopus* oocytes (Schmieden *et al.*, 1989; Grenningloh *et al.*, 1990a,b; Kuhse *et al.*, 1990a,b) or mammalian cell lines (Sontheimer *et al.*, 1989) indicate that these different GlyR subunits display significant functional and pharmacological differences upon assembly into agonist-gated chloride channels.

From autoradiography with the selective antagonist strychnine (Zarbin *et al.*, 1981; Frostholm and Rotter, 1985; Probst *et al.*, 1986; Frost White *et al.*, 1990) and immunocytochemistry with monoclonal antibodies (Triller *et al.*, 1985; Altschuler *et al.*, 1986; Araki *et al.*, 1988; Van den Pol and Gorcs, 1988), GlyRs are thought to be mainly concentrated in spinal cord, brain stem and other areas of the lower neuraxis (see also Aprison and Daly, 1978; Betz and Becker, 1988; Langosch *et al.*, 1990). Recent Northern blot hybridization experiments and amplification by polymerase chain reaction (PCR), however, indicate the presence of GlyR subunit mRNAs in higher regions of the central nervous system (Grenningloh *et al.*, 1990a; Kuhse *et al.*, 1990a). Here, we present an *in situ* hybridization analysis of GlyR α1, α2, α3 and β subunit transcripts in rat brain and spinal cord. Our data reveal marked differences in the regional and temporal expression of these inhibitory chloride channel proteins and suggest novel roles of GlyR proteins in the mammalian brain.

Results

Design and specificity of oligonucleotide probes

The probe sequences for α1, α2 and α3 antisense oligonucleotides were designed from regions of the corresponding cDNAs which encode the divergent intracellular region between transmembrane domains M3 and M4 (see Materials and methods). For detecting β subunit transcripts, an antisense oligonucleotide complementary to a portion of the extended N-terminal coding region characteristic of this subunit cDNA was used.

Northern blot analysis performed with the α1, α2 and β specific probes (Figure 1) revealed hybridizing RNAs of sizes characteristic of each GlyR subunit transcript (Grenningloh *et al.*, 1987, 1990a; Kuhse *et al.*, 1990a). Northern blot hybridization with the α3 specific

oligonucleotide gave no hybridization signal under the same conditions, indicating that this mRNA is under-represented in the central nervous system. Dot blot experiments, in which α1, α2, α3 and β subunit cDNAs were probed with the α3 oligonucleotide under stringency conditions comparable with those used for *in situ* hybridization, established the selectivity of this antisense probe (data not shown).

Distribution of GlyR subunit transcripts in the adult central nervous system

To determine the distribution of the four GlyR subunits known presently, we compared tissue hybridizations obtained with oligonucleotide probes specific for α1, α2, α3 and β mRNAs. Macroscopic transcript distributions in adult rat brain and spinal cord are shown in Figure 2. Each probe except that for β produced highly regionalized hybridization signals. While α2 (Figure 2B and F) and α3 (Figure 2C and G) transcripts were largely confined to higher regions of the central nervous system, α1 mRNA was expressed in spinal cord, brain stem and midbrain, thus pointing to a good correlation of the α1 subunit to the previously described rostrocaudal gradient of [^{3}H]strychnine binding sites (Zarbin *et al.*, 1981; Frostholm and Rotter, 1985; Probst *et al.*, 1986; Frost White *et al.*, 1990) and histochemically detected GlyR antigen (Triller *et al.*, 1985; Altschuler *et al.*, 1986; Araki *et al.*, 1988; Van den Pol and Gorcs, 1988). The pattern of β mRNA hybridization (Figure 2D and H) revealed a widespread expression of this subunit along the entire neuraxis.

Table I summarizes the regions of adult rat brain and spinal cord where α1, α2, α3 and β mRNA hybridization was observed. These data were compiled by evaluating both exposed X-ray films and emulsion-coated slides.

Telencephalon

Olfactory bulb. The only GlyR subunit mRNAs detected in olfactory bulb tissue were α3 and β. Cells giving a strong signal with the β probe were localized in the periglomerular region and may correspond to small interneurons surrounding the glomeruli as well as to external tufted cells. A few internal tufted cells in the external plexiform layer were also labelled by β antisense oligonucleotides. In contrast, only weak hybridization was found for α3 in the periglomerular region. Mitral cells were strongly labelled by the β, but not the α3 probe (Figure 3A), whereas the internal granular cell layer contained both mRNAs (Figure 2C and D). Consistently, β mRNA hybridization signals were more prominent than those produced by α3 transcripts.

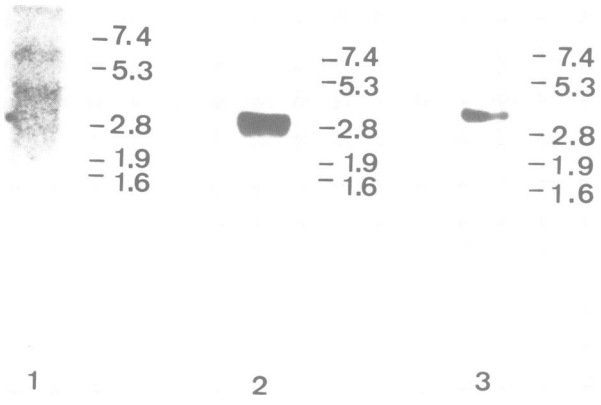

Fig. 1. Northern blot analysis showing specificity of oligonuceotide hybridization. Poly(A)$^+$ RNA isolated from rat spinal cord at different postnatal stages (P3, P20 and P40) was electrophoresed on a formaldehyde–agarose gel (5 μg/lane), transferred to a nylon filter and hybridized with the following GlyR subunit-specific probes. Lane 1, α1 antisense; lane 2, α2 antisense; and lane 3, β antisense oligonucleotides (see Materials and methods). The sizes of the transcripts observed after four days of exposure correspond to those previously reported for α1 (Grenningloh *et al.*, 1987), α2 (Kuhse *et al.*, 1990a) and β (Grenningloh *et al.*, 1990) subunit mRNAs. Only lanes giving strong hybridization signals (α1: P20, α2: P3, and β: P40, RNAs, respectively) are shown. Under the same conditions, no signal was obtained with an α3 antisense probe. RNA size markers (in kb) are indicated.

Cerebral cortex. The GlyR mRNAs detected in cortex were α2 and β. Expression of β transcripts was very high and seen in all layers, but notably in laminae IV and VI of the neocortex (Figure 2D). In contrast, α2 positive cells appeared to be largely confined to the deeper layer of lamina VI (Figures 2B and 3B).

In the posterior part of the cingulate cortex, labelling of β transcripts was also very prominent. Infralimbic cortex showed high hybridization to the β and, in its deeper layer, to the α2 probes as well as a moderate signal with the α3 antisense oligonucleotide. GlyR α2 and β transcripts were also found in the piriform cortex. Here, layers II and III showed strong hybridization to the β probe and low levels of α2 mRNA.

The levels of GlyR transcripts in the entorhinal cortex paralleled those observed in the other neocortical regions. Hybridization with the β probe was seen throughout this region, but notably in layer II, while α2 signals were detected in the internal layer of lamina VI (Figure 2, and data not shown).

Hippocampal formation. In the hippocampus, again distinct patterns of hybridization were observed for the different GlyR transcripts. A strong hybridization signal was produced by β probes: regions CA1–CA4, the dentate gyrus, the subiculum and the entorhinal cortex were all intensely labelled, with the granule cells of the dentate gyrus showing the highest expression levels (Figure 3D and Table I). Similarly, α2 mRNA was detected in the dentate gyrus and the CA2/CA3 and CA4 regions at moderate levels (Figure 2B and D). Expression of α3 mRNA was low, but consistently observed throughout the hippocampal formation including the subiculum (Figure 2C).

Striatum. Caudate putamen and globus pallidus are brain regions where many cells displayed only weak hybridization to the β antisense oligonucleotide (Figure 2D).

Diencephalon

Thalamus. The GlyR subunit mRNAs expressed in thalamic nuclei were α1, α2 and β. Anterodorsal and reticular nuclei expressed only β mRNA at high levels. Interestingly, cells of the parafascicular nucleus showed significant labelling by α1 and β probes (Figure 2A and D). In the supragenicular nucleus, expression of α2 and β mRNAs was seen (Table I).

Hypothalamus. In the ventromedial hypothalamic nucleus, a colocalization of α3 and β transcripts was found, while the dorsomedial hypothalamus was labelled by α1 and α3 probes. Very high levels of β transcripts were detected in the zona incerta (data not shown).

Geniculate nuclei. Both the dorso-lateral and the medial geniculate nuclei were moderately labelled by the $\alpha 2$ probe and showed high levels of β transcripts.

Mesencephalon

Colliculi. The inferior colliculus, in particular its central nucleus, displayed moderate expression of $\alpha 1$ and high levels of β transcripts (Figures 2A and D). In addition, β mRNA was seen in the brachium of the inferior colliculus. The $\alpha 1$ and β probes also labelled the superior colliculus in its intermediate grey layer (Figure 2A and D); β transcripts were in addition seen in the superficial grey and the optic nerve layers.

Other regions. The central grey is considered a relay station of motor, sensory and limbic pathways. Here it showed low hybridization signals with $\alpha 2$ and $\alpha 3$ probes and high expression of β transcripts (Figure 2). The substantia nigra and the interpeducular and red nuclei also displayed significant β signals .

Metencephalon

Cerebellum. An unusual distribution of GlyR mRNAs was found in the cerebellar cortex. Strong hybridization with the β probe was seen in all layers; however, Purkinje cells and the granular cell layer were particularly heavily labelled (Figures 2D and 3C). Of the other GlyR subunit probes

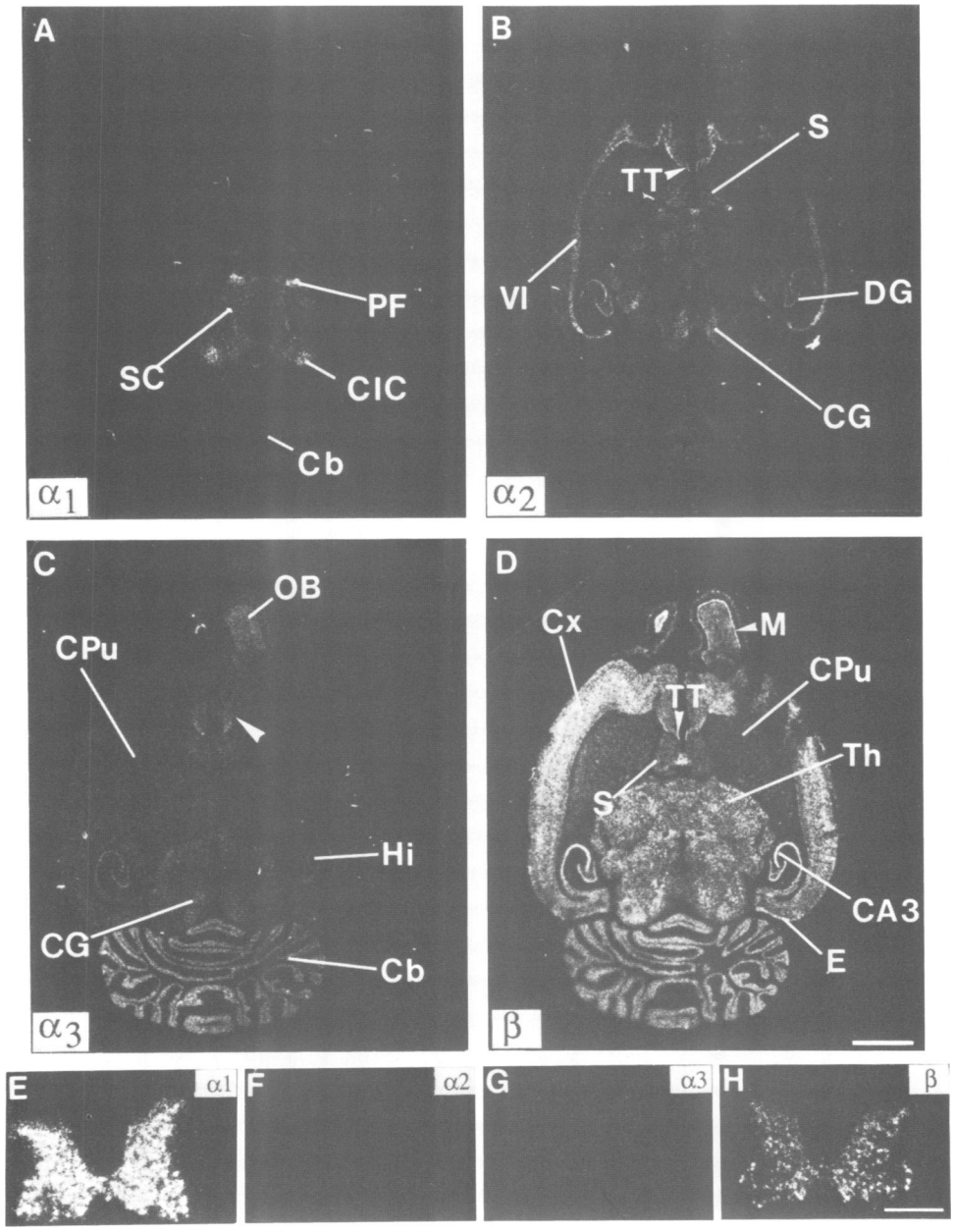

Fig. 2. *In situ* hybridization of GlyR subunit mRNAs in horizontal brain sections (A, B, C and D) and coronal spinal cord sections (E, F, G and H) from adult (P40) rats. CA3, CA3 region of the hippocampus; Cb, cerebellum; CG, central grey; CIC, central nucleus of the inferior colliculus; CPu, caudate putamen; Cx, cortex; DG, dentate gyrus; E, entorhinal cortex; Hi, hippocampus; OB, olfactory bulb; PF, parafascicular nucleus; S, septum; Th, thalamus; TT, tenia tecta; VI, sixth layer of cortex; arrowhead indicates supposed internal layers of the infralimbic cortex. (**A**, **E**) $\alpha 1$, (**B**, **F**) $\alpha 2$, (**C**, **G**) $\alpha 3$ and (**D**, **H**) β, subunit mRNA distributions. Exposure to Kodak X-Omat AR film was for eight weeks. Scale bars: 4 mm (D), 1 mm (H).

tested, only the α3 antisense oligonucleotide gave a moderate hybridization signal that was confined to granule cells (Figure 2C and Table I). None of the other α mRNAs was detected in significant amounts, although microscopic analysis of emulsion-coated slides revealed rare single cells expressing α1 transcripts in the granule cell layer (not shown).

Of the deep cerebellar nuclei analyzed, only the lateral nucleus displayed low to moderate hybridization signals for α1 and β subunit mRNAs (Table I).

Brain stem

Both α1 and β mRNAs were found in several brain stem nuclei (Table I). The dorsal and ventral cochlear nuclei, which receive direct inputs from the cochlea, showed high levels of hybridization for α1 and β transcripts, a finding consistent with the demonstration of [^{3}H]strychnine binding sites and GlyR antigen in these structures (Altschuler *et al.*, 1986). In the ventral nucleus of the lateral lemniscus, only β mRNA was detected. The trigeminal motor nucleus, which innervates the masticatory muscles, also displayed high levels of α1 and β probe hybridization. Similarly, the sensory trigeminal nuclei, e.g. the principal and spinal tract nuclei of the trigeminal nerve, were heavily labelled by the α1 and β antisense oligonucleotides, as were the reticular nucleus, the reticulo-tegmental nucleus, the reticular formation and the superior vestibular nucleus. The dorsal raphe gave low hybridization signals with the α2 probe and moderate ones with the β probe.

Table I. Distribution and relative abundance of GlyR subunit mRNAs in different regions of the adult rat central nervous system

AREA	mRNA			
	α1	α2	α3	β
Olfactory bulb				
Glomerular layer	n.d.	n.d.	n.d.	++
External plexiform layer	n.d.	n.d.	n.d.	+
Mitral cell layer	n.d.	n.d.	n.d.	+++
Internal granule cell layer	n.d.	n.d.	+	++
Tenia tecta	n.d.	+	n.d.	++
Cerebral cortex				
Layers II–V	n.d.	n.d.	n.d.	+++
Layer VI	n.d.	++	n.d.	+
Infralimbic cortex	n.d.	++	+	+++
Piriform cortex	n.d.	+	n.d.	+++
Cingulate cortex	n.d.	n.d.	n.d.	+++
Septum	n.d.	+	n.d.	++
Hippocampal complex				
Dentate gyrus	n.d.	+	+	+++
CA1	n.d.	+	+	++
CA3	n.d.	+	+	+++
CA4	n.d.	++	n.d.	+++
Subiculum	n.d.	n.d.	+	+++
Striatum	n.d.	n.d.	n.d.	+
Thalamus				
Anterodorsal nucleus	n.d.	n.d.	n.d.	++
Parafascicular nucleus	++	n.d.	n.d.	++
Suprageniculate nucleus	n.d.	+	n.d.	++
Reticular nucleus	n.d.	n.d.	n.d.	+++
Geniculate nuclei	n.d.	+	n.d.	+++
Hypothalamus	++	d	+	++
Superior colliculus	+	n.d.	n.d.	+++
Inferior colliculus	++	n.d.	n.d.	+++
Cerebellum				
Molecular cell layer	n.d.	n.d.	n.d.	+
Purkinje cell	n.d.	n.d.	n.d.	+++
Granule cell layer	n.d.	n.d.	++	+++
Lateral deep nucleus	+	n.d.	n.d.	++
Brain stem nuclei	+++	d	n.d.	+++
Spinal cord				
Dorsal horn	+++	+	n.d.	+++
Ventral horn	+++	n.d.	n.d.	+++

Relative expression levels were estimated by visual comparison of exposed X-ray films and emulsion-coated slides: n.d. = not detected; + = low; ++ = moderate; +++ = high; d = not determined.

Spinal cord

Throughout the different segments of spinal cord, α1 and β transcripts were highly expressed, motor neurons in the ventral horn being particularly heavily labelled (Figure 2E and H). Although not visible in Figure 2F, microscopic analysis of emulsion-coated spinal cord sections revealed some cells containing α2 mRNA which were scattered throughout the dorsal horn. Hybridization with α3 probes gave a very faint diffuse signal in both dorsal and ventral horns that was recognized only upon prolonged exposure.

Accumulation of GlyR transcripts during development

Horizontal sections of embryonic and postnatal rat brain and coronal sections of spinal cord were hybridized in parallel to α1, α2, α3 and β probes under identical conditions to allow comparison of the amount of transcripts at different stages of development (Figure 4 and Table II). Embryos were studied at days 14 and 19 (E14 and E19), and postnatal animals at days 0, 5, 15 (P0, P5 and P15) and 20 (P20, data not shown) after birth.

α1 RNA. In brain, significant levels of transcripts coding for the α1 subunit were detected at postnatal stage P5 (Figure 4). Their regional distribution at this stage was indistinguishable from that seen in adult animals (Figure 2A). Interestingly, a transient weak labelling of the lateral cerebellar nucleus was observed at P15 (Figure 4) and P20 (data not shown). At these stages, the dorso-lateral geniculate also transiently displayed higher amounts of α1 transcripts. In spinal cord, α1 mRNA was already seen at E14 and continously increased thereafter in both ventral and dorsal horn regions to reach high levels around P15 (Table II).

α2 RNA. Hybridization with the α2 probe revealed an early expression and significant changes in the regional distribution of the corresponding transcripts during development. In most brain areas, α2 mRNA was highly expressed at both prenatal (E14 and E19) and early postnatal (P0 and P5) stages, while later its distribution approached the adult hybridization pattern (Figure 4). Prenatally, α2 signals appeared localized in the telencephalon, diencephalon and in midbrain. Already at E14, i.e. when only layer I of the cortex is formed (Berry *et al.*, 1964), prominent labelling of this region was seen. All cell populations of the cortical layers originate at E19 and P0 (Angevine and Sidman, 1961; Berry *et al.*, 1964); more pronounced hybridization signals then became visible in a double-layered structure. At P5 the laminated structure of the cortex was clearly recognized, and layers I/II and IV were strongly labelled by the α2 probe. The adult hybridization pattern in cortex was achieved at P15 (Figure 4), with layer VI displaying strong labelling, which subsequently decreased to adult levels (Figure 2B).

The hippocampal formation contained high levels of α2 mRNA already at E19, i.e. at a time when only the Ammon's horn is macroscopically delineated. The granule

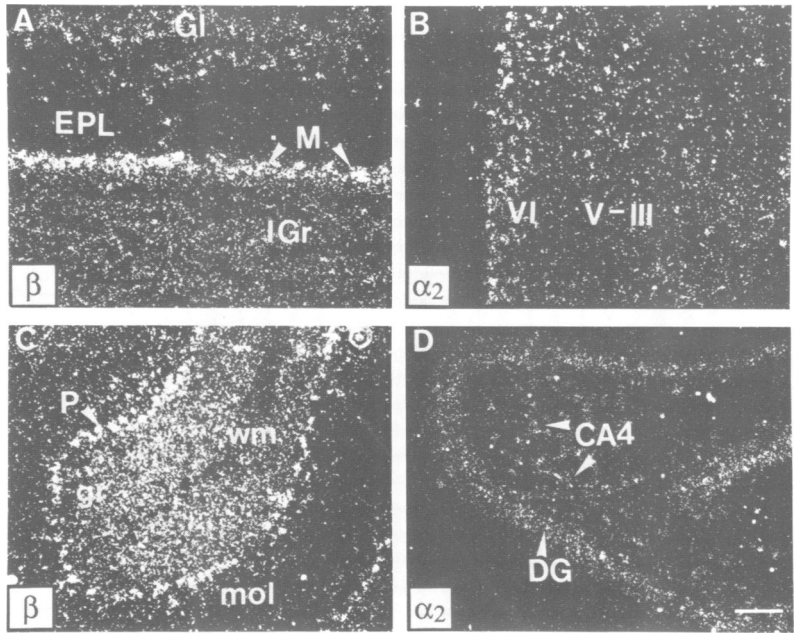

Fig. 3. Low-power dark field photomicrographs of olfactory bulb (**A**), cerebral cortex (**B**), cerebellum (**C**), and hippocampus (**D**). (A, C) β, and (B, D) $\alpha 2$, mRNA distributions. EPL, external plexiform layer; Gl, glomerular layer; M, mitral cell layer; IGr, internal granular cell layer; VI, V–III, layers of the neocortex; gr, granular cell layer; mol, molecular layer; P, Purkinje cell layer; wm, white matter; DG, dentate gyrus; CA4, CA4 region. Exposure of the emulsion-coated slides was for 8 weeks. Scale bar: 37 μm.

cells of the dentate gyrus, which continue to be formed until postnatal day 20, were labelled at stage P5. In addition, $\alpha 2$ mRNA was transiently expressed in the thalamus starting at E14 and reaching high levels around E19. Thereafter, thalamic labelling remained high until P5 and thereafter disappeared (Figure 2B), with some remaining hybridization at P20. In the cerebellar cortex, a structure that reaches maturity only very late after birth, transient $\alpha 2$ hybridization signals were seen at stages P0, P5 and P15. A strong transient hybridization was also seen in the lateral amygdala at P15 (Figure 4) and P20. The medial geniculate nucleus showed higher levels of $\alpha 2$ transcripts at this developmental stage (Figure 4) as compared with adult brain (Figure 2B).

In spinal cord, high levels of $\alpha 2$ transcript were found already at E14 which persisted in both ventral and dorsal horns until birth (Table II). Thereafter, signals decreased rapidly; with the exception of single cells in the dorsal horn, no label was seen after P15.

$\alpha 3$ RNA. Accumulation of $\alpha 3$ transcripts occurred late in development. Specific hybridization confined to the infralimbic cortex appeared only at P5 and persisted throughout all subsequent stages analyzed (Figures 4 and 2C). At P5, labelling of the colliculi and of the reticular thalamic nucleus also became significant. No hybridization signal was found in cerebellum before P40, indicating a late onset of $\alpha 3$ expression in this region. Only very weak hybridization was seen in spinal cord over dorsal and, to some extent, ventral horn regions from stages E19 to P20 (Table II).

β RNA. Transcripts of the GlyR β subunit gene showed a widespread distribution in many embryonic and early postnatal structures of rat brain (Figure 4). At E14, β mRNA was mainly found in the telencephalic region committed to develop into cortex. At E19, only the external cells (presumptive layers I and II) of the cortex were specifically labelled. Septum, the Ammon's horn of the hippocampal formation, thalamus and midbrain also displayed strong β hybridization signals at this stage. Interestingly, at E19 the cerebellum anlage was already labelled by β probes. At birth (P0), the cerebral cortex showed a diffuse distribution of β transcripts. Cortical layers I/II and VI were preferentially labelled at P5, although hybridization was seen throughout all layers of the cortex. The entorhinal cortex and hippocampus, in particular the subiculum and the Ammon's horn regions, also displayed intense labelling. Moreover, septum, thalamus and the colliculi showed high levels of β transcripts, whereas in the cerebellar cortex only a weak hybridization signal was found at this stage. The intensity and regional distribution of β mRNA at P15 closely resembled the adult situation. High levels of expression were then found in all layers of the cortex, olfactory bulb, hippocampus, cerebellum and thalamus. Moderate hybridization was detected in the septum and the striatum.

In spinal cord, β transcripts were first observed at E14 (Table II). Thereafter, their level increased rapidly to reach very high levels at birth in both dorsal and ventral horns. High expression then persisted postnatally up to adult stages.

Discussion

The *in situ* hybridization data presented in this study reveal two unexpected results. First, the distribution of known α subunit mRNAs does not match the pattern of β transcript expression. Second, we find GlyR subunit mRNAs in higher brain regions not previously known to contain GlyR proteins. Several investigators have used both [^{3}H]strychnine binding (Zarbin *et al.*, 1981; Frostholm and Rotter, 1985; Probst *et al.*, 1986; Frost White *et al.*, 1990) and monoclonal antibodies against affinity-purified GlyR preparations (Triller *et al.*, 1987; Akagi and Miledi, 1988; Van den Pol and Gorcs, 1988) to map the distribution of this receptor system in both the rodent and human central nervous system. Although many of the GlyR-positive areas identified in these

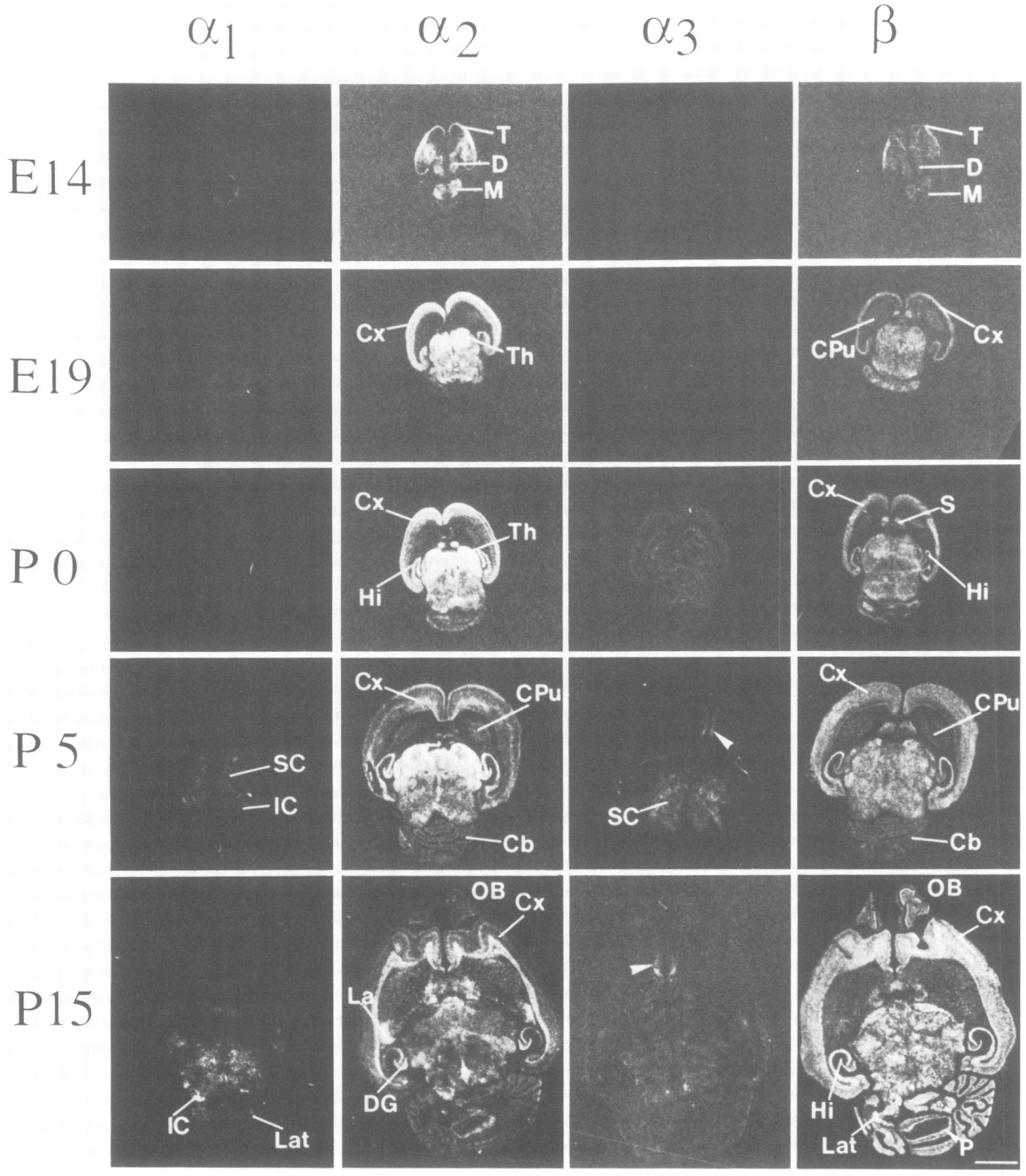

Fig. 4. *In situ* hybridization of GlyR subunit mRNAs in horizontal sections of rat brain during development. Sections from embryonic days E14 and E19, and of postnatal stages P0, P5, and P15, are shown. Cb, cerebellum; CPu, caudate putamen; Cx; cortex; D, diencephalon; DG, dentate gyrus; Hi, hippocampus; IC, inferior colliculus; La, lateral amygdaloid nucleus; Lat, lateral cerebellar nucleus; M, midbrain; OB, olfactory bulb; P, Purkinje cell layer; S, septum; SC, superior colliculus; T, telencephalon; Th, thalamus; arrowheads indicate putative internal layers of infralimbic cortex. Exposure to X-Omat AR film was for eight weeks. Scale bar: 3.4 mm.

studies overlap with the localizations of GlyR subunit transcripts described here, our data indicate a considerably wider distribution than seen previously. This probably reflects that (i) not all GlyRs bind strychnine with high affinity, as already shown by biochemical (Becker *et al.*, 1988; Hoch *et al.*, 1989) and molecular (Kuhse *et al.*, 1990a) studies and (ii) the monoclonal antibodies used were specific for the α1 subunit of the GlyR (Pfeiffer *et al.*, 1984; Schröder *et al.*, 1991) or directed against the 93 kDa peripheral protein of the post-synaptic GlyR complex (Pfeiffer *et al.*, 1984; Schmitt *et al.*, 1987; Becker *et al.*, 1989). In other words, not all GlyR polypeptides were disclosed by the mapping methods used previously.

High levels of α1 transcripts were found in spinal cord and brain stem, but also in the colliculi, and, with the exception of spinal cord, expression was seen only at postnatal stages of development. The regional and temporal expression pattern seen in spinal cord is consistent with

Table II. Relative abundance of GlyR subunit mRNAs in developing rat spinal cord

mRNA	stage					
	E14	E19	P0	P5	P15	P20
α1	+	(+)	++	++	+++	+++
α2	+++	+++	+++	+	(+)	(+)
α3	n.d.	(+)	(+)	(+)	(+)	(+)
β	+	+	+++	+++	+++	+++

Relative signal intensities are indicated by: (+), very low; +, low; ++, moderate; +++, high; n.d., not detected.

previous Northern blot (Grenningloh *et al.*, 1987) and S1 nuclease protection (Malosio *et al.*, 1991) data, which indicate a strong increase of α1 transcripts after birth. Moreover it largely resembles the previously described distribution of [^{3}H]strychnine binding sites and GlyR antigen in the rat central nervous system, indicating that the α1 subunit is indeed a component of the 'classical' adult-type strychnine-sensitive GlyR (see Becker *et al.*, 1988; Betz, 1990a).

In contrast to α1 transcripts, α2 mRNA was widely expressed in rat brain already at early embryonic stages (E14) and significantly decreased postnatally. In spinal cord, α2 transcripts were undetectable at adult stages, a finding which corroborates previous PCR amplification data (Kuhse *et al.*, 1990a). However, significant expression was still seen in many adult brain regions including cortex, hippocampus and thalamus, suggesting that GlyRs are involved in synaptic modulation of these structures. Interestingly, previous immunological studies have revealed significant levels of GlyR immunoreactivity in these regions (Araki *et al.*, 1988; Van den Pol and Gorcs, 1988; Naas *et al.*, 1991), although little or no [^{3}H]strychnine binding was detected (Young and Snyder, 1973; Zarbin *et al.*, 1981; Probst *et al.*, 1986). These observations are consistent with the properties of a recently described rat variant of the α2 cDNA, α2*, which generates strychnine-insensitive chloride channels upon heterologous expression (Kuhse *et al.*, 1990a). Moreover, the temporal expression pattern of α2 transcripts in spinal cord correlates with the presence of a 'neonatal' GlyR protein which binds strychnine only with low affinity (Becker *et al.*, 1988). Although in spinal cord this neonatal GlyR is replaced by the adult receptor during the first two weeks postnatally, it may persist in higher brain regions. However, not all early expressed GlyRs may harbour the same α2-type subunit, as a rat α2 cDNA generating strychnine-sensitive chloride channels in *Xenopus* oocytes has also been isolated in our laboratory (Kuhse *et al.*, 1991). Since the α2 and α2* cDNA sequences are nearly identical, we cannot distinguish these variants by *in situ* hybridization techniques. Furthermore, the α2 subunit in rat has been shown to occur in two isoforms generated by alternative splicing (Kuhse *et al.*, 1991), as does the previously described rat α1 subunit (Malosio *et al.*, 1991). Thus, heterogeneity may exist at the level of the GlyR transcripts defined by the α1 and α2 antisense oligonucleotide probes used in this study.

The early and transient prenatal expression of α2 transcripts suggests a function of glycinergic transmission in connectivity and/or synapse formation in distinct brain areas. Whether the decrease in α2 transcript levels seen after birth reflects elimination of glycinoceptive neurons during development or replacement of glycinergic inputs by GABA-containing terminals, is presently unclear. A detailed histochemical analysis of GlyR immunoreactive neurons using subunit-specific antibodies should allow to distinguish these possibilities.

The *in situ* hybridization patterns documented here establish α3 as a rare GlyR a subunit isoform. Low levels of α3 transcripts were found in olfactory bulb, cerebellum and spinal cord, i.e. regions involved in sensory or motor processing. Similarly to α1 mRNA, α3 transcripts accumulated only late during postnatal development, a conclusion supported by PCR amplification of α3 sequences from spinal cord RNA (Kuhse *et al.*, 1990b). As the α3 subunit forms strychnine-sensitive chloride channels upon expression in *Xenopus* oocytes (Kuhse *et al.*, 1990b), we consider α3 to be a ligand-binding subunit of a strychnine-sensitive adult-type GlyR. This is consistent with the demonstration of low levels of GlyR immunoreactivity in adult cerebellum (Triller *et al.*, 1987; Araki *et al.*, 1988; Van den Pol and Gorcs, 1988), but contrasts with the inability of several investigators to detect [^{3}H]strychnine binding in this brain region (Young and Snyder, 1973; Zarbin *et al.*, 1981; Frostholm and Rotter, 1985; Probst *et al.*, 1986). Presumably, cerebellar GlyR levels are too low to allow quantification by the comparatively insensitive method of radioligand binding (see also Becker *et al.*, 1989).

The most surprising result of our study is the early and abundant expression of β subunit transcripts in all major regions of the rat central nervous system. Indeed, β mRNA was seen already at E14 and thereafter increased continuously in most brain areas until adult stages. Importantly, the distribution of these transcripts was much wider than that covered by the individual α subunit mRNAs. Moreover, β mRNA levels were high in many brain regions devoid of [^{3}H]strychnine binding and GlyR immunoreactivity. This situation is reminiscent of that found for β2 subunit mRNAs of the neuronal nicotinic acetylcholine receptor (Wada *et al.*, 1989; Morris *et al.*, 1990) and may indicate that the β polypeptide assembles with yet unknown ligand binding (α-type) subunits, thus forming novel types of strychine-insensitive channels. Consistent with this view, genomic DNA fragments encoding a fourth isoform, α4, of GlyR α subunits have recently been identified in mouse genomic libraries (Y.Maulet, B.Matzenbach and H.Betz, unpublished data). However, since Northern blot and PCR experiments suggest low levels of α4 mRNA in the adult brain, other receptor proteins forming ion channels in combination with the β polypeptide must also be considered. Because of the high homology of $GABA_A$ receptor and GlyR proteins (Betz, 1990a,b), assembly of the β polypeptide with $GABA_A$ receptor subunits may be envisaged. Indeed, GlyR immunoreactivity apposed to GABAergic nerve terminals has been demonstrated at selected synapses using immunoelectron microscopy (Triller *et al.*, 1987). However, co-expression in *Xenopus* oocytes of the GlyR β polypeptide with $GABA_A$ receptor α1, α3 and α5 subunits so far has failed to reveal evidence for co-assembly of these proteins (T.Tagaki, I.Pribilla, M.Ewert, P.Seeburg and H.Betz, unpublished). Moreover, GlyR β subunit-specific antisera do not recognize detergent-solubilized brain GABA/benzodiazepine receptors under conditions where most of the [^{3}H]strychnine binding sites are precipitated from spinal cord extracts (I.Pribilla and H.Betz, unpublished). Thus, the

presence of GlyR β subunits in $GABA_A$ receptors currently appears unlikely.

The NMDA subtype of glutamate receptors displays high affinity binding of glycine, which acts as an obligatory co-agonist of the cation channel formed by this membrane protein (reviewed in Dingledine *et al.*, 1990). Autoradiographic mapping of NMDA receptor associated [^{3}H]glycine binding sites (Bristow *et al.*, 1986) has disclosed a distribution in brain which grossly is reminiscent of the *in situ* hybridization data shown for GlyR β subunit transcripts in this study. It therefore is tempting to speculate that the β polypeptide may serve as a 'joker' structural subunit of ligand gated ion channels which is capable of coassembly with polypeptides specific for the NMDA receptor, thus providing its glycine binding site. Indeed, the rather unusual carboxylate-rich M2 segment of this GlyR protein as well as its capacity to bind glycine as demonstrated by heterologous expression (Grenningloh *et al.*, 1990a) may be taken as support for such an interpretation. However, it should be noted here that there are consistent differences in the regional distributions of the GlyR β mRNA and high-affinity [^{3}H]glycine binding NMDA receptors. For example, the relative densities of [^{3}H]glycine binding sites and β transcripts in cortical layers and in the cerebellum are significantly different. Moreover, the cochlear nuclei contain high levels of β RNA and GlyR antigen (Altschuler *et al.*, 1986), but only weak [^{3}H]glycine binding is seen (Bristow *et al.*, 1986). Such differences cannot be solely attributed to a differential localization of receptor proteins on processes as compared to that of transcripts in neuronal somata. Therefore yet unknown types of glycine (or β-alanine/taurine; see Parker *et al.*, 1988) gated ion channels may exist in the mammalian central nervous system.

The most pertinent question raised by our data certainly concerns the role of GlyRs in higher structures of the brain. In view of the abundance of GABAergic terminals, post-synaptic inhibition in forebrain by the phylogenetically primitive amino acid glycine (Betz and Becker, 1988) may be viewed as an evolutionary remnant in areas controlling 'old' functions of the nervous system. Indeed, the α1 and α3 mRNAs encoding adult strychnine-sensitive GlyR proteins were found here in brain regions associated with sensory and motor processing, e.g. olfactory bulb, superior colliculus, vestibular nuclei, cerebellum and spinal cord. Interestingly, label was often seen over cell populations known also to express $GABA_A$ receptor mRNAs (cf. Wisden *et al.*, 1988). Thus, simultaneous use of both inhibitory neurotransmitter systems may provide better fine-tuning of neuronal activity by exploiting the different kinetic and conductance properties of GlyRs and $GABA_A$ receptors (Borman *et al.*, 1987). Moreover, it may allow a more precise seggregation of inhibitory inputs on somata and dendrites and, in the case of mixed receptor matrices (Triller *et al.*, 1987), facilitate heterosynaptic interactions (Changeux, 1986).

Materials and methods

Oligonucleotide probes

Oligonucleotide probes (45 bp) of unique sequence were synthesized on an automated DNA synthesizer (Applied Biosystems) and purified using a NaP-5 column (Pharmacia). The following antisense probe sequences derived from the rat GlyR subunit cDNAs were used for the hybridizations shown in this paper: α1 (GTT GGC ACC CTT GAC AGA GAT GCC ATC CTT GGC TTG CAG GCA GGC), complementary to nucleotides 1051–1095 of the α1 subunit cDNA (Grenningloh *et al.*, 1987); α2 (CTT TTG GGG GTT GCG GAA GTG GGT TGG CAG GTG TAG CCT TGA CAG), complementary to nucleotides 1682–1726 of the α2* cDNA sequence (Kuhse *et al.*, 1990a); α3 (GGC AGT GAA GCT GAG CCG ACT CTC CCT CAC CTC ATC ATC CGT GTC), complementary to nucleotides 1510–1554 of the α3 subunit cDNA (Kuhse *et al.*, 1990b); and β (GCA AGG TCC TCG GCC GAC TGT TGA GAT GGG CAC AAA TAC TGC TTC), complemetary to nucleotides 325–369 of the β subunit cDNA (Grenningloh *et al.*, 1990b). Sense oligonucleotides exactly complementary to the antisense probes were used as controls. Probes were 3′ end-labelled to identical specific activities (in the range of 5×10^8 d.p.m./μg to 1×10^9 d.p.m./μg) with 5′[α-^{35}S]dATP (1200 Ci/mmol, NEN) using terminal deoxynucleotidyl transferase (Gibco, BRL), at a 30:1 molar ratio of dATP:oligonucleotide.

In situ hybridization and Northern blot analysis

In situ hybridization with the unique ^{35}S-radiolabelled oligonucleotide probes was carried out as described previously (Wisden *et al.*, 1991; Marquèze-Pouey, *et al.*, 1991). Sections were exposed to Kodak X-Omat AR film for up to eight weeks at room temparature, or dipped in Kodak NTB 2 emulsion. No labelling over background was obtained in control hybridizations with antisense oligonucleotides in the presence of a large excess (50-fold) of unlabelled oligonucleotide, or with corresponding sense oligonucleotides (data not shown). As a further control of specificity, additional antisense probes complementary to different regions of the α1 (nucleotides 979–1014), α2 (nucleotides 741–785), α3 (nucleotides 1591–1635) and β (nucleotides 1393–1437) mRNAs were used; these produced *in situ* hybridization patterns identical to those obtained with the oligonucleotides specified above (data not shown).

Northern blot analysis of poly(A)$^+$ RNA fractions isolated from different brain regions using ^{32}P-labelled antisense oligonucleotides (specific activities 3×10^8 to 5.4×10^9 c.p.m./μg) as probes was performed as described (Grenningloh *et al.*, 1990a; B.Marquèze-Pouey, W.Wisden, M.-L.Malosio and H.Betz, submitted).

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