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Synaptic Clustering of $GABA_A$ Receptors

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Zusammenfassung

Die 'schnelle' inhibitorische Neurotransmission im Gehirn wird hauptsächlich durch den $GABA_A$-Rezeptor ($GABA_AR$) vermittelt, einen heteromeren Chlorid-Kanal, der durch γ-Aminobuttersäure (GABA) gesteuert wird. Die meisten Subtypen von $GABA_AR$ sind in der postsynaptischen Membran von GABAergen Synapsen konzentriert, und bestehen aus verschiedenen α- und β-Untereinheiten und der γ_2-Untereinheit. Während die γ_2-Untereinheit für die Bildung und den Transport der Rezeptoren zur Zell-Oberfläche entbehrlich ist, wird sie für die Modulation der $GABA_AR$ durch Benzodiazepine benötigt. Die synaptische Lokalisation ist eine Voraussetzung für die inhibitorische synaptische Wirkung von $GABA_AR$. Die zugrundeliegenden Mechanismen der postsynaptischen Aggregation dieser Rezeptoren sind jedoch weitgehend unbekannt.

Im ersten Teil der Arbeit wurde die potentielle Rolle der γ_2-Untereinheit für diese Aggregation der $GABA_AR$ *in vivo* untersucht. Die Analyse von Neuronen aus Mäusen, denen die γ_2-Untereinheit fehlt ($\gamma_2^{0/0}$) ergab, dass diese Untereinheit für die postsynaptische Aggregation von häufigen $GABA_AR$-Subtypen notwendig ist. Der Verlust von $GABA_AR$-Aggregaten in $\gamma_2^{0/0}$ Neuronen war vom Verlust des synaptischen Verankerungs-Proteins Gephyrin und dem Verlust von synaptischer GABAerger Aktivität begleitet. Umgekehrt bewirkte die Inhibition der Gephyrin-Expression in Wildtyp-Neuronen den Verlust von synaptischen $GABA_AR$-Aggregaten. Dies zeigt, dass die γ_2-Untereinheit und Gephyrin voneinander abhängige Komponenten des gleichen synaptischen Komplexes sind, und, dass sie von wesentlicher Bedeutung für die postsynaptische Aggregation der meisten $GABA_AR$-Subtypen *in vivo* sind.

Die γ_3-Untereinheit ist homolog zur γ_2-Untereinheit und hat ähnliche pharmakologische und physiologische Eigenschaften, wenn sie zusammen mit α- und β-Untereinheiten *in vitro* exprimiert wird. Im zweiten Teil der Arbeit wurde untersucht, inwieweit die γ_3-Untereinheit die γ_2-Untereinheit bei der synaptischen Aggregation der $GABA_AR$ *in vivo* ersetzen kann. Durch transgene Überexpression der γ_3-Untereinheit in $\gamma_2^{0/0}$ Mäusen wurde ein wesentlicher Anteil an Benzodiazepin-sensitiven, funktionellen, postsynaptischen $GABA_AR$ wiederhergestellt. Darüberhinaus konnte die γ_3-Untereinheit die γ_2-Untereinheit teilweise bei der Bildung von $GABA_AR$ ersetzen, die in Snapsen konzentriert waren und mit Gephyrin *in vivo* kolokalisierten. Die Fähigkeit zur Aggregation von $GABA_AR$ und Gephyrin wurde auch bei der endogenen γ_3-Untereinheit beobachtet, aber nur im Gehirn von $\gamma_2^{0/0}$ Mäusen. Die γ_2- und γ_3-Untereinheit stimmen also funktionell dahingehend überein, dass sie die postsynaptische Aggregation von $GABA_AR$ und Gephyrin *in vivo* vermitteln können.

Summary

Fast inhibitory neurotransmission in the brain is mediated mainly by γ-aminobutyric acid (GABA)-gating of heteromeric $GABA_A$ receptor chloride channels ($GABA_AR$). Most types of $GABA_ARs$ are preferentially localized in the postsynaptic membrane of GABAergic synapses, and consist of diverse α and β subunits together with the γ_2 subunit. Whereas the γ_2 subunit is dispensable for assembly and translocation of functional receptors to the cell surface, it is required for modulation of GABAergic function by benzodiazepines. Synaptic localization of $GABA_ARs$ is a prerequisite for inhibitory synaptic function, but the mechanisms by which the synaptic clustering of receptors is regulated are poorly understood.

In the first part of the project, a possible role of the γ_2 subunit for receptor clustering *in situ* was investigated. By analyzing γ_2 subunit-deficient ($\gamma_2^{0/0}$) neurons, it was found that this subunit was required for postsynaptic clustering of major $GABA_AR$ subtypes. Loss of $GABA_AR$ clusters in $\gamma_2^{0/0}$ neurons was paralleled by loss of the synaptic clustering molecule gephyrin and loss of synaptic GABAergic function. Conversely, inhibition of gephyrin expression in wild-type neurons resulted in loss of synaptic $GABA_AR$ clusters. The γ_2 subunit and gephyrin are thus interdependent components of the same synaptic complex and of major importance for postsynaptic clustering of abundant subtypes of $GABA_ARs$ *in vivo*.

The γ_3 subunit is a closely related homologue of the γ_2 subunit and shows similar pharmacological and physiological properties when coexpressed with α and β subunits *in vitro*. In the second part of the project, it was analyzed whether the γ_3 subunit could substitute for the γ_2 subunit in synaptic clustering of $GABA_ARs$ *in vivo*. Transgenic overexpression of the γ_3 subunit in $\gamma_2^{0/0}$ mice resulted in a significant restoration of benzodiazepine-sensitive, functional, postsynaptic $GABA_ARs$. Moreover, the γ_3 subunit was able to partially substitute for the γ_2 subunit in the formation of $GABA_ARs$ that are synaptically clustered and colocalized with gephyrin *in vivo*. The $GABA_AR$ and gephyrin clustering properties were also observed for the endogeneous γ_3 subunit, but only in the $\gamma_2^{0/0}$ brain. Thus, the γ_2 and γ_3 subunits are functionally similar in their ability to promote the clustering of $GABA_ARs$ and gephyrin at postsynaptic sites *in vivo*.

1. General Introduction

> "Insbesondere ist auch das Bewußtsein des Menschen und der höheren Tiere keineswegs ein besonderes übernatürliches 'Welträtsel'. ... Vielmehr beruht dasselbe ebenso auf der mechanischen Arbeit der Ganglienzellen im Gehirn, wie die übrigen Seelentätigkeiten."
> Ernst Haeckel, 1867
> (Natürliche Schöpfungsgeschichte)

Every perception and response of an animal to environmental or internal stimuli, from the jellyfish movement to complex processes like human consciousness, emotion, memory, or imagination, depends on the tightly regulated 'mechanical' (E. Haeckel) action of neurons. The - comparatively simple - underlying mechanism is the generation of an electrical signal, its transport along dendrites and axons, and its propagation across synapses to other neurons. The latter process relies largely on chemical neurotransmission. For this basic biological phenomenon, an electrical signal that arrives at a presynaptic terminal is transformed into a chemical signal, by release of neurotransmitters from the axon terminal into the synaptic cleft. Upon binding of the transmitter to its receptors in the postsynaptic membrane, the signal is retransformed to a change of membrane potential, by altering the conductance properties of ion channels, and is as such processed along the postsynaptic cell.

1.1 GABA and its receptors: Historical aspects

The hypothesis of chemical neurotransmission was first published by T. R. Elliot in 1905 with adrenaline as a transmitter (Elliot, 1905; von Euler, 1981), whereas its experimental proof was not demonstrated until 1921 (Loewi, 1921). Even beforehand, in 1883, the substance γ-aminobutyric acid (GABA), a non-protein amino acid (aa) was first synthesized and described under the name 'Piperidinsäure' (piperidic acid, Schotten, 1883; reviewed in Baxter, 1970). Its presence in the brain, however, was not discovered until 1950 (Awapara et al., 1950; Roberts and Frankel, 1950; Udenfriend, 1950). In 1954, the effect of an inhibitory factor, isolated from mammalian nervous system, on the crayfish stretch receptor was detected that emerged to consist largely of GABA (Florey, 1954; Bazemore et al., 1956). Although it had been suggested early on that GABA might be an inhibitory neurotransmitter, it took until the late sixties for such

a role to be generally accepted, in the invertebrate as well as vertebrate nervous system (Krnjevic and Schwartz, 1966). Much work with respect to this question was performed in the crustacean central nervous system (CNS, reviewed in Roberts, 1986; Rabow et al., 1995). Soon after, primary cultures of neurons were introduced into GABA research (Fischbach, 1972; Fischbach and Dichter, 1974). Since its manifestation as a neurotransmitter, the amount of scientific work dealing with GABA and its receptors has increased dramatically. A present-day search of the Medline database for the keyword 'GABA' reveals more than 2000 articles per year (6/98-6/99).

The first evidence for mammalian GABA receptors was obtained from electrophysiological studies (Curtis et al., 1968a; Curtis et al., 1968b). It took almost ten years till the discovery of high affinity binding sites for benzodiazepines (BZs) in the CNS, which were subsequently found to be intrinsic to $GABA_A$ receptors ($GABA_ARs$) (Mohler and Okada, 1977a; Mohler and Okada, 1977b; Braestrup and Squires, 1977; Schoch and Mohler, 1983; Schoch et al., 1984; Sigel and Barnard, 1984; Schoch et al., 1985). However, BZ modulation of synaptic GABAergic activity had already been demonstrated earlier (Schmidt et al., 1967; Polc and Haefely, 1976). Purification of the GABA/BZ receptor complex in the early eighties led to the first production of antibodies recognizing $GABA_AR$ subunits (α_1, β_2, and β_3) and their visualization in the brain (Schoch et al., 1985; reviewed in Enna and Karbon, 1986).

In 1987, the first two cDNAs for $GABA_AR$ subunits (α_1 and β_1) were cloned (Schofield et al., 1987), and this identified $GABA_ARs$ as members of the family of ligand-gated ion channels. Only recently, the cDNAS for the other type of GABA receptor, the metabotropic $GABA_B$ receptor have been isolated (Kaupmann et al., 1997; White et al., 1998; Jones et al., 1998; Ng et al., 1999; Kuner et al., 1999).

Heterologeous expression of functional $GABA_ARs$ by injection of chicken or rat brain mRNA in Xenopus oocytes was first reported in 1983 (Smart et al., 1983; Houamed et al., 1984). After cloning of the subunits, this approach has proven to be a very valuable tool to investigate the physiological and pharmacological profiles and functional properties of different receptor subtypes.

1.2 Inhibitory neurotransmission

Inhibitory neurotransmission in the CNS of most animal taxa is mediated by the neurotransmitters GABA and glycine. Glycine receptors (GlyR) are ionotropic ion channels, closely related to $GABA_A$Rs, but appear mainly restricted to spinal cord, brain stem, and retina (Kuhse et al., 1995). Thus, GABA is the main inhibitory neurotransmitter in the nervous system, which is present in many regions in the millimolar range. Local circuit GABAergic interneurons occur in most areas of gray matter in the CNS. They are prominent for instance in the cerebral cortex, where 20-50% of synapses are estimated to be GABAergic (Sieghart, 1995). These interneurons form synapses with 'symmetric' (type 2) morphology on somata and dendrites of cortical pyramidal cells (Parent, 1996) as well as interneurons. The evolutionary success and versatility of the neocortex could in part be attributed to the tightly balanced activity of excitatory and inhibitory neurons (Somogyi et al., 1998; Deutch and Roth, 1999). In some brain areas including the basal ganglia, cerebellar cortex or nucleus reticularis of the thalamus (RTN), GABAergic neurons are the prevalent cell type (Houser et al., 1980; Mugnaini and Oertel, 1985), and represent projection neurons mediating inhibition over longer distances. Examples are inhibitory neurons projecting from the striatum to the substantia nigra, or the Purkinje cells of the cerebellum, which project to the deep cerebellar nuclei (reviewed in Ottersen et al., 1995).

Two distinct types of receptors are known for GABA. The $GABA_A$R is a ionotropic chloride channel that mediates the main part of fast inhibitory neurotransmission in the brain, characterized by a main channel conductance of 27-30 pS (reviewed in Hevers and Luddens, 1998). A second, physiologically, pharmacologically and structurally different type of GABA receptor termed $GABA_B$ receptor ($GABA_B$R) was postulated to exist in 1981 (Hill and Bowery, 1981). Whereas $GABA_A$Rs can be blocked by bicuculline, but fail to be stimulated by the GABA analogue baclofen, $GABA_B$Rs are characterized by the antithetic pharmacology (Johnston, 1996). $GABA_B$Rs display a relatively 'slow' inhibitory action and gate potassium and/or calcium channels via G protein-coupled mechanisms. This was recently confirmed by cloning of two $GABA_B$R cDNAs, which exhibit the typical seven transmembrane structure of metabotropic, G-protein coupled receptors (Kaupmann et al., 1998; White et al., 1998; Jones et al., 1998; Ng et al., 1999; Kuner et al., 1999). $GABA_A$ and $GABA_B$Rs are both implicated in postsynaptic actions as well as presynaptic modulation of neurotransmitter release (Bettler et al., 1998; McGehee and Role, 1996; MacDermott et al., 1999).

The existence of a third type of GABA receptor called '$GABA_C$ receptor' had originally been proposed based on pharmacological properties. Recent cloning of the

corresponding subunits ρ_{1-3} revealed that they exhibit the same principle subunit structure as the $GABA_A$Rs (Cutting et al., 1991; Zhang et al., 1995; Ogurusu et al., 1995; Ogurusu and Shingai, 1996). Based on this observation, it was proposed that they should be considered specific subtypes of $GABA_A$Rs despite considerable functional differences (Barnard et al., 1998). For example, the receptors containing ρ subunits display a distinctly smaller main channel conductance of approx. 8 pS, and a longer mean open time (150 ms) than typical $GABA_A$Rs (Feigenspan and Bormann, 1998). Moreover, ρ subunit-containing receptors form homomeric complexes and differ from typical $GABA_A$Rs in that they are insensitive to bicuculline (Drew et al., 1984; Polenzani et al., 1991; Drew and Johnston, 1992; Johnston, 1996).

With respect to neuronal function, the term 'inhibitory' refers in general to a reduced probability of the affected neuron to generate action potentials. This inhibited state of a neuron is achieved by GABA through two different mechanisms, depending on the receptor-type involved. $GABA_A$Rs increase the Cl^- conductance of the postsynaptic neuron upon activation, whereas postsynaptically located $GABA_B$Rs might be responsible for 'long'-lasting increases in K^+ conductance. In a typical neuron, the equilibrium potential for Cl^- as well as K^+ is lower than the resting membrane potential of the cell. Upon activation of GABA receptors, Cl^- flows into the cell ($GABA_A$Rs) and/or K^+ flows outside ($GABA_B$Rs) along their concentration gradients, thus increasing the intracellular negative charges and hyperpolarizing the cell (Kandel et al., 1991; Mody et al., 1994). It is important to note that GABA can also mediate excitatory (depolarizing) functions, mainly during development of the nervous system. For example, in premature postnatal hippocampal neurons, GABAergic neurotransmission mediated by $GABA_A$Rs is excitatory and might be involved in trophic functions for the maturation of other receptor systems. The mechanism of depolarizing GABA function is not yet fully understood, but relies rather on altered Cl^- concentrations (elevated intracellular $[Cl^-]$), than on specific developmental $GABA_A$R subtypes (Cherubini et al., 1991; Ben-Ari et al., 1997; Belhage et al., 1998; Davies et al., 1998b; Rivera et al., 1999). In addition, GABA can act disinhibitory (i.e. excitatory in consequence), by activation of its receptors at GABAergic interneurons, resulting in inhibition of synaptic GABA release. For example, electrical stimulation of septo-hippocampal fibers in rat brain slices caused a maintained decrease in the frequency of spontaneous inhibitory postsynaptic potentials (IPSPs) recorded from CA3 pyramidal cells in the presence of glutamate receptors antagonists, suggesting that GABAergic septo-hippocampal afferents inhibit hippocampal inhibitory cells and so disinhibit pyramidal cells (Toth et al., 1997).

The main source for GABA biosynthesis in the presynaptic axon-terminal of GABAergic neurons is glutamate. A first important enzyme in the 'GABA shunt' is GABA α-oxoglutarate transaminase (GABA-T), catalyzing the transamination of α-ketoglutarate derived from the Krebs cycle, to L-glutamate. In the second step, GABA is synthesized from glutamate by decarboxylation, a reaction catalyzed by the enzyme glutamic acid decarboxylase (GAD). Two isoforms of GAD (65 and 67 kD in size) are known, whereby GAD65 is located at membranes and GABAergic terminals, thus providing a reliable marker for GABAergic synapses, and GAD67 is distributed throughout the neurons (Kaufman et al., 1991; Christgau et al., 1992; Soghomonian and Martin, 1998). GABA is stored in synaptic vesicles most likely through uptake via a vesicular GABA and glycine cotransporter (VGAT or VIAAT McIntire et al., 1997; Sagne et al., 1997; Chaudhry et al., 1998; Dumoulin et al., 1999) and, upon depolarization of the presynaptic GABAergic neuron, released into the synaptic cleft by exocytosis, where it binds to and activates GABA receptors. The action of GABA is terminated by cellular uptake with GABA transporters (GAT), located in the membrane of presynaptic terminals and surrounding glia cells (reviewed in Borden, 1996). Whereas re-uptake into the presynaptic terminal allows for re-utilization of GABA, glia cells metabolize GABA to succinic semialdehyde and succinic acid that can re-enter the Krebs cycle (reviewed in Hevers and Luddens, 1998; Olsen and DeLorey, 1999; Deutch and Roth, 1999).

The physiological importance of proper neuronal inhibitory function is most evident in the case of neuropathological conditions that are alleviated by the therapeutic action of drugs that enhance GABA transmission. Most notably, these include $GABA_AR$ ligands such as the BZs. Common human neuropathological and emotional disorders, such as epilepsy, Huntington's chorea, Parkinsonism, anxiety disorders, schizophrenia, and major depression are associated with changes in GABAergic transmission (Mohler et al., 1997a; Krogsgaard-Larsen et al., 1997). It is, however, often ambiguous, whether these changes are a prerequisite or a consequence of pathophysiological alterations. The involvement of GABAergic neurotransmission in epileptic disorders is one thoroughly studied focus of interest in GABA receptor research. For example, blockage or inhibition of GABAergic function is sufficient, but not necessary, to evoke epileptic seizures. Likewise, augmentation of GABA action by pharmacological means is used to treat acute seizures of epilepsy patients (Olsen and Avoli, 1997).

1.3 Structure, pharmacology, and diversity of the $GABA_A$ receptor

1.3.1 Structure and posttranslational modification of $GABA_A$ receptor subunits

Based on size and sequence similarities, the $GABA_AR$ subunits belong to the protein 'superfamily' of ionotropic ligand-gated ion channels. Other members of this family are the GlyR, the serotonin (5-HT_3) receptor and the nicotinic acetylcholine receptor (nAChR). The four types of ligand-gated ion channels derive in all probability from a common ancestor (Fig. 1, reviewed in Ortells and Lunt, 1995).

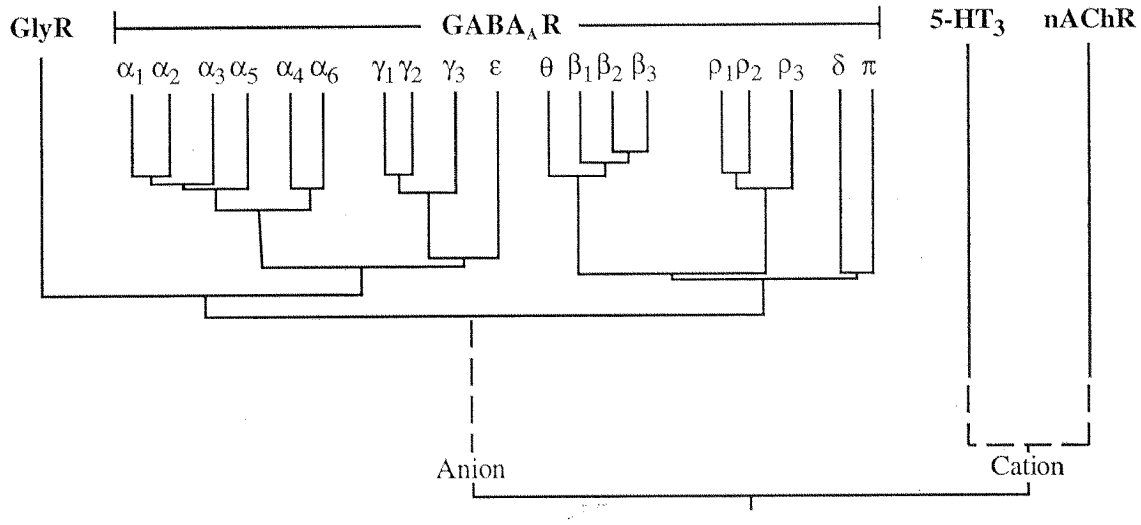

Fig. 1 Evolutionary tree of ligand-gated ion channels, derived from mammalian DNA-sequence comparisons; not to scale (i.e. the length of the branches represents only relative sequence similarities, modified from Ortells and Lunt, 1995; Barnard et al., 1998; Bonnert et al., 1999).

All proteins in this family are integral membrane complexes, formed most likely by assembly of five homologous subunits (Waxham, 1999). The protein complex forms a pore that regulates the flow of selective ions across the membrane. Whereas GlyRs and $GABA_ARs$ are selective for anions, 5-HT_3 and nAChR selectively gate cations. The ion channel opens upon binding of a receptor-specific ligand to the extracellular domain. The subunits typically exhibit four transmembrane domains (TM1-TM4), a large N-

terminal extracellular domain and a large intracellular loop between TM3 and TM4. An important property of these receptors - and probably one reason for their evolutionary success - is the high variability that can be achieved through different combinations of subunits, which is particularly evident for $GABA_AR$s, and allows for fine-tuned modulatory actions of the same ligand on different receptor subtypes[1]. The ionotropic glutamate receptors (NMDA = N-methyl-D-aspartate, AMPA = α-amino-3-hydroxy-5-methylisoxazole-propionic-acid, and kainate receptors) are distantly related to the super-gene family of ligand-gated ion channels and show significant differences in their transmembrane topology (Waxham, 1999).

For the mammalian $GABA_AR$, 19 subunit genes are known so far: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, ρ_{1-3}, and θ. In addition, several avian and invertebrate subunit variants were identified that seem to lack an obvious mammalian paralog. They include the chicken β_4 and γ_4 subunits (Bateson et al., 1991; Harvey et al., 1993), a molluscan β subunit (Harvey et al., 1991) and the rdl gene (resistant to the insecticide dieldrin) from *D. melanogaster*, which also appears to encode a $GABA_AR$ subunit (Ffrench-Constant et al., 1991). After the initial cloning of the bovine α_1 and β_1 subunits (Schofield et al., 1987), other subunits were identified by homology screening (reviewed by Burt and Kamatchi, 1991). The subunits within a given class (e.g. α_{1-6}) typically share aa sequence identities of 70-80%, whereas 30-40% identity are found in between different classes (α-ρ) (reviewed in Hevers and Luddens, 1998). The inter-species sequence homology for a given subunit is generally higher than 90% (Whiting et al., 1997).

The mature polypeptides of all subunits range in size between approx. 450-600 aa residues, which is reflected in a molecular weight of 40-60 kD for the subunits, and 240-290 kD for the pentameric receptors. The large extracellular N-terminal domain of the subunits (~ 220 aa) comprises 20-30 residues of a signal peptide, 2-4 putative N-linked glycosylation sites, and a disulfide-loop, which is present in all subunits of the protein-family and is presumed to be involved in ligand binding and assembly functions (see below). The four suggested membrane spanning regions (TM1-4) display the highest sequence homology between all $GABA_AR$ subunits and are characterized by approx. 20-25 lipophilic aa. Small intracellular and extracellular loops connecting TM1 with TM2, and TM2 with TM3, are followed by the large intracellular loop (TM3-4, ~ 100 aa) that shows the highest sequence variability among the subunits (Rabow et al., 1995; Hevers and Luddens, 1998). The C-terminus either ends within TM4 or is a very short

[1] In the following, the term 'subunit' refers to the single polypeptide, whereas 'subtype' shall define a oligomeric receptor with a given subunit-combination. A group of closely related subunits (e.g. the α subunits of the $GABA_AR$) is named 'subunit-class'.

extracellular domain that appears not associated with any obvious function. The exemplifying predicted secondary structure of the murine γ_2 subunit of the $GABA_AR$ is shown in Fig. 2 (Shivers et al., 1989; Pritchett et al., 1989; Kofuji et al., 1991).

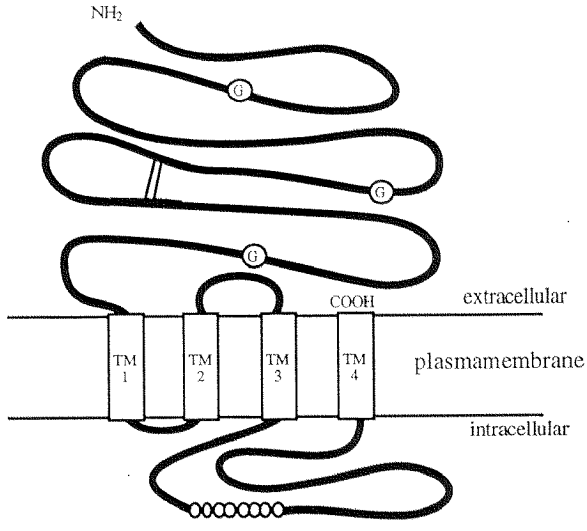

Fig. 2 Model of the predicted secondary structure of the murine γ_{2L} subunit isoform of the $GABA_AR$ (aa sequence from Swiss Prot, Kofuji et al., 1991). Putative glycosylation sites (G) and the disulfide-loop are indicated in the extracellular domain. The eight aa insert containing an additional PKC phosphorylation site and distinguishing the long from the short splice variant is marked in the intracellular loop.

In addition to the large number of identified subunits, alternative mRNA splicing of some $GABA_AR$ subunits was found to contribute further to the diversity of receptor subtypes. Two regions of alternative splicing on $GABA_AR$ subunits are know that result in two different isoforms, designated long and short (L, S). One affected domain is the N-terminal extracellular loop, resulting in isoforms of yet unknown function. Such variants exist for the human β_3 subunit (Kirkness and Fraser, 1993), the rat α_6 subunit (Korpi et al., 1994), and the ρ_1 subunit (Martinez-Torres et al., 1998). Furthermore, the ε subunit displays tissue-specific RNA-splicing, but only the short isoform has so far been found to be expressed in neurons (Whiting et al., 1997). The second variable domain is located in the large intracellular loop. Splice variants of this type are known for the chicken β_2 and β_4 subunits (Harvey et al., 1994; Bateson et al., 1991), the human β_2 subunit (McKinley et al., 1995), and the γ_2 subunit, where they are best characterized. The γ_{2S} and γ_{2L} subunit isoforms differ by 8 aa (Fig. 2) and exhibit a

partly overlapping but distinct expression pattern (Whiting et al., 1990; Kofuji et al., 1991; Gutierrez et al., 1994). Interestingly, the protein kinase C (PKC) phosphorylation site at Ser^{353} is only present in γ_{2L} and has been implicated in increased ethanol sensitivity of $GABA_A$Rs, however, with conflicting results (Sigel et al., 1993; Mihic et al., 1994a; Harris et al., 1997; Homanics et al., 1999). Furthermore, altered ratios of γ_{2S} and γ_{2L} mRNAs are found in schizophrenic patients (Wafford et al., 1991; Huntsman et al., 1998).

The genes for $GABA_A$R subunits are grouped together in distinct 'clusters' on different chromosomes (reviewed in Barnard et al., 1998), supporting the idea that they evolved by gene duplication (McLean et al., 1995). In general, these clusters contain one or two α, one β and one γ or ε subunit gene (e.g. α_1,α_6,β_2,γ_2, and π on human chromosome 5), but the expression of the different members in a $GABA_A$R gene cluster is not very well coordinated.

Phosphorylation might represent the most important posttranslational modification of $GABA_A$Rs and is generally thought to modulate $GABA_A$R function. Native $GABA_A$Rs can be phosphorylated *in vitro* (Sweetnam et al., 1988) and kinases copurify with them (Bureau and Laschet, 1995; Kannenberg et al., 1997; Kannenberg et al., 1999). Studies on recombinant receptors revealed that several putative phosphorylation sites for serine/threonine and tyrosine protein kinases are located in the large intracellular loop of $GABA_A$R subunits. The literature on the location of phosphorylation sites contains a considerable amount of conflicting results (Moss and Smart, 1996). Whereas putative PKC recognition sites are found on all subunits, targets of cAMP-dependent protein kinase (PKA) seem more restricted, and sites for tyrosine kinase appear limited to γ subunits (Macdonald and Olsen, 1994; McKernan and Whiting, 1996; Mohler et al., 1996b; Moss and Smart, 1996). Interestingly, long-term depression (LTD) of inhibitory postsynaptic currents (IPSCs) was shown to be mediated by $GABA_A$Rs through its sensitivity to the $GABA_A$R agonist THIP (4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol hydrochloride), and is reduced by phosphatase inhibitors (Morishita and Sastry, 1996). Although PKC seems to inhibit $GABA_A$R currents mostly by phosphorylation of serine residues on β subunits or the γ_2 subunit, augmentation of GABA currents was also found (reviewed in Moss and Smart, 1996; Smart, 1997). For example, PKA and PKC exhibited different effects on miniature IPSCs (mIPSCs), dependent on the cell-type investigated (Poisbeau et al., 1999). Recent work links the phosphorylation-dependent inhibition of GABAergic currents to an internalization of $GABA_A$Rs from the cell-surface (Chapell et al., 1998; Filippova et al., 1999, S. Penschuk and J.M. Fritschy, unpublished). Conversely, recruitment of

intracellular $GABA_AR$ subunits to postsynaptic sites in response to insulin is inhibited by tyrosine-kinase inhibitors (Wan et al., 1997). In addition, Ca^{2+}/calmodulin type II-dependent protein kinase (McDonald and Moss, 1997), and phosphatases such as calcineurin (Huang and Dillon, 1998) are also implicated in modulation of $GABA_ARs$.

1.3.2 Structure of the $GABA_A$ receptor complex

The number of subunits forming the $GABA_AR$ channel, and the subunit stoichiometry of individual receptor subtypes has not been determined conclusively yet. However, accumulating evidence confirms a pentameric structure for the $GABA_AR$, as initially predicted based on homology and functional analogy to the nAChR (Mohler et al., 1996a; Mohler et al., 1997a; Mohler et al., 1997b; Costa, 1998; Barnard et al., 1998). The molecular weight of purified native $GABA_ARs$ is consistent with the sum of five glycosylated subunits (Stephenson, 1995; Knight et al., 1998). Furthermore, analysis of electron microscopic (EM) images of native $GABA_ARs$ revealed data most consistent with the fivefold rotation symmetry expected of a pentameric receptor structure (Nayeem et al., 1994; Barnard et al., 1998). A schematic model of the $GABA_AR$ structure is shown in Fig. 3.

Much evidence for the subunit stoichiometry was obtained by analyses of recombinant receptors. Several studies took advantage of mutated subunits that resulted in quantifiable changes in recombinant receptor properties. For example, based on the quantification of outward rectification of GABA-evoked currents, which was altered by point mutations near the channel pore, Backus et al. (1993) suggested the most likely stoichiometriy to be 2α, 1β, 2γ. Another approach utilized coexpression of wildtype (wt) and mutated subunits where a conserved leucine in TM2 was replaced by serine. The resulting receptors displayed increased GABA sensitivities in proportion to the number of incorporated mutant subunits (Chang et al., 1996). Use of fluorescently labeled antibodies against tagged subunits similarly resulted in quantifiable changes of the maximal detected fluorescence signal. The signal was twice as high for tagged α_1 or β_2 than γ_2 subunits in coexpression with wt subunits (Farrar et al., 1999). In addition, tandemly linked subunits only gave rise to proper receptors when coexpressed with a single other subunit (Im et al., 1995). These experiments consistently suggested a stoichiometry of 2α, 2β, 1γ subunits, mostly for the $\alpha_1\beta_2\gamma_2$ subtype. Tretter et al. (1997) determined the affinities of $GABA_AR$ subunit specific antibodies directed against the N-terminus or cytoplasmic loop of different subunits, by use of chimeric subunits. The

antibodies were than applied in immunoblots of recombinant $\alpha_1\beta_3\gamma_2$ receptors and similarly suggested subunit ratios of $2\alpha_1{:}2\beta_3{:}1\gamma_2$.

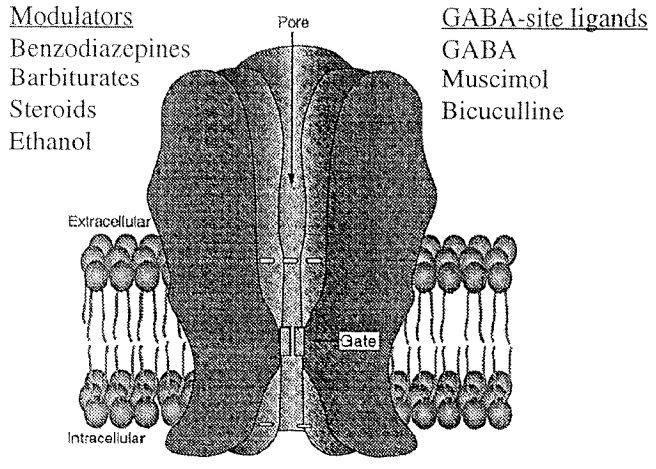

Fig. 3 Structural model of the $GABA_AR$. The receptor is shown as a pentameric complex. Various ligands of the GABA site and other modulators are indicated (modified from Waxham, 1999).

Numerous studies addressed the contribution of different subunits to recombinant and native $GABA_ARs$ mainly by means of subunit-specific antibodies. Immunoprecipitation and Western blotting of solubilized receptors with alternating combinations of antibodies, combined with radioligand-binding to determine the amount of receptors precipitated (e.g. [^{3}H]muscimol or [^{3}H]flumazenil for the GABA or BZ binding site respectively), allowed conclusions with regard to the subunit composition of $GABA_ARs$ (McKernan and Whiting, 1996). Additional information was obtained by immunohistochemical staining of brain sections and cultured neurons (Fritschy and Mohler, 1995; De Blas, 1996). The data indicate that two different types of α subunits often coexist in a single receptor complex, suggesting that the prevalent $GABA_AR$ subtypes contain two α subunits (Luddens et al., 1991; Duggan et al., 1991; Fritschy et al., 1992; Mertens et al., 1993; Fritschy and Mohler, 1995; Pollard et al., 1995; McKernan and Whiting, 1996; Barnard et al., 1998). The question as to whether different β subunits can coexist in the same receptor and whether the receptors contain two β or rather two γ subunits is still a matter of debate (Benke et al., 1994; but Li and De Blas, 1997; Fisher and Macdonald, 1997). Similar to the different α subunits, the γ_2 and γ_3 or the γ_{2S} and γ_{2L} subunits can apparently coexist in native receptor subtypes

(Backus et al., 1993; Khan et al., 1994; Benke et al., 1996), although again with conflicting results that suggest a separate existence of all 3 γ subunits (Mossier et al., 1994; Togel et al., 1994). The γ_1 subunit, however, does not appear to occur together with other γ subunits (Quirk et al., 1994a; Benke et al., 1996). The most prominent $GABA_AR$ subtypes in brain contain αβ subunit combinations together with the γ_2 subunit (Benke et al., 1990; Fritschy and Mohler, 1995; Benke et al., 1996; Somogyi et al., 1996). The δ subunit seems to replace the γ_2 subunit in a portion of $GABA_AR$ subtypes mainly in cerebellar granule cells (Quirk et al., 1994b; Quirk et al., 1995; Nusser et al., 1996a), but the γ_2 and δ subunits might also occur jointly (Mertens et al., 1993; Fritschy and Mohler, 1995). Finally, the ρ subunits are exceptional in that they don't seem to coexist with other $GABA_AR$ subunits (Cutting et al., 1991; Martinez-Torres et al., 1998; Koulen et al., 1998; Enz and Cutting, 1999). However, for the ρ subunits as well as for ε, π and θ the actual contribution to native receptors is not yet fully established.

1.3.3 Pharmacology of $GABA_A$ receptors

$GABA_ARs$ are of particular interest as a clinically important target for therapeutic modulation of various CNS functions such as vigilance, anxiety, epileptic activity, and memory. Classical BZs like diazepam are widely used as therapeutic agents with anticonvulsant, muscle-relaxant, sedative-hypnotic or anxiolytic activity. They act on $GABA_ARs$ as allosteric modulators, by enhancing the inhibitory function through an increase of the channel opening probability in response to GABA, reflecting an apparent increase in GABA-affinity (reviewed in Haefely, 1994). BZs do not open channels in the absence of GABA, which is an important aspect of the safety of this class of drugs (Smith and Olsen, 1995). A variety of other exogenous or endogenous compounds, such as the barbiturates, some neurosteroids, and ethanol, are known to modulate $GABA_AR$ function (Lambert et al., 1996; Mohler et al., 1997a). Several ligands that lack a clinical application are important tools in $GABA_AR$ research. For example, the convulsant bicuculline acts as a competitive antagonist of the GABA site, and picrotoxin blocks the channel pore.

The pharmacological heterogeneity of $GABA_ARs$ is mediated by a multitude of receptor subtypes, which differ in their subunit composition. Recombinant receptors with αβ subunit combinations are gated by GABA and are potentiated by barbiturates, but lack sensitivity to BZs (Schofield et al., 1987; Levitan et al., 1988). A γ subunit is required for the formation of the BZ binding site and potentiation of $GABA_AR$ currents

by BZs (Pritchett et al., 1989). Furthermore, only αβγ and αβδ combinations show the channel conductance of 27-30 pS, which corresponds to the main unitary conductance of native $GABA_A$Rs and cooperative GABA action (Bormann et al., 1987; Macdonald et al., 1989; Fisher and Macdonald, 1997), whereas the channel conductance of αβ combinations is smaller (10-12 or 17-20 pS, Macdonald and Olsen, 1994; Hevers and Luddens, 1998; Gunther et al., 1995). $GABA_A$R subtypes can be classified according to their different affinities to various BZs that largely depend on the α and γ subunits present (reviewed in Mohler et al., 1997a). Most $GABA_A$Rs (~ 80%) display high affinities (K_i 0.5-20 nM) for the classical BZ diazepam and are sensitive to zolpidem (~ 60%: $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$). The remaining subtypes are insensitive to zolpidem and split up into three groups that are discriminated by their sensitivity to diazepam (high: $\alpha_5\beta_x\gamma_2$; low: $\alpha_x\beta_x\gamma_1$, $\alpha_x\beta_x\gamma_3$; insensitive: $\alpha_4\beta_x\gamma_2$, $\alpha_6\beta_x\gamma_2$) (Mohler et al., 1997a). $GABA_A$Rs containing the δ subunit appear insensitive to BZs (Quirk et al., 1995; Araujo et al., 1998) with, however, some conflicting results that could be attributed to subtypes of αβγδ combinations (Mertens et al., 1993). The ε subunit which is distantly related to the γ subunits, fails to confer BZ sensitivity but renders recombinant αβε receptors insensitive to anaesthetic agents (Davies et al., 1997).

Crosslinking studies on native receptors and mutational analysis of recombinant receptors revealed that the GABA binding site is formed by α and β subunits and that the BZ site is located at the interface of α and γ subunits (Smith and Olsen, 1995). As expected, the relevant aa residues for several (but not all) ligand binding sites were found to be localized to the extracellular N-terminal domain of the subunits (reviewed in Smith and Olsen, 1995). For instance, Phe^{64} of different rat α subunits is most likely involved in formation of the GABA binding site, as mutation to leucine severely decreased binding affinities of agonists and antagonists for the GABA site (Sigel et al., 1992; Boileau et al., 1999). Similarly, the putative GABA binding pocket at the β_2 subunit was localized in proximity to the extracellular disulfide (Cys)-loop (Amin and Weiss, 1993; Smith and Olsen, 1995), a structure suggested to contribute to the ligand binding sites of all ionotropic receptors (Cockcroft et al., 1990). However, mutation of conserved aa within or adjacent to the Cys-loop of the β_2 subunit produced at best moderate effects on GABA sensitivity (Amin et al., 1994). The BZ binding site was equally examined. For example, the efficacy of different BZ agonists and inverse agonists was drastically altered when Thr^{142} of the human γ_2 subunit was mutated to serine (Mihic et al., 1994b). Buhr et al. found Phe^{77} of the γ2 subunit to be involved in BZ binding (Buhr et al., 1996; Buhr et al., 1997a), and Met^{130} to contribute to zolpidem sensitivity of γ_2 subunit-containing receptors, a property not present in γ_3 subunit-containing receptors (Buhr and Sigel, 1997c). Likewise, His^{101} of the α_1 subunit (or

corresponding residues of other α subunits), Tyr^{159} and Tyr^{209} are involved in diazepam sensitivity (Wieland et al., 1992; Amin et al., 1997; Buhr et al., 1997b; Davies et al., 1998a; Benson et al., 1998). Finally, the channel-lining sequences of the receptor that form the gate controlling ion-flux, were located to TM2 of several subunits, in analogy to the channel structure of the nAChR. Mutation of residues in this domain of α_1, β_2, and γ_2 subunits resulted in resistance for picrotoxin which blocks the channel directly at the pore (Gurley et al., 1995). Point-mutations adjacent to TM2 were shown to alter the rectification properties of $GABA_A$Rs which is also consistent with a channel-lining function of that domain (Backus et al., 1993).

1.3.4 Differential expression of $GABA_A$ receptor subunits

The diversity of $GABA_A$Rs became evident by numerous studies on the differential expression of $GABA_A$R subunit mRNAs (Wisden et al., 1992; Laurie et al., 1992a; Persohn et al., 1992; Ma et al., 1993; Boue-Grabot et al., 1998; Kultas-Ilinsky et al., 1998). More important, the subsequent availability of subunit specific antibodies enabled the direct comparison of protein expression patterns at the regional and cellular level, e.g. in the rat brain (Fritschy et al., 1992; Endo and Olsen, 1993; Gao et al., 1993), spinal cord (Bohlhalter et al., 1994; Bohlhalter et al., 1996), and the primate brain (Hornung and Fritschy, 1996). The most comprehensive immunohistochemical study so far compared the regional and cellular distribution of α_1, α_2, α_3, α_5, β_2 and β_3 ($\beta_{2/3}$, one antibody recognizes both subunits), γ_2 and δ subunits, i.e. all the major $GABA_A$R subunits in the adult rat brain (Fritschy and Mohler, 1995).

The *in situ* hybridization and protein expression patterns largely correlate, indicating that $GABA_A$R subunit expression is mainly determined at the transcriptional level. Consistent with immunopurification studies (see above), the α_1, $\beta_{2/3}$, and γ_2 subunits were found to be the major subunits in most areas of the brain, including olfactory bulb, cerebral cortex, interneurons of the hippocampus, basal ganglia, most nuclei of the thalamus, cerebellum, and brainstem. The α_2, α_3, and α_5 subunits also mostly coexist with the $\beta_{2/3}$, and γ_2 subunits, but are much less abundant and expressed largely complementary to the α_1 subunit. Whereas the α_2 subunit is mainly found in hippocampal pyramidal cells, striatum and olfactory bulb, the α_3 subunit is expressed in the lateral septum, the RTN, and several brainstem nuclei. The α_5 subunit is largely restricted to hippocampal pyramidal cells, olfactory bulb, hypothalamus, and the trigeminal sensory nucleus. $GABA_A$R subtypes containing α_4 and α_6 subunits are comparatively rare with the α_6 subunit selectively expressed in cerebellar granule cells

and cochlear nuclei, and the α_4 subunit mRNA, being most abundant in the thalamus and hippocampus. The combined expression pattern of the α_4 and α_6 subunits thus closely matches the cellular distribution of the δ subunit, suggesting that these α subunits and the δ subunit are often part of the same receptor (Wisden et al., 1992; Laurie et al., 1992a; Thompson et al., 1992; Fritschy and Mohler, 1995; Jones et al., 1997). *In situ* hybridization and immunoprecipitation indicated that the regional expression of the γ_3 subunit overlaps with the one of the γ_2 subunit in neocortex, basal nuclei and thalamus (Herb et al., 1992; Wisden et al., 1992). In contrast, γ_1 is present almost exclusively in amygdala, septum and hypothalamus (Wisden et al., 1992). Whereas the ε and θ subunit display a largely restricted expression pattern in brain (Davies et al., 1997; Whiting et al., 1997; Neelands et al., 1999; Bonnert et al., 1999), the π subunit is exclusively found in peripheral tissues (Hedblom and Kirkness, 1997).

Beside the regional diversity of $GABA_AR$s, temporal changes of subunit expression occur during development of the GABAergic system. Many $GABA_AR$ subunits are already abundantly expressed prenatally, although synaptogenesis takes place mainly after birth (Laurie et al., 1992b; Ma et al., 1993; Paysan and Fritschy, 1998). Some subunits display profound regulation during development. For instance γ_1 and γ_3 subunit mRNAs drop in expression after early development, whereas γ_2 transcripts are equally abundant in prenatal and postnatal brain (Laurie et al., 1992b; Ma et al., 1993). Probably the most striking change in expression is a developmental switch in expression of the α_1 and α_2 subunits. At birth, the α_2 subunit is expressed throughout the brain but disappears later on in numerous areas, whereas there is complementary upregulation of the α_1 subunit expression (Fritschy et al., 1994; Hornung and Fritschy, 1996). With respect to $GABA_AR$ subunit expression during development and adulthood, the cerebellum is probably the most extensively studied brain area, due to its relatively simple architecture, and clearly defined and limited number of cell types. For example, cerebellar granule cells express six $GABA_AR$ subunits abundantly (α_1, α_6, β_2, β_3, γ_2, and δ), which are coassembled into at least four to six distinct subtypes, whereas Purkinje cells contain $\alpha_1\beta_{2/3}\gamma_2$ receptors exclusively. A developmental switch from α_2 and α_3 subunits to the α_1 subunit is found in granule cells of the cerebellum (Gao and Fritschy, 1995; Nusser et al., 1998b; reviewed in Wisden et al., 1996; Siegel, 1998; Carlson et al., 1998).

In summary, present evidence suggests that $GABA_AR$s are pentameric channels that mostly consist of α and β subunits together with the γ_2 subunit, and that the major $GABA_AR$ subtype in the mammalian brain is $\alpha_1\beta_2\gamma_2$. The γ_2 subunit is the most abundant $GABA_AR$ subunit in the CNS, and contributes to the majority of BZ-sensitive

$GABA_AR$ subtypes. The stoichiometry of the major subtype is yet unclear and corresponds to either $2\alpha2\beta1\gamma$ or $2\alpha1\beta2\gamma$. Further subtypes with the δ subunit instead of - or together with - the γ_2 subunit may play an important role mainly in the cerebellum. Subtypes containing the ρ subunits are largely restricted to the retina and have so far not been demonstrated together with other $GABA_AR$ subunits. The role of the ε, π and θ subunits remains to be determined in more detail.

1.4 Assembly of $GABA_A$ receptors

The synthesis of ionotropic receptors from multiple subunit genes has to be regulated in a temporally and spatially controled manner. The expression of receptor subunit genes is mainly determined by cell-type specific gene transcription. In addition, there is accumulating evidence for segregation of different receptor subtypes to distinct neuronal surfaces. This might involve differential assembly from subunits, and targeting and anchoring mechanisms that are receptor subtype-specific, as well as mRNA transport to specific cellular compartments and translation *in situ* (see next chapter Barnard et al., 1984).

The well characterized assembly of the nAChR of the neuromuscular junction (NMJ) serves as a model for other ionotropic receptors. Like other integral membrane proteins, cell-surface receptor subunits are processed in the secretory pathway of cellular protein synthesis. They are translated at ribosomes of the rough endoplasmic reticulum (rER). N-terminal membrane translocation signal sequences direct the nascent polypetides to the rER by help of a signal recognition particle (SRP) and other proteins. After termination of translation, the signal sequence is cleaved off and the mature receptor subunit resides in the membrane in its final orientation with extracellular domains in the ER lumen and cytoplasmic domains directed towards the cytosol. For the muscle nAChR, five homologous subunits are known (α, β, γ, δ, and ε, whereby ε replaces the γ subunit in adult tissue), but only one configuration is typically found ($\alpha\beta\alpha\gamma/\varepsilon\delta$). Prior to assembly, posttranslational modifications like the formation of disulfide bonds and part of the glycosylation take place in the ER, and single subunits are stabilized and properly folded by interaction with chaperones like BiP (binding protein, also immunoglobulin heavy chain binding protein) and 'calnexin' in case of the nAChR (reviewed in Keller and Taylor, 1999). Unassembled α subunits of the nAChR are rapidly degraded (Blount and Merlie, 1990; Claudio et al., 1989), and calnexin appears to reduce this degradation during the initial oligomerization process. Folding

before assembly may be required to expose the appropriate aa for subunit contact (Keller and Taylor, 1999). Assembly of nAChRs in the ER is a sequential process with the initial formation of intermediate subunit-oligomers [$\alpha\varepsilon$, $\alpha\delta$ dimers (Gu et al., 1991), or $\alpha\beta\gamma$ trimers (Green and Claudio, 1993)]. The intermediates then bind additional subunits and to each other, to form the mature receptor. It has been shown that the N-terminal extracellular domain, especially the glycosylation sites, and the cystein disulfide-loop contribute to the specificity of subunit oligomerization of the nAChR (Yu and Hall, 1991; Verrall and Hall, 1992; Sumikawa, 1992). Beyond this, TM1 was also found to be involved in the assembly process (Wang et al., 1996). Properly assembled pentameric receptors are subsequently transported to the cell surface and inserted into the plasma-membrane via the Golgi apparatus and Golgi vesicles, whereas residual single subunits or intermediates are rapidly degraded in the ubiquitin-proteaosome pathway (Keller et al., 1998).

1.4.1 Assembly of $GABA_A$ receptors *in vitro*

The assembly of $GABA_A$Rs is less well characterized than for the nAChR. The capability of single $GABA_A$R subunits to form homomeric functional receptors appears controversial (reviewed in Hevers and Luddens, 1998). Whereas some groups described functional channels from single α subunits, or their appearance at the surface (e.g. Pritchett et al., 1988; Perez-Velazquez and Angelides, 1993; Bueno et al., 1998), others reported weak or no GABA-evoked currents (e.g. Sigel et al., 1990; Connolly et al., 1996a; Connor et al., 1998). In contrast, the formation of functional homomeric receptors of β_1 or β_3 subunits in heterologous expression systems is well established. These channels open spontaneously in the absence of ligand and are insensitive to GABA, but channel activity can be allosterically modulated e.g. by pentobarbitone and propofol (Pritchett et al., 1988; Sigel et al., 1990; Perez-Velazquez and Angelides, 1993; Sanna et al., 1995; Wooltorton et al., 1997). The same properties are not consistently seen with the β_2 subunit (Connolly et al., 1996b; Gorrie et al., 1997; but Verdoorn et al., 1990; Sigel et al., 1990; Taylor et al., 1999). $GABA_A$R α_1 and β_2 subunits that fail to assemble might be degraded rapidly similarly to nAChR subunits (Gorrie et al., 1997). Controversial findings also exist for the γ_2 and δ subunits (e.g. Shivers et al., 1989; but Sigel et al., 1990; Connolly et al., 1999), and the possible formation of homomeric receptors from γ_1 and γ_3 subunits was not investigated. The ρ_1 and ρ_2 subunits are able to form homomeric ('$GABA_C$') receptors, which in this case might well be indicative of native homomeric ρ subunit receptors (Hackam et al., 1997; Hackam et al., 1998; Enz

and Cutting, 1999). Finally, the ε, π and θ subunit seem not to form homomers *in vitro* (Davies et al., 1997; Hedblom and Kirkness, 1997; Bonnert et al., 1999).

Most binary combinations of recombinant α and β subunits form receptors with high affinity for GABA and robust picrotoxin- and bicuculline-sensitive currents. However, they are uniformly insensitive to BZs (for review see Hevers and Luddens, 1998). This has been demonstrated for $\alpha_1\beta_{(1\text{-}3)}$ combinations (e.g. Levitan et al., 1988; Ymer et al., 1989; Malherbe et al., 1990; Sigel et al., 1990; Angelotti et al., 1993), and other αβ combinations [$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_3\beta_2$ (Levitan et al., 1988; Sigel et al., 1990; Draguhn et al., 1990); $\alpha_4\beta_{1\text{-}2}$ (Khrestchatisky et al., 1989; Knoflach et al., 1996); $\alpha_5\beta_1$ (Sigel et al., 1990)]. Only small currents were detected with the $\alpha_5\beta_3$ (Burgard et al., 1996) and $\alpha_6\beta_3$ combinations (Saxena and Macdonald, 1996).

The situation is less clear for αγ and βγ combinations. Some reports describe significant GABA induced currents [$\alpha_1\gamma_1$, $\alpha_1\gamma_2$, $\alpha_3\gamma_2$ (Draguhn et al., 1990; Verdoorn et al., 1990), $\alpha_4\gamma_2$ (Knoflach et al., 1996)]; others found no or only weak currents [$\alpha_1\gamma_2$, $\beta_1\gamma_2$ (Angelotti et al., 1993; Connor et al., 1998); $\alpha_i\gamma_2$, $\beta_1\gamma_2$, $\beta_2\gamma_2$ (Sigel et al., 1990); $\alpha_1\gamma_2$, $\beta_3\gamma_2$ (Tretter et al., 1997); $\beta_2\gamma_2$ (Draguhn et al., 1990; Verdoorn et al., 1990)]. The known variability of the analytic systems might contribute to the discrepancy of the studies.

If α, β, and γ subunits are coexpressed, the formation of functional channels is consistently described [e.g. $\alpha_i\beta_j\gamma_2$ (Sigel et al., 1990); $\alpha_1\beta_1\gamma_2$ (Hadingham et al., 1992); $\alpha_1\beta_2\gamma_2$ (Connor et al., 1998); $\alpha_5\beta_i\gamma_2$ (Burgard et al., 1996); $\alpha_4\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ (Knoflach et al., 1996); $\alpha_1\beta_2\gamma_3$, $\alpha_5\beta_2\gamma_3$, $\alpha_3\beta_2\gamma_3$ (Knoflach et al., 1991)]. Receptor formation appears largely independent of the subunit ratio *in vitro*, indicating that the assembly of channels containing all three types of subunits is strongly prefered over formation of homomeric and binary receptors (Angelotti et al., 1993; Puia et al., 1990; Hartnett et al., 1996). This seems to be the case also for quadruple subunit combinations like $\alpha_i\alpha_j\beta_k\gamma_2$ (Sigel et al., 1990) which might also exist *in vivo* (Fritschy and Mohler, 1995). Functional $GABA_A$Rs containing the δ subunit are likewise predominantly formed in combination with αβ or αβγ subunits *in vitro* (Saxena and Macdonald, 1994; Saxena and Macdonald, 1996; but Shivers et al., 1989). The same minimal requirement of coexpression with α and β subunits for proper channel assembly appears to apply for ε and π subunits (Davies et al., 1997; Neelands et al., 1999; Hedblom and Kirkness, 1997), and the θ subunit seems to rely on coexpression with α, β and γ subunits to form functional receptors (Bonnert et al., 1999).

Connolly et al. showed evidence that the assembly of $GABA_A$R subunits (α_1, β_2, γ_2) occurs in the ER and that $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_2$, but not $\alpha_1\gamma_2$ and $\beta_2\gamma_2$ combinations are

transported to the cell-surface (Connolly et al., 1996a). Oligomers of all subunit combinations could be immunoprecipitated, even if retained in the ER (Connolly et al., 1996a; Ebert et al., 1996). In addition, assembly and membrane transport were independent from glycosylation (in contrast to the nAChR, see above), as suggested by the lack of an effect of tunicamycin, which inhibits glycosylation. Binding of single subunits to the ER chaperones BiP and calnexin was shown to occur like in the case of the nAChR (Connolly et al., 1996a). A possible role of the N-terminal extracellular domain of the $GABA_AR$ subunits for assembly is suggested by studies with ρ subunits (Hackam et al., 1997). By coexpression of the truncated N-terminal extracellular domain of ρ_1 ($N\rho_1$) with intact ρ_1 in Xenopus oocytes, GABA-mediated currents were abolished in a $N\rho_1$ dose-dependent manner, indicating that the extracellular domain contains a binding or assembly motif and interferes with the homomeric interaction of ρ_1 subunits. The other half of the subunit including all transmembrane domains did not show such an effect. However, $N\rho_1$ also interacted with the ρ_2 subunit. A chimeric protein consisting of the N-terminal extracellular β_1 subunit domain, fused to the C-terminal half of ρ_1 ($N\beta_1$-ρ_1) interacted with the α_1 subunit but not with ρ_2 or $N\rho_1$-β_1 (the inverse construct of $N\beta_1$-ρ_1). As expected, ρ_1, ρ_2, or $N\rho_1$ did not interact with α_1, α_5, or β_1 subunits (Hackam et al., 1998). Furthermore, the splice variant of the rat α_6 subunit (α_{6S}), which differs from the major α_6 subunit (α_{6L}) by a deletion of 10 aa in the extracellular loop, appears unable to assemble with β_1 and γ_2 subunits in Xenopus oocytes, in contrast to the α_{6L} isoform (Korpi et al., 1994). Mutating a cysteine to serine at positions presumably involved in the formation of the N-terminal disulfide-loop in α_1, β_2, or γ_2 subunits, resulted in receptors that lacked the mutated subunit in coexpression studies (Amin et al., 1994). All these findings support the notion that the N-terminus of most $GABA_AR$ subunits is involved in the assembly process.

1.4.2 Assembly of $GABA_A$ receptors *in vivo*

Compared to recombinant systems the assembly of $GABA_AR$ subunits appears further restricted *in vivo*. The most valuable information with respect to the rules that govern assembly of native receptors can therefore be expected from analyses of mutant mice that lack specific subunits (reviewed in Wisden and Moss, 1997; Mohler et al., 1998). Mice homozygous for a deletion of the α_5 subunit gene display reduced immunoreactivity (IR) for the $\beta_{2/3}$ and γ_2 subunits in neurons that normally express the α_5 subunit, whereas the distribution of the α_2 subunit was unaltered (Fritschy et al., 1997; Fritschy et al., 1998a). Similarly, mice devoid of the α_6 subunit of the $GABA_AR$

show a marked decrease in protein amount and membrane IR, but not mRNA levels, for the δ, β_2, β_3, and γ_2 subunits, and this phenomenon was again restricted to the neurons that normally express the α_6 subunit (Jones et al., 1997; Homanics et al., 1997b; Nusser et al., 1999). Both findings indicate a role for α subunits in the formation of $GABA_A$Rs. Mice lacking the β_3 subunit gene also display reduced numbers of $GABA_A$Rs, indicating that this subunit is likewise essential for receptor formation (Homanics et al., 1997a; Krasowski et al., 1998; DeLorey et al., 1998; Huntsman et al., 1999). As far as analyzed, inactivation of single subunit genes did not affect transcription of other subunit genes and loss of receptors appeared always due to posttranslational mechanisms (Mohler et al., 1998). Evidence for the existence of native αβ receptors is derived from mice that lack the γ_2 subunit of the $GABA_A$R ($\gamma_2^{0/0}$). They express almost unaltered numbers of $GABA_A$Rs on the neuronal surface that exhibit electrophysiological and pharmacological properties typical of αβ receptors (Gunther et al., 1995). Thus, the γ_2 subunit is not required for $GABA_A$R assembly *in vivo*. However, most $\gamma_2^{0/0}$ mice die shortly after birth with a few surviving to the second or third postnatal week, suggesting an essential role for γ_2 in postnatal development. The reduced channel conductance as well as the absence of BZ modulation in $GABA_A$Rs lacking the γ_2 subunit might contribute to this phenotype, but the role of the γ_2 subunit as well as the minor subunits γ_1 and γ_3 *in vivo* is not known. Interestingly, the death of $\gamma_2^{0/0}$ mice occurs during the period of synaptogenesis, which suggests a role for the γ_2 subunit in this process.

$GABA_A$R assembly can thus be summarized as follows. Transcription of $GABA_A$R subunit genes is followed by protein synthesis at the rER. Nascent subunit polypetides are inserted in the ER membrane and folded by interaction with BiP and calnexin. Subsequent assembly of subunits involves N-terminal recognition domains facing the ER lumen, whereas glycosylation does not seem essential. Homooligomeric intermediates might also form, but in most cases heterooligomers of α and β subunits are more stable and required for efficient transport to the neuronal membrane[2]. Other subunits such as γ_{1-3}, and probably δ, ε and θ are preferentially added to αβ oligomers. Properly assembled pentameric receptors are then transported to the surface membrane via the Golgi apparatus and vesicles.

[2] A clear exception for this 'rule' would be the ρ subunits, and probably to a limited extent the formation of homomeric β_1 and β_3 subunit receptors (Krishek et al., 1996b; Wooltorton et al., 1997).

1.5 Targeting and synaptic clustering of $GABA_A$ receptors

The distribution of neurotransmitter receptors in the plasma-membrane is not uniform. Firstly, neurons are polarized cells with functionally specialized domains like axons and dendrites, and different receptors and receptor subtypes are differentially distributed to these domains. For instance in hippocampal pyramidal cells, α_1 subunit-containing $GABA_AR$s are found in membranes of both, somata and dendrites, whereas the α_2 subunit is present preferentially in the axon initial segments (AIS, Nusser et al., 1996a; Fritschy et al., 1998b; Loup et al., 1998). Thus, the subcellular sorting of $GABA_AR$s is required for the precise localization of the receptors, a process that will be refered to as 'targeting' in the following. Secondly, within a given subcellular domain, the majority of neurotransmitter receptors are 'clustered' (i.e. concentrated, anchored and stabilized) at postsynaptic sites by interaction with the cytoskeleton. This uneven distribution is best documented for the nAChR. In mature tissue, the number of nAChRs at the postsynaptic membrane of the NMJ comprises ~ 10000/μm^2 compared to ~ 10/μm^2 at adjacent extrasynaptic sites (Fertuck and Salpeter, 1974; reviewed in Sanes and Lichtman, 1999). In addition, different types of receptors coexist in the same neuron and are segregated to different synapses. For example, postsynaptic glutamate receptors are located at dendritic spines of hippocampal neurons, whereas $GABA_AR$s are typically found at shaft-synapses (Craig et al., 1994). Thus, the subcellular targeting and synaptic clustering of neurotransmitter receptors are crucial for proper neuronal function.

1.5.1 Targeting of $GABA_A$ receptors

Two principal mechanisms are known to account for the targeting of proteins within neurons. Similar to non-neural cells, most neuronal proteins are translated at the rER of the perikaryon, and only later translocated to their final destination. However, for some proteins the mRNA is transported to dendrites and appears to be translated *in situ* by subsynaptic polyribosomes. For example, for several glutamate receptor subunits the presence of mRNA at dendritic subsynaptic domains has been demonstrated (Miyashiro et al., 1994; reviewed in Steward, 1995; Kirsch et al., 1996).

Connolly et al. (1996b) investigated the subcellular sorting of $GABA_AR$ subunits, expressed in Madin-Darby canine kidney (MDCK) cells. These polarized cells exhibit basolateral and apical surfaces, which are considered analogous to somatodendritic and axonal membranes of neurons (Dotti and Simons, 1990; Dotti et al., 1991; de Hoop et al., 1995). Expression of the β_3 subunit, but not of α_1, β_1, β_2, or γ_2 subunits, alone in

MDCK cells resulted in appearance at the apical ('axonal') surface and so does the $\alpha_1\beta_3$ combination. The $\alpha_1\beta_1$ combination showed a nonpolarized surface distribution and $\alpha_1\beta_2$ was targeted to the basolateral ('dendritic') membrane. Coexpression of the γ_2 subunit did not influence the respective distribution. A similar study by another group supported this finding, although in part contradictory (Perez-Velazquez and Angelides, 1993). The authors detected α_1 solely at the basolateral MDCK cell surface, and β_1 alone at the apical membrane. Coexpression, however, rerouted the α_1 subunit to the apical membrane. These data suggest a role for the β subunits in subcellular targeting of $GABA_A$Rs in neurons. Such a role for a distinct $GABA_A$R subunit in subcellular sorting of receptors might point to a vesicular transport rather than mRNA transport mechanisms, as it is unlikely that a certain subunit mRNA could control the subcellular targeting of other subunit mRNAs.

However, mRNA transport has been demonstrated to be involved in the site-specific expression of $GABA_A$Rs, at least during neuronal maturation in culture. Messenger RNAs and proteins of α_2, α_5, β_2, β_3, and γ_2 subunits were detected in the cellular processes and growth cones of neurons in culture after 1-3 days *in vitro* (DIV). After the onset of synaptogenesis, however, the subunit mRNA signal in the neuritic processes disappeared (Poulter and Brown, 1999). Similarly and more convincingly, the GlyR α_1 and α_2 subunit mRNAs were consistently found in dendritic and somatic locations in different CNS neurons, whereas GlyR β subunit and gephyrin (the GlyR anchoring protein, see below) mRNAs were predominantly found in the perikaryon (Racca et al., 1997; Racca et al., 1998). Interestingly, GlyR α subunit mRNAs form aggregates and appear associated with postsynaptic differentiatons in these neurons, suggesting processes of dendritic mRNA targeting and subsynaptic translation (Racca et al., 1997).

1.5.2 Synaptic clustering of neurotransmitter receptors

The concentration of ion channels and receptors at specialized membrane portions like synapses or nodes of Ranvier is crucial for neurons to spatially restrict and locally amplify their electrical and chemical signals (Sheng and Wyszynski, 1997). Proteins involved in the accumulation and anchoring of receptors at postsynaptic sites have received much attention in recent years due to their possible role in synaptic plasticity.

1.5.2.1 The nAChR

Mechanisms of nAChR clustering in the membrane of muscle fibers apposed to presynaptic motor nerve terminals are well examined (reviewed in Glass and Yancopoulos, 1997; Colledge and Froehner, 1998; Sanes and Lichtman, 1999). Rapsyn (receptor associated protein), a 43 kD protein, plays a crucial role in this process, and colocalizes precisely with nAChRs at the NMJ as soon as clusters form during synaptogenesis (Sobel et al., 1977; Sealock et al., 1984). nAChRs are diffusely distributed when expressed alone in heterologeous cells, but accumulate together with rapsyn upon coexpression (Froehner et al., 1990; Phillips et al., 1991). Furthermore, the absence of rapsyn in rapsyn$^{0/0}$ mice abolishes nAChR clustering (Gautam et al., 1995). Other proteins involved in nAChR clustering are 'agrin' and MuSK (muscle-specific receptor tyrosine kinase). Agrin is synthesized in motoneurons and transported to presynaptic terminals during neuronal maturation, where it is released and incorporated into the basal lamina of the synaptic cleft, a structure of the extracellular matrix. Nerve-derived agrin is essential for formation of the NMJ as demonstrated in agrin$^{0/0}$ mice, where the postsynaptic differentiation is significantly impaired, although levels of nAChR expression are not reduced (Gautam et al., 1996). Interestingly, addition of agrin to cultured muscle cells induces clustering of otherwise diffusely distributed nAChRs (Wallace, 1989). Among several myotube proteins that interact with agrin, MuSK appears to represent a receptor for agrin. In agreement with such a role, MuSK is selectively expressed by skeletal muscle and is colocalized with nAChR clusters at the postsynaptic differentiation (Valenzuela et al., 1995). Furthermore, MuSK$^{0/0}$ mice display neuromuscular deficits that are similar to those of agrin$^{0/0}$ mice (DeChiara et al., 1996). Despite this knowledge on components involved in clustering of the peripheral nAChR, mechanisms by which these proteins interact are poorly understood. In addition, it is questionable whether the NMJ provides a good model for central synapses, due to obvious morphological differences. For example the basal lamina is present in the NMJ only, where it interacts with the subsynaptic cytoskeleton (Sanes and Lichtman, 1999), but is absent at central synapses.

1.5.2.2 The NMDA and AMPA receptors

Much work with respect to synaptic clustering was also performed with the NMDA- and AMPA-type receptors (NMDA-R, AMPA-R) of glutamatergic synapses, where the pronounced 'postsynaptic density' (PSD, a morphologically distinct electron-

dense structure beneath the postsynaptic membrane) has turned out to be a complex network of cytoskeleton, anchoring, and regulatory proteins (Ehlers et al., 1996; Ziff, 1997). A new protein family was identified, termed PSD-95 proteins (or 'chapsyns', channel associated proteins from synapses, reviewed in Sheng and Kim, 1996; Colledge and Froehner, 1998; Kamboj and Huganir, 1998; Kirsch, 1999) that are involved in synaptic anchoring and stabilization functions for different neurotransmitter receptors. PSD-95 and other proteins of the family are predominantly, allthough not exclusively, found below the postsynaptic membrane and tightly associated with the cytoskeleton (Sheng and Wyszynski, 1997). They are characterized by three N-terminal PDZ domains (homology domains, first identified in PSD-95 or SAP90, Discs-large, ZO-1), C-terminal SH3 and guanylate kinase-like domains, and belong to the larger family of MAGUK proteins (membrane-associated guanylate kinases, Kennedy, 1997). PDZ domains are 90 aa sequences, which can engage in homomeric contacts or heteromeric protein-protein interactions, e.g. with the C-terminal tail of NMDA receptor NR2 subunits and K^+ channels. Other PDZ domain proteins are likewise involved in receptor clustering functions. For example GRIP (glutamate receptor interacting protein) comprises seven PDZ domains, of which the fourth and fifth mediate interaction with AMPA-R subunits (Dong et al., 1997; Dong et al., 1999) and 'Homer' (one PDZ domain) binds to a subset of metabotropic glutamate receptors (Brakeman et al., 1997). As expected, not only receptors, but also other proteins that accumulate at specialized surface portions exhibit typical anchoring proteins. For example, voltage-gated sodium channels that are clustered at nodes of Ranvier, bind to an axon specific isoform of 'ankyrin' (Sheng and Wyszynski, 1997). Recently, another protein was implicated in excitatory synaptogenesis. The immediate-early gene Narp (neuronal activity-regulated pentraxin) is enriched postsynaptically as well as presynaptically at excitatory synapses, and overexpression of recombinant Narp increases the number of excitatory but not inhibitory synapses in cultured spinal neurons. Moreover, expression of Narp in HEK 293 cells can induce the aggregation of neuronal AMPA-Rs in cocultures. Thus, Narp might function as an extracellular transsynaptic aggregating factor for AMPA-Rs similar to agrin for nAChRs at the NMJ (O'Brien et al., 1999; Fong and Craig, 1999).

In parallel to the nAChR, mechanisms of interaction are not clearly known for the glutamate receptor anchoring proteins. For example, PSD-95 is not required for synaptic localization of NMDA-Rs, as demonstrated in PSD-$95^{0/0}$ mice, although these mice show altered LTP and LDP, and impaired spatial learning (Migaud et al., 1998).

1.5.2.3 The glycine receptor and gephyrin

GlyRs represent presumably pentameric Cl^- channels that display much less heterogeneity than the $GABA_A$Rs (reviewed in Kuhse et al., 1995; Bechade and Triller, 1996b; Vannier and Triller, 1997). Four α subunits and one β subunit are known to contribute to the 250 kD GlyR channel with a stoichiometry of 3α:2β subunits (Schmitt et al., 1987; Langosch et al., 1988; Garcia-Calvo et al., 1989). In striking similarity to some of the $GABA_A$R subunits, alternative splicing occurs for the α_1 subunit (8 aa insert in the cytoplasmic loop, including a possible additional phosphorylation site, Malosio et al., 1991a), and for the α_2 subunit in the N-terminal extracellular domain (Kuhse et al., 1991). Pharmacologically, GlyRs are characterized by the agonistic action of the aa glycine, β-alanine, taurine, and L-alanine, and by the high affinity antagonist strychnine, a plant alkaloid that selectively blocks GlyRs at low concentrations (Vannier and Triller, 1997). The α subunits harbour the binding sites for glycine and strychnine (Graham et al., 1983). GlyRs in the CNS show a predominant incidence in the gray matter of the lower brain stem and spinal cord that decreases gradually from caudal to rostral regions of the neuraxis. *In situ* hybridization (Malosio et al., 1991b) and immunohistochemistry (Naas et al., 1991; Becker et al., 1993) revealed that the GlyR α_1 subunit is the predominant α subunit in the adult spinal cord and brainstem, whereas α_3 is sparsely expressed and α_2 is found mainly during early CNS development. The β subunit mRNA is prominent throughout brain and spinal cord even in areas where no α subunit is present (Grenningloh et al., 1990; Fujita et al., 1991; Malosio et al., 1991b). The physiological significance of this finding is not yet understood, as the β subunit (in contrast to the α subunits) fails to form homomeric receptors *in vitro* (Sontheimer et al., 1989; Grenningloh et al., 1990; Vannier and Triller, 1997). Low levels of functional GlyRs exist in higher brain regions as revealed by [^{3}H]strychnine-autoradiographies (Zarbin et al., 1981), and the detection of strychnine-sensitive and/or glycinergic currents in cerebellar Golgi cells (Dieudonne, 1995) and hippocampal cultures (Fatima-Shad and Barry, 1998). Beside spinal cord and brain stem, GlyRs are also prominent in the retina (Enz and Bormann, 1995; Koulen et al., 1996; Sassoe-Pognetto and Wassle, 1997; Wassle et al., 1998).

Strychnine affinity-purification of GlyRs identified a 93 kD protein, which was originally thought to be another receptor subunit (Pfeiffer et al., 1984). Subsequent EM analysis however, revealed that this protein is located in the cytoplasm beneath the glycinergic synapse, in contrast to the membrane-bound subunits (Triller et al., 1985; Triller et al., 1987). This non-glycosylated peripheral membrane protein binds tubulin with high affinity and is able to link GlyRs to brain microtubuli in a copolymerization

assay (Schmitt et al., 1987; Kirsch et al., 1991). It was named gephyrin (γεφνρα, greek: bridge) due to its putative role as a linker between GlyRs and the cytoskeleton (Prior et al., 1992). Gephyrin can be phosphorylated by a GlyR-associated kinase (Langosch et al., 1992) and alters the ligand-binding affinities of GlyRs when coexpressed in HEK 293 cells (Takagi et al., 1992). The protein is expressed in high amounts in the CNS, but its mRNA was also found in most other tissues like liver, kidney or lung. Cloning and expression studies revealed the existence of several gephyrin splice variants. In the adult rat CNS, one isoform is most abundant in brain and spinal cord, whereas two others are mainly restricted to cerebellar granule cells and the dentate gyrus (Prior et al., 1992; Kirsch et al., 1993b). The role of gephyrin for synaptic anchoring of GlyRs is well established. Coexpression with gephyrin in HEK 293 cells reroutes GlyR β subunits, but not α subunits, to intracellular gephyrin aggregates (Kirsch et al., 1995b), and a binding motif for gephyrin was identified in the large cytoplasmic loop of the β subunit (Meyer et al., 1995; Kneussel et al., 1999; Kins et al., 1999). Most important, gephyrin depletion by antisense oligonucleotide treatment *in vitro* (Kirsch et al., 1993c), or removal through gene targeting in mice ($gep^{0/0}$) (Feng et al., 1998), abolishes GlyR clustering at spinal cord synapses. In addition, gephyrin and GlyR clustering is disrupted by the treatment of spinal cord cultures with alkaloids that depolymerize microtubuli or microfilaments (Kirsch and Betz, 1995a). Immunohistochemical studies revealed the colocalization of punctate GlyR and gephyrin IR at postsynaptic sites in spinal cord (Kirsch and Betz, 1993a; Todd et al., 1995; Todd et al., 1996) and retina (Sassoe-Pognetto and Wassle, 1997; Zucker, 1998). During neuronal development, the accumulation of gephyrin at the surface membrane anticipates the clustering and colocalization of GlyRs in spinal cord cultures (Bechade et al., 1996a; Colin et al., 1996) and in the ventral horn of spinal cord tissue (Colin et al., 1998), suggesting a guiding role for gephyrin in the formation of glycinergic synapses.

Recent evidence indicates that gephyrin is a multifunctional protein that mediates several other actions that are not specific for the nervous system. For instance, gephyrin shares sequence homologies with proteins that are involved in the biosynthesis of molybdenum-cofactor (MoCo, Prior et al., 1992; Kamdar et al., 1994; Stallmeyer et al., 1995; Menendez et al., 1997). MoCo is required for the activity of molybdenum enzymes that mediate diverse metabolic processes like sulfur detoxification or purine catabolism (reviewed in Kisker et al., 1997; Moriwaki et al., 1997). Consistent with such a role, $gep^{0/0}$ mice show disturbed molybdoenzyme activities in non-neuronal tissues, a phenotype that resembles a hereditary MoCo deficiency in humans (Feng et al., 1998). Surprisingly, gephyrin expression can reconstitute MoCo biosynthesis in

MoCo-deficient bacteria, mouse cells, and plants. Conversely, inhibition of gephyrin expression in cultured murine cells reduces their MoCo content significantly (Stallmeyer et al., 1999). It remains to be determined, whether gephyrin's MoCo-biosynthetic activity could play a role at the neuronal inhibitory synapse as well.

Gephyrin also interacted with RAFT1 in a yeast two-hybrid assay and in copurification experiments. The immunophilin RAFT1 is an important regulator of mRNA translation and mediates the effects of the immunosuppressant rapamycin *in vivo*. Coexpression of both proteins in HeLa cells resulted in aggregation of otherwise evenly distributed RAFT1 in the cytoplasm, and RAFT1 mutants that could not associate with gephyrin failed to signal to downstream molecules that are involved in mRNA translation (Sabatini et al., 1999). Finally, several additional unrelated proteins were recently found to interact with gephyrin in a yeast two-hybrid screen, but are poorly characterized so far (Kins et al., 1998).

1.5.2.4 The $GABA_A$ receptor

Similar to the nAChR and the GlyR, most $GABA_AR$ subtypes are concentrated at GABAergic synapses (Craig et al., 1994; Somogyi et al., 1996; Fritschy et al., 1998b). However, the existence of extrasynaptic $GABA_AR$ subunits has been demonstrated by immunohistochemical analysis with light microscopy (Pinard et al., 1990; Fritschy et al., 1992) and EM (Baude et al., 1992; Nusser et al., 1995; Nusser et al., 1996b; Nusser et al., 1998b; Nusser et al., 1999). The δ subunit of the $GABA_AR$ has been found exclusively in extrasynaptic membranes of cerebellar granule cells where it may be associated with α_6, $\beta_{2/3}$, and γ_2 subunits (Nusser et al., 1999). Synaptic and extrasynaptic receptors in these cells were suggested to mediate different types of GABAergic inhibition (phasic and tonic, Brickley et al., 1996; Nusser et al., 1998b).

In contrast to the GlyR, synaptic clustering proteins of $GABA_ARs$ are not known. However, the principal candidate protein for $GABA_AR$ clustering is gephyrin. Several immunohistochemical studies investigating $GABA_AR$ and gephyrin expression in brain revealed that both proteins are frequently colocalized. Punctate gephyrin IR is colocalized with presynaptic GAD IR as well as GlyR and $GABA_AR$ IR in spinal cord, where a large portion of inhibitory synapses contains both neurotransmitters (Triller et al., 1987; Bohlhalter et al., 1994; Todd et al., 1995; Cabot et al., 1995; Maxwell et al., 1995; Bohlhalter et al., 1996; Todd et al., 1996; Jonas et al., 1998). In the brain, even in areas where GlyRs are absent or scarce, colocalization of GAD IR (Triller et al., 1987) and $GABA_AR$ subunit IR with gephyrin was found in the olfactory bulb (Giustetto et al.,

1998). Similarly in the retina, where GlyRs and $GABA_A$Rs are not colocalized, both codistribute with gephyrin IR (Sassoe-Pognetto et al., 1995; Sassoe-Pognetto and Wassle, 1997; Koulen et al., 1996; Zucker, 1998). Furthermore, in hippocampal cultures, gephyrin colocalizes with $GABA_A$R subunit IR at GABAergic, but not glutamatergic synapses (Craig et al., 1996). In addition, $GABA_A$R function (muscimol-stimulated Cl^- uptake, and GABA currents) can be inhibited by microtubule-depolymerizing agents like colchicine, possibly by disrupting the interaction of the receptor with the cytoskeleton, in parallel to GlyRs (Whatley et al., 1994). These data clearly suggest a role for gephyrin in $GABA_A$R clustering.

However, the notion that gephyrin would be involved in $GABA_A$R clustering was challenged by the absence of biochemical interactions of both proteins *in vitro*. In contrast to GlyRs, gephyrin was never copurified with $GABA_A$Rs, and coaggregation of gephyrin and $GABA_A$R subunits in heterologeous expression systems was not found, despite a weak interaction with the β_3 subunit (Kirsch et al., 1995b; Meyer et al., 1995).

Beside gephyrin, rapsyn has recently been implicated in synaptic clustering of $GABA_A$Rs by two studies. Heterologeous coexpression of rapsyn with different single and combined $GABA_A$R subunits resulted in colocalized accumulation (Yang et al., 1997; Ebert et al., 1999). It is interesting to note that agrin, the signaling molecule involved in synaptic clustering of the peripheral nAChR together with rapsyn, has been detected in close contact with gephyrin. Agrin mRNA is found broadly expressed in the CNS, including non-cholinergic regions, and agrin and gephyrin IR were found colocalized in primary cultures of rat embryonic dorsal horn neurons (Escher et al., 1996), and in chicken retina cells in culture and tissue (Mann and Kroger, 1996). Evidence for an agrin receptor is also found in cortical cultures (Hilgenberg et al., 1999). However, $agrin^{0/0}$ mice that lack ~ 90% of all agrin isoforms showed unaltered $GABA_A$R and gephyrin clustering in hippocampal neurons (Serpinskaya et al., 1999), and only low levels of rapsyn mRNA are detected in the brain (Yang et al., 1997). These findings make a significant role of these nAChR clustering molecules for synaptic clustering of $GABA_A$Rs unlikely.

1.5.2.5 Mechanisms of receptor clustering

Mechanisms by which synapses are established and neurotransmitter receptors accumulate at postsynaptic sites during neuronal development are poorly understood. Transsynaptic factors that are released from the presynaptic terminal are known to be involved in the formation of synaptic nAChR (agrin) and AMPA-R (Narp) clusters (see above). In addition, activity-dependent receptor signaling after contact of presynaptic axon-terminals at future synaptic sites appears to be be required for the formation and maintenance of receptor clusters (reviewed in Craig, 1998; Kamboj and Huganir, 1998; Kirsch, 1999).

Changes in receptor distribution that depend on activity were demonstrated for AMPA-Rs and NMDA-Rs. Blockage of AMPA-R activity in neuronal cultures increased glutamatergic currents, whereas enhanced avtivity reduced AMPA-R currents (Turrigiano et al., 1998; O'Brien et al., 1998), or induced the redistribution of AMPA-Rs away from synapses (Lissin et al., 1998; Lissin et al., 1999). Similar results are described for the NMDA-R (Rao and Craig, 1997). Whereas such changes may be important for regulatory feed-back mechanisms in mature synapses, similar effects were described in developing neurons in culture. AMPA-R or NMDA-R blockage increased the number, size and density of the respective receptor clusters, and NMDA-R blockage also decreased the number of AMPA receptors (Liao and Huganir, 1999).

GlyR clustering has been shown to rely on receptor activation (Kirsch and Betz, 1998; Levi et al., 1998). Blockage of GlyRs by strychnine-application during neuronal differentiation abolishes GlyR clustering. Interestingly, this effect can be mimicked by blocking voltage-dependent L-type calcium channels (Kirsch and Betz, 1998). Similar to $GABA_A$Rs, GlyRs are depolarizing during early neuronal development (Wang et al., 1994). Thus, activation of GlyRs could trigger the opening of voltage-dependent Ca^{2+} channels. Subsequent Ca^{2+} influx might then direct the aggregation of GlyRs into clusters (see Fig. 4). Whereas strychnine and tetrodotoxin (TTX) treatment affected gephyrin clustering similarly to the GlyR in one report (Kirsch and Betz, 1998), no such effect on gephyrin was found by others (Levi et al., 1998). TTX also failed to interfere with clustering of the receptors in the latter report. Whether similar mechanisms could account for $GABA_A$R clustering is not known. Available data however, favor no activation-triggered accumulation. TTX treatment of hippocampal neurons in culture revealed no changes in $GABA_A$R clustering (Craig et al., 1994), and chronic blockage of $GABA_A$Rs with gabazine in spinal cord cultures (Hussy et al., 1997; Levi et al., 1998; Levi, 1999a) did not significantly alter the distribution of gephyrin or $GABA_A$R clusters.

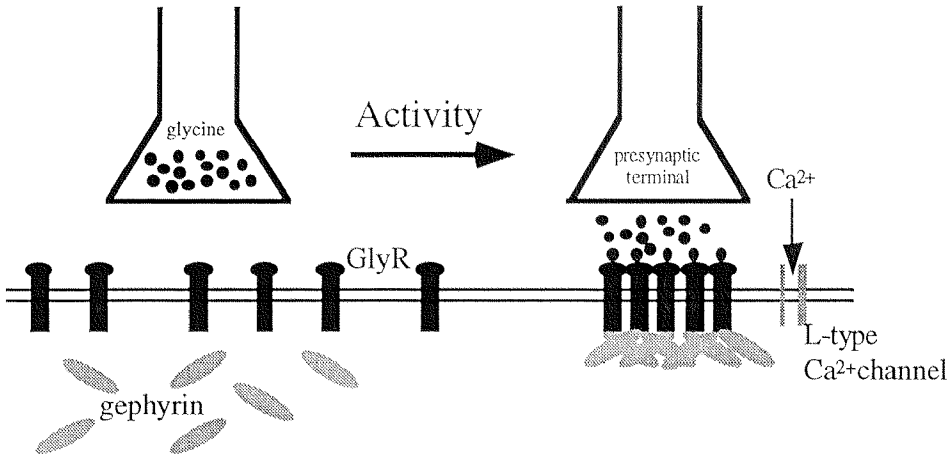

Fig. 4 Model of activity-dependent synaptic clustering of GlyRs (modified from Kirsch, 1999).

In summary, a comprehensive picture has emerged for synaptic clustering of GlyRs. It is clear that GlyRs are anchored at postsynaptic microtubules (and probably microfilaments) by interaction of their β subunit with gephyrin. During development, gephyrin accumulates prior to GlyRs which could be indicative of a guiding role for gephyrin, but GlyR activation and Ca^{2+} influx is also required for synaptic clustering.

A similar role for gephyrin in $GABA_AR$ clustering is possible but not known, and is a main topic of the work presented here.

2. Aim of the study

Postsynaptic clustering of $GABA_A$ receptors ($GABA_ARs$) is an important prerequisite for the functionality of GABAergic synaptic inhibition in the brain. It is assumed that the synaptic localization of $GABA_ARs$ depends at least in part on their specific contacts with cytoplasmic proteins which mediate their interaction with the cytoskeleton. Beside the anchoring and stabilization of receptors, such proteins might play a role in targeting of receptor subtypes to different postsynaptic sites and could be involved in the modulation of receptor density and synaptic plasticity. However, the receptor subunits and the anchoring proteins involved in the synaptic clustering of $GABA_ARs$ are unknown.

It was the aim of this study to address the role of the γ_2 subunit for synaptic clustering of $GABA_ARs$ *in vivo*. The γ_2 subunit is present in most $GABA_AR$ subtypes and may therefore provide a common motif required for synaptic clustering of $GABA_ARs$. To investigate this issue, a γ_2 subunit-deficient mutant mouse line was analyzed. In this mutant, the extent of $GABA_AR$ clustering was expected to be an indication for the relevance of the γ_2 subunit for this process. Moreover, it was investigated whether the γ_3 subunit, which is homologous to the γ_2 subunit but far less abundant, could play a similar role *in vivo*. To this end, the γ_3 subunit was overexpressed in γ_2 subunit-deficient mice by means of a transgene. Finally, the role of gephyrin, which was first identified as a synaptic clustering protein of the glycine receptor, was investigated as a putative clustering protein for $GABA_ARs$. It was found that the γ_2 subunit is required for postsynaptic clustering of $GABA_ARs$ and gephyrin. Furthermore, the γ_3 subunit could functionally replace the γ_2 subunit with regard to the clustering of $GABA_ARs$ and gephyrin. Finally, gephyrin was found to be a necessary requirement for postsynaptic clustering of $GABA_ARs$ in the brain.

3. Results

3.1 Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ_2 subunit and gephyrin

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Summary

Most fast inhibitory neurotransmission in the brain is mediated by $GABA_A$ receptors, which are mainly postsynaptic and consist of diverse α and β subunits together with the γ_2 subunit. Although the γ_2 subunit is not necessary for receptor assembly and translocation to the cell surface, we show here that it is required for clustering of major postsynaptic $GABA_A$ receptor subtypes. Loss of $GABA_A$ receptor clusters in mice deficient in the γ_2 subunit, and in cultured cortical neurons from these mice, is paralleled by loss of the synaptic clustering molecule gephyrin and synaptic GABAergic function. Conversely, inhibiting gephyrin expression causes loss of $GABA_A$ receptor clusters. The γ_2 subunit and gephyrin are thus interdependent components of the same synaptic complex that is critical for postsynaptic clustering of abundant subtypes of $GABA_A$ receptors *in vivo*.

Introduction

Nervous system signaling depends on the spatially and temporally coordinated function of a variety of voltage- and ligand-gated ion channels. Typically, these channels do not diffuse freely in the membrane but are concentrated at specific cellular membrane surfaces such as axon terminals, postsynaptic sites and nodes of Ranvier. This differential and regulated distribution of ion channels and neurotransmitter receptors on the neuronal surface seems to be critical for signaling within and between neurons (Kirsch et al., 1996; Colledge and Froehner, 1998; Craven and Bredt, 1998). Clustering and postsynaptic localization of voltage- and ligand-gated ion channels depends on interactions with intracellular proteins, which are enriched in the postsynaptic density and provide a link to the cytoskeleton. For example, at glycinergic inhibitory synapses of the spinal cord, clustering and postsynaptic localization of glycine receptors and their interaction with microtubules depend on direct protein-protein interaction of the glycine receptor β subunit with the 93-kD tubulin-binding protein gephyrin (Kirsch et al., 1993c; Kirsch et al., 1995b; Meyer et al., 1995). Gephyrin is also abundant in the brain, where inhibitory neurotransmission is mainly mediated by $GABA_A$ receptors. Postsynaptic gephyrin clusters are present at GABAergic synapses in the retina (Sassoe-Pognetto et al., 1995; Sassoe-Pognetto and Wassle, 1997), olfactory bulb (Giustetto et al., 1998), spinal cord (Bohlhalter et al., 1994; Cabot et al., 1995; Todd et al., 1995; Todd et al., 1996) and in cultured hippocampal neurons (Craig et al., 1996), in close association with various $GABA_A$ receptor subunits. Similarly to glycine receptors, $GABA_A$ receptors are associated with microtubules and microtubule-associated proteins (Whatley et al., 1996; Kannenberg et al., 1997) and are preferentially clustered in the postsynaptic membrane of symmetric (Type II) synapses, which lack a prominent postsynaptic density (Kirsch et al., 1996). However, direct interaction between gephyrin and $GABA_A$ receptor subunits has not been shown (Meyer et al., 1995), and the function of gephyrin at GABAergic synapses has remained elusive.

Structurally, $GABA_A$ receptors are closely related to glycine receptors. They are heteropentameric chloride channels assembled from a multitude of subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, ρ_{1-3}) (Macdonald and Olsen, 1994; Sieghart, 1995; Mohler et al., 1996b; Davies et al., 1997; Hedblom and Kirkness, 1997; Whiting et al., 1997) that are characterized by different spatiotemporal expression patterns. In principle, coexpression of varied subsets of these subunits in different neuronal cell types would allow the formation of a very large number of receptor subtypes. Receptor diversity is, however, significantly restricted by the rules that govern subunit assembly (Wisden and Moss, 1997; Mohler et al., 1998). The γ_2 subunit is by far the most abundant subunit in brain

and is incorporated in most $GABA_A$ receptor subtypes. Neonatal mice with a targeted disruption of the γ_2 subunit gene ($\gamma_2^{0/0}$) (Gunther et al., 1995) display almost normal numbers of $GABA_A$ receptors, but these receptors are functionally impaired, as indicated by a reduced channel conductance and a perinatally lethal phenotype in the majority of mutant mice (Gunther et al., 1995). Although the γ_2 subunit is completely absent, the expression levels, regional distribution and membrane localization of α and β subunits are essentially normal in newborn pups, and no compensatory upregulation of γ_1 or γ_3 subunits is observed (Gunther et al., 1995). These data, together with the observation that coexpression of recombinant α and β subunits in non-neural cells allows assembly and expression of functional $GABA_A$ receptors on the cell surface (Connolly et al., 1996a; Gorrie et al., 1997), indicate that the γ_2 subunit is not required for translocation of functional $GABA_A$ receptors to the cell membrane.

By analyzing cultured cortical neurons and brain from $\gamma_2^{0/0}$ mice, we now show that the γ_2 subunit of $GABA_A$ receptors is essential for clustering and postsynaptic localization of major subtypes of $GABA_A$ receptors and of gephyrin. Conversely, gephyrin is required for clustering and postsynaptic localization of $GABA_A$ receptors.

Results

Impaired $GABA_A$ receptor clustering in $\gamma_2^{0/0}$ neurons

Although most $\gamma_2^{0/0}$ mice do not survive beyond a few hours after birth, approximately 10% survive to the third postnatal week. This suggested that, in addition to the reduced channel conductance observed in dorsal root ganglion neurons (Gunther et al., 1995), there might be a molecularly distinct deficit in $\gamma_2^{0/0}$ mice that is specific for the mature central nervous system (CNS) or only manifested with advanced synaptogenesis in postnatal brain. To investigate the γ_2 subunit-deficient phenotype during and after synaptogenesis in CNS neurons, the cellular expression of $GABA_A$ receptors was analyzed in primary neurons from cerebral cortex of $\gamma_2^{+/+}$, $\gamma_2^{+/0}$ and $\gamma_2^{0/0}$ embryos cultured for 20 days in vitro (DIV, 18 independent sets of cultures). In wild-type neurons, immunocytochemical analyses of the γ_2, α_1 and α_2 subunits (three major $GABA_A$ receptor subunits expressed in neocortex) (Fritschy and Mohler, 1995) revealed strong staining of dendritic and somatic membranes. Approximately 70% of wild-type neurons stained positively for γ_2 and α_2 subunits (**Fig. 1a and g**), and the staining revealed intense immunofluorescent punctae on the dendritic and somatic membrane (γ_2, 16.0 ± 2.9; a2, 15.8 ± 1.0 punctae per 40 µm dendrite). Similarly, about 70% of neurons in these cultures stained for the α_1 subunit (**Fig. 2a**; 13.6 ± 0.4 punctae per 40 µm dendrite).

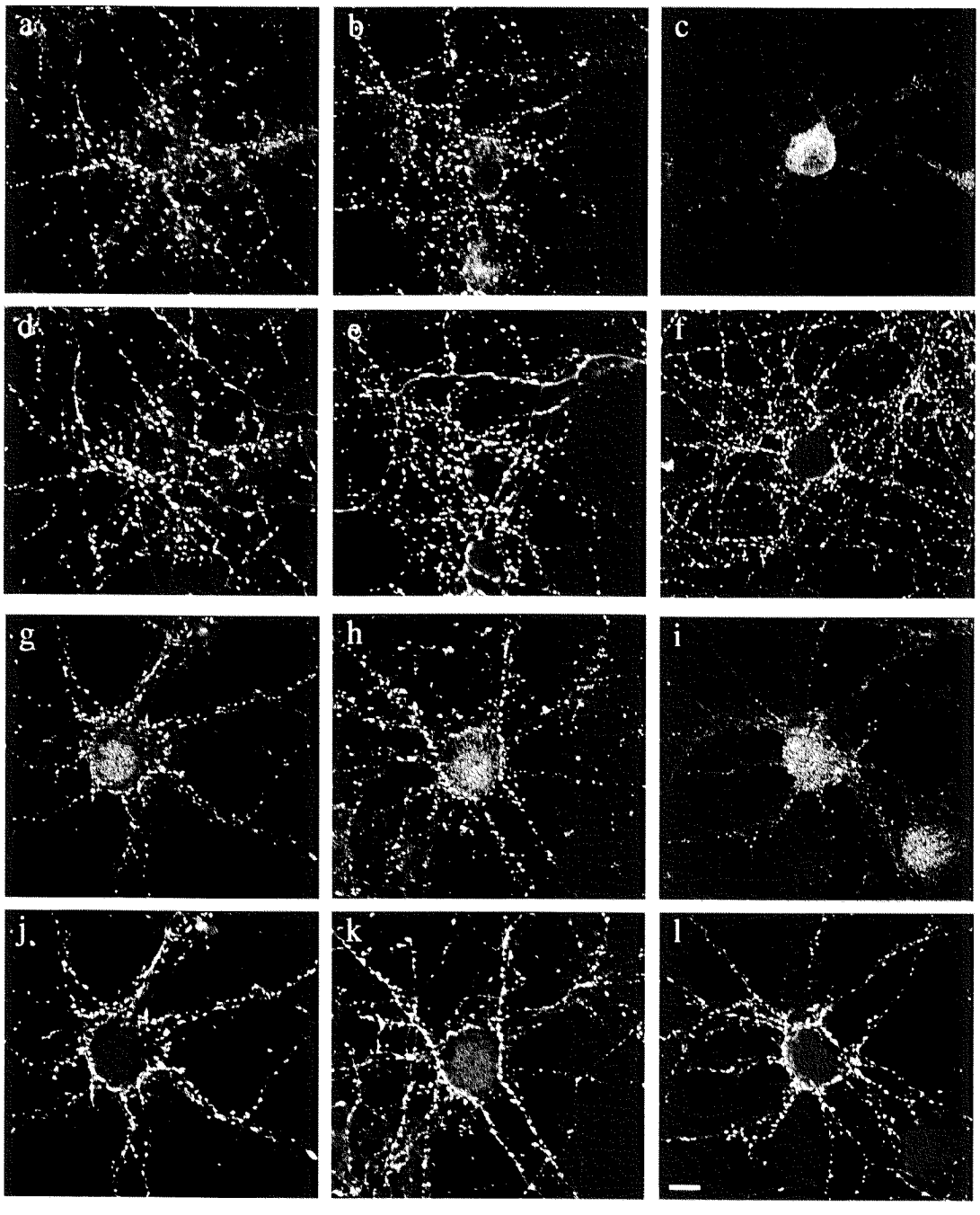

Fig. 1. Loss of clustered $GABA_A$ receptors containing the γ_2 or the α_2 subunit in $\gamma_2^{0/0}$ cortical neurons. At 20 DIV, cells cultured from single E14.5 mouse embryo cortices and representing different γ_2 subunit genotypes (+/+, a, d, g, j; 0/+, b, e, h, k; 0/0, c, f, i, l) were double labeled with antisera specific for the $GABA_A$ receptor γ_2 subunit (a-c) or the α_2 subunit (g-i) and for GAD (d-f, j-l, same cells as in a-c and g-i). Fluorescent images from double-labeled neurons were collected by confocal laser scanning microscopy. (Shown are stacks of three optical sections spaced by 800 nm; planar resolution 200 nm/pixel.) Note the selective loss of punctate γ_2 and α_2 subunit staining in neurons from $\gamma_2^{0/0}$ mice, whereas GAD staining was unaltered. Scale bar, 10 μm.

In most cells, the punctate staining on the membrane was more prominent for the α_2 than for the α_1 subunit, whereas diffuse membrane staining, probably representing extrasynaptic receptors, was more prominent for the α_1 subunit. Staining for $GABA_A$ receptor subunits is exactly juxtaposed to punctae immunoreactive for the presynaptic GABAergic marker glutamic acid decarboxylase (GAD, **Figs. 1a, d, g and j and 2a**) and to a subset of punctae immunoreactive for synaptophysin (**Fig. 6e**), suggesting that the large majority of the clustered receptors are localized in the postsynaptic membrane opposite GABAergic terminals. Immunohistochemical staining for intracellular antigens such as gephyrin and synaptophysin was significantly higher in permeabilized cells, whereas staining intensity for α_1, α_2 and γ_2 subunits (amino-terminal extracellular epitopes) was independent of prior permeabilization, thus confirming that the subunit immunoreactivity represents $GABA_A$ receptors localized in the membrane (not shown). Intracellular staining sometimes observed with the γ_2 subunit antiserum seems to be non-specific because it was largely unaffected by preadsorption of the antiserum with 10 µg/ml of the γ_2 peptide antigen. As expected, membrane staining for the γ_2 subunit was completely absent in $\gamma_2^{0/0}$ neurons (**Figs. 1c and 3a**).

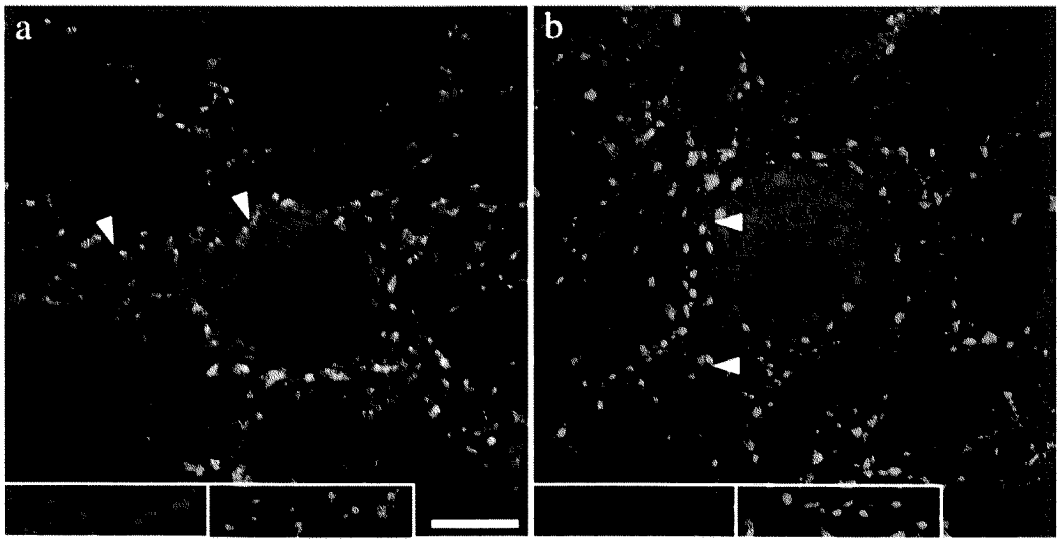

Fig. 2. Loss of clustered $GABA_A$ receptors containing the α_1 subunit in cultured $\gamma_2^{0/0}$ cortical neurons (20 DIV). Cortical neurons cultured from $\gamma_2^{+/+}$ (a) and $\gamma_2^{0/0}$ embryos (b) were double stained with antisera specific for the $GABA_A$ receptor α_1 subunit and GAD. Images were collected by confocal laser scanning microscopy. Juxtaposition of the α_1 subunit (red) and GAD (green) is demonstrated in the superimposed digital pictures. Insets at the bottom of each panel display color-separated images of the representative membrane segments marked with arrowheads in the color superimposed images. Note the absence of punctate staining for α_1 subunit-containing $GABA_A$ receptors but unaltered pattern of GABAergic terminals indicated by GAD immunoreactivity on the $\gamma_2^{0/0}$ neuron. Scale bar, 10 µm.

Remarkably, however, these neurons also showed a strong reduction of the punctate staining for the α_1 and α_2 subunits to 19.5 ± 2.8% and 21.4 ± 6.2% of $\gamma_2^{+/+}$ levels (for absolute values, see **Fig. 3a**), whereas the more diffuse and continuous membrane staining, probably representing extrasynaptic receptors, was unaffected. The morphology of $\gamma_2^{0/0}$ neurons (**Fig. 1c, f, i and l**) and staining for GAD (**Figs. 1f and l and 2b**), synaptophysin and the AMPA receptor GluR1 subunit were unaffected by the absence of the γ_2 subunit, suggesting that the mutant phenotype was limited to the postsynaptic apparatus of GABAergic synapses. Importantly, punctate dendritic staining in $\gamma_2^{+/0}$ neurons (α_1, 101.2 ± 12.1%; α_2, 106.2 ± 12.3% of $\gamma_2^{+/+}$) was normal, precluding a dominant negative effect by a putative truncated γ_2 polypeptide (**Figs. 1b and h and 3a**). These observations suggest that the absence of the γ_2 subunit results in a large and specific decrease in clustering and synaptic localization of $GABA_A$ receptors.

Deficit in postsynaptic currents in $\gamma_2^{0/0}$ neurons

If punctate staining observed with antisera directed against the α_1 and α_2 subunits represents $GABA_A$ receptors clustered at postsynaptic sites, reduced clustering in $\gamma_2^{0/0}$ neurons should be reflected functionally in alterations of miniature postsynaptic currents (mPSCs). Indeed, in $\gamma_2^{0/0}$ neurons, there was a marked increase in the number of neurons that showed no $GABA_A$ receptor-mediated mPSC activity at all ($\gamma_2^{0/0}$, 24 of 41; $\gamma_2^{+/+}$, 5 of 49), as well as a decrease in the level of activity in those neurons that did exhibit GABAergic mPSCs. The absence of the γ_2 subunit greatly reduced the average frequency of $GABA_A$ receptor-mediated mPSCs ($\gamma_2^{0/0}$, 0.74 ± 0.40 Hz, n = 41; $\gamma_2^{+/+}$, 4.14 ± 0.82 Hz, n = 49, p = 0.0007, Students t-test; **Fig. 3c**). In contrast, the average input frequency for AMPA receptor-mediated synaptic currents was not significantly altered ($\gamma_2^{0/0}$, 4.14 ± 1.12 Hz, n = 34; $\gamma_2^{+/+}$, 4.99 ± 1.07 Hz, n = 46, p = 0.59; **Fig. 3c**). Micro-application of GABA pulses evoked inward currents in all cortical neurons tested, independently of the genotype and in agreement with earlier data from dorsal root ganglion neurons (Gunther et al., 1995), indicating surface expression of functional receptors. Although a small part of the reduction in mPSC frequency in $\gamma_2^{0/0}$ neurons might be accounted for by a greater loss of mPSC in the electrical noise because of the reduced channel conductance of γ_2 subunit-deficient $GABA_A$ receptors (Gunther et al., 1995), we conclude that the reduced mPSC frequency is largely due to loss of receptor clustering. Thus, although the γ_2 subunit does not seem to be required for non-clustered surface expression of functional GABA-gated chloride channels in non-neural cells (Connolly et al., 1996a; Gorrie et al., 1997), in acutely dissociated dorsal root ganglion neurons (Gunther et al., 1995) and in cultured cortical neurons, it is required for

postsynaptic clustering and for normal GABAergic postsynaptic inhibitory function in cortical neurons.

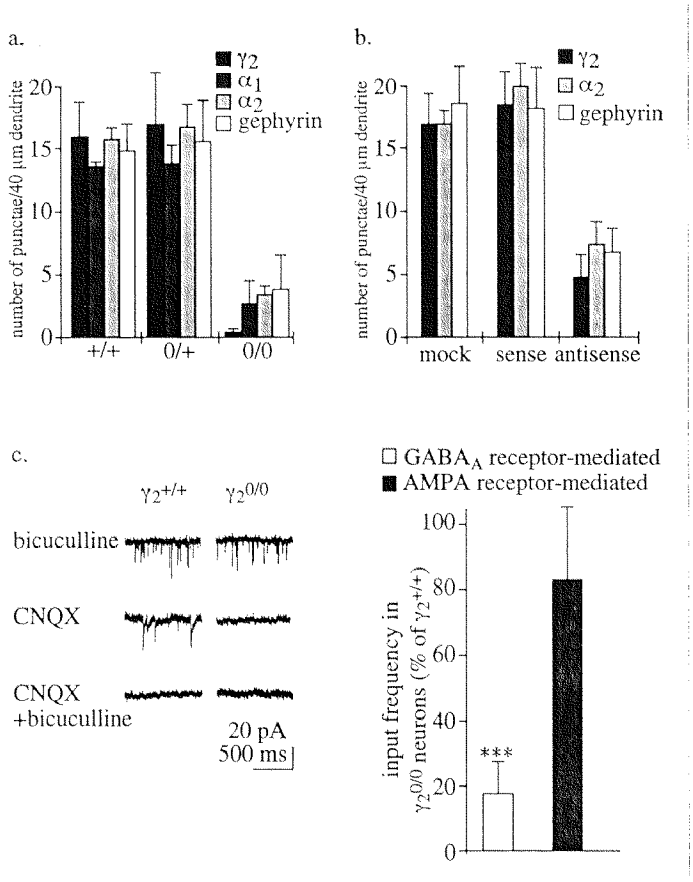

Fig. 3. Quantification of $GABA_A$ receptor and gephyrin clusters and functional deficit in $\gamma_2^{0/0}$ neurons. (a) Quantitative analysis of clustering deficit in $\gamma_2^{0/0}$ neurons (20 DIV). Cortical cultures were double stained for the γ_2, α_1 or α_2 subunit together with gephyrin. Immunoreactive punctae on dendrites of cultured $\gamma_2^{+/+}$ (n = 4), $\gamma_2^{+/0}$ (n = 3 or 4) and $\gamma_2^{0/0}$ neurons (n = 6 or 7) were quantified from video images as described in Methods. Note the very similar reduction in punctate staining for $GABA_A$ receptor α_1 and α_2 subunits and gephyrin. Error bars, standard deviation. (b) Quantitative analysis of clustering in antisense-treated cultures. Cultures from rat hippocampus were treated daily from 14 to 20 DIV with medium alone (mock), with gephyrin sense-strand oligonucleotide, or with gephyrin antisense oligonucleotide, using concentrations and other conditions as described (Kirsch et al., 1993c). The cells were fixed and double stained for the γ_2 or α_2 subunit and gephyrin (n = 3 for each condition), and the number of immunoreactive punctae per 40 µm dendrite was determined for each antigen as above. Note the parallel reduction of punctae for $GABA_A$ receptor subunits and gephyrin. Error bars, standard deviation. (c) GABAergic and glutamatergic miniature postsynaptic currents (mPSCs) in cortical neurons cultured from $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ embryos. Representative recordings from $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ cortical neurons (18 DIV) are shown with the relevant drug treatments indicated. Miniature PSCs were distinguished on the basis of current decay kinetics and pharmacological sensitivity. Fast-decaying, AMPA receptor-mediated mPSCs were blocked by CNQX, and slowly decaying $GABA_A$ receptor-mediated mPSCs were blocked by bicuculline. The average $GABA_A$ receptor-mediated mPSC frequency in $\gamma_2^{0/0}$ cortical neurons was strongly reduced (n = 49 for +/+ and 41 cells for 0/0) in $\gamma_2^{0/0}$ neurons, in contrast to the average input frequency of AMPA receptor-mediated spontaneous events, which was not significantly altered (n = 41 for +/+, n = 34 for 0/0, p = 0.59). Error bars, standard error.

Unaltered α_1 and α_2 subunit levels in $\gamma_2^{0/0}$ neurons

The loss of GABAergic inhibitory function observed in $\gamma_2^{0/0}$ mice is likely to have profound secondary effects on brain physiology and metabolism. To investigate whether altered cellular physiology might affect $GABA_A$ receptor gene expression, the levels of α_1 and α_2 subunit mRNAs in $\gamma_2^{0/0}$ neurons were assessed by quantitative RNase protection assays of RNA prepared from cortical cultures (**Fig. 4a and b**). The mRNA levels were found to be normal (in $\gamma_2^{0/0}$ neuron cultures as percent of values in $\gamma_2^{+/+}$ cultures, α_1, 95.5 ± 19.0; α_2, 111.6 ± 8.5, n = 4).

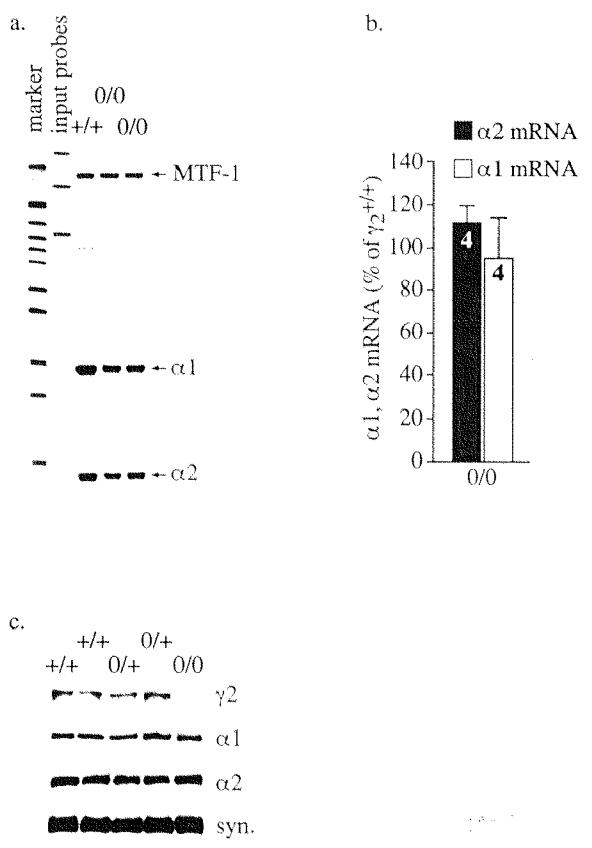

Fig. 4. Unaltered α_1 and α_2 mRNA and protein levels in cortical neurons cultured from $\gamma_2^{+/+}$, $\gamma_2^{+/0}$ and $\gamma_2^{0/0}$ embryos (20 DIV). (a) Representative RNase protection assays performed with mixtures of RNA probes specific for the mRNAs of the α_1 and α_2 subunits and the metallothionein I transcription factor (MTF-1, internal standard, Radtke et al., 1993), using cellular RNA prepared from neurons cultured from individual $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ embryos. RNase-resistant products for the α_1, α_2 and MTF-1 mRNAs are indicated. (b) Quantification of RNase-resistant products. RNase-resistant bands on sequencing gels from assays of four independent sets of cultures that each included both genotypes were quantified by phosphor image analysis. The MTF-1 signal (Radtke et al., 1993) was assumed to be constant and was used as an internal standard to normalize variations in RNA quality and sample recovery, and the results are represented in percent of $\gamma_2^{+/+}$ values. Error bars, standard error. (c) Western blot analysis of $GABA_A$ receptor α_1, α_2 and γ_2 subunits and of synaptophysin (syn.) in membranes isolated from $\gamma_2^{+/+}$, $\gamma_2^{+/0}$ and $\gamma_2^{0/0}$ neurons cultured from cortices of individual embryos. Equal amounts of protein (20 µg) were loaded per lane and probed with the antibodies indicated. The blot stained for the α_1 subunit was stripped and reprobed with antiserum against the γ_2 subunit.

Similarly, the steady-state levels of α_1 and α_2 subunits and synaptophysin were unaltered in the absence of the γ_2 subunit polypeptide, as shown by Western blot analyses of membranes isolated from cultured $\gamma_2^{+/+}$, $\gamma_2^{+/0}$ and $\gamma_2^{0/0}$ neurons (**Fig. 4c**). Thus, the deficit in receptor clustering in $\gamma_2^{0/0}$ neurons is not merely a consequence of reduced $GABA_A$ receptor gene expression. Unaltered steady-state levels of α_1 and α_2 subunits in $\gamma_2^{0/0}$ neurons are consistent with the notion that γ_2 subunit-deficient receptors are distributed to the extrasynaptic membrane and/or trapped intracellularly but not immediately degraded. Unaffected steady-state levels and cellular distribution of synaptophysin are in line with the unaltered GAD staining of GABAergic terminals and glutamatergic input. We conclude that the loss of the γ_2 subunit and the coincident changes in $GABA_A$ receptor clustering are causally related and not simply a result of nonspecific changes in cell physiology or gene expression.

Gephyrin, a clustering molecule for $GABA_A$ receptors

Accumulating evidence suggests that gephyrin might be involved in the anchoring of $GABA_A$ receptors at postsynaptic sites. In the cortical cultures analyzed here, gephyrin immunoreactivity revealed discrete punctae outlining dendritic and somatic membranes of the majority of neurons (14.9 ± 2.1 punctae per 40 μm dendrite). These punctae were almost completely colocalized with punctate staining of the $GABA_A$ receptor γ_2 (**Fig. 5a**), α_2 (**Fig. 5c**) or α_1 subunits (not shown). Similar results were obtained with hippocampal cultures, in which gephyrin staining is even more prominent. Interestingly, concomitant with the absence of the γ_2 subunit and reduction in punctate staining for the $GABA_A$ receptor α_1 and α_2 subunits in $\gamma_2^{0/0}$ neurons, the number of immunoreactive gephyrin punctae was markedly reduced to 25.7 ± 18.5% of $\gamma_2^{+/+}$ levels (**Figs. 3a and 5b and d**). The number of α subunit and gephyrin clusters remaining in the absence of the γ_2 subunit seemed to vary somewhat with the cell type (**Fig. 5b and d**). This likely reflects the heterogeneity of $GABA_A$ receptor subunit composition in different cortical neurons (see Discussion). Importantly, α subunit and gephyrin clusters always disappeared in parallel, as is reflected in similar quantitative losses (**Fig. 3a**). These observations extend previous evidence (Craig et al., 1996) and represent the first direct indication that gephyrin is important in anchoring abundant subtypes of $GABA_A$ receptors at GABAergic synapses.

To establish a causal relationship between gephyrin expression and $GABA_A$ receptor clustering, we treated wild-type hippocampal cultures with gephyrin antisense oligonucleotides. These cultures are more homogeneous than cortical cultures and consist mostly of pyramidal neurons expressing $\alpha_2\beta_{2/3}\gamma_2$ receptors, with few neurons expressing α_1 subunit-containing $GABA_A$ receptors.

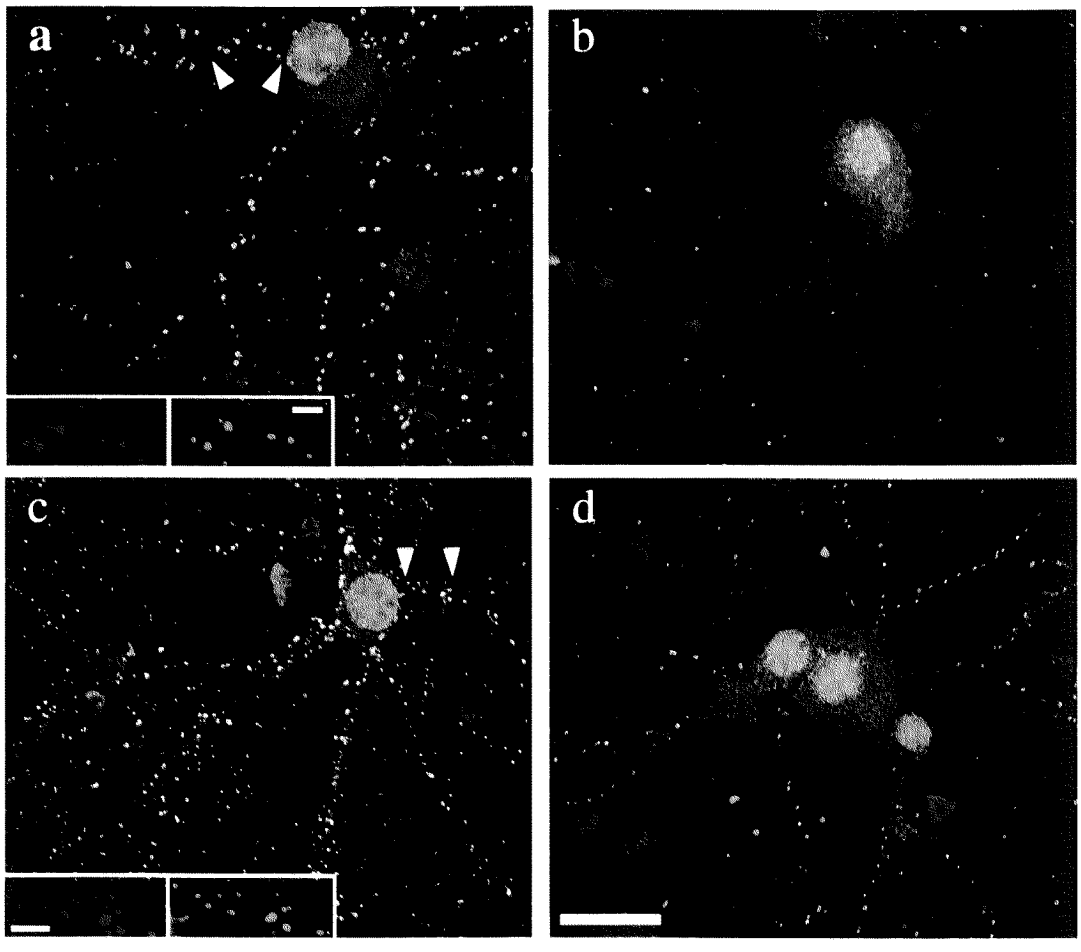

Fig. 5. Colocalization of gephyrin and $GABA_A$ receptor subunit immunoreactivity and loss of gephyrin staining in $\gamma_2^{0/0}$ neurons. Cortical neurons (20 DIV) from $\gamma_2^{+/+}$ (a, c) and $\gamma_2^{0/0}$ embryos (b, d) are double stained for gephyrin and either the γ_2 subunit (a, b) or the α_2 subunit (c, d). Images were collected by confocal laser scanning microscopy (stack of five optical sections spaced by 200 nm). Colocalization of gephyrin (green) and $GABA_A$ receptor subunits (red) in the superimposed digital pictures is represented in yellow. Insets in panels (a) and (c) display color-separated, enlarged images of representative dendrites marked with arrowheads in the superimposed images. Note the large degree of colocalization of punctate $GABA_A$ receptor subunits and gephyrin staining in $\gamma_2^{+/+}$ neurons and the almost complete absence of punctae on the dendritic and somatic membrane of $\gamma_2^{0/0}$ neurons. Scale bars, 20 μm (a-d), 2 μm (insets).

Gephyrin antisense treatment has previously been shown to disrupt clustering of glycine receptors in cultured spinal cord neurons (Kirsch et al., 1993c). In hippocampal cultures, similar treatment resulted in a strong reduction in punctate staining for gephyrin (36.0 ± 10.3% of mock-treated cell values; for absolute numbers of immunoreactive punctae, see **Fig. 3b**). Although the inhibition of gephyrin expression was not complete, it resulted in an equivalent reduction of the punctate staining for the $GABA_A$ receptor α_2 subunit to 43.4 ± 10.3% and of the γ_2 subunit to 27.9 ± 11.5% (mock-treated cells, 100%) in dendritic and somatic membranes (**Figs. 3b and 6b and d**). No effect on

clustering was observed upon treatment with solvent alone (**Figs. 3b and 6a, c and e**) or with gephyrin sense-strand oligonucleotide (**Figs. 3b and 6f**).

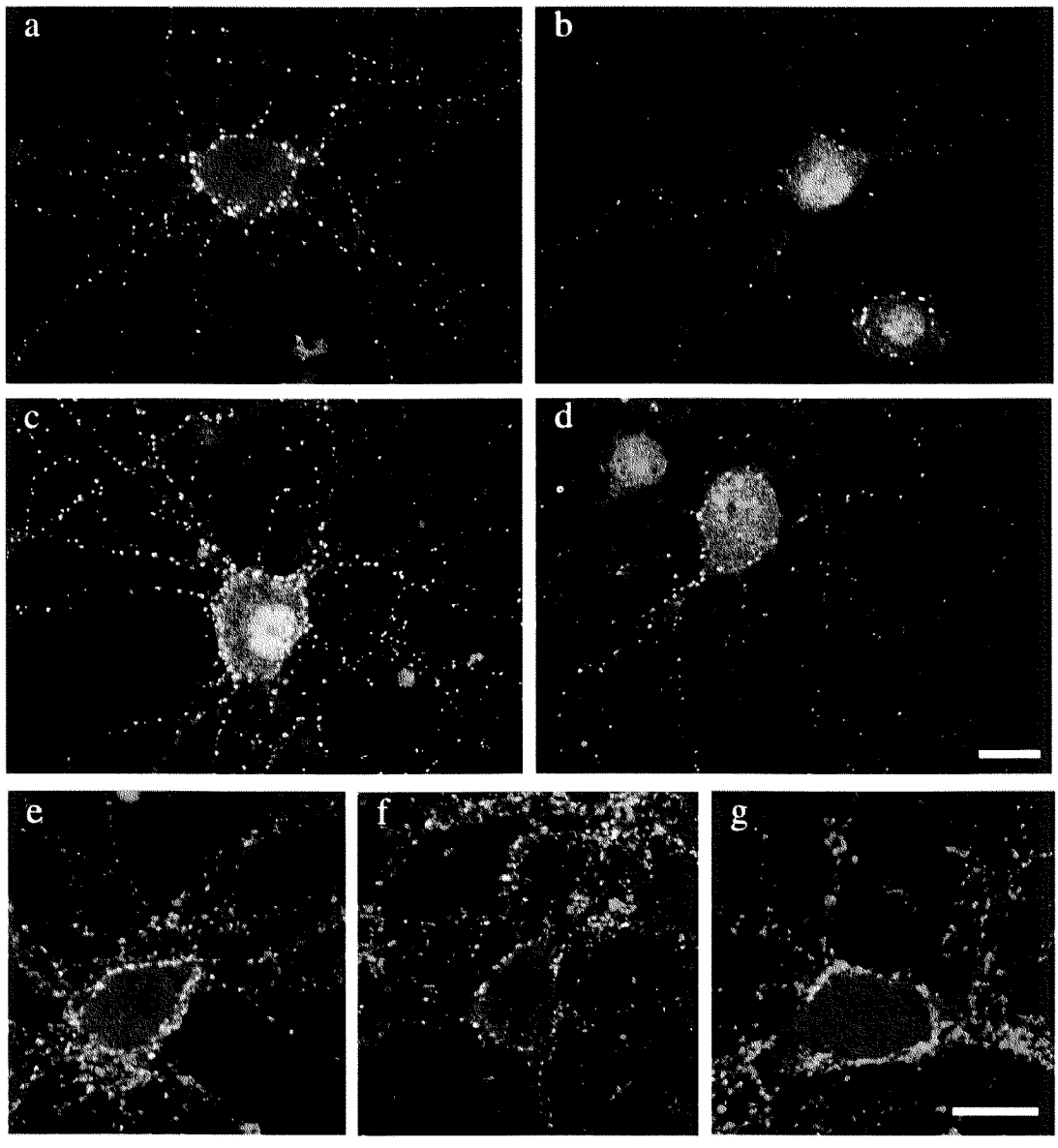

Fig. 6. Loss of punctate $GABA_A$ receptor subunit immunoreactivity upon antisense inhibition of gephyrin expression. Hippocampal cultures were treated daily from 14 to 20 DIV with medium alone (mock treatment, a, c, e) or with thio-substituted gephyrin sense (f) or antisense oligonucleotide (b, d, g) as described (Kirsch et al., 1993c). Cultures were double stained with antisera specific for the $GABA_A$ receptor γ_2 subunit (a, b) or the α_2 subunit (c-g) and gephyrin (a-d) or synaptophysin (e-g) and analyzed by confocal microscopy. Colocalization between $GABA_A$ receptor subunits (red) and gephyrin or synaptophysin (both in green) is represented in yellow. Note that almost all the immunoreactive punctate for gephyrin but only a fraction of synaptophysin punctae are colocalized with the γ_2 and α_2 subunits. Whereas staining for $GABA_A$ receptor subunits and gephyrin is colocalized (a, c), synaptophysin immunoreactivity appears apposed to $GABA_A$ receptor clusters (e, f), pointing to their postsynaptic localization. In the absence of gephyrin, punctate staining for the γ_2 (b) and the α_2 subunit (d, g) was strongly reduced, but staining for synaptophysin was retained (g). Scale bars, 20 μm.

Double staining for $GABA_A$ receptors subunits and synaptophysin revealed slight toxicity of sense and antisense oligonucleotides (**Fig. 6e-g**), but punctate gephyrin and $GABA_A$ receptor subunit immunoreactivity was reduced with antisense treatment only (**Figs. 3b and 6b, d and g**). Treatment with randomized oligonucleotides containing the nucleotide composition of the antisense probe revealed no change in gephyrin and α_2 subunit staining, although γ_2 subunit staining appeared reduced (not shown). Similar results were obtained with cortical neurons. We conclude that gephyrin is important for synaptic clustering of $GABA_A$ receptors in hippocampal and cortical neurons.

Loss of clustering and synaptic localization *in vivo*

$GABA_A$ receptor staining in the brain of newborn $\gamma_2^{0/0}$ mice, before the onset of synaptogenesis, is normal (Gunther et al., 1995). Even in older mice (P6-P14), the characteristic regional and laminar distribution of major subunits (α_1, α_2, α_3, α_5, $\beta_{2/3}$) in all parts of the brain was unaffected by the absence of the γ_2 subunit (data not shown). Similarly, the steady-state levels of α_1 and α_2 subunits were normal in postnatal brain of $\gamma_2^{0/0}$ mice. To determine whether the deficit in synaptic $GABA_A$ receptors observed in cultured neurons might be relevant *in vivo*, we used an antigen-retrieval procedure that was optimized to visualize clustered $GABA_A$ receptors in brain sections (Fritschy et al., 1998b). In the wild-type cortex (P14), immunofluorescent analysis of the α_2 subunit revealed intense punctate staining, together with more diffuse staining of apparently extrasynaptic receptors (**Fig. 7a**), as described earlier for the adult rat brain (Fritschy et al., 1998b). Punctate staining, thought to represent $GABA_A$ receptors clustered at postsynaptic sites (Fritschy et al., 1998b), was extensively colocalized with immunoreactive punctae for gephyrin (**Fig. 7c**). The few gephyrin punctae that were not colocalized with the α_2 subunit are most likely associated with $GABA_A$ receptors containing other α subunits. In equivalent cortical sections from $\gamma_2^{0/0}$ littermates, absence of the γ_2 subunit resulted in a strong reduction of immunoreactive punctae for the α_2 subunit (**Fig. 7b**) that was paralleled by a similar reduction in punctate gephyrin staining (**Fig. 7d**). Only a few $GABA_A$ receptor and gephyrin punctae remained in the absence of the γ_2 subunit, indicating a subset of receptors that is less or not affected by the loss of the γ_2 subunit, possibly by substitution of other subunits such as γ_1, γ_3 or ε for γ_2. Alternatively, some of the remaining clusters might be extrasynaptic and indicate that synaptogenesis in the cortex is not complete at P14. In agreement with the data from cultured neurons (**Fig. 1**), diffuse α_2 subunit staining seemed largely unaltered (**Fig. 7b**). These data indicate that the γ_2 subunit is required for clustering of α_2 subunit-containing $GABA_A$ receptors and their colocalization with gephyrin *in vivo*.

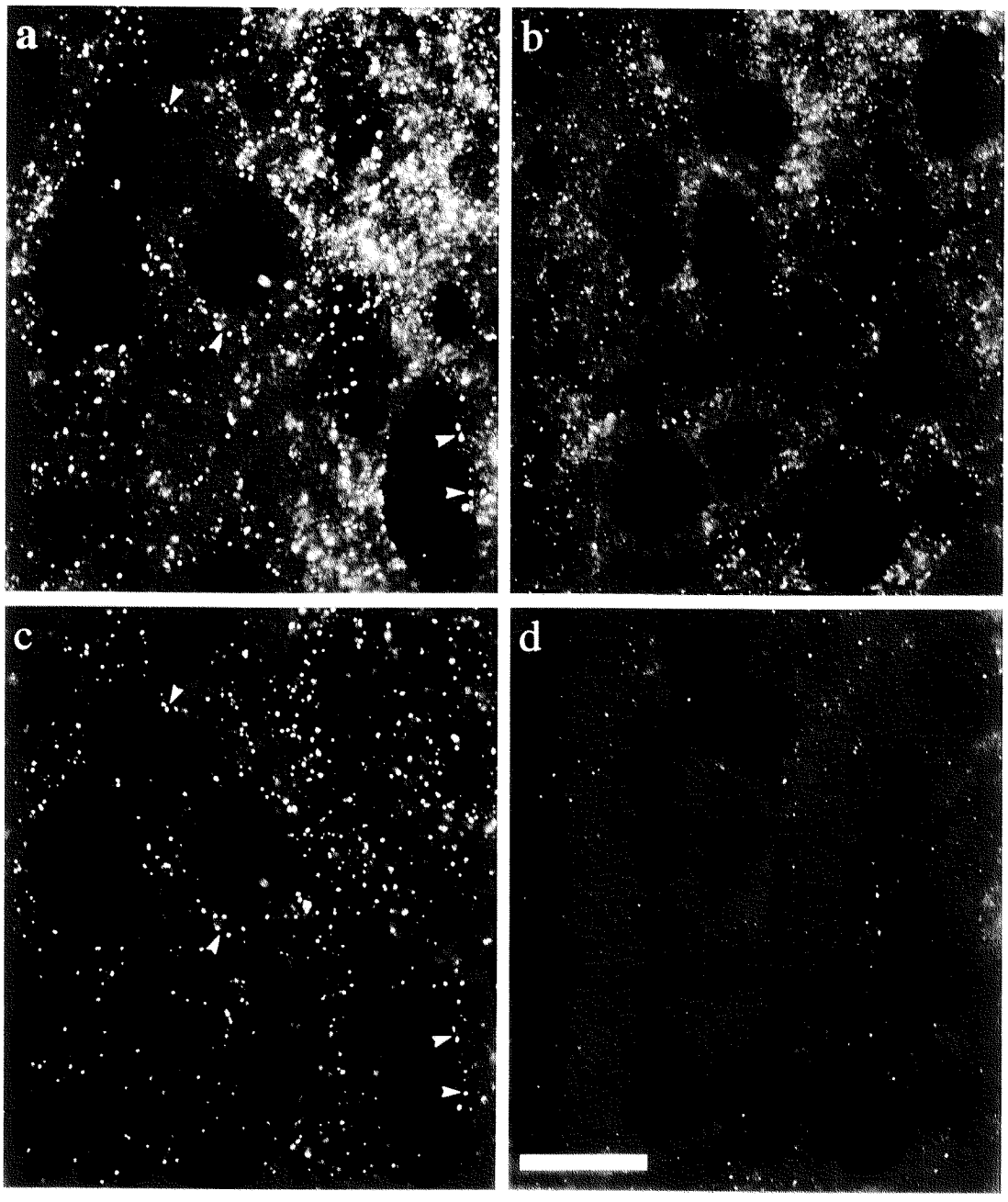

Fig. 7. Loss of clustered α_2 subunit-containing $GABA_A$ receptors and gephyrin in the cerebral cortex of the $\gamma_2^{0/0}$ brain (P14). Parasagital sections through the neocortex of a $\gamma_2^{+/+}$ (a, c) and a $\gamma_2^{0/0}$ mouse (b, d) were treated with an antigen-retrieval procedure (Fritschy et al., 1998b) and double stained with antibodies specific for the α_2 subunit (a, b) and gephyrin (c, d). Fluorescent micrographs of layer III reveal bright immunoreactive punctae for both the α_2 subunit (a) and gephyrin (c) in the $\gamma_2^{+/+}$ section, which are greatly reduced in $\gamma_2^{0/0}$ (b, d). In contrast, the more diffuse staining of the α_2 subunit appears unaltered. Arrowheads point out immunoreactive punctae for the α_2 subunit (a) that are extensively colocalized with punctate staining for gephyrin (c). Scale bar, 20 μm.

The molecular layer of the cerebellum is devoid of α_{2-6}, γ_1, γ_3, ε and δ subunits, such that all $GABA_A$ receptors have the $\alpha_1\beta\gamma_2$ subunit composition (Whiting et al., 1997; Fritschy and Mohler, 1995; Laurie et al., 1992a). Loss of the γ_2 subunit should therefore affect all postsynaptic $GABA_A$ receptors in this location. In the molecular layer of the wild-type cerebellum (P14), analysis of the α_1 subunit revealed strong punctate staining, probably representing postsynaptic receptors on dendrites of Purkinje, stellate and basket cells (**Fig. 8a**). In contrast, Purkinje cell somata showed mostly diffuse staining. Punctate α_1 subunit staining on dendrites was extensively colocalized with staining for gephyrin. In equivalent sections from the cerebellum of $\gamma_2^{0/0}$ littermates, punctate staining for both the α_1 subunit and gephyrin was lost entirely, whereas diffuse staining for the α_1 subunit, probably representing extrasynaptic receptors, appeared unaffected (**Fig. 8b**). Compared to the mutant phenotype in the neocortex, loss of clusters in the molecular layer of the cerebellum seems to be even more pronounced, reflecting the limited heterogeneity of $GABA_A$ receptor subunit composition and the notion that γ_2 is the only γ or γ-like subunit in this brain area. These results demonstrate that the γ_2 subunit is essential for clustering and postsynaptic localization of $GABA_A$ receptors and gephyrin on cerebellar Purkinje cells, stellate cells and basket cells.

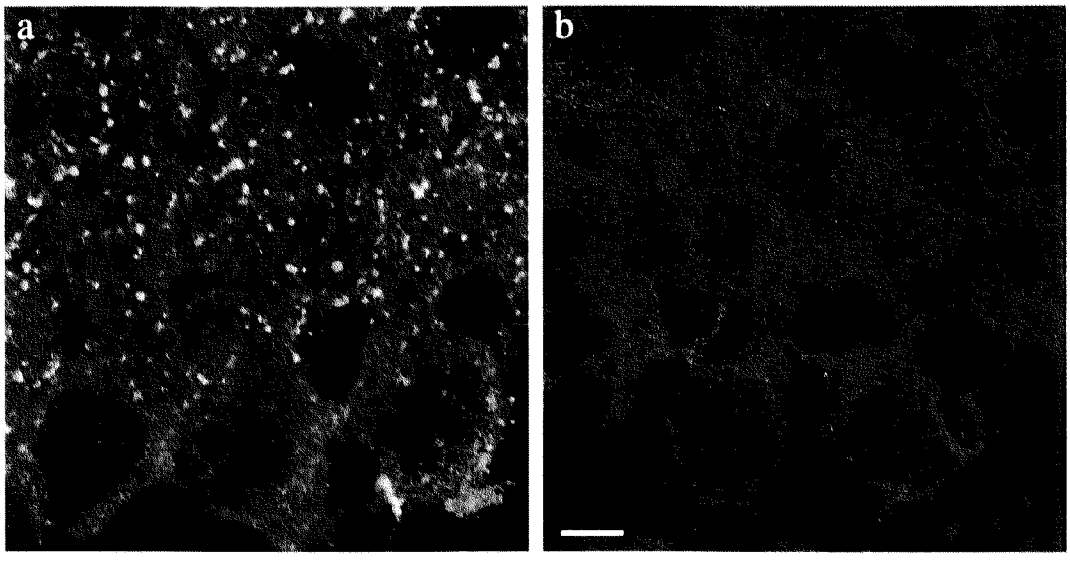

Fig. 8. Loss of clustered α_1 subunit-containing $GABA_A$ receptors and gephyrin in the cerebellum of the $\gamma_2^{0/0}$ brain (P14). Confocal images of sagittal sections through the molecular and Purkinje cell layers of cerebellar cortex of a $\gamma_2^{+/+}$ (a) and a $\gamma_2^{0/0}$ mouse (b). Sections were prepared for immunohistochemistry as described (Fritschy et al., 1998b) and double stained for the α_1 subunit (shown in red) and gephyrin (green). $GABA_A$ receptors in this region have a homogeneous $\alpha_1\beta\gamma_2$ subunit composition (Whiting et al., 1997; Fritschy and Mohler, 1995; Laurie et al., 1992a). Note the large degree of colocalization (indicated in yellow) between the α_1 subunit and gephyrin in the $\gamma_2^{+/+}$ section (a) and the complete loss of immunoreactive punctae for both the α_1 subunit and gephyrin in the $\gamma_2^{0/0}$ section (b). In contrast, the diffuse α_1 subunit staining is unaltered. Scale bar, 10 µm.

Discussion

The data presented here provide three lines of evidence that gephyrin and $GABA_A$ receptors are closely associated and interdependent components of the same postsynaptic complex of GABAergic inhibitory synapses. Firstly, the two proteins are extensively colocalized in cultured cortical and hippocampal neurons and *in vivo*; secondly, clustering of gephyrin depends on the presence of the γ_2 subunit in γ_2 subunit-containing $GABA_A$ receptors, and thirdly, postsynaptic clustering of $GABA_A$ receptors depends on expression of the clustering protein gephyrin. Interestingly, no corresponding mutant phenotype is detected on somata of dorsal root ganglion neurons, which lack postsynaptic specialization, nor in brain sections of newborn $\gamma_2^{0/0}$ mice before synaptogenesis has occurred (Gunther et al., 1995). Lethality at birth, which occurs in the majority of $\gamma_2^{0/0}$ mice, might be caused by the reduced channel conductance of γ_2 subunit-deficient $GABA_A$ receptors. Our data strongly suggest that in the few $\gamma_2^{0/0}$ mice that survive beyond birth, the additional deficit in postsynaptic $GABA_A$ receptor clustering is incompatible with survival beyond the third postnatal week, the time when synaptogenesis is reaching its peak.

The absence of the γ_2 subunit results in a drastic reduction in $GABA_A$ receptor and gephyrin clusters. However, some residual punctate immunoreactivity on cortical neurons suggests that a few receptors remain clustered in the absence of the γ_2 subunit (Figs 5b and d and 7b and d). These punctae might represent minor receptor subtypes in which other γ subunits (γ_1, γ_3) or the closely related δ, or ε subunits substitute for the γ_2 subunit. Accordingly, the mutant phenotype is most dramatic in the molecular layer of the $\gamma_2^{0/0}$ cerebellum, which most likely contains no γ or γ-like subunits at all. As a consequence, clustering of $GABA_A$ receptors and colocalization with gephyrin on Purkinje cells are absolutely dependent on the γ_2 subunit (Fig. 8b). Punctate staining for α_1 and α_2 subunits was reduced in all regions of the mutant brain, including the brainstem. In contrast, gephyrin immunoreactivity in the brainstem appeared only slightly reduced (data not shown), consistent with the notion that it is mostly associated with abundant glycinergic synapses in this brain area, and that glycinergic synapses seem not to be affected by the loss of clustered $GABA_A$ receptors. However, this interpretation awaits confirmation by detailed analysis of glycinergic synapses on γ_2 subunit-deficient neurons.

At least two distinct mechanisms could account for the deficit in $GABA_A$ receptor clustering in $\gamma_2^{0/0}$ neurons. In the case of the glycine receptor in spinal cord neurons, there is evidence that receptor activation is required for clustering (Kirsch and Betz, 1998; Levi et al., 1998). It is conceivable that the 2.5-fold reduction in chloride conductance observed in $\gamma_2^{0/0}$ neurons (Gunther et al., 1995) results in an analogous

partial impairment of clustering of $GABA_A$ receptors. However, there is reason to believe that clustering of $GABA_A$ and glycine receptors may depend on different mechanisms. Firstly, inhibition of synaptic neurotransmitter release by blocking voltage-dependent sodium channels by tetrodotoxin (TTX) can prevent clustering of glycine receptors in spinal cord neurons (Kirsch and Betz, 1998). In contrast, TTX does not prevent $GABA_A$ receptor clustering during maturation of hippocampal neuron cultures (Craig et al., 1994). Secondly, clustering of glycine receptors occurs rapidly after plating of spinal neurons *in vitro*, at a time when the receptors are still excitatory. In contrast, $GABA_A$ receptor clusters in cortical neurons formed much more slowly and were not detected in significant numbers before eight days in culture, by which time $GABA_A$ receptors are already inhibitory (Berninger et al., 1995). Although clustering of gephyrin can occur independently of glycine receptors in spinal neurons (Levi et al., 1998), gephyrin clusters were almost always colocalized with clustered $GABA_A$ receptors in wild-type and $\gamma_2^{0/0}$ cortical neurons (20 DIV). We therefore favor a second mechanism explaining the clustering deficit in $\gamma_2^{0/0}$ neurons, whereby absence of the γ_2 subunit directly impairs protein-protein interactions between $GABA_A$ receptors and other proteins underlying the postsynaptic specialization.

Gephyrin is a primary candidate to mediate interaction of $GABA_A$ receptors with the cytoskeleton, and the phenotype of γ_2 subunit-deficient neurons further supports this hypothesis. However, given the absence of gephyrin in purified $GABA_A$ receptor preparations (Kannenberg et al., 1997) and the lack of gephyrin binding to $GABA_A$ receptor subunits in overlay assays (Meyer et al., 1995), interaction between $GABA_A$ receptors and gephyrin is most likely indirect and/or may involve more than one type of subunit. Candidate factors that might stabilize interaction between $GABA_A$ receptors and gephyrin or function as bridging factors are so far unidentified. They may include ill-defined proteins that copurify with native $GABA_A$ receptors (Kannenberg et al., 1997) and a ubiquitous factor that interacts with the γ_2 subunit polypeptide *in vitro* (Wang, H. & Olsen, R., Soc. Neurosci. Abstr. 23, 107, 1997). At least some of these proteins are expected to be neuron specific, as coexpression of gephyrin and $GABA_A$ receptors in heterologous non-neural cells does not promote colocalization and cluster formation (Kirsch et al., 1995b).

Recent analyses of several lines of mutant mice lacking single $GABA_A$ receptor subunits indicate specific roles for different subunit classes in assembly, subcellular targeting and synaptic clustering of $GABA_A$ receptors. The α and β subunits are required for efficient assembly and surface expression of $GABA_A$ receptors *in vivo*. Evidence includes the loss of a major subset of $GABA_A$ receptors in mice that lack functional α_5 (Fritschy et al., 1997), α_6 (Jones et al., 1997) or β_3 subunit genes

(Homanics et al., 1997a). In addition, the α subunit variants are primary candidates for a role in segregation of $GABA_A$ receptor subtypes to different types of synapses, a prerequisite for fine tuning of the GABAergic input. In hippocampal pyramidal neurons, for example, receptors characterized by the α_1 subunit seem equally distributed to all inhibitory synapses, whereas receptors containing the α_2 subunit are preferentially localized at synapses on the axon initial segment, which is innervated by axo-axonic interneurons (Fritschy et al., 1998b; Nusser et al., 1996a). Similarly, formation of hippocampal $GABA_A$ receptors containing either the α_2 or α_5 subunit is largely independent, as shown by analysis of mice lacking the α_5 subunit (Fritschy et al., 1997), and receptors containing the α_1, α_2 or α_3 subunit have non-overlapping distribution in retinal ganglion cells (Koulen et al., 1996).

The γ_2 subunit and gephyrin are too abundant to determine differential subcellular targeting of $GABA_A$ receptors. Compared to other types of ligand-gated ion channel, a larger proportion of $GABA_A$ receptors seems to be localized extrasynaptically. Given the evidence presented here, the γ_2 subunit might be generally involved in specifying synaptic versus extrasynaptic localization of $GABA_A$ receptors. Consistent with such a role, the δ subunit was recently shown to replace the γ_2 subunit in extrasynaptic receptors of cerebellar granule cells (Nusser et al., 1998). Other candidate subunits that might also contribute preferentially to synaptic or extrasynaptic receptors include the γ_1 (Ymer et al., 1990), γ_3 (Knoflach et al., 1991; Herb et al., 1992; Benke et al., 1996) and ε (Davies et al., 1997) subunits. Consistent with such a role and much like the γ_2 and δ subunits, they form functional receptors only when coassembled with α and β subunits. Thus, although the heterogeneity of $GABA_A$ receptors has been appreciated for some time from a physiological and pharmacological point of view, it is equally important as a determinant of differential synaptic and extrasynaptic localization and synaptic efficacy.

Methods

Mice. The γ_2 subunit-deficient mice were described previously (Gunther et al., 1995). The animals used here were generations F2-F6 from the original chimeras in a 129/Ola x C57BL/6 mixed background. Animals were selected pathogen free and kept under optimal hygiene conditions. Timed matings between $\gamma_2^{+/0}$ mice were set up in the late afternoon and checked for vaginal plugs the next morning. The time when the plug was detected was designated embryonic day 0.5 (E0.5). All animal experiments were approved by the Veterinary Office of the Canton Zurich (licenses # 45/95 and 40/97) and conducted in accordance with the animal protection laws of the Canton Zurich.

Neuron cultures. Cortical hemispheres of single E14.5 mouse embryos derived from heterozygous intercrosses were dissected and collected on ice in phosphate-buffered saline (PBS, pH 7.4) containing 5.5 mM glucose. Livers were saved for genotyping by PCR (Gunther et al., 1995); cortical tissue was treated with papain [0.5 mg/ml (Sigma), in PBS containing DNase I (10 μg/ml), bovine serum albumin (1 mg/ml, cell culture grade, Sigma) and 10 mM glucose] for 15 min at room temperature and triturated with a fire-polished Pasteur pipette. The cells were plated at 4-5 x 10^4 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM, high glucose, with Glutamax I, Lifetechnologies) at 5% CO^2 in polylysine-coated polystyrene slide flasks (Lifetechnologies), and the medium was replaced after 30 min with DMEM containing 10% heat-inactivated fetal calf serum. The next day, the cells were washed with PBS, and the medium was replaced with serum-free B27-supplemented Neurobasal (Brewer et al., 1993). The cells were kept at 10% CO^2 without medium change for 20 days. Hippocampal neurons were prepared analogously from rat E17.5 embryos and plated at 2-3 x 10^4 cells/cm^2.

Immunochemistry. Cultures on polystyrene slide flasks were rinsed with PBS, fixed with 4% paraformaldehyde in 150 mM sodium phosphate buffer pH 7.4 (15 min) and divided into four equal areas to allow simultaneous monitoring of several antigens. The cells were permeabilized for 5 min in 0.2% Triton X-100 (in PBS, 10% normal donkey or goat serum), incubated (alone or in combinations), overnight at 4°C with primary antibodies to $GABA_A$ receptor α_1, α_2 or γ_2 subunits (Fritschy and Mohler, 1995), gephyrin (mAb 7a, Pfeiffer et al., 1984), synaptophysin (A0010, Dako) or glutamic acid decarboxylase (GAD-6, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa) in PBS containing 10% normal donkey or goat serum, rinsed with PBS and incubated with secondary antibody conjugated to CY2 or CY3 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania), Oregon Green or Alexa Green (Molecular Probes, Eugene, Oregon). Fluorescent images were captured from a Zeiss Axiophot microscope equipped with a 40x objective and a high resolution video camera linked to a microcomputer imaging device (MCID, Imaging Research, St. Catharines, Ontario) or using a confocal laser scanning microscope (Leica, TCS 4D). Cryostat sections were fixed as described (Fritschy et al., 1998b) and processed for immunofluorescent analysis as detailed above for cultured neurons.

Quantification of $GABA_A$ receptor and gephyrin clusters. For quantification of dendritic $GABA_A$ receptor and gephyrin clusters, digitally stored video images of fluorescent micrographs were processed with the MCID imaging system as follows.

Targets measuring an area between 0.1 and 10 μm^2 were automatically selected, applying a maximal target size of 2 μm^2 to digitally resolve and split larger target areas corresponding to adjacent or partly overlapping punctae. A segmentation range defining a density threshold was then set individually for each image such that targets corresponding to immunoreactive punctae on the membrane were selected specifically. The number of targets per 40 µm dendrite was determined by placing a 6 x 40 µm measuring field over a well isolated linear portion of a dendrite and automatically counting the selected targets in this field. Each cell was analyzed with four measuring fields; four cells corresponding to four independent images were analyzed of each culture. Independent cultures ($3 \leq n \leq 7$) were quantified for all antigens (γ_2, α_1, α_2, gephyrin) and each genotype (+/+, 0/+, 0/0). Quantification of clusters in antisense experiments was done analogously for the γ_2 and α_2 subunit and for gephyrin under the three conditions: mock treated, gephyrin sense treated and antisense treated (n = 3 independent cultures for each condition and each antigen).

Electrophysiological recordings. Whole-cell, patch-clamp recordings were made from individual cortical neurons cultured as described above from $\gamma_2^{+/+}$ and $\gamma_2^{+/0}$ embryos (14-21 DIV; holding potential -60 mV; superfusion saline, 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH 7.4; pipette saline, 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, pH 7.2). Bicuculline- or CNQX-sensitive mPSCs were isolated using a multi-channel superfusion microapplicator. The superfusion saline was supplemented with 0.5 mM TTX, 1 mM strychnine and either 40 mM bicuculline methochloride or 10 mM 6-cyano-7-nitroquinoxaline-2,3-dione sodium (CNQX). Application of 10 mM D-2-amino-5-phosphonopentanoic acid together with CNQX had no additional effect. A subset (32 of 80) of recordings of AMPA receptor-mediated PSCs were done without TTX. No difference in frequency of mPSCs was observed, and so the data were combined. Data were analyzed offline using an event-detection threshold of 10 pA. Neurons displaying input frequencies of no more than 0.05 Hz were designated inactive. Miniature synaptic currents were analyzed using Mini Analysis Program (V. 3.0.1, Jaejin Software, Leonia, New Jersey). The detection threshold was set to achieve low false-event rates (below 0.03 per s). Undetected events and false positives were corrected by visual inspection.

RNase protection assays. The plasmid used to generate the α_1 subunit-specific riboprobe was a gift from U. Rudolph and contained a 130-bp genomic fragment with exon 4 and flanking sequences subcloned into *EcoRV*-cut pBluescript SK+ (Stratagene), such that T7 RNA polymerase would transcribe the noncoding strand. To generate a

riboprobe specific for α_2 mRNA, a PCR amplified genomic *Dra I-Alu I* fragment containing exon 5 and flanking sequences of the α_2 gene was cloned into *EcoRV*-cut pBluescript SK+ such that T7 RNA polymerase would transcribe the noncoding strand. Riboprobes specific for α_1 and α_2 mRNA were prepared using the plasmids above linearized with *BamHI* for runoff transcription with T7 RNA polymerase. The MTF-1-specific probe identifying a ubiquitous metal-responsive transcription factor was as described (Radtke et al., 1993). RNase protection assays (Luscher and Schumperli, 1987) were done using mixtures of the three riboprobes and 8 µg of total cellular RNA per assay. RNase-resistant bands on sequencing gels from assays of four independent cultures for each genotype were quantified by phosphor image analysis. The MTF-1 signal (Radtke et al., 1993) was assumed to be constant and was used as an internal standard to normalize variations in RNA quality and sample recovery.

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3.2 Postsynaptic clustering of $GABA_A$ receptors by the γ_3 subunit *in vivo*

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Summary

Synaptic localization of $GABA_A$ receptors is a prerequisite for synaptic inhibitory function but the mechanism by which different receptor subtypes are localized to postsynaptic sites is poorly understood. The γ_2 subunit and the postsynaptic clustering protein gephyrin are required for synaptic localization and function of major $GABA_A$ receptor subtypes. We now show that transgenic overexpression of the γ_3 subunit in γ_2 subunit-deficient mice restores benzodiazepine binding sites, benzodiazepine-modulated whole cell currents, and postsynaptic miniature currents, suggesting the formation of functional, postsynaptic receptors. Moreover, the γ_3 subunit can substitute for γ_2 in the formation of $GABA_A$ receptors that are synaptically clustered and colocalized with gephyrin *in vivo*. These clusters were formed even in brain regions devoid of endogenous γ_3 subunit, indicating that the factors present for clustering of γ_2 subunit-containing receptors are sufficient to cluster γ_3 subunit-containing receptors. The $GABA_A$ receptor and gephyrin-clustering properties of the ectopic γ_3 subunit were also observed for the endogenous γ_3 subunit, but only in the absence of the γ_2 subunit, suggesting that the γ_3 subunit is at a competitive disadvantage with the γ_2 subunit for clustering of postsynaptic $GABA_A$ receptors in wild-type mice.

Introduction

Nervous system signaling depends on the coordinated function of a variety of cell surface ion channels and receptors which are typically concentrated at specific subcellular domains such as postsynaptic sites, axon terminals, or nodes of Ranvier (Colledge and Froehner, 1998; Kirsch et al., 1996). Clustering, postsynaptic localization and synapse-specific targeting of receptors are prerequisites for synaptic function and appear to characterize essential steps in synapse formation during neuronal development. In addition, modulation of synaptic clustering and localization of neurotransmitter receptors provide a potential mechanism for rapid adaptation of synaptic efficacy in mature neurons. This is particularly evident for fast neurotransmission mediated by receptors of the ligand-gated ion channel family.

Inhibitory neurotransmission in brain is mediated mainly by γ-aminobutyric acid (GABA) acting at heteropentameric $GABA_A$ receptor chloride channels. $GABA_A$ receptors comprise an ill-defined number of structurally and pharmacologically distinct receptor subtypes, which emerge by differential expression and assembly of a large number of subunits classified according to their primary structure as α, β, γ, δ, ε, π, or ρ subunits (Mohler et al., 1996b; Sieghart, 1995; Macdonald and Olsen, 1994). Most $GABA_A$ receptors are composed of variant α and β subunits together with the γ_2 subunit and are concentrated at postsynaptic sites. Specific receptor subtypes might be targeted to different types of synapses in distinct cellular domains. For example, in hippocampal pyramidal cells, $GABA_A$ receptors containing the α_1 subunit are found in all types of GABAergic synapses (on the soma, proximal and distal dendrites, and the axon initial segment), whereas receptors containing the α_2 subunit are selectively localized in the axon initial segment, which is innervated by axo-axonic interneurons (Nusser et al., 1996a; Fritschy et al., 1998b). Although the γ_2 subunit is largely dispensable for assembly and surface expression of $GABA_A$ receptors *in vivo*, it is required for normal channel conductance, postsynaptic clustering and postsynaptic function of major $GABA_A$ receptor subtypes (Gunther et al., 1995; Essrich et al., 1998; Brickley et al., 1999). Moreover, the γ_2 subunit is essential for proper aggregation of the clustering molecule gephyrin at sites apposed to GABAergic terminals (Essrich et al., 1998).

The γ_2 and γ_3 subunits are 64% identical at the level of their primary structure. They form recombinant receptors with similar unitary channel conductance (Herb et al., 1992) and both can contribute to the benzodiazepine (BZ) binding site (Herb et al., 1992; Knoflach et al., 1991). However, the γ_3 subunit-containing $GABA_A$ receptors are characterized by a very low affinity for the BZ agonist zolpidem (Benke et al., 1996; Togel et al., 1994; Quirk et al., 1994b). Due to the low abundance of the γ_3 subunit in brain (Herb et al., 1992; Benke et al., 1996; Togel et al., 1994), a functional analysis of

the corresponding receptors *in vivo* has been quite limited. In particular, it has remained elusive whether the γ_3 subunit contributes to receptors at postsynaptic sites. To determine whether the γ_3 subunit can substitute for the γ_2 subunit in the formation of functional postsynaptic $GABA_A$ receptors *in vivo*, a transgenic mouse strain overexpressing the γ_3 subunit throughout the brain has been established in a γ_2 subunit-deficient background. The results show that ectopically overexpressed γ_3 subunit partially restores the formation of BZ-modulated $GABA_A$ receptors *in vivo* and promotes the synaptic localization of gephyrin and $GABA_A$ receptors that contribute to miniature postsynaptic currents, similarly to the γ_2 subunit.

Results

A transgenic mouse line overexpressing the γ_3 subunit under control of the human β-actin promoter was established (γ_3^{tg}). In the adult brain, the endogenous γ_3 subunit gene is expressed primarily in the olfactory bulb, striatum, and in a subset of thalamic nuclei, but is absent, for example, in the cerebellum (Herb et al., 1992). Hence, to test for ectopic expression of the γ_3 subunit in transgenic mice, Western blots were performed using cerebellar and forebrain membrane preparations (**Fig. 1A**). Whereas no γ_3 subunit signal was present in cerebellum of non-transgenic, control mice, ectopic γ_3 subunit was readily detected as an intense band in γ_3^{tg} cerebellum. The electrophoretic mobility and band pattern of the transgene-encoded γ_3 subunit were indistinguishable from those of endogenous γ_3 subunit in forebrain, indicating that the protein was properly translated and efficiently processed. The expression level of the γ_3 subunit in forebrain was five to ten times higher in transgenic mice than in controls, indicating that it was expressed throughout the brain (**Fig. 1A**). The transgene-encoded γ_3 subunit was assembled with endogenous α_1 and $\beta_{2/3}$ subunits, as shown by Western blot analysis of $GABA_A$ receptors isolated by immunopurification (**Fig. 1B**). In the absence of the γ_2 subunit, an increased γ_3 subunit expression was noted, which was further increased in the presence of the γ_3 subunit transgene.

To test whether ectopic γ_3 subunit would compensate for loss of function in γ_2 subunit-deficient mice ($\gamma_2^{0/0}$), γ_3^{tg} mice were crossed with $\gamma_2^{+/0}$ mice (Gunther et al., 1995) and the $\gamma_3^{tg}/\gamma_2^{+/0}$ offspring were subsequently crossed with $\gamma_2^{+/0}$ mice to obtain γ_3-transgenic offspring lacking the γ_2 subunit ($\gamma_3^{tg}/\gamma_2^{0/0}$). Similarly to the $\gamma_2^{0/0}$ phenotype (Gunther et al., 1995), $\gamma_3^{tg}/\gamma_2^{0/0}$ mice displayed perinatal or postnatal lethality with no survivors beyond the third postnatal week, indicating that the γ_3 subunit could not fully substitute for the γ_2 subunit. The few $\gamma_2^{0/0}$ mice (γ_3^{tg} and non-transgenic) that survived to postnatal day 14 (P14) were used for analysis. The γ_3^{tg} mice also displayed

a phenotype in the $\gamma_2^{+/+}$ and $\gamma_2^{+/0}$ background, with a significant number of animals remaining smaller than their non-transgenic littermates (20 out of 143 $\gamma_3^{tg}/\gamma_2^{+/0}$ and $\gamma_3^{tg}/\gamma_2^{+/+}$ mice compared to three out of 214 $\gamma_2^{+/+}$ and $\gamma_2^{+/0}$; χ^2 (1) = 20.48, $p < 0.001$). In addition, fertility was reduced, and spontaneous or handling-induced seizures were sometimes observed in $\gamma_3^{tg}/\gamma_2^{+/0}$ mice, and more of these mice than $\gamma_2^{+/0}$ mice died unexpectedly (18.3% of $\gamma_3^{tg}/\gamma_2^{+/0}$ compared to less than 1% in $\gamma_2^{+/0}$ mice). Thus, ectopic γ_3 subunit expression in both $\gamma_2^{+/0}$ and $\gamma_2^{+/+}$ mice resulted in a functional deficit indicative of a dominant negative effect.

Restoration of BZ binding sites in γ_3 transgenic mice

$GABA_A$ receptors containing the γ_3 subunit are characterized by zolpidem-insensitive [^{3}H]flumazenil binding sites, similar to receptors containing the α_5 subunit in conjunction with the γ_2 subunit (Herb et al., 1992; Luddens et al., 1994). In $\gamma_2^{0/0}$ and $\gamma_3^{tg}/\gamma_2^{0/0}$ brain, the $\alpha_5\beta\gamma_2$ receptors are missing and zolpidem-insensitive flumazenil sites are indicative of γ_3 subunit-containing $GABA_A$ receptors. To determine whether the γ_3 subunit can substitute for the γ_2 subunit *in vivo*, the total number of BZ sites was analyzed by [^{3}H]flumazenil autoradiography of P14 brain sections (**Fig. 1C**) and quantified in hippocampus. Compared to wild-type (wt), the number of [^{3}H]flumazenil binding sites in $\gamma_2^{0/0}$ hippocampus was markedly reduced to 8.4 ± 1.6% (n = 3), in agreement with previous observations (Gunther et al., 1995). The large majority of the $\gamma_2^{0/0}$ [^{3}H]flumazenil sites (73.8%, corresponding to 6.2 ± 1.8% of total wt BZ sites) were insensitive to competition with zolpidem, suggesting that they represented receptors containing the endogenous γ_3 subunit (**Fig. 1C**). In hippocampus of $\gamma_3^{tg}/\gamma_2^{0/0}$ mice, [^{3}H]flumazenil binding was restored to 31.9 ± 2.3% of the wt level and nearly all of these sites were insensitive to zolpidem (31.5 ± 3.1% of wt level). A five- to ten-fold increase in γ_3 subunit steady state levels in $\gamma_3^{tg}/\gamma_2^{0/0}$ compared to $\gamma_2^{0/0}$ brain resulted in a corresponding five-fold increase in hippocampal BZ sites. Thus, the transgene-encoded γ_3 subunit assembles with endogenous α and β subunits similarly to endogenous γ_3 subunit and restores a substantial proportion of BZ site-containing $GABA_A$ receptors. These display the zolpidem-insensitive pharmacology characteristic of γ_3 subunit-containing receptors.

A similar analysis was carried out on cerebellum. Loss of the γ_2 subunit resulted in complete loss of [^{3}H]flumazenil sites in this location (**Fig. 1C**) as expected, since the cerebellum is normally devoid of γ_1 and γ_3 subunits. In contrast, BZ binding sites were clearly detectable in the cerebellum of $\gamma_3^{tg}/\gamma_2^{0/0}$ mice, albeit at a significantly lower level than in wt cerebellum. Thus, the transgene-encoded γ_3 subunit can contribute to

$GABA_A$ receptors in brain regions where it does not naturally occur.

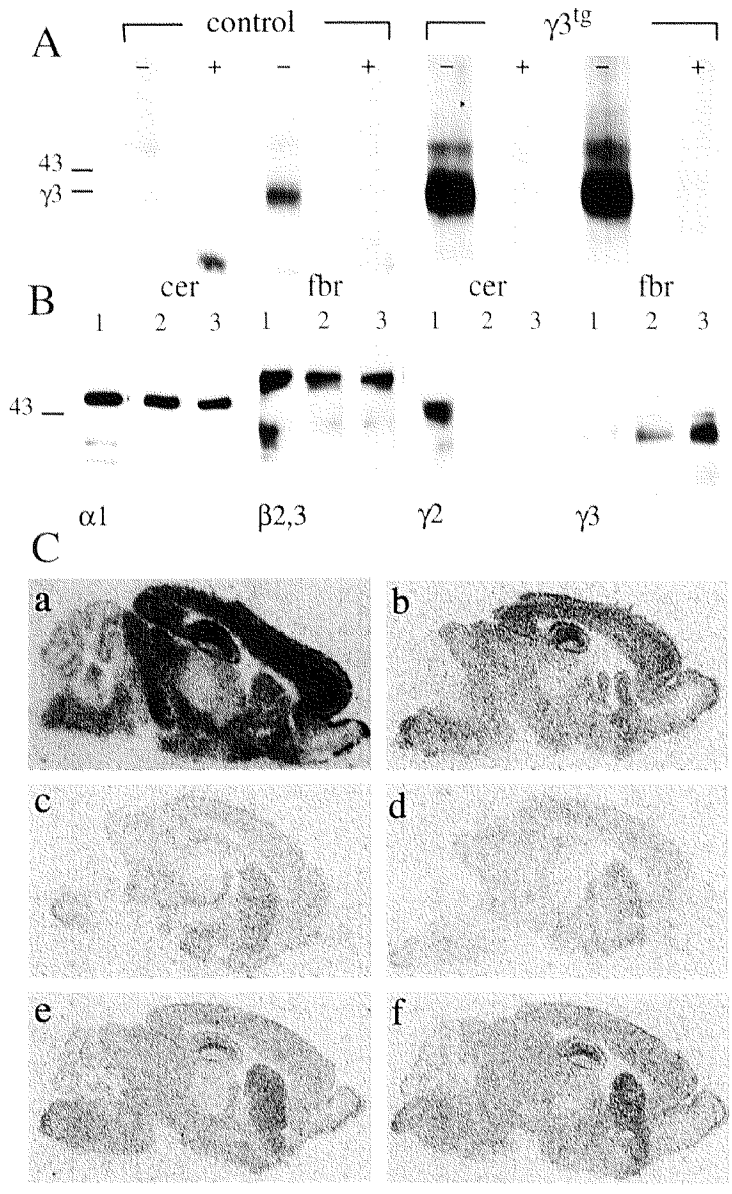

Fig. 1. Expression of γ_3 subunit and reconstitution of BZ binding sites in $\gamma_3^{tg}/\gamma_2^{0/0}$ brain. A) Western blot of γ_3 subunit in membranes from cerebellum (cer) and forebrain (fbr) of adult $\gamma_2^{+/+}$ (control) and $\gamma_3^{tg}/\gamma_2^{+/+}$ (γ_3^{tg}) mice using a γ_3 subunit specific antiserum. Lanes marked with (+) indicate competition by antigenic peptide (10 µg/ml). Note the low level of γ_3 subunit expression in forebrain and the lack of a signal in wt cerebellum. In contrast, the γ_3 subunit is abundant in both parts of transgenic brain. The molecular mass (in kD) of a standard and the position of the γ_3 subunit is indicated. B) Western blot of $GABA_A$ receptors immunoprecipitated from brain membranes with an α_1 subunit antiserum. Precipitated receptors from $\gamma_2^{+/+}$ (lane 1), $\gamma_2^{0/0}$ (lane 2), and $\gamma_3^{tg}/\gamma_2^{0/0}$ (lane 3) animals were analyzed with the antibodies indicated. Note the graded upregulation of the γ_3 subunit in $\gamma_2^{0/0}$ and $\gamma_3^{tg}/\gamma_2^{0/0}$ mice. C) Distribution of total and zolpidem-insensitive BZ binding sites in $\gamma_2^{+/+}$ (a, b), $\gamma_2^{0/0}$ (c, d), and $\gamma_3^{tg}/\gamma_2^{0/0}$ brain (e, f) as seen by autoradiography with [^{3}H]flumazenil (a, c, e) or with [^{3}H]flumazenil and 10 µM zolpidem (b, d, f). Note that nearly all BZ binding sites remaining in $\gamma_2^{0/0}$ mice are zolpidem-insensitive (d), suggesting that they represent $GABA_A$ receptors containing the γ_3 subunit. In $\gamma_3^{tg}/\gamma_2^{0/0}$ mice, there is a marked increase in BZ binding sites that are likewise zolpidem-insensitive (e, f).

The γ_3 subunit directs functional $GABA_A$ receptors to postsynaptic sites

To determine whether the increased number of BZ binding sites in $\gamma_3^{tg}/\gamma_2^{0/0}$ mice was reflected electrophysiologically, we first analyzed the effect of flunitrazepam on GABA-evoked responses in recordings from cultured cortical neurons (14-21 days *in vitro*, DIV). In $\gamma_2^{0/0}$ neurons, the GABA-gated currents were unaffected by flunitrazepam in most cases, or very slightly potentiated, as expected from the loss of most BZ sites (**Fig. 2A**). In striking contrast, $\gamma_3^{tg}/\gamma_2^{0/0}$ neurons displayed a potentiation by flunitrazepam similar to that observed in wt. Thus, the transgene-encoded γ_3 subunit contributes to the formation of BZ-sensitive $GABA_A$ receptors in γ_2 subunit-deficient neurons.

Postsynaptic localization of $GABA_A$ receptors in cultured cortical neurons requires the γ_2 subunit (Essrich et al., 1998). Consequently, the input frequency of GABAergic mIPSCs in $\gamma_2^{0/0}$ neurons was reduced to 33 ± 13.1% (n = 19) of wt (**Fig. 2B**), as described (Essrich et al., 1998). In $\gamma_3^{tg}/\gamma_2^{0/0}$ neurons, the input frequency was restored to 67 ± 8.9% (n = 21) of the wt level (**Fig. 2B**). Thus, in the absence of the γ_2 subunit, ectopic γ_3 subunit not only assembles with endogenous α and β subunits but also contributes to the formation of a major population of functional postsynaptic $GABA_A$ receptors.

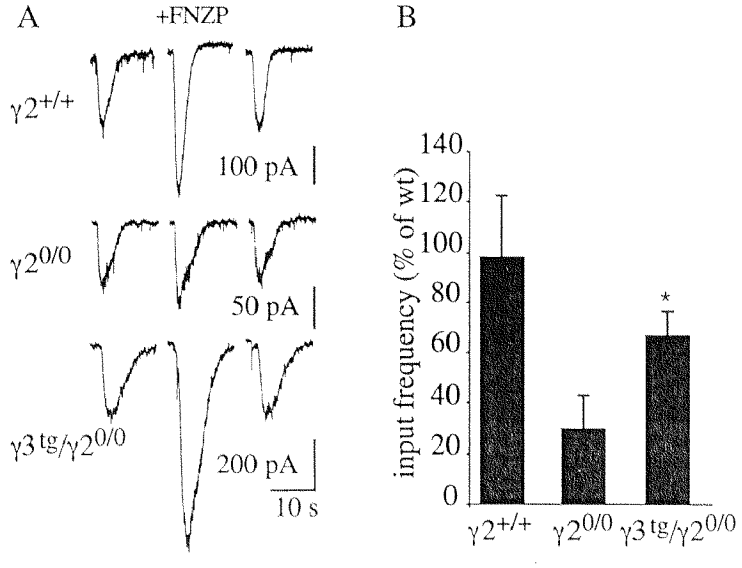

Fig. 2. Restoration of functional BZ-modulated and synaptic $GABA_A$ receptors in cultured cortical $\gamma_3^{tg}/\gamma_2^{0/0}$ neurons. A) Representative recordings illustrating the effect of flunitrazepam (1 μM) on GABA-evoked currents (GABA 1 μM, 2 s) recorded from $\gamma_2^{+/+}$, $\gamma_2^{0/0}$, and $\gamma_3^{tg}/\gamma_2^{0/0}$ cortical neurons (18 DIV). B) Relative frequency of GABAergic mIPSCs in cortical neurons cultured from $\gamma_2^{+/+}$, $\gamma_2^{0/0}$, and $\gamma_3^{tg}/\gamma_2^{0/0}$ embryos. GABAergic mIPSCs were identified based on current decay kinetics and pharmacological sensitivity (Essrich et al., 1998). The input frequency was reduced to 33 ± 13.1% of $\gamma_2^{+/+}$ in $\gamma_2^{0/0}$ neurons and restored to 67 ± 8.9% by overexpression of the γ_3 subunit in $\gamma_3^{tg}/\gamma_2^{0/0}$ neurons. Error bars, standard error.

Ectopic γ_3 subunit promotes synaptic clustering of α_1 and α_2 subunit-containing $GABA_A$ receptors *in vivo*

A major fraction of hippocampal $GABA_A$ receptors is characterized by the presence of the α_2 subunit. These receptors are clustered at gephyrin-rich, presumably postsynaptic sites, as indicated by the punctate and colocalized immunoreactivity (IR) for the α_2 subunit and gephyrin in the CA1 region (**Fig. 3a, d**) (Essrich et al., 1998; Craig et al., 1996). In the absence of the γ_2 subunit, punctate staining for the α_2 subunit and gephyrin was drastically reduced, indicating a major loss of α_2 subunit-containing $GABA_A$ receptors and gephyrin at postsynaptic sites (**Fig. 3b, e**) (Essrich et al., 1998). Little staining was detectable for the endogenous γ_3 subunit. Upon overexpression of the γ_3 subunit, a substantial recovery of punctate α_2 subunit IR was observed in $\gamma_3^{tg}/\gamma_2^{0/0}$ littermates. Moreover, α_2 subunit IR colocalized with punctate γ_3 subunit staining (**Fig. 3c**), indicating that the two subunits were located in the same synapse, and perhaps in the same $GABA_A$ receptor complex. This pattern was seen in all animals analyzed (n = 4 to 6 per genotype). Although the γ_3 subunit was expressed under control of a ubiquitously active promoter, no γ_3 subunit IR was detected in non-neural cells, which is consistent with the notion that the γ_3 subunit requires α and β subunits for assembly into stable complexes. The number of α_2 subunit clusters in $\gamma_3^{tg}/\gamma_2^{0/0}$ hippocampus (542 $\pm$ 18.1 clusters/10^4 μm^2) was as high as in wt (483.7 $\pm$ 22.3) and the average size of α_2 subunit clusters [0.198 μm^2 $\pm$ 0.104 (wt) and 0.194 $\pm$ 0.115 ($\gamma_3^{tg}/\gamma_2^{0/0}$)] was also restored. Interestingly, γ_3 subunit-containing receptors were colocalized with punctate IR for gephyrin (**Fig. 3f**), similarly to γ_2 subunit-containing receptors in wt hippocampus. Thus, in $\gamma_3^{tg}/\gamma_2^{0/0}$ hippocampus, the γ_3 subunit substitutes for the γ_2 subunit in the formation of clustered, presumably postsynaptic receptors. The two types of receptors are indistinguishable with regard to their colocalization with gephyrin clusters.

A similar analysis was conducted in the molecular layer of the cerebellum, which is largely devoid of $\alpha_{2\text{-}6}$, γ_1, γ_3, ε and π subunits, so that virtually all $GABA_A$ receptors conform to the $\alpha_1\beta\gamma_2$ subunit composition (Fritschy and Mohler, 1995; Whiting et al., 1997; Laurie et al., 1992a). Upon double immunofluorescence staining for the α_1 and γ_3 subunit in wt mice, strong punctate α_1 subunit IR was seen in the molecular layer of the cerebellum, probably representing postsynaptic receptors on dendrites of Purkinje, stellate and basket cells (**Fig. 3g**). In addition, diffuse staining of the neuropil was evident, indicating extrasynaptic receptors containing the α_1 subunit. No γ_3 subunit IR was detected in the cerebellum of non-transgenic mice (**Fig. 3g, h, j, k**).

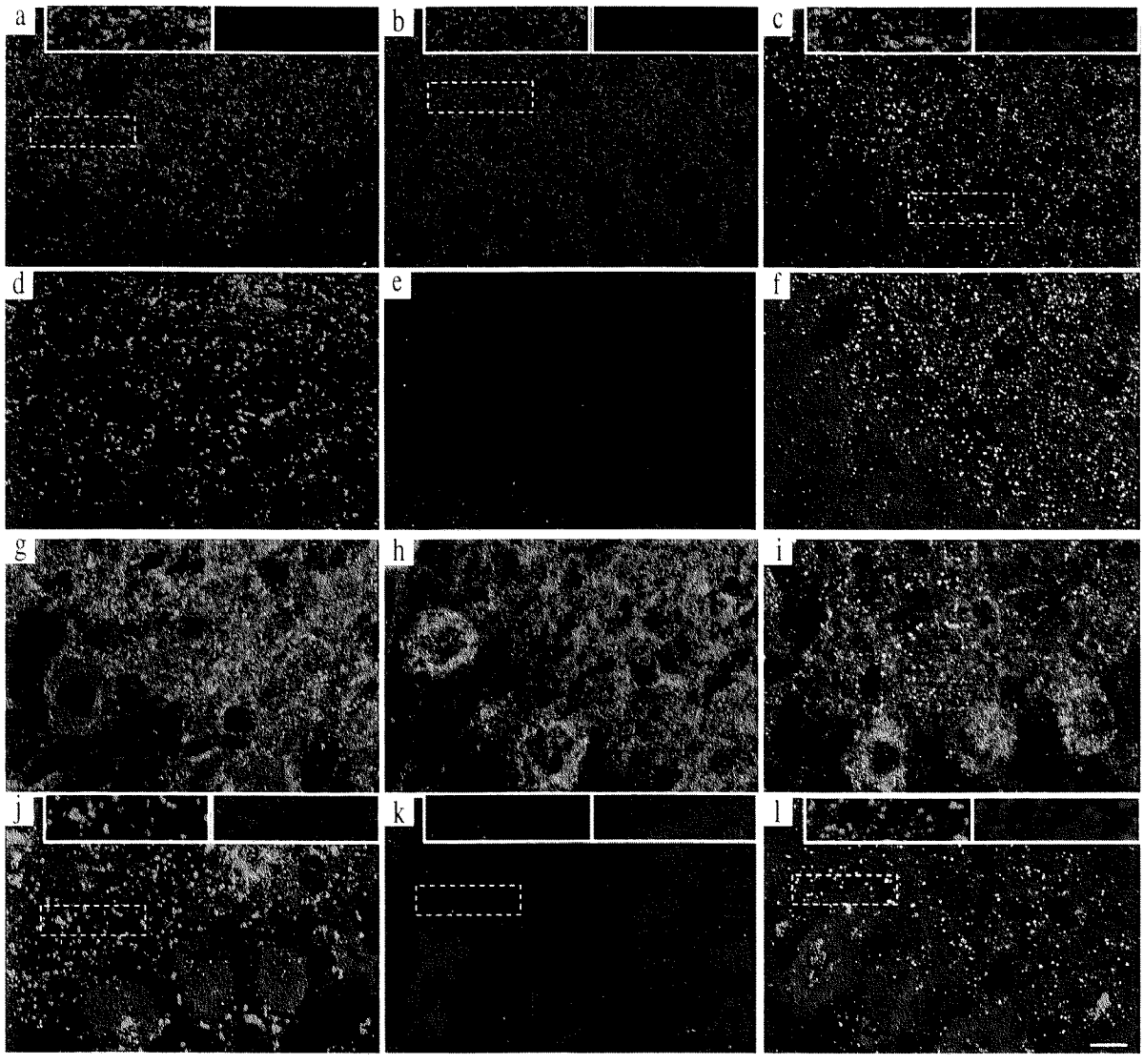

Fig. 3. Clustering of γ_3 subunit-containing receptors and gephyrin in $\gamma_3^{tg}/\gamma_2^{0/0}$ hippocampus and cerebellum. Parasagital sections through the CA1 region of P14 hippocampus (a-f, n = 4-6 per genotype) were stained with antibodies specific for the α_2 and γ_3 subunits (a-c; α_2 green, γ_3 red) and for gephyrin and the γ_3 subunit (d-f; gephyrin green, γ_3 red) and visualized by confocal microscopy. The images were digitally superimposed to illustrate the presence or absence of colocalization between the markers used (yellow punctae). The punctate α_2 (a) and gephyrin (d) staining, which is not colocalized with the scarce γ_3 subunit IR in wt mice, was completely lost in $\gamma_2^{0/0}$ mice (b, e) and largely restored in $\gamma_3^{tg}/\gamma_2^{0/0}$ mice (c, f). In the latter, it was extensively colocalized with the γ_3 subunit, as shown in yellow. Parasagital sections through the molecular layer of P14 cerebellum (g-l) were stained for the α_1 (green) and γ_3 subunit (red; g-i) and for gephyrin (green) and the γ_3 subunit (red; j-l). The punctate α_1 subunit (g) and gephyrin (j) staining in wt brain was completely lost in $\gamma_2^{0/0}$ (h, k) and largely restored in $\gamma_3^{tg}/\gamma_2^{0/0}$ brain (i, l). Whereas specific γ_3 subunit IR was absent in wt and $\gamma_2^{0/0}$ cerebellum, it was readily detected and extensively colocalized with the γ_3 subunit in $\gamma_3^{tg}/\gamma_2^{0/0}$ cerebellum. The data were reproduced with four to six animals per genotype. Insets at the top of panels a-c and j-l show enlargements of the boxed areas to illustrate the colocalization of clustered $GABA_A$ receptor and gephyrin IR in color-separated images. Scale bar, 10 μm.

In corresponding sections from $\gamma_2^{0/0}$ littermates, the punctate α_1 subunit staining was lost entirely, whereas the diffuse α_1 subunit staining appeared unaffected, as described (Essrich et al., 1998). Finally, in sections from $\gamma_3^{tg}/\gamma_2^{0/0}$ littermates, the punctate α_1 staining was largely re-established and was extensively colocalized with punctate IR for the γ_3 subunit (**Fig. 3i**). Recovery of $GABA_A$ receptor clusters in the cerebellum was again paralleled by a corresponding gain in gephyrin clusters that were colocalized with the γ_3 (**Fig. 3j-l**) and α_1 subunit IR (not shown). However, the number of α_1 subunit clusters in cerebellum was slightly reduced in $\gamma_3^{tg}/\gamma_2^{0/0}$ compared to wt tissue (243.8 ± 97.8 in $\gamma_3^{tg}/\gamma_2^{0/0}$ compared to 274 ± 9.8 clusters/10^4 μm^2 in $\gamma_2^{+/+}$), together with a reduction in the mean cluster size [0.28 ± 0.20 μm^2 ($\gamma_3^{tg}/\gamma_2^{0/0}$) and 0.35 ± 0.32 μm^2 (wt)]. Thus, ectopically expressed γ_3 subunit can substitute for the γ_2 subunit in the formation of clustered α_1 subunit-containing $GABA_A$ receptors in the cerebellar molecular layer, but the size and number of clusters does not reach wt levels.

$GABA_A$ receptor clustering mediated by the endogenous γ_3 subunit

Detailed examination of the [^{3}H]flumazenil autoradiograms revealed that the reticular thalamic nucleus (RTN) and the striatum of $\gamma_2^{0/0}$ P14 brain displayed increased levels of zolpidem-insensitive BZ binding sites compared to the surrounding tissue (**Fig. 1B**). This indicated that loss of BZ sites was not uniform across the brain, possibly due to higher than average levels of endogenous γ_3 subunit in some brain regions. In RTN of P14 $\gamma_2^{0/0}$ brain, staining for the γ_3 subunit revealed prominent punctate IR, indicative of synaptically clustered receptors. Moreover, γ_3 subunit staining was colocalized with the α_3 subunit (**Fig. 4a**), the most abundant α subunit in the RTN (Fritschy and Mohler, 1995), and with gephyrin (**Fig. 4b**). Thus, in $\gamma_2^{0/0}$ RTN the endogenous γ_3 subunit appears to contribute to postsynaptic $GABA_A$ receptors containing the α_3 subunit, thereby preventing the loss of $GABA_A$ receptor clusters.

In the wt RTN, the α_3 subunit and gephyrin IR were also colocalized (**Fig. 4d**). However, the γ_3 subunit IR was distinctly weaker than in $\gamma_2^{0/0}$ RTN, forming smaller punctae that were only partially colocalized with the α_3 subunit (**Fig. 4c**). These findings suggest that the γ_3 subunit contributes to only a fraction of postsynaptic $GABA_A$ receptors in wt RTN, the majority presumably being clustered by the γ_2 subunit. Thus, $GABA_A$ receptor clustering can be mediated by both the endogenous and the ectopic γ_3 subunit, a property best seen in the absence of the γ_2 subunit.

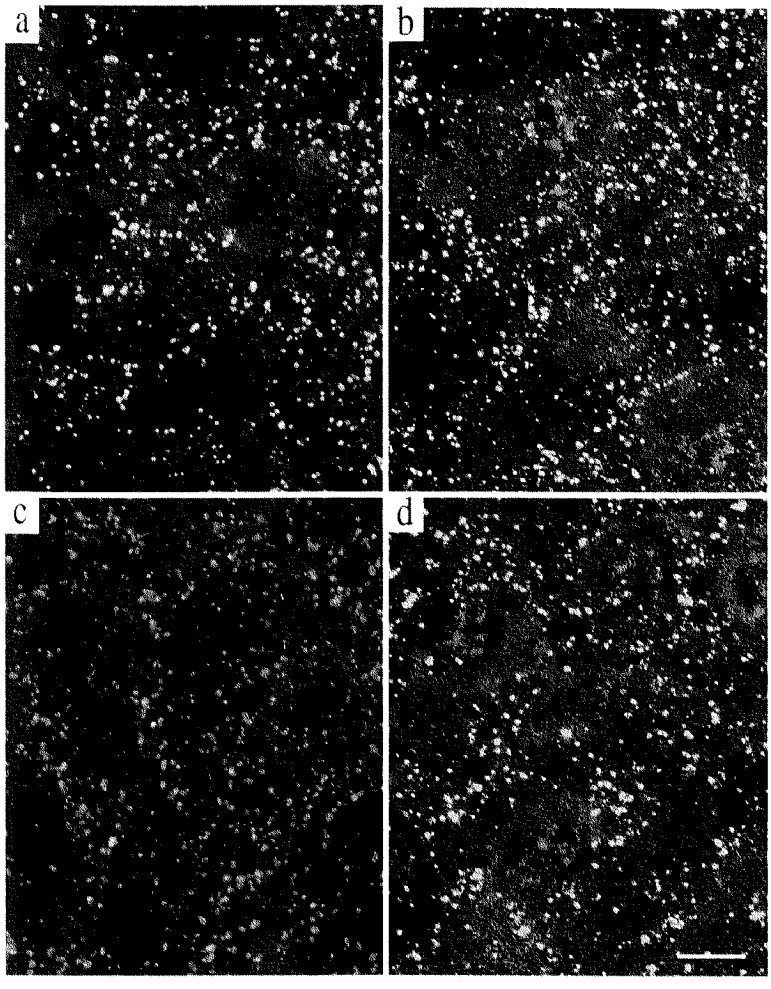

Fig. 4. Clustering of $GABA_A$ receptors mediated by the endogenous γ_3 subunit. The γ_3 subunit in the RTN promotes gephyrin and $GABA_A$ receptor γ_3 subunit clustering in $\gamma_2^{0/0}$ mice. Parasagital sections through the RTN of P14 mice (n = 4-6 per genotype) were double stained for the α_3 and γ_3 subunit (a, c; α_3 red, γ_3 green) or the α_3 subunit and gephyrin (b, d; α_3 red, gephyrin green) and visualized by confocal microscopy. Yellow punctae in the superimposed red and green images illustrate colocalization of the markers used. In $\gamma_2^{0/0}$ RTN, the α_3 subunit IR was colocalized with strong IR for the γ_3 subunit (a), as well as for gephyrin (b), whereas in $\gamma_2^{+/+}$ RTN, there was only a partial colocalization of the α_3 subunit IR and the weaker γ_3 subunit IR (c), but a prominent colocalization of the gephyrin and α_3 subunit IR (d). Scale bar, 10 μm.

Discussion

The γ_2 subunit and gephyrin are essential for postsynaptic localization of major γ_2 subunit-containing $GABA_A$ receptor subtypes. We now report that the γ_3 subunit is functionally equivalent to the γ_2 subunit in its contribution to postsynaptic localization and function of γ_3 subunit-containing $GABA_A$ receptors *in vivo*, and for recruitment of gephyrin to GABAergic synapses. In transgenic mice, these receptors were formed and

clustered even in brain regions devoid of endogenous γ_3 subunit, indicating that all the factors needed for clustering of γ_3 subunit-containing receptors are present and can be recruited in these neurons.

In $\gamma_2^{0/0}$ mice, γ_3 subunit-containing receptors appeared upregulated (Fig. 1B). A partial compensation for the lack of γ_2 subunit was even observed in the RTN, where the endogenous γ_3 subunit contributed to postsynaptic clusters containing the α_3 subunit and gephyrin. Since γ_3 subunit-containing clusters were much fewer in wt RTN, these data suggest that the γ_3 subunit is unable to displace the γ_2 subunit during formation of synaptically localized receptors. This might be due in part to structural differences or to a lower level of expression of the γ_3 subunit compared to the γ_2 subunit in wt RTN.

Despite the apparent functional similarity between the γ_2 and γ_3 subunits for the formation of postsynaptic receptors, the overexpressed γ_3 subunit did not rescue $\gamma_2^{0/0}$ mice from a lethal phenotype. Crossing of transgenic lines overexpressing the γ_2 subunit under control of the same ubiquitous promoter used here for the γ_3 transgene, allowed full rescue of mice without an overt behavioral phenotype (K. B. et al., unpublished). This indicates that the level of transgene expression with this promoter can be sufficient to fully restore $GABA_A$ receptor function and that ectopic expression of a γ subunit is not in itself toxic. However, the ectopic expression of the γ_3 subunit appeared to exert a dominant negative effect, as seen in some $\gamma_2^{+/0}$ and $\gamma_2^{+/+}$ transgenic animals.

The phenotype of $\gamma_3^{tg}/\gamma_2^{0/0}$ mice cannot be attributed simply to inefficient assembly of the γ_3 subunit with endogenous α and β subunits. In line with previous findings on $GABA_A$ receptors immunoprecipitated from brain extracts (Benke et al., 1996; Togel et al., 1994), the γ_3 subunit was found to assemble with α and β subunits (Fig. 1B). In the hippocampus, a five- to ten-fold increase in γ_3 subunit expression resulted in a roughly five-fold increase in BZ binding sites. Furthermore, γ_3 subunit-containing receptors show a unitary channel conductance similar to that of γ_2 subunit-containing receptors (Herb et al., 1992). However, synaptic $GABA_A$ receptor and gephyrin clusters appeared fully restored in hippocampus, but not in cerebellum. Incomplete restoration of synaptic receptors might in part be due to insufficient expression of the γ_3 subunit in the cerebellum and possibly other brain regions, possibly contributing to the lack of rescue of $\gamma_2^{0/0}$ mice by the γ_3 transgene.

Mechanisms of $GABA_A$ receptor clustering

The clustering of glycine receptors at postsynaptic sites requires both receptor activation and interaction with gephyrin (Kirsch et al., 1996; Kirsch and Betz, 1998; Levi et al., 1998). It is still unknown, however, whether this is also the case for $GABA_A$ receptors (Essrich et al., 1998; Betz, 1998), or whether protein interactions, involving at

least the γ_2 or γ_3 subunit and gephyrin, are sufficient for clustering at postsynaptic sites. In the absence of the γ_2 subunit, $GABA_A$ receptors are characterized by a markedly reduced channel conductance (Gunther et al., 1995), which might also account for the lack of clustering. The γ_3 subunit can substitute for the γ_2 subunit with respect to the conductance of the corresponding $GABA_A$ receptors (Herb et al., 1992). Thus, if receptor activation were required for clustering of $GABA_A$ receptors, then it would be the gain in conductance conferred by the presence of the γ_3 subunit that would trigger clustering of γ_3 subunit-containing $GABA_A$ receptors.

Independently of the outcome of further experiments addressing this issue, the γ_3 subunit-containing $GABA_A$ receptors were colocalized with gephyrin in $\gamma_3^{tg}/\gamma_2^{0/0}$ mice in a manner very similar to the γ_2 subunit-containing receptors in wt mice. Therefore, if protein interactions are important for this process, it is very likely that similar domains and factors are involved for both subunits. As the γ_2 and γ_3 subunits exhibit considerable heterogeneity in their intracellular cytoplasmic loop, it is remarkable that the gephyrin-clustering capability is also present in the γ_3 subunit. At glycinergic synapses, gephyrin is thought to mediate clustering of glycine receptors by direct protein-protein interaction with the receptor β subunit. Although gephyrin is also required for postsynaptic localization of $GABA_A$ receptors (Essrich et al., 1998), there is so far no evidence for a direct interaction between gephyrin and any $GABA_A$ receptor subunit. A candidate protein (GABARAP) that interacts directly with the γ_2 subunit and might mediate interaction between the γ_2 subunit and gephyrin has recently been identified (Wang et al., 1999). Interestingly, the portion of the putative γ_2 subunit cytoplasmic loop that interacts with GABARAP (residues 394-411) is highly conserved between all γ subunits, indicating that GABARAP might also interact with γ_3 subunit-containing receptors (Wang et al., 1999). These residues might be crucial for protein interactions underlying the clustering of $GABA_A$ receptors at postsynaptic sites.

Methods

Generation of γ_3 transgenic mice. The murine γ_3 cDNA (Wilson-Shaw et al., 1991) including the entire γ_3 subunit coding region, 23-bp of 5' flanking and 115-bp of 3' flanking sequence was reconstructed from two partial cDNAs generously provided by N. Walter and J. M. Sikela (University of Colorado, Denver). The cDNA was cloned into pBAP (Culiat et al., 1995) in-between 5' genomic sequences from the human β-actin gene and a 3' poly A signal from SV40. The expression cassette was separated from vector sequences and injected into the pronuclei of fertilized eggs of B6D2F1 hybrid donor mice and the eggs were transferred into foster mothers as described

(Hogan et al., 1994). Transgenic founder mice were identified by PCR analyses of tail biopsies and expanded into lines by crossing with mice that are heterozygous for the $GABA_A$ receptor γ_2 subunit gene ($\gamma_2^{+/0}$) (Gunther et al., 1995) on a 129/Ola x C57BL/6 background. The offspring were screened for expression of the transgene by Western blotting of brain membranes. One transgenic line was found to express substantial amounts of the γ_3 subunit and subsequently crossed with $\gamma_2^{+/0}$ mice. Offspring that were hemizygous for the γ_3 transgene and heterozygous for the null allele of the γ_2 subunit ($\gamma_3^{tg}/\gamma_2^{+/0}$) were crossed with $\gamma_2^{+/0}$ mice to obtain $\gamma_3^{tg}/\gamma_2^{0/0}$ homozygous mutants. This breeding scheme maintains hemizygozity at the transgene locus, thus eliminating possible effects due to insertion mutagenesis. The presence of the transgene and the genotype at the γ_2 locus were determined by PCR analyses of tail biopsies or embryonic tissue, using the primers 5'-CCGGC CCGGC TTCCT TTGTC C-3' (γ_3^{tg} upper primer) and 5'-TGCCT AATGT TGTTC TTGCT GGTGT CG-3' (lower primer) and those described (Gunther et al., 1995) for the γ_2 gene locus.

Immunoprecipitation. Affinity-purified α_1 subunit-specific antibodies were coupled to protein A-agarose beads and incubated overnight at 4°C with deoxycholate membrane extracts prepared from three P10-13 brains per genotype as described (Benke et al., 1996). Following extensive washing with 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.2% Triton X-100, the immunoprecipitates were subjected to Western blot analysis with antibodies against the α_1, $\beta_{2/3}$, γ_2, and γ_3 subunits (Benke et al., 1996).

Autoradiography. Cryostat brain sections (12 µm) of P14 mice were incubated with 6 nM [^{3}H]flumazenil in the absence or presence of 10 µM zolpidem as described (Olsen et al., 1990). Quantification in the hippocampal region was performed by densitometry in three mice per genotype using an MCID imaging system (Imaging Research, St. Catharines, Ontario). For each mouse, data were averaged from nine measurements of the CA1 region in three parasagital sections.

Immunohistochemistry. Cryostat brain sections (12 µm) of P14 mice were processed for double-immunofluorescence staining as described (Fritschy et al., 1998b; Essrich et al., 1998). $GABA_A$ receptor subunit expression was investigated with polyclonal antisera raised in guinea pig (α_1, α_2, α_3, γ_3) or rabbit (γ_3) (Benke et al., 1996; Fritschy and Mohler, 1995). Gephyrin was detected with the monoclonal antibody 7a (Connex, Martinsried, Pfeiffer et al., 1984). Following overnight incubation with a mixture of two primary antibodies, the sections were incubated for 30 min in the corresponding

secondary antibodies coupled to Cy3 (Jackson ImmunoResearch, West Grove, PA) or Oregon Green (Molecular Probes, Eugene, OR), washed, and coverslipped with buffered glycerol.

Data analysis. Fluorescent images were captured using a confocal laser scanning microscope (Leica, TCS 4D). For quantification of the number of synaptic receptor clusters, digital images were analyzed with the MCID software using a threshold segmentation algorithm for detection of clusters as described (Essrich et al., 1998). For each mouse (n = 3-4 per genotype) measurements were done in triplicate from three distinct sections. Results are given as mean ± SD. The size of clusters was averaged from individual measurements (n = 500-1300 per genotype).

Electrophysiological recordings and neuronal cultures. Miniature inhibitory postsynaptic currents (mIPSCs) and currents evoked by pulse application of GABA were recorded using the whole-cell patch-clamp method applied to neocortical neurons from $\gamma_2^{+/+}$, $\gamma_2^{0/0}$ and $\gamma_3^{tg}/\gamma_2^{0/0}$ embryos cultured for 2 to 3 weeks as previously described (Essrich et al., 1998).

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4. General Discussion

The γ_2 subunit is known as a major constituent of $GABA_AR$s in the brain. It represents an essential subunit that is present in most $GABA_AR$ subtypes in the brain. The γ_3 subunit is homologous to the γ_2 subunit and can substitute for it with respect to pharmacological and biophysical functions, as tested *in vitro*.

The present study shows that the γ_2 subunit is essential for the clustering of $GABA_AR$s at postsynaptic sites *in vivo*. In addition, the γ_2 subunit is required for the recruitment or maintenance of the putative clustering protein gephyrin at these sites, and gephyrin is likewise required for the accumulation of $GABA_AR$s at the synapse. The γ_3 subunit appears equivalent to the γ_2 subunit in its ability to cluster $GABA_AR$s and gephyrin. These findings are supported by five lines of evidence. **First**, the absence of the γ_2 subunit in $\gamma_2^{0/0}$ mice results in markedly reduced numbers of postsynaptic $GABA_AR$s, as demonstrated by a dramatic loss of immunoreactive punctae that colocalize with the presynaptic marker GAD, and a major loss of synaptic GABAergic currents. **Second**, the loss of postsynaptic $GABA_AR$s is paralleled by the loss of gephyrin at postsynaptic sites. **Third**, the inhibition of gephyrin expression in cultured wt neurons results in a corresponding loss of synaptically clustered $GABA_AR$s. **Fourth**, the transgenic overexpression of the $GABA_AR$ γ_3 subunit in $\gamma_2^{0/0}$ mice significantly restores the number of functional $GABA_AR$s that are synaptically clustered with gephyrin. **Fifth**, the endogenous γ_3 subunit is upregulated in the reticular thalamic nucleus (RTN) of $\gamma_2^{0/0}$ brain, and appears to mediate clustering of $GABA_AR$s and gephyrin there.

4.1 The role of γ subunits for synaptic clustering of $GABA_A$ receptors

The number of synaptic clusters of gephyrin and $GABA_AR$s in cultured $\gamma_2^{0/0}$ neurons is reduced to 20-25% of wt, as shown by immunocytochemistry and electrophysiology (Essrich et al., 1998). This demonstrates that the majority of synaptic $GABA_AR$s and gephyrin requires the γ_2 subunit for synaptic localization. The remaining clusters, however, suggest that a subunit other than γ_2 is also capable of mediating $GABA_AR$ and gephyrin clustering. The present work provided evidence that this remaining clustering may be mediated by the γ_3 subunit. In $\gamma_2^{0/0}$ brain, both the endogenous γ_3 subunit and the ectopically expressed γ_3 subunit were colocalized with synaptic $GABA_AR$ and gephyrin clusters, although in wt brain the γ_3 subunit IR is of very low abundance. This suggests that the γ_3 subunit is not efficiently incorporated into synaptic clusters when the γ_2 subunit is present in the same cell. In agreement with the low abundance of receptors containing the γ_3 subunit, mice that lack a functional γ_3

subunit gene by a chromosomal deletion show no overt phenotype (Culiat et al., 1993). Since the γ_3 subunit is most abundant before the onset of synaptogenesis (Laurie et al., 1992b; Ma et al., 1993), one could speculate that receptors containing the γ_3 subunit play an important role only during development. Based on sequence homologies, further candidate subunits that could mediate $GABA_AR$ clustering and could contribute to synaptic clusters of receptors include the γ_1 and ε subunits, although their expression is very limited in the wt brain.

A necessary requirement for the presence of a γ subunit for synaptic localization is also suggested by a study of the single channel conductances of neuronal $GABA_ARs$ in cerebellar granule cells (Brickley et al., 1999). Whereas currents recorded from extrasynaptic receptors revealed multiple conductance levels corresponding to recombinant $\alpha\beta$ and $\alpha\beta\gamma$ or $\alpha\beta\delta$ receptors, synaptic receptors were characterized by a single high conductance level that is typical for $\alpha\beta\gamma$ or $\alpha\beta\delta$ receptors (Fisher and Macdonald, 1997). Since δ subunit-containing receptors are known to be exclusively extrasynaptic (Nusser et al., 1998b), this suggests that a γ subunit is required for postsynaptic localization of $GABA_ARs$ *in vivo*, and that $\alpha\beta$ receptors are located at extrasynaptic sites. Based on their data, Brickley et al. could not distinguish whether the largest conductance in the extrasynaptic membrane originated from $\alpha\beta\gamma$ and/or $\alpha\beta\delta$ receptors. However, the presence of extrasynaptic $\alpha\beta\gamma$ receptors has been demonstrated in cerebellar granule cells (Nusser et al., 1998b) and hippocampal neurons (Somogyi et al., 1996). The extrasynaptic $\alpha\beta\gamma$ receptors might provide a supply pool for a short-term adaptive increase in synaptically localized $GABA_ARs$. Such an adaptation in synaptic efficiency has been reported to occur in the dentate gyrus of 'kindled' rats, an experimental model for temporal-lobe epilepsy (Nusser et al., 1998a). To account for extrasynaptic receptors containing a γ subunit, additional factors beyond the presence of a γ subunit appear to be required to specify a synaptic versus extrasynaptic localization of $GABA_ARs$. One factor essential for synaptic localization of $GABA_ARs$ is gephyrin (see below).

The mechanism by which $GABA_AR$ clustering is reduced in $\gamma_2^{0/0}$ mice remains unclear. It may be due to a lack of physical interaction of γ_2 subunit-deficient receptors with synaptic anchoring proteins such as gephyrin. Alternatively, synaptic receptor accumulation may fail due to the reduced channel conductance of γ_2 subunit-deficient $GABA_ARs$ (Gunther et al., 1995). Clustering of GlyRs depends on synaptic activity (Kirsch and Betz, 1998; Levi et al., 1998; Kirsch, 1999), which could similarly be important for $GABA_AR$ clustering. However, it is not conclusively determined so far whether the synaptic clustering of $GABA_ARs$ is regulated by receptor activation.

Preliminary data suggested that $GABA_AR$ clustering does not depend on receptor activation (see chapter 1.5.2.5). However, primary motoneurons were recently shown to develop $GABA_AR$ clusters at synaptic sites only when cocultured with dorsal root ganglion neurons that provided GABAergic innervation. In motoneurons receiving glycinergic and GABAergic input from spinal interneurons, both, GlyRs and $GABA_ARs$ formed mosaics at synaptic loci (Levi et al., 1999b). These results indicate that the presynaptic element controls the postsynaptic accumulation of these receptors, but the nature of this signal remains unknown. Whether deficits in synaptic clustering of $GABA_ARs$ and gephyrin could be due to impaired $GABA_AR$ activity needs to be examined in the future.

It has previously been shown that phosphorylation of $GABA_AR$ subunits can be involved in the alteration of $GABA_AR$ densities at the cell surface (see chapter 1.3.1), and that the γ_2 subunit - which is a target for different protein-kinases - might be involved in such processes. For example, the treatment of neuronal cultures with brain-derived neurotrophic factor (BDNF) causes a rapid downregulation of surface expression of $GABA_ARs$ that is accompanied by dephosphorylation of the γ_2 subunit (S. Penschuck and J. M. Fritschy, unpublished). Thus, the γ_2 subunit could also play an important role in controlling short-term, activity-dependent plasticity of $GABA_ARs$.

The critical role of the γ_2 subunit for clustering of synaptic $GABA_ARs$ was underscored by the analysis of mice which were heterozygous for the γ_2 subunit gene ($\gamma_2^{+/0}$). In these animals, a significant reduction in $GABA_AR$ and gephyrin clusters was observed (Crestani et al., 1999). This confirms that the γ_2 subunit is limiting with respect to synaptic clustering of $GABA_ARs$. Interestingly, the weak reduction in $GABA_AR$ clusters in $\gamma_2^{+/0}$ brain was accompanied by behavioral and cognitive deficits similar to anxiety disorders in humans (Crestani et al., 1999).

4.2 The significance of gephyrin for synaptic clustering of $GABA_A$ receptors

Antisense suppression of gephyrin expression resulted in a dramatic loss of $GABA_AR$ clusters in cultured hippocampal neurons (Essrich et al., 1998). This finding was recently confirmed by the analysis of gephyrin knock out mice ($gep^{0/0}$) (Feng et al., 1998). Beside the loss of synaptic GlyRs, these mice exhibit a severe deficit in $GABA_AR$ clustering in the retina, as well as in cultured neurons (Kneussel et al., 1999b, H. Wässle, personal communication). Therefore, a physical interaction of $GABA_AR$ subunits and gephyrin is very likely (see Fig. 5A). The failure to detect such interactions *in vitro* so far may indicate that they are weak, sensitive to detergent, or indirect. Recently, a candidate protein that might be involved in stabilizing the interaction between gephyrin and $GABA_ARs$ was discovered. GABARAP ($GABA_AR$-associated

protein) was identified by its interaction with a 18 aa sequence in the C-terminal part of the intracellular loop of the γ_2 subunit, which is conserved between all three γ subunits (Wang et al., 1999; reviewed in Passafaro and Sheng, 1999). GABARAP binds to $GABA_A$Rs *in vitro* and *in vivo*, and colocalizes with punctate $GABA_A$R staining in cultured cortical neurons (Wang et al., 1999). Thus, GABARAP could be a linker between the $GABA_A$R γ subunits and gephyrin. A general role for GABARAP in $GABA_A$R clustering would be in accordance with the clustering functions of the γ_2 and γ_3 subunits (Fig. 5A). Since GABARAP harbours a putative tubulin-binding motif at the N-terminus (Wang et al., 1999), a direct interaction with microtubuli, independent of gephyrin, is also conceivable. Such a role has been suggested for the microtubule-associated protein (MAP)-1B that is related to GABARAP and was recently found to interact with $GABA_A$R ρ_1 subunits at synaptic localizations in the retina (Hanley et al., 1999). MAP-1B fails to interact with other $GABA_A$R subunits (Hanley et al., 1999), and ρ subunits are not colocalized with gephyrin in the retina (Koulen et al., 1998). Therefore, MAP-1B is thought to directly mediate the anchoring of ρ_1 subunit-containing $GABA_A$Rs at the subsynaptic cytoskeleton.

The reduction of $GABA_A$R clusters in $gep^{0/0}$ mice in the retina is incomplete as in $\gamma_2^{0/0}$ neurons (H. Wässle, personal communication), and some $GABA_A$R clusters in wt rat brain do not colocalize with gephyrin IR, for instance at synapses of cerebellar Purkinje cell somata (J. M. Fritschy, unpublished). Therefore, different additional anchoring proteins beside gephyrin (or unknown isoforms of gephyrin, not detectable with the current gephyrin antibodies), might contribute to synaptic clustering of a minor population of $GABA_A$R subtypes. Such proteins are not known to date, but the cytoskeletal protein dystrophin, which is present at the NMJ together with rapsyn, has recently been implicated in clustering functions at GABAergic synapses. Dystrophin is colocalized with $GABA_A$R and gephyrin clusters in cerebral cortex, hippocampus, and cerebellum. Mdx mice, which lack dystrophin, showed reduced numbers of $GABA_A$R clusters containing the α_1 and α_2 subunits in these regions, but surprisingly gephyrin IR was unaltered (Knuesel et al., 1999). This provides further evidence that the interaction of $GABA_A$Rs with gephyrin is indirect and that dystrophin may be involved in the stabilization of $GABA_A$Rs in certain synapses. Similarly, rapsyn could play a restricted role in this process, as an interaction with $GABA_A$R subunits in heterologeous expression systems has been shown (see chapter 1.5.2.4).

4.3 Receptor clustering at excitatory and inhibitory synapses

The present knowledge suggests that different mechanisms apply for the formation of inhibitory and excitatory postsynaptic receptor clusters. Whereas receptor activity is required for the formation of GlyRs and possibly $GABA_AR$s, the implication of additional factors released from the presynaptic terminal has so far been demonstrated only for the nAChR and the AMPA-R. Such transsynaptic signals that induce receptor clustering are agrin in the case of the nAChR and Narp for the AMPA-R (Fig. 5B, see chapter 1.5.2). Furthermore, there is no homology between gephyrin, rapsyn, and postsynaptic proteins of the MAGUK family, such as GRIP, suggesting that the structural components for anchoring at the cytoskeleton are distinct between inhibitory and excitatory receptors.

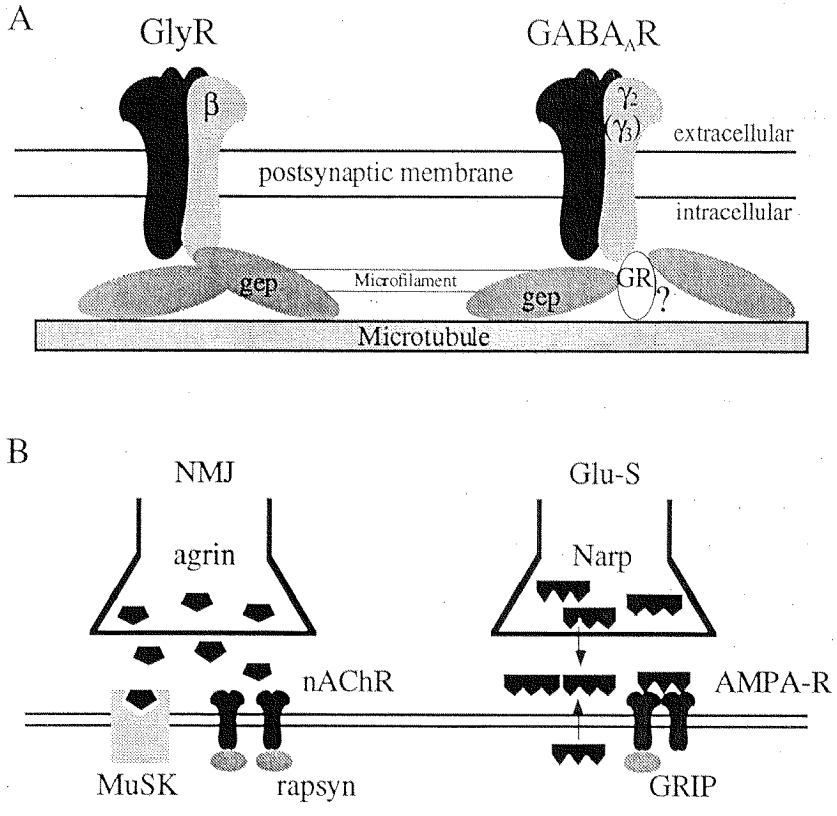

Fig. 5 A: Model for the anchoring of GlyRs and $GABA_AR$s at postsynaptic sites by gephyrin (gep=gephyrin, GR=GABARAP, modified from Kirsch, 1999). B: Model for agrin-induced nAChR clustering at the NMJ and Narp-induced AMPA-R clustering at glutamatergic synapses (Glu-S, see chapter 1.5.2, MuSK=muscle-type receptor tyrosine kinase, GRIP=glutamate receptor interacting protein, modified from Fong and Craig, 1999).

5. Outlook

Role of the γ subunits for GABA$_A$R clustering: To specify the structural motifs of the γ subunits required for GABA$_A$R clustering in more detail, a mutagenesis-study should be initiated. Ectopic expression of point-mutated, truncated or chimeric γ subunits, mainly altered in the intracellular loop, in brain or primary neuronal cultures of $\gamma_2^{0/0}$ mice could provide insights into γ subunit sequences essential for interaction with postsynaptic proteins.

Activity-dependence of GABA$_A$R clustering: A major open question concerns the mechanisms of synaptic clustering of GABA$_A$Rs. This process may either depend on receptor activation during neuronal maturation, similar to GlyRs, or rely on the physical interaction of GABA$_A$R subunits and cytoskeletal proteins. Possible effects of chronic blockage of GABA$_A$Rs and voltage-dependent Ca^{2+} channels on GABA$_A$R and gephyrin clustering could be investigated in neuronal cultures by immunocytochemical and electrophysiological analyses. Such experiments should be performed preferentially in cultures from brain tissue like hippocampus or cortex, since in spinal cord cultures GlyRs are abundant and may stabilize synaptic gephyrin aggregates.

GABARAP and additional proteins involved in GABA$_A$R clustering: Several lines of evidence suggest an indirect interaction of GABA$_A$R subunits with gephyrin. To characterize components involved in this process, the relationship of GABARAP to gephyrin and GABA$_A$Rs requires additional analysis. Immunohistochemical studies of GABARAP expression in wt and mutant mice that lack synaptic GABA$_A$Rs and gephyrin ($\gamma_2^{0/0}$, gep$^{0/0}$) could provide valuable information in this respect. If GABARAP is important for synaptic clustering of GABA$_A$Rs and gephyrin, absence of both proteins would predict a significant reduction of GABARAP IR *in vivo*. Disruption of the GABARAP gene in mice would directly highlight its possible role for synaptic clustering. In addition, coexpression studies of gephyrin and GABARAP in heterologeous cells could detect possible interactions *in vitro*. Similar approaches could be applied for dystrophin with respect to its GABA$_A$R and gephyrin interaction (Knuesel et al., 1999). Finally, the future characterization of so far poorly defined proteins that copurify with GABA$_A$R subunits (Kannenberg et al., 1997) or interact with gephyrin (Kins et al., 1998), may reveal other components of the complex that accumulate and stabilize GABA$_A$Rs at postsynaptic sites.

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7. Curriculum Vitae

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Born July 23rd 1963 in Stuttgart, Germany.

Education

1969 - 73 Primary school in Dusslingen near Tübingen, Germany

1973 - 82 Gymnasium (Secondary school) with Abitur (school-leaving exam) in Tübingen

1984 Study of Archeology at the University of Tübingen

1984 - 94 Study of Biology at the Universities of Konstanz and Freiburg, Germany

1994 Diploma student, Supervisor: Dr. F. A. M. Asselbergs,
at the Department of Biotechnology, Ciba-Geigy AG, Basel, Switzerland
Diploma thesis title: *Untersuchungen zur Etablierung eines Nachweis-verfahrens für TGFß mittels eines Luciferase-Reportergens* (Development of a detection method for TGFβ by use of a Luciferase-reportergene)

1995 - 99 Ph.D. student, Supervisor: Dr. B. Lüscher,
at the Institute of Pharmacology, University of Zürich, Switzerland
Ph.D. thesis title: *Synaptic clustering of $GABA_A$ receptors*
Examination for a doctor's degree, September 1999

Additional Training

1990 Student apprentice at the F. Hoffmann-La Roche AG, Switzerland
Optimierung eines Enzymimmunoassays zum Nachweis von TNF in Serum (Optimization of an ELISA for the detection of TNF in serum)

1991 - 92 Scientific assistant at the Fraunhofer Institute for Mechanics of Materials, Freiburg, Germany
Zweidimensionale und dreidimensionale numerische Beanspruchungs-analysen (Two-dimensional and three-dimensional numerical physical material stress analyses)

8. Publications

Original publications:

Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., Lüscher, B. (1998).
Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ_2 subunit and gephyrin. Nature Neuroscience, *1*, 7, 563-571.

Crestani, F., Lorez, M., Baer, K., Essrich, C., Benke, D., Laurent, J. P., Belzung, C., Fritschy, J. M., Lüscher, B., Möhler, H. (1999).
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Abstracts:

Essrich, C., Fritschy, J. M., Lüscher, B. (1997).
Dendritic targeting of $GABA_A$ receptors: A role for the γ_2 subunit.
Abstract, 29. annual meeting of USGEB, Geneva, Switzerland.

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Membrane targeting of $GABA_A$ receptors: Essential roles for the γ_2 subunit and gephyrin. Abstract, 30. annual meeting of USGEB, Lausanne, Switzerland.

Essrich, C., Fritschy, J. M., Lüscher, B. (1998).
Synaptic targeting of $GABA_A$ receptors: Essential roles for the γ_2 subunit and gephyrin.
Abstract, Forum of Euopean Neuroscience, Berlin, Germany.

Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., Lüscher, B. (1998).
Essential roles in postsynaptic clustering of $GABA_A$ receptors for the γ_2 subunit and gephyrin. Soc. Neurosci. Abstr., Vol. 24, Part 2, p. 1349.

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Simonian, S. X., Sim, J. A., Skinner, M. J., Essrich C., Lüscher, B., Herbison, A.E. (1998).
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Clustering and postsynaptic targeting of γ_3 subunit-containing $GABA_A$ receptors in γ_3-transgenic γ_2 subunit-deficient mice.
Abstract, 27th Göttingen Conference of the German Neuroscience Society.

Essrich, C., Baer, K., Johnson, D. K., Lüscher, B., Fritschy, J. M. (1999).
Clustering of α_3 subunit-containing $GABA_A$ receptors and gephyrin mediated by the γ_3 subunit in γ_2 subunit-deficient reticular thalamic nucleus.
Abstract, 27th Göttingen Conference of the German Neuroscience Society.

Baer, K., Essrich, C., Balsiger, S., Wick, M., Harris, R. A., Sieghart, W., Benson, J., Fritschy, J. M., Lüscher, B. (1999).
Rescue of γ_2 subunit-deficient mice by transgenic overexpression of the γ_{2S} or γ_{2L} subunit.
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Essrich, C., Baer, K., Johnson, D. K., Lüscher, B., Fritschy, J. M. (1999).
Clustering of $GABA_A$ receptors and gephyrin: The γ_3 subunit substitutes for the γ_2 subunit in the Reticular Thalamic Nucleus of $\gamma_2^{0/0}$ mice.
Soc. Neurosci. Abstr., 29th annual meeting, Miami, USA.

9. Abbreviations

aa	amino acids
AMPA	α-amino-3-hydroxy-5-methylisoxazole-propionic-acid
AMPA-R	AMPA receptor
BiP	Binding protein, also immunoglobulin heavy chain binding protein
BZ	benzodiazepines
cAMP	cyclic adenosine-monophosphate
cDNA	complementary DNA
CNS	central nervous system
DIV	days *in vitro*
E 1-20	embryonic day 1-20
EM	electron microscope, or electron microscopic
ER	endoplasmic reticulum
$\gamma_2^{0/0}$	mice homozygous for a deletion of the $GABA_A$ receptor γ_2 subunit gene
$\gamma_2^{+/0}$	mice heterozygous for a deletion of the $GABA_A$ receptor γ_2 subunit gene
$\gamma_2^{+/+}$	mice that are wildtype at the γ_2 subunit locus
γ_3^{tg}	mice carrying a $GABA_A$ receptor γ_3 subunit transgene
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
GABARAP	$GABA_A$R-associated protein
GAD	glutamic acid decarboxylase
GlyR	glycine receptor
HEK	human embryonic kidney cell-line
IR	immunoreactivity
kD	kilo Dalton
LTD	long-term depression
LTP	long-term potentiation
mAb	monoclonal antibody
MAP	microtubule-associated protein
MDCK	Madin-Darby canine kidney cells
mIPSC	miniature inhibitory postsynaptic current
MoCo	molybdenum cofactor
mRNA	messenger RNA
MuSK	muscle-type receptor tyrosine kinase
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NMDA-R	NMDA receptor
NMJ	neuromuscular junction
P 0-14	postnatal day 0-14
PCR	polymerase chain reaction
PKA	cAMP-dependant protein kinase
PKC	calcium-phospholipid-dependant protein kinase
PSD	postsynaptic density
rER	rough endoplasmic reticulum
RNase	ribonuclease
RTN	reticular thalamic nucleus
TM	transmembrane domain
wt	wildtype