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Identification and immunohistochemical mapping of $GABA_A$ receptor subtypes containing the δ-subunit in rat brain

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Synaptic inhibition in brain is mainly mediated via $GABA_A$ receptors which display a striking structural heterogeneity. A novel type of $GABA_A$ receptor subunit, the δ-subunit, has recently been described based on molecular cloning of its cDNA [1]. To identify the prevalence and distribution of $GABA_A$ receptors which contain the δ-subunit protein in situ, polyclonal site-directed antisera were developed against three synthetic peptides derived form the rat δ-subunit cDNA-sequence. All antisera specifically recognized a 54 kDa protein in $GABA_A$ receptor preparations. Nearly 30% of the $GABA_A$ receptors contained the δ-subunit immunoreactivity and displayed high affinity GABA and high affinity benzodiazepine binding sites as shown by immunoprecipitation. Receptors which contain the δ-subunit were immunohistochemically shown to be restricted to a few brain areas such as the cerebellum, thalamus and dentate gyrus of the hippocampal formation. Thus, those neurons which express $GABA_A$ receptors with a δ-subunit have now been visualized and made accessible for a functional analysis of this $GABA_A$ receptor subtype in situ.

$GABA_A$ receptor; δ-Subunit; Antipeptide antisera; Western blotting; Immunoprecipitation; Immunohistochemistry

1. INTRODUCTION

Inhibitory neurotransmission in mammalian brain is mainly mediated via $GABA_A$ receptors. They constitute GABA-gated hetero-oligomeric receptor-channel complexes which can be allosterically modulated by various clinically important neuroactive drugs, notably by ligands of the benzodiazepine receptor and by barbiturates [2]. Despite their physiological and pharmacological relevance, the structure of $GABA_A$ receptors is yet unresolved. Initial biochemical and immunochemical characterization of the $GABA_A$ receptor pointed to a 250 kDa receptor complex consisting of α- and β-subunits (reviewed in [3]). Subsequent molecular cloning of the α- and β-subunit cDNAs led to the discovery of not only multiple α- and β-subunit isoforms (α_{1-6}, β_{1-3}) but also of multiple γ-subunits (γ_{1-2}) and a δ-subunit (reviewed in [4–6]). To analyze the subunit composition of various $GABA_A$ receptor subtypes in situ, subunit-specific antibodies are being used. The α_1-, α_2-, α_3-, α_6-, γ_2- and $\beta_{2/3}$-subunits were identified as proteins of 50, 53, 60, 57, 43 and 56/57 kDa, respectively [7–18]. A frequent association of the α_1-, $\beta_{2/3}$- and γ_2-subunits was recently demonstrated for $GABA_A$ receptors in situ [13] suggesting that this subunit combination is of physiological relevance. Co-expression of this subunit combination (α_1, β_2, γ_2) resulted in GABA-gated chloride channels which were modulated by benzodiazepine receptor ligands [19,20]. In contrast to the α-, β- and γ-subunits, no information exists on the size, distribution and functional role of the δ-subunit protein in situ. In recombinant receptors, this subunit, which was identified by molecular cloning [1], formed GABA-sensitive homo-oligomeric ion channels but failed to convey benzodiazepine receptor modulation when co-expressed with α- and β-subunits [1]. However, it remained unclear to what extent the δ-subunit is a constituent of $GABA_A$ receptors in vivo, possibly denoting receptor subtypes which lack certain modulatory responses to drugs. To approach this question we developed antisera against three different peptide sequences unique to the δ-subunit and report here on the identification, size and distribution of the δ-subunit protein as integral component in a subset of $GABA_A$ receptors in vivo.

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2. MATERIALS AND METHODS

Three subunit-specific peptides derived from the rat δ-subunit cDNA sequence [1] were synthesized: peptide δ(1–17) (sequence pyroglutamyl-PHHGARAMNDIGNYVG), peptide δ(179–189) (sequence YWSENQEQIHG) and peptide δ(316–322) (sequence HFNADYR) [21,22]. An additional cysteine was added to the C-terminus (peptides δ(1–17) and δ(179–189)) or N-terminus (peptide δ(316–322)). For antibody production the peptides were coupled via the additional cysteine to keyhole limpet haemocyanine (KLH) [23] and about 100 μg conjugate emulsified 1:2 in Freund's complete adjuvant were used for immunization of rabbits (KOHA strain, BRL) as described previously [12–13]. The development of an immune response was monitored by enzyme-linked immunosorbent assay

(ELISA) using the synthetic peptides as antigen according to [24]. Western blot analysis, immunoprecipitation and [^{3}H]flumazenil and [^{3}H]muscimol radioligand binding to the $GABA_A$-receptor were performed as described previously [13–15]. Crude neuronal membranes and purified $GABA_A$ receptors were prepared from whole rat brain as described previously [13,25].

For immunohistochemistry male Wistar rats (120 g body weight) were anesthetized and fixed by vascular perfusion with 2% paraformaldehyde in 10 mM phosphate buffer, pH 7.4, and 150 mM NaCl for 20 min. The brains were quickly removed and processed for immunostaining as described [13,15] using the avidin-biotin complex method (Vectastain ABC, kit, Vector laboratories, Burlingame, USA). Affinity-purification of the δ(1–17)-antiserum was achieved on a thiopropyl-Sepharose 6B matrix (Pharmacia) to which the peptide δ(1–17) was coupled via the C-terminal cysteine as described previously [13].

3. RESULTS AND DISCUSSION

To identify the size and prevalence of the δ-subunit as constituent of $GABA_A$ receptors in vivo polyclonal antisera were raised against three different cDNA-derived peptide sequences unique to the rat δ-subunit. The N-terminal peptide (residues 1–17), as well as a peptide from the proposed extracellular domain (residues 179–189) and a peptide from the proposed intracellular loop located between the transmembrane spanning regions M3 and M4 (residues 316–322) were synthesized and coupled to KLH and used as antigens. High titer antisera were already obtained two weeks after the first booster injection as tested in ELISA using the respective peptides as antigens.

To identify the size of the δ-subunit protein $GABA_A$ receptors purified from whole rat brain were tested with the antisera on Western blots. All three antisera selectively recognized a 54 kDa polypeptide (Fig. 1, lanes 1,3,5). Co-incubation of the antisera with the respective peptides blocked the immune reaction in all three cases documenting the specificity of the antibody reactions (Fig. 1, lanes 2,4,6). The apparent size of the native protein (54 kDa) exceeds that of the deduced amino acid sequence of the δ-subunit cDNA (49 kDa) [1] suggesting that a carbohydrate moiety may be attached at one or both potential N-linked glycosylation sites within the δ-subunit sequence contributing to the apparent molecular size of the native protein.

Two of the antisera, the δ(1–17)- and the δ(179-189)-antiserum, recognized the δ-subunit in the native conformation as demonstrated by immunoprecipitation experiments. As illustrated for the δ(1–17)-antiserum, incubation of purified $GABA_A$ receptor preparations with increasing concentrations of the antiserum resulted in saturable immunoprecipitation of $GABA_A$ receptors as monitored by [^{3}H]flumazenil and [^{3}H]muscimol radioligand binding (Fig. 2A). A maximum of 28 ± 7% ($n = 7$) of [^{3}H]flumazenil binding and 26 ± 6% ($n = 5$) of [^{3}H]muscimol binding was precipitated with the δ(1–17)-antiserum. Similar results were obtained with the δ(179–189)-antiserum which yielded a maximum precipitation of 30 ± 2% ($n = 4$) of [^{3}H]flumazenil binding and 21 ± 6% ($n = 3$) of [^{3}H]muscimol binding. The specificity of the immune reaction was assessed in peptide competition experiments. When increasing concentrations of the peptide δ(1–17)- were added to the δ(1–17)-antiserum the immunoprecipitation of $GABA_A$ receptors was prevented in a dose-dependent manner (Fig. 2B). Thus, a subpopulation of nearly 30% of $GABA_A$ receptors appears to contain the δ-subunit.

To determine the regional distribution of the δ-subunit protein, crude membrane fractions from dif-

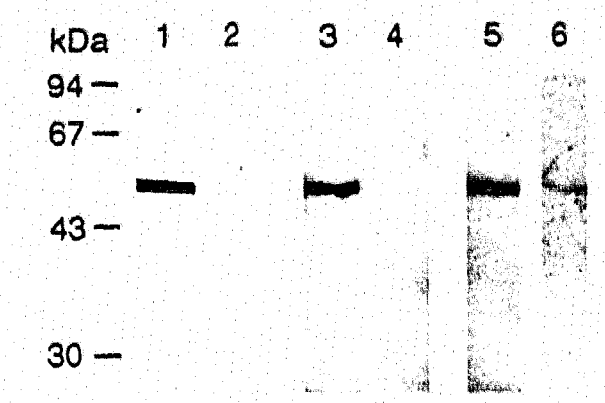

Fig. 1. Identification of the δ-subunit protein in $GABA_A$ receptor preparations by Western blotting. $GABA_A$ receptor purified from rat brain was subjected to SDS-PAGE (0.4 pmol [^{3}H]flumazenil binding sites per lane) and blotted onto nitrocellulose sheets for immunostaining with various δ-subunit-specific antisera. (Lane 1–2): δ(1–17)-antiserum (1:1000) in the absence (lane 1) or presence (lane 2) of 10 μg/ml peptide δ(1–17). (Lane 3–4): δ(179–189)-antiserum (1:1000) in the absence (lane 3) or presence (lane 4) of 30 μg/ml peptide δ(179–189). (Lane 5–6): δ(316–322)-antiserum (1:1000) in the absence (lane 5) or presence (lane 6) of 30 μg/ml peptide δ(316–322).

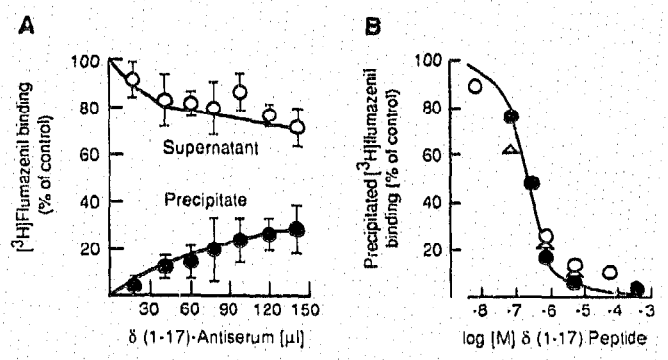

Fig. 2. Immunoprecipitation of purified $GABA_A$ receptors from rat brain with purified δ(1–17)-antiserum. (A) $GABA_A$ receptor (150–200 fmol [^{3}H]flumazenil binding) was incubated with increasing concentrations of antiserum and [^{3}H]flumazenil binding was determined in the supernatant (○) and the precipitate (●) in triplicate in at least three different experiments (means ± SD). Control values, for which the antiserum was replaced by preimmune serum or by buffer, were subtracted. (B) The specificity of the immunoprecipitation was determined by incubating the δ(1–17)-antiserum at a concentration leading to maximum immunoprecipitation in the presence of increasing concentrations of the δ(1–17)-peptide or in the absence of the peptide (100% value). Specific binding of [^{3}H]flumazenil was determined in three different experiments (●, ○, △).

ferent brain areas were analyzed for immunoreactivity in Western blots. In membranes of all brain regions examined, a single protein band of 54 kDa was labeled with the δ(1–17)-antiserum, confirming the results obtained with the purified $GABA_A$ receptors (Fig. 3). Although the δ-subunit immunoreactivity was present in all brain regions examined the intensities of signals varied. Strongest labeling was observed in the cerebellum followed by thalamus/hypothalamus, olfactory bulb and cerebral cortex, whereas weaker signals were detected in the striatum, hippocampal formation and brainstem.

To quantify the amount of $GABA_A$ receptors that contain the δ-subunit in the various brain regions immunoprecipitation experiments were performed using crude deoxycholate extracts of brain membranes (Table I). The amount of $GABA_A$ receptor precipitated from extracts of whole brain (21 ± 2% [^{3}H]flumazenil binding, $n = 3$) was slightly lower than that precipitated from purified $GABA_A$ receptor preparations (nearly 30%). In line with the regional prevalence of the δ-subunit detected in Western blots, highest immunoprecipitation was observed in extracts of the olfactory bulb, cerebellum and thalamus/hypothalamus, whereas in the cerebral cortex, striatum and hippocampal formation significantly less receptor was precipitated (Table I). These results suggest that the δ-subunit protein may be differentially expressed in various brain regions.

To identify those neuronal populations that express $GABA_A$ receptors containing the δ-subunit, immunohistochemical experiments were performed in parasagittal cryostat sections of rat brain. The δ-subunit immunoreactivity was most abundant in the granular layer of the cerebellum with mainly somatic staining in a subset of cells (Fig. 4D), followed by thalamus, dentate gyrus of the hippocampal formation and olfactory tubercle (Fig. 4A). In other brain regions, e.g. olfactory bulb, caudate putamen, ventral pallidum and cerebral cortex, only weak staining was detected (Fig. 4A). The specificity of immunostaining was assessed in peptide competition experiments. When the peptide δ(1–17) was added to the antiserum prior to the incubation procedure no immunostaining was observed (Fig. 4B). In general, the intensity of the δ-subunit signal observed immunohistochemically in various brain regions was in congruence with that obtained by Western blotting or immunoprecipitation. Furthermore, the immunostaining pattern of the δ-subunit protein (Fig. 4A) correlated well with the expression pattern of the δ-subunit mRNA [1]. For instance, in the hippocampal formation, the immunoreactivity of the dendritic area of the granule cells of the dentate gyrus (Fig. 4A,C) is correlated with the in situ hybridization signals in the corresponding cell body layer [1]. Likewise, in the cerebellum, strong immunoreactivity was displayed by the granular layer (Fig. 4A) which also showed very high in situ hybridization histochemistry signals for the δ-subunit mRNA [1].

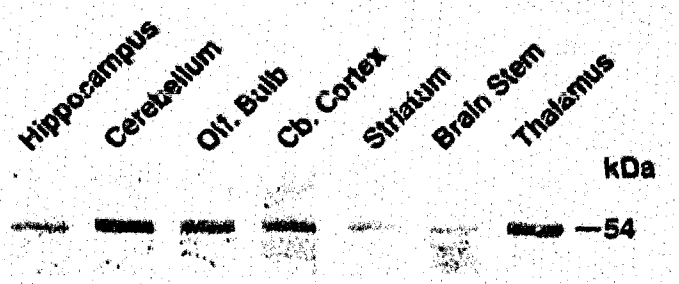

Fig. 3. Regional distribution of the δ-subunit immunoreactivity determined by Western blotting. An equal number of [^{3}H]flumazenil binding sites (~ 60 fmol) from crude membrane fractions isolated from several brain areas were applied to each lane and incubated with the δ(179–189)-antiserum (diluted 1:1000).

In the molecular layer no signals could be detected either immunohistochemically (Fig. 4D) or by in situ hybridization histochemistry [1].

The δ-subunit protein is most prominently expressed in the cerebellar granular layer, an area which is characterized by high levels of high affinity muscimol binding and low levels of benzodiazepine receptor binding [26]. It had therefore been suggested that the δ-subunit may constitute a $GABA_A$ receptor subtype that lacks high affinity benzodiazepine binding sites [1]. This was supported by the observation that the δ-subunit, when co-expressed with α- and β-subunits, was not able to confer high affinity benzodiazepine binding to recombinant $GABA_A$ receptors [1]. However, our immunoprecipitation data clearly show that $GABA_A$ receptors which contain the δ-subunit immunoreactivity display both high affinity GABA sites and high affinity benzodiazepine binding sites in all brain areas tested. Since functional benzodiazepine receptors are generated in recombinant receptors by co-expression of α-, β- and γ_2-subunits, it is conceivable that the δ-subunit may be associated with α-, β- and γ_2-subunits in

Table I

Immunoprecipitation of $GABA_A$ receptors containing the δ-subunit from crude deoxycholate extracts of various regions of rat brain

Brain Region	[^{3}H]Flumazenil binding sites precipitated (%)
Olfactory bulb	28 ± 5
Cerebellum	23 ± 3
Thalamus/hypothalamus	22 ± 3
Cerebral cortex	17 ± 3
Striatum	17 ± 3
Hippocampus	15 ± 3

Deoxycholate extracts from membranes of various brain areas (200 μl) were incubated with an amount of δ(179–189)-antiserum (10 μl) which leads to maximum immunoprecipitation. The results are expressed as percentage of specific [^{3}H]flumazenil binding; 100% values correspond to the sum of the radioligand binding in the immunoprecipitate and in the corresponding supernatant. Nonspecific immunoprecipitation was determined by replacing the antiserum by preimmune serum or buffer. The values are means ± SD from three experiments analysed in triplicates.

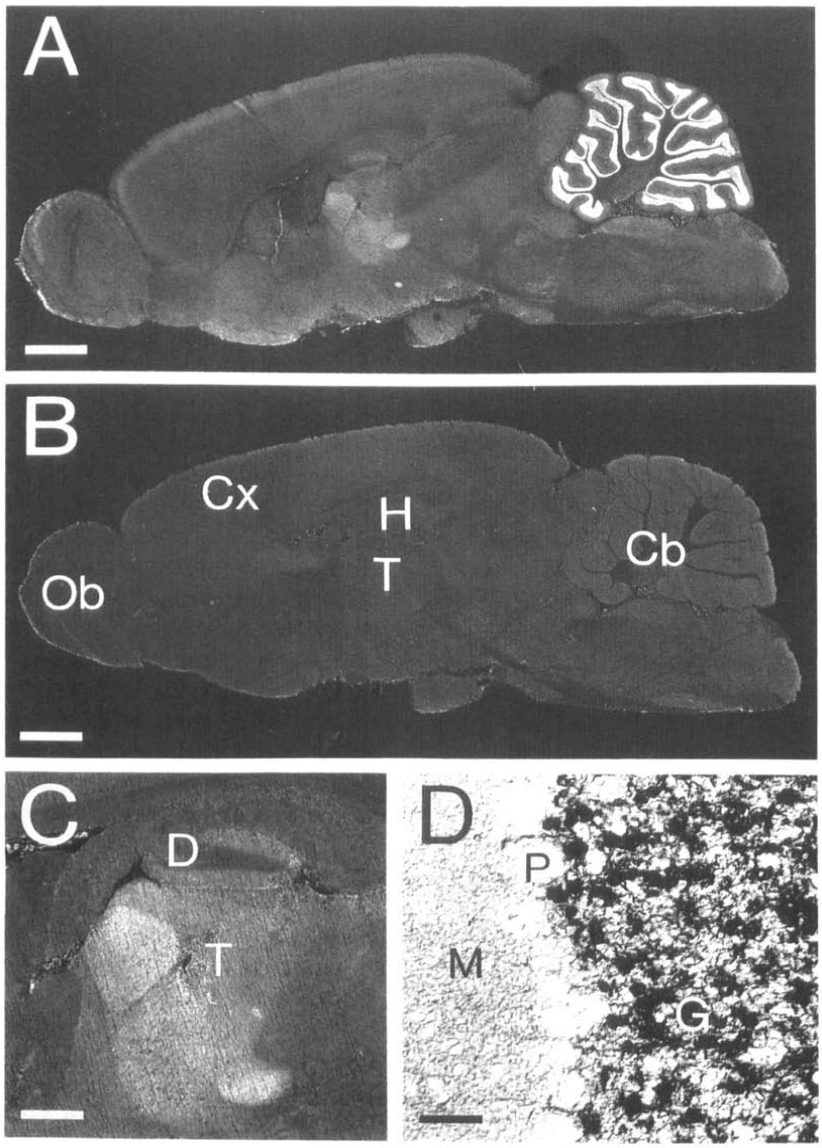

Fig. 4. Immunohistochemical localization of δ-subunit immunoreactivity in parasagittal rat brain sections incubated with affinity-purified δ(1–17)-antiserum (diluted 1:10). (A,B) Macroscopic view of staining in the absence (A) and presence (B) of 60 μg/ml peptide δ(1–17). (C) Immunostaining in the thalamus and the hippocampal formation. (D) Microscopic view of cerebellar staining. Note the intense staining in cerebellar granule cells and the lack of staining in the Purkinje cell layer and the molecular layer. Positive signals in (A,B,C) correspond to the white areas, in (D) to the black areas. Bars: (A,B) 2.0 mm; (C) 0.8 mm; (D) 20 μm. Abbreviations: Cx, cerebral cortex; D, dentate gyrus; H, hippocampus; G, granular layer; M, molecular layer; Ob, olfactory bulb; P, Purkinje cells; T, thalamus.

vivo. This view is supported by the strong immunoreactivity in the cerebellar granular layer observed not only for the δ-subunit but also for the α_1-, $\beta_{2/3}$ and γ_2-subunit proteins [13,15].

In summary, we have demonstrated that the δ-subunit is an integral protein of 54 kDa in a subset of native $GABA_A$ receptors comprising up to 30% of $GABA_A$ receptors in different brain areas. Neuronal populations which express the δ-subunit were identified immunohistochemically in situ and are now amenable to a functional analysis of this $GABA_A$ receptor subtype. Mapping the various subunit combinations is part of the attempt to establish a structure/function relationship for the various $GABA_A$ receptor subtypes in the brain.

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