The EMBO Journal vol.7 no.12 pp.3717-3726, 1988

Glycine receptor heterogeneity in rat spinal cord during postnatal development

Cord-Michael Becker, Werner Hoch and Heinrich Betz

Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

Communicated by H.Betz

Two different isoforms of the inhibitory glycine receptor were identified during postnatal development of rat spinal cord. A neonatal form characterized by low strychnine binding affinity, altered antigenicity, and a ligand binding subunit differing in mol. wt (49 kd) from that of the adult receptor (48 kd) predominates at birth (70% of the total receptor protein). Separation from the adult form could be achieved by either use of a selective antibody or glycine gradient elution of 2-aminostrychnine affinity columns. Both isoforms co-purify with the mol. wt 93 kd peripheral membrane protein of the post-synaptic glycine receptor complex.

Key words: CNS development/glycine receptor/receptor heterogeneity/spinal cord/strychnine

Introduction

Inhibitory transmission in the mammalian spinal cord is primarily mediated by the amino acid glycine (Curtis *et al.*, 1968; Aprison and Daly, 1978). The inhibitory action on spinal neurons of glycine and related α- and β-amino acids is effectively antagonized by the convulsant alkaloid strychnine. Conversely, binding of [^{3}H]strychnine to synaptosomal membranes from spinal cord and other CNS regions is inhibited by glycine and related amino acids (Young and Snyder, 1973, 1974). Glycine-displaceable [^{3}H]strychnine binding has been widely used as a reliable assay of the post-synaptic glycine receptor (GlyR). Scatchard analysis reveals a single class of high-affinity [^{3}H]strychnine binding sites with affinity constants of 3–10 nM (Young and Snyder, 1973, 1974; reviewed by Snyder and Bennett, 1976; Betz, 1987; Betz and Becker, 1988). Also, the pharmacological profile of [^{3}H]strychnine displacement by amino acids, alkaloids and synthetic compounds is consistent with a single class of binding sites (Betz, 1985; Betz and Becker, 1988).

The GlyR has been purified from spinal cord of different mammalian species by affinity chromatography on 2-aminostrychnine agarose. The pharmacological characteristics seen with membrane-bound GlyR are recovered in the isolated protein which contains three polypeptides of 48, 58 and 93 kd mol. wt (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985; Becker *et al.*, 1986). The antagonist binding site has been assigned to the 48 kd subunit by photoaffinity labelling using [^{3}H]strychnine (Graham *et al.*, 1981, 1983, 1985; Pfeiffer *et al.*, 1982; Becker *et al.*, 1986). Molecular cloning of a cDNA encoding the 48 kd subunit of the rat GlyR (Grenningloh *et al.*, 1987a) revealed a marked homology with subunits of the nicotinic acetylcholine (Noda *et al.*, 1983) and $GABA_A$ receptors (Schofield *et al.*, 1987). The genes encoding these ligand-gated post-synaptic ion channels thus belong to an evolutionary related receptor gene family (Grenningloh *et al.*, 1987b). Biochemical and immunological data indicate that the 58 kd subunit is a transmembrane polypeptide closely related to the 48 kd subunit (Pfeiffer *et al.*, 1984). The 93 kd GlyR polypeptide, in contrast, is a peripheral membrane protein (Schmitt *et al.*, 1987) located at the cytoplasmic face of the post-synaptic membrane (Triller *et al.*, 1985, 1987; Altschuler *et al.*, 1986).

Monoclonal antibodies (mAbs) raised against purified rat GlyR recognize epitopes localized on different subunits of the protein (Pfeiffer *et al.*, 1984). Both immunohistology (Triller *et al.*, 1985, 1987; Altschuler *et al.*, 1986) and quantitative immunoassays (Becker *et al.*, submitted) employing these antibodies have provided evidence for a co-distribution and close association of GlyR polypeptides in the post-synaptic membrane of the adult rat CNS.

Previous studies on GlyR development have exclusively relied on ligand binding techniques. The ontogenesis of [^{3}H]strychnine binding has been investigated in synaptic membrane preparations of spinal cord from chick embryo (Zukin *et al.*, 1975) and postnatal rat (Benavides *et al.*, 1981). The number of binding sites per mg protein in rat spinal cord is low at birth, but increases continuously to adult values at ~2 weeks postnatally. No developmental changes in [^{3}H]strychnine binding affinity have been detected (Benavides *et al.*, 1981).

Here, we show that immunoassays employing mAbs detect a GlyR isoform which is not seen by standard assays of high affinity [^{3}H]strychnine binding. This GlyR isoform exhibits a low affinity for strychnine and is abundant in the spinal cord of neonatal rats.

Results

Postnatal development of [^{3}H]strychnine binding to rat spinal cord membranes

At birth, glycine-displaceable [^{3}H]strychnine binding to crude spinal cord membranes as determined by filtration assay was ~20–30% of the adult level. Thereafter, the amount of [^{3}H]strychnine bound per mg of protein increased continuously to adult levels at the age of 2–3 weeks (Figure 1a). The [^{3}H]strychnine binding per rat spinal cord reached adult values even later (not shown). These observations are consistent with data reported earlier for synaptic membrane fractions from embryonic chick (Zukin *et al.*, 1975) and postnatal rat spinal cord (Benavides *et al.*, 1981).

Scatchard analysis of high affinity [^{3}H]strychnine binding to membrane preparations from neonatal and adult spinal cord yielded similar K_D values of 6.8 and 4.0 nM, respectively (Figure 1b). Corresponding B_{max} values were

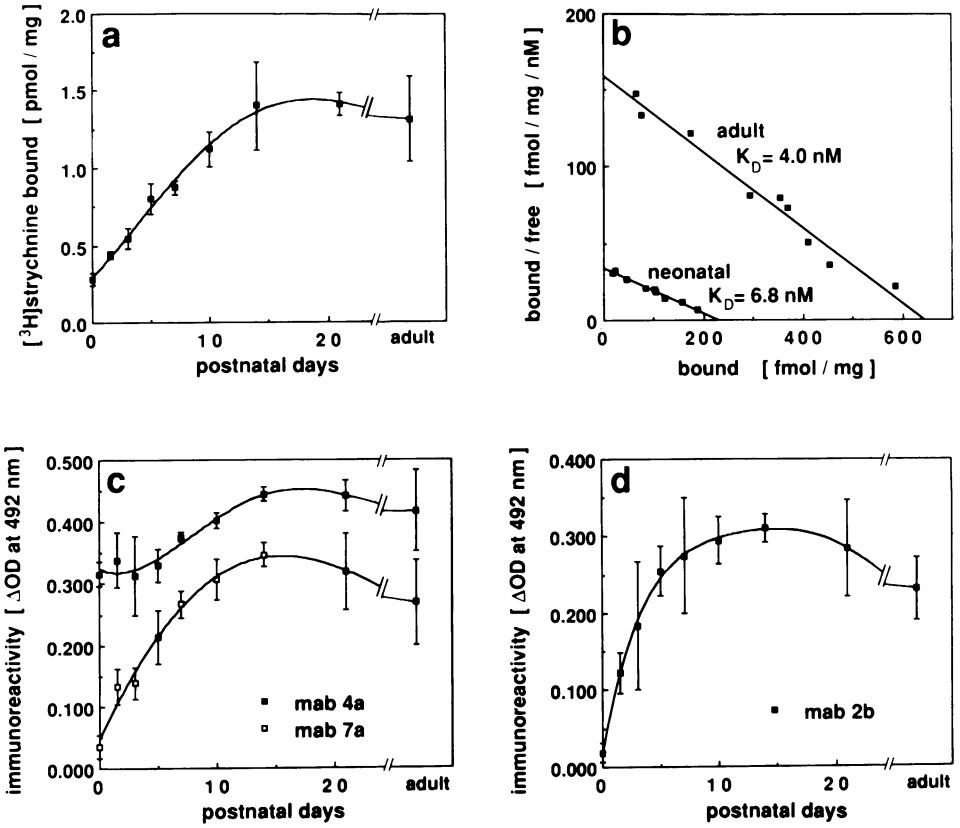

Fig. 1. [^{3}H]strychnine binding activity and GlyR-antigen content in rat spinal cord during postnatal development. (**a**) [^{3}H]strychnine binding to spinal cord membranes. Glycine-displaceable [^{3}H]strychnine binding per mg protein was determined by filtration assay. (**b**) Scatchard analysis of specific [^{3}H]strychnine binding to membranes from neonatal and adult spinal cord. Ligand binding was determined by filtration assay at protein concentrations of 2.1 and 2.4 mg/ml for neonatal and adult samples, respectively. (**c**) Content of mAb 4a- and 7a-antigens in spinal cord membranes. Binding of mAbs 4a and 7a to a constant amount of membrane protein (8 μg) was determined by DORA. Reactivity is expressed as specific absorbance using liver membranes as a background control. (**d**) Binding of mAb 2b to spinal cord membranes. Reactivity determined by MIA is expressed as specific absorbance for a constant amount of membrane protein (8 μg) with liver serving as a background control. Each data point shown in (a), (c) and (d) represents the mean ± SEM of three independent preparations each from spinal cord tissue of two rats.

229 and 643 fmol/mg protein, respectively. This gave a neonatal to adult ratio of 0.36 confirming the results reported in Figure 1(a).

GlyR-like immunoreactivity in rat spinal cord

The postnatal development of GlyR antigenic epitopes in rat spinal cord was investigated using sensitive quantitative immunoassays. The mAbs used were originally raised against GlyR from adult rat spinal cord (Pfeiffer *et al.*, 1984). As reported elsewhere (Becker *et al.*, submitted), GlyR-like immunoreactivity was found exclusively in membrane, but not soluble, fractions of spinal cord homogenates by dot receptor assay (DORA) (Table I).

The 48 kd polypeptide of isolated GlyR is recognised by mAbs 1a, 2b and 4a (Pfeiffer *et al.*, 1984). Reactivity of homogenates from rat spinal cord with mAb 2b in an immunoassay using native membrane fractions (MIA) increased during postnatal development and paralleled the accumulation of [^{3}H]strychnine binding (Figure 1d). A similar developmental increase was found with mAb 1a (not shown). In contrast, the antigen of mAb 4a was present at close to adult levels already at early postnatal stages (Figure 1c). Obviously, expression of the epitope recognized by this antibody does not correlate with other markers of the antagonist binding subunit, such as binding of [^{3}H]strychnine or mAb 2b.

Table I. Affinity purification of mAb 4a-antigen on 2-aminostrychnine agarose

Purification step	Neonatal (total units)	Adult (total units)	Ratio neonatal/adult
Membranes	1399 ± 82	1303 ± 67	1.07
Soluble fraction	ND[1]	ND[1]	—
Detergent extract	1344 ± 79	1340 ± 37	1.00
Column flow through	<5%	<5%	—
Column eluate	351 ± 7	304 ± 30	1.15

Spinal cord tissue of newborn and adult rats was homogenized and centrifuged as described in 'Materials and methods'. The first supernatant represented the soluble fraction, and the washed pellet the membrane fraction. Detergent extracts of the membranes were prepared and subjected to affinity purification on 2-aminostrychnine agarose columns. Immunoreactivity determined by DORA with mAb 4a is indicated in arbitrary units for the different steps of purification; data were corrected for differences in the amount of total protein of the membrane fractions. Values reported are means ± SEM of two independent GlyR purifications with both age groups. The relative size of the signal obtained for neonatal preparations is given as the ratio neonatal/adult.

[1]Not detected (ND) stands for a signal in the immunoassay not significantly different from background staining as defined by liver homogenates serving as a control.

Content of mAb 7-antigen was low at birth and showed a developmental increase which quantitatively correlated to

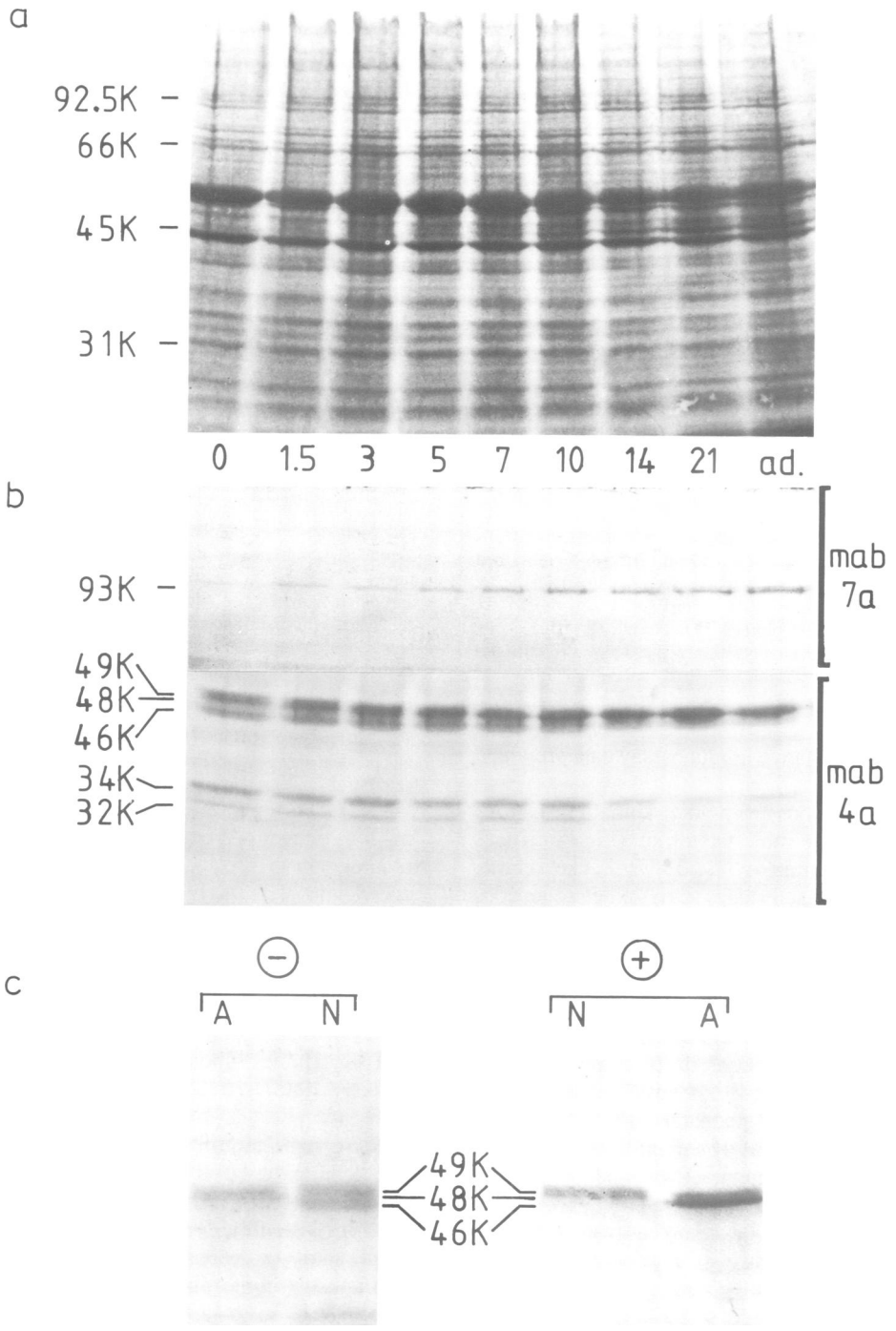

Fig. 2. Western blot analysis of postnatal GlyR development. Crude spinal cord membranes obtained from rats at different ages were electrophoresed and blotted onto nitrocellulose. (**a**) Staining of transferred proteins by Ponceau S. Positions of mol. wt markers are indicated.
(**b**) GlyR-like antigen was detected by mAbs 7a (upper panel) and 4a (lower panel). (**c**) Membranes were prepared from spinal cord of neonatal (N) and adult (A) rats in the absence (−) and presence (+) of an extended cocktail of protease inhibitors. Immunoblots of these were stained by mAb 4a. Apparent mol. wts of immunoreactive material are indicated in (b) and (c).

high affinity [^{3}H]strychnine binding. However, antagonist binding site and mAb 7a epitope are located on different GlyR polypeptides of 48 and 93 kd, respectively. Therefore, both changes may, in spite of a similar developmental time course, reflect independent processes.

Western blot analysis

To further characterize changes in GlyR antigens, spinal cord membranes from rats of different ages were subjected to Western blot analysis using mAbs 4a and 7a. mAb 2b, an antibody which binds native GlyR of adult rat spinal cord with high affinity, could not be used in this experiment, as it does not produce a reliable immunostaining of unpurified GlyR in SDS-denatured samples (Becker *et al.*, submitted). The staining intensity of the 93 kd mol. wt band recognized by mAb 7a increased continuously after birth (Figure 2b),

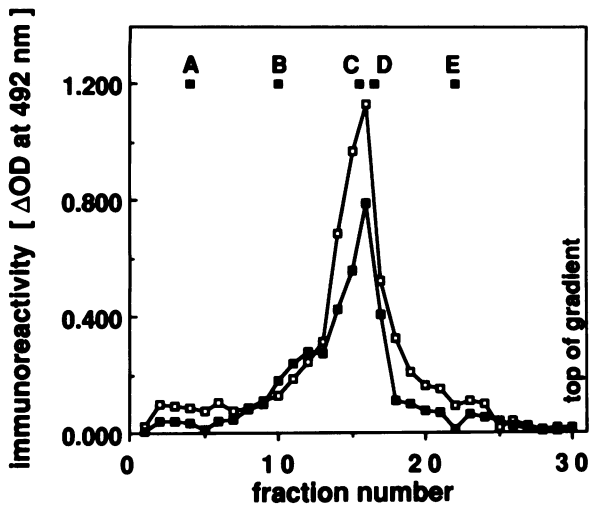

Fig. 3. Sedimentation of mAb 4a-antigen on sucrose density gradients. Detergent extracts of neonatal (■) and adult (□) spinal cord membranes were centrifuged on 5–20% sucrose gradients as described in 'Materials and methods'. Gradient fractions were analysed for antigen content by DORA using mAb 4a. The position in parallel gradients of the following marker proteins is indicated: (**A**) β-galactosidase (15.93S), (**B**) catalase (11.30S), (**C**) aldolase (7.70S), (**D**) lactate dehydrogenase (6.95S), (**E**) mitochondrial malate dehydrogenase (4.32S).

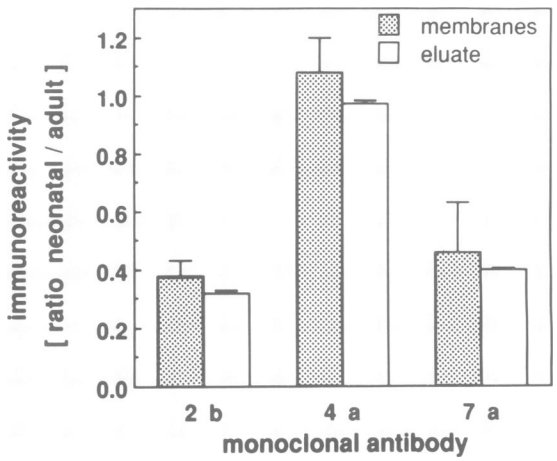

Fig. 4. Immunoreactivities of membranes and affinity purified GlyR preparations from neonatal and adult spinal cord with mAbs 2b, 4a and 7a. GlyR-like antigen from membranes of spinal cord of neonatal and adult rats was solubilized and subjected to affinity purification on 2-aminostrychnine agarose columns. The content of binding sites for mAbs 2b, 4a and 7a was determined by DORA for each membrane and eluate sample. The ratio neonatal/adult of reactivities was calculated with both membranes and column eluates. The data shown are the means ± SEM of these ratios observed in two independent purifications. Immunoreactivities were 45.3 ± 2.9 and 18.0 ± 1.9 U with mAb 2b, 130 ± 6.7 and 30.5 ± 2.9 U with mAb 4a, and 60.85 ± 9.82 and 4.0 ± 2.1 U with mAb 7a, for adult membranes and eluates, respectively [U = arbitrary units; mean ± SEM for two independent purifications]. Data were corrected for differences in the amount of total protein of the membranes.

thus confirming the quantitative immunoassay data presented in Figure 1(c).

In membrane preparations from adult spinal cord, mAb 4a recognized a band of mol. wt 48 kd (Figure 2b) as previously reported (Schmitt *et al.*, 1987; Becker *et al.*, submitted). At early postnatal stages, a more heterogeneous picture was obtained. A major band of mol. wt 46 kd and two minor ones of mol. wts 34 and 32 kd progressively disappeared during development (Figure 2b). This immunoreactive material most likely reflected partial proteolysis during sample preparation, as it was drastically reduced upon inclusion of an extended cocktail of protease inhibitors (Figure 2c). A band of mol. wt 49 kd was abundant at birth and most likely represented neonatal mAb 4a-antigen, indicating a heterogeneity of GlyR subunit mol. wt during early development. An immunoreactive band of mol. wt 48 kd present in neonatal preparations was contiguous to the 49 kd component, but could be resolved from the latter (Figure 2c; compare also Figure 6), and corresponded in size to that of adult tissue. Despite these mol. wt heterogeneities, the total amount of mAb 4a-antigen detected was not significantly altered during postnatal growth, a finding which is consistent with the results obtained by DORA. With isolated GlyR, mAb 4a also exhibits some low affinity binding to the 58 kd polypeptide (Pfeiffer *et al.*, 1984). Since no staining of bands corresponding to this mol. wt was found under the conditions used here (see also Schmitt *et al.*, 1987; Becker *et al.*, submitted), the 58 kd subunit does not account for the observed changes in immunoreactivity.

Sucrose density gradient centrifugation

GlyR antigen of both newborn and adult rats was efficiently solubilized from the membrane fraction of spinal cord homogenates using high salt and Triton X-100 as a detergent (Table I). The detergent extracts were subjected to centrifugation on 5–20% sucrose gradients. Gradient fractions were then analysed by DORA for their content of mAb 4a-antigen. At both developmental stages, a single peak of antigen was detected exhibiting an apparent sedimentation coefficient of 7.7S (Figure 3). This value is close to those reported earlier for the [^{3}H]strychnine binding GlyR complex (Pfeiffer and Betz, 1981; Pfeiffer *et al.*, 1982; Schmitt *et al.*, 1987) and its associated antigens (Becker *et al.*, submitted). Thus, neonatal mAb 4a-antigen exhibits the physical properties characteristic of the adult [^{3}H]strychnine binding GlyR (reviewed by Betz and Becker, 1988).

Affinity purification of neonatal and adult GlyR

Purification of GlyR from adult spinal cord membranes involves affinity chromatography on 2-aminostrychnine columns (Pfeiffer *et al.*, 1984). When detergent extracts from adult or neonatal spinal cord were passed over 2-aminostrychnine agarose, >95% of the GlyR antigen detectable by mAb 4a was removed from both extracts (Table I). At either stage of development, immunoreactive material could be eluted from the affinity column with similar yields using high concentrations of glycine. The ratio of neonatal to adult mAb 4a-antigen detected in membrane fractions and column eluate thus remained constant during receptor purification (Table I). Likewise, the ratio of antibody binding to neonatal samples versus that to adult samples was unaltered for membrane fractions and corresponding eluates regardless whether mAbs 2b or 7a were used (Figure 4). Under these conditions, affinity chromatography on 2-aminostrychnine agarose apparently failed to separate [^{3}H]strychnine binding and non-binding forms of GlyR-like antigen of neonates. Thus, the ability to bind immobilized strychnine appeared to reside in all of the mAb 4a-antigen, in spite of the low [^{3}H]strychnine binding activity of neonatal extracts.

Table II. Immunoprecipitation of mAb 4a-antigen by mAb 2b

Extract	mAb 4a-antigen		
	before IP (U/mg)	after IP (U/mg)	precipitated (%)
Adult	684 ± 28	158 ± 37	77
Neonatal	468 ± 43	395 ± 50	16

Detergent extracts of neonatal and adult spinal cord were incubated with mAb 2b, and immune-complexes were precipitated as described in 'Materials and methods'. Content of mAb 4a-antigen assessed in arbitrary units (U/mg protein) was determined in the supernatant before and after immunoprecipitation (IP). Data are means ± SEM of three independent experiments. The relative amount of antigen precipitated is given for both extracts.

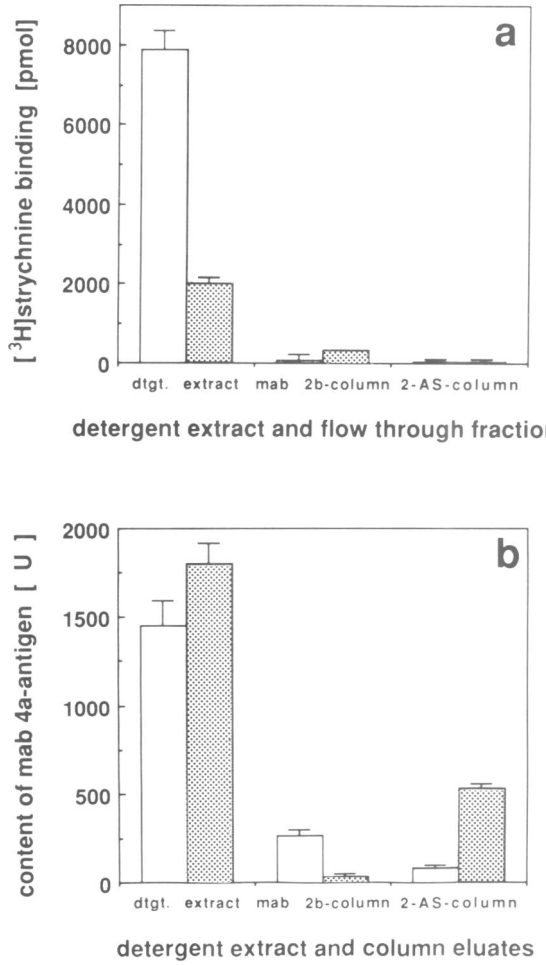

Fig. 5. Characterization of GlyR-subtypes by combined immuno- and ligand-affinity chromatography. Detergent extracts (5 ml each) from adult (□) and neonatal (▨) spinal cord were passed over a mAb 2b-column. The flow through fractions of the antibody column were subsequently subjected to adsorption on 2-aminostrychnine agarose (2-AS). (**a**)Detergent extracts and flow through fractions of both columns were assayed for [^{3}H]strychnine binding. Data represent total content in the extracts of specific ligand binding and are the mean ± SEM of triplicate determinations. (**b**) mAb 2b-affinity columns were eluted at alkaline pH, and 2-aminostrychnine columns by 200 mM glycine. Total content of mAb 4a-antigen (arbitrary units; U) was determined by DORA in extracts applied and column eluates for both developmental stages. The recovery in the eluates of mAb 4a-antigen applied was 25 and 30% for adult and neonatal extracts, respectively.

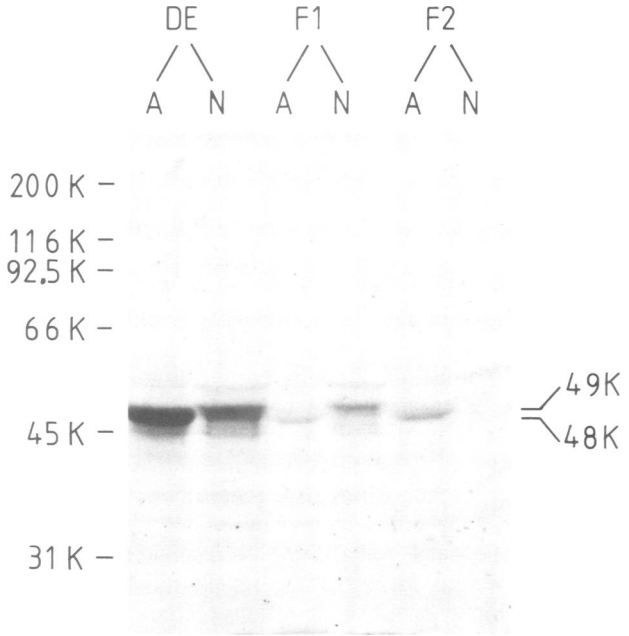

Fig. 6. Western blot analysis of mAb 4a-antigen during combined immuno- and ligand-affinity chromatography. Bands immunoreactive with mAb 4a were visualized in detergent extracts (DE) of adult (A) and neonatal (N) spinal cord as well as in the flow through fractions of consecutive affinity chromatography on mAb 2b- (F1) and 2-aminostrychnine columns (F2). Apparent mol. wts of marker proteins and immunoreactive material are indicated.

This observation suggested that the neonatal GlyR-like antigen recognized by mAb 4a differs from its adult counterpart in having a strychnine binding affinity too low to be readily detected by filtration assay, but still sufficient for retention on the highly derivatized affinity column. If so, the pivotal experiment should be a separation of two forms of mAb 4a-antigen both present in spinal cord extracts of newborn rats.

Immunoprecipitation experiments

Detergent extracts from spinal cord of both developmental stages were subjected to immunoprecipitation by mAb 2b (Table II). With adult spinal cord, mAb 2b cleared most of mAb 4a-antigen from the supernatant, but only 16% of the antigen was precipitated from extracts of neonatal spinal cord. Obviously in contrast to the adult stage, only a minor fraction of the mAb 4a-antigen is associated with the mAb 2b-epitope in neonatal spinal cord. Since precipitation of [^{3}H]strychnine binding activity from neonatal extracts was incomplete (not shown), GlyR was subsequently subjected to affinity chromatography on mAb 2b-columns.

Separation of GlyR-subtypes by combined immuno- and ligand-affinity chromatography

Upon passage over a mAb 2b-column of detergent extracts from adult and neonatal spinal cord, [^{3}H]strychnine binding sites were almost quantitatively cleared from the flow through (Figure 5a). Residual [^{3}H]strychnine binding was low, but repeatedly higher with samples from neonatal spinal cord tissue. After adsorption of the flow through fractions of the mAb 2b-columns on 2-aminostrychnine agarose,

virtually no [^{3}H]strychnine binding could be recovered in the flow through fractions of this second affinity column.

The flow-through fractions from both columns as well as the original detergent extracts of both adult and neonatal spinal cord were subjected to Western blot analysis. As already observed with membrane fractions, extracts from neonatal spinal cord displayed mol. wt heterogeneity of the mAb 4a-antigen, i.e. a doublet of mol. wt 48 and 49 kd (Figure 6). Their relative staining intensities were altered after passing the extract over the mAb 2b-column. The resulting flow-through was almost devoid of 48 kd polypeptide, arguing for its association with the mAb 2b-epitope. In contrast, the immunoreactive 49 kd polypeptide absent from adult spinal cord was recovered in the flow-through. It was, however, completely removed by chromatography on 2-aminostrychnine agarose in spite of the low [^{3}H]strychnine binding activity observed. We thus conclude that the different variants of GlyR-like antigen present in neonatal spinal cord correspond to different molecular weight forms of the ligand binding subunit. Chromatography on mAb 2b-affinity columns efficiently removed the mAb 4a-antigen of mol. wt 48 kd from detergent extracts of adult spinal cord (Figure 6). A minor fraction of the adult antigen which passed both the mAb 2b- and 2-aminostrychnine affinity columns probably represented inactivated GlyR.

The different antigenicity of neonatal and adult mAb 4a-antigens was confirmed by elution of mAb 2b-affinity columns at alkaline pH (Figure 5b). With extracts of adult spinal cord, the eluate contained about nine times more mAb 4a-antigen than that obtained with neonatal spinal cord extracts. In contrast, glycine elution of the following 2-aminostrychnine columns released significant amounts of mAb 4a-antigen after loading with neonatal extracts only. Recovery of GlyR-like antigen from 2-aminostrychnine columns loaded with the adult sample was only ~20% of that observed with the neonatal preparation.

Selective elution of GlyR subtypes from 2-aminostrychnine affinity columns

To further substantiate the differences in strychnine binding affinity of neonatal and adult GlyR, detergent extracts were

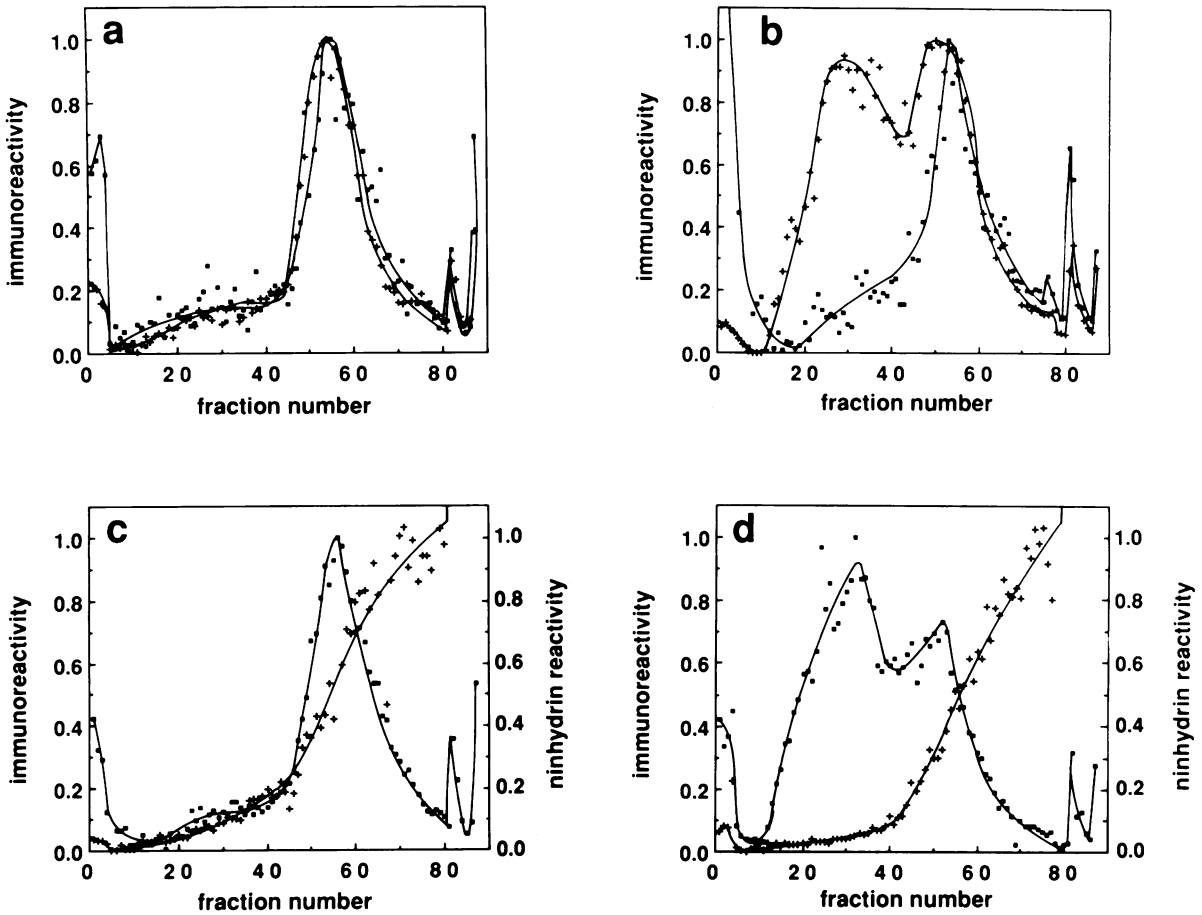

Fig. 7. Separation of GlyR-isoforms on 2-aminostrychnine affinity columns. Detergent extracts of spinal cord membranes from either neonatal or adult rats were passed over a 2-aminostrychnine column. Flow through was then collected in the first three fractions. Subsequently, the column was washed (fractions 4–8). Two consecutive glycine gradients ranging from 0 to 4 mM (fractions 9–44), and from 4 to 30 mM (fractions 45–80), were applied followed by step elution with 200 mM glycine starting at fraction 81. Nonspecifically bound protein was eluted by 6 M urea (fraction 88). Fractions were analysed for reactivity with mAbs 2b, 4a and 7a by DORA. Samples were not corrected for protein content. As can be seen with the first five fractions, this caused a high background staining of flow-through, especially by mAb 2b. The profiles obtained by the different immunoassays were normalized to peak absorbance values of 1.0. Note that the ratios neonatal/adult of GlyR antigen applied to the column were as given in Figure 4. (**a**,**b**) Elution profiles of immunoreactivities for mAbs 2b (■) and 4a (+) obtained with extracts of adult (**a**) and neonatal (**b**) spinal cord. (**c**,**d**) Elution profiles of mAb 7a-immunoreactivities (■) obtained with extracts of adult (**c**) and neonatal (**d**) spinal cord. A semi-quantitative, photometric assay of glycine concentration based on the ninhydrin reaction was performed on each fraction (+). Values were expressed as ratios of the absorbance observed with 30 mM glycine.

adsorbed on 2-aminostrychnine agarose, and the columns eluted by two consecutive glycine gradients ranging from 0 to 4 mM, and from 4 to 30 mM, respectively. With extracts from adult spinal cord, the first gradient eluted only little immunoreactive material (Figure 7a). Upon application of the second glycine gradient, fractions rich in GlyR-like antigen appeared at ~11–13mM glycine, as estimated by the ninhydrin reaction (Figure 7c). A subsequent step of 200 mM glycine yielded only minor amounts of GlyR-like antigen. Thus, no major high affinity-fraction of GlyR was retained on the 2-aminostrychnine column. No significant differences were found in the profile of reactivities with either mAb 2b or 4a.

A different picture emerged when detergent extracts from neonatal spinal cord were subjected to the same protocol (Figure 7b). Two populations of GlyR-like antigen present in neonatal spinal cord were eluted from the affinity column. At low concentrations of glycine, a peak reactive with mAb 4a only was observed followed at higher glycine concentrations by a second peak detectable by both mAbs 2b and 4a. In fact, the pattern produced by mAb 2b-analysis of GlyR from neonatal spinal cord resembled that obtained with adult material. Obviously, two peaks of mAb 4a immunoreactive material are present in neonatal spinal cord which differ in their affinities for the 2-aminostrychnine agarose matrix. The differences did not result from variations of the glycine gradient as judged by the ninhydrin reaction (Figure 7c and d).

The membrane-associated 93 kd polypeptide which reversibly binds to the transmembrane 'core' of the GlyR (Schmitt *et al.*, 1987; Becker *et al.*, submitted) accumulated postnatally as quantified by mAb 7a. To detect an eventual preferential association of this polypeptide with one of the two forms of mAb 4a-antigen separated on the affinity column, elution profiles were also determined for mAb 7a which is specific for the large GlyR polypeptide. Both with adult (Figure 7c) and neonatal (Figure 7d) spinal cord extracts, immunoreactivity patterns closely resembling that revealed by mAb 4a were obtained. The 93 kd protein thus can associate with both isoforms of the GlyR.

Discussion

The inhibitory GlyR from rat spinal cord is shown here to exhibit subtype heterogeneity. An isoform of low strychnine binding affinity and altered antigenicity occurs during early postnatal development. Different lines of evidence support this conclusion.

Shortly after birth, a neonatal GlyR-like antigen was found by quantitative immunoassays. Out of three mAbs directed against the 48 kd subunit of the GlyR from adult rat spinal cord (Pfeiffer *et al.*, 1984), only one, mAb 4a, detected the neonatal GlyR-like material. In contrast to the situation in adult tissue, this antigen could not be accounted for by the low amount of [^{3}H]strychnine binding found with spinal cord membranes of newborn rats. Immunoassays with mAbs 2b and 1a, which recognize the 48 kd polypeptides of the GlyR, respectively, produced a developmental pattern matching that seen by ligand binding assay. Thus, the increase in high affinity [^{3}H]strychnine binding coincides with expression of epitopes characteristic of the GlyR protein of adult spinal cord.

The use of mAb 2b allowed separation of the neonatal GlyR-like antigen detected by mAb 4a from the adult receptor species by either immunoprecipitation or affinity chromatography on antibody columns. Thus, the neonatal GlyR-like antigen represents a distinct protein which shares structural features with GlyR from adult spinal cord and behaves as a membrane protein in tissue homogenates. By Western blot analysis, immunoreactive bands corresponding to apparent mol. wt of 46, 48 and 49 kd were detected in the neonatal membrane preparation. The 46 kd band is thought to reflect a proteolytic degradation product, whereas the 48 kd band corresponds in size to the [^{3}H]strychnine binding subunit of adult GlyR (Pfeiffer *et al.*, 1982; Graham *et al.*, 1985; Becker *et al.*, 1986). The 49 kd polypeptide additionaly present in neonatal spinal cord was selectively enriched in the low [^{3}H]strychnine binding flow-through of mAb 2b-affinity chromatography. It most likely represents a variant of the ligand binding subunit which characterizes the neonatal isoform of the GlyR. A similar developmental heterogeneity of subunit mol. wt has previously been described for $GABA_A$ receptor polypeptides upon benzodiazepine photo-affinity labelling during postnatal development of rat cortex (Eichinger and Sieghart, 1986).

The neonatal mAb 4a-antigen is part of a macromolecular complex indistinguishable in sedimentation behaviour from adult GlyR (apparent sedimentation coefficient of 7.7S). After separation from the 93 kd associated protein on sucrose gradients (Schmitt *et al.*, 1987), the transmembrane core of the adult GlyR possesses a mol. wt of ~250 kd (Pfeiffer *et al.*, 1982). This excludes unassembled low affinity monomers of the ligand binding subunit to account for the GlyR antigen present in neonatal spinal cord. Monomers of low affinities for the cholinergic antagonists α-bungarotoxin and *d*-tubocurare have, however, been found as short-lived intermediates of nicotinic acetylcholine receptor synthesis (Carlin *et al.*, 1986; Smith *et al.*, 1987).

Adsorption onto 2-aminostrychnine columns showed that the neonatal GlyR-like antigen bound strychnine with low affinity even though this was not detectable by the standard filtration assay of [^{3}H]strychnine binding. Filtration assays are not suitable for determination of low affinity binding sites due to high losses of specifically bound ligand during the washing procedure (Bennett and Yamamura, 1985). Thus, a strychnine binding site of low affinity may remain undetected in the filtration assay, but will be retained on a highly substituted affinity column.

Inhibition of [^{3}H]strychnine binding by glycine is a characteristic feature of the adult GlyR (Young and Snyder, 1973, 1974). This antagonism was also conserved in the neonatal GlyR-like antigen, as it was eluted by glycine from the 2-aminostrychnine affinity column, albeit at concentrations lower than those required for elution of the adult GlyR. This suggests that the binding affinity for glycine, even though not directly assessable, was not, or to a lesser extent, decreased in the neonatal GlyR-form than that for strychnine. Furthermore, the clear separation by glycine gradient elution of both forms of GlyR-like antigen complements the immunoprecipitation data. It should be emphasized that the neonatal form of low strychnine binding affinity accounts for ~70% of the GlyR-like antigen in neonatal spinal cord whereas ~30% correspond to the adult antigen. This is evident from both immunoassays using mAb 2b, which is specific for the adult form of the GlyR, and

Scatchard analysis of high-affinity [^{3}H]strychnine binding to neonatal and adult membrane preparations. Furthermore, the glycine gradient elution profile of 2-aminostrychnine columns loaded with neonatal GlyR antigen indicates a similar ratio of both isoforms.

The molecular differences between neonatal and adult GlyR await to be described in more detail. The failure of mAb 2b to bind neonatal GlyR recognized by mAb 4a points to structural variations of the ligand binding subunit. Also, the apparent mol. wt of this subunit appears to be slightly higher in neonatal preparations. These changes are inconsistent with subunit rearrangements, like formation of homo-oligomeric complexes of the adult 48 kd polypeptide, being the cause of receptor diversity. For *Torpedo* nicotinic acetylcholine receptor, homo-oligomeric receptor forms have in fact been detected upon *in vitro* translation of individual subunit mRNAs (Anderson and Blobel, 1983). However, the high percentage of the neonatal isoform of the GlyR argues against its identity with biosynthetic intermediates of the adult GlyR. Differences in receptor properties due to post-translational modification of the protein can currently not be ruled out, but preliminary cDNA cloning data indeed suggest variations in primary structure of the ligand binding subunit (G.Grenningloh and C.-M.Becker, unpublished). Furthermore, GlyR antigen in dissociated cell cultures of embryonic mouse spinal cord almost exclusively consists of the neonatal type (Hoch *et al.*, in preparation) although these cultured neurons exhibit high glycine sensitivity in electrophysiological experiments (Nelson *et al.*, 1977; Bormann *et al.*, 1987). Based on these criteria, we conclude that the neonatal mAb 4a-antigen is indeed a functional GlyR which represents an embryonic or neonatal isoform. This conclusion is consistent with two remarkable 100-year-old reports on the relative 'immunity' to strychnine intoxication of newborn rodents (Falck, 1884, 1885). Accordingly, the lethal doses per body weight of this alkaloid decrease postnatally and, in the mouse, reach adult values at ~day 15 when, as shown here, the high affinity receptor isoform is fully expressed.

Our observations on GlyR isoforms find an analogy in the development of nicotinic acetylcholine receptors in rat skeletal muscle. An embryonic isoform of the nicotinic acetylcholine receptor is replaced postnatally by the adult receptor protein. These changes are accompanied by altered functional (reviewed by Schuetze and Role, 1987) and antigenic (Hall *et al.*, 1985) properties of muscle nicotinic acetylcholine receptors and have been attributed to the exchange of a non-ligand-binding subunit during muscle development (Mishina *et al.*, 1986; Gu and Hall, 1988).

Presently, it is unknown whether the neonatal and adult GlyR isoforms are expressed in the same set of neurons. Also, we cannot associate these isoforms with either different states of chloride channel function or alterations of receptor distribution in the neuronal plasma membrane. Due to a lack of suitable antibodies, redistribution of neurotransmitter receptors during synaptogenesis has not been investigated in CNS neurons. Immunohistological methods employing mAb 2b, however, have clearly demonstrated that the adult isoform of the GlyR is exclusively localized at synaptic complexes (Triller *et al.*, 1985, 1987; Altschuler *et al.*, 1986).

It is tempting to speculate that the occurrence of developmental isoforms of neuotransmitter receptors is correlated with the toplogical organization of the post-synaptic membrane. The 93 kd polypeptide of the GlyR is located at the cytoplasmic face of the post-synpatic membrane and has been implicated in post-synaptic anchoring of the GlyR, in analogy to the 43 kd protein associated with the synaptic nicotinic acetylcholine receptor (Schmitt *et al.*, 1987). Although postnatal expression of the 93 kd polypeptide detected by mAb 7a coincides with accumulation of the adult type receptor, it co-purifies with both GlyR isoforms on 2-aminostrychnine columns. *In vivo*, synaptic accumulation of the neonatal GlyR isoform thus may be achieved by association with newly expressed 93 kd protein. This process may be accompanied by a transition from neonatal to adult receptor isotype at the developing glycinergic synapses. Interestingly, a regulatory role of the 43 kd protein in synaptic clustering of nicotinic acetylcholine receptors is supported by biochemical and biophysical data (Burden, 1985; Bloch and Froehner, 1987). Considering the close evolutionary relationship of nicotinic acetylcholine receptor and GlyR subunits (Grenningloh *et al.*, 1987a), one may assume a general mechanism of synapse formation and stabilization (Changeux and Danchin, 1976) to underlie these observations. Such a mechanism could link regionalization of chemosensitivity in excitable membranes to the structural organization of the membrane skeleton.

Materials and methods

Materials

[^{3}H]strychnine (sp. act. 1.1 TBq/mmol) and horseradish peroxidase-linked anti-mouse immunoglobulin from sheep were purchased from Amersham-Buchler (Braunschweig, FRG); rabbit anti-mouse immunoglobulin antibodies coupled to alkaline phosphatase were from Promega (Madison, Wisconsin, USA). Affinity-purified rabbit anti-mouse immunoglobulin was obtained from Dako (Hamburg, FRG), fixed *Staphylococcus aureus* cells (Pansorbin) from Calbiochem (Frankfurt, FRG); Triton X-100, bacitracin, soybean and ovomucoid trypsin inhibitors from Serva (Heildelberg, FRG); low-mol.wt markers were from Bio-Rad (München, FRG) and nitrocellulose membranes (BA85) were from Schleicher und Schüll (Dassel, FRG). *o*-phenylene-diamine, polyethylene glycol 8000, egg yolk phosphatidylcholine (type X-E), and bovine serum albumine were from Sigma (München, FRG). All other chemicals were of analytical grade.

Preparation of membranes and solubilization of the GlyR

Spinal cord including medulla oblongata and pons were rapidly removed from the rats, frozen in liquid nitrogen, and stored at −70°C. Crude membranes were prepared by homogenization in 20 volumes ice-cold 50 mM Tris buffer, pH 7.4, containing 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethyleneglycolbis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), and a cocktail of protease inhibitors (Pfeiffer *et al.*, 1982). In some experiments, an extended cocktail of protease inhibitors was included which also contained bacitracin at a final concentration of 60 mg/l, and soybean and ovomucoid trypsin inhibitors (10 mg/l, each). After centrifugation at 50 000 *g* for 30 min at 4°C, pellets were rehomogenized in the same buffer and washed two more times. The final pellet was suspended in 25 mM KP_i, pH 7.4, containing 200 mM KCl and protease inhibitors, and immediately frozen in liquid nitrogen. Detergent extracts of spinal cord membranes were prepared in the presence of the extended protease inhibitor cocktail using a modification (Becker *et al.*, 1986) of the procedure of Pfeiffer and Betz (1981).

Ligand binding assay

Specific binding of [^{3}H]strychnine to crude membrane preparations and detergent extracts was determined according to published procedures (Pfeiffer and Betz, 1981; Becker *et al.*, 1986).

Immunomethods

DORA. GlyR-associated epitopes were quantified by DORA, a dot assay using mAbs raised against GlyR from adult spinal cord, as described elsewhere (Becker *et al.*, submitted). Briefly, samples were adsorbed onto

nitrocellulose membranes in the presence of application buffer VSOP [0.5% (w/v) sodium deoxycholate, 20% (v/v) methanol, and 150 mM NaCl in 50 mM Tris−HCl, pH 7.4]. The membranes were subsequently reacted with mouse anti-GlyR-mAbs and anti-mouse immunoglobulin antiserum coupled to horseradish peroxidase. Dots carrying the samples were punched out from the nitrocellulose sheets and reacted with peroxidase substrate solution. Colour development was quantitated photometrically. Non-specific antibody binding was determined by inclusion of liver membranes which do not contain GlyR, or by omission of first antibody. Unless stated otherwise, all values represent the mean ± SEM of triplicate determinations.

MIA. Binding of mAb 2b to native membranes from rat spinal cord was quantified by a membrane immunoassay to be published elsewhere (Hoch *et al.*, in preparation). Briefly, suspended spinal cord membranes were consecutively reacted with mAb 2b and horseradish peroxidase-coupled second antibody. Change of antibody solutions and washing of membranes were done by repeated centrifugation at 4000 *g* and resuspension. Liver membranes served as a background control. Antibody binding was quantified enzymatically. For mAb 2b, this assay was, where applicable, preferred to DORA as it produced better signal to background ratios.

Western blots. Crude membranes (125 μg of protein) or detergent extracts (100 μg) were subjected to electrophoresis on 10% SDS−polyacrylamide gels (Laemmli, 1970). Proteins were blotted onto nitrocellulose (Kyhse-Andersen, 1984) and stained after transfer with Ponceau S. Immunodetection of GlyR polypeptides was performed as described by Schmitt *et al.* (1987).

Immunoprecipitation of GlyR from Triton X-100 extracts of rat spinal cord membranes by affinity-purified mAb 2b. This was performed as described elsewhere (Becker *et al.*, submitted), except that pH was adjusted to 8.0. An unrelated mAb against β-bungarotoxin served as control. After incubation with anti-mouse immunoglobulin, immune-complexes were precipitated by adding a suspension of fixed *S.aureus* cells and centrifugation. The content of mAb 4a-antigen and [^{3}H]strychnine binding activity were determined in the supernatant. Appropriate corrections for background immunoreactivity by residual mAb 2b were made.

Affinity chromatography on 2-aminostrychnine agarose and glycine gradient elution of the GlyR. Affinity chromatography of the GlyR was performed as described by Pfeiffer *et al.* (1982) using 2-aminostrychnine agarose columns. Detergent extracts (8 ml containing ~3 mg protein/ml) were repeatedly passed over 1.5 ml of affinity resin. Following a wash of the columns (10 ml), step elution of GlyR-like antigen was performed by the application of 1.5 ml elution buffer containing 200 mM glycine. After extended cycling, the eluate was collected for further analysis. All experiments were conducted for neonatal and adult samples in parallel using two identical columns.

For glycine gradient elution, detergent extract was applied and the column washed as given above. Elution was performed by a gradient (60 ml) ranging from 0 to 4 mM glycine followed by a second gradient (60 ml) of 4 to 30 mM glycine in wash buffer. Finally, a step of 200 mM glycine was applied; thereafter, unspecifically bound material was eluted by 6 M urea dissolved in the same glycine containing buffer. Throughout the experiment, the flow rate was 1 ml/h, and each of the gradients lasted for 60 h to allow the matrix-bound GlyR to fully equilibrate with free glycine. Fractions of 1.5 ml were collected starting with the flow through. All steps were performed at 4°C. The same column was used in all gradient elution experiments for optimal reproducibility.

For analysis of antigen content by DORA, aliquots of eluate fractions were diluted 1:10 in application buffer, and volumes of 100 or 150 μl were adsorbed onto nitrocellulose. The concentration of glycine in the eluate fractions was determined by a semiquantitative photometric assay. Fractions were diluted by water (1:5) and aliquots (50 μl) added to 200 μl of a 1% (w/v) solution of ninhydrin in ethanol. Reactions were performed in microwell plates at room temperature, and colour development was quantified photometrically at 414 nm using an ELISA reader.

Sucrose gradient centrifugation

Sucrose gradient centrifugation of detergent extracts of rat spinal cord membranes was performed using a modification (Becker *et al.*, submitted) of the procedure of Pfeiffer and Betz (1981). Detergent extract (400 μl containing 1.2 mg protein) was applied to a 5−20% sucrose gradient (11.5 ml) underlayered by a 60% sucrose cushion (1.5 ml). After centrifugation in a SW40 rotor at 40 000 r.p.m. for 24 h at 4°C, fractions of 400 μl were collected. For DORA, aliquots were diluted 1:10 by VSOP-buffer and adsorbed onto nitrocellulose (100 μl per well). Marker proteins were detected in parallel gradients by their enzymatic activity.

Protein determination

Protein content was determined by a modification of the Lowry procedure (Larson *et al.*, 1986).

Acknowledgements

We thank C.Schroeder for expert technical assistance, our colleagues for critical reading of this manuscript, and I.Baro for skilful help during its preparation. This work was supported by the Deutsche Forschungsgemeinschaft (Be 718/8-1 and SFB 317), the Bundesministerium für Forschung und Technologie, and the Fonds der Chemischen Industrie.

References

Altschuler,R.A., Betz,H., Parakkal,M.H., Reeks,K.A. and Wenthold,R.J. (1986) *Brain Res.*, **369**, 316−320.
Anderson,D.J. and Blobel,G. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4359−4362.
Aprison,M.H. and Daly,E. (1978) *Adv. Neurochem.*, **3**, 203−294.
Becker,C.-M., Hermans-Borgmeyer,I., Schmitt,B. and Betz,H. (1986) *J. Neurosci.*, **6**, 1358−1364.
Benavides,J., Lopez-Lahoya,J., Valdivieso,F. and Ugarte,M. (1981) *J. Neurochem.*, **7**, 315−320.
Bennett,J.P. and Yamamura,H.I. (1985) In Yamamura,H.I., Enna,S.J.and Kuhar,M.J. (eds), *Neurotransmitter Receptor Binding*, 2nd edn. Raven Press, New York, pp. 61−89.
Betz,H. (1985) *Angew. Chem. Int. Ed. Engl.*, **24**, 365−370.
Betz,H. (1987) *Trends Neurosci.*, **10**, 113−117.
Betz,H. and Becker,C.-M. (1988) *Neurochem. Int.*, **13**, 137−146.
Bloch,R.J. and Froehner,S.C. (1987) *J. Cell Biol.*, **104**, 645−654.
Bormann,J., Hamill,O.P. and Sakmann,B. (1987) *J. Physiol.* (London), **385**, 234−286.
Burden,S.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8279−8273.
Carlin,B.E., Lawrence,J.C., Lindstrom,J.M. and Merlie,J.P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 498−502.
Changeux,J.P. and Danchin,A. (1976) *Nature*, **264**, 705−712.
Curtis,D.R., Hösli,L., Johnston,G.A.R.and Johnston,I.H. (1968) *Exp. Brain Res.*, **5**, 235−258.
Eichinger,A. and Sieghart,W. (1986) *J. Neurochem.*, **46**, 173−180.
Falck,F.A. (1884) *Archiv f. d. ges. Physiol.*, **34**, 530−575.
Falck,F.A. (1885) *Archiv f. d. ges. Physiol.*, **36**, 285−308.
Graham,D., Pfeiffer,F. and Betz,H. (1981) *Biochem. Biophys. Res. Commun.*, **102**, 1330−1335.
Graham,D., Pfeiffer,F. and Betz,H. (1983) *Eur. J. Biochem.*, **131**, 519−525.
Graham,D., Pfeiffer,F., Simler,R. and Betz,H. (1985) *Biochemistry*, **24**, 990−994.
Grenningloh,G., Rienitz,A., Schmitt,B., Methfessel,C., Zenssen,M., Beyreuther,K., Gundelfinger,E.D. and Betz,H. (1987a) *Nature*, **328**, 215−220.
Grenningloh,G., Gundelfinger,E.D., Schmitt,B., Betz,H., Darlison,M.G., Barnard,E.A., Schofield,P. and Seeburg,P.H. (1987b) *Nature*, **330**, 25−26.
Gu,Y. and Hall,Z. (1988) *Neuron*, **1**, 117−125.
Hall,Z.W., Gorin,P.D., Silberstein,L. and Bennett,C. (1985) *J. Neurosci.*, **5**, 730−734.
Kyhse-Andersen,J. (1984) *J. Biophys. Biochem. Methods*, **10**, 203−209.
Laemmli,U.K. (1970) *Nature*, **227**, 680−685.
Larson,E., Howlett,B. and Jagendorf,A. (1986) *Anal. Biochem.*, **155**, 243−248.
Mishina,M., Takai,T., Imoto,K., Noda,M., Takahashi,T., Numa,S., Methfessel,C. and Sakmann,B. (1986) *Nature*, **321**, 406−411.
Nelson,P.G., Ransom,B.R., Henkart,M. and Bullock,P.N. (1977) *J. Neurophysiol.*, **49**, 1178−1187.
Noda,M., Furutani,Y., Takahashi,H., Toyosato,M., Tanabe,T., Shimizu,S., Kikyotani,S., Kayano,T., Hirose,T., Inayama,S. and Numa,S. (1983) *Nature*, **305**, 818−823.
Pfeiffer,F. and Betz,H. (1981) *Brain Res.*, **226**, 273−279.
Pfeiffer,F., Graham,D. and Betz,H. (1982) *J. Biol. Chem.*, **257**, 9389−9393.
Pfeiffer,F., Simler,R., Grenningloh,G. and Betz,H. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7224−7227.
Schmitt,B., Knaus,P., Becker,C.-M. and Betz,H. (1987) *Biochemistry*, **26**, 805−811.
Schofield,P.R., Darlison,M.G., Fujita,N., Burt,D.R., Stephenson,F.A.,

Rodriguez,H., Rhee,L.M., Ramachandran,J., Reale,V., Glencorse,T.A., Seeburg,P.A. and Barnard,E.A. (1987) *Nature*, **328**, 221–227.
Schuetze,S.M. and Role,L.W. (1987) *Annu. Rev. Neurosci.*, **10**, 403–457.
Smith,M.M., Lindstrom,J. and Merlie,J.P. (1987) *J. Biol. Chem.*, **262**, 4367–4376.
Snyder,S.H. and Bennett,J.P. (1976) *Annu. Rev. Physiol.*, **38**, 153–175.
Triller,A., Cluzeaud,A., Pfeiffer,F., Betz,H. and Korn,H. (1985) *J. Cell Biol.*, **101**, 683–688.
Triller,A., Cluzeaud,F. and Korn,H. (1987) *J. Cell Biol.*, **104**, 947–956.
Young,A.B. and Snyder,S.H. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 2832–2836.
Young,A.B. and Snyder,S.H. (1974) *Mol. Pharmac.*, **10**, 790–809.
Zukin,S.R., Young,A.B. and Snyder,S.H. (1975) *Brain Res.*, **83**, 525–531.

Received on June 15, 1988; revised on August 31, 1988